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From adolescence to adulthood: characterizing the enduring consequences of adolescent adversities in rats

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TABLE OF CONTENTS

Seite

ABBREVIATION (IF REQUIRED)

Acb	Nucleus accumbens
AA	Adolescent adversity
AAV	Adeno-associated viruses
ACEs	Adverse childhood experiences
AMY	Amygdala
ANOVA	Analysis of variance
ASI	Adolescent social isolation
AVP	Vasopressin
BDNF	Brain derived neurotrophic factor
CD38	Cluster of differentiation thirty-eight
CeA	Central amygdala
c-Fos	Cellular fbj murine osteosarcoma viral oncogene homolog
ChR2	Channelrhodopsin-2
CORT	Corticosterone / Cortisol
CTLS	Controls
EASI	Early adolescent social isolation
EPM	Elevated plus maze
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
HIPPO	Hippocampus
LASI	Late adolescent social isolation
LE	Long Evans
LS	Lateral septum
MeA	Medial amygdala
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
NOR	Novel object recognition
OFT	Open field test

ROI	Region of interest
PBS	Phosphate-buffered saline
PR	Peer rejection
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular thalamic nucleus
PVA	Paraventricular thalamic nucleus, anterior part
PVP	Paraventricular thalamic nucleus, posterior part
PWSI	Post-weaning social isolation
SD	Sprague Dawley
SI	Social isolation
SIS	Social instability stress
SIT	Social interaction test
SRM	Social recognition memory
SON	Supraoptic nucleus
VMH	Ventromedial nucleus of the hypothalamus
VTA	Ventral tegmental area
WIST	Wistar

1 INTRODUCTION

In the mid 1990's the Center for Disease Control and Prevention together with Kaiser Permanente discovered an exposure that dramatically increased the risk of seven of the ten leading causes of death (Felitti et al. 1998). At high doses, this exposure can affect brain development (Brewer-Smyth 2022), immunity (Slopen et al. 2012), hormonal systems (Vejbaesya et al. 2007), and could even cause epigenetic changes (Lang et al. 2020; Tang et al. 2020). Individuals exposed to very high doses of this exposure have triple the lifetime risk of heart disease (Jakubowski et al. 2018) and lung cancer (Hu et al. 2021). However, the most striking finding is that this exposure could decrease life expectancy by 20-years (Brown et al. 2009).

The exposures I refer to here are adverse childhood experiences (ACEs) or adolescent adversity (AA) in the preclinical field. But what are ACEs? We all face adversity to a certain degree and facing some adversity is normal and needed for normal development, such as losing at games or failing a test. ACEs are threats that typically occur during the first two decades of life and are so severe or pervasive that they literally get "under our skin" and change our physiology. ACEs are a public health concern that have been estimated to cost $1.3 trillion per annum, demonstrating the immense financial burden ACEs cast (Bellis et al. 2019).

While clinical studies have revealed that ACEs lead to increase in risk behaviors, poor health outcomes and more recently neuroimaging studies have elucidated us on the neural correlates of ACEs. Nonetheless, there remains a significant knowledge gap on how ACEs lead to disrupted neural development and impairments in the social, cognitive, and emotional domains. Here, preclinical studies can help us elucidate the underlying mechanistic effects of AAs on behavioral and molecular outcomes not available in humans due to ethical reasons.

We use a reverse translational approach to identify the most appropriate preclinical models that resemble the effects of ACEs in humans in order to characterize long-term consequences of AAs in rats. The aim of this thesis is to understand the long-term behavioral effects of various ACE-like adversities in rodents on neurodevelopment and their behavioral sequelae in adulthood. Additionally, we are interested in delineating

sex differences within these models as well as general sex differences. Last, we want to understand what long-term effect adolescent social isolation has on the oxytocin (OT) system and whether their functional relevance can be uncovered.

1.1 Adverse Childhood Experiences

The early years of an individual's life are vital for fostering emotional bonding and physical growth, central for future well-being and development. Studies have shown that bonding (Winston and Chicot 2016), playing (Ginsburg et al. 2007) and learning (Pattwell and Bath 2017) are all notable features of normal development. However, ACEs disrupt aspects of normal development and have large, long-term detrimental effects on neurodevelopment that can become "biologically embedded" (Berens et al. 2017) changing our biology and altering health and quality of life across the lifespan (Herzog and Schmahl 2018). It can have deep-rooted effects on the neural, endocrine, immune, and metabolic physiology.

ACEs include (but are not exclusive to) stressors and adversity, such as physical, emotional, or sexual abuse; physical or emotional neglect; parental mental illness; addiction; incarceration; divorce; domestic violence; and frequency (Felitti et al. 1998). However, the terminology has continuously evolved and expanded, as our understanding of ACEs has improve and we have integrated new classifications such as low socioeconomic status and peer rejection (PR) as ACEs (Finkelhor et al. 2013). Over the past three decades, societal awareness and understanding of ACEs have increased significantly. Despite this increased awareness, the profound societal and financial implications of ACEs still remain (Bellis et al. 2019).

The contemporary belief is that ACE effects are likely initiated by changes in the hypothalamic-pituitary-adrenal (HPA) axis by cortisol, which play a role in our stress response (Berens et al., 2017). Disproportionate early activation of the HPA-axis during adolescence by ACEs can result in HPA dysregulation, abnormal peripheral and central cortisol levels, diminished oxytocin production and release, and elevated inflammatory markers (Herzog and Schmahl 2018). The neurobiological alterations following ACEs are shown to increase the risk of health issues directly and indirectly in adults (Hughes et al. 2017). The evidence is clear that ACEs lead to an increased

adoption of health-risk behaviors, disease, disability, social problems, and eventually early death. However, more research is needed to understand how ACEs disrupt critical features of neurodevelopment and how these alterations lead to social, emotional, and cognitive impairments (Figure 1).

Preclinical research offers an avenue to investigate these alterations. However, translation from preclinical research to humans is often difficult for several reasons (discussed later). Due to the significant failure in translation some researchers have proposed adapting a reverse translational approach (Sinha et al. 2011; Peleh et al. 2019). Reverse translation aims to use animal models that recapitulate critical aspects of clinical findings and try to replicate these findings in animals. This approach is proposed to better reflect the human condition and, has merit in enhancing our understanding of ACEs on behavior and specifically the underlying neurobiology in a robust manner. We want to focus on the most prevalent ACEs and their molecular targets and regions of interest that have already been identified in humans. To implement this approach effectively and robustly it is first vital inform us on the ACE landscape from an epidemiological perspective and their neural correlates.

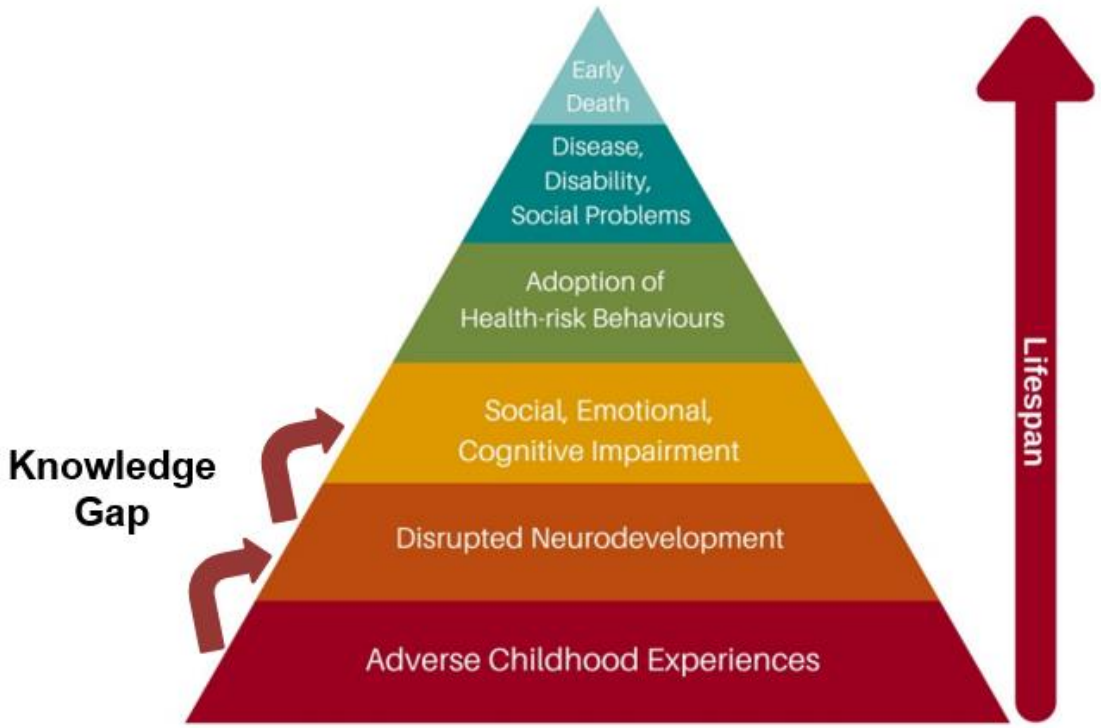


Figure 1: Adverse childhood experience pyramid. This represents the conceptual framework for studying ACEs. The knowledge gap in the field is represented by arrows, which are the focus areas of this dissertation. Adapted from www.wavetrust.org

1.1.1 Epidemiology

ACEs and other traumas are estimated to be among the largest unmet global challenges (Benjet et al. 2016). It is estimated that neuropsychiatric disorders cost almost € 800 billion annually in Europe (Gustavsson et al. 2011). Of these, €520 billion has been attributed to ACEs, which is estimated to be equivalent to 2.7% of Europe's regional gross domestic products (Hughes et al. 2017). A more recent estimate has placed the number past one trillion dollars per annum (Bellis et al. 2019). Which is higher than the combined cost of cardiovascular disease, diabetes, and cancer (Gustavsson et al. 2011).

The prevalence of ACEs is also extremely high worldwide. Epidemiological estimates in a USA estimated that almost 85% of children had been exposed to at least one ACE and more than 15% were exposed to two or more ACEs (DeBoer and Seaver 2019). A more heterogeneous population survey from 24 countries found that more than 70% reported at least one ACE. Astonishingly, almost 31% of participants reported exposure to four or more ACEs (Benjet et al. 2016). These findings have been replicated in low-, mid-, and high-income countries (Ramiro et al. 2010; Almuneef et al. 2014; Tang et al. 2020; Giano et al. 2020) suggesting that ACE exposure is high regardless of income.

In a comparable manner, there are differences in the geographic prevalence of ACE. For example, in the USA neglect (61%) is reported as the most common form of ACE, followed by physical abuse (10%), and sexual abuse (7%) (DeBoer and Seaver 2019). While in Germany results vary, with one survey study finding parental separation (19%) as the most common ACE, followed by alcohol and drug abuse (17%) and emotional neglect (Witt et al. 2019). However, when participants are interviewed instead a different picture emerges, with emotional neglect (51%) leading followed by emotional abuse (48%), and bullying (45%) (Seitz et al. 2022). While in Zambia, the most common ACEs reported were violence-related: physical violence (35%), community violence (30%), and emotional violence (20%) respectively (Lee et al. 2022). In Brazil, parental separation (42%) was the most prevalent ACE, followed by emotional neglect

(20%), and domestic abuse (10%) (Soares et al. 2016). These studies demonstrate some of the complex characteristics of ACEs and their geographically heterogeneity that need to be considered when trying to identify the most prevalent ACEs for reverse translation.

Neglect emerges as the most common type of ACE across various populations and geographical locations. Studies conducted in the USA, Germany, Zambia, and Brazil have highlighted the prevalence of emotional neglect, parental separation (social instability), and violence-related ACEs. However, the reporting and prevalence of ACEs varies depending on the study design and definitions used. While there has been a decrease in certain ACE domains over time, such as physical and sexual abuse, there are concerns about increases in non-ACE associated issues, such as suicides and obesity (Ogden et al. 2016; Ruch et al. 2019). ACEs also disproportionately affect certain populations, including females, individuals with multiracial backgrounds, and those from lower socioeconomic classes (Slopen et al. 2016; Mersky and Janczewski 2018; Liming 2019). The epidemiological data demonstrates the heterogeneity of ACEs and some of the important considerations that need to be accounted for when choosing an appropriate preclinical model. Next, we will review the neural correlates more commonly associated with ACEs. This will allow us to deduct our regions of interests from human research and allow us to compare results both from behavioral and neurobiological domains.

1.1.2 Neuroanatomical correlates

Neural adaptions to adversity may have evolutionarily served as adaptations that enhanced species survival and reproductive success but are now these adaptations to adversity have become toxic and are linked to considerable medical and neuropsychiatric disadvantages (Walker et al. 2017). ACEs result in specific brain alterations, depending on a variety of factors including type and timing of exposure. One consistent finding (> 180 studies) is a reduction in hippocampal volume across populations following ACEs (in adulthood), as well as a reduction in volume of the anterior cingulate and ventromedial and dorsomedial cortices (reviewed in; Teicher et al., 2016). Consistently, ACEs have also been shown to impact the developmental trajectory of fiber tracts, including the corpus callosum, superior longitudinal fasciculus,

uncinate fasciculus, and cingulum bundle, and appear to alter the development of sensory systems that process and convey stressful experiences (Teicher et al. 2016).

Furthermore, findings that are consistently shown in the literature are heightened amygdala (AMY) responses to threatening stimuli (Tottenham et al. 2011; Ji-Ann Lee et al. 2011; McCrory et al. 2011; Bogdan et al. 2012), diminished ventral striatal responding to anticipation of a reward (Takiguchi et al. 2015; Morey et al. 2016). There appears to be decreased connectivity between the medial prefrontal cortex (mPFC) and the AMY (Philip et al. 2013; Morey et al. 2016) and the ventral tegmental area (VTA) (Park et al. 2021). Additionally, increased volume and network centrality of the precuneus is observed in individuals with a history of ACEs (Li et al. 2013; Teicher et al. 2014; Jensen et al. 2015). There is also recent evidence that ACEs are negatively correlated with volume of seven thalamic nuclei (Xie et al. 2022) and of these nuclei the paraventricular nucleus of the thalamus (PVT) is a promising target due its activation in response to arousal, reward and stress (Flagel 2022). These findings provide important insights into how ACES alter neurodevelopment, and provide us with regions of interest for our studies, which include the mPFC, Nucleus accumbens (ACB) AMY, PVT and hippocampus (HIPPO)(Arcego et al. 2023).

It is crucial to underscore the role of adolescence as a pivotal stage in development, a period when such alterations to the brain can have significant and far-reaching implications as discussed. This period is marked by numerous biological, psychological, and social changes, and any adverse experiences or stressors during this time can greatly affect the individual's developmental trajectory and overall well-being. In the context of ACEs, understanding the impact of adversities during adolescence could unlock potential avenues for early interventions. In the following section, we will focus specifically on developmental milestones and critical periods which could be important to focus on.

1.2 Adolescence

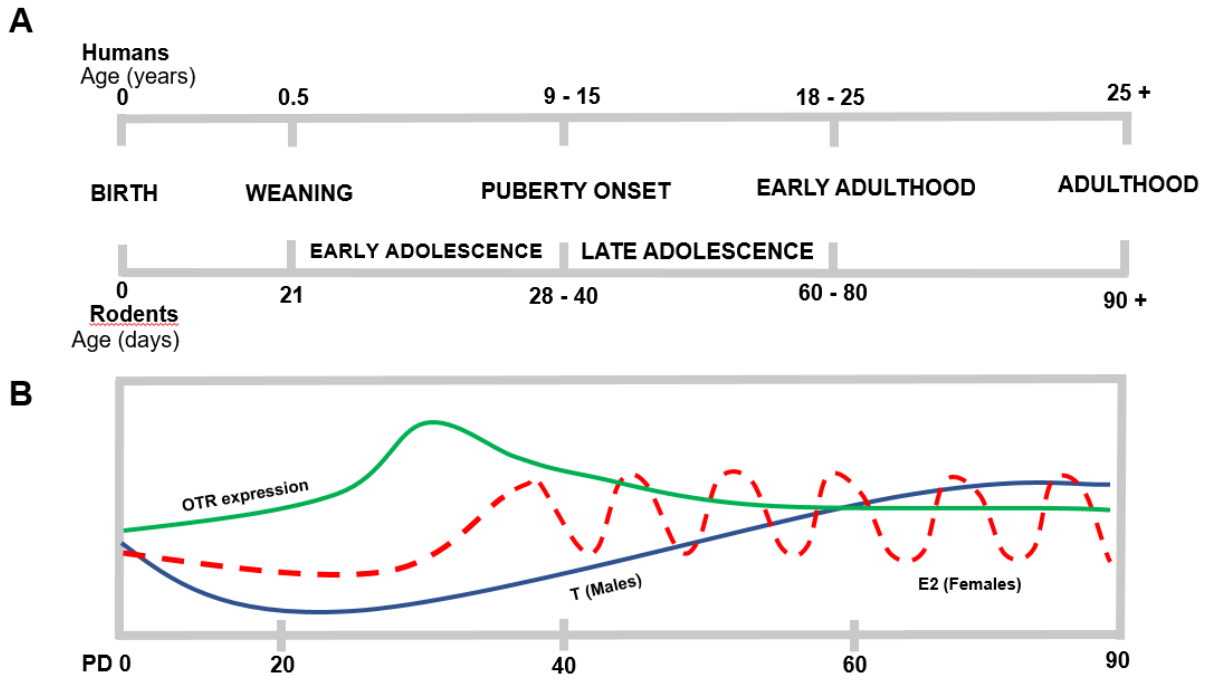


Figure 2: Timing of adolescence and developmental milestones. (A) Comparative developmental trajectory across adolescence in humans and rodents (B) Schematic of adolescent development of oxytocin receptor (OTR) (green), estrogen (red), and testosterone (blue) circuitry. Adapted from Brydges (2016) and Walker et al. (2017)

Adolescence typically refers to a gradual transition from childhood to adulthood, characterized by a sequence of "soft events" instead of being operationalize by specific, discrete transitions (Schneider 2013). Unlike, puberty which is operationalizable through the specific neuroendocrine changes, namely sexual maturation (Spear 2000). Adolescence is a tumultuous developmental period of notable change and development in social, emotional, and cognitive skills. It is widely accepted as a part of development in almost all mammals (Spear 2000). Both clinical and preclinical studies support the idea that adolescents are more sensitive to reward than adults, spend more time with peers, and show higher levels of risk taking, interest in exploration, and sensation seeking (Spear 2000).

These changes are believed to be important for the development of behavioral strategies that prepare for us adulthood (Gopnik et al. 2017). However, there is a flipside. While we undergo significant change in a highly plastic state, we are also significantly more vulnerable to stressors, which can change the developmental trajectory of neurodevelopment and have long-lasting effects on physiology and mental

health, as described previously. This increased vulnerability can be best demonstrated by the fact that over 50% of all neuropsychiatric disorders emerge by the age of 15 (Kessler et al. 2005). Similarly, adolescent rats are more vulnerable to adversity than adults suggesting comparisons may be (Walker et al. 2017).

To study these changes during adolescence, it is necessary to define when rats are adolescents as well as the different stages of adolescence (Schneider 2013). Additionally, we need to consider how well our constructs reflect human development to ensure external validity. Since adolescence is a construct rather than a well-defined developmental period, and there is a large variability between studies and mixed definitions of adolescent periods (reviewed in Burke et al., 2017). However, one-aspect researchers mostly agree on, is that adolescence arbitrarily begins on postnatal day (PD 21) when rodents are artificially weaned (Burke et al. 2017). Weaning on PD 21 is based on several factors. First, on PD 21 rats have developed sufficiently to survive independently. Second, by this age rats begin to demonstrate more peer-related social behavior such as play that can be studied in individuals. Last, weaning before PD 21 has been shown to increase mortality and health issues due to underdevelopment However others have suggested that the adolescent period begins at PD 28 and lasts until PD 42, when growth spurts and more typical adolescent behavioral characteristics appear (Spear 2000).

There is significant variability in how researchers define the various stages of adolescence. Some researchers refer to adolescence in rodents as three distinct periods: early PD 21 – 34, mid PD 34 – 46, and late PD 46 – 59, spanning from PD 21 to 60 (Lukkes et al. 2009b; Burke et al. 2017). Which are proposed to reflect 10 – 13, 14 – 16, and 17 – 21 years in human years (Burke et al. 2017). While other researchers have defined adolescence as early PD 21– 25; mid PD 28 – 32; and late PD 35 – 39 (Zoratto et al. 2018). Some researcher have defined adolescence as two time periods; early PD 30 - 42 and late PD 44 – 56 (Imhof et al. 1993; Lupien et al. 2009). There is a need for researchers to converge on agreed upon stages of adolescence to improve reliability and reproducibility. When are rats are typically seen as reaching adulthood? Most studies seem to refer to PD 60 as rats have achieved sexual maturity as measured by sperm motility (Chappell et al. 2013; McCormick et al. 2013; Schneider et al. 2014; Marcolin et al. 2019; Provensi et al. 2019a). However, some researchers

postulate males first attain sexual maturity at PD 70, as defined by testis morphology (Ojeda and Skinner 2006). There are simply no objective measures of adulthood in rats and therefore scientists have mostly decided to equate sexual maturity to adulthood. How well the proposed stages reflect human adolescent development remains debated (McCormick et al. 2017), as sexual maturity in humans does not equate to adulthood. Hence, this construct does not compare well with this aspect of the human condition. These studies highlight some of the challenges researchers have in studying adolescence and its associated stages which remains poorly defined and agreed upon in the field. More operationalizable frameworks of adolescence in rats is required, that better reflect aspects of human adolescence.

1.2.1 Operationalizing adolescence

In the interest of generating robust and reproducible scientific data, we emphasize the need to operationalize and quantify these transitional periods of adolescence using clearly identifiable developmental milestones such as puberty. Such an approach ensures that our research is grounded in measurable physiological phenomena, thereby enhancing the specificity and reproducibility of our findings. Hence, we propose splitting adolescence into two periods: one before and the other after the onset puberty. The onset of puberty is physically defined as balano-preputial separation in males (around PD 38 – 42, as observed in our Wistar (WIST) rats and by vaginal opening in females (around PD 33 – 39, as observed in our WIST rats (Ojeda & Skinner, 2006). The onset of puberty differs between strains and needs to be considered between strains when defining these periods (Rivest 1991; Campion et al. 2013). For WIST rats (which we used in our studies), we propose studying early adolescence between PD 21 and 42, which captures the time period between weaning and around the onset of puberty, when most adolescent behavioral characteristics have emerged (Spear 2000). And a post pubertal or late adolescent period between PD 42 – 63, a period after puberty onset and what is typically considered adulthood. A period when neural pruning is still in effect however adolescent characteristics progressively begin to fade (Vanderschuren et al. 2016).

Our proposed framework encapsulates the period around puberty onset for both sexes which incorporates a time dimension (early vs late), that is better operationalizable and therefore quantifiable. The framework can also be adapted to the threat and

deprivation framework which will be discussed later. Last, the onset of puberty is something that operationalizable in humans, making reverse translational comparisons possible. The early adolescent period covers a period of adolescence when most neuropsychiatric disorders emerge in humans (Kessler et al. 2005) and is also thought to be a period when the brain is more vulnerable to adversity(Burke et al. 2017). While the later adolescent period captures a period of cortical reorganization and heightened reward sensitivity (Friemel et al. 2010; Juraska and Willing 2017), which is also reflected in human development (Smith et al., 2012; Steinberg et al., 2009).

Preclinical research serves as a valuable tool in this regard, enabling us to study the neurobiological mechanisms and outcomes of AAs in a controlled setting. Aligning our preclinical models with human development is crucial, and our proposed framework centres around an operationalizable and quantifiable developmental milestone in humans offering us a more reliable and reverse translational approach to studying adolescence also in rats.

1.2.2 Sex differences

It is essential to acknowledge the inherent sex differences in the onset of puberty in rats and humans alike (Romeo 2010). This necessitates the comparison of distinct developmental stages between male and females. However, the problem of comparison cannot be addressed solely by aligning the ages of female and male rats to synchronize pubertal onset within one sex, as additional developmental milestones exhibit divergent trajectories between the sexes (Guily et al. 2022). In certain instances, female rats attain adult-typical levels earlier, while in other cases, they reach these levels later in comparison to their male counterparts (Beltz et al. 2019). Examples include a delayed qualitative change in performance on the elevated plus maze for females (Imhof et al. 1993) as well as differences in oxytocin receptor (OTR) binding levels going in both directions (Smith et al., 2017). These are just two examples of qualitative sex differences, where the functional form of the relationship varies by sex and age.

Sex differences have been historically overlooked and males have been the preferred sex in studies (Zucker and Beery 2010). This was largely because female rodents' behavior was seen as more variable due to their oestrus cycle; however, this hypothesis has since been disproven (Beery, 2018). To study sex differences in a valid

manner there is a need to recognize that these differences exist on various levels. We have already discussed qualitative difference, but there are also, quantitative sex differences which refer to both sexes demonstrating similar patterns of traits (i.e., alcohol intake), however, to a different extent, (i.e. females consuming more alcohol than males) (Elisa et al. 2018). The last sex difference to consider are latent differences which refer to differences in underlying mechanisms produce a trait (i.e., how specific neuropsychiatric disorders emerge more in women following ACEs) (Hakamata et al. 2022).

Sex differences are present in social behavior and stress response during adolescence (Adriani and Laviola 2004; Tanaka et al. 2019; Grinevich and Neumann 2021). Several brain regions involved in social behavior and the stress response are high in androgen and estrogen receptors where marked quantitative sex differences appear during adolescence (Figure 2) (Schulz and Sisk 2016). Some of these regions include the medial AMY (MeA), bed nucleus of the stria terminalis, lateral septum (LS), paraventricular nucleus of the hypothalamus (PVN) which are also high in OT and vasopressin (AVP) receptors whose roles have been well established in social behaviors (Oettl et al. 2016; Netser et al. 2020). Evidence shows androgen receptors have direct involvement in OT gene expression and androgen receptors have been shown to be altered by AAs in a sex-specific manner (Sinclair et al. 2014). Suggesting AA alters androgenic profiles in regions coupled with social behavior and stress, which in turn drive OT gene expression and potentially OT related behaviors.

OTR binding in the PVN and MeA and adolescent females higher OTR binding in the anterior insula and LS (Dumais and Veenema 2016). Other regions such as the HIPPO, mPFC, ACB contribute significantly to social behaviors, and interestingly, these brain regions also exhibit significant sex-specific changes across adolescence (for review see Premachandran et al. 2020; McCormick 2021).

Sex differences in social behavior become more apparent during adolescence as described earlier. In particular, males exhibit more frequent social play behavior than females (Pellis et al. 1997). Males display a higher preference for nose pokes leading to a social stimulus than females at P35-P41, suggesting a greater reward value of social interaction in males at least in the social interaction test (Schatz et al. 2019).

Additionally, adolescent males demonstrate a stronger social conditioned place preference, than females and adult males (Douglas et al. 2004; Weiss et al. 2015). Last, play-deprived adolescent males show a greater preference for play behaviors than do females (Pellis & Pellis, 2017).

Consequently, the observed sex differences in sensitivity to social rewards may have a profound impact on the neural circuitry involved in reward processing as well as on the development of sex-specific reward-associated behaviors in adult rodents. However, it should be noted that the degree to which these differences are apparent is also contingent on other factors such as test conditions, further complicating the exploration of sex differences in social behavior (Siviy and Panksepp 2011; Trezza et al. 2011; Himmler et al. 2013, 2014; Northcutt and Nwankwo 2018).

Sex difference in stress response (physical stressors) appear during adolescence, with female rats demonstrating higher HPA response to stressors, than males (Mifsud and Reul 2018). This is thought to be due the opposing role testosterone and estradiol, with the former attenuating and the later enhancing the HPA response (Bartlett et al. 2019). Furthermore, significant sex differences have been observed in the maturation of the HPA-axis during adolescence, with male WIST rats reaching a peak in their basal corticosterone level around PD 40, whereas female WIST rats first reach this peak by PD 60 (Rincón-Cortés et al. 2019). This difference in maturation likely plays a part in the divergent effects of stressor on the developing brain. Together, these findings underscore some of the intricate interplay of genes, hormones, and environmental factors in shaping social behavior and stress response in a sex-dependent manner. Using these findings on adolescent and sex differences in adolescence as a backdrop, we can now explore the theoretical landscape of ACEs and how these can be applied to preclinical models.

1.3 Theoretical framework

As our understanding of ACEs and their neurobiological consequences grows, we need to identify conceptual frameworks for organizing knowledge, guiding research, identifying knowledge gaps and limitations, facilitating comparisons, and promoting theory development. One of the earliest theories proposed was the dose-response model (Gilbert 1997), which posited that developmental changes depend on the level

of exposure and is the most commonly referred to model in the literature. However, this theory does not hold under all circumstances, as individuals exposed to high doses of ACEs do not always display negative consequences. Highlighting resilience as a variable that needs to be considered. The cumulative risk model (Evans et al. 2013), posits that the number of unique adversities affects experiential outcomes. While other frameworks emphasize the role of individual traits (Belsky and Pluess 2009), such as genotype, temperament, or biological sensitivity to context; however, categorization nonetheless shares its own pitfalls. It is improbable that one model alone can sufficiently explain the extensive connections between ACE exposure and neurodevelopment. However, one theory that has recently gained prominence is the dimensional approach.

Sheridan and McLaughlin (2014) proposed a framework that characterizes the underlying dimensions of environmental experiences associated with ACEs in order to understand their distinct effects on neural development and subsequent health outcomes. In short, the proposed framework divides ACEs across a continuum of deprivation and threat experiences (Figure 3). The authors suggest that deprivation and threat related ACEs represent seperately measurable (although they can co-occur) neurobiological effects (Sheridan and McLaughlin 2014). Threat-related ACEs encompass detrimental experiences, including physical abuse and domestic violence. These experiences are linked to dysregulated neural circuits involved in emotional responses, augmented physiological reactivity to stress (Weissman et al. 2022), and accelerated biological aging (Sumner et al. 2019). Neuronal changes in response to threat have been observed in the structure, function, and connectivity of the HIPPO, AMY, and mPFC (Teicher et al. 2016). Threat-related ACEs have also been found to alter hippocampal morphology and functionality (Ettcheto et al. 2017), and are characterized by increased dendritic spine density and arborization (Fox et al. 2020), as well as impaired performance in learning and memory tasks that rely on the hippocampus (Hawkins et al. 2021; Lombera et al. 2021). Conversely, deprivation-related ACEs are typified by the absence of cognitive stimulation and social inputs, such as neglect and parental unavailability (Mclaughlin et al. 2014). These deficiencies can impede the neural correlates responsible for executive functioning and social skills (Berzenski 2019; Raby et al. 2019; Lund et al. 2020).

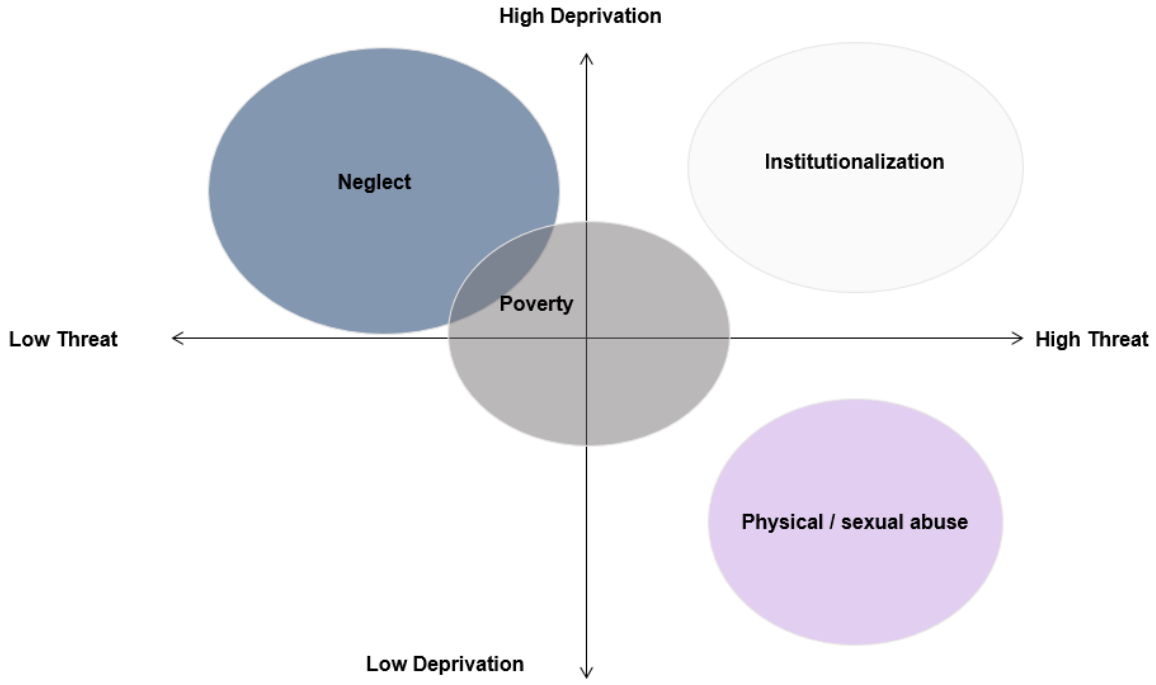


Figure 3: McLaughlin and colleagues proposed the dimensional framework of threat and deprivation for highly prevalent adverse childhood experiences (ACEs). These frameworks suggest that threat and deprivation differentially affect neurodevelopment. The authors propose that ACEs can co-occur, but can be measured separately. Adapted from the study by McLaughlin et al. (2014).

The proposed threat and deprivation framework effectively reverse translates into preclinical research, allowing us to examine the dimensions of ACEs that are typically unique to humans (Figure 4). For example the high deprivation, low threat dimension can be studied using social isolation (SI) models in rodents (Figure 4). While more moderate dimensions of deprivation and threat can be studied with the peer rejection (PR) or social instability stress (SIS) models across adolescence (Figure 4). To study the high threat and low deprivation dimensions, resident intruder or maternal separation (MS) models can be applied. Next, we pivot to a potential neurobiological targets, namely the oxytocin system, which plays an important role in social behavior, stress regulation and resilience (Grinevich and Ludwig 2021; Grinevich and Neumann 2021). Although the immediate effects of adversity on the oxytocin system have been studied the long term effects remain understudied.

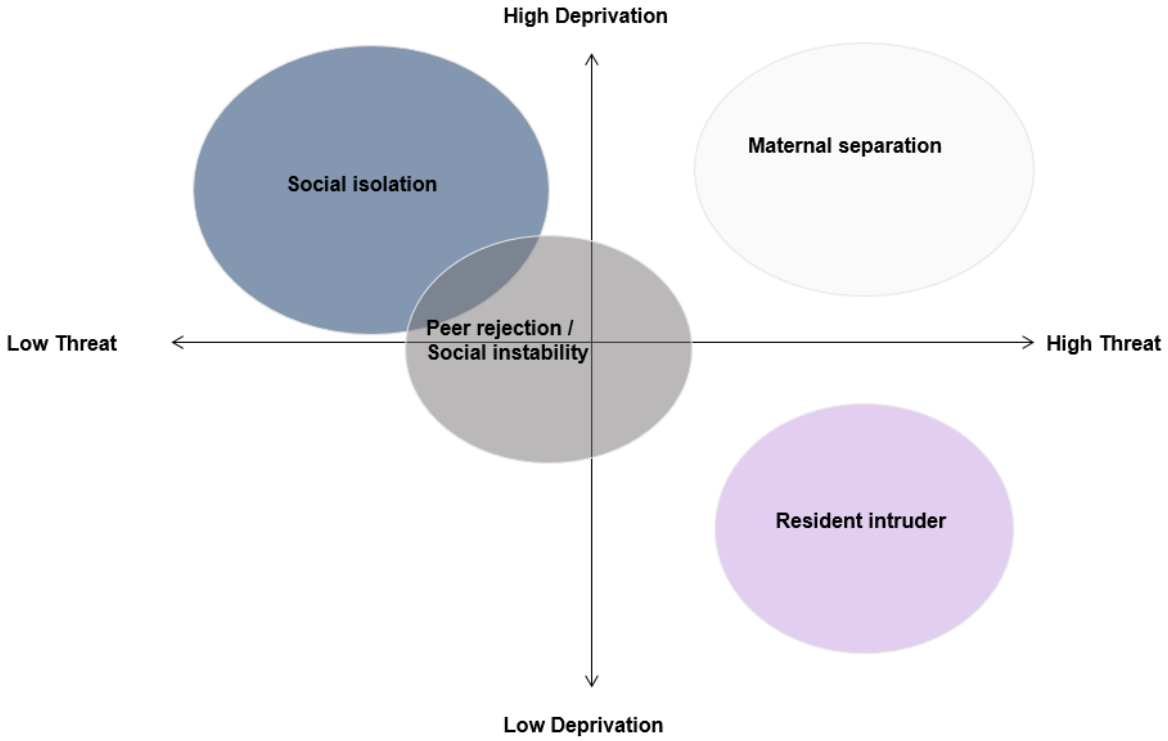


Figure 4: Alignment of reverse translation of the dimensional framework of threat and deprivation. We built on the proposed framework of McLaughlin et al. using a reverse translational approach for commonly used adversity paradigms in preclinical research. Adapted from the study by McLaughlin et al. (2014).

1.4 The oxytocin system

Oxytocin is best known as a prosocial neuropeptide that is involved in maternal behaviors and mate affiliation. In laymen's terms, is often referred to as the "love" hormone, however, a growing body of literature demonstrates that OT can have antagonistic effects on social behavior (Yong 2005; Bartz et al. 2011; Bethlehem et al. 2014; Harari-dahan and Bernstein 2014). These include increased aggression and fear in rodents (Knobloch et al. 2012; Anpilov et al. 2020). Hereafter, the OT system refers to alterations in OT and OTR in both the central and peripheral regions.

1.4.1 Synthesis, pathways, circuitry, and neurotransmission

OT is a 9 amino acid peptide hormone synthesized in three distinct hypothalamic regions: the PVN, supraoptic, and accessory nuclei of the hypothalamus (Swanson and Sawchenko 1983; Grinevich and Ludwig 2021). Classically, two populations of OT neurons have been classified because they differ in size, anatomical location, function,

projection sites, mode of release, and electrophysiological properties (Althammer and Grinevich 2018). Larger magnocellular OT neurons are found bilaterally in all the three nuclei, and these neuronal axons project through the pituitary to the neurohypophysis and are then secreted into the periphery. Only 10% of the magnocellular population is classified as OT neurons (Hasan et al. 2019). Magnocellular OT neurons in the PVN and SON locally release OT from dendrites (Berendzen et al. 2023). Parvocellular OT cells are smaller both in size and population and are mostly found bilaterally in the PVN (Althammer and Grinevich 2018). However, it has also been found to project to the brainstem (Eliava et al. 2016) and periaqueductal grey (Iwasaki et al. 2023). Populations of both magnocellular and parvocellular neurons form the OT system and provide the main mode of central OT release.

A conceptually groundbreaking study showed that OT neurons have projections to more than 50 brain regions (Knobloch et al. 2012) in addition to their projections to the neurohypophysis. Thus, OT neurons from the PVN and SON project to a large number of brain regions, including the mPFC, ACB, AMY, PVT, VTA, and several other areas (reviewed in Knobloch et al., 2012). Figure 5 summarizes the major projection sites from PVN and SON OT neurons (Grinevich and Neumann 2021). The schematic of OT projections indicates where OT is released, which is far more studied than how it is released. Current evidence points to a combination of synaptic and non-synaptic transmission through presynaptic terminals, axonal en passant, dendritic release, and somatic release (Grinevich and Neumann 2021).

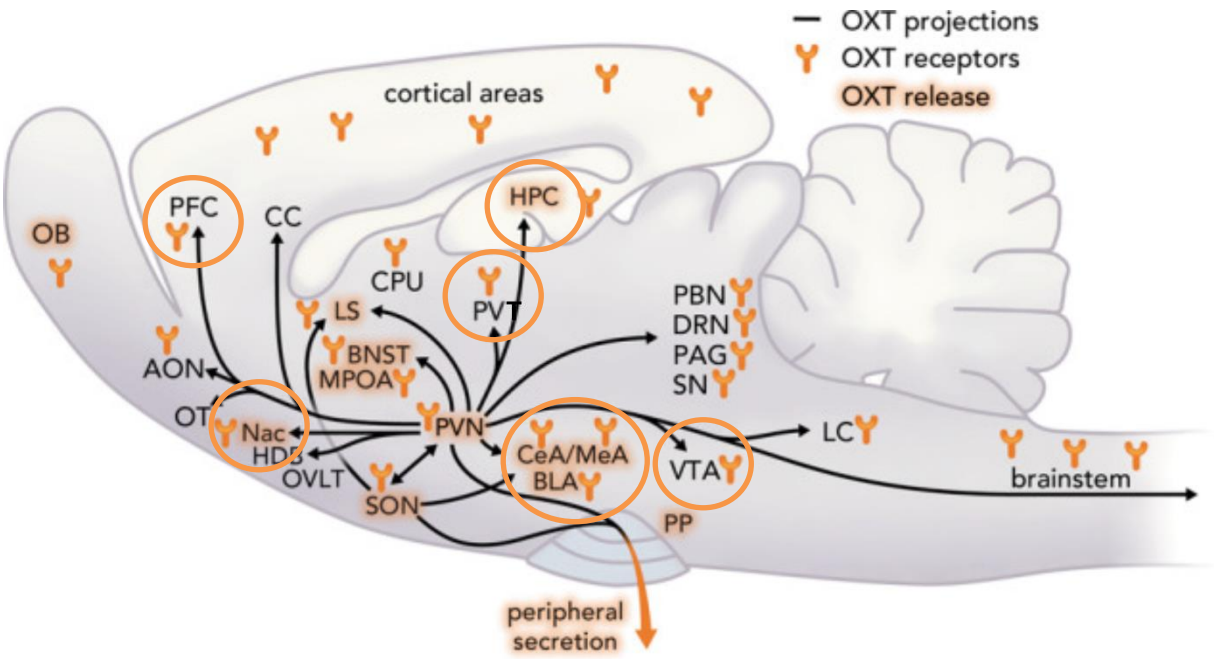


Figure 5: Sagittal schematic of OT neuronal projections, OTRs, and OT release sites. A summary of the related brain regions and projections is discussed in this section, as well as the regions of interest in our study (encircled): ON anterior olfactory nucleus, OB olfactory bulb, OT olfactory tubercle, ACB nucleus accumbens, OVLT organum vasculosum laminae terminalis, SON supraoptic nucleus, PVN paraventricular nucleus of the hypothalamus, PP posterior pituitary, PFC prefrontal cortex, CC cingulate cortex, MPOA medial preoptic area, BNST bed nucleus of the stria terminalis, LS lateral S septum, CPu caudate putamen, PVT paraventricular nucleus of the thalamus, CeA central amygdala, MeA medial amygdala, BLA basolateral amygdala, VTA ventral tegmental area, LC locus coeruleus, PBN parabrachial nucleus, DRN dorsal raphe nucleus, PAG periaqueductal gray, SN substantia nigra, HIPPO hippocampus, and HDB nucleus of the horizontal limb of the diagonal band. Adapted from Grinevich and Neumann, 2021.

1.4.2 Development of the oxytocin system

In rats, oxytocin has also been prenatally detected on E14 in the brain stem (Tribollet et al. 1989). Receptor autoradiography studies have shown that OT receptor distributions change markedly through postnatal development, but remain topographically similar to the adult brain (Tribollet et al. 1989). OTR binding peaks around PD 21 in the Acb, Anterior olfactory nucleus, caudate, and ventral subiculum. In the dorsal subiculum, cingulate cortex, LS, and anteroventral thalamus showed a peak in OT binding around PD 10. Finally, the ventromedial nucleus of the

hypothalamus (VMH) binding continued to increase throughout adolescence and peaked first at PD 60 (Shapiro and Insel 1989). These findings have been replicated more recently, where higher OTR binding density was observed in 15 brain regions in juvenile (PD35) than in adult (PD 84) male and female rats (Smith et al., 2016). Most of the sex differences in OTR binding were present during the juvenile period and occurred in regions with higher density OTR binding in adulthood (Smith et al., 2017). As these brain regions are sensitive to gonadal hormones, sex-dependent differences could induce puberty-related increases in gonadal hormone levels. These regions include the VMH, BNST, AMY, anterior insula , and perirhinal cortex (Smith et al., 2017).

A recent study in mice quantified postnatal OTR development using whole-brain imaging techniques combined with two-photon tomography and machine learning to detect OTR labelled cells (Newmaster et al. 2020). These results support previous receptor autoradiography, with most subcortical regions of OTR expression peaking around PD 21, with a subsequent reduction across adolescence into adulthood (Newmaster et al. 2020). Furthermore, sexually dimorphic results were found in the ventral premammillary nucleus in males, which showed higher levels of OTR expression throughout adolescence (PD 14 – 56) than females; in the anteroventral periventricular nucleus near the medial preoptic area, females showed higher levels of OTR expression compared to males (Newmaster et al. 2020). Additionally, the temporal distribution of the OTR neurons demonstrated region-specific differences. For example, in the PVT OT labelled cells increased and peaked at PD28 and then decreased until PD56 (Newmaster et al. 2020). While, in the ventrolateral part of the VMH OT labelled cells progressively increased between PD 7 and 56 (Newmaster et al. 2020). OT receptor mapping in mice mostly overlaps with previous rat studies and suggests that OTR expression peaks around PD21 and reduces across adolescence into adulthood through neural pruning, with the expectation of the ventromedial nucleus of the hypothalamus and the anterior ventral anteroventral periventricular nucleus, which progressively increased across adolescence and peaked in early adulthood (PD56).

OTRs seem to continue to decline with age (Arsenijevic et al. 1995), which is driven by age-related increases in CD38 (Jin et al. 2007; Camacho-Pereira et al. 2016). Cluster

of differentiation 38 (CD38) is a transmembrane glycoprotein with ADP-ribosyl cyclase activity that is directly related to OT release (Jin et al. 2007) and increases in CD38 have been shown to modulate not only oxytocin but also social behaviour (Jin et al. 2007). Overall, these studies highlight different regions of the brain that may be more sensitive to stress and, hence, alterations to the OT system across adolescence. Considering that OTR levels reach peak levels around PD 21, any adversity or stress after this point would most probably impact pruning of the OT system, and the effects of this modulation could potentially alter behavior.

1.4.3 Oxytocin receptor and behavior

The prevailing hypothesis on the influence of OT on social behavior posits that it selectively enhances the salience of socially relevant stimuli in brain regions with a high density of OTR-expressing neurons (Shamay-Tsoory and Abu-Akel 2016; Marlin and Froemke 2017). This hypothesis primarily stems from findings of studies on auditory and olfactory regions, where OT modulation alters the balance of excitation and inhibition within sensory circuits, thereby increasing the signal-to-noise ratio in favor of social stimuli and filtering out less relevant information (Marlin et al. 2015; Linster and Kelsch 2019). Although our understanding of how OT affects sensory systems has advanced, the precise mechanisms governing targeted axonal release of OT in brain regions associated with social relevance remain to be elucidated.

OTRs play a fascinating and multifaceted role in behavioral modulation, as evidenced by a rich body of scientific research. The diverse implications of OTR distribution are found across various regions of the brain, each corresponding with distinct behaviors and responses. For example, in the mPFC OTRs have been found to be negatively correlated with anxiety-like and maternal behaviors (Sabihi et al. 2014b, a). Similarly, in the LS showcases a unique relationship where OTRs negatively correlated with open-field locomotor activity and anxiety-like behaviors (Curley et al. 2012; Huang et al. 2021)

Meanwhile, MeA exhibits a positive correlation between OTR density and social interest in male and female rats (Dumais et al. 2016), highlighting their influence on social behaviors. In the VMH, OTR binding is positively correlated with sexual behavior (lordosis) while being negatively correlated with anxiety (Bale et al. 2001).

Moreover, in the PVT OTRs have been observed to regulate feeding motivation and fear memories (Penzo et al. 2015; Ye et al. 2022). Furthering our understanding of the OTRs role in social cognition, studies have shown that OTRs on glutamatergic neurons in the mPFC control social recognition (Tan et al. 2019) while hippocampal OTR activation is necessary for the discrimination of social stimuli (Raam et al. 2017).

Together, these findings demonstrate the heterogeneity of OTRs in modulating behaviors such as anxiety-like and maternal behaviors, social recognition, locomotor activity, social interest, sexual behaviors, feeding motivation, and discrimination of social stimuli. Moreover, it is important to note that various stressors and adversities can result in acute and persistent alterations in the oxytocin system, underlining the intricate dynamics between external stimuli and the neuroendocrine landscape.

1.4.4 Effects of adolescent adversity on the oxytocin system

There is abundant evidence that the OT system is modulated in response to early adversity (Veenema 2012; Burke et al. 2017; Jurek and Neumann 2018). The immediate effects of adolescent adversity on the OT system have been studied in more detail than their long-term effects. One model in which the OT system has been studied in detail is the MS paradigm. This is a widely used early adversity paradigm for assessing juvenile adversity and has been shown to modulate the OT system at both anatomical and functional levels (Lukas et al. 2010; Marlin et al. 2015).

Maternally separated pups are typically removed from their mother and placed in isolation from the dam for a certain period of time, typically 3 -6 hr/per/day starting any time after birth and lasting as long as PD21, when the rat pups can be weaned (Veenema et al. 2006).

Indeed, intriguing shifts in OT expression have been observed following maternal separation. Some studies have observed sex-specific decreases in OT expression in the PVN of adult females with no alterations in males (Veenema et al. 2007). While other researchers observed generalized decreases in OT expression in the PVN around puberty, following MS (Tsuda et al. 2011). If we turn to OTR expression, binding levels differ between regions. For example in the ventromedial hypothalamus and

medial preoptic area OTR binding increase while in the LS and caudate putamen it decreased (Lukas et al., 2010; Lukas et al., 2011). The decrease in the LS was associated with impaired social recognition (Lukas et al., 2010; Lukas et al., 2011).

Adding another layer of complexity, a recent study measured OTR binding in dams following pup-separation. Interestingly, the dams OTR binding levels were also higher in the medial preoptic area, corresponding to levels in maternally separated pups (Demarchi et al. 2023). All measurements were collected during adolescence and studies are lacking on long-term effects of MS on OT system.

Other adversity paradigms that have investigated adversity across adolescence have also found alterations in the OT system immediately following the stress procedures. Again, the long-term effects of AAs remain understudied in comparison to studies on the immediate effects of AAs. Here we describe the immediate effects of AAs on the OT system in rats.

Following the SIS paradigm (see Methods), OTR binding was increased in the dorsal LS and ACB, behaviorally this led to impairments in social interactions and social recognition memory in male rats (Hodges et al. 2017). In a separate study, SIS rats investigating the effects of SIS on OTR binding in the PVN, the researcher observed increased OTR binding but lower OT immunoreactivity than control males (Hodges et al. 2019). This increase in receptor binding, but a reduction in immunoreactivity, could suggest a compensatory mechanism of the OT system in response to reduced OT release following SIS. Increased receptor binding may be the result of an increase in the sensitivity of the OTRs to OT availability. On the other hand, the lower immunoreactivity of OT in the same region suggests that neurons produce and release less OT. This pattern of findings may indicate that the brain is attempting to compensate for a deficit in OT by increasing the number of available receptors while also reducing its production and release to better match the reduced availability of the neurotransmitter. Again, no studies, to our knowledge have been conducted on the long-term effects of SIS on OT systems.

A significant amount of data has been gathered on post-weaning social isolation (PWSI), and recently, a meta-analysis of 12 studies was published on the impact of

PWSI on the OT system (Krimberg et al. 2022). The main findings indicate that social isolation modulates OTR expression levels, but not OT levels. OT administration also alleviated the behavioral effects of PWSI. PWSI reduced OT cell activation in the PVN and SON in female rats (Tanaka et al., 2010; Tanaka et al., 2019). Furthermore, OT messenger ribonucleic acid (mRNA) levels were increased in the PVN and ACB of both male and female rats (Oliveira et al. 2019). This increase in OT mRNA levels indicates that PWSI influences OT synthesis; however, mixed results on the effect of PWSI on basal OT plasma make interpretation difficult. Only two studies have measured OT plasma levels following PWSI. One study observed decreases in both male and female rats immediately after PWSI (Harvey et al. 2019), whereas another study observed only an increase in male OT plasma immediately after PWSI (Neal et al. 2018).

The inconsistent findings could be explained by methodological differences between strains, length of social isolation, or time of sample collection (Butler-struben et al. 2022). Nonetheless, the existing literature on juvenile and AA paradigms, such as maternal separation, social instability stress, and PWSI, demonstrate that the OT system is malleable to stress (Oreland et al. 2010; Oliveira et al. 2019; Hodges et al. 2019). The long-term effects of adolescent adversities on the OT system remain largely unexplored. To our knowledge, no studies has investigated the long-term effects of adolescent social isolation on the OT system in rats.

1.5 Paraventricular nucleus of the thalamus

The paraventricular nucleus of the thalamus (PVT) is a midline thalamic structure that has garnered significant interest in the last decade due to its involvement in a wide array of behavioral processes and linked neuropsychiatric disorder. These behaviors include processing of emotionally salient experiences, reward, motivation, anxiety, fear response, top-down control and importantly it has been implicated in modulating the stress response (Barson et al. 2020). The PVT can be considered a relay node or control node, just like other thalamic nuclei, that both innervates and is innervated by many regions involved in the aforementioned behavioral processes (Noori et al. 2017).

The PVT can be subdivided both anatomically and functionally into the anterior (PVA) and posterior (PVP) parts. The PVA is more involved in arousal related behaviors such as the sleep wake cycle. While the PVP is more involved in stress response, anxiety, fear responses and cue-reward processing (Barson et al. 2020). More recently, the

PVT was proposed as a relay node for early life adversity, due to its role in processing emotionally salient experiences (Kooiker et al. 2021). For instance, a mouse study has shown that early life deprivation decreased cellular FBJ Murine Osteosarcoma viral oncogene homolog (c-Fos) activation of cells in the PVT when measured at PD 9 (Fenoglio et al. 2006). While electric shocks and restraint stress both produced a fast and long lasting reduction in the amplitude of inhibitory currents on D2R expressing neurons in the PVT in adult mice (Beas et al. 2018). Together these two studies suggest the PVT is acutely affected by stress both during the juvenile and adult time points. ASI specifically seems have to long lasting effects on the PVT, as it was shown to increase vasopressin fiber density in adulthood, when measured on PD 75-77 (Kinley et al. 2021). However, it should be noted that the authors' claim that the PVT is void of OT fiber is not accurate. Our collaborators have demonstrated that the PVT is rich in OTRs and receives significant innervation of OT fibers from the PVN (Figure 6, unpublished data). Long-term alterations following ASI have also been observed in mice. Where, ASI reduced sociability in adulthood and induced a smaller calcium response in the PVP than in controls (CTLs) (Yamamuro et al. 2020). Chemogenetic and optogenetic suppression of mPFC → PVT activity in group housed mice reduced sociability in adulthood to similar levels observed after ASI. Demonstrating a causal link between ASI and PVT modulation. These results indicate that ASI is effective in modulating the PVT persistently, whether the OT system would be impacted similarly remains unknown.

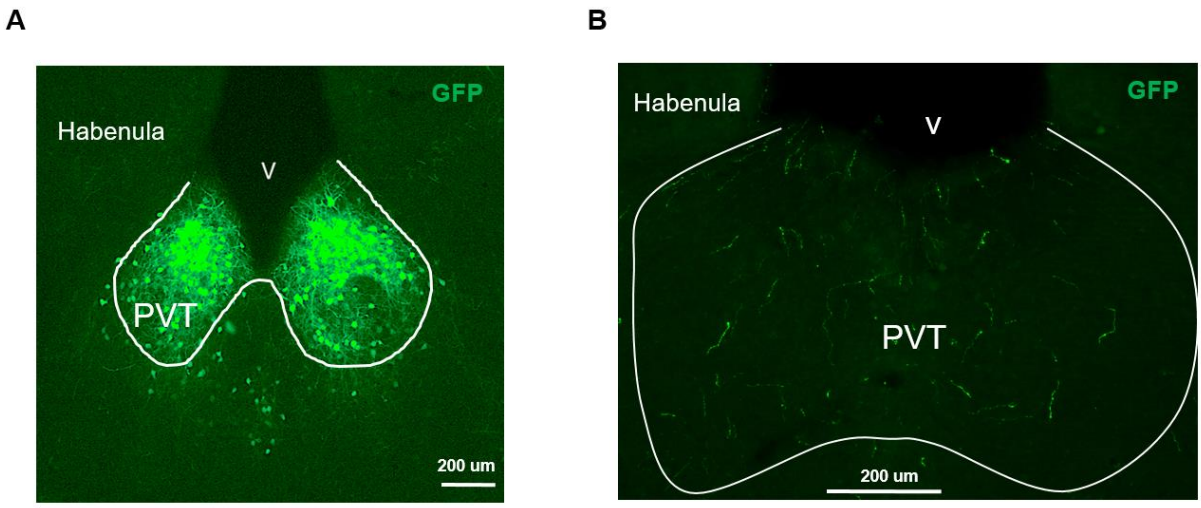


Figure 6: Confirmed PVN → PVT OT axon terminals and OTR expression in the PVT. (A) Representative immunostained image of GFP (green) localization in OTR

containing cells in the PVT. **(B)** Representative immunostained image of GFP (green) localization in OT fibers in the PVT derived from the PVN. Unpublished data, used with permission from Arthur Lefevre.

1.6 Preclinical models of adolescent adversity

Next, we shift our focus towards the profound impact of environmental factors of ASI, SIS, and PR. First, we focus on these AAs in rats due to their high prevalence in humans. Second, a significant amount of human studies have identified behavioral outcomes and brain regions of interest allowing us to employ a reverse translational approach to assess the validity of these models.

ASI represents an extreme form of social stress where the individual is deprived of any social interaction and somatic contact. Both humans and rodents are inherently social species, and the enforced absence of social contact can trigger significant physiological and psychological stress responses with long-term consequences, offering an impactful model of severe adversity (Le Mare and Audet 2006; Lukkes et al. 2009b; Sheridan et al. 2012; Lopizzo et al. 2021).

SIS is characterized by erratic changes in social environments and relationships during, which mirrors the unpredictability many adolescents face in their social environments, from fluctuating friendship groups to familial instability (i.e. parental separation) (Fowler et al. 2015; Baker et al. 2019; Marçal et al. 2023). This form of stress provides insights into the effects of unpredictability and change within social contexts.

Finally, PR is an unfortunately common experience in adolescence, involves exclusion or ostracism by an individual's social group. The detrimental impact of PR on adolescent mental health is well documented in humans (Eisenberger et al. 2003; Sebastian et al. 2010). Through the investigation of these distinct but interrelated AAs, we aim to gain a nuanced understanding of how various forms of AA can shape adult behavior and alter underlying neurobiological mechanisms, namely OT.

1.6.1 Adolescent social isolation

In the seminal publication almost 60 years ago Wiesel & Hubel (1965) demonstrated how early sensory deprivation influenced neural development. The mechanism that drives these changes is the initial pruning of the over-produced synaptic connections in the absence of stimuli (Huttenlocher et al. 1982). It has been proposed that similar mechanisms of synaptic prunning applies to animals that are socially deprived during adolescence, shaping their brain (Mclaughlin et al. 2014). The hypothesis postulates that without cognitive or social enrichment, the organism adapts to a low-complexity environment and is therefore less competent to handle complex social and cognitive tasks later in life. For example, social deprivation during the adolescent period, when social play behavior is heightened, would adapt the organism to low complex social situations due to the lack of social stimulation. This has been observed in children who have been institutionalized early in development in orphanges. These children show aberrant attachment styles, attachment disorders, increased emotional reactivity (Bos et al. 2011), and demonstrate significant social difficulties (Jones et al. 2022). Similar results have been observed in socially isolated rats during adolescence. Where ASI rats are less social in adulthood and demonstrate antisocial attributes (aggression) (Van Den Berg et al. 1999).

ASI is often studied in rats using the post-weaning social isolation paradigm. In short, rats are individually housed in their home cage with water and food but lacked somatosensory contact, but still had olfactory, auditory, and visual stimulation from the other rats in the colony room. This model has good face validity, as isolated humans typically have visual, olfactory and auditory stimulation from their surroundings (smart phones, social media) but often lack social touch or contact (Schulze et al. 2022). Additional similarity exists in modern society where adolescents have less direct social contact then before due to more smartphones and social media usage, which has been correlated with increased lonliness (Gao et al. 2016), and negative impact on cognitive control and socioemotional functioning (Abi-Jaoude et al. 2020). Features also observed after ASI in rats (Shao et al. 2013; Li et al. 2016). These societal changes in conjunction with COVID-19 related social isolation make elucidating the consequnces more important than ever.

Typically, the PWSI procedure begins directly after weaning between PD 21 – 28, which coincides with the emergence of social play behaviors and peaks around between PD 32 – 40 (Pellis 1990). During this developmental time social play is highly pleasurable and rewarding experience (Trezza et al., 2011; Vanderschuren, 2010) which displays predatory, aggressive and sexual characteristics accompanied by vocal, facial and physical signals that indicate the playful nature of the behavior. Rat take turns in attacking and defending and it has been postulated that these play behaviors are important in developiong social, emotional, cognitive and motor skills (Nijhof et al. 2018). Therefore, social deprivation during this period is thought to perturb several domains of development. The length of isolation varies between studies and majority of the studies rats are tested immediately after the isolation procedure which is typically for PWSI studies(reviewed in Fone & Porkess, 2008; Lukkes, 2009).

Most ASI paradigms also begin between PD 21 – 30 and last between three to eight weeks (reviewed in Walker et al., 2019). However, it is important to consider the initiation of isolation as there may be critial differences in development. For instance, initiating the isolation procedure when play behavior emerges may have a different impact on development than initiating isolation when play behavior is almost at its peak. A study found an increase in social interaction is observed in male WIST rats but not in their female counterparts, with this effect being more pronounced when isolation begins on PND 21 rather than PND 30.

Additionally, sex and strain differences in pubertal timing (Rivest 1991; Juraska and Willing 2017) need to be accounted for when comparing the isolation procedures, as different neurodevelopmental stages may be impacted across strains and sexes, as has previous been demonstrated (Provensi et al. 2019a; Begni et al. 2020b).

1.6.1.1 Behavioral findings

The long-term effects of adolescent social isolation (ASI) have been assessed through behavioral batteries on locomotor, anxiety, social and memory behaviors, and thermal pain after long-term ASI. Herein, ASI refers to an isolation period between PD 21 and 60 that lasts longer than two weeks, after which the rats are resocialized and allowed to recopperate until testing is performed in adulthood. Only studies on the long-term effects of ASI in outbred rats are described here. For a review of the immediate effects of post-weaning social isolation, see Fone & Porkess (2008) and Lukkes (2009).

The initial study linking ASI with enduring behavioral changes showed that rats isolated from postnatal day (PD) 25 to 45 exhibited a long-term decrease in novel object contact in adulthood (PD 90 and 180 (Einon and Morgan 1977). This finding was followed by more studies exploring ASI's impact on adult behavior in the open field test (OFT), a widely recognized test for evaluating exploratory and locomotor behaviors (Walsh and Cummins 1976).

The effects of ASI vary according to strain, ASI duration and time of testing. In the Long Evans (LE) rat strain the results are mixed. For instance, one study demonstrated increased locomotor activity in adulthood following ASI from PD 21 to 51 (Centre et al. 1991). In contrast, two other studies on LE rats showed no effects of ASI on locomotor activity (Joshi et al. 2017; Kinley et al. 2021). Interestingly, ASI increased locomotor activity in Sprague Dawley (SD) males (Pascual et al. 2006; Sun et al. 2017) but didn't affect SD females' locomotor activity (Lampert et al. 2017). In male WIST rats ASI had no effect on the distance travelled in the open field when assessed on PD 56, PD 60, PD 70, or PD 77, respectively (Bator et al., 2018; J. L. Lukkes et al., 2009; 2016; Van Den Berg et al., 1999). Finally, male Lister-hooded rats exposed to ASI (showed no differences in locomotor activity in adulthood (Begni et al. 2020a). The reported behavioral effects of ASI on locomotor activity seem to depend on strain, sex, housing group size. For example, Joshi et al (2017) and Kinley et al (2021) housed their rats in pairs or groups of three while Wright et al (1991) housed their rats in groups of four, which may have affected the outcomes. Other environmental factors, such as lighting conditions have also been shown to influence locomotor activity on the OFT (Bouwknecht et al. 2007). These findings underline the need for comprehensive reporting and inclusion of female for investigating sex differences.

The elevated plus maze (EPM) is another important behavioral paradigm used to measure anxiety-like behaviors by assessing the time spent on the open and closed arms of a plus-shaped maze for five minutes (Pellow et al. 1985). The validity of the EPM has been shown pharmacologically (Hogg 1996), whereby anxiolytic drugs increase open arm time and anxiogenic drugs have the opposite effect. Recently, a human version was validated; where EPM open arm time was negatively correlated with anxiety (Biedermann et al. 2022).

The results from the EPM following ASI are mixed. For example, in male LE rats ASI produced anxiogenic effects (Centre et al. 1991), while others have found no effects of ASI on anxiety-like behaviors in LE rats (Joshi et al. 2017). In male SD rats, ASI induces anxiolytic effects in males but had no effect on females (Weintraub et al. 2010). However, others have found ASI produces anxiogenic effects in SD males (Bledsoe et al. 2011; Sun et al. 2018). In WIST rats ASI (PD 22 – 35) does not appear to affect anxiety-like behavior when measured on PD 79 (Van Den Berg et al. 1999). Variability between strains suggest ASI may have strain specific effects on anxiety-like behaviour, or methodological differences may once again account for the variability. For instance, factors such as illumination intensities (lux), time of testing (light cycle), length of SI are important variables that have been shown to influence EPM outcomes (Violle et al. 2009; Arakawa 2018).

The social interaction test (SIT) assess social behaviors but originally developed to test for ethologically relevant sources of anxiety typically, for a five-minute period (File and Seth 2003). Few studies have investigated the effects of ASI on social interaction. Those that we found in the literature found ASI reduced social interactions following ASI. For example, male WIST rats that underwent ASI (PD 22 – 35) showed a reduction in social approach and an increase in social avoidance behaviors when tested on PD 84 (Hol et al. 1999). This pattern remained evident, even after repeated testing. However, other study in WIST rats found no effect of ASI on social behaviors on the SIT (Bator et al. 2018). A reduction in social interactions is also observed in ASI male SD rats tested in early adulthood (Lukkes et al. 2009b, a). Together, these studies suggest that when ASI is initiated before PD 30 (Hol et al. 1999; Lukkes 2009; Lukkes et al. 2009b) can have long-term effects on social interactions, while ASI initiated after PD30 does not appear to persistently affect social interactions (Bator et al. 2018). What is still lacking in the literature is a qualitative description of the reduction in social behaviors (i.e., a reduction in anogenital or non-anogenital sniffing). Other aspects of sociability have yet to be investigated in adulthood following ASI in rats.

Reports on novel object recognition memory are lacking, and to our knowledge, only one study has investigated object memory in adulthood following ASI. Here, the authors found no effect of ASI (PD 21 – 49) on object memory tested on PD 70 in male

and female Sprague Dawley rats (Begni et al. 2020b). Considering that social isolation in humans is associated with cognitive decline (Cardona and Andrés 2023), it is of utmost importance to study these long-term processes in rats to establish validity for the model and to better understand the mechanisms that may help in the development of future therapies.

Unfortunately, no studies have investigated the long-term effects of ASI on social recognition memory (SRM), which refers to a rat's ability to recognize and discriminate between familiar and unfamiliar conspecifics. This is an essential skill and not being able to recognize familiar conspecifics from novel ones may risk their lives. There is, however, significant evidence that PWSI, which refers to rats kept continuously in social isolation and tested in a state of social deprivation does impair SRM in rats (Leser and Wagner 2015; Almeida-Santos et al. 2019; Krupina et al. 2020). The long-term effect of other AAs has been shown to have long-term effects on memory in rats (Chaby et al. 2015; Provensi et al. 2019a; Begni et al. 2020b). Suggesting that AAs can have long-term effects on rats, however it remains unexplored whether these impairments are domain specific (to objects) or whether they extend into the social domain.

Studies on thermal pain following ASI are similarly lacking in the literature. Only two studies have investigated the effects of social isolation on pain, specifically visceral pain. Findings indicate that acute (2 or 24 h) and long-term (4-weeks) SI resulted in visceral hyper-analgesia, as demonstrated by the increased abdominal withdrawal reflex score when tested during SI (Zhou et al. 2022; Zhang et al. 2022). Other adolescent stress paradigms have been shown to induce persistent muscle hyperalgesia (Singaravelu et al. 2022). Taken together these studies suggest that adversity can have long-term effects on pain sensitization. Pain is one of the leading causes of disability worldwide (Vos et al. 2012). Hence, there is a growing need to understand how adolescent stress mediates pain sensitivity later in life.

1.6.1.2 Neurobiological findings

The long-term neurobiological effects of ASI have been described to alter the CORT, AVP, brain derived neurotrophic factor (BDNF), gamma-aminobutyric acid activity (GABA), and dopamine systems. Nevertheless, it remains unclear how ASI impacts the oxytocin (OT) system in rats, as no research has yet investigated this.

AVP fiber density increases were observed in the PVT following ASI (Kinley et al. 2021). Although the long-term effects of ASI on OT and AVP remain largely unknown, other neuroendocrine molecules such as CORT have been studied in more detail. For example, in SD rats ASI triggered a lagged CORT response in adulthood (Lukkes, 2009). The rats' initial stress response mimicked that of group-housed rats, but differences appeared when CORT was measured 2 h following restraint stress in adulthood. Indicating a heightened negative feedback of the HPA-axis (Lukkes et al., 2009). Moreover, alteration in mRNA of HPA-related genes were discovered. The NR3C1 gene which encodes glucocorticoid receptor proteins, showed upregulation in the PFC of ASI males and downregulation in the ventral HIPPO of females (Begni et al., 2020). These results suggest that ASI induces sex-dependent neurobiological responses in HPA-axis response that persists into adulthood.

Corticotropin-releasing factor (CRF), another neuroendocrine molecule, is also altered by ASI. CRF receptors in the dorsal raphe nucleus were found to mediate social anxiety after ASI (Lukkes et al., 2009). Interestingly, the administration of a CRF antagonist led to decreased social anxiety-like behavior in ASI rats. Suggesting that CRF receptors in dorsal raphe are required for social interactions which are altered persistently by ASI (Lukkes et al., 2009).

CRF has also been shown to increase GABA activity in the dorsal raphe (Kirby et al. 2008). These neurotransmitters are crucial for numerous neuronal processes, including the regulation of neuronal excitability, control of neuronal assembly size and propagation, determination of temporal aspects of spike trains, generation of oscillatory activity, and neuronal plasticity (Hou et al. 2020; Zhang et al. 2021). Despite the well-established shift from excitatory to inhibitory GABA(A) receptor-mediated responses during development, the extended postnatal maturation of the GABAergic system into adolescence is not thoroughly understood (Kilb 2012). Changes in the GABAergic system following ASI have been observed in GABA surface marker GAD65, which was increased in ASI rats (Bator et al. 2018). GAD65 is involved in producing GABA for fast release in an activity-dependent manner and is required for the maturation of the GABAergic system during adolescence (Kaufman et al. 1991). Increased GAD65 levels are associated with increased aggression (Grimes et al. 2003). As previously

described isolation-reared rats show heightened levels of aggression. Potentially, GAD65 levels are increased in isolation-reared rats, which could explain a part of the aggression observed in adult ASI rats (Oliveira et al. 2019).

Furthermore, dopamine system alteration has been reported in ASI studies. Dopamine communicates the perceived level of motivational significance, whether positive or negative, attached to a particular outcome (Wenzel et al. 2015; Hinton et al. 2019). This information then drives an organism's behavior towards or away from pursuing that outcome. In the dorsal striatum of adult ASI female rats, a reduction in dopamine receptor 2 (DR2) immunocontent and an increase in dopamine transporter (DAT) and tyrosine hydroxylase (TH) immunocontent were observed (Lampert et al. 2017).

During isolation, rats are deprived of sensory stimulation and physical activity, both of which are known to stimulate the expression of BDNF (Phillips et al. 2014; Sale et al. 2014). It has been postulated that ASI rats would demonstrate decreases in BDNF levels, which is a neurotrophic marker associated for neural plasticity, neuronal survival, and neurotransmitter regulation (Aid et al. 2007). Indeed, ASI induced long-term changes in BDNF mRNA and protein levels (Meng et al. 2011; Begni et al. 2020b). A transcriptional analysis of male SD rat brains collected at postnatal day 98 (PD 98) revealed lower BDNF mRNA levels in the mPFC and dorsal HIPPO BDNF exon VI levels (Begni et al. 2020b). On the contrary, increased numbers of BDNF-positive cells were found in the PFC and HIPPO of brains collected on PD 76 from ASI-exposed SD males (Meng et al. 2011). We postulate that these opposing findings are due to methodological differences reflecting sensitivity of measurements.

In conclusion, while no studies have yet looked at the long-term impact of ASI on the OT system in rats, the observed alteration in HPA axis-related hormones, AVP, BDNF, GABA, and dopamine systems might also affect the OT system due to their tight interplay. OT has been shown to interact with the HPA axis, playing a part in regulating CORT and CRF in response to stress (Torner et al. 2017). Changes in the CRF and GABA systems following ASI could impact social anxiety and aggression, both essential aspects of social behavior mediated by OT. OT also influences CRF and GABA release (Winter and Jurek 2019; Maniezzi et al. 2019). Additionally, the dopamine system, which signals motivational significance and drives reward

processing, interacts with the OT system in social behavior regulation (Baskerville and Douglas 2010). Finally, a connection between BDNF signaling in OT neurons and behavioral alterations has been suggested, implying a potential interaction between ASI-associated BDNF and OT (Maynard et al. 2018).

1.6.2 Social instability stress

Another adolescent stress paradigm that focuses on the intricacies of the social environment in development is the SIS paradigm. In humans, SIS refers to psychological and physiological stresses that arise from unpredictable and/or unstable social environments. SIS refers to a variety of factors, including unstable relationships, socially unstable environments (i.e., foster homes or half-way houses), residential uncertainty, or frequent moving. We refer to SIS as an umbrella term for the adolescent stressors in humans.

The impact of adolescent social instability in humans is best demonstrated by an increased propensity to develop depression (Glasheen et al. 2018), anxiety (Rumbold et al. 2012), deficits in social skills (Dupere et al. 2015), and increased levels of inflammatory markers (Schmeer and Yoon 2016). Frequent changes in housing or school mobility often coincide with alterations in family structure, and additional factors such as income loss, legal issues, and exposure to different peer groups also predict problematic behaviors in adolescents (Bakker et al. 2012). In a study involving children and adolescents who were part of child maltreatment investigations, housing mobility was found to predict worse behavioral outcomes over time (Fomby and Sennott 2013). Because SIS is frequently compounded by financial or environmental challenges, it can be difficult to separate it from other disruptive households or social circumstances.

Human studies suggest that SIS influences children differently depending on their initial health (Baker et al. 2019), making it difficult to study in humans, as the baseline conditions vary significantly and cannot be properly controlled. Additionally, limited research has examined the effects of SIS on neural development during adolescence. Appropriate SIS animal models can help us unravel the neural underpinnings of SIS. Preclinical SIS models also provide baseline conditions that are controlled for, and both immediate and long-term effects can be investigated.

1.6.2.1 Behavioral findings

The SIS paradigm combines features of unpredictability and short-term social isolation. In short, the paradigm relies on continuous changes in the cage hierarchy. During the procedure, the rats were socially isolated daily for 1 h and then returned to their home cage with a cage mate of the same sex. The repeated change in cage mate forces the rats to re-establish cage hierarchy daily in addition to isolation, which are both stressful for rats (McCormick & Green, 2013; Scharf et al., 2013). The model captures features of human SIS and is considered to have good face, construct, and predictive validity (McCormick & Green, 2013). For a comprehensive review of SIS mouse and rat literature, see Goñi-balentziaga et al., 2018; Koert et al., 2021.

SIS has been shown to influence various behavioral outcomes, both immediately and persistently, into adulthood. Only the findings in adulthood following SIS are described and will be referred to as SIS. Depressive-like behavior were assessed in the forced swim test (FST) in adulthood (PD70) in LE SIS male and female (Mathews et al., 2008). Male SS rats spent more timing climbing in compared to CTL, while no differences were observed in females. The increased duration of climbing was postulated to reflect a stress-induced bias towards active coping, which may have developed over time as it was not evident when SIS rats were evaluated immediately after the termination of the SIS procedure. The sucrose preference test (SPT) is another test used to assess anhedonia-like behavior. Here, neither SIS LE males nor females show differences in their sucrose preference compared to CTL (Herlehy et al. 2022). No differences in SPT have been reported in male WIST rats (Provensi et al. 2019a).

SIS LE males showed increases in anxiety-like behaviors on the EPM in adulthood (PD70) (McCormick et al. 2008; Green et al. 2013) whereas female rats were less anxious in the EPM when measured during the oestrus phase but not during the dioestrus phase of the cycle (McCormick et al. 2008). Recently, two studies in LE rats were unable to replicate previous findings and found no differences in anxiety-like behaviors on the EPM in adult males (Marcolin et al. 2019, 2020). Similar results were obtained in a recent study in WIST male rats, which found no effect of SIS on anxiety-like behaviors on the EPM in adulthood (Provensi et al. 2019a). The authors did not observe any persistent effects of SIS on locomotor activity or anxiety-like behaviors in the OFT.

Social skills are generally affected by SIS in LE rats. These effects are influenced in a sex-dependent manner, whereby SIS male LE rats show a reduction in social interactions (Green et al. 2013; Marcolin et al. 2020) but do not impact female social interactions (Asgari et al. 2021). Long-term effects on social memory have not yet been explored. However, object memory related deficits have been described in both female (McCormick et al., 2010) and male (McCormick et al. 2012) LE rats following SIS. Similar memory impairments have been observed in male WIST rats, whereby SIS induced long-term cognitive impairments in the novel object recognition test (Provensi et al. 2019a). Studies on fear learning following SIS have revealed sex-dependent effects. SIS male LE rats showed a reduction in their memory of the conditioned fear response (Morrissey et al. 2011), while the opposite was true for females (Green & McCormick, 2013). Cognitive impairment is the most consistent long-term finding in the SIS literature, followed by a reduction in social interactions in males.

Most of the SIS studies have been performed in the same lab over the last 15 years, and it is therefore surprising that the results are mixed. Some of these differences in findings could be explained by differences in procedures used in the part. For example, in early publications, rats were single-housed following SIS (Mathews et al., 2008; McCormick et al., 2008); however, since 2010, rats have been pair-housed post SIS (McCormick et al., 2010). This along with other methodological changes such as lighting (Jones & King, 2001), time of testing (Butler-struben et al.,2022) or genetic drift (Zeldovich 2017) in the rat line could account for the differences.

1.6.2.2 Neurobiological findings

Next, we explore the long-term neurobiological effects of SIS. The findings on CORT levels are mixed. Initial studies suggested no differences in basal CORT levels in either SIS male or female rats (McCormick et al. 2008; Mathews et al. 2008; Saaltink et al. 2020). However, recent findings in SIS male LE rats suggest that basal levels of CORT are persistently elevated even in adulthood (Marcolin et al. 2020). SIS rats also showed a slower recovery to baseline levels of CORT than CTLs (Marcolin et al. 2020). Further studies are required to better understand the relationship between SIS and basal CORT levels. Other neurobiological markers that have been studied in relation to SIS are neuronal markers of neuronal plasticity and neurogenesis. In one study, doublecortin was measured following SIS (McCormick et al., 2012). Doublecortin levels

were significantly higher in the dorsal hippocampus of SIS rats than in CTL, potentially reflecting increased survival of immature neurons in SIS rats (McCormick et al., 2012). Additionally, SIS rats were found to have higher basal calcium/calmodulin-dependent kinase II and lower expression of the phosphorylated CamKII subunit threonine 286, which are signaling molecules related to synaptic plasticity. Additionally, Zif268, a memory- and stress-related transcription factor, is modulated by SIS (Hodges et al. 2014). At basal levels, SIS male LE had a higher number of Zif268 immunoreactive cells in the arcuate nucleus than CTLs. While there was a significant decrease in Zif268 in the BLA, CEA and HIPPO following a short 1hr isolation in SIS male LE rats. SIS male WIST rats were found to have lower levels of BNDF in HIPPO than in CTLs (Provensi et al. 2019a).

Few studies have examined neurobiological differences across both sexes following SIS. One study found sex-specific changes in dendritic morphology, reduced apical branch number and length in SIS females, and reduced basilar length of dendrites in males (Breach et al. 2019). While a study on the neuroendocrine-gut axis found differences immediately following SIS, the difference dissipated with time and by adulthood no differences between stress conditions or sex remained (Mccormick et al. 2020). The long-term effects of oxytocin are yet to be studied in the SIS paradigm.

1.6.3 Peer rejection

As humans, we have an innate yearning to belong which gains significant importance during adolescence (Baumeister & Leary, 2017). The idea that we want to belong is grounded in the evolutionary theory of motivation, which posits that most social mammals are driven by innate biological drives that have evolved over time to help us survive as pack animals and helped us reproduce (Baumeister & Leary, 1995). For example, being shunned from a group challenges our fundamental need to belong as we have relied on social groups for survival. Remnants of these fundamentals have left deep evolutionary marks and consequences for modern psychological processes.

Peer rejection refers to the experience of being ostracized, ignored, bullied, or disliked by peers, which normally occurs during adolescence and often ostracized, social rejection or socially excluded are used interchangeably for PR. Too much PR has been shown to illicit negative emotions and consequences for psychological and social

functioning (Hatchel et al. 2019; Nepon et al. 2021). PR is particularly potent during adolescence, when the brain is still developing, and research has shown that PR can influence brain development (Masten et al. 2009). Females have been reported to be more sensitive to rejection, suggesting that sex is an important variable to consider when investigating PR (Schneider et al. 2016b; Stroud et al. 2017; Hance et al. 2018). Clinical studies have highlighted immediate and long-term negative consequences of peer rejection on social functioning and mental health. These include increased behavioral problems such as aggression, substance abuse, and delinquency as well as an increased risk for depression, anxiety, borderline personality, and social anxiety disorder as (Douglas et al. 2004; Masten et al. 2009; Murphy et al. 2013; Silk et al. 2014; Tomova et al. 2021)

1.6.3.1 Peer rejection and alcohol

Alcohol is one of the most commonly abused substances, and is frequently used in social contexts to cope with social stress (Bacon and Engerman 2018). Evidence indicates that rejection may increase alcohol use, and hence, alcohol-related problems. Only a few studies have investigated the relationship between PR and alcohol use, with mixed results. One study found a positive association between PR and increased alcohol consumption (Rabinovitz 2014). Here, participants were initially told whether their personality type was predictive of them “ending up alone” and those individuals drank more, indicating that they were willing to drink more than those in the social inclusion group. Similar results were observed in a study conducted among college students, where PR individuals consumed more beer than CTLs (Bacon and Engerman 2018). Some evidence suggests that rejection may increase alcohol consumption and related problems, highlighting the need for further investigation of this complex association. No studies in rodents have investigated the effects of PR on alcohol-related behaviors to date.

1.6.3.2 Preclinical findings

The novel PR-like paradigm was developed in our lab and capitulates features of borderline personality disorder (Schneider et al., 2014). The paradigm combines the features of play deprivation in a normal social environment. Unlike most stress paradigms, rats remain socially housed throughout their lives. In short, one WIST rat is group housed with less playful Fischer 344 across adolescence which moderates their ability to adequately engage in reciprocal social play. Specifically, WIST rats in

the inadequate social rearing condition received dramatically less playful responses, although they initiated more attacks and producing more prosocial 50kHz ultrasonic vocalizations (indicating their willingness) than their control counterparts (Schneider et al., 2014, 2016). Although PR is a complex phenomenon that can be induced in a multitude of ways in humans (Kawamoto et al., 2015), we found several similar features in our peer-rejected female rats and humans. These changes include deficits in social interactions, social recognition memory, pain sensitivity, emotional reactivity, processing of socially transmitted information, and neurochemical (endocannabinoid) system dysregulation (Bungert et al., 2015; Schaefer et al., 2014; Schneider et al., 2016a, 2014).

More specifically, even short periods (24 h) of PR during adolescence have been shown to increase latency to thermal pain (Schneider et al., 2014). Under basal conditions, CORT levels did not differ between PR and control rats during adolescence (Schneider et al., 2016). However, when basal CORT levels were measured in adulthood PR rats had significantly lower levels than CTLs (Schneider et al., 2014). Interestingly, CORT levels measured after an acute stressor in adulthood did not differ between the groups (Schneider et al., 2014). Several behavioral alterations persisted after the end of the PR procedure. For example, an increase in latency to a thermal stimulus that was apparent immediately after a short-term PR was also evident in adulthood (Schneider et al., 2014, 2016). Furthermore, the composition of social interactions was altered in PR rats. Whereby, PR rats performed less anogenital sniffing and showed fewer approach behaviors. These rats also show domain-specific deficits in social, but not object, memory (Schneider et al., 2016). Additionally, PR rats showed deficits in processing socially transmitted food preferences. Finally, PR rats demonstrated increased reactivity to predator odors in an acoustic state response test (Schneider et al., 2014).

Few studies have investigated the neurobiological sequelae of PR. The endocannabinoid and cannabinoid systems have been focal points of research. Western blot analysis of adult brains following PR revealed increases in cannabinoid receptor 1 levels in the thalamus and decreases in endogenous endocannabinoid ligand fatty acid amidase hydrolase (FAAH) levels in the AMY of PR rats (Schneider et al., 2014). The increase in CB1R protein levels in the thalamus was replicated in a

follow-up study, and CB1R protein levels were found to be upregulated in the AMY(Schneider et al., 2016). Similarly, FAAH findings were replicated, in addition to revealing decreases in monoacylglycerol lipase (MAGL) in the striatum (Schneider, et al., 2016). Additionally, endocannabinoid levels differed between PR and control rats. The endocannabinoid anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels were increased in the striatum of PR rats, with the former also increasing in the AMY of PR rats (Schneider et al., 2016). Pharmacological interventions during PR have revealed that responses to playful attacks are mediated by cannabinoid receptor 1 (CBR1) (Schneider et al., 2016). The CB1R antagonist, SR141716 (SR), prevented reciprocity to playful attacks to similar levels observed in PR rats (Schneider, et al., 2016). Incidentally, SR increased the number of playful attacks, imitating and attenuating the effects of PR on thermal-pain sensitivity.

Overall, the novel adolescent PR model developed in our lab has successfully replicated the key features of borderline personality disorder in rats, including social deficits, decreased pain sensitivity, increased emotional reactivity, and endocannabinoid dysregulation (Lieb et al. 2004; Schmahl et al. 2004). The model has also shed light on the neurobiological underpinnings of PR and its long-lasting effects on behavior and physiology and provides a novel approach to studying aspects of PR and borderline personality disorder. We wanted to apply the reverse translational apporach and test whether our paradigm would replicate the results observed in humans.

1.7 Summary of adolescent adversity paradigms

In summary, the ASI, PR, and SIS paradigms each provide unique insights into the long-term effects of different adolescent adversities in rats. All three paradigms share similar effects on social behavior and cognitive function. Specifically, all three paradigms showed alterations in social interaction. In the ASI and SIS paradigms, social interactions were altered in male rats and in females in the PR paradigm. Furthermore, all paradigms induced persistent impairments on some aspect of memory function. ASI resulted in mixed effects on anxiety-like behaviors but showed a reliable reduction in object memory. Finally, the ASI model had long-term effects on pain sensitivity. While the SIS model demonstrates sex-dependent effects on social interactions, anxiety-like behaviors, and long-term cognitive impairments. The PR

paradigm reveals deficits in social interactions, SRM, emotional reactivity, and neurochemical dysregulation. The long-term neurobiological effects of ASI, PR, and SIS in rats show both similarities and differences across various neurotransmitters, hormones, and peptides.

ASI has been linked to alterations in the HPA axis-related hormones, BDNF, GABA, dopamine, and AVP systems.

Studies on the neurobiological alterations following PR are lacking, but so far, evidence points to PR altering the endocannabinoid system. SIS, on the other hand, has revealed mixed findings regarding CORT levels, neuronal markers of plasticity, and neurogenesis. Despite some sex-differences in SIS, few studies have directly investigated neurobiological differences across sexes in adulthood, as most sex comparisons were compared across separate studies of the same paradigm.

1.8 Hypotheses and aims

The long-term effects of type and timing of AAs on the brain and behavior remain poorly understood. Few studies have investigated their effects and those that have been performed almost exclusively in male rats (Beery and Zucker 2011). Because data from half of the population is often missing, drawing conclusions is difficult. Hence, there is a need to study the how different timing and diverse types of AAs affects both males and females to elucidate their effects and potential therapeutic avenues that are currently lacking. Several studies have demonstrated that the OT system altered immediately following AAs and the developmental trajectory (most pruning during adolescence in rodents) suggest that it may be altered by AAs. However, whether these changes are persistent remain elusive.

1.8.1 Hypotheses

Hypothesis 1:
We hypothesize that type and timing of AAs will differentially impact behavior in adulthood.

Hypothesis 2:
We hypothesize that AAs in rats alters the OT system persistently

Hypothesis 3

We hypothesize that female rats are more affected by AAs both on the behavioral (i.e., alcohol seeking) and neurobiological level (oxytocin system).

1.8.2 Aims

Aim 1:

To systematically characterize the effects of timing and type of adolescent adversities and their sex-specific effects

Specific aim 1: To characterize the long-term effects of adolescent social isolation and social instability on adult behavior

Specific aim 2: To characterize the molecular sequelae of adolescent social isolation on the oxytocin system

Specific aim 3: Validate the functional role of potential behavioral and molecular changes

Aim 2:

To investigate whether PR during adolescence impacts alcohol seeking behaviors in a sex-dependent manner.

1.8.3 List of studies

Study 1 Longitudinal study characterizing of the persistent effects of early and late adolescent social isolation on adult social, memory, and anxiety-like behaviors in male and female rats (Aim 1, specific aim 1). Molecular characterization of OTR alterations in adult rats exposed early and late adolescent social isolation (Aim 1, specific aim 2) Functional validation by optogentically released endogenous OT in the PVT on behavior (Aim 1, specific aim 3)

Study 2 Longitudinal study characterizing the persistent impact of adolescent social instability stress on adult social, memory, and anxiety-like behaviors in male and female rats (Aim 1, specific aim 1)

Study 3 Longitudinal study characterizing the persistent impact of PR on adult alcohol self-administration and reinstatement of alcohol-seeking behaviors in male and female rats (Aim 2)

LIST OF PUBLICATIONS related to thesis

The author was wed during the PhD time and changed his surname from Surakka to Graf

1. **Surakka A**, Vengeliene V, Skorodumov I, et al (2021) Adverse social experiences in adolescent rats result in persistent sex-dependent effects on alcohol-seeking behavior. Alcohol Clin Exp Res 45:1468–1478. https://doi.org/10.1111/acer.14640
2. ***Graf A**, *Murray SH, Eltahir A, et al (2023) Acute and long-term sex-dependent effects of social instability stress on anxiety-like and social behaviours in Wistar rats. Behav Brain Res 438:114180. https://doi.org/10.1016/j.bbr.2022.114180

LIST OF PUBLICATIONS not related to thesis

1. Singaravelu SK, Goitom AD, **Graf A**, et al (2022) Persistent muscle hyperalgesia after adolescent stress is exacerbated by a mild-nociceptive input in adulthood and is associated with microglia activation. Sci Rep 12:18324. https://doi.org/10.1038/s41598-022-21808-x
2. **Graf A**, Giannone F, Bernardi RE, Spanagel R, The Effect of Nicotinamide Mononucleotide on Alcohol Seeking Behavior in Wistar Rats, Alcohol Clin Exp Res, Submitted.
3. Graf A, Riccio A, Spanagel R, Mixed results in a discrete choice paradigm between alcohol and social rewards. Addict Biol, in press

2 MATERIALS AND METHODS

All studies are reported according to the ARRIVE 2.0 guidelines to inform future studies (Percie Du Sert et al. 2020). We chose to work with outbred WIST rats as they reliably respond to various stressors both acutely and chronically, making them an appropriate model for studying the long-term effects of adversity (Tohei et al. 2003; Rex et al. 2004; Walker et al. 2009; Langen and Dost 2011; Djordjevic et al. 2012; Augier et al. 2014; Eskandari Sedighi et al. 2015). Additionally, WIST rats have been widely used in preclinical research and their behaviour has been thoroughly studied providing a rich knowledge base for interpreting results.

2.1 Adolescent Social Isolation (study 1)

2.1.1 Subjects

Male (*n* = 40) and female (*n* = 40) outbred **WIST:RccHan** rats were purchased from Envigo (Venray, Netherlands) and used to characterize the behavioral sequelae of ASI in adulthood (Study 1). A separate cohort of male (*n* = 24) and female (*n* = 24) from the same supplier was used to characterize the molecular alterations in OTRs in adult ASI rats (study 2). For the functional validation study, female WIST Rcc Han rats (n = 56) were again purchased from the same supplier. Rats in all studies arrived at our facility on postnatal day (PD) 21. On the day of arrival rats were randomly assigned to one of the experimental conditions, as shown in the schematic (Figure 7). Rats were housed either individually (Makrolon Type III cages) or in groups of four (Makrolon Type IV cages) under a standard diurnal 12 h light-dark cycle, temperature, and humidity with free availability of water and standard laboratory chow. Male and female rats were housed in separate colony rooms. All experiments were approved by the local animal care committee (Regierungspräsidium Karlsruhe, Referat 35, Karlsruhe, Germany) following the guidelines of the European Union (2010/63/EU) ethics number G-289/18).

2.1.2 Study Design

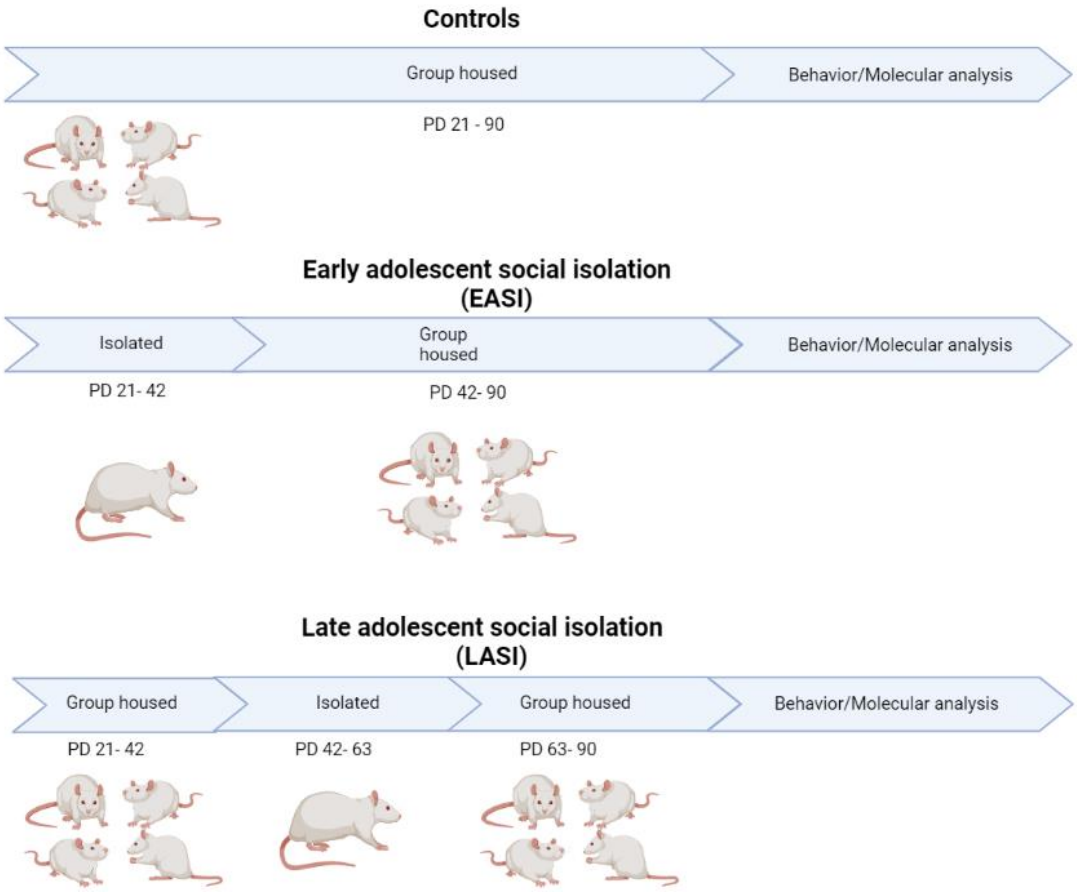


Figure 7: Timeline of adolescent social isolation procedures. EASI (PD21-42) and LASI (PD42-63) rats underwent three weeks of social isolation and were then rehoused with the same condition rats, whereas control rats remained group-housed throughout the study. In one study, rats underwent behavioral testing starting with PD90. In study two rats were sacrificed on PD90 to compare results with the same age.

Figure 7 illustrates the timeline of the experimental procedures and when behavioral testing began and tissue was collected. All rats were weaned prior to arrival at our facility on PD21 and were randomly selected for housing in either the early adolescent social isolation (EASI) or late adolescent social isolation (LASI) condition or control (CTL) condition where rats were housed in groups of four rats/cage. Each isolation condition lasted for three weeks. In the EASI condition, the rats were socially isolated from PD 21 to 42, and in the LASI condition, the rats were socially isolated from PD 42 to 63. For the duration of the social isolation, rats had no somatosensory contact but had olfactory, auditory, and visual stimuli of the other rats in the same colony room. At the end of the isolation period rats were rehoused with rats from the same condition.

Simultaneously, control rats were rehoused with other control rats to equalize potential rehousing stress among groups. Rats remained group housed for the remainder of the experiment. Behavioral testing (Study 1A) began with the EPM (PD 90), followed by the OFT (PD 92), NOR (PD 94), SIT/SRM (PD 96), and Hotplate test (PD 98) (Figure 8). A separate cohort of rats was used for the molecular characterization of OTR alterations following ASI (Study 1B). These rats underwent the same procedure as in Study 1 but did not undergo behavioral testing. Instead, the rats were sacrificed on PD90 within the first two hours of the light-ON cycle.

In Study 1C, female wild-type WIST RccHan rats underwent the same EASI procedure described above and were then group-housed for the remainder of the study. Additionally, rats underwent surgery between PD 70 and 77 and were left to recover for three weeks waiting for the virus to express. Behavioral testing began at approximately PD 110 ±2.

2.1.3 Behavioral Tests

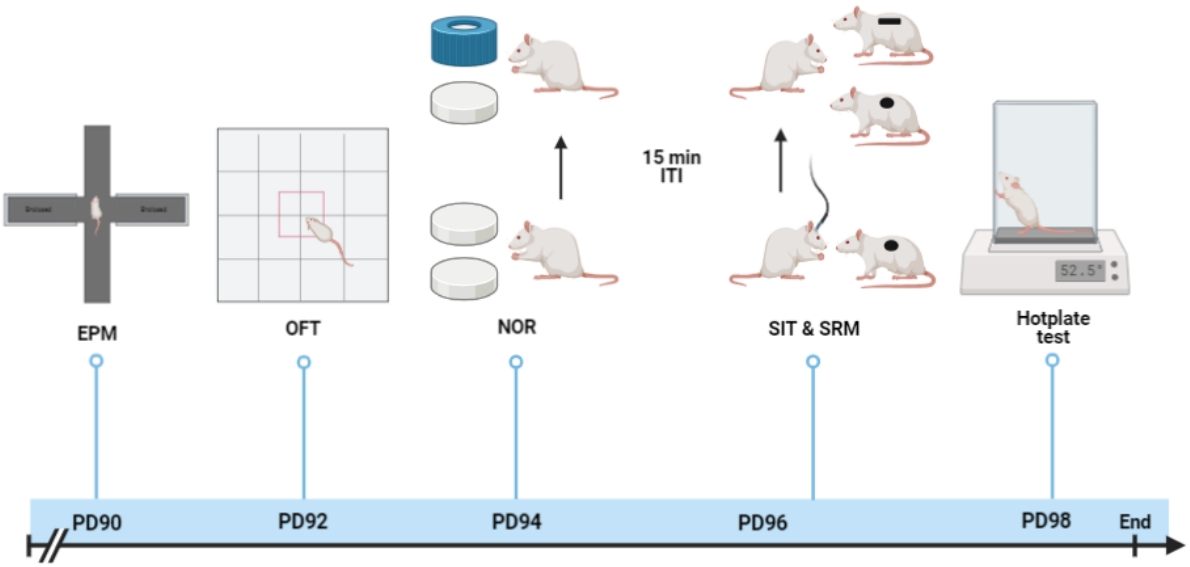


Figure 8: Timeline for behavioral experiments in Study 1. Behavioral experiments were performed in adulthood for EASI, LASI, and CTL rats starting from PD90 with elevated plus maze (EPM), open field test (OFT), novel object recognition (NOR), social interaction test (SIT), social recognition memory (SRM), and hotplate test.

Figure 8 shows the timeline for all behavioral experiments performed in Study 1. We chose commonly used behavioral tests to assess for anxiety-like, social and memory processes as well as pain sensitivity that had previously been shown to be altered

following AAs and had been previously validated in our lab. All behavioral tests were performed during the first five hours of the inactive phase (light ON) of the diurnal cycle. Rats were given at least 48 h between tests and all videos were recorded and evaluated offline by an expert blinded to the experimental manipulations. The estrous cycle of females was tracked after the EPM and HP tests because there are indications that anxiety-like behaviors (Lovick and Zangrossi 2021) and thermal pain sensitivity (Vinogradova et al. 2003; Ibironke and Aji 2011) are mediated by the estrous cycle. All behavioral apparatuses were first cleaned with 70% alcohol solution at the start of each day, between trials, and after each day of testing to prevent the transmission of olfactory cues. The next apparatuses were cleaned with water and allowed to dry because evidence suggests that strong scented solutions could influence behavioral results (Hershey et al. 2018).

2.1.3.1 Estrous cycle cytology

Cytological vaginal smears were collected immediately after the elevated plus-maze and hotplate test to monitor the estrous cycle, as evidence points to the estrous cycle influencing pain sensitivity in these two tests. The samples were analyzed under a light microscope (V300, Will Wetzlar) and characterized into two categories estrus/diestrus and proestrus/metestrus groups, where pain sensitivity differences have appeared (Vinogradova et al. 2003).

2.1.3.2 Elevated plus maze

To measure anxiety-like behaviors, we used the elevated plus maze (EPM), which is an apparatus shaped like a plus sign made of dark gray PVC. It has two open arms measuring 12 cm × 50 cm each and two enclosed arms measuring 12 cm × 50 cm × 50 cm each that surround a middle platform measuring 12 cm × 12 cm, 50 cm above the floor. At the beginning of each trial, a rat was gently placed on the middle platform facing an open arm and then allowed to explore the EPM (90 lx) for 5 min. The subsequent video analysis assessed the time spent in the open and closed arms, number of entries made into the open or closed arms (where an entry was defined as all four paws in a particular arm), head dips, and risk assessment. Risk assessment was defined as the act of placing only the head or forepaws in the open arm without any accompanying movement of the hind legs, even if the rat subsequently entered the arm. The percentage of time spent in the open arms was calculated using the following formula: open arm time / (open arm time + closed arm time) × 100.

2.1.3.3 Open field test

To assess the locomotor activity of the animals, we used the open field test, which measures the movement of test rats (Walsh and Cummins 1976). The apparatus comprised four uniformly sized arenas, each measuring 50 cm × 50 cm × 50 cm and was constructed from dark gray PVC. One day before testing, the rats were habituated to the experimental room for 15 min. On the test day, the rats were brought into the experimental room and habituated for 5 min before the test was started. The rats were gently placed in the center of the arena facing a random side, and locomotor activity was measured during a 30-minute test (50 lx). The distance travelled in the OFT was measured in meters.

2.1.3.4 Novel object recognition test

To assess object recognition memory in rats, we employed a test that comprised two phases, namely the initial 5 min acquisition phase (P1) and the 3 min discrimination phase (P2), separated by an inter-trial interval (ITI) of 15 min. The rats were habituated to the open field for 15 min one day prior to testing. The objects under investigation were made of ceramics or glass. To ensure the accuracy of the test results, all objects and the test arena were thoroughly cleaned and dried with 70% ethanol before and during the test. Our laboratory conducted preliminary tests that revealed that all the objects chosen for this test were equally attractive to the subjects (approximately 50% preference). During P1, the rat was placed in the center of the open field and exposed to two identical unknown objects (A), after which the rat was returned to its home cage and the objects were cleaned and dried. In P2, the rat was returned to the open field and presented with the familiar object A′ (an identical copy of the object presented in P1) and a novel test object (B). The duration of object exploration (sniffing, touching an object with whiskers, and licking) was recorded for both P1 and P2. The discrimination between the exploration time of the novel object and the familiar object was expressed as a percentage of the total exploration time of both objects during P2 [100/(A′+B) × B], whereas the discrimination index was calculated by subtracting the exploration time of the familiar object A′ from the novel object B in P2 (B − A′).

In the optogenetic behavioral study (1C), rats were connected to the patch cord during the acquisition phase and received 120 s of photostimulation during the first two minutes of the test

2.1.3.5 Social interaction and recognition memory

To evaluate social interactions and SRM in rats, we utilized an experimental design, as described previously (Schneider et al. 2008). The test involved exposing the test rat to an unfamiliar young adolescent same-sex social partner (5-6 weeks old) for a duration of 5 min in the open field. No habituation was required, as the rats had already been exposed to the open field across the OFT and NOR. The test rat was placed in the open field and allowed to explore for 1 min, after which the stimulus rat was placed in the open field, and the test began. The frequency of various social behaviors, including contact behavior such as social exploration including anogenital and non-anogenital investigations, were quantified for the test rat only. The frequency of social avoidance and self-grooming was also recorded.

In the second part of the test assessing social recognition memory, the initial 5-minute social interaction period with the unfamiliar social partner (A) served as the sample phase (P1) for the social recognition test (P2). In the subsequent test for social recognition memory, a second unfamiliar adolescent of the same sex (B) was introduced during the test (P2) after a 15-minute inter-trial interval. During P2, the familiar (A') and novel social partners (B) were presented to the experimental animal for 3 min, and the time for social investigation (anogenital, non-anogenital exploration, and approach/following) for the test rat was recorded. To calculate the social discrimination percentage, the exploration time of the novel conspecific was expressed as a percentage of the total exploration time of both conspecifics during P2 [100/(A'+B) × B].

In the optogenetic study, the rats were connected to the patch cord during the acquisition phase and received 120s of photostimulation during the first two minutes of the test.

2.1.3.6 Thermal pain sensitivity

Thermal pain sensitivity was quantified using a hot plate apparatus (Ugo Basil) with a fixed temperature of 52.5°C ± 0.1°C. This experimental setup was conducted in accordance with the methods established in previous studies (Schneider et al., 2014), and video recording of the behavior was analyzed offline frame-by-frame. The experiment was performed in the colony room of the experimental rats to reduce potential environmental stress-induced analgesia (Shankar et al. 1999). Briefly, rats

were gently placed onto the hotplate platform at the beginning of the experiment, and the test was terminated when the rats showed the first heat-provoked reaction or after a cut-off period of 30 s to avoid tissue damage. The first heat-evoked responses, including foot shake, stamping, paw licking, or jumping off the platform, were used as a cut-off measure of pain.

For study 1C, rats were first attached to the patch cord and placed in a type II cage (Makrolon ®) for 1 min prior to the initiation of the photo stimulation, and then exposed to one minute of blue-light stimulation in the type II cage before being gently picked up and placed on the hotplate platform. The same cutoff measures of pain were used as in Study 1.

2.1.3.7 Fear conditioning (study 1c)

Fear memory was measured using the fear conditioning paradigm described in previous studies (Knobloch et al. 2012), with slight modifications. Following the social recognition memory paradigm, rats underwent non-contextual fear conditioning for four consecutive days. On day 1, the rats were individually habituated to a conditioning box (45 cm × 18 cm × 25 cm) for 10 min. Days 2 and 3 served as the conditioning days, where rats were placed individually back in the conditioning box and after 5 min received five electrical stimulations (0.4 mA) at random intervals (15–120 s apart) over 5 min. After the last electric stimulation, the rat was left in the box for 5 more min. Rats were assessed for fear recall on day 4. Rats were placed back in the conditioning box, but no electrical stimulation was applied during the recall session. Instead, after 5 min, photostimulation was applied for over 120s and the freezing behavior was measured, rats where then left in the cage until the end of the session.

2.1.4 Surgical interventions

2.1.4.1 Stereotaxic surgery

All surgeries, injections, and implantation of optic fibers were performed as described in the STARS protocol (Tang et al., 2022). Stereotaxic coordinates used were obtained from the rat brain atlas (Paxinos and Watson 2007) and are summarized for study 1C in Table 1 and a schematic of the experimental protocols (Figure 17). Female wild-type WIST:RccHan rats underwent surgery between PD 70 and 77 and were left to recuperate and allow the virus to express for three weeks before behavioral experiments began.

Table 1 Stereotaxic coordinates for injection sites and optic fiber (study 1C)

Area	Hemisphere	X (M-L)	Y(A-P)	Z(D-V)	Angle	dm/vol	Misc
PVN	Both	±0.35	±1.8	-8	0	3 /450nL	
PVT	Right	1	-3.5	-5.1	11.1°	n/a	Optic fiber

2.1.4.1.1 Surgery preparation

To minimize stress, the experimenter transported the rat to the surgery room 30 min prior to administering anesthesia. Then, the rats were weighed on a scale and proper dosage was determined for the analgesic, a subcutaneous injection of bupivacaine (2 mg/kg) and meloxicam (2 mg/kg) or following veterinary recommendations. The rat was then placed in a small chamber that was slowly filled with perspired isoflurane (5%) until general anesthesia was induced. Surgery commenced once the rat exhibited no withdrawal reflex to a paw pinch. Following anesthesia, the rat was moved onto the surgical setup, where isoflurane was continuously inhaled by the rat throughout the surgery. The concentration of isoflurane began at 5% and progressively decreased to 1.5-2% (0.2 L/min) during surgery, depending on the animal's respiratory rate. The head of the rat was fixed in a stereotactic apparatus and then shaved, while its body was positioned on a heat-controlled plate (37°C, RWD, China). Blunt-tipped ear bars and an incisor adaptor with a nose clamp was used to secure the rat head to the stereotaxic frame.

2.1.4.1.2 AAV injections

The surgical procedure began by applying an eye ointment (Bepanthen, Bayer) to prevent eye dryness. Next, the shaved head was sterilized by applying a veterinary iodine solution (Vet-sept, Livisto) and lidocaine (Aspen) was applied for local analgesia. A single incision was made along the anterior-posterior-axis of the rat. The skin was kept open using four surgical clips. The skull surface was sterilized again by applying Vet-Sept iodine solution. Any bleeding was stopped using sterile cotton swabs and a high-speed dental drill. The surface was then cleaned with cotton swabs and lidocaine was applied and allowed to settle for 3-4 min. Next, Bregma and Lamda were taken, and the craniotomy positions were marked with a pen (1.8 mm posterior to Bregma and 0.35 mm lateral to the PVN). In study 1C, the craniotomy for the optic fiber was drilled at the same time as for the screws (3.5 mm posterior to Bregma and 1 mm

later for the PVT). Next, the craniotomy was performed in the marked positions using a high-speed dental drill (0.6 mm) drill tip with a ballpoint nose.

Next, the glass micropipettes were calibrated to 1 ml and used to inject the virus. A micropipette puller pulled a long and thin injection needle cut with scissors to create an opening of approximately 3 µm. The pipette had 1 mm check marks, with 1 mark corresponding to a 100 nl injection volume or 1 dm. The micropipette was inserted into the arm of the stereotactic apparatus and connected to plastic tubing connected to a 50 ml syringe. A small piece of parafilm was placed on the skull and 1 ml of the virus was placed on the parafilm. The tip of the micropipette was lowered into the virus solution, and suction was applied through a 50 ml syringe. The aspiration of the virus was stopped when the desired volume was reached in the micropipette. The parafilm was then removed and the micropipette was moved to Bregma and Lambda, and the X- and Y-coordinates for the injection site were recalculated. Next, the micropipette was moved above the injection site and lowered to the Z-coordinates (the dura was penetrated with the micropipette), and the virus was administered by slowly applying pressure to the syringe. The infusion rate was monitored visually, and the speed and volume were approximately 100 nl (1 mm across the shaft) per 10-15 seconds. The micropipette was kept in this position for 3 min to prevent virus backflow before being slowly withdrawn from the brain (10 µm/s).

2.1.4.1.3 Optic fiber implantation

Following the injection of the AAVs, the surgical procedure for optic fiber implantation commenced. As described above, the craniotomy for the optic fiber was drilled at the same time as the screw holes. The skull surface was cleaned and dried, and four steel screws (Knupfer) were positioned symmetrically on the skull surface. The screws were gently fastened into the skull bone and further fixed by applying dental glue and cement (Paladur) to create a mound around the screws. The dental cement was allowed to dry until the mounds hardened. Next, the optic fiber was inserted into the stereotaxic arm, and the Bregma and Lambda were recalculated. Then, the optic fiber was implanted at the desired X- and Y-coordinates and slowly lowered (10 µm/s) to the target Z-coordinate. Next, dental cement was poured around the optic fiber, and a round edgeless mound was formed around it. Once the mound hardened, the optic fiber was removed from the stereotaxic arm. The head wound was closed using surgical sutures, lidocaine, iodine solution was reapplied for local anesthesia and disinfection, and

analgesics were administered subcutaneously (5 mg/kg). The rat was then placed in a heated recovery cage to recuperate from anesthesia. The rats' health was assessed, and analgesics administered to the rats showed signs of pain.

2.1.4.2 Viral vectors

Different recombinant adeno-associated viruses (rAAVs) were used in Study 1C for this dissertation and are summarized in Table 2. The AAV_OTp_Venus was used as a control virus, the presence of the Venus fluorescent protein under the control of the OT promotor allowed for labeling and visualization of delivery and distribution of the vector. The AAV_OTp_ChR2-mCherry allowed for cell-specific expression of the vectors (ChR2 and mCherry) in OT producing cells. The expression of ChR2 allows for optogenetic activation of these targeted cells, while mCherry allows for the visualization of cells that had been transfected by the viral vector. Allowing us to confirm the viral vector delivery and mapping of the distribution of transfected cells. Both rAAVs were delivered into the PVN where there is a large OT neuron population which axons branch into the PVT (Figure 6). All rAAVs used in these studies were cloned into serotype 1/2 plasmids. All viral vectors were generated at our institute and were generously provided by Prof. Dr. Valery Grinevich.

Table 2 . rAAVs used in the thesis work

1	AAV_OTp_Venus (OT-V)
2	AAV_OTp_ChR2-mcherry

2.1.4.3 Optogenetics

For the in vivo optogenetic behavioral study, we used a blue laser (λ 473 nm, 100 mW/mm², DreamLasers) connected to an optical fiber patch cable (M128L01, Ø400 µm Core, 0.50 NA, FC/PC to Ø2.5 mm, Thorlabs). Channelrhodopsin-2 (ChR2), a light-sensitive protein, was used to stimulate the excitatory responses. ChR2 can convert bursts of blue light into electrical activity with a millisecond precision (Nagel et al. 2003). When ChR2 is stimulated with blue light (~470 nm), it undergoes a conformational change in the cation channel, causing an influx of cations (Ca2+, Na+, H+) into the cell, subsequently depolarizing the cell and initiating additional action potentials (Kato et al. 2012). The optical fiber probes were unilaterally implanted in the PVT as described below (2.1.5). The coordinates used were ML: -1, AP: -3.5, DV: -5.1, medio-lateral angle:11.1°. Photostimulation of the PVT was delivered using a series of

pulse trains with an intensity of ~ 6mW, frequency of 30 Hz, pulse width of 10 ms, and duration of 120s during the first two minutes of each behavioral assay, unless otherwise specified.

2.1.5 Tissue collection and preparation

2.1.5.1 Sacrifice

Study 1B: Rat brains were collected within the first two hours of the start of the inactive cycle. The rats were first dazed and then quickly and painlessly decapitated using a guillotine. The brains were quickly but carefully removed from the skull and flash frozen in 2-Methylbutane (-40°C) until completely frozen (~20-40s) and stored at -80°C until further processing.

Study 1C: Similar to Study 2, the brains were collected within the first 2 h of the inactive cycle. Rats were deeply anesthetized with isoflurane and first transcardially perfused with 150 ml of 1 × Phosphate-buffered saline (PBS) until they were completely bled out. Next, 200 ml of ice-cold 4% paraformaldehyde was perfused transcardially to fix the tissue. Once the liver had turned stiff and muscle contractions had stopped, the rats were decapitated, and the brains were carefully collected. The brains were post-fixed for 7 days at 4°C in 4% PFA to perfuse the tissue around the optic fiber for post-hoc verification. After fixing, the optic fiber was removed, and the brain was stored in 1× PBS and 0.01% sodium azide until further processing.

2.1.5.2 Brain section preparation

2.1.5.2.1 Cryosection preparation (study 1B)

To prepare the flash-frozen brains for sectioning, they were first removed from the freezer (-80°C) and placed in a cryostat-microtome (~ -20°C) (Leica CM 1950, Leica Biosystems) for 1 h for acclimatization prior to sectioning. After acclimatization, frozen brains were embedded in the specimen stage using O.C.T™ (Tissue-Tek) compound consisting of water-soluble glycols and resins. The brains were sectioned into 12 µm slices using a sharp blade, and brain sections were collected from the brain regions of interest using stereotaxic coordinates (Paxinos and Watson 2007). Brain sections from the following bregma levels were collected; mPFC, Bregma: +3.20 to +2.20, ACB, Bregma: +1.70 to +1.00, PVN), Bregma: AMY, PVT, Bregma: -2.12 to -3.2), and VTA, Bregma: -5.2 to -6.00) (Appendix 7). Slices were collected and embedded onto gelatin-

coated SuperFrost Plus slides (Thermo Fisher Scientific) and stored at - 20°C until further analysis.

2.1.5.2.2 Vibratome sectioning (study 1C)

To prepare PFA-fixed brains for immunohistochemical analysis, the brains were sliced using a vibrating blade microtome (Leica V400, Leica Biosystems). The brains were mounted on a specimen-mounting disc using superglue and secured to a vibratome stage. The buffer chamber was filled with 1 × PBS and 50 µm thick slices were cut (frequency 10) from the PVN, Bregma -1.3 – 2.00), and the PVT (Bregma: -2.12 – 3.7) were collected into 12-well plates containing 1 × PBS, 0.01% sodium azide, and stored at 4°C until further processing.

2.1.6 OT receptor autoradiography

Receptor autoradiography was performed for OTR using the [125I]-Ornithine Vasotocin Analog (d(CH2)5[Tyr(Me)2,Thr4,Orn8,[125I]Tyr9-NH2]-OVTA; (Perkin Elmer) as the hot ligand, while OT was used as the cold ligand to determine non-specific binding, as previously performed in our lab (Hansson et al. 2018). The specificity of these ligands has been previously reported (Uhrig et al. 2016; Hansson et al. 2018).

Prior to beginning the experiment, the frozen slides were kept at room temperature for 1 h for acclimatization. Slides were then incubated in room temperature pre-incubation buffer (50 mM Tris-HCl, pH 7.4) twice for 5 min before being transferred into cold pre-incubation buffer. Next, the sections were placed in a humidified chamber surrounded by ice, and 800 µL of reaction mix containing50 pM [125I]-OVTA (specific activity:2200 Ci/mmol (PerkinElmer), 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 0.1% bovine serum albumin, and 0.05% bacitracin was applied to each slide so that all sections were fully covered. Slides were incubated for 60 min at room temperature, and non-specific binding was determined by the addition of 2 µM OT (Tocris) into the incubation mix with [125I]-OVTA. Incubation was stopped by washing the sections three times with ice-cold washing buffer (50 mM Tris-HCl, 10 mM MgCl2) for 5 min, followed by dipping in ice-cold deionized water. Last, the sections were dried overnight under a stream of frigid air and left to dry overnight in the cold room (4-6 Celsius).

To visualize and analyze the data, phosphor imaging plates (FUJI imaging plates, Storage Phosphor BAS-IP SR2025 Screen, GE Healthcare Life Sciences) were exposed for 72 h to the slides with brain sections and scanned in a phosphoimager (Fuji Phosphoimager Typhoon FLA 700, GE Healthcare Life Sciences), as previously described (Hansson et al. 2018). Digital images of the phosphor imaging-generated data were analysed using MCID Image Analysis Software (InterFocus Imaging Ltd). Regions of interest (ROI) were defined based on anatomical landmarks, as illustrated in Figure 5 (circles). The total and non-specific binding (in the presence of the cold ligand) was determined for each ROI on adjacent sections, and the non-specific signal was subtracted from the total signal of each ROI. Similar to our previous work, [125I]-quantitation standard curves (Amersham, GE Healthcare Life Sciences) were used to extrapolate the measured optical densities (photostimulable luminescence per mm2) of the tissue-equivalent OXTR densities from sections into nCi/mg (Hansson et al. 2018). Binding in femtomoles per milligram (fmol/mg) was calculated according to the saturation binding equation (B = Bmax*[R]/(Kd +[R]), where Bmax represents the maximal bound receptor, Kd represents receptor affinity (Kd = 0.1 nM) in rat tissue (Liberzon and Young 1997), and [R] represents the concentration of the radioligand with which the specific activity of the radio ligand could be calculated. Data are expressed as fmol/mg protein (mean ± SEM). This methodology was adapted from (Hansson et al. 2018).

2.1.7 Immunohistochemistry

Immunohistochemistry was performed as previously described (Knobloch et al. 2012) on free-floating sections from regions of interest in 12-well plates (Corning Costar, Sigma-Aldrich). The following antibodies were used for immunohistochemical staining; anti-Ds-Red (1:1000; rabbit; Clontech, Cat#632397), Anti-oxytocin (1:1000, mouse, T-5021, 1:1000). Staining was detected using secondary antibodies, including Alexa 488 and Texas-Red 594 (Invitrogen). All secondary antibodies were diluted into a concentration of 1:500.

First, the sections were incubated in blocking buffer containing: 1 × PBS, 1% Triton, and 5% normal goat serum for 2 h at room temperature with gentle agitation. One milliliter of blocking buffer was used for every four slices. Second, the sections were incubated with the primary antibody together with: 1 × PBS, 1% Triton for 48 h at 4°C

with gentle agitation. Following primary antibody staining, the sections were washed in 1 × PBS three times for 10 min each with gentle agitation. Third, the sections were incubated with the fluorescent secondary antibody, together with: 1 × PBS and 1% Triton for 2 h at room temperature in the dark with gentle agitation. The sections were then washed again in 1 × PBS four times for 10 min each, with gentle agitation. Finally, the sections were mounted on Superfrost Plus microscope glass slides (VWR) (four sections per slide) and allowed to dry completely before being covered with glass coverslips (24 × 60 mm). High-quality images of the immunostained sections were obtained using a confocal laser-scanning microscope (Leica TCS SP5, Leica Biosystems).

2.1.8 Data analysis

The data analysis proceeded using univariate and mixed analysis of variance (ANOVAs) and statistically significant interactions, and main effects were followed up using Bonferroni-corrected pairwise comparisons, except when the interaction involved a within-group factor; paired t-tests were used. An alpha level of $p < 0.05$ (two-tailed) was set as the level of statistical significance, and we report partial eta squares as estimates of effect sizes along with individual data points for clarity. Statistical analyses were conducted using SPSS (29.0), and all graphs were illustrated in Graph Pad Prism.

2.2 Adolescent social instability stress (study 2)

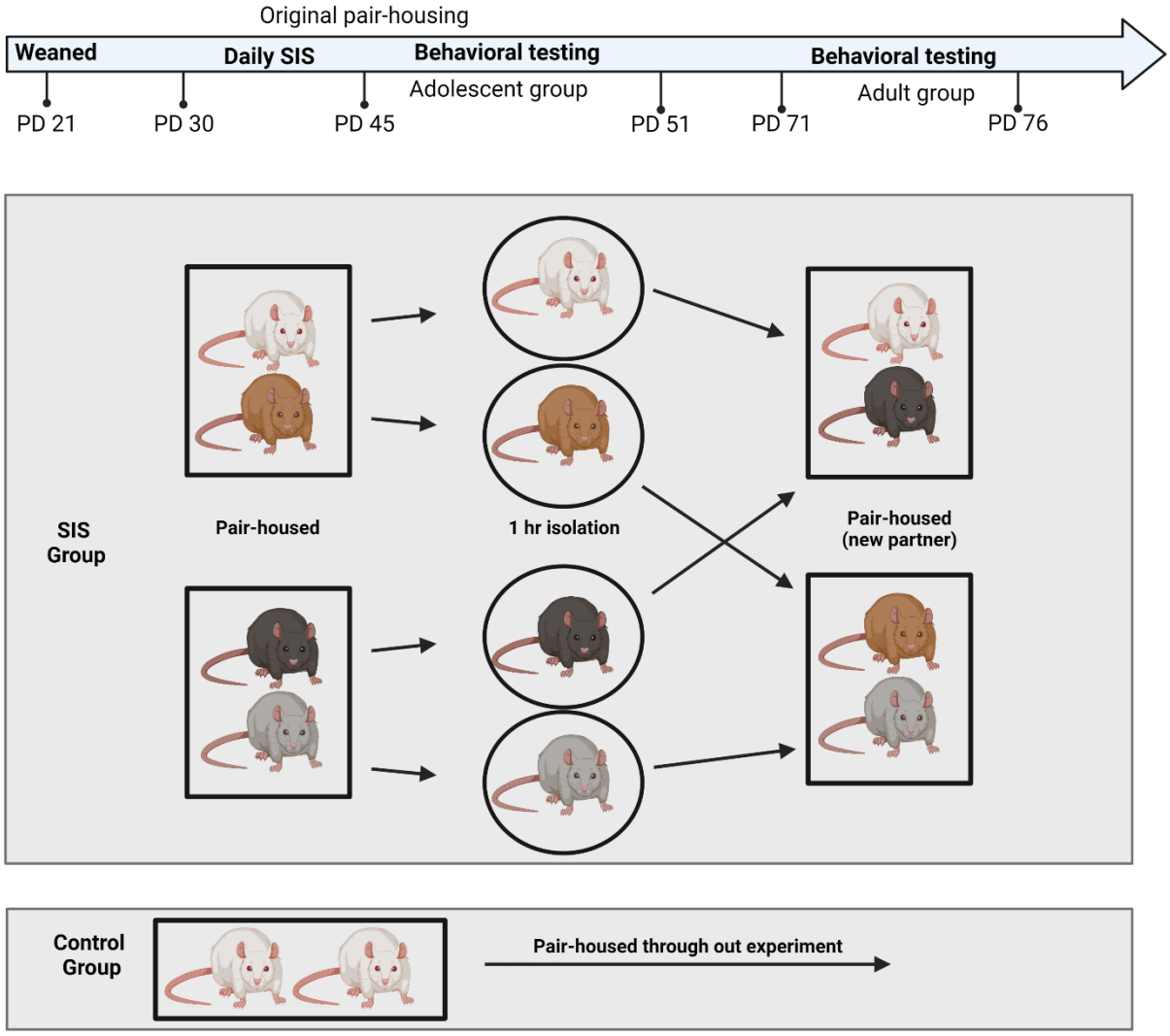
The methods for this study was recently published (Graf et al. 2023).

2.2.1 Subjects

Male (n = 64) and female (n = 64) outbred **WIST:RccHan** rats were obtained from Envigo (ON, Canada). Rats arrived at the institute on PD 22 and were transferred to the colony room for acclimatization until PD 30. Rats were housed in same-sex pairs in individually ventilated cages (IVCs) without enrichment. Male and female rats were housed in separate colony rooms. The colony rooms were maintained under an artificial 12 hr dark-light cycle (lights on: 05:00) with temperature (21 ± 1°C) and humidity (35%) control. Standard laboratory chow (Teklad 14% Protein Rodent Diet) and water were provided ad libitum throughout the study. Two male rats were excluded from the study because of tooth abnormalities. The experimental procedures were approved by the Brock University Institutional Animal Care Committee (ACC) and were

carried out in adherence to the Canadian Council on Animal Care, the ARRIVE and 3Rs guidelines.

2.2.2 Study design



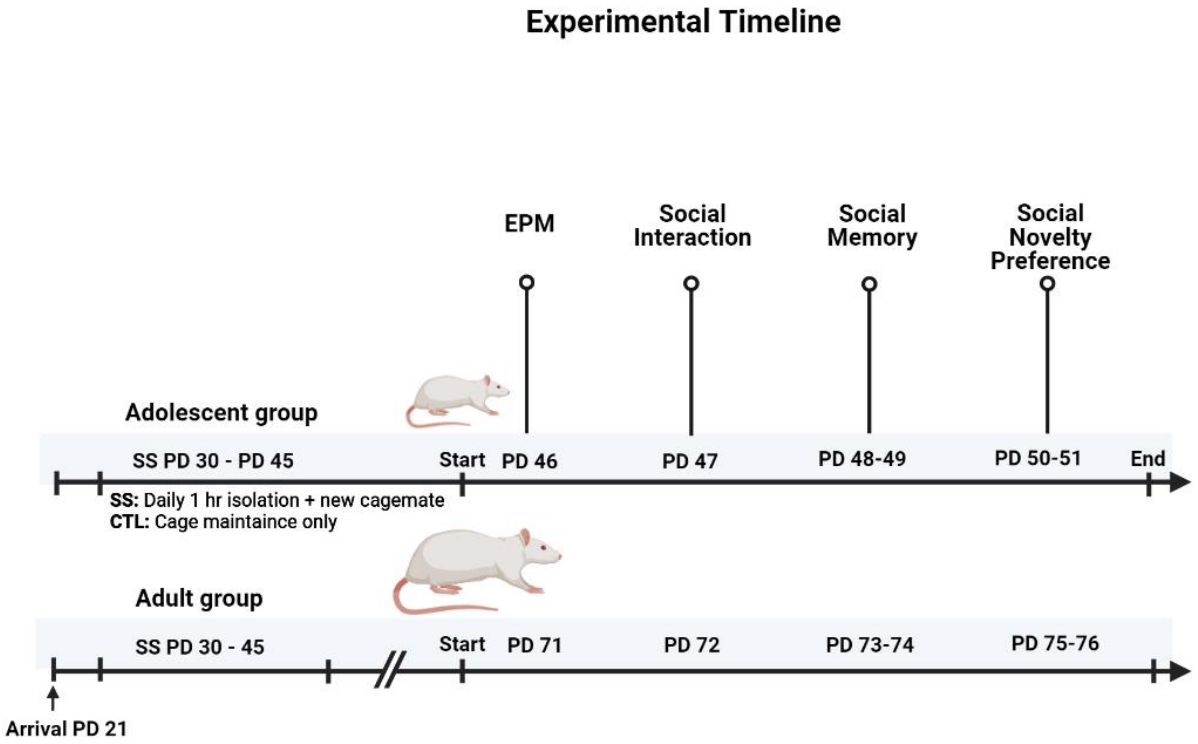


Figure 9: Timeline for the social instability procedure and behavioral testing. SIS rats underwent daily 1 h confinement and were rehoused with a previously unfamiliar cagemate, whereas CTL rats were pair-housed with the same cage mate. The adolescent group underwent behavioral testing between PD46-51 and the adult group between PD71-76.

Rats were randomly assigned to experimental groups of 16: stress group, (SIS vs. CTL) x sex (male vs female) x age (in adolescence vs adulthood). The SIS procedure was performed as previously described in (McCormick and Green 2013). On PD30, all rats were weighed, and SIS rats were confined in a 12.7 × 8.25 cm round ventilated plastic container in a separate experimental room for 1 h each day for 16 days. After confinement, rats were returned to the colony room and were rehoused in a new cage with an unfamiliar cage mate from the same condition. The SIS procedure was performed during the inactive cycle (light phase) at pseudo-random times to minimize habituation to stressors. After the end of SIS, the experimental rats were pair-housed with their original cage-partner, which had undergone the same SIS procedure. Meanwhile, CTL rats were kept pair-housed with the same cagemate and left undisturbed until time of testing except for cage changes and getting weighed. The adolescent group was assessed in a battery of behavioral tests immediately after the end of the SIS procedure (PD46 – 51), whereas the adult group was left undisturbed

until adulthood and assessed for the long-term effects of SIS (PD71 – 76). The experimental design and timeline are shown in Figure 9.

2.2.3 Behavioral tests

Behavioral tests were performed over six days (PD46-51 or PD71-76). The stimulus rats were novel conspecifics that were age- and sex-matched to the experimental rats. Stimulus rats were habituated to the test arenas for two days before testing. Tests were conducted during the dark phase (between 18:00 and 21:00) of the diurnal cycle under dim overhead red light. The test apparatus was cleaned using 70% ethanol for each trial. The exclusion criterion was set based on the tests. The tests were performed with n = 16 per group, unless otherwise stated. The videos were recorded with a video camera (Sony Handycam HDR-CX405) mounted overhead on the ceiling above the test apparatus. Behavioral testing and scoring of videos were conducted manually by experimenters blinded to the rats' stress condition.

2.2.3.1 Elevated plus maze

The EPM was used to assess anxiety-like behaviors in rats (reviewed in Hogg, 1996) and was performed as described in our previous reports under white light illumination (Hodges et al., 2018). The maze is a plus-shaped apparatus (64 × 64 cm2) raised 80 cm from the ground and consists of four equally long arms (51 cm) connected by a central platform (8 × 8 cm2). The two opposite arms are enclosed by walls (30 cm high), and the other arms are not enclosed (open arms). Rats were placed in the maze without habituation. At the start of each trial, the rat was gently placed on the central platform facing the open arm and allowed to freely explore the maze for 5 min. These behaviors were scored during video analysis: time spent on the open and closed arms and the number of open and closed arm entries (defined as two front paws crossing a border). The elevated plus maze was performed on PD 46 for the adolescent group and on PD 71 for the adult group. A video-recording problem excluded two rats from the analysis. A third was excluded as an outlier (spent 99% of time in the closed arm).

2.2.3.2 Social interaction test

The social interaction test is used to measure social anxiety (Johnston & File, 1991), and was performed as previously described (Hodges et al., 2017). The test rat was first placed in a polyvinyl chloride PVC arena (61 × 30 × 53 cm) followed by a novel conspecific from the same condition (i.e., SS rat with an unfamiliar SS rat, CTL rat with an unfamiliar rat). The rats interacted for 15 min and social interaction was scored

based on the time spent in social behaviors initiated by the experimental rat: (a) anogenital and (b) non-anogenital investigation of the partner (sniffing or licking the anogenital or any part except for the anogenital area, respectively; (c) approaching and/or following the social partner throughout the test arena; and (d) allogrooming, mechanical motion of grooming resembled scratching, picking, stroking, or rubbing (in some animals licking and nibbling) and is directed towards the outer body surface). Investigation bouts had to last for at least one second to be scored. The social interaction test was performed on PD47 for the adolescent group and on PD72 for the adult group.

2.2.3.3 Social Recognition Memory Test

The novel social recognition test was used to assess social recognition memory across 30- and 90- min retention interval, as described by Hodges et al. (2017), and capitalizes on rats' preference for novelty. Social recognition memory was assessed using a crossover design across two days, with each rat undergoing both the 30- and 90-min retention intervals, with the order of the intervals counter-balanced across rats. The adolescent group was tested in the social recognition memory test on PD48 and 49 and the adult group on PD73 and 74. Cagemates were tested simultaneously in opposing intervals in separate apparatuses. The apparatus consisted of three PVC chambers, with one large central arena (61 x3 1 x 52 cm) and at each end are two smaller chambers (22 x 24 x 20). The experimental rat was placed in the middle arena, with a stimulus rat in each of the smaller chambers. The middle arena and end chambers were separated by plastic mesh screens with many smaller holes (6 mm) for sniffing the conspecific and one larger hole (12.7 mm) for direct social contact.

The novel social recognition test consisted of two phases: a familiarization phase followed by an inter-trial interval and a test phase. Before the familiarization phase, two novel stimulus rats (age- and sex-matched) were placed in each end chamber. Experimental rats were placed in the middle chamber and allowed to explore freely for 5 min. After the familiarization phase, all rats were removed from the apparatus. The experimental rats were isolated for the duration of the inter-trial interval (30 or 90 min) in a separate room in a cage of similar dimensions to the home cage (37 cm × 20 cm × 13 cm) and bedding from the original home cage. The stimulus rats were returned to their home cage until required during the test phase. During the test phase, one side chamber contained a familiar stimulus rat from the familiarization phase and the other

contained a novel stimulus rat. The test rats were placed in the middle arena and allowed to explore for 5 min. The positions of the familiar and novel conspecifics were counterbalanced to minimize any bias of side preference. After the test phase, all the rats were returned to their home cages. The time spent investigating the stimulus rats was measured in both phases. Rats that spent less than 10 s investigating both stimulus rats during the familiarization or test phase were excluded from the analysis (six rats were excluded; two adolescent control females and four adult SIS males) to ensure sufficient investigation time for memory formation. More time spent investigating the novel stimulus rat than the familiar stimulus rat was measured as the social recognition memory.

2.2.3.4 Social novelty preference test

The social novelty preference test was used to assess the preference of rats to investigate a novel versus familiar conspecific. The test was based on a previous study (Smith et al., 2015) with some alterations. The cagemate of the test rat was used as the familiar conspecific; thus, testing was conducted over two days such that both rats could be tested. The same three-chambered apparatus was used in the previous novel social recognition test. Similar to the novel social recognition test, a novel stimulus rat and familiar rat (cagemate) were placed in the end chambers. The side of the novel and familiar rat were counterbalanced. The experimental rats were then placed in the middle arena and allowed to explore for 15 min. The measures scored were social novelty preference (time spent investigating novel vs. familiar peers), social vigilance (Williams et al., 2020) (time spent facing conspecific, novel vs. familiar peers), social avoidance (Williams et al., 2020) (time spent huddling in corners of novel vs. familiar peers), rearing frequency, and grooming frequency. The social novelty preference test was performed on PD50-51 for the adolescent group and PD75-76 for the adult group. Rats that spent less than 10 s investigating the conspecifics during the test were excluded from the analysis (six rats were excluded).

2.2.4 Data analysis

Statistical analyses were conducted using SPSS (28.0), and all graphs were illustrated in Graph Pad Prism 8 and edited with Photoshop. The analysis of the data proceeded using univariate and mixed ANOVAs as statistically significant interactions, and main effects were followed up using Bonferroni-corrected pairwise comparisons, except when the interaction involved a within-group factor; paired t-tests were used. An alpha

level of $p < 0.05$ (two-tailed) was used to determine statistical significance, and we report partial eta squares as estimates of effect sizes.

2.3 Adolescent peer rejection (study 3)

The following methods were published (Surakka et al. 2021) and only small modifications for improving clarity have been made.

2.3.1 Subjects

Male (n = 24) and female (n = 24) outbred **WIST.RccHan** rats and female (n = 120) and (n = 72) male **Fischer 344** rats with the former obtained from Envigo (Horst, Netherlands) and the latter from Charles River (Sulzfeld, Germany). All the rats were delivered to our institute on pd 21. On arrival, the rats were assigned to either condition. All rats were group-housed (four rats per cage) throughout adolescence into adulthood (unless otherwise stated) in standard type IV rat cages (Ehret, Emmendingen, Germany). Male and female rats were kept in separate colony rooms. The colony rooms were maintained under an artificial 12 hr dark-light cycle (lights on: 08:00) with temperature (21 $\pm$ 2°C) and humidity (35-50%) control. Standard laboratory chow (Ssniff, Soest, Germany) and tap water were provided ad libitum throughout the study. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe, Germany) and were conducted in accordance with the local Animal Welfare Act and the European Communities Council Directive of 22 September 2010 (2010/63/EU).

2.3.2 Study design

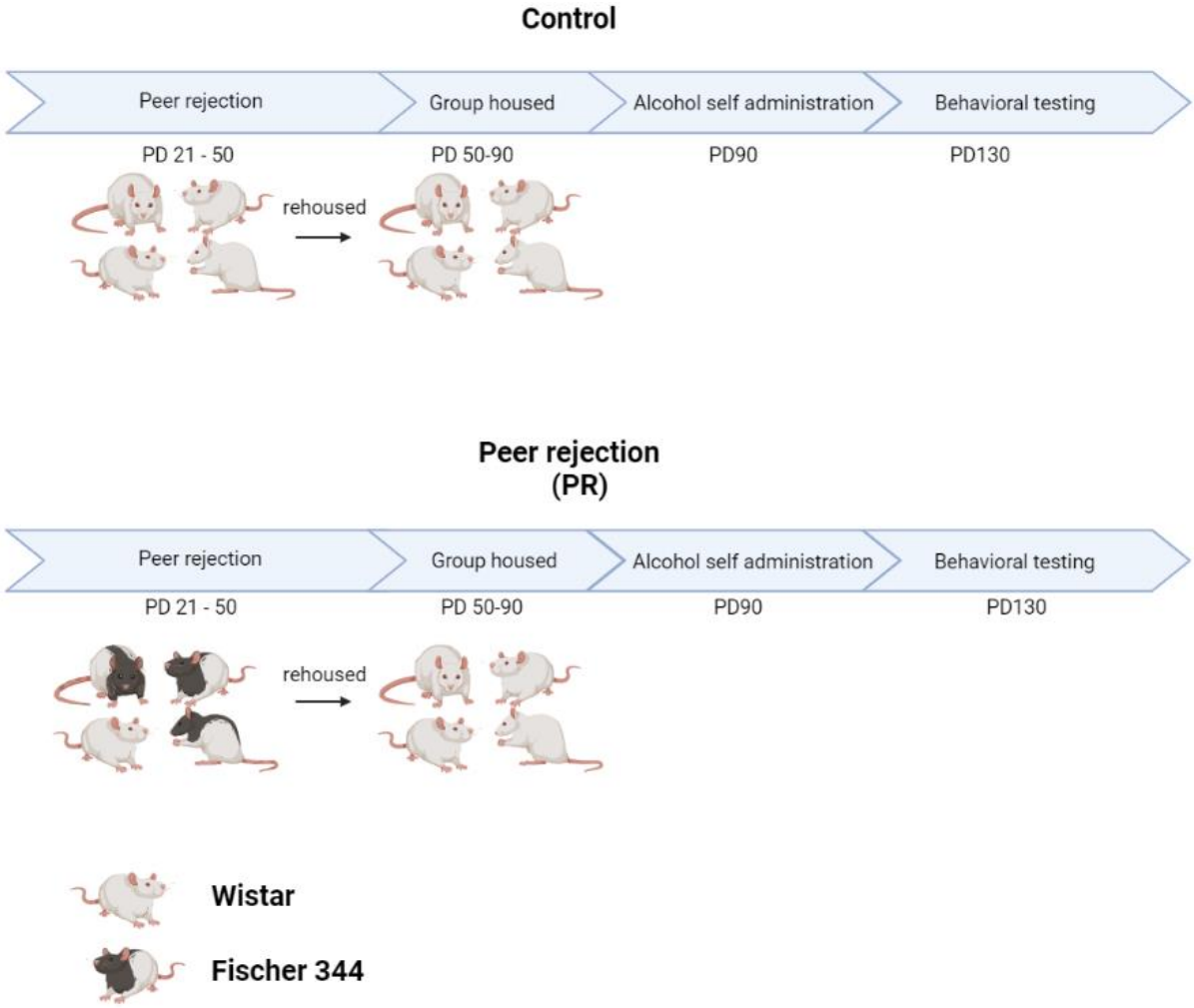


Figure 10: Timeline for PR procedure and behavioral testing. Three Fischer 344 rats were housed with one WIST rat in the PR condition, whereas CTL rats (WIST) were group-housed with the same cagemates throughout the study. Rats were tested for relapse-like behaviors using the alcohol self-administration paradigm and other behavioral tests. Adapted from the study by Surakka et al. (2021).

The study design was based on our previous studies on PR (Schneider et al., 2016a). In the present study, WIST rats were subjected to either control or PR conditions during adolescence, immediately after weaning, into late adolescence (days 21–50). Male and female WIST rats were housed in the same sex groups of four under standard housing conditions in separate rooms. In the PR condition, one WIST rat was housed with three age- and sex-matched Fischer 344 rats. WIST rats and the less playful Fischer 344 exhibit a mismatch in their social play behaviors. Fischer 344 rats inadequately reciprocate social play initiated by WIST rats, mimicking aspects of PR.

On pd 50, control WIST rats were re-grouped with unfamiliar WIST rats and peer rejected WIST rats were grouped with other peer rejected rats, terminating the PR procedure. After the PR period, rats were allowed to recuperate with other peer-rejected rats of the same sex. All rats underwent alcohol self-administration training during adulthood (Figure 11).

2.3.3 Behavioral experiments

Following PR, rats were left alone until PD 90 (besides weekly cage changes) when they began alcohol self-administration acquisition, followed by extinction and cue-induced reinstatement. We also tested rats in the home-cage locomotor activity and sucrose consumption following the self-administration paradigm.

2.3.3.1 Cue-induced reinstatement of alcohol-seeking

Figure 11: Timeline of alcohol cue-induced reinstatement paradigm.

2.3.3.1.1 Operant alcohol self-administration apparatus

Cue-induced reinstatement of alcohol-seeking behavior was performed in operant chambers (MED Associates Inc.) enclosed in ventilated sound-attenuating cubicles in control/PR female (n=20/20) and male (n=12/12) rats. Each side panel of the chamber was equipped with a response lever. Responses at the active lever activated a syringe pump that delivered a ~30µl drop of fluid into a liquid receptacle next to it. Responses at the inactive lever were recorded but had no programmed consequences. A light stimulus (house light) was mounted above the left and right response levels of the self-administration chamber and served as a discriminative stimulus. The house light indicated the availability of the reward (alcohol/water) as well as an indicator of “time-out” (blinking light), during which lever presses did not result in reward delivery, although lever responses were recorded. Fluid delivery, presentation of stimuli and data recording were controlled with Med-PC-V software (MED Associates Inc., St. Albans, VT).

2.3.3.1.2 Alcohol self-administration acquisition and extinction phase

Rats were trained to self-administer 10% (v/v) alcohol with initial fluid deprivation as previously described (Surakka et al., 2021). During the first two days of acquisition, the rats were fluid-deprived for 20 h per day. After the initial two days, alcohol and water sessions were conducted without fluid deprivation. Acquisition sessions were a pseudo-random performed with a maximum of three consecutive training days of one solution, until the rats had performed 10 alcohol (excluding the two fluid deprivation acquisition sessions) and 10 water sessions. Rats trained to lever press on a fixed ratio 1 (FR1) to self-administer 10% (v/v) alcohol and water over a 30-minute session once per day, five days a week. Orange olfactory stimuli served as the discriminative stimulus (S+) for alcohol, whereas water availability was indicated by lemon-grass olfactory stimuli (S-). These olfactory stimuli were generated by dripping a few drops of the respective extract into the bedding of the operant chamber prior to each session. A lever press on the alcohol-associated lever during alcohol sessions was accompanied by a 5-s blinking-light conditioned stimulus (CS+), whereas a 5-s constant-light stimulus (CS-) was presented with water delivery during water sessions. In response to the lever press (CS+ or CS- sessions), a reward of 30 µl was delivered into the liquid receptacle, followed by a 5 s time-out during which responses were recorded but had no consequence. Rats that did not achieve <50 lever presses during the last three days of acquisition were excluded from the study.

Following the acquisition phase, rats underwent daily 30-minute unreinforced extinction sessions for five consecutive days. These sessions were sufficient to reduce response rates, approximating the extinction criterion of 20% of the last three days of alcohol acquisition. All rats met this criterion. No discriminative stimuli were presented during the extinction sessions. Responses to the previously active lever activated the syringe pump without resulting in the delivery of either alcohol or water or the presentation of discrete cues (stimulus blinking light or constant light).

2.3.3.1.3 Alcohol cue-induced reinstatement

Reinstatement testing began two days after the final extinction session and was conducted under the same conditions as the acquisition phase without reward delivery. Discriminative and discrete cues predicting alcohol or water availability were presented during the cue-induced reinstatement session. The reinstatement tests were initiated by extending both levers. Responses to the active lever activated the syringe pump

and triggered presentation of the appropriate conditioned stimulus. Reinstatement testing was counterbalanced, with half of the rats assessed under the alcohol-associated condition on day one and the water-associated condition on day two, and vice versa. This was performed to account for the potential effects of the testing day. The number of responses to the active and inactive levers was recorded.

2.3.3.2 Circadian locomotor activity measurements by the E-motion system

Home cage locomotion was measured after alcohol reinstatement in control and peer rejected female (n=12/12) and male (n=12/12) rats that were single housed for the duration of the test (3 days). The number of movements was monitored using an infrared sensor connected to a recording and data storing system (Infra-e-motion). An Infra-e-motion device was placed above each cage (30 cm from the bottom) so that the rat could be detected at any position inside the cage. The device was sampling every second whether the rat was moving or not. The sensor detected body movements of the rat of at least 1.5 cm from one sample point to the next. The data measured by each Mouse-E-Motion device were downloaded onto a personal computer and processed with Microsoft Excel.

2.3.3.3 Sucrose consumption test

To study potential alterations in reward sensitivity, sucrose consumption was measured in control/peer-rejected female (n=12/12) and male (n=12/12) rats that were housed individually. All the rats in this experiment were housed in separate single-house cages. Two pre-weighed bottles, one containing tap water and the other containing a sucrose solution, were placed in each cage. The positions of the bottles were counterbalanced to avoid side bias subsequent 24-hour intake of water and sucrose solution was measured. After 24 h, the bottles were removed and re-weighed, and the liquid consumption was measured as the difference in the weight (g) of each bottle before and after the test. Sucrose preference was calculated using the following formula: Sucrose preference = [consumed sucrose solution/total liquid consumed (sucrose solution + water)] × 100. All rats were given ad libitum access to one bottle of tap water and another bottle of sucrose solution for 24 h. Male rats received 0.35% (w/v) sucrose solution, whereas female rats were given 0.15% (w/v) sucrose. We found that females consumed more sucrose solution than males at equal concentrations to sweet tasting solutions and therefore used a lower concentration for testing females (unpublished data).

2.3.4 Data analysis

Data obtained from the self-administration and cue-induced alcohol-seeking experiments were analyzed using a mixed multifactorial ANOVA with repeated measures for sex (female vs. male), condition (control vs. peer-rejected), lever (active vs. inactive), and session (extinction vs. reinstatement]). Locomotor activity data was analyzed using 1-hour recordings using three-way ANOVA with repeated measures of sex (female vs. male), group (control vs. peer-rejected) and day (1,2,3). Furthermore, 24-hour locomotor activity and dark- and light-phase data were analyzed using two-way ANOVA. One-way ANOVA was used to analyze sucrose intake and preference conditions (control vs. peer-rejected). Post-hoc testing (Tukey's HSD) was performed when appropriate. Statistical significance was set at $p < 0.05$. All analyses were conducted using SPSS Statistics version 26.0, and figures were illustrated using GraphPad Prism version 6.0.

3 RESULTS

To address the first aim of the thesis we characterized how the timing of ASI (early or late) influenced adult social and anxiety-like behavior in male and female rats (study 1A). In a separate cohort, we proceeded with an OTR autoradiography study in target areas known to influence social and anxiety-like behavior (study 1B). The experiment (1B) was performed together with the master student Rafi Bor. We identified the PVN and PVT in EASI female rats as the two regions of interest with significant increase in OTR binding.

Our initial findings demonstrated alterations in OTR availability within the PVN and PVT, simultaneously yet independently our collaborators using viral vectors and immunohistochemistry confirmed a vast population of OTRs in the PVT and projections from the PVN to the PVT (Figure 6). We therefore performed a validation experiment with female EASI rats which should the largest molecular alteration across condition and sex and compared them to female control rats. Specifically, we investigated how axonal OT release in the PVT influenced behavior. These rats received PVN injections of either rAAV1/2-OTp-ChR2-mCherry or rAAV1/2-Otp_Venus and optic fiber implantation into the PVT. We then tested the effects of optogenetic-evoked axonal OT release in the PVT on the novel object recognition, social interaction, social recognition memory tests, hotplate with CFA, and fear conditioning. Arthur Lefvere and Stephanie Küppers from the Grinevich lab helped with surgeries and data collection.

To build on the first aim, we characterized the short- and long-term behavioral outcomes of SIS in WIST male and female rats (study 2). The study was performed in collaboration with the McCormick Lab at Brock University. The social interaction data were scored by Akif Eltahir, half of the social recognition memory data were scored by my Shealin Murray, and Smit Patel scored the EPM data. The author of the dissertation led the design of the study, collected, scored, and analyzed the remaining data, and wrote the manuscript. The results were recently published (Graf et al. 2023), and only small alterations to the figure design have been made for consistency.

To address the second aim of the thesis, we used our novel adolescent PR model to investigate the long-term effects on alcohol seeking behavior in both male and female

rats (study 3). Data was collected by Ivan Skorodumov and Marcus Meinhardt. The data was analysed, and the manuscript was written by the author. The data from PR study was published recently (Surakka et al. 2021). The figures were modified from the publication to show individual data points for more clarity and consistency.

3.1 Adolescent social isolation (study 1)

3.1.1 Behavioral characterization of the persistent effect of adolescent social isolation (study 1A)

In short, we observed a general decrease in social recognition memory in both stress groups (EASI and LASI) and sexes. Male EASI rats demonstrated heightened thermal pain sensitivity, whereas the opposite was true for females. Both male and female LASI rats showed reduced social interactions. In the hotplate test, male LASI rats showed increased thermal pain sensitivity compared to CTLs, while female LASI rats displayed faster latencies than female EASI rats. All data collected and analyzed by the author.

3.1.1.1 Elevated plus maze

ASI did not influence adult anxiety-like behavior in the EPM ($p > 0.058$), as indicated by more time spent in the open arm, although EASI males spent more time in the center zone compared to CTL males, which has been suggest to reflect an indirect of anxiety-like behavior (Rico et al. 2017) (see discussion). Female LASI rats showed more general activity in the EPM, as indicated by the total number of arm entries. The EPM data were analyzed using stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVAs. The ANOVA for time spent in the center zone revealed an interaction between stress group and sex ($F_{(1, 68)} = 3.199$, $p = 0.047$, $\eta p2 = 0.086$). Pairwise comparisons revealed that male EASI rats spent more time in the central zone than female CTL rats (Figure 12A). Additionally, males spent more time in the center zone than did females ($F_{(1, 68)} = 11.396$, $p = 0.001$, $\eta p2 = 0.144$) (Figure 12B). The other main effects were not significant ($p > 0.159$). The interaction between the stress group and sex was statistically significant for the total number of arm entries ($F_{(1, 68)} = 5.390$ $p = 0{,}0004$, $\eta p2 = 0.165$) and the main effect of sex ($F_{(1, 68)} = 13.395$, $p = 0.001$, $\eta p2 = 0.144$). Female LASI rats made more total entries compared to EASI ($p < 0.009$), CTL ($p < 0.027$), and male LASI ($p < 0.00001$) rats (Figure 12C). In general, female rats made more total arm entries than did male rats ($p < 0.0004$). The different phases of the estrous cycle did not influence anxiety-like behavior (data not shown). Summary statistics in Appendix 1.

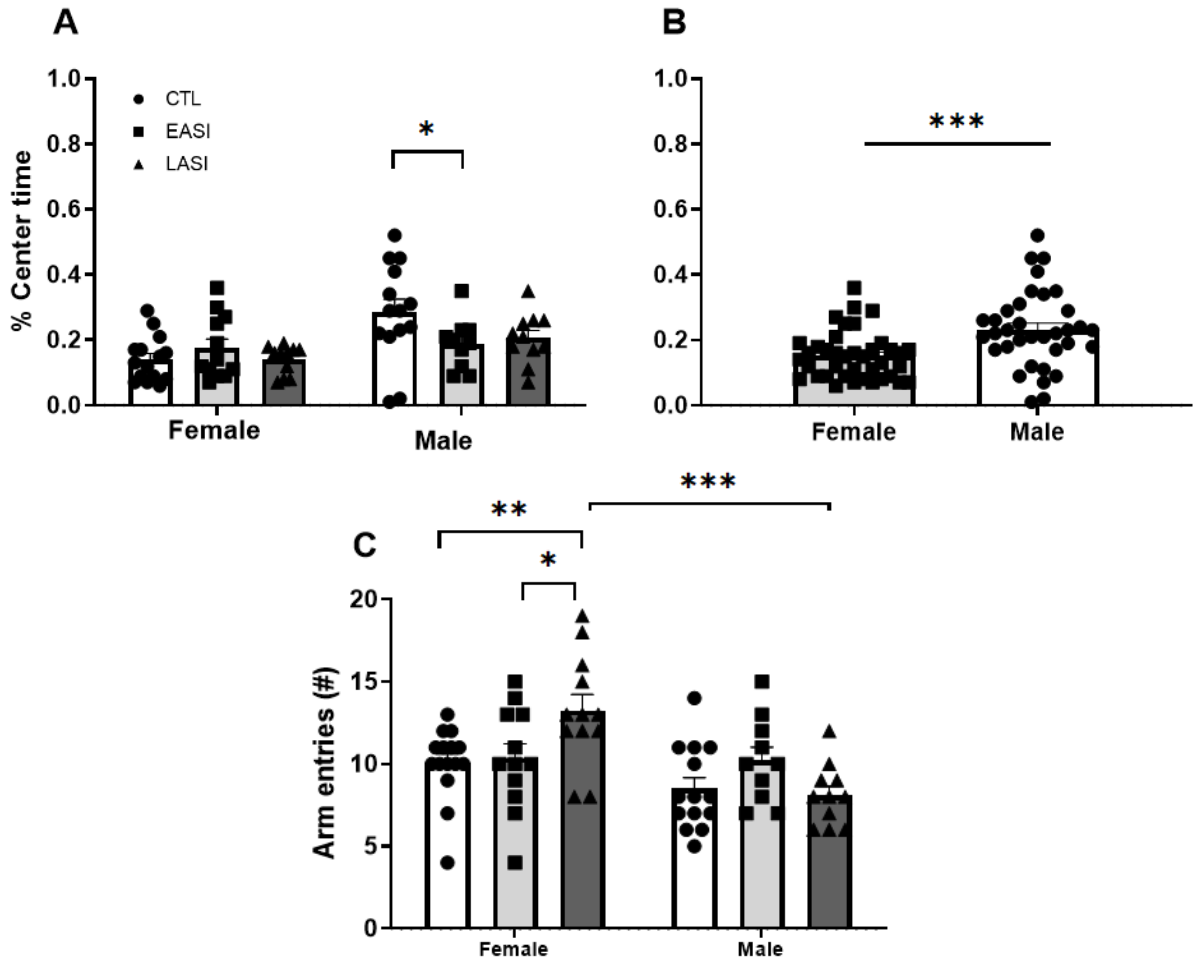


Figure 12: Behavioral performance in the elevated plus maze (A) percent time spent in the center zone, (B) sex effect (all conditioned collapsed into two columns) of percent time spent in the center zone, and (C) total arm entries in CTL (control), early (EASI), and late (LASI) adolescent social isolation groups tested in adulthood (PD 90). Data are shown as individual data points with the mean ±SEM. *p < 0.5, **p < 0.01.

3.1.1.2 Open field

We observed no long-term effects of ASI on the adult locomotor activity in the OFT. The OFT data were analyzed using a stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVA, which revealed a sex effect. Female rats travelled a longer distance during the 30 min OFT compared to males ($F_{(1, 74)} = 51.395$ $p < 0.000001$, $\eta p2 = 0.410$) (see Appendix 2). No effect was observed in the stress group ($F_{(1, 74)} = 0.705$, $p = 0.498$, $\eta p2 = 0.019$). Summary statistics in Appendix 2.

3.1.1.3 Novel object recognition memory

Adult ASI rats did not differ in object recognition ability. NOR data were analyzed using stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVA. The ANOVA revealed

that ASI rats did not differ from CTL in time spent investigating the objects during the acquisition phase ($F_{(1, 74)} = 0.911$, $p = 0.407$, $\eta p2 = 0.024$). All rat preferences during the test phase differed from chance ($t_{(9)} = 12.095$, $p < 0.000001$; Hedge's $g = 11.115$). No differences were observed between the discrimination ability of ASI and CTL rats for novel objects during the test phase ($F_{(1, 74)} = 51.395$, $p < 0.000001$, $\eta p2 = 0.410$). Summary statistics in Appendix 3.

3.1.1.4 Social interaction

ASI rats spent less time in social interactions (per individual based on which rat initiated the interaction) than CTL rats, which was largely driven by a reduction in interaction times in the LASI groups of both sexes. SIT data were analyzed using a stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVA. The main effect of stress group was statistically significant ($F_{(1, 74)} = 9.036$, $p = 0.0003$, $\eta_p2 = 0.196$). Pairwise comparisons revealed LASI rats spent less time in social investigation than EASI ($p = 0.003$) and CTL ($p < 0.0005$) rats (Figure 13A). Further analysis demonstrated a decrease in non-anogenital sniffing bouts between stress groups ($F_{(1, 74)} = 5.483$, $p = 0.006$, $\eta_p2 = 0.129$). LASI rats performed fewer non-anogenital social investigation bouts than CTLs ($p = 0.013$) and EASI ($p = 0.031$) rats (Figure 13B). Additionally, male rats performed more non-anogential social bouts than did female rats ($F_{(1, 74)} = 24.670$, $p = 0.000004$, $\eta_p2 = 0.250$) (Figure 13D). We observed no effect of ASI on the frequency of anogenital sniffing bouts ($F_{(1, 74)} = 3.001$, $p = 0.056$, $\eta_p2 = 0.075$ (Appendix 4). However, we did observe an interaction effect of the stress group and sex on rearing behaviors during the SIT ($F_{(1, 74)} = 4.341$, $p = 0.017$, $\eta_p2 = 0.105$) and a main effect of the stress group ($F_{(1, 74)} = 8.887$, $p = 0.0003$, $\eta_p2 = 0.194$). Post-hoc analysis of the interaction effect revealed that male EASI rats reared less than LASI ($p = 0.00001$) and CTL ($p = 0.012$) rats, and male LASI rats reared more than LASI female rats ($p = 0.008$). Summary statistics in Appendix 4.

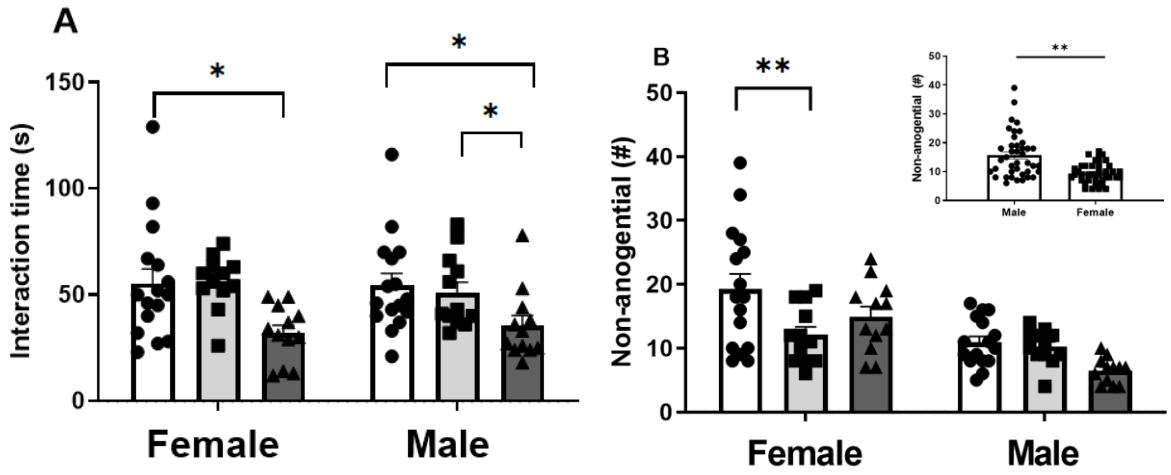


Figure 13: Behavioral performance in the social interaction test. (A) Total social interaction time in the social interaction test (SIT) and (B) number of non-anogenital sniffing bouts in the CTL (control), early (EASI), and late (LASI) adolescent social isolation groups tested in adulthood (PD 96). Data are shown as individual data points with the mean ±SEM. *p < 0.5, **p < 0.01. Main effect of sex on the small figure.

3.1.1.5 Social recognition memory

ASI rats demonstrated impaired social recognition memory compared to CTL rats. Social recognition memory data were analyzed using a stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVA. No differences were observed in the acquisition phase of the test ($F_{(1, 74)} = 0.794$, $p = 0.456$, $\eta_p2 = 0.021$). We tested rat preference against chance during the test phase before further analysis. The one sample t-test was statistically significant ($t_{(9)} = 10.115$, $p < 0.000001$; Hedge's $g = 12.735$). Analysis of the test phase revealed a main effect of stress group ($F_{(1, 74)} = 11.241$, $p = 0.00003$, $\eta_p2 = 0.241$) (Figure 14). Pair-wise comparison showed that EASI ($p = 0.00002$) and LASI ($p = 0.018$) rats showed impaired social recognition ability compared to CTL rats (Figure 14, small figure). Summary statistics in Appendix 5.

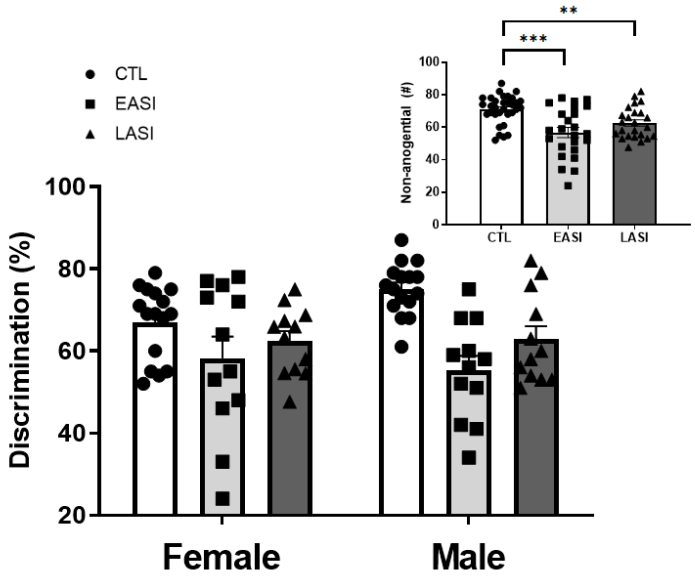


Figure 14: Behavioral performance on the social recognition memory test: The ability of rats to discriminate between social partners was indicated by a decrease in % discrimination compared to CTL. Data are shown as individual data points with the mean ±SEM. ***p** < 0.5, **p < 0.01, ***p < 0.001. Main effect of stress group in small figure (top left).

3.1.1.6 Hotplate test

Male EASI and LASI rats showed heightened thermal pain sensitivity, while female EASI rats demonstrated a reduction in thermal pain sensitivity in the hotplate test. The hotplate test data were analyzed using stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVA. The interaction effects of stress group and sex ($F_{(1, 74)} = 11.843$, $p = 0.00003$, $\eta_p2 = 0.242$) (Figure 15) and the main effects of stress group ($F_{(1, 74)} = 3.380$, $p = 3.380$, $\eta_p2 = 0.039$) and sex ($F_{(1, 74)} = 7.812$, $p = 0.007$, $\eta_p2 = 0.095$) were statistically significant. Pairwise comparisons showed that both EASI ($p = 0.0005$) and LASI ($p = 0.001$) rats demonstrated lower thermal pain sensitivity than male CTL rats. Female EASI rats showed reduced pain sensitivity compared to LASI ($p = 0.031$) and CTL ($p = 0.031$) rats. Male CTL had longer thermal latencies than female CTL. No differences were observed between the estrous cycle phases in female rats. Summary statistics in Appendix 6.

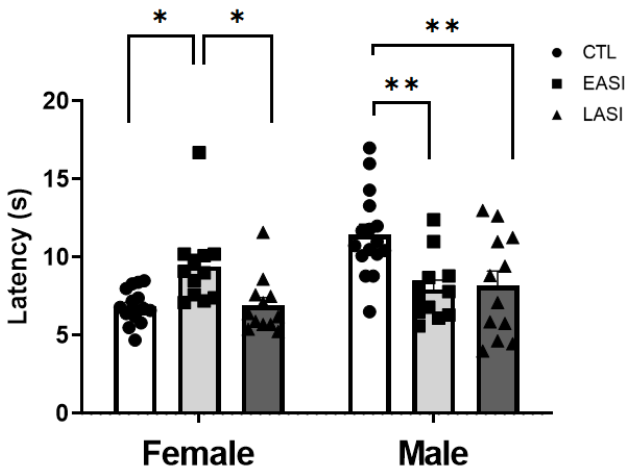


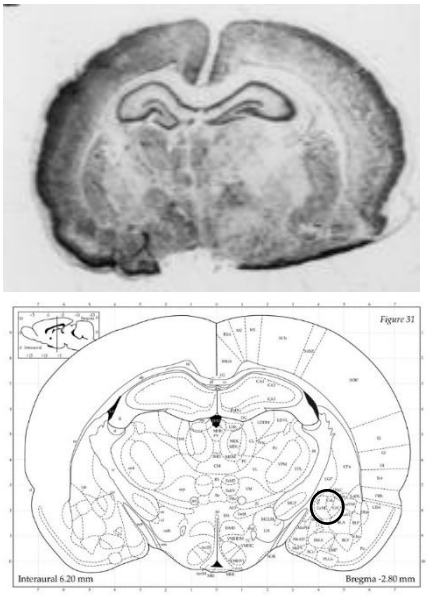
Figure 15: Behavioral performance on the hotplate test (52.5°C). Latency to react to thermal pain stimuli in the hotplate test compared with CTL. Data are shown as individual data points with the mean ±SEM. ***p** < 0.5, **p < 0.01, ***p < 0.001.

3.1.2 Molecular characterization of the OTR binding in adulthood following adolescent social isolation (study 1B)

We observed sex-dependent effects of ASI on OTR binding in the CeA, PVN, and PVT. In the PVT OTR binding increased by 154% in LASI and 141% in EASI rats compared to CTL (Figure 16E). PVN OTR binding in female EASI and LASI rats increased by 136 and 54%, respectively (Figure 16D). Male EASI rats showed a 52% increase in OTR binding in the CeA compared to CTL (Figure 16B). Data were analyzed using stress group (CTL, EASI, LASI) and sex (Male, Female) ANOVA. For the PVT, we observed a statistically significant interaction effect between the stress group and sex ($F = 15.791_{(1, 29)}$, $p = 0.00003$, $\eta_p2 = 0.194$), and the main effect of treatment ($F = 7.870_{(1, 29)}$, $p = 0.001$, $\eta_p2 = 0.351$). Pairwise comparisons revealed that both EASI ($p = 0.0008$) and LASI ($p = 0.0003$) females had higher OTR binding levels in the PVT compared to CTL, and male EASI demonstrated significantly less OTR binding compared to their female counterparts ($p = 0.0001$). The interaction between stress group and treatment ($F = 8.267_{(1, 21)}$, $p = 0.002$, $\eta_p2 = 0.440$) was significant in the PVN. Pair-wise comparisons demonstrated that female EASI had significantly more OTR binding than CTL ($p = 0.006$). In CEA, we found a significant stress group and sex interaction ($F = 3.519_{(1, 21)}$, $p = 0.048$), and the main effects of stress group ($F = 3.904_{(1, 21)}$, $p = 0.036$, $\eta_p2 = 0.271$) and sex ($F = 6.771_{(1, 21)}$, $p = 0.016$, $\eta_p2 = 0.243$). Post-hoc analysis revealed that male LASI had significantly more OTR binding than female LASI ($p = 0.026$). Post-hoc analysis for the stress group revealed no significant difference

between the groups, suggesting that the interaction effect was driven largely by sex differences. All statistical values are shown by sex for clarity but were analyzed together. Summary statistics in Appendix 7.

A



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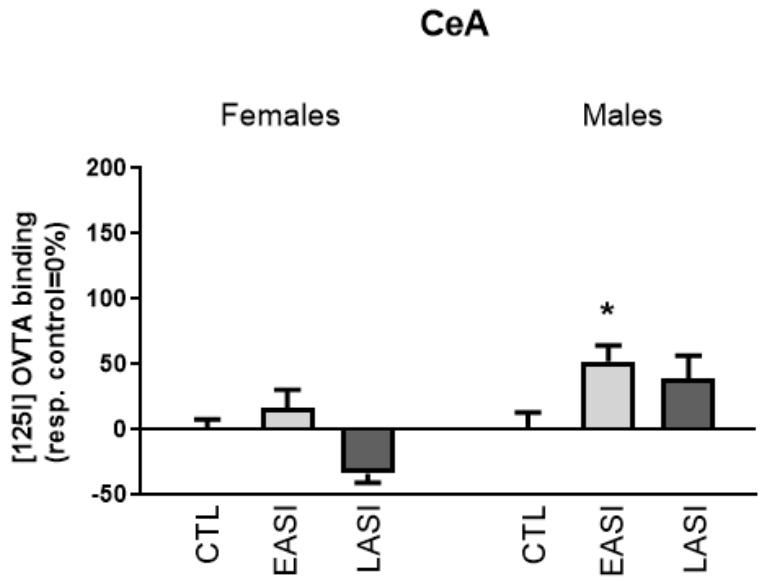

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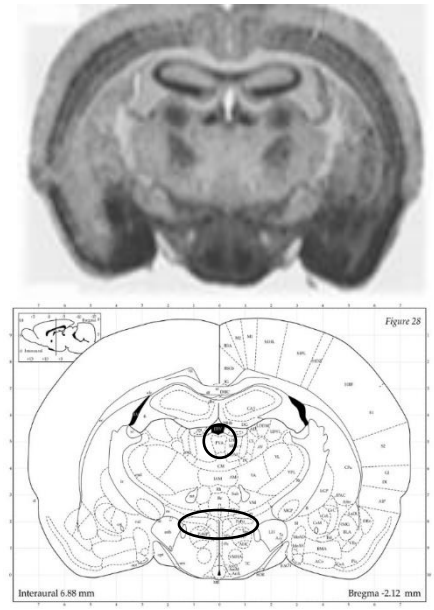

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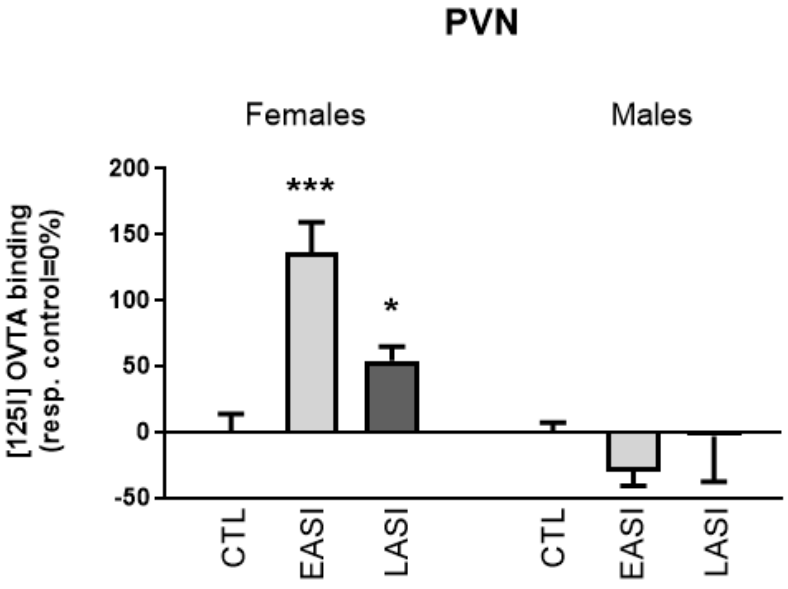

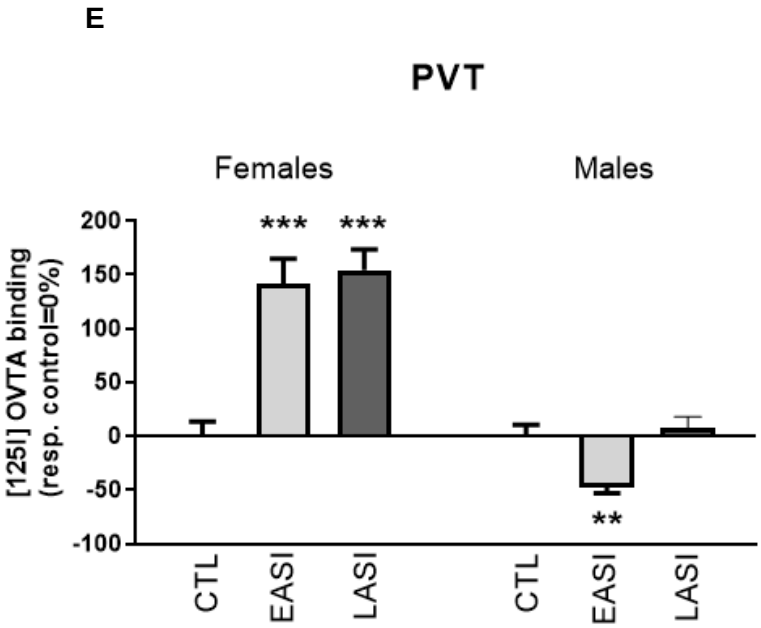

Figure 16: OTR binding sites in the CeA, PVN and PVT in adult rats (PD90) (A, C) representative autoradiograph and bregma coordinates for regions of interest. (B, D, E) OTR bindings sites in the (B) central amygdala (CeA), (D) paraventricular nucleus of the hypothalamus (PVN), and (E) paraventricular nucleus of the thalamus (PVT) measured by saturated [125I] OVTA receptor autoradiography. Data shown as µ±SEM. *****p < 0.5, **p < 0.01, ***p < 0.001. n= 4-8/group.

3.1.3 Validation of axonal OT release in the PVT (Study 1C)

The aim was to determine the functional significance of the OTR changes in the PVT on behavior using optogenetics. We performed the validation experiment in female EASI rats who showed the largest OTR alterations in the PVN as well as the PVT following ASI. Rats received PVN injections of either rAAV1/2-OTp-ChR2-mCherry or rAAV1/2-Otp_Venus and optic fiber implantation into the PVT. We then compared the effects of optogenetic-evoked axonal OT release in the PVT on the NOR, SIT, SRM. Additionally, we performed the Hotplate test with CFA injections, and contextual fear conditioning paradigm as our collaborators from the Grinevich group had previously shown (unpublished data) that optogenetic OT release into the PVT enhanced thermal pain sensitivity and increased freezing behavior. We wanted to try and replicate these findings with a second cohort of rats. Arthur Lefvere and Stephanie Küppers helped with surgeries and data collection for the hotplate test.

The main finding was that optogenetic stimulation during the familiarization phase reduced social recognition memory in a virus-specific manner. Whereby, ChR2 rats showed impaired social recognition memory. All the data presented in Study 1C were collected from two cohorts of rats. Fear conditioning and CFA hot-plate test data were collected from control rats as part of our collaboration with the Grinevich lab.

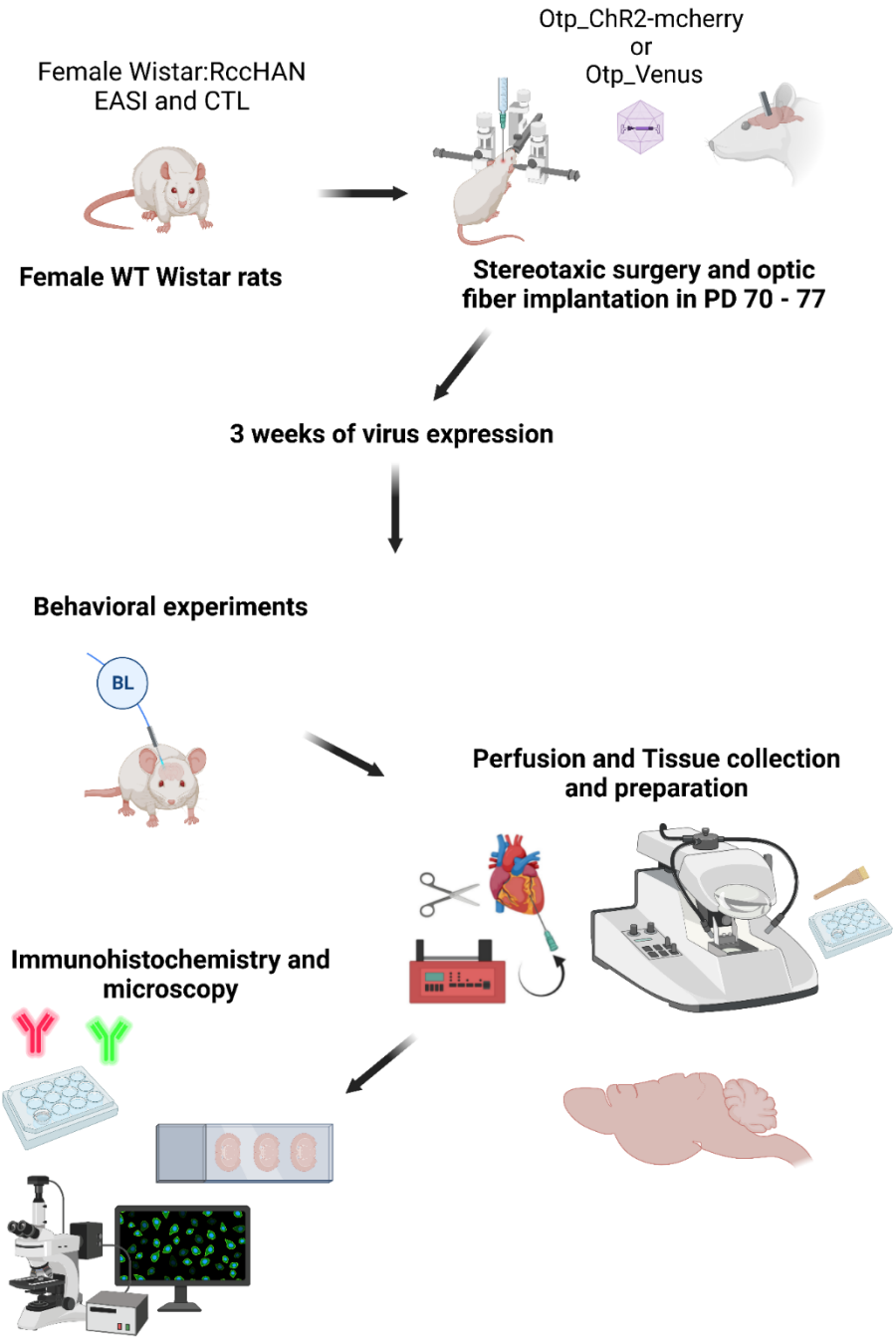

Figure 17: Schematic of the experimental procedures for the functional validation study in female WIST:RccHan rats Female EASI or CTL rats underwent stereotaxic surgery between PD 70 and 77, during which half of the EASI and CTL rats received Otp_ChR2 or Venus. The virus was allowed to be expressed for three weeks before the behavioral experiments started. After the last experiment, rats were left undisturbed for two weeks before tissue was collected, processed with immunohistochemistry using fluorescent probes, and analyzed with a confocal microscope. We observed significant transduction of the virus injected into the PVN in

OT neurons exclusively (Figure 18A). We verified good optic fiber placement in the PVT in 26/30 Otp_ChR2 injected rats. However, due to optic fibers coming off, this number reduced as the experiment ran (Figure 18B). We verified OT fibers from the PVN extending into the PVT (Figure 18C).

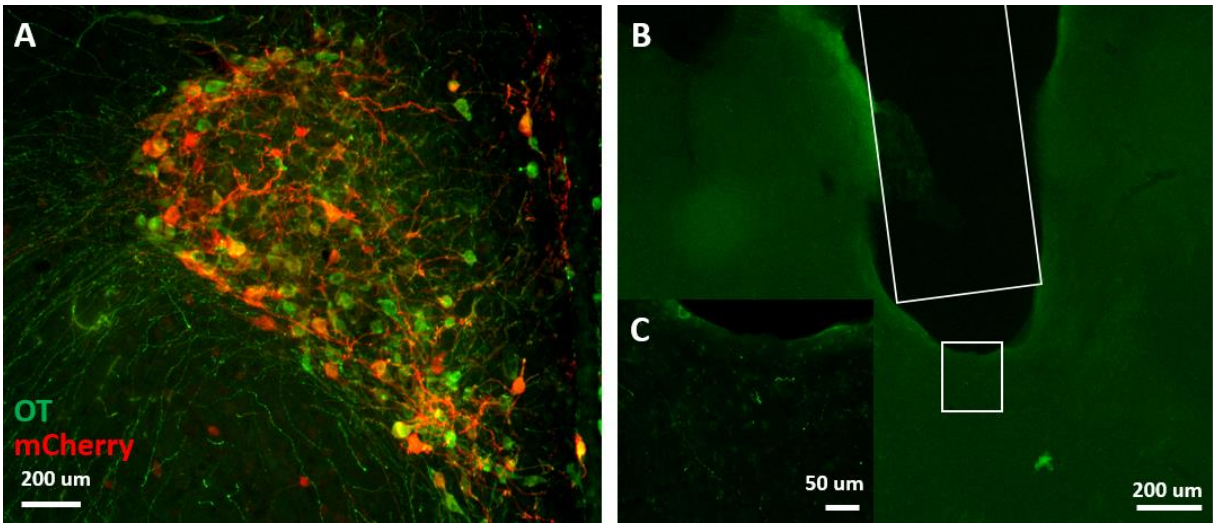

Figure 18: OT neurons transfection in the PVN and fiber placement in the PVT. (A) Representative immunostained PVN section image showing co-labeling of GFP OT neurons (green) and mCherry (red) with the AAV, **(B, C)** Immunostained image of PVT with representative fiber placement and PVN collateral into the PVT.

3.1.3.1 Novel object recognition

Optogenetic stimulation during the acquisition phase did not lead to differences in object discrimination between groups during the test phase ($p = 0.699$) in our first cohort of rats ($N = 9$). Therefore, we did not pursue this in our second cohort. The data were analyzed using a stress group (CTL, EASI) and virus (ChR2, Venus) ANOVA and a one-sample t-test. The discrimination ability of all rats was first assessed against chance ($t_{(8)} = 5.124$, $p = 0.0004$). No difference was observed between groups in the time spent familiarizing with the target object during the acquisition phase ($p = 0.446$). Summary statistics in Appendix 8.

3.1.3.2 Social interaction

Optogenetic stimulation did not influence total social interaction time but did influence the time spent in anogenital sniffing. The data were analyzed using stress group (CTL, EASI) and virus (ChR2, Venus) ANOVA. ChR2 and Venus EASI rats spent 41±5 s and 52±6 s, respectively, in social interactions. CTL ChR2 spent 47±7 s and Venus rats spent 40±7 s interacting with each other. These differences are not statistically

significant (p = 0.179) (see Appendix 9). Data were dissected and analyzed for anogenital and non-anogenital behaviors by time and frequency. ANOVA on time spent in anogenital sniffing revealed a stress group and virus interaction ($F = 5.070_{(1, 22)}$, p = 0.035, $\eta_p 2 = 0.18$) (Figure 19). However, pairwise comparison of the estimated marginal means revealed no statistically significant differences between the groups (p > 0.096). Furthermore, groups did not differ in the number of anogenital (p = 0.082), non-anogenital (p = 0.416) bouts, or time spent in non-anogenital sniffing (p = 0.775). Finally, the time-bin analysis of the blue light stimulation period did not reveal the effects of optogenetic stimulation on social interactions (p = 0.242). Summary statistics in Appendix 9.

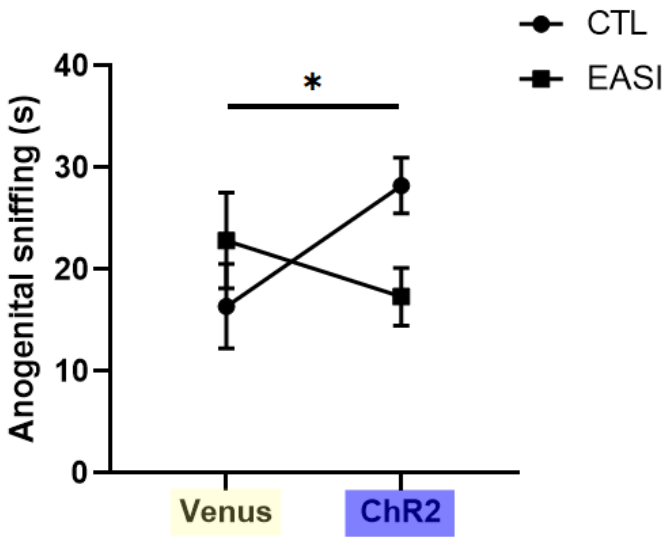

Figure 19: Line graph depicting the interaction effect of anogenital sniffing during the social interaction test. Optogenetic stimulation increased anogenital sniffing in CTL rats and had the opposing effects in EASI rats. Data shown as μ±SEM. ***p < 0.5**

3.1.3.3 Social recognition memory

ChR2 rats demonstrated a reduction in social recognition memory but not in the stress group (p = 0.073). The data was analysed using a stress group (CTL, EASI) and virus (ChR2, Venus) ANOVA. This revealed a main effect of virus ($F = 5.599_{(1, 21)}$, p = 0.028, $\eta_p 2 = 0.21$), with ChR2 rats showing a reduction in discrimination ability (Figure 20). ChR2 rats were able to discriminate between novel and familiar conspecifics 53% of the time compared to Venus rats (66%). The groups did not differ in their total amount

of time spent investigating both partners (p = 0.518) or time spent investigating novel conspecifics (p = 0.892). Summary statistics in Appendix 10.

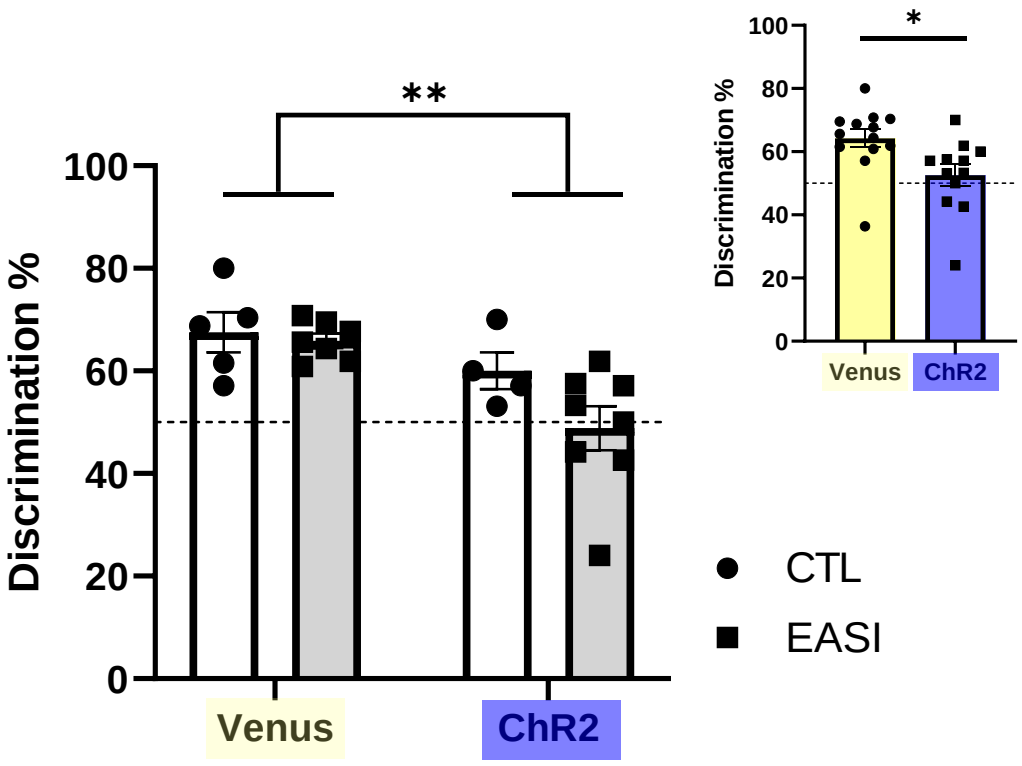

Figure 20: Behavioral performance on the social recognition memory test Rats ability to discriminate between social partners as indicated by % discrimination compared to CTL. Data are shown as individual data points with the mean ±SEM. *p < 0.5, **p < 0.01, ***p < 0.001. Main effect of the virus in small figure.

3.1.3.4 Fear conditioning

Previously collected data by our collaborator demonstrated in a pilot study that axonal OT release in the PVT increases freezing behaviors during the recall of fear memory in female rats. We employed fear conditioning only on CTL ChR2 and Venus rats to replicate previous findings. However, we observed no effect of blue-light stimulation during the recall phase (min 5-7) on freezing behavior ($F = 0.901_{(1, 21)}$, $p = 0.028$, $\eta_p 2 = 0.21$). Summary statistics in Appendix 11).

3.1.3.5 Thermal pain sensitivity

Optogenetic stimulation did not influence the thermal pain sensitivity. Data were analyzed using a stress group (CTL, EASI) × virus (ChR2, Venus) ANOVA. The

interaction effect of stress group and sex (p = 0.334) and the main effects of stress group (p = 0.615) and virus (p = 0.444) were all statistically non-significant (see Appendix 12).

3.2 Adolescent social instability (study 2)

Briefly, in Study 2, WIST rats exposed to SIS showed less anxiety-like behavior in the EPM and initiated fewer interactions in the social interaction test, irrespective of sex or time of testing (immediately or long after SIS exposure). SIS rats had better social recognition memory than control rats after the 30 min inter-trial interval, irrespective of sex and age. Male SIS rats had a higher preference for social novelty than did control rats.

3.2.1.1 Body weight

SIS had no effect on body weight. The weight data were analyzed using a stress group and sex and age (PD30, PD45) ANOVA, which revealed an interaction between sex and age ($F_{(1,118)} = 480.34$, $p < 0.001$, $\eta_p^2 = 0.802$) and stress group and age ($F_{(1,118)} = 9.33$, $p = 0.002$, $\eta_p^2 = 0.073$) (see Appendix X). The SIS and CTL groups did not differ in weight on either PD30 or PD45 (both $p > 0.19$), and both groups increased in weight from PD30 to PD45 (both $p < 0.001$). The interaction of age and sex obviated the main effect of sex ($p < 0.001$), whereby there was no sex difference in weight at PD30 ($p = 0.495$), and males weighed more than females at PD45 ($p < 0.001$). A stress group and sex ANOVA at PD70 indicated only an effect of sex $F_{(1,58)} = 680.39$, $p < 0.001$, $\eta_p^2 = 0.92$) (effect of stress group and interactions $p > 0.68$). Summary statistics in Appendix 13.

3.2.1.2 Elevated plus maze

SIS rats displayed less anxiety-like behavior in the EPM, as indicated by more time in the open arm than CTL rats and did not differ from CTL rats in general activity, as indicated by the total number of arm entries. The EPM data were analyzed using stress group (CTL, SIS), sex (Female, Male), and age (adolescence, adulthood) ANOVAs. SIS rats spent more time on the open arm than CTL rats (Figure 21B) ($F_{(1,115)} = 7.13$, $p = 0.009$, $\eta_p^2 = 0.058$), and female rats spent more time on the open arm than male rats ($F_{(1,115)} = 6.04$, $p = 0.015$, $\eta_p^2 = 0.05$) (Figure 21C) and adult rats more than adolescent rats ($F_{(1,115)} = 40.25$, $p < 0.001$, $\eta_p 2 = 0.26$) (all other interactions $p > 0.13$).

Female rats made more open arm entries than male rats ($F_{(1,115)} = 8.90$, $p = 0.003$, $\eta_p^2 = 0.072$), as did adult compared to adolescent rats ($F_{(1,115)} = 4.53$, $p = 0.035$, $\eta_p^2 = 0.038$) (effect of stress group and all interactions $p > 0.21$) (Figure 21D). The total number of arm entries was higher for females than for males ($F_{(1,115)} = 9.58$, $p = 0.002$, $\eta_p^2 = 0.076$) (other main effects and interactions, $p > 0.12$, Appendix 14) (Figure 21E).

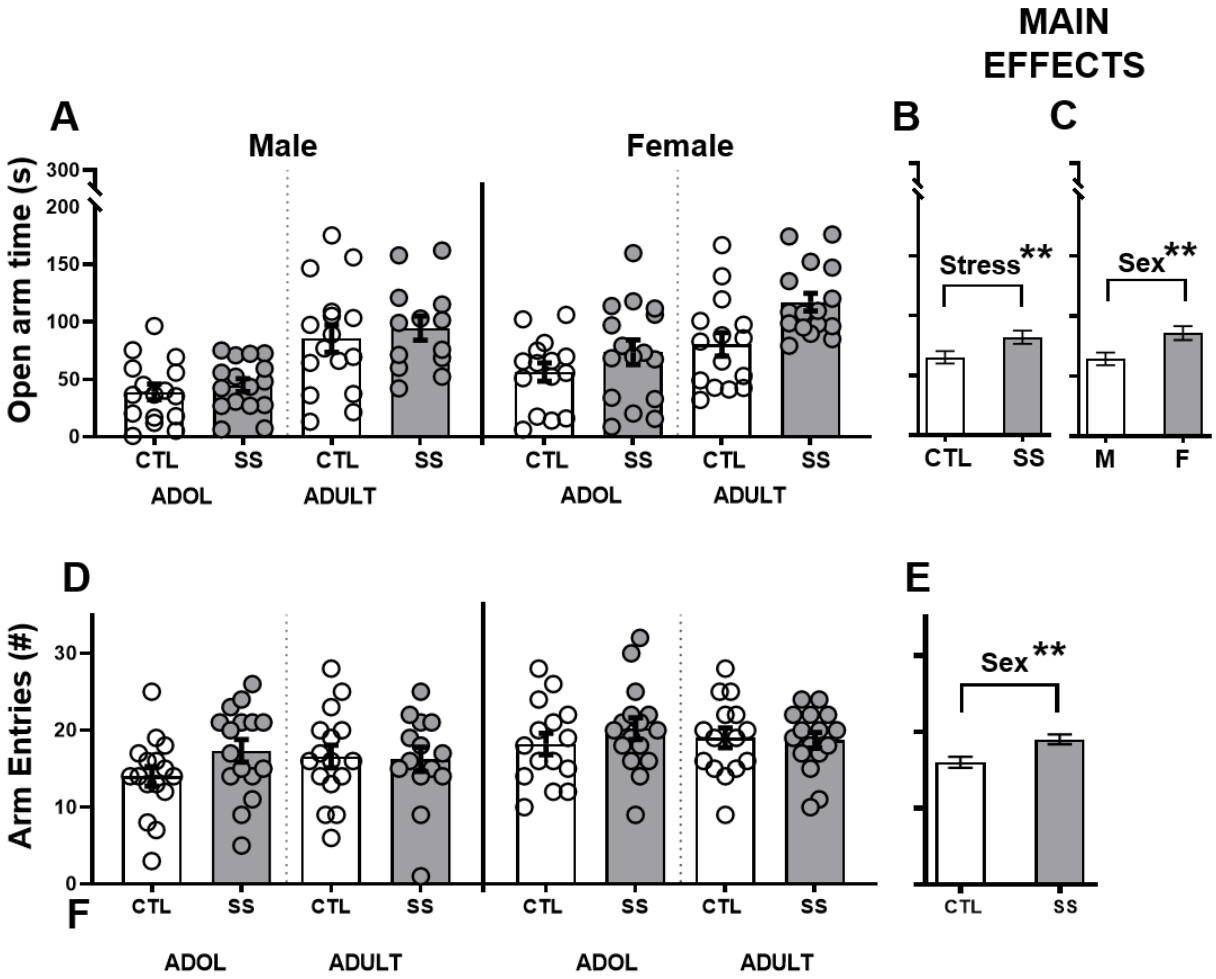

Figure 21: Behavioral tests were performed using the elevated plus maze in adult CTL and SIS rats, both during adolescence and adulthood. (A) Time spent in the open arms of the elevated plus maze, main effect of (B) stress, (C) sex, and (D) total number of arm entries and main effect of (E) sex in CTL (control) and SIS (social instability stress) rats tested either as adolescents or adults. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.2.1.3 Social interaction test

SIS rats spent less time in social interactions (per individual based on which rats initiated the interaction) than CTL rats ($F_{(1,118)} = 6.147$, $p = 0.015$, $\eta_p 2 = 0.050$) (Figure

22B), while female rats spent less time interacting than male rats ($F_{(1,118)} = 4.46$, $p = 0.0367$, $\eta_p2 = 0.036$) (Figure 22C). (main effect of age and interactions, $p > 0.11$). When the time spent in social interaction of the pair was analyzed rather than the time each individual initiated social interaction, the effect of stress group remained significant ($F_{(1,55)} = 5.69$, $p = 0.021$, $\eta_p2 = 0.094$), although the effect of sex did not ($F_{(1,55)} = 3.68$, $p = 0.06$, $\eta_p2 = 0.063$) (Appendix 15).

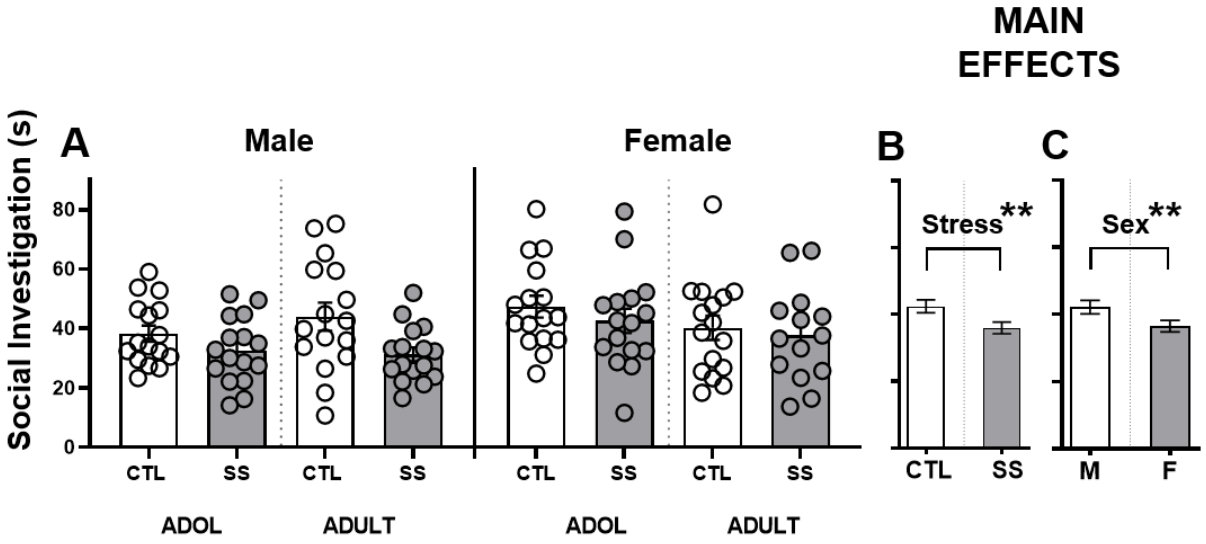

Figure 22: Behavioral performed on the social interaction test (A) Time spent in social investigation by the experimental rat's and main effects of (B) stress and (C) sex between CTL (control) and SIS (social instability stress) rats tested either during adolescence or adults. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.2.1.4 Social recognition memory test

3.2.1.4.1 Familiarization phase

SIS and CTL rats did not differ in the time spent investigating the stimulus rats during the familiarization phase. A stress group, sex, age, and trial (30 min, 90 min) ANOVA on the time spent investigating the stimulus rats during the familiarization phase revealed an interaction of stress group, sex, and age ($F_{(1,112)} = 4.01$, $p = 0.047$, $\eta p2 = 0.034$) (Figure 23A). No pairwise comparison was significant between SIS and CTL rats (all $p > 0.058$). Females spent more time in investigation than males in the CTL adult group ($p < 0.001$; CTL adolescent group, $p = 0.062$), SIS adolescent group ($p = 0.004$), and SIS adult group ($p < 0.001$) (Figure 23A). Adolescents in the CTL male group spent more time investigating than did adults ($p < 0.001$; other comparisons $p >$

0.08). The main effect was statistically significant ($p < 0.001$) (Figure 23B) (other main effects and interactions, $p > 0.13$).

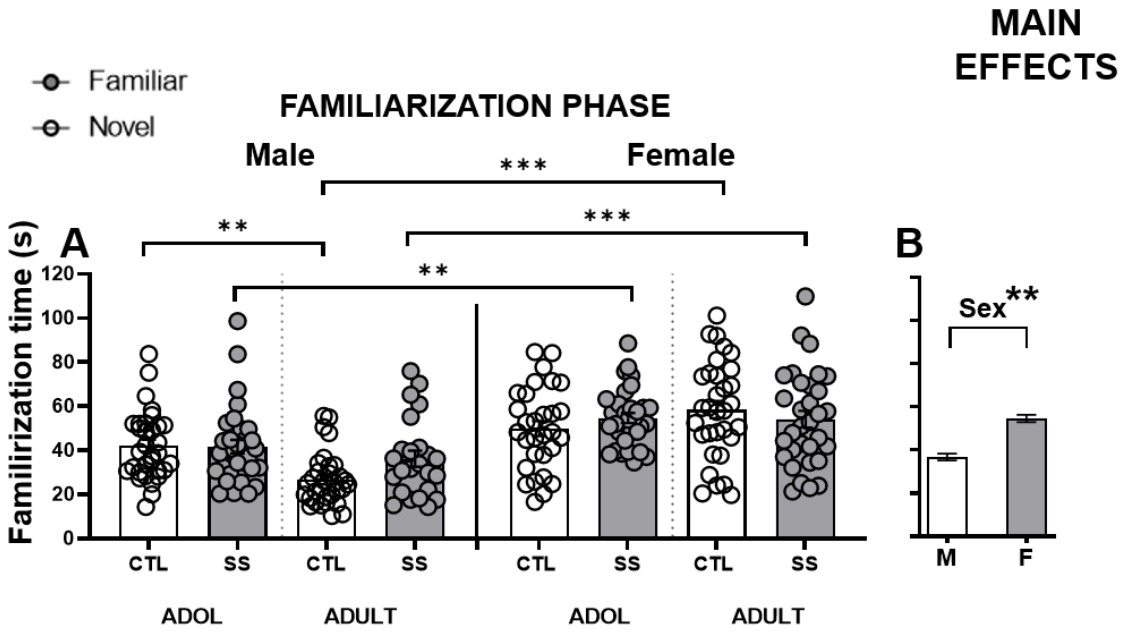

Figure 23: Behavioral performance on the social recognition memory test.

(A) Time spent investigating the conspecifics during the familiarization phase (P1) of the social recognition memory test, (B) sex main effect between CTL (control) and SIS (social instability stress) rats tested during adolescence or adulthood. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.2.1.4.2 Test phase

The effect of stress group on social recognition memory depended on the trial. After the 30 min trial, SIS rats had better social recognition memory (spent more time with the novel than the familiar rat) than the control rats and they did not differ in the 90 min trial. A stress group, sex, age, trial (30 min, 90 min), and familiarity (familiar rat, novel rat) ANOVA on the time spent investigating the stimulus rats during the test phase revealed main effects that were obviated by the interaction of stress group, trial, and familiarity ($F_{(1,112)} = 4.67$, $p = 0.033$, $\eta p2 = 0.040$). The two trials were analyzed separately to interpret this interaction (Appendix 16 and 17). For the 30 min trial (Figure 24A), the interaction between the stress group and familiarity was significant ($F_{(1,112)} = 5.39$, $p = 0.022$, $\eta p2 = 0.042$), whereby SIS rats spent more time investigating the novel than familiar rats ($p < 0.001$), and SIS rats spent more time investigating the novel than CTL rats ($p = 0.007$, other comparisons $p > 0.45$). For the 90 min trial, only the effect of familiarity was significant, whereby more time was spent investigating the novel rat than the familiar rat ($F_{(1,112)} = 6.70$, $p = 0.011$, $\eta p2 = 0.05$) (Figure 24B).

Summary statistics in Appendix 17

Other main effects from the initial ANOVA were obviated by the interaction between sex, age, and familiarity ($F_{(1,112)} = 11.70$, $p < 0.001$, $\eta p2 = 0.095$) (Figure 24B). To interpret the interaction of sex, age, and familiarity, separate ANOVAs for familiar and novel investigations were performed, and the differences were analyzed with paired t-tests by sex and age group separately. Adolescents spent more time investigating familiar rats than did adult rats ($p < 0.001$). Regarding the time spent investigating novel rats, the age difference was significant for both sexes ($p < 0.001$); however, females spent more time investigating novel stimulus rats than males ($p = 0.016$). Paired t-tests indicated that adolescent females and adult males spent more time investigating novel peers versus familiar peers (both $p < 0.001$; other comparisons $p > 0.45$, Appendix 16 and 17).

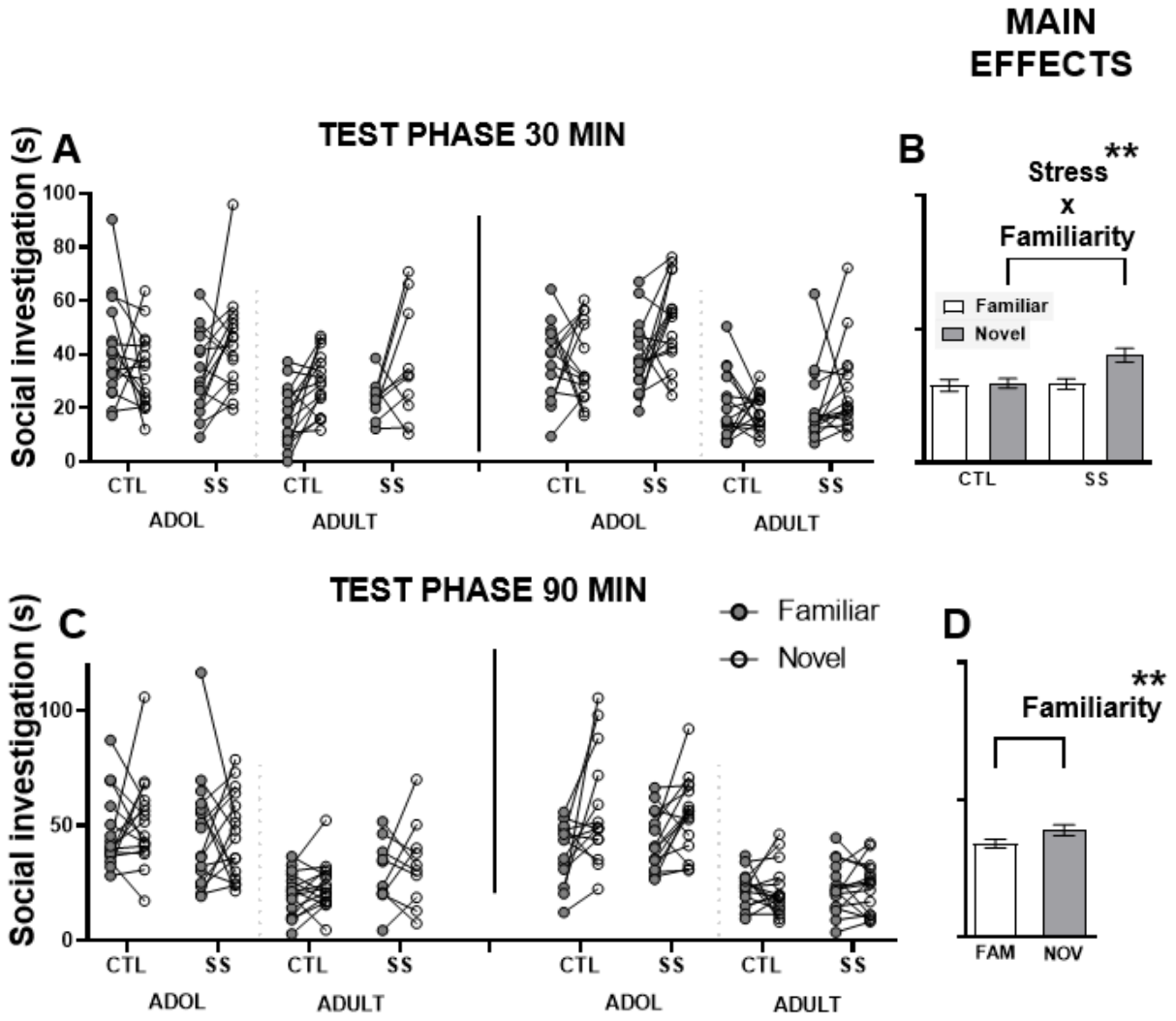

Figure 24: Behavioral performance during the social recognition memory test phase. Time spent investigating the novel and familiar conspecifics during the test phase, after the (A) 30 and (C) 90 min inter-trial-interval in the social recognition

memory test for CTL (control) and SIS (social instability stress) rats tested either during adolescence or adulthood, (B) shows the stress group and familiarity (preference for novel or familiar conspecifics) interaction, and (D) denotes the familiarity main effect. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.2.1.5 Social novelty preference test

3.2.1.5.1 Novelty preference

SIS males spent more time in social approach towards a novel peer than cagemates, while females spent more time in social approach towards their cagemate than the novel peers. A stress group, sex, age and familiarity (familiar rat, novel rat) ANOVA revealed a four-way interaction between stress group, sex, age, and familiarity ($F_{(1,112)} = 5.58$, $p = 0.019$, $\eta_p2 = 0.047$), interaction effects of stress group and familiarity ($F_{(1,112)} = 8.91$, $p = 0.003$, $\eta_p2 = 0.073$), sex and familiarity ($F_{(1,112)} = 12.71$, $p < 0.001$, $\eta_p2 = 0.101$), and main effect of sex ($F_{(1,112)} = 8.58$, $p = 0.004$, $\eta_p2 = 0.071$), all other interactions and main effects ($p > 0.056$, Appendix 18).

Follow-up analyses were conducted separately for both sexes. For males, the interaction of stress group, age and familiarity was significant ($F_{(1,56)} = 6.775$, $p = 0.011$, $\eta_p2 = 0.107$); SIS adult males spent more time in social approach towards the novel peer than did CTL adults ($p = 0.004$) and SIS adolescents ($p = 0.001$) (Figure 25A). The increase in time spent with the novel peer over the cagemate was statistically significant only for adolescent ($p = 0.005$) and adult ($p = 0.009$) SIS males. For females only the main effect of familiarity was significant ($F_{(1,56)} = 5.632$, $p = 0.021$, $\eta_p2 = 0.021$), with all female rats preferring to spend more time near their cagemate than the novel conspecific ($p = 0.014$) (Figure 25B). No other statistically significant differences were observed between the female groups ($p > 0.11$, Appendix 19 and 20).

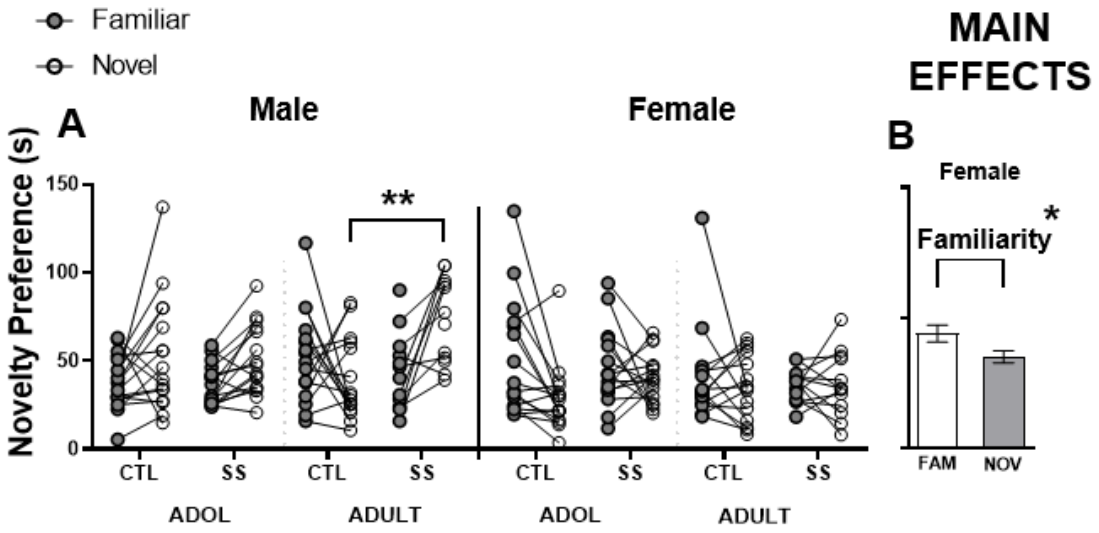

Figure 25: Behavioral performance in the social novelty preference test. (A) Time spent investigating novel and familiar conspecifics during the 15 min social novelty preference test in CTL (control) and SIS (social instability stress) rats tested either during adolescence or adulthood. (B) Main effect of familiarity on female rats. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.2.1.5.2 Social vigilance and avoidance

SIS females spent more time vigilant of a novel rat than a cagemate, whereas the other groups did not show this difference. SIS rats spent more time on social avoidance (exploring corners) than did CTL rats. For time spent in vigilance, the interaction of stress group, sex, and familiarity was significant ($F_{(1,112)} = 5.031$, $p = 0.026$, $\eta_p2 = 0.042$); all other interactions and main effects $p > 0.22$) (Figure 26A). The sexes were analyzed separately to interpret the interaction. There were no significant main effects or interactions among males ($p > 0.13$). For females, the stress group and familiarity interaction were significant ($F_{(1,112)} = 5.041$, $p = 0.028$, $\eta_p2 = 0.028$), whereby SIS females spent more time vigilant of the novel rat than cagemates ($p = 0.013$; all other comparisons $p > 0.07$). With respect to time spent on social avoidance (Figure 26B), SIS rats spent more time on social avoidance than CTL rats ($F_{(1,112)} = 10.519$, $p = 0.001$, $\eta_p2 = 0.085$; other interactions and main effects ($p > 0.08$). Summary statistics in Appendix 19 and 20)

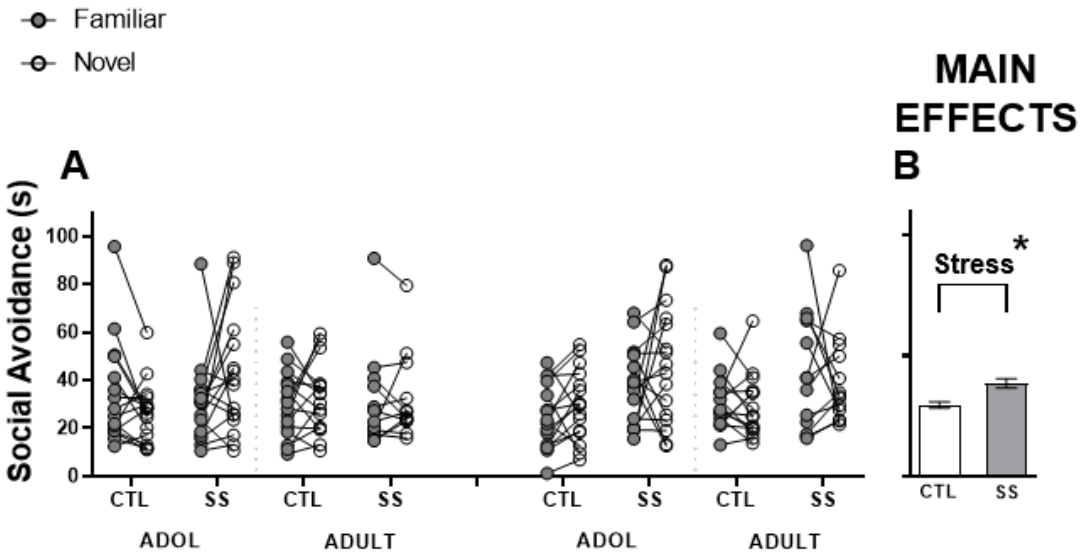

Figure 26: Social avoidance behavioral performance during the social novelty preference test (A) Time spent on social avoidance in CTL (control) and SIS (social instability stress) rats tested either during adolescence or adulthood, (B) main effect of stress group. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.2.1.5.3 Rears and grooming behavior

SIS rats reared more than CTL rats ($F_{(1,112)} = 11.78$, $p < 0.001$, $\eta_p2 = 0.095$) (Figure 27B), and more rears were made on the side of the arena near the novel rat than on the side near the familiar side ($F_{(1,112)} = 5.84$, $p = 0.017$, $\eta_p2 = 0.044$). The interaction of age and sex was significant ($F_{(1,112)} = 4.19$, $p = 0.042$, $\eta_p2 = 0.034$, all pairwise comparisons $p > 0.05$), and other main effects and interactions ($p > 0.27$). SIS rats had a higher frequency of grooming than CTL rats ($F_{(1,112)} = 5.77$, $p = 0.017$, $\eta_p2 = 0.047$; other main effects and interactions, $p > 0.055$).

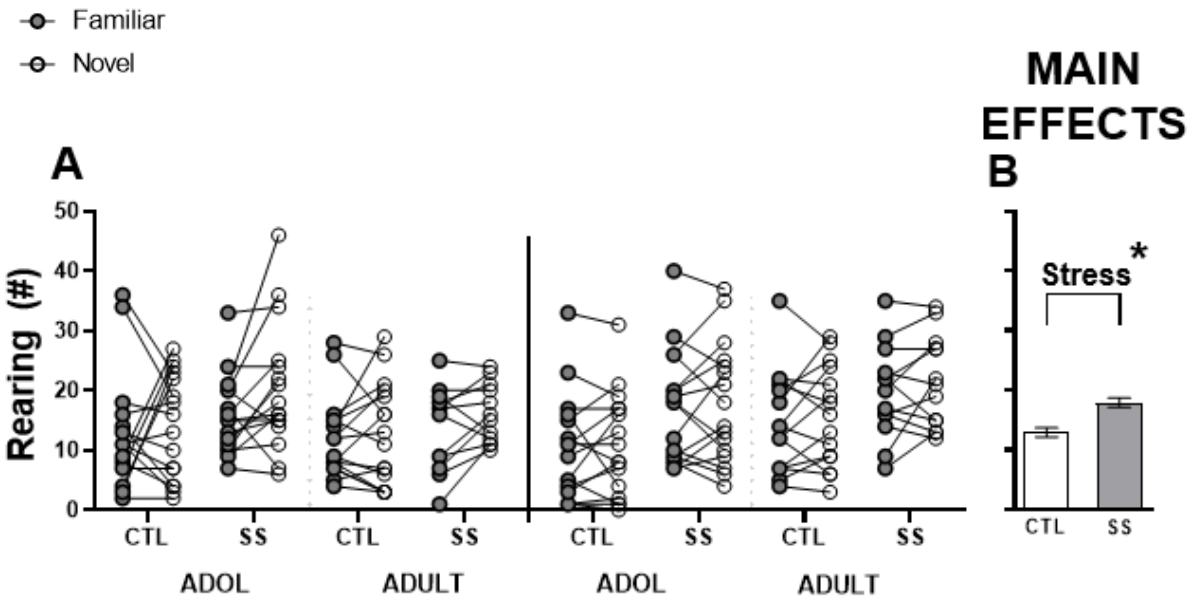

Figure 27: (A) Rearing behavioral performance during the social novelty preference test. Time spent rearing in CTL (control) and SIS (social instability stress) rats tested during adolescence or adulthood; (B) main effect of stress group. Data are shown as individual data points and means ±SEM. *p < 0.5, **p < 0.01.

3.3 Adolescent peer rejection (study 3)

In short, PR induced persistent sex-specific changes to alcohol cue-induced reinstatement, with females showing an increase in reinstatement and males a decrease. Sex differences were observed in locomotor activity.

3.3.1 Cue-induced reinstatement of alcohol seeking

Female control rats reached 97±13 alcohol-associated lever responses (S+/CS+ condition) and 21±2 water-associated lever responses (S-/CS- condition), whereas male control rats had 100±21 alcohol-associated lever responses (S+/CS+ condition) and 28±6 water-associated lever responses (S-/CS- condition) by the end of the acquisition phase. During acquisition peer-rejected females consumed the most alcohol 0.9 g/kg (control females 0.8 g/kg), while peer-rejected males consumed the least amount of alcohol per body weight 0.43 g/kg (control males 0.54 g/kg). On average female peer-rejected rats reached 124±12 alcohol-associated lever responses (S+/CS+ condition) and 29±4 water-associated lever responses (S-/CS- condition) and male peer-rejected rats had 80±16 alcohol-associated lever responses (S+/CS+ condition) and 16±2 water-associated lever responses (S-/CS- condition) by the end of the acquisition phase. Neither alcohol nor water self-administration acquisition differed significantly between the conditions (peer rejected vs. control) for

water ($F_{(1,60)}$ = 0.053, p = 0 .81), or ethanol ($F_{(1,58)}$ = 0.15, p = 0.69). No differences were observed between the sexes in the acquisition of alcohol ($F_{(1,60)}$ = .55, p = .45) (Figure 28) or water ($F_{(1,60)}$ = 3.44, p = 0.06) self-administration. Analysis of alcohol intake (g/kg) across acquisition demonstrated a significant main effect of sex ($F_{(1,60)}$ = 9.983 p = 0.02), which was driven by female. Summary statistics in Appendix 21 and 22.

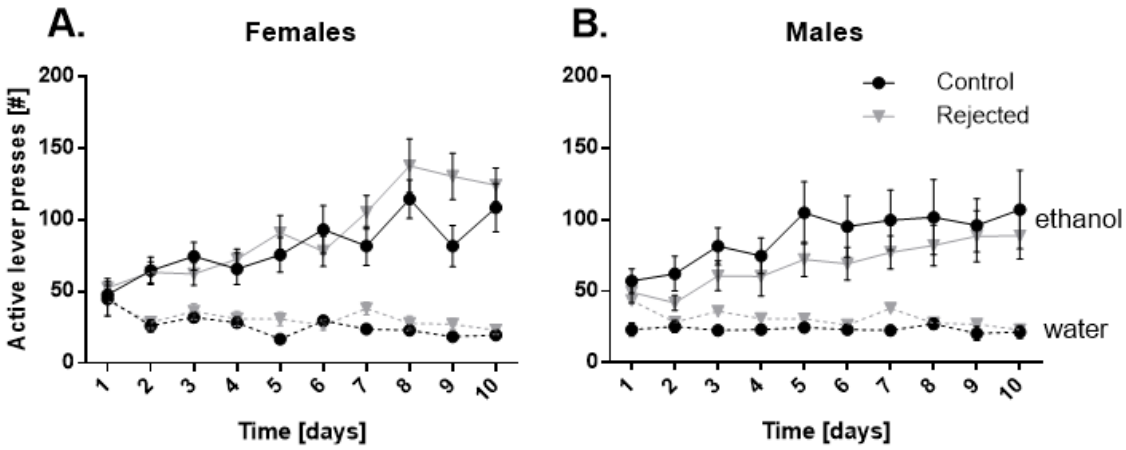

Figure 28: Alcohol self-administration. Average number of alcohol-paired (S+/CS+) and water-paired lever responses for each session (day) of self-administration under an FR1 reinforcement schedule in females (n=20/20) **(A)** and males (n=12/12) **(B).** The alcohol-paired (ethanol) lever responses were greater than the (water) lever responses but no group differences were observed in alcohol or water consumption between CTL and PR animals Data shown as µ±SEM. *p < 0.5, **p < 0.01.

For the reinstatement test, a mixed multifactorial ANOVA with repeated measures revealed a main effect of session ($F_{(1,60)}$ = 106.43, p < 0.001), lever ($F_{(1,60)}$ = 149.08, p < 0.001), sex ($F_{(1,60)}$ = 4,80, p = 0.03), condition and sex ($F_{(1,60)}$ = 23,83, p < .001), condition and sex and lever ($F_{(1,60)}$ = 10,79, p = 0.002), and session and lever and condition and sex ($F_{(1,60)}$ = 11.32, p < 0.01) interactions, indicating that all animals increased their pressing for the alcohol lever during the S+/CS+ test compared to the last extinction session (Figure 29). Post-hoc analysis of the interaction effects revealed that female peer-rejected (vs. control) rats responded significantly more to the active lever during the S+/CS+ test condition ($F_{(1,38)}$ = 7.35, p < 0.001) (Figure 29A), while the opposite effect was observed in peer-rejected (vs. control) males; condition ($F_{(1,22)}$

= 4.87, p = 0.038) (Figure 29C). No significant differences were observed in responding on the inactive lever during the S+/CS+ tests between the groups (Figure 29B/D). Furthermore, a main effect of condition ($F_{(1,60)}$ = 5.28, p = 0.003) was observed in the S-/CS-test (water responding). This effect was mainly driven by a lower response rate to the active lever in the peer-rejected male rats (see Appendix 21 and 22).

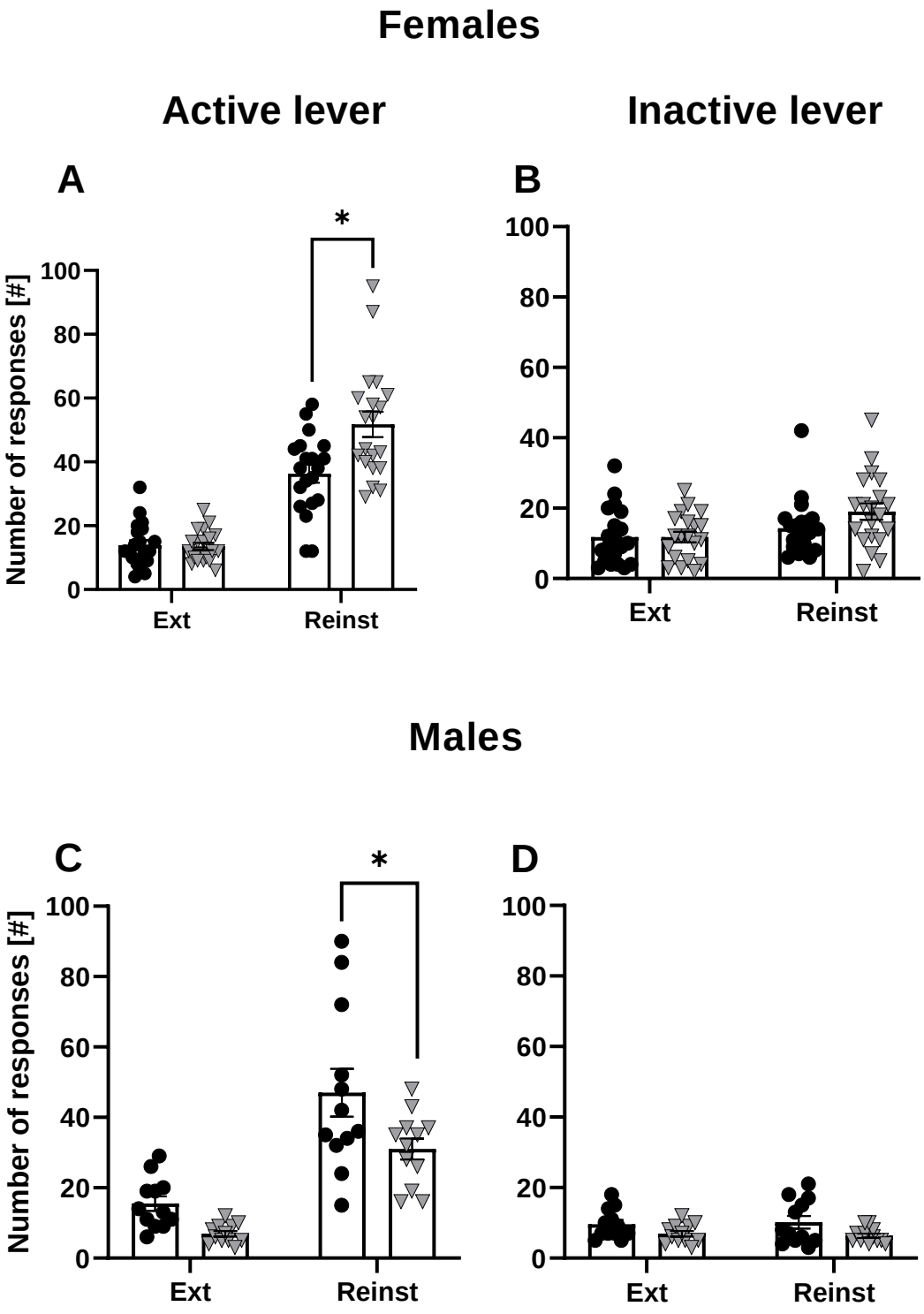

Figure 29: Alcohol-and water cue-induced reinstatement. Data are shown as the average number of lever presses on the active (A, C) and inactive (B, D) levers during the last three extinction sessions (Ext), and as the number of responses after the presentation of stimuli previously paired with either alcohol (Reinst) in control and

peer-rejected female (A-B, n=20/20) and male (C-D, n=12/12) rats. Data are shown as mean ±SEM. *p < 0.5, **p < 0.01.

3.3.2 Home-cage circadian locomotor activity

A three-way ANOVA with repeated measures revealed a significant main effect of day ($F_{(1,42)} = 5.92$, $p = 0.004$), sex ($F_{(1,42)} = 11.705$, $p = 0.001$), and day, sex, and group interaction ($F_{(1,42)} = 6.814$, $p = 0.001$). These data indicate that locomotor activity increased on day three which was mostly driven by female (vs. male) locomotor activity ($F(1, 42) = 11.705$, $p = 0.001$). Average 24 h activity differed between the sexes ($F_{(1,42)} = 0.009$, $p = 0.92$ (Figure 30A, B), where the effect was driven by increased locomotion in female rats. All the rats showed a typical increase in locomotion during the dark phase and a reduction in activity during the inactive light-ON phase. We did not observe differences in the dark- (04:00 – 16:00) or light-phase (16:00 – 04:00) locomotor activity (Figure 30A, B). Summary statistics in Appendix 23.

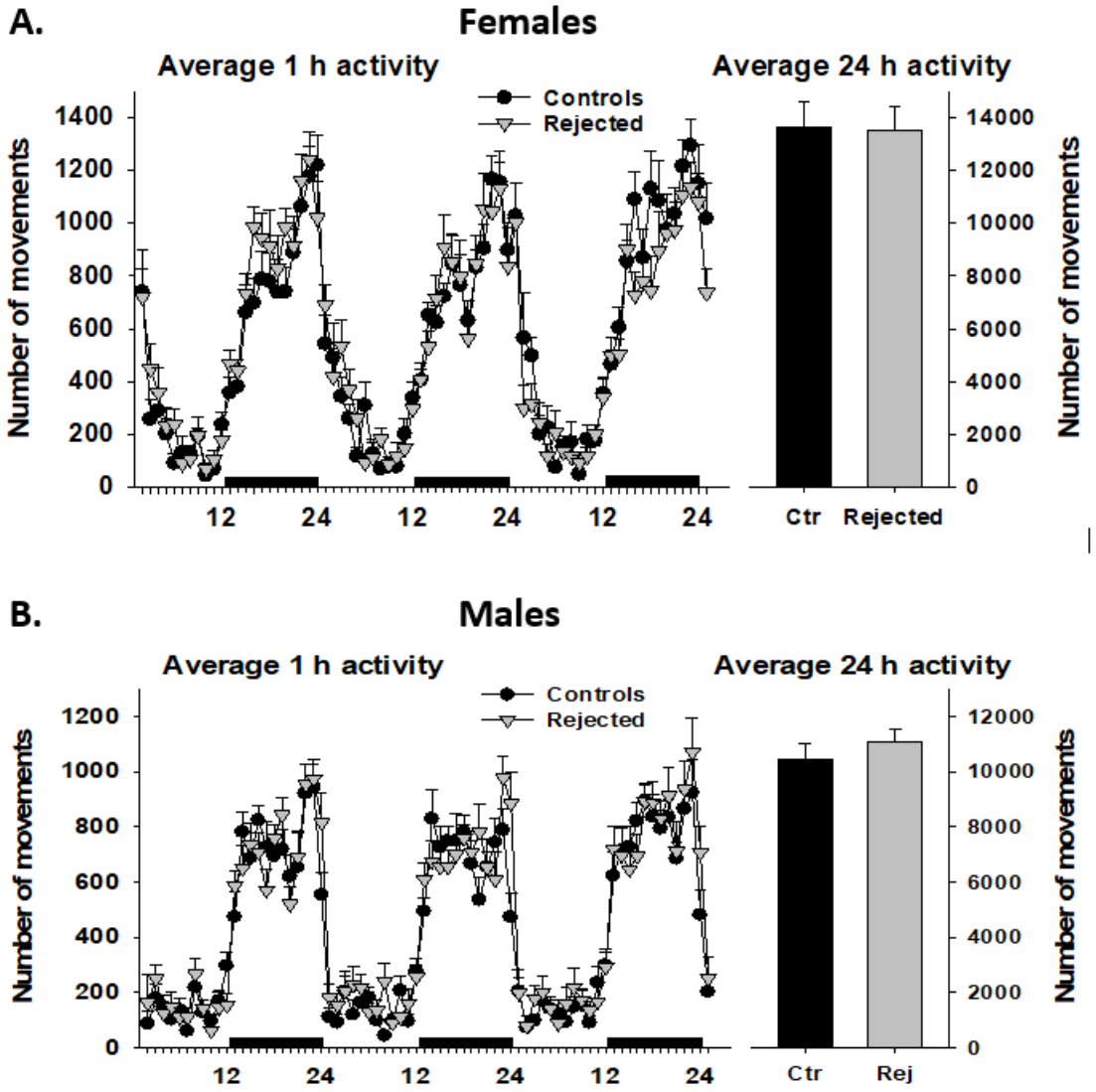

Figure 30: Circadian home-cage locomotor activity. Data from control and peer-rejected female (A) (n=12/12) and (B) (n=12/12) male rats were measured as the number of movements during three consecutive days in the home-cage (black horizontal bars mark the dark (active) phases of the circadian cycle). The figures on the left show the total number of movements during each consecutive hour, and the figures on the right represent the total number of movements during a 24 h period.

3.3.3 Sucrose consumption test

Analysis of sucrose preference did not reveal significant differences between control and peer-rejected animals in either females ($F_{(1,22)} = 2.847$, $p = 0.106$) or males ($F_{(1,22)} = 0.687$, $p = 0.416$). Further analysis of sucrose solution (ml/kg) intake during the 24-hour free-choice sucrose did not differ between rejected and control animals in females

($F_{(1,22)} = 3.235$, $p = 0.086$) or males ($F_{(1,22)} = 0.143$, $p = 0.709$). Summary statistics in Appendix 24 and 25.

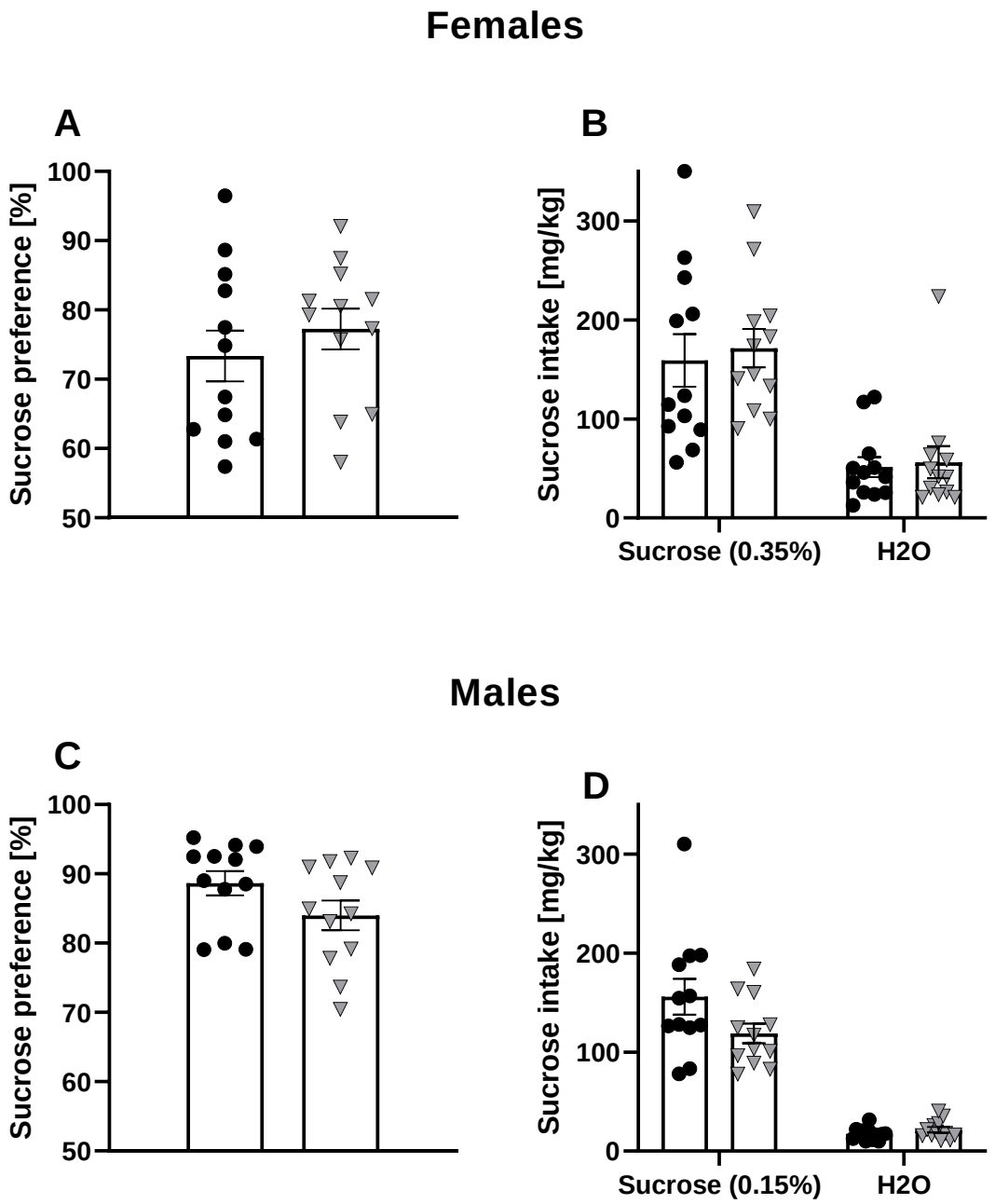

Figure 31: Behavioral performance on the sucrose consumption test. Intake of water and sucrose solution (mg/kg) in control and peer-rejected rats by (A) female (n=12/12) and (B) male (n=12/12) rats. All rats had free 24-hour access to a water bottle and a bottle containing either 0.15% sucrose solution (female rats) or 0.35% sucrose solution (male rats).

4 DISCUSSION

Briefly, this dissertation characterized the long-term sequelae AAs on male and female rats. In study one, we characterized the effects of ASI on anxiety-like, social and memory behaviors in adulthood. Additionally, we assessed the effects of ASI on the OT system in various brain regions using receptor autoradiography and demonstrated how both behavioral and brain changes were differentially modulated in a time-, and sex-dependent manner. Our findings provide novel insights on the effects of timing of ASI which inform about the potential neurobiological changes and can also warn us about the potential long-term consequences we may see in the years to come in adolescents who experiences social isolation during the COVID-19 pandemic. Furthermore, we attempted to validate the functional relevance of endogenous OT release in the PVT of female rats. However, we did not observe optogenetic stimulation of OT fibres in the PVT to rescue the behavioral changes observed following ASI. Instead, we observed non-specific effects of PVT photostimulation on impairing social recognition memory in female rats.

Additionally, we established the adolescent social instability model in WIST male and female rats and characterized the immediate and long-term effects on anxiety-like, social and memory related behaviors. Allowing us to characterize and compare the behavioral phenotypes of induced by EASI and SIS in adulthood.

Finally, in study three, we demonstrated how adolescent peer rejection modulated relapse-like behaviors in a sex-dependent manner. Whereby, female rejected rats demonstrated heightened relapse-like behaviors, while males showed a reduction. Together these studies characterized the unique and long-lasting effects AAs on the OT system and various behaviors in a timing-, type-, and sex-dependent manner.

4.1 Long-term effects of adolescent social isolation

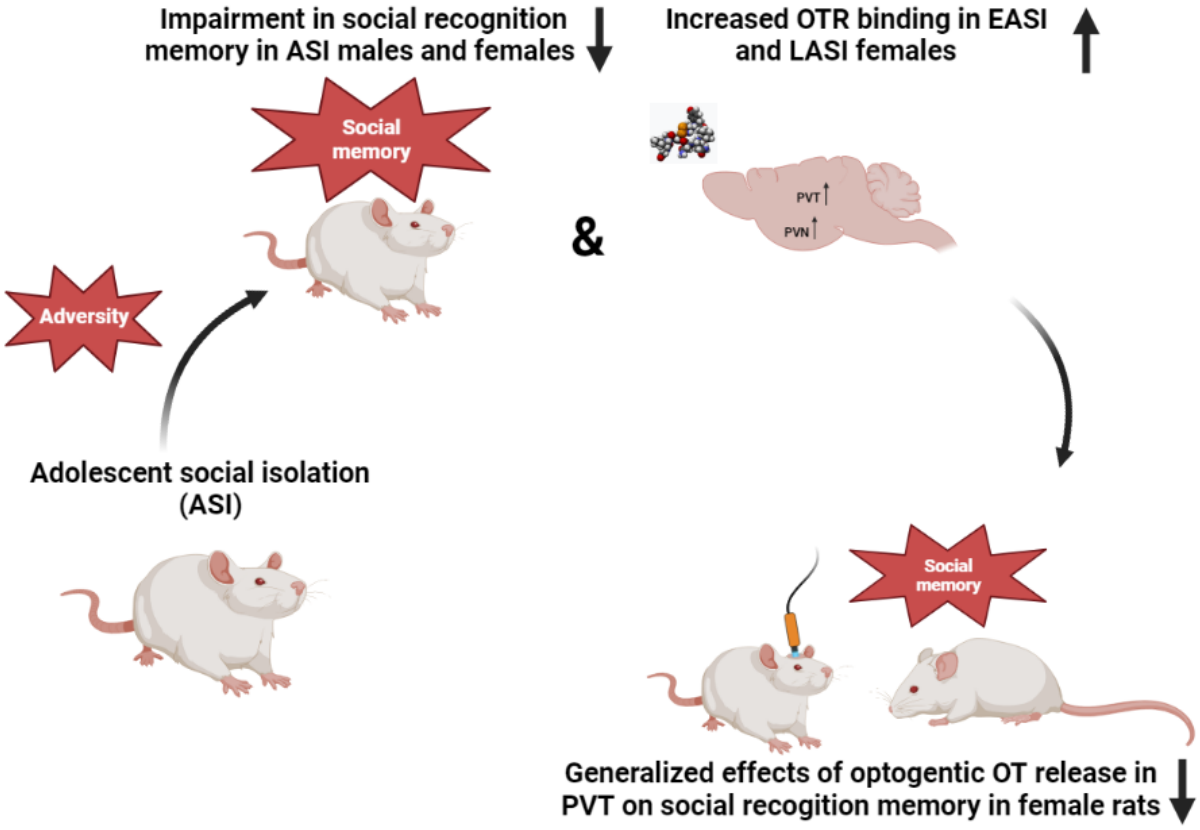

Figure 32: Main finding from the ASI studies 1A-C. All ASI groups demonstrated impairments in social recognition memory in adulthood. EASI and LASI female rats showed increases in OTR binding in the PVT and PVN. Functional validation of OT release into the PVT lead to generalized (regardless of stress group) decrease in social recognition memory.

In study 1A, adolescent ASI induced long-lasting changes to behavioral performance across several tests in both male and female WIST rats when compared with age- and sex-matched control (group housed) rats. Male EASI rats spent less time in the center zone of the EPM. Male and female LASI rats spent less time interacting in the social interaction test. We observed a general decrease in SRM in both stress groups (EASI and LASI) and across sexes. Both male stress groups demonstrated heightened thermal pain sensitivity, whereas EASI females showed decreased pain sensitivity.

4.1.1 Interpreting the effect of ASI across behavioral outcomes

Male EASI rats spent less time in the center zone of the EPM than CTLs (Figure 12A). The decrease in time spent in the center zone has been suggested to represent less impulsiveness (Langen and Dost 2011; Pawlak et al. 2012). Impulsiveness is a multifaceted trait which includes the ability to inhibit responses when faced with negative consequences (Evenden 1999). This is evidenced by the finding that rats that typically spent more time in the center zone of the EPM were less effective in lever pressing for a sweet reward (lever pressed more than required to obtain a reward) under a fixed-interval 60 ratio (Rico et al. 2017). However, it is still debated whether a one-time test such as the EPM, is sensitive enough to detect impulsivity (Pawlak et al. 2012).

Our findings on the EPM align with previous research on ASI in male WIST rats (Van Den Berg et al. 1999), where an increase in time spent in the center zone was observed. To our knowledge, no studies have investigated the effects of ASI on anxiety-like behaviors in female WIST rats. However, comparable results were observed in female SD rats following ASI (Weintraub et al. 2010). Additionally, LASI rats made more total arm entries on the EPM compared to CTLs. Total arm entries are used as indirect measure of locomotor activity on the EPM that has been shown to correlates with OFT locomotor activity in male WIST rats following ASI (Van Den Berg et al. 1999). However, we did not observe similar effects of ASI on male locomotor activity on the open field test.

Our interpretation of this finding is that male EASI rats show signs of increased anxiety-like behavior, as they are less willing to spend time in assessing risk in the open and unprotected center zone like the open arms. This hypothesis is supported by evidence that the center zone elicits risk assessment behavior in rats and more anxious rats are less willing to spend time in risk assessment or the center zone (Gerlai et al. 2006). Furthermore, less anxious (spent more time in the open arm) rats spend more time in the center zone (Frye et al. 2000), suggesting that a reduction in center zone time could infer increased anxiety-like behavior. Although, male LASI rats did not differ statistically from CTL rats there was a trend in the reduction in the time spent in the center zone (Figure 11A) that may be indicative that anxiety-like behavior irrespective of timing of ASI in males.

These anxiogenic effects could reflect changes in the OTR binding (Neumann and Slattery 2016). It has been postulated that low OT activity, PVN OT gene expression, low levels of central OT release and/or low OTR expressing and binding in brain regions relevant to anxiety-like behavior lead to higher anxiety states (Neumann and Landgraf 2012). These states can be shifted to lower anxiety states by increasing any of these OT parameters through environmental stimuli (positive social interactions) or vice versa for higher anxiety states (Alaerts et al. 2021). Interestingly, we observed changes in OTR binding in the CeA and PVT of EASI male rats, which could be mediating the observed changes in anxiety-like behavior. We observed increased OTR binding in the CeA, which would go against the proposed hypothesis. In the PVT we observed decreased binding, which could be driving the behavior. This remains to be explored in future studies.

We observed significant increases in OTR binding in female ASI rats in the PVN and PVT yet saw no difference anxiety-like behavior. Anxiety-like behavior is complex, and we need to keep in mind that there is complex circuitry involved. Where there are balancing mechanisms, neurotransmitter interplay and threshold effects that together interact with anxiety-like states. One interpretation could be that other regions (i.e., amygdala or mPFC) may be exerting overall effects on anxiety that are overshadowed by the changes in OTR receptors expression differences in the PVN and PVT. Hence, not being physiologically relevant to anxiety-like behavior.

Alternatively, interrelated neurotransmitter systems can modulate each other's effects. For instance, the OT system might interact with the serotonin or GABA systems, which are also implicated in anxiety regulation, as has been shown (Han et al. 2018). Ultimately, complex circuitry and neurotransmitters interplay can lead to seemingly contradictory results between altered receptor expression and behavioral outcomes.

In general, ASI has been found to have differential long lasting effects on anxiety-like behavior on the EPM and locomotor activity on the OFT that are attributable to several factors. The first and most common attribute is strain difference. Rat strains display differences in their stress response, that can lead long-term behavioral and neuroendocrine alterations, which may explain the divergence in anxiety-like behavior

on the EPM (Ellenbroek et al. 2005; Walker et al. 2009; Jung et al. 2020). For example, SD rats are less affected by restraint stress than LE rats (Faraday 2002), which could explain some of the anxiolytics effect observed in SD (Weintraub et al. 2010) and anxiogenic effects observed in LE rats (Centre et al. 1991).

However, before strain difference can be identified we need to consider several other parameters first. A second, important parameter is the difference in the timing of social isolation and time of testing between studies could explain some of the variance observed across studies even within strain (LE: Wright et al. 1991; Joshi et al. 2017, SD: Weintraub et al. 2010; Bledsoe et al. 2011; Sun et al. 2018). Third, differences in the illumination plays an important role in effectively inducing anxiogenic effects (Violle et al. 2009). Unfortunately, none of the above studies indicated the intensity of the illumination on the EPM or OFT. Fourth, the time of day when the rats were tested (dark/light cycle) can further explain some of the variance observed between studies (Arakawa 2018; Tsao et al. 2022).

These four factors highlight the importance of considering all possible variables prior to inferring strain differences as the causal difference. However, if all parameters align then strain differences should be considered. Last, we need to acknowledge that anxiety states are transient and there may be significant day to day variation in anxiety-like behavior. Repeatability of a measure would improve reliability of results.

4.1.2 LASI reduces social interactions in adulthood

LASI rats of both sexes demonstrated a reduction in social interactions, which has been interpreted as increased social anxiety (File et al. 1999), but could also reflect reduced social interest or motivation (Netser et al. 2020). Our findings do not converge with previous research in WIST rats. For example, in one study found that EASI males WIST rats showed reduced social approach in the SIT (Hol et al. 1999). While another study in WIST rats found no effect of ASI on adult social interactions at either PD 60 or 64 (Bator et al., 2018). This pattern remained evident, even after repeated testing. Methodological difference could account for differences observed. For example, in the study by Hol and colleauges (1999) investigated social behaviors across 20 min sessions in the SIT, while Bator and colleauges (2018), used a 7 min test compared to

our 5 min test. Additionally, rats where tested during the dark phase of the light cycle in the prior study. Last, the time of testing differs between studies which could additionally add variance. Hence, direct comparisons are difficult to make. Previous ASI research has mostly been conducted in males and to the best of our knowledge we are the first to report of ASI reducing social interaction in females WIST rats in adulthood.

Other studies in different rat strains have generally found a reduction in social interactions following ASI. For example, male SD rats that underwent ASI and were tested in early adulthood (PD56) demonstrated shorter social interaction times (J. Lukkes et al., 2009; J. L. Lukkes et al., 2009). Together, these studies in other rat strains suggest that when ASI is initiated before PD 30, it has a long-term effect on reducing social interactions. However, this rule does not seem to apply to WIST rats.

An alternate interpretation of the results is that LASI in both male and female rats is a more critical for the development of social behaviors than EASI. LASI covers a period between late adolescence and young adulthood a time when social interactions become more complex in nature and important for social development (Walker et al. 2017). Isolation during late adolescence may interfere with this development and lead to less sociability. While the EASI group may have had time to recuperate from their ASI after resocialization on PD42 and hence, mitigated some of the effects. Alternatively, LASI represent a period when individuals still undergo a significant amount of neuronal pruning in brain regions known to modulate social behaviors such as the mPFC and AMY (Shapiro et al. 2017). The authors in this study observed a significant reduction in dendritic spine between PD42 – 56 but not PD 31–39 in the PFC. Hence, the absence of social contact during LASI may lead to different patterns of neural pruning in the PFC or AMY which could account for the reduction in social interactions in adulthood.

What is still lacking in the literature is a qualitative description of social behaviors (i.e., a reduction in anogenital or non-anogenital sniffing). Furthermore, other aspects of sociability remain understudied in adulthood following ASI.

4.1.3 ASI impairs social recognition memory in adulthood

We found that both EASI and LASI rats regardless of sex showed impairments in their SRM, suggesting that ASI has negative impact social cognition. We also demonstrated that this effect was domain specific, as we did not observe differences in novel object recognition. Again, to the best of our knowledge we are the first to demonstrate these deficits on social recognition memory.

No significant differences were observed in the acquisition phase of the social recognition test, which suggests that the ability to recognize a novel conspecific was not affected by the SI. However, during the test phase, both EASI and LASI groups displayed a notable impairment in social discrimination ability relative to CTL rats. This suggests that while initial processing and recognition of social cues may remain intact following ASI, the ability to retrieve this information and apply it to discriminate between familiar and unfamiliar conspecifics is compromised.

As there are no direct comparisons within the ASI field, we turn to other AA models (chronic unpredictable stress and social instability stress). Here, we find that our findings align when compared to other AA paradigms where memory related impairs have been shown to persist into adulthood (Chaby et al. 2015; Provensi et al. 2019b). Findings from early life stress models also align with those we observed. Whereby, adult rats that had been maternally separated, demonstrated deficits particularly within the social recognition after a 60min inter-trial-interval (Lukas et al. 2011).

A plausible neurobiological explanation for impaired social recognition could be led due to alterations in hippocampal OTRs, as both have been shown to be critical to social recognition memory (Ferguson et al. 2000; Raam et al. 2017). However, we did not observe generalized effects of ASI on OTR binding in the HPC, which makes the hypothesis unlikely. Instead, we observed sex-specific alterations in OTR binding in the PVT and PVN in females and CeA males. This could suggest that PVN and PVT related changes are driving impairments in SRM in female while CeA and potential other mechanisms beyond OTR could be involved in males SRM. Most likely, this impairment in social recognition is a result of a combination of factors such as changes in neuroplasticity, neurotransmitter alterations, and HPA-axis alterations that then lead to impaired social recognition (Markham and Juraska 2007).

Further studies are needed to understand the neural mechanisms underlying these social recognition memory deficits. It would be interesting to investigate changes in pairs of rats, as social behavior is complex interaction between individuals, their internal states, and the environment. In summary, our results underscore the profound effect of ASI on social recognition memory in adulthood and provide a foundation for future exploration of the underlying mechanisms.

4.1.4 ASI alters thermal pain sensitivity in a time- and sex-dependent manner

We observed pronounced sex and stress group differences in thermal pain sensitivity on the hotplate test (52.5C). Both male EASI and LASI rats showed heightened sensitivity to thermal pain compared to control males. Conversely, female EASI rats exhibited a reduction in thermal pain sensitivity compared to both LASI and CTL females.

A plausible explanation for the heightened pain sensitivity in males exposed to ASI may be associated with OTR alterations in the CeA, which functioning is important in modulating pain (Neugebauer et al. 2004; González-Hernández et al. 2014). Indeed, OT administration into the CeA have been shown to attenuate thermal pain sensitivity in rats (Han and Yu 2009). In this context, our findings of lower OTR binding in the EASI male group is intriguing. This could potentially imply that reduced OTR binding in the CEA could be driving the heightened thermal pain sensitivity. Hence, if OTs pain relieving effects are facilitated through OTRs in the CeA, lower OTR binding could essentially lead to the exacerbate pain sensitivity. Alternately, the increased pain sensitivity in males could be also linked with changes in the functioning of the endogenous opioid systems, which are crucial in modulating pain modulation (Bodnar 2021).

Interestingly, we observed the opposite pattern in female EASI rat's thermal pain sensitivity and a trend in increased OTR binding in the CeA. This trend in increased OTR could hint towards opposite effects of OTR upregulation in females, with increases in binding decreasing pain sensitivity. Remarkably, males and females could have different compensatory mechanisms in response to ASI, with time specific mechanisms in females that lead to these different effects. However, caution should

be inferred into this interpretation as the OTR difference in females were not statistically significant. Finally, we observed that thermal pain sensitivity did not vary across different phases of the estrous cycle in females, implying that cyclical hormonal changes are not the primary driver of the observed sex differences.

In the broader context of existing literature, our findings align with several studies indicating differential response to adolescent, demonstrating that stress experienced during critical developmental periods can have long-lasting effects on pain perception (LaCroix-Fralish et al. 2005; Yohn and Blendy 2017). Despite the significant findings, it is crucial to acknowledge our study's limitations. While the hotplate test is a widely recognized measure of thermal pain sensitivity, it offers a somewhat narrow view of pain. Future studies could benefit from utilizing complementary assessments (i.e., Hargraves Test) to capture a more comprehensive picture of pain processing and sensitivity, including different types of pain (e.g., neuropathic, inflammatory).

In terms of future research, it would be valuable to dissect the neurobiological mechanisms underlying the sex-specific impacts of ASI on pain. Additionally, examining the effects of interventions aimed at mitigating these impacts would provide useful insights for the development of effective pain management strategies.

In conclusion, our study demonstrates that ASI can lead to sex-specific alterations in thermal pain sensitivity, emphasizing the importance of considering both sex and ASI experiences in understanding pain sensitivity and developing targeted pain management strategies in the future.

4.1.5 Sex differences independent of ASI

Regardless of the stress condition, sex differences were apparent on the EPM, OFT, SIT, and hotplate test. Male rats spent more time in the central zone than females on the EPM, as previously discussed, this may be an indirect indicator of impulsivity. Interestingly, we observed no sex differences in anxiety-like behavior on the EPM, a finding also previously reported in WIST rats (Flores et al. 2020). However, most rat studies in different strains report female rats spending more time on the open arms, indicating a less anxious phenotype (Johnston and File 1991; Imhof et al. 1993; Scholl et al. 2019; Knight et al. 2021). These results suggest that strain differences also play

a vital role in mediating sex differences in anxiety-like behaviour on the EPM. One critic of the EPM and OF sex differences in rats is that this picture does not reflect the human condition. Where, women, not men, were more found to be anxious under baseline conditions when tested on a virtual reality EPM which recapitulates the rodent experience on the EPM (Biedermann et al. 2017). Women are also more likely to develop anxiety disorders (Boivin et al. 2017) and experience more symptoms of anxiety more than men (Pigott 2003).

Next, female rats made more total number of arm entries, indicating females travelled a longer distance in the EPM than males. This finding is one of the most consistent sex differences found in the literature (Johnston and File 1991; Imhof et al. 1993; Scholl et al. 2019; Knight et al. 2021; Börchers et al. 2022). This increase in locomotor activity is also observed on the open field consistently (Hyde and Jerussi 1983; van Hest et al. 1987; Bishnoi et al. 2021; Börchers et al. 2022) , a result we also observed in our study. However, other researchers have suggested that this increased locomotion on both the OFT and EPM should be considered a potential confound (Börchers et al. 2022) and could consequently explain why female rats appear less anxious than males. Together, these findings would suggest that the predictive validity of the EPM is poor because the results do not recapitulate the human condition and hence the predictive validity of the test has come into question.

An alternative anxiety test that demonstrates better predictive validity is the acoustic startle response test. Using the acoustic startle response test for measuring anxiety-like states as several advantages, first, it eliminates the locomotor aspect. Second, the results from the acoustic startle response test in rodents aligns with human clinical findings on anxiety (Lehmann et al. 1999; Bianchin and Angrilli 2012; Blanch et al. 2018). Hence, future studies should consider running additional anxiety tests like the acoustic startle response test to measure various aspects of anxiety-like behaviour that demonstrate good predictive validity. We should also continue using the EPM and OFT, so we can decipher the potential neural mechanisms driving female behaviour on these tests.

On the SIT male rats made more non-anogenital sniffing bouts than females, interestingly they did not differ in the time spent in social investigation. The number of

non-anogenital bouts are hard to dissect, as we did not categorize social behaviors in more detail, which we recognize is a limitation (more on this later). Future studies need to consider categorizing social behaviors across modalities which include not only interactive cues (sniffing, climbing, allogrooming, crawling, following, approach) but also olfactory cues (i.e., urination, feces), visual cues (freezing, grooming, facial expression, paw scratching) and auditory cues (ultrasonic vocalizations). A multimodal approach would capture more subtle differences in specific social behaviors that in turn may reflect affective states of the rat. This in turn, would allow us to identify the underlying neural circuits for the various behavioral responses observed with certainty.

Last, males demonstrated a higher thermal pain threshold compared to females on the hotplate test. Our finding aligns with the current literature on thermal pain in both rodents and humans. Where typically, women and female rats demonstrate a lower thermal pain threshold and higher pain sensitivity than males (Fillingim et al. 2009; Mogil 2012). In rodents, thermal pain sensitivity on the hotplate test is mediated by the second X chromosome but not gonadal hormones (Gioiosa et al. 2008). However, factors other than sex can also influence pain sensitivity, such as affective states (Mohammadi et al. 2020), past pain experiences (Chang et al. 2022), as well as history of AA (Singaravelu et al. 2022). Where we previously showed that adolescent restraint stress primed muscle hyperalgesia in adulthood (Singaravelu et al. 2022). A further consideration is the multimodality of pain, where increased pain sensitivity in one modality (thermal) does not always imply increases in another pain modality (mechanical) (Modi et al. 2023). For example reports have shown that electroacupuncture attenuates mechanical but not thermal hyperalgesia (Huang et al. 2004).

In conclusion, we were able to replicate the most consistent sex differences (increased locomotor activity and higher thermal pain sensitivity in females). However, we also observed divergent results on the EPM the SIT on non-anogenital sniffing bouts. This divergence in results could reflect methodological differences and can suggest that these sex differences may not be as reliable as the ones consistently replicated. As more studies continue to investigate sex differences, we will begin to see which sex differences are true and which are not, due the law of large numbers. This mathematical law dictates that as the number of observation increases obtained from

a large number of studies increases the mean should approach the true mean of the population (Devore 2006).

4.1.6 Interpreting the effects of oxytocin release in the PVT

We found a generalized effect of optogenetic stimulation of endogenous OT in the PVT during the first two minutes of the familiarization phase on reducing social recognition memory during the test phase. To the best of our knowledge, we are the first to report on endogenous OT release in the PVT impairing social recognition memory in rodents and showing the critical involvement of OT in the PVT in mediating these effects. It is remarkable that endogenous OT release impaired social recognition but had no effect on other behaviors irrespective of stress group. OTs role in social recognition is vital and well established in rodents (Popik et al. 1992; Ferguson et al. 2000). Where the effects of OT depend on several factors such as the region of interest, the length of the test, and the mode of OT manipulation.

Typically, systemic and intracerebral injections of OT typically improve SRM (Popik et al. 1992; Gur et al. 2014), while OT antagonist and genetic tools that knockout OTRs impairs social recognition (Popik and Vetulani 1991; Ferguson et al. 2000). Interestingly, optogenetic activation of OTR containing glutamatergic neurons projecting to the PVT have been shown to impair social recognition (Tan et al. 2019). Supporting the idea, that OTR excitation can have antagonistic effects on social cognition.

Our interpretation is that endogenous OT release in the PVT, a region implicated in stress responses and social isolation, leads to heightened arousal or stress that interferes with the encoding or retrieval of social memories. An alternative hypothesis is that the PVT regulates memory through other non-stress related mechanisms, as suggested previously for fear related memories (Penzo et al. 2015). Perhaps, a balance in OT levels is required for successful encoding and supraphysiological levels facilitate encoding (i.e. ICV administration of OT) while endogenous OT release may impair memory related processes as observed in our study and by Penzo et al. (2015).

To establish the specificity of our initial results are OTR specific we will need to perform a series of control experiments. Unfortunately, we do not have a well working opsin to

inhibit endogenous OT release. Therefore, we plan to perform a series of control experiments using the OTR-Cre rat line and Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to verify the results of our optogenetic experiment. This experiment will be performed in both male and female rats to understand if this effect is sex-specific or more generalized. This chemogenetic approach will allow us to both excite and inhibit OTR activity over a longer period. A challenge with a chemogenetic approach is that we lose the temporal precision of optogenetic stimulation and hence, will need to modify the social recognition test so we do not have the OT excitation/inhibition onboard during the test phase. In conclusion, our results emphasize the complexity of OT in social recognition memory in female rats, where the timing and mode of OT release are an important consideration for future research.

4.1.7 Summary

These findings collectively underline the profound effect of ASI on social interactions, social recognition memory, and thermal pain sensitivity in adulthood and laid a foundation for follow up studies on identifying molecular targets. We chose the OT system because of its crucial role in the social domain, where we found alterations in the CeA, PVN and PVT receptor binding. We followed up these results in EASI female rats which showed the largest alterations in OTR bindings level and behavior. Using an optogenetic approach we stimulated endogenous OT in the PVT to try and understand the functional relevance of these molecular changes. Unfortunately, we did not observe any effect of photostimulation on normalizing the behavior following EASI in female rats. We did however observe a generalized effect of optogenetic stimulation during the initial two minutes of the familiarization phase impairing social recognition memory during the test phase, with no impact on other behaviors, regardless of stress group. This is a novel finding in the field, which underscores a significant role of the PVT in social cognition.

4.2 Immediate and long-term effect of adolescent social instability

In this study, we demonstrated how adolescent SIS induced long-term alterations in behavioral performance across several tests in both male and female WIST rats when compared with age- and sex-matched control rats. The main findings comparing SIS to CTL was that SS rats showed a reduction in anxiety-like behavior in the EPM and interacted less in the social interaction test, irrespective of sex or age. Additionally, SIS

rats demonstrated better social recognition memory than control rats following a 30 min ITI, regardless of sex or age. SIS rats also demonstrated heighten levels of social avoidance, rearing and grooming in the social novelty preference test. In addition, male SIS rats showed a higher preference for social novelty than male CTL rats.

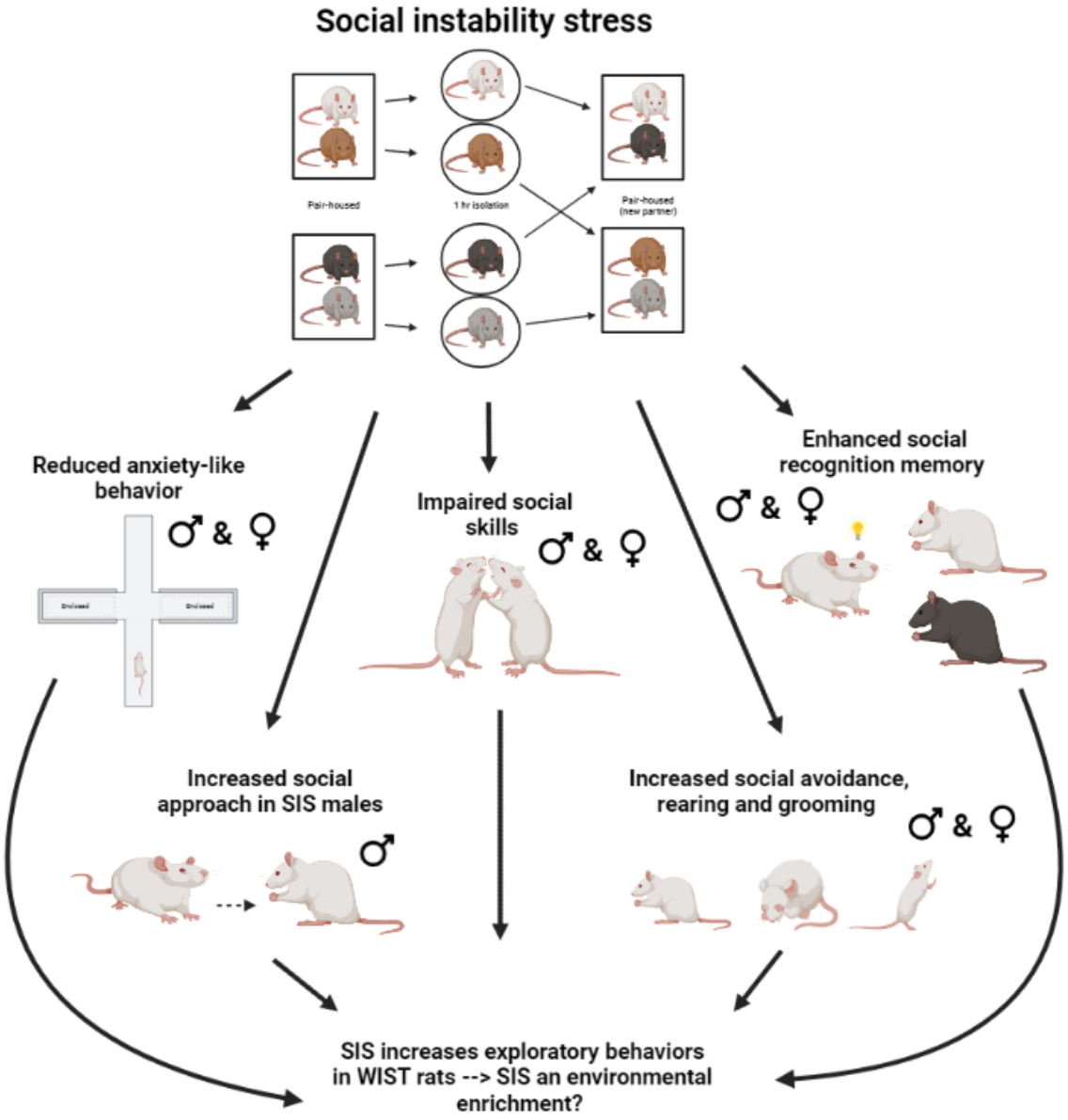

Figure 33: Main findings from our SIS study in WIST rats. SIS led to reduced anxiety-like behaviors and social interactions across all stress groups in dependent of time of testing. We also observed an improvement in SRM in the 30 min ITI. Male rats spent more time in social approach in the social novelty test, while all stress groups show increase social avoidance, rearing and grooming. Our interpretation is that SIS rats show increases in exploratory behaviors regardless of time of testing.

4.2.1 Interpreting the effects of SIS on behavioral outcome across measures

SIS rats regardless of sex or age spent more time on the open arm of the EPM, a standard measure of anxiety-like behavior. Notably, these differences were not attributable to overall activity levels, as both the SIS and control groups exhibited similar number of total arm entries, a standard measure of locomotor activity.

SIS rats engaged less in social interactions compared to the control group, irrespective of sex or age. Our finding that SIS rats spend less time in social interactions aligns with previous reports in Long Evans rats (Asgari et al., 2021; Green & McCormick, 2013; Hodges et al., 2017, 2018; Marcolin et al., 2020). Although the SIT is often considered to measure ethnological indicator of social anxiety, it may provide a broader understanding of anxiety, as factors such as lighting conditions and familiarity with the testing arena can influence the degree rats interact during the test (File & Seth, 2003).

Our interpretation of the results is that the observed decrease in social interactions following adolescent SIS suggests factors other than social anxiety. This hypothesis is supported by the fact that there were no noticeable differences in social conditioned place preferences between SIS and CTL LE rats (Hodges et al. 2017). Additionally, male SIS rats typically spend more time in social approach behaviors (Green et al., 2013; Hodges et al., 2017) converging with our findings in the social novelty preference test. Whereas female rats were more inclined to approach their cage mates over than novel conspecific, independent of their stress group. Together, we interpret these findings as SIS impacting social interactions in a non-social anxiety related manner in males.

4.2.2 SIS impacts social vigilance and avoidance

Social vigilance and avoidance behaviors in rodents are typically seen as manifestations of social anxiety, however, vigilance may serve as an adaptive mechanism for coping with environmental challenges (Wright et al., 2020). We observed sex-differences in vigilance behavior, whereby, males SIS did not differ from CTL males, but female SIS rats showed increased social vigilance towards the novel but not the familiar cage mate when compared to CTL rats. Furthermore, SIS rats, regardless of their age, sex, or area of the arena (close to the cage mate or the novel peer), spent more time displaying social avoidance (e.g., remaining in corners) than

their control counterparts. However, such corner-dwelling behavior may also be indicative of exploratory tendencies rather than anxiety, as rats were typically observed stretching upright against the walls. A behavior known as supported rears which is suggested to reflect active exploration rather than anxiety (Sturman et al. 2018) Our hypothesis is further backed by reports that supported rearing is decreased under stressful conditionings (Sturman et al. 2018). Additionally, due to sufficient habituation to the arena, it is unlikely that the rats perceived the environment as threatening or unpredictable.

Moreover, SIS rats exhibited more grooming behaviors than the CTL. Evidence suggests rats displays an inverted-U relationship with anxiety, being lower under both extremely high and low stress conditions, while increasing as rats habituate to novel or anxiety-inducing environments (Fernández-Teruel & Estanislau, 2016; Veloso et al., 2016). Considering SIS rats exhibited reduced anxiety in the EPM, they may be less anxious and better habituated to the test arena, thus grooming more frequently than CTL rats. However, further studies investigating grooming behavior under various stress conditions would help clarify whether increased grooming indicates higher or lower anxiety levels in SIS rats. Our findings suggest that SIS in WIST rats may enhance exploratory behavior and social approach. This finding contradicts conventional findings from human studies, where adverse childhood experiences such as SIS are considered risk factors for anxiety (Anda et al. 2006; Hughes et al. 2016), our results also differ from previous findings in other rat strains (Al-Rahbi et al. 2013). This divergence in results could imply that the SIS procedure, at least in WIST rats may not perceive SIS as aversive in this context as LE rats do.

4.2.3 Improved social recognition memory performance following SIS

We observed no notable differences in time spent investigating the two new conspecifics during the familiarization phase of the social recognition memory test between SIS and CTL rats. SIS rats exhibited better social recognition memory than CTL, as evidenced by their preference for the novel rat over the familiar rat after a 30-minute ITI. However, this difference disappeared when the ITI extended to 90-minutes. Our results contradict previous findings, which generally suggest AA impairs social recognition memory in rats (Eagle et al. 2013; Patki et al. 2013; Schaack et al. 2021), including a reduction in social recognition memory performance in LE rats (Hodges et

al. 2017). In line with our previous conclusion that adolescent SIS enhances exploratory behavior, the data on social recognition memory align with the idea that SIS may not be as severe an adversity for WIST rats as for LE rats. Instead, it may serve as a form of enrichment, leading to reduced anxiety and increased exploratory behaviors. One explanation for these differences in behavioral outcomes between rat strains may be due to variations in social behaviors during adolescence; LE display more play behaviors than WIST (Northcutt and Nwankwo 2018) rendering changes in cage partners potentially more disruptive to their development. Conversely, WIST rats may be less sensitive to alterations in their social environment than LE that show higher HPA activation in response to stressors at least in males (Sanchís-Ollé et al. 2021).

Studies have shown WIST rats exhibit a lower hypothalamic-pituitary-adrenal response to stressors compared to LE in several stress measures. For instance, WIST show less anxiety-like behavior in the EPM (Hughes & Hancock, 2016) and freeze less after receiving foot shocks (Ambrogi et al., 1987). Unlike LE rats, WIST rats did not exhibit the typical reduction in weight gain after SIS (McCormick et al., 2004, 2005). Collectively, these findings might elucidate why WIST rats respond differently to SIS compared to the LE strain.

4.2.4 Sex differences independent of SIS

Regardless of the stress condition, sex differences were evident in EPM, social interaction, social recognition memory, and social novelty tests. Females exhibited less anxiety-like behaviour in the EPM compared to males, spending more time on the open arm, making more open arm entries, and performing more head dips. These findings align with prior studies involving non-stressed, group-housed WIST and Long Evans rats (Johnston and File 1991; Imhof et al. 1993; Pohl et al. 2007; Scholl et al. 2019). However, some studies have also reported no sex differences on anxiety-like behaviour in the EPM (Steenbergen et al. 1991; Ou et al. 2019; Pavlova et al. 2020) suggesting that additional factors such as lighting conditions during testing and pre-test housing conditions might influence results. Female rats also recorded a higher number of total arm entries, corroborating the broader evidence suggesting higher locomotor activity in females than males in both WIST and Long Evans rats (Hyde and Jerussi 1983; van Hest et al. 1987; Bishnoi et al. 2021). Consequently, the observed

sex differences in anxiety-like behavior may stem more from females' higher locomotor activity than anxiety.

In the social interaction test, predominantly used as a measure of ethnological anxiety behavior, male rats dedicated more time to social investigation than females, a consistent finding in literature (File & Seth, 2003). However, in the social recognition memory test, female rats spent more time examining a novel conspecific during both the familiarization and test phase than males. A crucial difference between the test was that the conspecifics for the social recognition memory test were confined behind a plastic mesh but could interact freely during the social interaction test. These data indicate that females interact more with confined conspecifics than males, potentially leading to better social recognition memory in females. These findings align with observations in Long Evans rats, where adult females (3-5 months old) demonstrated better social recognition than males (Markham and Juraska 2007). However, null-findings have also been reported with no sex differences in either adolescence or adulthood in WIST rats (Veenema et al., 2012). Comparing these studies is challenging due to the variations in retention intervals and procedures across studies.
Interestingly, the sex difference in social approach were not apparent in the social novelty preference test conducted in the same apparatus, suggesting that the sex difference may be unreliable or dependent on the peer (familiar versus novel) or test duration (5 min versus 15 min). The role of sex-hormones are a likely driver of these directly by estrogen which have been consistently shown to improve SRM in rodents (Karlsson et al. 2016) which aligns with our findings.

4.2.5 Age differences independent of SIS

Adolescence is typically characterized by higher risk-taking, sensation-seeking, and exploratory behaviors compared to adults in both rodents and humans (Spear 2000; Walker et al. 2017). This leads to stark differences in a variety of behavioral outcomes. Unexpectedly, we observed that adult rats spent more time on the open arm of the EPM, indicating less anxiety-like behavior than adolescent rats. A similar age effect on anxiety in the EPM was documented when both male and female SD rats were tested either on PD 33-38 or PD 83-88 (Przybysz et al. 2020) (However, more frequently, evidence points to increases in anxiety with age (Imhof et al. 1993; Doremus et al. 2004; Sotoudeh et al. 2020). Several factors may account for these divergent findings.

First, whether rats are tested during the active or inactive part of the light cycle can influence anxiety measures (Huynh et al. 2011; Aslani et al. 2014). Second, lighting conditions and the transparency of the closed wall can also affect EPM anxiety metrics (Violle et al. 2009) with rodents making more open-arm entries under red light conditions compared to white light (Violle et al., 2009), and heightened illumination intensity typically reduces open-arm time (Garcia et al. 2005; Pawlak et al. 2012). However, a significant limitation with most studies, even recent ones, don't report the testing time or lighting conditions (Imhof et al. 1993; Doremus et al. 2004; Przybysz et al. 2020) thereby obscuring the specific influences of circadian rhythm and lighting intensity on anxiety-like behaviors.

In the social recognition memory test, adolescent rats spent more time examining the familiar rat than adult rats did. Another study found no difference in SRM between adolescent and adult male rats (Markham and Juraska 2007). Unfortunately, there is a lack of studies investigating age effects on SRM across sexes, and comparisons are often difficult to make due to changes in social behaviors across adolescence (Spear 2000; Crews et al. 2007; Walker et al. 2017). The discrepancy in findings may be attributed to strain differences (the study was performed in LE rats) or variations in the inter-trial interval within the paradigm(Bonapersona et al. 2019).

4.2.6 Summary

Adolescent exposure to SIS modifies the behavioral repertoire in a lasting way, resulting in decreased social interaction, heightened social approach, amplified exploratory behavior, and diminished anxiety-like behavior in both male and female rats. Consequently, we propose that the SIS paradigm may be perceived as less stressful by WIST rats compared to LE rats. The diminished anxiety and stress response observed in WIST rats (compared to LE rats) might suggest that SIS acts more as a form of environmental enrichment in the former than a stressor.

Investigating the differences in sex and age across various rat strains is crucial in understanding the translatability and generalizability of the findings to achieve biological robustness adversity models (Spanagel 2022). A direct comparative study of SIS impact on LE and WIST rats may unveil factors leading to distinct phenotypes following adolescent social instability and could enhance the translation of these

findings to the more heterogeneous human population. We summary our findings in comparisons to LE rats below (Table 3).

Table 3: Summary of results for the effects of social instability stress (SIS) depicted relative to control (CTL) rats, with a comparison of results in the literature in Long Evans (LE) rats.

	PRESENT RESULTS IN WISTAR RATS				**RESULTS IN THE LITERATURE in LE RATS**
	Adolescent SIS males	**Adult SIS males**	**Adolescent SIS females**	**Adult SIS females**	
Weight	**=**	**=**	**=**	**=**	↓ in LE ♂ at PD45, not ♀; no difference at PD 70 (McCormick et al. 2005)(McCormick et al. 2004, 2005; McCormick and Ibrahim 2007) 3
EPM					
Open arm time	↑	↑	↑	↑	↑open arm time in LE ♂, not ♀ at PD45, and ↓ in LE ♂ at PD70 and ↑in estrous ♀ at PD70 (McCormick et al. 2008) ↑open arm time in LE ♂, not ♀ at ~PD47 (Roeckner et al. 2017) **=** in LE ♀ at PD45, PD70 (Asgari et al. 2021)
Open arm entries	**=**	**=**	**=**	**=**	
Total arm entries	**=**	**=**	**=**	**=**	
Social Interaction	↓	↓	↓	↓	↓ in LE ♂ + ♀ at PD45, PD70 (Asgari et al. 2021) ↓ in LE ♂ at PD45 (Hodges et al. 2017, 2018, 2019; McCormick et al. 2020; Asgari et al. 2021) ↓ in LE ♂ at PD70 (Green et al. 2013)
Social Recognition Memory					
Familiarization	**=**	**=**	**=**	**=**	↓ in LE ♂ at ~ PD47 (Hodges et al. 2017)
30 min Trial	↑	↑	↑	↑	↓ in LE ♂ at ~ PD47 (Hodges et al. 2017)
90 min Trial	**=**	**=**	**=**	**=**	**=** in LE ♂ at ~ PD47(Hodges et al. 2017)
Social Novelty Preference	↑	↑	**=**	**=**	**=** in LE ♂ + ♀ at PD45, PD70 (Herlehy et al. 2022)
Social approach	↑	↑	**=**	**=**	↑ in LE ♂ at PD45 (Hodges et al. 2017, 2019) **=** in LE ♂ at PD70 (Green et al. 2013)
Social vigilance	**=**	**=**	↑ **toward novel**	↑ **toward novel**	
Social avoidance	↑	↑	↑	↑	
Rearing	↑	↑	↑	↑	
Grooming	↑	↑	↑	↑	

4.3 Long-term effects of PR on alcohol seeking and reward related behaviors

This study showed how adolescent PR induced long-lasting effects on alcohol-seeking behaviors in a sex-dependent manner, without affecting general homecage locomotor activity or sucrose preference in adulthood. The PR paradigm did not affect acquisition of alcohol, water or extinction. Nevertheless, adolescent PR manifested as increased alcohol cue-induced reinstatement in female rats while having the opposing effect in males. This is a notable discovery, highlighting the complex interplay between AAs and vulnerability to in relapse-like behaviors, which is influenced by sex. These findings somewhat converge with human data. Where women, but not men a history of ACEs is predictive of relapse to cocaine. This would be interesting lead to follow-up on for future studies to better understand the generalizability of our findings to other drugs.

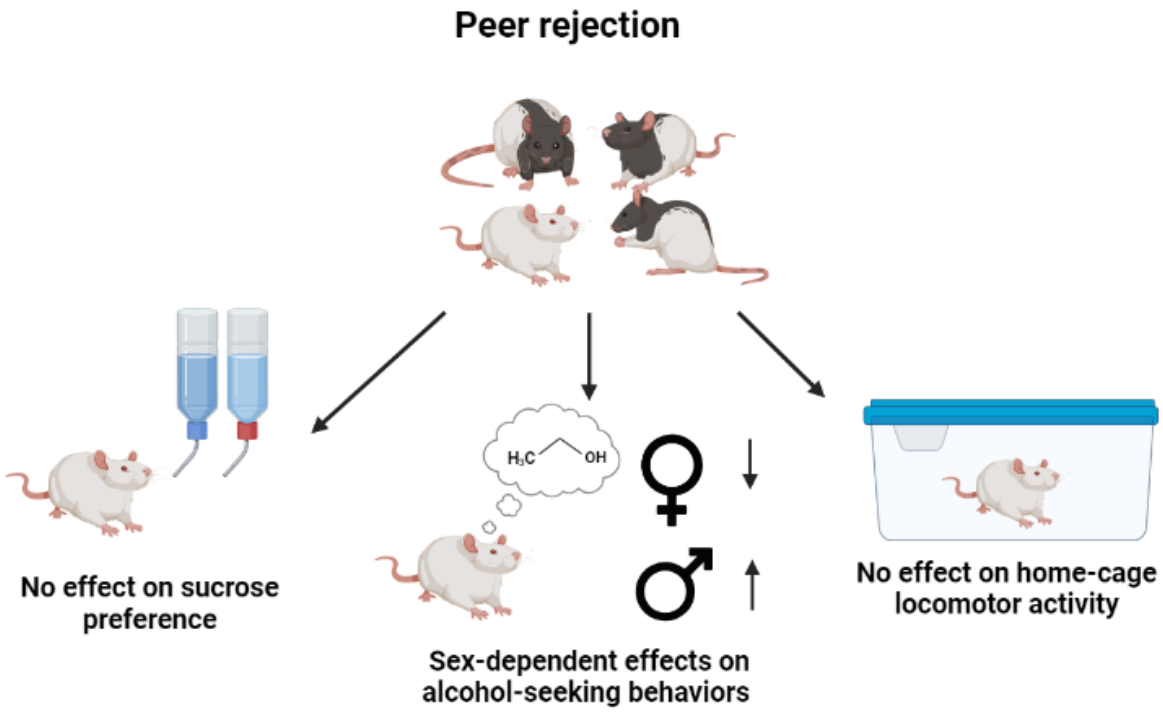

Figure 34: Summary figure demonstrating the main findings from the peer rejection paradigm. PR increased alcohol-seeking behavior in a sex-dependent manner without influencing secondary reward paths (sucrose preference) or home-cage locomotion.

4.3.1 Sex differences in stress responsiveness and alcohol use following PR

We postulate that the sex difference observed could be due to adolescent female rats exhibiting a higher physiological response to PR compared to their male counterparts. Although, sex differences have not yet been assessed in the PR model, human studies have shown that girls show a heightened alpha amylase and cortisol peaks in response to PR, compared to boys (Stroud et al. 2002, 2017).

Whether PR leads to higher rates of alcohol-related problems remains elusive in humans. However, if we look at individuals who have experiences ACES, we find that women are more likely to develop AUD (Dube et al. 2006). Additionally, studies report women having a higher tendency to escalate their rate of alcohol consumption and progression to alcoholism faster than men (Diehl et al. 2007). The data on alcohol relapse is mixed, with some studies finding women relapse faster in the first few months of abstinence, however this difference disappears with after longer abstinence periods (Greenfield et al. 2007). While other researchers have found that men relapse faster after treatment, they found that women's relapse rates were reliant on negative emotional states and relationship problems (Walitzer and Dearing 2006). We need to remember that these difference may also be compounded by adversity, which typically increases the likelihood of developing alcohol related problems (Becker and Chartoff 2019). Since, girls are more affected by PR, this may compound overtime and lead the increased relapse-like behavior like what we observed in our female rats.

Given the substantial intersection between PR and relapse-like behaviors observed in both humans and rats, it is imperative to underscore the apparent resilience of male rats to the PR process. Specifically, we observed no alterations in their social interactions or pain thresholds (unpublished data, Appendix 26). In contrast, female rats that experienced PR (Schneider et al. 2016a), paralleling pre-pubertal girls, exhibit increased sensitivity to PR and its enduring effects (Benenson et al. 2013; Stroud et al. 2017).

4.3.2 Sex differences in the underlying neurobiological mechanisms following PR

Several candidate mechanisms may underlie the observed sex-dependent differences in alcohol cue-induced reinstatement. Notably, PR leads to marked changes in the endocannabinoid system, specifically in increased expression of CB1R and CB1

agonist stimulated binding in the AMY and thalamus (Schneider et al., 2014; Schneider et al., 2016). Studies have shown that blocking CB1R with an antagonist can reduction relapse-like behaviors in rats (Economidou et al. 2006), while antagonist increase alcohol seeking (Getachew et al. 2011). Additionally, we have demonstrated in our lab how enhanced CB1R signaling in a mutant rat strain leads to augmented sensitivity to drug rewards (Schneider et al., 2015). A plausible explanation for the increased relapse-like behavior in female PR rats, maybe that they have higher levels of CB1R in the AMY as a compensatory mechanism to PR. This higher level of CB1Rs could lead to more endocannabinoids binding to promoting alcohol-seeking. This would also postulate that males show the opposite pattern in CB1R expression in the AMY following PR. Future studies should administer CB1 agonist and antagonist into the AMY to validate our claim.

Preliminary data also suggests OTR mRNA levels are altered in the thalamus following PR (Unpublished data, Appendix 27). OT is known to play a pivotal roles in relapse-like behavior (Spanagel, 2020), and previous research has described OTs sex-dependent modulation relapse-like behavior (Hansson et al. 2018; Hansson and Spanagel 2021). The previously observed increase in thalamic OT mRNA (~60%) following PR, suggests long-lasting changes to the OT system. What this increased OT mRNA implies remains to be studied. Potentially, the increase in OT mRNA may indicate an upregulation of OTRs as a compensatory mechanism to PR, which may entail that more OT is required to reach similar effects as in CTL females.

A region which has shown context dependent activation to PR is the dorsal anterior cingulate cortex (dACC) (Eisenberger et al. 2003) and abnormal signaling have been correlated to alcohol-craving (Huang et al., 2018). Additionally, dACC functional connectivity is associated with future relapse in alcoholics. Suggesting that abnormal connectivity and signaling in a vulnerable population such as alcoholics or rejected women may be more pre-disposed to alcohol relapse. The sex-differences highlighted demonstrate the importance of considering biological sex as a factor when studying the neurobiological underpinnings of substance use disorders and their relation to AAs.

4.3.3 Peer rejection alters circadian dysregulation

PR has been associated with sleep disturbances during adolescence (Tu et al. 2019). These sleep irregularities, in turn, are linked to a multitude of health risks, one of which

includes heightened alcohol consumption in both rodents and humans (Jagannath et al. 2013; Logan et al. 2018). Furthermore, alcohol influences circadian-related gene expression (Perreau-Lenz and Spanagel 2015) so PR together with alcohol could potentially disrupt the normal circadian rhythmicity. However, we did not observe any effect of PR on circadian rhythms or locomotion. We did observe a sex-typical effect on daily locomotor activity, with females travelling more than males (Rosenfeld 2017).

Our findings contrasts those of other studies reporting on circadian dysregulation following a AAs (Wells et al. 2017). However, these studies typically employ more severe physical stressors, such as chronic social defeat. This difference in severity, coupled with the fact that our circadian measurements were assessed three weeks following the termination of alcohol self-administration, may account for the lack of findings in our study. It is important to further investigate the relationship between PR and circadian rhythms under different conditions, such as varying the timing of measurements or adjusting the severity of the stressor. Exploring these interactions might provide a more nuanced understanding of how social stressors such as PR may influence physiological processes, including sleep and circadian rhythms, and how these changes may relate to health outcomes such as substance use disorders.

4.3.4 Peer rejection does not alter reward sensitivity to sucrose

In previous research, we have noted a decreased affinity for social reward in peer-rejected rats (Schneider, et al., 2016). Following these findings, we investigated whether these deficits were generalizable to other rewards such as palatable foods. In this context, we examined alterations in reward sensitivity by implementing the sucrose consumption test. Our findings, however, indicated no discernible differences in sucrose consumption or preference among peer-rejected rats. This would suggest that PR does not result in a general alteration in reward sensitivity, results which align with the current literature. This dysfunction seems to be specific to the social domain, which is corroborated by our previous findings (Schneider, et al., 2016). Parallels can be drawn from individuals from alcohol related problem who have altered effective connectivity in the reward network and often find themselves in social problems(Lewis and Nixon 2014).

4.3.5 Summary

Our study investigated the long-term effects of PR during adolescence on various aspects such as alcohol-seeking behavior, circadian rhythms, and reward sensitivity in rats. The findings indicated that PR has sex-dependent effects on alcohol-seeking behavior, increasing alcohol cue-induced reinstatement in females, while decreasing in males. PR had no significant effect on circadian rhythms or sucrose consumption, suggesting the impact of social stressors like PR may be restricted to alcohol-seeking and social domain, as previously demonstrated (Schneider et al. 2016a). Further research is necessary to understand these interactions better, potentially informing interventions for substance use disorders.

4.4 General discussion

Our findings reveal significant insights into the long-term behavioral and molecular changes induced by AA using a reverse translational approach. We will first discuss how our findings align with the human adversity/ACE findings. Second, we discuss future prospects. Last, we discuss how we can improve translatability of our findings and how this can help improve the field of adolescent adversity research.

Table 4: Summary behavioral results for the long-term effects of adolescent social isolation (ASI), social instability stress (SIS) and peer rejection (PR) depicted relative to control (CTL) rats in male and female rats. Includes unpublished PR data from our lab. ↓ = reduction, ↑= increase, ≠ = no difference, empty = no data

COMPARSION vs. CTL	ASI				SIS		PR	
TIMING OF ADVERSITY	Early		Late		Early		Early + late	
SEX	♂	♀	♂	♀	♂	♀	♂	♀
ANXIETY	≠	≠	≠	≠*	↑	↑	≠	≠
SOCIAL MEMORY	↓	↓	↓	↓	↓	↓	?	↓

OBJECT MEMORY	≠	≠	≠	≠	?	?	≠	≠
SOCIAL INTERACTION	≠	≠	↓	↓	↑	↑	↓	≠
THERMAL PAIN SENSITIVITY	↓	↑	↓	↓	?	?	↓	≠

4.4.1 Translatability of models

It is important to consider the translatability of our models to understand how well our reverse translational approach can be applied in future studies.

4.4.1.1 Adolescent social isolation

In humans, early deprivation/isolation has been primarily studied in cohorts of Romanian orphans. This was because of the immense number of orphans attributable to pro-natality policies placed by the Romanian dictator Nicolae Ceaușescu in the 1960s. It has been estimated that over 500,000 children were institutionalized between the 1960s and the 1990s (Odobescu 2015). These children were reared in deplorable conditions where they were extremely deprived in all respects, malnourished, spending most parts of the day alone in cribs/beds lacking in social and physical contact, and lacking auditory and visual stimulation (Le Mare and Audet 2006). This is similar to rat model of ASI, where rats are deprived in the same manner. This deprivation period adversely affected cognitive and social development (Rutter et al. 1998). Notably, those who spent more than six months in such institutions showed an increased incidence of neurodevelopmental disorders, including attention deficit/hyperactivity disorder, autism spectrum disorder, and disinhibited social engagement disorder, which persisted into young adulthood (Kennedy et al. 2016; Sonuga-Barke et al. 2017). Additionally, these institutionalized children showed lower intelligence quotients (IQ) and impairments in learning to speak, write, and read than non-institutionalized children (Fox et al. 2011). This, in turn, influenced school and work performance, making socialization more difficult.

Although, our model cannot completely recapitulate human neuropsychiatric disorders or make direct comparisons, we can draw parallels from similarities in behavioral domains and neurobiological domains following ASI. For instance, we observed that the ASI model lead to persistent impairments in the social domain, in both social skills (social interactions) and cognition function (social recognition memory) as well as pain sensitivity (thermal), which are reminiscent of social problems, cognitive and chronic pain conditions in humans exposed to AA (Herzog and Schmahl 2018). These data suggest that the ASI model has good face validity as it brings about similar impairments as in humans and at least moderate construct validity as similar symptomology as in some neuropsychiatric disorder such as schizophrenia (Robbins 2016). Furthermore, neurobiological findings converge on changes in HPC and AMY in both human and rodent studies following ASI (Mehta et al. 2009; Silvers et al. 2016; Begni et al. 2020b). Taken together, these data suggest that the ASI paradigm has merit in the preclinical AA field as a valid model that recapitulates key features of the human condition.

4.4.1.2 Social instability stress

SIS in LE rats has previously been shown to be an etiologically valid paradigm leading to similar impairments in humans (Koert et al. 2021). The SIS model demonstrates good construct validity as social instability stressors have been shown to strongly influence the development of stress-related disorders in humans (van Praag 2004). Our data suggested that WIST rats may perceive the SIS paradigm as an environmental enrichment rather than a pure stressor, compared to LE rats which shown more detrimental effects of SIS. Hence, it may be more advantageous for future studies to investigate resilience to social stressor in the SIS WIST model.

In humans, social instability has been shown to increase in anxiety (Rumbold et al. 2012) and depression (Glasheen et al. 2018) rats, as well as lower social skills (Dupere et al. 2015). we observed a decrease in anxiety-like behavior across sexes in our WIST rats, further suggesting that WISTs may not be the best model to investigate the detrimental effects of SIS. On the other hand, we observed impaired social a result comparable to the human data. To the best of our knowledge, novelty, seeking and social cognition has not been investigated in this context in humans. Only one other study has investigated SIS in WIST rats (Provensi et al. 2019a), which found no change in anxiety-like behaviors but observed impairments in memory. Hence, more studies

are required before we can truly determine the translational value of this model in WIST rats.

4.4.1.3 Peer rejection

Our study showed how PR during adolescence leads to long-term changes relapse-like behavior in a sex-dependent manner. If we compare our findings to the human literature similarities appear. For example, women with a history of ACEs show greater vulnerability for alcohol and cocaine relapse as well as greater cue reactivity to alcohol in an experimental setting (Hyman et al. 2008; Heffner et al. 2011; Hartwell and Ray 2013). These findings suggest a good degree of reverse translation and it can inform human studies as they parallel some behavioral patterns observed in humans who have experienced PR or bullying, including increased social anxiety, depression, and other mental health issues(Silk et al. 2014; Hance et al. 2018; Nepon et al. 2021).

To translate these findings into human research, future studies should focus on developing valid and reliable tools for assessing experiences of PR in rodents. For example, neuroimaging studies, could provide insights into whether PR affects the dACC in a similar manner in rodent as in humans who are rejected (Eisenberger et al. 2003). However, this is a significant challenge to do in rodents with neuroimaging but could be overcome by using fiber photometry approach to measure neural activity in the dACC during the PR exposure.

The changes observed in the dACC were first demonstrated in the virtual ball tossing game (cyber ball) which mimics PR and activated the brain regions associated with the experience of physical pain (Eisenberger et al. 2003) and have since been replicated in several studies. This study was the first to highlight the significant impact social rejection could have on an individuals' well-being. Specifically, the dACC as well as the AI were activated in response to virtual rejection, both regions have been shown to be involved in processing emotional (Gu et al. 2012) and physical (Liu et al. 2021) pain. Another cyber-ball study found similar brain activation in response to PR. Where, post pubertal adolescents showed the highest neural activity in the subgenual anterior cingulate cortex and AMY in response to rejection (Silk et al. 2014). The subgenual anterior cingulate cortex is involved in emotional responses to negative stimuli, whereas the AMY processes a wide variety of emotional information (Sergerie et al.

2008). These findings in the dACC, AI and AMY would be important to replicate in peer rejected rats to further improve the reverse translational value of the model in respect to their neural correlates observed in humans.

Rejection by peers also leads to negative emotions such as sadness, anger, and shame (Rudolph 2002). Adolescents who experience peer rejection show more anger- and impulsivity-related issues (Prinstein and La Greca 2004), internalization of problems (Reijntjes et al. 2010), and sleep disturbances (van der Wilt et al. 2019). A recent study found a positive association between PR and social withdrawal (Ran et al. 2022). The negative cascade of peer rejection is also correlated with higher incidences of anxiety and depression in adolescence as well as difficulties in regulating emotions in social situations (Rudolph 2002). We did not observe differences in anhedonia-like behavior or sleep disturbances, which would suggest differences in the behavioral outcomes of PR in humans and rats. Nevertheless, this is only a single test, and a more in-depth assessment of depression-like behaviors and sleep dysregulation would be required to understand the effects of PR on these domains.

Some aspects of the PR model have shown good face validity (i.e. impaired social skills and cognition and increased pain sensitivity) which also reflects good construct validity as these are domains often impaired in borderline personality disorder patients (Lieb et al. 2004; Bungert et al. 2015).

4.4.2 Future prospects and next steps

We will perform further validation experiments in order to understand the functional role of OTRs in the PVT on social, anxiety, fear and pain related behaviors. First, we want to manipulate OTRs in the PVT using a chemogenetic approach. Second, we plan to validate the role of OTR activation and inhibition in the PVT using optogenetics in order to validate that OTR action is responsible for the observed effects

First, future ASI studies should first focus on trying to replicate our findings with a multi-site collaboration to increase reliability. Second, studies should shift focus to a single behavioral domain (i.e., social) and perform a battery of tests within this domain to determine validity of our findings. Third, there is a need determine the region-specific functional relevance of the OTR binding changes in the PVN, CeA, PVT in EASI males and females, which showed the largest behavioral and molecular changes. From a

translational perspective, we hope our research guides clinicians to investigate, social recognition, thermal pain sensitivity and OT alterations in adults exposed to social isolation/deprivation during adolescence to evaluate the rigor of the reverse translational approach. Alternatively, more in-depth ASI studies are required focusing on replicating outcomes that have already been demonstrated in individuals exposed to ASI. For example, flattening of the cortisol awaking curve (Flannery et al. 2017) or changes in grey and white matter volumes in the PFC and hippocampus (Hodel et al., 2015; Mackes et al., 2020; Sheridan et al., 2012; VanTieghem et al., 2021) to improve the predictive validity of our animal model.

Future SIS studies should focus on similar approaches suggested for ASI: a multi-level approach that combines behavioral and biological measures that have already been shown to be altered in humans exposed to social instability. This includes, examining stress hormone levels, preclinical brain imaging data studies (non-exist so far), or trying to establish genetic markers (i.e., OT) could provide deeper insights into the physiological effects of SIS. OT is a plausible target due to the social interaction and memory changes often observed following SIS.

Finally, for the PR model, which is quite a new model there is little data on males. We, propose a large-scale longitudinal behavioral characterization study to first elucidate what other sex differences may exists. There is also a need to introduce a method for the assessment of the PR severity to determine how well rats are being peer rejected and as mentioned the use of fiber photometry could be used to assess this in the dAAC.

4.4.3 Improving translatability of preclinical adolescent adversity models

Despite our results aligning with human findings, the likelihood of our findings land in the “valley of death” is high (Seyhan 2019). The “valley of death” refers to important (perceived at least by us as such) preclinical findings that end up in the widening gap between funding for basic science and development of therapeutic interventions (Butler 2008). There have been several efforts from the scientific community to improve this, from the ARRIVE and PREPARE guidelines (Smith et al. 2018; Percie Du Sert et al. 2020), research domain criteria (RDoC) project, to scientists laying out guidelines to improving preclinical research (Spanagel 2022). Nonetheless, these large scale efforts have been slow and progress is needed faster considering there is significant pressure from animal activists and politicians in Europe to completely eliminate animal

research (Vanderbemden 2022). There is sense of urgency to make preclinical research more productive we think the reverse translational approach can help speed up the process. Collaborative efforts between clinicians and preclinical scientists are a necessity, along with collaborations between academia and industry, if we are to succeed

From a more methodical perspective, reverse translation could push us towards streamlining research goals and methods as a field, because several researchers could be working to replicate human findings, and this could be done within a smaller field. For example, we could bring together researchers from the AA field to create a scientific consortium. A consortium where AA researchers can agree on which time points to investigate together, when to assess the rodents, and establish collaboration with clinicians. This harmonization of scientific efforts would allow us to achieve unified goals more effectively and increase our collective knowledge as a field. This harmonization could play an important part in improving reproducibility, data sharing, traceability, and making comparisons more reliable. Such efforts have taken place in various fields with success (Miller and Greenberg 2021; Rinaldi et al. 2022; Plebani and Lippi 2023). The success of such efforts will rely on our willingness as researchers to improve our own quality of work.

Although one of the biggest advantages with reverse translation is the potential of identifying underlying neurobiological mechanism of the human condition in rodents. There is a need to use comparable research tools and methods (i.e., neuroimaging and genomics) across organisms to improve external validity. We also need to remember that reverse translation is a two-way street, and the quality of the humans' studies guides the quality of the preclinical work.

4.5 Limitations

4.5.1 Adolescent social isolation study (Study 1)

There are several limitations with our ASI study that need to be considered. Our aim was to characterize the long-term effects which meant we had to rely on a significant number of behavioral tests. This could create fatigue effects. We tried our best to negate these with how we controlled the order of our experiments. Fatigue effect refers to rats becoming tired or less motivated in longer experiments. We therefore gave rats

48 hrs to recover between each test. Another consideration is a lack of other ASI comparisons for most tests, which led us to compare other adversity models. This highlights the need for replication of our findings so a direct comparison can be made to validate our results. Another caveat to with our study is that although we demonstrate that endogenous OT release in the PVT impairs social recognition, this finding is not yet causally established. We plan to run a control study using a genetic OTR Cre line and optogentically activate and inhibit OTRs in the PVT in order to validate our initial findings.

4.5.2 Social instability study (study 2)

One of the main limitations of our study is that we only have behavioral readout and there would be a need for biomarkers of SIS. We had planned to investigation of the OT system as previous research in LE rats has shown immediate effect of SIS on the OTR binding in males (Hodges et al. 2017). Additionally, we had planned to assess basal CORT levels, unfortunately due to technical failure of the freezer resulting in the loss of all biological material. Another potential caveat with our study was that rats were tested on consecutive days which may lead to order effects; however, this is hard to overcome when testing in adolescence as behavior repertoire changes rapidly during this time as evidenced in the previous section. Additionally, to keep our study comparable to the those performed in LE rats it was important to keep the experiment as similar as possible.

4.5.3 Peer rejection study (study 3)

It is important to note a potential limitation in our methodology for assessing reward sensitivity. The ability of the sucrose consumption test to detect subtle changes in consumption or preference may be constrained. Using an operant paradigm aligned with the matching law principle (Sanchis-Segura et al., 2005), or intracranial self-stimulation, could potentially offer improved sensitivity and discrimination to uncover any nuanced changes between peer-rejected and control animals. As with any animal model, caution must be exercised when generalizing results to human populations. Peer rejection in rats may not fully encapsulate the complexity of human social interactions and the accompanying psychological effects. Additionally, the assessment of peer rejection in rats might not mirror the actual psychological experience in humans, limiting the interpretation of the results. The definition and measurement of peer

rejection in rodents can be somewhat subjective and lacks a standardized approach. Finally, the severity and duration of PR can vary, which may influence the outcomes. Hence, more studies are needed to better understand the effects of timing and duration of PR

5 CONCLUSION

The present work demonstrated some of the profound and multifaceted long-term impact of adolescent adversities on adult behaviour in both of male and female rats. Through careful study of adolescent social isolation (ASI), social instability stress (SIS), and peer rejection models in rats, we have shed light on their behavioral and neurobiological effects that mirror some of the symptoms and outcomes of humans who were exposed to ACEs.

The behavioral alterations observed in ASI rats — social and cognitive dysfunction, and heightened pain sensitivity — reflect the repercussions observed in humans who experienced prolonged periods of deprivation (i.e., Romanian orphans) throughout their adolescence. Furthermore, we demonstrated how endogenous OT release is able to impair the social recognition memory of female rats regardless of adversity condition. Further validation experiments are planned to establish causality. The translational value of our findings into human research lies largely in the exploration of the role of the PVT following ASI but could be used to guide clinicians in their research questions on understanding how ASI influences social and cognitive dysfunction, and heightened pain sensitivity. The use of neuroimaging techniques could be used to identify similarities with humans, specifically the mPFC and hippocampus are affected in humans (Hodel et al., 2015; Mackes et al., 2020; Sheridan et al., 2012; VanTieghem et al., 2021). Replicating these finding would improve the external validity of the ASI model and improve translatability.

Studies on adolescent SIS in rats have revealed significant behavioral alterations, including decreased social interaction, heightened exploratory behavior, and improved social recognition memory. These findings align with behavioral patterns observed in humans exposed to unstable social environments during their early years. We tested this paradigm in WIST rats in order to test the external validity of the model and compare results with previously published research on the SIS model in LE rats. We found that WIST rats were more resilient than LE for SIS stress. Future studies could investigate neurobiological strain differences (i.e., OT system differences) in order to better understand what may be driving this difference in resilience.

Finally, our novel adolescent PR paradigm showed that such experiences have enduring changes in relapse-like behaviors in a sex-dependent manner. These findings suggest the PR model is a valid model of relapse-like behaviour. Although more work is required in the development of valid tools for assessing severity of PR experiences and further supplemented by investigations into the neurobiological ramifications of these behavioral shifts. The dorsal part of the anterior cingulate cortex remains region of interest, due to the activation observed following PR in the cyberball task (Eisenberger et al. 2003).

In summary, animal models such as those presented here provide vital insights into the mechanisms underlying adversity-related disorders. The ultimate goal is that these mechanistic understanding leads to the development of effective preventions and intervention strategies that mitigate the adverse effects of ACEs, thereby fostering improved human health and well-being.

The gravity of ACEs, with their far-reaching societal, financial, and individual health costs, underscores the urgency of this mission. The risk for negative mental, somatic, and behavioral outcomes escalates with each adverse experience, impacting not just individuals but communities, families, and societal structures. To address these profound challenges, society must commit to building robust infrastructure for health promotion, disease prevention, and research into vulnerable populations exposed to ACEs. Although we as preclinical researcher are crucial in understanding how the underlying neural circuitry is altered by AA. The onus falls not only on researchers but on the broader public too, reaffirming the crucial role of collective responsibility in preventing and mitigating the harmful effects of adverse childhood experiences.

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7 TABLED APPENDIX (IF REQUIRED)

7.1 Adolescent social isolation summary statistics

Appendix 1: Summary statistics for percent time spent on the open, closed arms, center zone and total number of arm entries on the elevated plus maze performed on PD 90 in study 1A

Percent time spent on open arms

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	0.218[a]	5	0.044	2.428	0.044	0.151
Intercept	3.709	1	3.709	206.330	<0.001	0.752
Treatment	0.105	2	0.053	2.924	0.061	0.079
Sex	0.023	1	0.023	1.280	0.262	0.018
Treatment * Sex	0.095	2	0.048	2.647	0.078	0.072
Error	1.222	68	.018			
Total	5.097	74				
Corrected Total	1.441	73				

a. R Squared = 0.151 (Adjusted R Squared = 0.089)

Percent time spent in center zone

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	0.197[a]	5	0.039	4.523	0.001	0.250
Intercept	2.599	1	2.599	297.967	<0.001*	0.814
Treatment	0.023	2	0.012	1.324	0.273	0.037
Sex	**0.099**	**1**	**0.099**	**11.396**	**0.001**	**0.144**
Treatment * Sex	**0.056**	**2**	**0.028**	**3.199**	**0.047**	**0.086**
Error	0.593	68	0.009			
Total	3.450	74				
Corrected Total	0.790	73				

a. R Squared = 0.250 (Adjusted R Squared = 0.194)

Percent time spent in closed arms

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	0.168[a]	5	0.034	1.267	0.288	0.085

Intercept	24.542	1	24.542	925.523	<0.001	0.932
Treatment	0.052	2	0.026	0.989	0.377	0.028
Sex	0.021	1	0.021	0.774	0.382	0.011
Treatment * Sex	.094	2	0.047	1.769	0.178	0.049
Error	1.803	68	0.027			
Total	27.202	74				
Corrected Total	1.971	73				

a. R Squared =.0,085 (Adjusted R Squared = 0.018)

Total number of arm entries

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	199.925[a]	5	39.985	5.643	<0.001	0.293
Intercept	7370.017	1	7370.017	1040.057	<0.001	0.939
Treatment	26.745	2	13.372	1.887	0.159	0.053
Sex	**94.916**	**1**	**94.916**	**13.395**	**<0.001**	**0.165**
Treatment * Sex	**76.386**	**2**	**38.193**	**5.390**	**0.007**	**0.137**
Error	481.859	68	7.086			
Total	8162.000	74				
Corrected Total	681.784	73				

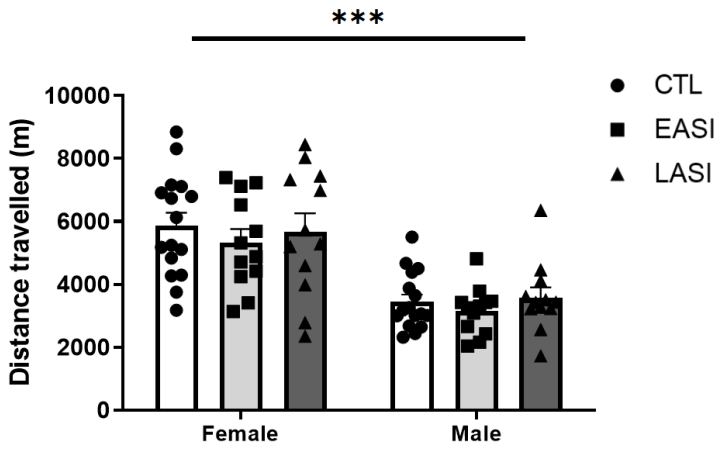
a. R Squared = 0.293 (Adjusted R Squared = 0.241)

Appendix 2 Summary statistics and figure for distance travelled on the open field test on PD 92 in study 1A

Total distance travelled in centimeters

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	104544676.325[a]	5	20908935.265	10.990	<0.001	0.426
Intercept	1599865722.946	1	1599865722.946	840.902	<0.001	0.919
Treatment	2681346.934	2	1340673.467	0.705	0.498	0.019
Sex	**98016872.555**	**1**	**98016872.555**	**51.518**	**<0.001**	**0.410**
Treatment * Sex	384375.104	2	192187.552	0.101	0.904	0.003
Error	140789289.024	74	1902557.960			
Total	1885351120.590	80				
Corrected Total	245333965.349	79				

a. R Squared = 0.426 (Adjusted R Squared = 0.387)



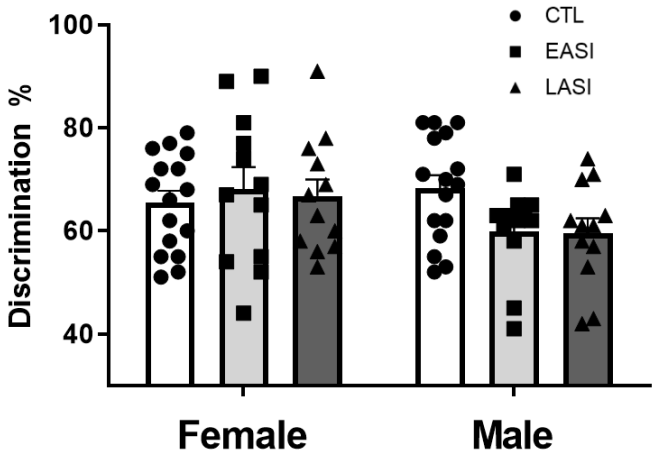
Adolescent social isolation did not influence adult performance on the open field test. Females rats travelled a longer distance during the 30 min OFT compared to males (F (1. 74) = 51.395 p < 0.000001. ηp2 = 0.410) in a sex-typical manner. Data shown with individual data points and μ±SEM.

Appendix 3: Summary statistics and figure for rats' discrimination ability as a percent of time spent investigating the novel vs. the familiar object during the test phase of the novel object recognition test performed on PD 94 in study 1A

Object discrimination ability

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	984.050[a]	5	196.810	1.695	0.146	0.103
Intercept	328494.910	1	328494.910	2829.237	<0.001	0.975
Treatment	212.435	2	106.218	.915	0.405	0.024
Sex	342.046	1	342.046	2.946	0.090	0.038
Treatment * Sex	530.102	2	265.051	2.283	0.109	0.058
Error	8591.937	74	116.107			
Total	346407.000	80				
Corrected Total	9575.988	79				

a. R Squared = 0.103 (Adjusted R Squared = 0.042)



Adolescent social isolation did not influence adult novel object recognition ($F_{(1.\ 74)}$ = 51.395 p < 0.000001. ηp2 = 0.410). Data shown with individual data points with μ±SEM.

Appendix 4: Summary statistics for time spent in social interactions, frequency of anogenital sniffing, non-anogenital sniffing and rearing bouts in the social interaction test performed on PD 96 in study 1A

Total time spent in social interactions

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	7349.117[a]	5	1469.823	3.736	0.005	0.202
Intercept	176054.190	1	176054.190	447.494	<0.001	0.858
Treatment	**7110.002**	**2**	**3555.001**	**9.036**	**<0.001**	**0.196**
Sex	12.963	1	12.963	0.033	0.856	0.000
Treatment * Sex	225.502	2	112.751	.287	0.752	0.008
Error	29113.271	74	393.423			
Total	221455.000	80				
Corrected Total	36462.387	79				

a. R Squared = 0.202 (Adjusted R Squared = 0.148)

Anogenital sniffing bouts

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	141.217[a]	5	28.243	1.334	0.260	0.083
Intercept	6465.690	1	6465.690	305.283	<0.001	0.805
Treatment	127.102	2	63.551	3.001	0.056	0.075

Sex	9.281	1	9.281	0.438	0.510	0.006
Treatment * Sex	6.302	2	3.151	0.149	0.862	0.004
Error	1567.271	74	21.179			
Total	8535.000	80				
Corrected Total	1708.488	79				

a. R Squared = 0.083 (Adjusted R Squared = 0.021)

Non-anogenital bouts

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1332.179[a]	5	266.436	8.743	<0.001	0.371
Intercept	11927.463	1	11927.463	391.408	<0.001	0.841
Treatment	**334.190**	**2**	**167.095**	**5.483**	**0.006**	**0.129**
Sex	**751.781**	**1**	**751.781**	**24.670**	**<0.001**	**0.250**
Treatment * Sex	178.790	2	89.395	2.934	0.059	0.073
Error	2255.021	74	30.473			
Total	16288.000	80				
Corrected Total	3587.200	79				

a. R Squared = 0.371 (Adjusted R Squared = 0.329)

Rearing bouts

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	3348.800[a]	5	669.760	5.939	<0.001	.0.286
Intercept	56774.668	1	56774.668	503.473	<0.001	0.872
Treatment	**2004.352**	**2**	**1002.176**	**8.887**	**<0.001**	**0.194**
Sex	326.296	1	326.296	2.894	0.093	0.038
Treatment * Sex	**978.935**	**2**	**489.468**	**4.341**	**0.017**	**0.105**
Error	8344.688	74	112.766			
Total	69421.000	80				
Corrected Total	11693.488	79				

a. R Squared = 0.286 (Adjusted R Squared = 0.238)

Appendix 5: Summary statistics for duration spent in investigating the novel, familiar, both conspecifics and social discrimination ability in the social recognition memory test performed on PD 96 in study 1A

Duration spent investigating the novel conspecific

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	228.029[a]	5	45.606	.314	0.903	0.021
Intercept	39176.727	1	39176.727	269.984	<0.001	0.785
Treatment	104.363	2	52.181	0.360	0.699	0.010
Sex	34.186	1	34.186	0.236	0.629	0.003
Treatment * Sex	74.054	2	37.027	0.255	0.775	0.007
Error	10737.958	74	145.108			
Total	51421.000	80				
Corrected Total	10965.988	79				

a. R Squared = 0.021 (Adjusted R Squared = 0.045)

Duration spent investigating familiar conspecific

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1163.533[a]	5	232.707	3.360	0.009	0.185
Intercept	14131.410	1	14131.410	204.030	<0.001	0.734
Treatment	**930.085**	**2**	**465.043**	**6.714**	**0.002**	**0.154**
Sex	2.660	1	2.660	0.038	0.845	0.001
Treatment * Sex	222.935	2	111.468	1.609	0.207	0.042
Error	5125.354	74	69.262			
Total	19887.000	80				
Corrected Total	6288.888	79				

a. R Squared = 0.185 (Adjusted R Squared = 0.130)

Total interaction duration for both conspecifics

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	636.596[a]	5	127.319	0.415	0.837	0.027
Intercept	100366.501	1	100366.501	327.166	<0.001	0.816
Treatment	487.523	2	243.761	0.795	0.456	0.021
Sex	17.774	1	17.774	0.058	0.810	0.001
Treatment * Sex	134.623	2	67.311	0.219	0.804	0.006
Error	22701.354	74	306.775			
Total	124300.000	80				

Corrected Total	23337.950	79				

a. R Squared = 0.027 (Adjusted R Squared = 0.038)

Social discrimination ability

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	3468.638[a]	5	693.728	5.641	<0.001	0.276
Intercept	316774.486	1	316774.486	2575.626	<0.001	0.972
Treatment	**2896.597**	**2**	**1448.298**	**11.776**	**<0.001**	**0.241**
Sex	67.145	1	67.145	.546	0.462	0.007
Treatment * Sex	449.974	2	224.987	1.829	0.168	0.047
Error	9101.210	74	122.989			
Total	342966.495	80				
Corrected Total	12569.848	79				

a. R Squared = 0.276 (Adjusted R Squared = 0.227)

Appendix 6: Summary statistics for latency (s) to a thermal pain stimulus (52.5°C) on the hotplate test performed on PD 98 in study 1A

Latency to a thermal pain stimulus

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	227.299[a]	5	45.460	8.432	<0.001	0.363
Intercept	5622.039	1	5622.039	1042.781	<0.001	0.934
Treatment	**36.449**	**2**	**18.225**	**3.380**	**0.039**	**0.084**
Sex	**42.116**	**1**	**42.116**	**7.812**	**0.007**	**0.095**
Treatment * Sex	**127.695**	**2**	**63.848**	**11.843**	**<0.001**	**0.242**
Error	398.963	74	5.391			
Total	6447.134	80				
Corrected Total	626.262	79				

a. R Squared = ,363 (Adjusted R Squared = ,320)

Appendix 7: Summary statistic for [^{125}I]-OVTA receptor autoradiography from male and female rats separately from study 1B

Males

Binding sites	Region	CTL ♂	EASI ♂	LASI ♂	F-value	P-value
([^{125}I]-OVTA	DLS	0.619±0.4 N = 8	0.546±0.05 N = 8	0.608±0.02 N= 8	1.301 (1. 37)	0.284
	DMS	0.678±0.01 N = 8	0.632±0.03 N = 8	0.655±0.02 N = 8	1.430 (1. 36)	0.237
	CPU	0.291±0.03 N = 7	0.273±0.04 N = 8	0.250±0.05 N = 6	0.571 (1. 28)	0.721
	ACB	0.291±0.03 N = 8	0.210±0.02 N = 7	0.26±0.07 N = 4	0.817 (1. 30)	0.547
	PVN	**0.205±0.01** N = 5	**0.143±0.02** N = 3	**0.199±0.07** N = 3	**8.267 (1. 21)**	**0.002**
	CEA	**0.663±0.08** N = 4	**1.008±0.08** N = 5	**0.919±0.11** N = 6	**3.519 (1. 21)**	**0.048**
	BLA	0.121±0.00 N =4	0.193±0.05 N = 5	0.164±0.02 N = 6	2.018 (1. 19)	0.1220.
	PVT	**0.239±0.02** N = 6	**0125±0.01** N = 6	**0.257±0.02** N = 4	**15.791 (1. 29)**	**0.00003**
	vCA1	0.869±0.08 N = 5	1.173±0.13 N = 6	1.070±0.04 N = 4	1.520 (1. 25)	0.219

Females

Binding sites	Region	CTL ♀	EASI ♀	LASI ♀	F-value	P-value
([^{125}I]-OVTA	DLS	0.614±0.3 N = 6	0.628±0.02 N = 7	0.688±0.03 N = 6	1.301 (1. 37)	0.284
	DMS	0.696±0.02 N = 5	0.643±0.02 N = 8	0.711±0.02 N = 6	1.430 (1. 36)	0.237
	CPU	0.359±0.04 N = 4	0.261±0.04 N = 4	0.295±0.03 N = 5	0.571 (1. 28)	0.721
	ACB	0.308±0.04 N = 4	0.234±0.06 N = 6	0.284±0.03 N = 6	0.817 (1. 30)	0.547
	PVN	**0.124±0.01** N = 3	**0.295±0.02** N = 6	**0.192±0.01** N = 7	**8.267 (1. 21)**	**0.002**
	CEA	**0.705±0.05** N =4	**0.822±0.09** N = 4	**0.465±0.04** N =4	**3.519 (1. 21)**	**0.048**

BLA	0.182±0.04	0.179±0.00	0.089±0.00	2.018 (1. 19)	0.1220.
	N = 4	N = 4	N = 4		
PVT	**0.124±0.01**	**0.299±0.02**	**0.316±0.02**	**15.791 (1. 29)**	**0.00003**
	N = 4	N = 8	N = 7		
vCA1	1.072±0.07	1.032±0.09	1.205±0.09	1.520 (1. 25)	0.219
	N = 6	N = 5	N = 5		

Table 2: [^{125}I]-OVTA receptor autoradiography in ASI male and female rats. All receptor/transporter autoradiography was performed under saturated conditions. Kd-values, the dissociation equilibrium constant describing the affinity for a specific receptor, as well as Bmax-values, describing the maximum density of the receptor in the specified regions DLS. dorsal-lateral striatum; DMS. dorsal-medial striatum; CPU. caudate putamen; ACB. nucleus accumbens core; PVN. paraventricular nucleus of the hypothalamus.; CeA. central amygdala; BLA. basolateral amygdala; PVT. paraventricular nucleus of the thalamus; vCA1. ventral cornu Ammonis 1. Statistics performed across all groups and both sexes. All values shown in fmol/g with Mean±SEM.

Appendix 8: Summary statistics for the familiarization phase and the object discrimination ability during test phase on the novel object recognition test in study 1C

Time spent investigating objects during the familiarization phase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	125.889[a]	3	41.963	1.846	0.256	0.526
Intercept	9552.061	1	9552.061	420.179	<0.001	0.988
Condition	62.061	1	62.061	2.730	0.159	0.353
Virus	58.242	1	58.242	2.562	0.170	0.339
Condition * Virus	15.515	1	15.515	0.682	0.446	0.120
Error	113.667	5	22.733			
Total	10173.000	9				
Corrected Total	239.556	8				

a. R Squared = 0.526 (Adjusted R Squared = 0.241)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	31.807[a]	3	10.602	0.085	0.965	0.049
Intercept	40725.488	1	40725.488	328.310	<.001	0.985
Condition	6.621	1	6.621	0.053	0.826	0.011
Virus	5.199	1	5.199	0.042	0.846	0.008
Condition * Virus	20.849	1	20.849	0.168	0.699	0.033
Error	620.229	5	124.046			
Total	42452.812	9				
Corrected Total	652.036	8				

a. R Squared = 0.049 (Adjusted R Squared = -0.522)

Appendix 9: Summary statistics for the social interaction test, duration and frequency of anogenital sniffing, non-anogenital sniffing, total social interaction duration and rearing in study 1C

Anogenital sniffing duration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	718.560[a]	3	239.520	1.782	0.180	0.196
Intercept	11572.900	1	11572.900	86.107	<0.001	0.796
Condition	0.054	1	0.054	0.000	0.984	0.000
Virus	6.175	1	6.175	0.046	0.832	0.002
Condition * Virus	**682.632**	**1**	**682.632**	**5.079**	**0.035**	**.188**
Error	2956.825	22	134.401			
Total	16084.000	26				
Corrected Total	3675.385	25				

a. R Squared = 0.196 (Adjusted R Squared = 0.086)

Anogenital sniffing time (pair-wise comparison)

Virus	Condition	Condition	Mean Difference	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
Venus	CTL	eASI	-10.725	6.609	0.119	-24.431	2.981
	eASI	CTL	10.725	6.609	0.119	-2.981	24.431

ChR2	CTL	eASI	10.917	6.967	0.131	-3.531	25.365
	eASI	CTL	-10.917	6.967	0.131	-25.365	3.531

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Anogenital sniffing duration (pair-wise comparison)

Condition	Virus	Virus	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
CTL	Venus	ChR2	-11.850	7.777	0.142	-27.978	4.278
	ChR2	Venus	11.850	7.777	0.142	-4.278	27.978
eASI	Venus	ChR2	9.792	5.633	0.096	-1.891	21.474
	ChR2	Venus	-9.792	5.633	0.096	-21.474	1.891

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Anogenital sniffing frequency

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	91.216[a]	3	30.405	1.215	0.328	0.142
Intercept	2473.668	1	2473.668	98.833	<0.001	0.818
Condition	1.466	1	1.466	0.059	0.811	0.003
Virus	0.089	1	0.089	0.004	0.953	0.000
Condition * Virus	83.142	1	83.142	3.322	0.082	0.131
Error	550.631	22	25.029			
Total	3282.000	26				
Corrected Total	641.846	25				

a. R Squared = .142 (Adjusted R Squared = .025)

Non anogenital sniffing duration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	122.385[a]	3	40.795	0.275	0.843	0.036
Intercept	12448.188	1	12448.188	83.813	<0.001	0.792
Condition	60.010	1	60.010	0.404	0.532	0.018
Virus	73.128	1	73.128	0.492	0.490	0.022

Condition * Virus	12.399	1	12.399	0.083	0.775	0.004
Error	3267.500	22	148.523			
Total	17937.000	26				
Corrected Total	3389.885	25				

a. R Squared = 0.036 (Adjusted R Squared = -0.095)

Non anogenital sniffing frequency

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	41.983[a]	3	13.994	0.856	0.478	0.105
Intercept	2922.339	1	2922.339	178.808	<0.001	0.890
Condition	.882	1	0.882	0.054	0.818	0.002
Virus	39.748	1	39.748	2.432	0.133	0.100
Condition * Virus	11.246	1	11.246	0.688	0.416	0.030
Error	359.556	22	16.343			
Total	3726.000	26				
Corrected Total	401.538	25				

a. R Squared = .105 (Adjusted R Squared = -.018)

Total social investigation duration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	759.675[a]	3	253.225	0.954	0.432	0.115
Intercept	48026.224	1	48026.224	181.019	<0.001	0.892
Condition	56.479	1	56.479	0.213	0.649	0.010
Virus	36.803	1	36.803	0.139	0.713	0.006
Condition * Virus	511.033	1	511.033	1.926	0.179	0.081
Error	5836.825	22	265.310			
Total	60423.000	26				
Corrected Total	6596.500	25				

a. R Squared = 0.115 (Adjusted R Squared = -0.005)

Rearing frequency

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	258.324[a]	3	86.108	0.837	0.488	0.102
Intercept	15914.462	1	15914.462	154.678	<0.001	0.875
Condition	.016	1	0.016	0.000	0.990	0.000
Virus	189.231	1	189.231	1.839	0.189	0.077
Condition * Virus	145.911	1	145.911	1.418	0.246	0.061
Error	2263.522	22	102.887			
Total	19994.000	26				
Corrected Total	2521.846	25				

a. R Squared = ,102 (Adjusted R Squared = -,020)

Appendix 10: Summary statistics for duration spent investing the novel and familiar conspecific separately and the discrimination percentage in the social recognition memory test in study 1C

Duration spent investigating novel conspecific

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	72.040[a]	3	24.013	0.664	0.583	0.087
Intercept	8251.429	1	8251.429	228.300	<0.001	0.916
Condition	22.857	1	22.857	0.632	0.435	0.029
Virus	17.500	1	17.500	0.484	0.494	0.023
Condition * Virus	17.500	1	17.500	0.484	0.494	0.023
Error	759.000	21	36.143			
Total	10124.000	25				
Corrected Total	831.040	24				

a. R Squared = 0.087 (Adjusted R Squared = -0.044)

Duration spent investigating familiar conspecific

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	216.010[a]	3	72.003	1.097	0.373	0.135
Intercept	4073.657	1	4073.657	62.038	<0.001	0.747

Condition	160.514	1	160.514	2.444	0.133	0.104
Virus	40.889	1	40.889	0.623	0.439	0.029
Condition * Virus	.032	1	.032	0.000	0.983	0.000
Error	1378.950	21	65.664			
Total	6523.000	25				
Corrected Total	1594.960	24				

a. R Squared = 0.135 (Adjusted R Squared =0 .012)

Discrimination percentage

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1274.413[a]	3	424.804	3.840	0.025	0.354
Intercept	81347.814	1	81347.814	735.375	<0.001	0.972
Condition	394.927	1	394.927	3.570	0.073	0.145
Virus	**619.413**	**1**	**619.413**	**5.599**	**0.028**	**0.211**
Condition * Virus	48.649	1	48.649	0.440	0.514	0.021
Error	2323.039	21	110.621			
Total	89572.711	25				
Corrected Total	3597.452	24				

a. R Squared = 0.354 (Adjusted R Squared = 0.262)

Appendix 11: Summary statistics for fear conditioning familiar conspecific separately and the discrimination percentage in the social recognition memory test in study 1C

Freezing during photostimulation (5 – 7 min)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	510.202[a]	3	170.067	0.197	0.897	0.034
Intercept	40778.045	1	40778.045	47.227	<0.001	0.735
Condition	217.551	1	217.551	0.252	0.622	0.015
Virus	13.810	1	13.810	0.016	0.901	0.001

Condition * Virus	224.385	1	224.385	0.260	0.617	0.015
Error	14678.464	17	863.439			
Total	64247.000	21				
Corrected Total	15188.667	20				

a. R Squared = 0.034 (Adjusted R Squared = -0.137)

Percent total freezing

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1245.907[a]	3	415.302	1.310	0.303	0.188
Intercept	34063.025	1	34063.025	107.486	<0.001	0.863
Condition	1.483	1	1.483	0.005	0.946	0.000
Virus	542.851	1	542.851	1.713	0.208	0.092
Condition * Virus	1072.640	1	1072.640	3.385	0.083	0.166
Error	5387.417	17	316.907			
Total	46791.895	21				
Corrected Total	6633.324	20				

a. R Squared = 0.188 (Adjusted R Squared = 0.045)

Appendix 12: Summary statistics for latency (s) to a thermal pain stimulus (52.5°C) on the hotplate test performed in study 1C

Latency to a thermal pain stimulus

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1.201[a]	3	0.400	0.252	0.859	0.051
Intercept	288.964	1	288.964	181.592	<0.001	0.928
Condition	.294	1	0.294	0.185	0.674	0.013
Virus	.702	1	0.702	0.441	0.517	0.031
Condition * Virus	.228	1	0.228	0.143	0.711	0.010
Error	22.278	14	1.591			
Total	403.256	18				

Corrected Total	23.479	17				

a. R Squared = 0.051 (Adjusted R Squared = -0.152)

7.2 Social instability stress summary statistics

Appendix 13: Body weight data from adolescent social instability stress study two

Parameters	Sex	Age					
		PD30		PD45		PD70	
Stress group		CTL	SIS	CTL	SIS	CTL	SIS
Body Weight (g)	Male	78.25 (14.41)	80.70 (8.60)	170.22 (15.13)	166.37 (11.90)	289.06 (14.41)	292.36 (19.57)
	Female	79.03 (8.86)	77.28 (8.93)	138.25 (10.45)	134.19 (12.31)	186.06 (17.54)	185 (11.51)

Appendix 14: Summary statistics for time and frequency spent on the open, total arm entries, and rearing frequency on the elevated plus maze performed in study 2

Time spent on the open arm

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	75722.878[a]	7	10817.554	8.482	<0.001	0.340
Intercept	669905.569	1	669905.569	525.259	<0.001	0.820
Stress_group	**9101.527**	**1**	**9101.527**	**7.136**	**0.009**	**0.058**
Age	**51331.767**	**1**	**51331.767**	**40.248**	**<0.001**	**0.259**
Sex	**7711.643**	**1**	**7711.643**	**6.047**	**0.015**	**0.050**
Stress_group * Age	983.228	1	983.228	0.771	0.382	0.007
Stress_group * Sex	2869.828	1	2869.828	2.250	0.136	0.019
Age * Sex	1530.841	1	1530.841	1.200	0.276	0.010
Stress_group * Age * Sex	486.296	1	486.296	0.381	0.538	0.003
Error	146669.001	115	1275.383			
Total	887898.028	123				
Corrected Total	222391.880	122				

a. R Squared = 0.340 (Adjusted R Squared = 0.300)

Frequency open arm entries

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	152.974[a]	7	21.853	2.449	0.022	0.130
Intercept	6257.000	1	6257.000	701.053	<0.001	0.859
Stress_group	13.774	1	13.774	1.543	0.217	0.013
Age	40.478	1	40.478	4.535	0.035	0.038
Sex	79.377	1	79.377	8.894	0.003	0.072
Stress_group * Age	11.718	1	11.718	1.313	0.254	0.011
Stress_group * Sex	.893	1	0.893	0.100	0.752	0.001
Age * Sex	1.549	1	1.549	0.174	0.678	0.002
Stress_group * Age * Sex	.097	1	0.097	0.011	0.917	0.000
Error	1026.392	115	8.925			
Total	7461.000	123				
Corrected Total	1179.366	122				

a. R Squared = 0.130 (Adjusted R Squared = 0.077)

Total arm entries

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	412.920[a]	7	58.989	2.035	0.056	0.109
Intercept	37980.812	1	37980.812	1310.459	<0.001	0.919
Stress_group	41.609	1	41.609	1.436	0.233	0.012
Age	1.129	1	1.129	0.039	0.844	0.000
Sex	277.727	1	277.727	9.582	0.002	0.076
Stress_group * Age	68.689	1	68.689	2.370	0.126	0.020
Stress_group * Sex	3.214	1	3.214	0.111	0.740	0.001
Age * Sex	9.056	1	9.056	0.312	0.577	0.003
Stress_group * Age * Sex	3.580	1	3.580	0.124	0.726	0.001
Error	3362.007	116	28.983			
Total	41855.000	124				
Corrected Total	3774.927	123				

a. R Squared = 0.109 (Adjusted R Squared = 0.056)

Rearing frequency

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	940.961[a]	7	134.423	3.437	0.002	0.173
Intercept	34629.920	1	34629.920	885.451	<0.001	0.885
Stress_group	82.972	1	82.972	2.122	0.148	0.018
Age	150.195	1	150.195	3.840	0.052	0.032
Sex	20.695	1	20.695	0.529	0.468	0.005
Stress_group * Age	76.466	1	76.466	1.955	0.165	0.017
Stress_group * Sex	**397.904**	**1**	**397.904**	**10.174**	**0.002**	**0.081**
Age * Sex	**218.907**	**1**	**218.907**	**5.597**	**0.020**	**0.046**
Stress_group * Age * Sex	.000	1	0.000	0.000	0.998	0.000
Error	4497.641	115	39.110			
Total	39839.000	123				
Corrected Total	5438.602	122				

a. R Squared = 0.173 (Adjusted R Squared = 0.123)

Appendix 15: Summary statistics social interaction test.

Total social interaction duration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	3390.324[a]	7	484.332	2.295	0.031	0.120
Intercept	193757.392	1	193757.392	918.272	<0.001	0.886
Stress_group	**1297.030**	**1**	**1297.030**	**6.147**	**0.015**	**0.050**
Age	109.419	1	109.419	0.519	0.473	0.004
Sex	**941.869**	**1**	**941.869**	**4.464**	**0.037**	**0.036**
Stress_group * Age	44.237	1	44.237	0.210	0.648	0.002
Stress_group * Sex	247.312	1	247.312	1.172	0.281	0.010
Age * Sex	532.264	1	532.264	2.523	0.115	0.021
Stress_group * Age * Sex	163.302	1	163.302	0.774	0.381	0.007
Error	24898.255	118	211.002			
Total	222662.519	126				
Corrected Total	28288.580	125				

a. R Squared = 0.120 (Adjusted R Squared = 0.068)

Appendix 16: Summary statistics social recognition memory.

Duration spent investigating familiar conspecific during test phase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	10346.195[a]	7	1478.028	7.602	<0.001	0.322
Intercept	98215.134	1	98215.134	505.151	<0.001	0.819
Stress_group	2.190	1	2.190	0.011	0.916	0.000
Age	**8853.374**	**1**	**8853.374**	**45.536**	**<0.001**	**0.289**
Sex	16.472	1	16.472	0.085	0.772	0.001
Stress_group * Age	342.612	1	342.612	1.762	0.187	0.015
Stress_group * Sex	44.501	1	44.501	0.229	0.633	0.002
Age * Sex	28.827	1	28.827	0.148	0.701	0.001
Stress_group * Age * Sex	491.309	1	491.309	2.527	0.115	0.022
Error	21775.876	112	194.427			
Total	133672.352	120				
Corrected Total	32122.071	119				

a. R Squared = 0.322 (Adjusted R Squared = 0.280)

Duration spent investigating novel conspecific during test phase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	11955.035[a]	7	1707.862	7.442	<0.001	0.317
Intercept	141782.830	1	141782.830	617.803	<0.001	0.847
Stress_group	**2813.571**	**1**	**2813.571**	**12.260**	**<0.001**	**0.099**
Age	**5936.260**	**1**	**5936.260**	**25.867**	**<0.001**	**0.188**
Sex	124.405	1	124.405	0.542	0.463	0.005
Stress_group * Age	188.980	1	188.980	0.823	0.366	0.007
Stress_group * Sex	52.051	1	52.051	0.227	0.635	0.002
Age * Sex	**1829.814**	**1**	**1829.814**	**7.973**	**0.006**	**0.066**
Stress_group * Age * Sex	18.086	1	18.086	0.079	0.779	0.001
Error	25703.450	112	229.495			
Total	182023.005	120				
Corrected Total	37658.486	119				

a. R Squared = 0.317 (Adjusted R Squared = 0.275)

Social discrimination ability

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	3687.940[a]	7	526.849	2.336	0.029	0.127
Intercept	354148.384	1	354148.384	1570.453	<0.001	0.933
Stress_group	547.385	1	547.385	2.427	0.122	0.021
Age	637.564	1	637.564	2.827	0.095	0.025
Sex	215.303	1	215.303	0.955	0.331	0.008
Stress_group * Age	760.690	1	760.690	3.373	0.069	0.029
Stress_group * Sex	94.091	1	94.091	0.417	0.520	0.004
Age * Sex	525.531	1	525.531	2.330	0.130	0.020
Stress_group * Age * Sex	661.602	1	661.602	2.934	0.090	0.026
Error	25256.800	112	225.507			
Total	391427.648	120				
Corrected Total	28944.741	119				

a. R Squared = 0.127 (Adjusted R Squared = 0.073)

Appendix 17: Summary statistics for social investigation during the test phase (novel and familiar conspecific) and social discrimination ability in the social recognition memory test with a 90 min inter-trial-interval performed in study 2B

Duration spent investigating familiar conspecific during test phase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	14385.721[a]	7	2055.103	9.767	<0.001	0.379
Intercept	134588.477	1	134588.477	639.608	<0.001	0.851
Stress_group	345.363	1	345.363	1.641	0.203	0.014
Age	**11958.223**	**1**	**11958.223**	**56.829**	**<0.001**	**0.337**
Sex	467.101	1	467.101	2.220	0.139	0.019
Stress_group * Age	42.044	1	42.044	0.200	0.656	0.002
Stress_group * Sex	36.433	1	36.433	0.173	0.678	0.002
Age * Sex	86.013	1	86.013	0.409	0.524	0.004
Stress_group * Age * Sex	375.600	1	375.600	1.785	0.184	0.016
Error	23567.423	112	210.423			

Total	176933.026	120				
Corrected Total	37953.144	119				

a. R Squared = 0.379 (Adjusted R Squared = 0.340)

Duration spent investigating novel conspecific during test phase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	24853.881[a]	7	3550.554	12.611	<0.001	0.441
Intercept	176943.496	1	176943.496	628.452	<0.001	0.849
Stress_group	18.129	1	18.129	0.064	0.800	0.001
Age	**21372.100**	**1**	**21372.100**	**75.908**	**<0.001**	**0.404**
Sex	13.022	1	13.022	0.046	0.830	0.000
Stress_group * Age	594.692	1	594.692	2.112	0.149	0.019
Stress_group * Sex	46.816	1	46.816	0.166	0.684	0.001
Age * Sex	**1275.874**	**1**	**1275.874**	**4.532**	**0.035**	**0.039**
Stress_group * Age * Sex	221.788	1	221.788	0.788	0.377	0.007
Error	31534.100	112	281.554			
Total	237582.835	120				
Corrected Total	56387.981	119				

a. R Squared = 0.441 (Adjusted R Squared = 0.406)

Social discrimination ability

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1791.297[a]	7	255.900	1.630	0.134	0.092
Intercept	332859.501	1	332859.501	2119.603	<0.001	0.950
Stress_group	127.980	1	127.980	0.815	0.369	0.007
Age	69.190	1	69.190	0.441	0.508	0.004
Sex	1.511	1	1.511	0.010	0.922	0.000
Stress_group * Age	1.030	1	1.030	0.007	0.936	0.000
Stress_group * Sex	117.497	1	117.497	0.748	0.389	0.007
Age * Sex within subjects)	**1105.101**	**1**	**1105.101**	**7.037**	**0.009**	**0.059**
Stress_group * Age * Sex	275.187	1	275.187	1.752	0.188	0.015
Error	17588.327	112	157.039			
Total	360105.228	120				

Corrected Total	19379.624	119				

a. R Squared = 0.092 (Adjusted R Squared = 0.036)

Appendix 18: Summary statistics for social novelty preference in study 2

Social novelty preference (within subjects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	81.179	1	81.179	0.185	0.668	0.002
Familiarity * Stress_group	**3901.682**	**1**	**3901.682**	**8.905**	**0.003**	**0.074**
Familiarity * Age	59.299	1	59.299	0.135	0.714	0.001
Familiarity * Sex	**5567.760**	**1**	**5567.760**	**12.708**	**<0.001**	**0.102**
Familiarity * Stress_group * Age	1052.329	1	1052.329	2.402	0.124	0.021
Familiarity * Stress_group * Sex	335.055	1	335.055	0.765	0.384	0.007
Familiarity * Age * Sex	1117.167	1	1117.167	2.550	0.113	0.022
Familiarity * Stress_group * Age * Sex	**2442.918**	**1**	**2442.918**	**5.576**	**0.020**	**0.047**
Error(Familiarity)	49071.815	112	438.141			

Social novelty preference (between subjects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	456270.757	1	456270.757	827.033	<0.001	0.881
Stress_group	489.028	1	489.028	0.886	0.348	0.008
Age	158.763	1	158.763	0.288	0.593	0.003
Sex	**4735.546**	**1**	**4735.546**	**8.584**	**0.004**	**0.071**
Stress_group * Age	360.248	1	360.248	0.653	0.421	0.006
Stress_group * Sex	313.643	1	313.643	0.569	0.452	0.005
Age * Sex	2043.356	1	2043.356	3.704	0.057	0.032
Stress_group * Age * Sex	1609.481	1	1609.481	2.917	0.090	0.025

Error	61789.933	112	551.696			

Social novelty preference (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	18146.252[a]	7	2592.322	5.439	<0.001	0.254
Intercept	234261.983	1	234261.983	491.521	<0.001	0.814
Stress group	**3576.671**	**1**	**3576.671**	**7.504**	**0.007**	**0.063**
Age	206.059	1	206.059	.432	0.512	0.004
Sex	**10286.474**	**1**	**10286.474**	**21.583**	**<0.001**	**0.162**
Stress group * Age	1321.998	1	1321.998	2.774	0.099	0.024
Stress group * Sex	648.522	1	648.522	1.361	0.246	0.012
Age * Sex	69.378	1	69.378	0.146	0.704	0.001
Stress group * Age * Sex	**4009.083**	**1**	**4009.083**	**8.412**	**0.004**	**0.070**
Error	53379.875	112	476.606			
Total	301422.800	120				
Corrected Total	71526.127	119				

a. R Squared = 0.254 (Adjusted R Squared = 0.207)

Appendix 19: Summary statistics for males social novelty preference, vigilance, avoidance and rearing on the social novelty preference test performed in study 2

Social novelty preference (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	275839.107	1	275839.107	519.159	<0.001	0.903
Stress group	789.690	1	789.690	1.486	0.228	0.026
Age	1663.708	1	1663.708	3.131	0.082	0.053
Stress group * Age	1739.084	1	1739.084	3.273	0.076	0.055
Error	29753.878	56	531.319			

Social novelty preference (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	3482.284	1	3482.284	7.070	0.010	0.112
Familiarity * Stress group	3248.221	1	3248.221	6.595	0.013	0.105
Familiarity * Age	329.478	1	329.478	0.669	0.417	0.012
Familiarity * Stress group * Age	3337.100	1	3337.100	6.775	0.012	0.108
Error(Familiarity)	27583.144	56	492.556			

Social vigilance (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	2.477	1	2.477	0.027	0.870	0.000
Familiarity * Stress group	80.063	1	80.063	0.875	0.354	0.015
Familiarity * Age	2.227	1	2.227	0.024	0.877	0.000
Familiarity * Stress group * Age	89.377	1	89.377	0.976	0.327	0.017
Error(Familiarity)	5125.571	56	91.528			

Social vigilance (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	12059.712	1	12059.712	98.873	<0.001	0.638
Stress group	42.299	1	42.299	0.347	0.558	0.006
Age	8.100	1	8.100	0.066	0.798	0.001
Stress group * Age	23.507	1	23.507	0.193	0.662	0.003
Error	6830.436	56	121.972			

Avoidance (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	117.975	1	117.975	0.548	0.462	0.010

Familiarity * Stress group	595.512	1	595.512	2.765	0.102	0.047
Familiarity * Age	.031	1	0.031	0.000	0.991	0.000
Familiarity * Stress group * Age	**878.960**	**1**	**878.960**	**4.080**	**0.048**	**0.068**
Error(Familiarity)	12062.859	56	215.408			

Avoidance (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	125695.400	1	125695.400	256.643	<0.001	0.821
Stress group	478.381	1	478.381	0.977	0.327	0.017
Age	122.069	1	122.069	0.249	0.620	0.004
Stress group * Age	230.764	1	230.764	0.471	0.495	0.008
Error	27426.966	56	489.767			

Rearing (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	**145.413**	**1**	**145.413**	**4.134**	**0.047**	**0.069**
Familiarity * Stress group	50.885	1	50.885	1.447	0.234	0.025
Familiarity * Age	13.962	1	13.962	0.397	0.531	0.007
Familiarity * Stress group * Age	3.490	1	3.490	0.099	0.754	0.002
Error(Familiarity)	1969.938	56	35.177			

Rearing (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	25524.628	1	25524.628	252.061	<0.001	0.818
Stress group	385.926	1	385.926	3.811	0.056	0.064
Age	126.926	1	126.926	1.253	0.268	0.022
Stress group * Age	36.013	1	36.013	0.356	0.553	0.006
Error	5670.771	56	101.264			

Appendix 20: Summary statistics for females social novelty preference, vigilance, avoidance and rearing on the social novelty preference test performed in study 2

Social novelty preference (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	**2161.160**	**1**	**2161.160**	**5.632**	**0.021**	**0.091**
Familiarity * Stress group	979.078	1	979.078	2.552	0.116	0.044
Familiarity * Age	849.149	1	849.149	2.213	0.142	0.038
Familiarity * Stress group * Age	144.869	1	144.869	0.378	0.541	0.007
Error(Familiarity)	21488.671	56	383.726			

Social novelty preference (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	184788.495	1	184788.495	323.016	<0.001	0.852
Stress group	9.738	1	9.738	0.017	0.897	0.000
Age	533.710	1	533.710	0.933	0.338	0.016
Stress group * Age	224.344	1	224.344	0.392	0.534	0.007
Error	32036.056	56	572.072			

Vigilance (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	184.019	1	184.019	2.045	0.158	0.035
Familiarity * Stress group	**453.593**	**1**	**453.593**	**5.042**	**0.029**	**0.083**
Familiarity * Age	.600	1	0.600	0.007	0.935	0.000
Familiarity * Stress group * Age	27.491	1	27.491	0.306	0.583	0.005
Error(Familiarity)	5038.251	56	89.969			

Vigilance (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	14690.184	1	14690.184	100.367	<0.001	0.642
Stress group	27.947	1	27.947	0.191	0.664	0.003
Age	5.917	1	5.917	0.040	0.841	0.001
Stress group * Age	226.363	1	226.363	1.547	0.219	0.027
Error	8196.463	56	146.365			

Avoidance (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	111.638	1	111.638	0.430	0.515	0.008
Familiarity * Stress group	0.396	1	0.396	0.002	0.969	0.000
Familiarity * Age	620.344	1	620.344	2.390	0.128	0.041
Familiarity * Stress group * Age	43.065	1	43.065	0.166	0.685	0.003
Error(Familiarity)	14535.026	56	259.554			

Avoidance (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	147675.209	1	147675.209	408.575	<0.001	0.879
Stress group	5308.579	1	5308.579	14.687	<0.001	0.208
Age	37.969	1	37.969	0.105	0.747	0.002
Stress group * Age	91.225	1	91.225	0.252	0.617	0.004
Error	20240.624	56	361.440			

Rearing (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Familiarity	28.143	1	28.143	1.703	0.197	0.030
Familiarity * Stress group	0.459	1	0.459	0.028	0.868	0.000
Familiarity * Age	5.787	1	5.787	0.350	0.556	0.006

Familiarity * Stress group * Age	0.719	1	0.719	0.044	0.836	0.001
Error(Familiarity)	925.220	56	16.522			

Rearing (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	30683.770	1	30683.770	221.501	<0.001	0.798
Stress group	**1124.409**	**1**	**1124.409**	**8.117**	**0.006**	**0.127**
Age	419.409	1	419.409	3.028	0.087	0.051
Stress group * Age	17.299	1	17.299	0.125	0.725	0.002
Error	7757.489	56	138.527			

7.3 Peer rejection summary statistics

Appendix 21: Summary statistics for alcohol extinction and reinstatement in study three

Alcohol cue-induced reinstatement (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Lever	**16854.694**	**1**	**16854.694**	**227.555**	**<0.001**	**0.791**
Lever * Group	.138	1	0.138	0.002	0.966	0.000
Lever * Sex	273.600	1	273.600	3.694	0.059	0.058
Lever * Group * Sex	**383.169**	**1**	**383.169**	**5.173**	**0.027**	**0.079**
Error(Lever)	4444.129	60	74.069			
Session	**13013.219**	**1**	**13013.219**	**128.799**	**<0.001**	**0.682**
Session * Group	13.896	1	13.896	0.138	0.712	0.002
Session * Sex	**495.219**	**1**	**495.219**	**4.901**	**0.031**	**0.076**
Session * Group * Sex	**1307.834**	**1**	**1307.834**	**12.944**	**<0.001**	**0.177**
Error(Session)	6062.096	60	101.035			
Lever * Session	**9043.607**	**1**	**9043.607**	**179.183**	**<0.001**	**0.749**
Lever * Session * Group	9.700	1	9.700	0.192	0.663	0.003
Lever * Session * Sex	10.732	1	10.732	0.213	0.646	0.004
Lever * Session * Group * Sex	**615.200**	**1**	**615.200**	**12.189**	**<0.001**	**0.169**

Error(Lever*Session)	3028.279	60	50.471			

Alcohol cue-induced reinstatement (between subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	92365.075	1	92365.075	490.609	<0.001	0.891
Group	8.532	1	8.532	0.045	0.832	0.001
Sex	**884.544**	**1**	**884.544**	**4.698**	**0.034**	**0.073**
Group * Sex	**1718.688**	**1**	**1718.688**	**9.129**	**0.004**	**0.132**
Error	11295.979	60	188.266			

Appendix 22: Summary statistics for average lever presses across extinction and reinstatement in study 3

Extinction and cue-induced reinstatement

Factors		Extinction		Reinstatement	
Sex	**Condition**	**Active**	**Inactive**	**Active**	**Inactive**
Female	**Control**	12 ± 1.80	14 ± 1.78	14 ± 1.78	16 ± 1.39
	Peer-rejected	12 ± 1.48	13 ± 1.39	19 ± 1.78	15 ± 1.35
Male	**Control**	10 ± 1.20	15 ± 1.90	10 ± 2.29	13 ± 1.67
	Peer-rejected	7 ± 0.74	15 ± 1.90	11 ± 1.80	16 ± 1. 79

Table 1. Water extinction and reinstatement data from female (N=20/20) and male (N=12/12) rats. Data are indicated as mean ± SEM.

Appendix 23: Summary statistics for home cage locomotion across 72 hrs in study three

Locomotor activity (within subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Day	21050810.886	1	21050810.886	5.104	0.029	0.108
Day * sex	401497.523	1	401497.523	0.097	0.757	0.002
Day * group	15128991.407	1	15128991.407	3.668	0.062	0.080

Day * sex * group	28101766.957	1	28101766.957	6.814	0.012	0.140
Error(Day)	173213865.156	42	4124139.647			

Locomotor activity (between-subjects effects)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	20662178889.120	1	20662178889.120	1023.225	<0.001	0.961
sex	**236363905.031**	**1**	**236363905.031**	**11.705**	**0.001**	**0.218**
group	121246.500	1	121246.500	0.006	0.939	0.000
sex * group	1118695.518	1	1118695.518	0.055	0.815	0.001
Error	848114370.194	42	20193199.290			

Appendix 24: Summary statistics for sucrose preference and sucrose intake (g/kg) of males on the sucrose preference test in study three

Sucrose preference

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	129.845[a]	1	129.845	2.847	0.106	0.115
Intercept	178840.899	1	178840.899	3921.326	<0.001	0.994
Condition	129.845	1	129.845	2.847	0.106	0.115
Error	1003.360	22	45.607			
Total	179974.104	24				
Corrected Total	1133.205	23				

a. R Squared = 0.115 (Adjusted R Squared = 0.074)

Sucrose intake (g/kg)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	8257.781[a]	1	8257.781	3.235	0.086	0.128

Intercept	453551.438	1	453551.438	177.696	<0.001	0.890
Condition	8257.781	1	8257.781	3.235	0.086	0.128
Error	56152.896	22	2552.404			
Total	517962.116	24				
Corrected Total	64410.677	23				

a. R Squared = 0.128 (Adjusted R Squared = 0.089)

Appendix 25: Summary statistics for sucrose preference and total fluid intake of females on the sucrose preference test in study three

Sucrose preference

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	91.345[a]	1	91.345	0.687	0.416	0.030
Intercept	136086.219	1	136086.219	1023.975	<0.001	0.979
Condition	91.345	1	91.345	0.687	0.416	0.030
Error	2923.798	22	132.900			
Total	139101.362	24				
Corrected Total	3015.143	23				

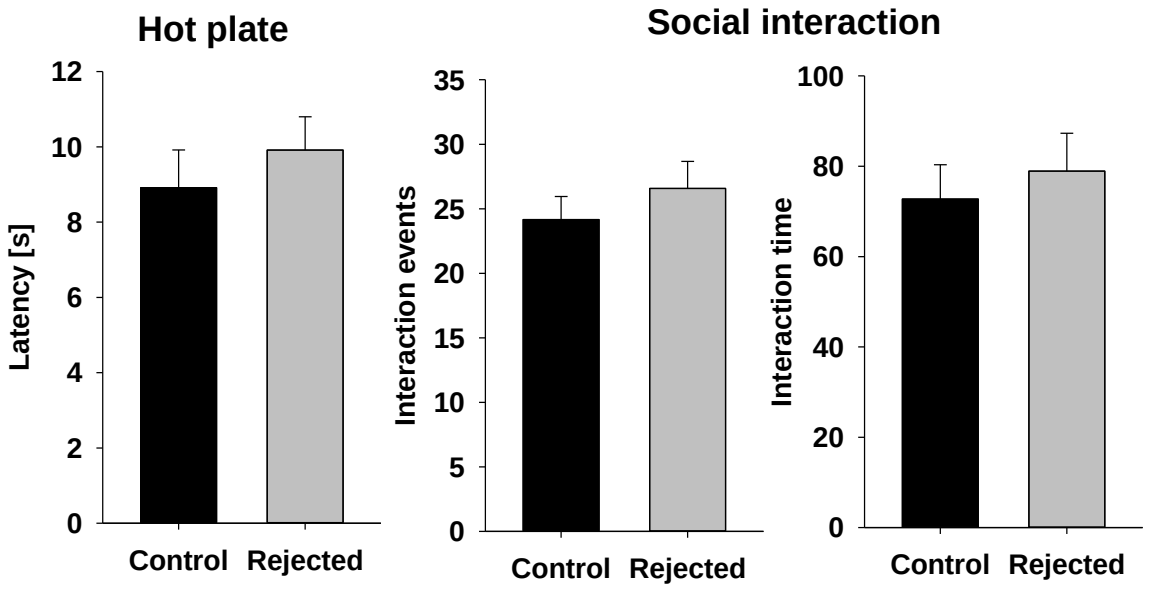
a. R Squared = 0.030 (Adjusted R Squared = -0.014)

Sucrose intake (g/kg)

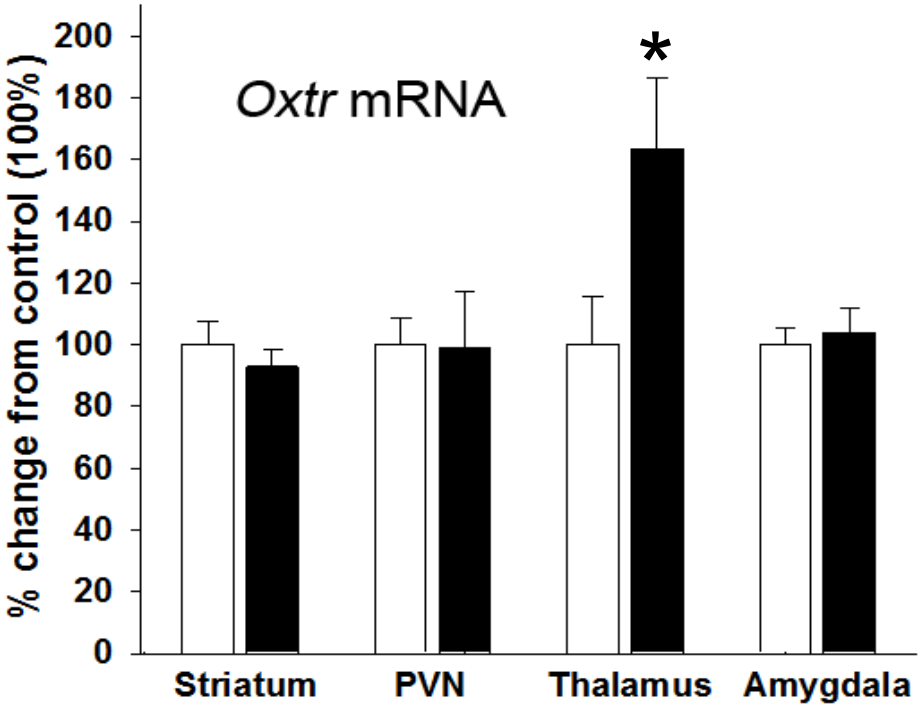
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	923.252[a]	1	923.252	0.143	0.709	0.006
Intercept	655513.907	1	655513.907	101.397	<0.001	0.822
Condition	923.252	1	923.252	0.143	0.709	0.006
Error	142225.486	22	6464.795			
Total	798662.646	24				
Corrected Total	143148.738	23				

a. R Squared = 0.006 (Adjusted R Squared = -0.039)

Appendix 26: Behavioral data from male WIST rats on the Hotplate test and SIT performed in adulthood following the PR paradigm performed by Dr. Valentina Vengeleine



Appendix 27: OT mRNA from the N, PVN, Thalamus and AMY from female peer rejected rats collected and analysed by Dr. Peggy Schneider.



8 CURRICULUM VITAE

PERSONALIEN

Name and Surname:	Graf, Akseli Petteri
Date of birth:	29.05.1989
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SCHOOL EDUCATION

(2005) – (2008)	Upper secondary school, International IT College of Sweden
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UNIVERSITY EDUCATION

(2011) – (2014)	B.Sc. Psychology, University of Groningen, Groningen, Netherlands
(14.9.2014)	Bachelor thesis: Modulating Cues Influences Color Fusion in a Missing Element, Task – Merit (7/10)
(2016) – (2017)	MSc Neuroscience, King's College London, London, United Kingdom
(18.01.2018)	Master thesis: The Effects of Ach and GABA Co-release on Layer I Interneurons – Distinction (73%)
Since 01.04.2018	Doctoral student at the University of Heidelberg, Medical Faculty Mannheim, Germany

PUBLICATIONS

Surakka A, Vengeliene V, Skorodumov I, Meinhardt M, Hansson AC, Spanagel R. Adverse social experiences in adolescent rats result in persistent sex-dependent

effects on alcohol-seeking behavior. Alcohol Clin Exp Res. 2021 Jul;45(7):1468-1478. doi: 10.1111/acer.14640. Epub 2021 Jul 17. PMID: 34273113.

Singaravelu SK, Goitom AD, **Graf A**, Moerz H, Schilder A, Hoheisel U, Spanagel R, Treede RD. Persistent muscle hyperalgesia after adolescent stress is exacerbated by a mild-nociceptive input in adulthood and is associated with microglia activation. Sci Rep. 2022 Oct 31;12(1):18324. doi:10.1038/s41598-022-21808-x.

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Graf A, Bernardi RE. ChatGPT in Research: Balancing Ethics, Transparency and Advancement. Neuroscience. 2023 Apr 1;515:71-73. doi: 10.1016/j.neuroscience.2023.02.008.

POSTER AND TALKS

Presentation: The impact of adolescent social isolation on social behavior and the oxytocin system Akseli Graf, AC Hansson, R Spanagel GRK2350 Final symposium, Mannheim, Germany, 2022

Presentation: Boozy Bias: Rats Show a Surprising Preference in Operant Choice Task. Akseli Graf, A Riccio, R Spanagel, EWCBR, Les Diablerets, Switzerland, 2023

Poster: Axonal oxytocin release in the paraventricular nucleus of the thalamus impairs social memory. Akseli Graf, A Lefevre, S Küppers, V Grinevich, R Spanagel, EBPS 2022 Biennial Workshop, Rome, Italy, 2022

Poster: The Effect of Nicotinamide Mononucleotide on Alcohol Seeking Behaviors in Wistar Rats. Akseli Graf, F Gianonne, RE Bernardi, R Spanagel, Addiction 2022, Sardinia, Italy 2022

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