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Of Mice and Men: A Translational Study Investigating Depressive-Like Behavior Induced by Early Life Adversities

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"The best-laid plans of mice and men oft go awry"
(*Of Mice & Men*, John Steinbeck, 1993)

To all adults who once were wounded children.

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Introduction

Depression is a widely spread disorder that has a great cost both in terms of health to the individual and to society. Among the main risk factors for depression are Early Life Adversities (ELA), which occur in a time window of great development for the brain, creating non-linear developmental trajectories that can predispose to psychopathology.

The objective of this thesis is to investigate ELA-related depression, splitting ELA into two macro categories: the alteration of the attachment bond and the experiences of childhood trauma (Adverse Childhood Experiences, ACE) to understand if these experiences induce differences in the depressive-like phenotype and if they are accompanied by different neurobiological alterations.

To achieve this objective, we first investigated the presence of ELA in a non-clinical population and verified whether, in the ELA group, experiences of Trauma or Attachment Bond Alteration were present. We then carried out a regression analysis to observe any relationships between the type of experience lived and the presence of depressive symptoms. In addition, in a subpopulation of the sample, we analyzed the levels of Oxytocin in plasma, being the Oxytocin a social neuropeptide widely involved in both early adverse experiences and depression.

Next, to further investigate the neurobiological substrates underlying the effects of Trauma and Attachment Bond Alteration, we leveraged the preclinical model. We used two mouse models of ELA in male mice of the inbred strain C57BL/6J: the Repeated Cross Fostering (RCF) model, which reproduces the alteration of the attachment bond in humans, and the Scarcity model, which mimics the Neglect condition (traumatic experience) in humans.

In these two models, we first investigated the quality of maternal care to highlight aspects related to the presence or absence of abuse in pups. Then, we investigated the attachment behavior of pups towards their mothers through the recording of ultrasonic vocalization distress calls (which are an index of separation anxiety) and through the Mouse Strange Situation Procedure (which evaluate Attachment behavior). Following, we investigated the depressive-like behaviors, both in adolescence and adulthood, in these experimental groups through three tests: the Saccharin Preference Test (to evaluate anhedonia), the Forced Swimming Test (to evaluate coping style), and the Social Interaction Test (to evaluate social impairment).

Finally, we evaluated the alteration of two different social neuropeptides within the mesocorticolimbic circuit: Oxytocin and Endogenous Opioids. In fact, these two neuropeptides are modulated by ELA and are altered in subjects affected by depression. In addition, their joint action has a protective role against stress on the mesocorticolimbic circuit, a circuit critical for the creation of the bond with the caregiver, which is also altered by ELA due to the temporal overlap between the occurrence of ELA and its development in the brain.

The aim of this work is therefore double: to investigate a link between different ELA and depression in a non-clinical sample; to investigate similarities and differences in the depressive-like phenotype induced by different ELA in a preclinical model, with the purpose of highlighting some neurobiological alterations.

1. Depression in Humans

The World Health Organization has identified major depressive disorder (MDD) as the principal contributor to the global burden of disease (Buch & Liston, 2021). Millions of people worldwide are affected by depressive disorders, and up to 40% of patients show inadequate responses to current antidepressant treatments. MDD is a symptomatically heterogeneous condition, encompassing cognitive, emotional, motivational, and physiological dimensions (Fox & Lobo, 2019). In the absence of treatment, many patients experience multiple depressive episodes throughout their lifetimes, which often increase in frequency and severity over time (Wang et al., 2021).

Revealing the precise etiology of MDD remains challenging, as patients typically exhibit a constellation of symptoms unlikely to be explained by a single unifying mechanism (Fox & Lobo, 2019). Historically, most research has conceptualized depression as a unitary disease entity due to the lack of better alternatives. However, this approach has recently been recognized as a potential barrier to scientific progress. This is particularly relevant for efforts to uncover the neurobiological mechanisms underlying depression, which may vary between individuals, as well as for optimizing existing pharmacotherapies and developing new antidepressant strategies. Distinct pathophysiological pathways may, in fact, require tailored treatment approaches (Buch & Liston, 2021).

Given the substantial health and economic burden associated with a chronic and recurrent course of depressive illness, it is essential to identify factors that predict vulnerability to persistent or relapsing depressive episodes, as well as poor response or remission during treatment. Childhood maltreatment has emerged as a significant predictor of an unfavorable course and poor treatment outcomes. Compared with individuals without a history of maltreatment, those who experienced childhood adversities are at significantly higher risk of developing depressive episodes across the lifespan (Nanni et al., 2012). Moreover, exposure to early life stress has been linked to reduced responsiveness to current antidepressant therapies in adulthood, highlighting the urgent need for innovative treatment strategies (Portugalov et al., 2022).

Table 1. Diagnostic and Statistical Manual of Mental Disorders (DSM-5-TR) diagnostic criteria for a major depressive episode.

A. Five (or more) of the following symptoms have been present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.
<i>NOTE: Do not include symptoms that are clearly attributable to another medical condition.</i>
1) Depressed mood most of the day, nearly every day, as indicated by either subjective report (eg, feels sad, empty, hopeless) or observations made by others (eg, appears tearful). (<i>NOTE: In children and adolescents, can be irritable mood.</i>)
2) Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
3) Significant weight loss when not dieting or weight gain (eg, a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. (<i>NOTE: In children, consider failure to make expected weight gain.</i>)
4) Insomnia or hypersomnia nearly every day.
5) Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
6) Fatigue or loss of energy nearly every day.
7) Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
8) Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by their subjective account or as observed by others).
9) Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.
B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
C. The episode is not attributable to the direct physiological effects of a substance or to another medical condition.
D. The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.
E. There has never been a manic or hypomanic episode.

The current edition of the DSM recognizes at least 256 distinct symptom combinations that meet diagnostic criteria for MDD. This reflects the considerable heterogeneity inherent in the disorder. Multiple factors contribute to this diagnostic complexity: depression frequently co-occurs with anxiety disorders, substance use disorders, ADHD, and other psychiatric comorbidities. Individuals with depression also display substantial variability in the temporal dynamics of their illness, including the frequency and duration of episodes as well as the stability of remission. Furthermore, the mechanisms behind “spontaneous” depressive episodes may differ from those triggered by identifiable antecedent psychosocial stressors, such as a history of adversity, trauma, or abuse (Buch & Liston, 2021).

To address some of the inherent challenges in studying psychiatric disorders characterized by high rates of comorbidity and limited alignment with biological underpinnings, the National Institute of Mental Health (NIMH) launched the Research Domain Criteria (RDoC) initiative. The RDoC framework integrates multiple levels of analysis to better capture the multidimensional nature of mental illness, including self-reported symptoms, objective behavioral data, neurophysiology, neural circuitry, cellular and molecular neurobiology, and genetics (Buch & Liston, 2021). The primary aim of the RDoC project is to identify research domains that link and integrate higher-order components of psychopathology. By doing so, it encourages the combination of clinical and experimental findings from different approaches, such as behavioral, neurophysiological, and genetic studies. Overall, RDoC is intended to provide a framework for identifying valid and reliable phenotypes that incorporate both biological and psychological elements, guided by the systems neuroscience perspective (Sanislow et al., 2010). The RDoC initiative represents an effort to build an experimental classification system in which symptoms or behavioral domains, rather than entire mental disorders, are categorized based on evidence of their etiology and pathophysiology (Lo Iacono et al., 2021). Currently, the framework is organized around 5 behavioral domains: (1) positive valence systems, (2) negative valence systems, (3) arousal and regulatory systems, (4) systems for social processes, and (5) cognitive systems. These domains, along with their associated constructs, can be used to comprehensively characterize behavior (Anderzhanova et al., 2017).

Rather than seeking neurobiological features that distinguish a heterogeneous population of MDD patients from healthy controls, researchers adopting the RDoC approach aim to develop models that predict individual differences in clinically relevant dimensions (for example, anhedonia) across multiple diagnostic categories (Buch & Liston, 2021).

The complexities in defining and classifying depression are further compounded by sociocultural factors, such as gender norms, which can strongly influence how depressive symptoms are expressed, perceived, and diagnosed.

Although current epidemiological data suggest that women experience depression at higher rates than men, a deeper examination reveals important diagnostic blind spots. Men are diagnosed with depression only about half as often as women, yet they die by suicide three to four times more frequently, highlighting the potentially severe consequences of unrecognized depressive symptoms (Swetlitz, 2021). Gendered socialization processes (which encourage boys and men to express emotions through behaviors like overworking, substance use, or aggression rather than sadness or hopelessness) may mask depressive states and lead to their underdiagnosis. When studies incorporate these “male-typical” symptoms into diagnostic criteria, the apparent sex difference in depression prevalence narrows significantly, suggesting that traditional gender roles and masculine norms may actively hinder both recognition and treatment of depression in men. Moreover, depression research has historically neglected the experiences of gender-nonconforming individuals, leaving important gaps in our understanding of how diverse masculinities influence mental health trajectories. The longstanding cultural narrative that depression primarily affects women (reinforced by public health messaging and even clinical guidelines) may further discourage men from acknowledging depressive symptoms or seeking care (Swetlitz, 2021).

The coronavirus disease 2019 (COVID-19) pandemic has further exacerbated these vulnerabilities, amplifying social isolation and economic stressors that heighten the risk of depressive symptoms (Swetlitz, 2021). Since the onset of the pandemic, evidence has emerged of significant psychopathological consequences, both during acute infection and in the post-acute phase among COVID-19 survivors. Multiple independent meta-analyses have consistently demonstrated a high prevalence of depressive symptoms following COVID-19, although considerable heterogeneity remains, highlighting the need to identify relevant risk and protective factors. Contributing elements, such as prolonged social isolation, psychological stress, medical comorbidities, and COVID-19 severity, are all thought to interact with neuroimmune pathways and increase vulnerability to post-COVID depressive symptomatology (Mazza et al., 2022).

At the same time, however, the rapid expansion of telehealth and other virtual mental health resources during the pandemic may represent an important opportunity to better reach individuals (particularly men) whose depression has remained unrecognized and untreated due to traditional gender norms and stigma (Swetlitz, 2021).

1.1 Early Life Adversities-induced Depression in Humans

Early Life Adversities (ELA) are strongly associated with increased risk for multimorbidity across both mental and physical health domains (Antoniou et al., 2023). Indeed, childhood adversity (including abuse, neglect, parental loss, exposure to domestic violence, and other significant stressors) has been estimated to account for approximately 45% of the variance in childhood-onset psychopathology and 26–32% of the risk for later-onset psychiatric disorders (Green et al., 2010; Baracz et al., 2020). Adults who experienced early trauma frequently report a higher prevalence of depression, suicidality, anxiety, post-traumatic stress disorder, and aggressive behavior (Baracz et al., 2020). Several studies have pointed out ELA as particularly detrimental to depression (Anda et al., 2002; Gilman et al., 2003; Chapman et al., 2004; Björkenstam et al., 2017a; Björkenstam et al., 2017b). Among ELA, the relationship between traumatic experiences (Adverse Childhood Experiences, ACEs) and depression has been extensively documented, showing a robust linear association: the greater the number of ACEs reported, the higher the likelihood of developing depression in adulthood (Chapman et al., 2004; Giano et al., 2021). Two main mechanisms have been proposed to explain this link. First, the cumulative impact of adversity may induce neurobiological and epigenetic changes, altering brain structure and increasing vulnerability to psychiatric disorders. Second, ACEs often occur during sensitive developmental windows, leading to chronic dysregulation of key physiological systems (including the endocrine, immune, metabolic, and nervous systems), thereby contributing to long-term allostatic load and increased mental health risk (Giano et al., 2021).

Studies have shown that the associations between different ACEs and depression vary depending on the type of ACEs (Anda et al., 2002; Chapman et al., 2004; Björkenstam et al., 2017b). Moreover, ACEs tend to occur in clusters rather than as single or separate events (Dong et al., 2004), and clustered ACEs have a strong graded relationship to depression (Chapman et al., 2004; Björkenstam et al., 2017b). Particularly, Chapman et al. (2004) found that the majority of individuals report at least one ACE, with approximately one-third of adults reporting two or more. These findings (in line with earlier research from the original ACE Study (Felitti et al., 1998)) suggest that identification of a single ACE should prompt clinicians to further assess for additional exposures. Notably, a strong graded relationship has been observed between the number of ACEs and the risk of both current and lifetime depressive disorders in both sexes. Given the high degree of interrelation among different forms of childhood adversity, ACEs should be considered as a cumulative set of experiences that collectively influence vulnerability to depression across the lifespan (Chapman et al., 2004).

It is equally crucial to emphasize the role of disrupted attachment bonds, beyond the exclusive impact of ACEs, in the emergence of depression-related symptoms following early life adversity. Indeed, children with insecure attachment patterns may come to perceive later experiences of loss or disappointment as reflections of personal inadequacy and may doubt their own capacity for recovery. This cognitive vulnerability fosters feelings of hopelessness, which in turn predisposes individuals to clinical depression. The withdrawal and emotional disengagement characteristic of depressive states can further impair the ability of young individuals to form meaningful peer relationships and sustain social bonds (Fahs et al., 2022).

A variety of tools have been employed to assess the quality of parent–child relationships. Among the most influential, Parker and colleagues (1979) identified two key dimensions underlying parenting behavior: care and control. The "care" dimension captures the degree of affection, warmth, and emotional attunement provided by the parent, while "control" reflects parental overprotection and intrusiveness, in contrast to fostering autonomy. Based on these two axes, Parker et al. proposed four distinct parenting styles: Optimal Bonding (high care, low control), Affectionate Constraint (high care, high control), Neglectful Parenting (low care, low control), and Affectionless Control (low care, high control). To operationalize these constructs, they developed the Parental Bonding Instrument (PBI), a retrospective self-report measure of perceived parental care and control during childhood. Research using the PBI has consistently linked specific bonding patterns, particularly those characterized by low levels of parental care, to increased risk of adult psychopathology, including depression, anxiety disorders, obsessive-compulsive disorder, and schizophrenia. In particular, low parental care has emerged as a significant predictor of lifetime depression (Fahs et al., 2022).

The evidence reviewed above highlights ELA as a central etiological factor in depression, prompting the need for a precise characterization of these experiences. In the following section we will provide a detailed overview of the concept of ELA, its dimensions, and its relevance for neurodevelopment.

2. Early Life Adversities (ELA)

Human development takes place within a social environment that typically addresses the basic needs of offspring, such as feeding, sensory stimulation, and emotional warmth. Consequently, it is not unexpected that early-life environmental quality, particularly the nature of parent–child interactions, can exert long-term biological effects and influence developmental trajectories. Research in this area has extensively examined how parental behaviors (ranging from attachment quality and sensitivity to infant signals to the effects of absence, neglect, or maltreatment) can shape outcomes across the lifespan (Kundakovic & Champagne, 2015).

The term Early Life Adversities (ELA) has been introduced to describe the extensive and multifaceted challenges that may affect the developing brain, beyond the conventional framework of stress. Indeed, unlike classical stress responses that aim to restore homeostasis (Selye, 1936), development is inherently dynamic and entails continuous change, making it particularly vulnerable to disruptions. Even in settings where basic physical needs are met and a nuclear family is present, inconsistency in caregiving or emotional signals may still be perceived by infants as adverse (Birnie & Baram, 2025). Understanding how adverse developmental experiences can cascade into long-term risk is essential for designing early interventions, which are often more effective and economically efficient when applied during early childhood (Webster, 2022).

In neurobiological terms, ELA include conditions that results in alteration of normal maturation of neural circuits and cellular processes (Birnie & Baram, 2025). The postnatal period represents a particularly sensitive phase, during which environmental inputs play a crucial role in shaping neurodevelopmental outcomes (Lo Iacono et al., 2021). Empirical evidence from both human and animal studies indicates that adverse experiences during these sensitive windows are associated with increased vulnerability to psychiatric conditions later in life (Di Segni et al., 2019; Malave et al., 2022). Early interactions with caregivers are especially important, as they guide the infant’s neural development. In caregiving-dependent species, infants form strong preferences for their caregivers regardless of the quality of care. Even when maltreatment occurs, children often develop intense, though disorganized, attachments, making them more susceptible to emotional dysregulation, psychiatric symptoms, and changes in stress-related neural pathways (Roth & Sullivan, 2005; Krugers et al., 2017; Di Segni et al., 2019; Goodwill et al., 2019).

The dynamic nature of postnatal brain development allows individuals to flexibly adapt to changing environmental conditions. However, this same plasticity also makes the developing brain particularly vulnerable to adverse environmental influences (Krugers et al., 2017). A substantial body of research indicates that traumatic stress, especially when experienced early in life, constitutes a major risk factor for the onset of nearly all psychiatric disorders (Raine et al., 2012; Daskalakis et al., 2013). Rates of maltreatment are typically highest during the first four years of life (Baracz et al., 2020), a period characterized by rapid neurological development and heightened sensitivity to environmental inputs.

Research into ELA in humans is complicated by several moderating factors, such as the type, intensity, frequency, and timing of the stressor, as well as the co-occurrence of multiple adversities. In addition, many adverse experiences, such as abuse linked to parental psychopathology, may reflect heritable influences, complicating the disentanglement of genetic and environmental contributions to psychopathological outcomes (Tyrka et al., 2013). Despite the extensive empirical literature, key questions remain unresolved. It is still unclear which specific combinations of stressful life events are most predictive of psychopathological trajectories or how such events interact across developmental stages. Importantly, some developmental periods may constitute windows of heightened vulnerability, during which the impact of adversity is overstated. Variables such as individual personality traits, pre-existing psychiatric symptoms, and coping mechanisms must also be considered when evaluating the effects of adversity (Daskalakis et al., 2013). Notably, both clinical and preclinical studies also suggest that ELA may, in some cases, foster resilience by promoting adaptation to future stressors (D'Addario et al., 2021).

The “three-hit” model proposed by Daskalakis offers a conceptual framework for understanding interindividual differences in vulnerability to psychopathology. According to this model, mental health outcomes result from the interaction of: genetic predispositions (Hit 1), early-life environmental exposures (Hit 2), and later-life environmental stressors (Hit 3). Early-life experiences modulate gene expression during sensitive periods of brain development, shaping a neurobiological phenotype that may be more or less vulnerable to subsequent environmental stress. If this phenotype encounters further adversity later in life, the risk of psychopathology may increase (vulnerability); however, in the presence of supportive environments, the same individual may demonstrate resilient functioning, despite earlier adversities (resilience) (Daskalakis et al., 2013). This model underscores the interactive and dynamic nature of genetic, environmental, and developmental factors in determining mental health trajectories.

2.1 Traumatic Experiences in Humans

World Health Organization (WHO) describes child maltreatment as:

“The abuse and neglect of people under 18 years of age. It includes all forms of physical and/or emotional ill-treatment, sexual abuse, neglect or negligent treatment or commercial or other exploitation, resulting in actual or potential harm to the child’s health, survival, development or dignity in the context of a relationship of responsibility, trust or power. Four types of child maltreatment are generally recognized: physical abuse, sexual abuse, psychological (or emotional or mental) abuse, and neglect.”

The data available in 2024 reports that in the world, the most widespread type of abuse is psychological, with 36% of boys and girls victims of psychological abuse, followed by physical abuse affecting 23% of children, sexual abuse affecting 18% of girls and 8% of boys, and finally, 16% of children are neglected by their parents and caregivers. In an already worrying picture, it emerges that maltreatment begins very early: 300 million children between the ages of 2 and 4 have suffered violent disciplinary measures from their caregivers. In Europe it is estimated that 55 million boys and girls suffer forms of maltreatment: in 9.6% of cases they have suffered sexual abuse, in 22.9% of cases physical abuse, in 16.3% of cases children are instead victims of physical neglect, in 18.4% of cases they were emotionally neglected, and, finally, in 29.6% of cases children are victims of emotional abuse. As far as Italy is concerned, the maltreatment of minors in the family takes place in a framework in which crimes against minors are generally increasing: in 2021, there was an increase in crimes against minors of 8% compared to 2020, with a total number of 6,248 cases, the highest number ever recorded (Badalassi & Gentile, 2024).

Child maltreatment is, unfortunately, a widespread phenomenon throughout the world. The USA’s most recent data from 2023 indicate that 546,159 children were confirmed victims of abuse or neglect, representing a national rate of 7.4 per 1,000 children. The prevalence is slightly higher among females (52.0%) compared to males (47.6%). The youngest age groups are particularly vulnerable: children aged 0–2 years accounted for 26.6% of all cases, with infants under the age of one comprising 14.2% of victims (U.S. Department of Health and Human Services, Children’s Bureau, 2023).

The study of Adverse Childhood Experiences (ACEs) is important for developing interventions to help mitigate additional risks associated with ACE exposure. Research into Adverse Childhood Experiences (ACEs) has become essential for understanding the long-

term effects of early trauma and for informing the development of effective interventions (Webster, 2022).

The initial ACE study by Felitti et al. (1998) revealed a robust and graded association between childhood exposure to abuse, neglect, and household dysfunction and an increased likelihood of numerous health risk factors in adulthood (Figure 1). Adults who reported 4 or more ACEs were found to be 12 times more likely to experience outcomes such as alcoholism, substance use, depression, and suicide attempts (Webster, 2022). This study also demonstrated a dose-response relationship, whereby the accumulation of adverse experiences correlated directly with worsening physical and psychological health across the lifespan. These findings underscore the cumulative and enduring nature of early adversity (Felitti et al., 1998). While the ACE framework focuses on objective measures of trauma, it often fails to account for subjective interpretations of these experiences, factors that are critical to understanding the complex dynamics of vulnerability and resilience. Participants may display maladaptive behaviors in some areas of functioning while simultaneously demonstrating competence in others (Webster, 2022).

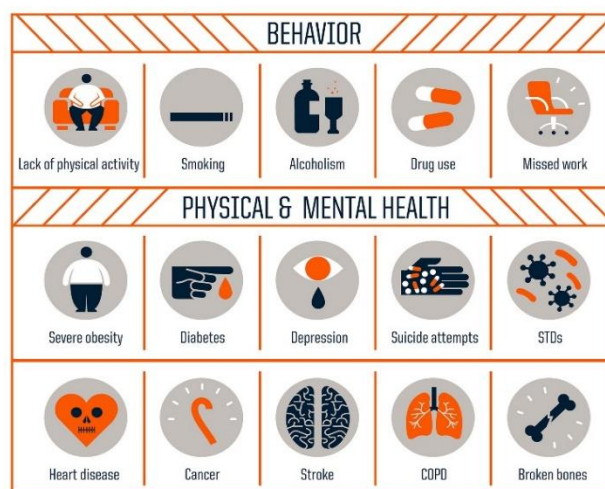


Figure 1. ACEs multiple health risk factors
(Centers for Disease Control and Prevention)

The original 10-item ACE questionnaire assesses exposure to traumatic events across three primary domains (Figure 2): abuse (physical, emotional, and sexual), neglect (physical and emotional), and household dysfunction (including mental illness, domestic/interparental violence, parental separation, incarceration, and substance abuse) (Webster, 2022).

Emotional abuse involves manipulative behaviors such as inducing guilt, shame, or fear to meet the caregiver's emotional needs, leading children to engage in inappropriate acts, devaluing their possessions or identity, or exposing them to harmful environments like

interparental violence (Teicher et al., 2016). Despite being less visible than physical abuse, emotional abuse can have deeply corrosive effects on a child's developing self-concept and emotional regulation.

Child neglect, the most frequently reported and fastest-growing form of maltreatment, is particularly damaging due to its insidious nature and lasting consequences. Approximately 60% of the nearly 700,000 cases of child maltreatment reported annually in the United States are attributed to neglect (U.S. Department of Health and Human Services, Children's Bureau). Neglect refers to the failure to meet a child's fundamental physical and emotional needs. Physical neglect may involve insufficient access to nutrition, hygiene, healthcare, or education, while emotional neglect is characterized by a lack of warmth, affection, and responsiveness to the child's needs for comfort and attention. Most children who suffer from neglect are exposed to a combination of both forms. Maternal neglect in particular represents a profound disruption of one of the most fundamental human bonds. It undermines biological systems that have evolved to support infant development, posing serious risks to attachment formation, emotional regulation, and neurodevelopment (Strathearn, 2011). Paradoxically, even in the presence of abuse or neglect, many children maintain strong emotional ties to their caregivers. However, such disorganized attachment often results in maladaptive patterns of emotional and social behavior, cognitive impairment, and increased vulnerability to mental health disorders in adolescence and adulthood (Perry & Sullivan, 2014).

Understanding the multifaceted impact of ACEs is crucial not only for clinical intervention but also for public policy aimed at prevention. It requires a comprehensive approach that considers both the objective severity and the subjective meaning of childhood adversities.

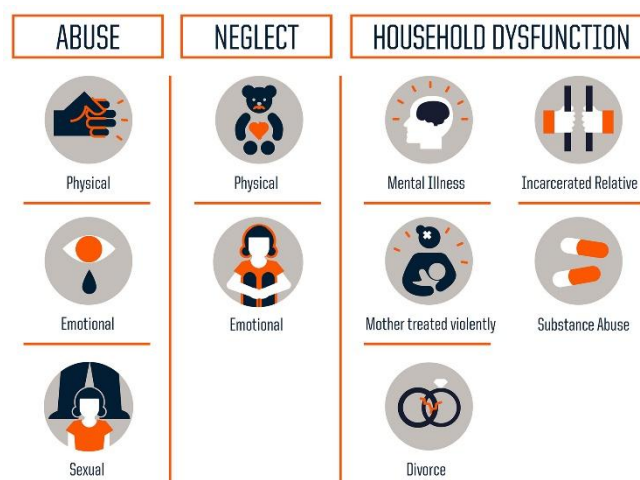


Figure 2. Adverse Childhood Experiences
(Centers for Disease Control and Prevention)

2.2 Attachment Bond Alteration in Humans

In mammals, the early relationship between infant and caregiver is fundamental for emotional regulation and long-term psychological well-being (Bowlby, 1969; 1982; 1973; Mancini et al., 2024). Maternal caregiving during the postnatal period not only involves physical nurturing but also provides the emotional, social, and environmental inputs essential for healthy neurodevelopment (Čater & Majdič, 2021). When these early interactions are stable and sensitive, they facilitate the formation of a secure attachment bond, which promotes the optimal organization of neural circuits and supports emotional resilience. Conversely, disruptions in these interactions can lead to insecure attachment patterns, which are associated with heightened vulnerability to emotional and psychiatric difficulties later in life (Lo Iacono et al., 2021; Mancini et al., 2024).

From an evolutionary perspective, the attachment bond plays a critical role in species whose newborns are highly dependent on caregivers for survival. In the first year of life, infants exhibit selective attachment behaviors directed toward specific individuals, typically their primary caregivers. These early bonds provide not only physical protection but also emotional scaffolding, laying the foundation for the child's social, cognitive, and emotional development (Szepeswol & Simpson, 2018; Hornor, 2019).

Attachment theory, originated from the studies of John Bowlby (1973), is based on the principle that human beings have evolved to develop mental representations based on their experiences of attachment relationships. These models help individuals gather and interpret information related to various social agents (such as parents, peers, and romantic partners) (Bowlby, 1969/1982). Children are biologically predisposed to form attachment bonds with available adult caregivers (Dykas & Cassidy, 2011), who serve as a secure base for exploring their environment during everyday activities.

According to Ainsworth, who significantly contributed to expanding the literature on attachment, the term "attachment" refers to an emotional bond such that the figure with whom it develops cannot be replaced or exchanged by another, even though it is possible to form new bonds (Ainsworth, 1989). The attachment bond leads to the development of a specific attachment style over time, based on the key characteristics of the attachment figure. Since the 1980s, hundreds of studies have shown that an individual's attachment style is a powerful predictor of various socio-relational phenomena (such as the quality of romantic relationships, sexual motivation, and reactions to loss) as well as psychological ones (such as the development of personal and social schemas and the ability to self-regulate stress and emotions) (Gillath & et al., 2005).

The nature of early caregiving experiences gives rise to distinct attachment styles, which have been shown to predict a range of outcomes-ranging from interpersonal functioning to emotional regulation and mental health (Gillath et al., 2005).

One of the most widely adopted methods for assessing attachment in young children is Ainsworth's "Strange Situation Procedure" (SSP). This standardized assessment exposes a child to a series of brief separations and reunions with their caregiver in an unfamiliar environment, allowing researchers to evaluate how the child uses the caregiver for reassurance and regulation under mild stress (van Rosmalen et al., 2015). Based on observed behaviors, children are categorized into four main attachment styles: secure (B), insecure-avoidant (A), insecure-ambivalent/resistant (C), and disorganized (D) (Ainsworth, 1979; Main, 2000). Securely attached children typically explore their environment freely when the caregiver is present and seek comfort upon reunion. In contrast, avoidant children show emotional detachment, minimizing their interactions with the caregiver. Ambivalent children exhibit conflicting behaviors, like seeking closeness but also resisting contact. The disorganized attachment style, later added by Main and colleagues, is marked by contradictory and disoriented behaviors, often observed in children exposed to trauma, fear, or maltreatment (Main, 2000).

Although the SSP remains an indispensable part of attachment research, it has limitations. Cultural differences can influence the interpretation of behaviors, and the procedure is generally not suitable for children older than two years. Moreover, the artificial nature of the laboratory setting may reduce ecological validity, and the stress induced by the procedure has raised ethical concerns in recent years (van IJzendoorn et al., 2004).

3. Animal Models of ELA-induced Depression

Preclinically, a range of animals, from non-human primates to zebrafish, have been and continue to be used in the study of depression. Nevertheless, rodents remain the gold-standard organisms for many experimental manipulations (Gururajan et al., 2019). Preclinical rodent models for studying depression have been useful for identifying the cell-type-specific mechanisms that are otherwise inaccessible in human studies (Fox & Lobo, 2019). Historically, rodent models of major depression have been generated by an inducing manipulation based on a specific aspect or different aspects of its pathophysiology. Rather than aiming to replicate the full syndrome, most contemporary models seek to reproduce specific pathophysiological aspects or endophenotypes of depression, in line with frameworks such as the Research Domain Criteria (RDoC). Endophenotype-focused investigations could provide information in identifying novel pathophysiological mechanisms with potential transdiagnostic relevance (Gururajan et al., 2019).

Within the domain of early life stress research, significant challenges persist in delineating the mechanisms through which ELA leads to both immediate and long-term pathophysiological outcomes, such as depression. A range of preclinical models has been developed to experimentally induce ELA under controlled conditions.

Exploring ELA in rodents is possible because, among all mammals, the bond between the newborn and the caregiver is universally recognized as the most important, gradually transforming into the child–caregiver relationship and ultimately supporting the offspring’s path toward physical independence (Pryce et al., 2005). In species where parental care is essential for survival, evolutionary processes have supported the development of strong and rapid attachment to caregivers, even when the quality of care is suboptimal (Perry & Sullivan, 2014). Although translating findings from rodent models to humans poses certain challenges, there is significant cross-species similarity in the attachment system and its responses to trauma (Opendak et al., 2017). Rodent models offer, indeed, a valuable tool for studying the mother–infant relationship from a translational perspective. Research on maternal behavior in rodents has underscored the crucial influence of early parent–offspring interactions on the developing brain, cognitive functions, emotional regulation, and sensitivity to stress (Krugers et al., 2016).

While human studies have demonstrated the link between ELA and psychopathology, they are intrinsically limited by their correlational nature. In contrast, preclinical research is essential for uncovering causal relationships between ELA, brain function, and behavior.

These insights hold significant promise for identifying new therapeutic strategies aimed at restoring neurobiological mechanisms disrupted by ELA (Malave et al., 2022).

During the first 15 Postnatal Days (PND), mouse pups exhibit a limited behavioral repertoire due to their immaturity; consequently, the mother represents the primary environmental factor with which they interact, playing a critical role in their development. To modulate mother-pup interactions during development, various strategies have been proposed in rodent models (as reviewed in Di Segni et al., 2018). Indeed, natural variations in maternal care from PND 1 to PND 6 are critical for the long-term development of pups' stress sensitivity and emotional development (Liu et al., 1997). The effects of these manipulations underscore the mediating role of parental care in neural development during early life, including the maturation of systems involved in stress responsivity (Caldji et al., 2000).

3.1 Traumatic Experience in Mice: a Neglect model

Employing an early-life animal model that integrates maternal abuse within the framework of attachment offers a valuable strategy for investigating how maltreatment impacts brain development, particularly given the shared neural circuits involved in attachment and the emergence of pathological phenotypes (Roth & Sullivan, 2005).

One such model in rodents simulates infant trauma within the attachment system by placing mothers in conditions with inadequate nesting material, inducing rough handling of their pups (Ivy et al., 2008; Rainecki et al., 2010; Roth & Sullivan, 2005; Perry & Sullivan, 2014). This ethologically relevant paradigm mirrors the complexity of abusive caregiver-child relationships observed in humans and demonstrates how environmental stress can increase the risk of infant maltreatment. Remarkably, despite repeated exposure to maternal abuse, pups continue to seek proximity to their mother and display attraction to abuse-associated odors, even into adulthood (Perry & Sullivan, 2014). This paradoxical behavior underscores a key feature of early attachment: abused pups develop a preference for their caregiver and stimuli linked to the abuse (Roth & Sullivan, 2005).

Over a decade ago, the Baram Laboratory developed the Limited Bedding and Nesting (LBN) model in rodents to simulate low-resource home environments (Shupe & Clinton, 2021). Originally established in rats, this model has since been successfully adapted for use in mice by various research groups to induce chronic early-life stress (as reviewed in Walker et al., 2017). The primary aim of the LBN paradigm is to alter maternal caregiving patterns while minimizing direct experimenter interference. Both continuous and intermittent

procedures have been designed to produce fragmented, unpredictable, or adverse maternal behavior, closely mirroring caregiving seen in humans experiencing depression, severe stress, or substance abuse. A major strength of the LBN model lies in its adaptability: researchers can modulate maternal disruption and stress intensity (e.g., by adjusting nesting material availability) to examine how variations in care influence developmental outcomes. Additionally, the model allows for manipulation of the timing of exposure to determine sensitive periods in development. In its original form, the LBN model involved continuous exposure to the impoverished environment from PND 2 to 9 (Walker et al., 2017).

A variation of this model, known as the “Scarcity Model”, was later introduced by Roth and Sullivan. This approach involves a less extreme reduction in bedding and elicits maternal behaviors such as stepping on, dragging, or roughly handling the pups. It serves as a valuable tool for studying early attachment learning and the effects of caregiver maltreatment (Shupe & Clinton, 2021). Unlike the standard LBN, the Scarcity Model does not use a wire mesh floor but still reduces nesting material, impairing the dam’s ability to build a stable nest. This leads to increased nest-building attempts, less time spent nursing, and more distress vocalizations from the pups. Interestingly, while body weight remains unaffected in this model (as opposed to the weight reduction observed in LBN), basal corticosterone levels are elevated (Walker et al., 2017).

The Scarcity Model has been applied over various postnatal timeframes (such as PND 2–9, 1–7, 3–8, 8–12, or 10–14) to evaluate the long-term consequences of maltreatment across different developmental stages. Notably, the most pronounced neurobehavioral effects occur when exposure begins during the sensitive attachment period, which concludes around PND 10. This model captures critical aspects of abusive maternal behavior, including rough handling (e.g., stepping on or transporting pups by limbs), which is less commonly observed in the standard LBN protocol (Walker et al., 2017). It is really important to highlight that a hallmark of maternal behavior in neglect/abuse situations is its unpredictable and fragmented quality (Ivy et al., 2008).

Pups reared in a low bedding environment exhibit disrupted attachment behaviors toward their mother during the preweaning period (Raineke et al., 2010); the behavior exhibited by adversity-reared rat pups paralleled that exhibited by the children with high maltreatment risk (classified as disorganized) (Opendak et al., 2020). Moreover, these animals show social deficits with peers during adolescence and adulthood (Raineke et al., 2012; Perry & Sullivan, 2014; Rincón-Cortés & Sullivan, 2016) and long-term alterations in fear and anxiety-related behaviors, providing a useful framework for investigating how early-life stress increases

vulnerability to psychopathologies. The usefulness of LBN rearing as a model of rodent depressive-like behavior has been demonstrated in several studies, and in particular using the Scarcity model between PND8-12. Pups raised in an LBN environment show dysfunctional social attachment behaviors, followed by deficits in social behaviors with peers during adolescence and adulthood (Raineke et al., 2010; Raineke et al., 2012; Rincón-Cortés & Sullivan, 2016; Walker et al., 2017). Moreover, adult rat offspring raised under the Scarcity Model displayed increased time immobile in the Forced Swim Test (Raineke et al., 2012; Walker et al., 2017), a behavioral marker of depressive-like behavior, which is accompanied by heightened activation of the central, lateral, and basal nuclei of the amygdala (Raineke et al., 2012). Notably, these amygdala-dependent depressive-like behaviors do not emerge until after weaning, once pups attain independence, and persist into adulthood (Raineke et al., 2012). Another key symptom of depressive-like behavior was found in rodents raised under the LBN paradigm: anhedonia. Anhedonia is the reduced ability to experience pleasure, and it is an important emotional variable linked to depression. Indeed, rats raised under the LBN paradigm exhibit reduced sucrose preference during adolescence, an indication of anhedonia (Molet et al., 2016; Walker et al., 2017).

The LBN and Scarcity models serve as robust and translationally relevant examples of early-life stress paradigms in animal research. Their strength lies in their methodological simplicity and high reproducibility, consistently inducing changes in maternal behavior across different rodent species, laboratories, and geographic locations (Walker et al., 2017).

3.2 Attachment Bond Alteration in Mice: The Repeated Cross-Fostering

Gaining insight into the psychobiological mechanisms linking early insecure attachment to adult mental disorders holds considerable clinical significance. Importantly, attachment represents a phylogenetically conserved behavioral system, exhibiting striking parallels across altricial species. This evolutionary continuity makes it particularly well-suited for comparative, cross-species investigations. Consequently, animal models play a crucial role in clarifying the causal pathways between early attachment experiences and the emergence of maladaptive behaviors in adulthood (Mancini et al., 2024).

The Repeated Cross-Fostering (RCF) protocol was designed to disrupt early mother–infant bond during the first days of life, thereby predisposing offspring to separation anxiety (Di Segni et al., 2018). This is evidenced by an increased number of separation-induced ultrasonic vocalizations (USVs) in RCF pups, without inducing neglectful behavior by the caregivers, as shown by the absence of differences in body weight and temperature between

RCF and control pups (D'Amato et al., 2011). Thus, the procedure selectively affects the mother–infant bond without compromising maternal orienting behaviors toward the pups (Di Segni et al., 2018).

In this protocol, pups spend PND0 with their biological mother. On PND1, litters are randomly assigned to either the RCF or control group. Unlike standard cross-fostering procedures, RCF pups are transferred to a new caregiver every 24 hours: this occurs 4 times between PND1 and PND4, following a rotational pattern (Figure 3). Each time, the entire litter is moved into the cage of a foster mother whose own pups have just been relocated to another adoptive dam. At PND4, RCF pups remain with the last foster mother until weaning at PND28, after which they are separated by sex (D'Amato et al., 2011).

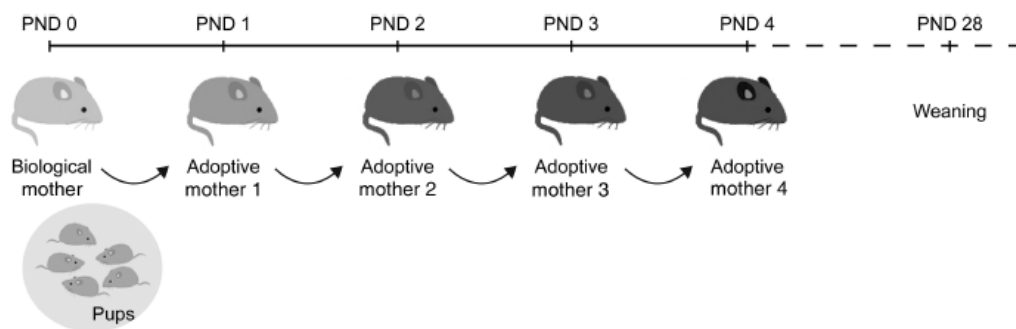


Figure 3. Schematic representation of RCF procedure (Di Segni et al., 2016)

Research has shown that mouse pups fostered by lactating adoptive mothers are generally well accepted and nourished. Furthermore, this procedure exerts minimal impact on the functioning of the offspring's hypothalamic–pituitary–adrenal (HPA) axis (D'Amato et al., 2011). Indeed, the RCF manipulation is not considered a stressor, as both basal and stress-induced corticosterone levels in adulthood are comparable between RCF and control animals, suggesting the disruption is specific to the attachment bond (D'Addario et al., 2021). It is also hypothesized that repeated changes in maternal cues (particularly olfactory ones, which are crucial in rodents) may interfere with associative learning processes essential for forming a stable attachment relationship (Di Segni et al., 2018).

The RCF manipulation also modify adults' inclination to seek rewarding stimuli and increases their sensitivity to negative events in adulthood. However, these effects can differ depending on the animal's genotype and the environmental conditions they are exposed to (Di Segni et al., 2016). This was clearly demonstrated in a study by Di Segni and colleagues (2016), which examined female mice from two inbred strains, C57BL/6J (C57) and DBA/2J

(DBA). RCF-exposed females from the DBA and C57 strains exhibited opposite behavioral coping strategies in the Forced Swim Test (FST), as well as divergent patterns of c-Fos activation across various cortical and subcortical regions. They also showed contrasting tendencies in natural reward-seeking behavior, assessed using the Saccharin Preference Test (SPT), and displayed opposite dopaminergic responses in the prefrontal–accumbens circuitry following exposure to acute stress. Overall, these findings suggest that RCF leads DBA females toward a depression-like phenotype, whereas C57 females show increased sensitivity to rewarding experiences in adulthood (Di Segni et al., 2016).

Interestingly, a sex-dependent response to RCF has also been observed. In C57 mice, early postnatal environmental disruption through RCF enhances sensitivity to both natural and pharmacological rewards in females, while promoting a more pronounced depression-like phenotype in males (Di Segni et al., 2019). A sex-specific pattern is also seen in DBA mice: while RCF-exposed DBA females show passive coping behaviors in the FST and reduced sensitivity to natural rewards (Di Segni et al., 2016; Mancini et al., 2024), DBA males appear largely unaffected by the long-term consequences of the RCF manipulation.

These findings highlight not only the complex interplay between early attachment disruption and emotional processing but also the critical role of the genotype–environment interaction in shaping vulnerability or resilience to depressive phenotypes in adulthood.

4. Neurobiological Effects of ELA-induced Depression in Humans and Mice

ELA contributes to stress-induced brain dysfunction that in turn may lead to mental health problems (Björkenstam et al., 2017b). Indeed, a growing body of evidence highlights how early life experiences, particularly those involving abuse or neglect, can profoundly affect brain development and increase the risk of depression. Although brain maturation is genetically programmed, it is critically shaped by environmental input during sensitive developmental windows (Teicher et al., 2016).

Early life adversity impacts key brain regions implicated in stress and depression (Heim & Binder, 2012). Specifically, a growing body of behavioral and neurobiological studies in humans suggests ELA-related alterations in reward-processing and connected brain circuitry. Overall, these findings suggest that reward circuit physiology is particularly vulnerable to the effects of ELA, likely due to the ongoing maturation of dopaminergic reward circuitry in the postnatal period when these ELA manipulations are being implemented (Hanson et al., 2021).

Findings include reduced medial prefrontal cortical volume in adults with emotional maltreatment experiences (van Harmelen et al., 2010), reduced orbital-frontal cortical volume in previously institutionalized children (Pollak et al., 2010), and increased amygdala volume in previously institutionalized children with increased amygdala response to face cues (Tottenham et al., 2011). Increased amygdala reactivity to emotional cues has been reported as a function of early adversity in adult patients with depression (Grant et al., 2011). Recent evidence further suggests that ELA is associated with altered cortical affective processing in patients with various psychiatric disorders. It may be hypothesized that early life stress leads to changes in a connected neural network that fails to adapt and compensate in response to additional challenge, thereby leading to behavioral and physiological changes that form the clinical phenotype of depression (Heim & Binder, 2012). These neurobiological changes secondary to early life stress, taken together, likely reflect risk of developing depression and a host of other disorders in response to additional challenge. In several studies, these changes were not present in depressed persons without early life stress, suggesting the existence of biologically distinguishable subtypes of depression as a function of early life stress. Hence, consideration of early life stress might be critical for clinical decisions (Heim & Binder, 2012).

Neuroimaging studies have demonstrated that exposure to different forms of childhood maltreatment is associated with structural and functional alterations in specific brain regions in humans. For instance, repeated verbal abuse is linked to increased grey matter volume in the superior temporal gyrus (Tomoda et al., 2011), while visually witnessing interparental violence correlates with thinning in visual regions such as the lingual gyrus and occipital pole (Tomoda et al., 2012). Similarly, childhood sexual abuse has been associated with volume reductions in somatosensory areas involved in processing genital sensation, as well as in visual regions linked to facial recognition (Teicher et al., 2016).

Functional imaging studies consistently show that individuals with a history of maltreatment exhibit heightened amygdala reactivity to emotionally salient or threatening stimuli, even in the absence of clinical psychopathology (Tottenham et al., 2011; Dannlowski et al., 2012; Teicher et al., 2016). In contrast, striatal responses to reward, particularly in the ventral striatum, are blunted in maltreated individuals, suggesting a dysregulation of the reward circuitry that may underlie anhedonia, a core symptom of depression. These disruptions are especially pronounced when maltreatment occurs between birth and nine years of age (Takiguchi et al., 2015; Hanson et al., 2016). Further, reductions in corpus callosum area or integrity, often more severe in males, have also been widely reported in both children and adults exposed to early abuse (Paul et al., 2008).

At the network level, maltreatment has been shown to alter cortical connectivity patterns. Graph-theoretical analyses indicate reduced centrality in nodes such as the anterior cingulate cortex, temporal pole, and middle frontal gyrus, while the anterior insula and precuneus show increased centrality (Teicher et al., 2014). These findings suggest that childhood adversity can lead to long-lasting disruptions in the architecture of large-scale brain networks implicated in emotional regulation and self-referential processing (Teicher et al., 2016).

Taken together, these findings suggest that different subtypes of early life stress may exert distinct and lasting neurobiological effects, contributing to heterogeneous trajectories of depression and influencing both disease course and treatment response.

While human studies have revealed the impact of ELA on psychopathology, they are limited by correlational inferences. Preclinical studies are invaluable in establishing causal relationships between adversity, brain function, and behavior, which may bring great potential to discover novel opportunities for therapeutic interventions aimed at targeting neurobiological mechanisms that are impaired by ELA (Malave et al., 2022).

Pups exposed to limited bedding environment have accelerated amygdala development, which prematurely ends the sensitive period for attachment learning (Roth et al., 2013) and disrupts social behavior with siblings (Moriceau et al., 2009; Rainecki et al., 2012). Amygdala activation in central, lateral, and basal nuclei is associated with increased immobility in the Forced Swim Test displayed by adult rats raised under the Scarcity Model (Rainecki et al., 2012; Walker et al., 2017). The amygdala-dependent depressive-like behaviors do not emerge until pups reach independence after weaning, and persist into adulthood (Rainecki, et al., 2012; Walker et al., 2017). Moreover, growing up in an LBN environment was associated with changes in the hippocampus and prefrontal cortex, key structures for cognition and regulation of the HPA axis (Baram et al., 2012). The LBN model appears to impair higher-order cognitive functions while amplifying emotional processes, particularly the expression of conditioned fear (Krugers et al., 2016).

The long-term impact of early environmental instability induced by RCF is evident in the behavioral and neurochemical responses to acute stress. Di Segni et al. (2016), using for the first time a new “dual probe” *in vivo* microdialysis procedure, found that the RCF procedure induces an opposite response of prefrontal-accumbal dopaminergic transmission to acute stress in adult C57 and DBA female mice. Indeed, restraint exposure induces a higher dopamine release in the prefrontal cortex and lower dopamine outflow in the Nucleus Accumbens of RCF DBA and an opposite effect in RCF C57 (Di Segni et al., 2016). Moreover, Di Segni et al. (2019) analyzed sex differences in C57 mice induced by RCF, highlighting that the reduction in active coping strategies in male RCF mice was linked to impaired activation in the prefrontal cortex, Nucleus Accumbens, and Caudate-Putamen complex. In contrast, the increase in active coping strategies in RCF female mice was related to greater c-Fos activation (Di Segni et al., 2019).

In conclusion, converging evidence from animal models and human studies would suggest that early life stress is a general risk factor leading to increased stress vulnerability and depression risk. It should be noted that there is increasing evidence for variability in the effects of early life stress among humans. Certainly, not all individuals exposed to early life stress go on to develop depression and remain resilient even when exposed to additional stressors in adulthood (Heim & Binder, 2012). This raises the important question as to what factors may determine outcome variability in the effects of early life stress. Such factors may include differential effects of early life stress experienced at different times during early development, i.e., there might be critical time windows of vulnerability or enhanced plasticity during which a stressor or trauma has a particularly strong or specific effect, depending on the developmental stage of brain regions at the time of exposure. Variability

in the neurobiological and behavioral outcomes of early life stress is further dependent on interactions of early life stress with the genotype, which may be differentially vulnerable to the effects of early life stress, perhaps mediated by differential vulnerability to direct epigenetic programming effects of early life stress (Heim & Binder, 2012).

4.1. Neurobiological Effects of ELA: a Focus on Social Neuropeptides

Environmental factors interact with innate factors and result in a specific response depending on the individual genotype. So far, the molecular mechanisms underlying these interactions are unclear, and likewise, the mediators of the environmental influences are not fully known. There are a number of different proteins associated with peptide transmission, and they are all putative targets for environmental influence (Nylander & Roman, 2012). Two main peptides that act as neuromodulators in the brain are candidates as a link between ELA and adult psychopathologies, specifically depression: Oxytocin (Oxt) and Endogenous opioids.

Oxt, which can act as a neuromodulator in the brain (Grinevich et al., 2016; Marlin et al., 2017; Bakos et al., 2018; Putnam & Chang, 2022), robustly affects social behaviors in both humans and animal models (Ross & Young, 2009; Gothard et al., 2018; Putnam & Chang, 2022) and modulates behaviors such as learning, maternal bonding and social interaction (Putnam & Chang, 2022). This evidence strongly point toward the Oxt system being a core component in the neural substrates of social cognition (Ross & Young, 2009; Chang & Platt, 2014; Putnam et al., 2018; Putnam & Chang, 2022), and have prompted interest in the potential of Oxt as a therapeutic strategy to augment socio-behavioral deficits observed in psychiatric disorders (Modi & Young, 2012; Carter et al., 2020; Putnam & Chang, 2022). Oxt pathways and receptors modulate the activity of different brain regions including Amygdala (Amy), Nucleus Accumbens (NAc) and Ventral Tegmental Area (VTA), which are typically associated with fear, aggression and social behaviors (Heinrichs & Domes, 2008; Febo & Ferris, 2014; Sharma et al., 2020), as well as emotion, cognition and responses to trauma (Phelps, 2006; Sharma et al., 2020).

However, it is evident that endogenous Oxt does not work alone in the brain; instead, interaction effects between neuromodulator systems may critically account for many of the behavioral and neural effects of Oxt. Interactions between Oxt and serotonin (Pagani et al., 2015; Lefevre et al., 2017), endocannabinoids (Wei et al., 2015), or opioids (Bicknell et al., 1988; Dal Monte et al., 2017) have been demonstrated to underlie or modulate many behavioral effects once attributed to just a single neuromodulator (Putnam & Chang, 2022).

Specifically, oxytocinergic and opioidergic interactions have been postulated to modulate corticostriatal circuits in a protective response against stress (Putnam & Chang, 2022).

The endogenous opioid peptides are present in the Central Nervous System (CNS) well before birth and are among the first neurochemical markers to be detectable in the developing brain (Nylander & Roman, 2012). During the first few weeks of neonatal life, a significant developmental reorganization of the opioid systems occurs, accompanied by regional divergence within the brain (McDowell and Kitchen, 1987; Smith et al., 1993). The endogenous μ -opioid (Oprm) and κ -opioid (Oprk) receptors are the first binding sites to appear within the rodent CNS at the embryonic stage. The presence of opioids when neurogenesis, neuronal migration, process outgrowth, and synaptogenesis occur suggests a role for opioids in neuronal developmental processes (Nylander & Roman, 2012).

Inside the reward system, Opioid and Oxt neuronal fibers and receptors are distributed very closely together within the rodent Amy and hypothalamus, suggesting physiological and behavioral interactions between the two systems (Zanos et al., 2018; Sharma et al., 2020). Studies examining the effect of Oxt on the opioid system have suggested two potential roles for Oxt: stimulating the release of endogenous opioids and binding to Oprm as a positive allosteric modulator, which may account for its ability to also improve analgesic responses in both animals and humans (Meguro et al., 2018; Sharma et al., 2020). Moreover, the endogenous μ -opioid receptor system or naturally occurring peptides that act on Oprm (principally β -endorphin, but also enkephalins and dynorphin) have been shown to mediate reward processing (Le Merrer et al., 2009; Resendez et al., 2013; Fields & Margolis, 2015; Castro et al., 2021) through mesocorticolimbic reward circuitry. The Oprk system has been strongly implicated in mediating stress responses that are strongly interlinked with social behavior (Knoll & Carlezon, 2009; Beery & Kaufer, 2015; Putnam & Chang, 2022).

It is important to highlight that exposure to ACE and early life trauma has been found to impact not only responses to Oxt administration, but also to behavioral outcomes based on specific Oxt Receptor (OxtR) gene polymorphisms that may be associated with increased vulnerability, as well as higher adaptation to early life trauma exposure. Human brain-imaging studies have shown that variations in OxtR gene polymorphisms are related to structural and functional variations in the Amy, hypothalamus, and cingulate gyrus (Tost et al., 2010). In particular, Oxt may play a role in modulating morphological features of these structures and, thereby, social behavioral outcomes in situations of repeated childhood trauma and emotional neglect. There is evidence suggesting possible associations between the OxtR gene and certain personality or cognitive characteristics that convey either

resilience or vulnerability to stress-related disorders. This effect emerges primarily following exposure to ACE, resulting in disordered attachment behaviors in adulthood (Lucht et al., 2009; Lucas-Thompson & Holman, 2013; Tollenaar et al., 2017; Sharma et al., 2020).

Moreover, the quality of childhood family environment may have long-term influences on the opioid system, which is known to play a crucial role in experiencing social reward during social interaction and affective pain after social exclusion (Saarinen et al., 2025). Chronic stressors may change the regulation of the opioid system under stress, which, in turn, may result in stress-related disorders. A crucial region where childhood stress events were linked to Oprm availability was the orbitofrontal cortex. Thus, childhood stress-induced dysregulation of the Oprm transmission in the orbitofrontal cortex may imply altered perceptions of reward and pleasantness of social interaction with close others (Saarinen et al., 2025). A PET study found that when compared to individuals not exposed to stressful life events in childhood, individuals who had been exposed to these had lower Oprm availability in the orbitofrontal cortex, hippocampus, putamen, Amy, insula, thalamus, anterior cingulate cortex, and dorsal caudate in adulthood (Saarinen et al., 2025).

Besides the modulations in Oxt and opioids related to trauma, it is also important to highlight their role in the consequences of altering the attachment bond. Indeed, individual differences in adult attachment modulate activation of the corticolimbic circuits that encode social and affective information (Vrticka & Vuilleumier, 2012). As is widely known in the literature, the Oxt system contributes significantly to attachment behavior (Insel & Young, 2001; Johnson & Young, 2015). Animal studies suggest that the endogenous opioid system could be another candidate neurochemical pathway for the human attachment circuit. Indeed, exogenous opioid agonists alleviate separation distress in pups (Panksepp et al., 1978), whereas μ -opioid receptor (Oprm1) gene knockout mice pups display a deficit in maternal attachment behavior (Moles et al., 2004). Pharmacological manipulations in primates further show that opioid receptor antagonists increase the frequency of both social grooming (Fabre-Nys et al., 1982) and grooming solicitations (Keverne et al., 1989), suggesting that endogenous opioid tone modulates attachment behavior (Nummenmaa et al., 2015). Furthermore, activation of the endogenous Oprm is essential for bonding in monogamous adult voles (Resendez et al., 2013), suggesting the role of Oprm in long-term adult social bonds (Nummenmaa et al., 2015). Finally, human behavioral studies provide indirect evidence for the role of Oprm system in bonding, by showing that pain threshold—a proxy of endogenous opioid release—is increased by social behavior promoting intragroup affiliation (Dunbar et al., 2012).

In 2015 a PET study shows for the first time that individual differences in the attachment style of human adults are associated with baseline Oprm availability, with high attachment avoidance being associated with low Oprm availability both in regions belonging to the putative social distress circuit and in the medial frontocortical regions that support functions such as emotions and theory-of-mind that are critical for social interaction (Nummenmaa et al., 2015). These finding accords with primate work showing that the opioid system modulates pair bonding and attachment in primates and other mammals (Nelson & Panksepp, 1998; Machin & Dunbar, 2011), in addition to the well-known oxytocin system (Insel & Young, 2001; Nummenmaa et al., 2015)

Overall, striking results highlight that oxytocinergic and opioidergic interaction strongly influences social behaviors beyond the contributions of the individual neuromodulators alone. However, the precise mechanism of this interaction is still unknown (Putnam & Chang, 2022).

5. Hypothesis

Early Life Adversities (ELA) are highly prevalent worldwide, affecting millions of children every year. As highlighted in the previous chapters, children exposed to such adversities are at a significantly higher risk of developing depressive episodes across the lifespan.

Adopting a translational approach, in this study, we first examine the association between exposure to ELA and vulnerability to depressive symptoms in a non-clinical human cohort. To do that, we administered the ELAX questionnaire, a self-report questionnaire that aims to investigate the presence of different ELA as well as symptoms of depression in a non-clinical population.

Although the relationship between ELA and depressive symptoms is well established in the literature, relatively few studies have explored whether heterogeneity in depressive symptoms relates to specific subtypes of ELA, largely due to the intrinsically correlational nature of human research.

To test the hypothesis that distinct patterns of depressive symptoms may be linked to different kinds of early aversive experiences - through their impact on partially overlapping neurobiological pathways - we next conduct experiments in animal models. Specifically, we build on our well-established model of attachment bond interference (RCF) (D'Amato et al., 2011; Ventura et al., 2013; Di Segni et al., 2017; Lo Iacono et al., 2021; Di Segni et al., 2016; Di Segni et al., 2019; Babicola et al., 2023; D'Addario et al., 2021) and employ a widely used rodent model of neglect (the Scarcity Model) (Ivy et al., 2008; Raineke et al., 2010; Roth & Sullivan, 2005; Perry & Sullivan, 2014). We hypothesize that distinct early-life experiences (attachment bond disruption vs. neglect) may be associated with different depressive symptom profiles in adult mice. Moreover, we start to investigate the hypothesis that attachment bond disruption and neglect differentially affect the developmental trajectories of neuropeptides critically implicated in depression - oxytocin and opioids - within one of the brain circuits most sensitive to ELA, the mesocorticolimbic system.

6. Methods

6.1 Human Study

6.1.1 *The ELAX questionnaire in humans*

To investigate the link between ELA and depressive symptoms in humans, we collected a set of surveys from 2062 people in the age range of 18-65 years, through word of mouth and flyers. Only surveys without missing data were included ($n = 1025$; 759 women, age: $25,34 \pm 0,27$; 269 men, age: $26,84 \pm 0,58$ years; 7 other/non binary, age: $23,0 \pm 1,73$ years).

We created a self-report questionnaire (The ELAX questionnaire) composed by:

1. The Childhood Trauma Questionnaire- Short Form (CTQ-SF; 28 items combined in 5 subscales: 1. Emotional abuse, 2. Physical abuse, 3. Sexual abuse; 4. Emotional neglect and 5. Physical neglect) (Bernstein, et al., 1994). The CTQ responses are provided on a 5-point Likert scale, where 1 = never, 2 = almost never, 3 = sometimes, 4 = often, 5 = very often (Bernstein et al., 1994). Regarding the scales used, the subscales analyzed in the CTQ are: Emotional Abuse (5 items; e.g., "I thought that my parents wished I had never been born."; $\alpha = .871$); Physical Abuse (5 items; e.g., "Someone in my family hit me so hard that it left marks or bruises."; $\alpha = .808$); Emotional Neglect (5 items; e.g., "I felt loved."; $\alpha = .883$); Physical Neglect (2 items; e.g., "I didn't have enough to eat."; $\alpha = .485$; correlation between the two items $r = .321^*$).
2. The Parental Bonding Inventory (PBI; 50 items assessing maternal and paternal care, with 4 subscales: 1. Maternal care, 2. Paternal care, 3. Maternal overprotection, 4. Paternal overprotection) (Parker, 1990). The PBI uses a 4-point Likert scale, where 0 = very untrue, 1 = somewhat untrue, 2 = somewhat true, 3 = very true (Parker, 1990). The analyzed subscales are: Maternal Care (12 items; e.g., "Spoke to me in a warm and friendly voice."; $\alpha = .902$) ; Maternal Overprotection (13 items; e.g., "Did not want me to grow up."; $\alpha = .893$) ; Paternal Care (12 items; e.g., "Spoke to me in a warm and friendly voice."; $\alpha = .910$) ;Paternal Overprotection (13 items; e.g., "Did not want me to grow up."; $\alpha = .873$).
3. The Relationship Questionnaire (RQ; 4 items (Bartholomew & Horowitz, 1991)). In the RQ, participants are asked to rate how much they identify with each attachment prototype, using a 7-point Likert scale (1 = not at all like me, 7 = very much like me). The 4 items correspond to the 4 most common relational styles (Bartholomew & Horowitz, 1991).

4. The Patient Health Questionnaire-9 (PHQ-9; self-report scale: 0-4, Absent; 5-9, Minimal; 10-14, Mild; 15-19, Moderate; ≥ 20 , Severe) (Kroenke, Spitzer, & Williams, 2001). The PHQ-9 consists of 9 items aimed at assessing depressive symptomatology. The PHQ-9 score ranges from 0 to 27. Scores between 5 and 9 indicate the presence of subthreshold depression. A score of 10 is the optimal cut-off to identify clinically significant depression with three levels of severity (mild, moderate, and severe). Responses are based on a 0–3 Likert scale measuring the frequency of symptoms: 0 = Never, 1 = Several days, 2 = More than half the days, 3 = Nearly every day (Kroenke, Spitzer, & Williams, 2001).

Prior to enrolment, all participants were given a complete description of the study and signed a written informed consent (Prot. n. 0000268;17/02/2022). The study was approved by the Department of Psychology, Sapienza University Rome, Ethical Committee. This study complies with the Declaration of Helsinki.

6.1.2 Plasma oxytocin quantification

Plasma Oxytocin (Oxt) quantification was measured by Enzyme-linked Immunosorbent Assays (ELISA). For ELISA experiments, total protein content was extracted from plasma samples using C18 Spin Columns (C18 Spin Columns, G-Bioscience, 786-931). Columns were placed in collection tubes and 200ul of Activation Solution (50% ACN) was added, then they were briefly centrifuged (1500xg for 1 min). The residue was discarded, and the Activation Solution wash was repeated. 200ul of Equilibration Solution (0.5% TFA in 5% ACN) was added, the columns were centrifuged as reported before, and the solution was discarded. The equilibration Solution wash was repeated once. The columns were then transferred to clean collection tubes, and the sample was applied. After a brief centrifugation, the flow-through was recovered and reapplied to ensure complete binding before repeating the centrifugation. Columns were transferred to clean collection tubes, and 200ul of Wash Solution (0.5% TFA in 5% ACN) was applied before centrifugation. The flow-through was discarded, and the Wash Solution was repeated. The columns were transferred to clean collection tubes, and 20ul of Elution Buffer (70% ACN) was added before centrifugation. The elution step was then repeated, and the final volume of 40ul was collected and stored at -20 °C when not immediately processed for the assay. The ELISA kit was used to analyze OXT content in samples, following the manufacturer's instructions (Oxytocin ELISA kit, Enzo Lifescience, ADI-900-153A).

6.2 Animal Study

6.2.1 Animals

6-8 weeks old C57BL/6J (C57) female and male mice (purchased from Charles River Laboratories, Italy) were housed in standard conditions with water and food available ad libitum, at constant room temperature ($21 \pm 1^\circ\text{C}$) and in a 12:12 h light-dark cycle (lights on at 07:00 a.m.). At 12 weeks old, C57 female and male mice were mated (Di Segni, et al., 2016). Briefly, two females and one male were housed in transparent polysulfone cages with water and food ad libitum. After 15 days, pregnant females were isolated in clean cages with nesting material and inspected twice daily. Adequate measures were taken to minimize pain or discomfort of mice. All test were conducted only in male mice.

All experiments were carried out in accordance with Italian national laws on the use of experimental animals (DL116/92 and DL26/2014; Experimental Protocol 768/2025-PR approved by the Italian Ministry of Health), in line with the European Communities Council Directives (86/609/EEC and 2010/63/UE).

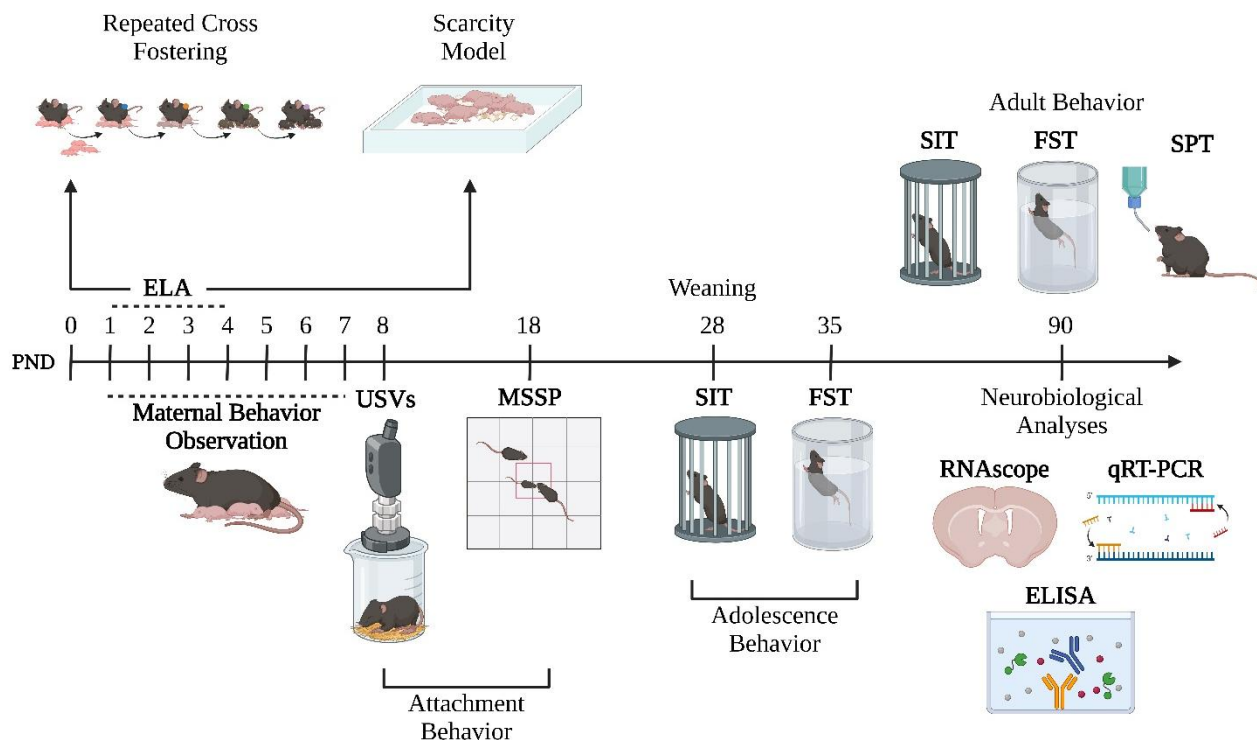


Figure 4. Experimental timeline of Animal Study: pups are exposed to ELA (RCF or Scarcity) or Control condition from PND1 to PND4; Maternal Behavior was observed from PND1 to PND7; Attachment behaviors of pups was measured through Ultrasound Vocalization Distress Calls (USVs) (PND8) and Mouse Strange Situation Procedure (MSSP) (PND18); adolescence behavior was measured on PND28 through Social Interaction Test (SIT) and Forced Swimming Test (PND35); in adulthood animals were tested in SIT, FST and Saccharin Preference Test (SPT) and neurobiological analyses were conducted.

6.2.2 Early Life Adversities in Mice: The Repeated Cross Fostering (RCF)

The RCF manipulation was performed as previously described (D'Amato, et al., 2011; Di Segni, et al., 2016; Di Segni, et al., 2019; Lo Iacono, et al., 2021; D'Addario, et al., 2021; Babicola, et al., 2023). Briefly, C57 pups from the same litter spent the first postnatal day (PND0) with their biological mother. On PND1, litters were randomly assigned to experimental (RCF) or Control (Cont) conditions. RCF pups were fostered (daily until PND4, between 10:00 and 10.30 a.m.) by moving the entire litter from the biological to the adoptive mother's cage. Pups were left with the last adoptive mother until weaning.

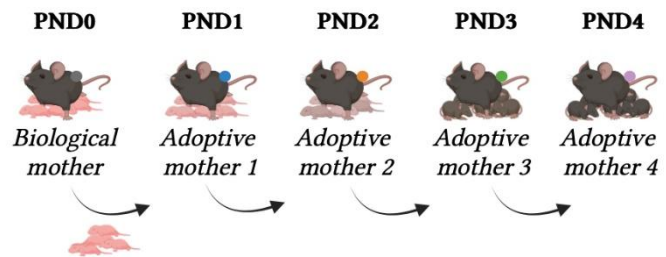


Figure 5. The RCF procedure. Pups change adoptive mother daily from PND1 to PND4.

Cont pups were only picked up daily and reintroduced to their home cage within 30 seconds. Animals were weaned at P28, sorted by sex, and housed in groups of 4 littermates (Di Segni, et al., 2016; Di Segni, et al., 2019).

6.2.3 Early Life Adversities in Mice: The Scarcity Model

This procedure was adapted from the Scarcity model (Rainecki et al., 2012). In this study, the execution window was modified, and the procedure was carried out from PND1 to PND4, to reproduce the same time window as the RCF manipulation.

For the Scarcity procedure, the dam and her pups were left undisturbed on PND0. On PND1, Scarcity pups (Scar) were housed in a cage with limited nesting and bedding materials until PND4 (the same temporal window of RCF). The cage was provided with 100 mL of sawdust bedding (one-fifth of the amount provided to control animals) and 1.8 g of nesting material (one-fifth of the amount provided to control animals). After the end of the protocol (in the morning of PND5), the dam and her pups were transferred to a new cage containing the standard amount of material provided to control animals (500 mL of sawdust bedding and 9 g of nesting material). Animals were weaned at PND28, separated by sex, and housed in groups of four littermates.

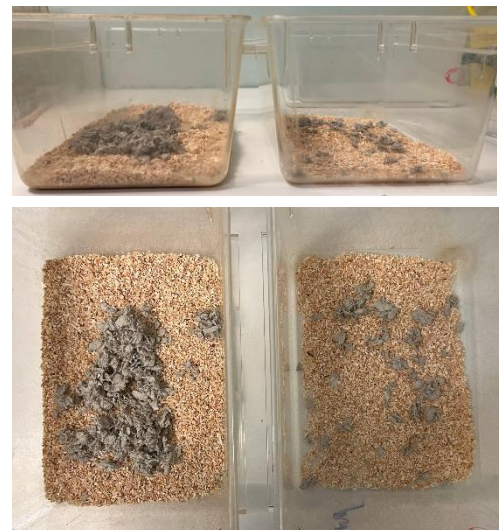


Figure 6. Control bedding and nesting material compared to Scarcity bedding and nesting material.

6.2.4 Evaluation of Maternal Behavior

The Maternal Behavior observation was conducted as previously described (Ventura, et al., 2013). Maternal Behavior observation took place daily, from PND1 to PND7, twice a day (from 12 to 12:30 and from 16 to 16:30), with an instantaneous sampling (2 minutes of sampling/rate) for 16 observations/sampling in each session.

The Maternal Behavior observation analysis considered for Cont, RCF, and Scar mothers the following behaviors: Nursing (N), Liking/Grooming (L/G), Nesting (B), and Self-grooming (SG) (Shoji & Kato, 2006; D'Amato, et al., 2011). Moreover, due to the presence of abusive behavior under conditions of scarcity (Roth & Sullivan, 2005), we also analyzed the presence of such behaviors in Cont, RCF, and Scar mothers, including stepping or jumping on pups (S/J), rough handling (RH), aggressive grooming (AG), retrieval of pups (Ret) and sitting on pups (St).

Table 2 - Description of Behaviors observed during Maternal Behavior Observation

Behaviors observed	Description
Nursing (N)	The mother feeds her pups in an arched-back or lateral lying position.
Liking/Grooming (L/G)	The mother licks and cleans her pups, often focusing on their anogenital area to stimulate elimination and hygiene.
Nesting (B)	The mother organizes or builds the nest, either from within or outside, by gathering and arranging nesting materials.
Self-grooming (SG)	The mother grooms herself by licking or scratching her body, typically for hygiene or stress relief.
Stepping or jumping on pups (S/J)	The mother steps-on or jumps-over the pups, potentially causing discomfort and/or harm.
Rough handling (RH)	The mother manipulates the pups with excessive force, such as pushing, nudging, or lifting them in a harsh or careless manner.
Aggressive grooming (AG)	The mother licks or handles the pups roughly, often with excessive force and/or agitation.
Retrieval of pups (Ret)	The mother frequently retrieves pups scattered outside the nest due to her repeated exits and re-entries disrupting their position.
Sitting on pups (St)	The mother sits on the pups without engaging in nursing or other caregiving behaviors, potentially causing distress and/or harm.

6.2.5 Evaluation of separation anxiety in pups: Ultrasonic Vocalization Calls (USV's)

Pup's separation anxiety was measured by Ultrasonic Vocalization Calls (USVs) on PND8, during the separation from the mother (biological mother for Control (Cont) and Scarcity (Scar) mice; last adoptive mother for RCF mice), as previously described (D'Amato et al., 2011; Lo Iacono et al., 2021; Mancini et al., 2024).

Each pup was individually placed into a sterilized beaker containing home cage bedding (Home) or clean bedding (Clean). Vocalizations were recorded with an UltraSoundGate microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) placed 1 cm above the beaker (5 minutes) and analyzed with a dedicated software (Avisoft Bioacoustics, Berlin, Germany). No more than 4 pups x litter were individually tested; pups of each group (Cont, RCF, Scar) belonged to 4-5 different litters (Ventura et al., 2013).

6.2.6 Evaluation of attachment behavior in pups: The Mouse Strange Situation Procedure (MSSP)

The Mouse Strange Situation procedure (MSSP) was conducted in a grey arena (60×60×60cm³ cm³) at PND18 as reported by Lassi and Tucci (2017) and previously described (Mancini et al., 2024). This procedure consists of three consecutive "episodes", 3-min-long each, preceded by a pre-test phase during which the mother becomes familiar with another age-matched virgin female. Pups were tested in random order. In the first episode, the pup was located in the arena with the mother (M1) and the stranger (S1). In the second episode, the mother was removed (M2) from the arena, and the pup was left alone with the stranger (S2). In the third episode, the mother (M3) returned to the arena with the stranger (S3). We evaluated the attachment-like style of mice by defining three possible behavioral responses:

1. the "Maternal preference", in which we analyzed pups' preference for mother compared to a stranger in the third episode (M3 vs S3);
2. the "Reunion", during which we observed the exploration of the mother by the pup in episode 3 (M3) compared to 1 (M1) (M3 vs M1);
3. and, the "Stranger Effect", during which we evaluated the time the pup spent exploring the stranger during episode 2 compared to the other episodes (S2 vs S1,S3).

All MSSP sessions were video-recorded with a webcam and scored manually by a trained observer for each episode/mouse/litter. For each episode, we quantified the time (seconds)

spent by the pup actively exploring (sniffing and touching) the stranger (S) and the mother (M) (Lassi & Tucci, 2017).

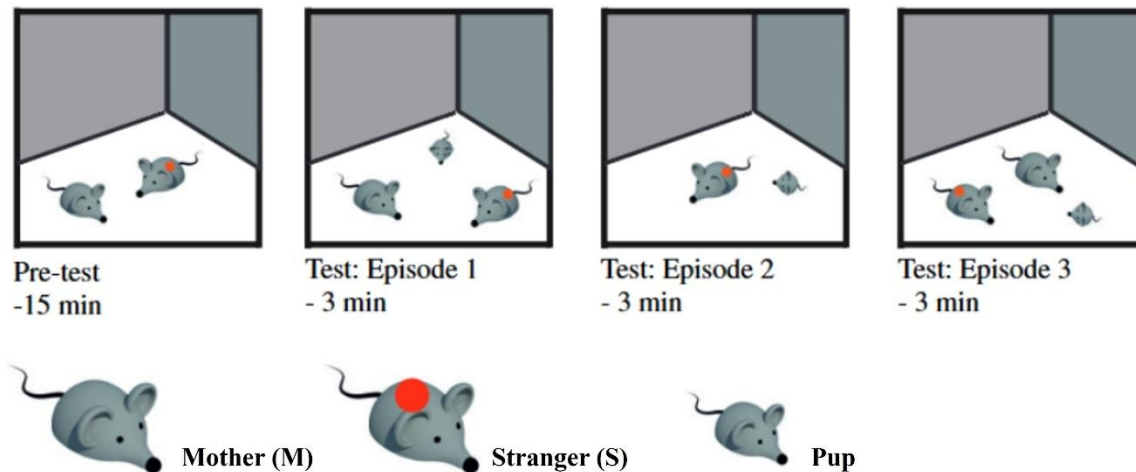


Figure 7. Representation of MSSP episodes, adapted by Lassi and Tucci (2017).

6.2.7 Evaluation of Depressive-like behaviors: The Social Interaction Test

The Social Interaction Test (SIT) was conducted at PND 28 (adolescence) and PND 90 (adulthood). Animals were tested in a gray Plexiglas rectangular box (60 × 40 × 24 cm) consisting of an empty central “starting chamber” interconnected with two “stimulus chambers” where two identical clear Plexiglas cylinders (8 cm in diameter) with multiple small holes were placed (Cinque et al., 2012; Fiori et al., 2015; Lo Iacono et al., 2021). During the habituation session (10 min) the mouse was placed in the central chamber and allowed to freely explore the whole apparatus. An age- and sex-matched mouse was then introduced as a social stimulus into one cylinder (pseudo-randomly chosen), whereas an object was introduced into the other, for the 10-minute test session. Both sessions were recorded and the time the subject mouse spent in each chamber was measured by a video-tracking system. Time spent sniffing each cylinder in the test session was manually scored by a blind trained observer (Fiori et al., 2015).

6.2.8 Evaluation of Depressive-like behaviors: The Forced Swimming Test

The Forced Swimming Test (FST) was conducted as previously described (Di Segni et al., 2019; Lo Iacono et al., 2021; Mancini et al., 2024) both at PND35 (adolescence) and at PND 90-100 (adulthood). Mice were individually introduced for 10 min into a glass cylinder (diameter 18 cm), filled with water (up to 20 cm; 28 ± 2 °C). The duration of immobility (seconds) was manually scored with Boris by a trained observer in blind (Friad & Gamba, 2016; Di Segni et al., 2019; Lo Iacono et al., 2021; Mancini et al., 2024).

6.2.9 Evaluation of Depressive-like behaviors: The Saccharin Preference Test

The saccharin preference test (SPT) was carried out as previously described (Di Segni et al., 2016, 2019; Lo Iacono et al., 2021) only at PND 90, to avoid social isolation during adolescence. During the habituation day, mice were singly moved in a cage with two bottles containing water; after 24h, they were exposed to a double-choice drinking test (saccharin solution [0.5%] or drinking water) from graduated tubes (10 ml volume). Intake was measured to the nearest 0.1 ml. The test cage was the same throughout the experiment. Only mice drinking at least 0.1 ml on day 1 were included in the study. The percentage of saccharin intake was evaluated.

6.2.10 Neurobiological Analyses: Brain and Plasma Collection

For neurobiological analyses at PND90, the mice's brain was collected after decapitation and immediately frozen at -80 °C until the day of punch collection. For plasma collection, blood samples were collected after decapitation and immediately centrifuged (10 minutes, 1500 RCF, 4 °C). The supernatant was taken and stored at -80 °C until the day of the assay.

6.2.11 Neurobiological Analyses: Enzyme-linked Immunosorbent Assays (ELISA): Oxytocin quantification

For ELISA experiments, total protein content was extracted from all samples using C18 Spin Columns (C18 Spin Columns, G-Bioscience, 786-931). Columns were placed in collection tubes and 200ul of Activation Solution (50% ACN) was added, then they were briefly centrifuged (1500xg for 1 min). The residue was discarded, and the Activation Solution wash was repeated. 200ul of Equilibration Solution (0.5% TFA in 5% ACN) was added, the columns were centrifuged as reported before, and the solution was discarded. The equilibration Solution wash was repeated once. The columns were then transferred to clean collection tubes, and the sample was applied. After a brief centrifugation, the flow-through was recovered and reapplied to ensure complete binding before repeating the centrifugation. Columns were transferred to clean collection tubes, and 200ul of Wash Solution (0.5% TFA in 5% ACN) was applied before centrifugation. The flow-through was discarded, and the Wash Solution was repeated. The columns were transferred to clean collection tubes, and 20ul of Elution Buffer (70% ACN) was added before centrifugation. The elution step was then repeated, and the final volume of 40ul was collected and stored at -20 °C when not immediately processed for the assay. The ELISA kit was used to analyze OXT content in samples, following the manufacturer's instructions (Oxytocin ELISA kit, Enzo Lifescience, ADI-900-153A).

6.2.12 Neurobiological Analyses: Quantitative Real-Time PCR (qRT-PCR)

Brain tissue micro-punches of Prefrontal Cortex (pFC), Nucleus Accumbens (NAc), and amygdala (Amy) were obtained from frozen brain slices (coronal sections according to the atlas of Paxinos and Franklin (Paxinos and Franklin, 2019)) not thicker than 300 μ m using stainless-steel tubes of different inner diameters (1.0 mm for pFC, 0.7 mm for NAc and Amy) and stored at -80 °C until the day of the assay.

RNA extraction and qRT-PCR was performed for the evaluation of Oxytocin receptor (OxtR) and μ (Oprm) and κ (Oprk) Opioid Receptors mRNA levels in pFC, NAc and Amy of Cont, RCF and Scar mice at PND90. RNA from punches was extracted using microcolumn from Total RNA purification Plus Kit (Norgen Biotek, Canada), and RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-VIS spectrophotometer. Complementary DNA (cDNA) was obtained using the High-Capacity Reverse Transcription kit (Applied Biosystems, USA). Around 10 ng of cDNA templates were amplified by qPCR using the 7900HT thermal cycler apparatus equipped with SDS software version 2.3 (Applied Biosystems, AB) for data collection. Each data point was obtained from 5 to 6 biological replicates. Ct values were normalized to measures of Tata Binding Protein (TBP, Taqman assay ID Mm00446973_m1) for OxtR (ID Mm01182684_m1) and Oprm1 (ID: Mm01188089_m1) and Oprk1 (Mm01230885_m1 oprk1) to achieve Δ Ct values. Relative expression was calculated as $2^{-\Delta Ct}$. All data were run in triplicate and expressed as means $\pm$ SE.

6.2.13 Neurobiological Analyses: RNAscope

At PND90 animals were perfused transcardially with 50 ml of phosphate buffer (PB; 0.1 M; pH 7.4) followed by 50 ml of 4% paraformaldehyde in PB, under deep anesthesia. Each brain was removed immediately, post-fixed in the same fixative for 24h, and, after three washes in PB, transferred to 30% sucrose in PB solution at 4 °C. 20 μ m coronal sections were mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Pittsburgh, PA). RNAscope was performed by using RNAscope Multiplex Fluorescent Detection Reagents v2 (cat#323110; Advance Cell Diagnostics, Hayward, CA). The following probes were used: OxtR-C3 (Mm-OxtR-C3 cat# 412171-C3; Advance Cell Diagnostics, Hayward, CA) in combination with Oprm-C2 (Mm-Oprm1-C2 cat# 315841-C2) and Oprk (Mm-Oprk1 cat# 316111). Sections were processed for RNAscope by following the company protocol. To label the amplified hybridizations with fluorophores the TSA Vivid Fluorophores 520 (for C2 or C1 probes), 570 (for C3 probe) were used. Images were taken by using Nikon Eclipse Ti1 confocal microscope. Densitometric analysis (optical density, O.D.) on neurons were performed on digital images taken by using the confocal microscope by maintaining

confocal settings constant throughout the acquisition of sections from the different experimental groups. After background subtraction, OxtR/Oprm/Oprk-associated signals were quantified by manually outlining individual neurons at the level of the PFC, NAc shell and Amy (central Amygdala, CeA) by measuring cell-associated fluorescence intensity with the ImageJ software.

6.3 Statistical Analyses

For the human study, we conducted the analyses using the statistical software SPSS 28.0. At a preliminary level, we performed descriptive analyses (mean, standard deviation, skewness, and kurtosis) for all variables of interest. Next, we conducted bivariate correlation analyses using Pearson's r coefficient among all variables of interest, separated by gender (see supplementary Data). We then examined, through two hierarchical regression models, the effects of: "Gender," "Age," "Emotional Abuse," "Physical Abuse," "Emotional Neglect," "Physical Neglect," "Maternal Care," "Maternal Overprotection," "Paternal Care," "Paternal Overprotection," "Secure Attachment," "Dismissing–Avoidant Attachment," "Preoccupied Attachment," and "Fearful–Avoidant Attachment" in explaining "Depressive Symptomatology". Oxt Plasma levels in humans were analyzed by "SuperANOVA Statistical Software", performing a two-way ANOVA (gender, 2 levels: females and males; group, 4 levels: high attachment, low attachment, trauma, trauma + low attachment).

For animal study, we checked the normality of the data. Normally distributed data were analyzed using the "SuperANOVA Statistical Software", and when necessary, multiple comparisons were performed using the Bonferroni post-hoc test. Non-normally distributed data were analyzed using the "Prism" software with the Kruskal–Wallis test followed by Dunn's post-hoc test. We considered a statistically significant p -value <0.05 . Data are presented as mean $\pm$ SEM:

- Maternal behaviors: one-way ANOVA was performed for nurturing behaviors (group, 3 levels: Cont $n=5$, RCF $n=9$, Scar $n=10$) and non-maternal behaviors (group, 3 levels: Cont $n=6$, RCF $n=10$, Scar $n=10$).
- Pups' vocalizations (USVs): Kruskal-Wallis test (group, 6 levels: Cont clean $n=8$, Cont home $n=7$, RCF clean $n=14$, RCF home $n=17$, Scar clean $n=7$, Scar home $n=10$) was used to evaluate differences in pups' vocalizations (USVs).
- Mouse Strange Situation Procedure (MSSP): "Maternal Preference", "Strange Effect", and "Reunion" were analyzed in the single group time spent in interaction

with mother and/or stranger in different sessions (for “Maternal Preference” M3, S3; for “Reunion” M1, M3; for “Strange effects” S1, S2, S3 ; Cont n=9, RCF n=9 and Scar n=13). For “Maternal Preference” and “Reunion” we performed t-test and for “Stranger Effect” we performed one-way ANOVA (3 levels: S1, S2, S3).

- Forced Swimming Test (FST): one-way ANOVA was performed for the percentage of time spent in immobility at both PND35 (group, 3 levels: Cont n=6, RCF n=6, Scar n=6) and PND90 (group, 3 levels: Cont n=6, RCF n=6, Scar n=12) ($Immobility = \left(\frac{immobility}{(immobility+active\ coping)} \right) * 100$).
- Social Interaction Test (SIT): one-way ANOVA was performed for the percentage of time spent interacting with the subject ($Time\ spent\ interacting\ with\ subject = \left(\frac{Subject\ interaction}{(subject\ interaction+object\ interaction)} \right) * 100$) at PND 28 (group, 3 levels: Cont n=4, RCF n=4, Scar n=5) and Kruskal-Wallis test (group, 3 levels: Cont n=6, RCF n=6, Scar n=12) at PND90.
- Saccharin Preference Test (SPT): one-way ANOVA (group, 3 levels: Cont n=5, RCF n=4, Scar n=8) was performed for the percentage of Saccharin intake at PND90 ($Saccharin\ intake = \left(\frac{Saccharin\ intake}{(water\ intake+Saccharin\ intake)} \right) * 100$).
- Quantitative Real-Time PCR (qRT-PCR): one-way ANOVA was performed in pFC for mRNA OxtR (group, 3 levels: Cont n=5, RCF n=4, Scar n=7) and oprk levels (group, 3 levels: Cont n=5, RCF n=4, Scar n=7), in NAc for mRNA oprm (group, 3 levels: Cont n=5, RCF n=4, Scar n=7) and oprk (group, 3 levels: Cont n=6, RCF n=4, Scar n=8) levels and in Amy for mRNA OxtR (group, 3 levels: Cont n=4, RCF n=4, Scar n=8) and oprm (group, 3 levels: Cont n=6, RCF n=4, Scar n=8) levels. Kruskal-Wallis test was performed for oprm mRNA levels in pFC (group: Cont n=6, RCF n=5, Scar n=8), oprk mRNA levels in Amy (group: Cont n=5, RCF n=4, Scar n=7) and OxtR mRNA levels in NAc (group: Cont n=4, RCF n=4, Scar n=5).
- RNAscope: one-way ANOVA (group, 3 levels: Cont, RCF, Scar) was performed for OxtR, oprm and oprk levels in pFC, NAc and AMY. Quantification was done on n = 4 sections/mice; N = 3 mice/group.
- Enzyme-linked Immunosorbent Assays (ELISA): Kruskal-Wallis test (group: Cont n=9, RCF n=7, Scar n=7) was performed for Oxt levels in plasma.

7. Results

7.1 Human Study

7.1.1 Demographic information

The sample consisted of women, men, and individuals identifying as other/non-binary. The mean age was 25.34 years (SD $\pm$ 0.27) for women (n = 1025), 26.84 years (SD $\pm$ 0.58) for men (n = 269), and 23.00 years (SD $\pm$ 1.73) for other/non-binary (n = 7) participants. Regarding educational level, among women, 0.97% reported having a middle school diploma, 51.14% a high school diploma, 40.91% a bachelor's degree, and 6.98% a postgraduate degree.

Among men, 0.51% held a middle school diploma, 50.25% a high school diploma, 42.13% a bachelor's degree, and 7.11% a postgraduate degree. Finally, among other/non-binary participants, 50.00% reported having a bachelor's degree and 50.00% a postgraduate degree.

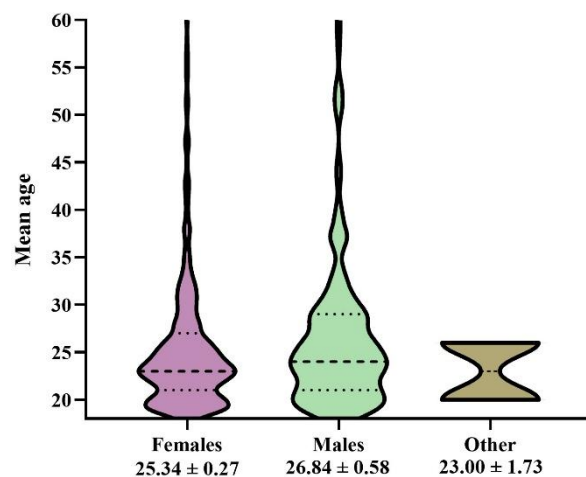


Figure 8. Mean age of the sample

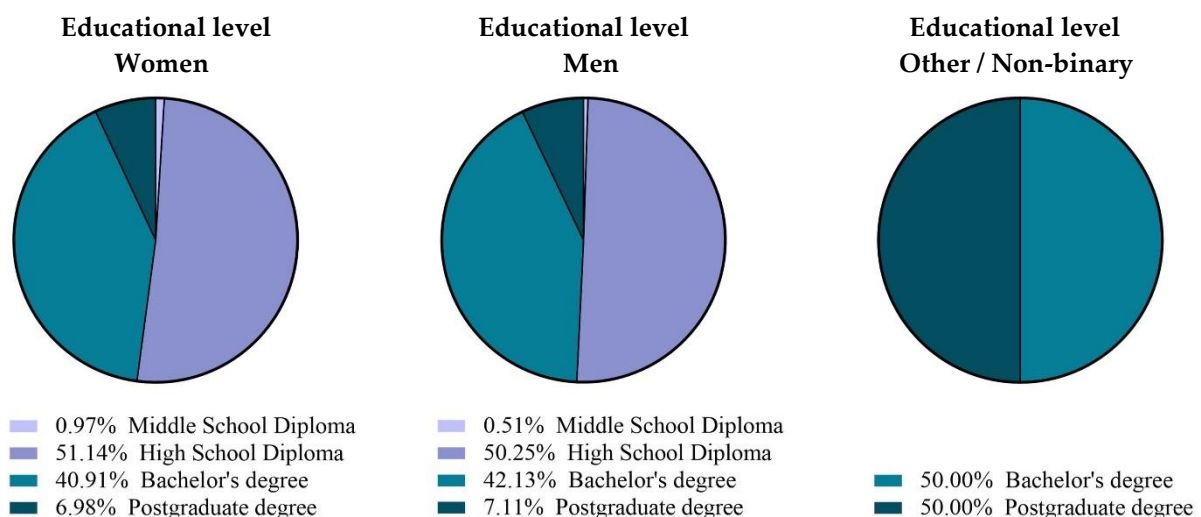


Figure 9. Education level of Women, Men and other / non binary

People who identify as other/non-binary were excluded from the statistical analysis because the sample size was too small to perform the analysis (n = 7).

7.1.2 Distribution of the sample depending on ELA and depression

A graphical representation of the distribution of our sample can be seen in the following graphs. We first investigated the presence of ELA (Figure 10) in the sample (gray) and found that half the participants experienced ELA. We also represented the division between ELA and non-ELA separately for men (green) and women (purple).

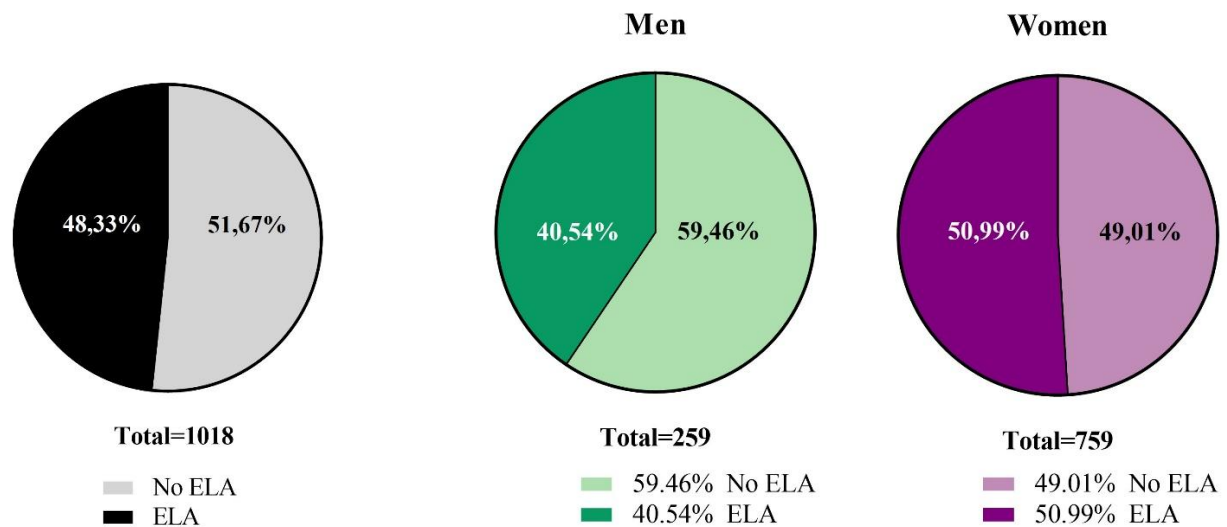


Figure 10. Prevalence of ELA in our sample.

We then analyzed the prevalence of ELA in the distribution of PHQ-9 symptoms (severity of depressive symptoms), considering these categories: Absent, Minimum, Mild, Moderate, Severe (Figure 11). In the upper line (green) are represented men and in the lower line (purple) women.

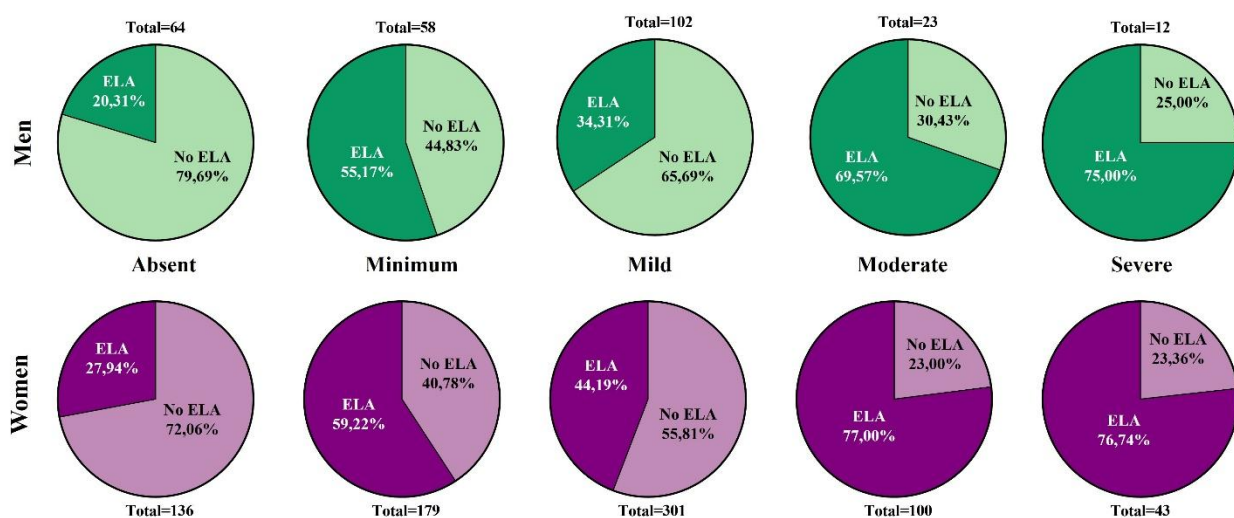


Figure 11. Distribution of the sample (split in ELA and no ELA) in men and women through PHQ-9 severity symptoms.

In order to separate the role of Trauma from the Attachment bond alteration in the development of depressive symptoms, we divided the ELA groups into three subcategories (Figure 12) based on the test medians:

- **Trauma (T)** = Σ abuse and neglect (CTQ) > 29 + secure attachment (RQ) > 5
- **Low Secure Attachment (LS)** = Σ abuse and neglect (CTQ) < 29 + secure attachment (RQ) < 5
- **Trauma + Low Secure Attachment (T+LS)** = Σ abuse and neglect (CTQ) > 29 + secure attachment (RQ) < 5

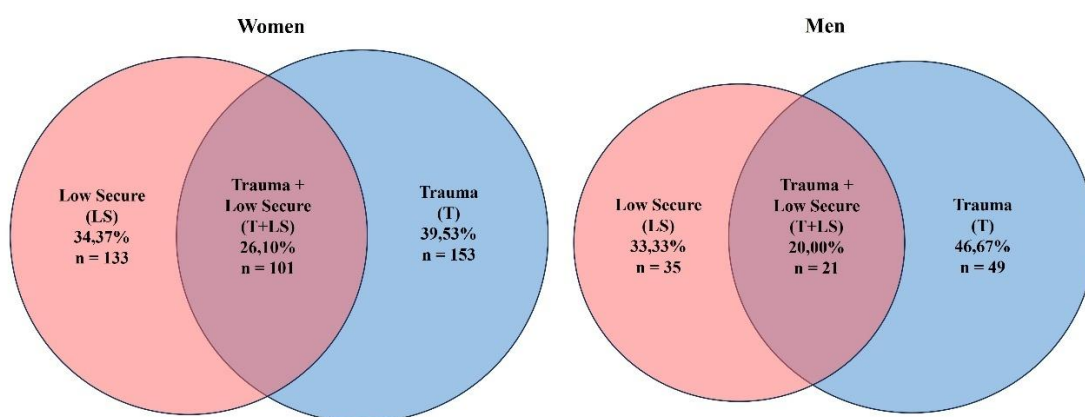


Figure 12. Distribution of different ELA (Low Secure, Trauma and Trauma + Low Secure) in ELA group of Women and Men.

Subsequently, we analyzed again the prevalence of ELA in the distribution of PHQ-9 symptoms (Absent, Minimum, Mild, Moderate, Severe) considering the distribution of our sample in No ELA, Low Secure (LS), Trauma (T) and Trauma + Low Secure (T+LS) (Figure 13) in men (upper line) and women (lower line).

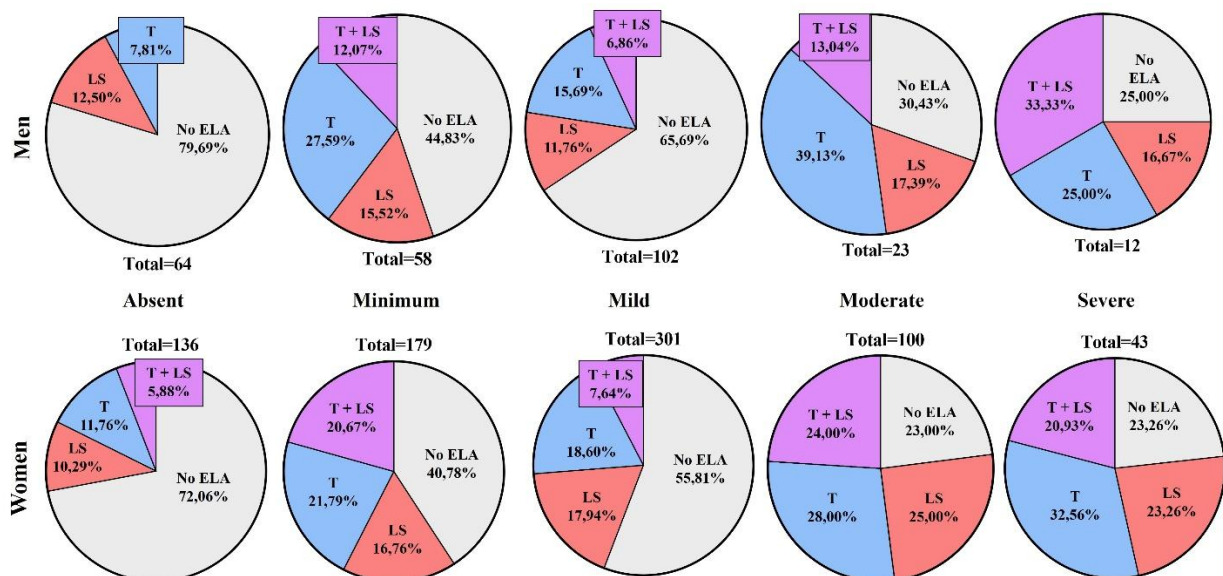


Figure 13. Distribution of the sample in women and men trough PHQ-9 symptoms severity.

7.1.3 Descriptive analysis

Table 3 reports the descriptive analysis (means, standard deviations, skewness, and kurtosis) of the variables “Emotional Abuse,” “Physical Abuse,” “Emotional Neglect,” “Physical Neglect,” “Maternal Care,” “Maternal Overprotection,” “Paternal Care,” “Paternal Overprotection,” “Secure Attachment,” “Dismissing-Avoidant Attachment,” “Preoccupied Attachment,” “Fearful-Avoidant Attachment,” and “Depressive Symptoms,”. Values lower than 2 for univariate skewness and lower than 5 for univariate kurtosis were used as criteria for the evaluation of univariate normality (Curran, West, & Finch, 1996).

Table 3. Descriptive Statistics

	Means	Standard deviation	Skewness	Kurtosis
Emotional Abuse	9,32	4,27	1,21	1,12
Physical Abuse	6,11	2,31	3,10	12,15
Emotional Neglect	10,22	4,13	0,87	0,51
Physical Neglect	2,89	1,27	1,69	2,67
Maternal Care	25,71	8,06	-1,04	0,35
Maternal Overprotection	13,57	8,72	0,70	-0,18
Paternal Care	21,76	8,79	-0,47	-0,71
Paternal Overprotection	11,66	7,81	0,90	0,47
Secure Attachment	4,16	1,52	0,01	-0,49
Dismissing-Avoidant Attachment	3,51	1,88	0,36	-1,01
Preoccupied Attachment	3,52	2,00	0,35	-1,10
Fearful-Avoidant Attachment	2,08	1,41	1,45	1,53
Depressive symptoms (PHQ9)	9,31	5,45	0,78	0,20

As reported in Table 3, all the variables under investigation are normally distributed except for “Physical Abuse.” In line with the recommendations of Barbaranelli (2003), the variable was transformed through inverse transformation ($X = 1/X$). The result proved effective in normalizing the distribution; in fact, the normalized variable shows the following descriptive statistics:

Table 4. Descriptive statistics of Physical Abuse after inverse transformation

	Means	Standard deviation	Skewness	Kurtosis
Physical Abuse after inverse transformation	0,17	0,03	-1,53	1,23

In the analyses, the variable obtained through the inverse transformation of “Physical Abuse” will be considered. It is important to take into account that this variable must be interpreted by reversing the direction of the results under investigation. Indeed, the correlation between the original (skewed) Physical Abuse variable and the inversely transformed Physical Abuse variable is negative.

7.1.4 Correlation Analysis

Table 5. Correlation matrix between Depressive Symptom (PHQ9) and constructs of interest, divided by Total, Women and Men

		PHQ9 Total	PHQ9 Women	PHQ9 Men
Emotional Abuse	r	.378**	.341**	.495**
Emotional Neglect	r	.318**	.273*	.475**
Physical Neglect	r	.280*	.275*	.288*
Physical Abuse (inverse)	r	-.224*	-.229*	-.209*
Secure Attachment	r	-.393**	-.410**	-.314*
Dismissing-Avoidant Attachment	r	.458**	.463**	.419**
Preoccupied Attachment	r	.451**	.441**	.473**
Fearful-Avoidant Attachment	r	.165*	.187*	.113
Maternal Care	r	-.256*	-.240*	-.287*
Paternal Care	r	-.287**	-.246*	-.427**
Maternal Overprotection	r	.270*	.286*	.237*
Paternal Overprotection	r	.247*	.233*	.263*

Note: * = $p < .05$; ** = $p < .01$

In table 5, the correlation between Depressive Symptoms (PHQ-9) and the constructs of interest is reported, divided by Total subjects, women, and men. We also carried out correlation analyses between the constructs in the individual scales, which are reported in the supplementary data section.

Table 5 shows that in females, all the variables of interest correlate with the Depressive Symptoms, while in males, “Fearful-Avoidant Attachment” does not correlate (.113). In particular, “Depressive Symptoms” has a strong positive association with:

- “Emotional Abuse” in women (.341**; $p < .01$) and men (.495**; $p < .01$);
- “Emotional Neglect” in women (.273*; $p < .05$) and men (.475**; $p < .01$);
- “Physical Neglect” in women (.275*; $p < .05$) and men (.288*; $p < .05$);
- “Physical Abuse (inverse)” in women (-.229*; $p < .05$) and men (-.209*; $p < .05$) (to be interpreted by reversing the emerged direction);
- “Dismissing-Avoidant Attachment” in women (.463**; $p < .01$) and men (.419**; $p < .01$);
- “Preoccupied Attachment” in women (.441**; $p < .01$) and men (.473**; $p < .01$);
- “Fearful-Avoidant Attachment” only in women (.187*; $p < .05$);
- “Maternal Overprotection” in women (.286*; $p < .05$) and men (.237*; $p < .05$);
- “Paternal Overprotection” in women (.233*; $p < .05$) and men (.263*; $p < .05$).

The “Depressive Symptoms” have, instead, a strong negative association with

- “Safe attachment” in women (-.410**; $p < .01$) and men (-.314*; $p < .05$);
- “Maternal care” in women (-.240*; $p < .05$) and men (-.287*; $p < .05$);
- “Paternal care” in women (-.246*; $p < .05$) and men (-.427**; $p < .01$).

7.1.5 Regression analysis

Based on the results obtained from the analysis of correlations, which highlight significant associations between the variables under investigation, we have deepened these relationships, testing a hierarchical regression model considering as dependent variable the Depressive Symptoms and examining the effects of “Age” (Step 1) and of “Emotional Abuse”, “Physical Abuse”, “Emotional Neglect”, “Physical Neglect”, “Maternal Care”, “Maternal Overprotection”, “Paternal Care”, “Paternal Overprotection”, “Secure Attachment”, “Dismissing-Avoidant Attachment”, “Preoccupied Attachment”, “Fearful-Avoidant Attachment” (Step 2). In addition, we conducted the same analyses separately for women and men, as shown in Tables 6 and 7.

All lower-order and interactive terms will be mean-centered in order to facilitate a proper interpretation of lower-order terms and to decrease nonessential multicollinearity (Cohen et al., 2003).

In particular, the regression model provides a theoretically established variable entry model, which highlights each time the independent variables that present the highest correlation with the dependent variable.

Table 6. Analysis of regressions: dependent variable “Depressive Symptoms” in women

	VD: Depressive Symptoms	β	R^2	p
Step 1	Age	-.181	.031**	.000**
Step 2	Emotional Abuse	.054	.346**	.373
	Emotional Neglect	-.052		.421
	Physical Neglect	.133		.002**
	Physical Abuse (inverse)	-.058		.143
	Secure Attachment	-.143		.000**
	Dismissing-Avoidant Attachment	.221		.000**
	Preoccupied Attachment	.202		.000**
	Fearful-Avoidant Attachment	.038		.261
	Maternal Care	.015		.758
	Paternal Care	.042		.369
	Maternal Overprotection	.087		.033*
	Paternal Overprotection	.081		.043*

Note: * = $p < .05$; ** = $p < .01$. β = standard regression coefficient. R^2 = Coefficient of determination.

The regression analysis (Table 6) highlighted that the predictors considered make a significant contribution capable of explaining 35% of depressive symptoms in women.

Specifically, the direct contribution of “Age” ($\beta = -.181$) was significant (Step 1).

In addition, the contribution of “Physical Neglect” ($\beta = .133$), “Secure Attachment” ($\beta = -.143$), “Dismissing-Avoidant Attachment” ($\beta = .221$), “Preoccupied Attachment” ($\beta = .202$), “Maternal Overprotection” ($\beta = .087$) and “Paternal Overprotection” ($\beta = .081$) (step 2) was significant.

Table 7. Analysis of regressions: dependent variable “Depressive Symptoms” in men

	VD: Depressive Symptoms	β	R ²	p
Step 1	Age	-.146	.017*	.033*
Step 2	Emotional Abuse	.181	.410**	.039*
	Emotional Neglect	.222		.009**
	Physical Neglect	.113		.072
	Physical Abuse (inverse)	.020		.750
	Secure Attachment	-.039		.542
	Dismissing-Avoidant Attachment	.119		.092
	Preoccupied Attachment	.328		.000**
	Fearful-Avoidant Attachment	.004		.944
	Maternal Care	.075		.295
	Paternal Care	-.096		.206
	Maternal Overprotection	.027		.724
	Paternal Overprotection	-.078		.369

Note: * = $p < .05$; ** = $p < .01$. β = standard regression coefficient. R² = Coefficient of determination.

The regression analysis (Table 7) highlighted that the predictors considered make a significant contribution capable of explaining 41% of depressive symptoms in men.

Specifically, the direct contribution of “Age” ($\beta = -.146$) was significant (Step 1).

Furthermore, the contribution of “Emotional Abuse” ($\beta = .181$), “Emotional Neglect” ($\beta = .222$), and “Preoccupied Attachment” ($\beta = .328$) was significant (Step 2).

7.1.6 Oxytocin quantification in plasma

Two-way ANOVA for “Oxt pg/mL” highlights a significant “Gender x Group” effect ($F_{(3,59)} = 16,162$; $p = .0001$) (Figure 14). Post-hoc analysis (Bonferroni) highlighted a significant Gender effect (*) in all Groups: in gray no ELA ($p = .0001$), in red Low Secure ($p = .0038$), in light blue Trauma ($p = .0019$), in purple Trauma + Low Secure ($p = .0005$). Moreover, in Women (#) there is a reduction of Oxt plasma levels in Low Secure ($p = .0001$), Trauma + Low Secure ($p = .0003$) and Trauma groups ($p = .0089$) compared to No ELA group, and there is significant difference in Trauma group compared to Low Secure group ($p = .0026$). In Men (§) Oxt plasma levels are increased in Trauma ($p = .0002$) and Trauma + Low Secure ($p = .0005$) groups compared to No ELA, with a non-significant trend between No ELA and Low Secure group ($p = .0645$).

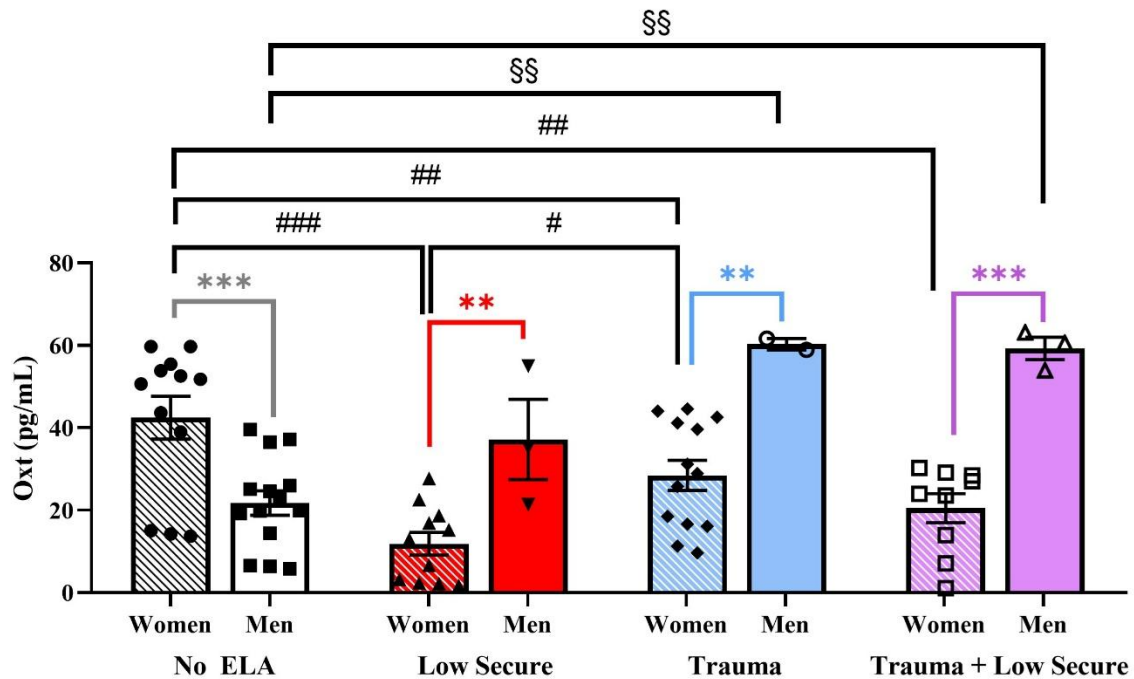


Figure 14. Oxt plasma levels in Women and Men, grouped in No ELA, Low Secure, Trauma and Trauma + Low Secure.

To sum up, in Women, attachment bond alteration and Trauma decrease Oxt plasma levels, with a stronger effect of the attachment bond alteration compared to Trauma; in Men, there is an increase in Oxt plasma levels linked to Trauma (alone or in combination with Low Secure). Altogether, these data indicate that plasma Oxt levels are altered in both Women and Men, but in a different manner in terms of both directionality and specificity of the ELA experienced.

Moreover, Pearson's correlations were conducted to examine the relationships between Oxt plasma levels and our constructs of interest, separately for the women and man samples (Table 8).

In the women group ($n = 46$), no statistically significant correlations emerged (all $p > .17$). However, some descriptive trends were observed, suggesting a possible positive association between oxytocin and secure attachment, and oxytocin and physical abuse (inverse) and negative associations with maternal overprotection. In the man group ($n = 20$), no correlations reached statistical significance (all $p > .10$), although several moderate trends were found. Specifically, Oxt levels showed a negative association with dismissing-avoidant attachment ($r = -.39$, $p = .10$), a positive association with physical neglect ($r = .37$, $p = .11$), and a negative association with depressive symptoms ($r = -.25$, $p = .29$). Although not

significant, these findings may suggest differential patterns of association between Oxt and relational functioning across genders.

Although none of the correlations reached statistical significance, it is important to note that the relatively small sample sizes (particularly in the men group) may have limited the statistical power to detect effects of small to moderate magnitude. Future research with larger samples is needed to clarify whether these trends represent genuine associations between oxytocin and constructs of interest.

Table 8. Correlation matrix between Oxt Plasma levels (Oxt) and constructs of interest in women and men

		Oxt Women	Oxt Men
Emotional Abuse	r	-,152	.018
Emotional Neglect	r	-,096	-.095
Physical Neglect	r	,060	,370
Physical Abuse (inverse)	r	-,177	-,247
Secure Attachment	r	,153	-,133
Dismissing-Avoidant Attachment	r	-,114	-,388
Preoccupied Attachment	r	-,058	,020
Fearful-Avoidant Attachment	r	-,157	-,155
Maternal Care	r	,006	,060
Paternal Care	r	,072	,159
Maternal Overprotection	r	-,204	-,022
Paternal Overprotection	r	-,176	-,188
PHQ-9 (depressive symptoms)	r	-,072	-,247
GAD-7 (anxiety symptoms)	r	,059	-,198

7.2 Animal Models Data

7.2.1 Maternal Behavior

Maternal Behavior Analysis was conducted for “Nurturing Behaviors” (Nursing and Licking/Grooming) and “Other Behaviors” (Non-Maternal Behaviors).

One-way ANOVA for “Nurturing Behaviors” shows a significant Group Effect (Control, RCF and Scarcity) ($F_{(2,21)} = 18,836$; $p = ,0001$). Post-hoc analysis (Bonferroni) highlighted a significant reduction in Nurturing behavior in Scarcity Dams compared to Control Dams ($p = ,0060$) and RCF Dams ($p = ,0001$) (Figure 15).

One-way ANOVA for “Other Behaviors” shows a significant Group Effect (Control, RCF and Scarcity) ($F_{(2,23)} = 6,939$; $p = ,0044$). Post-hoc analysis (Bonferroni) highlighted a significant increase in “Other behaviors” in Scarcity Dams compared to Control Dams ($p = ,0125$) and RCF Dams ($p = ,0020$).

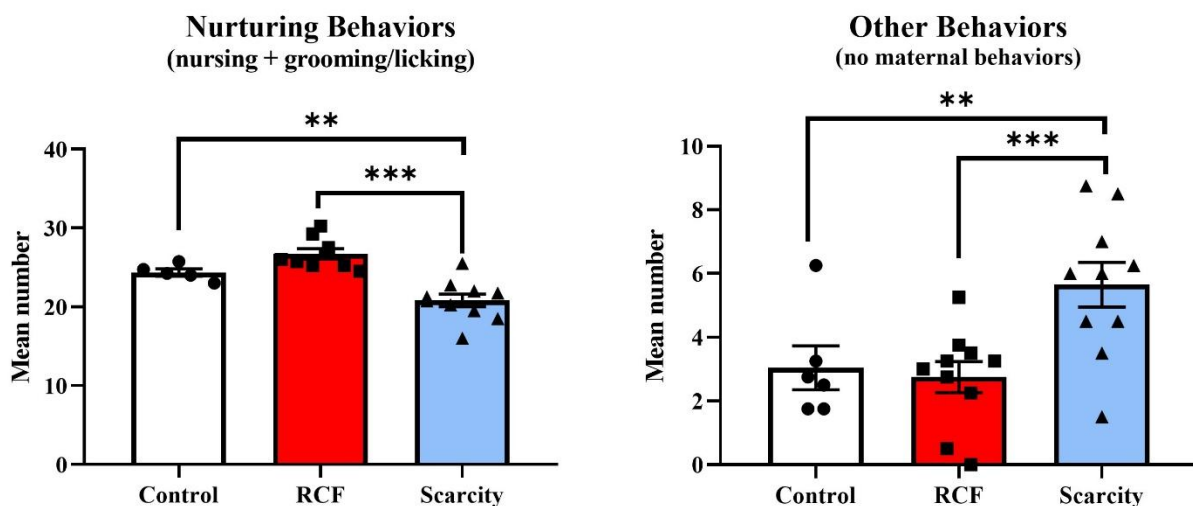


Figure 15. Mean number of Nurturing Behaviors and Other Behaviors in Control, RCF and Scarcity dams.

We also represented the frequency of maternal behaviors in a heatmap (Figure 16). Each row represents a mother, while the columns correspond to the days of observation. Color intensity reflects the frequency of behaviors, with red indicating higher frequencies and blue indicating lower frequencies. RCF and Control mothers display predominantly warm colors (reds and oranges), suggesting a generally higher frequency of maternal behaviors. Scarcity mothers show more cool colors (greens to blues), indicating lower behavioral frequencies. This pattern suggests that maternal behavior frequency is lower in the Scarcity group.

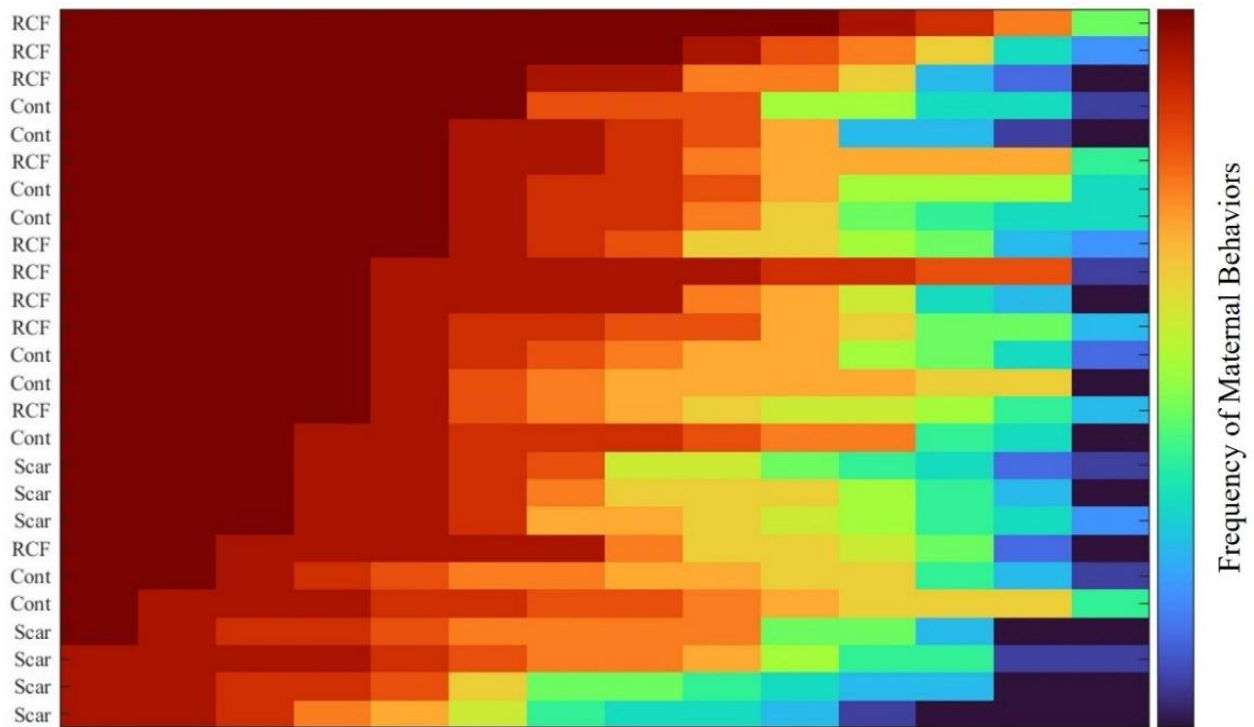


Figure 16. Heatmap representing the frequency of maternal behaviors (greater in red).

For this reason, we represented the presence of “abusive behaviors” in mothers with less frequency of maternal behaviors (Figure 17). The increasing presence of abusive behaviors corresponds to a darker shade of blue.

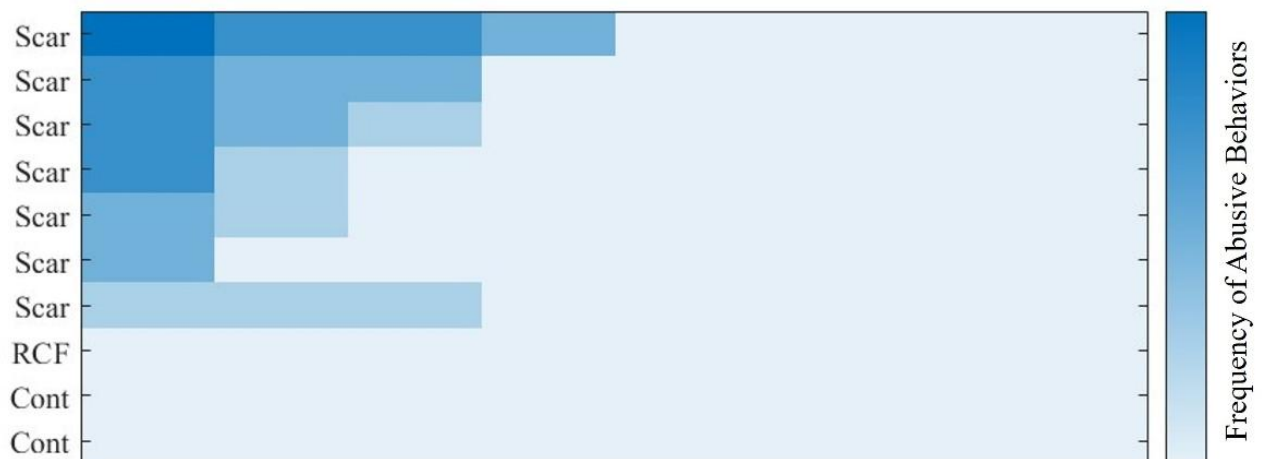


Figure 17. Heatmap representing the frequency of Abusive behaviors (greater in blue).

Considering the presence of abusive behaviors only in Scar mothers, we calculated the behaviors that those mothers engage during "Non-Maternal Behaviors". As can be seen from the distribution (Figure 18), in addition to spending a lot of time outside the nest, these mothers carry out real abusive behaviors, such as sitting on the pups (preventing them from

attaching themselves to the nipples), frequent retrieval behaviors, walking or jumping on puppies and aggressive grooming (accompanied by audible vocalizations of the puppies).

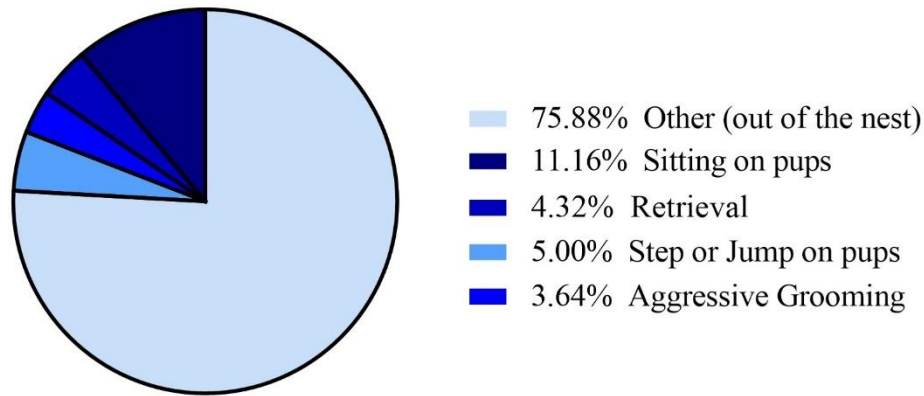


Figure 18. Abusive behaviors in Scar Dams.

7.2.2 Evaluation of separation anxiety and attachment behavior in pups: Ultrasonic Vocalization Calls (USVs) and Mouse Strange Situation Procedure (MSSP)

Kruskal-Wallis Test for “USVs” shows a significant effect ($H_{(6, N=63)} = 20,70$; $p = ,0009$) (Figure 19). Post hoc Dunn’s tests highlighted a significant difference between the number of USVs in the two conditions (clean or home litter) in the Cont ($p = ,0380$) and Scar ($p = ,0345$) groups. RCF pups show no significant differences between the number of USVs in the two conditions ($p > ,9999$).

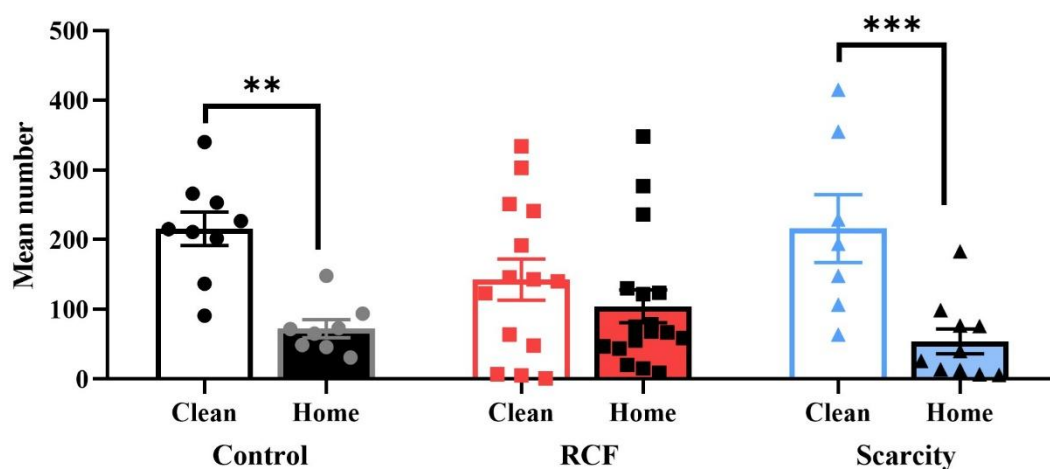


Figure 19. Mean number of USVs in Cont, RCF and Scar pups in Clean or Home condition.

For MSSP we present the three parameters (“Maternal Preference” $M3 > S3$; “Reunion” $M1 < M3$; “Stranger effect” $S1 > S2 < S3$) for every group.

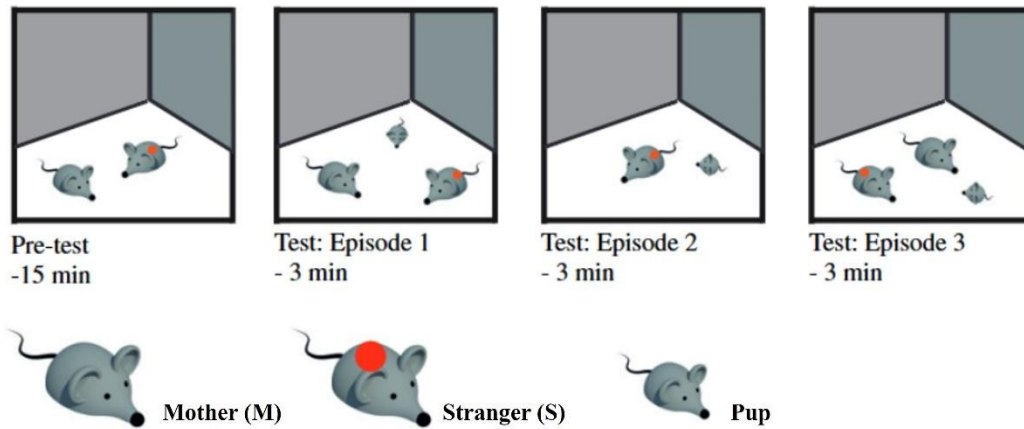


Figure 20. Representation of MSSP episodes, adapted by Lassi and Tucci (2017).

Control animals show all three parameters of Secure Attachment (Figure 21). Indeed, we can observe a significant effect for “Maternal Preference” ($t = 3,715$; $p = ,0019$), “Reunion” ($t = 5,171$; $p < ,0001$) and “Stranger Effect” ($F_{(2,21)} = 5,302$; $p = ,0137$) with a significant increase in time interacting with the stranger in session 2 (S2) compared both to both session 1 (S1 vs S2 $p = ,0421$) and session 3 (S2 vs S3 $p = ,0181$).

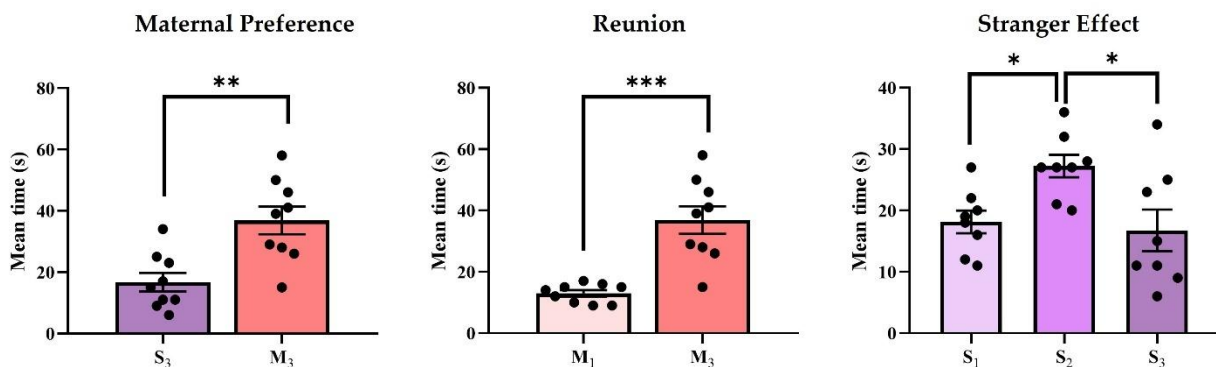


Figure 21. MSSP parameters in Cont group.

RCF animals (Figure 22) show only one significant parameter: “Reunion” ($t = 3,742$; $p = ,0018$), but no significant effect for “Maternal Preference” and “Stranger Effect”.

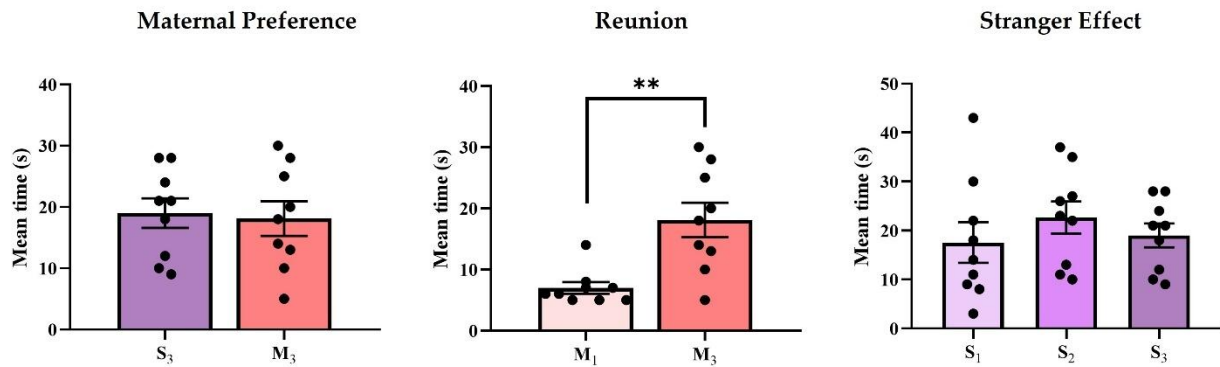


Figure 22. MSSP parameters in RCF group.

Scarcity animals show two of the three parameters of Secure Attachment (Figure 23). Indeed, we observe a significant effect for “Reunion” ($t = 2,479$; $p = ,0206$) and “Stranger Effect” ($F_{(2,27)} = 5,311$; $p = ,0113$) with a significant increase in time interacting with stranger in session 2 (S2) compared both to session 1 (S1 vs S2 $p = ,0419$) and session 3 (S2 vs S3 $p = ,0144$), but no significant effect for “Maternal Preference”.

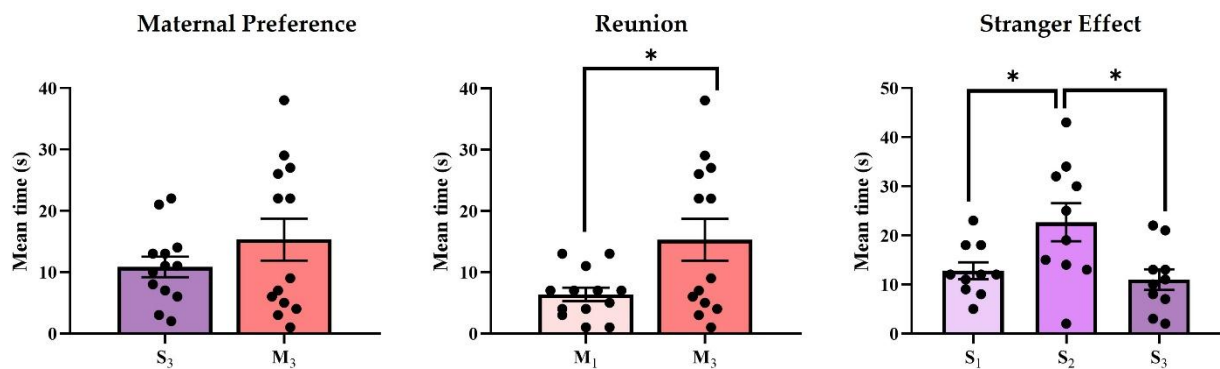


Figure 23. MSSP parameters in Scar group.

To summarize, the Control group presents all three parameters (Maternal Preference, Reunion, and Stranger Effect), indicating an attachment bond with the mother. The Scarcity group shows both the “Reunion” and the “Stranger Effect” parameters, but not the “Maternal Preference” parameter. In the RCF group, only the “Reunion” parameter is evident.

7.2.3 Depressive-like behavior induced by Traumatic experiences or Attachment bond disruption

Depressive-like behavior was analyzed by three behavioral patterns: Anhedonia, Passive Coping, and Social Impairment, measured respectively by Saccharin Preference Test (SPT), Forced Swimming Test (FST), and Social Interaction Test (SIT). We analyzed depressive like behavior in two different time points: during adolescence (PND 28 – PND 35) and in adulthood (PND 90-100). During adolescence, animals were exposed only to SIT on PND28 and FST on PND35, but not to SPT, to avoid social isolation.

One-way ANOVA for “Subject interaction” (Figure 24) at PND28 highlights a significant group effect ($F_{(2,10)} = 6,454$; $p = ,0159$). Specifically, there is no difference between RCF and Control groups ($p = ,6068$) while there is a strong decrease in time spent interacting with the subject in Scarcity group compared both to Control ($p = ,0208$) and RCF ($p = ,0080$) groups. Figure 25 graphically represents the difference in % time interacting with the subject compared to the Control group (mean $\pm$ SEM of % of change with the Control group).

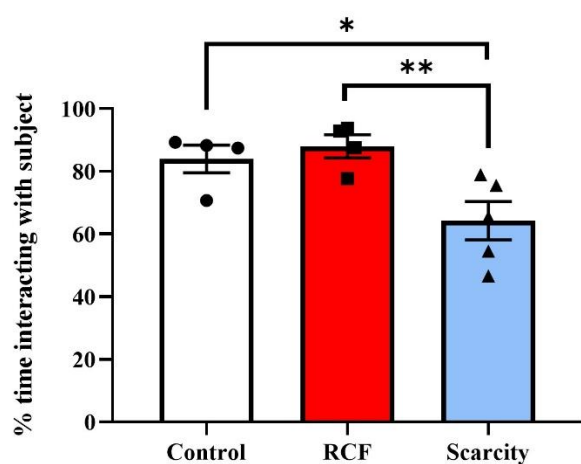


Figure 24. SIT during adolescence (PND28) in Control, RCF and Scarcity groups.

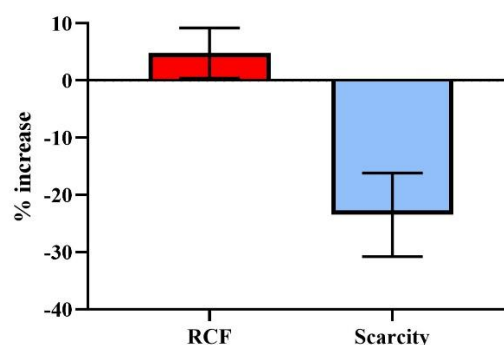


Figure 25. % increase in time spent interacting with subject compared to Control.

One-way ANOVA for “Immobility” (Figure 26) at PND35 highlights a significant group effect ($F_{(2,13)} = 6,600$; $p = ,0105$). Specifically, RCF group shows an increase in time spent in immobility compared to both Control group ($p = ,0201$) and Scarcity group ($p = ,0047$). Figure 27 graphically represents the difference in % time in immobility compared to the Control group (mean $\pm$ SEM of % of change with the Control group).

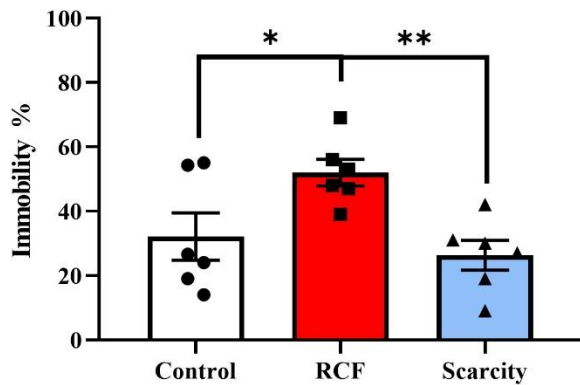


Figure 26. FST during adolescence (PND 35) in Control, RCF and Scarcity groups.

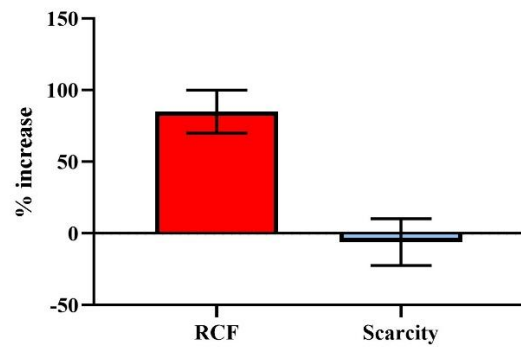


Figure 27. % increase in time spent in immobility compared to Control.

Kruskal-Wallis Test for “Subject interaction” at PND 90-100 (Figure 28) shows a significant effect ($H_{(3, N=24)} = 15,75$; $p = ,0004$). Post hoc Dunn’s tests highlighted a significant difference between time spent interacting with the subject in both Control ($p = ,0121$) and RCF ($p = ,0012$) groups compared to Scarcity group (#).

Moreover, a single T-test to analyze the preference between subject and object within groups (*) highlights that both Control ($p < 0001$) and RCF ($p < 0001$) strongly prefer interacting with the subject, while the Scarcity group shows a deep social impairment ($p = ,1124$).

In Figure 29, the difference in % time spent interacting with the subject compared to the Control group (mean ± SEM of % of change with the Control group) is graphically represented.

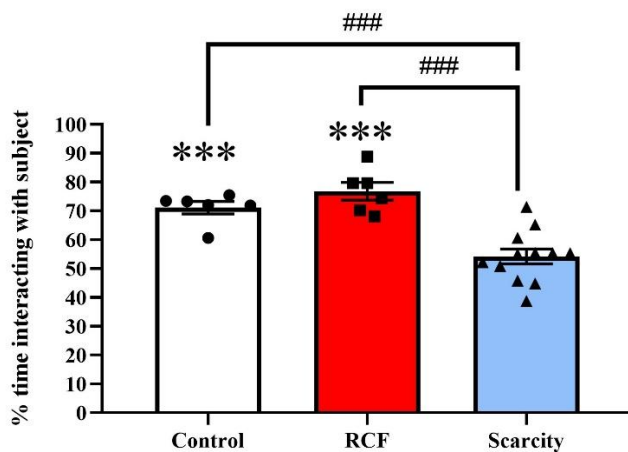


Figure 28. SIT during adulthood in Control, RCF and Scarcity groups.

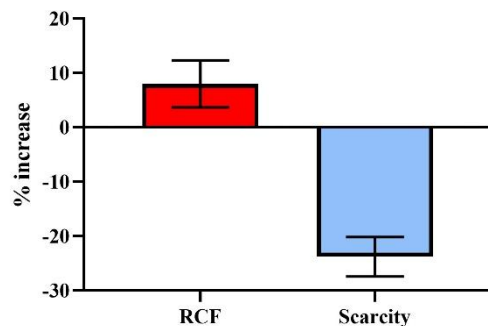


Figure 29. % increase in time spent interacting with subject compared to Control.

One-way ANOVA for “Immobility” (Figure 30) at PND 90-100 highlights a significant group effect ($F_{(2,21)} = 4,154$; $p = ,0302$). Specifically, the RCF group shows an increase in time spent in immobility compared to the Control group ($p = ,0350$), while there are no differences between the Control and Scarcity groups ($p = ,7595$).

Figure 31 graphically represents the difference in % time in immobility compared to the Control group (mean $\pm$ SEM of % of change with the Control group).

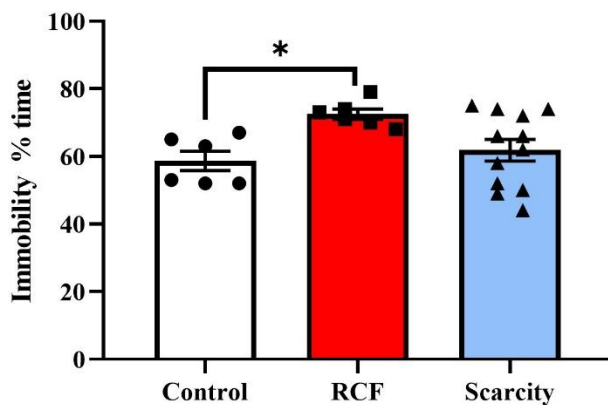


Figure 30. FST in adulthood (PND90-100) in Control, RCF and Scarcity groups.

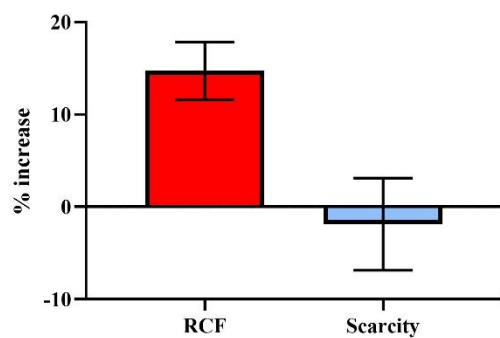


Figure 31. % increase in time spent in immobility compared to Control.

One-way ANOVA for “Saccharin intake” (Figure 32) highlighted a significant group effect ($F_{(2,14)} = 10,59$; $p = ,0016$). Specifically, both RCF and Scarcity groups show a reduction in Saccharin intake compared to Control (Control vs RCF $p = ,0054$; Control vs Scarcity $p = ,0023$), showing an anhedonic behavior.

In Figure 33, the difference in % saccharin intake compared to the Control group (mean $\pm$ SEM of % of change with the Control group) is graphically represented.

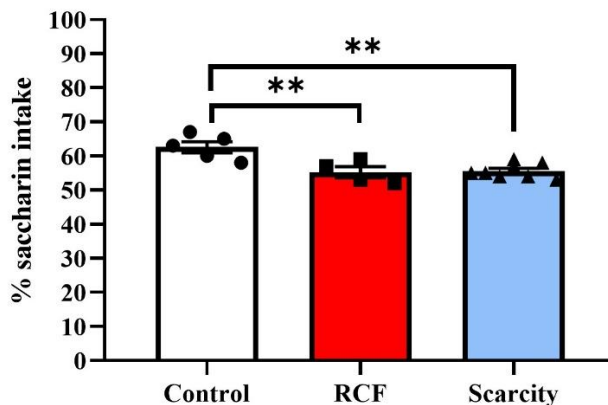


Figure 32. SPT in adulthood (PND90-100) in Control, RCF and Scarcity groups.

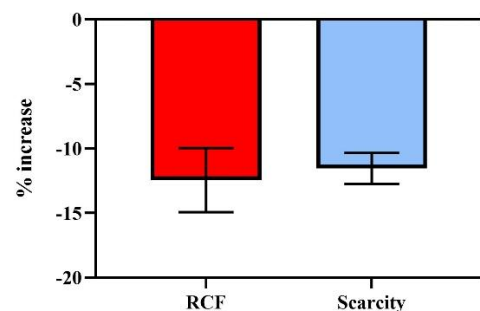


Figure 33. % increase in saccharin intake compared to Control.

To summarize, although anhedonia is evident in both RCF and Scar groups, high levels of passive coping behavior are evident only in the RCF group, while social impairment is observed only in Scar mice, both during adolescence and in adult life.

7.3 Neurobiological Analyses

7.3.1 qRT-PCR

To start to investigate neurobiological substrates of these phenotypes, we performed qRT-PCR on pFC, NAc, and Amy punches from Control, RCF, and Scarcity mice to analyze the mRNA expression levels of *OxtR*, *oprm*, and *oprk*.

In pFC (Figure 34), one-way ANOVA for *OxtR* mRNA levels highlight a significant “Group” effect ($F_{(2,13)} = 10,42$; $p = .0020$). Specifically, Scarcity group shows increased *OxtR* mRNA levels compared to Control ($p = .0305$) and RCF ($p = .0020$) groups.

Kruskal-Wallis Test for *oprm* mRNA levels reveals a significant “Group” effect ($H_{(3, N=19)} = 7,632$; $p = .0151$). Post hoc Dunn’s tests highlight a significant increase in *oprm* mRNA levels in Scarcity group compared to RCF ($p = .0191$) but not to Control group.

Regarding *oprk* mRNA levels, one-way ANOVA shows a significant group effect ($F_{(2,12)} = 16,14$; $p = .0004$) with a reduction in RCF group ($p = .0326$) and an increase in Scarcity group ($p = .0385$) compared to Control group. Moreover, an increase in Scarcity group compared to RCF group is observed ($p = .0003$).

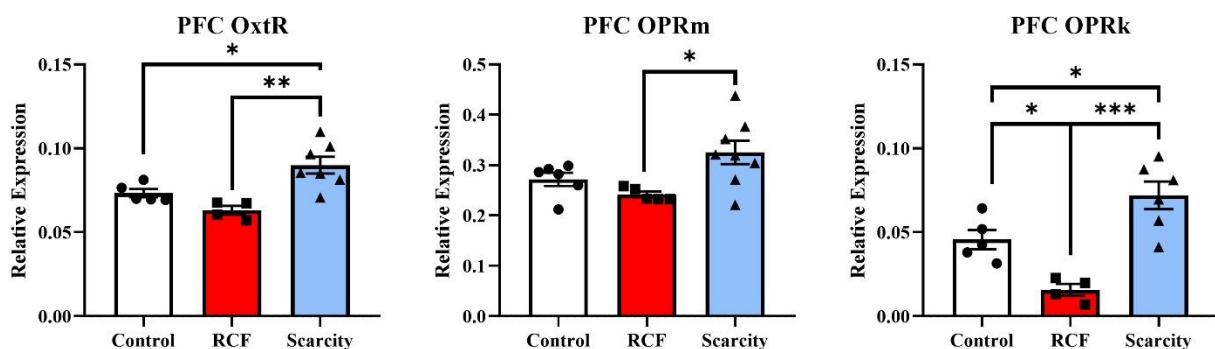


Figure 34. Neuropeptide receptor alterations in the PFC in Control, RCF, and Scarcity mice.

In NAc, we found a significant “Group” effect only for *oprm* mRNA levels (Figure 35) ($F_{(2,13)} = 4,659$; $p = ,0298$) with a reduction in RCF group compared to Control group ($p = ,0312$).

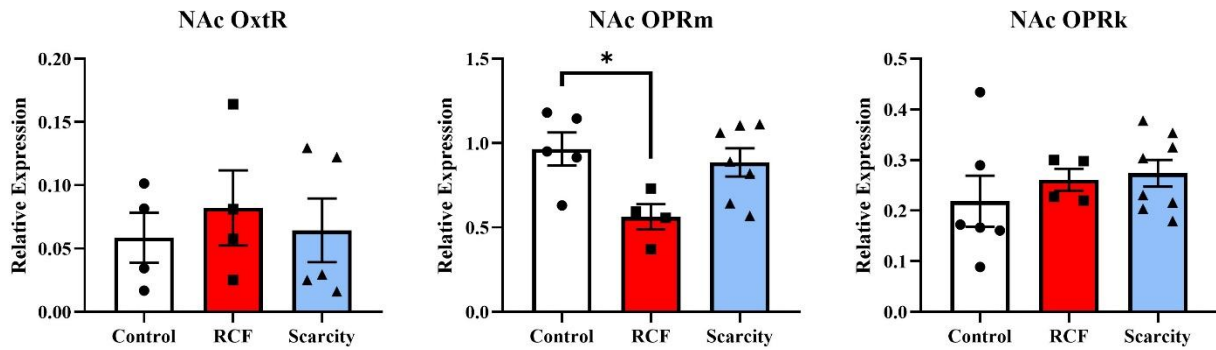


Figure 35. Neuropeptides receptor alterations in NAc in Control, RCF and Scarcity mice.

In Amy, we found a significant “Group” effect for *OxtR* ($F_{(2,13)} = 8,982$; $p = ,0035$) and *oprm* ($F_{(2,15)} = 5,560$; $p = ,0156$) mRNA levels (Figure 36). Specifically, Scarcity group shows a reduction in *OxtR* mRNA levels compared to Control ($p = ,0042$) and RCF ($p = ,0440$) groups. Scarcity group also shows a reduction in *oprm* mRNA level compared to Control group ($p = ,0214$).

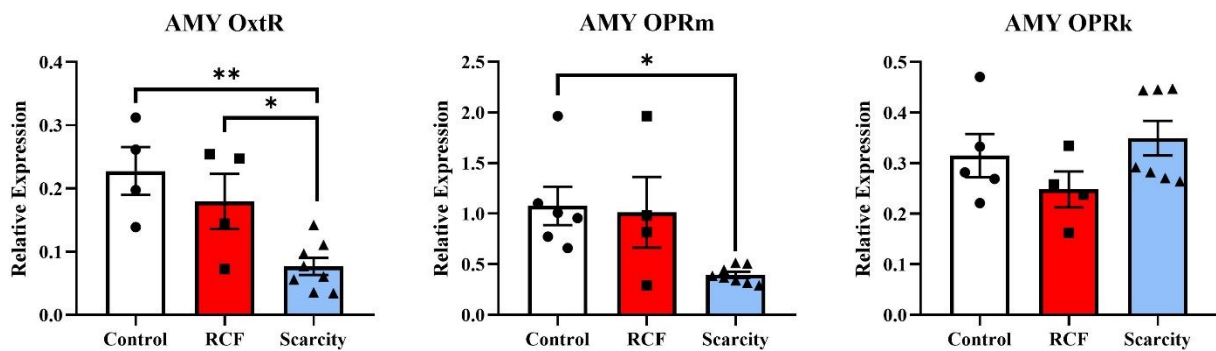


Figure 36. Neuropeptides receptor alterations in Amy in Control, RCF and Scarcity mice.

7.3.2 RNAscope

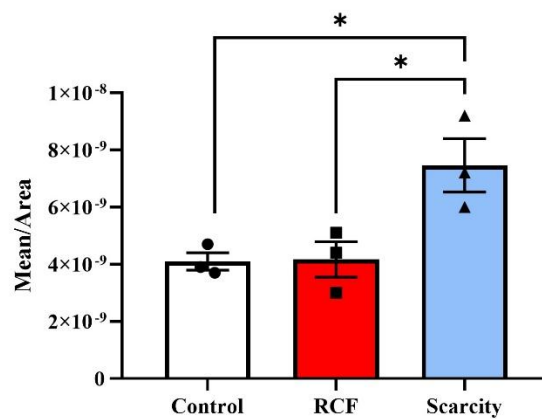
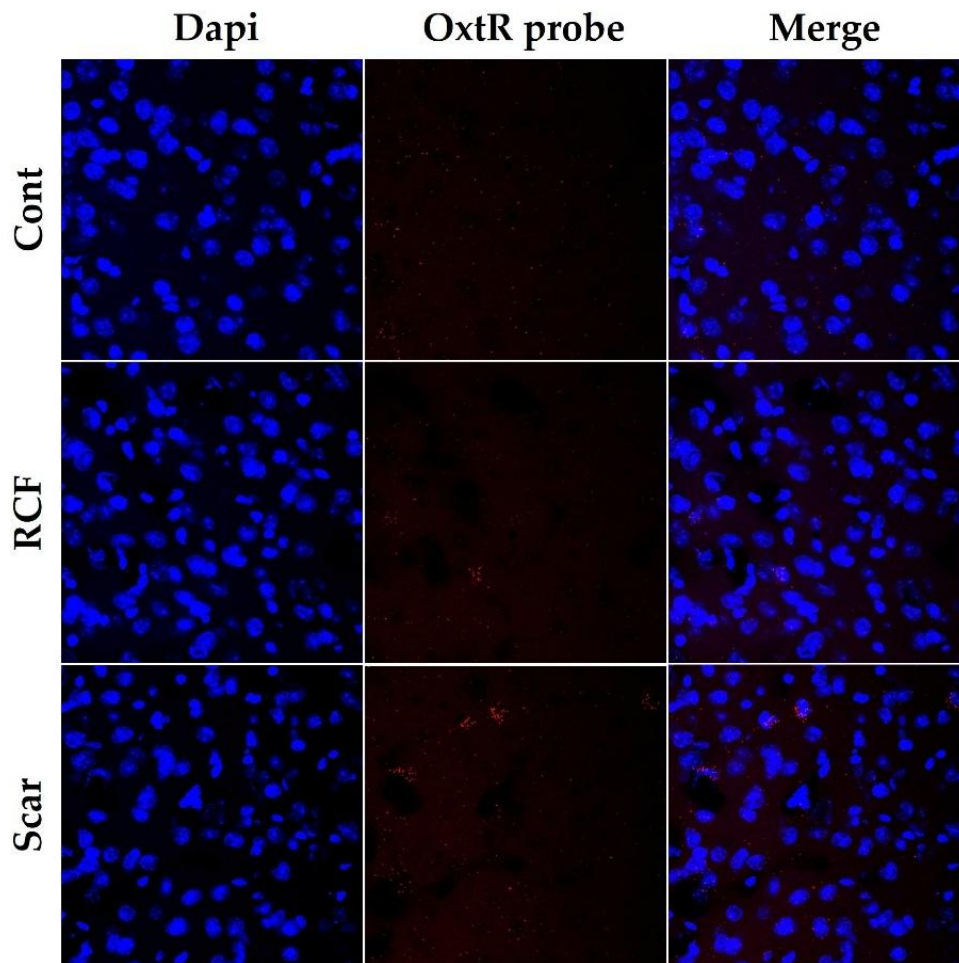


Figure 37. RNAscope for OxtR in PFC in Control, RCF and Scarcity groups.

One-way ANOVA for OxtR in PFC (Figure 37) highlights a significant Group effect ($F_{(2,6)} = 8,260$; $p = 0.0189$). Specifically, Scarcity group shows an increase in OxtR compared to Control ($p = .0279$) and RCF ($p = .0303$) groups.

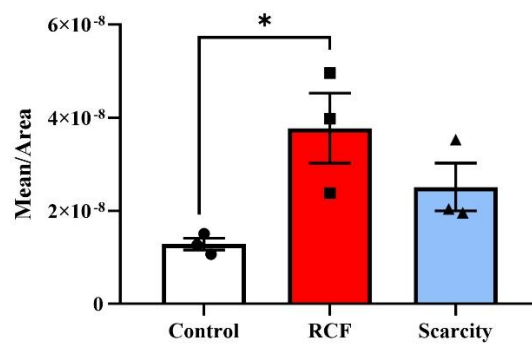
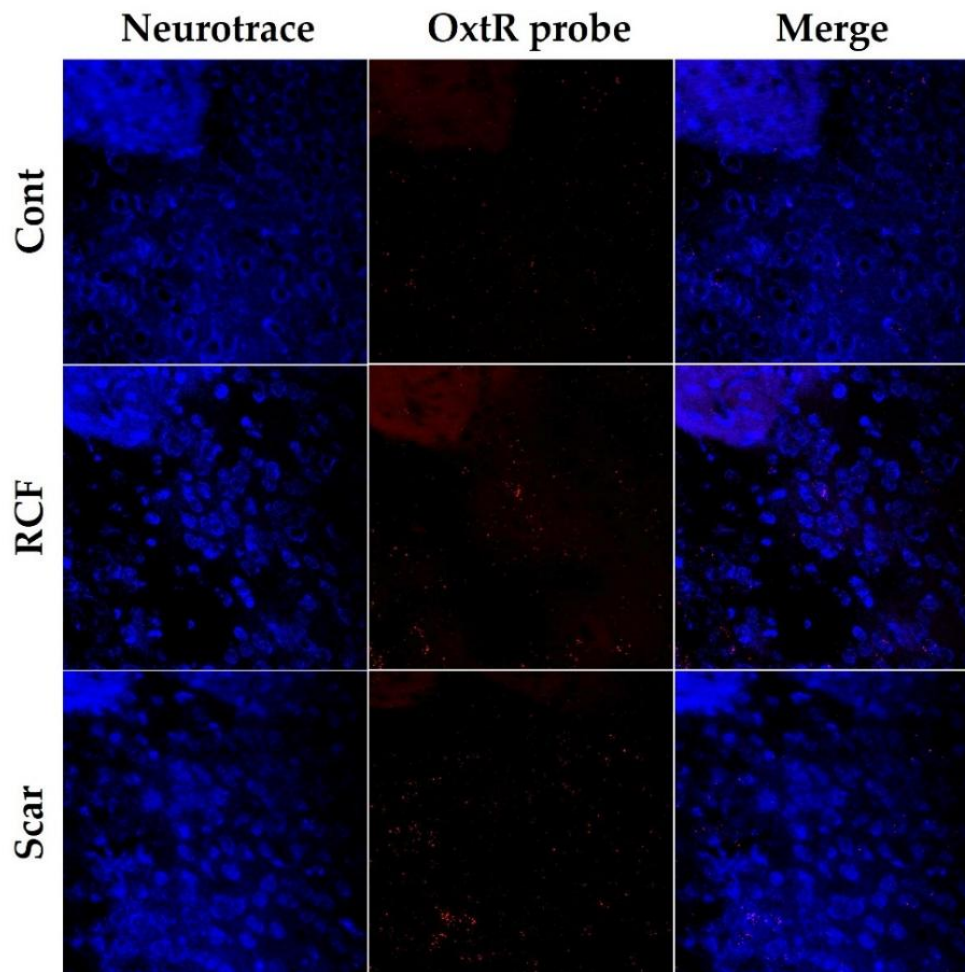


Figure 38. RNAscope for OxtR in NAc in Control, RCF and Scarcity groups.

One-way ANOVA for OxtR in NAc (Figure 38) highlights a significant Group effect ($F_{(2,6)} = 5,522$; $p = .0436$). Specifically, RCF group shows an increase in OxtR compared to Control group ($p = .0367$). A non-significant increase is also observed in the Scarcity group compared to the Control mice.

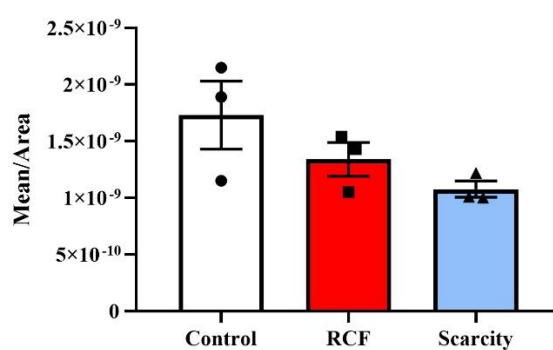
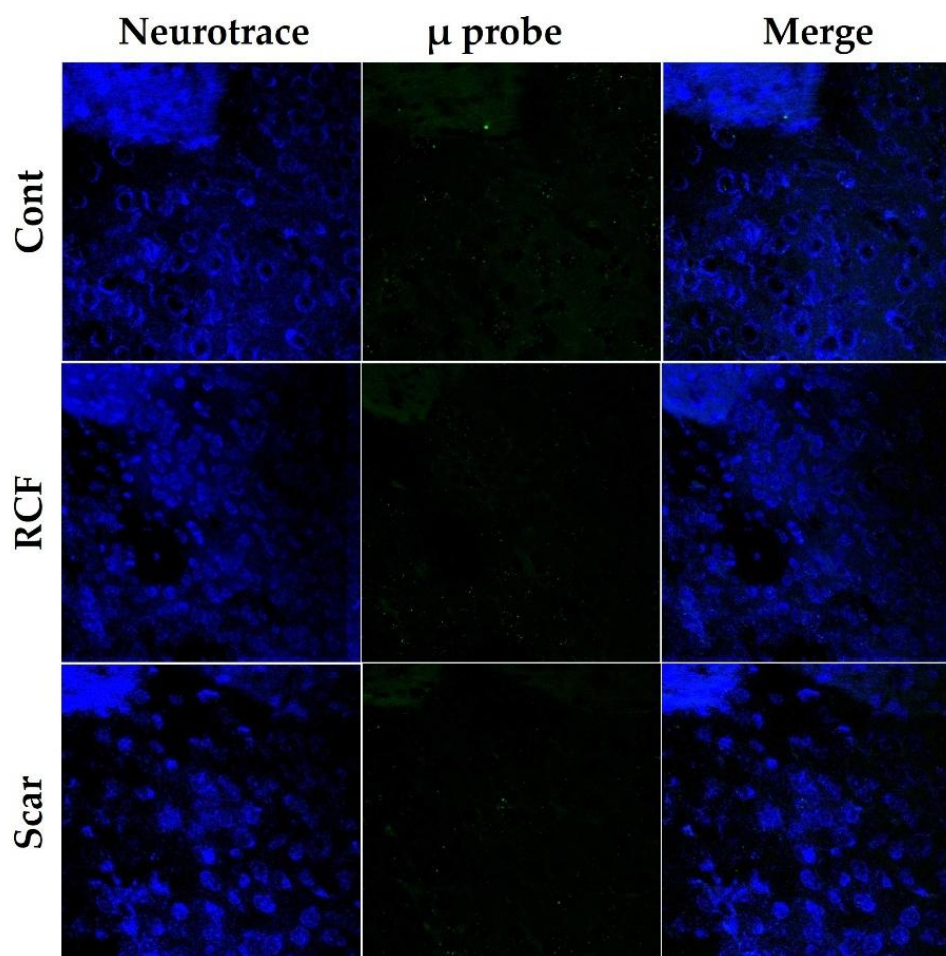


Figure 39. RNAscope for MOPR in NAc in Control, RCF and Scarcity groups.

One-way ANOVA for oprm in NAc (Figure 39) highlights no significant Group effect ($F_{(2,6)} = 2,773$; $p = ,1404$).

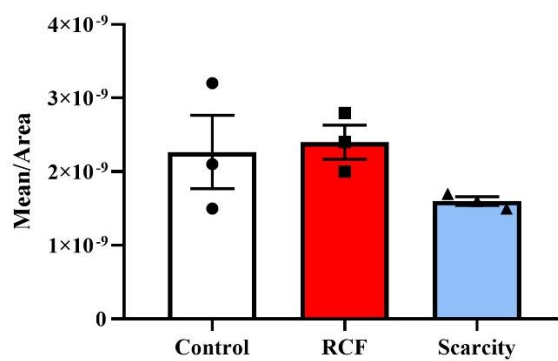
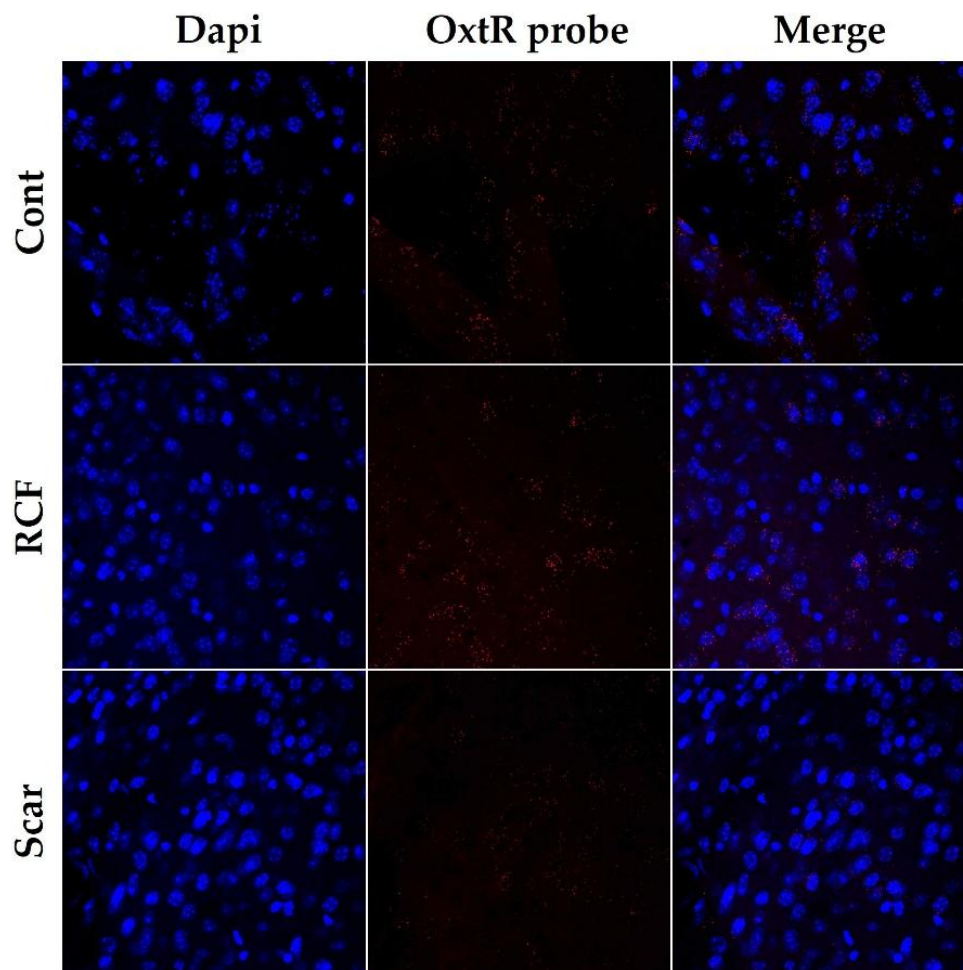


Figure 40. RNAscope for OxtR in AMY in Control, RCF and Scarcity groups

One-way ANOVA for OxtR in Amy (Figure 40) highlights no significant Group effect ($F_{(2,6)} = 1,810$; $p = ,2426$).

The remaining areas/targets that are not presented here are still being analysed.

7.3.3 ELISA

The Kruskal–Wallis test indicated a significant “Group” (Control, RCF, Scarcity) effect on Oxt (pg/mL) plasma levels ($H_{(2, N = 25)} = 9,42$; $p = ,009$) (Figure 41). Post hoc Dunn’s tests showed that RCF group differed significantly from Control group ($p = ,0135$). Moreover, also Scarcity group seems to show an increase in Oxt plasma levels, but probably due to the distribution of the samples it does not reach statistical significance ($p = ,055$). Further analyzes will be carried out to increase the size of the sample and verify whether this will reveal genuine significance.

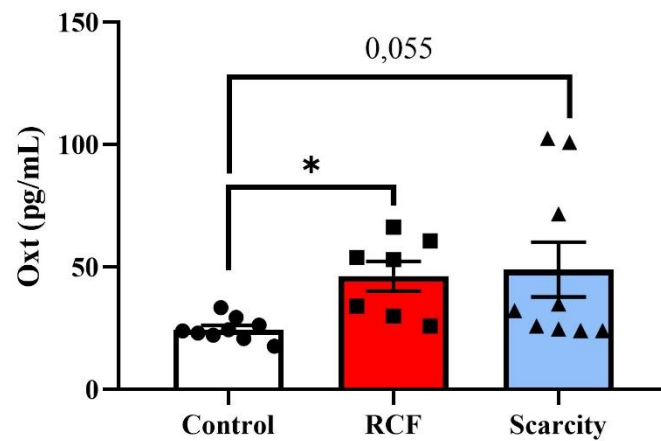


Figure 41. Oxt plasma levels in Control, RCF and Scarcity groups.

8. Discussion

As has been extensively discussed in the first chapters, having experienced ELA represents a risk factor for the onset of depressive symptoms, contributing to stress-induced brain dysfunction that, in turn, may lead to depression. The objective of this work was to investigate such a relationship both in a non-clinical population and in the mouse model, with the purpose of separating the role of two different ELA (Trauma and Attachment bond alteration) in the onset of depressive symptoms.

First, we find that in a non-clinical population, the presence of ELA is positively associated with the presence and severity of depressive symptoms, both in women and men, even if with important differences related to gender. Our findings suggest that women are more affected by ELA than men, both in terms of vulnerability to negative outcomes and in the influence of protective relational factors. Indeed, in women, we identify several risk factors for the onset of depressive symptoms, as physical Neglect, insecure attachment (Dismissing-Avoidant and Preoccupied), and overprotection, both maternal and paternal, while Secure attachment acts, in contrast, as a protective factor. Accordingly, increased sensitivity to (aversive and positive) early environment has also been reported in females in comparison with males, in animal models of ELA (Barko et al., 2019; Lo Iacono et al., 2021; Mancini et al., 2024). In men, instead, we find only Preoccupied Attachment, Emotional Abuse, and Emotional Neglect to correlate with vulnerability to depressive symptoms, with no protective factor emerging as significant. Surprisingly, from the regression analysis, it also emerges that Trauma has a marginal role in the onset of vulnerability to depressive symptoms in women, emphasizing the stronger contribution of Attachment Bond Alteration. Unlike women, in men, Trauma acts as the main risk factor for depressive symptoms vulnerability. All together, these data indicate that women are more sensitive to the quality of early attachment bonds and caregiving dynamics, whereas men are more affected by trauma exposure (Heim et al., 2009; Kuehner, 2017).

The protective role of secure attachment style against the development of mental health problems has been reported (Okello et al., 2014; McGuire et al., 2018), showing that secure individuals show higher well-being and more adaptive forms of coping with stress, as compared to individuals with attachment characterized by anxiety or avoidance (Cassidy & Shaver, 2016). Interestingly, in our study, secure attachment emerges as a protective factor against depressive symptoms vulnerability only among women, further supporting the idea that relational security plays a pivotal role in female psychological adjustment (Mikulincer

& Shaver, 2016; Zhang et al., 2022). The differences observed in our study between women and men may reflect gender-related differences in socio-emotional processing and stress reactivity. Previous research has, indeed, shown that women tend to exhibit greater interpersonal sensitivity and stronger physiological responses to relational stressors, partly mediated by oxytocinergic and affect-regulation systems (Taylor et al., 2000; Carter, 2014). However, further studies are necessary to investigate in depth this link.

Taken together, our data highlight three aspects worthy of subsequent in-depth analysis: 1. higher sensitivity to early (aversive and positive) experiences in women than men; 2. the possibility to differentiate the selective role of Trauma and Attachment bond alteration in individuals with ELA; 3. the differential weight of Trauma and Attachment bond alteration on the onset of depressive symptoms vulnerability related to gender. Specifically, our findings indicate that for women, relational environment (both positive and negative) plays a pivotal role in shaping vulnerability or resilience to later depressive outcomes, whereas men's responses appear to be more influenced by the presence or absence of traumatic experiences (Tolin & Foa, 2006; McLaughlin et al., 2010).

However, it is important to highlight a limitation inherent in the data collection of our questionnaire: the data were produced by a self-report questionnaire, which is accompanied by a lower possibility of delving into life events than an interview. Moreover, we measure attachment patterns in adulthood: according to Attachment theory, attachment maintains some stability over time due to the hardness of expectations about relationships (Bowlby, 1969; 1982; 1973). However, a longitudinal study that aims to evaluate attachment style at different stages of life would allow us to understand more accurately whether the first attachment patterns are maintained in adulthood.

Furthermore, given the known link between alterations of the oxytocinergic system and ELA, as well as depression, we analyzed plasma Oxt levels in our non-clinical sample. Also in this case, interesting gender effects emerged, according to those found in the regression. In fact, we find that Oxt levels are modulated by the experience of both Low Attachment and Trauma in a different manner in women and men. In women, lower plasma Oxt levels are observed in both the Low Attachment and Trauma groups compared with the Control group. However, we also observe decreased Oxt levels in the Low Attachment group in comparison with the Trauma group, suggesting that attachment bond alteration has a critical role in women. By contrast, in men, Oxt plasma levels are increased by the Trauma experience (alone or in combination with Low Attachment), while the experience of Low Attachment alone increases the Oxt levels in a non-significant manner. These data,

consistent with regression results, support the point of view that early trauma exposure has a greater weight in mediating the vulnerability to depressive symptoms in men, while the alteration of the attachment bond affects such vulnerability mainly in women. In addition, our data also show that ELA exposure modulates in the opposite direction the plasma Oxt levels in women and men, reducing and increasing them, respectively.

Yuen et al. (2014) have previously reported a similar pattern related to gender, with a reduction in depressed women's Oxt plasma levels compared to healthy women and an (non-significant) increase in depressed men compared to gender-matched healthy controls. One possible explanation for this gender effect is that, in women, Oxt primarily facilitates social bonding (Pedersen and Prange, 1979; Pedersen et al., 1982; Lim and Young, 2006). When women experience social isolation or loss—conditions often preceding the onset of depression (Bruce and Hoff, 1994)—they may show reduced Oxt release. In contrast, in men, Oxt appears to function more as a stress-regulating hormone: exogenous Oxt has been shown to suppress cortisol secretion in response to psychological stress when accompanied by social support (Heinrichs et al., 2003). Thus, men may exhibit increased Oxt release as a mechanism to mitigate stress through enhanced social behavior, greater social support, and reduced cortisol levels. This interpretation could help to clarify the inconsistent findings in previous studies reporting both elevated (Parker et al., 2010) and decreased (Frasch et al., 1995) plasma Oxt concentrations among individuals affected by depression.

In order to investigate the correlation between depressive symptoms and Oxt levels in our non-clinical sample, we next performed a correlational analysis. In the women's group, no statistically significant correlations emerged. However, some descriptive trends were observed, suggesting a possible positive association between oxytocin and secure attachment, oxytocin and physical abuse, and negative associations with maternal overprotection. Also in the men's group, no correlations reached statistical significance, although several moderate trends were found. Specifically, Oxt levels showed a negative association with dismissing-avoidant attachment, a positive association with physical neglect, and a negative association with depressive symptoms. Although not significant, these findings may suggest differential patterns of association between oxytocin and relational functioning across genders.

Although the lack of correlation observed in our study is an apparent contrast with previous reports (Ozsoy et al., 2009; Yuen et al, 2014; Massey et al., 2016), different hypotheses could be proposed. First, differently from previous studies, we investigated the Oxt levels-depressive symptoms correlation in a non-clinical sample. Second, the reduced-size sample

(for Oxt evaluation) could take into account the lack of effects. Increasing the sample size will be, therefore, necessary to clarify this point.

Overall, results from our study strongly indicate that Oxt levels modulation is mainly related to ELA exposure - differentially in women and men - rather than to depressive symptoms. Our data suggest that attachment alteration and trauma exposure could affect Oxt functionally, which, in turn, could affect the vulnerability to depressive symptoms in adult life. Longitudinal studies should be important to test this hypothesis. A critical open question posed by our results is the functional significance of the opposite direction of Oxt levels observed between women and men exposed to ELA. How can an opposite modulation underlie similar behavioral (depression vulnerability) effects? Finally, another important effect that emerged from our study concerns the different Oxt levels observed between women and men belonging to the control groups, with women having higher Oxt levels in comparison with men, a difference frequently reported in human literature (Marazziti et al., 2019; Roels et al., 2021; Marazziti et al., 2022).

Marazziti and colleagues (2019) reported a sexual dimorphism concerning plasma Oxt levels, with women generally having higher plasma Oxt concentrations than men in the control condition (no psychopathological or medical issues). This sexual dimorphism suggests that male and female oxytocin systems are tuned differently even in the absence of external challenge, likely reflecting modulation by gonadal steroids (Gabor & Choleris, 2012; Carter, 2017), sex-specific receptor distribution across key limbic and hypothalamic regions (Dumais & Veenema, 2016; Johnson & Young, 2017), and partially independent central-peripheral coupling mechanisms (Jurek & Neumann, 2018; Lee et al., 2009). Consequently, what may appear as an “increase” relative to male baselines could represent a return to a normal physiological range in females, and vice versa. In this sense, the concept of homeostasis, which assumes a single universal set-point, is insufficient.

Physiological systems are often described in terms of homeostasis: the maintenance of internal stability through negative feedback mechanisms. However, growing evidence supports that many neuroendocrine responses function allostatically rather than homeostatically: they shift their set-points to promote adaptive flexibility in changing environments (Quintana & Guastella, 2020; Takayanagi & Onaka, 2021). Within this framework, the Oxt system exemplifies how a “departure from homeostasis” can reflect an adaptive recalibration of physiological state rather than a breakdown of regulation. According to this point of view, in humans, peripheral Oxt levels show marked variability across individuals and contexts, and, crucially, exhibit consistent sex differences at baseline

(Marazziti et al., 2019; Roels et al., 2021; Marazziti et al., 2022). Such differences mean that men and women have distinct “homeostatic” set-points, and any perturbation that elicits a change in Oxt concentrations should be interpreted differently across sexes, according to their respective starting point, as a potential attempt to reach a functional allostatic condition adequate to cope with challenging events.

Consistent with this framework, the Oxt system function should be, indeed, viewed as maintaining sex-specific allostatic ranges, where adaptive responses are contingent upon both biological sex and contextual demands (Quintana & Guastella, 2020). Empirical findings reinforce that stress, or social perturbations, can elicit opposite peripheral Oxt changes in men and women, consistent with the notion of adaptive divergence (Taylor et al., 2010). This divergence supports the idea that male and female neuroendocrine systems employ distinct allostatic strategies for coping with social stress. For example, Rubin et al. (2011) reported that peripheral Oxt correlated with positive affect and social closeness in women but not in men, again pointing to sex-specific functional associations. From an allostatic perspective, these patterns represent functional recalibrations to maintain adaptive equilibrium within sex-typical neuroendocrine architectures.

Quintana and Guastella (2020) explicitly frame Oxt within an allostatic theory, proposing that Oxt serves as a neuromodulator of adaptive change rather than of fixed balance. Under this model, acute or chronic social challenges trigger temporary “departures” from baseline Oxt activity, which in turn reshape behavior and physiology to meet new environmental demands, and over time these departures can consolidate into new stable states. Takayanagi and Onaka (2021) extend this reasoning, suggesting that Oxt contributes to social allostasis by integrating internal and external cues to maintain organismal stability through change. Importantly, since sex hormones shape both Oxt release and receptor sensitivity, allostatic recalibrations are expected to differ across sexes. Thus, when peripheral Oxt changes appear opposite between men and women, these may not represent conflicting or anomalous findings, but rather distinct pathways toward sex-specific adaptive stability. Based on these observations, rather than asking whether Oxt levels “increase or decrease,” the critical question becomes whether the direction and magnitude of change are consistent with adaptive goals (e.g., promoting affiliation, buffering stress, or facilitating vigilance). The recognition that each sex maintains its own dynamic equilibrium aligns with contemporary models of neuroendocrine regulation that prioritize flexibility, context sensitivity, and anticipatory control over static balance (Quintana & Guastella, 2020).

Research on ELA in humans is complicated by numerous moderating factors, including the type, intensity, frequency, and timing of stressors, as well as the frequent co-occurrence of multiple adversities. Moreover, many adverse experiences in humans may reflect heritable influences, making it difficult to disentangle genetic and environmental contributions to psychopathological outcomes (Tyrka et al., 2013). Despite extensive empirical work, key questions remain unresolved: it is still unclear which specific combinations of stressful life events most strongly predict psychopathological trajectories, or how these experiences interact across developmental stages.

In contrast, preclinical research allows for the investigation of causal relationships between ELA, brain function, and behavior. Such studies hold great promise for identifying novel therapeutic strategies aimed at restoring neurobiological mechanisms disrupted by ELA (Malave et al., 2022). Investigating ELA in rodent models is, indeed, fundamental. In species where parental care is essential for survival, evolutionary pressures have favored the development of strong and rapid attachment to caregivers, even when the quality of care is suboptimal (Perry & Sullivan, 2014). Although translating findings from rodents to humans presents challenges, there are substantial cross-species similarities in the attachment system and its responses to trauma (Opendak et al., 2017). Rodent models, therefore, provide a valuable translational framework for studying the mother–infant relationship. Research on maternal behavior in rodents has highlighted the crucial role of early parent–offspring interactions in shaping brain development, cognitive functions, emotional regulation, and stress reactivity (Krugers et al., 2016).

Here, we decided to investigate different kinds of ELA using two different paradigms in the mouse model: a model known to alter the attachment bond and one known to be a traumatic experience (Neglect). These models allow us to understand similarities and differences in the depressive-like phenotype induced by these two different ELA, both in terms of behavioral phenotype and neurobiological alterations.

We chose to adapt the Scarcity model to the RCF model by performing this manipulation during the same RCF time window. By presenting both experiences in the same time window, it is in fact possible to eliminate any effects due to the different sensitive periods. As far as we know, this is the first time the Scarcity model has been presented in the PND1-4-time window. Furthermore, this study was conducted only in male mice of the C57BL/6J (C57) strain, as it is known in the literature that while males of this strain develop a depressive-like phenotype following RCF, females of this strain develop a resilient phenotype with an increased preference for both natural and pharmacological rewarding

stimuli (Di Segni et al., 2019). Moreover, even with regard to the Scarcity model, whenever sex-dependent differences were observed, the majority (64%) of the studies reported significant effects in males but not in females (Walker et al., 2017). Since our goal was to focus on ELA-induced depression, we used male C57 mice.

The first important difference that emerges between the RCF model and the Scarcity model concerns maternal care. In fact, mothers of RCF pups do not present differences in maternal behavior compared to control mothers. This is consistent with the known effect of the RCF of absence of neglect response by mothers (D'Amato et al., 2011) as the RCF alters the mother-pups bond without interfering with maternal orientation behavior towards the pups (Di Segni et al., 2018). On the other hand, regarding dams that are in a Scarcity condition, these present an alteration of care: in fact, they show a reduction in maternal behaviors and an increase in non-maternal behaviors. This increase in non-maternal behaviors is associated with the enactment of abusive behaviors. Among the observed abusive behaviors (entirely described in table 1, page 38), "Retrieval" was also included, which in itself does not represent abusive behavior. However, it must be considered that a hallmark of maternal behavior in neglect/abuse situations is its unpredictable and fragmented quality, which is characterized by shortened bouts of each nurturing behavior and frequent shifts between behaviors as well as an apparent reduction in the drive or ability of the dam to keep the pups in the nest area (Ivy et al., 2008). Having observed these abusive behaviors is consistent with what has been observed in the literature (albeit in other time windows) concerning the Scarcity model: rough handling of the pups by the dam (i.e. stepping or jumping on pups, mother aggressively grooms pups, transports pups by gripping a limb) represents a strong element of maternal behavior in Scarcity model (Moriceau, et al., 2009; Rainecki, et al., 2012; Rainecki, et al., 2010) that is less often reported in the classical Limited Bedding and Nesting (LBN) condition.

This difference in maternal behavior between RCF and Scarcity dams confirms that, in the RCF model, there is no trauma or abuse condition, whereas such a condition is markedly present in the Scarcity model.

The next step is therefore to evaluate whether RCF and Scarcity pups exhibit separation anxiety and attachment behaviors towards their mothers. To evaluate separation anxiety and attachment bond, we used two different tests: the recording of Ultrasonic Vocalizations distress calls (USVs) at PND8 and the Mouse Strange Situation Procedure (MSSP) at PND18. USVs are recorded during separation from the mother and are an index of separation anxiety (Lo Iacono et al., 2021). Moreover, at PND18 we tested pups in the MSSP test, an

experimental paradigm with translational validity (Lassi & Tucci, 2017) developed to match the Strange Situation Procedure and quantified common features of secure attachment, as: “Maternal Preference”, “Reunion” and “Stranger Effect” (Mancini et al., 2024).

In USVs, RCF pups emit the same number of USVs in home or clean beddings, seeming not to benefit from the calming effect of maternal odor. Our results confirm and expand previous results obtained in outbred and inbred mice as well as in rats, showing that RCF impairs the formation of attachment bond (D’Amato et al., 2011; Ventura et al., 2013; Di Segni et al., 2018; Dulor Finkler et al., 2020; Lo Iacono et al., 2021; Mancini et al., 2024). Instead, Scarcity pups show the same pattern of Cont pups: they emit fewer USVs when exposed to home bedding, compared to clean bedding, showing no separation anxiety. This pattern seems to take up a phenomenon that is known in human literature, whereby even in the presence of abuse or neglect, many children maintain strong emotional ties to their caregivers. However, such (disorganized) attachment often results in maladaptive patterns of emotional and social behavior, cognitive impairment, and increased vulnerability to mental health disorders in adolescence and adulthood (Perry & Sullivan, 2014). This paradoxical behavior is observed even in mice, and underscores a key feature of early attachment: abused pups develop a preference for their caregiver and stimuli linked to the abuse (Roth & Sullivan, 2005).

In the MSSP, RCF pups show only one feature of Secure Attachment, the “Reunion”. However, they do not show “Maternal Preference” and “Stranger Effect”. Instead, Scarcity pups show “Reunion” and “Stranger Effect” but no “Maternal Preference”. Overall, RCF animals present only one element indicative of secure attachment; they show, indeed, an increase in separation anxiety in USVs and do not present two out of three indices in MSSP. In contrast, Scarcity animals show all the signs of secure attachment, except for the “Maternal Preference” parameter in the MSSP. Although the presence of this index does not allow us to state that they present a form of secure attachment, we can conclude that they present a much milder attachment impairment than RCF animals.

An explanation for the presence of “Reunion” in RCF could be linked to the well-known fact that, from an attachment-theoretical perspective, separation from a primary caregiver is a natural cue to danger. This separation leads to activation of the attachment system, followed by behavioral changes. Behavioral reactions to separations and reunions are expected in all children, although they may be less evident in some, such as avoidantly attached children, or appear awkward in others, such as disorganized attached children (Ainsworth et al., 1978; Main & Solomon, 1990; Oosterman & Schuengel, 2007). Probably,

although we cannot define the type of attachment in the mouse model, the presence of this single attachment index (considering both USVs and MSSP) could indicate not an absence of binding (biologically necessary) but a strongly dysfunctional form of it. In the Scarcity pups, we can observe the presence of all attachment indices (USVs and MSSP) except for “Maternal Preference”. The absence of interest in the mother when she returns after a separation is a phenomenon that in humans is observed in children who have experienced traumatic experiences (ACE) and is an index of disorganized attachment (Solomon et al., 2017; Reisz et al., 2018). Disorganization is signaled through a variety of behaviors, including stilling or freezing, disorientation or confusion, stereotypies, and interrupted, incomplete, or contradictory movements when in their caregiver’s presence (Main & Solomon, 1990). Another important index of attachment quality is the interaction with a stranger, the “Stranger Effect”. The ability to maintain exploratory behavior and to interact positively with the stranger, even during temporary separations from the caregiver, is considered an indicator of attachment security, as it reflects the internalization of the caregiver as a secure base (Ainsworth et al., 1978; Cassidy & Shaver, 2016).

The observation in the Scarcity pups of only one index (Maternal Preference), associated with disorganized attachment in children, could indicate that Scarcity pups show a dysfunctional attachment with the abusive figure, reminiscent of disorganized attachment. Overall, considering the indices considered in this study to evaluate the attachment bond in our animal models (USVs, Reunion, Maternal Preference, and Stranger Effect), we can speculate that the attachment bond is strongly disrupted in RCF than in Scarcity mice.

Regarding depressive-like behavior, this has been investigated through different symptom categories: anhedonia, the use of passive coping strategies (immobility), and social impairment. Indeed, animal models do not typically fully recapitulate depression per se, but rather display behavioral and physiological endophenotypes that may have face, construct, and predictive validity relevant to depression (Bosch et al., 2016).

Anhedonia is a core feature of major depressive disorder reflecting deficits in interest and the experience and pursuit of pleasure (American Psychiatric Association, 2013) and has been identified as a predictor of poor response to antidepressant medications in adults (Uher et al., 2012; Vinckier et al., 2017) and in youth (McMakin et al., 2012). Reward processing deficits have been associated with chronicity and severity in depression in multiple investigations (Morgan et al., 2013; Vrieze et al., 2013; Hall et al., 2014; Satterthwaite et al., 2015; Kelly et al., 2022).

We measured anhedonia through the Saccharin Preference Test (SPT) in which animals are exposed to a double choice of water or saccharin. What we observed is that both RCF animals and Scarcity animals show a marked reduction in saccharin preference compared to the control group. Both groups appear, therefore, to exhibit an anhedonic behavior.

In humans, a symptom of the passive coping response can be described as hopelessness, which is a core feature of major depressive disorder (Yin et al., 2021). The forced-swimming test (FST) is widely used to evaluate the despair aspect of depression-like behavior and to screen antidepressants in rodents. At the outset of a rodent swimming in a cylinder, a brief period of vigorous activity occurs; subsequently, a passive state emerges when the rodent finds it is impossible to escape from the cylinder (Warden et al., 2012). Furthermore, transitions between active (swimming) and passive (immobility) coping responses in the FST are clearly demarcated; therefore, the FST is an appropriate model to imitate challenging conditions involving acute and intense stress (Yin et al., 2021).

In FST, we found an increase in immobility (an index of passive coping) in the RCF group compared to the control, but not in the Scar group.

Social withdrawal is defined as the behavioral tendency to isolate oneself from peers (Rubin & Coplan 2004). Prior work has provided evidence in support of a direct, positive relationship between social withdrawal and depression. A concurrent association between social withdrawal and depression has been found in children (Strauss et al., 1986), adolescents (Vargo 1996), and young adults (Alfano et al., 1994). Interestingly, human literature highlights how emotional neglect is more strongly associated with problems in social behaviors than other types of maltreatment (Derin et al., 2022). Generally, social behaviors refer to behaviors that occur when two or more individuals of the same species interact (Katz et al., 2011). Rodents are highly social animals, and when placed into an area in which territory has not been established, they socially engage with one another displaying a number of behavioral acts that can be quantitatively measured (Sams-Dodd, 1995). These include both playful and aggressive acts such as pouncing, chasing, social grooming, crawling over/under, charging, boxing, wrestling, pinning, anogenital sniffing, and biting (Vanderschuren et al., 1997). Further, social interaction appears to be rewarding to rodents, with behaviors increased by social deprivation and reduced by social satiation (Panksepp & Beatty, 1980; Calcagnetti & Schechter, 1992).

We evaluate social impairment in mice through Social Interaction Test (SIT), measuring time interacting with a conspecific animal (paired for age and sex) compared to a plastic object.

Both Cont and RCF animals show a strong preference for the conspecific animal, but Scar do not show a preference between subject and object.

Overall, while anhedonia is a symptom present in both the attachment bond alteration model (RCF) and the neglect model (Scarcity), increased passive coping is present only in RCF animals, while social impairment is only present in Scarcity animals. Moreover, we observed this symptomatic difference already in adolescence, exposing animals to SIT at PND28 (weaning day) and FST a week later (PND35). The difference in symptoms characterizing the depressive phenotype between RCF and Scarcity, already since adolescence, represents a characterization that does not appear to be present in the literature and which seems to confirm our hypothesis on the difference in depression induced by Trauma or Alteration of the attachment bond.

Furthermore, we also analyzed the expression of receptors of two different social neuropeptides (Oxytocin and Endogenous Opioids) in the mesocorticolimbic circuit, as their joint role on corticostriatal circuits is known (Putnam & Chang, 2022). We focused on this circuit because it represents a key neural substrate involved in the long-term effects of ELA and in the pathophysiology of depression. The main effects we observed in these receptors are in different areas regarding RCF animals and Scarcity animals. Specifically, in RCF animals, we observe receptor alterations in NAc, while in Scarcity in Amy and pFC.

OxtR in pFC, hippocampus, and Amy is fundamental for short-term social recognition (Yashima & Sakamoto, 2023). GABAergic interneurons in the pFC receive excitatory input from the ventral hippocampus (Sun et al., 2020) and glutamatergic neurons in the pFC project to the basolateral Amy (BLA) (Tan et al., 2019), both pathways being involved in social recognition. GABAergic interneurons in the pFC, which receive input from the ventral hippocampus, do not express OxtR (Sun et al., 2020); in contrast, glutamatergic neurons in the pFC abundantly express OxtR (Tan et al., 2019). Moreover, optical activation of OxtR-expressing pFC neurons reduces social recognition memory without affecting sociability or anxiety-like avoidance behavior. Furthermore, directly stimulating OxtR-expressing pFC axons within the BLA recapitulated this effect (Mack et al., 2024). Moreover, a recent study (Nisbett & Koob, 2025) demonstrates three previously undescribed populations of cells in the Central Amy: cells that co-express *oprm1* and OxtR; cells that co-express *oprm1*, OxtR, and *oprk1*; and cells that co-express *oprm1* and *oprk1*. The authors hypothesize that *oprm1* + OxtR cells may play a role in the previously described interaction between OxtR agonists and *oprm* antagonists and provide morphological support for an interaction between *oprm* and OxtR within central Amy cells and its broader

network (Nisbett et al., 2024). These alterations are consistent with what we found in animals of the Scarcity group and may be associated, in particular, with the social impairment observed in this group.

In contrast, in RCF animals, we found alterations mainly in NAc, an area that is known to be linked to the formation and maintenance of attachment bonding, both in relation to OxtR (Young et al., 2001; Keebaugh et al., 2015; Borie et al., 2022) and oprm (Moles et al., 2004). Multiple evidence identifies the NAc as a key region involved in modulating passive coping strategies in response to stress or social adversity and highlight the role of Oxt and opioids in this brain area. For instance, Bosch et al. (2016) demonstrated that intra-accumbal administration of Oxt in male prairie voles reversed the increase in passive coping (immobility in the FST) induced by partner loss, whereas blockade of Oxt signaling within the NAc enhanced passive coping behaviors. This suggests that reduced oxytocinergic activity in this area promotes behavioral withdrawal and passive stress responses. Moreover, activation of the oprk system in the NAc shell has been linked to negative affective states and stress-related behavioral suppression (Massaly et al., 2019), while dopaminergic signaling within the same circuit regulates the balance between active and passive coping under inescapable stress (Cui et al., 2020). Together, these findings support the view that alterations in oxytocin and opioid receptor expression within the NAc may underlie the emergence of passive coping behaviors in animal models of attachment disruption or social stress.

One alteration we found to be oppositely modulated in RCF and Scar animals concerns oprk in pFC. Oprk alteration is implicated in depression: drugs acting on oprk have been shown to have strong antidepressant effects (Emrich et al., 1982; Bodkin et al., 1995) and oprk abnormalities have been reported in the cingulate and prefrontal cortices in subjects affected by major depression (Peckys & Hurd, 2001). Moreover, in humans, oprk is thought to regulate neural systems associated with anhedonia (Pizzagalli et al., 2020), and mouse studies highlight divergent effects of early life stress paradigms on oprk mRNA, probably determined by the severity of the stressor employed (Browne et al., 2022).

Finally, we analyzed Oxt levels in plasma with the aim of identifying a peripheral index that could also be investigated in humans. Indeed, studies have shown that administration of Oxt increases social interactions and behaviors in humans and animals (Russell, 2018). The Yamamoto group has recently reported that Oxt can bind to the receptor for advanced glycation end-products (RAGE), suggesting that RAGE may function as the direct transporter of Oxt in brain vascular endothelial cells (Yamamoto et al., 2019). Based on this perspective, Oxt has therapeutic potential for conditions such as autism spectrum disorder,

schizophrenia, posttraumatic stress disorder, anxiety, and depression (Carter et al., 2020). In 2025, C4a, a cleaved fragment of the complement component C4, was identified as an Oxt-binding protein in plasma (Yamamoto et al., 2025). It is highly likely that C4a modulates the free form of Oxt dynamics in the plasma and subsequent Oxt transport through the Brain Blood Barrier and brain Oxt availability, contributing to the promotion of sociability and prosocial behavior (Yamamoto et al., 2025). For these reasons, analyzing plasma Oxt levels could have a strong translational value, with a view to increasingly personalized medicine.

Interestingly, according to men results, we find increased plasma Oxt levels in the RCF group and a non-significant trend in the Scarcity group. While in the literature it is known that in females, depression is associated with a reduction in Oxt levels (Yuen et al., 2014), in males, the results are often conflicting (Parker et al., 2010; Frasch et al., 1995). This could depend on the fact that, as already discussed for Oxt levels on ELAX subjects, in males, Oxt appears to function more as a stress-regulating hormone (Heinrichs et al., 2003). These data suggest that having experienced an ELA induces an increase in plasma Oxt levels in both male humans and animal models.

In conclusion, what emerges from our study is that in a non-clinical sample, having experienced ELA is associated with a greater probability of developing depressive symptoms. In addition, we report that ELA affects plasma Oxt levels in an opposite manner in women and men. Specifically, insecure attachment mainly correlates with reduced Oxt levels in women, while trauma exposure mainly correlates with increased Oxt levels in men. In the preclinical models, we were able to observe differences between the experiences of alteration of the attachment bond (RCF) and neglect (Scarcity). In fact, RCF animals, despite receiving maternal care comparable to that of controls, show a disrupted attachment to their mother. Conversely, Scar pups, even if they receive maternal care characterized by abusive behavior, seem to present a better attachment. Furthermore, these animals present, already from adolescence, different depressive symptoms: both groups present anhedonia, while the increase in passive coping characterizes the RCF group and the social impairment the Scar group. These phenotypic differences are also associated with differences in social neuropeptides in the mesocorticolimbic circuit.

However, it is important to highlight how this study has limitations. In fact, with regard to the human study, the use of self-report questionnaires prevents us from delving into some aspects that emerged and that could have been investigated in the case of an interview. Concerning the animal study, we did not investigate a potential gender effect, and, in addition, we did not carry out experiments to highlight a possible causal link between the

behaviors shown and the neurobiological alterations. However, future studies will aim to address these limitations.

Despite these limitations, the study presents interesting results both in Human Study and Animal Study and can be classified as an interesting increase in knowledge on ELA-induced depression.

9. Supplementary Data

9.1 Correlation Analysis in Human

In the following tables, the correlation matrices of the constructs of interest are reported.

Table 9. Correlation matrix among the CTQ variables

		(1)	(2)	(3)	(4)
Emotional Abuse	(1) r	1			
Emotional Neglect	(2) r	.746**	1		
Physical Neglect	(3) r	.524**	.615**	1	
Physical Abuse (inverse)	(4) r	-.557**	-.434**	-.348**	1

Note: * = $p < .05$; ** = $p < .01$

As we can observe from Table 8 the CTQ dimensions are all strongly correlated with each other. Table 9 shows a strong negative association between “Physical abuse (inverse)” and “Emotional abuse” ($r = -.557$; $p < .01$), “Emotional neglect” ($r = -.434$; $p < .01$) and “Physical neglect” ($r = -.348$; $p < .01$). This result must be interpreted by reversing the directions that have emerged. Therefore, high physical abuse is positively associated with other forms of abuse.

Table 10. Correlation matrix between CTQ variables, separated into women (in bold below diagonal) and men (above diagonal)

		(1)	(2)	(3)	(4)
Emotional Abuse	(1) r	1	.635**	.381**	-.485**
Emotional Neglect	(2) r	.771**	1	.458**	-.338**
Physical Neglect	(3) r	.556**	.651**	1	-.271**
Physical Abuse (inverse)	(4) r	-.585**	-.462*	-.372*	1

Note: * = $p < .05$; ** = $p < .01$

As we can observe from Table 10 the CTQ dimensions are all strongly correlated with each other in both women and men. In both cases, a strong negative association emerges between “Physical abuse (inverse)” and the other forms of abuse and, even in this case, this result must be interpreted by reversing the directions that emerged. Therefore, high physical abuse is positively associated with other forms of abuse, both in females and males.

Table 11. Correlation matrix between RQ variables (Attachment)

		(1)	(2)	(3)	(4)
Secure Attachment	(1) r	1			
Dismissing-Avoidant Attachment	(2) r	-.506**	1		
Preoccupied Attachment	(3) r	-.389**	.446**	1	
Fearful-Avoidant Attachment	(4) r	-.167*	.284*	.043	1

Note: * = $p < .05$; ** = $p < .01$

Table 11 shows a strong negative association between “Secure Attachment” and “Dismissing-Avoidant Attachment” (-.506**; $p < .01$), “Preoccupied Attachment” (-.389**; $p < .01$) and “Fearful-Avoidant Attachment” (-.167*; $p < .05$). A strong positive association also emerges between “Dismissing-Avoidant Attachment” and “Preoccupied Attachment” (.446**; $p < .01$) and “Fearful-Avoidant Attachment” (.248*; $p < .05$). However, no association emerges between “Preoccupied Attachment” and “Fearful-Avoidant Attachment” (.043).

Table 12. Correlation matrix between RQ variables (Attachment), separated into women (in bold below diagonal) and men (above diagonal)

		(1)	(2)	(3)	(4)
Secure Attachment	(1) r	1	-.531**	-.362**	-.177*
Dismissing-Avoidant Attachment	(2) r	-.491**	1	.464**	.236*
Preoccupied Attachment	(3) r	-.395**	.438**	1	.041
Fearful-Avoidant Attachment	(4) r	-.169*	.303*	.048	1

Note: * = $p < .05$; ** = $p < .01$

Table 12 shows that, in both Women and Men, there is a strong negative association between “Secure Attachment” and all types of insecure attachment. A strong positive association also emerges between “Dismissing-Avoidant Attachment” and “Preoccupied Attachment” and “Fearful-Avoidant Attachment”. However, no association emerges between “Preoccupied Attachment” and “Fearful-Avoidant Attachment”.

Table 13. Correlation matrix between PBI variables

		(1)	(2)	(3)	(4)
Maternal Care	(1) r	1			
Paternal Care	(2) r	.455**	1		
Maternal Overprotection	(3) r	-.469**	-.309**	1	
Paternal Overprotection	(4) r	-.362**	-.415**	.539**	1

Note: * = $p < .05$; ** = $p < .01$

Table 13 shows a strong positive association between “Maternal Care” and “Paternal Care” (.455**; $p < .01$) and between “Maternal Overprotection” and “Paternal Overprotection” (.539**; $p < .01$). In contrast, the constructs of Care and Overprotection show a strong negative association. In fact, “Maternal Care” associates negatively with both “Maternal Overprotection” (-.469**; $p < .01$) and “Paternal Overprotection” (-.362**; $p < .01$). At the same time, “Paternal Care” also associates negatively with both “Maternal Overprotection” (-.309**; $p < .01$) and “Paternal Overprotection” (-.415**; $p < .01$).

Table 14. Correlation matrix between PBI variables, separated into women (in bold below diagonal) and men (above diagonal)

		(1)	(2)	(3)	(4)
Maternal Care	(1) r	1	.440**	-.472**	-.349**
Paternal Care	(2) r	.459**	1	-.272*	-.397**
Maternal Overprotection	(3) r	-.471**	-.326**	1	.631**
Paternal Overprotection	(4) r	-.361**	-.415**	.533**	1

Note: * = $p < .05$; ** = $p < .01$

Table 14 shows a strong positive association, in both women and men, between “Maternal Care” and “Paternal Care” and between “Maternal Overprotection” and “Paternal Overprotection”. In contrast, the constructs of Care and Overprotection show a strong negative association.

9.2 Anxiety in Humans

Given the strong comorbidity between Anxiety and Depression, we checked our sample for anxiety symptoms with General Anxiety Disorder-7 (GAD-7).

The GAD-7 consists of 7 items, each assessing a specific symptom of anxiety experienced during the past two weeks. Responses are based on a 0–3 Likert scale measuring the frequency of symptoms: 0 = Never, 1 = Several days, 2 = More than half the days, 3 = Nearly every day. The following score ranges correspond to anxiety severity levels: Score 0–4 = Minimal anxiety; Score 5–9 = Mild anxiety; Score 10–14 = Moderate anxiety; Score ≥ 15 = Severe anxiety (Spitzer et al., 2006).

As for PHQ-9 (Results 7.1 Human Study) we analyzed the prevalence of ELA in the distribution of GAD-7 symptoms (severity of anxiety symptoms), considering these categories: Minimal, Mild, Moderate, Severe (Figure 42). In the upper line (green) are represented men and in the lower line (purple) women.

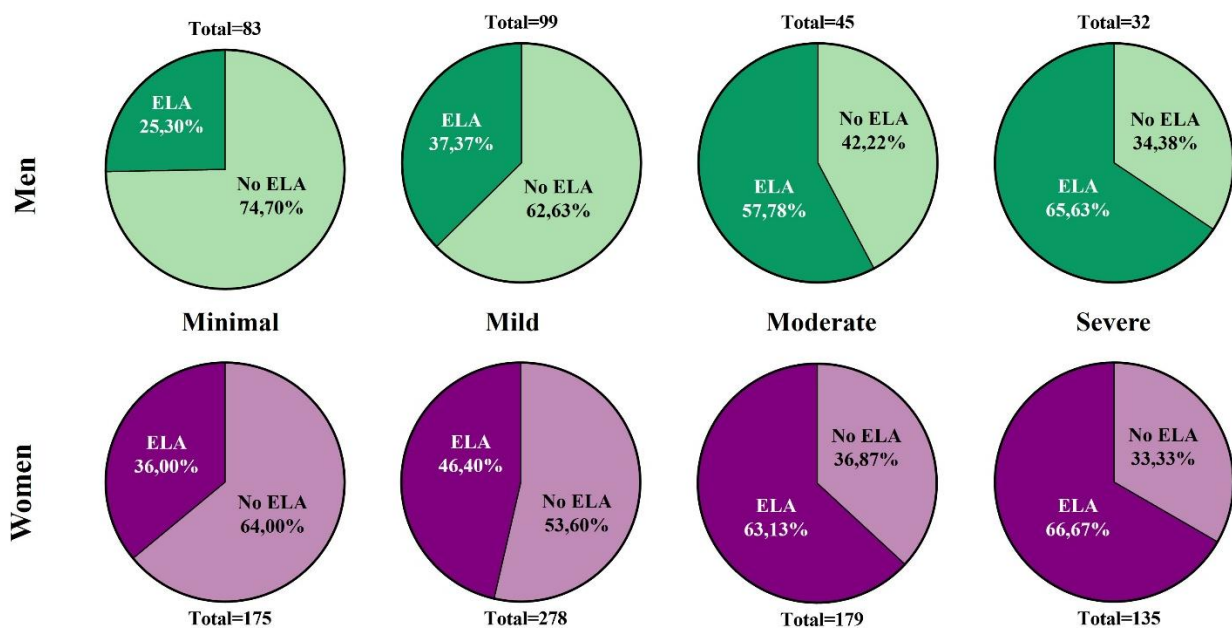


Figure 42. Distribution of the sample (split in ELA and no ELA) in men and women through GAD-7 severity symptoms.

Subsequently, we analyzed again the prevalence of ELA in the distribution of GAD-7 symptoms (Minimal, Mild, Moderate, Severe) considering the distribution of our sample in No ELA, Low Secure (LS), Trauma (T) and Trauma + Low Secure (T+LS) (Figure 43) in men (upper line) and women (lower line).

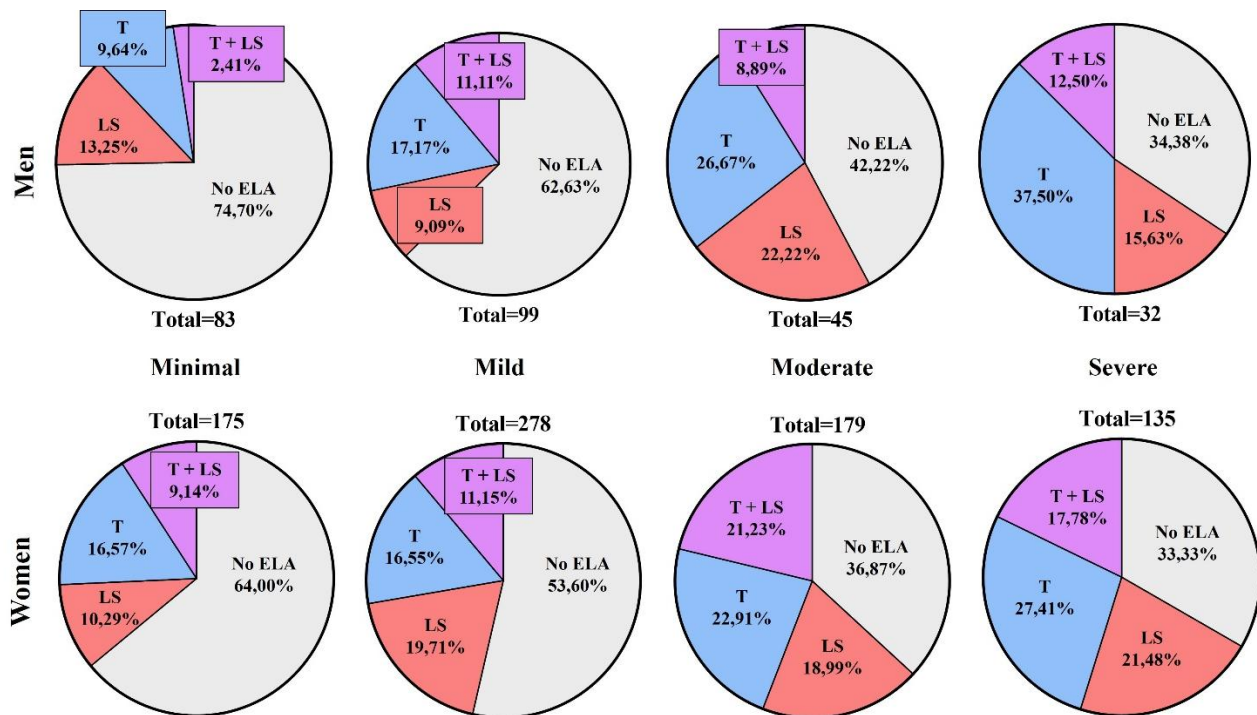


Figure 43. Distribution of the sample in women and men through GAD-7 symptoms severity.

As for “Depressive symptoms” we checked means, standard deviations, skewness, and kurtosis of “Anxious symptoms”. Values lower than 2 for univariate skewness and lower than 5 for univariate kurtosis were used as criteria for the evaluation of univariate normality (Curran, West, & Finch, 1996).

The variable “Anxious symptoms” is normally distributed, as you can see in Table 15.

Table 15. Descriptive Statistics for “Anxious Symptoms”

	Means	Standard deviation	Skewness	Kurtosis
Anxious Symptoms (GAD-7)	8,65	5,29	0,56	-0,56

We then performed a correlation matrix between anxious symptomatology (GAD-7) and constructs of interest, divided by Total, Women and Men (Table 16).

Table 16. Correlation matrix between Anxious Symptom (GAD-7) and constructs of interest, divided by Total, Women and Men

		GAD-7 Total	GAD-7 Women	GAD-7 Men
Emotional Abuse	r	.299**	.273*	.337**
Emotional Neglect	r	.230*	.208*	.280**
Physical Neglect	r	.221*	.239*	.137*
Physical Abuse (inverse)	r	-.185*	-.205*	-.124
Secure Attachment	r	-.325**	-.339**	-.248*
Dismissing-Avoidant Attachment	r	.412**	.412**	.384**
Preoccupied Attachment	r	.428**	.392**	.525**
Fearful-Avoidant Attachment	r	.130*	.157*	.079
Maternal Care	r	-.176*	-.176*	-.120
Paternal Care	r	-.201*	-.175*	-.260*
Maternal Overprotection	r	.197*	.222*	.145*
Paternal Overprotection	r	.228*	.204*	.241*

Note: * = $p < .05$; ** = $p < .01$

Table 16 shows that only in females all the variables of interest correlate with the Anxious Symptoms. In particular, “Anxious Symptoms” has a strong positive association with:

- “Emotional Abuse” in Women (.273*; $p < .05$) and Men (.337**; $p < .01$);
- “Emotional Neglect” in Women (.208*; $p < .05$) and Men (.280**; $p < .01$);
- “Physical Neglect” in Women (.239*; $p < .05$) and Men (.137*; $p < .05$);
- “Physical Abuse (inverse)” just in Women (-.205*; $p < .05$) (to be interpreted by reversing the emerged direction);
- “Dismissing-Avoidant Attachment” in Women (.412**; $p < .01$) and Men (.384**; $p < .01$);
- “Preoccupied Attachment” in Women (.392**; $p < .01$) and Men (.525**; $p < .01$);
- “Fearful-Avoidant Attachment” just in Women (.157*; $p < .05$);
- “Maternal Overprotection” in Women (.222*; $p < .05$) and Men (.145*; $p < .05$);
- “Paternal Overprotection” in Women (.204*; $p < .05$) and Men (.241*; $p < .05$).

The “Anxious Symptoms” has, instead, a strong negative association with:

- “Secure attachment” in Women (-.339**; $p < .01$) and Men (-.248*; $p < .05$);
- “Maternal care” in Women (-.176*; $p < .05$);
- “Paternal care” in Women (-.175*; $p < .05$) and Men (-.260*; $p < .05$).

Based on the results obtained from the analysis of correlations, which highlight significant associations between the variables under investigation, we have deepened these relationships, testing a hierarchical regression model considering as dependent variable the Anxious Symptom and examining the effects of “Age” (Step 1) and of “Emotional Abuse”, Physical Abuse”, “Emotional Neglect”, “Physical Neglect”, “Maternal Care”, “Maternal Overprotection”, “Paternal Care”, “Paternal Overprotection”, “Secure Attachment”, “Dismissing-Avoidant Attachment”, “Preoccupied Attachment”, “Fearful-Avoidant Attachment” (Step 2). In addition, we conducted the same analyses separately for Women (Table 17) and Men (Table 18).

All lower-order and interactive terms will be mean-centered in order to facilitate a proper interpretation of lower-order terms and to decrease nonessential multicollinearity (Cohen et al., 2003). In particular, the regression model provides a theoretically established variable entry model which highlights from time to time the independent variables that present the highest correlation with the dependent variable.

Table 17. Analysis of regressions: dependent variable “Anxious Symptoms” in Women

	VD: Anxious Symptoms	β	R²	p
Step 1	Age	-.125	.014**	.001**
Step 2	Emotional Abuse	.049	.263**	.447
	Emotional Neglect	-.056		.415
	Physical Neglect	.151		.001**
	Physical Abuse (inverse)	-.074		.081
	Secure Attachment	-.114		.006**
	Dismissing-Avoidant Attachment	.225		.000**
	Preoccupied Attachment	.198		.000**
	Fearful-Avoidant Attachment	.026		.462
	Maternal Care	.057		.277
	Paternal Care	.085		.090
	Maternal Overprotection	.042		.330
	Paternal Overprotection	.103		.015*

Note: * = $p < .05$; ** = $p < .01$. β =standard regression coefficient. R²= Coefficient of determination.

The regression analysis (Table 17) highlighted that the predictors considered make a significant contribution capable of explaining 26% of anxious symptoms in Women. Specifically, the direct contribution of “Age” ($\beta = -.125$) was significant (Step 1).

In addition, the contribution of “Physical Neglect” ($\beta = .151$), “Secure Attachment” ($\beta = -.114$), “Dismissing-Avoidant Attachment” ($\beta = .225$), “Preoccupied Attachment” ($\beta = .198$) and “Paternal Overprotection” ($\beta = .103$) (step 2) was significant.

Table 18. Analysis of regressions: dependent variable “Anxious Symptoms” in Men

	VD: Anxious Symptoms	β	R^2	p
Step 1	Age	-.048	-.002	.489
Step 2	Emotional Abuse	.173	.320**	.066
	Emotional Neglect	.156		.085
	Physical Neglect	.069		.305
	Physical Abuse (inverse)	.029		.668
	Secure Attachment	.017		.809
	Dismissing-Avoidant Attachment	.129		.088
	Preoccupied Attachment	.457		.000**
	Fearful-Avoidant Attachment	-.016		.787
	Maternal Care	.168		.030*
	Paternal Care	.017		.836
	Maternal Overprotection	-.020		.805
	Paternal Overprotection	.025		.764

Note: * = $p < .05$; ** = $p < .01$. β = standard regression coefficient. R^2 = Coefficient of determination.

The regression analysis (Table 18) highlighted that the predictors considered make a significant contribution capable of explaining 32% of depressive symptoms in Men.

Only the contribution of “Preoccupied Attachment” ($\beta = .457$) and “Maternal Care” ($\beta = .168$) was significant (Step 2).

9.3 Anxiety behavior in Mice

To analyze the presence of Anxiety-like behaviors, that could interfere with the phenotype of interest, we perform at PND 90-100 the Open Field Test (OF) and the Elevated Plus Maze Test (EPM) on Cont, RCF and Scar groups.

The Open Field Test (OF) was performed as previously reported (Di Segni et al., 2016 ; Lo Iacono et al., 2021 ; Mancini et al., 2024). Distance moved (cm) and time spent in the external part of the apparatus (percentage of total time spent in exploration) were recorded and analyzed with the fully automated tracking video system “EthoVision” (Noldus, The Netherlands).

The Elevated Plus Maze Test (EPM) was conducted as previously described (Di Segni et al., 2016 ; 2019 ; Lo Iacono et al., 2021 ; Mancini et al., 2024). Mice were individually tested for 5 min in an apparatus consisting of four arms (two open, two closed) connected by a central platform (starting point), 38.5 cm above the floor. The percentage of time spent in the closed arms was estimated using the “EthoVision” (Noldus, The Netherlands) fully automated video tracking system.

For the OF the percentage time in the periphery of the apparatus was analyzed. One-way ANOVA for “Open Field” (Figure 44) highlighted no differences between the three groups ($F_{(2,30)}=1,017$; $p = ,3739$).

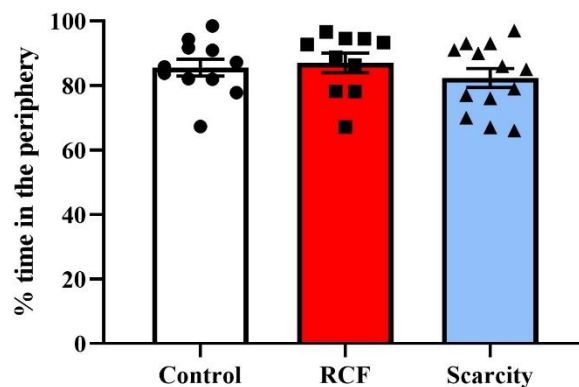


Figure 44. OF in Control, RCF and Scarcity groups.

For the EPM the percentage time in the closed arms of the apparatus was analyzed. One-way ANOVA for “Elevated Plus Maze” (Figure 45) highlighted a significant group effect ($F_{(2,22)} = 8,678$; $p = ,0017$). Specifically, even if there are no differences between the Control group and the other two groups (Control vs RCF $p = ,0730$; Control vs Scar $p = ,0689$),

there is a difference in percentage time in the closed arms between RCF and Scarcity groups ($p = ,0005$).

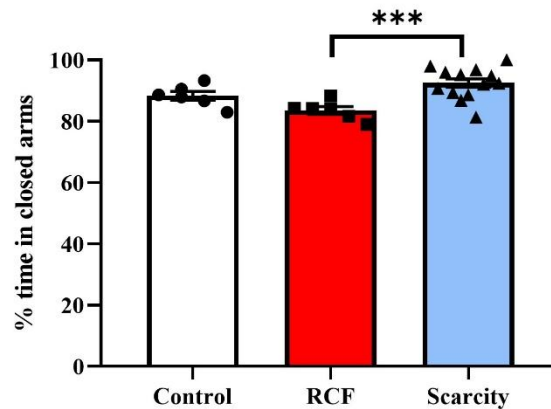


Figure 45. EPM in Control, RCF and Scarcity groups.

9.4 Immunohistochemistry for Oxt positive neurons

Animals at PND90 were perfused transcardially with 50 ml of saline followed by 50 ml of 4% paraformaldehyde in a phosphate buffer (PB; 0.1 M; pH 7.4) under deep anesthesia. Each brain was removed immediately, post-fixed in the same fixative for 24h and, after three washes in PB, transferred to 30% sucrose in PB solution at 4 °C. Brains were cut into four series of 30 µm -thick transverse sections using a freezing microtome and collected in PB. Every fourth section was processed for immunohistochemistry. Before incubation with the primary antibody, free-floating sections were incubated in a blocking solution (5% normal donkey serum, 0.3% Triton X-100, PB) for 2 h at room temperature. Rabbit anti-Oxytocin-neurophysin1 (1:1000; #AB212193; Abcam) was prepared in PB and 0.3% Triton X-100 and incubated for 48 hours at 4 °C. Each incubation step was followed by three, 5-min rinses in PB. Then, sections were incubated with a biotinylated donkey anti-rabbit IgG as secondary antibody (1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by the avidin-biotin-peroxidase method (Vectastain, ABC kit, Vector, Burlingame, CA, USA), and 3,3'-diaminobenzidine (Sigma) as a chromogen. Sections were mounted on chrome alum-gelatinized slides and air-dried for at least 24 h, dehydrated and coverslipped with Entellan (Sigma). The immunoperoxidase material was examined under a light-transmission microscope (Zeiss, Axioskop 2) equipped with a CCD camera (ProgRes C10 plus, Zeiss). Qualitative observations were limited to paraventricular (PVN) and Supraoptic (SON) nucleus. Cell counting of oxytocin+ neurons was performed on 10 sections regularly spaced throughout the rostro-caudal extent of the two nuclei by using a 10X objective. Only positive neurons with the nucleus in the focal plane were counted.

One-way ANOVA for Oxt positive cells highlights no significant effect both for PVN ($F_{(2,9)} = 0,1654$; $p = ,8500$) (Figure 46) and SON (Figure 47) ($F_{(2,9)} = 1,107$; $p = ,3717$).

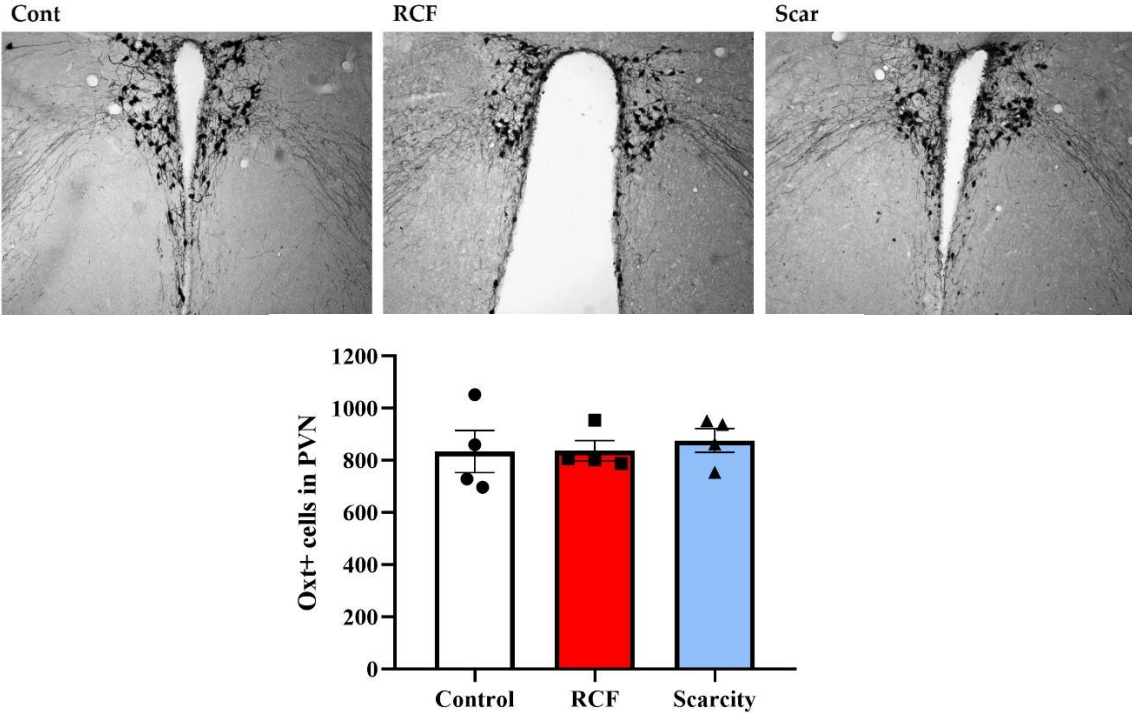


Figure 46. Oxt+ cells in PVN in Control, RCF and Scarcity groups.

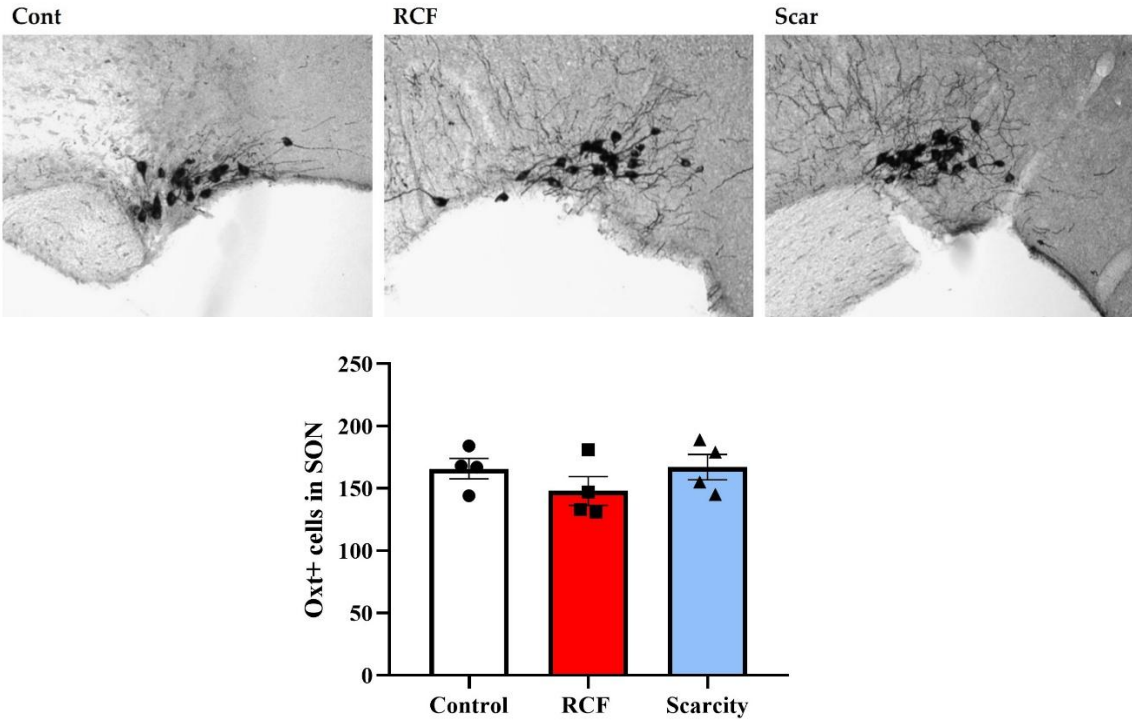


Figure 47. Oxt+ cells in SON in Control, RCF and Scarcity groups.

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