

Mesocortical dopamine hypofunction and prefrontal plasticity deficits in the cuprizone model

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system frequently associated with cognitive impairment, which critically depends on the integrity of the prefrontal cortex (PFC) and its mesocortical dopaminergic inputs. However, direct experimental evidence linking prefrontal dopamine dysfunction to MS-related cognitive deficits is limited. Here, we investigated the contribution of mesocortical dopaminergic transmission to cognitive and behavioral alterations using the cuprizone model of demyelination in male Wistar rats. After six weeks of 1% cuprizone administration, we combined *ex vivo* electrophysiology in the rostral ventral tegmental area (rVTA) and medial PFC (mPFC), *in vivo* microdialysis of extracellular dopamine in the mPFC, behavioral assessment of cognition and anxiety-like behavior, gait analysis, and plasma corticosterone measurements. Cuprizone reduced the spontaneous firing rate of rVTA dopaminergic neurons and decreased glutamatergic miniature excitatory postsynaptic current frequency. In the mPFC, cuprizone increased pyramidal neuron excitability and presynaptic glutamatergic transmission, while impairing long-term potentiation and reducing NMDA receptor-mediated currents. Microdialysis showed reduced basal extracellular dopamine levels in the mPFC which, together with the results on the EPM and the marked decrease observed in plasma corticosterone, indicate blunted mesocortical and hypothalamic–pituitary–adrenal axis responsiveness. Cuprizone-treated rats exhibited working memory deficits with preserved performance in a task not strictly PFC-dependent, reduced anxiety-like behavior, and subtle gait changes. These findings suggest that cuprizone-induced intoxication disrupts VTA–mPFC circuitry, leading to mesocortical dopamine hypofunction, altered cortical plasticity, and executive dysfunction, and support targeting mesocortical dopamine transmission as a potential strategy for MS-related cognitive symptoms.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS), traditionally characterized by motor, sensory, and autonomic dysfunction (Compston and Coles, 2008). However, a growing body of evidence has revealed that cognitive impairments are highly prevalent among individuals with MS, affecting approximately half of people with MS (pwMS) and involving domains such as attention, working memory, executive functions, and

information processing speed (Chiaravalloti and DeLuca, 2008; Benedict et al., 2002). These deficits are prevalent in late stages of the disease and can significantly impact quality of life, even in the early stages of the disease, often progressing independently of physical disability (Rocca et al., 2015).

Working memory, response initiation and inhibition, problem solving, strategic planning, and conceptual ability are the primary functions affected (Foong et al., 1997). Cognitive deficits have been consistently correlated with magnetic resonance lesions in specific cerebral areas

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(Arnett et al., 1994; Benedict et al., 2002; Foong et al., 1997; Sweet et al., 2004). The prefrontal cortex (PFC), a region critical for higher-order cognitive processes, has been increasingly implicated in the pathophysiology of MS-related cognitive decline (Schoonheim et al., 2015). Functional and structural abnormalities in the PFC have been documented in pwMS through neuroimaging and postmortem studies (Calabrese et al., 2010; Petracca et al., 2021; Popescu and Lucchinetti, 2012). However, the neurochemical mechanisms underlying these alterations remain poorly understood.

The involvement of PFC is further supported by evidence of significant comorbidity of anxious and depressive symptoms with the pathology (Feinstein, 2007). Dopamine, a critical neuromodulator in this brain area, plays a central role in regulating attention, working memory, and goal-directed behavior (Cools and D'Esposito, 2011). Dysregulation of dopaminergic signaling in the PFC has been associated with cognitive deficits in various neurological and psychiatric disorders, including Parkinson's disease, schizophrenia, and attention deficit hyperactivity disorder (Arnsten and Rubia, 2012; Robbins and Arnsten, 2009). In the context of MS, recent studies suggest that neuroinflammatory processes and white matter damage may disrupt fronto-striatal and mesocortical dopaminergic pathways, which could contribute to cognitive symptoms (Centonze et al., 2010; Pardini et al., 2014).

Moreover, preclinical data suggest that dopaminergic depletion induced by MPTP administration exacerbates experimental autoimmune encephalomyelitis (EAE)-induced symptoms (Bałkowiec-Iskra et al., 2007). Dysfunction of the dopaminergic system is associated with mood changes induced by IL-1 β administration in the same experimental model (Gentile et al., 2015), and treatment with dopaminergic drugs prevents or reduces the severity of symptoms induced by EAE (Benson et al., 2013; O'Sullivan et al., 2014; Lieberknecht et al., 2017). However, direct experimental evidence investigating mesocortical dopamine transmission in preclinical models of demyelination or MS remains limited. Chanaday et al. (2015) analyzed the functional and morphological changes in isolated presynaptic terminals (synaptosomes) from the PFC of mice with EAE, revealing impairment in the presynaptic release machinery of glutamatergic neurons. Because glutamatergic transmission in the PFC is essential for regulating dopaminergic neuronal function, these findings further highlight the critical role of the mesocortical dopaminergic pathway in MS symptoms and progression.

Clinical data also suggest that pwMS exhibit marked cognitive-motor interference (that is the phenomenon for which the simultaneous performance of a motor and a cognitive task leads to a deterioration in performance on one or both tasks compared with performing them separately) during dual-task activities, with greater dual-task-related impairments observed particularly in those with cognitive deficits and higher levels of disability (Wajda and Sosnoff, 2015). Decreased performance on dual tasks in pwMS correlates with cognitive fatigue, but not with the disease severity or duration of the pathology, suggesting that alterations in working memory may explain the observed difficulties they experience when performing dual tasks (Hamilton et al., 2009).

The present study aims to investigate the role of mesocortical dopaminergic pathway in cognitive dysfunctions associated with an animal model of demyelination. Using the oral administration of cuprizone [bis(cyclohexanone)oxalldihydrazone, CPZ], a copper chelator that causes oligodendroglial cell death, we assessed, in male Wistar rats, changes in mesocortical dopaminergic transmission and their correlation with behavioral performance in cognitive tasks. We examined potential alterations in putative dopaminergic neurons and the GABA/glutamate transmission in the ventral tegmental area (VTA), as well as the effects on membrane properties, GABAergic and glutamatergic transmission, and synaptic plasticity in glutamatergic neurons in the PFC. To further investigate the relationship between PFC function and motor impairments, we analyzed the gait patterns of cuprizone-treated and control animals to determine their potential association with extracellular dopamine levels in the PFC.

The selection of the cuprizone model is justified by the opportunity to investigate the consequences of cortical demyelination and localized, innate neuroinflammation (characterized by robust microglial and astrocytic activation) in the complete absence of systemic T-cell infiltration and the confounding physical paralysis typical of active autoimmune models (Ciccarelli et al., 2024; Dedoni et al., 2023).

Elucidating the link between neurochemical changes in the PFC and cognitive impairments in a model of demyelination could provide new insights into the pathophysiological mechanisms of MS and inform the development of targeted therapeutic strategies.

2. Materials and methods

2.1. Animals

Male Wistar rats (Envigo, Italy) were used in this study. They were born in the animal facility of the University of Cagliari (CeSAS) and, after weaning (at about 21 postnatal days, PND), were randomly assigned to either the control (CTRL) or cuprizone-treated group (CPZ). All animals were housed in groups of 4 per cage and maintained at a constant temperature of 22 ± 2 °C, a relative humidity of 65%, and an artificial light/dark cycle of 12/12 h (light on from 7:00 to 19:00). Experiments were conducted during the light phase. All animals had free access to water and standard food unless otherwise indicated. Every effort was made to minimize animal suffering and reduce the number of animals used, in accordance with the "3R" principles that govern animal experimentation. The experimental protocols were reviewed and authorized by the Italian Ministry of Health (authorization #786/2019-PR) in compliance with the European Communities Council Directives (86/609/EEC and 63/2010) and the Italian law (D.L. 26/2014) for the care and use of experimental animals and with policies issued by the Organism for Animal Welfare (OPBA) of the University of Cagliari.

2.2. Randomization, blinding, exclusion criteria, and sample-size determination

Animals were randomly assigned after weaning to either the control (CTRL) or cuprizone-treated (CPZ) group. Group allocation was maintained throughout the study, and all animals were processed according to identical experimental procedures.

Behavioral scoring was performed by investigators blinded to the experimental groups whenever feasible. In particular, videos from the Elevated Plus Maze and gait analysis were analyzed offline without knowledge of treatment allocation. Electrophysiological recordings and microdialysis analyses were performed using predefined experimental protocols, and data analysis was carried out using standardized criteria.

Pre-established exclusion criteria were applied before data analysis. For electrophysiological recordings, cells were excluded when access resistance changed by more than 20% during the recording session. Normal distribution of data was verified by Skewness and Kurtosis evaluation with Graph Pad Prism 9.0 before applying statistical evaluation. Animals or samples were excluded only in cases of technical failure (e.g., incorrect probe placement, tissue damage, or poor-quality recordings) that prevented reliable data acquisition. All exclusions were determined before statistical analysis.

Sample sizes were based on previous studies using the same experimental procedures in our laboratory and on published studies employing the cuprizone model, and a formal a priori power calculation was performed in order to obtain authorization for the experimentation. The number of animals and, where appropriate, the number of recorded cells are reported in the corresponding figure legends.

2.3. Treatment

Immediately after weaning on PND 21, the animals in the cuprizone (CPZ) group were fed standard food supplemented with 1% cuprizone

(Oakden et al., 2017). Cuprizone was added to the pulverized food pellets at a ratio of 1 g to 100 g of food. Pasteurized egg white and water were added to create a malleable mixture, which was then formed into new pellets and dried in an oven at 50 °C until completely dry. The animals in the CPZ group were fed only with the cuprizone-supplemented food for six weeks. CTRL animals received the same food without cuprizone. Throughout the entire treatment, food intake was measured daily for both experimental groups, while the animals were weighed once a week. At the end of the six-week treatment period, the animals were allocated to different experimental cohorts. The treatment was prolonged for the entire duration of the experiments without interruption. Whenever possible, animals from the same cohort were used for multiple experiments to minimize the total number of animals used, in accordance with the 3Rs principles. Specifically, the same cohort of animals was used to perform the behavioral tests (EPM, Y-maze, T-maze and Gait analysis). A total number of 88 CTRL and 90 CPZ animals were used for the entire study.

2.4. Ex vivo electrophysiology

2.4.1. Preparation of brain slices

Brain slices were prepared as previously described (Talani et al., 2016). In brief, CTRL and CPZ male rats (PND 63–73) were euthanized after reaching deep anesthesia with vapors of isoflurane (5%). The brains were quickly removed from the skull and transferred to a beaker containing an ice-cold modified artificial cerebrospinal fluid (aCSF), which was composed of the following (in mM): 220 sucrose, 2 KCl, 0.2 CaCl₂, 6 MgSO₄, 26 NaHCO₃, 1.3 NaH₂PO₄, and 10 D-glucose (pH 7.4, set by aeration with 95% O₂ and 5% CO₂). Horizontal brain slices containing the rostral part of the ventral tegmental area (rVTA) or coronal brain sections containing the medial prefrontal cortex (mPFC) (260 µm thick), were cut in ice-cold modified aCSF using a VT1200S vibratome (Leica, Heidelberg, Germany). The slices were then immediately transferred to a nylon net submerged in standard aCSF containing (in mM): 126 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 D-glucose (pH 7.4, set by aeration with 95% O₂ and 5% CO₂), with a final osmolarity of 330. After incubating for at least 40 min at a controlled temperature of 35 °C and waiting at least 1 h at room temperature, the hemislices were transferred to the recording chamber and continuously perfused with standard aCSF at a constant flow rate of ~2 ml/min. The temperature of the bath was maintained at 33 °C for all recordings.

2.4.2. Electrophysiology experiments

Patch-clamp recordings were performed from rVTA putative dopaminergic neurons or mPFC pyramidal neurons as previously described (Bassareo et al., 2019). Recording pipettes were prepared from borosilicate capillaries containing an internal filament using a P-97 Flaming Brown micropipette puller (Sutter Instruments, Novato, CA, USA). The resistance of the micropipettes ranged from 4.5 to 6.0 MΩ. We used two different internal solutions. For cell-attached and whole-cell current-clamp recordings, we used an internal solution containing (in mM): 140 K-gluconate, 0.5 CaCl₂, 5 EGTA, 10 Hepes-KOH (pH 7.3), and 1 ATP disodium salt, with a final osmolarity of 286 Osm. For whole-cell voltage-clamp recordings of excitatory and inhibitory postsynaptic currents, we used an internal solution containing (in mM): 140 CsCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 HEPES, 3 ATP Na⁺ (single bond), pH 7.3 (adjusted using concentrated CsOH). Bioelectrical signals were recorded with the use of an Axopatch 200B amplifier (Axon Instruments Inc., San Jose, CA, USA), filtered at 2 kHz, and digitized at 5 kHz. We used the pClamp 10.7 software (Molecular Devices, Union City, CA, USA) to measure and analyze the firing rate and postsynaptic current kinetic parameters of rVTA neurons as well as the occurrence of HCN-mediated hyperpolarization-activated currents (I_h) (see below). After obtaining a micropipette-membrane seal with a GΩ resistance, at least 10 min were allowed for a stable and regular baseline firing rate to be established.

GABA receptor- or glutamate receptor-mediated miniature postsynaptic currents (mIPSCs, and mEPSCs), respectively, were recorded in whole-cell mode after breaking the membrane seal in the presence of lidocaine (500 µM), to block voltage-gated Na⁺ channels and to prevent action-potential-dependent neurotransmitter release, and of the non-selective glutamate receptor antagonist kynurenic acid (1 mM), or the GABAA receptor antagonist bicuculline (20 µM), respectively. Access resistance, which was monitored throughout the recording by injecting 10 mV hyperpolarizing pulses, ranged from 15 to 30 MΩ. If access resistance changed by more than 20% during recordings, the cell was automatically discarded from the analysis. Quantitative analysis of mIPSCs and mEPSCs was performed offline using Mini Analysis Software (Synaptosoft, Inc., version 6.0.2) with a noise amplitude threshold of 5 pA. The amplitude, decay time, and frequency of mIPSCs and mEPSCs were then analyzed in both experimental groups. Evoked EPSCs (eEPSCs) mediated by NMDA receptors were recorded in the presence of the AMPA receptor selective antagonist NBQX (5 µM) and bicuculline (20 µM) in single mPFC pyramidal neurons by holding the membrane potential at +40 mV in response to presynaptic stimulation of increasing intensity (from 0.0 to 1.0 mA, in 0.1 mA intervals).

To elicit the HCN-mediated I_h, voltage-clamp recordings were performed by using a protocol with incremental hyperpolarizing voltage steps of −10 mV of the membrane, from −50 to −110 mV.

For the current-clamp experiments, we used a protocol that involved injecting current steps ranging in intensity from −80 to 200 pA in 20 pA increments for 1 s, to hyperpolarize or depolarize the cell membrane and measure the relative voltage changes. The parameters analyzed included the resting membrane potential, the action potential (AP) threshold, the rheobase (the minimum current required to evoke the first AP), AP latency (the time required for the first AP to occur in response to depolarization), and AP frequency (the number of APs observed during the 1-s step).

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed at the dendritic level in the layer 5 of the mPFC through electrical stimulation of excitatory afferents. fEPSPs were recorded by filling the recording electrode with 3 M KCl. They were digitally triggered every 20 s by the application of a constant current pulse of 0.2–0.4 mA for 60 µs, which usually yielded a half-maximal response, using a bipolar concentric electrode (FHC, ME, USA) and a stimulator (Digitimer Ltd., UK). To induce long-term potentiation (LTP), a single high-frequency stimulation (HFS, 100 stimuli at 250 Hz) was applied after 10 min of stable baseline. During this time, fEPSPs were evoked every 20 s at the current stimulation intensity that triggered approximately 50% of the maximal response. The effect of HFS was evaluated for the following 60 min. The magnitude of LTP was calculated by averaging the slope of fEPSPs recorded from 50 to 60 min post-HFS and comparing this value to the pre-HFS baseline. The paired-pulse protocol was used to evaluate possible changes in presynaptic activity induced by CPZ intoxication. This protocol involved delivering two consecutive electrical stimuli of the same intensity that could yield a half-maximal response of fEPSP, with an inter-event interval of 100 ms. Then, the ratio of the slope of the second fEPSP to the first was calculated.

2.5. Microdialysis

After six weeks of cuprizone treatment, animals from both experimental groups were deeply anesthetized with isoflurane to undergo stereotaxic surgery allowing the implant of a microdialysis probe into the mPFC according to the Paxinos & Watson atlas coordinates (AP +3.2; ML +0.5; DV −5.3 relative to bregma) (Paxinos and Watson, 2004). The active length of the dialysis membrane was 4 mm allowing sampling from infralimbic and prelimbic cortices as previously described (Dazzi et al., 2014). Experiments were performed in freely moving rats 24 h after probe implantation to allow recovery from surgical procedures. Ringer's solution [3 mM KCl, 125 mM NaCl, 1.3 mM

CaCl₂, 1 mM MgCl₂, 23 mM NaHCO₃, 1.5 mM potassium phosphate (pH 7.3)] was pumped through the dialysis probe at a constant rate of 2 µl/min. From each rat at least 6 dialysate samples were collected every 20 min and immediately analyzed with HPLC coupled to electrochemical detection for the determination of dopamine content as previously described (Dazzi et al., 2014).

2.6. Elevated plus maze

The elevated plus maze (EPM) consisted of four arms (50 cm in length × 10 cm in width) elevated 55 cm above the ground. Two of the arms were closed by 40 cm-high black walls (closed arms) while the other two arms were open. The rats were habituated to the conditions in the testing room for 60 min before being placed in the center of the apparatus facing an open arm. All subsequent activity was recorded for 5 min via the use of a Sony HDR-SR5E digital camera recorder, and the following parameters were measured: number of entries into closed arms; number of entries into open arms, then expressed as percent of total number of entries into both arms; time spent in open arms, then expressed as percent of total time spent in both arms. Arm entries were considered only when the rats had all four limbs inside the arm. Videos were analyzed by an observer blind to the animal condition.

2.7. Y-maze spontaneous alternation test

Spatial working memory was evaluated by a maze with the shape of a capital letter 'Y' (arm size: 50 cm × 10 cm; middle choice area size: 10 cm × 10 cm). All the experiments were performed at the same time range (from 9:00 to 14:00 h) to avoid confounding effects due to changes in circadian rhythm and each animal performed only one trial. The animals were habituated to the experimental room for at least 30 min before the experiment. The animals were placed in the center of the maze and allowed to freely explore the three arms for 5 min. Spontaneous Alternation Performance (SAP) and Alternate Arm returns (AAR) were recorded. Specifically:

SAP corresponds to the number of correct triads, with correct triad being a sequence of three different arm choices (e.g. identified the arms of the T maze as A, B and C, the sequence ABCABCABC corresponds to 7 correct triads). The percentage of correct triads was calculated by using the following formula:

$$SAP = \frac{\text{number of correct triads}}{\text{total number of entrances} - 2} \times 100$$

AAR refers to the number of times the animal returns to a newly explored arm (e.g. the sequence ABCBACABA corresponds to 3 alternating entries in the same arm). AAR was calculated with the following formula:

$$AAR = \frac{\text{number of alternate entries}}{\text{total number of entrances}} \times 100$$

2.8. T-maze delayed non matched to target (DNMT)

CTRL and CPZ rats were habituated to the maze for two consecutive days, by allowing them to freely explore the maze that was baited in both arms with honey cereal pieces as reward. Rats were handled daily for four consecutive days prior to behavioral testing, followed by a two-day habituation period and a 14-day training phase. During training and testing, food availability was restricted to 85% of animals' daily intake to enhance motivation for the food reward.

During the DNMT task, food rewards were positioned at the end of each goal arm of the T-maze. Each rat was initially placed in the start arm to begin the trial. Training consisted of five trials per day over 14 consecutive days, with each trial comprising a forced-choice phase followed by a free-choice phase.

At the start of each trial, rewards were placed in both goal arms,

although access was restricted to a single open arm during the forced-choice phase. Rats were therefore required to enter the accessible arm. Subsequently, animals were returned to the start arm, and after a 10-s delay, the free-choice phase was conducted with both arms accessible. Consecutive trials were separated by 10-min intervals, and the number of correct choices was recorded for each animal. Body weight was measured once a week after training sessions to avoid potential interference with behavioral performance.

Once all the animals of both experimental groups reached 70% of correct alternation on 2 out of 3 consecutive days as previously described (Spellman et al., 2015), the performance of the animals was measured with increasing delays between the two trials (0, 10, 20, 40 and 60 s). The maze was cleaned with a diluted solution of sodium hypochlorite between trials to avoid olfactory cues. During the task, each rat performed multiple trials at each training day and delay condition. The percentage of errors and latency were first averaged for each animal, and these per-animal values were used for statistical analyses.

2.9. Gait analysis

The apparatus used for the gait analysis consisted of an acrylic plastic walkway (110 cm L × 8 cm W × 10 cm H) similar to what previously described by Bounds et al. (2019). The floor was made translucent by covering it with a 16 LB cotton fiber drafting vellum cut to the same width as the walkway. Two strips of 12 V LED lights with 18 LEDs/m were secured 5 cm away and 2.5 cm above the floor of the walkway to illuminate the track. A digital camera (Sony Alpha6000) operating at a frame rate of 50 frame per second (fps) was placed approximately in central position below the walkway, at distance suitable to include the entire track in the camera field of view. Animals were allowed to familiarize with the apparatus and allowed to walk from the experimenter's hand across the whole walkway length to its end. Once the animals were able to cross smoothly the walkway without any interruption at a steady pace, at least 3 valid runs for each animal were recorded and subsequently analyzed. A trial was considered valid, and thus included in the subsequent analysis, when the animals walked consistently and without pause for at least 4 consecutive steps at a quite constant pace.

2.9.1. Data processing

The acquired videoclip were segmented into frames with a 0.33 s interval using the free software VLC media player (<https://www.videolan.org/>) and each frame was then post-processed using the public domain software ImageJ (<https://imagej.net/ij/index.html>). Following a spatial calibration which was performed by including in the field of view of the camera a metric ruler, the position of each limb was marked in each frame belonging to a specified trial and the correspondent position annotated. Then, the following spatio-temporal parameters were calculated:

- stride length: the distance between two consecutive contacts of the same limb on the walkway (cm);
- step width: the distance between the posterior limbs perpendicular to the direction of travel (cm);
- gait speed: the mean velocity of progression (cm/s);

In order to remove the transient effects associated with acceleration and deceleration possibly occurring at the two extremities of the walkway, the first and last two strides were not considered. The average values calculated across the 3 trials of the previously listed spatio-temporal parameters were employed for the subsequent analysis.

2.10. Measurement of corticosterone levels

Plasma corticosterone levels were measured as previously described (Biggio et al., 2014) using an enzyme-linked immunosorbent assay

(ELISA). Briefly, animals were sacrificed between 10:00 a.m. and 12:00 p.m. by decapitation and blood was rapidly collected into K3-EDTA tubes, centrifuged at 900 $\times g$ for 10 min at 4 °C and stored at –80 °C until use. The enzyme immunoassay was used to quantify plasma levels of corticosterone. ELISA was performed according to the manufacturer's instruction using a 96-well plate that was pre-coated with a polyclonal antibody against an antigenic site on the corticosterone molecule. The kit also provided a seven-point standard curve using two-fold serial dilutions. Each sample was run in duplicate.

2.11. Statistical analysis

The data are presented as mean $\pm$ SD. They were compared using one- or two-way analysis of variance (ANOVA) and Tukey's post hoc test or by unpaired *t*-test when needed using GraphPad Prism software (version 9.0). A *p*-value of less than 0.05 was considered statistically significant. For the electrophysiology experiments, the sample size is defined as *n*. For experiments in which multiple measurements (e.g., cells, brain slices, dialysate samples, or repeated behavioral observations) were obtained from the same animal, the animal was considered the experimental unit. Individual measurements collected from each animal were averaged to obtain a single representative value per animal before statistical analysis, thereby avoiding pseudoreplication. All statistical comparisons were therefore performed using animal-based mean values, and the data are presented accordingly in the figures.

3. Results

3.1. Effect of cuprizone treatment on food intake and body weight

Fig. 1 shows the differences in food intake (Panel A) and body weight (Panel B) between CTRL and CPZ animals. CPZ rats consumed significantly less food compared to CTRL, but both groups reached a plateau in food consumption after about 14 days of treatment (CTRL: about 24 g/day; CPZ: about 18 g/day). Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,9)} = 73.90$; $p < 0.0001$]; a significant effect of time [$F_{(2.636, 23.72)} = 15.48$; $p < 0.0001$]; and a not significant interaction between factors [$F_{(2.636, 23.72)} = 1693$; $p = 0.1994$].

Despite a lower food intake, CPZ rats showed a growth curve that, although significantly lower than CTRL animals, showed the same trend over time. Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,346)} = 420.4$; $p < 0.0001$]; a significant effect of time

[$F_{(7,346)} = 488.6$; $p < 0.0001$]; and a significant interaction between factors [$F_{(7,346)} = 15.38$; $p < 0.001$].

3.2. Effect of cuprizone treatment on basal firing rate of rVTA neurons

We identified single putative dopaminergic neurons in the rostral VTA (rVTA) by verifying, in agreement with previous reports (Ungless and Grace, 2012), the presence of a regular spontaneous firing rate (4.34 ± 0.4 Hz, $n = 16$ cells tested from 5 animals) (Fig. 2A), and the occurrence of hyperpolarization-activated cyclic nucleotide-gated (HCN) cationic currents (I_h) in response to hyperpolarizing the cell membrane from –50 to –110 mV (Fig. 2B) although we acknowledge that these criteria do not fully establish dopaminergic identity. To quantitatively evaluate the effects of cuprizone treatment on the spontaneous basal firing activity of putative dopaminergic neurons in rat rVTA slices, spike discharge was recorded in the cell-attached configuration. The firing rate of rVTA putative dopaminergic neurons was markedly reduced by 49% in CPZ rats compared to CTRL animals [unpaired *t*-test, $t = 4.784$ $p = 0.0014$, $n = 5$] (Fig. 2A).

Since hyperpolarization-activated I_h are a major determinant of intrinsic excitability in putative dopaminergic neurons of the VTA (Bosque-Cordero et al., 2020), we evaluated whether the cuprizone treatment altered I_h activation in rVTA putative dopaminergic neurons. To do so, we applied voltage steps ranging from –50 to –110 mV (in 10 mV increments) for 1 s each to single rVTA dopaminergic neurons under voltage-clamp conditions. This evoked a slowly activating inward current whose amplitude increased as the membrane voltage was progressively raised to –110 mV (see Fig. 2B). These currents could be abolished by the bath application of the HCN channel inhibitor ZD7288 (10 μ M) (result not shown). The *I*-*V* curve revealed that I_h activation occurred at an apparent membrane potential higher than –60 mV in both experimental groups, with a linear relationship from –70 to –110 mV. Under these conditions, we found no apparent effect of cuprizone treatment on I_h amplitude, with steady-state currents, measured by hyperpolarizing the cell membrane at –110 mV, that were not significantly different from those recorded in dopamine neurons of the CTRL group (CTRL = 153.8 ± 59.5 pA; CPZ = 187.8 ± 70.9 pA; unpaired *t*-test $t = 0.8226$ $p = 0.43$, $n = 5$) (Fig. 2B).

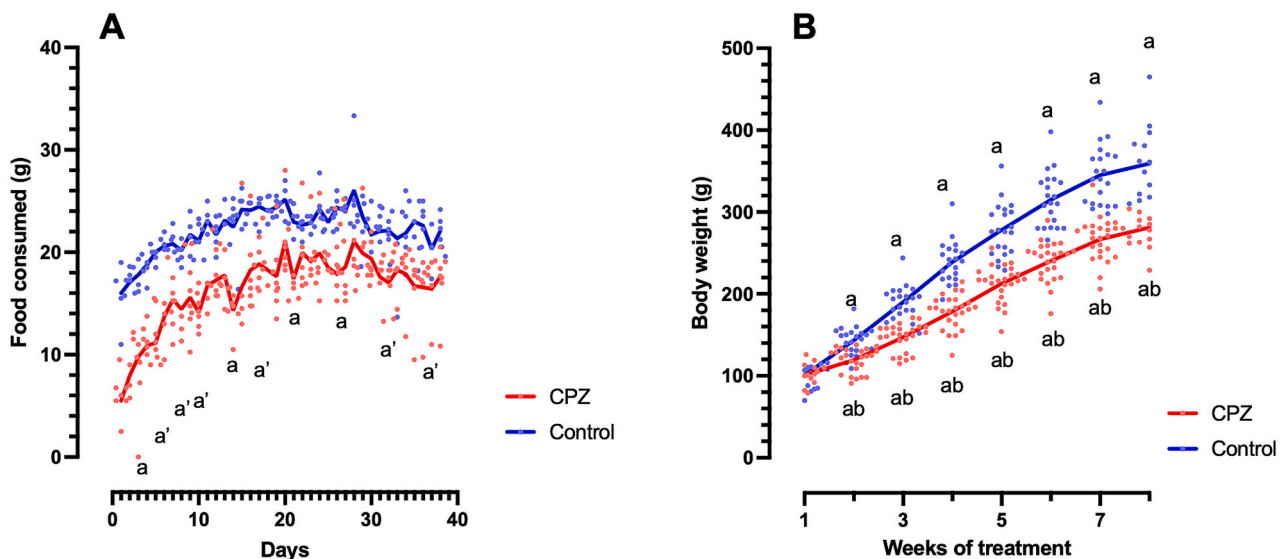


Fig. 1. Effect of cuprizone treatment on food consumption (A) and rats' body weight (B). Food intake was calculated daily; animals were weighted once a week. Data are presented as individual values for 21 CTRLs and 25 CPZs animals. ^a*p* < 0.05, ^{a'}*p* < 0.01 vs day1; ^b*p* < 0.05 ^{b'}*p* < 0.01 vs same time-points of CTRL.

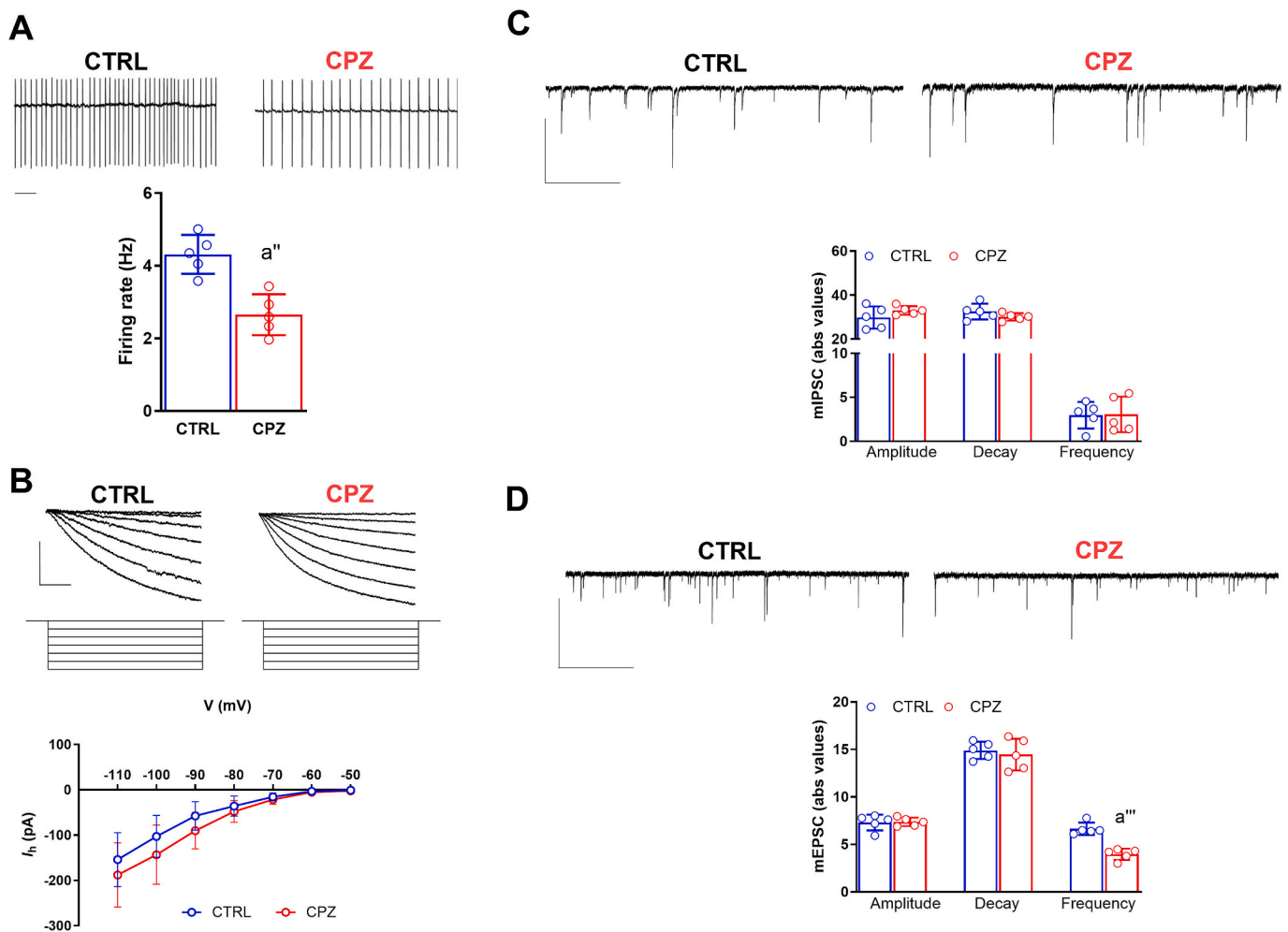


Fig. 2. Effect of CPZ treatment on spontaneous excitability, I_h currents and inhibitory/excitatory synaptic GLUT transmission in VTA DA neurons. (A) On top, sample traces recorded from single putative dopaminergic neurons in the VTA, showing spontaneous action potential firing. Scale bar 1 s. On the bottom: bar graph representing the averaged firing rate recorded from different putative dopaminergic neurons in CTRL and CPZ rats. a'' $p = 0.0043$ vs. CTRL; two-tailed t -test; $n = 5$. (B) On top: sample I_h recorded in single VTA putative dopaminergic neurons in response to membrane voltage steps from -50 to -110 mV. Scale bar 50 pA/200 ms. On the bottom: graph representing the averaged I_h amplitudes in response to membrane voltage steps. $n = 5$. (C) On top: sample traces of mIPSCs recorded in single VTA putative dopaminergic neurons in the presence of lidocaine (500 μ M) and kynurenic acid (1 mM). Scale bar: 50 pA/10 s. On the bottom: bar graph representing the average values (amplitude in pA, decay time in ms and frequency in Hz) recorded from different dopaminergic VTA neurons in CTRL and CPZ animals. $n = 5$. (D) On top, sample traces of mEPSCs recorded in single dopaminergic neurons from the VTA in the presence of lidocaine (500 μ M) and the GABAA receptor antagonist bicuculline (20 μ M). Scale bar: 50 pA/10 s. Below: bar graph representing the averaged values (amplitude in pA, decay time in ms and frequency in Hz) recorded from different dopaminergic neurons in CTRL and CPZ animals. a''' $p = 0.0003$ vs. CTRL; two-tailed t -test; $n = 5$.

3.3. Effect of treatment with cuprizone on inhibitory and excitatory transmission in VTA neurons

Next, we evaluated the effect of cuprizone treatment on inhibitory GABAergic and excitatory glutamatergic transmission in rVTA putative dopaminergic neurons. In both cases, we voltage-clamped the neuronal membrane at -65 mV, and recorded mIPSCs and mEPSCs separately in the presence of kynurenic acid (1 mM), a glutamate receptor antagonist, or bicuculline (20 μ M), a GABAA receptor antagonist and in the presence of lidocaine (500 μ M), to block voltage-gated Na^+ channels and to prevent action-potential-dependent neurotransmitter release. Under these recording conditions, activation of GABAA receptor or AMPA/kainate receptors generates inward currents that reflect an outflow of Cl^- and a net inflow of Na^+ , respectively. We analyzed current properties, such as amplitude, decay time, and frequency, to detect possible alterations in the presynaptic and/or postsynaptic compartments.

Analysis of the GABAergic current parameters revealed no significant differences in the three parameters (Fig. 2C) of mIPSCs in CPZ rats compared with CTRL animals.

Analysis of glutamatergic mEPSCs revealed that cuprizone treatment did not alter amplitude or decay time but it caused a significant decrease in event frequency [unpaired t -test $t = 6.81$ $p = 0.0001$, $n = 5$] (Fig. 2D).

3.4. Effects of cuprizone treatment in mPFC pyramidal cell excitability

Based on the results obtained in rVTA putative dopaminergic neurons and considering that the mPFC is a crucial brain structure involved in executive functions, receives prominent dopaminergic projections from the VTA (mesocortical pathway), and in turn regulates the firing pattern of VTA dopaminergic neurons (Lodge, 2011; Wee et al., 2019), we explored whether cuprizone treatment may also affect mPFC function. First, we investigated the effect of cuprizone on the basal passive properties and excitability of mPFC pyramidal cells. Current-clamp experiments conducted in pyramidal neurons of the mPFC revealed a significant [unpaired t -test $t = 5.059$ $p = 0.0001$] shift to a more depolarized resting membrane potential in CPZ rats compared with CTRL animals. The resting membrane potential was -67.0 ± 1.9 mV in CTRL rats ($n = 8$) and -61.3 ± 2.6 mV in CPZ animals ($n = 9$) (Fig. 3A,

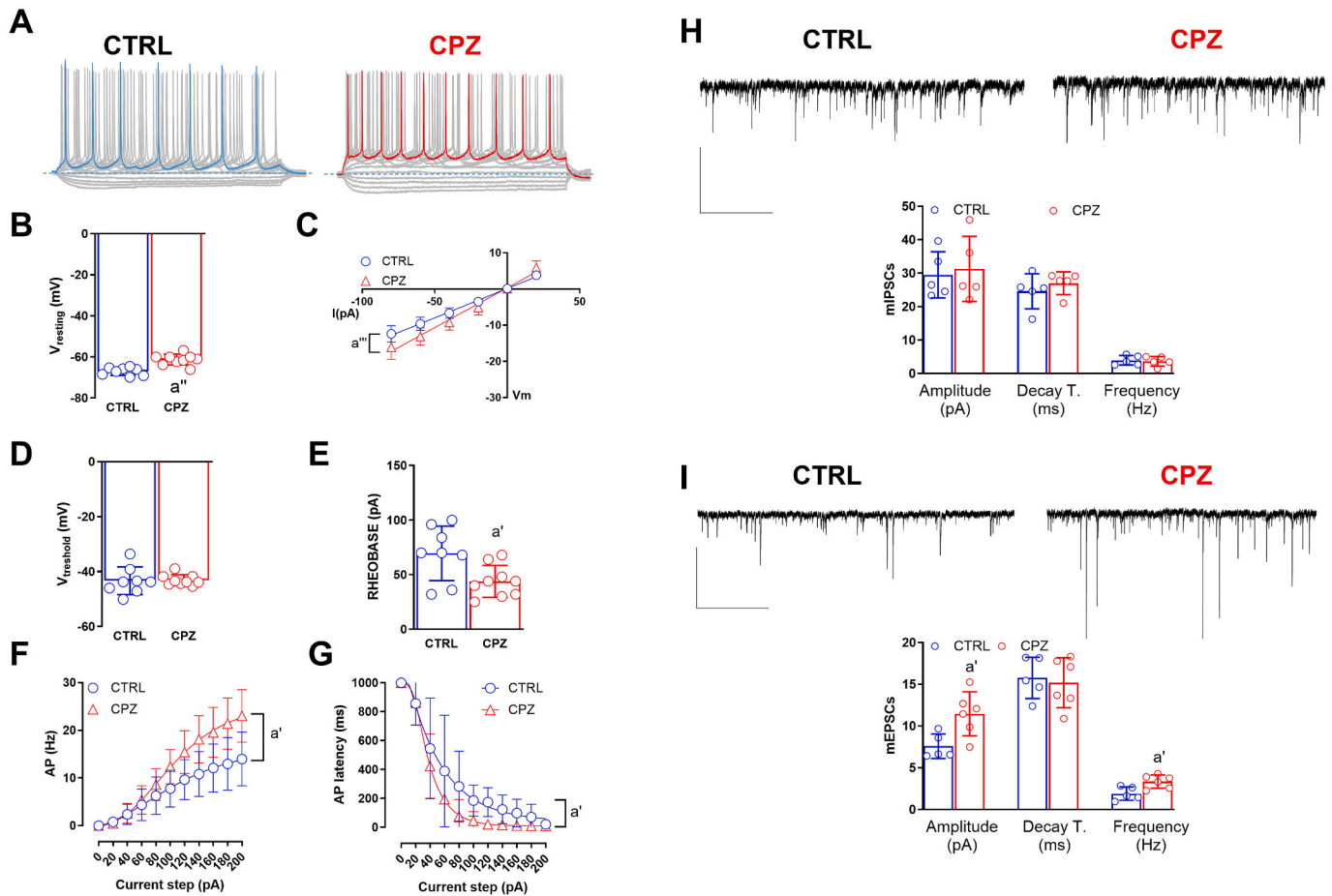


Fig. 3. Effect of CPZ treatment on passive membrane properties, evoked excitability, and inhibitory/excitatory synaptic transmission in pyramidal neurons of the mPFC. (A) Sample traces, recorded from single mPFC pyramidal neurons, showing changes in membrane potential in response to hyperpolarising and depolarising current steps (from -80 mV to 200 pA). All steps are shown in gray, with the colored line highlighting the membrane response relative to the 60 pA step. (B) Bar graph showing the averaged resting membrane potential values recorded in mPFC neurons from both experimental groups. Values are mean $\pm$ SD ($n = 8$ for CTRL, and 9 for CPZ rats). $a'' p = 0.0001$. (C) V/I curves showing evoked changes in membrane voltage in response to current steps (from -80 to 20 pA in 20 pA increments). $a' p = 0.001$ vs. CTRL; two-way ANOVA; $n = 8-9$. (D) Bar graph showing the mean V-threshold values. Values are reported as the mean $\pm$ SD ($n = 8$ for CTRL, and 9 for CPZ rats). (E) Bar graph representing the mean rheobase values, i.e. the minimum depolarising current intensity required to evoke the first action potential. $a'' p = 0.004$ vs. CTRL; two-tailed t -test. $n = 8-9$. Scatter plots showing AP frequency (F) and AP latency (G) in response to positive current steps (from 0 to 200 pA, in increments of 20 pA). a'' Frequency: $p = 0.0001$ vs. CTRL; a' Latency: $p = 0.03$ vs. CTRL. $n = 8-9$. (H) At the top are sample traces of mIPSCs recorded in single neurons from the mPFC in the presence of lidocaine (500 μ M) and kynurenic acid (1 mM). Scale bar 50 pA/ 10 s. Below is a bar graph representing the averaged values (amplitude in pA, decay time in ms and frequency in Hz) recorded from different dopaminergic neurons in CTRL and CPZ animals. $n = 5-6$. (I) At the top are sample traces of mEPSCs recorded in single neurons from the mPFC in the presence of GABA_A receptor antagonist bicuculline (20 μ M). The scale bar is 50 pA/ 10 s. On the bottom is a bar graph representing the averaged values (amplitude in pA, decay time in ms and frequency in Hz) recorded from different dopaminergic neurons in CTRL and CPZ animals. $a' p = 0.01$ vs CTRL, two-tailed t -test; $a'' p = 0.007$ vs CTRL, $n = 5-6$.

B). In addition, analyzing the voltage change in response to the current step injection (from -80 to 20 pA), revealed a significant increase in the R_{in} , represented by the slope of the straight line in the V/I graph (Fig. 3C). Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,15)} = 8.117$; $p = 0.012$, $n = 8-9$]; a significant effect of current step [$F_{(1,455, 21.82)} = 318.4$; $p = 0.0001$]; and a significant interaction between factors [$F_{(5, 75)} = 7.745$; $p = 0.0001$]. To study membrane excitability, AP firing was evoked by injecting depolarizing currents of higher intensity ($20-200$ pA, in 20 pA increments). Cuprizone treatment failed to alter the AP threshold (Fig. 3D), but it significantly decreased the rheobase value (the minimum depolarizing current intensity needed to evoke the first AP) [unpaired t -test $t = 2.617$, $p = 0.019$, $n = 8-9$] (Fig. 3E). Additionally, cuprizone treatment produced a significant step-dependent increase in AP frequency. Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,15)} = 7.460$, $p = 0.016$, $N = 8-9$]; a significant effect of current step [$F_{(1,467,22.01)} = 123$; $p = 0.0001$]; and a significant interaction between factors [$F_{(10,150)} = 8.859$; $p = 0.0001$] (Fig. 3F). A significant reduction in AP latency (the time delay from

current injection to the appearance of the first AP) was also observed. Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,16)} = 6.281$, $p = 0.023$, $n = 8-9$]; a significant effect of current step [$F_{(1,880, 30.08)} = 120.4$; $p = 0.0001$]; and a significant interaction between factors [$F_{(10,160)} = 2.052$; $p = 0.031$] (Fig. 3G).

3.5. Effect of treatment with cuprizone on GABAergic and glutamatergic transmission on mPFC pyramidal neurons

To investigate whether cuprizone treatment altered inhibitory GABAergic and excitatory glutamatergic transmission in the mPFC, pyramidal neurons in this brain region were voltage-clamped at -65 mV, and mIPSCs and mEPSCs were then recorded separately under the same conditions as in rVTA experiments.

Analysis of GABAergic mIPSCs revealed no significant differences in any of the three parameters between the two experimental groups of rats (Fig. 3H). However, cuprizone treatment significantly increased the amplitude and frequency of glutamatergic mEPSCs [amplitude,

unpaired *t*-test $t = 2.93$ $p = 0.017$ vs. CTRL; frequency, *t*-test $t = 2.988$ $p = 0.0153$ vs. CTRL, $n = 5$ –6] (Fig. 3I). These data suggest that cuprizone treatment may influence glutamatergic signaling at both the pre- and postsynaptic levels.

3.6. Effect of treatment with cuprizone on NMDA-mediated currents and long-term synaptic plasticity in mPFC glutamatergic synapses

Changes in GABAergic and glutamatergic transmission, as observed at the mPFC level following cuprizone treatment, may predict a potential impact on the long-term plasticity of glutamatergic synapses in this area. However, it is still unclear whether cuprizone treatment can modify synaptic plasticity in the mPFC, or if these changes are correlated with alterations in executive cognitive functions. First, AMPA/kainate receptor-mediated fEPSPs were extracellularly recorded in layer 5 of the mPFC, and the input-output (I–O) relationship was examined by

stimulating the excitatory afferents with increasing current intensity (from 0 to 1.0 mA in 0.1 mA increments). Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,8)} = 4.126$, $p = 0.023$, $n = 8$ –9]; a significant effect of stimulating step [$F_{(10,80)} = 165.3$; $p = 0.0001$]; and a significant interaction between factors [$F_{(10, 80)} = 4.571$; $p = 0.0001$] (Fig. 4A, B). While this shift suggests an apparent increase in postsynaptic AMPA/kainate receptor sensitivity, this finding alone remains inconclusive. Indeed, functionally equivalent leftward shifts can independently arise from alternative physiological alterations, such as shifted axonal fiber recruitment, elevated presynaptic release probability, increased postsynaptic membrane resistance, or heightened intrinsic excitability. Consistent with this result and the previously observed increase in mEPSC frequency, the paired pulse (PP) protocol revealed a significant decrease in the PP ratio. This suggests an increased probability of glutamate release from presynaptic terminals [*t*-test $t = 3.995$ $p = 0.004$ vs. CTRL, $n = 5$] (Fig. 4C, D). To evaluate long-term plasticity,

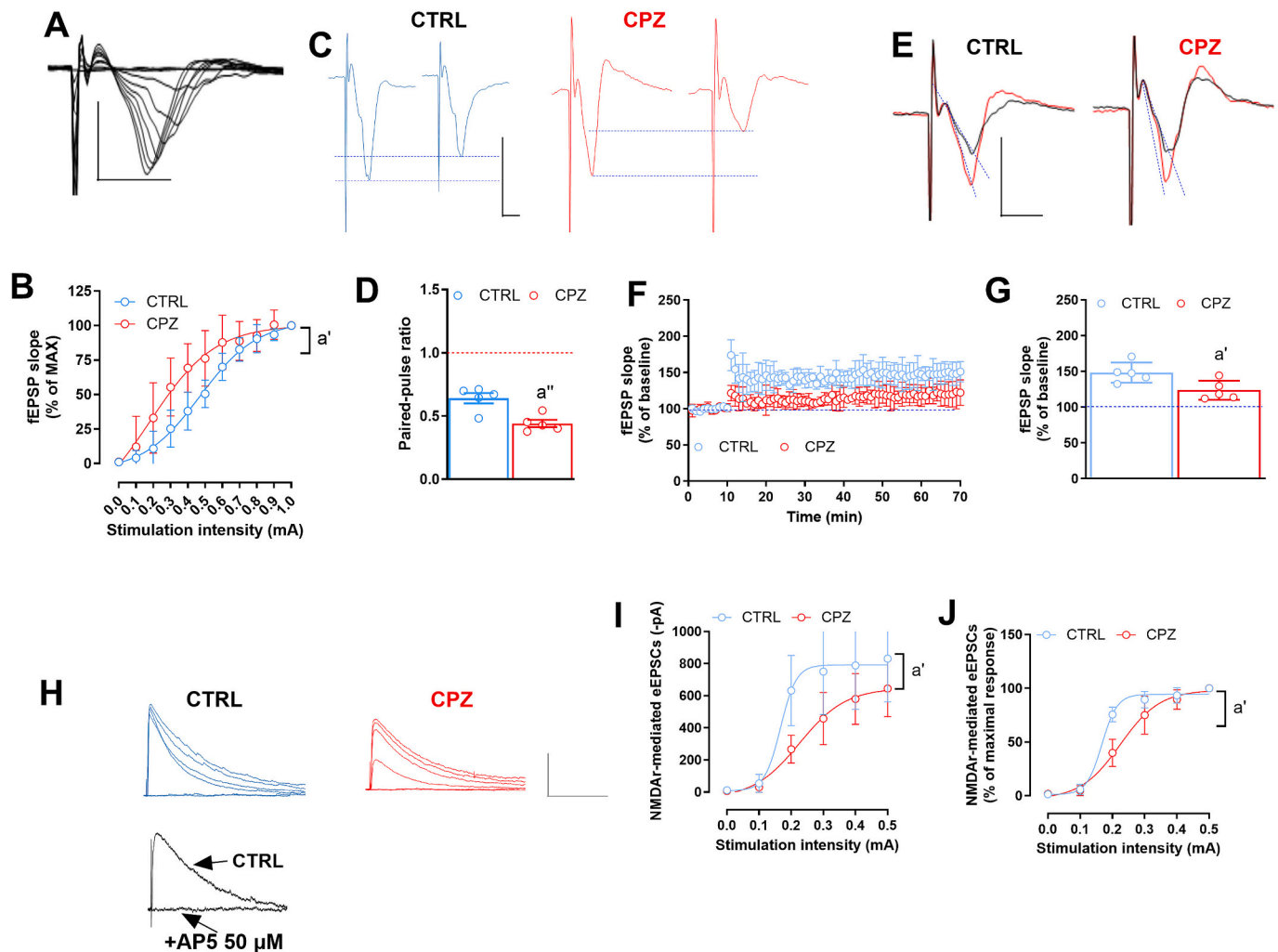


Fig. 4. Effect of CPZ treatment on short- and long-term plasticity in pyramidal neurons of the mPFC. (A) Sample traces of fEPSPs recorded in the mPFC, representing potential responses to increasing electrical stimulation. Scale bar: 1 mV/5 ms. (B) A scatter plot showing the averaged fEPSP slope values as a percentage of the maximal response. $a^* p = 0.001$ vs. CTRL, two-way ANOVA; $n = 8$ –9. (C) Sample traces of fEPSPs recorded in the mPFC representing the paired pulse protocol consisting of two equal stimulations separated by 100 ms. Scale bar: 1 mV/5 ms. (D) Bar graph representing the averaged values of the paired-pulse ratio (obtained by dividing the slope of the second response by the slope of the first). $a^* p = 0.001$ vs. CTRL, unpaired *t*-test, $n = 5$. (E) Sample traces of fEPSPs recorded in the mPFC before (black trace) and 1 h after (red trace) HFS stimulation. Scale bar: 1 mV/5 ms. (F) Scatter plot showing the changes in fEPSP slope induced by HFS. Values are expressed as percentage of baseline in both experimental groups. (G) Bar graph summarising the magnitude of LTP, considering the fEPSP slope values recorded between 50 and 60 min after HFS. Data are expressed as mean percent of fEPSP slope $\pm$ SD from baseline. $a^* p = 0.001$ vs. CTRL, unpaired *t*-test, $n = 5$. (H) Sample traces of NMDA-mediated eEPSC recorded in mPFC neurons of CTRL and CPZ animals at +40 mV in response to increasing electrical stimulation (0–1 mA, 0.2 mA intervals). Scale bar: 50 pA/50 ms. (I, J) Scatter plots representing the averaged values of eEPSC, expressed as absolute values (in pA), or as a % change vs. maximal response (J). $a^* p = 0.001$ vs. CTRL; two-way ANOVA; $n = 5$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

we extracellularly recorded dendritic fEPSPs in layer 5 of the mPFC before and after delivering high-frequency stimulation (HFS) to the excitatory glutamatergic afferents to elicit a stable LTP. We quantified LTP magnitude by comparing the averaged slope value of fEPSPs recorded during the last 10 min (i.e., from 50 to 60 min post-HFS), as a percentage of the baseline value. In CTRL rats, LTP resulted in an apparent increase of $48.3 \pm 14.1\%$ ($n = 5$). In CPZ rats, LTP was significantly less pronounced ($23.8 \pm 13.5\%$, $n = 5$) than in CTRL animals [unpaired t -test $t = 2.817$ $p = 0.0226$ vs. CTRL, $n = 5$] (Fig. 4E–G).

The clear effect of cuprizone treatment on LTP observed at the mPFC level, prompted us to investigate the influence of such treatment on NMDA receptor function in these pyramidal neurons. The involvement of NMDA receptor in this form of long-term synaptic plasticity has been widely demonstrated (Bartlett et al., 2007; Liu et al., 2004; Massey et al., 2004; Morishita et al., 2007). LTP in the mPFC depends on NMDA receptors containing the GluN2A subunit. It has been suggested that subpopulations of NMDA receptors containing either the GluN2A or GluN2B subunit could be used to study recognition memory formation and changes in LTP formation in these areas (Warburton et al., 2013). To explore this aspect, we recorded evoked EPSCs (eEPSCs) mediated by NMDA receptors in the presence of the AMPA receptor selective antagonist NBQX (5 μ M) and the GABAA receptor antagonist bicuculline (20 μ M) in single mPFC pyramidal neurons by holding the membrane potential at +40 mV in response to presynaptic stimulation of increasing intensity (from 0.0 to 1.0 mA, in 0.1 mA intervals). The subsequent bath-perfusion of the selective NMDA receptor antagonist APV (50 μ M) completely blocked eEPSCs confirming that they were mediated by this glutamate receptor subtype (Fig. 4H black traces). The resulting I–O curve of NMDA receptor-mediated EPSCs revealed a decrease in the maximal response. Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,8)} = 4.131$; $p = 0.023$, $n = 5$]; a significant effect of stimulating step [$F_{(1,327,10.61)} = 83.94$; $p = 0.0001$]; and a significant interaction between factors [$F_{(5,40)} = 4.160$; $p = 0.0039$] (Fig. 4I), and a consequent rightward shift in the curve when values were expressed as a percentage of the maximal response. Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,8)} = 16.59$, $p = 0.036$, $n = 5$]; a significant effect of stimulating step [$F_{(1,917,15.34)} =$

304.9; $p = 0.0001$]; and a significant interaction between factors [$F_{(5,40)} = 8.171$; $p = 0.0001$] (Fig. 4J). This suggests a reduction in NMDA receptor function consequent to the cuprizone treatment.

3.7. Effect of treatment with cuprizone on basal extracellular concentration of dopamine in the mPFC and basal blood levels of corticosterone

Animals treated with cuprizone show a significant reduction in the basal extracellular concentration of dopamine in the mPFC (Fig. 5A). The mean dopamine concentration in this group was almost half of that observed in CTRL animals (8.685 ± 5.064 pmol/40 μ l sample in CPZ rats vs. 14.32 ± 5.815 pmol/40 μ l sample in CTRL animals) [$t = 2.871$; $df = 27$; $p = 0.0077$; $n = 15$ animals per group].

Plasma levels of corticosterone were also markedly decreased in CPZ rats compared to CTRL animals (–77%) [unpaired t -test $t = 4.69$ $p = 0.0003$ vs. CTRL] (Fig. 5C). Corticosterone levels were 109.8 ± 17.8 ng/ml in CTRL rats and 23.95 ± 4.2 ng/ml in CPZ animals.

3.8. Elevated plus maze (EPM)

To better evaluate whether treatment with cuprizone might reduce anxiety as suggested by our present results, we tested the behavior of CPZ and CTRL rats in the EPM.

Treatment with cuprizone altered the distribution of arm exploration, increasing the proportion of entries (Fig. 6A) and time (Fig. 6B) in the open arms while reducing the entries in the closed arms. Accordingly, two-way ANOVA revealed a significant main effect for the number of arm entries [$F_{(1,28)} = 9.255$; $p = 0.0051$]; a not significant effect of treatment [$F_{(1,28)} = 1.959$; $p = 0.1726$]; and a significant Arm X Treatment interaction [$F_{(1,28)} = 46.06$; $p < 0.0001$].

Two-way ANOVA revealed a significant main effect the time spent in open vs closed arm [$F_{(1,56)} = 78.09$; $p = 0.0051$]; a not significant effect of treatment [$F_{(1,56)} = 0.00035$; $p = 0.9849$]; and a significant Arm X Treatment interaction [$F_{(1,56)} = 6.4696$; $p < 0.0138$].

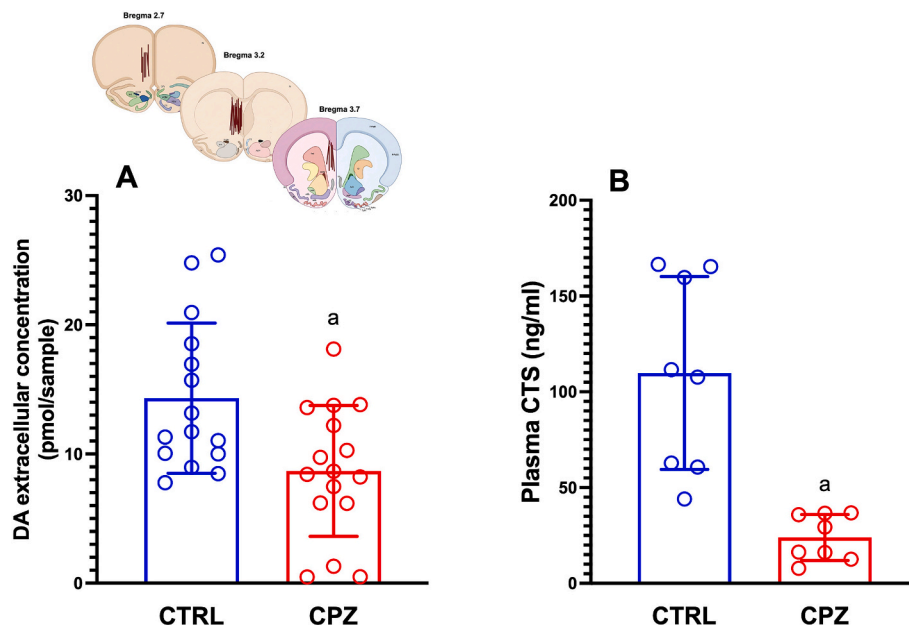


Fig. 5. Effect of cuprizone treatment on dopamine extracellular concentration in the mPFC and on corticosterone levels in plasma. (A) Basal extracellular concentration of dopamine in the PFC of controls and CPZ-treated rats measured by in vivo microdialysis. The experimental unit was the individual animal. Data are expressed as the mean $\pm$ SD of 15 CTRL and 15 CPZ animals, with four samples collected from each animal and averaged before statistical analysis; data are expressed as pmol/40 μ l sample. (B) Bar graph indicating the blood levels of corticosterone in CTRL and CPZ animals. Data are reported as mean $\pm$ SD. $n = 8$ animals for each group. ^a $p < 0.001$ vs. CTRL.

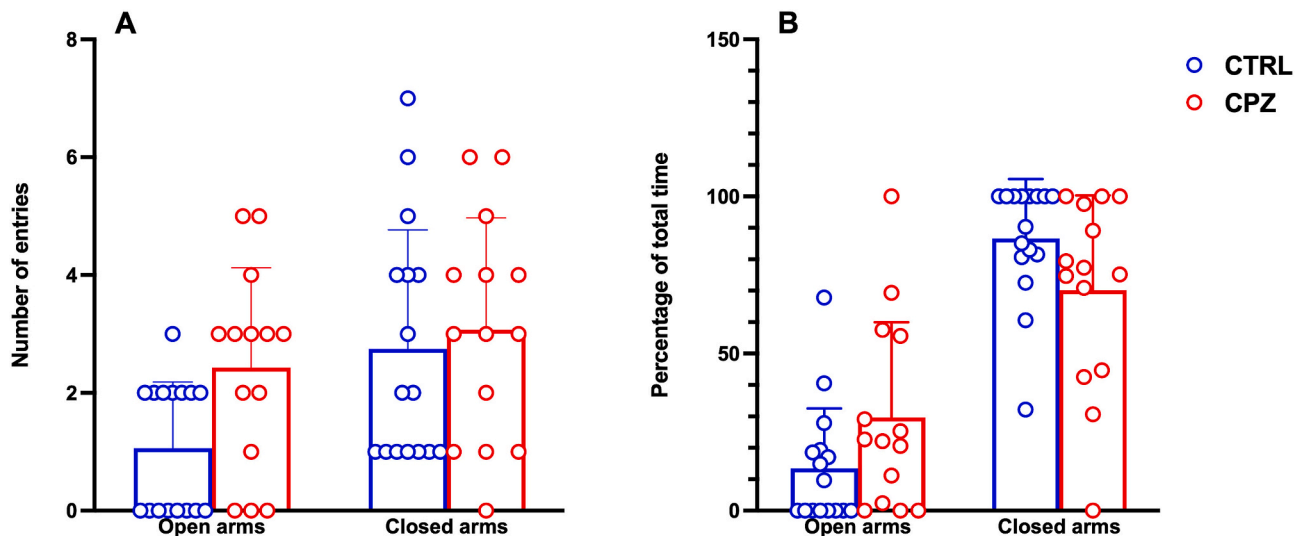


Fig. 6. Performance of rats in the Elevated Plus Maze (EPM) test. Data are presented as time spent in the open and closed arms (A), and percentage of time spent in open and closed arms (B) during a 5-min test session. Experiments were performed on CTRL and CPZ animals after 6 weeks of treatment. Data are expressed as mean $\pm$ SD of 16 CTRL and 14 CPZ animals.

3.9. Effect of treatment with cuprizone on Y-maze Spontaneous Alternation Performance (SAP) and Alternate Arms Returns (AAR)

To evaluate possible differences in spatial memory performance between the experimental groups, we measured the Spontaneous Alternation Performance (SAP) and the Alternate Arms Returns (AAR). As shown in Fig. 7 we did not observe any significant difference in either paradigms (SAP: $67.74 \pm 3.69\%$ vs. $64.49 \pm 5.28\%$ in CTRL and CPZ group, respectively; AAR: $27.16 \pm 4.136\%$ vs. $25.53 \pm 4.34\%$, in CTRL and CPZ group, respectively).

T-test revealed no significant differences for SAP [$t = 0.4693$; $df = 24$; $p = 0.6431$] or for AAR [$t = 0.2397$; $df = 24$; $p = 0.8126$] between the experimental groups.

3.10. Effect of treatment with cuprizone on T-maze DNMT performance

In order to evaluate differences in learning ability and working memory performance in the two experimental groups, we measured the

percentage of errors and latency to reach the reward during training (Fig. 8A, B) and in a DNMT test (Fig. 8C, D). CTRL animals gradually decrease the percentage of errors through the days of training (from 40% to about 10%) suggesting a good ability to learn the alternation between the arms of the maze to obtain the reward. On the contrary, CPZ animals showed a higher percentage of errors since day 1 of training compared to CTRL rats (50% vs 40%), and their learning curve did not show a significant improvement over time. Two-way ANOVA revealed a significant main effect of treatment [$F_{(1,18)} = 8.116$; $p = 0.0107$]; a significant effect of time [$F_{(4,74,73.34)} = 3.928$; $p = 0.0058$]; and a not significant interaction between factors [$F_{(4,74,73.34)} = 1.791$; $p = 0.1386$]. CPZ animals were also slower than CTRL in reaching the reward (Fig. 8A) although the difference was not statistically significant. Two-way ANOVA revealed a not significant main effect of treatment [$F_{(1,26)} = 0.4861$; $p = 0.4919$]; a significant effect of time [$F_{(5,309,138.0)} = 2.741$; $p = 0.0193$]; and a not significant interaction between factors [$F_{(5,309,138.0)} = 1.775$; $p = 0.1176$].

Introducing an increasing delay between the two trials requires the

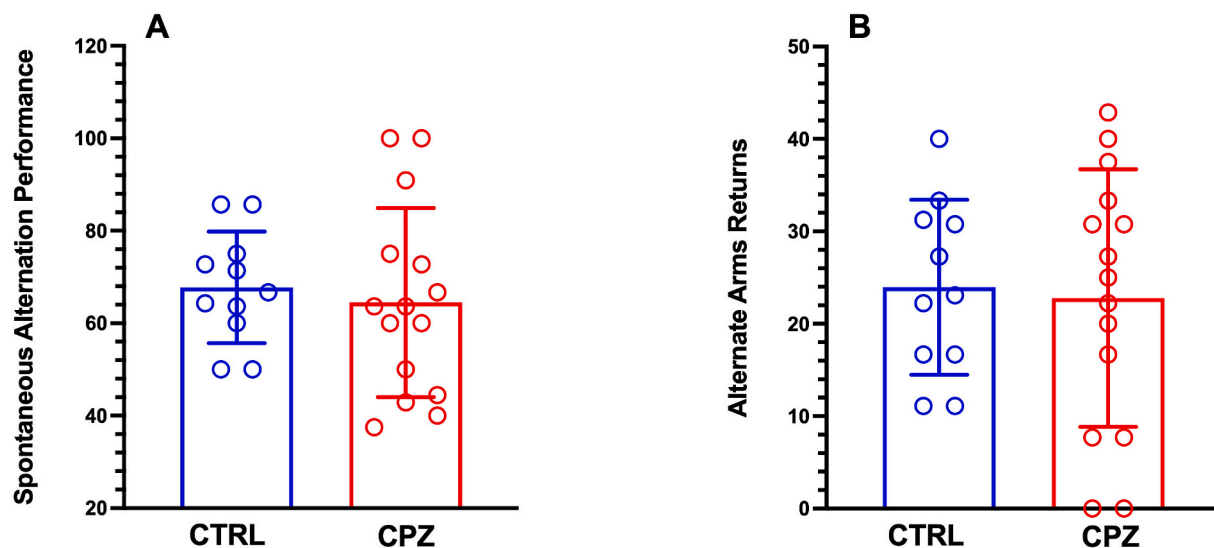


Fig. 7. Effect of cuprizone treatment on spatial memory. (A) Spontaneous Alternation Performance (SAP) and (B) Alternation Arm Returns (AAR) were measured in the Y-maze in CTRL and CPZ animals after 6 weeks of treatment. Data are mean $\pm$ SD of 11 CTRL and 15 CPZ animals.

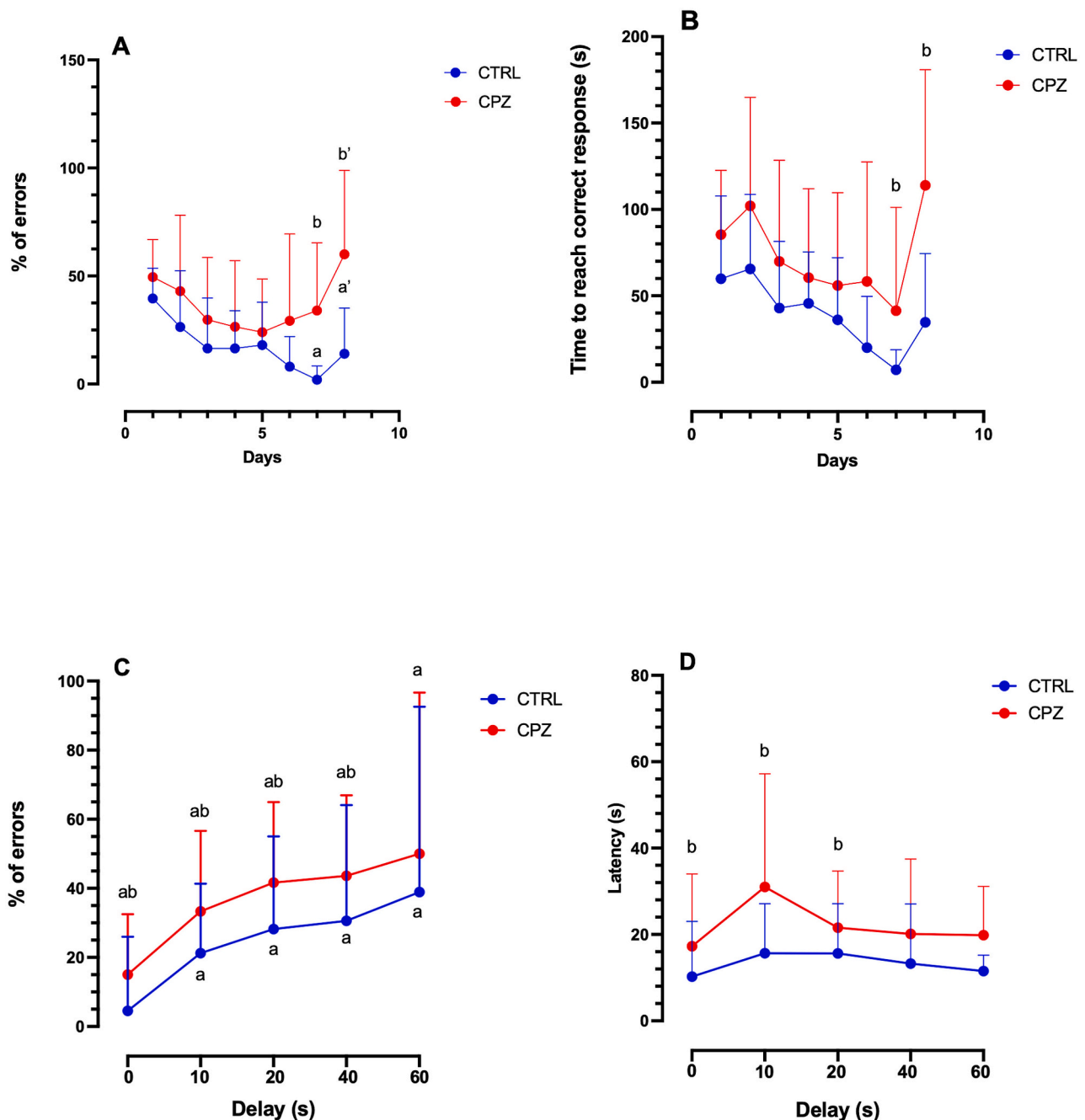


Fig. 8. Effect of cuprizone treatment on working memory. % of errors (A) and time to reach the response (B) were measured in CTRL and CPZ-treated animals after 6 weeks of treatment during the alternation training in the T-maze. % of errors in the DNMT (C) and latency to reach the response in the DNMT (D) in CTRL and CPZ animals. Animals were trained in the T-maze to alternate between the two opposite arms of the maze to obtain a reward. For panels A and B the alternation was forced by closing the left arm during the first trial and leaving both arm open in the second one. For panels C and D an increasing delay (0, 10, 20, 40 and 60 s) was added between the two trials. During the DNMT task, each rat performed multiple trials at each training day and delay condition. The percentage of errors and latency were first averaged for each animal, and these per-animal values were used for statistical analyses. Data are mean $\pm$ SD of 20 CTRL and 10 CPZ animals. ^a $p < 0.05$; ^a $p < 0.01$ vs. time 0; ^b $p < 0.05$; ^b $p < 0.01$ vs. same time point of CTRL.

animals to remember the previous choice in order to make the correct alternation and is therefore considered a good measure of working memory abilities. Cuprizone treatment consistently disrupts the ability of the animals to reach a correct response and animals showed, at all the delays except the last, a higher percentage of errors (Fig. 8C) and a higher latency to reach the reward (Fig. 8D) compared to CTRL rats. Two-way ANOVA of error percentage revealed a significant main effect of treatment [$F_{(1,28)} = 8.175$; $p = 0.0079$]; a significant main effect of delay [$F_{(3,081,106.4)} = 15.16$; $p < 0.0001$]; and a not significant

interaction between factors [$F_{(3,081,106.4)} = 0.1128$; $p = 0.9742$]. Two-way ANOVA of latency to reach the reward revealed a significant main effect of treatment [$F_{(1,46)} = 21.61$; $p < 0.0001$]; a significant effect of delay [$F_{(4,184)} = 2.878$; $p = 0.024$]; and a not significant interaction between factors [$F_{(4,184)} = 0.7817$; $p = 0.5384$].

3.11. Gait analysis

To deeply investigate the effect of cuprizone treatment on motor

skills, we performed a gait analysis on CPZ and CTRL animals using a validated system (Bounds et al., 2019) that allows for a more precise measurement of this parameter. Our results (Fig. 9) showed that animals treated with cuprizone undergo a significant reduction in stride length (13.65 cm vs. 15.99 cm for the CTRL group) [$t = 3.941$; $df = 14$; $p = 0.0015$] and step width (6.15 cm vs. 6.94 of the CTRL group) [$t = 3.417$; $df = 14$; $p = 0.0037$]; while speed was similar across groups (26.59 cm/s vs. 24.96 cm/s for the CTRL group) [$t = 0.3099$; $df = 14$; $p = 0.7612$].

4. Discussion

In the contemporary therapeutic era of MS, the widespread use of highly effective disease-modifying therapies (DMTs) has successfully mitigated focal inflammatory relapses, yet cognitive impairment remains highly prevalent and progresses insidiously as Progression Independent of Relapse Activity (PIRA) (Amato and Portaccio, 2026; Ciccirelli et al., 2024; Sumowski et al., 2026).

While active autoimmune models of MS (e.g., EAE) are valuable for studying peripheral immune infiltration, severe motor impairment can confound cognitive assessment. In contrast, the non-autoimmune cuprizone model provides a targeted approach to isolate key neurobiological features of PIRA, including cortical demyelination and localized microglial activation, without systemic T-cell-mediated motor weakness (Ciccirelli et al., 2024; Dedoni et al., 2023; Zhan et al., 2020). A standardized 6-week protocol reliably inducing cortical myelin loss and glial activation (Zhan et al., 2020) therefore enabled us to investigate the effects of frontocortical cuprizone toxicity on synaptic pathology.

The present study demonstrates that chronic treatment with cuprizone profoundly alters dopaminergic function in the rVTA and disrupts the excitatory/inhibitory balance and synaptic plasticity in the mPFC. These two regions are critically involved in motivation, cognitive control, and stress responsivity (Grace et al., 2007; Arnsten and Rubia, 2012). These neurophysiological alterations were accompanied by marked impairments in PFC-dependent cognitive functions and by a reduction in basal extracellular dopamine levels in the mPFC, indicating a hypodopaminergic mesocortical state. Moreover, cuprizone intoxication also decreased the sensitivity of mesocortical dopaminergic neurons to an acute exposure to a stressful stimulus (see Supplemental material). These results align with the view of MS as a network disease with prominent prefrontal involvement (Chiaravalloti and DeLuca, 2008; Benedict et al., 2002; Foong et al., 1997) and suggest that changes in

mesocortical dopamine signaling induced by long-term administration of cuprizone may be a critical contributor to MS-related cognitive symptoms.

A major strength of our study is the detailed anatomical and functional identification of the affected mesocortical circuitry. While recent clinical literature conceptualizes MS-associated cognitive decline as a ‘network disease’ and hypothesizes that localized neuroinflammation downregulates dopaminergic transmission in the prefrontal cortex (Amato and Portaccio, 2026; Sumowski et al., 2026), direct circuit-level mapping in preclinical models has remained scarce. By combining in vivo microdialysis of extracellular dopamine in the mPFC with ex vivo electrophysiology of both rVTA dopaminergic neurons and mPFC pyramidal cells, we provide robust neuroanatomical and functional evidence that chronic intoxication with cuprizone specifically disrupts the VTA–mPFC axis. Our finding of a ~50% reduction in basal prefrontal dopamine extracellular concentration, coupled with decreased rVTA firing and impaired mPFC LTP, supports a biologically plausible mechanism underlying the correlation between localized structural damage and a defined network synaptopathy (Amato and Portaccio, 2026; Di Filippo et al., 2018).

Our electrophysiological data revealed convergent cuprizone-induced functional changes in both the rVTA and the mPFC, two regions whose reciprocal connectivity is essential for the regulation of motivational and cognitive domains (Zhong et al., 2020). A six-week cuprizone regimen significantly reduced the spontaneous firing rate of rVTA dopaminergic neurons without affecting the amplitude of HCN channel mediated I_h currents. Although I_h has been proposed to contribute to the pacemaker activity of VTA dopaminergic neurons (Seutin et al., 2001), other studies have challenged this view, suggesting that I_h are not a major determinant of their spontaneous firing properties (Neuhoff et al., 2002). Consistent with the latter evidence, the lack of cuprizone effects on I_h in our experiments implies that alternative mechanisms underlie the reduced dopaminergic neuron firing. Our analysis of miniature synaptic events in putative dopaminergic neurons provides a plausible explanation: cuprizone intoxication decreased the frequency, but not the amplitude or decay time, of glutamatergic mEPSCs, with no detectable changes in mIPSC parameters. This pattern is consistent with a presynaptic reduction in glutamate release probability, resulting in a shift of the excitatory/inhibitory balance toward inhibition and an overall reduction in VTA dopaminergic neuron excitability. A limitation of the present study is that VTA dopamine

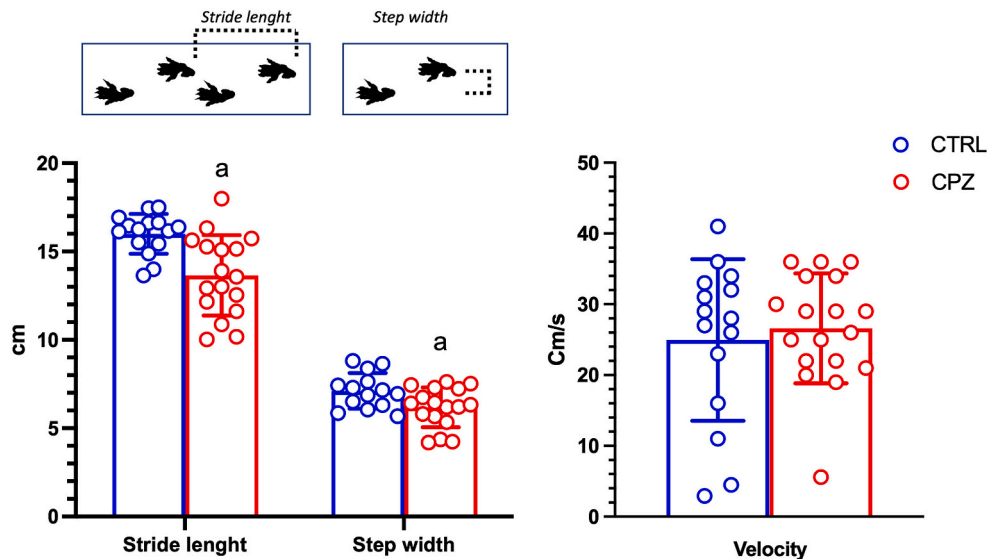


Fig. 9. Effect of cuprizone treatment on gait analysis. Stride length, step width and velocity were evaluated. Stride length was considered as the distance from the heel print of one foot to the next heel print of that same foot; step width was considered as the mediolateral distance between the left and right hind limbs; velocity was expressed as cm/s. Data are mean $\pm$ SD of 14–15 CTRL and 17 CPZ animals. ^a $p < 0.01$ vs. CTRL.

neurons were identified solely using electrophysiological criteria. Consequently, these cells should be considered putative dopamine neurons, as anatomical confirmation of dopaminergic identity and projection specificity fell outside the scope of this investigation.

In contrast to the rVTA, cuprizone increased the intrinsic excitability of PFC pyramidal neurons and enhanced presynaptic glutamatergic transmission, as indicated by the more elevated frequency of mEPSCs, while inhibitory mIPSCs remained unchanged, when compared with control rats. Thus, cuprizone appears to induce an opposite shift in excitability along the VTA–mPFC axis: reduced excitatory drive and firing in dopaminergic neurons, but heightened excitability and glutamatergic input in PFC pyramidal cells. Given the major role of dopamine in the PFC in the regulation of the excitation–inhibition balance (Di Domenico and Mapelli, 2023) such a pattern is compatible with a mesocortical hypodopaminergic state that fails to restrain cortical glutamatergic activity, with potential consequences that could influence network stability and information processing.

Overall, these changes in PFC physiology led to a pronounced impairment of long-term synaptic plasticity. Cuprizone treatment caused a robust reduction of LTP in the mPFC pyramidal neurons, associated with a marked decrease in NMDA evoked currents. This result is in line with previous work showing that D1 receptor activation, and thus dopaminergic input, is crucial for the induction and modulation of LTP in the PFC through regulation of NMDA receptor function (Gurden et al., 1999; Gurden et al., 2000). Our findings therefore support the notion that cuprizone-induced reductions in mesocortical dopaminergic tone may contribute to the impaired NMDA receptor-dependent plasticity and, consequently, to functional deficits within this key cortical region.

Behavioral assessments provide further insight into the functional consequences of these synaptic, cellular, and neurochemical alterations within the VTA–mPFC circuit. Overall, our data indicate that cuprizone-induced toxicity not only perturbs connectivity and neurophysiology but also leads to cognitive, emotional, and motor abnormalities that recapitulate features of neuropsychiatric and neurodegenerative disorders. In the EPM, CPZ rats showed an altered distribution of arm exploration, suggested by an increased proportion of entries and time spent in the open arms and reduced entries in the closed arms, a profile usually interpreted as reduced anxiety like behavior (Pellow et al., 1985). However, in the context of mesocortical dopaminergic hypofunction, this phenotype is more parsimoniously interpreted as reduced emotional reactivity rather than genuine anxiolysis. The reduction in basal and stress-stimulated extracellular dopamine levels in the mPFC, as revealed by microdialysis (see Supplemental Material), is consistent with impaired mesocortical dopaminergic function and aligns with previous evidence linking prefrontal dopamine hypoactivity to altered stress responsiveness and affective blunting (King et al., 1997; Scornaienko et al., 2009; Finlay and Zigmond, 1997; Datta and Arnsten, 2019).

This view is supported by endocrine data. CPZ rats displayed markedly reduced basal plasma corticosterone levels, suggesting reduced hypothalamic–pituitary–adrenal (HPA) axis function, a phenotype often observed in chronic stress and neuroinflammatory models (Calcia et al., 2016). The combination of low corticosterone and mesocortical dopamine dysfunction likely underlies the apparent decrease in anxiety like behavior, suggesting a maladaptive hyporesponsive stress phenotype. Such a profile is highly relevant to conditions including schizophrenia, depression, and MS, where altered HPA axis activity and prefrontal dopamine dysregulation frequently co-occur (Pham et al., 2018).

Our behavioral results in the T-maze DNMT task showed a significant learning and acquisition impairment in CPZ rats starting from the training phase, with parallel performance curves across delays and a notable deficit even at the 0-s delay. This delay-independent pattern, paired with the completely intact spontaneous spatial alternation in the Y-maze, suggests that chronic cuprizone exposure does not induce a generalized, simple memory decay. Instead, it points toward a specific

executive dysfunction, characterized by deficits in rule acquisition, cognitive flexibility, or sustained attention. This interpretation aligns seamlessly with the paradigm shift currently emerging in MS clinical research. Recent landmark studies by Sumowski et al. (2026) and Leavitt (2026) have dismantled the traditional ‘speed-centric’ model of MS cognition, demonstrating that while basic processing speed is often preserved in the modern treatment era, deficits in executive control, attention, and complex working memory maintenance remain prominent. Biologically, these executive deficits are known to be modulated by prefrontal dopaminergic tone and NMDA receptor-dependent long-term synaptic plasticity (Cools and D’Esposito, 2011; Robbins and Arnsten, 2009).

Importantly, cuprizone did not impair performance in the Y maze spontaneous alternation (SAP) or alternate arm return (AAR) paradigms, which are generally considered less dependent on PFC integrity alone and more reliant on the function of distributed hippocampal-prefrontal networks and overall locomotor activity. Preservation of performance in these tasks suggests that cuprizone does not produce a generalized cognitive or motivational deficit but is rather consistent with a preferential impairment of mPFC dependent working memory processes. This dissociation strengthens the link between mesocortical dopamine dysfunction, PFC synaptic alterations, and specific executive deficits.

Motor function was also subtly but detectably affected. Earlier reports using Wistar rats suggested that cuprizone does not cause overt motor deficits (Basoglu et al., 2013; Kolo et al., 2025); demyelination and the significant reduction in nerve impulse conduction velocity in the spinal motor nerves, observed following prolonged cuprizone treatment (Basoglu et al., 2013), lead to mild motor alterations that are not always detectable using standard tests for the assessment of locomotor abilities (Kolo et al., 2025). However, to the best of our knowledge, to date data obtained from instrumental quantitative assessment of motor skills are not available from the literature. Our data extracted from gait analysis revealed a significant reduction in stride length and in step width, without concomitant changes in overall locomotor speed. These findings argue against generalized lethargy or motor suppression and instead point to mild but measurable coordination impairments. Instead, this high-frequency, narrow-stance gait pattern might be indicative of sub-clinical muscle stiffness or mild pyramidal tract rigidity, consistent with the early sensorimotor consequences of cuprizone-induced demyelination along the corticospinal tracts (Basoglu et al., 2013). Such subtle gait alterations may reflect early sensorimotor consequences of cuprizone induced toxicity in corticospinal or cerebellar pathways that are not detectable with coarse measures such as open field locomotion. Similar changes in gait patterns have been reported in pwMS and schizophrenia, disorders characterized by compromised white matter integrity and disrupted frontocortical and subcortical connectivity (Coca-Tapia et al., 2021; Topa et al., 2025).

Taken together, the combination of reduced basal and stress-induced mesocortical dopamine release, reduced basal corticosterone levels, increased cortical excitability, and working memory deficits suggests a broader disruption of executive and motivational control systems. These phenotypes are particularly pertinent for modeling aspects of schizophrenia, depression, and MS, all of which involve varying degrees of cognitive impairment, emotional blunting, and motor disturbances linked to mPFC and VTA dysfunction (Chiaravalloti and DeLuca, 2008; Benedict et al., 2002; Foong et al., 1997). Notably, our data reveal a dissociation between emotional and cognitive outcomes: cuprizone induces an apparent anxiolytic like profile, which might be judged beneficial in isolation, yet concurrently impairs cognitive flexibility, working memory, and motivational engagement. This underscores the complex, domain specific impact of neuroinflammation and demyelination on brain function and cautions against simplistic interpretations of single behavioral readouts.

The lack of pair-fed control might represent a limitation of our study. In fact, CPZ-treated animals consumed substantially less food and displayed reduced body weight. Since treatment began at PND21,

nutritional restriction and altered post-weaning development may have independently affect dopamine function, corticosterone levels, synaptic physiology, and cognition. Future studies incorporating an appropriate pair-fed control group will be required to distinguish the effects of cuprizone exposure from those associated with reduced caloric intake and altered growth.

Moreover, in the present study, we investigated the effects of cuprizone-induced intoxication exclusively in male rats to minimize the variability associated with the estrous cycle in females. However, given that MS has a marked sex-bias in prevalence, clinical presentation, and disease progression, we are currently conducting the same experiments in female rats to determine whether the observed effects are sex specific (Dazzi et al., in preparation).

In summary, cuprizone treatment produced: (i) a hyporesponsive stress phenotype characterized by reduced basal dopamine and corticosterone secretion and attenuated dopamine responses to challenge; (ii) an impairment in PFC dependent working memory with hippocampal-prefrontal dependent tasks as spatial memory more preserved; (iii) subtle gait abnormalities indicative of early motor coordination deficits; and (iv) profound physiological and synaptic alterations within the VTA–mPFC circuit, including decreased dopaminergic neuron firing, altered glutamatergic transmission, impaired NMDA receptor function, and reduced LTP. Altogether, our results validate the cuprizone model as a valuable tool for probing the neurobehavioral consequences of disrupted mesocortical function and for testing therapeutic strategies aimed at restoring cognitive–emotional balance in neuroinflammatory and neurodegenerative diseases, including MS.

Within this framework, cuprizone-induced reductions in rVTA putative dopaminergic neuron firing, glutamatergic drive, and mPFC dopamine extracellular concentration, together with impaired mPFC LTP and NMDA receptor function, may provide a mechanistic link between cuprizone intoxication and executive deficits. The parallel reduction of corticosterone basal levels suggests coordinated disruption of mesocortical dopamine and HPA-axis function, both highly relevant to MS, where chronic inflammation, oxidative/mitochondrial stress, and diffuse white- and gray-matter injury shape long-term cognitive decline (Melief et al., 2021; Meyer-Arndt et al., 2020; Cercignani et al., 2021).

These converging data raise the possibility that targeting mesocortical dopamine transmission—pharmacologically (e.g., PFC-selective dopamine enhancers) or via interventions that normalize PFC–VTA feedback and glia–dopamine interactions—could ameliorate MS-related executive dysfunction without exacerbating mesolimbic side effects.

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CRediT authorship contribution statement

Laura Dazzi: Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giuseppe Talani:** Writing – original draft, Formal analysis, Data curation. **Francesca Biggio:** Writing – review & editing, Formal analysis, Data curation. **Maria Cristina Mostallino:** Writing – review & editing, Formal analysis, Data curation. **Roberta Puliga:** Writing – review & editing, Formal analysis, Data curation. **Massimiliano Pau:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Eleonora Cocco:** Writing – review & editing, Funding acquisition, Conceptualization. **Enrico Sanna:** Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics declaration

Animal subject

This study was conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

This study was approved by the Italian Ministry of Health.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

Data availability

Data will be made available on request.

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