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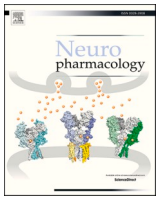

Sex-specific changes in voluntary alcohol consumption and nucleus accumbens synaptic plasticity in C57BL/6J mice exposed to neonatal maternal separation

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ABSTRACT

The long-term influence of early-life stress on brain neurophysiology has been extensively investigated using different animal models. Among these, repeated maternal separation (RMS) in rodents is one of the most commonly adopted. In this study, we elucidated the long-lasting effects of exposure to postnatal RMS in C57BL/6J adult mice on voluntary alcohol consumption and nucleus accumbens (NAc) neurophysiology. Mice were separated from their dam for 360 min daily from postnatal day 2 (PND2) to PND17, and experiments were then performed in adult (PND60) animals. In addition, as recent evidence showed that circulating estrogens may play a protective role against stress effects on brain function, including the organization and activation of neuronal structures, we also evaluated the effect of a single injection of β-estradiol 3-benzoate (EB) at PND2, which is known to disrupt male sex differentiation, in male RMS mice. The RMS exposure was associated with an increased voluntary alcohol consumption and preference in male mice, but not in female mice or male mice treated with a single injection of EB. Patch clamp experiments conducted in NAc medium spiny neurons (MSNs) revealed that excitatory but not inhibitory synaptic transmission and long-term plasticity of glutamatergic synapses were significantly impaired in male but not in female mice exposed to the RMS protocol. This effect was again prevented in RMS male mice treated with EB. Our findings strengthen the idea of a sex-dependent influence of early-life stress on long-lasting modifications in synaptic transmission and plasticity in brain areas involved in goal-directed behavior and alcohol intake.

1. Introduction

It has been demonstrated in numerous studies that there is a strong correlation between adverse events occurring early in life and an enhanced vulnerability to develop neurological and psychiatric diseases in adulthood, including drug abuse in humans (Palma-Gudiel et al., 2020; Hegde and Mitra, 2020; Sarkar et al., 2019; Liu et al., 2017) and increased substance consumption in rodents (Jin et al., 2023; Smith et al., 2023; Kirsch and Lippard, 2022).

To investigate the long-term effects of early life stress, a variety of animal models have been utilized, predominantly in rodents, that include different stress paradigms applied during the early postnatal period (Murthy and Gould, 2018). Irrespective of the employed experimental protocol, impairment in memory formation and cognitive performance (Talani et al., 2023; Li et al., 2013; Reincke and Hanganu-Opatz, 2017), increased anxiety (Brunton, 2015), depression-like symptoms (Vetulani, 2013), vulnerability for drug consumption (de Almeida Magalhães et al., 2017; Delavari et al., 2016), as well as neurochemical, neurophysiological and epigenetic alterations (Silberman et al., 2016; Zhang and Meaney, 2010), have been described.

Among the various paradigms used to induce early life stress, the repeated maternal separation (RMS) in rodents is one of the most reliable methods for studying the consequent alterations at the endocrine, neurophysiological, and behavioral levels (Lee and Jung, 2024; Alves et al., 2022; Mejía-Chávez et al., 2021; Nishi et al., 2014). Among different protocols of RMS, the repeated daily separation of pups deprived of their mother's care during the first three weeks of life [from postnatal day 1 (PND1) to PND21] is a frequently reported separation

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paradigm in the literature (Cirulli et al., 2009; Marco et al., 2009; Plotsky and Meaney, 1993). Moreover, the duration of each daily separation episode may vary consistently between the different studies, ranging from a few minutes to several hours (Bailoo et al., 2014), with different outcomes and severity of the induced effects such as a long-lasting detrimental modification of hippocampal neuronal morphology and function as well as alterations in spatial memory in male rats (Sousa et al., 2014). In addition, the RMS-induced behavioral impairments, including increased anxiety-like behavior, may be dependent on sex (Bondar et al., 2018). In particular, Bondar and colleagues demonstrated that female but not male mice exposed to maternal separation show an increase in social behavior and increased anxiety while there was an RMS-induced disruption of individual behavior, including locomotor and exploratory activity detected only in male mice. Interestingly, handling had positive effects on RMSinduced deficits in social behavior both sexes (Bondar et al., 2018). However, other studies report opposite results (Veenema et al., 2007; Tsuda and Ogawa, 2012; Cui et al., 2020), suggesting that the influence of sex as a factor for vulnerability to RMS needs further investigation.

Interesting, early life stress induced by RMS exposure was shown to significantly promote excessive alcohol (EtOH) consumption in adulthood. Although several studies are consistent with this idea (Delavari et al., 2016), other reports showed no RMS-induced effects on EtOH intake or preference (Nylander and Roman, 2013), suggesting that also this aspect may deserve more in-depth consideration.

Recently, a general consensus has emerged to view EtOH abuse as a substance-induced aberrant form of neural plasticity that occurs in key neuronal circuits (Kalivas and Brady, 2012), including those involved in drug seeking behavior and reward effects such as the mesolimbic system (Koob, 2024; Spiga et al., 2014; Cannizzaro et al., 2019; Koob and Volkow, 2010). In this regard, the nucleus accumbens (NAc) plays a central role in the neural circuits that are responsible for the development and progression of addictive states (Koob and Volkow, 2010; Edwards and Koob, 2010). Previous work has shown that impairments at glutamatergic synapses of medium spiny neurons (MSNs) at the level of the NAc shell may be critical for the abnormal response in EtOH-dependent rats during withdrawal, and restoring dopaminergic function may help to restore the altered synaptic physiology (Spiga et al., 2014; Cannizzaro et al., 2019).

The present study aimed to investigate the long-term effects of neonatal exposure of C57BL/6J mice to RMS. The two-bottle free choice paradigm was employed to investigate the impact of RMS on voluntary EtOH consumption and preference. Given the potential role of the balance between excitatory and inhibitory synaptic transmission in ensuring proper synaptic signaling information by finely tuning neural activity, we further investigated the effect of RMS on the function of NAc excitatory glutamatergic and inhibitory GABAergic synapses in adult (PND60) animals, with a particular focus on the potential impact on synaptic plasticity in the form of long-term depression (LTD). Additionally, we sought to determine whether the effects induced by RMS exhibit sex-specific characteristics. Indeed, since in female mice changes in circulating estrogens were associated with altered dendritic spine density in various brain regions, suggesting the crucial physiological role of such endogenous compounds in the organization and activation of neural structures (Sheppard et al., 2019), we extended our evaluation to both male and female mice. A separate group of male mice was treated at postnatal day 2 (PND2) with a single injection of β-estradiol 3-benzoate (EB), which has been shown to impair male sex differentiation (Nef and Parada, 2000; Toyama et al., 2001; Delbès et al., 2006). Subsequently, these mice were exposed to RMS.

2. Methods

2.1. Animals

For this study, 46 female and 73 male C57BL/6J mice (Charles River, Como, Italy) were used. Female and male mice were maintained under standard conditions with an artificial 12-hr light/dark cycle, a constant temperature of 22 ± 2 °C, and a relative humidity of 65%. They had free access to water and standard food at all times. For the purpose of breeding, animal cages were established containing a single male together with two female mice. During pregnancy, females were housed individually and observed daily until delivery, which was designated as postnatal day 0 (PND0). A scheme representing the timeline of the whole experimental design is reported in Fig. 1A.

The animal care and handling procedures employed throughout the entire experimental period were reviewed by the Organism for Animal Care and Wellness of the University of Cagliari (OPBA-UniCA) and approved by the Italian Ministry of Health (authorization no. 581/2016-PR) in accordance with the guidelines for the care and use of experimental animals set forth in the European Communities Council Directives (2010/63/UE, October 20, 2010) and the Italian law (D.L. 26, March 04, 2014). In compliance with the three Rs principles governing animal experimentation, all feasible measures were taken to minimize animal suffering and reduce the number of animals used.

2.2. Repeated maternal separation (RMS)

Litters consisted of a similar number of male and female pups (5–6 of each sex); if their number was dissimilar, the pups were redistributed among the different litters. Each entire litter was then randomly assigned to one of the two experimental groups: male and female controls (CO) that were never separated from their mothers, and male and female mice that were exposed to the repeated maternal separation (RMS) procedure. Within each litter of these two experimental groups, male mice were again randomly assigned to the EB-treated and vehicle-treated groups, but they were kept together throughout the RMS exposure and until weaning. This resulted in the following 4 experimental groups of male mice: CO (vehicle-treated and not exposed to RMS), CO + EB (EB-treated and not exposed to RMS), RMS (vehicle-treated and exposed to RMS), and RMS + EB (EB-treated and exposed to RMS). Females in the two CO and RMS experimental groups were not exposed to EB or vehicle treatment. For the RMS protocol, from PND2 until PND17, both female and male pups were separated from their dams for 360 min daily, from 9:00 a.m. to 3:00 p.m., in order to induce a robust effect by dam separation (Talani et al., 2023). To avoid experimenter-induced behavioral variance in mice, handling was performed by a single experimenter. The RMS protocol was implemented, in agreement with previous studies (Plotsky and Meaney, 1993; Lundberg et al., 2017; Talani et al., 2023), with the removal of the pups from their nest and their placement in a different room with other pups (males and females) in a controlled temperature (30–32 °C) by the addition of cotton wool to create a comfortable nest. During this period, the dams were left undisturbed in their home cage until the reunion. The control group (CO) was handled twice daily, with the pups being moved from one side of the cage to the other. However, they were left with dams in the same cage. At PND17, all pups were returned to standard housing until weaning (PND21), at which point males and females belonging to the different experimental groups were definitively separated from the dams and housed in numbers of 5 per cage.

For the EB treatment, at PND2, male pups were injected subcutaneously (s.c.) with 25 μl of sesame oil (Sigma-Aldrich, Milan, Italy) containing 10 mg of EB (Sigma-Aldrich, Milan, Italy), or with 25 μl of sesame oil only as a vehicle in accordance with previous reports (Talani et al., 2023).

2.3. Voluntary ethanol consumption

The modified sucrose-fading technique (Samson, 1986) applied in our previous report (Sanna et al., 2011) was utilized in the present investigation. In brief, female and male C57BL/6J mice at PND 60 were provided with 2-h access to EtOH in their home cage, beginning at 0.5 h

prior to the commencement of the dark period. During this period, the animals were presented with a two-bottle free choice (EtOH *vs*. water), with the position of the bottles alternating daily. For the duration of the 2-h EtOH/water period, animals were housed individually to accurately quantify their fluid intake. Each day, the bottle of tap water was replaced with two 250 ml bottles. One bottle contained EtOH/sucrose solution and the other contained water/sucrose at a matching sucrose concentration. This sucrose-fading procedure lasted 10 days, during which the animals were given 10% EtOH/5% sucrose for 2 days, 12% EtOH/5% sucrose for 2 days, 15% EtOH/5% sucrose for 2 days, 15% EtOH/2% sucrose for 2 days, 15% EtOH/1% sucrose for 2 days, and then 15% EtOH/0% sucrose as the final solution for the remainder of the 6-week study. At the conclusion of the 2-h access period, the EtOH and water bottles were removed, and the single bottle of tap water was reintroduced to the animals. At the conclusion of the experimental procedure, all animals were regrouped into 5 per cage. Although C57BL/6J mice are known to readily drink EtOH (Yoneyama et al., 2008), the sucrose-fading procedure was employed to further stabilize their daily intake. The different EtOH and sucrose solutions were prepared as a volume-to-volume (v/v) and weight-to-volume (w/v) solutions, respectively. Both the EtOH and sucrose solutions were presented at room temperature. The EtOH (2 h) and water (2 h) intake was quantified daily by weighing the bottles and calculating the difference in weight from the initial weight to determine the amount of fluid consumed during the 2-h period. The EtOH preference ratio was calculated as the volume of EtOH divided by the total volume (EtOH + water) consumed daily. All the mice that were exposed to voluntary ethanol consumption were not used for any other experiments.

2.4. Preparation of brain slices

Coronal brain slices containing the NAc shell region were prepared from mice of the different experimental groups ranging in age from PND 60 to 75, as previously described for rats (Spiga et al., 2014; Cannizzaro et al., 2019). In summary, following the induction of deep anesthesia with isoflurane vapors (4%), the animals were sacrificed. The brains were rapidly removed from the skull and transferred to a modified artificial cerebrospinal fluid (aCSF) containing the following (in mM): 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2). Coronal brain slices (with a thickness of 250 μm) containing the NAc were cut using a vibratome (Leica, Germany) and then immediately transferred to a submerged nylon net for at least 40 min at a controlled temperature of 35 °C in standard aCSF, which contained the following components (in mM): 126 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, and 10 D-glucose (pH 7.4, set by aeration with 95% O2 and 5% CO2). Subsequently, hemi-slices were incubated for a minimum of 1 h at room temperature. Thereafter, they were transferred to a recording chamber with a constant flow rate of approximately 2 ml/min of aCSF. For all recordings, the temperature of the bath was maintained at 33 °C.

2.5. Electrophysiological recording

Whole-cell patch-clamp recordings from medium spiny neurons (MSNs) of the NAc shell were performed as previously described (Spiga et al., 2014; Cannizzaro et al., 2019). The identification of MSNs was based on the measurement of several parameters of the cell membrane, in particular the resting membrane potential and the pattern of action potential firing evoked by a depolarizing current injection, according to the study of Taverna et al. (2007). During the experiments, only a small fraction (about 2%) of the recorded cells were excluded from the analysis, mainly because of the depolarization-induced action potential discharge pattern showed strong adaptation, which is more typical for FS interneurons (Taverna et al., 2007). However, we did not use precise values of these parameters as exclusion criteria, because we assumed that these parameters could be modified by the different treatments to which the animals were subjected.

Recording microelectrodes were prepared from borosilicate capillaries with an internal filament with the use of a Fleming Brown puller (Molecular Devices). Resistance of the pipettes ranged from 4.5 to 6.0 MΩ when they were filled with either of the two following internal solutions: for current-clamp experiments we used an internal solution containing 135 mM potassium gluconate, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM EGTA, 10 mM Hepes-KOH (pH 7.3), and 2 mM ATP (disodium salt); for voltage-clamp experiments the internal solution contained, in mM, 140 CsCl, 2 MgCl, 2 $CaCl_2$, 10 EGTA, 10 Hepes, 2 ATP-Na, pH 7.3 with CsOH (5 N). The access resistance ranged from 15 to 30 MΩ and was monitored throughout all patch-clamp recordings by injection of 10 mV hyperpolarizing pulses. Series resistance was not compensated, and cells were excluded from a further analysis if access resistance changed by more than 20% during the recording. Recording of the different neurophysiological parameters usually started at least 10 min after the whole-cell configuration (membrane patch break-in) was reached. Membrane potentials and membrane currents were recorded with the use of an Axopatch 200-B amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 5 kHz. The pClamp 10.7 software (Molecular Devices) was used, which allowed us to measure and analyze various kinetic parameters of the neuronal membrane, membrane potential and currents.

For current-clamp experiments, we applied a protocol consisting of the injection of currents of 1 s duration and ranging in intensity from −80 to 160 pA, with steps of 20 pA, in order to hyperpolarize or depolarize the cell membrane and thus measure voltage changes starting from physiologic resting membrane potential. Bridge balance compensation as well as pipette capacitance neutralization were applied for these recordings. The parameters analyzed included resting membrane potential, membrane capacitance (determined from the least-squares fit of an exponential current decay in the response to voltage clamped 10 mV rectangular pulses), action potential (AP) threshold (defined as the corresponding V value for a dV/dt of the AP of 15 mV/ms) (Yu et al., 2008; Talani et al., 2016), minimum injected current (rheobase) capable of evoking the first AP, AP latency (time required for the first AP to occur in response to depolarization) and AP frequency (number of events detected during the 1 s depolarizing current step). In addition, membrane input resistance (Rin) was measured in voltage-clamp mode, through the analysis of I/V relation; hyperpolarizing voltage steps, ranging from 20 to −85 mV, were imposed and steady-state currents required for holding the membrane potential were measured. Rin was calculated as the $slope^{-1}$ of the linear regression.

Miniature inhibitory and excitatory postsynaptic currents (mIPSCs and mEPSCs, respectively) were recorded in voltage-clamp mode in the presence of the voltage-gated Na^+ channel blocker lidocaine (500 μM) to suppress AP-dependent neurotransmitter release, and the non-selective glutamate receptor antagonist kynurenic acid (1 mM) to isolate mIPSCs, or the GABAAr antagonist bicuculline (20 μM) to isolate mEPSCs. Under these recording conditions, the activation of GABAAr or AMPAr results in the generation of inward currents that reflect an outflow of Cl^- or a net inflow of Na^+, respectively.

Evoked NMDAr-mediated responses were recorded at a holding potential of +40 mV in the presence of the AMPA/kainate receptor antagonist CNQX (5 μM). The analysis of mEPSCs and mIPSCs was conducted manually using Mini analysis software (Synaptosoft, Inc., version 6.0.2) with a noise amplitude threshold of 2 pA. Amplitude and frequency of events were averaged for each individual cell. NMDAr-mediated eEPSCs were analyzied using pClamp 10.7 (Molecular Devices).

For the experiments in which the long-term depression (LTD) at glutamatergic synapses was measured, a stable baseline recording of electrically evoked AMPA-mediated EPSCs, evoked every 20 s, was conducted at a stimulating current intensity that triggered 50% of the maximal response, and at a holding potential fixed at −65 mV. This was achieved through the application of a constant current pulse of 0.2–0.4

mA with a duration of 60 μs. The half-maximal response was calculated in advance, for each cell, through an input-output (I-O) relation. To induce LTD, low frequency stimulation (LFS, 500 stimuli at 1 Hz) paired with membrane depolarization (holding potential −50 mV) was applied and the eEPSC amplitude was then monitored for another 60 min, with fEPSCs evoked every 20 s and recorded at −65mV. For evoked EPSCs, a bipolar concentric stimulating electrode was placed at the bottom of the NAc shell while recorded MSNs were always located ~400 μm above the stimulating electrode tip (see Fig. 5E for the precise location of the recording microelectrode). The magnitude of LTD was calculated by

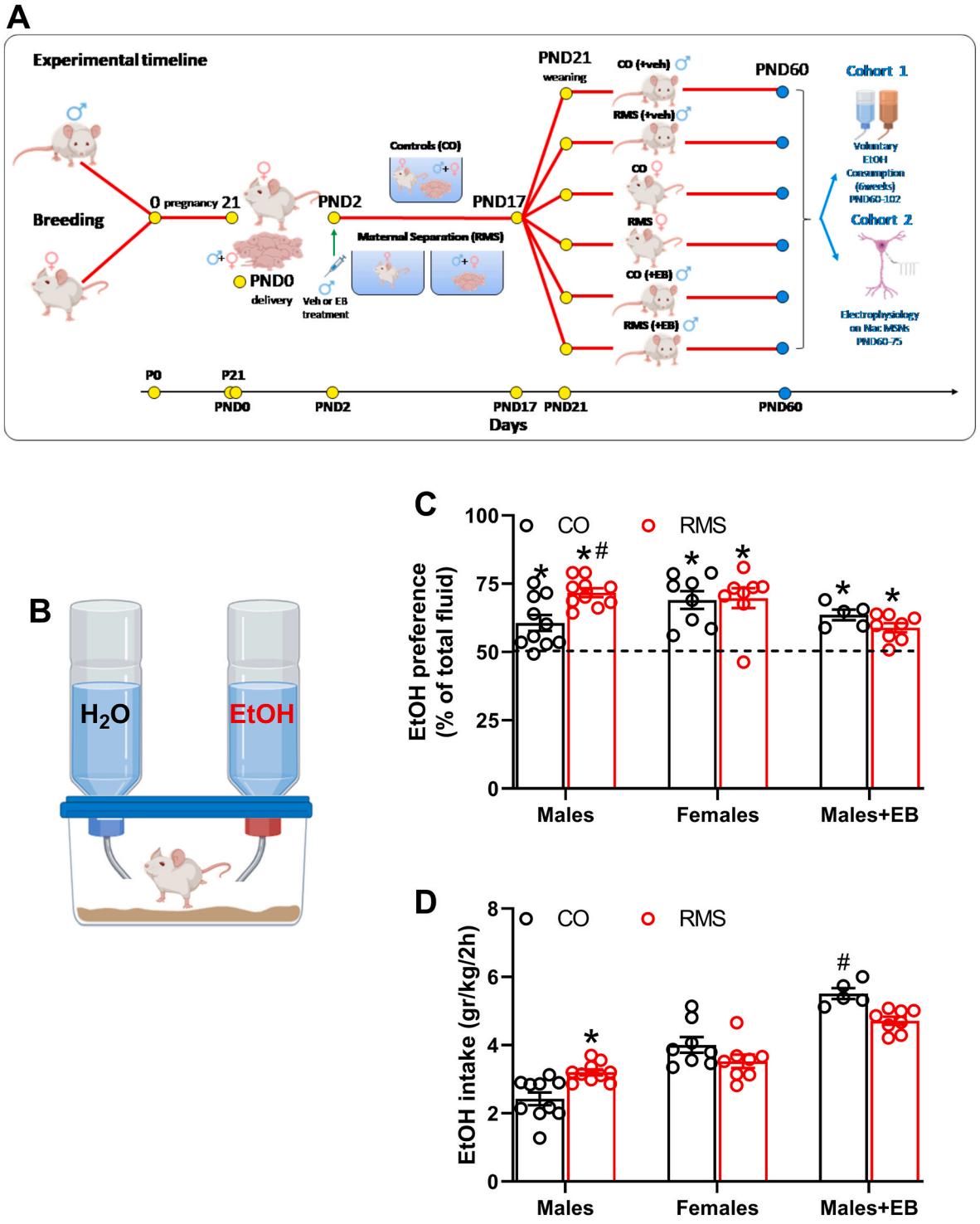

Fig. 1. Effects of RMS on voluntary EtOH consumption and preference in male and female C57BL/6J mice. (A) Schematic representation of the timeline of the whole experimental procedure. The image was obtained using the software BioRender. **(B)** Schematic representation of the two-bottle free-choice model for the 2-h daily intake of EtOH *vs*. H2O. The image was obtained using the software BioRender. **(C)** The graphs show the average per animal of voluntary EtOH preference *vs.* 50% of total fluid consumed in CO and RMS treated animals from all experimental group, calculated during the 6-weeks of the two-bottle free-choice test. Values are expressed as average per animal ± ESM, *p < 0.05 *vs.* 50% of total fluid consumed; # < 0.01 *vs.* CO males. **(D)** The graphs show the amount of voluntary EtOH intake calculated during the 6-weeks of the two-bottle free-choice test. Values are expressed as the average per animal ± ESM, *p < 0.05 *vs.* CO males. Male CO and RMS mice, N = 10 for both groups; female CO and RMS mice, N = 8; male CO and RMS mice treated with a single injection of EB at PND2, CO N = 5, RMS N = 8.

averaging the eEPSC amplitude during the interval between 50 and 60 min after LFS and comparing this value to averaged baseline values recorded for 10 min before applying LFS.

2.6. Statistical analysis

The data are presented as mean ± SEM and compared by *t*-Test or two-way analysis of variance (ANOVA) and Sidak's post hoc test with the use of GraphPad Prism software (version 8.0). A *p*-value of less than 0.05 was considered statistically significant. The sample size (number of cells), defined as "n", and the number of animals per each group, defined as "N", were clearly indicated in the figure legends for every graph. For electrophysiology results, the indication of N/n was used, while for behavioral observation, only N was indicated. Treatment with EB and relative vehicle was restricted to males only, thus the group males + EB has been considered as third "sex" in the statistical analysis.

3. Results

3.1. Effects of RMS on voluntary EtOH intake

To determine whether neonatal RMS was capable of altering EtOH preference over water and voluntary intake in adult mice, we exposed mice to 2 h of free access to EtOH and water drinking in a free-choice paradigm (Fig. 1B) over a 6-week period beginning at PND60. As expected (Yoneyama et al., 2008), C57BL/6J mice of all experimental groups showed an average daily preference for EtOH over water [*t*-test vs. 50% of total fluid consumed (water + EtOH); CO males, t = 3.669, df = 9, p = 0.005; RMS males, t = 13.51, df = 9, p = 0.0001; CO females, t = 5.876, df = 7, p = 0.0006; RMS females, t = 5.463, df = 7, p = 0.0009; CO + EB males, t = 6.989, df = 4, p = 0.002; RMS + EB males, t = 5.317, df = 7, p = 0.001] (Fig. 1C). In addition, in line with recent evidence that circulating estrogens may play a protective role against stress effects on brain function and synaptic plasticity (Kurata et al., 2004; Simpkins and Dykens, 2008; Liu and Mauvais-Jarvis, 2010) including the organization

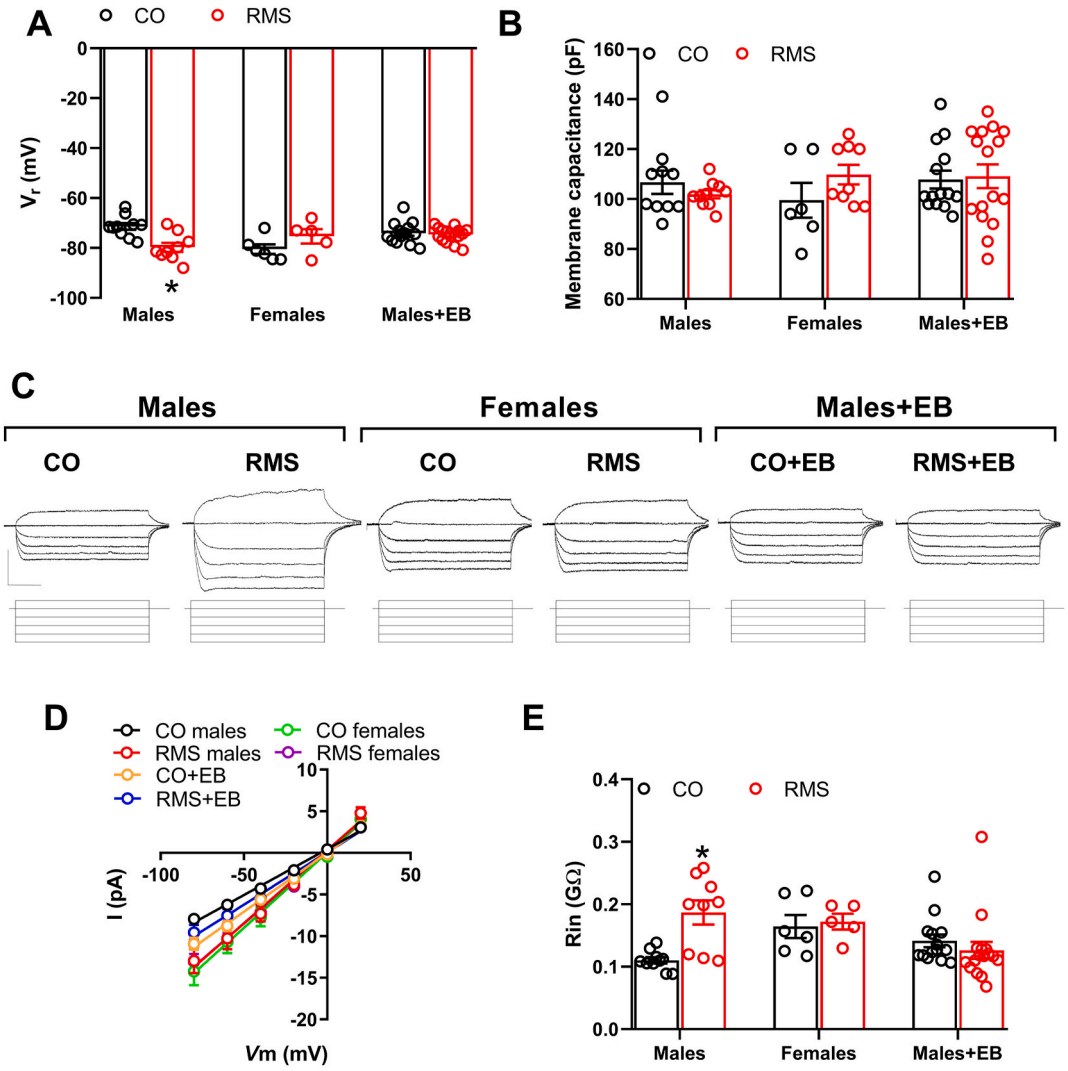

Fig. 2. Effects of RMS on intrinsic passive membrane properties of NAc MSNs from male and female mice. (A) Bar graph showing the averaged values of resting membrane potential recorded in MSNs from all experimental groups. Values are mean ± SEM and are derived from different "N" animals and recorded from "n" MSNs indicated as N/n (Male CO, 5/10; Male CO + EB, 5/14; Male RMS, 5/9; Males RMS + EB, 5/16; Female CO, 5/6; Female RMS 5/5). *p < 0.05 vs. CO. **(B)** Bar graph representing the averaged values of cell capacitance recorded in different MSNs of all experimental groups. **(C)** Representative traces of membrane potential change in response to current steps (from −80 to +20 pA, Δ 20 pA) applied in the current-clamp mode in single MSNs of all experimental groups. Scale-bar 10 mV/200 ms **(D)** *V/I* curves of evoked changes of membrane potentials in response to current steps (from −80 to +20 pA, Δ 20 pA). **(E)** Bar graph representing the changes of R_{in} values calculated from the *V/I* curves showed in panel D. Male CO, 5/10; Male CO + EB, 5/14; Male RMS, 5/9; Males RMS + EB, 5/16; Female CO, 5/6; Female RMS 5/5. *p < 0.05 vs CO males.

and activation of neuronal structures (Sheppard et al., 2019), we extended our evaluation to male mice exposed to RMS and pretreated with a single injection of EB, which is known to disrupt male sex differentiation (Nef and Parada, 2000; Toyama et al., 2001; Delbès et al., 2006). Two-way ANOVA revealed a not significant main effect of RMS on EtOH preference [F (1, 20) = 1.283; p = 0.27], but a significant main effect of sex [F (2, 23) = 3.540; p = 0.04], and a significant interaction between factors [F (2, 20) = 5.109; p = 0.02]. Sidak post hoc analysis indicated that RMS affected EtOH preference in males (p = 0.007), but not in females (p = 0.99) or in males treated with EB at PND2 (p = 0.61) (Fig. 1C). In parallel with these results, RMS influenced strongly the daily intake of EtOH in RMS males but not in RMS females or male treated with EB and exposed to RMS. Two-way ANOVA revealed a significant main effect of sex on the intake of EtOH [F (2, 43) = 70.94; p = 0.0001], a not significant main effect of RMS [F (1, 43) = 0.4397; p = 0.51], and a significant interaction between factors [F (2, 43) = 9.645; p = 0.0001]. Sidak post hoc analysis indicated that RMS affected EtOH intake only in males (p = 0.004), but not in females (p = 0.19) or in males treated with EB at PND2 (p = 0.13). Furthermore, CO + EB treated animals showed a significant increase in EtOH intake when compared to CO males administered with vehicle (p = 0.0001).

3.2. Effects of RMS on passive membrane properties and excitability of NAc shell MSNs in male and female mice

Consistent with previous reports (Spiga et al., 2014; O'Donnell and Grace, 1993), MSNs of the NAc shell are moderately hyperpolarized, with a mean resting membrane potential (V_r) of −71.4 ± 1.3 mV (n = 10) in CO males, −79.8 ± 1.8 (n = 9) in RMS males, −80.4 ± 1.9 mV (n = 9) in CO females, −75.3 ± 2.8 mV (n = 5) in RMS females, −74 ± 1.1 mV (n = 14) in CO + EB males, and −74.6 ± 0.7 mV (n = 16) in RMS + EB males (Fig. 2A). In contrast, RMS did not alter V_r values in either females or RMS males treated with EB. Overall, two-way ANOVA revealed a not significant main effect of RMS [F (1, 54) = 1.083; p = 0.31] or sex [F (2, 54) = 2.714; p = 0.07] on V_r values, but a significant interaction between factors [F (2, 54) = 9.027; p = 0.0004]. Sidak post-hoc analysis indicated that RMS causes a significant hyperpolarization of the V_r only in males (p = 0.0003), but not in females (p = 0.16) or in males treated with EB at PND2 (p = 0.97) (Fig. 2A). We also found no significant changes in cell capacitance among experimental groups [two-way ANOVA, not significant main effect of RMS, F (1, 27) = 0.3355; p = 0.56; sex F (2, 32) = 0.5664; p = 0.57; and not significant interaction between factors F (2, 27) = 1.244; p = 0.3] (Fig. 2B). Injection of current steps from −80 to +20 pA (increments of 20 pA) resulted in a current-dependent change in V_m. Two-way ANOVA revealed a significant main effect of RMS [F (1.138, 17.07) = 514.3; p = 0.0001] but not of sex [F (2.658, 39.87) = 2.1; p = 0.12] with a significant interaction between factors [F (2.611, 16.60) = 3545; p = 0.04]. Sidak post hoc analysis indicated that RMS causes a significant (p = 0.002) increase in the in the slope of the curve only in males compared to CO animals (Fig. 2C and D). In parallel, we observed an increase in the input resistance (R_{in}) only in RMS males compared to the CO group. Two-way ANOVA revealed a significant main effect of RMS [F (1, 25) = 7.025; p = 0.01] but not of sex [F (2, 29) = 2.238; p = 0.12] with a significant interaction between factors [F (2, 25) = 9.873; p = 0.0007]. Sidak post hoc analysis indicated that RMS causes a significant (p = 0.0002) increase in R_{in} only in males compared to CO animals (Fig. 2C–E).

Conversely, injection of depolarizing currents (from 20 to 200 pA) resulted in a depolarization-dependent firing of action potentials (APs) in all experimental groups (Fig. 3A and D; note that in panel A only the representative traces related to the 140 pA step are shown). We found no significant difference between groups in $V_{threshold}$ [two-way ANOVA, not significant main effect of RMS, F (1, 25) = 1.406; p = 0.25; sex, F (2, 29) = 1.806; p = 0.18; or significant interaction between factors, F (2, 25) = 1.554; p = 0.23] (Fig. 3B), or rheobase, the minimum current required to trigger the first AP [two-way ANOVA, not significant main effect of RMS, F (1, 25) = 1041; p = 0.32; sex, F (2, 29) = 3.617; p = 0.15; and not significant interaction between factors, F (2, 25) = 0.8245; p = 0.45] (Fig. 3C). In addition, we analyzed both depolarization-induced AP frequency and latency. No between-group difference was found in depolarization-induced AP frequency [two-way ANOVA, not significant main effect of RMS, F (1, 54) = 0.064; p = 0.8; sex, F (2, 54) = 2.247; p = 0.11; and not significant interaction between factors, F (2, 54) = 0.57; p = 0.45] (Fig. 3D), or latency [two-way ANOVA, not significant main effect of RMS, F (1, 54) = 0.47; p = 0.49; sex, F (1.830, 49.42) = 2.481; p = 0.09; and not significant interaction between factors, F (2, 54) = 0.5908; p = 0.55] (Fig. 3E).

3.3. Effects of RMS on excitatory and inhibitory synaptic transmission in NAc shell MSNs in male and female mice

The next step was focused to assess whether postnatal RMS might influence GABAergic and glutamatergic transmission in NAc shell MSNs of male and female mice. The membrane potential of MSNs neurons was maintained at − 65 mV during whole-cell patch clamp recordings. Analysis of mIPSC properties revealed no significant difference between MSNs of all experimental groups in either amplitude [two-way ANOVA, not significant main effect of RMS, F (1, 8) = 3.286; p = 0.1; sex, F (1.904, 15.23) = 0.2953; p = 0.73; and not significant interaction between factors, F (1,542, 12.33) = 0.9418; p = 0.39], and frequency [two-way ANOVA, not significant main effect of RMS, F (1, 8) = 0.00034; p = 0.98; sex, F (1.678, 13.43) = 0.1102; p = 0.86; and not significant interaction between factors, F (1,267, 10,14) = 0.77; p = 0.43]. This suggests that neonatal exposure to RMS does not alter GABAergic synaptic transmission in both adult male and female mice (Fig. 4A–C). Conversely, a statistically significant decrease in mEPSC frequency was observed only in RMS male mice [two-way ANOVA, significant main effect of RMS, F (1, 9) = 110.5; p = 0.0001; significant main effect of sex, F (1.576, 14,18) = 4.978; p = 0.03; significant interaction between factors [F (1.588, 14,29) = 13.47; p = 0.0009]. Sidak post hoc analysis indicated that RMS causes a significant (p = 0.0001) decrease in the frequency of mEPSCs only in males, indicating a decrease in the probability of glutamate release from presynaptic terminals as a result of neonatal RMS exposure. Notably, the effect induced by RMS was no longer detectable in RMS males treated with EB at PND2 before the start of the RMS procedure (p = 0.89 *vs.* CO) (Fig. 4D–F). No changes in mEPSC amplitude were observed between groups [two-way ANOVA, not significant main effect of RMS, F (1, 9) = 0.0040; p = 0.95; sex, F (1,976, 17,79) = 4.046; p = 0.36; and not significant interaction between factors, F (1,228, 11,05) = 0.4042; p = 0.57] (Fig. 4D–E).

We therefore explored the possibility that RMS could be associated with an altered function of NMDAr, which have a very low conductance for cations at resting V_m. Therefore, patch clamp recordings were conducted at a V_m of +40 mV, and an input-output (I-O) paradigm was established by stimulating the excitatory afferents with increasing current intensity (from 0 to 1.0 mA, with 0.2 mA increments) and recording the NMDAr-mediated currents in single MSNs in the presence of the AMPAr antagonist CNQX (5 μM). As expected, while two-way ANOVA showed that NMDAr-mediated currents were dependent on the stimulation intensity in all experimental groups, the I-O curve was significantly altered only in males exposed to RMS [two-way ANOVA, significant main effect of RMS, F (1, 5) = 7.737; p = 0.04; significant main effect of sex, F (1,853, 9267) = 5.235; p = 0.03; significant interaction between factors, F (1,315, 6573) = 19.03; p = 0.003] in the stimulation intensity to evoke the half maximal NMDA current response. Sidak post hoc analysis indicated that RMS causes a significant (p = 0.02) increase in the half maximal stimulation intensity only in males (Fig. 4G and H). This effect was not observed in females (p = 0.51 *vs.* CO) (Fig. 4G and H) or in RMS males treated with EB (p = 0.74 *vs.* CO) (Fig. 4G and H).

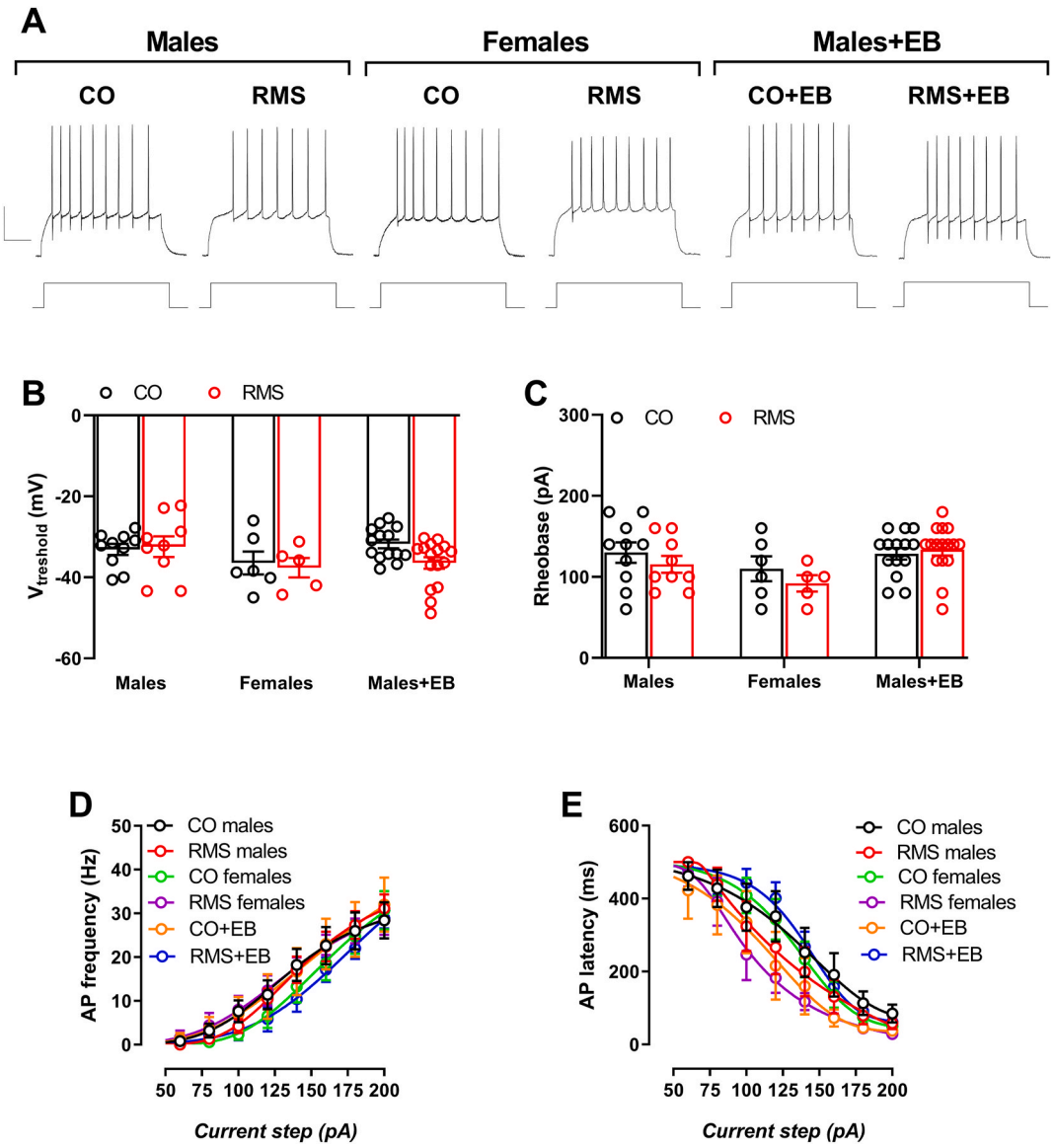

Fig. 3. Effects of RMS on NAc MSNs excitability of NAc MSNs from male and female mice. (A) Representative traces of membrane potential change in response to a positive current step (+140 pA) recorded in the current-clamp mode in single MSNs from all experimental groups. Scale-bar 10 mV/200 ms. **(B)** Bar graph representing the averaged values of $V_{threshold}$ in different MSNs of all experimental groups. Values are reported as average ± SEM and were obtained in (n) neurons from different (N) animals indicated as N/n (Male CO, 5/10; Male CO + EB, 5/14; Male RMS, 5/9; Males RMS + EB, 5/16; Female CO, 5/6; Female RMS 5/5). *p < 0.05 *vs.* CO. **(C)** Bar graph representing the averaged values of rheobase, i.e. the minimum depolarizing current intensity required to evoke the first action potential. **(D, E)** Scatter plots reporting the action potential frequency (D) and latency (E) in response to the application of positive current steps (from 0 to +200 pA, Δ 20 pA). N/n (Male CO, 5/10; Male CO + EB, 5/14; Male RMS, 5/9; Males RMS + EB, 5/16; Female CO, 5/6; Female RMS 5/5).

3.4. RMS impairs LTD formation on NAc MSN excitatory synapses

The alterations induced by neonatal exposure to RMS on AMPAr- and NMDAr-mediated postsynaptic currents observed in NAc MSNs of adult mice may be indicative of a potential dysfunction in long-term synaptic plasticity. In the NAc, the long-term depression (LTD) of excitatory glutamatergic synapses has been shown to involve NMDAr-mediated signaling, a mechanism that is associated with a reduction in post-synaptic AMPAr membrane expression and function (Turner et al., 2018; Spiga et al., 2014; Cannizzaro et al., 2019). Low frequency presynaptic stimulation (LFS, 500 stimuli at 1 Hz) can readily induce a consistent LTD in the NAc. The anatomical location of the stimulating electrode is illustrated in Fig. 5D. LTD was quantified by comparing the averaged amplitude of eEPSCs recorded from 50 to 60 min after LFS with that observed during the 10 min of baseline before LFS. Consistent with previous reports (Spiga et al., 2014; Cannizzaro et al., 2019), LFS-induced LTD was completely prevented when the NMDAr antagonist D-2-amino-5-phosphonovaleric acid (APV, 50 μM) was added to the recording extracellular aCSF solution (data not shown). Comparing the effect of LTD in all experimental groups, LTD resulted dramatically decreased only in RMS male mice compared to the respective CO, without any significant alteration in either females or RMS + EB treated males [two-way ANOVA, significant main effect of RMS, $F(1, 12) = 20.09$; $p = 0.0008$; significant main effect of sex, $F(2, 14) = 5.172$; $p = 0.02$; significant interaction between factors, $F(2, 12) = 13.53$; $p = 0.0008$]. Sidak post hoc analysis indicate that RMS causes a significant ($p = 0.0001$) decrease in the measured levels of LTD only in males, with no change in females ($p = 0.52$) or RMS males treated with EB ($p = 0.98$), suggesting a sex-dependent effect of RMS as well as a protective role of estrogens in the early postnatal period by preventing the negative effects of RMS.

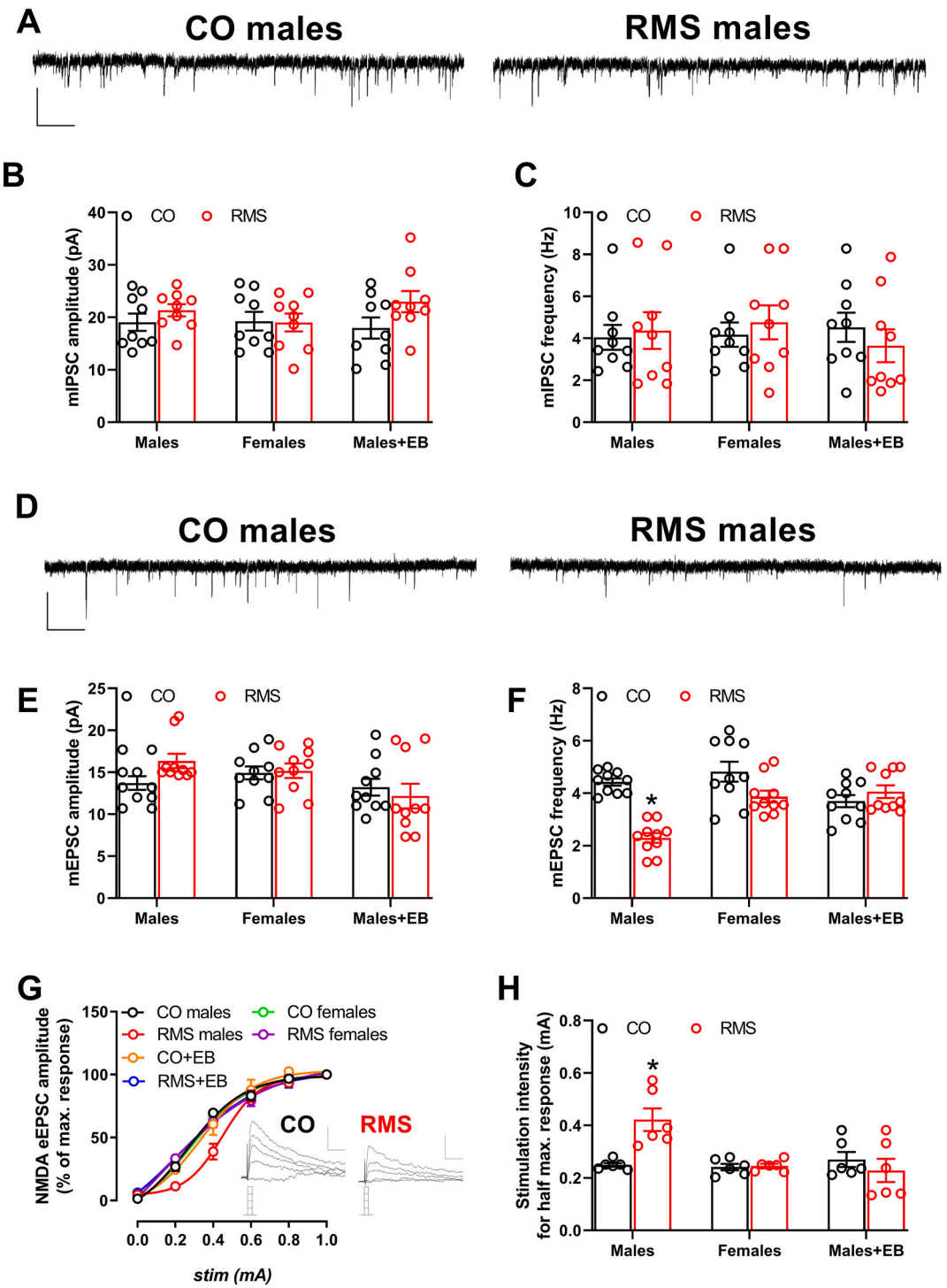

Fig. 4. Effect of RMS on GABAergic and glutamatergic miniature currents in NAc MSNs of male and female mice. (A, D) Representative traces of miniature IPSCs (A) and mEPSC (D) recorded from single voltage-clamped MSNs of CO male and RMS male mice. Scale bar, 5 pA/500 ms. **(B, C)** Averaged mIPSC amplitude (B) and frequency (C) were calculated from the recording of a 3-min period. The bar graphs summarize the changes in amplitude and frequency and data are expressed as the mean of absolute values ± SEM. N/n, Male CO, 5/9; Male CO + EB, 5/9; Male RMS, 5/9; Males RMS + EB, 5/9; Female CO, 5/9; Female RMS 5/9. **(E, F)** Averaged mEPSCs amplitude (E) and frequency (F) were calculated from the recording of a 3-min period. The bar graphs summarize the changes in amplitude and frequency and are expressed as the mean of absolute values ± SEM. N/n, Male CO, 5/10; Male CO + EB, 5/10; Male RMS, 5/10; Males RMS + EB, 5/10; Female CO, 5/10; Female RMS 5/10. *p < 0.05 *vs.* CO males. **(G)** Scatter plot representing the input-output curves of NMDAr-mediated eEPSCs in all experimental groups. The values represent current amplitude and are normalized to the maximal response. In the insert are reported the representative traces of evoked NMDAr-mediated EPSCs recorded at +40 mV in MSNs from CO and RMS male mice in the presence of the GABAergic antagonist bicuculline (20 μM) and AMPAr antagonist NBQX (5 μM), and elicited by current pulses from 0 to 1 mA (Δ 0.2 mA). Scale bar, 100 pA/50 ms. **(H)** Bar graph that summarize the values of the stimulus intensity that evoked a half-maximal response of the NMDA evoked EPSCs in the different experimental groups. Data are expressed as mean ± SEM. **p < 0.001 *vs.* CO, *p < 0.05 *vs.* RMS. *p < 0.05 *vs.* CO males.

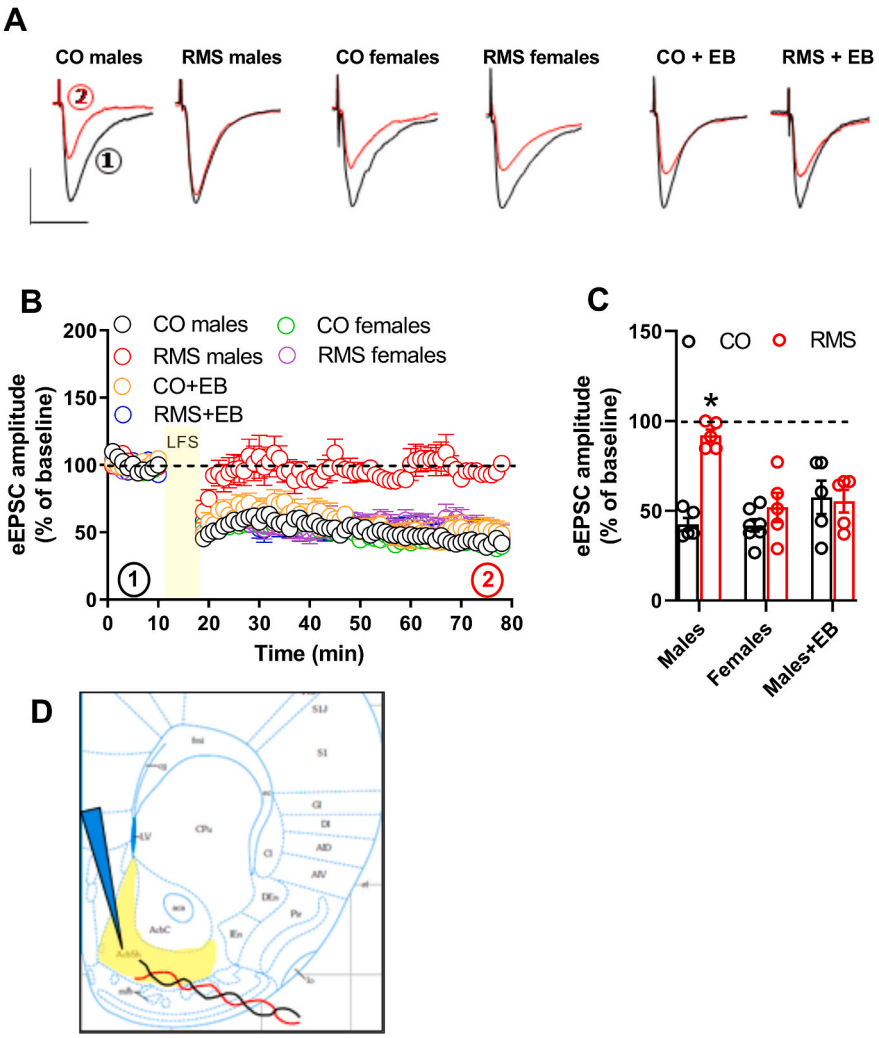

Fig. 5. RMS alters the LTD formation in NAc MSNs in male but not female mice. (A) Representative traces of evoked EPSCs recorded in slices obtained from males or females of the different groups. Traces were recorded before (baseline) (1) and after (2) LTD induction by delivery of LFS (500 stimuli at 1 Hz), scale bar, 1 mV/5 ms. **(B)** Scatter plots showing the percentage of change of eEPSC amplitude values induced before and after LFS (indicated by the yellow bar) and calculated with respect to baseline (before LFS), in all experimental groups. **(C)** The graph summarizes the magnitude of LTD, calculated by averaging the percentage change in eEPSC amplitude from baseline considering the values between 50 and 60 min after LFS, obtained from graphs B. Data are expressed as mean percent change of eEPSC amplitude ±SEM from baseline. *$p < 0.05$ *vs.* CO males. **(D)** Schematic representation of the position of recording and stimulating electrodes in a coronal section of the NAc (intraneural 10.2 mm, bregma 1.2 mm).

4. Discussion

The results of the present study provide new evidence for a sex-specific response to early life stress associated with RMS in C57BL/6J mice. Our findings indicate that the aforementioned stress protocol, when applied during the first two weeks of life, was able to enhance the voluntary EtOH consumption and preference in adult male but not female mice. These changes were accompanied by marked alterations in glutamatergic signaling and long-term synaptic plasticity in MSNs of the NAc shell. Notably, all of these changes were prevented by the pre-treatment of male pups with EB.

Genetic influence (Enoch, 2012) together with environmental factors (Kirsch and Lippard, 2022) are thought to play a critical role in the development of alcohol use disorders. From a preclinical perspective, maternal separation is a commonly employed model for investigating the impairments induced by early life stress in rodents and their impact on a range of behavioral responses including the propensity for EtOH consumption (Hanson et al., 2021) observed in adulthood. However, the mechanisms underlying this effect and the potential role of sex as a discriminating factor remain poorly understood. For these reasons, the main goal of the present study was to assess whether exposure of newborn C57BL/6J mice to RMS could alter the propensity for EtOH voluntary consumption in adult males and females, directing our attention on understanding the possible changes of NAc neurophysiology.

Consistent with previous reports conducted in the same mouse strain (Sanna et al., 2011; Lopez et al., 2011), our results show that naïve C57BL/6J mice exposed to a 6-week two-bottle free-choice paradigm show a significant preference for EtOH over water. Interestingly, the preference and averaged amount of daily session for EtOH consumption were significantly increased in male mice exposed to RMS compared to the CO counterpart, an effect that was not evident in female animals. These results support the evidence that males exposed to RMS, which have also been shown to exhibit behavioral expression of moderate stress levels in adulthood (Kim et al., 2023), reflect the increased propensity to consume EtOH and that sex may clearly influence this specific outcome. Consistent with these findings, previous reports have shown that RMS causes an increased EtOH consumption in adult rats (Huot et al., 2001; Ploj et al., 2003; Roman and Nylander, 2005; Gustafsson and Nylander, 2006) and mice (Cruz et al., 2008). The stress response

that occurs during the daily 6 h of separation may be related to the lack of maternal care, although it has been found that there is an increase in caregiving immediately after the pups are reunited with the dam (Macrí et al., 2004). The present results, together with our previous report (Talani et al., 2023), reinforce the notion of the importance of sex as a factor that plays a role in the response to early life stress, with males being more vulnerable than females in terms of their propensity to consume EtOH (de Souza et al., 2018; Loi et al., 2014; Roman and Nylander, 2005). In contrast, a recent study failed to observe a sex difference in Sprague-Dawley rats that were separated for 6 h daily (3 h + 3 h in two separate daily rounds), demonstrating that the depressive-like behavior and impairment of synaptic plasticity caused by RMS were similar between sexes (Cui et al., 2020). On the other hand, a more recent report, also in rats, showed that adolescent female rats exposed to RMS consumed more EtOH, which may be associated with an increase in the NMDAr GluN1 subunit expression in the prefrontal cortex and ventral striatum (Filarowska-Jurko et al., 2022). These discrepancies may be related to the maternal separation protocol employed, the time points examined, and the animal species used, suggesting that the precise mechanisms underlying the impairments caused by RMS warrant further investigation. Consistent with this idea, a recent work suggests that the window of vulnerability to early life stressors may be sexually dimorphic because of profound sex differences in many aspects of neural development, including neural structures such as dopaminergic and GABAergic systems that are known to be more sensitive to stress and alcohol exposure (McElroy et al., 2023; Ortelli et al., 2023).

Furthermore, we investigated the possible role of the different hormonal patterns between male and female mice by treating newborn male RMS mice with a single injection of EB at PND2, immediately before the start of the RMS protocol. Indeed, circulating estrogens have been shown to play a protective role against stress effects on brain function and synaptic plasticity (Kurata et al., 2004; Simpkins and Dykens, 2008; Liu and Mauvais-Jarvis, 2010). We have previously shown that EB treatment did not affect the change in body weight of male mice induced by RMS (Talani et al., 2023), but was able to produce a significant decrease in testicular weight as well as adult male fertility when compared to those treated with vehicle alone at PND2 (Talani et al., 2023), a result consistent with other findings showing the negative influence of EB treatment on male sex differentiation (Toyama et al., 2001; Delbès et al., 2006; Nef and Parada, 2000). This estrogen was remarkably effective in preventing the behavioral changes observed in adult males related to EtOH consumption, suggesting a possible protective effect of EB against the negative influence of RMS on EtOH consumption, as reported in some studies in females (Bondar et al., 2018; Mehta and Schmauss, 2011; Dombret et al., 2020; Romeo et al., 2003; Kundakovic et al., 2013; Bailoo et al., 2014), but not in others (Veenema et al., 2007; Tsuda and Ogawa, 2012).

Drug self-administration has been used extensively as a consistent paradigm for studying drug-seeking behavior and reinforcement induced by drugs of abuse, including EtOH (Koob, 2024). In the scenario of mechanisms associated with drug abuse, the NAc is fundamental in driving the motivated behavioral outcomes. Long-term changes in synaptic strength within the NAc underlie experience-dependent neural plasticity (Koob, 2024; Everitt and Robbins, 2005). These synaptic adaptations include epigenetic, molecular, biochemical, electrophysiological, and morphological changes in neurons, including a remodeling of synaptic function (Turner et al., 2018). In addition, a more recent study assessing the transcriptome in the NAc, revealed and confirmed that early life adversity has lasting effects on brain areas involved in the reward circuitry, highlighting the importance of further exploring the impact of early life stress on motivational processes (Benoit et al., 2023).

Our patch-clamp data collected in NAc MSNs show that neonatal exposure to RMS is associated with an increase in membrane resistance in parallel with an increase in membrane resting potential only in male mice, an effect that does not appear to contribute to overall neuronal excitability, as neither depolarization-induced action potential firing nor the latency between current application and detection of the first action potential is affected by RMS treatment or sex. Regulation of the electrical membrane properties of MSNs has been reported to be critical for the integration and processing of drug-induced changes that converge on the NAc. It has been well described that MSNs exhibit at least two membrane potential states, a hyperpolarized down-state and a more depolarized up-state, which are directly related to action potential discharge (for review, see Marty and Spigelman, 2012). Dysregulation of this mechanism has been proposed as one of the major neuroadaptations that may underlie addiction (Kim et al., 2011). The NAc is primarily composed of GABAergic MSNs that project massively to the ventral pallidum, substantia nigra, and the ventral tegmental area (Xia et al., 2011) and receive extensive excitatory glutamatergic inputs from limbic areas such as the medial prefrontal cortex, hippocampus, and basolateral amygdala (Sesack and Grace, 2010) and inhibitory inputs from the subpopulations of local circuit GABAergic interneurons. Changes in the balance of excitatory/inhibitory inputs may affect the steady state of MSNs, thereby altering membrane excitability, with consequences for behavior associated with addictive states. These findings are consistent with others showing that early life stress caused by environmental challenges, such as those produced by RMS, affects long-term microanatomical, neurochemical, and structural reorganization of corticostriatal circuits, with atrophy in the prefrontal cortex and hypertrophy in the NAc (Romano-López et al., 2016). All of these changes may contribute to create a so-called “pre-addict brain”, with multiple and consequential implications for the development of neuropsychiatric disorders such as addiction to various drugs of abuse, including EtOH (Hanson et al., 2021). In addition, taking into account other findings (Brake et al., 2004) as well as our results on the decrease in glutamate release and NMDAr function, we believe that alterations in the glutamatergic inputs in the NAc MSNs may promote a reorganization of various neurotransmitter and neuromodulatory systems, such as the dopamine and endocannabinoid systems, and a consequent modification of the entire reward system (Hanson et al., 2021). Consistent with the ability of such early life stress to alter the function of specific brain areas in a long-lasting manner, with the potential to transform a “normal” into a “pre-addicted” brain, we are led to consider that our findings appear similar to those obtained in our previous studies in EtOH-dependent rats (Spiga et al., 2014; Cannizzaro et al., 2019). Indeed, in these studies, EtOH-dependent rats undergoing withdrawal exhibited changes in the NAc MSNs that are partially consistent with those observed in male RMS mice, including a decrease in NMDAr function and a marked decrease in LTD (Spiga et al., 2014; Cannizzaro et al., 2019). Altered glutamatergic transmission and reduced NMDAr-dependent long-term plasticity in MSNs of the NAc following chronic EtOH exposure are cell type specific, as these changes occur selectively on dopamine D1 receptor-expressing MSNs (Renteria et al., 2017) and are critically linked to the expression of EtOH drinking behavior (Becker and Lopez, 2004; Jeanes et al., 2011; Kircher et al., 2019). Consistent with these data, EtOH-dependent rats that were withdrawn for 14 days, and showed no overt behavioral signs of withdrawal hyperexcitability, exhibited increased EtOH voluntary drinking, an effect that was paralleled by a sustained decrease in NAc LTD (unpublished results, Talani and Sanna).

These studies suggest that chronic alcohol and early life stress lead to specific adaptations in the NAc, including impaired neuron excitability, altered glutamatergic activity, decreased long-term plasticity, and changes in dopamine signaling, which collectively influence motivation, reward processing, and susceptibility to alcoholism and anxiety disorders. A recent review by Hanson et al. (2021) discusses the impact of early life stress on the function of reward circuit structure, including the NAc in both human and animal models, and links the findings to emerging theoretical models of the indirect and direct detrimental effects of early life on alterations in motivated and reward-seeking behavior directed toward drugs of abuse such as psychostimulants, ethanol, and opiates. In this regard, early life stress paradigms, including the RMS, are associated with altered VTA morphology, a decreased

GABAergic inhibition onto dopaminergic neurons with a consequent increase in excitability of dopaminergic neurons, and an increased release of dopamine into the NAc. Consistent with our findings, some studies have reported a decrease in presynaptic glutamate release in NAc MSNs only in male rats, while others demonstrate that RMS is associated with a decreased GluA2 AMPA subunit expression in the NAc of male but not female rats (see Hanson et al., 2021, and references cited therein). An interesting key point for future investigation will be directed to understand how early life stress may alter the activity of D1 vs D2 expressing MSNs, since it has been found that these cell types respond differently to stress applied in adults.

Although not directly tested in the present study, our data demonstrating the marked decrease in LTD in male RMS mice may be related to a possible decreased spine density, shrinkage, and synaptic loss in these animals, again similar to what found in EtOH-dependent rats undergoing withdrawal (Spiga et al., 2014; Cannizzaro et al., 2019). On the other hand, the decrease in NMDAr-mediated synaptic responses could clearly be a contributing factor for such LTD loss in male RMS mice. Indeed, LTD formation in the NAc appears to be dependent on NMDAr activation, as bath perfusion with (2R)-amino-5-phosphonopentanoate (APV) (50 μM) completely suppressed the long-term decrease in EPSC amplitude (Spiga et al., 2014; Jeanes et al., 2011). In addition, our present data indicate that RMS exposure is associated with a significant decrease in the release of glutamate from afferents impinging on NAc MSNs. This alteration may be another factor contributing to the decreased LTD formation observed in RMS mice, with the implication that RMS is associated with a profound impairment involving both pre- and postsynaptic mechanisms. This aspect certainly deserves further investigation, and future studies will be directed at analyzing the potential role of specific presynaptic receptors, including endocannabinoid CB1 and certain subpopulations of metabotropic glutamate receptors, which are known to regulate the glutamate release in the NAc (Robbe et al., 2002, 2003). Since we failed to find changes in glutamatergic transmission and LTD impairments in females, our data suggest that RMS causes critical changes in the glutamatergic system in different brain areas in a sex-specific manner (Ganguly et al., 2019).

Taken together, the different effects induced by RMS in males and females not only confirm the sex-specific sensitivity to RMS, but also suggest that sex hormones, particularly estrogens, may play a critical role in stress protection during this critical period of early life. Wei and coworkers (2014) demonstrated that females and males exhibit a clear dimorphic stress response, with female rats showing no negative effects on temporal order recognition memory after 1 week of repeated restraint stress (Wei et al., 2014). Interestingly, the detrimental effects of such stress exposure were present in females when estrogen signaling was blocked or occluded in males when estrogen signaling was activated, suggesting that such potent endogenous hormones may significantly protect against the detrimental effects of a repeated stress on glutamatergic transmission in specific brain areas, thereby accounting for the stress resilience observed only in female rodents. Consistent with these findings, our results obtained in male RMS mice treated with EB at PND2 suggest that altering the normal estrogenic pattern in male mice during the first days of life may thus reduce the vulnerability to RMS-induced changes at the central level, as often observed in females (Bondar et al., 2018; Dombret et al., 2020; Wei et al., 2014). It should be noted, however, that because we focused on the effect of altering the physiological hormonal composition of the male sex on RMS responses, treatment with EB and relative vehicle was restricted to males only. We recognize that this may be a limitation in the interpretation of our results, as the comparison between the male and the female groups is confounded by the fact that females were not exposed to the EB treatment. Therefore, future experiments will be conducted to evaluate whether EB can also alter RMS responses in female mice.

In conclusion, our data support the idea that RMS induces long-lasting changes in the reward system, involving glutamatergic transmission and long-term synaptic plasticity and, ultimately, the behavioral propensity for EtOH consumption only in male mice.

The neurobiological mechanisms underlying these changes still require further multidisciplinary investigation to explore in depth the sex-specific sensitivity/resilience to early life stress. However, the reported results seem to support the idea that early life adversity is a triggering environmental factor for changes occurring in the developing brain that shape the function of specific neuronal circuits (and neurotransmitter systems) and may act as a switch in the transition from a "normal" to a "preaddicted" state.

CRediT authorship contribution statement

Giuseppe Talani: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesca Biggio:** Writing – review & editing, Data curation. **Maria Cristina Mostallino:** Writing – review & editing, Data curation. **Elisabetta Batzu:** Writing – review & editing, Data curation. **Giovanni Biggio:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Enrico Sanna:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

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