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Early remyelination in the murine model exposed to cuprizone: are there any behavioral alterations?

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Early remyelination in the murine model exposed to cuprizone: are there any behavioral alterations?

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1. Summary

The administration of cuprizone (CPZ) to C57BL/6 male mice during 5 weeks induces extensive and generalized central nervous system demyelination similar to what is found in human multiple sclerosis (MS). However, if the intoxication is stopped, there will be spontaneous remyelination. This unique phenomenon associated with the inexistence of an overt immunologic response makes this model one of the most used for the study of MS-related remyelination. We tried to disclose what is the animals' phenotype during CPZ administration and at the edge of toxic demyelination (week 5) and 2 weeks after its cessation (week 7), which was considered a very early phase of spontaneous remyelination. Using a wide battery of tests comprising rotarod, open field, elevated-plus maze, Y-maze, novel object recognition and splash tests, we evaluated animals' locomotor ability, coordination, anxiety levels, explorative behavior, spatial and long term memory and mood state. We did find trends for a difference at week 5 (W5) between animals exposed to CPZ and healthy controls, namely considering explorative and depressive-like behavior. These trends were reversed at week 7 (W7), meaning that a resolution of CPZ-induced neurotoxicity was achieved at this early phase of spontaneous remyelination. Despite needing further confirmation, in larger samples of animals, these data are relevant, when considering the usage of this model for the study of possible pharmacological interventions promoting remyelination. It produces an early stage of the condition (and literature highlights biochemical mechanisms disrupted by CPZ intoxication that may become therapeutic targets in such an early disease course), which is critical for drugs to be effective. As it is frequently said, in clinical practice, "time is brain" and therapeutic interventions in early disease courses are more likely to have an impact in chronic conditions such as MS.

2. Keywords

Animals

Behavior, Animal/drug effects*

Cuprizone/toxicity*

Demyelinating Diseases/chemically induced

Disease Models, Animal

Multiple Sclerosis

Male

Mice

Mice, Inbred C57BL

3. Introduction

Cuprizone (CPZ) is a copper chelator that has been known for its neurotoxic effects in rodents since 1966[1]. In the past, it was viewed has an invaluable tool for the study of demyelination, however, upon discovery of spontaneous remyelination[2] in mice after suppression of CPZ intoxication, the importance of this chemical compound has been revived. The CPZ-induced demyelination mouse model is being used as the playground of studies focused on augmenting remyelination and therefore providing a new therapeutic approach[3] to the treatment of human diseases were central nervous system (CNS) demyelination is present, such as multiple sclerosis (MS)[4,5] and, to a lesser extent, schizophrenia[6]. CPZ intoxication induces specific oligodendrocyte cell death[7] by a mechanism believed to be related to disturbances in energy metabolism[2,8]. This oligodendrocytopathy leads, unequivocally and directly, to demyelination without axonal damage[9,10], unless there is chronic exposure to the toxic (>12weeks)[11], not only in the corpus callosum and cerebral peduncles[9] as it was previously thought, but also to the whole cortex, mimicking the extensive subpial demyelination[12] found in MS.

Hiremath *et al.*, in 1998[13], were the first group to use C57BL/6 mice instead of Swiss mice and also methodically determined the dose of CPZ related to de- and remyelination processes without depletory hepatic secondary effects – 0,2% w/w. Males were used because female mice were apparently more resistant to CPZ-induced demyelination, possibly due to genetic and hormonal differences[14]. The best age to initiate demyelination was defined to be 6 to 9 weeks of age, as younger mice did not completely recover from CPZ exposition[15] and CPZ should be administered for 5 to 6 weeks in order to achieve peak demyelination[9,10,16], still allowing robust remyelination 3 to 5 weeks after toxicant withdrawn. Some features of this model make it very attractive: it is simple, reliable and easily reproducible; intoxication

can easily be stopped as to induce spontaneous remyelination and, because blood-brain barrier is intact[17], it circumvents the heavy inflammatory component associated with lesions (which is not common to other animal models, such as the experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus mouse models), greatly simplifying biochemical studies of those yet-to-know pathways; CPZ effects are different depending on the rodent strains due to idiosyncratic effects, however they are best characterized in C57BL/6 mice, which is the strain most commonly used for gene knockout studies, making possible single gene studies[18]. The CPZ model also presents some limitations: as it does not induce an overt inflammatory response, it must not be used for studying the impact of inflammatory cells on remyelination processes. Also, it is not possible to evaluate the state of demyelination/remyelination without sacrificing the animals and there are no clear-cut behavioral signs such as paresis (which are present in other animal models[19]) to clearly measure disease burden *in vivo*.

Previous behavioral studies involving this animal model were mainly focused on the demyelination phase and the few works addressing remyelination period neglected the early remyelination period (2 weeks), which is very important in biochemical terms, since it is when oligodendrocytes and dependent pathways are most active and elicit the start of myelin deposition. Most authors evaluated the impact of CPZ in locomotor ability, explorative behavior, anxiety-levels and memory function, essentially with rotarod[20,23], open-field (OF)[15,20,21,22,23,24], elevated plus-maze (EPMT)[15,22,23,25] and y-maze[15,22,25] tests. There are contradictory findings all over the literature: there is no consensus if the treated animals have in fact locomotor impairment or decreased anxiety levels. Furthermore, memory testing is also contradictory: some studies point out decreased Y-maze performance while others not; long-term memory, as tested by novel object recognition test (NORT)[15], seems to be preserved.

Attending to the lack of a behavioral marker of demyelination, to the inconsistent findings in literature, to the inexistence of mood evaluation reports and to the scarcity of studies concerning the remyelination period and, more importantly, the early remyelination phase, our group planned a large battery of tests comprising rotarod and OF tests, EPMT, y-maze test, NORT and one depressive-like behavior assessment test (the splash test) with the purpose of untangling CPZ impairments in treated C57BL/6 mice and reversibility of such deficits during an early remyelination period.

4. Materials and methods

4.1. Animals and treatments

A total of 40 C57BL/6 male mice were acquired from Charles River Laboratories, Barcelona, Spain. They were housed two or four per cage in a temperature- and humidity-controlled animal facility under a reversed light-dark cycle (12:12h). After 2 weeks of acclimatization, on standard chow and water *ad libitum*, the animals were randomly divided into two groups: the control (Ctrl) group, with 20 animals, continued to receive tap water while the CPZ group, with 20 animals, received water mixed with CPZ (C9012 Sigma-Aldrich®, Sintra, Portugal) at 0.2% (w/v). Both groups were fed with standard chow through all the experience. After 5 weeks (13 weeks of age), 10 animals from each group (Ctrl and CPZ W5 groups) were submitted to behavioral testing and sacrificed. All the 20 remaining animals (Ctrl and CPZ W7 groups) were given normal tap water for 2 more weeks in order to allow spontaneous remyelination in the CPZ group (Fig. 1). At this point (W7) they were submitted to the same behavioral protocols and subsequently sacrificed. The animals sacrifice took place two days after behavioral testing: they were subjected to intraperitoneal anesthesia with a 2 mg/kg of a 50 mg/kg pentobarbital (Sigma-Aldrich, Sintra, Portugal) solution and then sacrificed by decapitation. Animal's body weight was registered (KERN® CB 6 K1, Germany) in three time points: beginning of the experiments, W5 and W7. All experiments were conducted according to the European Community Council Directive on Animal Care 2010/63/EU, transposed to Portuguese law in Decreto Lei nº 113/2013.

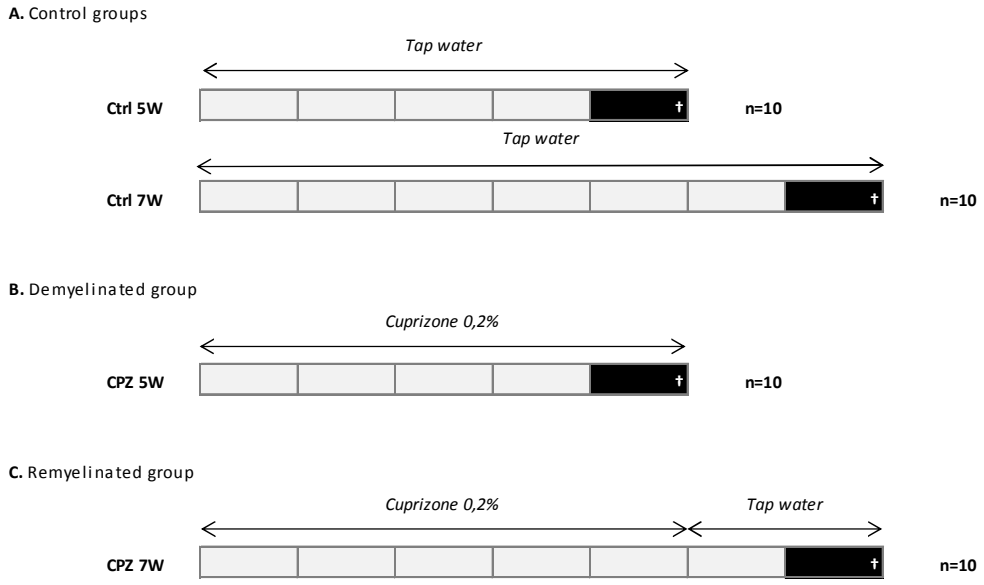

Figure 1. Experimental protocol. A. Control groups. Ctrl W5 and Ctrl W7 were given normal tap water for 5 and 7 weeks, respectively. **B. Demyelinated group.** CPZ W5 was given water with CPZ 0,2% (w/v) for 5 weeks to induce demyelination. **C. Remyelinated group.** In order to study the early remyelination period, CPZ W7 group was given CPZ 0,2% (w/v) for 5 weeks and then normal tap water for 2 weeks more. Behavioral tests were conducted in the 5^{th} and 7^{th} weeks (black rectangles in the image).

4.2. Behavioral tests

A total of 6 behavioral tests were distributed during 4 days at W5 and W7. The most anxiogenic tests were performed in last place. All the tests took place during the light phase of the day and the animals were brought to the testing room 30 min before the trials. All tests were conducted in a low–intensity light environment, with the exception of the elevated-plus maze test which was conducted with maximum-light intensity. The devices were cleaned between experiences with ethanol (Aga®, Prior Velho, Portugal) at 60% (w/v). OF test,

EPMT, y-maze test, NORT and splash test were recorded with a Microsoft® LifeCam Cinema and, with exception of the last, were analyzed using the behavior tracking software Any-Maze®.

4.2.1. Rotarod test

Motor coordination, balance and learning ability were evaluated through a series of 4 protocols of increasing difficulty executed in a speed-regulated rotarod device (Panlab Harvard Apparatus®, Cornella, Spain). The first protocol consisted on 5 rotations per minute (rpm) during 60 seconds (s); the second of 10 rpm during 60 and the third of 10 rpm during 60s followed by 15 rpm during 100s. The last protocol was executed 4 times, a first attempt in the third day of experiences and three attempts in the last day. It consisted of 15 rpm during 60s followed by 25 rpm during 100s. The speed transition had place in a 10s-period with an increase of 1 rpm per second. Animals were placed on the rotating cylinder at an angle of 45°. The number of falls and the time the animal stayed on the cylinder per essay were recorded.

4.2.2. Open-field test

Motor ability, anxiety, explorative behavior and habituation pattern[26,27] were measured in an OF apparatus, which consisted of a 45x45cm light-grey box surrounded by 40cm high walls. Two areas were defined: the peripheral zone (PZ), which consisted on the area 10 cm from the wall, and central zone (CZ), comprising the remaining area. Mice were placed in the center of the arena at the beginning of the test and allowed to move feely during 15 minutes. The tests were recorded and posteriorly evaluated by the behavior tracking software Any-

Maze®. Total travelled distance during the test was calculated, as well as the central travelled distance. These calculations were made considering the whole 15 minutes period and also 3 time segments (5, 10 and 15 minutes), in order to evaluate the habituation pattern of the animal to the new environment.

4.2.3. Elevated-plus maze test

The EPMT was used as a measure of anxiety and general activity levels[28]. The maze consisted of two opposed open arms (OA) (30x5cm) and two closed arms (CA) with the same size at 90° angles to the open arms. They were connected by a central area (5x5cm) and enclosed by 15cm-high walls. The room light was at maximum intensity and the device was elevated 1,25m above the floor. At the beginning of each test, a single animal was placed in the central area facing an open arm and allowed to freely explore the maze for 10 minutes. The test was recorded and posteriorly analyzed by the tracking software Any-maze®. Animal's entry into any arm was considered only when 95% of its body crossed from the central area into that arm. The number of entries in the open arms, the time spent in the open and closed arms and also the total travelled distance in the device were calculated. The amount of time spent in the open arms was expressed as a ratio to the test time of 10 minutes.

4.2.4. Y-maze test

Y-maze test measures the spatial working memory based on simple recognition of the surrounding environment[29]. The y-maze consists of three similar arms (30x6,5cm) with 15cm walls diverging from their central connection at 120°. The arms were labeled as 1, 2 and 3. At the beginning of the test a single animal was placed at the end of arm 1, facing the center and

allowed to freely move in the maze for 8 minutes. The test was recorded and then arms entries sequence was registered by the tracking software Any-maze®. The number of complete alternations was manually calculated. An alternation was defined as successive entries into three different arms on consecutive triple sets (e.g. 123 is one alternation, as any other combination of these 3 arms; so the sequence 123231231 has 5 complete alternations – 123, 231, 312, 123 and 231). The spontaneous alternation behavior (SAB) was presented as a percentage according to the following formula: SAB=number of alternations/(arms entries-2)x100.

4.2.5. Novel object recognition test

The long-term memory was evaluated through the novel object recognition test (NORT)[30].This test comprises two sessions. In the first session, the animal was presented to two objects that were similar in size, but different in shape and color, disposed in a box (45x45x45 cm), diagonally at 18 cm from the nearest corner. The animal was placed at the center of the box at the beginning of the test and allowed to explore it for 10 min. Twenty-four hours after the first session, the animal was presented to the same box, with one novel object replacing an object used in the first session. The trails were recorded and analyzed by the tracking soft-ware Any-maze®. The mouse was considered to be exploring the object when its head was within 8 cm of the object. The ratio of time exploring the novel object to the total time exploring both objects in the second session and also the total time exploring both objects in the first session were determined.

4.2.6. Splash test

The splash test is based on the evaluation of the grooming behavior, which is a form of motivational behavior related to self-care[31]. Its decrease is considered as the counterpart of some depression symptoms as apathetic behavior. The test consists in the application of 10% (w/v) sucrose (Sigma-Aldrich®, Sintra, Portugal) on the back of the mouse. Because of its viscosity, the solution dirties animal's fur and compelling him to initiate a grooming behavior. The test was performed individually in an opaque box (30x30x18cm) with wooden chips at the bottom. A single animal was placed in the center of the box and was given 2 minutes for habituation to the new environment. Then the sucrose solution was added and the animal behavior was recorded for 5 min. Posteriorly, time spent in grooming was counted and presented as a percentage of the total time.

4.3. Statistical analysis

The normal distribution of the data was assessed by Shapiro-Wilk test. According to the result, differences between treatments (Ctrl and CPZ) were tested by independent Student's t- test or Mann-Whitney U test. The mixed-ANOVA analysis was used to test for differences in the rotarod and OF tests performance evolution. Differences were considered for a 95% confidence interval ($p<0.05$). The results were presented as means ± standard deviation (SD). Statistical analysis was obtained using IBM® SPSS® Statistics version 20.

5. Results

5.1. Body weight and general outcome

Mice body weights were measured at three time points (at the beginning of the experiment, week 5 and week 7). All the animals increased weight from the basal point, however at W5 the treated animals had gained less 2.41% than controls ($p=0.422$). By W7, both Ctrl and CPZ groups increased weight compared to W5 and the difference between these groups reduced to 1.54%. The gain in weight for the Ctrl W7 and CPZ W7 groups since the beginning of the experiment was 14%.

It was noticed that some animals have been barbered. They had their back fur removed in a pattern that was unique to each one[32]. There were 9 barbered animals, 2 from Ctrl W5, 3 from CPZ W5 and 4 from Ctrl W7. No deaths were registered during the experimental period.

5.2. Rotarod test

CPZ-treated animals showed preserved locomotor and motor coordination abilities as there were no differences in the time on the device between treated (CPZ W5 and CPZ W7) and control animals (Ctrl W5 and Ctrl W7) in any essay (t-test, *p>0,05*). CPZ-demyelinated mice also revealed preserved learning ability, as mixed-ANOVA test did not find altered temporal evolutions (**Fig. 2. A.** Ctrl W5 vs CPZ W5, *p=0,219* and **B.** Ctrl W7 vs CPZ W7, *p=0,457*).

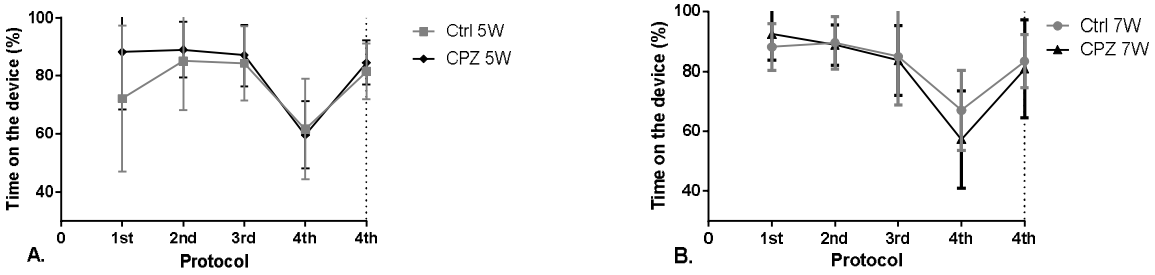

Figure 2. Rotarod test – A. 5th week and B. 7th week tests. There were no differences between Ctrl and CPZ groups in motor coordination and locomotor ability when considering individual protocols (t-student, *p>0,05*) or temporal evolution (mixed-ANOVA week 5, *p=0,219* and week 7, *p=0,457*). The learning ability also seems to be preserved in treated animals, as it can be assessed by the increased time on the device in the last 4th protocol, when compared to the first attempt (**A.** CPZ 5W 59,68±11,68 vs. 84,59±7,67; **B.** CPZ 7W 57,25±16,37 vs. 80,90±16,47). Data are expressed as mean±SD.

5.3. Open-field test

OF test studied animals' locomotor ability and their anxiety levels by measuring the total traveled distance and the percentage of distance traveled in the center of the field, respectively. These variables were analysed considering the whole 15-min test (**Table 1.**) and 5-min periods (**Fig. 3**). The former analysis (**Table 1.**) showed no differences between Ctrl W5 and CPZ W5 groups. The segment evaluation analysis showed Ctrl and CPZ groups had similar profiles and, therefore, similar adaptation/habituation to the new environment. We also tested for differences in immobile time, but there were not any (data not shown).

		Ctrl W5	**CPZ W5**	***p-value***	**Ctrl W7**	**CPZ W7**	***p-value***
Open-field	Total distance (m)	**48,713** ± 6,661	**47,216** ± 10,445	*0,707*	**49,887** ± 9,161	**50,917** ± 11,184	*0,824*
	Ratio central:total traveled distance (%)	**11,57** ± 2,361	**10,361** ± 4,646	*0,473*	**11,629** ± 3,088	**11,398** ± 2,220	*0,850*

Table 1. Open-field test. CPZ effect in the total distance traveled by the animals during the 15 min essay was assessed. The ratio of traveled distance in the center of the OF device was calculated. At the end of demyelination period (W5), treated animals (CPZ 5W) presented a normal locomotor ability and anxiety levels compared to the control group (Ctrl W5). Data are expressed as mean±SD.

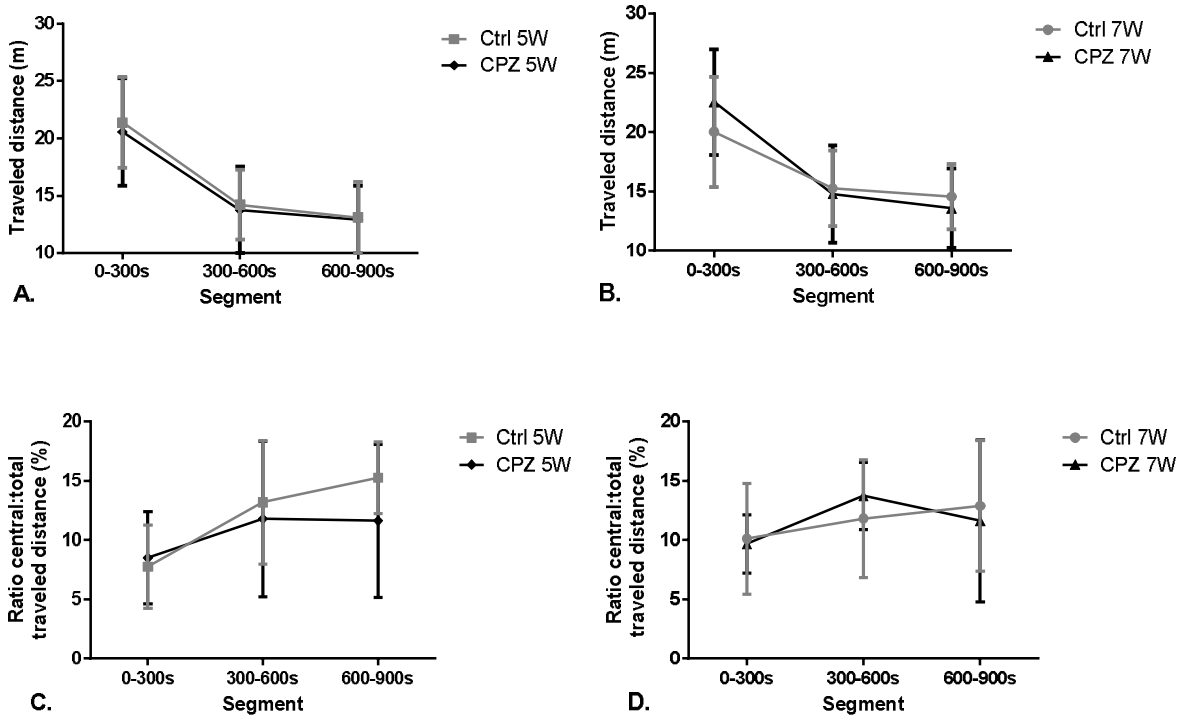

Figure 3. OF test – segment evaluation. We considered the traveled distance (A. and B.) and the ratio central:total traveled distance (C. and D.) performed in each block of 5 minutes of the 15 minute-OF assay. Controls and treated animals presented similar profiles (mixed-Anova test, *p>0,05*) at the end of demyelination period (A and C.) and at the early remyelination period (B. and D.). Data are expressed as mean±SD.

5.4. Elevated-plus maze test

Ctrl W5 and CPZ W5 animals spent almost the same time in the OA (Ctrl W5=55,69±31,60s vs CPZ W5=55,41±46,10s) and traveled similar distances (Ctrl W5=15,58±2,26m vs CPZ W5=14,35±3,22m) (**Fig. 4**). CPZ-treated animals spent less time than controls in the CA (Ctrl W5=350,59±75,39s vs CPZ W5=324,46±70,70s), but this difference did not achieve statistical significance. At week 7, both groups spent the same time in the OA (Ctrl W7=363,43±81,30s vs CPZ W7=372,65±61,73s).

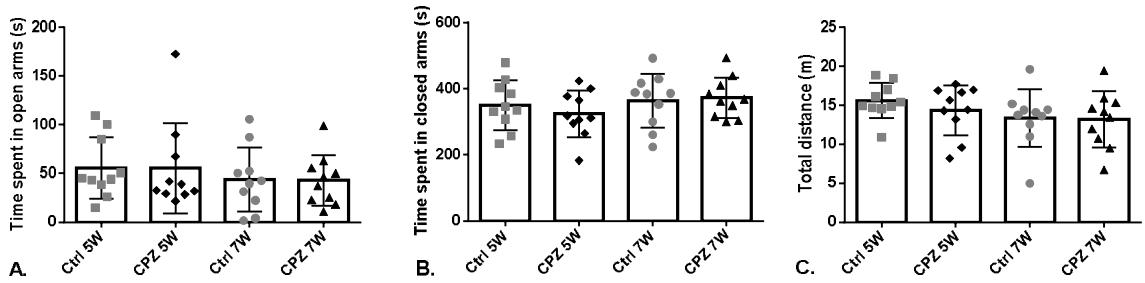

Figure 4. Elevated-plus maze test. Treated animals did not show increased levels of anxiety (A. and B.) or impairment of the locomotor ability (C.). Data are expressed as mean±SD.

5.5. Y-maze test and NORT

CPZ did not induce impairment of short- or long-term memory, as assessed by Y-maze test and NORT, respectively (Fig. 5). However, despite not reaching statistical significance, demyelinated animals explored the objects longer than controls and this contrast was reversed at W7 (Fig. 5. B.)

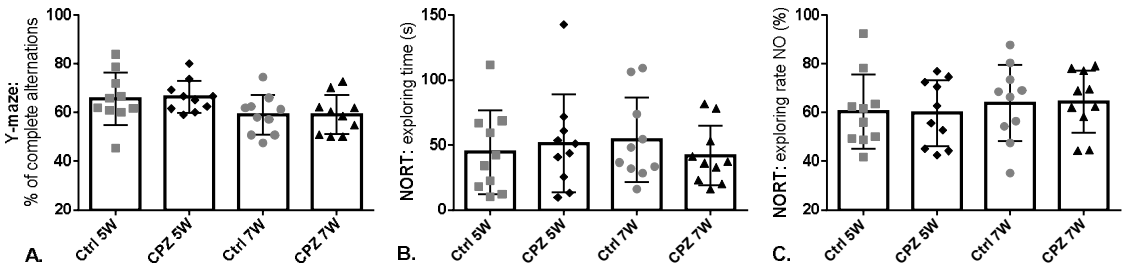

Figure 5. Memory testing. A. Spatial working memory was tested using Y-maze test. The ratio of complete alternations was similar between Ctrl and CPZ groups in both assessments. **B. and C. NORT evaluated animals' long term memory and explorative behavior.** There were no statistical differences between treated and non-treated mice. However CPZ W5 animals explored the objects during more time, while at W7 this was not detected (B. Ctrl W5=44,56±32,21s vs CPZ W5=51,23±37,81s; Ctrl W7=53,84±32,55s vs CPZ W7=41,88±22,76s). Data are expressed as mean±SD.

5.6. Splash test

The splash test assessed animals' mood. The CPZ W5 animals showed a slightly anhedonic state, when compared to controls (Fig. 6. A.). This tendency for depressive behavior disappeared with the withdrawal of CPZ from the diet and by week 7 treated animals showed increased grooming behavior.

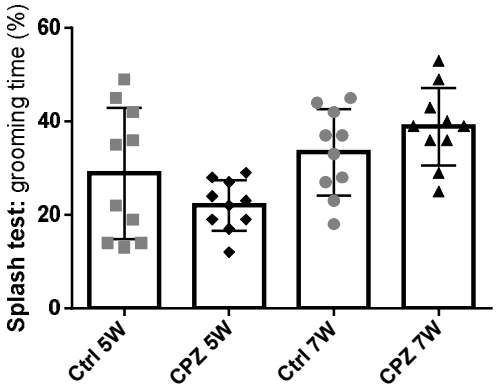

Figure 6. Mood evaluation (a decreased grooming time is associated with an anhedonic state). There were no statistically significant differences (t-student and Mann-Whitney tests, *p>0,05*). However CPZ W5 animals spent less time grooming than controls (Ctrl W5=28,90±14,04% vs CPZ W5=22,00±5,35%). By week 7, those animals previously exposed to CPZ presented an increased grooming time (Ctrl W7=33,40±9,20% vs CPZ W7=38,90±8,34%). Data are expressed as mean±SD.

6. Discussion

The main aim of this study was to behaviorally characterize the CPZ induced-demyelination C57BL/6 mouse model and to identify differences between controls and treated animals that could act as longitudinal phenotypical markers of demyelination and remyelination in progress. In order to achieve this goal, we performed a large battery of tests focused in several skills. More importantly, the same ability was evaluated by different tests, looking for coherence among results, which are summarized in **Table 2**.

	Locomotor activity	**Anxiety-like behavior**	**Explorative behavior**	**Memory**	**Depressive-like behavior**
Rotarod test	↔	−	−	−	−
OF test	↔	↔	−	−	−
EPMT	↔	↔	−	−	−
Y-maze test	−	−	−	↔	−
NORT	−	−	↑	↔	−
Splash test	−	−	−	−	↓

Table 2. CPZ W5 versus Ctrl W5. There was a marked coherence among different tests evaluating the same skill. Demyelinated animals presented preserved motor ability, anxiety levels and memory. They had increased explorative behavior, as assessed by NORT. Accordingly to splash test's results, CPZ W5 animals were also more depressed than controls. (−) the skill was not evaluated by that test; (↔) there were no differences between Ctrl W5 and CPZ W5. (↑) CPZ W5 presented increased ability compared to controls; (↓) CPZ W5 presented decreased levels of performance, when compared to controls.

Overall, despite not reaching statistical significance, we observed a tendency towards a difference regarding the explorative behavior and animals' mood (as revealed by NORT and splash test) between Ctrl W5 and CPZ W5 that not only disappeared at W7, but even reversed. When considering locomotor ability, memory and anxiety levels, demyelinated animals presented a similar behavior to controls. Although the decreased time in closed arms found at W5 (Fig. 4 B) could mean demyelination-induced decreased anxiety-levels, this cannot be effectively assumed. If the animals were not in the CA, they had to be in the OA or in the central square. As there were no differences in OA time, we must assume increased time in the central square, a behavior that is not associated with decreased anxiety.

It is difficult to compare these results with the current *state of the art,* since former studies were not coherent: some authors found decreased locomotor ability while others did not. Xu H. *et al.*[22,25] found increased OA time in EPMT suggesting decreased anxiety levels, though they did not find increased central distance travelled in OF test, as it was expected. Also Makinodan *et al.*[15] and Zainana A. *et al.*[23] did not find increased OA time in EPMT, yet the former used female mice and the later a higher CPZ concentration (0.3% w/w). Makinodan *et al.*[15] were also the only group, so far, that had studied CPZ effects in NORT performance and they did not find differences. When considering Y-maze test performance, the literature points out a decreased number of alternations performed by demyelinated animals. There are no studies describing splash test. This controversial situation is quite surprising, given the relevance of the CPZ-induced demyelination mouse model.

Our results point out that probably there are no phenotypical differences after 5 weeks of demyelination between treated and non-treated animals regarding motor skills, short-term memory dysfunction or anxiety. However, long-term memory and anhedonia seem to be the first domains to be affected by CPZ intoxication and with larger groups of animals maybe we will be able to evidence differences with statistical significance. This is quite relevant for

future research and it is congruent with what we know to be the most appropriate approach for the study of remyelination in MS clinical trials, using new magnetic resonance imaging techniques – cortical lesions are those that present the best properties for measuring remyelination outcomes (such was water magnetic transfer imaging) and those cortical lesions are mainly involved in cognitive dysfunction associated with the disease. Thus, our model seems to reproduce a very early phase of the disease, with associated cognitive dysfunction, but with no physical impairment. Since "time is brain", this will be of notorious relevance to test the effects of early pharmacological interventions in the future, promoting precocious brain repair.

During the early remyelination period, besides the reversion of the differences found in W5, we did not find any other dissimilarity between groups. As we were the first group to test for differences in such a short period of remyelination (2 weeks), it is impossible to compare these results with previous studies.

These findings were congruent with the hypothesis of a CPZ-induced demyelinating process that mimics MS-subpial demyelination. Instead of the affection of distinct pathways with a straightforward implication in observed behavior, the identified alterations suggest a more general and subtle process that, more importantly, can be reversed by ending the toxic stimulus. But CPZ W7 animals presented not only levels similar to controls: they were less depressed and presented a decreased exploration behavior, probably related with a proper functioning of long-term memory, as assessed by NORT. It seems there was a "rebound effect" during the remyelination period. Can this be related to the rebound found in the levels of subventricular cells during the remyelination phase[33]? This is a question to be addressed in future studies.

Another thought-evoking aspect of this work is the huge standard-deviation found for almost all the tests. We were not expecting such variability. There are some factors we can

indicate as putative causes of such findings: 1) the effect of the barbed animals; 2) the different number of animals per cage and 3) CPZ model intrinsic variability. The presence of barbed animals, also known as "the Dalila effect", is an intrinsic aspect to several mouse strains, including C57BL/6. Normally, in C57BL/6 its presence is associated with dominant, sexual or maternal behavior[34]. Since we only used male mice in our study the most plausible cause for barbering is the social dominance effect. In this situation the dominant males, usually heavier, with their fur and whiskers preserved, will pluck receiver's fur and whiskers during periods of mutual grooming. Sarna J. *et al.*[32] showed that barbered animals had preserved brain size but presented reduced dendritic density which will have further impact in brain function and organization. It is not known what triggers barbering or how the dominant male is selected. It is also not known if CPZ increases "the Dalila effect". In our study, we had 9 barbered animals (23%), a number that is similar to what is described in literature[32,35]. Regarding the second point, we knew from the start that different housing conditions would lead to a bigger standard deviation[36,37,38], however due to logistical limitations this was the only available possibility. Finally, considering the third aspect, there are reports about the variability found in the CPZ mouse model[39], but nothing can be done to circumvent it, since it is inherent to the model.

Looking forward, our group's next step will be a biochemical study to understand the level of demyelination and remyelination in weeks 5 and 7, respectively. If we prove that these processes are in fact happening at a molecular level at the same time the phenotypical alterations are being established, we may open a new rational approach of the CPZ mouse model: MS-like subpial demyelination[12] with no lesion of major pathways may be tracked by splash test and NORT, at least in an early phase of the disease. This would be a precious contribution for the role of CPZ model in therapeutic studies focused in developing strategies to treat MS before irreversible lesions of major pathways occur.

In conclusion, despite not reaching statistical significance, we did find behavioral aspects that had not been previously described and that, at least, deserve to be further explored, in larger samples of animals, since they make biological sense and are in line with what is expected in the human condition. If those aspects further prove to be different between groups, we may establish explorative behavior and mood as the most sensitive behavior markers of demyelination and early remyelination in this animal model. This may open new paths of research, trying to find molecular and therapeutic targets to promote remyelination in MS, which is widely desired, both for patients and for physicians taking care of people diagnosed with this neurological condition.

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