Check for updates

Developmental Psychobiology

WILEY

Developmental Psychobiology

RESEARCH ARTICLE

The Predictive Value of Infant Ultrasonic Vocalizations in a Longitudinal Study of Maternal Separation in Rats

Bahar Yuksel | Gunes Unal

Behavioral Neuroscience Laboratory, Department of Psychology, Boğaziçi University, Istanbul, Turkey

Correspondence: Gunes Unal (gunes.unal@bogazici.edu.tr)

Received: 20 January 2026 | **Revised:** 23 July 2026 | **Accepted:** 14 October 2026

Keywords: amygdala | c-Fos | corticosterone | habenula | maternal separation | ultrasonic vocalization

ABSTRACT

Maternal separation (MS) is a well-established rodent model of early-life stress, implicated in the development of various neuropsychiatric conditions. Infant ultrasonic vocalizations (USVs) serve as key communicative signals and have been used to assess early emotional and social development. Here, we examined the long-term behavioral and neurobiological effects of MS across adolescence and adulthood, focusing on the predictive potential of separation-induced infant USVs (40–60 kHz). MS was conducted between postnatal days (PND) 2–14, followed by a maternal potentiation test on PND 15, during which USVs were recorded. Behavioral assessments included anxiety and social play in adolescence (PND 35–36); and anxiety, sociability, anhedonia, and fear memory in adulthood (PND 80–88). MS reduced USVs in the potentiation paradigm, suppressed social play, and ameliorated anxiety-like behavior in adolescence. In adulthood, MS induced anhedonia, impaired fear memory, and decreased c-Fos-immunopositive cells in the lateral amygdala. Path and regression analyses revealed that infant USVs predicted open arm duration in the elevated plus maze in adolescence. USVs were negatively correlated with stress-induced neuronal activation in the habenula in control, but not MS animals. These findings indicate that infant USVs constitute a biobehavioral marker for early-life adverse experiences and predict the development of later affective conditions.

1 | Introduction

Early-life experiences play a crucial role in shaping behavioral and physiological responses to stress in the subsequent stages of life. Early-life stress (ELS), or exposure to adverse events during the early developmental period, is associated with various neuropsychiatric conditions including clinical depression (Colman et al. 2014; Heim et al. 1997), anxiety and post-traumatic stress disorder (Carr et al. 2013; Chaby et al. 2017). Maternal separation (MS) is a widely used rodent model of ELS that disrupts the mother-pup bond, leading to long-term neurobiological and behavioral alterations in adulthood (Ellenbroek et al. 1998; Enthoven et al. 2008). MS has been extensively studied in developmental research (Gunnar et al. 2009; Tractenberg et al. 2016). Yet, identifying early biobehavioral markers that may predict future affective outcomes remains a challenge. In addition, earlier studies produced varying effects on behavior and neural processes, highlighting the need for a comprehensive approach that assesses different processes in a longitudinal design. Here, we implement a longitudinal MS paradigm with male and female Wistar rats, assessing several social and affective responses in adolescence and adulthood. We test the predictive value of early-life ultrasonic vocalizations (USVs) for subsequent affective states, corticosterone (CORT) levels and associated neuronal activity in different limbic structures.

Rodents exposed to MS in early periods of life exhibited anxiety- (Huot et al. 2001; Lee et al. 2007; Maniam and Morris 2010) and depressive-like behaviors (Frank et al. 2019; Huot et al. 2001; Lambás-Señas et al. 2009; Lee et al. 2007; Maniam and Morris

© 2026 Wiley Periodicals LLC.

2010). However, it is important to note that the effects of MS on defensive behaviors appear to be highly heterogeneous and may be influenced by publication bias (Wang et al. 2020). Other studies also indicated alterations in social behaviors across development following MS (Gazzo et al. 2021; Kentrop et al. 2018; Lundberg et al. 2017; Mansouri et al. 2021; Veenema and Neumann 2009). Its complex impact on emotional processes is further emphasized by the bidirectional effects observed in fear memory studies (Chocyk et al. 2014; Diehl et al. 2014; Guijarro et al. 2007; Mishra et al. 2019; Sampath et al. 2014; Sanguino-Gómez and Krugers 2024; Shin and Lee 2023; Toda et al. 2014). These behavioral alterations are linked to MS-induced activation of the hypothalamo–pituitary–adrenal (HPA) axis (Ladd et al. 1996; McCormick et al. 1998), resulting in long-term changes in several limbic structures, including the amygdala (Qin et al. 2021; Raineki et al. 2012; Sanders and Anticevic 2007; Zhang et al. 2021) and habenula (Tchenio et al. 2017; Webster et al. 2023), both of which have been implicated in the processing of aversive experiences (Chastrette et al. 1991; Duvarci and Pare 2014; LeDoux 2000; Nagao et al. 1993; Ressler and Maren 2019; Webster et al. 2023).

Rodent USVs, which serve as a key indicator of affective state (Brudzynski 2013; Burgdorf et al. 2008; Demaestri et al. 2019; Knutson et al. 2002; Wöhr and Schwarting 2013), have been tested with the MS paradigm in both infants (Ploj et al. 2003; Yin et al. 2016) and adult animals (Dimatelis et al. 2016). Rodent pups emit USVs at the 40–60 kHz frequency band (Boulanger-Bertolus et al. 2017; Brudzynski et al. 1999), when they are separated from their mother and littermates, which serve as a species-specific behavior to seek maternal care (Allin and Banks 1972; Brunelli et al. 1994; Smotherman et al. 1974). Several mechanisms have been proposed to explain the generation of these vocalizations. Early studies suggested that pup USVs represent an autonomic response to cold exposure during isolation, as increasing ambient temperature markedly reduces call rates (Allin and Banks 1972). In addition, infant calls have been proposed to arise as an acoustic consequence of laryngeal braking, a respiratory maneuver that facilitates oxygen exchange in the lungs (Blumberg and Alberts 1990). While these mechanisms likely contribute to USV production, individual differences in infant call rates have also been linked to affective processes and later behavioral outcomes.

The rate of separation-induced USV calls is thought to indicate the anxiety levels of the pups (Winslow and Insel 1991), and it decreases with various anxiolytic and antidepressant treatments (Hodgson et al. 2008; Kehne et al. 2000; Olivier, Molewijk, van Oorschot, et al. 1998, Olivier, Molewijk, van der Heyden, et al. 1998). Animals bred for low USV emission in infancy display lower anxiety and depression-like behavior in adolescence (Zimmerberg et al. 2005). Similarly, a higher rate of separation-induced USVs on PND 8–10 was associated with increased freezing to a shock-predicting cue on PND 24, which, in turn, has been linked to fear responses and anxiety-like behavior in adulthood (Harmon-Jones 2023; Harmon-Jones et al. 2020). Together, these findings suggest a shared relationship between infant USV emission and behavioral responses to aversive experiences. Alternatively, infant USVs can be interpreted as an active coping mechanism, rather than an indicator of anxiety, that emerge in response to the distress caused by the mother's absence (Cordeiro et al. 2024). Earlier reports supported this perspective, showing that higher separation-induced USVs are associated with lower anxiety-like behavior later in life (Cordeiro et al. 2024), correlate negatively with freezing and USV emission during fear conditioning (Wöhr and Schwarting 2008) and predict social play in adolescence (Granata et al. 2022). These findings suggest that infant USVs serve not only as indicators of immediate distress but also as potential predictors of long-term behavioral outcomes; however, whether they also forecast specific neural changes or activity patterns remains to be determined.

Infant USVs diminish over time and disappear at the end of infancy around PND 20 in rats (Allin and Banks 1972; Noirot 1968). Stress exposure also has an effect on the number of infant calls, as repeated exposure to MS increase USV emission rates in rat pups (Burenkova et al. 2020; Ploj et al. 2003; Yin et al. 2016), but decrease these calls in late infancy in a sex specific manner (Granata et al. 2021). In the maternal potentiation paradigm, in which there are two rounds of brief isolation from the home cage, (Brunelli et al. 1998; Hofer et al. 1998), pups produce higher rates of USVs in the second isolation period after a brief exposure to their mother. This increase is considered to be a reflection of the infant animal's bond to the caregiver (Shair 2007). An earlier study revealed infant USV emissions in maternal potentiation following MS, and reported increased calls in the second isolation period as compared to control animals (Kaidbey et al. 2019). To date, no previous study has examined the effects of ELS on USVs within the maternal potentiation paradigm or investigated whether these vocalizations have long-term predictive value.

This work addresses these gaps using a longitudinal design to examine the impact of early MS on emotional and social behaviors across development, focusing on infant USVs and the predictive value of these vocalizations for later outcomes. Based on previous findings, we hypothesized that repeated MS would alter infant USVs and produce long-term behavioral and neurobiological alterations. The behavioral assays were selected a priori to assess the principal behavioral domains previously shown to be affected by MS, including anxiety-like behavior, anhedonia, social behavior, and fear memory. We further hypothesized that individual differences in infant USVs would predict later behavioral outcomes. In contrast, the associations between infant USVs and neuronal activation or CORT responses were considered exploratory. To achieve this, we conducted a maternal potentiation USV test in infancy, followed by the open field test (OFT), elevated plus maze (EPM), and social play test in adolescence. In adulthood, we assessed sociability and sucrose preference test (SPT), along with OFT and EPM, and performed an auditory fear conditioning paradigm with USV recordings. Additionally, we measured CORT release and neuronal activation in the amygdala, habenula, paraventricular nucleus of the thalamus (PVT), and paraventricular nucleus of the hypothalamus (PVN) during acute restraint stress. Finally, we conducted regression analyses to examine the relationships among these measures and determine whether infant USVs predict later emotional and social processes.

2 | Materials and Methods

2.1 | Subjects

The experimental subjects were obtained from Boğaziçi University Life Sciences Animal Care and Production Unit, and

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

all procedures were approved by the Bogăziçi University Ethics Committee for the Use of Animals in Experiments (2024-08). Pregnant Wistar rats were housed individually with ample bedding under controlled laboratory conditions (21 ± 1°C; 40%–60% humidity; 12:12 day/night) with ad libitum access to food and water. Parturition was checked daily, and the day of birth was designated as postnatal day (PND) 0. A total of eight male and eight female pups were used in each experimental group to ensure balanced sex distribution across conditions. Offspring from four litters (6–10 pups per litter) were included in the study. Pups were weaned on PND 21 and subsequently housed in groups of four with same-sex conspecifics.

2.2 | Experimental Design

Animals in the MS group underwent the MS procedure between PND 2 and 14 (Figure 1A), while control pups were only acclimated to the test room and handled for 1 min between PND 12 and 14 to minimize potential handling stress in subsequent test sessions. On PND 15, the maternal potentiation test was carried out, and thereafter, animals remained undisturbed until weaning (PND 21) and were given 2 additional weeks before the behavioral testing in adolescence. The OFT and EPM in adolescence were performed on PND 35 to assess locomotor activity and anxiety-like behavior, respectively. These were followed by the social play test on PND 36.

In adulthood, OFT and EPM were repeated on PND 80 as the first behavioral tests. The sociability test was conducted on PND 81 in two sessions to evaluate interactions with a social stimulus versus an empty chamber. On PND 82–83, sucrose preference was assessed over 48 h in individual cages. Fear conditioning took place on PND 85–87, including fear acquisition, contextual exposure, and cued extinction, with a 24-h interval between sessions. On PND 88, animals were restrained for 20 min, and blood samples were collected for CORT analysis, followed by perfusion-fixation. USVs were recorded during maternal potentiation and fear conditioning. All behavioral procedures were recorded using a video camera (Sony FDR-AX100E or HDR-CX405) and analyzed offline in the freezing module of ezTrack (Pennington et al. 2019; for freezing) or EthoVision XT 17 (Noldus, the Netherlands).

2.3 | Behavioral Procedures

2.3.1 | Maternal Separation

MS (and isolation) was applied between PND 2 and 14 (Figure 1A). The procedure began with transferring the mother rat to an individual cage, which was then moved to a separate room. Each pup was subsequently isolated for 180 min per day during the light phase. From PND 2 to 10, pups were placed in cups (50 mm base diameter, 70 mm height) with fresh bedding inside an incubator maintained at 32–34°C (Plotsky et al. 2005). Between PND 11 and 14, pups were isolated in individual cages with fresh bedding at room temperature (RT). Each session concluded with gathering the pups, clustering them together, and returning the dam to the home cage.

2.3.2 | Maternal Potentiation Test

The maternal potentiation procedure used in this study was adapted from the protocol of Branchi et al. (2006). On PND 15, the mother rat was transferred to a new cage which served as the dam cage for the reunion procedure. The test pup was separated from its littermates, placed in a small clean cage, and brought to the test room within 5 s. USV recordings were conducted for 3 min (premom session). Next, the dam cage was brought to the room, and the 3-min reunion session began immediately after the pup was placed inside. The pup was then returned to the previous cage, and the dam cage was removed from the room. After the final 3-min recording session (postmom session), the pup was returned to its littermates, and the next pup was tested.

2.3.3 | Open Field Test

The test apparatus used in adolescent and adult sessions consisted of square arenas with high opaque walls (a 45 × 45 × 45 cm white maze for adolescence, PND 35; and a 70 × 70 × 45 cm black maze for adulthood, PND 80). Animals were acclimated to the test room for 30 min before the experiment. Each session lasted 10 min under dim lighting, and the maze was cleaned with 70% ethanol between trials to eliminate olfactory cues. Locomotor activity was assessed by measuring the total distance traveled (cm) and the time spent in the center as an indicator of exploratory behavior (Valle 1970).

2.3.4 | Elevated Plus Maze

An EPM (arm length: 50 cm, width: 10 cm, height from the floor: 70 cm) with opaque black walls in the closed arms was used to assess anxiety-like behavior in both adolescence (PND 35) and adulthood (PND 80). Animals were acclimated to the test room for 30 min before each session. The test lasted 10 min under dim lighting, with greater illumination in the open arms than in the closed arms. Anxiety-like behavior was assessed based on the time spent in the open and closed arms (Walf and Frye 2007), while locomotor activity was evaluated by measuring the total distance traveled (cm).

2.3.5 | Social Play

The social play test (PND 36) took place in the same arena as the OFT to ensure the animals were familiar with the context. We isolated the animals for 3 h prior to the test to enhance their motivation to socialize (Vanderschuren et al. 1995). Pairs were selected from the same cage and matched for weight, with the weight difference not exceeding 10 g. Animals were brought to the test room in individual cages 15 min prior to testing. They were then placed in the test arena for another 15 min, during which camera recordings were captured. Social play behaviors were identified based on an adapted version of Meaney and Stewart (1981) for the entire session. For each animal, we manually recorded instances of pouncing (attempting to engage the partner's nose or neck to initiate play), pinning (positioning itself over the partner with forepaws on the partner), supine

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

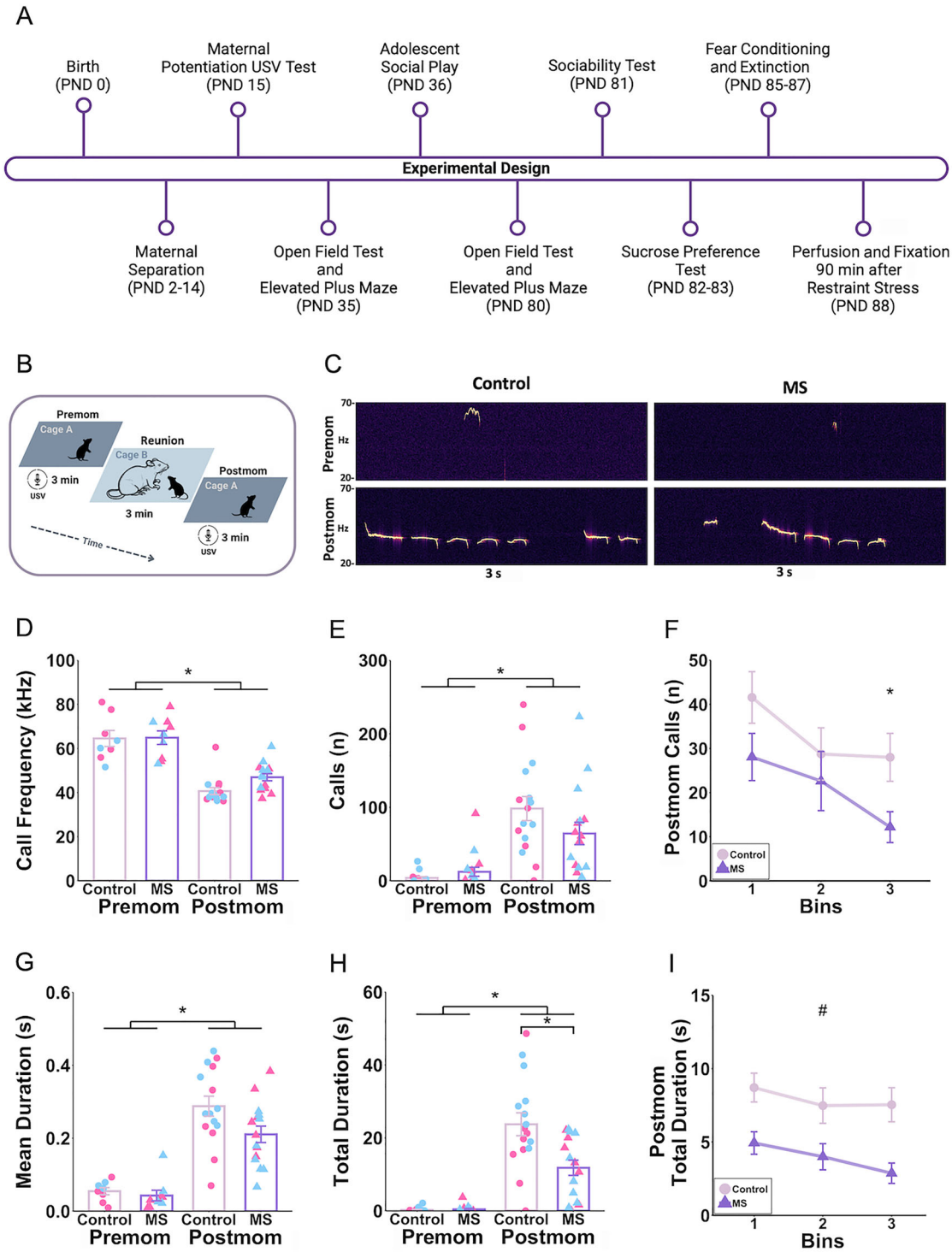


FIGURE 1 | Experimental timeline and maternal potentiation test results. (A) Illustration of the experimental manipulations and assessments, from the animals' birth (PND 0) to perfusion-fixation (PND 88). (B) Design of the maternal potentiation test. (C) USV profiles of a representative animal from each group during both sessions. (D) Mean call frequency and (E) number of USV calls during both the premom and postmom sessions. (F) Number of USV calls in the postmom session across each minute (bins). (G) Average call duration and (H) total call duration in both sessions. (I) Total duration of USV calls in the postmom session. All animals were included in the call number and total duration analyses ($n = 16$), but animals producing no USVs were excluded from frequency and mean duration analyses ($n = 8$ in control, $n = 9$ in MS group during premom, $n = 15$ for each group during postmom session). Data points represent individual animal scores (pink: female, blue: male). Error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$), and hashtags denote a main effect of group.

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

behavior (lying on its back and exposing its ventral surface to maintain the play), and sniffing (as nonplayful exploration).

2.3.6 | Sociability Test

The sociability test was conducted on PND 81 to assess adult social-agent preference, and the procedure was adapted from the design of Jacqueline and Crawley (2007) for mice. Two test sessions were conducted in the same open field used for the adult OFT. In the first session, animals interacted with an empty cylindrical chamber (diameter: 15 cm) placed in one corner of the arena, while in the second session, the chamber contained an unfamiliar, same-sex and same-age animal. The test began by placing the experimental animal in the arena and lasted for 10 min in both sessions. The arena was virtually divided into quadrants, and the quadrant containing the chamber, along with a contact zone (a circular area extending 10 cm from the edges of the chamber), was defined as the target zone in EthoVision XT 17 (Noldus, the Netherlands). We analyzed the time spent in and the number of entries into each zone, as well as the overall locomotion of the animals within the entire arena.

2.3.7 | Sucrose Preference Test

We conducted a SPT to assess anhedonic responses in the animals as an indicator of depression-like behavior. On PND 82, animals were housed in individual cages and given access to two bottles of water (50 mL each), one of which contained 1% sucrose. The test lasted for 48 h, during which the bottles were renewed, and their positions were counterbalanced at the 24-h mark. We measured the amount of sucrose water and the total liquid consumption each day, and calculated the sucrose preference as the ratio of sucrose consumption to total liquid consumption.

2.3.8 | Fear Conditioning

A modified Pavlovian fear conditioning protocol, adapted from Totty et al. (2023) was used in this study. Fear acquisition (PND 85) and context exposure (PND 86) took place in a conditioning chamber (21 × 45 × 27 cm) with transparent gray acrylic walls and a floor made of 32 metal grids (Context A; Figure 6A). On each day, animals were transferred to the test room in single cages and were given 5 min for acclimation. During the fear acquisition session, five pairings of a neutral tone (10 s, 80 dB, 8 kHz) as the conditioned stimulus (CS) and a coterminating foot shock (1 mA, 2 s) as the unconditioned stimulus (US) were presented, with a randomly generated intertrial interval (ITI) ranging from 50 to 70 s (average of 60 s). These pairings followed a 3-min baseline period, and animals were given an additional minute after the last pairing before being returned to their home cages. During the context exposure session, animals were placed in the conditioning chamber for 20 min without any CS or US presentation.

We performed a cued fear extinction session in a different chamber (Context B; described in Yuksel et al. 2024; Figure 6A); which had transparent acrylic walls and an opaque black floor. This chamber was located in another part of the test room, and the session was conducted under dim light (41 × 43 × 31 cm). Animals were given 5 min to acclimate to the experiment room, followed by a 3-min stimulus-free baseline period once placed in the test chamber. Subsequently, 30 CSs were presented with a fixed ITI of 30 s, and the last CS was followed by an additional minute without stimulus before the session ended. In all sessions, chambers were cleaned with 70% ethanol to eliminate any olfactory cues left by previous animals. We calculated the conditioned response as the percentage of freezing during CS presentations, while contextual fear was assessed during the baseline periods and for each 5-min block of the contextual extinction phase. The freezing percentages during the three CS presentations in cued fear extinction were averaged and used as blocks for analysis. Active coping behaviors, such as jumping and darting responses, were encoded during fear acquisition. USVs were recorded throughout all sessions of fear conditioning.

2.3.9 | USV Recordings and Analysis

USVs were captured using an ultrasound-sensitive microphone positioned 15 cm above individual pups or next to the chambers in fear sessions (sampling rate: 250 kHz, frequency range: 5–120 kHz), which was connected to a computer via the UltraSoundGate 116 USB audio device (Avisoft Bioacoustics, Glienicke, Germany). Vocalizations were monitored using Avisoft Recorder (version 4.2) during the test sessions, and were analyzed with DeepSqueak 2.0 software (Coffey et al. 2019) in MATLAB.

2.4 | Sample and Tissue Preparation

Animals were placed into cylindrical plexiglass restraint tubes (10 cm in diameter) for 20 min on PND 88. In the second half of the restraint stress, ~400 μL of blood was collected from each animal with tail clip. The blood samples were left at RT for 30–60 min to allow clotting, and then centrifuged for 15 min (Dlab D1008 microcentrifuge, 7000 rpm, RT). The serum was carefully removed and immediately stored at −80°C for further processing.

Animals were anesthetized with isoflurane and a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine, then transcardially perfused with 0.9% saline followed by 4% depolymerized paraformaldehyde (PFA) 90 min after the restraint stress. The brain tissue was postfixed in the same PFA solution for two overnight periods and stored in phosphate-buffered saline (PBS) with azide. The brains were sliced into coronal sections (~50 μm thick) using a vibratome (Leica, VT1000S). A rat brain atlas (Paxinos and Watson 2007) was used to locate the target regions, and the sections were stored in PBS-azide for subsequent immunohistochemistry processing.

2.5 | Immunohistochemistry and Cell Counting

We performed c-Fos immunohistochemistry on selected sections to examine neuronal activity levels during restraint stress. Sections were washed three times for 10 min in PBS-Triton X (PBS-Tx) solution to enable tissue penetration and then blocked with 10% normal horse serum (NHS) in PBS-Tx for 1 h at RT. The sections were then incubated with guinea-pig monoclonal c-Fos antibody

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

(Synaptic Systems, 226308, 1:5000 in 1% NHS PBS-Tx) for 48 h at 4°C. Secondary incubation was carried out with Alexa Fluor (AF) 488 donkey anti-guinea pig antibody (Jackson Immunoresearch, AB_2340472, 1:500, in 1% NHS PBS-Tx) for 4 h at RT. The sections were washed three times and transferred into PBS solution for mounting. Immunohistochemistry for parvalbumin (PV) and choline acetyltransferase (ChAT) was performed to obtain reference images for identifying BLA nuclei. Primary antibodies used were rabbit anti-PV (Abcam, ab11427, 1:2000) and goat anti-ChAT (Merck-Millipore, AB144P, 1:250), followed by secondary antibodies: donkey anti-rabbit AF 488 (1:250, ab150073, Abcam) and donkey anti-goat AF 555 (1:250, ab150134, Abcam).

C-Fos immunopositive (+) cells were counted from two sections of each animal. Sections containing the lateral amygdala (LA), anterior (BLa) and posterior nuclei of the basal amygdala (BLp), the medial (MHb) and lateral habenula (LHb), and the PVT were taken between −3.00 mm and −3.36 mm from the Bregma point (Paxinos and Watson 2007). PVN sections were obtained from −1.72 mm to −2.00 mm from Bregma. Cell quantification was performed using an epifluorescence microscope (Olympus BX53) equipped with a monochrome CCD camera (Olympus XM10), and regions were captured using cellSens (Olympus LS, MIA module) on processed sections.

2.6 | Corticosterone Analysis

CORT concentrations in serum samples collected during restraint stress were measured using an ELISA kit (Cayman Chemical, 501320) according to the manufacturer's instructions. The samples were plated in duplicates and read at a wavelength of 412 nm using a microplate reader (Multiskan Go, ThermoFisher). The standard curve was verified, and the concentration of the samples was calculated using the manufacturer's spreadsheet. One animal from each group was excluded due to having a concentration above 3 standard deviations from the mean and falling outside the suggested absorbance range of the kit.

2.7 | Statistical Analysis

2.7.1 | Group Comparison Analyses

All statistical analyses were performed using R version 4.3.1. Paired samples *t*-tests were used to analyze within-subjects measurements for comparing variables (e.g., baseline freezing comparison across two sessions in fear conditioning). Between-group differences were assessed using independent samples *t*-tests or analysis of variance (ANOVA), with sex included as an additional independent variable. We first examined whether there were any sex-related differences—either as significant main effects or interactions—in the behavioral tests. To do this, we conducted the appropriate ANOVAs using the aov() function in base R (2 × 2 Group × Sex or 2 × 2 × A Group × Sex × Sessions designs). We reported these results only when a significant sex difference was observed. Otherwise, independent samples *t*-tests were reported for simplicity and to stay focused on the main research question. For analyses with fewer levels of a within-subject variable (e.g., potentiation sessions), we used the aov_car() function from the afex package with Type III sums of squares. The aov() function was used for fear conditioning analyses involving higher levels of within-group parameters, given the balanced nature of the data. Variables were screened for potential outliers using a ± 3 SD criterion. One influential observation was identified in the postmaternal USV call data that exceeded this threshold and was Winsorized to the 95th percentile to reduce the influence of the extreme value while retaining the observation in the dataset (for total calls and for the first and second time bins). Tukey's correction was applied for multiple pairwise comparisons following ANOVAs. Effect sizes are reported as partial eta-squared for ANOVAs and Cohen's *d* for independent samples *t*-tests.

2.7.2 | Path and Regression Analysis

We investigated the associations of pup USVs and adolescence measurements with (1) adult behavioral assessments and (2) neuronal activity and CORT levels during acute stress in two groups of analyses. The variables included both direct measurements (USV call number in the postmom session, time spent in the open arm of the EPM, sucrose preference index, cell density in the PVN and PVT) and merged variables to simplify the analyses. These merged variables included social play (the sum of three types of play behaviors; $r = 0.86$, $p < 0.001$ for pouncing and pinning, $r = 0.76$, $p < 0.001$ for pouncing and supine, and $r = 0.74$, $p < 0.001$ for pinning and supine), sociability (the increase in time spent in the interaction zone), active coping (total jumping and darting behaviors in response to shock during fear acquisition, $r = 0.43$, $p < 0.015$), fear memory (the average freezing percentages during the contextual exposure and in response to the CSs in the extinction session based on their correlation: $r = 0.51$, $p = 0.003$), and the average of c-Fos+ cell density in the MHb and LHb ($r = 0.57$, $p = 0.002$), and in the LA and BL ($r = 0.69$, $p < 0.001$).

To investigate the associations of infant and adolescent measurements with adult behavioral data, we first conducted partial correlation analyses for all animals, controlling for sex and group. Next, we combined a model of the hypothesized relationships among the primary developmental domains of interest and tested how well this model corresponded to our data through path analysis. We used the sem() function in the lavaan package, an extension of multiple regression that enables the evaluation of more complex relationships (Streiner 2005). To obtain a parsimonious model appropriate for the available sample size, the analysis focused on adolescent anxiety-like behavior and social play, and adult anxiety-like behavior, anhedonia, and fear memory. The model was estimated using maximum likelihood (ML), and we assessed the fit of the model for each experimental group separately to evaluate the relationships between variables comparatively ($n = 16$ for MS and 15 for control). For each path, we also included sex as a predictor to control for its effect on the assessed relationships. The complete tested model was illustrated as a path diagram in Figure 7.

The data used for path analysis contained no missing values, except for one animal lacking the cued fear score needed to calculate the average fear response, which was removed from the partial correlation and path analysis listwise. Prior to analysis, we scaled the data to obtain comparable levels of variance, with

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

a mean of 0 and a standard deviation of 1. We checked the distribution of the scores for each group, noting slight deviations from normality in the sucrose preference of the control group and the USV number of the MS group. To address this and the small sample size, we applied bootstrapping with 5000 resamples to obtain robust estimates of effect sizes and p values. We also applied a minimal ridge regularization penalty ($\lambda = 0.001$) to stabilize the coefficient estimates. The unstandardized (B) and standardized (beta) coefficients, referring relative effects in the model, are reported in Table 1 (Supporting Information).

We conducted regression analysis to evaluate the predictive role of pup USVs, adolescent EPM data, and social play data on biological assessments related to acute restraint stress in adulthood. We included the group as a second predictor, along with its interaction with the main predictor, to test whether the relationship with the outcome variable differed between groups. We used scaled data in these analyses, and the missing values in the outcome variable were removed listwise. For each analysis, we checked the assumptions of multiple regression through residual plots, Q–Q plots, normality tests, and GVIF. Model statistics, regression coefficients, and Bonferroni-corrected p values are reported in Table 2 (Supporting Information).

3 | Results

3.1 | Maternal Potentiation USV Calls

Potentiation test included three experimental phases, where the pups meet their mother between two isolation periods, named as premom and postmom sessions (Figure 1B). After a brief exposure to the dam, pups showed a significant decrease in the mean call frequency in the postmom session ($F(1, 15) = 67.467$, $p < 0.001$, $\eta 2_p = 0.54$, 2 × 2 two-way mixed ANOVA; Figure 1C,D), while producing a higher number of calls ($F(1, 30) = 44.964$, $p < 0.001$, $\eta 2_p = 0.60$, 2 × 2 two-way mixed ANOVA; Figure 1C,E). However, MS did not alter the call frequency ($F(1, 15) = 0.676$, $p = 0.424$) and the call number ($F(1, 30) = 0.426$, $p = 0.519$), and no group-session interaction was observed (call frequency: $F(1, 15) = 1.044$, $p = 0.323$; call number: $F(1, 30) = 1.851$, $p = 0.184$).

We divided the USV recording sessions into three 1-min bins to analyze the temporal changes during the postmom session. The number of calls during the postmom session decreased across time ($F(2, 60) = 15.784$, $p < 0.001$, $\eta 2_p = 0.34$, 3 × 2 two-way mixed ANOVA; Figure 1F); with no difference between the groups ($F(1, 30) = 2.668$, $p = 0.113$) or a group-bin interaction ($F(2, 60) = 1.825$, $p = 0.170$). Interestingly, control animals showed the decrease in the call number from the first (M = 41.56, SD = 23.39) to the second bin (M = 28.75, SD = 23.72; $t(30) = 3.245$, $p = 0.008$, Cohen's $d = 0.544$, Tukey corrected t-test; Figure 1F), while it remained at a similar level in the third bin (M = 28.00, SD = 21.73; $t(30) = 0.195$, $p = 0.978$, Tukey corrected t-test). In contrast, MS animals did not show a significant change between the first (M = 32.5, SD = 35.27) and second bins (M = 26.69, SD = 38.62; $t(30) = 1.530$, $p = 0.202$, Tukey corrected t-test; Figure 1F); but exhibited a significant decrease in the third bin (M = 12.19, SD = 13.99; $t(30) = 2.712$, $p = 0.029$, Cohen's $d = 0.499$; Tukey corrected t-test). MS animals also produced lower number of calls compared to the control group within the last 1-min bin ($t(30) = 2.447$, $p = 0.021$, Cohen's $d = 0.865$, independent samples t-test; Figure 1F).

The mean duration of each call ($F(1, 15) = 57.669$, $p < 0.001$, $\eta 2_p = 0.77$, 2 × 2 two-way mixed ANOVA; Figure 1G), as well as the total duration of the calls increased in the postmom period ($F(1, 30) = 89.988$, $p < 0.001$, $\eta 2_p = 0.75$, 2 × 2 two-way mixed ANOVA; Figure 1H). However, there was no group-level difference in the mean duration ($F(1, 15) = 3.021$, $p = 0.103$) and no interaction effect with the sessions ($F(1, 15) = 1.605$, $p = 0.225$). Notably, MS reduced the total duration of USVs ($F(1, 30) = 9.327$, $p = 0.005$, $\eta 2_p = 0.24$; Figure 1H) and this decrease interacted with the sessions ($F(1, 30) = 10.804$, $p = 0.003$, $\eta 2_p = 0.26$). MS animals exhibited a shorter total duration of USVs after exposed to their dam (M = 11.81, SD = 8.34) when 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 1H).

The total call duration in the postmom period was also analyzed across the three 1-min bins, which replicated the group effect ($F(1, 30) = 10.082$, $p = 0.003$, $\eta 2_p = 0.25$, 3 × 2 two-way mixed ANOVA; Figure 1I), and revealed an effect of time ($F(2, 60) = 5.702$, $p = 0.005$, $\eta 2_p = 0.16$) with no significant interaction ($F(2, 60) = 0.805$, $p = 0.452$, $\eta 2_p = 0.03$). The pairwise comparisons of bins for each group indicated that the total duration of USVs decreased from the first bin (M = 4.93, SD = 3.08) to the last bin (M = 2.87, SD = 2.77) in the MS group ($t(30) = 2.899$, $p = 0.019$, Cohen's $d = 0.703$, Tukey corrected t-test; Figure 1I), which was not observed 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; Figure 1I). Altogether, these results suggest that MS reduced the USVs of pups in a maternal potentiation paradigm, with this change partially driven by the decreased vocalizations of MS animals over time during the second isolation.

3.2 | Locomotor Activity and Anxiety-Like Behavior

In the OFT conducted during adolescence (PND 35), the MS group (M = 2234.90, SD = 664.25) traveled a shorter distance than the control group (M = 2942.56, SD = 574.33; $t(30) = 3.224$, $p = 0.003$, Cohen's $d = 1.140$, independent samples t-test; Figure 2A). However, both groups spent a similar amount of time in the center of the open field (control: M = 68.75, SD = 38.70; MS: M = 85.03, SD = 38.63; $t(30) = -1.191$, $p = 0.243$, Cohen's $d = 0.421$, independent samples t-test; Figure 2B,I). In the adult OFT (PND 80), MS did not affect the total distance traveled in the maze ($F(1, 28) = 0.279$, $p = 0.602$) and both groups spent similar time in the center ($F(1, 28) = 1.156$, $p = 0.292$). Yet, female animals traveled more than male animals, indicating a sex effect on locomotion in adulthood ($F(1, 28) = 27.503$, $p < 0.001$, $\eta 2_p = 0.50$; 2 × 2 two-way ANOVA; Figure 2C). Similarly, female animals spent more time in the center area than males ($F(1, 28) = 4.837$, $p = 0.036$, $\eta 2_p = 0.15$, 2 × 2 two-way ANOVA; Figure 2D,K), possibly due to their higher locomotor activity in the maze.

Control (M = 2431.79, SD = 545.93) and MS animals (M = 2030.23, SD = 619.04) showed similar locomotor activity in the EPM in adolescence (PND 35) ($t(30) = 1.946$, $p = 0.061$, Cohen's $d = 0.688$, independent samples t-test; Figure 2E). However, the MS group

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

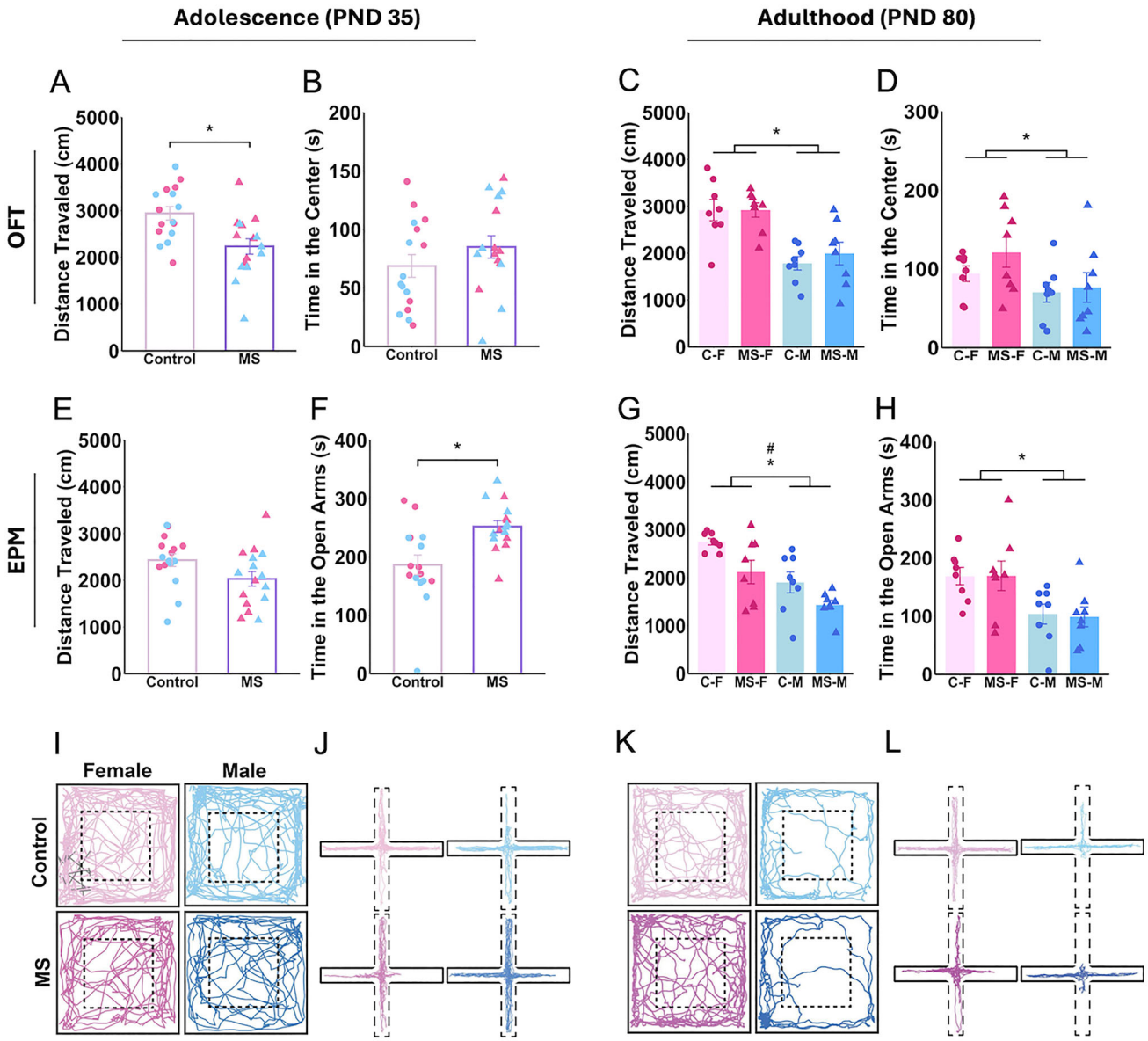


FIGURE 2 | Locomotor activity and anxiety-like behavior in adolescence (left) and adulthood (right). (A–D) Open field test (OFT). (A) Total distance traveled and (B) time spent in the center of the arena in adolescence. (C) Traveled distance for each subgroup (C-F: control female, MS-F: MS female, C-M: control male, MS-M: MS male) and (D) time spent in the center of the open field in adulthood. (E–H) Elevated plus maze (EPM). (E) Total distance traveled and (F) time spent in the open arms in adolescence. (G) Traveled distance for each subgroup and (H) time spent in the open arms in adulthood. (I–L) Path trajectories of representative animals in OFT and EPM in adolescence (I, J) and adulthood (K, L). Note that subgroups are plotted only when there is a statistically significant difference at the subgroup level. Data points represent individual animal scores ($n = 16$ for each group, $n = 8$ per subgroup). Error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$), and hashtags denote a main effect of group.

(M = 252.13, SD = 40.01) spent more time in the open arms compared to the control group (M = 186.49, SD = 68.01; $t(30) = -3.327$, $p = 0.002$, Cohen's $d = 1.176$; independent samples t-test; Figure 2F,J), suggesting an anxiolytic effect. In the second EPM conducted on PND 80, a sex effect emerged in locomotion and female animals exhibited higher activity ($F(1, 28) = 19.288$, $p < 0.001$, $\eta 2_p = 0.41$; 2 × 2 two-way ANOVA; Figure 2G). Notably, MS decreased the total distance traveled in the maze ($F(1, 28) = 9.877$, $p = 0.004$, $\eta 2_p = 0.26$), but it did not interact with the sex effect ($F(1, 28) = 0.221$, $p = 0.642$). Female animals also spent more time in the open arms of the maze ($F(1, 28) = 12.666$, $p = 0.001$, $\eta 2_p = 0.31$; 2 × 2 two-way ANOVA; Figure 2H,L), while MS did not change this duration ($F(1, 28) = 0.012$, $p = 0.912$). Overall, MS appeared to reduce locomotion, manifesting in the OFT during adolescence and the EPM during adulthood, while also producing an anxiolytic-like effect during adolescence.

3.3 | Social Play in Adolescence

Male animals exhibited pouncing behavior, which initiates play, more frequently than females ($F(1, 28) = 12.532$, $p = 0.001$, $\eta 2_p = 0.31$; 2 × 2 two-way ANOVA; Figure 3A). While there was no group-level difference on the frequency of pouncing ($F(1, 28) =$

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

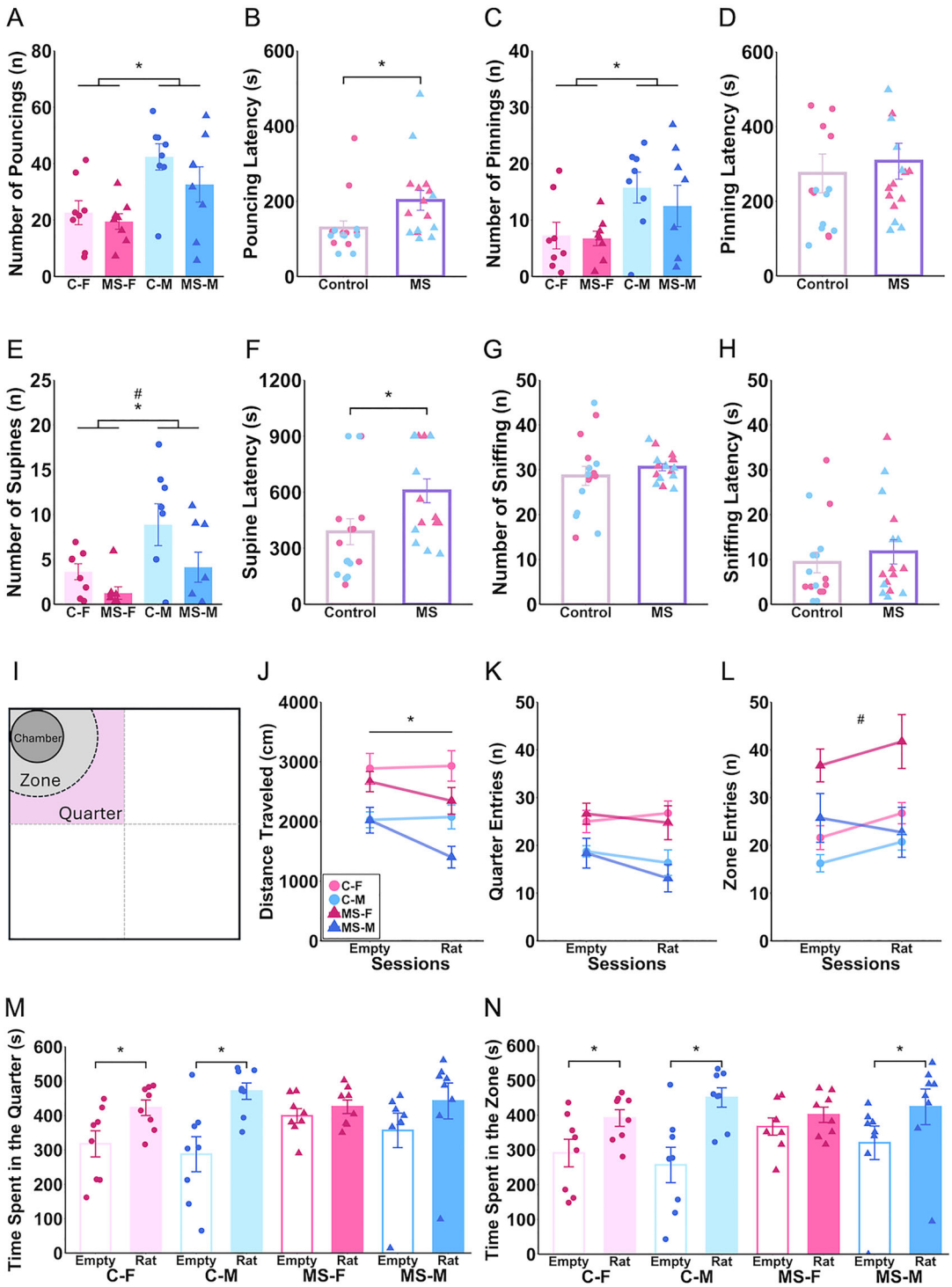


FIGURE 3 | Social play in adolescence (A–H) and sociability in adulthood (I–N). (A) Instances of pouncing behavior and (B) pouncing latency. (C) Frequency of pinning and (D) pinning latency. (E) Frequency of supine and (F) latency to display supine. (G) Frequency of sniffing and (H) sniffing latency. (I) Illustration of the sociability test arena. (J) Traveled distance for each subgroup during each session, with the chamber either empty or containing a rat. (K) Number of entries to the interaction quarter and (L) zone. (M) Time spent in the interaction quarter and (N) zone. Data points represent individual animal scores ($n = 16$ for each group, $n = 8$ per subgroup). Note that subgroups are plotted only when there is a statistically significant difference at the subgroup level. Error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$), and hashtags denote a main effect of group.

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

1.922, $p = 0.177$) or an interaction with sex ($F(1, 28) = 0.509$, $p = 0.482$), the MS group (M = 202.25, SD = 104.42) displayed a longer latency to exhibit this behavior compared to control animals (M = 129.02, SD = 75.13; $t(30) = -2.277$, $p = 0.030$, Cohen's $d = 0.805$; independent samples t-test; Figure 3B).

Similarly, pinning frequency was higher in males ($F(1, 28) = 7.213$, $p = 0.012$, $\eta 2_p = 0.20$; 2 × 2 two-way ANOVA; Figure 3C) with no effect of MS ($F(1, 28) = 0.500$, $p = 0.486$). Unlike pouncing, the latency of pinning was comparable between the experimental groups (control: M = 274.56, SD = 208.02; MS: M = 307.16, SD = 192.70; $t(30) = -0.460$, $p = 0.649$; independent samples t-test; Figure 3D).

MS animals exhibited fewer instances of supine behavior ($F(1, 28) = 5.332$, $p = 0.029$; $\eta 2_p = 0.16$; 2 × 2 two-way ANOVA; Figure 3E), with males displaying this behavior more frequently, as indicated by the main effect of sex ($F(1, 28) = 6.934$, $p = 0.014$, $\eta 2_p = 0.20$). In addition, the MS group showed a longer latency to exhibit supine behavior (M = 607.17, SD = 255.44) compared to the control group (M = 388.06, SD = 277.78; $t(30) = -2.323$, $p = 0.027$, Cohen's $d = 0.821$; independent samples t-test; Figure 3F). These results indicate that the MS group had reduced engagement in supine behavior, despite the lack of difference in pinning frequency, since the pinning behavior of an animal may not always end up with the supine behavior of the other animal. This pattern suggests that MS selectively decreased the motivation to maintain an ongoing play.

We also analyzed sniffing as an indicator of social exploration but found no significant differences in either the frequency of sniffing between the control (M = 28.625, SD = 8.44) and MS groups (M = 30.56, SD = 3.16; $t(30) = -0.859$, $p = 0.397$; independent samples t-test; Figure 3G) or the latency to start 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 3H). These results indicate that MS reduces playful behaviors in adolescence and delays their onset while also highlighting a sex difference, with males exhibiting more pronounced behaviors.

3.4 | Sociability in Adulthood

Presence of the social object (rat) in the chamber resulted in a general decrease in locomotion ($F(1, 28) = 6.760$, $p = 0.015$, $\eta 2_p = 0.19$, 2 × 2 × 2 three-way mixed ANOVA; Figure 3I,J). Female animals, however, exhibited greater locomotion ($F(1, 28) = 10.009$, $p = 0.004$, $\eta 2_p = 0.40$), regardless of their experimental group ($F(1, 28) = 0.028$, $p = 0.869$) or session type ($F(1, 28) = 0.800$, $p = 0.379$). Although the main effect of group did not reach significance ($F(1, 28) = 3.776$, $p = 0.062$, $\eta 2_p = 0.12$), a group-session interaction ($F(1, 28) = 10.009$, $p = 0.004$, $\eta 2_p = 0.26$) indicated that MS animals (M = 1874.43, SD = 740.11) traveled less than control animals (M = 2504.21, SD = 767.17) when the social object was present ($t(28) = 2.900$, $p = 0.034$, Cohen's $d = 0.836$, Tukey corrected t-test; Figure 3J).

The number of quarter entries did not differ between sessions ($F(1, 28) = 3.260$, $p = 0.082$, 2 × 2 × 2 three-way mixed ANOVA; Figure 3K). However, female animals exhibited more quarter entries ($F(1, 28) = 14.308$, $p < 0.001$, $\eta 2_p = 0.34$), likely reflecting their higher locomotion, while MS did not influence this measure ($F(1, 28) = 0.172$, $p = 0.682$). The number of entries into the interaction zone was not affected by session type either ($F(1, 28) = 2.460$, $p = 0.128$, 2 × 2 × 2 three-way mixed ANOVA; Figure 3L). Similar to the sex effect observed in quarter entries, female animals entered the interaction zone more frequently ($F(1, 28) = 9.818$, $p = 0.004$, $\eta 2_p = 0.26$). Notably, MS further increased the number of interaction zone entries ($F(1, 28) = 9.937$, $p = 0.004$, $\eta 2_p = 0.26$).

Presence of the social object also increased the time in the interaction quarter ($F(1, 28) = 35.862$, $p < 0.001$, $\eta 2_p = 0.56$, 2 × 2 × 2 three-way mixed ANOVA; Figure 3M). This effect depended both on sex ($F(1, 28) = 4.223$, $p = 0.049$, $\eta 2_p = 0.13$) and experimental group ($F(1, 28) = 6.987$, $p = 0.013$, $\eta 2_p = 0.20$). Pairwise comparisons revealed that only 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; Figure 4M) and control 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; Figure 3M) increased their time in the interaction quarter in the second session, showing enhanced sociability in stress-free groups. These results generally remained unchanged when the analysis were repeated for the interaction zone ($F(1, 28) = 39.404$, $p < 0.001$, $\eta 2_p = 0.58$, 2 × 2 × 2 three-way mixed ANOVA; Figure 3N). However, here, male MS animals performed better (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) than females (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; Figure 3N). Overall, MS appeared to decrease locomotion in the presence of the social object while increasing interaction zone entries across both sessions. However, MS animals failed to show a preference for the session containing the social object, potentially because they already exhibited greater interaction with the empty object during the first session.

3.5 | Anhedonia

The total liquid (sucrose + water) consumption of the animals over 48 h did not reveal a group-level difference ($F(1, 28) = 3.846$, $p = 0.060$; 2 × 2 two-way ANOVA; Figure 4A). However, male animals consumed more liquid than females ($F(1, 28) = 16.910$, $p < 0.001$, $\eta 2_p = 0.38$), with no significant group-sex interaction ($F(1, 28) = 1.268$, $p = 0.270$). Likewise, sucrose consumption did not differ between the MS and control groups ($F(1, 28) = 2.834$, $p = 0.103$; 2 × 2 two-way ANOVA; Figure 4B). In contrast, the sucrose preference index, calculated as the proportion of sucrose water to total liquid consumption, revealed that the MS group exhibited a lower sucrose preference (M = 0.57, SD = 0.29) compared to the control group (M = 0.78, SD = 0.15; $t(30) = 2.503$, $p = 0.018$, Cohen's $d = 0.885$, independent samples t-test; Figure 4C), indicating an anhedonic response in the MS animals.

3.6 | Fear Memory and Active Coping

The baseline freezing responses of the animals in the fear acquisition phase (Figure 5A) did not differ between the groups

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

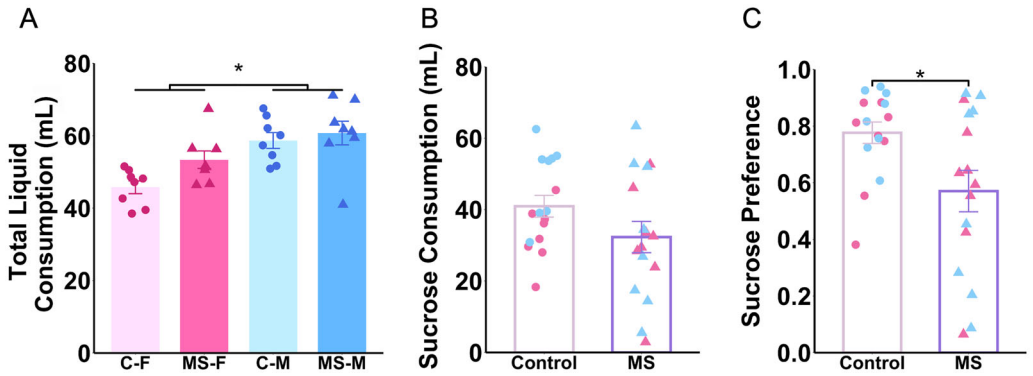


FIGURE 4 | Sucrose preference test. (A) Total liquid intake over 48 h. (B) Amount of sucrose water consumed. (C) Sucrose preference, calculated as the proportion of sucrose water to total liquid consumption. Data points represent individual animal scores ($n = 16$ for each group, $n = 8$ per subgroup). Note that subgroups are plotted only when there is a statistically significant difference at the subgroup level. Error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$).

($F(1, 28) = 0.307$, $p = 0.584$, 2 × 2 two-way mixed ANOVA; Figure 5B). Similarly, no sex effect was observed either as main ($F(1, 28) = 0.531$, $p = 0.472$) or interaction effects ($F(1, 28) = 1.099$, $p = 0.304$). Animals displayed higher rates of freezing behavior across the pairings ($F(4, 135) = 12.735$, $p < 0.001$, $\eta 2_p = 0.27$; 2 × 2 × 5 three-way mixed ANOVA; Figure 5B), indicated successful cued conditioning. The experimental groups did not differ in their freezing rates to the CS ($F(1, 135) = 0.001$, $p = 0.976$; Figure 5B), while male animals exhibited greater freezing (M = 42.99, SD = 33.32) compared to females (M = 31.08, SD = 31.64; $F(1, 135) = 13.512$, $p < 0.001$, $\eta 2_p = 0.09$).

The freezing percentages of the animals increased in the first 5-min block of the context exposure (M = 38.10, SD = 17.35) compared to baseline levels in acquisition (M = 0.69, SD = 2.30; $t(31) = -11.882$, $p < 0.001$, Cohen's $d = 3.023$; paired samples t-test; see Figure 5B) and remained stable across the session ($F(3, 108) = 0.642$, $p = 0.589$, 4 × 2 × 2 three-way mixed ANOVA, Figure 5B). Similar to fear acquisition, there was a major sex difference in conditioned contextual fear ($F(1, 108) = 11.762$, $p < 0.001$, $\eta 2_p = 0.10$). Notably, MS also decreased freezing responses to the context ($F(1, 108) = 5.303$, $p = 0.023$, $\eta 2_p = 0.05$), and a group-sex interaction was found ($F(1, 108) = 6.298$, $p = 0.014$, $\eta 2_p = 0.06$): control male animals (M = 49.43, SD = 24.44) showed more freezing compared to MS male (M = 38.77, SD = 17.98; $t(108) = 3.340$, $p = 0.006$, Cohen's $d = 0.497$, Tukey corrected t-test), control female (M = 30.84, SD = 18.19; $t(108) = -4.585$, $p < 0.001$, Cohen's $d = 0.863$, 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; Figure 5B).

In the cued extinction session (Context B), the baseline freezing responses were not different between the groups ($F(1, 28) = 2.643$, $p = 0.115$, 2 × 2 two-way mixed ANOVA; Figure 5B) or sexes ($F(1, 28) = 3.324$, $p = 0.079$; interaction: $F(1, 28) = 0.419$, $p = 0.523$). The freezing response of the animals varied across cue blocks ($F(9, 260) = 3.186$, $p = 0.001$, $\eta 2_p = 0.09$, 10 × 2 × 2 three-way mixed ANOVA; Figure 5B). MS did not alter the fear response ($F(1, 260) = 1.872$, $p = 0.172$; Figure 5B), whereas sex influenced freezing behavior ($F(1, 260) = 18.275$, $p < 0.001$, $\eta 2_p = 0.08$) and interacted with the experimental groups ($F(1, 260) = 10.972$, $p = 0.001$, $\eta 2_p = 0.05$). Pairwise comparisons across blocks revealed that control male animals (M = 62.90, SD = 22.11) exhibited higher freezing levels than 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), as well as compared to control females (M = 42.72, SD = 25.48; $t(260) = -5.475$, $p < 0.001$, Cohen's $d = 0.846$, Tukey corrected t-test) and MS females (M = 43.54, SD = 25.36; $t(260) = -3.619$, $p = 0.002$, Cohen's $d = 0.814$, Tukey corrected t-test). These findings indicate that the reduction in cued fear memory due to MS occurred exclusively in male animals.

Darting and jumping responses to the foot shock during fear acquisition were analyzed as indicators of active coping behavior. Female animals displayed significantly more darting ($F(1, 28) = 26.938$, $p < 0.001$, $\eta 2_p = 0.49$, 2 × 2 two-way ANOVA; Figure 5C) and jumping ($F(1, 28) = 10.869$, $p = 0.003$, $\eta 2_p = 0.28$, 2 × 2 two-way ANOVA; Figure 5D) compared to males, consistent with their lower freezing levels in this session. No group-level differences were observed for either behavior (darting: $F(1, 28) = 1.263$, $p = 0.271$; jumping: $F(1, 28) = 1.475$, $p = 0.235$).

We also tested the emission of alarm calls (~22 kHz) during fear acquisition, context exposure, and cued extinction. Female animals did not emit USVs in any session. During fear acquisition, seven of the eight male animals in the MS group produced alarm calls, whereas only three males in the control group exhibited this response (Figure 5E). In each group, only one male emitted USVs during the 20-min context exposure. During cued extinction, USVs were recorded in five control males and two MS males (Figure 5F).

3.7 | Neuronal Activity and Corticosterone Levels

BLA nuclei were delineated with ChAT and PV labeling (Figure 6A) and immunohistochemistry for c-Fos revealed activated neurons in limbic regions of interest (Figure 6B–D) following acute restraint stress. MS decreased the density of c-Fos+ cells in the LA (M = 353.54, SD = 64.39) compared to 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 6E). However, no such difference was observed in the BL (control: M = 312.99, SD = 59.24; MS: M = 297.08, SD = 48.08; $t(27) = 0.806$, $p = 0.427$, independent

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

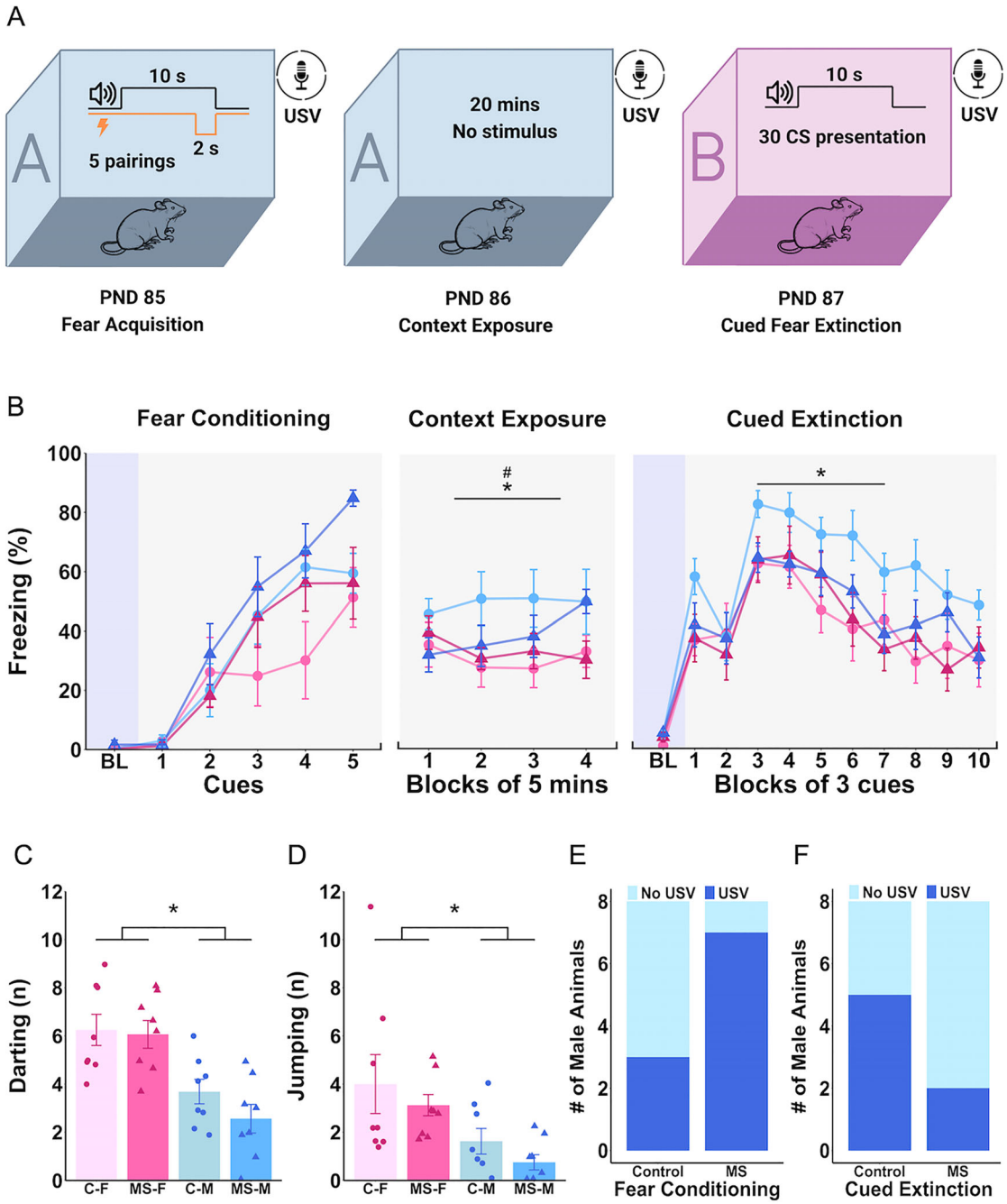


FIGURE 5 | Cued fear conditioning. (A) Schematic representation of the conditioning paradigm showing fear acquisition in Context A (left), exposure to the same context (middle), and extinction in Context B (right). (B) Freezing percentages during baseline (BL) and in response to the CS in fear acquisition (left), context exposure (middle), and extinction (right). (C) Darting behavior instances in response to the foot shock during fear acquisition. (D) Jumping responses to the foot shock in fear acquisition. (E) Distribution of male animals that produce and do not produce USVs during fear acquisition and (F) extinction. Data points represent individual animal scores ($n = 8$ per subgroup). Error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$), flat lines show statistical interaction between group and sex, and hashtags denote a main effect of group.

samples *t*-test). We then analyzed the BLp and BLa subdivisions separately and found higher activation in the BLa (M = 398.77, SD = 70.02) compared to the BLp (M = 210.89, SD = 54.05; $t(28) = 13.549$, $p < 0.001$, Cohen's $d = 3.004$, paired samples *t*-test; see Figure 6F,G). However, no group-level differences were detected in either the 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 6F) or the 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 6G).

The c-Fos+ cell density did not reveal a group-level difference in the PVN (control: M = 2016.94, SD = 219.38; MS: M = 2054.08, SD = 240.49; $t(29) = -0.448$, $p = 0.657$, independent samples *t*-test; Figure 6H), PVT (control: M = 783.33, SD = 135.13; MS: M = 717.65, SD = 101.46; $t(24) = 1.402$, $p = 0.174$, independent samples

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

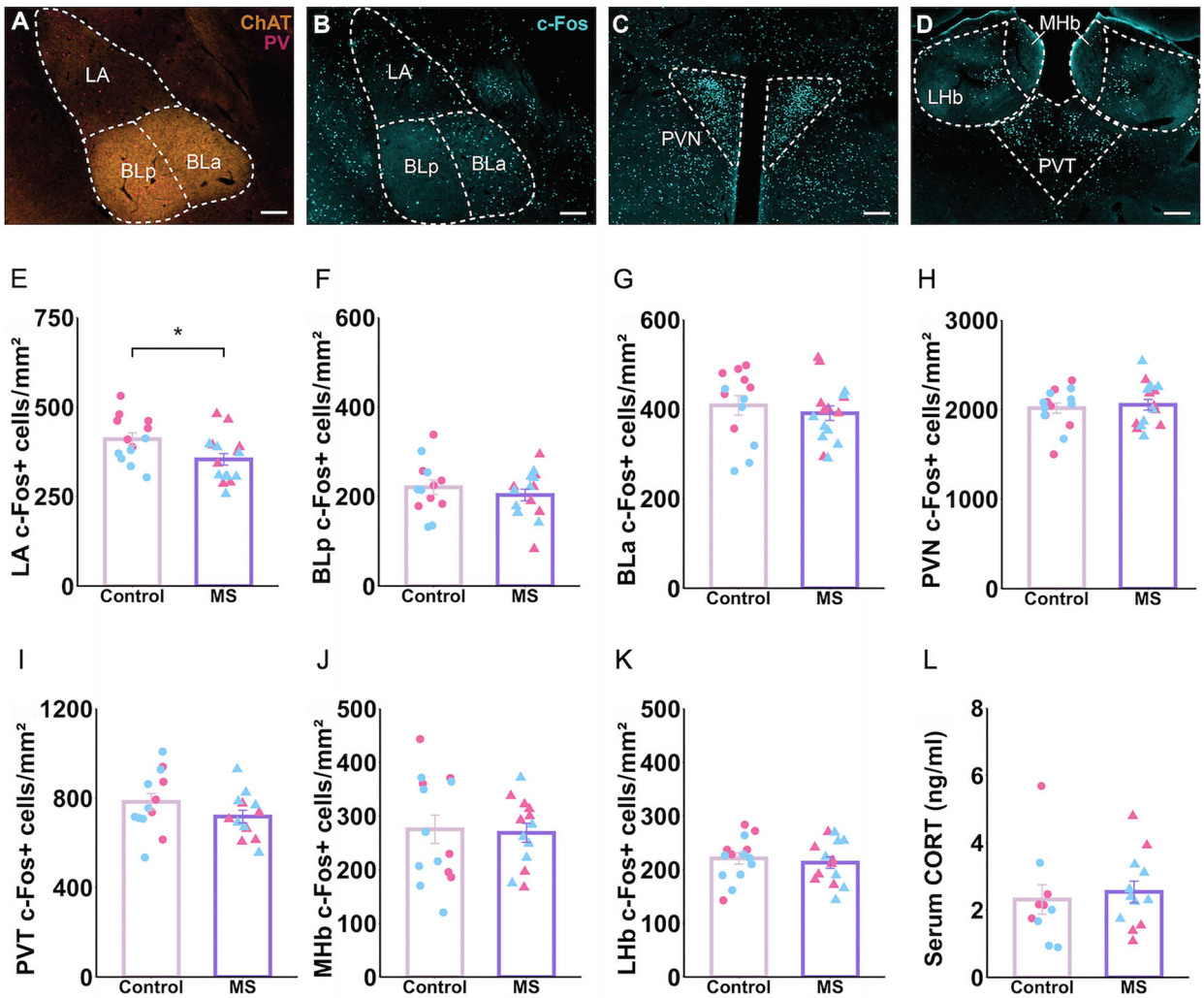


FIGURE 6 | Neuronal activation and CORT release during acute restraint stress. (A) Choline acetyltransferase (ChAT, orange) and parvalbumin (PV, magenta) immunoreactivity in the BLA delineate the borders of LA, BLa, and BLp nuclei. (B) Representative images of c-Fos (cyan) labeled cells in the same BLA section, (C) PVN, (D) MHb, LHb, and PVT. Scale bars depict 200 μm. (E–K) c-Fos+ cell density in (E) the LA, (F) BLp, (G) BLa, (H) PVN, (I) MHb, (J) LHb, and (K) PVT. (L) Serum CORT levels ($n = 10$ for control and $n = 12$ for MS). Data points represent individual animal scores (pink: female, blue: male; $n = 13$–16 per group). Error bars represent SEM. Asterisks indicate statistical significance ($p < 0.05$).

t-test; Figure 6I), 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 6J) or 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 6K).

We also analyzed serum CORT levels during restraint stress, but no group differences were found (control: M = 2.31, SD = 1.39; MS: M = 2.54, SD = 1.10; $t(20) = -0.426$, $p = 0.675$, independent samples *t*-test; Figure 6L). This result aligns with the comparable levels of activation in the PVN observed between the control and MS groups.

3.8 | USV Calls Predict Subsequent Behavior and Biological Processes

The partial correlation matrix examining the relationships between pup USVs and behavioral data revealed a positive correlation, approaching statistical significance, between the number of USV calls emitted during the postmother session of the maternal potentiation test and the time spent in the open arms of the EPM during adolescence (EPM 1; $r = 0.406$, $p = 0.054$; Figure 7A). Likewise, the number of postmother USV calls was negatively correlated with adult fear memory (average percentage of freezing across contextual and cued fear tests), also at the threshold of statistical significance ($r = -0.412$, $p = 0.051$).

We performed path analysis with all the hypothesized relationships (the final version of the model is shown in Figure 7B) and verified the model fit for each group via multiple fit indices. The chi-square test for the user model was not significant for either the control ($\chi^2 = 0.009$, $p = 0.924$) or MS groups ($\chi^2 = 0.011$, $p = 0.916$), indicating a good fit of the model to the data. The comparative fit index (CFI) and Tucker–Lewis index (TLI) both demonstrated a high level fit, with values of 1.000 and 1.620 for the control group, and 1.000 and 2.778 for the MS group, respectively. Additionally, the root mean square error of approximation (RMSEA) was 0.000 for both groups (90% CI, 0–0.237 for control and 0–0.255 for MS), and the standardized root

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

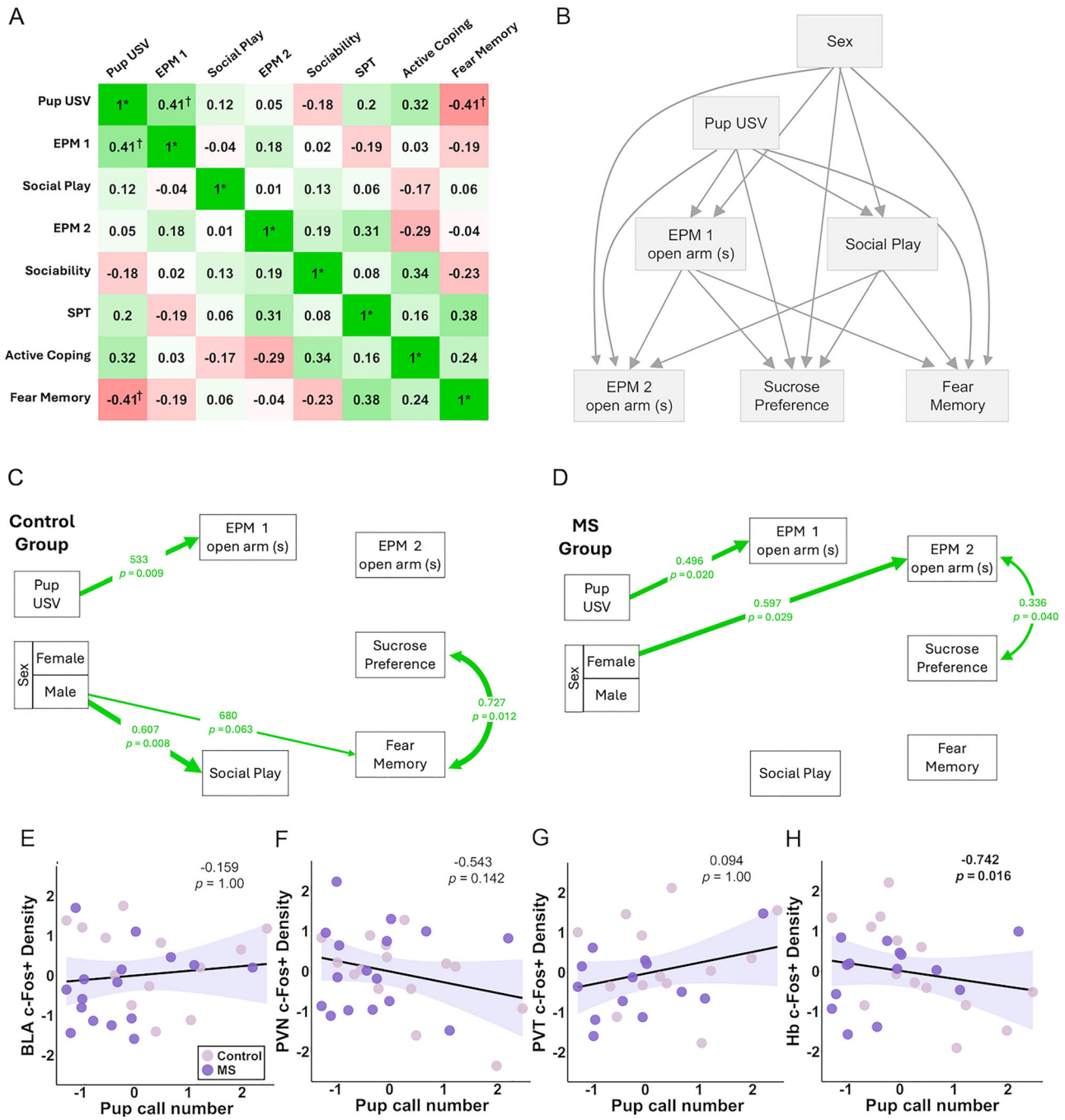


FIGURE 7 | The associations of pup USVs and adolescence measurements with adult behavioral and biological outcomes. (A) The partial correlation analysis results, controlling for group and sex effects, depict *r* values. The color density illustrates the magnitude of the *r* value, with green and red squares indicating positive and negative relationships, respectively. † represents $p < 0.06$. (B) Diagram illustrating the variables and tested relationships in the path analysis. (C) Results of path analysis with standardized regression coefficients and *p* values for each relationship tested in the control group and (D) the MS group. Arrows represent positive relationships between variables when $p < 0.1$. (E–H) Regression plots displaying the relationship between pup USV call numbers and c-Fos+ cell density in (E) the BLA, (F) PVN, (G) PVT, and (H) habenula. The black line represents the overall regression with a shadowed confidence interval. Regression coefficients and *p* values (bold when significant) are presented.

mean square residual (SRMR) was 0.003 and 0.004 for control and MS groups, respectively, which are below the recommended threshold of 0.08. These results further support the models' good fit to the data. The path coefficients, standard errors and *p* values for all paths in the models are reported in Table 1 (Supporting Information).

In line with the partial correlation results, pup USV numbers during the second isolation period predicted the open arm duration in the EPM in adolescence for both control ($B = 0.554$, $p = 0.009$; Figure 7C) and MS animals ($B = 0.329$, $p = 0.020$; Figure 7D). Infant USV numbers were not associated with any other model variables (Figure 7C,D; Table 1, Supporting Information). When testing the covariances between adulthood

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

measurements, sucrose preference in the control animals was positively correlated with fear memory ($B = 0.208$, $p = 0.031$; Figure 7C), which was lost with MS ($B = 0.240$, $p = 0.177$). However, in adult MS animals, time spent in the open arms of the EPM was positively related to sucrose preference ($B = 0.336$, $p = 0.040$; Figure 7D), while control animals did not exhibit this association ($B = 0.024$, $p = 0.720$).

Finally, we examined the predictive role of pup USVs, social play, and EPM data in adolescence on stress-induced neuronal activation and HPA axis output in adulthood. Infant USV calls were negatively correlated with activity in the habenula ($B = -0.742$, $p = 0.016$; Figure 7H). Notably, the group effect interacted with pup USVs in predicting habenula activation, and the direction of this relationship was positive ($B = 1.048$, $p = 0.018$; Figure 7H). Hence, the negative prediction of pup calls on habenula c-Fos expression was abolished in the MS group. We did not observe a predictive role of pup calls in neuronal activation in the BLA ($B = -0.159$, $p = 1.000$; Figure 7E), PVN ($B = -0.543$, $p = 0.142$; Figure 7F), PVT ($B = 0.094$, $p = 1.000$; Figure 7G), or CORT release in response to restraint stress ($B = 0.060$, $p = 1.000$). Other models were also not significant (Table 2, Supporting Information).

4 | Discussion

This longitudinal study identified several significant effects of MS, including reduced USVs in the maternal potentiation test, anxiolytic behavior, and delayed play initiation in adolescence, as well as anhedonia and impaired fear memory in adulthood. These behavioral alterations were accompanied by decreased activation in the LA during acute restraint stress in adult MS animals compared to controls, demonstrating a long-term neuronal impact of the MS model. Notably, we found that pup USV emissions have predictive value for adolescent anxiety-like behavior across all animals. Additionally, in control animals, early USVs predicted stress-induced activity in the habenula, a relationship disrupted by MS, highlighting how this potent developmental stress model interferes with the link between species-specific vocalizations and neuronal activity in the habenula.

Earlier studies reported that rat pups subjected to MS displayed greater separation-induced USVs (Burenkova et al. 2020; Ploj et al. 2003; Yin et al. 2016). However, these studies employed a single separation protocol during the first 2 postnatal weeks, a period when USV emission rates are naturally higher (Insel et al. 1986; Noirot 1968). Moreover, this increase was later found to reverse in the preweaning period, leading to a reduction in USVs following separation, which was observed only in females (Granata et al. 2021). In our work, no difference was observed during the initial isolation period, as most pups did not emit calls. However, after contact with the mother, the profile of USVs (i.e., frequency and duration) significantly shifted toward the typical 40–60 kHz range (Boulanger-Bertolus et al. 2017; Brudzynski et al. 1999; Wöhr and Schwarting 2008).

The increased USV emission following reunion with the mother, known as maternal potentiation (Brunelli et al. 1998; Hofer et al. 1993), may represent a distinct state from the calls produced during the initial isolation, which have been proposed as indicators of an infant's bond with the caregiver (Shair 2007). A detailed analysis of pup calls on PND 12 revealed higher rates of high-frequency vocalizations during the second isolation after separation (Kaidbey et al. 2019). In that study, pups also emitted a substantial number of vocalizations during the first isolation, suggesting that MS may have influenced their rates of separation-induced calls. In contrast, in our study, a greater proportion of calls during the second isolation was likely driven by prior maternal contact. Thus, the reduction in potentiated calls suggests a weakened bond with the mother due to repeated separation.

We examined the predictive role of these vocalizations and found that they positively predicted the amount of time animals spent in the open arms of the EPM during adolescence, suggesting increased exploration and reduced anxiety-like behavior (Walf and Frye 2007). Interestingly, although infant calls and open arm exploration were altered in animals subjected to MS in our work, the predictive relationship between the USVs and behavior remained unchanged for this group. This finding is consistent with a previous report showing that the association between infant USVs and adult anxiety-like behavior, assessed using the light–dark box paradigm, was not altered by a history of MS, although they did not find infant USVs to be predictive of adult anxiety-like behavior (Harmon-Jones 2023). Our findings suggest that separation-induced USVs may serve as early biobehavioral markers for later affective behaviors, rather than ELS-driven behavioral changes.

Previous work using selective breeding for animals emitting high and low USVs in infancy suggested that higher levels of separation-induced vocalization in infancy might be an early indication of later anxiety-like behavior (Brunelli 2005; Zimmerberg et al. 2005). However, our study indicated a different pattern along with a prior study showing that single separation-induced 40 kHz calls at PND 11 predicted open arm exploration in adulthood (Cordeiro et al. 2024). Notably, this relationship did not persist into adulthood, as infant calls no longer predicted behavior in the second EPM, in either control or MS animals. Similarly, Harmon-Jones et al. (2020) found no association between infant USV emission and adulthood anxiety-like behavior or contextual fear memory. Together, these findings suggest that the predictive relationship between individual variability in isolation-induced infant vocalizations and later defensive behavior may be limited to adolescence. Yet, in adulthood, we observed a negative correlation between infant USVs and conditional fear memory, supporting earlier findings (Wöhr and Schwarting 2008). Furthermore, in our analyses, the control group exhibited significant positive associations between sucrose preference and fear memory, while this relationship was not observed in the MS group, suggesting that ELS may disrupt this coherence of the traits they measure. In contrast, a positive relationship emerged between anhedonia and anxiety-like behavior in the MS group, indicating that ELS may enhance the comorbidity of these symptoms.

We also examined the predictive role of these vocalizations on the stress-induced c-Fos expression in multiple regions and revealed a negative link with the habenula of control animals. The hyperactivity of habenula is well associated with depressive-like behaviors (Cameron et al. 2024), and elevated neuronal activity was reported in this region following various rodent chronic stress

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

models (Greenwood et al. 2005; Kingir et al. 2023; Li et al. 2021). In particular, c-Fos expression in habenular nuclei increases in response to an acute stressor (Durieux et al. 2022; Kovács et al. 2022; Sood et al. 2018); while an earlier stress exposure modifies this response (Li et al. 2021; Webster et al. 2023). In the current study, MS and control animals exhibited comparable levels of c-Fos+ cells in both the MHb and LHb, with MS selectively reducing the total duration of USV calls while leaving call frequency and number unaffected. However, MS abolished the predictive power of USVs, highlighting how ELS disrupts the relationship between species-specific vocalizations and stress-related neuronal activity. Additionally, in the amygdala, MS decreased c-Fos+ LA neuron activation following restraint stress, suggesting that this ELS model alters functional connectivity within the amygdala circuitry and modifies its activation patterns under stress.

MS has been linked to behavioral despair (Lambás-Señas et al. 2009; Lee et al. 2007) and anhedonia (Frank et al. 2019; Huot et al. 2001; Maniam and Morris 2010), with prolonged separation inducing depressive-like behaviors across multiple rodent tests (Frank et al. 2019; Huot et al. 2001; Lambás-Señas et al. 2009; Lee et al. 2007; Maniam and Morris 2010). Particularly, a 3 h MS paradigm decreased sucrose preference in rats (Frank et al. 2019; Huot et al. 2001), whereas brief separation (15 min) was insufficient to elicit this effect (Huot et al. 2001; Maniam and Morris 2010). The 3 h/day MS model used in this study similarly led to anhedonic symptoms. Regarding anxiety-like behavior, meta-analytic evidence indicates that the effects of MS are highly heterogeneous. The same analysis also identified evidence of publication bias, suggesting that effect sizes may be overestimated (Wang et al. 2020). In addition, the effects of MS appear strain-dependent. While MS is known to induce anxiolytic effects in Wistar rats (León Rodríguez and Dueñas 2013; Marmendal et al. 2006; Mavrenkova et al. 2023; Ploj et al. 2002), studies in other strains have reported increased anxiety-like behavior (Huot et al. 2001; Lee et al. 2007; Maniam and Morris 2010). In line with findings in Wistar rats, we observed an anxiolytic effect of MS during adolescence, which diminished in adulthood.

MS resulted in significant changes in sociability. In adolescent social play, it led to a reduction in supine behavior, which typically sustains ongoing play interactions (Meaney and Stewart 1981). ELS models have been shown to either increase (Lundberg et al. 2017; Veenema and Neumann 2009) or decrease play behaviors (Cuarenta et al. 2021; Kentrop et al. 2018), with increased play often linked to aggression in male animals exposed to MS (Veenema and Neumann 2009). In our study, MS animals engaged in social play, showing a play-type specific difference. The reduction in supine behavior was not accompanied by changes in pouncing or pinning, which are considered aggressive play behaviors (Panksepp et al. 1984; Veenema and Neumann 2009). Thus, our results suggest a decrease in motivation to sustain social play in MS animals, while the aggressive play was not altered. Adult sociability levels of the tested animals were also suppressed by MS. In line with a previous report indicating higher vulnerability for female animals (Gazzo et al. 2021), we observed relatively unaltered sociability in males exposed to MS.

In fear conditioning, MS impaired both contextual and cued fear memory in adulthood, consistent with extensive literature indicating that ELS can disrupt fear memory processes (Chocyk et al. 2014; Guijarro et al. 2007; Mishra et al. 2019; Sanguino-Gómez and Krugers 2024; Shin and Lee 2023; Sun et al. 2014). However, some studies have reported no effect or even enhanced 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 procedural differences and the age at testing may significantly influence outcomes. In our design, a sex difference was evident across all sessions, with males exhibiting higher freezing levels overall, whereas females displayed more active coping behaviors in response to shock during fear acquisition. Notably, since the MS-induced impairment manifested in freezing behavior, its effects were primarily observed in males, while females may have shifted toward active coping strategies (Gruene et al. 2015).

Similar levels of c-Fos expression in the PVN and CORT release during restraint stress suggest that the acute HPA axis response was comparable between the MS and control groups. While several studies have reported ELS-induced increases in PVN activation (Fóscolo et al. 2022; Sanders and Anticevic 2007; Trujillo et al. 2016) and CORT levels (Lajud et al. 2012; Lehmann et al. 2002; Plotsky et al. 2005; Raineki et al. 2019), others have found no effect (Banihashemi et al. 2011; Renard et al. 2010), further emphasizing the variability across experimental conditions. Interestingly, we observed a decrease in c-Fos expression in the LA of MS animals, despite several studies reporting increased activation in the BLA following MS (Qin et al. 2021; Raineki et al. 2012; Sanders and Anticevic 2007). In our study, the animals were perfused following acute restraint stress, suggesting that the reduced neuronal activity observed in the LA may reflect changes in amygdaloid circuits due to ELS, altering their engagement in response to subsequent acute stress. In fact, an ELS model involving low bedding diminished activity in multiple amygdaloid nuclei, including the LA, in response to an acute threat (Junod et al. 2019). In that study, freezing responses to foot shock were also reduced in the ELS group, and this reduction was correlated with c-Fos expression levels in the LA. This finding suggests a positive relationship between passive stress responses and neuronal activation in the LA. The reduced freezing behavior in MS animals in our study aligns with the decreased LA activity observed in response to restraint stress.

5 | Conclusion

This study utilized a range of physiological and behavioral assessments in both male and female rats to investigate the long-term effects of MS and the predictive role of infant USV calls on subsequent social and affective behaviors across adolescence and adulthood. MS resulted in several behavioral changes, including reduced locomotion, decreased social play, heightened exploration in adolescence, and increased anhedonia and impaired fear memory in adulthood, as well as diminished stress-induced neuronal activation in the LA. Additionally, MS led to significant changes in pup USVs within a maternal potentiation paradigm. Partial correlation and path analysis revealed that these vocalizations predicted later exploratory behavior during adolescence and stress-induced neuronal activity in the habenula. Overall, these findings provide a longitudinal perspective on the lasting impact of MS as an ELS model and demonstrate that pup USVs serve not only as communicative or active coping mechanisms, but also as

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

potent biobehavioral markers for future behavioral patterns and stress-induced neuronal activity.

Acknowledgments

This research was funded by a grant from the Scientific and Technological Research Council of Türkiye (grant no: 224K875) to Gunes Unal.

Ethics Statement

All procedures were approved by the Bogăziçi University Ethics Committee for the Use of Animals in Experiments (approval no: 2024-08).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data will be made available to anyone upon request.

References

Allin, J. T., and E. M. Banks. 1972. "Functional Aspects of Ultrasound Production by Infant Albino Rats (*Rattus norvegicus*)." *Animal Behaviour* 20, no. 1: 175–185. https://doi.org/10.1016/S0003-3472(72)80189-1.

Banihashemi, L., E. J. O'Neill, and L. Rinaman. 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.

Blumberg, M. S., and J. R. Alberts. 1990. "Ultrasonic Vocalizations by Rat Pups in the Cold: An Acoustic By-Product of Laryngeal Braking?" *Behavioral Neuroscience* 104, no. 5: 808–817. https://doi.org/10.1037/0735-7044.104.5.808.

Boulanger-Bertolus, J., M. Rincón-Cortés, R. M. Sullivan, and A.-M. Mouly. 2017. "Understanding Pup Affective State Through Ethologically Significant Ultrasonic Vocalization Frequency." *Scientific Reports* 7, no. 1: 13483. https://doi.org/10.1038/s41598-017-13518-6.

Branchi, I., D. Santucci, and E. Alleva. 2006. "Analysis of Ultrasonic Vocalizations Emitted by Infant Rodents." *Current Protocols in Toxicology* 30, no. 1: 13.12.1–13.12.14. https://doi.org/10.1002/0471140856.tx1312s30.

Brudzynski, S. M. 2013. "Ethotransmission: Communication of Emotional States Through Ultrasonic Vocalization in Rats." *Current Opinion in Neurobiology* 23, no. 3: 310–317. https://doi.org/10.1016/j.conb.2013.01.014.

Brudzynski, S. M., P. Kehoe, and M. Callahan. 1999. "Sonographic Structure of Isolation-Induced Ultrasonic Calls of Rat Pups." *Developmental Psychobiology* 34, no. 3: 195–204. https://doi.org/10.1002/(SICI)1098-2302(199904)34:3⟨195::AID-DEV4⟩3.0.CO;2-S.

Brunelli, S. A. 2005. "Selective Breeding for an Infant Phenotype: Rat Pup Ultrasonic Vocalization (USV)." *Behavior Genetics* 35, no. 1: 53–65. https://doi.org/10.1007/s10519-004-0855-6.

Brunelli, S. A., J. R. Masmela, H. N. Shair, and M. A. Hofer. 1998. "Effects of Biparental Rearing on Ultrasonic Vocalization (USV) Responses of Rat Pups (*Rattus norvegicus*)." *Journal of Comparative Psychology* 112, no. 4: 331–343. https://doi.org/10.1037/0735-7036.112.4.331.

Brunelli, S. A., H. N. Shair, and M. A. Hofer. 1994. "Hypothermic Vocalizations of Rat Pups (*Rattus norvegicus*) and Direct Maternal Search Behavior." *Journal of Comparative Psychology* 108, no. 3: 298–303. https://doi.org/10.1037/0735-7036.108.3.298.

Burenkova, O. V., A. A. Averkina, E. A. Aleksandrova, and I. Y. Zarayskaya. 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.

Burgdorf, J., R. A. Kroes, J. R. Moskal, J. G. Pfaus, S. M. Brudzynski, and J. Panksepp. 2008. "Ultrasonic Vocalizations of Rats (*Rattus norvegicus*) During Mating, Play, and Aggression: Behavioral Concomitants, Relationship to Reward, and Self-Administration of Playback." *Journal of Comparative Psychology* 122, no. 4: 357–367. https://doi.org/10.1037/a0012889.

Cameron, S., K. Weston-Green, and K. A. Newell. 2024. "The Disappointment Centre of the Brain Gets Exciting: A Systematic Review of Habenula Dysfunction in Depression." *Translational Psychiatry* 14, no. 1: 499. https://doi.org/10.1038/s41398-024-03199-x.

Carr, C. P., C. M. S. Martins, A. M. Stingel, V. B. Lemgruber, and M. F. Juruena. 2013. "The Role of Early Life Stress in Adult Psychiatric Disorders." *Journal of Nervous & Mental Disease* 201, no. 12: 1007–1020. https://doi.org/10.1097/NMD.0000000000000049.

Chaby, L. E., L. Zhang, and I. Liberzon. 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., D. W. Pfaff, and R. B. Gibbs. 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, no. 1–2: 339–344. https://doi.org/10.1016/0006-8993(91)91559-J.

Chocyk, A., A. Przyborowska, W. Makuch, I. Majcher-Maślanka, D. Dudys, and K. Wędzony. 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.

Coffey, K. R., R. E. Marx, and J. F. Neumaier. 2019. "DeepSqueak: A Deep Learning-Based System for Detection and Analysis of Ultrasonic Vocalizations." *Neuropsychopharmacology* 44, no. 5: 859–868. https://doi.org/10.1038/s41386-018-0303-6.

Colman, I., P. B. Jones, D. Kuh, et al. 2014. "Early Development, Stress and Depression Across the Life Course: Pathways to Depression in a National British Birth Cohort." *Psychological Medicine* 44, no. 13: 2845–2854. https://doi.org/10.1017/S0033291714000385.

Cordeiro, N., J. A. Pochapski, W. S. Luna, et al. 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., S. L. Kigar, I. C. Henion, L. Chang, V. P. Bakshi, and A. P. Auger. 2021. "Early Life Stress During the Neonatal Period Alters Social Play and Line1 During the Juvenile Stage of Development." *Scientific Reports* 11, no. 1: 3549. https://doi.org/10.1038/s41598-021-82953-3.

Demaestri, C., H. C. Brenhouse, and J. A. Honeycutt. 2019. "22 kHz and 55 kHz Ultrasonic Vocalizations Differentially Influence Neural and Behavioral Outcomes: Implications for Modeling Anxiety via Auditory Stimuli in the Rat." *Behavioural Brain Research* 360: 134–145. https://doi.org/10.1016/j.bbr.2018.12.005.

Diehl, L. A., N. de Sá Couto Pereira, D. P. Laureano, et al. 2014. "Contextual Fear Conditioning in Maternal Separated Rats: The Amygdala as a Site for Alterations." *Neurochemical Research* 39, no. 2: 384–393. https://doi.org/10.1007/s11064-013-1230-x.

Dimatelis, J. J., I. M. Vermeulen, K. Bugarith, D. J. Stein, and V. A. Russell. 2016. "Female Rats Are Resistant to Developing the Depressive Phenotype Induced by Maternal Separation Stress." *Metabolic Brain Disease* 31, no. 1: 109–119. https://doi.org/10.1007/s11011-015-9723-8.

Durieux, L., K. Herbeaux, C. Borcuk, et al. 2022. "Functional Brain-Wide Network Mapping During Acute Stress Exposure in Rats: Interaction Between the Lateral Habenula and Cortical, Amygdalar, Hypothalamic

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

and Monoaminergic Regions." *European Journal of Neuroscience* 56, no. 8: 5154–5176. https://doi.org/10.1111/ejn.15803.

Duvarci, S., and D. Pare. 2014. "Amygdala Microcircuits Controlling Learned Fear." *Neuron* 82, no. 5: 966–980. https://doi.org/10.1016/j.neuron.2014.04.042.

Ellenbroek, B. A., P. T. J. M. van den Kroonenberg, and A. R. Cools. 1998. "The Effects of an Early Stressful Life Event on Sensorimotor Gating in Adult Rats." *Schizophrenia Research* 30, no. 3: 251–260. https://doi.org/10.1016/S0920-9964(97)00149-7.

Enthoven, L., M. S. Oitzl, N. Koning, M. van der Mark, and E. R. de Kloet. 2008. "Hypothalamic-Pituitary-Adrenal Axis Activity of Newborn Mice Rapidly Desensitizes to Repeated Maternal Absence but Becomes Highly Responsive to Novelty." *Endocrinology* 149, no. 12: 6366–6377. https://doi.org/10.1210/en.2008-0238.

Fóscolo, D. R. C., P. M. A. Lima, G. V. Rodovalho, and C. C. Coimbra. 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., A. Zlotnik, O. Kofman, et al. 2019. "Early Life Stress Induces Submissive Behavior in Adult Rats." *Behavioural Brain Research* 372: 112025. https://doi.org/10.1016/j.bbr.2019.112025.

Gazzo, G., M. Melchior, A. Caussaint, C. Gieré, V. Lelièvre, and P. Poisbeau. 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.

Granata, L., A. Valentine, J. L. Hirsch, J. Honeycutt, and H. Brenhouse. 2021. "Trajectories of Mother-Infant Communication: An Experiential Measure of the Impacts of Early Life Adversity." *Frontiers in Human Neuroscience* 15: 632702. https://doi.org/10.3389/fnhum.2021.632702.

Granata, L. E., A. Valentine, J. L. Hirsch, and H. C. Brenhouse. 2022. "Infant Ultrasonic Vocalizations Predict Adolescent Social Behavior in Rats: Effects of Early Life Adversity." *Developmental Psychobiology* 64, no. 3: 22260. https://doi.org/10.1002/dev.22260.

Greenwood, B. N., T. E. Foley, D. Burhans, S. F. Maier, and M. Fleshner. 2005. "The Consequences of Uncontrollable Stress Are Sensitive to Duration of Prior Wheel Running." *Brain Research* 1033, no. 2: 164–178. https://doi.org/10.1016/j.brainres.2004.11.037.

Gruene, T. M., K. Flick, A. Stefano, S. D. Shea, and R. M. Shansky. 2015. "Sexually Divergent Expression of Active and Passive Conditioned Fear Responses in Rats." *Elife* 4: 11352. https://doi.org/10.7554/eLife.11352.

Guijarro, J. Z., P. A. Tiba, T. L. Ferreira, S. E. Kawakami, M. G. M. Oliveira, and D. Suchecki. 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, no. 2: 101–108. https://doi.org/10.1016/j.bbr.2007.06.020.

Gunnar, M. R., N. M. Talge, and A. Herrera. 2009. "Stressor Paradigms in Developmental Studies: What Does and Does Not Work to Produce Mean Increases in Salivary Cortisol." *Psychoneuroendocrinology* 34, no. 7: 953–967. https://doi.org/10.1016/j.psyneuen.2009.02.010.

Harmon-Jones, S. 2023. "Examining the Relation Between Individual Differences in Early Memory and Adulthood Anxiety Across Species." Doctoral diss., University of New South Wales. https://doi.org/10.26190/unsworks/24897.

Harmon-Jones, S. K., C. S. M. Cowan, N. Shnier, and R. Richardson. 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., M. J. Owens, P. M. Plotsky, and C. B. Nemeroff. 1997. "Persistent Changes in Corticotropin-Releasing Factor Systems Due to Early Life Stress: Relationship to the Pathophysiology of Major Depression and Post-Traumatic Stress Disorder." *Psychopharmacology Bulletin* 33, no. 2: 185–192.

Hodgson, R., D. Guthrie, and G. Varty. 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, no. 3: 341–348. https://doi.org/10.1016/j.pbb.2007.09.005.

Hofer, M. A., S. A. Brunelli, and H. N. Shair. 1993. "Ultrasonic Vocalization Responses of Rat Pups to Acute Separation and Contact Comfort Do Not Depend on Maternal Thermal Cues." *Developmental Psychobiology* 26, no. 2: 81–95. https://doi.org/10.1002/dev.420260202.

Hofer, M. A., J. R. Masmela, S. A. Brunelli, and H. N. Shair. 1998. "The Ontogeny of Maternal Potentiation of the Infant Rats' Isolation Call." *Developmental Psychobiology* 33, no. 3: 189–201. https://doi.org/10.1002/(SICI)1098-2302(199811)33:3⟨189::AID-DEV1⟩3.0.CO;2-N.

Huot, R., K. Thrivikraman, M. Meaney, and P. Plotsky. 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, no. 4: 366–373. https://doi.org/10.1007/s002130100701.

Insel, T. R., J. L. Hill, and R. B. Mayor. 1986. "Rat Pup Ultrasonic Isolation Calls: Possible Mediation by the Benzodiazepine Receptor Complex." *Pharmacology Biochemistry and Behavior* 24, no. 5: 1263–1267. https://doi.org/10.1016/0091-3057(86)90182-6.

Jacqueline, N., and J. N. Crawley. 2007. "Mouse Behavioral Assays Relevant to the Symptoms of Autism." *Brain Pathology* 17, no. 4: 448–459. https://doi.org/10.1111/j.1750-3639.2007.00096.x.

Junod, A., M. Opendak, J. E. LeDoux, and R. M. Sullivan. 2019. "Development of Threat Expression Following Infant Maltreatment: Infant and Adult Enhancement but Adolescent Attenuation." *Frontiers in Behavioral Neuroscience* 13: 00130. https://doi.org/10.3389/fnbeh.2019.00130.

Kaidbey, J. H., M. Ranger, M. M. Myers, et al. 2019. "Early Life Maternal Separation and Maternal Behaviour Modulate Acoustic Characteristics of Rat Pup Ultrasonic Vocalizations." *Scientific Reports* 9, no. 1: 19012. https://doi.org/10.1038/s41598-019-54800-z.

Kehne, J. H., S. Coverdale, T. C. McCloskey, D. C. Hoffman, and J. V. Cassella. 2000. "Effects of the CRF1 Receptor Antagonist, CP 154,526, in the Separation-Induced Vocalization Anxiolytic Test in Rat Pups." *Neuropharmacology* 39, no. 8: 1357–1367. https://doi.org/10.1016/S0028-3908(00)00043-5.

Kentrop, J., C. R. Smid, E. J. M. Achterberg, et al. 2018. "Effects of Maternal Deprivation and Complex Housing on Rat Social Behavior in Adolescence and Adulthood." *Frontiers in Behavioral Neuroscience* 12: 00193. https://doi.org/10.3389/fnbeh.2018.00193.

Kingir, E., C. Sevinc, and G. Unal. 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., J. Burgdorf, and J. Panksepp. 2002. "Ultrasonic Vocalizations as Indices of Affective States in Rats." *Psychological Bulletin* 128, no. 6: 961–977. https://doi.org/10.1037/0033-2909.128.6.961.

Kovács, L. Á., N. Füredi, B. Ujvári, A. Golgol, and B. Gaszner. 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: 862098. https://doi.org/10.3389/fnagi.2022.862098.

Ladd, C. O., M. J. Owens, and C. B. Nemeroff. 1996. "Persistent Changes in Corticotropin-Releasing Factor Neuronal Systems Induced by Maternal Deprivation." *Endocrinology* 137, no. 4: 1212–1218. https://doi.org/10.1210/endo.137.4.8625891.

Lajud, N., A. Roque, M. Cajero, G. Gutiérrez-Ospina, and L. Torner. 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."

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Psychoneuroendocrinology 37, no. 3: 410–420. https://doi.org/10.1016/j.psyneuen.2011.07.011.

Lambás-Señas, L., O. Mnie-Filali, V. Certin, et al. 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, no. 2: 262–268. https://doi.org/10.1016/j.pnpbp.2008.11.017.

LeDoux, J. E. 2000. "Emotion Circuits in the Brain." *Annual Review of Neuroscience* 23, no. 1: 155–184. https://doi.org/10.1146/annurev.neuro.23.1.155.

Lee, J.-H., H. J. Kim, J. G. Kim, et al. 2007. "Depressive Behaviors and Decreased Expression of Serotonin Reuptake Transporter in Rats That Experienced Neonatal Maternal Separation." *Neuroscience Research* 58, no. 1: 32–39. https://doi.org/10.1016/j.neures.2007.01.008.

Lehmann, J., H. Russig, J. Feldon, and C. R. Pryce. 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, no. 1: 141–145. https://doi.org/10.1016/S0091-3057(02)00788-8.

León Rodríguez, D. A., and Z. Dueñas. 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, no. 6: e68010. https://doi.org/10.1371/journal.pone.0068010.

Li, Z. L., Y. Wang, H.-W. Zou, X.-Y. Jing, Y.-J. Liu, and L.-F. Li. 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.

Lundberg, S., M. Martinsson, I. Nylander, and E. Roman. 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., and M. J. Morris. 2010. "Palatable Cafeteria Diet Ameliorates Anxiety and Depression-Like Symptoms Following an Adverse Early Environment." *Psychoneuroendocrinology* 35, no. 5: 717–728. https://doi.org/10.1016/j.psyneuen.2009.10.013.

Mansouri, M., H. Pouretemad, G. Wegener, et al. 2021. "Dual Profile of Environmental Enrichment and Autistic-Like Behaviors in the Maternal Separated Model in Rats." *International Journal of Molecular Sciences* 22, no. 3: 1173. https://doi.org/10.3390/ijms22031173.

Marmendal, M., C. Eriksson, and C. Fahlke. 2006. "Early Deprivation Increases Exploration and Locomotion in Adult Male Wistar Offspring." *Pharmacology Biochemistry and Behavior* 85, no. 3: 535–544. https://doi.org/10.1016/j.pbb.2006.10.005.

Mavrenkova, P. V., N. N. Khlebnikova, I. B. Alchinova, M. S. Demorzhi, B. B. Shoibonov, and M. Y. Karganov. 2023. "Effects of Maternal Separation and Subsequent Stress on Behaviors and Brain Monoamines in Rats." *Brain Sciences* 13, no. 6: 956. https://doi.org/10.3390/brainsci13060956.

McCormick, C. M., P. Kehoe, and S. Kovacs. 1998. "Corticosterone Release in Response to Repeated, Short Episodes of Neonatal Isolation: Evidence of Sensitization." *International Journal of Developmental Neuroscience* 16, no. 3–4: 175–185. https://doi.org/10.1016/S0736-5748(98)00026-4.

Meaney, M., and J. Stewart. 1981. "Neonatal Androgens Influence the Social Play of Prepubescent Rats." *Hormones and Behavior* 15, no. 2: 197–213. https://doi.org/10.1016/0018-506X(81)90028-3.

Mishra, P. K., B. M. Kutty, and T. R. Laxmi. 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, no. 1: 181–190. https://doi.org/10.1007/s00221-018-5411-3.

Nagao, M., H. Kamo, I. Akiguchi, and J. Kimura. 1993. "Induction of c-Fos-Like Protein in the Lateral Habenular Nucleus by Persistent Noxious Peripheral Stimulation." *Neuroscience Letters* 151, no. 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, no. 1: 129–134. https://doi.org/10.1016/0003-3472(68)90123-1.

Olivier, B., E. Molewijk, R. van Oorschot, J. van der Heyden, E. Ronken, and J. Mos. 1998. "Rat Pup Ultrasonic Vocalization: Effects of Benzodiazepine Receptor Ligands." *European Journal of Pharmacology* 358, no. 2: 117–128. https://doi.org/10.1016/S0014-2999(98)00603-7.

Olivier, B., H. E. Molewijk, J. A. M. van der Heyden, et al. 1998. "Ultrasonic Vocalizations in Rat Pups: Effects of Serotonergic Ligands." *Neuroscience & Biobehavioral Reviews* 23, no. 2: 215–227. https://doi.org/10.1016/S0149-7634(98)00022-0.

Panksepp, J., S. Siviy, and L. Normansell. 1984. "The Psychobiology of Play: Theoretical and Methodological Perspectives." *Neuroscience & Biobehavioral Reviews* 8, no. 4: 465–492. https://doi.org/10.1016/0149-7634(84)90005-8.

Paxinos, G., and C. Watson. 2007. *The Rat Brain in Stereotaxic Coordinates*, hard cover edition. Elsevier.

Pennington, Z. T., Z. Dong, Y. Feng, et al. 2019. "ezTrack: An Open-Source Video Analysis Pipeline for the Investigation of Animal Behavior." *Scientific Reports* 9, no. 1: 19979. https://doi.org/10.1038/s41598-019-56408-9.

Ploj, K., E. Roman, and I. Nylander. 2002. "Effects of Maternal Separation on Brain Nociceptin/Orphanin FQ Peptide Levels in Male Wistar Rats." *Pharmacology Biochemistry and Behavior* 73, no. 1: 123–129. https://doi.org/10.1016/S0091-3057(02)00778-5.

Ploj, K., E. Roman, and I. Nylander. 2003. "Long-Term Effects of Short and Long Periods of Maternal Separation on Brain Opioid Peptide Levels in Male Wistar Rats." *Neuropeptides* 37, no. 3: 149–156. https://doi.org/10.1016/S0143-4179(03)00043-X.

Plotsky, P. M., K. V. Thrivikraman, C. B. Nemeroff, C. Caldji, S. Sharma, and M. J. Meaney. 2005. "Long-Term Consequences of Neonatal Rearing on Central Corticotropin-Releasing Factor Systems in Adult Male Rat Offspring." *Neuropsychopharmacology* 30, no. 12: 2192–2204. https://doi.org/10.1038/sj.npp.1300769.

Qin, X., X.-X. Liu, Y. Wang, et al. 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.

Raineki, C., M. R. Cortés, L. Belnoue, and R. M. Sullivan. 2012. "Effects of Early-Life Abuse Differ Across Development: Infant Social Behavior Deficits Are Followed by Adolescent Depressive-Like Behaviors Mediated by the Amygdala." *Journal of Neuroscience* 32, no. 22: 7758–7765. https://doi.org/10.1523/JNEUROSCI.5843-11.2012.

Raineki, C., M. Opendak, E. Sarro, et al. 2019. "During Infant Maltreatment, Stress Targets Hippocampus, but Stress With Mother Present Targets Amygdala and Social Behavior." *Proceedings of the National Academy of Sciences* 116, no. 45: 22821–22832. https://doi.org/10.1073/pnas.1907170116.

Renard, G. M., M. A. Rivarola, and M. M. Suárez. 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, no. 1: 71–80. https://doi.org/10.1159/000280102.

Ressler, R. L., and S. Maren. 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.

Sampath, D., K. R. Sabitha, P. Hegde, et al. 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., and A. Anticevic. 2007. "Maternal Separation Enhances Neuronal Activation and Cardiovascular Responses to Acute Stress in Borderline Hypertensive Rats." *Behavioural Brain Research* 183, no. 1: 25–30. https://doi.org/10.1016/j.bbr.2007.05.020.

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Sanguino-Gómez, J., and H. J. Krugers. 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.

Shair, H. N. 2007. "Acquisition and Expression of a Socially Mediated Separation Response." *Behavioural Brain Research* 182, no. 2: 180–192. https://doi.org/10.1016/j.bbr.2007.02.016.

Shin, S., and S. Lee. 2023. "The Impact of Environmental Factors During Maternal Separation on the Behaviors of Adolescent C57BL/6 Mice." *Frontiers in Molecular Neuroscience* 16: 1147951. https://doi.org/10.3389/fnmol.2023.1147951.

Smotherman, W. P., R. W. Bell, J. Starzec, J. Elias, and T. A. Zachman. 1974. "Maternal Responses to Infant Vocalizations and Olfactory Cues in Rats and Mice." *Behavioral Biology* 12, no. 1: 55–66. https://doi.org/10.1016/S0091-6773(74)91026-8.

Sood, A., K. Chaudhari, and V. A. Vaidya. 2018. "Acute Stress Evokes Sexually Dimorphic, Stressor-Specific Patterns of Neural Activation Across Multiple Limbic Brain Regions in Adult Rats." *Stress* 21, no. 2: 136–150. https://doi.org/10.1080/10253890.2017.1422488.

Streiner, D. L. 2005. "Finding Our Way: An Introduction to Path Analysis." *Canadian Journal of Psychiatry* 50, no. 2: 115–122. https://doi.org/10.1177/070674370505000207.

Sun, X.-M., W.-Q. Tu, Y.-W. Shi, L. Xue, and H. Zhao. 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., S. Lecca, K. Valentinova, and M. Mameli. 2017. "Limiting Habenular Hyperactivity Ameliorates Maternal Separation-Driven Depressive-Like Symptoms." *Nature Communications* 8, no. 1: 1135. https://doi.org/10.1038/s41467-017-01192-1.

Toda, H., S. Boku, S. Nakagawa, et al. 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, no. 5: e97421. https://doi.org/10.1371/journal.pone.0097421.

Totty, M. S., T. Tuna, K. R. Ramanathan, J. Jin, S. E. Peters, and S. Maren. 2023. "Thalamic Nucleus Reuniens Coordinates Prefrontal-Hippocampal Synchrony to Suppress Extinguished Fear." *Nature Communications* 14, no. 1: 6565. https://doi.org/10.1038/s41467-023-42315-1.

Tractenberg, S. G., M. L. Levandowski, L. A. de Azeredo, et al. 2016. "An Overview of Maternal Separation Effects on Behavioural Outcomes in Mice: Evidence From a Four-Stage Methodological Systematic Review." *Neuroscience & Biobehavioral Reviews* 68: 489–503. https://doi.org/10.1016/j.neubiorev.2016.06.021.

Trujillo, V., P. E. Durando, and M. M. Suárez. 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, no. 1: 91–103. https://doi.org/10.3109/10253890.2015.1105958.

Valle, F. P. 1970. "Effects of Strain, Sex, and Illumination on Open-Field Behavior of Rats." *American Journal of Psychology* 83, no. 1: 103. https://doi.org/10.2307/1420860.

Vanderschuren, L. J. M. J., E. A. Stein, V. M. Wiegant, and J. M. Van Ree. 1995. "Social Play Alters Regional Brain Opioid Receptor Binding in Juvenile Rats." *Brain Research* 680, no. 1–2: 148–156. https://doi.org/10.1016/0006-8993(95)00256-P.

Veenema, A. H., and I. D. Neumann. 2009. "Maternal Separation Enhances Offensive Play-Fighting, Basal Corticosterone and Hypothalamic Vasopressin mRNA Expression in Juvenile Male Rats." *Psychoneuroendocrinology* 34, no. 3: 463–467. https://doi.org/10.1016/j.psyneuen.2008.10.017.

Walf, A. A., and C. A. Frye. 2007. "The Use of the Elevated Plus Maze as an Assay of Anxiety-Related Behavior in Rodents." *Nature Protocols* 2, no. 2: 322–328. https://doi.org/10.1038/nprot.2007.44.

Wang, D., J. L. S. Levine, V. Avila-Quintero, M. Bloch, and A. Kaffman. 2020. "Systematic Review and Meta-Analysis: Effects of Maternal Separation on Anxiety-Like Behavior in Rodents." *Translational Psychiatry* 10, no. 1: 174. https://doi.org/10.1038/s41398-020-0856-0.

Wang, R.-H., Y.-F. Chen, S. Chen, et al. 2018. "Maternal Deprivation Enhances Contextual Fear Memory via Epigenetically Programming Second-Hit Stress-Induced Reelin Expression in Adult Rats." *International Journal of Neuropsychopharmacology* 21, no. 11: 1037–1048. https://doi.org/10.1093/ijnp/pyy078.

Webster, J. F., S. Beerens, and C. Wozny. 2023. "Effects of Early Life Stress and Subsequent Re-Exposure to Stress on Neuronal Activity in the Lateral Habenula." *Neuropsychopharmacology* 48, no. 5: 745–753. https://doi.org/10.1038/s41386-022-01493-0.

Winslow, J. T., and T. R. Insel. 1991. "The Infant Rat Separation Paradigm: A Novel Test for Novel Anxiolytics." *Trends in Pharmacological Sciences* 12, no. 11: 402–404. https://doi.org/10.1016/0165-6147(91)90616-z.

Wöhr, M., and R. K. W. Schwarting. 2008. "Maternal Care, Isolation-Induced Infant Ultrasonic Calling, and Their Relations to Adult Anxiety-Related Behavior in the Rat." *Behavioral Neuroscience* 122, no. 2: 310–330. https://doi.org/10.1037/0735-7044.122.2.310.

Wöhr, M., and R. K. W. Schwarting. 2013. "Affective Communication in Rodents: Ultrasonic Vocalizations as a Tool for Research on Emotion and Motivation." *Cell and Tissue Research* 354, no. 1: 81–97. https://doi.org/10.1007/s00441-013-1607-9.

Yin, X., L. Chen, Y. Xia, et al. 2016. "Maternal Deprivation Influences Pup Ultrasonic Vocalizations of C57BL/6J Mice." *PLoS ONE* 11, no. 8: e0160409. https://doi.org/10.1371/journal.pone.0160409.

Yuksel, B., Z. Sen, and G. Unal. 2024. "Ketamine Differentially Affects Implicit and Explicit Memory Processes in Rats." *Psychopharmacology* 242: 1245–1258. https://doi.org/10.1007/s00213-024-06720-8.

Zhang, W.-H., J.-Y. Zhang, A. Holmes, and B.-X. Pan. 2021. "Amygdala Circuit Substrates for Stress Adaptation and Adversity." *Biological Psychiatry* 89, no. 9: 847–856. https://doi.org/10.1016/j.biopsych.2020.12.026.

Zimmerberg, B., S. A. Brunelli, A. J. Fluty, and C. A. Frye. 2005. "Differences in Affective Behaviors and Hippocampal Allopregnanolone Levels in Adult Rats of Lines Selectively Bred for Infantile Vocalizations." *Behavioural Brain Research* 159, no. 2: 301–311. https://doi.org/10.1016/j.bbr.2004.11.009.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information: dev70212-sup-0001-SuppMat.docx

10982302, 2026, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/dev.70212 by Gunes Unal - Koc University , Wiley Online Library on [29/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License