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Original research

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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Abstract

The repeated maternal separation (RMS) is a useful experimental model in rodents to study the long-term influence of early-life stress on brain neurophysiology. We here investigated the influence of RMS exposure on hippocampal inhibitory and excitatory synaptic transmission, long-term synaptic plasticity and 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, and experiments were performed at PND60. Patch-clamp recordings in hippocampal CA1 pyramidal neurons revealed a significant enhancement of GABAergic miniature IPSC (mIPSC) frequency and a decrease in the amplitude of glutamatergic mEPSCs in male mice exposed to RMS. Only a slight but significant reduction 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 and in the response to the CB1r agonist win55,212 were detected in RMS male but not female mice. An impaired spatial memory and a reduced preference for novelty were observed in males exposed to RMS but not in females. A single injection of β-ethynyl estradiol at PND2 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 influence of RMS on long-lasting modifications in synaptic transmission, effects that may be relevant to cognitive performance.

Highlights

- The repeated maternal separation causes a significant imbalance between GABAergic and glutamatergic transmission in male but not in female mice
- RMS alters long-term depression at CA3-CA1 glutamatergic synapses and response to CB1r agonist in males but not in females.
- RMS impairs spatial memory and reduces preference for novelty in male mice exposed to RMS but not in females.
- A single injection of b-ethynyl estradiol at PND2, prevented in males the changes induced by RMS.

Keywords: Maternal separation, Stress, Neuronal plasticity, GABA, Glutamate, CB1r, Cognition

Introduction

Traumatic and stressful events occurring early in life are considered as a predictive factor of enhanced vulnerability to develop brain diseases in human 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 induced by early-life stress have been described, 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).

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, Mejia-Chavez et al., 2021). 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 (PND1) to PND21 (Cirulli et al., 2009; Hall, 1998; Marco et al., 2009) or from PND3 to PND15 (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 on gender, hinting that certain outcomes related to sex, such as different hormonal pattern, could be predictive for vulnerability to early-life stress (Bondar et al., 2018). Accordingly, in some reports, male but not female mice showed 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 opposite results (Veenema et al., 2007; Tsuda and Ogowa, 2012; Cui et al., 2020), suggesting, altogether, that the influence of sex as a factor for vulnerability to RMS needs further investigation.

Since the balance between excitatory and inhibitory synaptic transmission is essential to ensure proper synaptic information by finely tuning 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 explored whether the neurophysiological alterations produced by RMS may correlate with parallel changes in cognitive responses in adult C57BL/6J mice. In addition, in female rodents, changes in circulating estrogens were associated with altered dendritic spine density in various brain regions, including the hippocampus, suggesting the crucial physiological role of such endogenous compounds in the organization and activation of neural structures (Sheppard et al., 2019). Furthermore, it has been reported that robust LTD can be reliably induced in vitro in CA1 when estradiol levels are low (Zamani et al., 2000). Thus, based on previous observations showing 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 mice exposed to RMS and treated with a single injection of β-ethinyl estradiol (EB), which causes disruption of male sex differentiation (Nef and Parada, 2000; Toyama et al., 2001; Delbès et al., 2006). Finally, endogenous cannabinoids (eCBs) have a long-term influence on neural function by mediating the so-called eCB-LTD (Heifets and Castillo, 2009). This form of plasticity has been observed in different brain structures, including the hippocampus, at both excitatory and inhibitory synapses. Regardless of the stimulation protocol, nearly every synapse examined expresses eCB-LTD, which therefore represents one of the best examples of presynaptic forms of long-term plasticity (Abush and Akirav 2010; Chevaleyre et al., 2007). Thus, we considered it of relevance also to evaluate whether eCB signaling may be altered in adult mice that were exposed to RMS during adolescence.

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 conducted following the guidelines for the 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 number of animals used.

Repeated maternal separation

Animals were weighed at post-natal day 2 (PND2), PND18, and PND60, and litters were composed of a similar number of males and females (5–6 for each sex). From PND2 until PND17, litters were separated from their dams for 6 h daily, from 9:00 a.m. to 3:00 p.m., to induce a robust effect by dam separation, with their handling performed by the same experimenter. The RMS protocol was in agreement with previous 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 in a controlled temperature (30–32°C) by adding cotton wool to create a comfortable nest. During this time, the dams were left undisturbed in their home cage until the reunion. Control pups (CTRL) were handled twice a day, moving them from one side of the cage to the other, but left with dams in the same cage. At PND 17, all pups were returned to ordinary housing until weaning (PND21), when males and females were separated definitively from the dams and randomly housed in groups of 5 per cage. At PND2, a group of male pups was 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 a vehicle in agreement with previews reports (Calza et al., 2010). To

evaluate the effect of EB on primary sexual characters, testicle 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 PND60, as previously described (Talani et al., 2016). Briefly, after reaching deep anesthesia with vapors of 5% isoflurane, animals were sacrificed. Brains were rapidly removed from the skull and transferred to a modified artificial cerebrospinal 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 μm for patch clamp experiments and 400 μm for field potential extracellular recordings) 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 a 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 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; it was monitored throughout the recordings by injection of 10 mV depolarizing pulses, and if it changed during recording more than ± 20%, the cell was discarded from the analysis. For all patch-clamp and extracellular experiments, a period of at least 10 min was allowed before the start of recordings to ensure stable control conditions.

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. 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 then calculated. 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, comprising computation of amplitude, rise time, decay time, and event frequency, was performed using Mini Analysis software (Synaptosoft, Inc., version 6.0.2) with a noise amplitude threshold of 2 pA. 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). fEPSPs were recorded by filling the recording electrode with KCl (3 M). They 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, applied for evaluating the presynaptic effect of the activation of CB1rs by the perfusion of the selective agonist win55,212 (5 μM) for 15 min, consisted in delivering two consecutive electrical stimuli able to yield a half-maximal response, with an inter-event interval of 100 ms; the ratio of the slope of the second to the first fEPSP was calculated. In a different set of experiments, the effect of win55,212 on the paired-pulse ratio could be prevented by the concomitant application of the CB1r antagonist SR141716 1 μM (Fig. S1 panel B). 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 50% of the maximal response, a low-frequency stimulation (LFS, 900 stimuli at 1 Hz) was applied, and its effect was evaluated for 60 min. The magnitude of LTD was calculated by averaging the slope of fEPSPs between 50 and 60 min post-LFS and comparing this value to the pre-LFS baseline.

Barnes maze

At PND 60, the Barnes maze was used in order to evaluate their spatial learning and memory performances that are related to the hippocampal activity (Talani et al., 2020; Rosenfeld and Ferguson, 2014). Five mice from each experimental group were tested, and the experiment 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 defined as the “escape room”. Animals were driven to find the escape room by being exposed to a bright light (1400 lumen) fixed 50 cm up of the maze, using 4 different cue panels. Animals underwent a spatial acquisition phase that lasted 4 days, consisting of a single trial of 3

min. The position of the escape room was rotated by 90° before each consecutive trial and the same cue was associated to the escape room. Short-term retention (probe) was evaluated on day 5. During each trial test, the time needed to identify the escape room (latency), the number of errors and the distance traveled (monitored by Mousetron 12.1) were recorded by a digital camera and analyzed by the same operator.

Novel Object Recognition (NOR)

With the NOR test, we can obtain rapid information on the influence produced by RMS on cognition in adult mice. The NOR test is a paradigm based on the natural tendency of rodents for novelty, exploring more a novel object than a familiar one (more than 50% of the total time of objects exploration). As previously reported (Talani et al., 2020), one day before the test, each animal was placed in a plexiglass arena (50 x 50 x 50 cm) for 30 min to acclimate to the environmental condition. Dim light (120 lumen) was used during each session. Five mice from each experimental group were tested. On the day of the test, two identical objects (Obj1 and Obj2) were placed into the arena, and each mouse was allowed to explore them for 5min (familiarization). After 6 h (Talani et al., 2020) each animal was returned to the arena and was allowed to explore the two objects, with one of them being exchanged by a new one (novel) with a different shape and color, for a single session of 3 min (test) (modified by Bolz et al., 2015). The objects were plastic tools of different shape and dimensions (a ball and a little kid toy, see insert on Fig. 4), weighted so rats would not move them around, and available in triplicate copies. The test trial was administered by the afore mentioned protocol with the presentation of one familiar sample object and novel object. Both apparatus and objects were thoroughly cleaned with a 20% methanol solution to avoid olfactory cues between each trial. Memory was assessed by comparing the time spent exploring the novel object and the time spent

exploring the familiar object. We calculated the recognition index as the percentage of the time spent to each object compared to the total time spent in object exploration (Time Obj1 or 2/(Time Obj1+Obj2 x 100) in both the familiar and test phases and the discrimination index (Time Obj nov – Time Obj fam)/(Time Obj nov + Time Obj fam), that represents the difference in exploration time expressed as a proportion of the total time spent exploring the novel object after the retention trial. The discrimination ratio ranges from −1 to +1, with negative scores indicating a preference for the familiar object and positive scores signifying preference for the novel object.

Statistical analysis

Data are presented as mean ± SEM and compared by one- or two-way analysis of variance (ANOVA) and Dunnet's test or Student's *t*-test with the use of GraphPad Prism software (version 7). 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 body weight of both control mice and those that were exposed to RMS, at the beginning and at the end of the separation procedure (PND2 and PND17), as well as when the adult age was reached (PND60). As shown in **Tab. 1**, at PND2, the body weight was similar between groups, but after 16 days of RMS (PND18), 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 the change in body weight induced by RMS in male mice [$F_{(5, 24)} = 17.26$, $p = 0.0029$ vs. respective controls].

Moreover, 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 influence of EB treatment on male sex differentiation (Toyama et al., 2001; Delbès et al., 2006; Nef and Parada, 2015). Ten male mice were single-breeded 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 held at − 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 **(Fig. 1).**

Analysis of the GABAergic current kinetic properties revealed no significant difference in the amplitude **(Fig. 1A-E)** 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, C)**. Interestingly, this effect was no longer detectable in RMS males treated with EB at PND2 before the start of RMS **(Fig. 1A, E)**. Furthermore, we observed a decrease in event amplitude in RMS females [$F_{(5, 36)} = 3.94$, $p = 0.027$] with no changes in the frequency **(Fig. 1E)**.

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. 1F, G, J)**. Interestingly, the treatment with EB completely abolished the effect of RMS on mEPSC amplitude observed in male mice **(Fig. 1F, G, J)**.

RMS increases LTD at hippocampal CA1-CA3 excitatory synapses

Changes induced by RMS on GABAergic and glutamatergic transmission at CA1 may be predictive of a possible influence on long-term plasticity of glutamatergic synapses in the hippocampus. While the effect of RMS on hippocampal synaptic plasticity has been explored by analyzing more frequently LTP (Sousa et al., 2014), relatively less is known about the effect of RMS on LTD. This form of plasticity was shown to be crucial for spatial memory formation, especially during the first steps of learning (Ashby et al., 2021). We first recorded dendritic fEPSPs in the CA1 field. We applied an input-output paradigm (I–O) to construct a related curve by stimulating the Schaffer's collateral afferents with increasing current intensity (from 0 to 1.0 mA). Two-way ANOVA revealed an effect of males exposed to RMS [$F_{(3,25)} = 5.229$, $p = 0.0061$] with a significant decrease of the maximal effect of

stimulation compared to CTRL and RMS-EB groups **(Fig. 2A)**, an effect that was not observed in females (**Fig. 2D**). 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 50 to 60 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. S1A)**, 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 %) as indicated by two-way ANOVA [$F_{(3, 28)} = 8.92$, $p = 0.006$ vs. CTRL] **(Fig. 2B, C)**. Furthermore, LTD measured in RMS males treated with EB was indistinguishable from that obtained in CTRL and CTRL+EB groups, suggesting that treatment with EB could selectively prevent the increase of LTD associated with exposure of mice to RMS with no significant effect on CTRL animals **(Fig. 2B, C)**. In CTRL female mice, LFS produced a decrease (-22 ± 4.8) in fEPSP slope value that was like that observed in males. Interestingly, when LFS was applied to hippocampal slices obtained from female mice exposed to RMS, LTD was suppressed and we could detect a slight but significant (p = 0.0002) increase in fEPSP slope values compared to baseline **(Fig. 2E, F).**

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 detail 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 by holding the membrane potential at -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 in AMPA/NMDA ratio compared to CTRL male mice as indexed by the two-way ANOVA [$F_{(3,26)} = 6.67$, $p = 0.015$ vs. CTRL] **(Fig. 2G, H)**. The treatment with EB treatment was ineffective in modifying this parameter in CTRL animals, while it completely abolished the effect of RMS **(Fig. 2G, H)**. Interestingly, RMS failed to affect the AMPA/NMDA ratio in females compared to CTRL animals **(Fig. 2G, I)**. For a more detailed comparison, we reported the absolute values of AMPA and NMDA currents used to obtain the graphs shown in panels “H” and “I” relative to males and females, respectively; 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. Two-way ANOVA comparison highlighted a significant decrease of the current amplitude of both AMPA [$F_{(3, 48)} = 14.1$, $p = 0.0006$ vs. CTRL] and NMDA [$F_{(3,24)} = 1.24$, $p = 0.02$ vs. CTRL] in RMS male mice **(Fig. 2J)** with no significant effect observed in females **(Fig. 2K)**. Interestingly, the treatment with EB, while ineffective in altering AMPAr- or NMDAr-mediated currents in CTRL animals, completely abolished the reduction of these glutamatergic excitatory currents induced by RMS (2J).

The decrease in the NMDAr component detected in CA1 pyramidal of male mice exposed to RMS might not have been predicted based on the higher LTD levels detected in these mice. To explore these findings further, 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). This modulatory system was reported to have a long-term influence on neural function by mediating the so-called eCB-LTD (Heifets and Castillo, 2009). In agreement with this idea, when LFS was applied in the presence of the CB1r antagonist SR141716 (1 μM), the magnitude of LTD measured in hippocampal slices obtained from CTRL mice was markedly reduced **(Fig. S1B)**, indicating that the CB1r activity is required for the full LTD formation, at least in our experimental conditions, and suggesting that mechanisms related to this form of plasticity at the CA3-CA1 synapses may involve both NMDA and CB1r activation. To explore the possible involvement of this system in the synaptic effects of RMS, we measured the activity 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) in the presence of win55,212. Consistent with the predominant presynaptic location of CB1r (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, as revealed by two-way ANOVA analysis [$F_{(3,19)}$ = 10.47, p = 0.0043 vs CTRL] **(Fig. 3A- D)**, suggesting an increased function and/or expression of CB1r. 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 controls **(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. This test has been used to examine spatial learning and memory, exploiting the fact that rodents avoid bright illuminated areas and instead favor dark, confined spaces (Talani et al., 2020; Rosenfeld and Ferguson, 2014). Animals from all experimental groups were trained once a day for four consecutive days, during which the time needed and the errors made before hitting the escape room, and the distance traveled to find it 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-F)**. 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 [$F_{(3, 80)} = 9.80$, $p = 0.001$ vs. CTRL], committed more errors than CTRL at day 5 [$F_{(3,16)} = 12.6$, $p = 0.0001$ vs. CTRL] and traveled and increased distance [$F_{(3,14)} = 9.8$, $p = 0.001$ vs. CTRL] before hitting the escape room **(Fig. 4A, C)**. 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, C)**. RMS failed to alter all three parameters in female mice compared with the relative CTRL animals **(Fig. 4D, F)**.

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 in demonstrating the specific contribution of the

rodent hippocampus on object memory and preference for novelty (Antunes and Biala, 2012; Talani et al., 2020). During familiarization, animals were exposed to the presence of two identical objects. We found no differences in both male and female mice in terms of the recognition index during this phase **(Fig. 4G, J)**, which mirrored the expected similar 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 and the positive rate of the discrimination index, indicating a marked preference for the novel object **(Fig. 4H- L)**. Interestingly, when RMS male mice were exposed to the NOR test, during the test phase they revealed a marked impairment in the recognition index and negative rate of discrimination index, resulting in a higher preference for the familiar object relative to the novel one, as revealed by two-way ANOVA [recognition index, $F_{(3, 16)} = 15.19$, $p = 0.0013$ vs. CTRL; discrimination index, $F_{(3, 16)} = 15.19$, $p = 0.0013$ vs. CTRL] **(Fig. 4H-I)**. Such effect was no longer detectable in RMS animals treated with EB, while EB treatment per se did not alter both indexes for novelty in CTRL mice **(Fig. 4H-I)**. Furthermore, RMS did not cause such impairments for novelty in female mice. Rather a trend of improvement was seen in the preference for the novel object as indicated by both indexes **(Fig. 4K-L)**.

Discussion

In the present work, we provide new evidence for a sex-dependent responsiveness of adult C57BL/6J mice that were exposed to repeated maternal separation during the first two weeks of postnatal life. Male but not female mice showed a marked difference in the neurophysiological changes in hippocampal inhibitory and excitatory synaptic transmission, and long-term plasticity at excitatory synapses. Furthermore, 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 limitation in the time dedicated to maternal caregiving, consequent to the separation period in our experimental condition, could be responsible for the observed effects on neurochemical and behavioral parameters. However, several reports showed that immediately after the pups and the dam are reunited, there is an increase in caregiving (Macrí et al., 2004; Biggio et al., 2014). This observation suggests that the exposure to RMS during a crucial time window in which animals are particularly vulnerable produces long-lasting effects, which the increased maternal care cannot effectively reverse in the post-RMS period. In addition, different studies have demonstrated that specific effects that appear as a consequence of RMS exposure, such as impairment of dopamine transmission and hippocampal neurogenesis, as well as increased ethanol intake in adulthood, are dependent on sex, with males resulting in 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 daily rounds) from PND1 to PND21; the depressive-like behavior and impairment in synaptic plasticity were similar in male and female rats (Cui et al., 2020). Compared to our present data, such differential outcomes produced by maternal separation observed in these studies may depend on the separation paradigm employed, time points examined, and animal species used, suggesting that the precise mechanisms underlying these modifications need further exploration.

Similar to other reports (Maghami et al., 2018), we found that after RMS, males but not female presented a significant decrease in body weight, even though 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 again that differences in the maternal separation protocols can be critical in determining such different 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 in the excitatory/inhibitory balance, which may be predictive of decreased activity in this hippocampal neuronal circuit. Furthermore, the decrease in the amplitude of glutamatergic mEPSCs detected in patch-clamp recordings reflects the rightward shift of the I-O curve observed in RMS animals when compared with controls in extracellular recordings. The effect of RMS was different in females, with an apparent change at the post-synaptic site indexed by a decrease in GABAergic mIPSC amplitude and no significant difference in glutamatergic activity. 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 influence on long-term synaptic plasticity.

Like long-term potentiation (LTP), LTD is a form of hippocampal synaptic plasticity crucial for spatial recognition, especially during the first steps of learning as reported by a recent study from Ashby et al. (2021). The authors report that in the hippocampus, LTD is required for consolidation of spatial memories mediating performance in behavioral tasks such as the Morris water maze and habituation to a novel object configuration, suggesting a core role of LTD in encoding novel information. Such behaviors may thus trigger neurochemical responses that, in turn, elicit LTD in the hippocampus. In our experimental

conditions, 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 possibly consequent to the decreased activity in this neuronal circuit. Furthermore, these findings also agree with those showing the involvement of inhibitory transmission in long-term synaptic depression in hippocampal CA3-CA1 synapses in rats (Steele and Mauk, 1999). This study demonstrated that either application of the GABAAr agonist muscimol or increasing endogenous recurrent GABAergic input facilitates the expression of LTD. This effect that is consistent with the increase of GABA release observed in our RMS male mice with the consequent rise in the LTD extent. In addition to these effects, in male mice exposed to RMS, we also detected a decrease in both AMPAr- and NMDAr-mediated postsynaptic currents in CA1 pyramidal neurons with a marked increase in the AMPA/NMDA ratio.

RMS's neurochemical and neurophysiological effects on inhibitory and excitatory signaling in the hippocampal CA1 region could also be related to or may involve an increase in the pruning mechanism. 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 on NMDAr activation (Henson et al., 2017). Furthermore, RMS may also affect the expression of several neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), that are involved in neuronal growth, survival, and plasticity. 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, LTD measured in female mice exposed to RMS resulted slightly but significantly increased, indicative of long-term potentiation. Important observations were

made when analyzing the effects of estrogens on LTP and LTD in the hippocampus of female rodents (Sheppard et al., 2019). Hippocampal excitability and seizure threshold increase significantly with high 17β-estradiol, and the hippocampus is more prone to seizure during proestrus when high circulating estrogens are found in the blood (Sheppard et al., 2019). Since we found no alterations in the glutamatergic transmission in females, but we observed a significant reduction in the amplitude of mIPSCs, the change of the inhibitory transmission at the level of the CA1 synapses might be crucial for the altered synaptic plasticity, as reported by other authors (Nishiyama et al. 2010), although other neurochemical mechanisms may undoubtedly be involved as well. However, other experiments will be needed to characterize further 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 impairment, 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 explore further 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 LTD 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. Endogenous cannabinoids have a long-term influence on neural function by mediating the so called eCB-LTD (Heifets and Castillo, 2009). This form

of plasticity has been observed in different brain structures, including the hippocampus at both excitatory and inhibitory synapses. Despite the variety of stimulation protocols, nearly every synapse studied expresses eCB-LTD. eCB-LTD is one of the most significant examples of presynaptic forms of long-term plasticity (Abush and Akirav 2010; Chevaleyre et al., 2007). The alteration in eCBr response to agonists was observed again only in male but not in female mice and consisted of a more substantial 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 was downregulated. Moreover, by subjecting rats to a single maternal separation for 24 h at PND9, Suàrez et al. (2009) showed dysregulation of CB1rs in adult males but not 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 aspect is particularly significant given that sex differences in eCB signaling have been widely established (Hill et al., 2019; Suàrez et al., 2009).

Exploration of a novel environment triggers behavioral and neurochemical responses that elicit LTD in the hippocampus (Ashby et al., 2021). Consistent with the data on hippocampal synaptic signaling and plasticity, our behavioral studies indicate that RMS is associated with a significant decrease in cognitive performance in male mice. RMS completely impaired the preference for novelty in males, as shown in the NOR test, and reduced spatial learning in the Barnes maze test. At the same time, we found no significant alterations in female mice in both behavioral tests. In line with our data, a vast number of experimental and clinical evidence confirms that early-life stress, including that associated with 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, our data

demonstrating that RMS failed to affect spatial memory in female mice differ from other reports showing 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 supports the "mismatch hypothesis" that consists in the idea that early-life aversive experiences may promote some adaptive processes that make the subject more resilient and fit to tolerate better 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 worth mentioning that female rats were resilient to developing depressive-like symptoms following exposure to RMS (Dimatelis et al., 2016). Considering these results, a more detailed evaluation of the response to subsequent stress exposure in animals exposed to RMS may be relevant for a future study.

Sex hormones, particularly estrogens, play a fundamental role in sexual differentiation in early postnatal life, and estrogens have long been considered 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, paralleled by increased locomotor activity and reduced or unaffected anxiety-state level. Interestingly, the marked changes in GABA/glutamate balance, synaptic plasticity, and behavioral performances induced by RMS in our study, are all remarkably prevented in male animals when exposed to a single injection of EB at PND2, 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 females by some studies (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 Ogowa, 2012). Furthermore, the treatment with EB failed to alter the decrease in body weight observed in RMS male mice, suggesting that the mechanism associated with this effect does not involve this hormonal aspect and raises the possibility that the nature of such outcome is not limited to the role of estrogens.

Overall, our data strengthen the idea that RMS produces long-lasting modifications at hippocampal levels at both excitatory and inhibitory synaptic signaling and long-term plasticity in a manner dependent on sex. Furthermore, these effects are associated with altered cognitive performances in adult male but not female mice. These results herein add to previous evidence supporting a sex-dependent sensitivity to stress associated with RMS, and confirm that hormones, such as estrogens, may affect the outcome of early-life stress. However, the neurobiological mechanisms underlying these changes still need further investigation to examine further the idea that females are more resilient to the effects of early-life stress.

While these findings may have potential clinical implications, the neurobiological mechanism underlying these changes still need to be investigated more thoroughly to elucidate further the role of sex in the sensitivity to the long-lasting effects of early-life stress.

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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 revised 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 by mating one male and one female for 4 consecutive days and waiting 21 days to ascertain pregnancy. 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. veh, 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) were recorded from single voltage-clamped CA1 pyramidal neurons of the different experimental groups. Scale bar, 10 pA/1s. Averaged mIPSCs **(B, C)** and eEPSC **(G, H)** were recorded during a period of 3 min, from which amplitude and event frequency analysis was performed. The bar graphs summarize the changes in mIPSC or mEPSC amplitude 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). **(D, I)** Sample traces of averaged mIPSCs (D, scale bar 2 pA/ 5 ms) and mEPSCs (**I**, scale bar 2 pA/ 5 ms) recorded from a sample neuron obtained from CTRL and RMS male mice; baseline (green line), peak (red “x”), and decay time (purple line, fitted by a two-component exponential equation) are represented. **(E, G)** Frequency distribution by Kolmogorov-Smirnov test of mIPSCs (E) and mEPSCs (G) from a sample neuron that highlights the change in event amplitude induced by RMS.

Figure 2. RMS alters the LTD formation and AMPA/NMDA components in hippocampal CA1-CA3 excitatory synapses. (A, D) Scatter plot representing the I-O relation obtained in male (A) and female mice (D). **(B, E)** Scatter plot depicting the percentage of change in fEPSP slope values induced by LFS with respect to baseline, in male mice of the different experimental groups. The insert shows the representative traces of dendritic fEPSPs recorded in slices obtained from males (a) or females (b) the different groups. Traces were recorded before (black) and after (red) LTD conditioning (LFS, 900 stimuli at 1 Hz), scale bar, 1 mV/ 5 ms. **(C, F)** The graphs summarize the magnitude of LTD, calculated by averaging the percentage change in fEPSP slope from baseline 50–60

min after LFS obtained from graphs A and B. Data are expressed as mean percent change of fEPSP slope ± SEM from baseline. Two-way ANOVA, *p < 0.05 vs. CTRL, $^{\#}p$ < 0.05 vs. baseline, n = 6-12, for males; n = 11, for females. (**G)** Representative traces of evoked glutamatergic EPSCs were recorded in the presence of the GABAergic antagonist bicuculline (20 μM) in CA1 neurons voltage-clamped at -65 mV (lower trace) for AMPA currents, and at +40 mV (upper trace) for NMDA currents in the presence of the AMPA antagonist NBQX (5 μM) in slices obtained from both sexes of the different groups. Scale bar, 100 pA / 50 ms. 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). (**H, I)** The graph summarizes the AMPA/NMDA ratio obtained from CA1 neurons of the different experimental groups of males (H) and females (**I**). Data are expressed as mean ± SEM of obtained ratios for every recording. One-way ANOVA, *p < 0.001 vs. CTRL, n = 5-12. (**J, K)** 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. Two-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 recorded with a protocol of paired-pulse stimulation (black arrows indicate both stimuli with interstimulus interval of 100 ms) in the absence (black trace) and presence (red trace) of the CB1r agonist win55,212 (5 μM) perfused for 15 min; scale bar, 1 mV/ 5 ms. The paired-pulse ratio was calculated as the ratio between the second response's slope and the first's slope. **(B, C)** Scatter plot representing the effect of 15-min win55,212 perfusion on the paired-pulse ratio in males

(B) and females **(C)**. **(D)** The bar graph summarizes the effect of win55,212 on the paired-pulse ratio averaged during the latest 3 min of drug perfusion in males (blue bars) and females (pink bars). Data are expressed as mean ± SEM of obtained ratios for every recording. Two-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-F) Scatter plots representing the change in latency needed **(A)**, errors made **(B)**, and distance traveled **(C)** before finding the escape room in the Barnes maze test by males **(A-C)** and females **(D, F)** 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. **(G, L)** The bar graphs summarize the effect of RMS on the recognition index and discrimination index during the familiarization and test phase in the novel object recognition test. The insert shows the type of object used during the different phases. The recognition index was calculated as the percentage of the time dedicated to a single object compared to the total time of objects exploration. The discrimination index for the novel object was calculated as the ratio of the difference between the time dedicated to the novel and the familiar object divided by the total time dedicated in objects exploration. Data are expressed by mean ± SEM. Two-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. (A) Scatter plot representing the percentage of change in fEPSP slope values induced by LFS with respect to baseline in male mice in the absence and presence of AP5 50 μM and SR 141716 1 μM, antagonists of NMDAr and CB1r, respectively. (LFS, 900 stimuli at 1 Hz). **(B)** Scatter plot representing the percentage of

change in paired-pulse ratio calculated in the presence of win55,212 and the antagonism produced by the concomitant application of the CB1r antagonist SR141716 (1 μM).

Figure 1R1

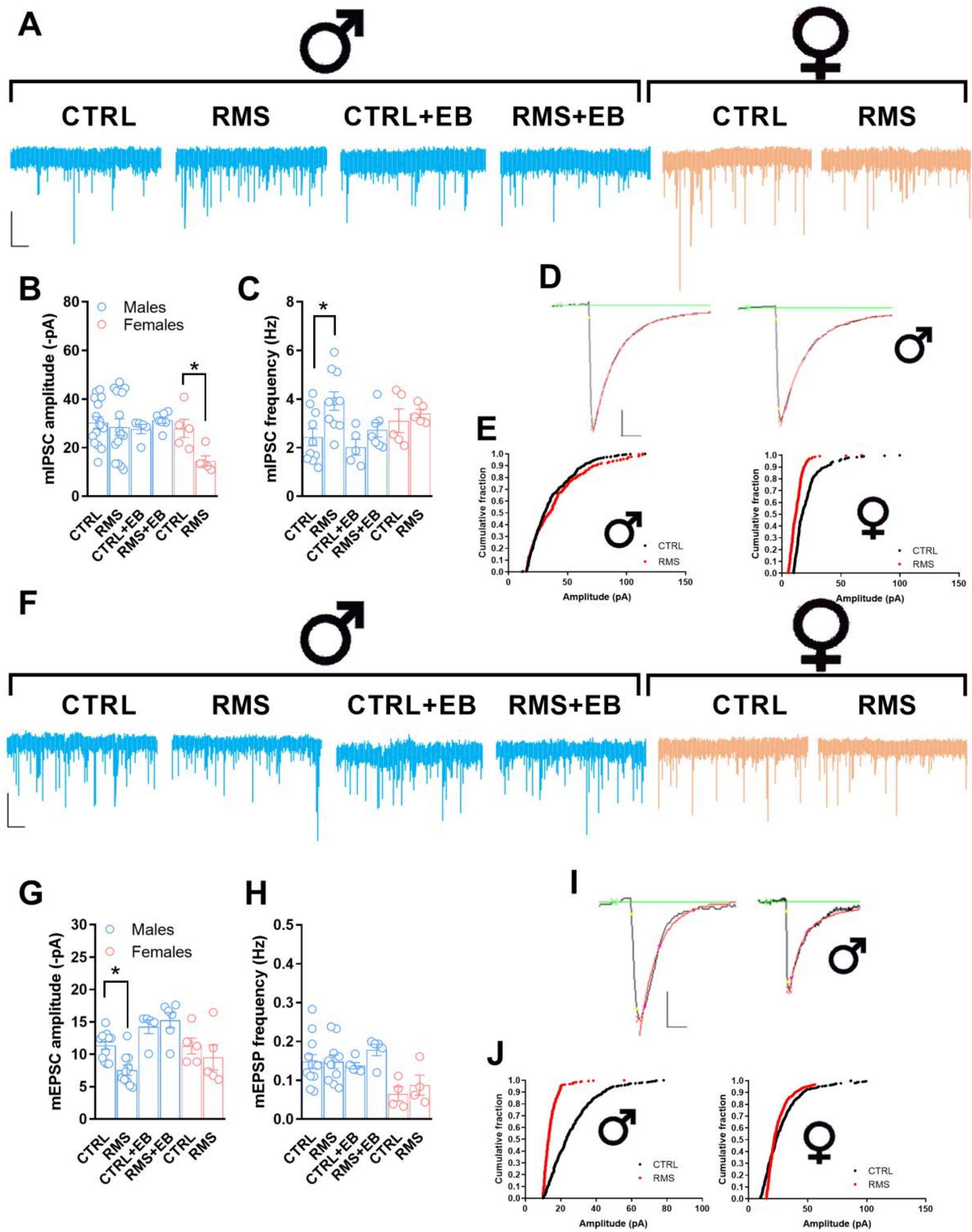


Figure 2R2

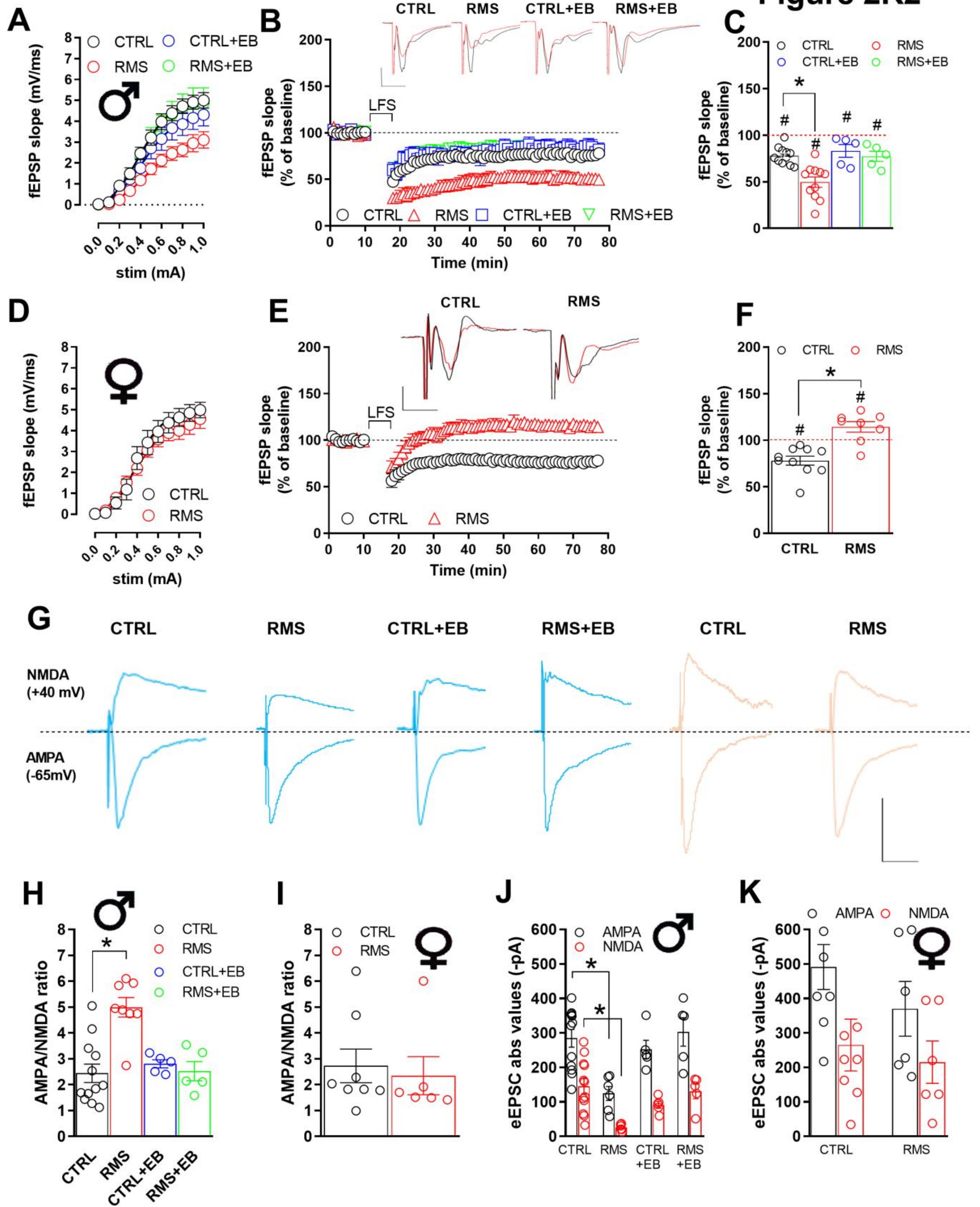


Figure 3R2

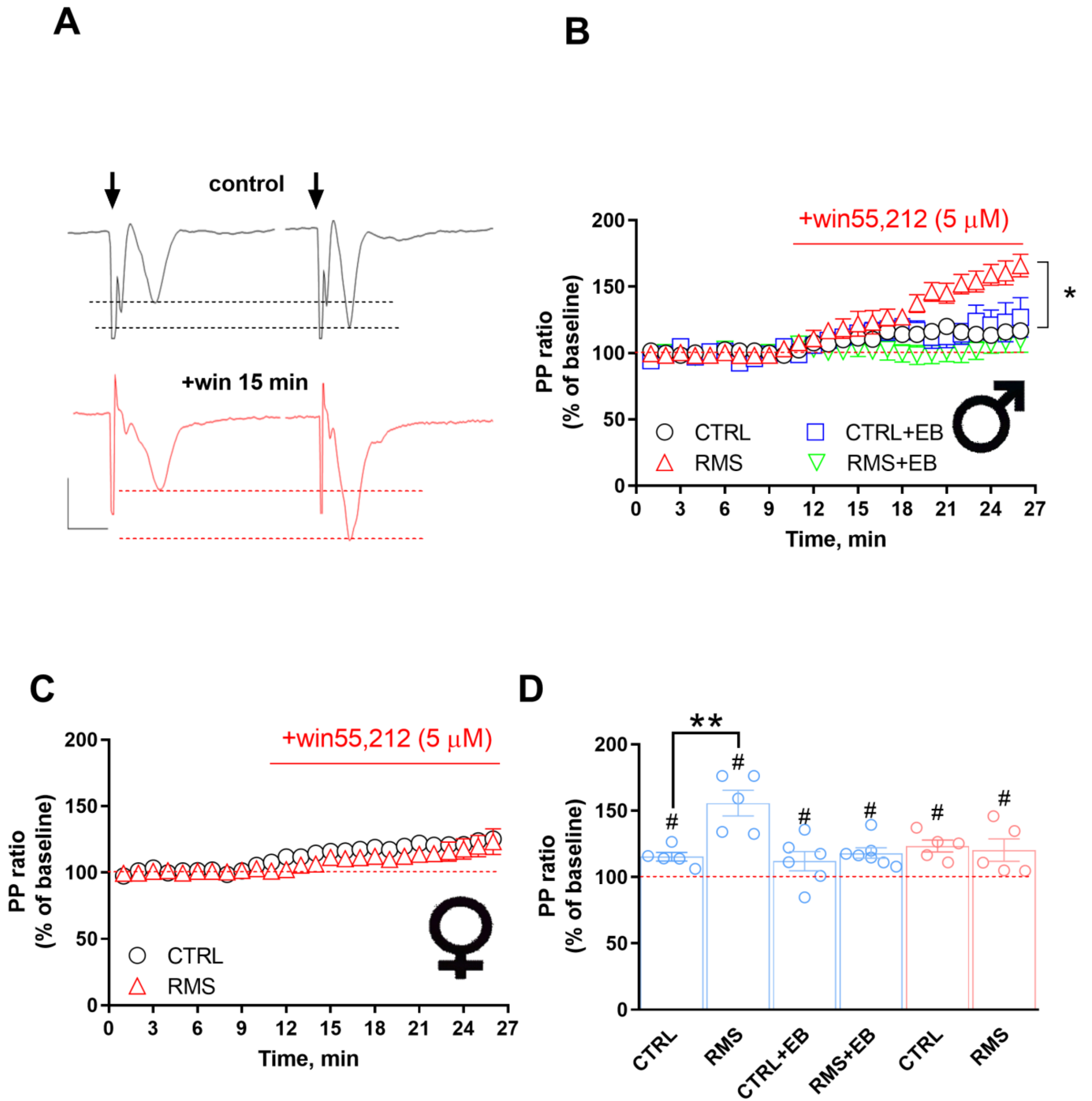
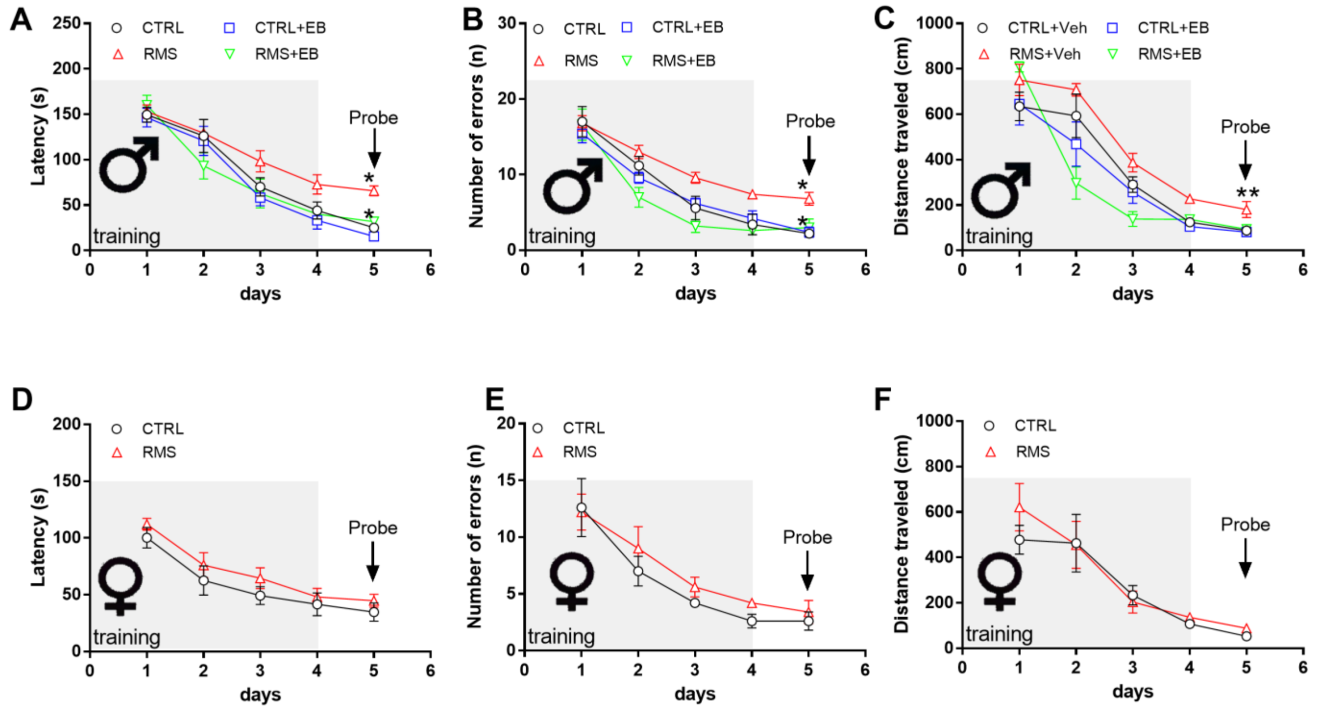


Figure 4R2

Barnes maze



Novel object recognition

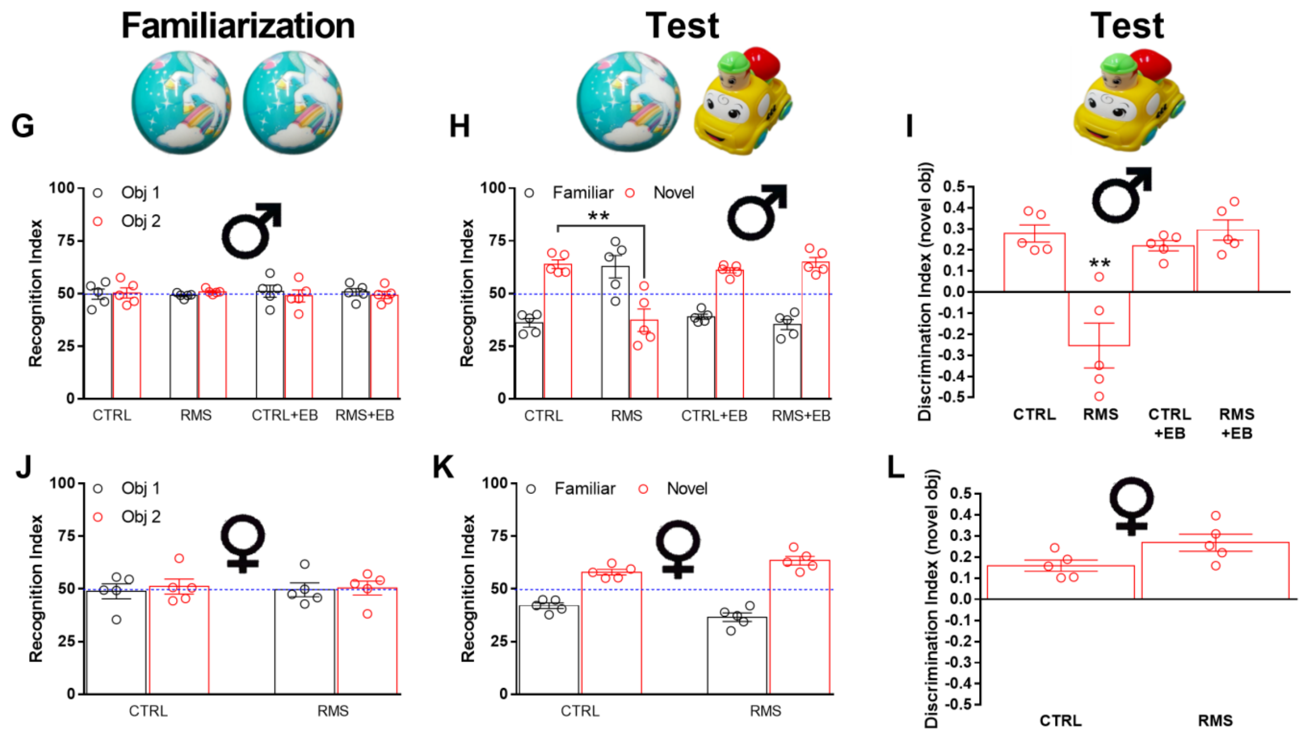
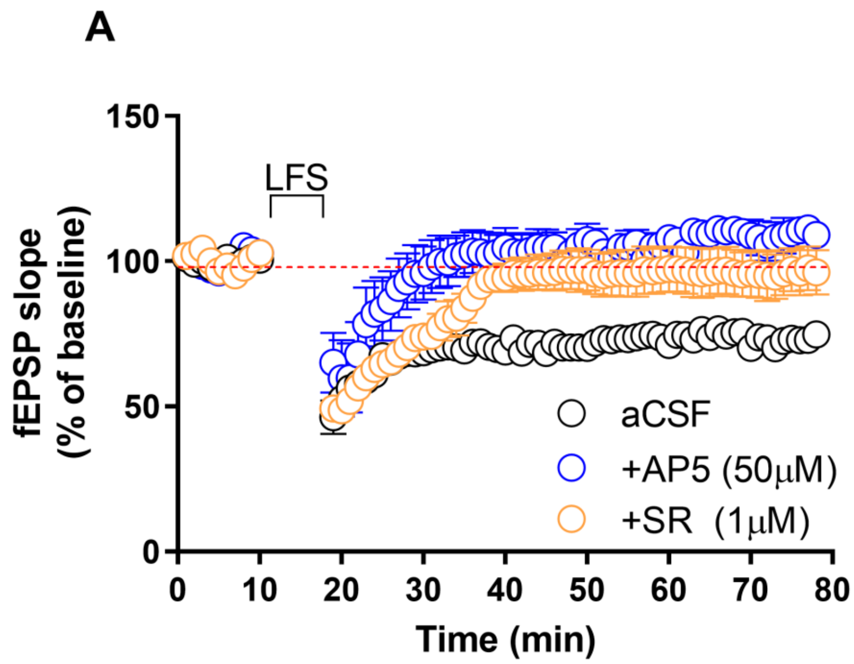


Figure S1R1

A



B

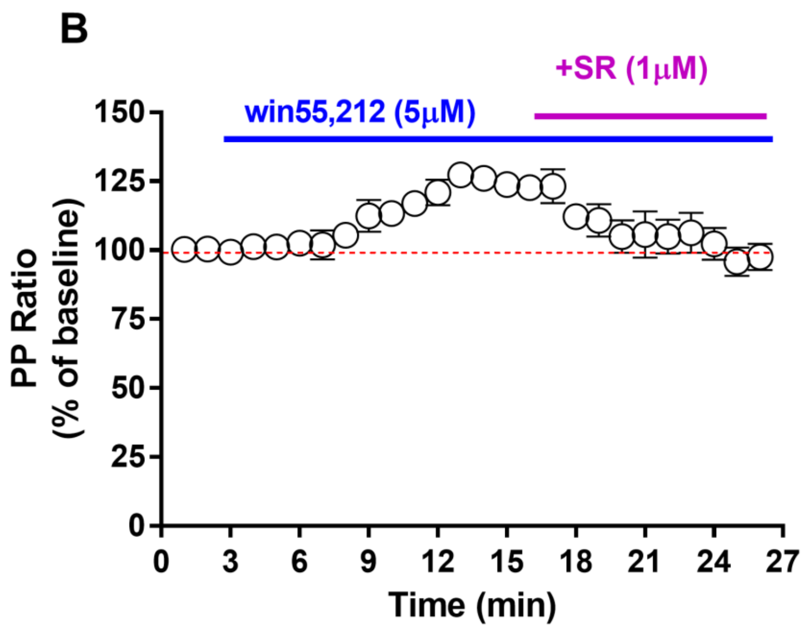


Table 1

	Body weight (g)					
Post Natal Days	**CTRL-M**	**RMS-M**	**CTRL-EB-M**	**RMS-EB-M**	**CTRL-F**	**RMS-F**
2	1.1 ± 0.06	1.1 ± 0.06	1.1 ± 0.05	1.1 ± 0.06	1 ± 0.02	1 ± 0.01
18	8.4 ± 0.08	7.1 ± 0.14*	8.4 ± 0.13	7.5 ± 0.17*	7.2 ± 0.19	7.2 ± 0.13
60	31.5 ± 1.12	30 ± 0.59	30 ± 1.24	31 ± 1.17	23.4 ± 0.44	23.3 ± 0.49

Table 2

Parameter	CTRL	RMS	CTRL-EB	RMS-EB
Testicles weight (mg)	210.8 ± 1.32	208.8 ± 2.89	165.8 ± 4.56*	163.9 ± 6.24*
Fertility	9/1	8/2	3/7*	2/8*