EARLY LIFE STRESS AND INFANT ULTRASONIC VOCALIZATIONS AS INDICATORS OF LONG-TERM AFFECTIVE PROCESSES IN RATS

BAHAR YÜKSEL

BOĞAZİÇİ UNIVERSITY

2025

EARLY LIFE STRESS AND INFANT ULTRASONIC VOCALIZATIONS AS INDICATORS OF LONG-TERM AFFECTIVE PROCESSES IN RATS

Thesis submitted to the

Institute for Graduate Studies in Social Sciences

in partial fulfillment of the requirements for the degree of

Master of Arts

in

Psychological Sciences

by

Bahar Yüksel

Boğaziçi University

2025

Early Life Stress and Infant Ultrasonic Vocalizations

as Indicators of Long-Term Affective Processes in Rats

The thesis of Bahar Yüksel

has been approved by:

Assoc. Prof. Güneş Ünal _____
(Thesis Advisor)

Assoc. Prof. Yasemin Sohtorik İlkmen _____

Assist. Prof. Mehmet Kocatürk _____
(External Member)

May 2025

DECLARATION OF ORIGINALITY

I, Bahar Yüksel, certify that

- I am the sole author of this thesis and that I have fully acknowledged and documented in my thesis all sources of ideas and words, including digital resources, which have been produced or published by another person or institution;
- this thesis contains no material that has been submitted or accepted for a degree or diploma in any other educational institution;
- this is a true copy of the thesis approved by my advisor and thesis committee at Boğaziçi University, including final revisions required by them.

Signature …….

Date …….

ABSTRACT

Early Life Stress and Infant Ultrasonic Vocalizations

as Indicators of Long-Term Affective Processes in Rats

Early life stress (ELS) takes a crucial role in shaping long-term behavioral and physiological processes, and maternal separation (MS) is a well-established rodent model of ELS. Ultrasonic vocalizations (USVs) are closely linked to the emotional states of animals; however, the predictive value of infant USVs for subsequent affective processes remains poorly understood, especially in relation to ELS. The present thesis examines the behavioral and neurobiological effects of MS in Wistar rats across adolescence and adulthood, while investigating the predictive potential of pup USVs on measured outcomes. A repeated MS procedure was employed during postnatal days (PND) 2–14 followed by a maternal potentiation test with USV recordings on PND 15. Animals were subjected to a range of behavioral evaluations including anxiety-like and social behaviors during adolescence (PND 35–36) and adulthood (PND 80–87), along with additional measurements of anhedonia and fear conditioning in adulthood. At the end of behavioral testing, corticosterone release and neuronal activation in several limbic regions were assessed following restraint stress. MS reduced infant USVs, suppressed social and anxiety-like behavior in adolescence, and led to impairments in hedonic response and fear memory in adulthood, associated with decreased stress-related activation in the lateral amygdala. Infant USVs inversely predicted anxiety-like behavior in adolescence for both groups, while relating with neuronal activation in habenula in control, but not MS animals. These results show that pup USVs not only reflect early stressful experiences but also possess a predictive value for subsequent affective processes.

ÖZET

Sıçanlarda Uzun Vadeli Duygulanımsal Süreçlerin Göstergeleri Olarak Erken Yaşam Stresi ve Bebeklik Ultrasonik Vokalizasyonları

Anne ayrılığı (AA), çeşitli davranışsal ve fizyolojik süreçleri düzenlemede rol alan erken yaşam stresini modelleyen bir kemirgen modelidir. Ultrasonik vokalizasyon (USV) sinyalleri ise kemirgenlerin duygulanımsal durumları ile ilişkilendirilmektedir. Ancak yavru kemirgen USV'lerinin erken yaşam stresi ile ilişkisi ve erişkinlikteki duygulanımsal süreçleri öngörmedeki (prediktif) rolü yeterince anlaşılamamıştır. Bu tez çalışması, Wistar sıçanlarında AA'nın uzun vadeli nörobiyolojik ve davranışsal etkileri ile beraber, yavru USV'lerinin daha sonra ortaya çıkan duygulanımsal süreçler üzerindeki prediktif işlevini araştırmaktadır. Bu çerçevede, ikinci ve 14. postnatal günler (PNG) arasında tekrarlı bir AA prosedürü uygulanmış ve 15. PNG'de USV'lerin kaydedildiği bir anne etkisi testi gerçekleştirilmiştir. Hayvanlar, ergenlik (PNG 35–36) ve erişkinlik (PNG 80–87) dönemlerinde anksiyete benzeri davranışlar ile sosyal davranışları da içeren çeşitli testlere tabi tutulmuştur. Erişkinlik döneminde anhedoni ile korku koşullanması üzerine ilave ölçümler yapılmıştır. Deney sonunda kısıtlama stresine bağlı kortikosteron salınımı ve nöronal etkinlik değerlendirilmiştir. AA gelişim boyunca belirgin davranışsal etkiler yaratmış; yavru USV'lerinde azalma, ergenlikte baskılanmış sosyal ve anksiyete benzeri davranışlar, erişkinlikte ise anhedoni ile korku belleği bozuklukları gözlemlenmiştir. AA grubunda, kısıtlama stresi ile lateral amigdala nöronal etkinliğinin azaldığı saptanmıştır. Yavru USV sinyalleri, her iki grup için de ergenlikteki anksiyete benzeri davranışları ters yönde öngörmüş; ancak sadece kontrol grubu habenula çekirdeğindeki nöronal etkinlik ile ilişkilenmiştir. Bu

sonuçlar, yavru USV'lerinin yalnızca erken dönemdeki stresli deneyimleri yansıtmakla kalmayıp, sonraki duygulanımsal süreçler üzerinde prediktif bir potansiyel taşıdığını ortaya koymaktadır.

ACKNOWLEDGEMENTS

As I reach the end of my master's studies, which was a long and transformative period of time, I owe a deep sense of appreciation to the many people who have supported, inspired, and walked beside me throughout this journey.

I would like to begin by thanking my principal investigator, Assoc. Prof. Güneş Ünal, who introduced me to the field and recognized my potential when I transferred the lab as an undergraduate student. His guidance has been invaluable in my growth, and his trust gave me the confidence to move forward in the face of challenges. I feel incredibly fortunate to have been mentored by someone whose expertise, patience, and encouragement were truly inspiring. I am deeply grateful for his support and belief in me.

I sincerely thank the committee members Assoc. Prof. Yasemin Sohtorik İlkmen and Assist. Prof. Mehmet Kocatürk for their valuable time, feedback and support for the thesis.

Very special thanks go to my incredible labmates, who have filled both my undergraduate and graduate years with friendship, laughter, and unforgettable memories. Zeynep, you were there at the very beginning and I cannot imagine this journey without you. No doubt, you've been more than just a lab buddy for me; you've been a genuine friend, and working with you has been the best part of it all. Dilan and Cem, thank you for guiding me and turning me into a true lab member. I will always cherish your friendship with inspiring conversations and constant support. I am sincerely grateful for the frienship of Sinem and Defne. You both made the most mundane tasks enjoyable, and your presence added color to each lab day. And to Mert, thank you for always being there with a helping hand or a solution. I

thank Dr. Beenish Asrar for warm conversations and her guidance during sample processing. And Sude, thank you for all kinds of help and I wish we had more time to work together.

I want to thank my parents who have stood by me through the highs and lows of this journey. Your support to my every decision has been my anchor. And my lovely brother, you always manage to pull me out of my existential crises; first with your playful teasing, then by making me laugh while I'm going through it. Your presence means the world to me. Sincere thanks to my wonderful girls; Elif, Melike, Ece and Betül, for your endless support and patience, especially during the times when I disappeared into a mess of experiments and deadlines.

I also thank TÜBİTAK for financially supporting me via 2210-A Scholarship Program. This research was also supported by COST-TÜBİTAK (no: 224K875) and EMBO (no: 4432) grants to Assoc. Prof. Güneş Ünal.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AchE	Acetylcholinesterase
AF	Alexa Fluor
BLA	Basolateral nuclear complex of amygdala
BL	Basal nucleus of amygdala
Bla	Anterior division of basal nucleus of amygdala
BLp	Posterior division of basal nucleus of amygdala
ChAT	Cholinacetyltransferase
CORT	Corticosterone
CS	Conditioned stimulus
ELS	Early life stress
EPM	Elevated plus maze
ITI	Inter stimulus interval
LA	Lateral nucleus of amygdala
LHb	Lateral habenula
MHb	Medial habenula
MS	Maternal separation
NHS	Normal horse serum
OFT	Open field test
PBS	Phosphate buffered-saline
PBS-Tx	Phosphate buffered-saline TritonX
PFA	Paraformaldehyde
PND	Postnatal day
PV	Parvalbumin

PVN	Paraventricular nucleus of hypothalamus
PVT	Paraventricular nucelus of thalamus
RT	Room temperature
SPT	Sucrose preference test
US	Unconditioned stimulus
USV	Ultrasonic vocalization

CHAPTER 1

INTRODUCTION

1.1 Early life stress (ELS)

Early life adversities take a crucial role in shaping long-term behavioral and physiological processes (Cicchetti, 2010; Pollak, 2005). Particularly, severe and chronic stressful experiences in sensitive periods, such as childhood trauma and maltreatment, have deteriorating effects on later cognitive and affective functions (Pechtel & Pizzagalli, 2011). Moreover, diverse psychiatric conditions are frequently found to be related to early life stress (ELS), including major depression (Chaby et al., 2017; Colman et al., 2014), and anxiety disorders (Carr et al., 2013; Gibb et al., 2007; Hovens et al., 2010). While stress is defined as a healthy reaction with a range of physiological and behavioral responses to ensure adaptation to a challenging situation (Smith & Pollak, 2020), the long-lasting activation of the stress systems cause dysregulation of normal physiological and behavioral processes, and triggers negative psychological consequences (Frodl & O'Keane, 2013; Heim & Nemeroff, 2001). In addition to the extensive clinical research on this topic, various animal models are utilized to obtain experimental investigation, which provide detailed perspective on the neurobiological and behavioral effects of ELS.

Children may be exposed to a different types of maltreatments, such as physical, sexual and psychological abuse or a combination of these (Clemmons et al., 2007; Higgins, 2004), which often lead to a wide range of adverse experiences. A similar variety can also be obtained in rodent models of ELS. The manipulations aimed at disrupting the maternal care are widely used in the literature (Maras & Baram, 2015; Schmidt et al., 2011). Maternal separation (MS) in the neonatal period

is a common procedure of preventing mother-pup interaction for either a single or repeated period of time (Ellenbroek et al., 1998; Enthoven et al., 2008; Maras & Baram, 2015; Figure 1). Another way to intervene maternal care is to complicate the nesting environment with limited bedding, which naturally changes the behavior of the mother (Maras & Baram, 2015; Schmidt et al., 2011; Figure 1). In addition, several studies utilize different stressors such as inescapable foot shocks (Moriceau, 2009; Perry et al., 2025) and predator odor exposure as a form of ELS (Chen et al., 2014; Cuarenta et al., 2021). These studies mimic different forms of early life adversities in humans, contributing to the rich literature on the experimental outcomes of ELS on affective processes in animals.

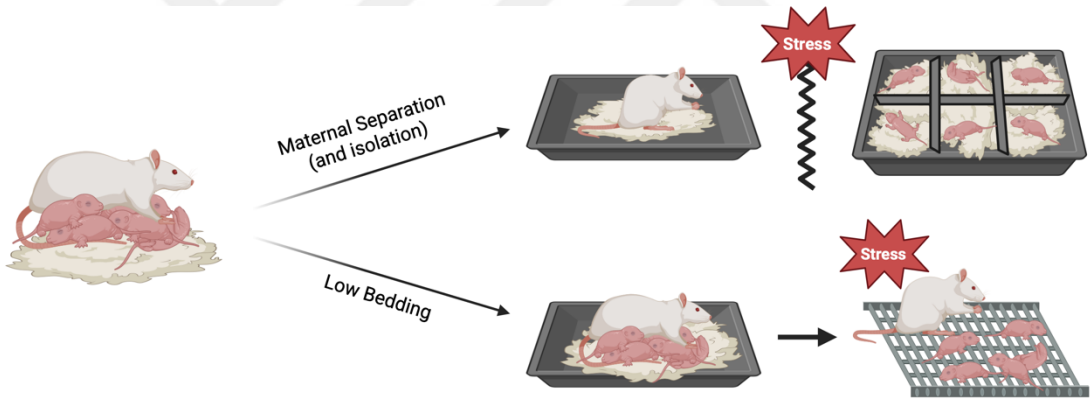

Figure 1. Rodent models of ELS targeting mother-pup interaction.

1.2 Neurobiological and behavioral effects of maternal separation (MS)

MS triggers the HPA axis in early life, a period characterized by stress hyporesponsiveness (Levine, 2001), and leads to hyperactivation of the stress-related systems (Ladd et al., 1996; McCormick et al., 1998). Although its impact on basal corticosterone (CORT) levels is reported to be variable in the literature (Lajud et al., 2012; Pinheiro et al., 2011; Veenema & Neumann, 2009), several studies showed that

animals exposed to MS display higher CORT release during stress in adulthood (Lajud et al., 2012; Lehmann et al., 2002; Plotsky et al., 2005). In similar conditions, a number of studies reported higher levels of neural activation in the paraventricular nucleus of hypothalamus (PVN), the nucleus triggering the HPA axis (Fóscolo et al., 2022; Sanders & Anticevic, 2007; Trujillo et al., 2016). These observations, however, could not be supported in some other studies (Banihashemi et al., 2011; Renard et al., 2010).

The aforementioned mechanisms are regulated by several limbic structures, including thalamic and amygdaloid nuclei (Feldman et al., 1995; Herman et al., 2020; Ziegler & Herman, 2002), some of which also indicate altered neural activity patterns by MS. For instance, the central and basolateral nuclei of amygdala (BLA), which are well-associated with fear processes and response (Duvarci & Pare, 2014; LeDoux, 2000; Ressler & Maren, 2019), show altered activity in either basal conditions or following acute stress when the animals were previously exposed to MS (Qin et al., 2021; Sanders & Anticevic, 2007). Similarly, the neural activity in the paraventricular nucleus of thalamus (PVT) reduced in response to acute restraint stress following chronic variable stressors (Radley & Sawchenko, 2015), low maternal care (Spencer & Tilbrook, 2009) and MS (James et al., 2014). In addition, the monoamine systems affected by ELS and implicated in depression (Elhwuegi, 2004; Heim & Nemeroff, 2001) are regulated by the medial (MHb) and lateral divisions of habenula (LHb) (Aizawa et al., 2012; Cameron et al., 2024), limbic structures associated with encoding of aversive experiences (Chastrette et al., 1991; Nagao et al., 1993; Webster et al., 2023). Particularly, the hyperactivation of LHb is reported in various stress models including MS (Lecca et al., 2016; B. Li et al., 2011; Tchenio et al., 2017), while experimentally activating LHb neurons are shown to be

sufficient to induce depressive-like symptoms (Stamatakis & Stuber, 2012; Yang et al., 2018). Moreover, a recent study highlighted MS-induced sensitization in LHb neurons in response to an acute stressor in adulthood, contrary to the hypofunction reported at rest (Webster et al., 2023). Taken together, these findings point to the variety in long-term neurobiological influences of MS, and the importance of further investigation into these mechanisms in relation to its behavioral influences.

The deteriorating effects of MS particularly on affective processes were previously highlighted by different rodent behavioral paradigms. In brief, MS increased immobility in the forced swim test (Lambás-Señas et al., 2009; Lee et al., 2007) while decreasing the natural preference of the rodents for sucrose in several studies (Frank et al., 2019; Huot et al., 2001; Maniam & Morris, 2010), indicating behavioral despair and anhedonia, respectively (Porsolt et al., 1978; Scheggi et al., 2018; Unal & Canbeyli, 2019). Similarly, anxiety manifested itself in MS animals through spending more time in the closed arms of the elevated plus maze (Frank et al., 2019; Huot et al., 2001; Lee et al., 2007; Maniam & Morris, 2010), but an opposite effect was also noted by other studies (León Rodríguez & Dueñas, 2013; Ploj et al., 2002). This may emphasize the other potential factors influencing animal behavior in this test (Jaimes-Hoy et al., 2016; Wang et al., 2020).

The behavioral effects of MS can be also extended to rodent social paradigms in both adolescence and adulthood. Decreased social play behavior in adolescence was observed in animals subjected to predator odor in infancy (Cuarenta et al., 2021) or a single 24h-long MS (Kentrop et al., 2018), in contrast to the increase in play behaviors in males induced by repeated MS applications (Lundberg et al., 2017; Veenema & Neumann, 2009), where both changes may associate with later stress-coping behaviors (Granata et al., 2022; Veenema & Neumann, 2009). A similar

impairment was also observed in adulthood through decreased sociability in MS animals (Gazzo et al., 2021; Mansouri et al., 2021), while sex-specific effects were noted in the literature. Male animals exposed to MS displayed more aggressive social contacts in adulthood (Mavrenkova et al., 2023), whereas a brief separation was sufficient to decrease sociability in females (McClafferty et al., 2024).

Lastly, the impact of MS on adult fear learning has also yielded different results. It elevated conditioned fear response in adulthood assessed via auditory and contextual learning paradigms (Diehl et al., 2014; Mishra et al., 2019; Sampath et al., 2014; Toda et al., 2014; Wang et al., 2018), and also increased fear generalization to a novel tone and context (Elliott & Richardson, 2019; Sampath et al., 2014). However, these findings were conflicted by the reports of decreased freezing response by MS animals in fear retrieval sessions and facilitated extinction paradigms (Chocyk et al., 2014; Guijarro et al., 2007; Kosten et al., 2005; Lehmann et al., 1999; Mishra et al., 2019; Sanguino-Gómez & Krugers, 2024; Shin & Lee, 2023; Sun et al., 2014). Altogether, these reports of bidirectional MS-induced effects highlight the necessity of utilizing multiple assessments to comprehensively study the neurobiological and behavioral alterations by MS.

1.3 Rodent ultrasonic vocalizations (USVs)

Rodents emit USVs above the hearing threshold of humans (~20 kHz), which were previously recorded in several species (Blanchard et al., 1990; Ehret, 2005; Faure et al., 2017; Hennessy et al., 2006; Simola & Brudzynski, 2018). Rat USVs are classified into three major categories based on their morphometric features and the context they are recorded (Wöhr & Schwarting, 2013), as visualized in Figure 2 (Lenell et al., 2021).

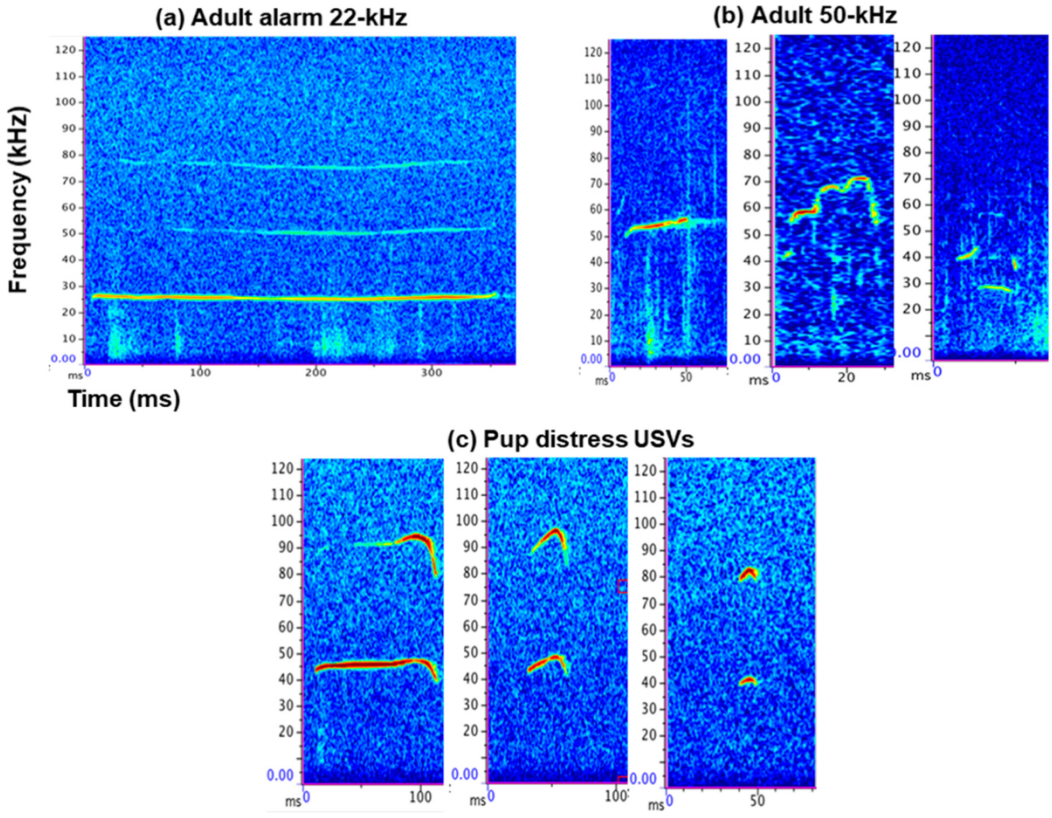

Figure 2. Spectrograms of the three types of rat USVs. Adult (a) aversive USVs in ~22 kHz, (b) appetitive calls around 50 kHz and (c) isolation induced pup USVs. Please refer Lenell et al. (2021) as the figure source.

The first type is the ~22 kHz alarm calls emitted in aversive situations by adult rats, such as in the contexts of anxiety and fear (Brudzynski, 2019; Wöhr & Schwarting, 2013). Rats produce these calls when they are exposed to predator odor (Bedoya-Pérez et al., 2025; Blanchard et al., 1990; Fendt et al., 2018) and following fear conditioning in laboratories (Wöhr et al., 2005; Wöhr & Schwarting, 2008; Zheng et al., 2025). In addition, the USV emission in these paradigms are modulated by the anxiety levels of the animals (Zheng et al., 2025), further supporting the investigation of rodent USVs as a signature of the animals' affective state (Brudzynski, 2019).

The second class of rat USVs is the 50 kHz calls emitted in appetitive conditions such as social play (Knutson et al., 1998; Lukas & Wöhr, 2015), mating (Sales, 1972; Thomas & Barfield, 1985) and in response to tickling by the experimenter (Panksepp & Burgdorf, 2000). Similarly, the animals isolated prior to the tickling produced more appetitive calls during the test phase indicating the modulatory role of social motivation in play-related 50 kHz USVs (Panksepp & Burgdorf, 2000).

The third type of USV is only emitted by pups when they are removed from the nest (Hofer et al., 1993). These have a frequency range of 30-70 kHz, but often referred as 40 kHz calls (Shair, 2018). These vocalizations are considered as significant communicative signals since they trigger maternal care (Allin & Banks, 1972; Brunelli et al., 1994; Smotherman et al., 1974). A very early study in mice reported that mothers specifically search for pups that emit these vocalizations, but not for those anesthetized and unable to vocalize (Zippelius & Schleidt, 1956). Furthermore, mothers also respond to playback calls of separated pups but not other sounds (Sewell, 1970), highlighting the survival role of USV emission for the infants in natural conditions. The rate of these vocalizations follows a curved pattern across the neonatal days, with a higher rate in the middle and decreasing to zero around 18-23 days (Allin & Banks, 1972; Hofer et al., 1998; Shair, 2018). And other environmental conditions of the test environment, such as the temperature, also affect these vocalizations and the rate of emission across days (Allin & Banks, 1972; Hofer et al., 1998; Shair, 2018).

1.4 Infant USVs and ELS

The interpretation of individual variability in separation-induced infant USVs remained as a challenge. At first, the rate of pup USVs was considered as an

indicator of anxiety levels (Winslow & Insel, 1991). Therefore, various studies have tested the potential of pharmacological treatments with anxiolytic and antidepressant effects on these vocalizations (Hodgson et al., 2008; Kehne et al., 2000; Olivier et al., 1998). These reports indicated that the call rates decrease with benzodiazepines, serotonin receptor agonists or reuptake inhibitors, as well as corticotropin-releasing factor receptor antagonists (Hodgson et al., 2007; Kehne et al., 2000). Additionally, Zimmerberg et al. (2005) tested the anxiety and depression-like behavior of animals bred for emitting low levels of USV in infancy and showed that they exhibited less of such behaviors in adolescence compared to high-USV line. In another study, isolation call rates predicted the freezing response to a conditioned stimulus in late-infancy (Harmon-Jones et al., 2020). However, pup USV calls may also be interpreted as an active coping mechanism in response to the distress caused by the mother's absence, therefore differing from the passive behavior assessments (e.g. freezing) in adulthood (Cordeiro et al., 2024). This is further supported by the recent reports on separation calls that negatively correlate with adult anxiety-like behavior in the elevated plus maze (Cordeiro et al., 2024) as well as freezing and USV emissions during fear conditioning in adulthood (Wöhr & Schwarting, 2008), while predicting higher social play in adolescence (Granata et al., 2022). Altogether, these studies point to the potential for infant USVs to serve as an indicator of later affective states.

The number of USV emission decrease in rat pups subjected to an acute 4h MS (Zimmerberg et al., 2003) or low bedding ELS (Granata et al., 2022). However, repeated exposure to MS in different studies resulted in elevated infant USV rates (Burenkova et al., 2020; Ploj et al., 2003; Yin et al., 2016). Pups produce higher rates of USVs after a brief exposure to their litter, referred as maternal potentiation

(Brunelli et al., 1998; Hofer et al., 1998). This increase is considered to be a sign of the infant bond to the mother (Shair, 2007), indicating a different affective aspect. However, only a single study investigated the effects of MS on USV emission of pups in a maternal potentiation paradigm (Kaidbey et al., 2019). MS increased USV calls in the second isolation period (Kaidbey et al., 2019). In addition to the lack of sufficient number of reports on the MS-induced USV changes in this paradigm, no study has examined the potentiated vocalizations in relation to later social and affective measurements.

1.5 Present study

The present study aims to investigate the long-term effects of an early MS on pup USVs as well as several affective processes in adolescence and adulthood, concentrating on the predictive potential of infant calls in the maternal potentiation paradim for the subsequent behavioral and neurobiological assessments. To achieve this, I implemented a longitudinal paradigm starting with maternal potentiation USV test in infancy. In adolescence, open field test (OFT), elevated plus maze (EPM) and social play tests were conducted. The same tests were performed in adulthood with a slight modification to test sociability instead of social play, in addition to sucrose preference test (SPT) and auditory fear conditioning paradigm with USV recordings. At the end of behavioral testing, I measured serum CORT levels and neuronal activation markers in the PVN, PVT, BLA and habenular nuclei following acute restraint stress. Eventually, I tested the predictive value of infant USVs on behavioral and neurobiological assessments through path and multiple regression analyses.

CHAPTER 2

METHODS

2.1 Subjects

Animals were housed under standard laboratory conditions with controlled temperature (21 ± 1°C), humidity (40-60%) and equal day/night pattern (12:12 hours) with lights turned on at 08:00. All animals had *ad libitum* access to food and water, and all the procedures were conducted in day time. Four pregnant Wistar rats housed individually untill parturition (postnatal day 0, PND 0). Cages were randomly attributed to control or experimental conditions. Each group contained 16 pups (8 male, 8 female) selected by checking their weights and ensuring a balanced sex ditribution across groups. The offspring was weaned on PND 21 and housed in groups of four. All the experimental procedures were approved by the Boğaziçi University Ethics Committee for the Use of Animals in Experiments (Appendix A).

2.2 Experimental design

Maternal separation procedure was carried out between PND 2 and 14, followed by the potentiation USV test on PND 15 (Figure 3). The pups were left undistrubed for two weeks between weaning and adolescence tests (PND 35–36). On the first day, the OFT and EPM were conducted, during the first and second half of the day time, respectively. Animals underwent the social play test on PND 36, in which the free interaction of pairs was examined. Afterwards, no intervention was made to the animals except the weekly cage cleaning and renewal of tail marks. On PND 80, behavioral tests in adulthood has began with OFT and EPM. Next day, sociability test was conducted where the approach behavior of the animals to a chamber which

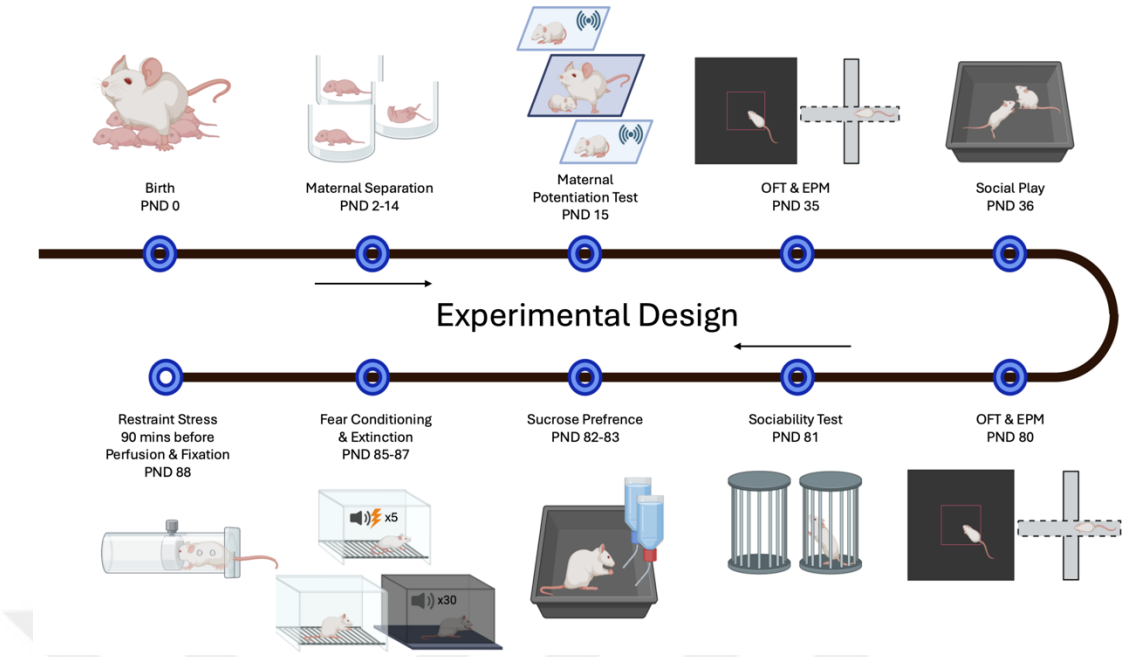

Figure 3. The timeline of the experimental procedures.

was empty vs. containing a rat was evaluated. I conducted a SPT for 48 hours in individual cages on PND 82 and 83. After a day of break, animals turned back to their group cages and tested in a three-day fear conditioning paradigm (PND 85–87), including a context exposure and a cued extinction following auditory fear acquisition. On the last day, animals were exposed to a restraint stress, during which the blood samples were collected for CORT analysis, approximately 90 mins prior to perfusion and fixation procedure to investigate neuronal activation in several limbic regions following acute stress. I captured USVs during maternal potentiation and fear conditioning phases, and all the behavioral tests were recorded by a video camera (Sony FDR-AX100E or HDR-CX405) for further analysis. I used the freezing module of EzTrack (Pennington et al., 2019) for fear conditioning analysis and Ethovision XT 17 (Noldus, Netherlands) for tracking the animals to obtain their activity in the OFT, EPM and sociability tests. Illustrative figures were created in BioRender.

2.3 MS procedure

Maternal separation and isolation procedure was carried out between the PND 2 and 14 for 3 hours a day, and the timing of MS randomly changed within day time (08:00 am – 08:00 pm). I first removed the mothers from the nest, placed them in a transient cage moved to another room to prevent any olfactory or auditory cue. I captured 3 mins of USV of each pup at the beginning of their isolation, to be used in another study. Then, I labeled pups with a non-toxic marker to differentiate them for tracking their weight and USV recordings, which applied as a tail mark after PND 9. I placed each pup to individual cups (50 mm base diameter, 70 mm height) with fresh bedding and placed them in an incubator set at 32-34°C (Plotsky et al., 2005) to maintain necessary temperature until PND 10 (Figure 4). Between PND 11 and 14, pups were separated to individual cages in room temperature (RT, 21 ± 1°C). When 3 hours of isolation was completed, the pups were culled together, and the mother was transferred to the litter cage. Control group was left undisturbed during this time except the brief handling applied for three days between PND 12-14, to eliminate the handling stress during the maternal potentiation test. The mother was transferred to another cage and pups were handled for 1 min before their mom was placed back.

Figure 4. Photographs showing pups exposed to MS on PND 2.

2.4 Behavioral assessments

2.4.1 Maternal potentiation USV test

I used a maternal potentiation protocol adapted from Branchi et al. (2006), with three experimental sessions, each lasting 3 mins. The pups in the same cage tested one after another. At the beginning, the mother was transferred to a big novel cage and remained there untill the all the pups were tested. USV and video recordings were immediately started when an infant was placed in the test cage (pre-mom session). After 3 mins, the mother cage is brought to the room and the pup was moved to that cage, initiating the reunion phase, enabling 3 mins of mother-pup interaction. At the end of reunion, the pup was placed back to the first isolation cage and the mother was moved from the test room, which immediately starts the third session (post-mom). When the three sessions were completed, the pup isolation cage was cleaned with 70% ethanol prior to the testing of another pup from the same nest.

2.4.2 Open field test

A white opaque square arena (45 x 45 x 45 cm) was used as an open field in adolescence on PND 35 (Figure 5). I checked the temperature and humidity of the test room to ensure standard conditions for each day of behavioral testing. The animals were brought to the test room in individual cages to enable 30 mins of acclimation. The OFT was conducted under dim light and lasted for 10 minutes. Animals were gently placed to the center of the open field and recorded untill the end of the session. The same procedure was repeated for the OFT in adulthood on PND 80, except that the test arena was changed to a black opaque square (70 x 70 x 45 cm; Figure 5). The maze was cleaned with 70% ethanol or isopropanol between each session to remove any olfactory cue of the previous test animal.

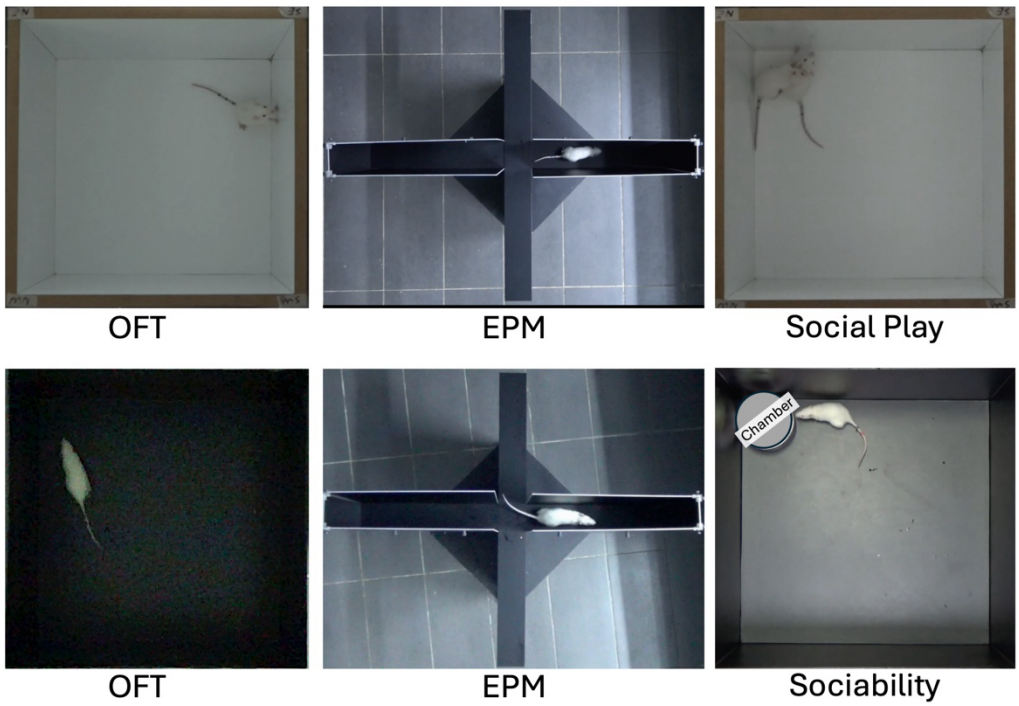

Figure 5. The OFT, EPM and social behavior tests conducted in adolescence (top) and adulthood (bottom).

The video recordings were analyzed in Ethovision XT 17 (Noldus, Netherlands). Locomotor activity levels were measured as total distance traveled (cm) in the arena, with a 2 cm-smoothing applied to fix jittering. A center area was virtually defined (~40 x 40 cm), and the time that animals spent in this area (s) was also examined to assess the exploratory and anxiety-like behavior (Valle, 1970).

2.4.3 Elevated plus maze

A plus shaped black apparatus with two open and two enclosed arms, elevated 70 cm from the floor, was utilized for EPM in both adolescence (PND 35) and adulthood (PND 80; Figure 5). The arm length was 50 cm while the width was 10 cm in each arm. Animals were acclimated to the test environment for 30 mins between the transfer to the test room in individual cages and the beginning of the test. The test was carried out under dim light, and the maze was positioned in a way that the open

arms had higher illumination. The test animal was gently placed to the center of the arms and allowed 10 mins to explore the maze. The maze was cleaned with 70% ethanol or isopropanol between the sessions. The videos were analyzed via Ethovision to obtain the locomotor activity in the maze (traveled distance in cm, 2 cm smoothed) and the time animals spent in open vs. closed arms of the maze (s) to assess exploratory activity and anxiey-like behavior (Walf & Frye, 2007).

2.4.4 Social play

Social play was conducted in the open field arena to ensure that the animals were familiar with the test environment (PND 36; Figure 5). In addition, they were isolated for 3 hours before testing, to increase the motivation to engage in social play (Vanderschuren et al., 1995). Again, animals were transferred to the test room in individual cages 15 mins prior to the session. Afterwards, same-sex and same-cage animals were tested in pairs, ensuring that the weight difference was not more than 10 grams. Animals were placed to the test arena one after the other, and had 15 mins before the session ends. The video recordings were analyzed through the manual scoring in Ethovision for the defined play behaviors adapted from Meaney and Stewart (1981) and sniffing as non-playful social exploration. The pouncing behavior was counted when one animal approaches to the neck of the other aiming to initiate a play. The pinning was defined as biting with two paws placed on the other animal. Supine behavior was defined as the instances of lying on the back and opening the ventral side to the partner to maintain the play. The frequency of each play type and the latency to exhibit that behavior was analyzed in addition to the same parameters for sniffing.

2.4.5 Sociability test

The sociability test was conducted on PND 81 using the open field arena utilized in adulthood to present a familiar environment for the animals, except for the increased lighting (Figure 5). This test aimed to assess the social preference of the animals in adulthood. I implemented an adapted version of Jacqueline and Crawley's (2007) protocol designed for mice. The procedure consisted of two sessions, each lasting for 10 mins. In the first session, the animals were allowed to explore the arena including an empty grey circular chamber (15 cm in diameter) placed in one corner. In the second session, animals underwent the same procedure but the chamber in the corner contained a social agent, a same-sex same-age unfamiliar rat. While the video recordings were analyzed, a circular area around the chamber (extending 10 cm from the borders of the chamber) was defined as the interaction zone in Ethovision. In addition, the square quarter including the chamber was also defined as the target quarter. The locomotor activity of the animals in the maze through distance traveled (cm), the entries to and the time spent in the defined zones were analyzed.

2.4.6 Sucrose preference test

Hedonic responses of the animals was measured on PND 82–83 through a 48 hour SPT. Animals were housed in individual cages and two bottles of water were presented *ad libitum*. One of the bottles was filled with 1% sucrose water while the other contained regular tap water. The waters were renewed and the place of the bottles was counterbalanced at the end of the first day. The liquid amounts in the bottles was measured twice a day. I analyzed the sucrose water consumption and total liquid consumption over 48 hours, as well as the sucrose preference through the ratio of sucrose water consumption to total liquid consumption.

2.4.7 Fear conditioning

An auditory fear conditioning procedure was carried out including fear acquisition (Context A; PND 85), context exposure (Context A; PND 86) and a cued extinction in a different context (Context B; PND 87; Figure 6). The conditioning chamber (21 x 45 x 27 cm; Figure 6) was composed of transparent acrylic walls and 32 metal grid at the bottom to provide electrical foot shock. Animals were acclimed to the test environment for 5 mins prior to each session. On PND 85, they were placed to the chamber and given 3 mins before the first sound presentation to test the baseline freezing levels. Afterwards, 5 pairings of tone (10 s, 80 dB, 8 kHz, conditioned stimulus, CS) and a co-terminating shock (2 s, 1.0 mA, unconditioned stimulus, US) were presented. The inter trial interval (ITI) was randomly generated with a range of 50-70 secs. After the last pairing, animals were given another minute before being gently transferred to home cage. USV recordings were captured in each session.

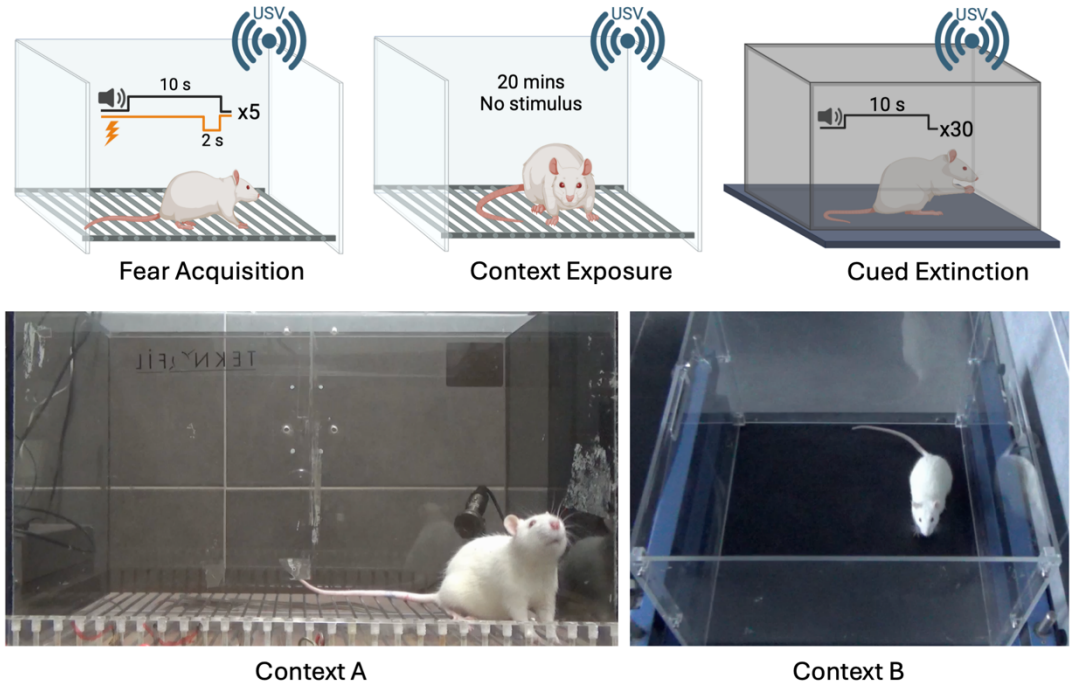

Figure 6. Fear conditioning procedure (top) and the two experimental chambers: Context A and Context B (bottom).

Exposure to the conditioning context was applied for 20 mins on PND 86, during which no US or CS were presented, to assess the contextual fear response of the animals (Figure 6). After 24 hours, cued extinction was conducted in another chamber (41 x 43 x 31; Context B; Figure 6), with black opaque floor and transparent walls. The chamber was located in a different part of the room and the session was carried out under dim light, in order to provide visual differences. Animals had a three mins baseline period in the chamber without any stimulus, similar to the conditioning phase. Thirty CSs followed the baseline with a 30 secs fixed ITI to test the tone-associated fear response. The chambers were cleaned with 70% ethanol between each session to prevent any olfactory cue remained by the previous animal. The conditioned response was analyzed with the freezing module of EzTrack (Pennington et al., 2019) for each session, and the active coping behaviors such as jumping and darting in response to the foot shock in the acquisition was counted manually. Context exposure was divided into 5-mins blocks, while the freezing response to three CSs were blocked in the cued extinction session and analyzed accordingly.

2.4.8 USV analysis

An UltraSoundGate 116 USB audio device was utilized to capture USVs in experiments (Avisoft Bioacoustics, Glienicke, Germany), which was connected to a computer and an ultrasound microphone placed approximately 15 cm above the mazes. I monitored the vocalizations during the experimental sessions through an Avisoft Recorder (Avisoft Bioacoustics, version 4.2) and analyzed the calls with DeepSqueak 2.0 software (Coffey et al., 2019) run with MATLAB.

2.5 Neurobiological assessments

2.5.1 Sample and tissue preparation

All animals were placed into restrainers (cylindirical plexiglass tubes with 10 cm diameter) at the end of behavioral testing. They stayed in the restrainers for 20 minutes, and blood samples (~400 µl) were collected via tail clips in the second half of this period. The samples were kept 30–45 mins in RT (21 ± 1°C) to ensure clotting before being centrifuged for 15 mins (Dlab D1008 microcentrifuge, 7000 rpm, RT). Then, the supernatant was carefully placed into a clean tube and stored in -80°C for subsequent processes.

Animals were transiently anesthesized with isofluorane, followed by IP injection of a 100 mg/kg ketamine-10 mg/kg xylazine mixture for terminal anesthesia. The lost of foot and eye reflexes was confirmed before the perfusion and fixation procedure has started. The animals were transcardially administered 0.9% saline and 4% paraformaldehyde (PFA) via a peristaltic pump, 90 mins after the restraint stress. The removed brain tissues were kept in PFA for 2 more days for post-fixation and the solution was then replaced with phosphate-buffered saline (PBS) with azide. Afterwards, the tissues were sliced using a vibratome (Leica, VT1000S) into coronal sections in 50 µm thickness (Figure 7). The target locations were detected utilizing a rat brain atlas (Paxinos & Watson, 2007) and the sections were stored in PBS-azide for further processing.

2.5.2 Corticosterone analysis

I assessed the corticosterone levels in the serum samples stored at -80°C via an ELISA kit (Cayman Chemical, 501320). To identify different levels of CORT in the samples, the kit utilizes the competition between CORT found in medium and

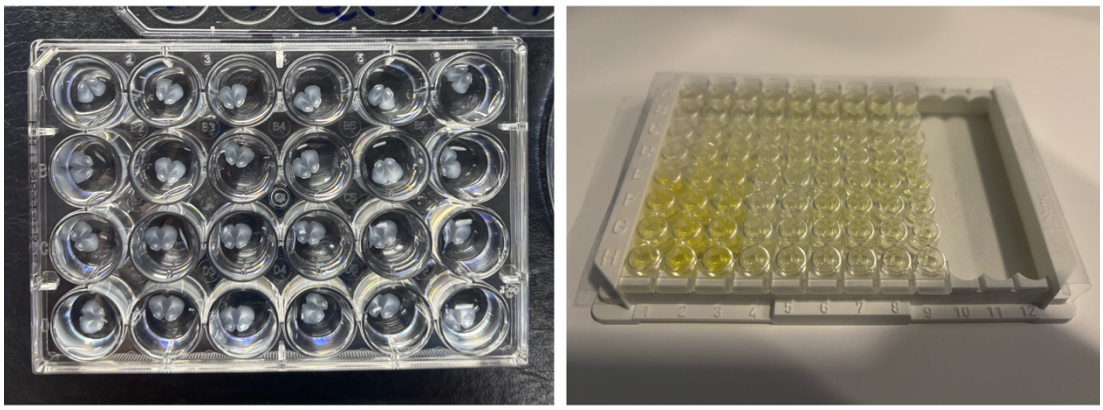

Figure 7. Coronal brain sections (left) and ELISA well plate (right).

CORT-acetylcholinesterase (AChE) conjugate with a certain concentration to bind the antiserum (Pradelles et al., 1985). Therefore, if a sample includes high levels of CORT, the binding of CORT-AChE to the serum will be less compared to another sample with a lower CORT amount. To test the CORT levels in my samples, I followed the manufacturer's instructions while conducting the analysis. Briefly, I prepared the standards through a serial dilution of CORT ELISA standard provided in the kit. At the end, I obtained 8 dilutions of standards from 8.2 to 5000 pg/ml and placed these solutions to the second and third columns of the 96-well plate, while the first column included controls such as minimum (blank) and maximum levels of activity (Figure 7). Starting from the 4th column, I placed the samples (50 µl each) in duplicates and removed the last three columns that I did not use in this analysis. Afterwards, I added 50 µl of CORT-AChE solution and 50 µl of antiserum to each well prior to an overnight incubation of the plate in 4°C. Next day, the plate was washed 5 times with buffer solution and the 200 µl of Ellman's Reagent was added to the wells to obtain a yellow color as a result of the enzymatic reaction with AChE. Following 2 hours of development, the plate was read under 412 nm via a microplate reader (Multiskan Go, ThermoFisher) to assess the absorbance of each sample. In

Figure 7, it is seen that the color intensity of the lower wells in the 2nd and 3rd columns, including the standards with lower CORT concentrations, is higher than the upper wells with higher concentration, supporting the expected enzymatic activity of Ellman's Reagent and higher levels of CORT-AChE. I calculated the CORT concentration of each well using the manufacturer's spreadsheet and verified the curve of standards. I excluded one animal from each group in the analysis, with a value greater than 3 standard deviations from the mean.

2.5.3 Immunofluorescence and cell counting

I performed c-Fos immunofuorescence on the selected sections to examine neuronal activation during restraint stress. For each target region, I processed two sections to obtain data from four hemispheres, and used the averaged data for each animal. The sections of BLA, habenula and PVT were taken between the Bregma points of -3.00 and -3.36 (Paxinos & Watson, 2007), while the sections between -1.72 and -2.00 Bregma points were processed for PVN. The sections were washed in PBS-Triton X (PBS-Tx) for 10 mins three times to enable tissue penetration. Then, I used 10% normal horse serum (NHS) in PBS-Tx for blocking which lasted an hour in RT. The primary incubation was carried out with Guinea-pig c-Fos monoclonal antibody (Synaptic Systems, 226308) with a 1:5000 dilution in PBS-Tx. The incubation solution also included 1% NHS. The sections were incubated for two days in 4°C and then washed again for 3x10 mins with PBS-Tx. The secondary incubation solution included a donkey anti-guinea pig antibody (Jackson Immunoresearch, 2340472, Alexa Fluor (AF) 488) with 1:500 dilution in PBS-Tx and 1% NHS. Then, the sections were exposed to another washing procedure before mounting for microscopic observation. I also performed another procedure for choline

acetyltransferase (ChAT) and parvalbumin (PV) to obtain sections as reference for the BLA nuclei identification. For those, I used the same procedure with the primary antibodies of goat anti-ChAT (Merck-Millipore, AB144P, 1:250) and rabbit anti-PV (Abcam, 11427, 1:2000); with a combination of the secondary antibodies as donkey anti-goat (1:250, Abcam, 150134, AF 555) and donkey anti-rabbit (1:250, Abcam, 150073, AF 488). I used an epifluorescence microscope (Olympus BX53) with a monochrome CCD camera (Olympus XM10) to obtain photographs of processed sections through cellSens (Olympus LS, MIA module) and the immunopositive cells were semi-automatically counted using Cellpose (Stringer et al., 2021).

2.6 Statistical analysis

I conducted the statistical analyses and produced graphs in R (version 4.3.1). For group-level comparisons, I used independent-samples *t*-test or analysis of variance (ANOVA) including sex as another variable. When the behavioral assessments pointed a sex-related effect, I reported a 2x2 (Group x Sex; using aov() function in base R) or 2x2xA ANOVA (Group x Sex x Session; using aov_car() fuction with Type III sums of squares in afex package). For higher levels of within group conditions in mixed ANOVAs (e.g., fear conditioning sessions), aov() function was utilized given the balanced nature of tha data. Independent samples t-tests were reported when no sex effect was observed, and paired samples t-tests were performed for within group analysis to compare variables such as baseline freezing levels in fear acquisition and context exposure. The USV numbers of an MS animal was an outlier in the post-mom period of maternal potentiation paradigm, with a greater value than 3 standard deviations from the mean. It was Winsorized to the 95th percentile in the total calls, and the first and second bins data.

To assess the predictive role of pup USVs for behavioral and biological measurements, I conducted path and multiple regression analyses. These analses included both the collected data and some merge variables. In brief, I directly used the number of infant calls in the post-mom potentiation session, the time they spent in the open arms of EPM, sucrose preference in terms of calculated index, CORT levels and immunopositive cell density in PVN and PVT. On the other hand, I used the sum of the three play behaviors (pouncing, pinning and supine) as social play data, the increase in the time spent in defined interaction zone across sessions as sociability index, the total of jumping and darting instances in response to shock as active coping, the average of freezing levels in the context exposure and cued extinction as fear memory based on the positive correlation between these variables ($r = 0.51$, $p = 0.003$), average c-Fos density in the lateral (LA) and basal amygdaloid nuclei (BL) ($r = 0.69$, $p < 0.001$) as well as in MHb and LHb ($r = 0.57$, $p = 0.002$).

There was no missing value for the behavioral measurements except the freezing data of a single animal in the cued extinction session. For that animal, I directly used the contextual freezing value instead of the average of two values. I first conducted a partial correlation analysis including the behavioral assessments in adolescence and adulthood with the infant USV data, and added group and sex as additional variables to control for their effect ($n = 32$). Further, I conducted a path analysis, as an extension of multiple regression for detailed and complex models (Streiner, 2005), to test the relations of these variables via sem() function of lavaan() package in R. The path diagram in Figure 8 illustrates all the hypothesized relations defined in the model, with sex included as a predictor to control for its effects. The model was estimated using maximum likelihood and tested for control and MS group separately ($n = 16$) in order to obtain a comparative investigation of the relationships.

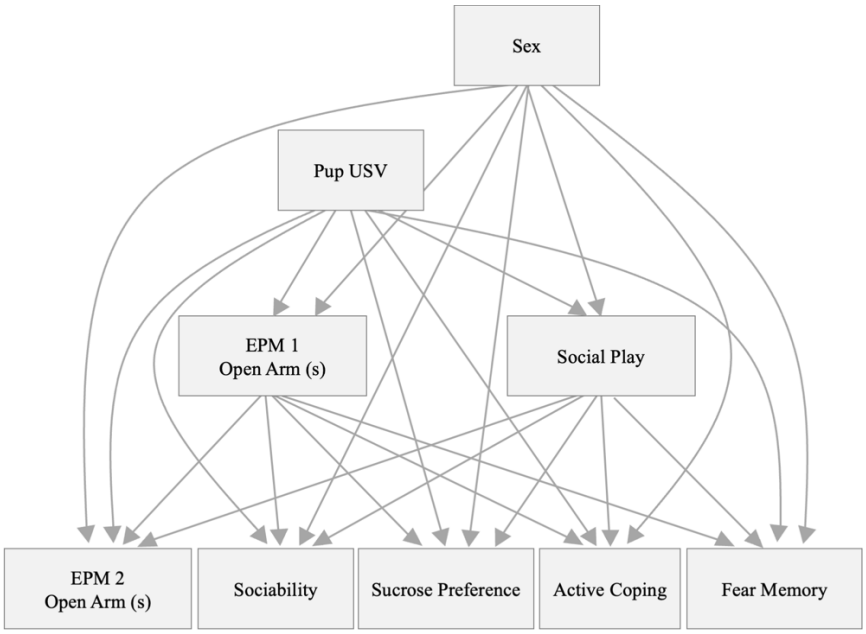

Figure 8. The diagram of hypothesized relations in the path analysis.

Before conducting the analysis, I scaled the data to a mean of 0 with a standard deviation of 1, to have similar levels of variance in different measurements. When I checked the assumptions of regression analysis for each group, I observed slight deviations in the sucrose preference of control and USV calls in the MS group. Then, I applied bootstrapping (5000 resamples) to make the estimation of *p* values and effect sizes more robust to address these violations and the small sample size. In addition, I applied a regularization penalty at a minimum level ($\lambda = 0.001$) to obtain more stabilized estimation of the coefficients.

I conducted multiple regression analyses to examine the predictive value of infant USVs, EPM and social play in adolescence on the neurobiological assessments during acute stress in adulthood. These analyses were conducted for all animals, however the group was added as the second predictor to test any difference between MS and control groups. I used scaled data similar to the path analyses, and checked the assumptions via residual and Q-Q plots, normality tests and GVIF, while the missing values excluded listwise when they are in the outcome variable.

CHAPTER 3

RESULTS

3.1 Behavioral test results

3.1.1 Maternal potentiation USVs

The potentiation test consisted of two isolation periods and a reunion with the mother in between. The reunion induced significant changes in the USVs of infants (Figure 9A). Particularly, the frequency of the calls decreased in the second session and turned into 40 kHz calls ($F(1, 15) = 67.467$, $p < 0.001$, $\eta 2_p = 0.54$, 2x2 two-way mixed ANOVA; Figure 9A-B), and the number of calls increased ($F(1, 30) = 44.964$, $p < 0.001$, $\eta 2_p = 0.60$, 2x2 two-way mixed ANOVA; Figure 9A, C). MS did not affect either the frequency ($F(1, 15) = 0.676$, $p = 0.424$) or the USV number ($F(1, 30) = 0.426$, $p = 0.519$), and the interaction between group and session was also not significant for the call frequency ($F(1, 15) = 1.044$, $p = 0.323$), and the number ($F(1, 30) = 1.851$, $p = 0.184$).

In order to analyze the temporal changes within the post-mom session, in which the USV emission was higher, I further divided the call numbers into three single minutes (bins), and found that the call number decreases across bins ($F(2, 60) = 15.784$, $p < 0.001$, $\eta 2_p = 0.34$, 3x2 two-way mixed ANOVA; Figure 9D). However, no effect of MS ($F(1, 30) = 2.668$, $p = 0.113$) and no interaction effect was observed ($F(2, 60) = 1.825$, $p = 0.170$). Notably, the decrease in the control group was detected between the first ($M = 41.56$, $SD = 23.39$) and second bins ($M = 28.75$, $SD = 23.72$; $t(30) = 3.245$, $p = 0.008$, Cohen's $d = 0.544$, Tukey corrected t-test; Figure 9D), but not between the second and third bins ($M = 28.00$, $SD = 21.73$; $t(30) = 0.195$, $p = 0.978$, Tukey corrected t-test). On the other hand, MS animals exhibited this decrease between the second ($M = 26.69$, $SD = 38.62$) and third bins (M = 12.19, SD = 13.99;

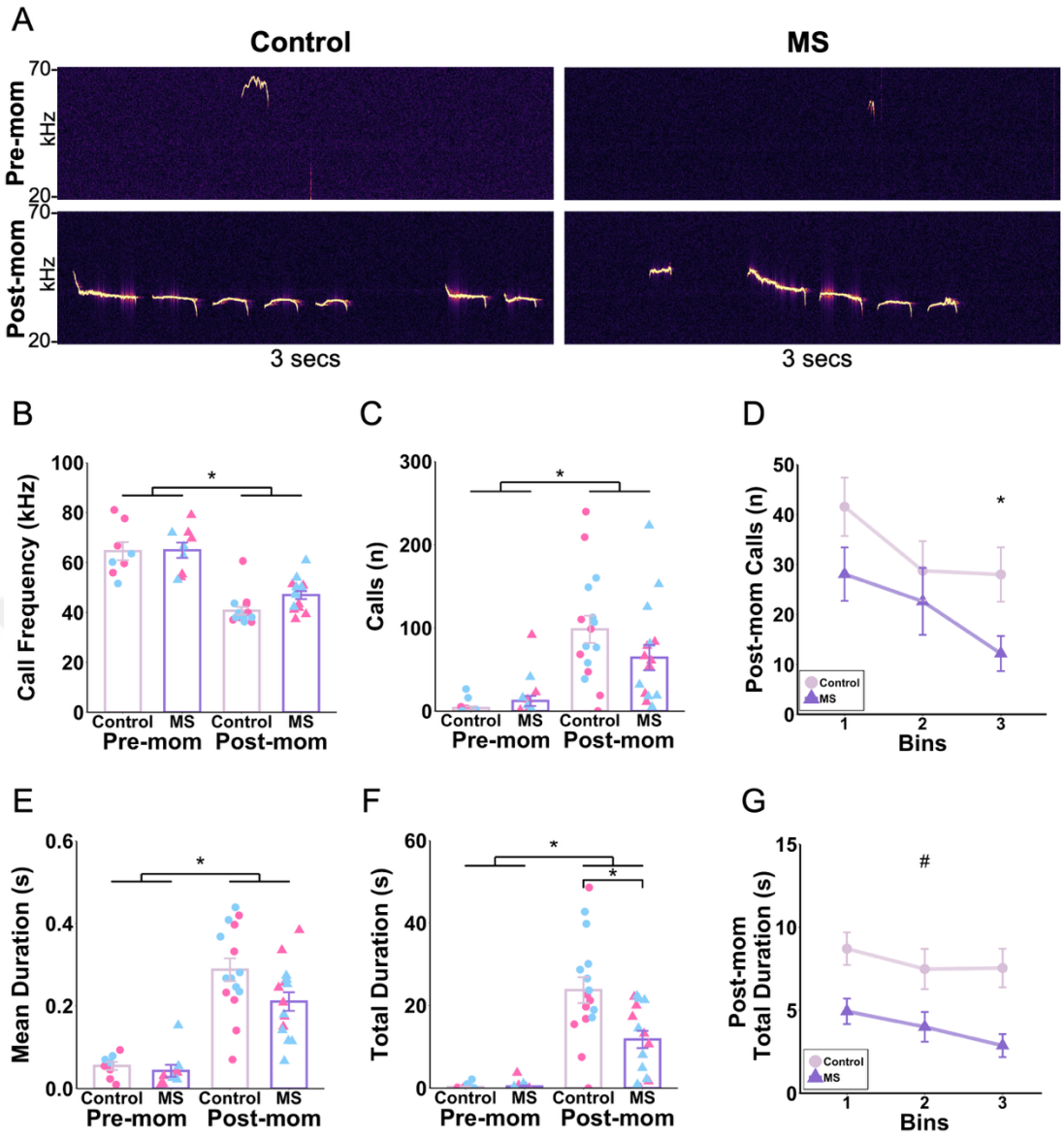

Figure 9. The results of maternal potentiation USV test. (A) Visualizations of infant USVs in the potentiation test. (B) Call frequency and (C) call number results. (D) Number of USVs in the postmom session across three mins (bins). (E) The mean and (F) total duration of the calls. (G) The total duration of USVs in the post-mom session across three mins. Error bars represent SEM and asterisks (*) depict statistical significance ($p < .05$) while hashtags (#) refer to the main effect of group.

$t(30) = 2.712$, $p = 0.029$, Cohen's $d = 0.499$; Tukey corrected t-test; Figure 9D), while no such reduction is found between the first ($M = 32.5$, $SD = 35.27$) and second bins ($t(30) = 1.530$, $p = 0.202$, Tukey corrected t-test). An additional t-test showed that MS decreased the USV numbers in the last bin of the post-mom session ($t(30) = 2.447$, $p = 0.021$, Cohen's $d = 0.865$, independent samples t-test; Figure 9D).

Similar to the call numbers, the mean duration ($F(1, 15) = 57.669$, $p < 0.001$, $\eta 2_p = 0.77$, 2x2 two-way mixed ANOVA; Figure 9A, E), and the total duration of the USVs increased in the second session ($F(1, 30) = 89.988$, $p < 0.001$, $\eta 2_p = 0.75$, 2x2 two-way mixed ANOVA; Figure 9A, F). MS did not affect the mean duration of the calls either directly ($F(1, 15) = 3.021$, $p = 0.103$), or through an interaction with the sessions ($F(1, 15) = 1.605$, $p = 0.225$). However, the total duration of calls was lower in MS group ($F(1, 30) = 9.327$, $p = 0.005$, $\eta 2_p = 0.24$; Figure 9F). In addition, the interaction effect was also significant for the total duration ($F(1, 30) = 10.804$, $p = 0.003$, $\eta 2_p = 0.26$). In particular, the difference was revealed in the post-mom session in which MS animals emitted less vocalizations ($M = 11.81$, $SD = 8.34$) compared to the control animals ($M = 23.73$, $SD = 12.50$; $t(30) = 3.175$, $p = 0.017$, Cohen's $d = 1.120$, Tukey corrected *t*-test; Figure 9F).

I also divided the total call duration in the second session into three bins (minutes). This analysis showed that the call duration has changed across bins ($F(2, 60) = 5.702$, $p = 0.005$, $\eta 2_p = 0.16$; 3x2 two-way mixed ANOVA; Figure 9G), in addition to the group-level effect ($F(1, 30) = 10.082$, $p = 0.003$, $\eta 2_p = 0.25$). However, no interaction between these variables was revealed ($F(2, 60) = 0.805$, $p = 0.452$, $\eta 2_p = 0.03$). Notably, when the effect of bins is examined by post-hoc tests, a decrease in total duration was obtained in MS group from the first bin ($M = 4.93$, $SD = 3.08$) to the last bin ($M = 2.87$, $SD = 2.77$; $t(30) = 2.899$, $p = 0.019$, Cohen's $d = 0.703$, Tukey corrected *t*-test; Figure 9G), but no such decrease was revealed in the control group (first bin: $M = 8.71$, $SD = 3.93$; last bin: $M = 7.54$, $SD = 4.66$; $t(30) = 1.640$, $p = 0.245$, Tukey corrected *t*-test). These additional analyses suggest that the within-session decrease in the USV emission of MS animals contributed the reduction of USVs in this group observed in the post-mom session.

3.1.2 Locomotor activity and anxiety-like behavior

The baseline locomotor activity and the anxiety-like behaviors of the animals were examined through the OFT and EPM in adolescence and adulthood. The MS group exhibited lower rates traveled distance in OFT (M = 2234.90, SD = 664.25) compared to the control group (M = 2942.56, SD = 574.33) in adolescence (PND 35; $t(30) = 3.224$, $p = 0.003$, Cohen's $d = 1.140$, independent samples t-test; Figure 10A, I), indicating decreased locomotion. However, no difference was observed in the time animals spent in the center of the maze between control (M = 68.75, SD = 38.70) and MS groups (M = 85.03, SD = 38.63; $t(30) = -1.191$, $p = 0.243$, Cohen's $d = 0.421$, independent samples t-test; Figure 10B, I). In the OFT conducted in adulthood (PND 80), the locomotion of the animals was comparable between groups ($F(1, 28) = 0.279$, $p = 0.602$; 2x2 two-way ANOVA; Figure 10C, K), with females displaying more activity in the maze ($F(1, 28) = 27.503$, $p < 0.001$, $\eta 2_p = 0.50$; Figure 10C, K). The amount of time animals stayed in the center was also not different between the groups ($F(1, 28) = 1.156$, $p = 0.292$; 2x2 two-way ANOVA; Figure 10D, K), with a sex effect in the same direction ($F(1, 28) = 4.837$, $p = 0.036$, $\eta 2_p = 0.15$; Figure 10D, K). This may be explained by the higher activity of females in the maze.

The groups were comparable in terms of their locomotor activity in the EPM in adolescence (PND 35; control: M = 2431.79, SD = 545.93; MS: M = 2030.23, SD = 619.04; $t(30) = 1.946$, $p = 0.061$, Cohen's $d = 0.688$, independent samples t-test; Figure 10E, J), assessed through the total distance traveled in the maze. However, the time spent in the open arms of the maze was higher in MS group (M = 252.13, SD = 40.01), compared to the controls (M = 186.49, SD = 68.01; $t(30) = -3.327$, $p = 0.002$, Cohen's $d = 1.176$; independent samples t-test; Figure 10F, J).

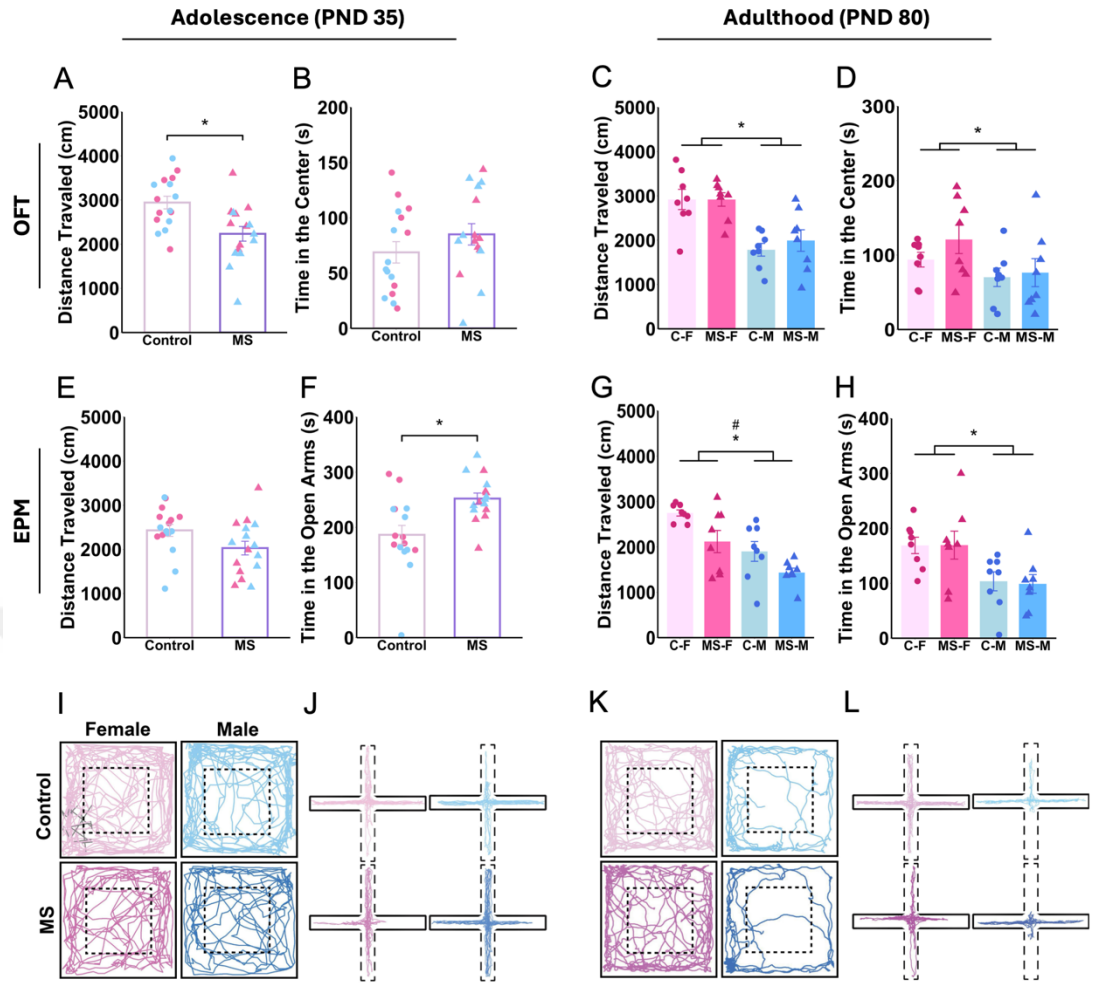

Figure 10. The locomotor activity and anxiety-like behavior. (A) The total distance traveled in and (B) the time spent in center of OFT in adolescence and (C, D) in adulthood. (E) The distance traveled in and (F) the time spent in the open arms of EPM in adolescence and (G, H) in adulthood (pink dots: females, blue: males). (I-L) The path trajectories of representative animals. Error bars show SEM and asterisks (*) depict statistical significance, while hashtags (#) refer to the main effect of group.

In adulthood (PND 80), the locomotion in the EPM was decreased in MS group ($F(1, 28) = 9.877$, $p = 0.004$, $\eta 2_p = 0.26$; 2x2 two-way ANOVA; Figure 10G, L), and a sex effect was revealed ($F(1, 28) = 19.288$, $p < 0.001$, $\eta 2_p = 0.41$; 2x2 two-way ANOVA; Figure 10G, L), without an interaction ($F(1, 28) = 0.221$, $p = 0.642$). Similarly, the time spent in the open arms was higher in female animals ($F(1, 28) = 12.666$, $p = 0.001$, $\eta 2_p = 0.31$; 2x2 two-way ANOVA; Figure 10H, L), however no effect of MS was observed in this measurement ($F(1, 28) = 0.012$, $p = 0.912$).

3.1.3 Social Play

The analysis revealed a sex effect in the number of pouncing behaviors with males displaying more pouncing behavior than females ($F(1, 28) = 12.532$, $p = 0.001$, $\eta 2_p = 0.31$; 2x2 two-way ANOVA; Figure 11A). There was no effect of MS on this measurement ($F(1, 28) = 1.922$, $p = 0.177$) similar to the lack of interaction with sex ($F(1, 28) = 0.509$, $p = 0.482$). However, MS animals had higher latency to exhibit this behavior ($M = 202.25$, $SD = 104.42$) when compared to controls ($M = 129.02$, $SD = 75.13$; $t(30) = -2.277$, $p = 0.030$, Cohen's $d = 0.805$; independent samples t-test; Figure 11B).

A similar sex difference was also observed in the number of pinning instances ($F(1, 28) = 7.213$, $p = 0.012$, $\eta 2_p = 0.20$; 2x2 two-way ANOVA; Figure 11C), with no effect of MS ($F(1, 28) = 0.500$, $p = 0.486$). However, the latency for pinning was

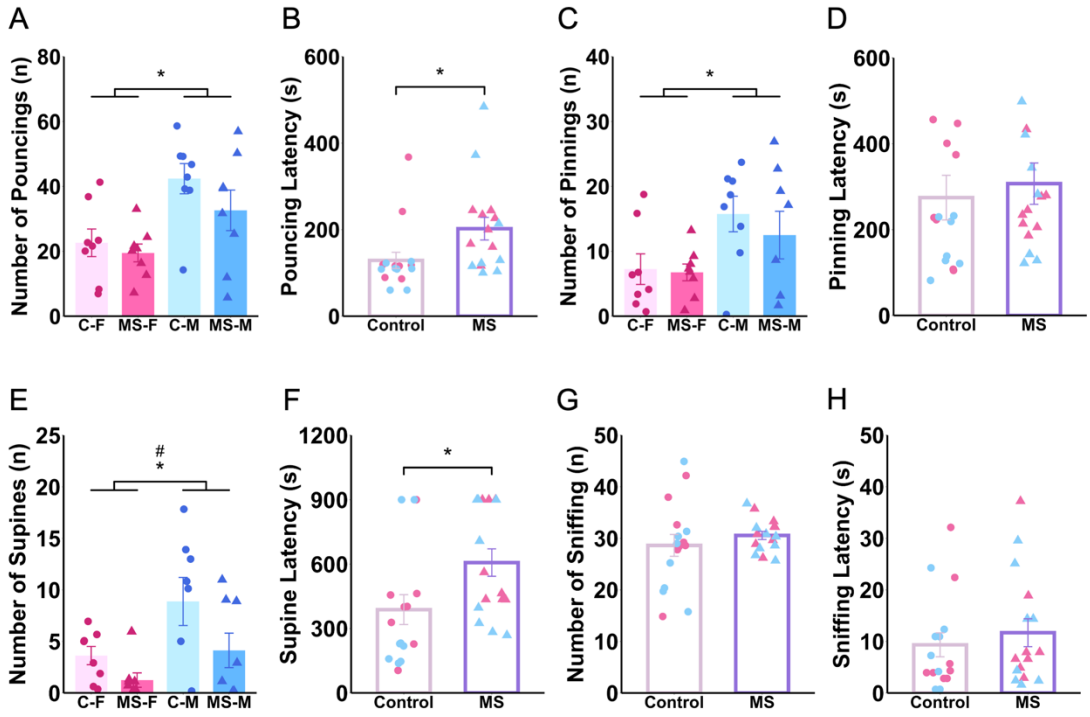

Figure 11. Social play behaviors. The instances of (A) pouncing, (C) pinning, (E) supine and (G) sniffing, as well as the latency to display these behaviors (B, D, F, H respectively). Error bars represent SEM, asterisks (*) depict statistical significance, while hashtags (#) refer to the main effect of group.

not different between control (M = 274.56, SD = 208.02) and MS groups (M = 307.16, SD = 192.70; $t(30)$ = -0.460, p = 0.649; independent samples t-test; Figure 11D), contrary to the findings in pouncing.

The number of supine behaviors were decreased in MS group ($F(1, 28)$ = 5.332, p = 0.029; $\eta 2_p$ = 0.16; 2x2 two-way ANOVA; Figure 11E). And a sex effect was revealed showing that the male animals exhibit this behavior more than the females ($F(1, 28)$ = 6.934, p = 0.014, $\eta 2_p$ = 0.20). Additionally, the latency for supine was longer in MS group (M = 607.17, SD = 255.44) when compared to the control animals (M = 388.06, SD = 277.78; $t(30)$ = -2.323, p = 0.027, Cohen's d = 0.821; independent samples t-test; Figure 11F), similar to the increased latency for pouncing behavior.

Sniffing behavior was also analyzed for this session as a behavior of non-playful social exploration. However, control (M = 28.625, SD = 8.44) and MS groups (M = 30.56, SD = 3.16) did not differ in terms of sniffing counts ($t(30)$ = -0.859, p = 0.397; independent samples t-test; Figure 11G), as well as the latency to show sniffing (Control: M = 9.35, SD = 9.27; MS: M = 11.70, SD = 10.8; $t(30)$ = -0.661, p = 0.514; independent samples t-test; Figure 11H). Altogether, the findings suggest that animals subjected to MS have reduced levels of play-type-specific social play, with observed sex effects indicating that males engage in play behaviors more frequently in adolescence.

3.1.4. Sociability

The virtually defined zones in the test arena are depicted in Figure 12A. Animals decreased their locomotor activity in the second session, when there was a social object in the chamber ($F(1, 28)$ = 6.760, p = 0.015, $\eta 2_p$ = 0.19, 2x2x2 three-way

mixed ANOVA; Figure 12B). In addition, females had higher locomotion in the maze ($F(1, 28) = 10.009$, $p = 0.004$, $\eta 2_p = 0.40$), and this effect did not interact with the groups ($F(1, 28) = 0.028$, $p = 0.869$) or sessions ($F(1, 28) = 0.800$, $p = 0.379$). The effect of group on distance traveled remained unsignificant ($F(1, 28) = 3.776$, $p = 0.062$, $\eta 2_p = 0.12$), with an interaction with the session number ($F(1, 28) = 10.009$, $p = 0.004$, $\eta 2_p = 0.26$). This shows that MS animals reduced their locomotor activity ($M = 1874.43$, $SD = 740.11$) compared to the controls ($M = 2504.21$, $SD = 767.17$) when the social object was in the chamber ($t(28) = 2.900$, $p = 0.034$, Cohen's $d = 0.836$, Tukey corrected t-test; Figure 12B).

The groups had comparable numbers of quarter entries ($F(1, 28) = 0.172$, $p = 0.682$; 2x2x2 three-way mixed ANOVA; Figure 12C), while females displayed more

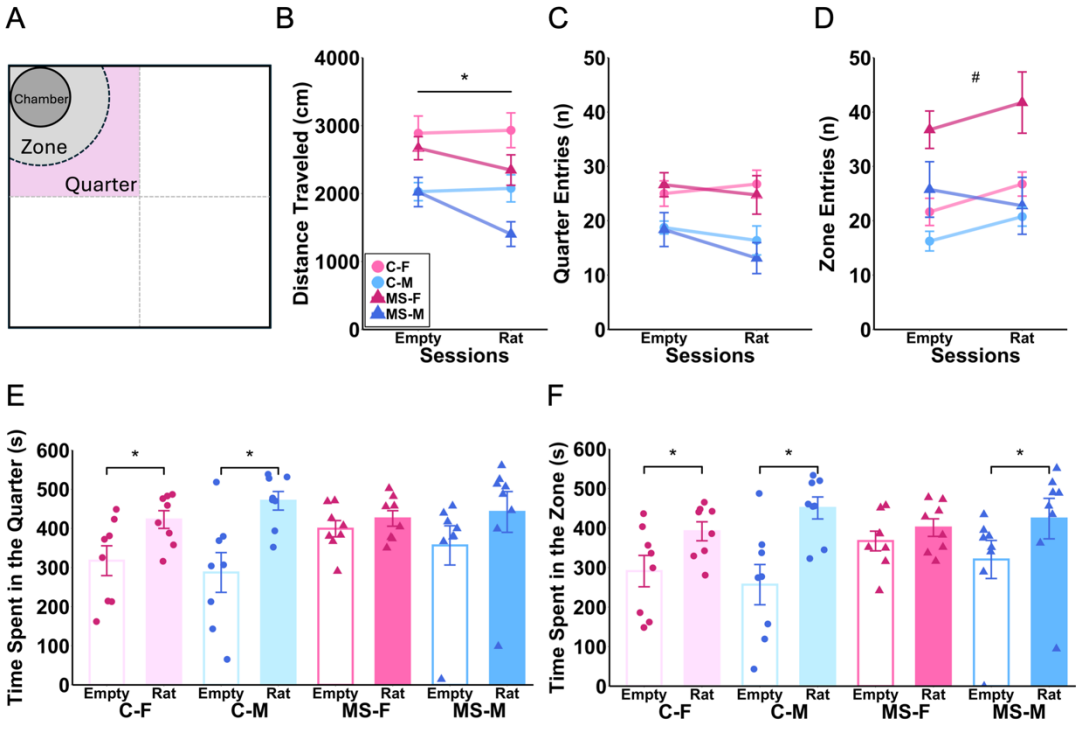

Figure 12. Sociability test. (A) The representation of defined target zones. (B) the distance traveled in the maze. The number of entries to the (C) target quarter and (D) the interaction zone. The time spent in the (E) target quarter and (F) the interaction zone. Error bars represent SEM, asterisks (*) depict statistical significance, while hashtags (#) refer to the main effect of group.

entries ($F(1, 28) = 14.308$, $p < 0.001$, $\eta 2_p = 0.34$). However, this measure was not affected by the session ($F(1, 28) = 3.260$, $p = 0.082$). Similarly, the entries to the interaction zone were not different between sessions either ($F(1, 28) = 2.460$, $p = 0.128$, 2x2x2 three-way mixed ANOVA; Figure 12D). In addition, female animals entered to the interaction zone more frequently ($F(1, 28) = 9.818$, $p = 0.004$, $\eta 2_p = 0.26$). Interestingly, MS also increased the zone entries ($F(1, 28) = 9.937$, $p = 0.004$, $\eta 2_p = 0.26$).

The animals spent more time in the target quarter when the social object was in the chamber ($F(1, 28) = 35.862$, $p < 0.001$, $\eta 2_p = 0.56$, 2x2x2 three-way mixed ANOVA; Figure 12E). This increase separately interacted with the sex of the animals ($F(1, 28) = 4.223$, $p = 0.049$, $\eta 2_p = 0.13$) and the group effects ($F(1, 28) = 6.987$, $p = 0.013$, $\eta 2_p = 0.20$). However, pairwise comparisons only indicated the interaction of group and the session, revealing that control female (session 1: $M = 317.52$, $SD = 107.79$; session 2: $M = 422.61$, $SD = 64.05$; $t(28) = -3.147$, $p = 0.019$, Cohen's $d = 1.185$, Tukey corrected t-test) and male animals (session 1: $M = 287.37$, $SD = 143.82$; session 2: $M = 470.53$, $SD = 67.15$; $t(28) = -5.485$, $p < 0.001$, Cohen's $d = 1.632$, Tukey corrected t-test) increased their time in the second session, while MS female (session 1: $M = 399.17$, $SD = 58.72$; session 2: $M = 425.40$, $SD = 56.20$; $t(28) = -0.785$, $p = 0.861$, Cohen's $d = 0.456$, Tukey corrected t-test) and male animals did not (session 1: $M = 356.58$, $SD = 141.76$; session 2: $M = 442.06$, $SD = 147.33$; $t(28) = -2.560$, $p = 0.072$, Cohen's $d = 0.591$, Tukey corrected t-test).

An increase in the second session was also revealed for the time spent in the interaction zone ($F(1, 28) = 39.404$, $p < 0.001$, $\eta 2_p = 0.58$, 2x2x2 three-way mixed ANOVA; Figure 12F). While sex and group did not exhibit a direct effect on this duration ($F(1, 28) = 0.000$, $p = 0.995$; $F(1, 28) = 0.814$, $p = 0.375$, respectively); they

separately interacted with the increase in this time across sessions ($F(1, 28) = 5.596$, $p = 0.025$, $\eta 2_p = 0.17$; $F(1, 28) = 5.233$, $p = 0.030$, $\eta 2_p = 0.16$; respectively). In the control group, an increased time spent around the chamber when included the social object was observed in both female (Session 1: $M = 290.96$ $SD = 112.19$; Session 2: $M = 391.65$, $SD = 67.70$; $t(28) = -2.927$, $p = 0.032$, Cohen's $d = 1.090$, Tukey corrected t-test) and male animals (Session 1: $M = 256.73$, $SD = 143.92$; Session 2: $M = 450.63$, $SD = 78.63$; $t(28) = -5.637$, $p < 0.001$, Cohen's $d = 1.670$, Tukey corrected t-test); however this increase was exhibited by only male (Session 1: $M = 320.39$, $SD = 136.20$; Session 2: $M = 423.76$, $SD = 144.53$; $t(28) = -3.005$, $p = 0.027$, Cohen's $d = 0.736$, Tukey corrected t-test) but not female animals in MS group (Session 1: $M = 367.12$, $SD = 70.41$; Session 2: $M = 400.98$, $SD = 62.66$; $t(28) = -0.984$, $p = 0.759$, Cohen's $d = 0.508$, Tukey corrected t-test).

3.1.5 Sucrose Preference

There was no group-level difference in the total liquid consumption over 48h ($F(1, 28) = 3.846$, $p = 0.060$; 2x2 two-way ANOVA; Figure 13A). The analysis revealed a sex effect with males consuming more liquids ($F(1, 28) = 16.910$, $p < 0.001$, $\eta 2_p = 0.38$), but no interaction effect ($F(1, 28) = 1.268$, $p = 0.270$). The groups also had comparable levels of sucrose consumption (Control: $M = 40.94$, $SD = 12.17$; MS: $M = 32.32$, $SD = 17.56$; $t(30) = 1.615$, $p = 0.117$, Tukey corrected t-test; Figure 13B). Notably, MS group exhibited a lower sucrose preference ($M = 0.57$, $SD = 0.29$) compared to the control group ($M = 0.78$, $SD = 0.15$), calculated as the ratio of sucrose consumption over the total liquid consumption ($t(30) = 2.503$, p = 0.018, independent samples t-test; Figure 13C), indicating decreased hedonic response in MS animals.

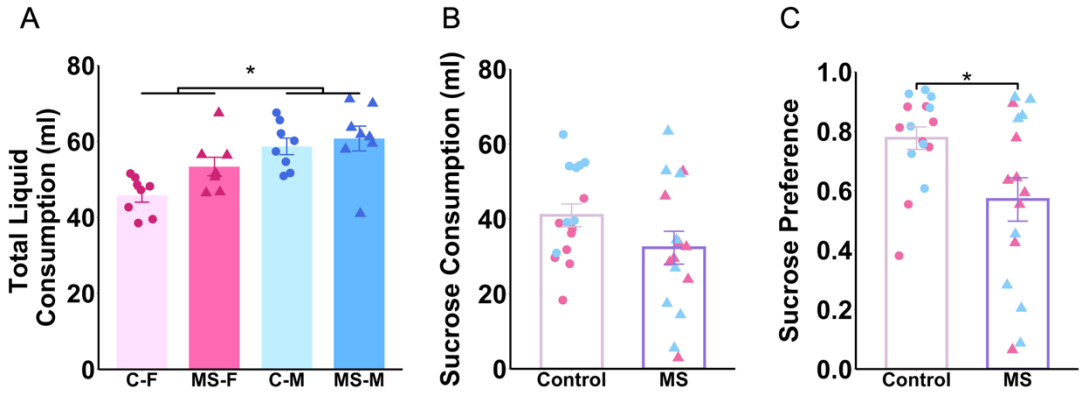

Figure 13. Sucrose preference. (A) Total liquid amounts consumed, (B) levels of sucrose water consumption and (C) sucrose preference index. Error bars represent SEM and asterisks (*) depict statistical significance.

3.1.6 Fear Conditioning

Fear conditioning started with an acquisition phase. Baseline freezing levels of the animals were similar between the control (M = 0.46, SD = 1.01) and MS groups in the fear acquisition (M = 0.92, SD = 3.13; $t(30) = 0.558$, $p = 0.581$, independent samples t-test; Figure 14A), indicating no differential response to the context before the conditioning. Freezing responses to the CS increased across the pairings ($F(4, 135) = 12.735$, $p < 0.001$, $\eta 2_p = 0.27$; 2x2x5 three-way mixed ANOVA; Figure 14A), as a sign of successful auditory conditioning. And there was an effect of sex on these responses ($F(1, 135) = 13.512$, $p < 0.001$, $\eta 2_p = 0.09$), pointing to higher freezing levels in male animals. However, the groups did not differ in their freezing rates to the conditioned stimulus ($F(1, 135) = 0.001$, $p = 0.976$; Figure 14A).

In the first block of context exposure (i.e., 5 mins) the freezing percentages of the animals were higher (M = 38.10, SD = 17.35) compared to the baseline of fear acquisition (M = 0.69, SD = 2.30; $t(31) = -11.882$, $p < 0.001$, Cohen's $d = 3.023$; paired samples t-test; Figure 14A), indicating successful contextual conditioning. In addition, the freezing levels did not change across the bins of context exposure ($F(3, 108) = 0.642$, $p = 0.589$, 4x2x2 three-way mixed ANOVA). Male animals exhibited

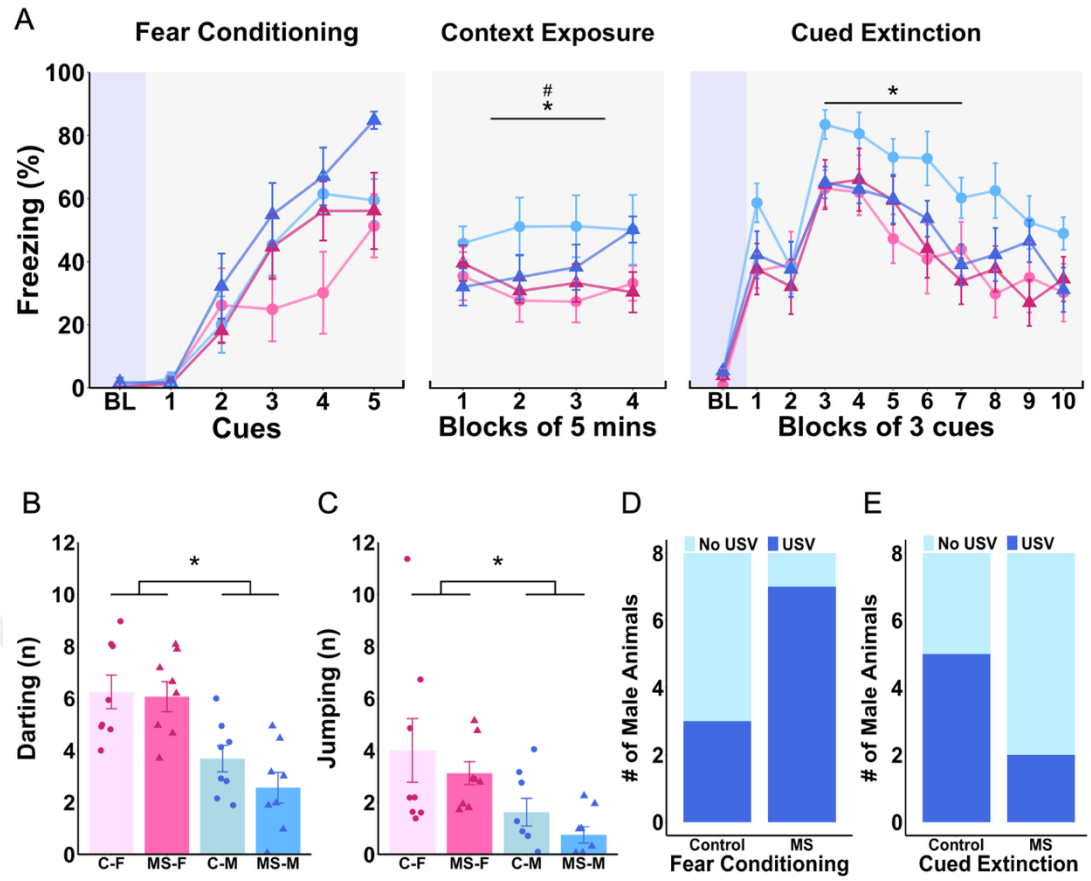

Figure 14. Fear Conditioning. (A) Freezing percentages across fear conditioning sessions. (B) Darting and (C) jumping behaviors in response to foot. The number of animals emitting USVs in (D) conditioning and (E) extinction sessions. Error bars represent SEM, asterisks (*) depict statistical significance, flat lines show significant interaction of group and sex, while hashtags (#) refer to the main effect of group.

stronger freezing behavior during this session ($F(1, 108) = 11.762$, $p < 0.001$, $\eta 2_p = 0.10$), similar to the previous phase. Notably, animals exposed to MS displayed less freezing behavior in the conditioning context ($F(1, 108) = 5.303$, $p = 0.023$, $\eta 2_p = 0.05$), and an interaction between sex and group effects was revealed ($F(1, 108) = 6.298$, $p = 0.014$, $\eta 2_p = 0.06$). In particular, the freezing levels of the control male animals ($M = 49.43$, $SD = 24.44$) were higher than all the other groups: control female ($M = 30.84$, $SD = 18.19$; $t(108) = -4.585$, $p < 0.001$, Cohen's $d = 0.863$, Tukey corrected t-test), MS male ($M = 38.77$, $SD = 17.98$; $t(108) = 3.340$, $p = 0.006$, Cohen's $d = 0.497$, Tukey corrected t-test) and MS female animals ($M = 33.37$, $SD =$

15.35; $t(108) = -3.909$, $p < 0.001$, Cohen's $d = 0.787$, Tukey corrected t-test). This follow-up comparison supports the revealed main effect of group and sex, indicating that the group-level difference was evident in male animals.

The freezing responses of control ($M = 2.53$, $SD = 3.18$) and MS animals ($M = 4.62$, $SD = 4.20$) were not different in the baseline of the cued extinction session conducted in a novel context ($t(30) = -1.581$, $p = 0.125$, independent samples t-test; Figure 14A). As expected, there was a main effect of blocks ($F(9, 260) = 3.186$, $p = 0.001$, $\eta 2_p = 0.09$, 10x2x2 three-way mixed ANOVA; Figure 14A), indicating that the freezing response of the animals changed across blocks. The sex of the animals altered the freezing behavior both by itself ($F(1, 260) = 18.275$, $p < 0.001$, $\eta 2_p = 0.08$) and through an interaction with the groups ($F(1, 260) = 10.972$, $p = 0.001$, $\eta 2_p = 0.05$). MS did not alter the fear response by itself in this session ($F(1, 260) = 1.872$, $p = 0.172$; Figure 14A). Post-hoc comparisons over the blocks showed that control males ($M = 62.90$ $SD = 22.11$) displayed higher freezing response compared to MS male animals ($M = 47.87$, $SD = 21.39$; $t(260) = 3.083$, $p = 0.012$, Cohen's $d = 0.691$, Tukey corrected t-test); control female ($M = 42.72$, $SD = 25.48$; $t(260) = -5.475$, $p < 0.001$, Cohen's $d = 0.846$, Tukey corrected t-test) and MS female animals on average ($M = 43.54$, $SD = 25.36$; $t(260) = -3.619$, $p = 0.002$, Cohen's $d = 0.814$, Tukey corrected t-test). These results suggest that MS group showed reduced cued fear memory, but this effect was restricted to the male animals.

Additional parameters such as active coping in response to foot shock and USV emission during these sessions were also analyzed. The darting response to the shock was higher in female animals ($F(1, 28) = 26.938$, $p < 0.001$, $\eta 2_p = 0.49$, 2x2 two-way ANOVA; Figure 14B), similar to the number of jumping behaviors ($F(1, 28) = 10.869$, $p = 0.003$, $\eta 2_p = 0.28$, 2x2 two-way ANOVA; Figure 14C), which is in

line with the lower freezing levels of females in this session. However, no group-level effect was observed in these analyses (darting: $F(1, 28) = 1.263$, $p = 0.271$; jumping: $F(1, 28) = 1.475$, $p = 0.235$), similar to the lack of interaction effects (darting: $F(1, 28) = 0.644$, $p = 0.429$; jumping: $F(1, 28) = 0.000$, $p = 1.000$).

In terms of the USV analysis, the number of observations was not sufficient to run a frequency analysis, therefore the prevalence of USV emission is only described here. Female animals in each group did not produce alarm calls in any session. The number of males emitting USVs was 3 for the control and 7 for MS animals, out of 8 animals in each group during fear acquisition (Figure 14D). Interestingly, no USVs were emitted when the animals were re-exposed to the conditioning context. In the cued extinction, five male animals in control group and 2 male animals in MS group emitted USVs.

3.2. Neuronal activity and corticosterone levels

Immunofluorescence for ChAT and PV delignated the borders of BL and LA as reference labelings (Figure 15A) and the regions of interest in which the neuronal activation was examined through c-Fos immunopositive cell density can be seen in Figure 15B-D. Notably, MS decreased the density of c-Fos+ cells in LA ($M = 353.54$, $SD = 64.39$) when compared to the control group ($M = 409.82$, $SD = 63.73$; $t(27) = 2.352$, $p = 0.026$, Cohen's $d = 0.879$, independent samples t-test; Figure 15E); but had no effect on BL c-Fos (Control: $M = 312.99$, $SD = 59.24$; MS: $M = 297.08$, $SD = 48.08$; $t(27) = 0.806$, $p = 0.427$, independent samples t-test). I further separated the posterior (BLp) and anterior nuclei of BL (BLa) to examine possible differences and observed higher neuronal activation in the anterior nuclei ($M = 398.77$, $SD = 70.02$) than the posterior one ($M = 210.89$, $SD = 54.05$; $t(28) = 13.549$, $p < 0.001$, Cohen's

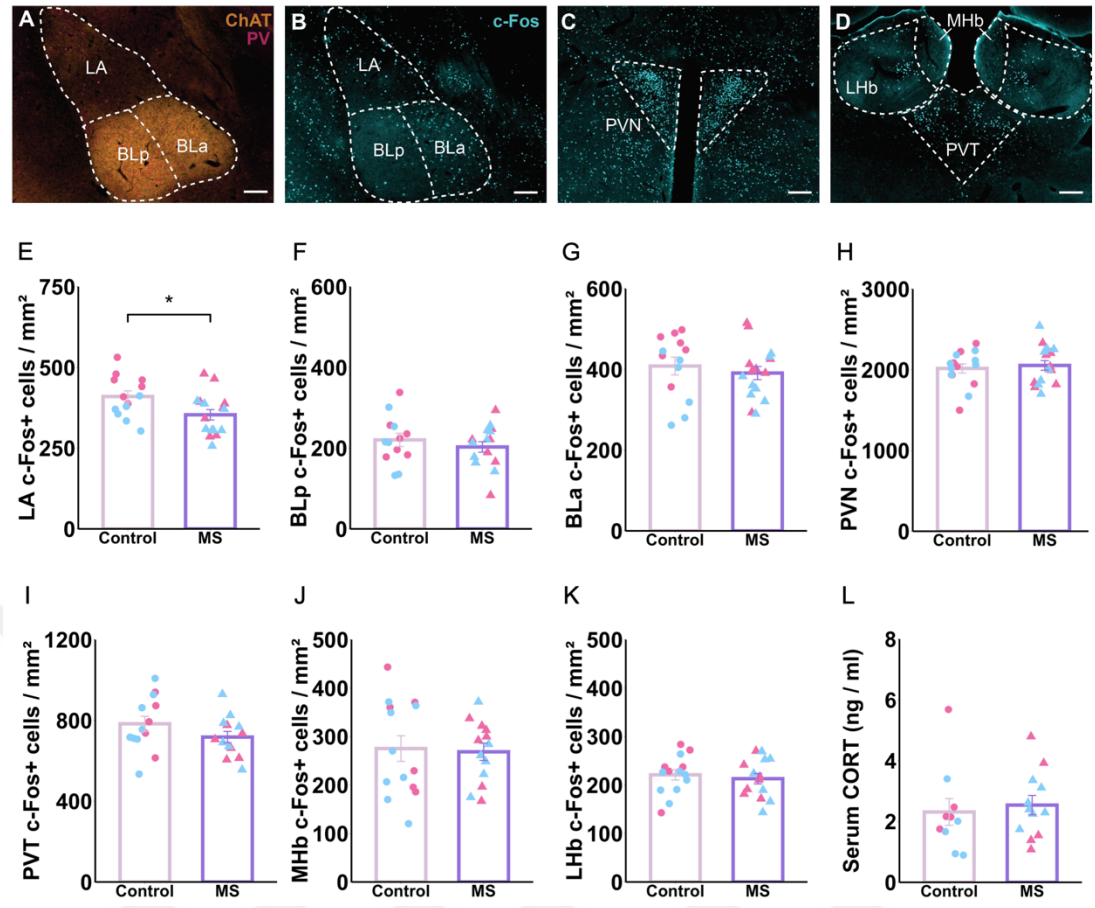

Figure 15. Neuronal activation and CORT release. (A-D) The borders of target regions. Scale bars represent 200 µm. (E-K) c-Fos cell density in each region and (L) serum CORT levels during acute stress. Error bars represent SEM, asterisks (*) depict statistical significance.

d = 3.004, paired samples t-test; see Figure 15F-G). However the lack of group-level effect remained true for both BLp (control: *M* = 220.52, *SD* = 59.05; MS: *M* = 203.06, *SD* = 52.09; *t*(27) = 0.845, *p* = 0.405, independent samples *t*-test; Figure 15F), and BLa (control: *M* = 408.39, *SD* = 79.42; MS: *M* = 390.96, *SD* = 65.47; *t*(27) = 0.648, *p* = 0.522, independent samples *t*-test; Figure 15G).

The c-Fos+ cell density in the PVN did not reveal a difference between control (*M* = 2016.94, *SD* = 219.38) and MS groups (*M* = 2054.08, *SD* = 240.49; (*t*(29) = -0.448, *p* = 0.657, independent samples *t*-test; Figure 15H), suggesting comparable levels of activation following acute stress for both samples. Similarly,

the group-level difference in PVT c-Fos+ density remained unsignificant (control: M = 783.33, SD = 135.13; MS: M = 717.65, SD = 101.46; $t(24) = 1.402$, $p = 0.174$, independent samples t-test; Figure 15I). The analyses of neuronal activation in medial and lateral habenula did not indicate an alteration in MS animals (MHb: control: M = 275.28, SD = 98.86; MS: M = 268.59, SD = 63.88; $t(25) = 0.207$, p = 0.838, independent samples t-test; Figure 15J; LHb: control: M = 221.08, SD = 39.95; MS: M = 213.16, SD = 40.59; $t(26) = 0.520$, $p = 0.607$, independent samples t-test; Figure 15K).

The serum CORT assessment during restraint stress in adulthood did not reveal a difference between control (M = 2.31, SD = 1.39) and MS groups (M = 2.54, SD = 1.10; $t(20) = -0.426$, $p = 0.675$, independent samples t-test; Figure 15L), which is consistent with the similar levels of c-Fos immunopositive neurons in the PVN of control and MS groups.

3.3 The predictive role of infant USVs

The partial correlation matrix investigating the relations of infant vocalizations with adolescent and adult behavioral measurements while controlling for sex and group is depicted in Figure 16. In this analysis, the number of USV calls in the post-mom session of the potentiation test were positively correlated with the time spent in the open arms of EPM in adolescence (EPM 1; $r = 0.442$, $p = .031$), while it was negatively correlated with the fear memory in adulthood ($r = -0.460$, $p = .024$). In addition, the sucrose preference index was also correlated with fear memory (r = 0.464, $p = .023$). No other relationship was significant (Figure 16).

For the path analysis, I first verified the model fit indexes for each group's analysis. The chi-square test was not significant for the control ($\chi^2 = 0.017$, p =

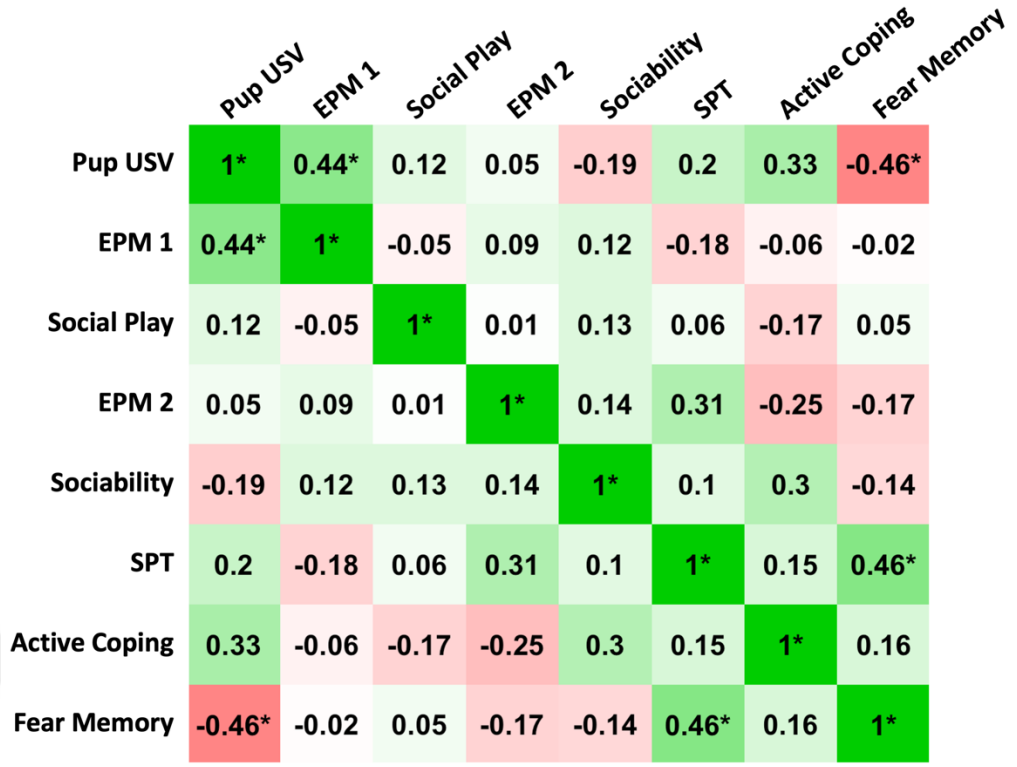

Figure 16. Partial correlation matrix for the behavioral measurements. Numbers in the cells indicate R values for each pair, the colors depict the direction of the relation, and asterisks (*) represent statistical significance at alpha < .05.

0.897) and MS group ($\chi^2 = 0.011$, $p = 0.916$), which points to that the model was good fit to the data. I also checked comparative fit index (CFI) and Tucker-Lewis index (TLI) and they also indicated good fit for both control (1.000 and 1.794) and MS groups (1.000 and 2.039, respectively). The Root Mean Square Error of Approximation (RMSEA) of both analyses was 0.000, with 95% confidence intervals of 0–0.301 for control and 0–0.255 for MS group. In addition, Standardized Root Mean Square Residual (SRMR) was 0.003 for both groups. Therefore, the model seems as a good fit to the data of each group. The unstandardized and standardized path coefficients with standard errors and p values for all paths can be found in Appendix B. I reported unstandardized coefficients in the text, while the coefficients in the figures were standardized to enable between-analysis comparisons.

Pup USV numbers following brief maternal contact predicted the time animals spent in the open arms of the EPM conducted in adolescence for both control (B = 0.579, p = 0.015; Figure 17A), and MS groups (B = 0.329, p = 0.008; Figure 17B). Together with the reported partial correlation effect, these findings highlight a positive relationship between infant USVs and exploratory behavior in adolescence. However, no relation between infant vocalizations and fear memory was observed in either group (Control: B = -0.533, p = 0.223, MS: B = -0.365, p = 0.416), contrary to the observed effect in partial correlation. Similarly, the USV numbers were not associated with any other variable in the model for either group (Figure 17A–B, Appendix B). The revealed sex effects in these analyses can be observed in Figure 17A–B, and the sex the arrows derive from shows the group exhibiting target behavior more frequently. The covariances between adulthood measurements indicated a positive relation between active coping behaviors in response to shock and the fear memory in the subsequent sessions in the control animals (B = 0.220, p = 0.026, Figure 17A), while no such effect was observed for MS group (B = 0.050, p = 0.469). In addition, the sucrose preference of the animals was a positive covariant for both active coping (B = 0.165, p = 0.005, Figure 17A) and fear memory in controls (B = 0.350, p = 0.012, Figure 17A), but not in MS group (sucrose preference vs. active coping: B = 0.114, p = 0.182; sucrose preference vs. fear memory: B = 0.234, p = 0.178). However, the time spent in the open arms of the EPM by adult MS animals was positively related to the sucrose preference (B = 0.336, p = 0.039, Figure 17B), and control animals did not show this association (B = -0.029, p = 0.685). These findings suggest that animals' hedonic responses are associated with their active and conditioned behavior toward the aversive stimulus in the control group, whereas in MS group, they are linked to exploration in the EPM.

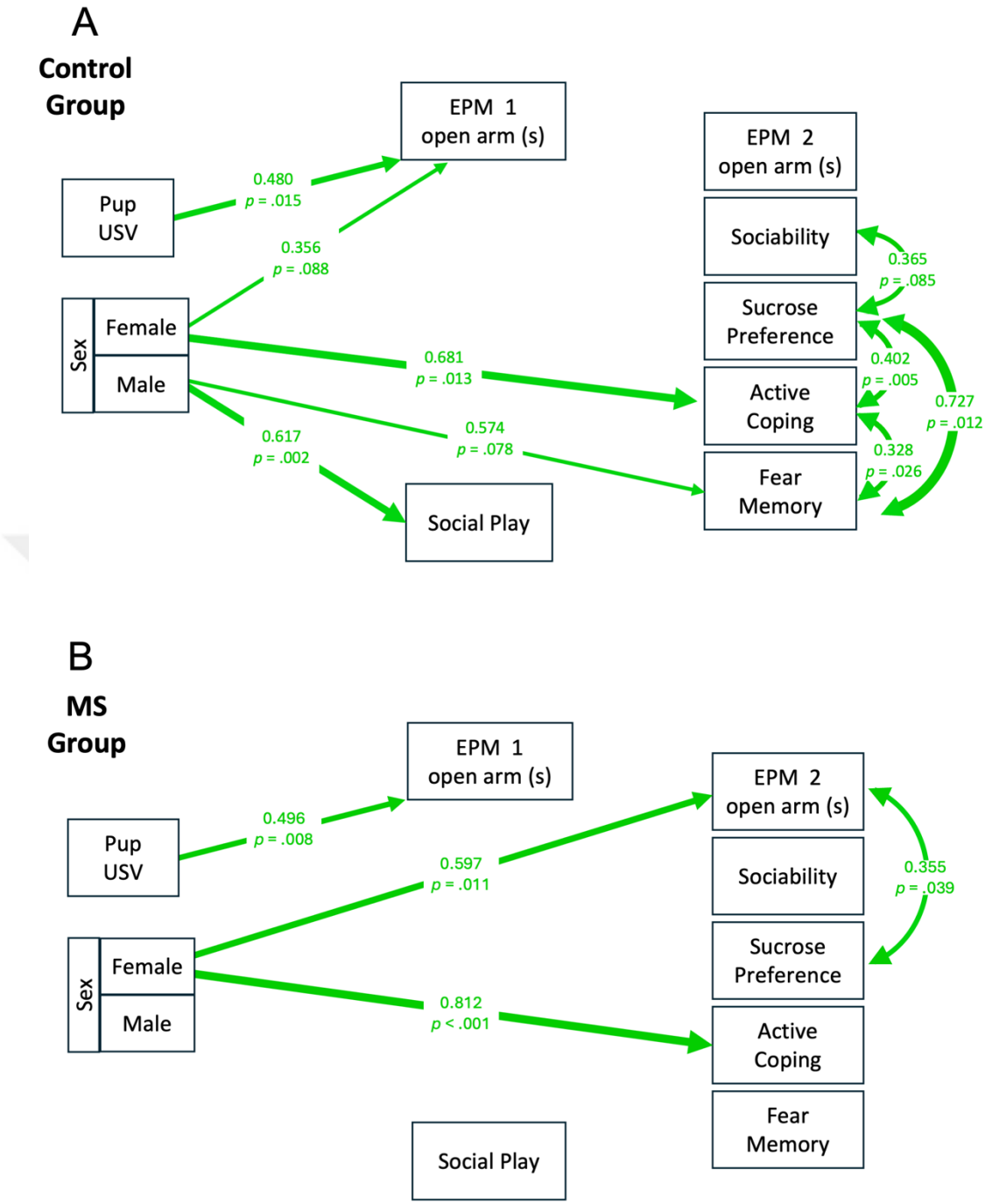

Figure 17. Path analyses. (A) Results of path analysis with standardized regression coefficients and *p* values for each relationship tested in the control group and (B) MS group. Arrows represent positive relationships between variables when $p < 0.1$.

Eventually, multiple regression analyses were utilized to investigate the predictive value of infant USVs with EPM and social play in adolescence for the neurobiological assessments during acute stress in adulthood, through c-Fos+ cell

density and serum CORT levels. The model statistics, adjusted R^2 values and predictor coefficients with bonferroni corrected *p* values can be found in Appendix C. No predictive role of pup USVs was revealed for neuronal activation in BLA (B = -0.159, *p* = 1.000; Figure 18A), PVN (B = -0.543, *p* = 0.142; Figure 18B), PVT (B = 0.094, *p* = 1.000; Figure 18C) or CORT release in response to restraint stress (B = 0.060, *p* = 1.000). Furthermore, the overall models were also not significant (Appendix C), indicating that the predictors failed to explain the variance in the outcome variables. However, the model for habenula neuronal activation was significant (Appendix C) and infant vocalizations predicted the neuronal activation in this region with a negative relation (B = -0.742, *p* = 0.016; Figure 18D). Notably, the interaction of pup USVs and the group was observed with a positive direction (B = 1.048, *p* = 0.018; Figure 18D). This indicates that the negative prediction of infant calls on habenula c-Fos density was abolished in MS group. No other models with the time spent in the open arms of the EPM and social play behaviors in adolescence as predictors showed an association with biological outcomes of acute restraint stress (Appendix C).

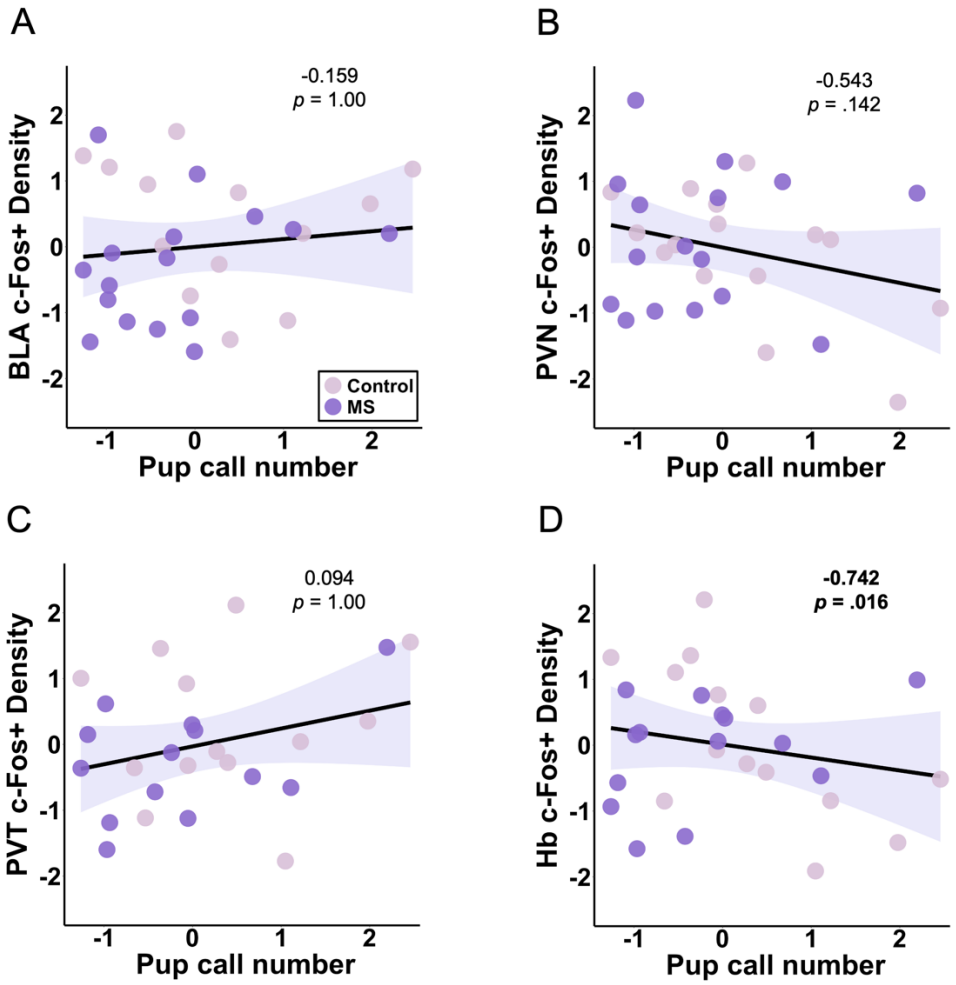

Figure 18. Regression plots. The relationship between pup USV call numbers and c-Fos+ cell density in (A) BLA (n = 13 for control and 16 for MS), (B) PVN (n = 15 for control and 16 for MS), (C) PVT (n = 13 for control and 13 for MS) and (D) habenula (n = 14 for control and 14 for MS), respectively. The black line represents the overall regression with a shadowed confidence interval. The regression coefficients and *p* values (bold when significant) were presented on the plots.

CHAPTER 4

DISCUSSION

I conducted a longitudinal investigation of behavioral and physiological effects of MS as well as the associations of these measurements obtained at multiple life stages. I showed a decrease in the pup USV emission in animals subjected to MS, via the maternal potentiation paradigm, and the predictive role of these vocalizations on adolescent anxiety-like behavior and stress-induced neuronal activity in the habenula. Behavioral assessments indicated substantial alterations induced by MS. MS gave rise to anxiolytic behavior and delayed play initiation in adolescence, and anhedonia and impaired fear memory in adulthood. I also showed decreased neuronal activation in the LA during acute stress in adult MS animals compared to controls; while the groups lacked a difference in HPA axis-related measurements, the neuronal activation in PVN and CORT release. Altogether, this work provides valuable insights into the diverse effects of MS as an ELS model across developmental stages and highlights the predictive role of infant vocalizations on subsequent affective processes.

Previous studies reported that rat pups exposed to ELS displayed higher rates of USVs when separated from the mother (Burenkova et al., 2020; Ploj et al., 2003; Yin et al., 2016). Notably, these experiments recorded USVs on a single separation session within the first two weeks of life, and the USV emission rates in the following days are known to be lower (Insel et al., 1986; Noirot, 1968). Furthermore, a recent study found that this effect is reversed in the late infancy, with a decreased USV emission in MS group (Granata et al., 2021). In this study, MS did not affect the infant vocalizations in the first isolation on PND 15, as the call emission rates

were close to zero. However, the pup USVs substantially increased in the second isolation, following the reunion with the mother, and switched to the typical 40 kHz calls (Wöhr & Schwarting, 2008), indicated by the changes in frequency and mean duration of calls.

The elevated USV emission after reunion with the mother, known as maternal potentiation (Brunelli et al., 1998; Hofer et al., 1993), may reflect a different state than the calls in the first separation (Shair, 2007). A detailed investigation of the pup calls on PND 12 indicated elevated rates of high frequency calls in the second isolation following MS (Kaidbey et al., 2019). I included all the calls in the analysis and showed that the total USV duration in the second session decreased in MS group and this change was partially driven by the within session reduction in the calls. The conflict may be explained by the fact that animals also had a good level of USV emission in the first isolation on PND 12 (Kaidbey et al., 2019), suggesting that potentiation may have interacted with their initial separation-induced calls. In contrast, a larger proportion of calls during the second isolation in this study was likely driven by the preceding litter exposure. Therefore, the decrease in the potentiated calls in the current study may reflect a disruption in the pups' bond to their litter due to the history of repeated MS.

The longitudinal design of the current study enabled examining the predictive role of these vocalizations which may provide additional and significant information on how these calls can be associated to the affective state of the animals. The results showed that infant USVs positively predicted the amount of time animals spend in exploring the open arms of the EPM in adolescence, suggesting elevated exploration and decreased anxiety-like behavior (Walf & Frye, 2007). This is consistent with a previous finding showing that separation-induced 40 kHz calls in a single session on

PND 11 predicted EPM open arm time in adulthood (Cordeiro et al., 2024). However, the lack of a relationship between infant calls and adult behavior in the EPM in this work, may be due to potentiation calls being more closely linked to attachment to the litter (Shair, 2007) rather than general distress from home cage separation because reunion with littermates does not induce potentiation (Hofer et al., 1994). As a result, the relationship between potentiated infant USVs and behavior in EPM may be more pronounced in adolescence but weaken or lose its direct association in adulthood. In addition, this also showed that even if the infant calls and the open arm exploration were altered in animals subjected to MS, the predictive relationship of USVs remained unchanged. This means that, an ELS model reduced the pup USV emission in a potentiation paradigm, but those producing fewer calls still exhibited elevated anxiety-like behavior in adolescence compared to the ones emitting more USVs, similar to control animals. This further supports the hypothesis that pup USV levels not only reflect the early life stress but also function as a predictor for behaviors in later life.

Infant vocalizations are previously reported to negatively predict the freezing and USV emission of animals in fear conditioning (Wöhr & Schwarting, 2008). In this experiment, the partial correlation analysis indicated a similar link between pup calls and fear memory in adulthood, in a way that the rats producing fewer USVs in infancy exhibited higher freezing levels in response to conditioned stimuli. However, this relationship disappeared in the models conducted for each group separately, possibly because the explained variance was distributed across indirect paths in the model, reducing the strength of the direct effect. Furthermore, the control group showed significant positive associations among adult behavioral outcomes, particularly in sucrose preference, active shock response, conditioned fear, and

sociability. In contrast, these relations were not observed in the MS group, suggesting that ELS may disrupt the natural interconnection of these behaviors and the coherence of the traits they measure. In addition, a positive relation between anhedonia and anxiety-like behavior emerged in the MS group, suggesting that ELS enhance the co-occurrence of these symptoms compared to the natural variance in the control group.

The predictive value of pup USVs on the neuronal activation in multiple regions during acute restraint stress in adulthood was further examined and found a negative association with habenula c-Fos+ cell density. Elevated activity in habenula was frequently reported following various rodent chronic stress models (Greenwood et al., 2005; Kingir et al., 2023; Li et al., 2021), and its hyperactivity is shown to be associated with depressive-like behaviors (Cameron et al., 2024). Particularly, the density of c-Fos+ cells in habenular divisions increases with acute stress (Durieux et al., 2022; Kovács et al., 2022; Sood et al., 2018); and several studies showed that this response can be altered by an earlier stress experience (Li et al., 2021; Webster et al., 2023). Other studies found that animals resilient to the effects of stress in early life lacked this increase in habenular activity in response to the stressor (Li et al., 2021) or even had lower levels of c-Fos expression (Febbraro et al., 2017). Furthermore, LHb lesions enhanced the active threat avoidance behavior and fear extinction (Antunes et al., 2023), suggesting a relation between hypoactivity in habenula and adaptive behavior in aversive situations. Therefore, the suggested interpretation of infant vocalizations as an active coping behavior (Cordeiro et al., 2024) may explain their potential relationship with habenular c-Fos+ density during acute stress in adulthood. Notably, there was no effect of MS in c-Fos expression in habenular nuclei in the current study, similar to the total USV numbers used in prediction

analyses. However, this relationship was absent in animals subjected to MS, likely due to ELS disrupting the developmental connection between infant vocalizations and habenular function. Interestingly, MS animals showed alterations in the LA c-Fos expression during stress, suggesting that changes in neural connectivity or compensatory mechanisms may interfere with the involvement of the habenula in these processes, weakening its link to early-life infant behaviors.

Prolonged MS induces depressive-like behaviors assessed through multiple rodent tests (Frank et al., 2019; Huot et al., 2001; Lambás-Señas et al., 2009; Lee et al., 2007; Maniam & Morris, 2010). Particularly, a 3h MS was shown to decrease the sucrose preference of rats (Frank et al., 2019; Huot et al., 2001), while brief separation protocols do not produce such effects (Huot et al., 2001; Maniam & Morris, 2010). The results of the current work also supported the MS-induced anhedonic responses in the literature. In terms of anxiety-like behavior, this study revealed a decrease in locomotor activity in animals with a MS history that was consistent with several previous reports but generally attributed to the higher anxiety levels (Biswas et al., 2024; Scopano et al., 2023). However, an anxiolytic effect of MS was observed in EPM in adolescence through increased time spent with exploring the open arms of the maze, and this effect was lost in adulthood. Contrary to the frequently reported MS-induced anxiety-like behaviors in the literature (Frank et al., 2019; Huot et al., 2001; Lee et al., 2007; Maniam & Morris, 2010) a group of studies showed an effect in opposite direction (León Rodríguez & Dueñas, 2013; Marmendal et al., 2006; Mavrenkova et al., 2023; Ploj et al., 2002). Interestingly, these studies used Wistar rats, whereas increased anxiety has been reported in other strains, supporting the hypothesis that MS may differentially influence behavioral

outcomes in this test across genetic backgrounds, in addition to the effects of varying MS paradigms (Mavrenkova et al., 2023).

In adolescent social play, MS induced a decrease specifically in supine behavior, which serves to sustain an ongoing play interaction (Meaney & Stewart, 1981), consistent with the elevated latency to initiate pouncing and supine behaviors. ELS has been shown to induce an increase (Lundberg et al., 2017; Veenema & Neumann, 2009) or decrease in play behaviors (Cuarenta et al., 2021; Kentrop et al., 2018), and the increase has been associated to aggressive play in male animals subjected to MS (Veenema & Neumann, 2009). In this experiment, the engagement of MS animals with social play indicated a play-type specific difference, the decrease in supine behavior was not observed in the instances of pouncing or pinning that are considered as aggressive play behaviors (Panksepp et al., 1984; Veenema & Neumann, 2009). Hence, the findings point to a decrease in motivation to continue social play in animals with an MS history, while the aggressive play behaviors were not affected. Social behavior in adulthood has also been shown to decrease following MS (Gazzo et al., 2021; Mansouri et al., 2021). However, sex-specific effects were also reported in sociability, indicating a decrease in either female (Gazzo et al., 2021) or male animals subjected to MS (Mavrenkova et al., 2023). In this study, female MS animals spent a similar amount of time with the empty and social chambers across both quarter and zone analyses. However, in this design, animals interacted with the chambers in two separate sessions. This setup is different from the typical three-chamber test, where animals divide their time between the two chambers presented at the same time. Therefore, the lack of preference for the social chamber in MS female animals should be attributed to this interaction pattern rather than a reduced interest in the social chamber.

Fear memory was examined through contextual and auditory conditioning, and MS animals exhibited reduced freezing response in both sessions, with a sex-dependent effect. This aligns with a substantial body of literature demonstrating that early-life stress can disrupt fear memory processes (Chocyk et al., 2014; Guijarro et al., 2007; Mishra et al., 2019; Sanguino-Gómez & Krugers, 2024; Shin & Lee, 2023; Sun et al., 2014). However, some studies have reported no effect or heightened fear memory following MS (Diehl et al., 2014; Mishra et al., 2019; Sampath et al., 2014; Toda et al., 2014; Wang et al., 2018), suggesting that various factors such as including the age at testing, differences in MS protocols, and variations in fear conditioning procedures may substantially influence outcomes. Overall, male animals displayed higher levels of freezing, while females exhibited more active coping behaviors in response to shock in fear acquisition. This highlights a key aspect of the findings that since the MS-induced impairment was observed in freezing behavior, its effects were primarily limited to males, while females may exhibit a shift toward active coping (Gruene et al., 2015).

The results of the neurobiological assessments indicated that MS did not alter c-Fos expression in the PVN or affect CORT release following acute restraint stress, suggesting that the HPA axis response was similar between the control and experimental groups. Previous studies often reported increased activity in the PVN following MS (Fóscolo et al., 2022; Sanders & Anticevic, 2007; Trujillo et al., 2016) as well as heightened CORT release (Lajud et al., 2012; Lehmann et al., 2002; Plotsky et al., 2005), while others have found no effect (Banihashemi et al., 2011; Renard et al., 2010), highlighting variability across experimental designs. Notably, a reduction was revealed in neuronal activity in the LA of MS animals, contrary to several earlier reports (Qin et al., 2021; Raineki et al., 2012; Sanders & Anticevic,

2007). Given BLA's critical role in processing aversive stimuli (Duvarci & Pare, 2014; LeDoux, 2000; Ressler & Maren, 2019), this reduction suggests ELS-induced alterations in neural engagement of the BLA in acute stress, potentially indicating long-term modifications in amygdala function. A low bedding ELS weakened the neuronal activity in multiple amygdaloid divisions in response to an acute stressor (Junod et al., 2019). In addition, freezing response to this stimulus (i.e., foot shock) was also decreased in the ELS group, which was associated with the c-Fos density in LA. This finding suggests a positive relation between the passive responses to a threatening stimulus and neural activation in the LA. Therefore, the decrease in the freezing levels of MS animals in the current work may be a supporting finding to the reduction in LA neuronal response to restraint stress.

In conclusion, this thesis employed a group of neurobiological and behavioral measurements in both male and female rats to examine the longitudinal effects of early life MS stress and reveal the predictive value of infant USVs on various affective processes across adolescence and adulthood. I demonstrated MS-induced changes in infant USV calls through a maternal potentiation paradigm and identified these vocalizations as predictors of exploratory behavior in adolescence and stress-induced neuronal activity in the habenula. Moreover, these findings highlighted significant behavioral effects of the MS procedure, including decreased locomotor activity, reduction in social play, and elevated exploration in adolescence. In adulthood, MS induced anhedonia, impaired fear memory, and alleviated neuronal activation in the LA following acute stress. Overall, this research provides detailed insights into the predictive value of pup USVs in subsequent affective processes, while also offering a longitudinal perspective on the effects of MS as an ELS model.

APPENDIX A

ETHICS COMMITTEE APPROVAL

T.C.
BOĞAZİÇİ ÜNİVERSİTESİ REKTÖRLÜĞÜ
Kurumsal Hayvan Deneyleri Yerel Etik Kurulu (Bühadyek)

Sayı : E-44697215-050.04-200809 08.10.2024
Konu : 2024-08 Kayıt Numaralı Başvurunuz Hakkında

Sayın Doç. Dr. Güneş ÜNAL

Yürüttücülüğünü üstlendiğiniz "Behavioral, neurobiological and hormonal effects of maternal separation stress in rats (Sıçanlarda anneden ayrılma stres modelinin davranışsal nörobiyolojik ve hormonal etkileri)" adlı ve 2024-08 kodlu başvurunuz, Boğaziçi Üniversitesi Kurumsal Hayvan Deneyleri Yerel Etik Kurulu'nun (BÜHADYEK) 24.09.2024 tarih ve 2024/09 sayılı kurul toplantısında görüşülmüş ve projenize etik onay verilmesi uygun bulunmuştur.

Bu karar tüm üyelerin toplantıya on-line olarak katılımıyla ve oybirliği ile alınmıştır. Onay mektubu tüm üyeler adına Komisyon Başkanı tarafından imzalanmıştır. Bilgilerinize rica ederiz.

Saygılarımızla,

Not : Komisyon üyelerinden Doç. Dr. Güneş Ünal proje ekibinde bulunduğundan görüş bildirmemiştir.

Prof. Dr. Naime Hale SAYBAŞILI
Kurul Başkanı

Adres: Boğaziçi Üniversitesi 34342 Bebek/ İstanbul
Telefon No: Faks No:
e-Posta: İnternet Adresi: www.bogazici.edu.tr
Kep Adresi: bogaziciuniversitesi@hs01.kep.tr
Bilgi İçin: Nurşen MUNAR
Mühendis
Telefon No:

APPENDIX B

STATISTICS OF PATH ANALYSES

Control Group						
Outcome	Predictor	B	SE	Z	Std. B	*p*
EPM 1	Pup USV	0.579	0.237	2.443	0.480	0.015
	Sex	-0.730	0.428	-1.705	-0.356	0.088
Social Play	Pup USV	-0.092	0.215	-0.426	-0.077	0.670
	Sex	1.244	0.400	3.110	0.617	0.002
EPM 2	Pup USV	0.261	0.336	0.776	0.256	0.438
	EPM 1	0.025	0.320	0.077	0.029	0.938
	Social Play	0.108	0.345	0.312	0.125	0.755
	Sex	-1.167	0.804	-1.451	-0.671	0.147
Sociability	Pup USV	0.348	0.341	1.022	0.334	0.307
	EPM 1	-0.046	0.427	-0.107	-0.053	0.914
	Social Play	0.102	0.412	0.247	0.116	0.805
	Sex	0.564	0.948	0.595	0.318	0.552
SPT	Pup USV	0.160	0.272	0.589	0.234	0.556
	EPM 1	-0.086	0.223	-0.387	-0.152	0.699
	Social Play	0.007	0.291	0.025	0.012	0.980
	Sex	0.282	0.581	0.485	0.242	0.628
Active Coping	Pup USV	0.339	0.369	0.919	0.282	0.358
	EPM 1	-0.134	0.260	-0.516	-0.135	0.606
	Social Play	0.015	0.278	0.053	0.015	0.958
	Sex	-1.393	0.560	-2.486	-0.681	0.013
Fear Memory	Pup USV	-0.533	0.437	-1.220	-0.386	0.223
	EPM 1	-0.075	0.388	-0.194	-0.065	0.847
	Social Play	-0.196	0.404	-0.485	-0.168	0.628
	Sex	1.350	0.766	1.762	0.574	0.078
Covariant 1	Covariant 2	B	SE	Z	Beta	*p*
Sociability	EPM 2	0.113	0.118	0.960	0.231	0.337
SPT	EPM 2	-0.029	0.072	-0.405	-0.083	0.685
	Sociability	0.151	0.087	1.725	0.365	0.085
Active Coping	EPM 2	-0.118	0.081	-1.456	-0.242	0.145
	Sociability	0.163	0.122	1.342	0.284	0.180
	SPT	0.165	0.059	2.787	0.402	0.005
Fear Memory	EPM 2	-0.173	0.127	-1.366	-0.302	0.172
	Sociability	-0.069	0.139	-0.497	-0.102	0.619
	SPT	0.350	0.140	2.505	0.727	0.012
	Active Coping	0.220	0.099	2.223	0.328	0.026

MS Group						
Outcome	Predictor	B	SE	Z	Std. B	*p*
EPM 1	Pup USV	0.329	0.125	2.633	0.496	0.008
	Sex	0.260	0.250	1.370	0.215	0.300
Social Play	Pup USV	0.247	0.255	0.966	0.248	0.334
	Sex	0.674	0.509	1.325	0.372	0.185
EPM 2	Pup USV	-0.113	0.466	-0.243	-0.095	0.808
	EPM 1	0.362	0.834	0.434	0.201	0.664
	Social Play	0.074	0.503	0.148	0.062	0.883
	Sex	-1.298	0.534	-2.432	-0.597	0.015
Sociability	Pup USV	-0.500	0.400	-1.250	-0.436	0.211
	EPM 1	0.314	0.515	0.609	0.181	0.542
	Social Play	0.263	0.384	0.684	0.228	0.494
	Sex	0.905	0.642	1.409	0.433	0.159
SPT	Pup USV	0.045	0.670	0.067	0.036	0.946
	EPM 1	-0.743	0.723	-1.028	-0.398	0.304
	Social Play	0.221	0.602	0.367	0.178	0.713
	Sex	0.102	0.835	0.122	0.045	0.903
Active Coping	Pup USV	0.120	0.313	0.384	0.121	0.701
	EPM 1	0.139	0.324	0.431	0.093	0.667
	Social Play	-0.232	0.309	-0.749	-0.233	0.454
	Sex	-1.464	0.299	-4.895	-0.812	<.001
Fear Memory	Pup USV	-0.365	0.448	-0.814	-0.464	0.416
	EPM 1	-0.189	0.560	-0.337	-0.159	0.736
	Social Play	0.211	0.382	0.552	0.267	0.581
	Sex	0.451	0.397	1.136	0.315	0.256
Covariant 1	Covariant 2	B	SE	Z	Beta	*p*
Sociability	EPM 2	-0.020	0.180	-0.112	-0.026	0.911
SPT	EPM 2	0.336	0.163	2.065	0.355	0.039
	Sociability	0.012	0.189	0.067	0.014	0.947
Active Coping	EPM 2	-0.115	0.088	-1.302	-0.294	0.193
	Sociability	0.115	0.125	0.915	0.317	0.360
	SPT	0.114	0.085	1.335	0.254	0.182
Fear Memory	EPM 2	0.050	0.105	0.473	0.094	0.636
	Sociability	0.037	0.083	0.443	0.075	0.657
	SPT	0.234	0.175	1.348	0.384	0.178
	Active Coping	0.050	0.069	0.725	0.199	0.469

APPENDIX C

STATISTICS OF REGRESSION ANALYSES

Outcome	Predictor	Model statistics				Predictor		Group		Predictor* Group	
		F	*df*	*p*	R^2_{adj}	B	*p*	B	*p*	B	*p*
BLA	Pup USV	1.416	3, 25	0.262	0.043	-0.159	1.000	-0.639	0.430	0.392	1.000
	EPM 1	1.324	3, 25	0.289	0.034	0.198	1.000	-0.644	0.618	-0.391	1.000
	Social Play	1.002	3, 25	0.408	<.001	-0.029	1.000	-0.657	0.390	0.000	1.000
PVN	Pup USV	1.724	3, 27	0.186	0.068	-0.543	0.142	0.042	1.000	0.601	0.430
	EPM 1	1.885	3, 27	0.156	0.081	-0.510	0.154	0.584	0.688	0.177	1.000
	Social Play	0.566	3, 27	0.642	-0.045	0.059	1.000	0.104	1.000	-0.398	1.000
PVT	Pup USV	1.220	3, 22	0.326	0.026	0.094	1.000	-0.448	1.000	0.263	1.000
	EPM 1	1.777	3, 22	0.181	0.086	-0.127	1.000	-0.830	0.295	0.938	0.352
	Social Play	0.755	3, 22	0.531	-0.030	0.087	1.000	-0.542	0.859	-0.289	1.000
Habenula	Pup USV	4.007	3, 24	0.019	0.250	-0.742	0.016	-0.328	1.000	1.048	0.018
	EPM 1	0.688	3, 24	0.568	-0.036	-0.336	0.755	-0.039	1.000	0.484	1.000
	Social Play	0.159	3, 24	0.923	-0.103	-0.086	1.000	-0.225	1.000	-0.089	1.000
CORT	Pup USV	0.034	3, 19	0.991	-0.152	0.060	1.000	0.078	1.000	0.022	1.000
	EPM 1	0.025	3, 19	0.994	-0.153	0.038	1.000	-0.032	1.000	0.115	1.000
	Social Play	0.760	3, 19	0.530	-0.034	0.418	0.721	0.125	1.000	-0.224	1.000

REFERENCES

Aizawa, H., Kobayashi, M., Tanaka, S., Fukai, T., & Okamoto, H. (2012). Molecular characterization of the subnuclei in rat habenula. *Journal of Comparative Neurology*, *520*(18), 4051–4066. https://doi.org/10.1002/cne.23167

Allin, J. T., & Banks, E. M. (1972). Functional aspects of ultrasound production by infant albino rats (Rattus norvegicus). *Animal Behaviour*, *20*(1), 175–185. https://doi.org/10.1016/S0003-3472(72)80189-1

Antunes, G. F., Campos, A. C. P., Martins, D. de O., Gouveia, F. V., Rangel Junior, M. J., Pagano, R. L., & Martinez, R. C. R. (2023). Unravelling the role of habenula subnuclei on avoidance response: Focus on activation and neuroinflammation. *International Journal of Molecular Sciences*, *24*(13), 10693. https://doi.org/10.3390/ijms241310693

Banihashemi, L., O'Neill, E. J., & Rinaman, L. (2011). Central neural responses to restraint stress are altered in rats with an early life history of repeated brief maternal separation. *Neuroscience*, *192*, 413–428. https://doi.org/10.1016/j.neuroscience.2011.06.052

Bedoya-Pérez, M. A., Rahman, M. F., & McGregor, I. S. (2025). Conspecific presence facilitates 22-kHz ultrasonic calls in male rats exposed to cat odor. *Scientific Reports*, *15*(1), 7658. https://doi.org/10.1038/s41598-025-90272-0

Biswas, B., Eapen, V., Morris, M. J., & Jones, N. M. (2024). Combined effect of maternal separation and early-life immune activation on brain and behaviour of rat offspring. *Biomolecules*, *14*(2), 197. https://doi.org/10.3390/biom14020197

Blanchard, R. J., Blanchard, D. C., Rodgers, J., & Weiss, S. M. (1990). The characterization and modelling of antipredator defensive behavior. *Neuroscience & Biobehavioral Reviews*, *14*(4), 463–472. https://doi.org/10.1016/S0149-7634(05)80069-7

Branchi, I., Santucci, D., & Alleva, E. (2006). Analysis of ultrasonic vocalizations emitted by infant rodents. *Current Protocols in Toxicology*, *30*(1). https://doi.org/10.1002/0471140856.tx1312s30

Brudzynski, S. M. (2019). Emission of 22 kHz vocalizations in rats as an evolutionary equivalent of human crying: Relationship to depression. *Behavioural Brain Research*, *363*, 1–12. https://doi.org/10.1016/j.bbr.2019.01.033

Brunelli, S. A., Masmela, J. R., Shair, H. N., & Hofer, M. A. (1998). Effects of biparental rearing on ultrasonic vocalization (USV) responses of rat pups (Rattus norvegicus). *Journal of Comparative Psychology*, *112*(4), 331–343. https://doi.org/10.1037/0735-7036.112.4.331

Brunelli, S. A., Shair, H. N., & Hofer, M. A. (1994). Hypothermic vocalizations of rat pups (Rattus norvegicus) and direct maternal search behavior. *Journal of Comparative Psychology*, *108*(3), 298–303. https://doi.org/10.1037/0735-7036.108.3.298

Burenkova, O. V., Averkina, A. A., Aleksandrova, E. A., & Zarayskaya, I. Yu. (2020). Brief but enough: 45-min maternal separation elicits behavioral and physiological responses in neonatal mice and changes in dam maternal behavior. *Physiology & Behavior*, *222*, 112877. https://doi.org/10.1016/j.physbeh.2020.112877

Cameron, S., Weston-Green, K., & Newell, K. A. (2024). The disappointment centre of the brain gets exciting: a systematic review of habenula dysfunction in depression. *Translational Psychiatry*, *14*(1), 499. https://doi.org/10.1038/s41398-024-03199-x

Carr, C. P., Martins, C. M. S., Stingel, A. M., Lemgruber, V. B., & Juruena, M. F. (2013). The role of early life stress in adult psychiatric disorders. *Journal of Nervous & Mental Disease*, *201*(12), 1007–1020. https://doi.org/10.1097/NMD.0000000000000049

Chaby, L. E., Zhang, L., & Liberzon, I. (2017). The effects of stress in early life and adolescence on posttraumatic stress disorder, depression, and anxiety symptomatology in adulthood. *Current Opinion in Behavioral Sciences*, *14*, 86–93. https://doi.org/10.1016/j.cobeha.2017.01.001

Chastrette, N., Pfaff, D. W., & Gibbs, R. B. (1991). Effects of daytime and nighttime stress on Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus, the habenula, and the posterior paraventricular nucleus of the thalamus. *Brain Research*, *563*(1–2), 339–344. https://doi.org/10.1016/0006-8993(91)91559-J

Chen, L., Shen, B., Liu, D., & Li, S. (2014). The effects of early-life predator stress on anxiety- and depression-like behaviors of adult rats. *Neural Plasticity*, *2014*, 1–10. https://doi.org/10.1155/2014/163908

Chocyk, A., Przyborowska, A., Makuch, W., Majcher-Maślanka, I., Dudys, D., & Wędzony, K. (2014). The effects of early-life adversity on fear memories in adolescent rats and their persistence into adulthood. *Behavioural Brain Research*, *264*, 161–172. https://doi.org/10.1016/j.bbr.2014.01.040

Cicchetti, D. (2010). Resilience under conditions of extreme stress: a multilevel perspective. *World Psychiatry*, *9*(3), 145–154. https://doi.org/10.1002/j.2051-5545.2010.tb00297.x

Clemmons, J. C., Walsh, K., DiLillo, D., & Messman-Moore, T. L. (2007). Unique and combined contributions of multiple child abuse types and abuse severity to adult trauma symptomatology. *Child Maltreatment*, *12*(2), 172–181. https://doi.org/10.1177/1077559506298248

Coffey, K. R., Marx, R. E., & Neumaier, J. F. (2019). DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations. *Neuropsychopharmacology*, *44*(5), 859–868. https://doi.org/10.1038/s41386-018-0303-6

Colman, I., Jones, P. B., Kuh, D., Weeks, M., Naicker, K., Richards, M., & Croudace, T. J. (2014). Early development, stress and depression across the life course: pathways to depression in a national British birth cohort. *Psychological Medicine*, *44*(13), 2845–2854. https://doi.org/10.1017/S0033291714000385

Cordeiro, N., Pochapski, J. A., Luna, W. S., Baltazar, G., Schwarting, R. K., Andreatini, R., & Da Cunha, C. (2024). Forty-kHz ultrasonic vocalizations of rat pups predict adult behavior in the elevated plus-maze behavior but not the effect of cocaine on 50-kHz ultrasonic vocalizations. *Behavioural Brain Research*, *458*, 114759. https://doi.org/10.1016/j.bbr.2023.114759

Cuarenta, A., Kigar, S. L., Henion, I. C., Chang, L., Bakshi, V. P., & Auger, A. P. (2021). Early life stress during the neonatal period alters social play and Line1 during the juvenile stage of development. *Scientific Reports*, *11*(1), 3549. https://doi.org/10.1038/s41598-021-82953-3

Diehl, L. A., Pereira, N. de S. C., Laureano, D. P., Benitz, A. N. D., Noschang, C., Ferreira, A. G. K., Scherer, E. B., Machado, F. R., Henriques, T. P., Wyse, A. T. S., Molina, V., & Dalmaz, C. (2014). Contextual fear conditioning in maternal separated rats: The amygdala as a site for alterations. *Neurochemical Research*, *39*(2), 384–393. https://doi.org/10.1007/s11064-013-1230-x

Durieux, L., Herbeaux, K., Borcuk, C., Hildenbrand, C., Andry, V., Goumon, Y., Barbelivien, A., Mathis, C., Bataglia, D., Majchrzak, M., & Lecourtier, L. (2022). Functional brain-wide network mapping during acute stress exposure in rats: Interaction between the lateral habenula and cortical, amygdalar, hypothalamic and monoaminergic regions. *European Journal of Neuroscience*, *56*(8), 5154–5176. https://doi.org/10.1111/ejn.15803

Duvarci, S., & Pare, D. (2014). Amygdala microcircuits controlling learned fear. *Neuron*, *82*(5), 966–980. https://doi.org/10.1016/j.neuron.2014.04.042

Ehret, G. (2005). Infant rodent ultrasounds? A gate to the understanding of sound communication. *Behavior Genetics*, *35*(1), 19–29. https://doi.org/10.1007/s10519-004-0853-8

Elhwuegi, A. S. (2004). Central monoamines and their role in major depression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *28*(3), 435–451. https://doi.org/10.1016/j.pnpbp.2003.11.018

Ellenbroek, B. A., van den Kroonenberg, P. T. J. M., & Cools, A. R. (1998). The effects of an early stressful life event on sensorimotor gating in adult rats. *Schizophrenia Research*, *30*(3), 251–260. https://doi.org/10.1016/S0920-9964(97)00149-7

Elliott, N. D., & Richardson, R. (2019). The effects of early life stress on context fear generalization in adult rats. *Behavioral Neuroscience*, *133*(1), 50–58. https://doi.org/10.1037/bne0000289

Enthoven, L., Oitzl, M. S., Koning, N., van der Mark, M., & de Kloet, E. R. (2008). Hypothalamic-Pituitary-Adrenal axis activity of newborn mice rapidly desensitizes to repeated maternal absence but becomes highly responsive to novelty. *Endocrinology*, *149*(12), 6366–6377. https://doi.org/10.1210/en.2008-0238

Faure, A., Pittaras, E., Nosjean, A., Chabout, J., Cressant, A., & Granon, S. (2017). Social behaviors and acoustic vocalizations in different strains of mice. *Behavioural Brain Research*, *320*, 383–390. https://doi.org/10.1016/j.bbr.2016.11.003

Febbraro, F., Svenningsen, K., Tran, T. P., & Wiborg, O. (2017). Neuronal substrates underlying stress resilience and susceptibility in rats. *PLOS ONE*, *12*(6), e0179434. https://doi.org/10.1371/journal.pone.0179434

Feldman, S., Conforti, N., & Weidenfeld, J. (1995). Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli. *Neuroscience & Biobehavioral Reviews*, *19*(2), 235–240. https://doi.org/10.1016/0149-7634(94)00062-6

Fendt, M., Brosch, M., Wernecke, K. E. A., Willadsen, M., & Wöhr, M. (2018). Predator odour but not TMT induces 22-kHz ultrasonic vocalizations in rats that lead to defensive behaviours in conspecifics upon replay. *Scientific Reports*, *8*(1), 11041. https://doi.org/10.1038/s41598-018-28927-4

Fóscolo, D. R. C., Lima, P. M. A., Rodovalho, G. V., & Coimbra, C. C. (2022). Early maternal separation alters the activation of stress-responsive brain areas in adulthood. *Neuroscience Letters*, *771*, 136464. https://doi.org/10.1016/j.neulet.2022.136464

Frank, D., Zlotnik, A., Kofman, O., Grinshpun, J., Severynovska, O., Brotfain, E., Kut, R., Natanel, D., Melamed, I., & Boyko, M. (2019). Early life stress induces submissive behavior in adult rats. *Behavioural Brain Research*, *372*, 112025. https://doi.org/10.1016/j.bbr.2019.112025

Frodl, T., & O'Keane, V. (2013). How does the brain deal with cumulative stress? A review with focus on developmental stress, HPA axis function and hippocampal structure in humans. *Neurobiology of Disease*, *52*, 24–37. https://doi.org/10.1016/j.nbd.2012.03.012

Gazzo, G., Melchior, M., Caussaint, A., Gieré, C., Lelièvre, V., & Poisbeau, P. (2021). Overexpression of chloride importer NKCC1 contributes to the sensory-affective and sociability phenotype of rats following neonatal maternal separation. *Brain, Behavior, and Immunity*, *92*, 193–202. https://doi.org/10.1016/j.bbi.2020.12.010

Gibb, B. E., Chelminski, I., & Zimmerman, M. (2007). Childhood emotional, physical, and sexual abuse, and diagnoses of depressive and anxiety disorders in adult psychiatric outpatients. *Depression and Anxiety*, *24*(4), 256–263. https://doi.org/10.1002/da.20238

Granata, L. E., Valentine, A., Hirsch, J. L., & Brenhouse, H. C. (2022). Infant ultrasonic vocalizations predict adolescent social behavior in rats: Effects of early life adversity. *Developmental Psychobiology*, *64*(3). https://doi.org/10.1002/dev.22260

Granata, L., Valentine, A., Hirsch, J. L., Honeycutt, J., & Brenhouse, H. (2021). Trajectories of mother-infant communication: An experiential measure of the impacts of early life adversity. *Frontiers in Human Neuroscience*, *15*. https://doi.org/10.3389/fnhum.2021.632702

Greenwood, B. N., Foley, T. E., Burhans, D., Maier, S. F., & Fleshner, M. (2005). The consequences of uncontrollable stress are sensitive to duration of prior wheel running. *Brain Research*, *1033*(2), 164–178. https://doi.org/10.1016/j.brainres.2004.11.037

Gruene, T. M., Flick, K., Stefano, A., Shea, S. D., & Shansky, R. M. (2015). Sexually divergent expression of active and passive conditioned fear responses in rats. *ELife*, *4*. https://doi.org/10.7554/eLife.11352

Guijarro, J. Z., Tiba, P. A., Ferreira, T. L., Kawakami, S. E., Oliveira, M. G. M., & Suchecki, D. (2007). Effects of brief and long maternal separations on the HPA axis activity and the performance of rats on context and tone fear conditioning. *Behavioural Brain Research*, *184*(2), 101–108. https://doi.org/10.1016/j.bbr.2007.06.020

Harmon-Jones, S. K., Cowan, C. S. M., Shnier, N., & Richardson, R. (2020). Is good memory always a good thing? An early offset of infantile amnesia predicts anxiety-like behavior throughout development in rats. *Behaviour Research and Therapy*, *135*, 103763. https://doi.org/10.1016/j.brat.2020.103763

Heim, C., & Nemeroff, C. B. (2001). The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biological Psychiatry*, *49*(12), 1023–1039. https://doi.org/10.1016/S0006-3223(01)01157-X

Hennessy, M. B., Miller, E. E., & Shair, H. N. (2006). Brief exposure to the biological mother “potentiates” the isolation behavior of precocial Guinea pig pups. *Developmental Psychobiology*, *48*(8), 653–659. https://doi.org/10.1002/dev.20180

Herman, J. P., Nawreen, N., Smail, M. A., & Cotella, E. M. (2020). Brain mechanisms of HPA axis regulation: neurocircuitry and feedback in context Richard Kvetnansky lecture. *Stress*, *23*(6), 617–632. https://doi.org/10.1080/10253890.2020.1859475

Higgins, D. J. (2004). The importance of degree versus type of maltreatment: A cluster analysis of child abuse types. *The Journal of Psychology*, *138*(4), 303–324. https://doi.org/10.3200/JRLP.138.4.303-324

Hodgson, R., Guthrie, D., & Varty, G. (2008). Duration of ultrasonic vocalizations in the isolated rat pup as a behavioral measure: Sensitivity to anxiolytic and antidepressant drugs. *Pharmacology Biochemistry and Behavior*, *88*(3), 341–348. https://doi.org/10.1016/j.pbb.2007.09.005

Hodgson, R., Higgins, G. A., Guthrie, D. H., Lu, S. X., Pond, A. J., Mullins, D. E., Guzzi, M. F., Parker, E. M., & Varty, G. B. (2007). Comparison of the V1b antagonist, SSR149415, and the CRF1 antagonist, CP-154,526, in rodent models of anxiety and depression. *Pharmacology Biochemistry and Behavior*, *86*(3), 431–440. https://doi.org/10.1016/j.pbb.2006.12.021

Hofer, M. A., Brunelli, S. A., & Shair, H. N. (1993). Ultrasonic vocalization responses of rat pups to acute separation and contact comfort do not depend on maternal thermal cues. *Developmental Psychobiology*, *26*(2), 81–95. https://doi.org/10.1002/dev.420260202

Hofer, M. A., Brunelli, S. A., & Shair, H. N. (1994). Potentiation of isolation-induced vocalization by brief exposure of rat pups to maternal cues. *Developmental Psychobiology*, *27*(8), 503–517. https://doi.org/10.1002/dev.420270804

Hofer, M. A., Masmela, J. R., Brunelli, S. A., & Shair, H. N. (1998). The ontogeny of maternal potentiation of the infant rats' isolation call. *Developmental Psychobiology*, *33*(3), 189–201. https://doi.org/10.1002/(SICI)1098-2302(199811)33:3<189::AID-DEV1>3.0.CO;2-N

Hovens, J. G. F. M., Wiersma, J. E., Giltay, E. J., Van Oppen, P., Spinhoven, P., Penninx, B. W. J. H., & Zitman, F. G. (2010). Childhood life events and childhood trauma in adult patients with depressive, anxiety and comorbid disorders vs. controls. *Acta Psychiatrica Scandinavica*, *122*(1), 66–74. https://doi.org/10.1111/j.1600-0447.2009.01491.x

Huot, R., K., T., Meaney, M., & Plotsky, P. (2001). Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. *Psychopharmacology*, *158*(4), 366–373. https://doi.org/10.1007/s002130100701

Insel, T. R., Hill, J. L., & Mayor, R. B. (1986). Rat pup ultrasonic isolation calls: Possible mediation by the benzodiazepine receptor complex. *Pharmacology Biochemistry and Behavior*, *24*(5), 1263–1267. https://doi.org/10.1016/0091-3057(86)90182-6

Jacqueline, N., & Crawley, J. N. (2007). Mouse behavioral assays relevant to the symptoms of autism*. *Brain Pathology*, *17*(4), 448–459. https://doi.org/10.1111/j.1750-3639.2007.00096.xx

Jaimes-Hoy, L., Gutiérrez-Mariscal, M., Vargas, Y., Pérez-Maldonado, A., Romero, F., Sánchez-Jaramillo, E., Charli, J.-L., & Joseph-Bravo, P. (2016). Neonatal maternal separation alters, in a sex-specific manner, the expression of TRH, of TRH-degrading ectoenzyme in the rat hypothalamus, and the response of the thyroid axis to starvation. *Endocrinology*, *157*(8), 3253–3265. https://doi.org/10.1210/en.2016-1239

James, M. H., Campbell, E. J., Walker, F. R., Smith, D. W., Richardson, H. N., Hodgson, D. M., & Dayas, C. V. (2014). Exercise reverses the effects of early life stress on orexin cell reactivity in male but not female rats. *Frontiers in Behavioral Neuroscience*, *8*. https://doi.org/10.3389/fnbeh.2014.00244

Junod, A., Opendak, M., LeDoux, J. E., & Sullivan, R. M. (2019). Development of threat expression following infant maltreatment: Infant and adult enhancement but adolescent attenuation. *Frontiers in Behavioral Neuroscience*, *13*. https://doi.org/10.3389/fnbeh.2019.00130

Kaidbey, J. H., Ranger, M., Myers, M. M., Anwar, M., Ludwig, R. J., Schulz, A. M., Barone, J. L., Kolacz, J., & Welch, M. G. (2019). Early life maternal separation and maternal behaviour modulate acoustic characteristics of rat pup ultrasonic vocalizations. *Scientific Reports*, *9*(1), 19012. https://doi.org/10.1038/s41598-019-54800-z

Kehne, J. H., Coverdale, S., McCloskey, T. C., Hoffman, D. C., & Cassella, J. V. (2000). Effects of the CRF1 receptor antagonist, CP 154,526, in the separation-induced vocalization anxiolytic test in rat pups. *Neuropharmacology*, *39*(8), 1357–1367. https://doi.org/10.1016/S0028-3908(00)00043-5

Kentrop, J., Smid, C. R., Achterberg, E. J. M., van IJzendoorn, M. H., Bakermans-Kranenburg, M. J., Joëls, M., & van der Veen, R. (2018). Effects of maternal deprivation and complex housing on rat social behavior in adolescence and adulthood. *Frontiers in Behavioral Neuroscience*, *12*. https://doi.org/10.3389/fnbeh.2018.00193

Kingir, E., Sevinc, C., & Unal, G. (2023). Chronic oral ketamine prevents anhedonia and alters neuronal activation in the lateral habenula and nucleus accumbens in rats under chronic unpredictable mild stress. *Neuropharmacology*, *228*, 109468. https://doi.org/10.1016/j.neuropharm.2023.109468

Knutson, B., Burgdorf, J., & Panksepp, J. (1998). Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *Journal of Comparative Psychology*, *112*(1), 65–73. https://doi.org/10.1037/0735-7036.112.1.65

Kovács, L. Á., Füredi, N., Ujvári, B., Golgol, A., & Gaszner, B. (2022). Age-dependent FOSB/ΔFOSB response to acute and chronic stress in the extended amygdala, hypothalamic paraventricular, habenular, centrally-projecting edinger-westphal, and dorsal raphe nuclei in male rats. *Frontiers in Aging Neuroscience*, *14*. https://doi.org/10.3389/fnagi.2022.862098

Ladd, C. O., Owens, M. J., & Nemeroff, C. B. (1996). Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation. *Endocrinology*, *137*(4), 1212–1218. https://doi.org/10.1210/endo.137.4.8625891

Lajud, N., Roque, A., Cajero, M., Gutiérrez-Ospina, G., & Torner, L. (2012). Periodic maternal separation decreases hippocampal neurogenesis without affecting basal corticosterone during the stress hyporesponsive period, but alters HPA axis and coping behavior in adulthood. *Psychoneuroendocrinology*, *37*(3), 410–420. https://doi.org/10.1016/j.psyneuen.2011.07.011

Lambás-Señas, L., Mnie-Filali, O., Certin, V., Faure, C., Lemoine, L., Zimmer, L., & Haddjeri, N. (2009). Functional correlates for 5-HT1A receptors in maternally deprived rats displaying anxiety and depression-like behaviors. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *33*(2), 262–268. https://doi.org/10.1016/j.pnpbp.2008.11.017

Lecca, S., Pelosi, A., Tchenio, A., Moutkine, I., Lujan, R., Hervé, D., & Mameli, M. (2016). Rescue of GABAB and GIRK function in the lateral habenula by protein phosphatase 2A inhibition ameliorates depression-like phenotypes in mice. *Nature Medicine*, *22*(3), 254–261. https://doi.org/10.1038/nm.4037

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience*, *23*(1), 155–184. https://doi.org/10.1146/annurev.neuro.23.1.155

Lee, J.-H., Kim, H. J., Kim, J. G., Ryu, V., Kim, B.-T., Kang, D.-W., & Jahng, J. W. (2007). Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. *Neuroscience Research*, *58*(1), 32–39. https://doi.org/10.1016/j.neures.2007.01.008

Lehmann, J., Russig, H., Feldon, J., & Pryce, C. R. (2002). Effect of a single maternal separation at different pup ages on the corticosterone stress response in adult and aged rats. *Pharmacology Biochemistry and Behavior*, *73*(1), 141–145. https://doi.org/10.1016/S0091-3057(02)00788-8

Lenell, C., Broadfoot, C. K., Schaen-Heacock, N. E., & Ciucci, M. R. (2021). Biological and acoustic sex differences in rat ultrasonic vocalization. *Brain Sciences*, *11*(4), 459. https://doi.org/10.3390/brainsci11040459

León Rodríguez, D. A., & Dueñas, Z. (2013). Maternal separation during breastfeeding induces gender-dependent changes in anxiety and the GABA-A receptor alpha-subunit in adult Wistar rats. *PLoS ONE*, *8*(6), e68010. https://doi.org/10.1371/journal.pone.0068010

Levine, S. (2001). Primary social relationships influence the development of the hypothalamic–pituitary–adrenal axis in the rat. *Physiology & Behavior*, *73*(3), 255–260. https://doi.org/10.1016/S0031-9384(01)00496-6

Li, B., Piriz, J., Mirrione, M., Chung, C., Proulx, C. D., Schulz, D., Henn, F., & Malinow, R. (2011). Synaptic potentiation onto habenula neurons in the learned

helplessness model of depression. *Nature*, *470*(7335), 535–539. https://doi.org/10.1038/nature09742

Li, Z. L., Wang, Y., Zou, H.-W., Jing, X.-Y., Liu, Y.-J., & Li, L.-F. (2021). GABA(B) receptors within the lateral habenula modulate stress resilience and vulnerability in mice. *Physiology & Behavior*, *230*, 113311. https://doi.org/10.1016/j.physbeh.2021.113311

Lukas, M., & Wöhr, M. (2015). Endogenous vasopressin, innate anxiety, and the emission of pro-social 50-kHz ultrasonic vocalizations during social play behavior in juvenile rats. *Psychoneuroendocrinology*, *56*, 35–44. https://doi.org/10.1016/j.psyneuen.2015.03.005

Lundberg, S., Martinsson, M., Nylander, I., & Roman, E. (2017). Altered corticosterone levels and social play behavior after prolonged maternal separation in adolescent male but not female Wistar rats. *Hormones and Behavior*, *87*, 137–144. https://doi.org/10.1016/j.yhbeh.2016.11.016

Maniam, J., & Morris, M. J. (2010). Palatable cafeteria diet ameliorates anxiety and depression-like symptoms following an adverse early environment. *Psychoneuroendocrinology*, *35*(5), 717–728. https://doi.org/10.1016/j.psyneuen.2009.10.013

Mansouri, M., Pouretemad, H., Wegener, G., Roghani, M., Afshari, M., Mallard, C., & Ardalan, M. (2021). Dual profile of environmental enrichment and autistic-like behaviors in the maternal separated model in rats. *International Journal of Molecular Sciences*, *22*(3), 1173. https://doi.org/10.3390/ijms22031173

Maras, P. M., & Baram, T. Z. (2015). Early-Life Stress: Rodent models, lessons and challenges. In *Neuroendocrinology of Stress* (pp. 265–286). Wiley. https://doi.org/10.1002/9781118921692.ch12

Marmendal, M., Eriksson, C., & Fahlke, C. (2006). Early deprivation increases exploration and locomotion in adult male Wistar offspring. *Pharmacology Biochemistry and Behavior*, *85*(3), 535–544. https://doi.org/10.1016/j.pbb.2006.10.005

Mavrenkova, P. V., Khlebnikova, N. N., Alchinova, I. B., Demorzhi, M. S., Shoibonov, B. B., & Karganov, M. Yu. (2023). Effects of maternal separation and subsequent stress on behaviors and brain monoamines in rats. *Brain Sciences*, *13*(6), 956. https://doi.org/10.3390/brainsci13060956

McClafferty, S. R., Paniagua-Ugarte, C., Hannabass, Z. M., Jackson, P. A., & Hayes, D. M. (2024). Comparing the effects of infant maternal and sibling separation on adolescent behavior in rats (Rattus norvegicus). *PLOS ONE*, *19*(8), e0308958. https://doi.org/10.1371/journal.pone.0308958

McCormick, C. M., Kehoe, P., & Kovacs, S. (1998). Corticosterone release in response to repeated, short episodes of neonatal isolation : evidence of

sensitization. *International Journal of Developmental Neuroscience*, *16*(3–4), 175–185. https://doi.org/10.1016/S0736-5748(98)00026-4

Meaney, M., & Stewart, J. (1981). Neonatal androgens influence the social play of prepubescent rats. *Hormones and Behavior*, *15*(2), 197–213. https://doi.org/10.1016/0018-506X(81)90028-3

Mishra, P. K., Kutty, B. M., & Laxmi, T. R. (2019). The impact of maternal separation and isolation stress during stress hyporesponsive period on fear retention and extinction recall memory from 5-week- to 1-year-old rats. *Experimental Brain Research*, *237*(1), 181–190. https://doi.org/10.1007/s00221-018-5411-3

Moriceau, S. (2009). Enduring neurobehavioral effects of early life trauma mediated through learning and corticosterone suppression. *Frontiers in Behavioral Neuroscience*, *3*. https://doi.org/10.3389/neuro.08.022.2009

Nagao, M., Kamo, H., Akiguchi, I., & Kimura, J. (1993). Induction of c-Fos-like protein in the lateral habenular nucleus by persistent noxious peripheral stimulation. *Neuroscience Letters*, *151*(1), 37–40. https://doi.org/10.1016/0304-3940(93)90039-N

Noirot, E. (1968). Ultrasounds in young rodents. II. Changes with age in albino rats. *Animal Behaviour*, *16*(1), 129–134. https://doi.org/10.1016/0003-3472(68)90123-1

Olivier, B., Molewijk, E., van Oorschot, R., van der Heyden, J., Ronken, E., & Mos, J. (1998). Rat pup ultrasonic vocalization: effects of benzodiazepine receptor ligands. *European Journal of Pharmacology*, *358*(2), 117–128. https://doi.org/10.1016/S0014-2999(98)00603-7

Panksepp, J., & Burgdorf, J. (2000). 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. *Behavioural Brain Research*, *115*(1), 25–38. https://doi.org/10.1016/S0166-4328(00)00238-2

Panksepp, J., Siviy, S., & Normansell, L. (1984). The psychobiology of play: Theoretical and methodological perspectives. *Neuroscience & Biobehavioral Reviews*, *8*(4), 465–492. https://doi.org/10.1016/0149-7634(84)90005-8

Paxinos, G., & Watson, C. (2007). The rat brain in stereotaxic coordinates, hard cover edition. *Elsevier*.

Pechtel, P., & Pizzagalli, D. A. (2011). Effects of early life stress on cognitive and affective function: an integrated review of human literature. *Psychopharmacology*, *214*(1), 55–70. https://doi.org/10.1007/s00213-010-2009-2

Pennington, Z. T., Dong, Z., Feng, Y., Vetere, L. M., Page-Harley, L., Shuman, T., & Cai, D. J. (2019). ezTrack: An open-source video analysis pipeline for the

investigation of animal behavior. *Scientific Reports*, *9*(1), 19979. https://doi.org/10.1038/s41598-019-56408-9

Perry, T. W., Carvour, H. M., Reichert, A. N., Sneddon, E. A., Roemer, C. A. E. G., Gao, Y. Y., Schuh, K. M., Shand, N. A., Quinn, J. J., & Radke, A. K. (2025). Early life stress paired with adolescent alcohol consumption reduces two-bottle choice alcohol consumption in mice. *Alcohol, Clinical and Experimental Research*, *49*(3), 678–691. https://doi.org/10.1111/acer.70004

Pinheiro, M. L., Ferraz-de-Paula, V., Ribeiro, A., Sakai, M., Bernardi, M. M., & Palermo-Neto, J. (2011). Long-term maternal separation differentially alters serum corticosterone levels and blood neutrophil activity in A/J and C57BL/6 mouse offspring. *Neuroimmunomodulation*, *18*(3), 184–190. https://doi.org/10.1159/000323516

Ploj, K., Roman, E., & Nylander, I. (2002). Effects of maternal separation on brain nociceptin/orphanin FQ peptide levels in male Wistar rats. *Pharmacology Biochemistry and Behavior*, *73*(1), 123–129. https://doi.org/10.1016/S0091-3057(02)00778-5

Ploj, K., Roman, E., & Nylander, I. (2003). Long-term effects of short and long periods of maternal separation on brain opioid peptide levels in male Wistar rats. *Neuropeptides*, *37*(3), 149–156. https://doi.org/10.1016/S0143-4179(03)00043-X

Plotsky, P. M., Thrivikraman, K. V, Nemeroff, C. B., Caldji, C., Sharma, S., & Meaney, M. J. (2005). Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring. *Neuropsychopharmacology*, *30*(12), 2192–2204. https://doi.org/10.1038/sj.npp.1300769

Pollak, S. D. (2005). Early adversity and mechanisms of plasticity: Integrating affective neuroscience with developmental approaches to psychopathology. *Development and Psychopathology*, *17*(03). https://doi.org/10.1017/S0954579405050352

Porsolt, R. D., Anton, G., Blavet, N., & Jalfre, M. (1978). Behavioural despair in rats: A new model sensitive to antidepressant treatments. *European Journal of Pharmacology*, *47*(4), 379–391. https://doi.org/10.1016/0014-2999(78)90118-8

Pradelles, P., Grassi, J., & Maclouf, J. (1985). Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay. *Analytical Chemistry*, *57*(7), 1170–1173. https://doi.org/10.1021/ac00284a003

Qin, X., Liu, X.-X., Wang, Y., Wang, D., Song, Y., Zou, J.-X., Pan, H.-Q., Zhai, X.-Z., Zhang, Y.-M., Zhang, Y.-B., Hu, P., & Zhang, W.-H. (2021). Early life stress induces anxiety-like behavior during adulthood through dysregulation of neuronal plasticity in the basolateral amygdala. *Life Sciences*, *285*, 119959. https://doi.org/10.1016/j.lfs.2021.119959

Radley, J. J., & Sawchenko, P. E. (2015). Evidence for involvement of a limbic paraventricular hypothalamic inhibitory network in hypothalamic-pituitary-adrenal axis adaptations to repeated stress. *Journal of Comparative Neurology*, *523*(18), 2769–2787. https://doi.org/10.1002/cne.23815

Raineki, C., Cortés, M. R., Belnoue, L., & Sullivan, R. M. (2012). Effects of early-life abuse differ across development: Infant social behavior deficits are followed by adolescent depressive-like behaviors mediated by the amygdala. *The Journal of Neuroscience*, *32*(22), 7758–7765. https://doi.org/10.1523/JNEUROSCI.5843-11.2012

Renard, G. M., Rivarola, M. A., & Suárez, M. M. (2010). Gender-dependent effects of early maternal separation and variable chronic stress on vasopressinergic activity and glucocorticoid receptor expression in adult rats. *Developmental Neuroscience*, *32*(1), 71–80. https://doi.org/10.1159/000280102

Ressler, R. L., & Maren, S. (2019). Synaptic encoding of fear memories in the amygdala. *Current Opinion in Neurobiology*, *54*, 54–59. https://doi.org/10.1016/j.conb.2018.08.012

Sales, G. D. (1972). Ultrasound and mating behaviour in rodents with some observations on other behavioural situations. *Journal of Zoology*, *168*(2), 149–164. https://doi.org/10.1111/j.1469-7998.1972.tb01345.x

Sampath, D., Sabitha, K. R., Hegde, P., Jayakrishnan, H. R., Kutty, B. M., Chattarji, S., Rangarajan, G., & Laxmi, T. R. (2014). A study on fear memory retrieval and REM sleep in maternal separation and isolation stressed rats. *Behavioural Brain Research*, *273*, 144–154. https://doi.org/10.1016/j.bbr.2014.07.034

Sanders, B., & Anticevic, A. (2007). Maternal separation enhances neuronal activation and cardiovascular responses to acute stress in borderline hypertensive rats. *Behavioural Brain Research*, *183*(1), 25–30. https://doi.org/10.1016/j.bbr.2007.05.020

Sanguino-Gómez, J., & Krugers, H. J. (2024). Early-life stress impairs acquisition and retrieval of fear memories: sex-effects, corticosterone modulation, and partial prevention by targeting glucocorticoid receptors at adolescent age. *Neurobiology of Stress*, *31*, 100636. https://doi.org/10.1016/j.ynstr.2024.100636

Scheggi, S., De Montis, M. G., & Gambarana, C. (2018). Making sense of rodent models of anhedonia. *International Journal of Neuropsychopharmacology*, *21*(11), 1049–1065. https://doi.org/10.1093/ijnp/pyy083

Schmidt, M. V., Wang, X.-D., & Meijer, O. C. (2011). Early life stress paradigms in rodents: potential animal models of depression? *Psychopharmacology*, *214*(1), 131–140. https://doi.org/10.1007/s00213-010-2096-0

Scopano, M. R., Jones, H. E., Stea, S. G., Freeman, M. Z., & Grisel, J. E. (2023). Age, β-endorphin, and sex dependent effects of maternal separation on

locomotor activity, anxiety-like behavior, and alcohol reward. *Frontiers in Behavioral Neuroscience*, *17*. https://doi.org/10.3389/fnbeh.2023.1155647

Sewell, G. D. (1970). Ultrasonic communication in rodents. *Nature*, *227*(5256), 410–410. https://doi.org/10.1038/227410a0

Shair, H. N. (2007). Acquisition and expression of a socially mediated separation response. *Behavioural Brain Research*, *182*(2), 180–192. https://doi.org/10.1016/j.bbr.2007.02.016

Shair, H. N. (2018). Infantile vocalizations in rats. In S. M. Brudzynski (Ed.), *Handbook of ultrasonic vocalization: A window into the emotional brain* (pp. 129–137). Elsevier Academic Press. https://doi.org/10.1016/B978-0-12-809600-0.00012-3

Shin, S., & Lee, S. (2023). The impact of environmental factors during maternal separation on the behaviors of adolescent C57BL/6 mice. *Frontiers in Molecular Neuroscience*, *16*. https://doi.org/10.3389/fnmol.2023.1147951

Simola, N., & Brudzynski, S. M. (2018). Repertoire and biological function of ultrasonic vocalizations in adolescent and adult rats. In S. M. Brudzynski (Ed.), *Handbook of ultrasonic vocalization: A window into the emotional brain* (pp. 177–186). Elsevier Academic Press. https://doi.org/10.1016/B978-0-12-809600-0.00017-2

Smith, K. E., & Pollak, S. D. (2020). Early life stress and development: potential mechanisms for adverse outcomes. *Journal of Neurodevelopmental Disorders*, *12*(1), 34. https://doi.org/10.1186/s11689-020-09337-y

Smotherman, W. P., Bell, R. W., Starzec, J., Elias, J., & Zachman, T. A. (1974). Maternal responses to infant vocalizations and olfactory cues in rats and mice. *Behavioral Biology*, *12*(1), 55–66. https://doi.org/10.1016/S0091-6773(74)91026-8

Sood, A., Chaudhari, K., & Vaidya, V. A. (2018). Acute stress evokes sexually dimorphic, stressor-specific patterns of neural activation across multiple limbic brain regions in adult rats. *Stress*, *21*(2), 136–150. https://doi.org/10.1080/10253890.2017.1422488

Spencer, S. J., & Tilbrook, A. (2009). Neonatal overfeeding alters adult anxiety and stress responsiveness. *Psychoneuroendocrinology*, *34*(8), 1133–1143. https://doi.org/10.1016/j.psyneuen.2009.02.013

Stamatakis, A. M., & Stuber, G. D. (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nature Neuroscience*, *15*(8), 1105–1107. https://doi.org/10.1038/nn.3145

Streiner, D. L. (2005). Finding our way: An introduction to path analysis. *The Canadian Journal of Psychiatry*, *50*(2), 115–122. https://doi.org/10.1177/070674370505000207

Stringer, C., Wang, T., Michaelos, M., & Pachitariu, M. (2021). Cellpose: a generalist algorithm for cellular segmentation. *Nature Methods*, *18*(1), 100–106. https://doi.org/10.1038/s41592-020-01018-x

Sun, X.-M., Tu, W.-Q., Shi, Y.-W., Xue, L., & Zhao, H. (2014). Female-dependent impaired fear memory of adult rats induced by maternal separation, and screening of possible related genes in the hippocampal CA1. *Behavioural Brain Research*, *267*, 111–118. https://doi.org/10.1016/j.bbr.2014.03.022

Tchenio, A., Lecca, S., Valentinova, K., & Mameli, M. (2017). Limiting habenular hyperactivity ameliorates maternal separation-driven depressive-like symptoms. *Nature Communications*, *8*(1), 1135. https://doi.org/10.1038/s41467-017-01192-1

Thomas, D. A., & Barfield, R. J. (1985). Ultrasonic vocalization of the female rat (Rattus norvegicus) during mating. *Animal Behaviour*, *33*(3), 720–725. https://doi.org/10.1016/S0003-3472(85)80002-6

Toda, H., Boku, S., Nakagawa, S., Inoue, T., Kato, A., Takamura, N., Song, N., Nibuya, M., Koyama, T., & Kusumi, I. (2014). Maternal separation enhances conditioned fear and decreases the mRNA levels of the neurotensin receptor 1 gene with hypermethylation of this gene in the rat amygdala. *PLoS ONE*, *9*(5), e97421. https://doi.org/10.1371/journal.pone.0097421

Trujillo, V., Durando, P. E., & Suárez, M. M. (2016). Maternal separation in early life modifies anxious behavior and Fos and glucocorticoid receptor expression in limbic neurons after chronic stress in rats: effects of tianeptine. *Stress*, *19*(1), 91–103. https://doi.org/10.3109/10253890.2015.1105958

Unal, G., & Canbeyli, R. (2019). Psychomotor retardation in depression: A critical measure of the forced swim test. *Behavioural Brain Research*, *372*, 112047. https://doi.org/10.1016/j.bbr.2019.112047

Valle, F. P. (1970). Effects of strain, sex, and illumination on open-field behavior of rats. *The American Journal of Psychology*, *83*(1), 103. https://doi.org/10.2307/1420860

Vanderschuren, L. J. M. J., Stein, E. A., Wiegant, V. M., & Van Ree, J. M. (1995). Social play alters regional brain opioid receptor binding in juvenile rats. *Brain Research*, *680*(1–2), 148–156. https://doi.org/10.1016/0006-8993(95)00256-P

Veenema, A. H., & Neumann, I. D. (2009). Maternal separation enhances offensive play-fighting, basal corticosterone and hypothalamic vasopressin mRNA expression in juvenile male rats. *Psychoneuroendocrinology*, *34*(3), 463–467. https://doi.org/10.1016/j.psyneuen.2008.10.017

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*, *2*(2), 322–328. https://doi.org/10.1038/nprot.2007.44

Wang, D., Levine, J. L. S., Avila-Quintero, V., Bloch, M., & Kaffman, A. (2020). Systematic review and meta-analysis: effects of maternal separation on anxiety-like behavior in rodents. *Translational Psychiatry*, *10*(1), 174. https://doi.org/10.1038/s41398-020-0856-0

Wang, R.-H., Chen, Y.-F., Chen, S., Hao, B., Xue, L., Wang, X.-G., Shi, Y.-W., & Zhao, H. (2018). Maternal deprivation enhances contextual fear memory via epigenetically programming second-hit stress-induced Reelin expression in adult rats. *International Journal of Neuropsychopharmacology*, *21*(11), 1037–1048. https://doi.org/10.1093/ijnp/pyy078

Webster, J. F., Beerens, S., & Wozny, C. (2023). Effects of early life stress and subsequent re-exposure to stress on neuronal activity in the lateral habenula. *Neuropsychopharmacology*, *48*(5), 745–753. https://doi.org/10.1038/s41386-022-01493-0

Winslow, J. T., & Insel, T. R. (1991). The infant rat separation paradigm: a novel test for novel anxiolytics. *Trends in Pharmacological Sciences*, *12*(11), 402–404. https://doi.org/10.1016/0165-6147(91)90616-z

Wöhr, M., Borta, A., & Schwarting, R. K. W. (2005). Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: A dose–response study in the rat. *Neurobiology of Learning and Memory*, *84*(3), 228–240. https://doi.org/10.1016/j.nlm.2005.07.004

Wöhr, M., & Schwarting, R. K. W. (2008). Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behavioral Neuroscience*, *122*(2), 310–330. https://doi.org/10.1037/0735-7044.122.2.310

Wöhr, M., & Schwarting, R. K. W. (2013). Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. *Cell and Tissue Research*, *354*(1), 81–97. https://doi.org/10.1007/s00441-013-1607-9

Yang, Y., Cui, Y., Sang, K., Dong, Y., Ni, Z., Ma, S., & Hu, H. (2018). Ketamine blocks bursting in the lateral habenula to rapidly relieve depression. *Nature*, *554*(7692), 317–322. https://doi.org/10.1038/nature25509

Yin, X., Chen, L., Xia, Y., Cheng, Q., Yuan, J., Yang, Y., Wang, Z., Wang, H., Dong, J., Ding, Y., & Zhao, X. (2016). Maternal deprivation influences pup ultrasonic vocalizations of C57BL/6J mice. *PLOS ONE*, *11*(8), e0160409. https://doi.org/10.1371/journal.pone.0160409

Zheng, B., Rao, J., Bao, L., Yu, D., & Yin, B. (2025). Differential modulation of freezing and 22-kHz USVs by shock intensity, tone-duration matching, and anxiety levels in rodent fear-conditioning paradigms. *Learning & Behavior*. https://doi.org/10.3758/s13420-025-00671-6

Ziegler, D. R., & Herman, J. P. (2002). Neurocircuitry of stress integration: anatomical pathways regulating the Hypothalamo-Pituitary-Adrenocortical axis of the rat. *Integrative and Comparative Biology*, *42*(3), 541–551. https://doi.org/10.1093/icb/42.3.541

Zimmerberg, B., Brunelli, S. A., Fluty, A. J., & Frye, C. A. (2005). Differences in affective behaviors and hippocampal allopregnanolone levels in adult rats of lines selectively bred for infantile vocalizations. *Behavioural Brain Research*, *159*(2), 301–311. https://doi.org/10.1016/j.bbr.2004.11.009

Zimmerberg, B., Rosenthal, A. J., & Stark, A. C. (2003). Neonatal social isolation alters both maternal and pup behaviors in rats. *Developmental Psychobiology*, *42*(1), 52–63. https://doi.org/10.1002/dev.10086

Zippelius, H.-M., & Schleidt, W. M. (1956). Ultraschall-laute bei jungen M usen. *Die Naturwissenschaften*, *43*(21), 502–502. https://doi.org/10.1007/BF00632534