Sex-dependent changes of hippocampal synaptic plasticity and cognitive performance in C57BL/6J mice exposed to neonatal repeated maternal separation

Running title: Changes in hippocampal plasticity in mice exposed to maternal separation

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Summary

The repeated maternal separation (RMS) model in rodents is useful to study the long-term impact of early-life stress on brain neurophysiology. We here investigated the effects of RSM exposure on hippocampal inhibitory and excitatory synaptic transmission and plasticity as well as the related potential alterations in learning and memory performance in adult male and female C57Bl/6J mice.

Mice were separated daily from their dam for 360 min, from postnatal day 2 (PND2) to PND17. Electrophysiological and behavioral experiments were then conducted at PND 60. Patch-clamp recordings in hippocampal CA1 pyramidal neurons revealed a significant enhancement of GABAergic miniature IPSC (mIPSC) frequency, and a decrease in the

amplitude and decay of glutamatergic mEPSCs in male mice exposed to RMS. Only a slight but significant decrease in the amplitude of GABAergic mIPSCs was observed in females exposed to RMS compared to the relative controls. A marked increase in long-term depression (LTD) at CA3-CA1 glutamatergic synapses was detected in RMS male, but not female, mice. At behavioral level, an impaired spatial memory as well as reduced preference for novelty was observed in males only. Interestingly, a single injection of β-ethynyl estradiol at PND2, completely prevented the changes observed in RMS male mice, suggesting that estrogens may play a protective role early in life against the exposure to stressful conditions. Our findings strengthen the idea of a sex-dependent effect of RMS on long-lasting modifications in both excitatory and inhibitory synapses, effects that may be relevant for the altered synaptic plasticity and thus cognitive performance.

Introduction

Traumatic and stressful events occurring early in life in humans are considered as a predictive factor of enhanced vulnerability to develop brain diseases in adulthood (Palma-Gudiel et al., 2020; Hegde and Mitra, 2020; Sarkar et al., 2019; Liu et al., 2017; Andersen et al., 2015; Bramon et al., 2001; Ellenbroek et al., 1998; Walker and Diforio, 1997). Several animal models have been employed to evaluate the vast array of long-lasting pathophysiogical alterations that were described in adult rodents exposed to various stress paradigms in the early post-natal life (Murthy and Gould, 2018). Several changes, including impairments in memory formation and cognitive performances (Li et al., 2013; Reincke and Hanganu-Opatz, 2017; Hedges and Woon, 2011; Kosten et al., 2012), increased anxiety (Brunton, 2015), depression-like symptoms (Vetulani, 2013), vulnerability for drug abuse (de Almeida Magalhães et al., 2017; Delavari et al., 2016), as

well as epigenetic alterations (Silberman et al., 2016; Zhang & Meaney, 2010), have been described.

The maternal separation (MS) is one of the most widely used experimental models to study the alterations at the endocrine, neurophysiological, and behavioral level consequent to early-life stress exposure in rodents (Nishi et al., 2014; Levine, 2005; Gutman and Nemeroff, 2002). Protocols of MS may comprise a single episode in which pups are deprived of their mother's cares (Fabricius et al., 2008) or repeated daily separations (repeater maternal separation, RMS) during the first weeks of life, for example from postnatal day 1 (PND 1) to PND 21 (Cirulli et al., 2009; Hall, 1998; Marco et al., 2009) or from PND 3 to PND 15 (Plotsky & Meaney, 1993). In addition, the duration of each daily separation episode can also be variable, ranging from a few minutes to several hours with different outcomes and severity of the observed effects (Bailoo et al., 2014). Particularly relevant are the detrimental long-lasting effects of prolonged RMS on hippocampal neuronal morphology and function (Lai and Huang, 2011) as well as alterations in spatial memory in male rats (Sousa et al., 2014).

Recent evidence suggests that some of the RMS-induced impairments, including increased anxiety-like behavior and changes in hippocampal neuronal morphology, may depend by gender, hinting that certain outcomes related to sex, such as different hormonal pattern, could be predictive of vulnerability to early-life stress (Bondar et al., 2018). Accordingly, in some reports, male but not female mice have shown an increase in anxiety-like behavior after RMS (Mehta and Schmauss, 2011; Romeo et al., 2003; Kundakovic et al., 2013; Bailoo et al., 2014; Romeo et al., 2003) when compared with controls. Conversely, other studies report negative results (Veenema et al., 2007; Tsuda and Ogowa, 2012; Cui et al., 2020), suggesting, altogether, that the influence of sex as a factor of vulnerability to RMS needs further investigation.

Since the balance between excitatory and inhibitory synaptic transmission is essential to ensure proper synaptic information finely tune the neural activity and, conversely, excitatory/inhibitory imbalance contributes to numerous neuropsychiatric phenotypes, including anxiety and depression (Kim et al., 2020), in the present study we aimed to investigate the effects of RMS on the function of hippocampal CA1 inhibitory GABAergic and excitatory glutamatergic synapses. In addition, it is known that both acute and chronic stress may affect dramatically hippocampal synaptic plasticity (Sousa et al., 2014), impairing LTP and facilitating LTD formation and that such changes may correlate with memory impairments that to some extent may be influenced by sex (Luine et al., 2002). Thus, we studied the long-term hippocampal plasticity of glutamatergic synapses in both male and female mice, and, we have also explored whether the neurophysiological alterations produced by RMS correlate with parallel changes in cognitive responses in adult C57BL/6J mice. Furthermore, because changes in circulating estrogens were associated with altered dendritic spine density in various brain regions, including the hippocampus, in female rodents suggesting the crucial physiological role of estradiol in the organization and activation of neural structures (Sheppard et al., 2019), and considering that some report have shown a relative resilience against the depressive-like symptoms induced by RMS in female rats (Dimatelis et al., 2016), we also studied the potential outcome in male RMS mice treated with a single injection of β-ethinylestradiol (EB) which disrupts male sex differentiation (Nef and Parada, 2000; Toyama et al., 2001; Delbès et al., 2006;). Finally, since endogenous cannabinoids (eCB) are physiological modulators of synaptic transmission and are involved in long-term depression (LTD) in the hippocampus (Abush and Akirav 2010; Chevaleyre et al., 2007), we also evaluated whether eCB signaling was altered in adult mice that were exposed to RMS.

METHODS

Animals

C57BL/6J mice (Charles River, Como, Italy) were maintained under an artificial light-dark cycle (12 h on-12 h off), constant temperature of 22 ± 2°C, and humidity of 65%. Animal care and handling, throughout the whole experimental procedures and protocols, were in accordance with the guidelines for care and use of experimental animals of the European Communities Council (2010/63/UE L 276 20/10/2010) and the Italian law (DL: 04/03/2014, n° 26) as well as approved by the Organism for Animal Care and Wellness of the University of Cagliari (OPBA-UniCA), and performed in accordance with the Ministry of Health with authorization number 581/2016-PR. Furthermore, every effort was made to minimize suffering and reduce the numbers of animals used.

Repeated maternal separation

Animals were weighed at post-natal day 2 (PND2), PND18, and PND60, and litters were composed by a similar number of males and females (5–6 each sex). From PND 2 until PND 17, litters were separated from their dams for 6 h daily, from 9:00 a.m. to 3:00 p.m., with their handling performed by the same experimenter. The RMS protocol was in agreement with previews studies (Plotsky and Meaney et al., 1993; Lundberg et al., 2017), and consisted in removing the pups from their nest and placing them together (males and females) in a different room at a controlled temperature (30–32 °C) by adding cotton wool in order to create a comfortable nest. During this time the dams were left undisturbed in their home cage until reunion. Control pups (CTRL) were handled twice a day, moving them to one side of the cage to the other, but left with dams in the same cage. At PND 17, all pups were returned to normal housing until weaning (PND21), when males and females were separated definitely from the dams and randomly housed in groups of 5 per cage. At PND2, a group of male pups were injected subcutaneously (s.c.) with 25 µl of sesame oil (Sigma-Aldrich, Milan, Italy) containing 10 µg of β-estradiol 3-benzoate (EB; Sigma-

Aldrich, Milan, Italy), or with 25 µl of sesame oil only as vehicle in agreement with previews reports (Calza et al., 2010). In order to evaluate the effect of EB on primary sexual characters, testicles weight was measured in adults (PND 60) after sacrifice. In addition, fertility was tested in 10 males by mating them with 10 females and checking for pregnancy after 21 days.

Preparation of brain slices

Coronal brain slices containing the hippocampus were prepared from mice at PND 60 as previously described (Talani et al., 2016). Briefly, after reaching deep anesthesia with vapors of 5% isoflurane, animals were sacrificed and brains rapidly removed from the skull and transferred to a modified artificial cerebro-spinal fluid (aCSF) containing (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 (thickness of 250 or 300 µm) were cut using a vibratome (Leica, Germany) and then immediately transferred to a nylon net submerged for at least 40 min at a controlled temperature of 35°C in standard aCSF containing (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). After subsequent incubation for at least 1 h at room temperature, hemi-slices were transferred to a recording chamber with a constant flow rate of ~2 ml/min of aCSF at controlled temperature of 33°C.

Electrophysiology experiments

For patch-clamp recordings on hippocampal CA1 pyramidal neurons, GABAergic miniature inhibitory postsynaptic currents (mIPSCs), and GLUergic miniature and evoked excitatory postsynaptic currents (mEPSCs, eEPSCs) were recorded with an Axopatch 200B amplifier, filtered at 2 kHz, and digitized at 5 kHz. Resistance of the micropipettes

ranged from 4.5 to 6.0 MΩ when they were filled with an internal solution composed by (in mM): 140 CsCl, 2 MgCl, 2 CaCl, 10 EGTA, 10 HEPES, 2 ATP-Na, pH 7.3 with CsOH 5 N. Access resistance ranged from 15 to 30 MΩ and was monitored throughout the recordings by injection of 10 mV depolarizing pulses and if it changed during recording more than ±20%, the cell was automatically discarded from analysis. GABAAr-mediated mIPSCs were recorded in the presence of the non-selective glutamate receptor antagonist kynurenic acid (1 mM). AMPAr-mediated eEPSCs were recorded in the presence of the GABAAr antagonist bicuculline (20 μM) at a holding potential of −65 mV, whereas NMDAr-mediated responses were recorded at a holding potential of +40 mV in the presence of the AMPA/kainate receptor antagonist NBQX (5 μM). The AMPA/NMDA ratio was calculated for some experiments. For both AMPAr- and NMDAr-mediated glutamatergic components, the evoked currents were obtained using a constant current pulse of 0.2–0.4 mA with a duration of 60 μs, which yielded a half-maximal response, using a bipolar concentric electrode (FHC, ME, USA) and a stimulator (Digitimer Ltd, UK). Analysis of mIPSCs and mEPSCs was performed using Mini Analysis software (Synaptosoft, Inc., version 6.0.2) with a noise amplitude threshold of 2 pA, whereas analysis of eIPSC was conducted using Clampfit 10.7 software.

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed in the stratum radiatum of the CA1 hippocampal region through stimulation of the Schaffer collateral afferents as previously described (Sanna et al, 2011; Talani et al, 2011). For fEPSPs, responses were recorded by filling the recording electrode with KCl (3 M) and were triggered digitally every 20 s by application of a constant current pulse of 0.2–0.4 mA with a duration of 60 μs, which usually yielded a half-maximal response, using a bipolar concentric electrode (FHC, ME, USA) and a stimulator (Digitimer Ltd, UK). The paired-pulse protocol for evaluating the presynaptic effect of the activation of CB1rs by the perfusion of the selective agonist win55, 212 (5 μM), consisted of delivering two

consecutive electrical stimuli with an inter-event interval of 100 ms, and the ratio of the slope of the second to the first fEPSC was calculated. For induction of LTD, after 10 min of stable baseline, where fEPSPs were evoked every 20 s at the current stimulation intensity that triggered about the 50% of the maximal response, a low frequency stimulation (LFS, 500 stimuli at 1 Hz) was applied and its effect was evaluated for 60 min. The extent of LTD was calculated by averaging the slope of fEPSPs during the interval between 50 and 60 min after LFS and comparing this value vs. baseline.

Barnes maze

At PND 60, a set of animals were subjected to the test of Barnes maze in order to evaluate their spatial learning and memory performances that are related to hippocampal activity. Five mice of each experimental group were used and the test was conducted at 3:00 p.m. Animals were placed in the center of a circular platform with 40 equally spaced holes (platform: 122 cm of diameter; holes: 5 cm diameter; 5 cm between each hole). Only one of the holes was connected to a dark chamber called the “target box”. Animals were driven to find this box by being exposed to a bright light (200 W, 1400 lumen) fixed 50 cm up of the maze, using 4 different cue panels. Animals went through a spatial acquisition phase that lasted 4 days, consisting of a single trial of 3 min. The position of the target hole was rotated by 90° before each consecutive trial and the same cue was associated to the target hole. Short-term retention (probe) was evaluated on day 5. The time needed to identify (latency) and number of errors to find the target hole during each trial were recorded by a digital camera and analyzed by the same operator during the entire test.

Novel Object Recognition (NOR)

With the NOR test we obtained a rapid information on the impact produced by RMS on cognition in adult mice. NOR is a paradigm based on the natural tendency of rodents to

investigate a novel object compared to a familiar one; the more the exploration of the novel object vs. the old object is increased the more learning and recognition memory is enhanced. One day before the test each animal was placed in the Plexiglas arena (50x50x50 cm) in order to acclimate with the environmental condition. Soft light was used during each session. NOR test was performed using a different set of animals for all experimental groups. The day of the test, two identical objects (Obj1 and Obj2) were placed into the arena during a 5 min sample phase (familiarization). After 6 h each animal was returned to the arena and was exposed to the two objects with one of those that was exchanged by a new one with different shape and color (novel) for one single session of 3 min (test) (modified by Bolz et al., 2015). Objects were cleaned with ethanol 70% after every session. Memory was assessed by comparing the time spent exploring the novel object and the time spent exploring the familiar object. We calculate the recognition index as the percentage time spent to each object compared to the total time spent in objects exploration (Time Obj1 or 2/(Time Obj1+Obj2 x 100) in both familiar and test phase.

Statistical analysis

Data are presented as mean ± SEM and compared by one-way analysis of variance (ANOVA) and Bonferroni's test or Student's t test with the use of Prism software (version 7, Graphpad). A p value of < 0.05 was considered statistically significant.

RESULTS

Effects of RMS on body weight and male fertility

To verify whether RMS exposure might affect animal growth, we measured the bodyweight of both control mice and mice that were exposed to RMS, at the beginning and

at the end of the separation procedure (PND 2 and PND 17), as well as when the adult age was reached (PND 60). As shown in Tab. 1, at PND 2 the bodyweight was similar between groups, but after 16 days of RMS (PND 18), males but not females showed a significant decrease [$F_{(5, 24)} = 17.26$, $p < 0.0001$ vs CTRL] in body weight when compared with CTRL. Furthermore, the treatment with EB failed to affect body weight in either CTRL and RMS male mice [$F_{(5, 24)} = 17.26$, $p = 0.0029$ vs CTRL+EB]. The RMS-induced decrease in body weight observed in male mice at PND18 was longer detectable at PND 60 **(Tab. 1)**.

It is well known that treatment with supra-physiological doses of estrogens during the crucial neonatal differentiation results in male rodents in a series of impairments of the reproductive system, which include atrophy of testes as well as sexual accessory glands (Tena-Sempere et al., 2000; Toyama et al., 2001; Delbès et al., 2006; Nef and Parada, 2015). We have thus evaluated whether, in our experimental conditions, treatment with EB might have affected testes development and fertility. EB treatment caused a significant ($p = 0.013$) decrease in testes weight compared to that of males treated with vehicle only, while exposure to RMS alone failed to affect this parameter **(Tab. 2)**, a result that is in agreement with other findings showing the negative impact of EB treatment on male sex differentiation (Toyama et al., 2001; Delbès et al., 2006; Nef and Parada, 2015). Ten male mice were single breaded with one female and after 21 days, the occurrence of pregnancy was verified. As shown in Tab. 2, only 3 out of 10 CTRL mice ($p = 0.013$ vs CTRL + Veh) and 2 out of 10 of RMS mice ($p = 0.013$ vs RMS + Veh) treated with EB were fertile.

Effects of RMS on synaptic transmission in CA1 hippocampal pyramidal neurons

We next evaluated whether neonatal RMS treatment might affect GABAergic and glutamatergic transmission in hippocampal CA1 pyramidal cells of C57BL/6J mice.

Neurons were voltage-clamped (− 65 mV) and miniature inhibitory and excitatory postsynaptic currents (mIPSCs and mEPSCs, respectively) were recorded separately. Under these recording conditions, activation of GABAAr or AMPAr generates inward currents that reflect an outflow of Cl^- and a net inflow of Na^+, respectively.

Analysis of the GABAergic current kinetic properties revealed that there was no significant difference in the amplitude, rise or decay-time of mIPSCs in male mice exposed to RMS. However, a significant increase in mIPSC frequency was observed in RMS male mice compared with CTRL [$F_{(5, 36)} = 3.7$, $p = 0.019$] (Fig. 1a, e). Interestingly, this effect was no longer detectable in RMS males treated with EB before the start of RMS **(Fig. 1a, e)**. Furthermore, we observed a decrease in event amplitude in RMS females with no changes in the other kinetic parameters [$F_{(5, 36)} = 3.94$, $p = 0.027$] **(Fig. 1a, b)**.

Analysis of glutamatergic mEPSCs revealed only a significant decrease of event amplitude in RMS males with no apparent alteration in the other parameters when measured in all experimental groups of both sex [$F_{(5, 37)} = 7.9$, $p = 0.024$] (Fig. 1A, B) (Fig. 1c, d). Interestingly, the treatment with EB completely abolished the effect of RMS on mEPSC amplitude observed in male mice **(Fig. 1c, d)**.

RMS increases LTD at hippocampal CA1-CA3 excitatory synapses

Changes induced by RMS on GABAergic and glutamatergic transmission at CA1 inhibitory and excitatory synapses respectively, may be predictive of a possible impact on long-term plasticity of glutamatergic synapses in the hippocampus. While the effect of RMS on hippocampal synaptic plasticity has been explored more in details particularly with respect to LTP (Sousa et al., 2014), relatively less is known about to the effect of RMS on LTD. To evaluate further this latter form of synaptic plasticity, we recorded extracellularly

dendritic fEPSPs in the hippocampal CA1 subregion before and after the delivery of a low frequency stimulation (LFS) to the Schaffer's collateral glutamatergic afferents, to elicit LTD. In male mice, LTD, quantified by comparing the averaged slope value of fEPSPs recorded during the last 10 min (i.e., from 67 to 77 min post LFS), as a percent of baseline, was apparent in all experimental groups. As expected, LFS-induced LTD was completely prevented when the NMDAr antagonist AP5 (50 μM) was added in the recording extracellular solution **(Fig. S1)** suggesting a crucial involvement of NMDA receptors. However, in RMS male mice LTD was more pronounced (-50 ± 5.57%) when compared to that in CTRL mice (-22 ± 2.85 %) [$F_{(3, 28)}$ = 10.21, p = 0.0001] **(Fig. 2a, b)**. Furthermore, LTD measured in RMS males treated with EB was indistinguishable from that obtained in CTRL and CTRL+EB group, suggesting that treatment with EB was capable of selectively preventing the increase on LTD associated with exposure to RMS animals with no significant effect on CTRL animals **(Fig. 2a, b)**. In CTRL female mice, LFS produced a decrease (-22 ± 4.8) in fEPSP slope value, similar to that observed in males. Interestingly, when LFS was applied to hippocampal slices obtained from female mice that were exposed to RMS, not only LTD was suppressed but we could detect a slight but significant (p = 0.0002) increase in fEPSP slope values compared to baseline **(Fig. 2c, d).**

RMS alters the AMPA/NMDA ratio and endocannabinoid signaling in hippocampal CA1 neurons

The contrasting effects of RMS on LTD at hippocampal CA1 glutamatergic synapses observed in male and female mice prompted us to investigate in more details the influence of RMS on the function of AMPAr and NMDAr in these pyramidal neurons. Evoked EPSCs (eEPSCs) mediated selectively by AMPAr or NMDAr were recorded in single CA1 pyramidal neurons at resting membrane potential of -65 and +40 mV,

respectively. For isolating NMDAr-mediated eEPSCs, the AMPA selective antagonist NBQX (5 μM) was added to the recording solution, and the AMPA/NMDA ratio was calculated. RMS caused a significant increase of AMPA/NMDA ratio compared to CTRL male mice [$F_{(3,26)}$ = 10.93, p = 0.0001 vs CTRL] **(2e, f)**. The treatment with EB treatment was ineffective in modifying this parameter in CTRL animals, while it completely abolished the effect of RMS **(2e, f)**. Interestingly, RMS failed to affect the AMPA/NMDA ratio in females when compared with CTRL animals **(Fig. 2e, g)**. For a more detailed comparison, we reported the absolute values of AMPA and NMDA currents used to obtain the graphs in panels “f” and “g” for both males and females, respectively; ~~one should consider that,~~ for each recording, a constant current pulse of 0.2–0.4 mA with a duration of 60 μs was used to yield a half-maximal response. This comparison highlights a significant decrease of current amplitude of both AMPA [$F_{(7,48)}$ = 14.1, p = 0.0006] and NMDA [$F_{(7,48)}$ = 14.1, p = 0.02] in RMS male mice **(Fig. 2h)** ~~without any~~ with no significant effect observed in females **(Fig. 2i)**.

The decrease in the NMDAr component detected in CA1 pyramidal of male mice exposed to RMS might have not been predicted based on the higher LTD levels revealed in these mice. In order to explore further these findings, we studied the potential role of another possible mechanism that may contribute to the regulation of LTD formation in the hippocampus, namely the endocannabinoid system (Xu et al. 2010). In agreement with this idea, when LFS was applied in the presence of the CB1r antagonist SR141716 (1 μM) the amount of LTD measured in hippocampal slices obtained from CTRL mice was markedly reduced **(Fig. S1)**, suggesting that the CB1r activity is required for the full LTD formation, indicating that mechanisms related to this form of plasticity at the CA3-CA1 synapses may involve both NMDA or CB1r activation. To explore the possible involvement of this system in the synaptic effects of RMS, we measured the action of the CB1r selective agonist win55,212 on glutamatergic fEPSPs recorded in CA1 pyramidal neurons

by applying the paired-pulse protocol and calculating the paired-pulse ratio (PP ratio). Consistent with the predominant presynaptic location of CB1rs (Johnson and Lovinger, 2016), 15 min bath-perfusion of win55,212 (5 μM) caused a significant increase of the PP ratio in all experimental groups, confirming that stimulation of CB1r decreases the probability of presynaptic release of glutamate [$F_{(5,27)}$ = 5.8, p = 0.0035] **(Fig. 3a-d)**. However, when win55,212 was perfused in slices of RMS animals, the increase of PP ratio was significantly greater than that in CTRL mice, suggesting an increased function and/or expression of CB1r **(Fig. 3a- d)**. As shown in Fig. 3b and d, the treatment with EB completely abolished the enhanced effect of win55,212 observed in RMS mice without modifying the PP ratio in CTRL animals. Finally, this parameter did not change when measured in female mice that were exposed to RMS compared to the relative control female mice **(Fig. 3c, d)**.

Effects of RMS on spatial learning and memory

The changes in LTD formation detected in RMS mice led us to explore whether the altered hippocampal long-term glutamatergic synaptic plasticity could be correlated with parallel changes at the behavioral level in both sexes. We thus measured spatial learning in the Barnes maze test. In fact, this test has been used to examines spatial learning and memory exploiting the fact that rodents avoid bright illuminated areas, and instead favor dark confined spaces. Animals from all experimental groups were trained once a day for four consecutive days during which the time needed for, and the errors made before, hitting the target hole decreased significantly in both males and females [males, $F_{(5, 80)}$ = 92.9, p = 0.0001; females $F_{(5, 48)}$ = 16.78, p = 0.0001] **(Fig. 4a-d)**. Animals were then tested on day 5 for their short-term memory. The comparison between groups indicates that the performance of male mice exposed to RMS was significantly altered with respect

to CTRL animals. Two-way ANOVA revealed that RMS male mice had a higher latency [$F_{(3, 80)}$ = 9.80, p = 0.001] and committed more errors than CTRL at day 5 [$F_{(3,16)}$ = 12.6, p = 0.0001] **(Fig. 4a, b)**. However, the treatment with EB antagonized the effect of RMS in male mice with numbers of errors and latency resulting substantially similar to those observed in CTRL **(Fig. 4a, b)**. RMS failed to alter the latency and the number of errors in female mice when compared with CTRL animals **(Fig. 4c, d)**.

RMS alters learning for novelty in male mice.

In this set of behavioral experiments, we used the novel object recognition test (NOR), a paradigm that has been useful to demonstrate the specific contribution of the rodent hippocampus on object memory and preference for novelty (Antunes and Biala, 2012). During familiarization, animals were exposed to two identical objects. As expected, we found no differences in both male and female mice, in terms of the recognition index **(Fig. 4e, g)**, which mirrored the identical interest of the animal for both objects placed in the arena. However, when one of the two objects was replaced with a novel one (test phase), the preference for the novel object was higher in almost all experimental group of both sexes as revealed by the percentage of recognition index, indicating a marked preference for the novel object **(Fig. 4f, h)**. Interestingly, when RMS male mice were exposed to NOR test during the test phase they revealed an impairment in the recognition index, resulting in a high preference for the familiar object relative to the novel one [$F_{(3, 16)}$ = 17.19, p = 0.0001] **(Fig. 4f)**. Such effect is not present in RMS animals treated with EB while EB treatment per se did not alter the recognition index for novelty in CTRL mice **(Fig. 4f)**. Furthermore, RMS did not cause such impairments for novelty in female mice rather a trend of improvement has seen in the preference of the novel object **(Fig. 4h)**.

Discussion

In the present work, we provide new evidence for a sex-dependent responsiveness of adult C57BL/6J mice that were exposed to ~~a~~ repeated maternal separation during the first two weeks of postnatal life. Male and female mice showed marked difference in the neurophysiological changes in hippocampal inhibitory and excitatory synaptic transmission as well as long-term plasticity at excitatory synapses. These effects were associated, in males only, to alterations of spatial learning and propensity for novelty, which involve the activity of the hippocampal formation (Lisman et al., 2018; Antunes and Biala, 2012), and could be prevented by a single injection of EB.

The neurochemical and behavioral effects produced by RMS appear to be a consequence of a reduced maternal caregiving (Macrí et al., 2004; Biggio et al., 2014), and different studies have demonstrated that certain effects, produced as a consequence of RMS exposure, such as impairment of dopamine transmission and hippocampal neurogenesis as well as increased ethanol intake in adults, are dependent on sex, with males resulting more vulnerable than females (de Souza et al., 2018; Loi et al., 2014; Roman and Nylander, 2005). In contrast, a very recent study failed to observe sex difference in Sprague-Dawley rats that were separated daily from their mother for 6 hours (3 hours + 3 hours in two separate rounds daily) from PND1 to PND21; the depressive-like behavior and impairment in synaptic plasticity were similar in male and female rats (Cui et al., 2020). Such differential outcomes produced by maternal separation observed in these studies, compared to our present data, may depend by the separation paradigm employed, time points examined, and animal species used.

Similar to other reports (Maghami et al., 2018), we found at completion of RMS, that male, but not female, presented a significant decrease in body weight, but this effect was no longer evident in adult animals in all experimental groups. Other authors, however, found that repeated maternal separation for 3 h daily produced no change in body weight

(Zimmerberg and Sageser, 2011) suggesting, once again, that differences in the maternal separation protocol can be critical in determining various outcomes.

Our patch-clamp data demonstrate that RMS in male mice is associated with an increased probability of GABA release from presynaptic terminals impinging on CA1 pyramidal neurons, with a parallel decrease in the amplitude of glutamatergic EPSCs recorded in the same pyramidal neurons. These results suggest a change of the excitatory/inhibitory balance which may be predictive of a decrease activity in this neuronal circuit. The effect of RMS was ~~slightly~~ different in females, with an apparent change at post-synaptic site indexed by a decrease in GABAergic mIPSC amplitude, but no change ~~was observed~~ in glutamatergic transmission was observed. These data indicate that, in our experimental conditions, neonatal exposure of mice to RMS produces long-lasting alterations of inhibitory/excitatory signaling that are different between males and females,and suggest that this may have a differential impact on on long-term synaptic plasticity. In fact, the magnitude of LTD was significantly enhanced in RMS male, but not female, mice, a result that is consistent with previous reports (Xu et al., 1998; Kim and Diamond, 2002; Sousa et al., 2014; Richter-Levin and Xu, 2018). The greater extent of LTD observed in RMS male mice can be then consequent to the decreased activity in this neuronal circuit. Furthermore, these findings are also in agreement with those showing the involvement of the inhibitory transmission in long-term synaptic depression in hippocampal CA3-CA1 synapses in rats (Steele and Mauk, 1999). In fact, this study demonstrated that either application of the GABAAr agonist muscimol or increasing endogenous recurrent GABAergic input facilitates the expression of LTD, an effect that is consistent with the increase of GABA release observed in our RMS male mice with the consequent increase in the LTD extent. In addition to these effects, we also detected a decrease in both AMPAr- and NMDAr-mediated postsynaptic currents in CA1 pyramidal neurons consequent to RMS in male mice with a marked increase of the AMPA/NMDA ratio.

The neurochemical and neurophysiological effects of RMS on inhibitory and excitatory signaling in the hippocampal CA1 region could also be related to or may involve an increase of the pruning mechanism; in fact, a similar activity-dependent reduction in synaptic strength may occur in the developing brain, representing an essential step in synaptic pruning and postnatal development of neural circuits dependent by NMDAr activation (Henson et al., 2017). Furthermore, RMS may affect also the expression of several neurotrophic factors, such as the brain derived neurotrophic factor (BDNF), that are involved in both neuronal trophism and plasticity. In fact, different studies have reported that RMS affects BDNF expression in the hippocampus of adult and aged rodents (Daskalakis et al. 2015; MacQueen et al. 2003; Aisa et al. 2019; Solas et al. 2010; Biggio et al., 2014). On the other hand, the LTD levels measured in female mice that were exposed to RMS showed a slight but significant increase in fEPSP slope above baseline, indicative of a long-term potentiation. Since we found no alterations of the glutamatergic transmission in females, but we observed a significant reduction in the amplitude of the mIPSCs, the change of the inhibitory transmission at the level of the CA1 synapses may be crucial, although other mechanisms may certainly be involved, for the such modification of synaptic plasticity as reported by others (Nishiyama et al. 2010). However, it is clear that other experiments will be needed to further characterize the effect of RMS on synaptic plasticity in females. Nonetheless, these data are in line with a recent report demonstrating that RMS causes critical modifications of the glutamatergic system in a sex-specific manner in different brain areas (Ganguly et al., 2019). These authors showed that RMS affects in a sex-dependent manner the glutamatergic system with a cortical and striatal AMPAR impairments, an effect that involves systemic inflammation with the expression of cytokines, the tumor necrosis factor (TNF) signaling and GluA2 expression (Ganguly et al., 2019).

To further explore the molecular determinants involved in the modification of long-term plasticity at glutamatergic synapses induced by RMS we focused our attention on the endocannabinoid system that, in the hippocampus, is involved in LDT formation (Araque et al., 2017). This evidence has been confirmed by the marked decrease of LTD formation in control animals when the CB1r antagonist SR141716 (1 μM) was perfused during the application of LFS. RMS produced a significant change in the modulatory action of the CB1r agonist win55,212 on excitatory synapses at CA1 pyramidal neurons, suggesting an involvement of the eCB system. This alteration was observed only in male, but not in female, mice and consisted of a stronger CB1r-mediated decrease of glutamate release from presynaptic terminals. This finding appears in contrast to the study by Hill et al. (2019) who found that, in adult male rats exposed to RMS, the CB1r density across all brain regions is downregulated. Moreover, by exposing rats to a single maternal separation for 24 h at PND9, Suàrez et al. (2009) showed a dysregulation of CB1rs in adult males but not in females. To date, there is only a limited number of published studies on sex-dependence of the effects of early-life stress on the eCB system. This is particularly relevant given that sex differences in eCB signaling have been widely established (Hill et al., 2019; Suàrez et al., 2009).

Consistent with the data on hippocampal synaptic signaling and plasticity, our behavioral studies indicate that RMS is associated in male mice with a significant decrease in the cognitive performance. In fact, RMS impaired completely the preference for novelty in males as shown in the NOR and reduced spatial learning in the Barnes maze test, while we found no significant alterations in female mice in both behavioral tests. In line with our data, a wide number of experimental and clinical evidences confirm that early-life stress, including the maternal separation, might exert deleterious effects on brain structure and function later in life (Reincke and Hanganu-Opatz, 2017; Hedges and Woon, 2011; Kosten et al., 2012; Levine, 2005; Gutman and Nemeroff, 2002). Moreover, whereas

our data demonstrated that RMS failed to affect the performance in the spatial memory test of female mice, other reports demonstrated that early-life stress leads to cognitive impairments, reduced numbers of CA3 neurons and altered maternal behavior in adult female mice (Reshetnikov et al., 2020). Furthermore, some evidence showed that early-life stress events may support the “mismatch hypothesis” that consists in the idea that early aversive experiences may predict some adaptive processes that make the subject more resilient and able to better tolerate uncomfortable events occurring in adulthood (Schmidt, 2011; Nederhof and Schmidt, 2012, Scharf and Schmidt, 2012; Biggio et al., 2014). In agreement with these findings, it is also worthwhile mentioning that female rats were shown to be resilient to the development of depressive-like symptoms following exposure to RMS (Dimatelis et al., 2016).

Sex hormones, particularly estrogens, play a fundamental role in sexual differentiation in the early postnatal life, and estrogens have long been considered to be female hormones, but are also crucial for the normal development of the male reproductive system (Weinbauer and Nieschlag, 1995). Dombret et al. (2010) demonstrated that male mice lacking the estrogen gene exhibited reduced social interaction and impaired aggressive behavior, effects that were paralleled by an increased locomotor activity, and reduced or unaffected anxiety-state level. The marked changes induced by RMS on GABA/glutamate balance as well as synaptic plasticity and behavior aspects are prevented remarkably in male animals exposed EB treatment, suggesting that altering the normal hormonal pattern in male mice during the first days of life, may affect the vulnerability to RMS-induced alterations at central level, an effect that has been reported in some studies for 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 other (Veenema et al., 2007; Tsuda and Ogowa, 2012). Furthermore, the treatment of male pups with a single injection of β-ethinyl estradiol (EB) on PND2 before animals were

exposed to RMS failed to alter the decrease of body weight observed in RMS male mice, suggesting that the mechanism associated to this effect does not involve this hormonal aspect and raises the question that the nature of such effect is not limited to the role of estrogens. On the other hand, it is known that the hormone testosterone can be reduced into 5α-dihydrotestosterone (DHT) or aromatized to generate estrogens. DHT and estrogens have opposite effects, while androgens virilize, estrogens feminize (Nef and Parada, 2015).

Overall, our data strengthen the idea that RMS produces long-lasting modifications at both excitatory and inhibitory synaptic signaling and long-term plasticity in a manner dependent on sex. These effects are associated with altered cognitive performance in adulthood found only in male but not female mice. These results, herein add to previous evidence supporting a sex-dependent sensitivity to stress associated to RMS, confirm that hormones such as estrogens, may affect the outcome of stress occurred during early life, although the neurobiological mechanisms behind these changes need still further investigation, but may justify the trend in resilience for early life stress showed by females. These findings may have promising clinical implications and emphasizes the need for comparative studies directed to elucidate the effect of early life stress in both sexes.

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Credit authorship contribution statement

GT wrote the manuscript, designed and supervise all experiments; **FB** supervised behavioral experiments and helps to revise the final version of the manuscript**; ES, DC,**

GS, MP, FV, AAG, performed part of behavioral and electrophysiological experiments; **ES** and **GB** designed the experiments and contributed to the final revision of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon needed request.

Declaration of competing interest

None.

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Table and figure legends

Table 1. Change of body weight in mice exposed to RMS. values representing the change of body weight in C57BL/6J mice of both sexes, detected at PND 2, 18 and 60. One-way ANOVA, *$p < 0.05$ vs respective CTRLs, n = 5.

Table 2. Change in fertility and testicles weight in male mice exposed to RMS. Fertility was evaluated mating one male and one female for 4 consecutive days and waiting 21 days in order to have a pregnant female that match with a fertile male. One-way ANOVA, *$p < 0.001$ vs non EB treated, n = 10. After sacrifice, both testicles of males were weighted (weight is relative to both testicles). One-way ANOVA, *$p < 0.05$ vs non EB treated, n = 8

Figure 1. Effect of RMS on GABAergic and GLUergic transmission in CA1 hippocampal neurons. (a, f) Representative traces of miniature IPSCs (a) and mEPSC (f) recorded from single voltage-clamped CA1 pyramidal neuron of the different experimental groups. Scale bar 10 pA/1s. Averaged mIPSCs **(b-e)** and eEPSC **(g-j)** recorded during a period of 3 min from which analysis of the kinetic properties and event frequency has been conducted. The bar graphs summarize changes in mIPSC or mEPCS amplitude (indicated as absolute value), rise and decay time constants, and frequency, and are expressed as the mean of absolute values ± SEM. One-way ANOVA, $*p < 0.05$, n = 5-15 neurons (for GABAergic currents) and n = 5-12 (for glutamatergic currents).

Figure 2. RMS alters the LTD formation and AMPA/NMDA components in hippocampal CA1-CA3 excitatory synapses. a, c) Scatter plot representing the percentage of change in fEPSP slope values induced by LFS with respect to baseline in male mice of the different experimental groups. In the insert are shown the representative traces of field EPSPs that were recorded in the dendritic portion of CA1 neurons in slices obtained from the different groups of males (a) or females (b). Trace are recorded before (black) and after (red) LTD conditioning (LFS, 500 stimuli at 1 Hz). **b, d)** The graph summarizes the degree of LTD, calculated by averaging the percentage change in fEPSP slope from baseline 50–60 min after LFS obtained from graph a and b. Data are expressed as mean percent change of fEPSP slope ± SEM from baseline. One-way ANOVA, $*p < 0.05$ vs CTRL, $^{\#}p < 0.05$ vs baseline, n = 6-12 for males and n = 11 for females. **e)** Representative traces of evoked glutamatergic EPSCs recorded in the presence of the GABAergic antagonist bicuculline (20 μM) in CA1 neurons clamped at -65 mV (lower trace) for AMPA current, and at +40 mV (upper trace) for NMDA currents in the presence of the AMPA antagonist NBQX (5 μM) in slices obtained from the different groups of both sexes. Currents were evoked using

a constant current pulse of 0.2–0.4 mA with a duration of 60 μs, which yielded a half-maximal response, using a bipolar concentric electrode (FHC, ME, USA) and a stimulator (Digitimer Ltd, UK) (**f, g)** The graph summarize the AMPA/NMDA ratio obtained from CA1 neurons of the different experimental groups of both males (f) and females (g). Data are expressed as mean ± SEM of obtained ratios for every single recording. One-way ANOVA, **p* < 0.001 vs CTRL, n = 5-12. **(h, i)** Bar graphs representing the averaged absolute values of AMPA (black bar) and NMDA (red bars) currents, used to obtain ratios in graphs f and g for males and females, respectively. Data are expressed as mean ± SEM. One-way ANOVA, **p* < 0.001 vs CTRL, n = 5-12.

Figure 3. RMS alters endocannabinoid signaling in CA1 hippocampal neurons. (a) Representative traces of evoked glutamatergic fEPSPs that were recorded with a protocol of paired pulse stimulation (black arrows indicate both stimuli with inter-event stimulus of 100 ms) in the absecnce (black trace) and presence (red trace) of the CB1r agonist win55, 212 (5 μM) perfused for 15 min. Paired pulse ratio was calculated as the ratio between the slope of the second response and the slope of the first. **(b, c)** Scatter plot represents the effect of 15 min of win perfusion in the change of fEPSP ratio in both males (b) and females (c). **(d)** The bar graph summarizes the effect of win on fEPSP ratio averaged during the latest 3 min of drug perfusion in both males (blue bars) and females (pink bars). Data are expressed as mean ± SEM of obtained ratios for every single recording. One-way ANOVA, **p* < 0.001 vs CTRL, $^{\#}p$ < 0.05 vs baseline, n = 5-7.

Figure 4. Effect of RMS on spatial memory and propensity for novelty in mice. (a, b) Scatter plots representing the change in latency needed (a) and errors made (b) before find the target hole in the Barnes maze test by males **(a, b)** and females **(c, d)** during days of training, and in the test (Probe, day 5). Data are expressed as mean of values for single

animals ± SEM. Two-way ANOVA, *p < 0.001 vs CTRL, n = 6. **(e, g)** The bar graphs summarize the recognition index for the two identical objects during the familiarization phase by all experimental groups. The recognition index was calculated as the percentage of the time dedicated to a single object compared to the total time of objects exploration. **(f, h)** The bar graphs summarize the recognition index during the test when one of the two familiar object was replaced with a non–familiar one. Data are expressed by mean ± SEM. One-way ANOVA, *p < 0.001 vs CTRL, n = 6.

Figure S1. LTD at CA3-CA1 excitatory synapses is reduced in the presence of NMDA or CB1 receptors antagonists. Scatter plot representing the percentage of change in fEPSP slope values induced by LFS with respect to baseline in male mice in the absence or presence of AP5 and SR 141716, an antagonist of NMDA and CB1rs respectively. (LFS, 500 stimuli at 1 Hz).

Figure 1

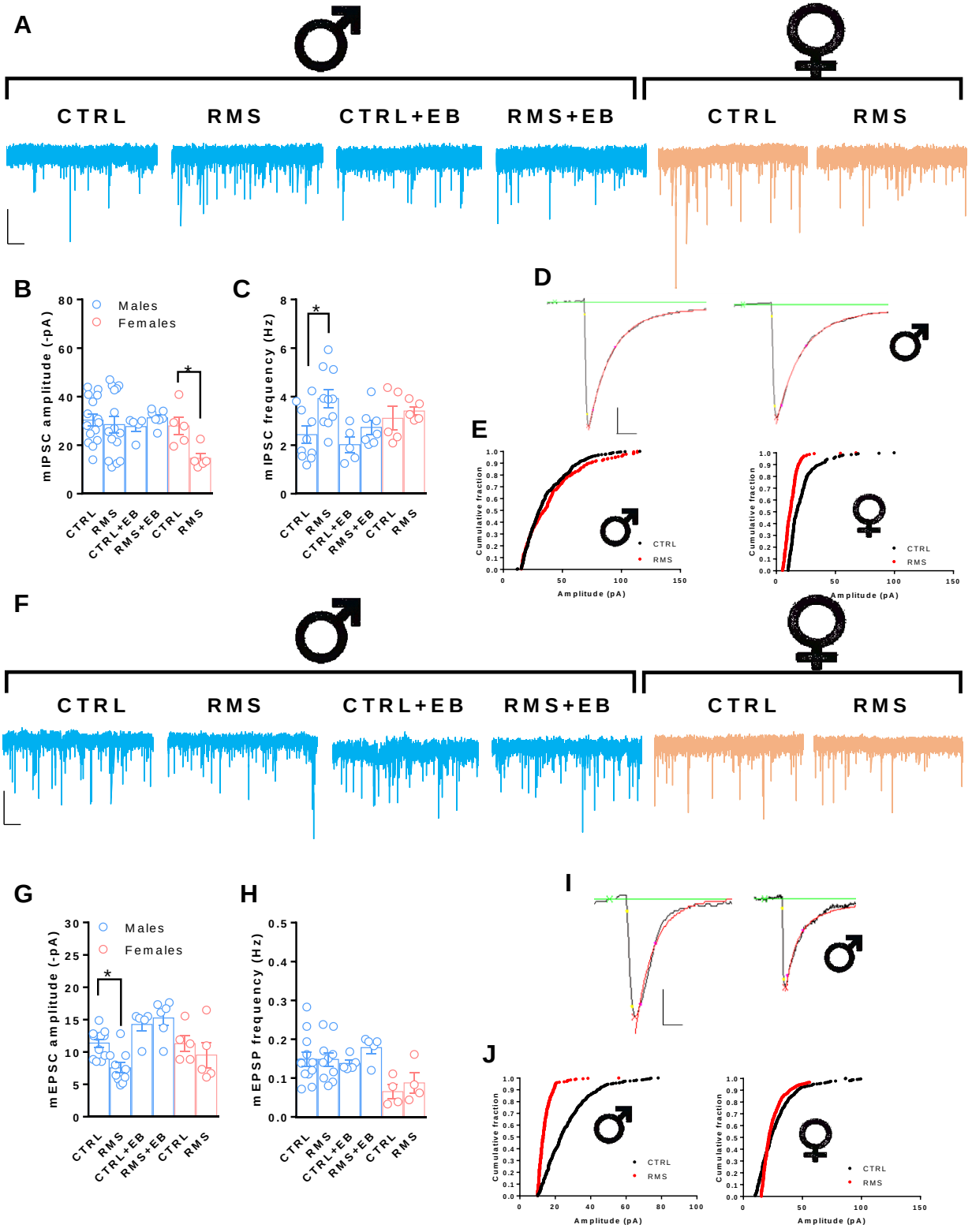

A
CTRL
RMS
CTRL+EB
RMS+EB
CTRL
RMS
B
Males
Females
mIPSC amplitude (-pA)
C
mIPSC frequency (Hz)
D
E
Cumulative fraction
Amplitude (pA)
CTRL
RMS
F
CTRL
RMS
CTRL+EB
RMS+EB
CTRL
RMS
G
Males
Females
mEPSC amplitude (-pA)
H
mEPSP frequency (Hz)
I
J
Cumulative fraction
Amplitude (pA)
CTRL
RMS

Figure 2

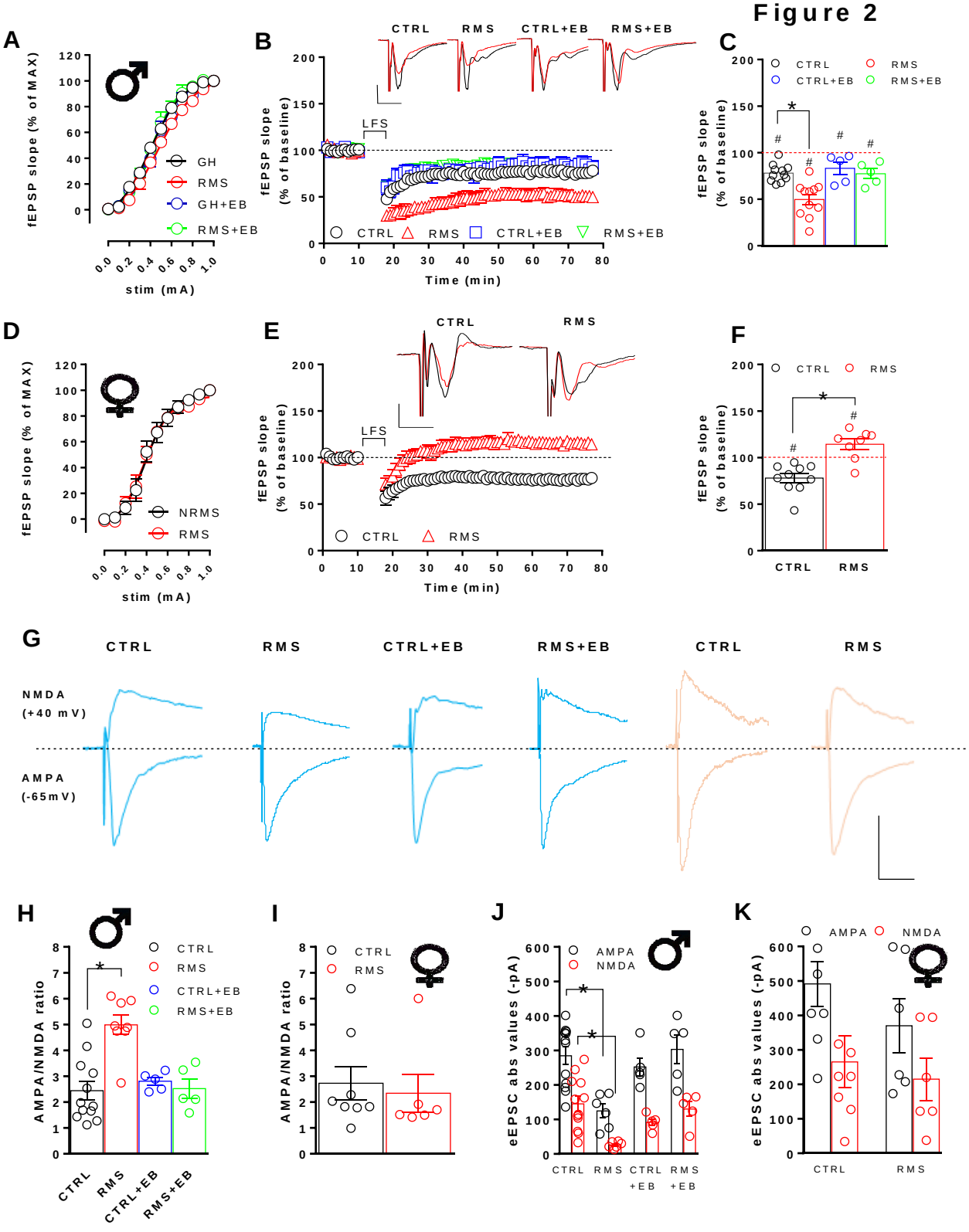

A
fEPSP slope (% of MAX)
GH
RMS
GH+EB
RMS+EB
stim (mA)
B
CTRL
RMS
CTRL+EB
RMS+EB
fEPSP slope (% of baseline)
LFS
Time (min)
C
fEPSP slope (% of baseline)
D
fEPSP slope (% of MAX)
NRMS
RMS
stim (mA)
E
CTRL
RMS
fEPSP slope (% of baseline)
LFS
Time (min)
F
fEPSP slope (% of baseline)
G
CTRL
RMS
CTRL+EB
RMS+EB
CTRL
RMS
NMDA (+40 mV)
AMPA (-65mV)
H
AMPA/NMDA ratio
I
AMPA/NMDA ratio
J
eEPSC abs values (-pA)
AMPA
NMDA
K
eEPSC abs values (-pA)
AMPA
NMDA

Figure 3

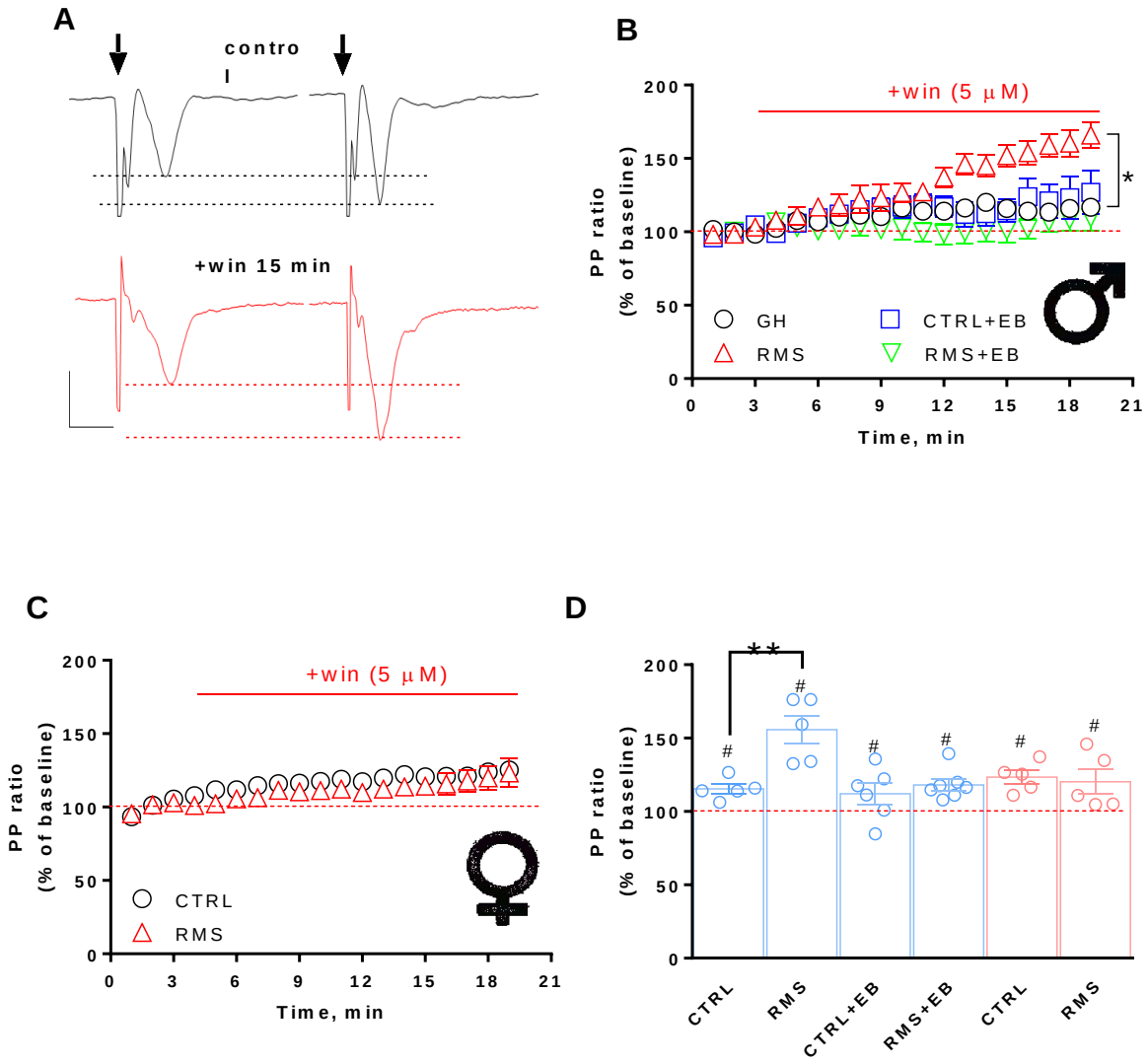
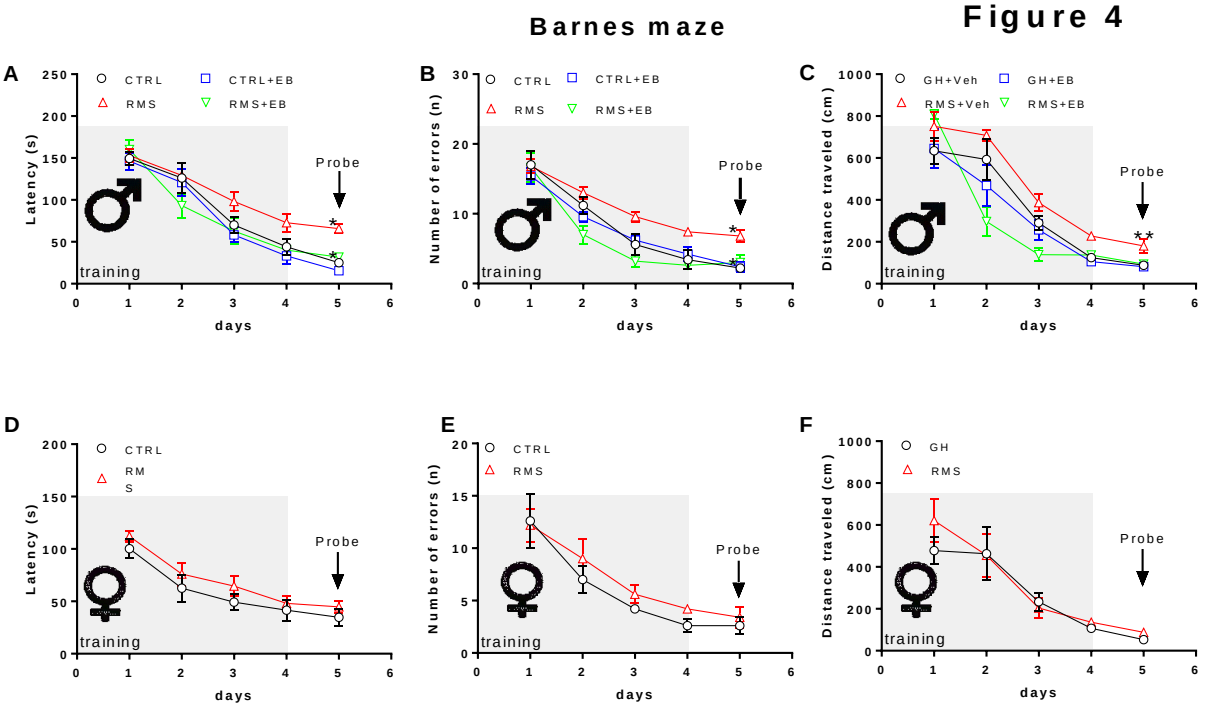

A
control
+win 15 min
B
+win (5 μM)
PP ratio
(% of baseline)
GH
RMS
CTRL+EB
RMS+EB
Time, min
C
+win (5 μM)
CTRL
RMS
D
**
#
CTRL
RMS
CTRL+EB
RMS+EB
CTRL
RMS

Figure 4


Barnes maze
A
CTRL
CTRL+EB
RMS
RMS+EB
Latency (s)
Probe
training
days
B
Number of errors (n)
C
GH+Veh
GH+EB
RMS+Veh
RMS+EB
Distance traveled (cm)
D
E
F
GH

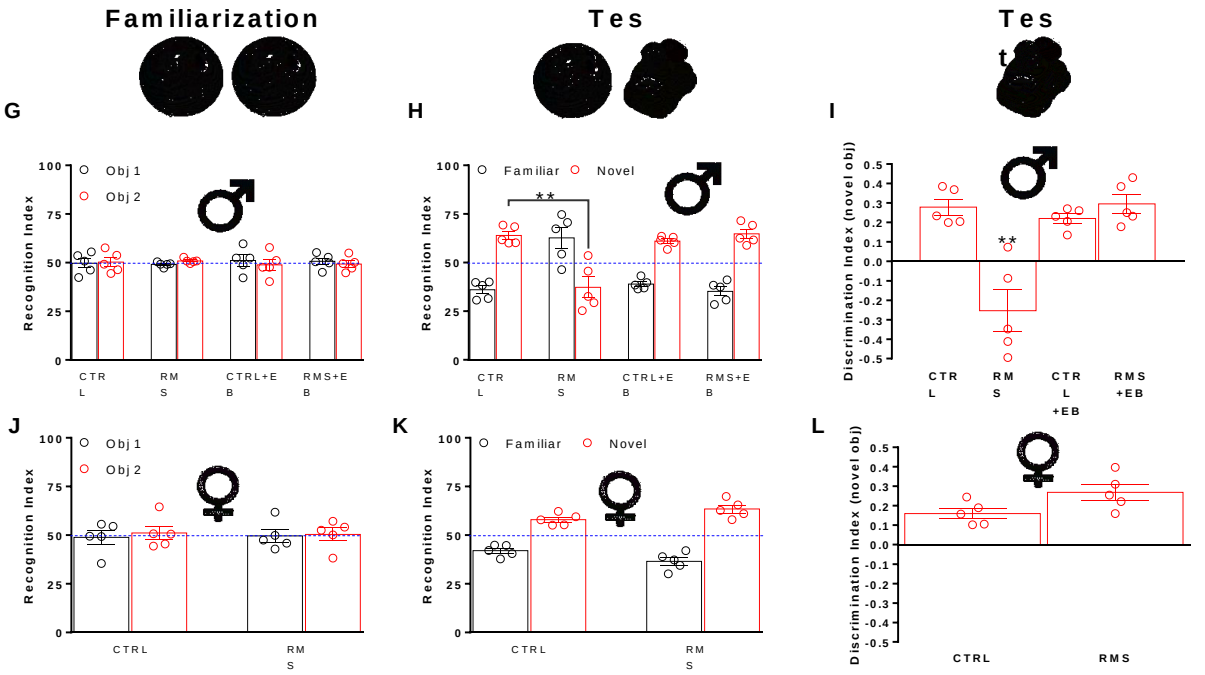
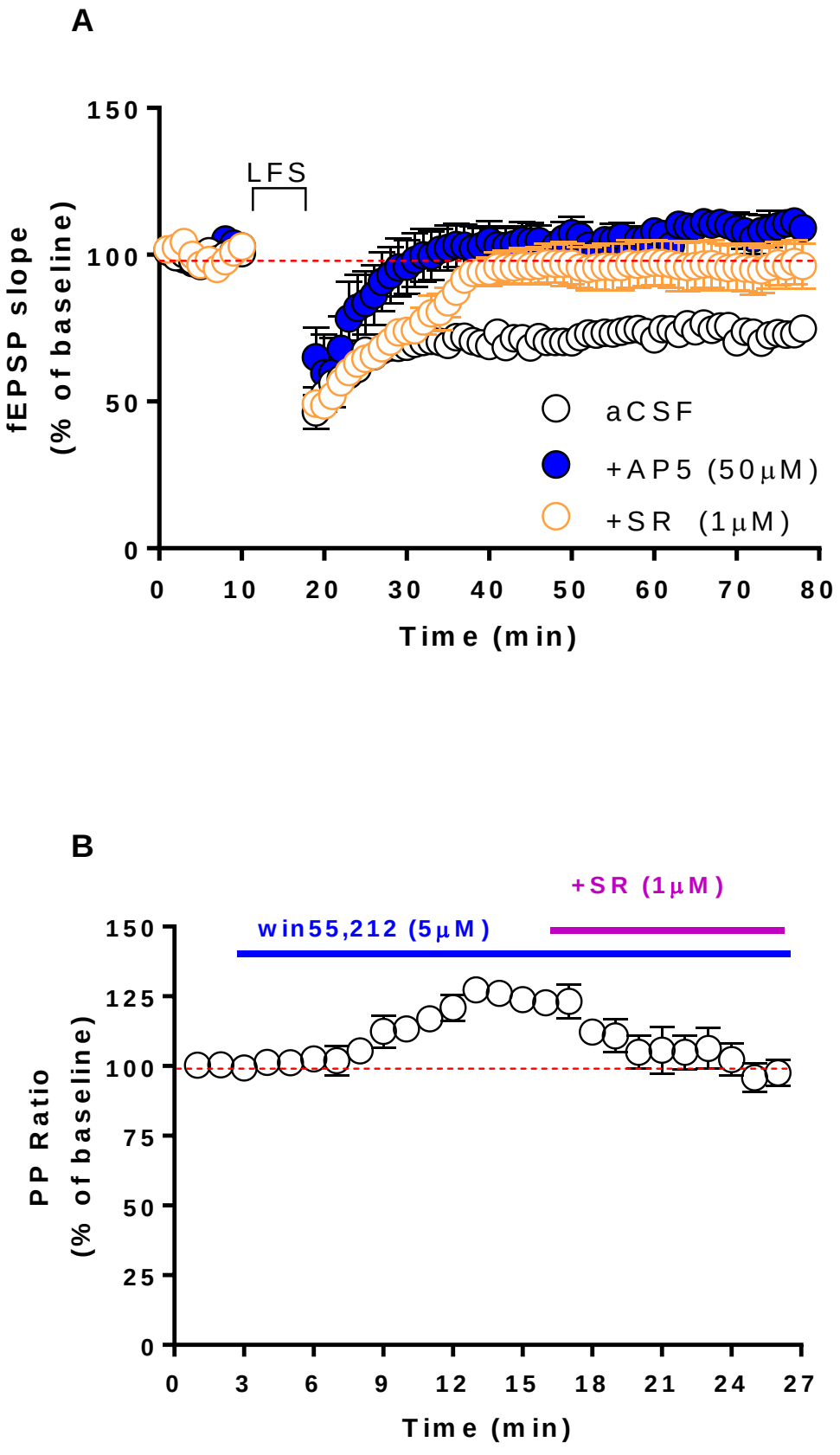

Novel object recognition
Familiarization
Tes
t
G
H
I
Obj 1
Obj 2
Recognition Index
Familiar
Novel
Discrimination Index (novel obj)
CTR L
RM S
CTRL+EB
RMS+EB
J
K
L
CTRL
RMS

Figure S1


A
150
100
50
0
fEPSP slope
(% of baseline)
LFS
aCSF
+AP5 (50μM)
+SR (1μM)
0
10
20
30
40
50
60
70
80
Time (min)
B
+SR (1μM)
win55,212 (5μM)
150
125
100
75
50
25
0
PP Ratio
(% of baseline)
0
3
6
9
12
15
18
21
24
27
Time (min)