Long lasting behavioural effects on cuprizone fed mice after neurotoxicant withdrawal.

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Keywords: cuprizone, demyelination, remyelination, neurobehavioral screening, motor coordination and anxiety.

Abstract

Destruction of the myelin sheath in the central nervous system (CNS) is prominent in many clinico-pathologic conditions. Among animal models that reproduce the pathological features of de- and remyelination processes, the mouse model of cuprizone administration is widely used. Both hyperactivity and motor impairment have been reported upon cuprizone exposure. The aim of the present study was to assess behaviour in mice after CPZ withdrawal.To summarize, animals showed hypo-activity and deficits in motor coordination when they were subjected to acute demyelinating insult while minor exploratory activity, impairment in motor coordination and lower anxiety levels emerged when remyelination was reached following cuprizone withdrawal. A recovery period of 6 weeks after removal of CPZ was not accompanied by a similar return of normal activity indicating long lasting behavioural effects caused by this neurotoxicant. Specifically, the recovery group showed impairments in neurological functions involved in sensorimotor, neuromuscular, motor coordination and the capacity to cope with a stress-inducing event.

Highlights

1. Demyelinated animals showed less grooming activity and worse motor coordination.
2. At 6 weeks, CPZ-fed animals showed impairments in sensorimotor and exploratory behaviour.
3. Animals undergoing remyelination displayed less rearings, worse motor coordination, gait abnormalities and anxiety.
4. At 10 weeks, CPZ-fed mice displayed worse limb coordination and an anxiogenic response.
5. The recovery group showed dysfunctions in sensorimotor, neuromuscular, motor coordination and the capacity to cope with a stress event.

1. Introduction

Progressive loss of myelin in the central nervous system (CNS) is a common hallmark in the physiopathology of many clinically relevant diseases [1]. Several animal models such as those developed for the evaluation of experimental autoimmune encephalomyelitis (EAE) and infection with Theiler's virus are currently available for the study of de- and remyelination processes (for review see [2]). One of the best well characterized demyelinating models is the cuprizone (CPZ) murine model [3]. Several hypotheses have been proposed [3,4,5] but it is still unclear why this neurotoxicant causes oligodendrocyte cell death [6]. The physiopathology of the CPZ model has been widely studied with respect to variations in the timing and dose (for review see [7]). Intoxication with 0.2% of CPZ causes copper metabolic disruption and death of myelinating oligodendrocytes [4,5,8] which leads to lower levels of myelin proteins [5] originating severe demyelination in multiple white matter tracts [9,10]. As a result of demyelination, there is an activation of innate immunity [11,12], astrogliosis [4] and the migration of oligodendrocyte precursors cells (OPCs) to the demyelinated lesion where proliferate to mature oligodendrocytes [5,13,14]. Subsequently, a spontaneous remyelination while ongoing CPZ feeding is observed [3]. After 2 weeks of CPZ withdrawal, a second attempt at remyelination occurs [3]. In a previous study, we reported pronounced behavioural dysfunctions when mice were exposed to CPZ [15]. Indeed, we demonstrated that concurrent with the start of demyelination, animals showed an increase in CNS activity and lower anxiogenic response to the novelty. At 5 weeks of CPZ exposure, when maximum demyelination occurs, equilibrium was affected and sensorimotor reactivity was also overwhelmed. Further, rota-rod analysis revealed that the CPZ group had weaker motor co-ordination than the controls. Finally,

we reported that the recovery group showed an impairment in the rota-rod learning task [15].

Thus, with the rationale that behavioural deficits are markedly present when mice given a diet containing CPZ, we directed the present investigation to characterize the mice behaviour once CPZ is removed from the diet.

2. Material and methods

2. 1. Animals

A total of 144 C57BL/6 male mice at age of 6–7 week were purchased from Charles River Laboratories (Barcelona, Spain). Upon arrival, the animals were housed 12 mice per cage and kept under standard conditions (12 h light/dark cycle with 6:00/18:00 lights on/off, room temperature of 21±2ºC and food and water *ad libitum*). All procedures were approved by University Rovira i Virgili Institutional Animal Care and Use Committee and were in accordance with NIH guidelines for the use of animals in research and the Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (2010/63/EU).

2. 2. The cuprizone mouse model

After 1 week of habituation period, mice were sorted in animals fed with regular diet (controls) and animals subjected to a diet supplemented with 0.2% CPZ supplied by Harlan laboratories (Madrid, Spain) for either 3 or 6 weeks (treated). In the recovery group, mice were fed with CPZ for 6 weeks, followed by a 6 weeks period fed on a regular chow diet free of CPZ. At certain time points (0, 3, 6, 8, 10 and 12 weeks), mice were tested for 2 consecutive days to measure motor activity, anxiety, motor resistance, coordination and motor learning and sensorimotor reactivity. Thus, animals were classified as controls (C0, C3, C6, C8, C10 and C12) or treated animals (T3, T6, T8, T10 and the recovery group). Tests were administered as follows: the first day animals were evaluated by an open field, black and white box, body suspension wire, functional observational battery (FOB) and a rota-rod (training trials); the next day animals were evaluated in a rotarod (accelerated rotarod task), a body suspension wire and in a

footprint task. Animals were sacrificed the day after the last behavioral testing. Figure 1 shows a schematic drawing of the experiment.

2. 3. Brain sample collection and tissue evaluation

After behavioral assessment mice were deeply anesthetized by an i.p. injection of ketamine and xilacine at 80 and 120 mg/kg, respectively (Sigma-Aldrich, Madrid, Spain); and then transcardially perfused with 0.1% phosphate buffered saline (PBS) or 4% paraformaldehyde (PFA) (Serva, Heidelberg, Germany). Brains perfusion-fixed with 4% PFA were surgically removed, fixed for longer time with 4% paraformaldehyde (PFA) and postfixed in 2.5 % glutaraldehyde (Science Services, Munich, Germany). Finally, the brains were postfixed with 2% osmium tetroxide (Science Services) in 0.1 M phosphate buffer pH 7.3 and embedded in EPON (Serva) after dehydration with ethanol and propylene oxide. EPON blocks with embedded tissue were then trimmed, using a Leica EM TRIM (Leica, Vienna, Austria), to the size of the corpus callosum. In the following, ultrathin sections were stained with an aqueous solution of 4% uranyl acetate followed by lead citrate [16]. The pictures were taken in an unbiased random fashion with a Zeiss EM900 electron microscope (Zeiss, Oberkochen, Germany) using a side-mounted 2k CCD camera (TRS, Waakirchen, Germany) (n=4). In parallel, the corpus callosum (Bregma 0.62mm; Interaural 4.42mm) was freshly isolated from PBS-perfused brains and then frozen in liquid nitrogen for quantitative RT-PCR analysis (n=3 mice/group). We chose this brain region because it is a white matter structure severely affected by use of the CPZ [3].

2.4. Behavioural assessment

We used a set of behavioural paradigms including the FOB, open field, rota-rod, body suspension, black and white box and footprint analyses to determine the main dysfunctions in CPZ-fed mice throughout the experiment. N= 12 mice/group.

2.4.1. Functional observation battery

The FOB was originally used to assess neurotoxic effects in rats [17] and later was adapted for mice [18,19] . The protocol consisted of 18 end-points for the study of CNS activity and excitability, neuromuscular and autonomic effects, and sensorimotor reactivity [19]. Following a brief assessment of the mouse in its home cage, each mouse was handled by the observer to score ease-of-removal, handling reactivity and general appearance. Finally, each mouse was placed in a experimental white box (60 cm×90 cm) while the observer evaluated CNS activity, autonomic effects, muscle tone/equilibrium and sensorimotor reactivity [15].

2.4.2. Open-field assessment

Exploratory activity and behavioural responses to a novel environment were assessed in an open-field apparatus consisting of a 1m×1m wooden square with two differentiated areas, the periphery (the area 15 cm from the wall) and the centre (comprising the rest of the open field). The mice were placed in the centre at the time of testing and left undisturbed for 15 min. The movements of the animals were recorded by a video camera device located above the square and analyse by Ethovision® software (Noldus Information Technologies, Wageningen, The Netherlands). The number of faecal boli and the distance travelled in the centre were used as measures of anxiety-like behaviour ([15,20].

2.4.3. Motor co-ordination and learning: rotarod test

Motor coordination and balance were tested by using an accelerating rotarod (Letica Scientific Instruments). The rotarod test was performed by placing the animal on a rotating drum and recording every time the mouse was not able to maintain its balance and walk on top of the rod. The number of the whole flips on the rod and the latency of the first fall were also recorded. This procedure was repeated for 2 days. During the first sessions (day 1), the speed of the rotarod was set at 16 rpm. Every time the animal fell off, the timer was stopped, then the animal was placed again on the rod and the timing continued up to 60 s. At day 2, animals were placed on the rotating drum subjected to linear acceleration (16 to 32 rpm) and latency to fall from the rotating drum was registered [15].

2.4.4. Body suspension

Forelimbs resistance was assessed by suspending the animals to an elastic band and measuring the time until the animals dropped down. In a second trial, we also quantified the use of the hindlimbs suspending the animal to a 4 mm diameter wooden bar. The animals were required to curl the body and swing their hindlimbs up to contact the bar in order to stabilise body position. The second trial was scored using a three-category scale: a score of 0 indicates no ability to use the hindlimbs, a score of 1 means ability to use one hindlimb while 2 points indicated the use of both hindlimbs [21].

2.4.5. Black and white box test

The black and white test is based on the innate aversion of rodents to brightly illuminated areas [22]. Thus, this test is used as an index of anxiety. The apparatus consists of two compartments; the white part is illuminated while the black

compartment is darkened. The parameters such as latency to enter in the white compartment, number of crossing transitions and time spent in either white or black compartment were accurately registered.

2.4.6. Footprint analyses

Footprint analyses were first assessed in rats de Medinaceli et al. [23] and then adapted to mice [21]. The test allows the study of limb coordination and body weight support. The hind paws of the animals were inked and foot prints were made on paper covering a 5 cm narrow, 50 cm long runway. The runway was used to standardize the direction of the steps. A series of at least 10 sequential steps, recorded per session, were used to determine mean values of stride length. Stride length was measured between the central pads of two consecutive prints on each side. Additionally, for assessments of limb coordination footprints of fore- and hindlimbs were recorded synchronously, as forelimbs were inked with another colour. The distance between the central pads of the fore- and hindlimb on each side of the body was measured [21].

2.5. RNA Isolation

The corpus callosum from each of the CPZ-exposed and control mice was immersed in RNAlater™-ICE Frozen Tissue Transition Solution (ThermoFisher, Barcelona, Spain) and then homogenized with TRI Reagent® RNA Isolation Reagent (Merck, Barcelona, Spain**)**. Total RNA was obtained following a TRIzol protocol (Invitrogen Ltd., NY, USA) and finally digested with DNA-free™ DNA Removal Kit (ThermoFisher, Barcelona, Spain). N= 3 mice/group.

2.6. Quantitative RT-PCR

cDNA was synthesized from 1 μg RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Darmstadt, Germany). Then, mRNA expression was quantified by qRT-PCR using CXF96TM Real-Time PCR system (Bio-Rad, Hercules, USA). GAPDH mRNA was our endogenous control. TaqMan gene expression assays for mouse allograft inflammatory factor 1 (*Aif1*), glial fibrillary acidic protein(*Gfap*) and *paranodine* cDNAs were obtained from validated and predesigned Assays-on-Demand (Applied Biosystems, Darmstadt, Germany) and used in real time PCR amplifications to detect the expression of these genes. *Gfap* was used as a marker of reactive glia while *Aif1* as a marker of microglia. *Paranodine* was selected as an axonal integrity marker. The reactions were performed in triplicate using 2 μl of cDNA in a 10 μl volume. mRNA expression for each sample was determined using the comparative cycle threshold (Ct) method according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The quantification was based on $2^{-\Delta\Delta Ct}$ method was performed relative to a "calibrator" control sample, which was arbitrarily set to 1.

2.7. Statistical analysis

Data were analyzed with the SPSS Statistics 21.0 software (IBM Corp, Chicago, USA). A two-way ANOVA was used to examine the effects of time and CPZ treatment on our cohort of animals. The mean differences among the levels of one factor were determined by one-way ANOVA or Brown-Forsythe test when applicable. Bonferroni or nonparametric Tamhane post-hoc test was applied for multiple comparisons. Analysis of simple main effects was done whether there was a significant interaction of the two main factors. Individual comparisons were done using the Student's t -test. Repeated-measure analysis of variance was also used to evaluate habituation to the

open-field apparatus and learning in the rota-rod motor task. Categorical variables were analyzed by Person χ^2. Body weight was introduced as a covariant. Significance was set at $p < 0.05$. In all figures and text, data are represented as mean ± sem.

3. Results

3.1. Validation of the cuprizone mouse model

Body weight was measured in grams and registered weekly throughout the experimental period. It remarkably diminished after 3 week undergoing exposure to CPZ (T3) ($t(118)=3.44$; $p<0.001$) and then restored to control levels after 6 weeks of CPZ withdrawal (the recovery group) ($t(22)= -0.78$; $p=0.44$) (Figure S1). Myelinated fibres tracts were followed-up for CPZ feeding and withdrawal periods to corroborate both de- and remyelination processes. We reported a clear demyelination in mice treated for 3 weeks (T3) with CPZ which was associated with a smaller axonal calibre ($t(4)=-7.42$; $p<0.05$) and a thinner myelin sheath ($t(4)=-4.56$; $p<0.01$) (Figure S2). Myelin thickness and axonal area in CPZ-fed mice were restored to control levels during the spontaneous remyelination phase after CPZ withdrawal (T8) (Figure S2).

3.2. Neurobehavioral screening

Gross sensory and motor deficits in mice were determined by a FOB. Self-grooming activity in mice's homecage declined in treated-animals at 3 weeks (T3) ($t (16) = 2.26$; $p<0.05$) (Table 1). Later on, we found a markedly elevated grooming behaviour when CPZ-fed animals for 6 weeks were allowed to recover on a regular diet for 6 extra weeks (the recovery group) ($t (21)=-2.73$; $p<0.05$) (Table 1). Treated animals displayed less reactivity to local pressure skin stimulation by the end of the CPZ feeding (T6) and after a withdrawal period of 4 (T10) and 6 (T12) weeks respectively ($\chi^2(1, 24)=4.80$; $p<0.05$; $\chi^2(2, 24)=7.06$; $p<0.05$; $\chi^2(1, 23)=4.44$; $p<0.05$) (Table 1). At 8 weeks, 2 weeks after a recovery period with regular diet (T8), animals showed lower exploratory activity as indicated by less vertical movements (rearings) ($t (22)=2.15$;

$p<0.05$) (Table 1). Finally, CPZ exposed mice after a recovery period of 4 weeks (T10) performed better than their respective controls in the inverted screen ($\chi^2(1, 24)=5.04$; $p<0.05$) (Table 1).

3.3. Muscular strength and motor coordination

The rotarod and footprint analyses were designed to evaluate motor coordination while neuromuscular function was assessed by the body suspension test. At the third week on CPZ diet, when maximum demyelination is achieved, treated mice showed worse performance in the rota-rod (Table 2). In brief, animals fed with CPZ for 3 weeks (T3) spent less time on the rotating rod (Latency first trial, $t(22)=2.21$; Latency second trial, $t(22)=2.63$; $p<0.05$, respectively) and fell off more frequently ($t(22)=-3.06$; $p<0.01$) during the training sessions (Table 2). On the other hand, CPZ exposed animals for 6 weeks followed by 2 weeks of CPZ withdrawal (T8) fell off less frequently from the rotating rod ($t(22)=2.36$; $p<0.05$), exhibited worse fore- and hindlimbs resistance when they were suspended to a bar ($\chi^2(2,24)=6.35$; $p<0.05$) and displayed shorter distance between their two forelimbs ($t(21)=3.68$; $p<0.01$) (Table 2). Moreover, CPZ treated-animals for 6 weeks that were allowed to recover on a regular diet for 4 weeks (T10) displayed weaker grip strength than their controls ($t(22)=2.12$; $p<0.05$) (Table 2). Finally, CPZ treated-animals for 6 weeks subjected to CPZ withdrawal for 6 weeks (the recovery group) showed worse fore- and hindlimbs coordination ($\chi^2(2,23)=8.02$; $p<0.05$) and also smaller distance between their two forelimbs ($t(21)=3.68$; $p<0.01$) (Table 2).

3.4. Locomotor activity, exploratory and anxiety-like behaviour

Open field and black and white box were used to assess activity and anxiety-like behaviour. The open field test was used to measure exploratory activity in a novel environment; the centre of the open field is considered anxiogenic and supposes a conflict between the natural tendency to the exploration and the aversion generated by open spaces. All the animals were habituated to the novel space and no significant differences were observed between CPZ exposed and control groups. Animals exposed to CPZ for 6 weeks (T6) travelled less distance in the open field arena ($F(1,23)=4.92$; $p<0.05$), showed a depressed rearing activity ($F(1,23)=12.29$; $p<0.01$) and travelled smaller distances in the centre ($F(1,23)=4.92$; $p<0.05$) (Table 3). These effects are more pronounced at the initial period 0-5 min of the test in total distance travelled which was significantly lower in CPZ exposed mice and centre exploration where the number of entries ($F(1,23)=12.57$; $p<0.05$) and the time spent in this zone ($F(1,23)=9.67$; $p<0.05$) were lower in CPZ exposed mice. Once CPZ was removed from the diet, a transient increase in corrected distance travelled in the centre ($F(1,23)=5.31$; $p<0.05$) during the period 10-15'min was observed in mice 4 weeks after CPZ withdrawal (T10). Finally, 6 weeks after CPZ withdrawal (the recovery group) only activity in the centre was decreased in exposed mice compared to controls as observed in distance ($F(1,23)=5.85$; $p<0.05$), corrected distance ($F(1,23)=4.45$; $p<0.05$), vertical activity ($F(1,23)=4.98$; $p<0.05$), the number of entries into the centre ($F(1,23)=5.81$; $p<0.05$) and the time spent in the centre ($F(1,23)=7.01$; $p<0.05$).

Regarding the white and dark box, animals exposed to CPZ and recovered for 2 weeks with standard diet (T8) showed minor number of entries into the light compartment than the control group ($F(1,23)=7.14$); $p<0.05$) (Table 3). At ten weeks (T10), treated animals showed greater number of entries into the light ($F(1,23)=5.25$; $p<0.05$) and spent less time in the darkness ($F(1,23)=8.93$; $p<0.01$) (Table 3). Treated animals fed

with regular diet free of CPZ for 6 weeks (the recovery group) spent less time to enter for the first time into the light compartment ($F(1,23)=5.22$; $p<0.05$), performed more entries into the light ($F(1,23)=6.37$; $p<0.05$) and spent less time in the darkness ($F(1,23)=6.99$; $p<0.05$) (Table 3).

3.5. Gene expression analysis

Two-way ANOVA revealed a significant effect of the group ($F(1,40)=34.37$; $p<0.001$) and the interaction between group and time ($F(3,40)=10.50$; $p<0.001$) on Aif expression. Particularly, pairwise comparisons revealed that animals subjected to acute demyelination (T3) showed a strong increase in the expression of *Aif1* when compared to controls ($t(8)=4.97$; $p<0.001$) (Figure 2). Later on, in the course of the experiment the transcription levels for *Aif1* were reduced but still remains significantly higher than controls (T6, $t(8)= 7.66$; T8, $t(9)= 2.28$; $p<0.001$, $p<0.05$, respectively) (Figure 2). CPZ-treated mice fed with regular diet free of CPZ for 6 weeks (the recovery group) did not show differences in the expression of *Aif1* ($t(8)= -2.13$; ns) (Figure 2). Gene expression analysis for *paranodine* marker did not reveal significant differences between groups pointing out no damage on axonal integrity after the CPZ experiment ($F(1, 45)=0.82$; ns) (Figure 2). A two-way ANOVA showed a significant effect of the factor group in *Gfap* marker ($F(1,42)=45.14$; $p<0.001$) but did not reveal where the differences became significant. No interactions between *treatment and time* were found ($F(3,42)=0.47$; ns).

4. Discussion

In the present study, we used mice body weight and morphological changes in myelin to validate the model. Current results underwent the same histological time-course of de- and remyelination as previously reported by Matsushima and Morell [3] and Tomas-Roig and colleagues [16]. Likewise, in a previous study performed in our laboratory, behavioural dysfunctions in mice (from a moderate hyperactivity to prominent motor impairment) when mice were exposed to CPZ for 6 weeks [15] were reported. The present study extends the evaluation to intermediate time points at the recovery period and assesses gene expression related to neural degeneration.

Animals subjected to acute demyelination (T3) showed less grooming activity than their counterparts. Self-grooming in rodents is an innate response involved in hygiene, thermoregulation, social interaction, de-arousal and also is considered as an index of motor activity [24]. In line with the results presented here, Paumier and colleagues [25] found significant deficits in overall grooming behaviour in an experimental model of demyelination. We also observed that demyelinated animals (T3) showed worse motor coordination than the control group. Similarly, Liebetanz and Merkler [26] showed that animals fed with CPZ for 3 weeks exhibited deficits in motor coordination when they were tested by the motor skill sequence (MOSS). Exposure to acute demyelinating event upon CPZ administration (T3) was accompanied by a prominent increase of *Aif1* expression [3,16]. The characterization of astrocyte reactivity in the cuprizone mouse model contributes to study CNS damage [3]. An overall increase in astrogliosis measured by *Gfap* marker was observed in CPZ-fed mice when compared to their controls however, these differences became no significant at 12 weeks of the experiment (the recovery group) indicating an attenuation of the astrocyte activity. At

week 6, when mice were still undergoing exposure to CPZ (T6), we assessed elevates levels of Aif1 expression, these can be related to an increased number of macrophages to clear myelin debris [3,16]. These gene expression changes observed during CPZ treatment and after withdrawal are in agreement with behavioural observations. In this regard, by the end of CPZ feeding (T6), animals were less reactive to local skin stimulation-induced by mechanical pressure which persisted long in the course of the experiment in agreement with a previous study [27]. We also demonstrated that treated animals for 6 weeks (T6) showed a hypoactive exploratory behaviour in the open field arena as indicated by less time in the centre, smaller distance travelled and minor rearing activity. These changes are compatible with either less arousal, an anxiogenic response to the novelty, an increased inhibition of impulsivity or can be associated with dysfunctions in white matter tracts [27, 28, 29].

Once CPZ was removed from the diet, animals undergo a remyelinating event (T8), which is paralleled with a decrease of rearing activity, worse motor coordination, gait abnormalities and a prominent anxiety trait. Likewise, Li and colleagues [28] reported a decrease of exploratory activity and also an anxiogenic-like effect in remyelinated animals after CPZ withdrawal. Similarly, Skripuletz and colleagues [30] demonstrated that during myelin repair in the CNS, animals fed with the neurotoxicant CPZ presented deficits in motor coordination. Furthermore, Franco-Pons and co-workers [15] found remarkable gait abnormalities in CPZ-induced remyelination mice which supports the findings found here.

Animals fed with CPZ and left undisturbed with regular diet for 4 weeks (T10) displayed better performance when they were tested by inverted screen but not when animals were experimentally evaluated by body suspension test indicating dysfunctions

in the coordination of mice limbs. In contrast to the results previously obtained in the time course of the CPZ model (T6 and T8), we found that recovered animals at 10 weeks (T10) showed a blunted anxiogenic response. From the current data, we cannot conclude that behavioural changes observed here are direct effects of demyelination or related to other side effects such as inflammation or derived from CPZ chelating-effects elsewhere.

The recovery group still displayed persistent behavioural deficits which in turn, point out to long-lasting effects of the neurotoxicant CPZ. Concretely, these animals showed considerable impairments in neurological functions involved in sensorimotor, neuromuscular, motor coordination and the capacity to cope with a stress-inducing event. Briefly, the recovery group was less reactive to local skin stimulation when animals were subjected to mechanical pressure [27] and also showed deficits in motor-coordination [15]. The recovery group was also less active in the centre of an open field arena than controls indicating an anxiogenic response to novel environment. However when tested in the light and dark paradigm the recovery group displayed lower latency and more frequent entries into the light compartment which is compatible with a less anxious or a more impulsive behaviour. Taken into account data obtained by Franco-Pons and co-workers [15] in a previous study, referring a lack of habituation and a more active behaviour in the centre of an open field, we speculate that our data is more compatible with an impulsive trend. In this line, a recent study found an association between compulsive behaviour and demyelination [31]. Apparently discrepancies between results obtained from the open field paradigm and the black and white compartments could be related to different frightening properties which lead to different sensitivity to assess anxiety behaviour as pointed by Heredia and colleagues [32]. In this sense, the more aversive conditions on the open field let us observe the anxiety trait

while the more safe conditions in the light and dark test favours the emergence of an impulsive or hyperactive trait. Therefore, as both anxiety and impulsivity can be persistent after a demyelinating/remyelinating process more specific tests must be conducted to characterize this effects and its reversibility. Furthermore, since some of these behavioural changes remain after CPZ withdrawal we can speculate that alterations at structural either axonal or myelin sheath or at glial reactivity persist after the recovery period.

In conclusion, exposure to CPZ causes from a moderate to severe behavioural dysfunctions in mice even once CPZ is replaced from the diet. To summarize, animals showed reduced motor activity and deficits in motor coordination when they were subjected to acute demyelinating insult (T3) while minor exploratory activity and impairment in motor coordination were assessed when spontaneous remyelination was reached following cuprizone withdrawal (T8). Interestingly, we demonstrated that the recovery group displayed considerable impairments in neurological functions involved in sensorimotor, neuromuscular, motor coordination and the capacity to cope with a stress-inducing event, a trend to a progressive increase in impulsivity is also suggested and deserve more investigations. Therefore, we conclude that cuprizone had a long-lasting behavioural effect even when animals were recovered for 6 week with regular diet. Therefore, further investigation should be accompanied by an extent analysis of gene expression in order to understand the underlying mechanisms associated to the neurological deficits observed in recovered animals.

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6. Acknowledgments

The authors would like to thank Dr. Lourdes Martorell from Hospital Universitari Institut Pere Mata (Spain) and Maria Buxó (IDIBGI, Spain) for excellent technical support. Dr. Tomas-Roig was supported by Deutsche Forschungsgemeinschaft fellowship (#TO 977/1-1). The work was supported by the Stanely Research Institute (#03R-392 and #05R-896).

The author reports no potential conflicts of interest.

7. Figure and table captions

Figure 1. Schematic drawing of the experiment. Mice were fed with regular diet (control group) or subjected to CPZ for either 3 or 6 weeks (treated group). At certain time points, as indicated vertical arrows, mice were behaviorally tested for 2 consecutive days (n=12 mice/group). Once animals were behaviorally tested, they were deeply anesthetized and sacrificed. Finally, the corpus callosum was processed for transmission electron microscopy (n=4) and quantitative RT-PCR (n=3). CTR, control; CPZ, cuprizone.

Figure 2. Gene expression analysis. *Gfap* was used as a marker of reactive glia while *Aif1* as a marker of monocyte-macrophages. *Paranodine* was selected as an axonal integrity marker. Animals subjected to demyelination showed an increase in *Aif1* when compared to controls ($p<0.001$). Later on, levels for *Aif1* were reduced but still significantly higher than controls (t= 6 weeks, $p<0.001$; t= 8 weeks, $p<0.05$). The recovery group did not show differences in the expression of *Aif1*. Gene expression analysis for *paranodine* did not reveal significant differences between groups and thus, we can assume that the administration of CPZ did not alter axonal integrity. ANOVA revealed a significant effect of the group (control *vs* CPZ) in *Gfap* but no interaction between *treatment x time* was found. N=3. Aif1, allograft inflammatory factor 1; Gfap, glial fibrillary acidic protein; CPZ, cuprizone.

Table 1. Gross sensory and motor activity. Self-grooming in mice's homecage declined in treated-animals at 3 weeks ($p<0.05$). At the end of the experiment, we found an elevated grooming behaviour in CPZ-fed animals when compared to the controls ($p<0.05$). Treated animals displayed less reactivity to local pressure skin stimulation by the end of the CPZ feeding (week 6) and after a recovery period of 4 (week 10) and 6

weeks (the recovery group) when compared to controls ($p<0.05$). After 2 weeks of recovery period, treated animals showed lower exploratory activity (less rearing activity) ($p<0.05$). Inverted screen test revealed that CPZ-fed mice after a recovery period of 4 weeks (week 10) had the ability to turn around and climb the inverted screen more efficiently than the controls ($p<0.05$). Data are expressed as mean ± SEM. N=12 mice/group.

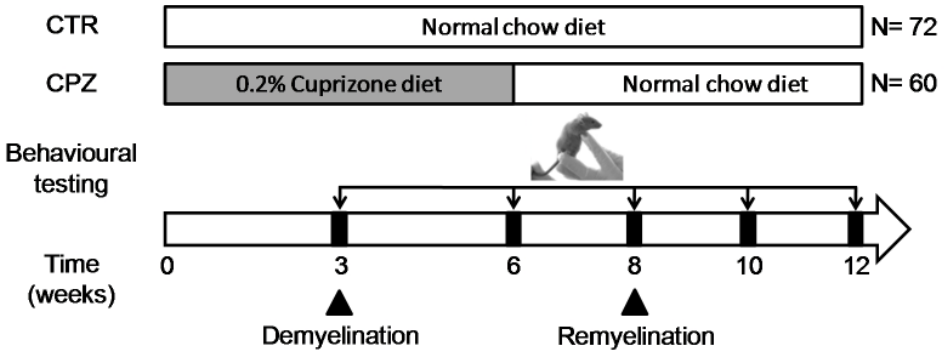
Table 2. Motor coordination and neuromuscular function. At the third week, when maximum demyelination is achieved, treated mice displayed worse results in the rota-rod. In brief, CPZ-fed mice spent less time on the rotating rod ($p<0.05$) and also fell off more frequently ($p<0.01$) during the training sessions. Recovered animals fed with standard diet for 2 weeks (week 8) fell off less frequently from the rotating rod ($p<0.05$), exhibited worse fore- and hindlimbs resistance when suspended to a bar ($p<0.05$) and displayed shorter distance between their two forelimbs ($p<0.01$). Treated-animals recovered on a regular diet for 4 weeks (week 10) displayed weaker grip strength than their controls ($p<0.05$). Finally, the recovery group (12 weeks) showed worse fore- and hindlimbs coordination ($p<0.05$) and also smaller distance between their two forelimbs ($p<0.01$). Data are expressed as mean ± SEM. N=12 mice/group.

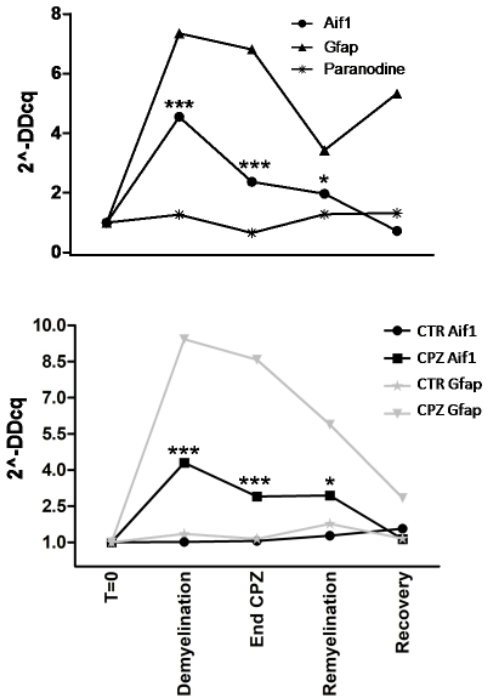
Table 3. General activity and anxiety-like behaviour. By the end of the CPZ feeding, animals displayed minor number of entries, spent less time and travelled shorter distance in the centre during the first 5 minutes interval of testing ($p<0.01$). Rearing activity was reduced after CPZ withdrawal in either 0-5', 5-10' or 10-15' interval of time ($p<0.05$). In whole interval of testing (0-15'), CPZ-fed mice for 6 weeks showed depressed rearing activity ($p<0.01$), travelled smaller distances in the centre ($p<0.05$) and globally travelled less distance ($p<0.05$). Treated animals recovered for 2 weeks

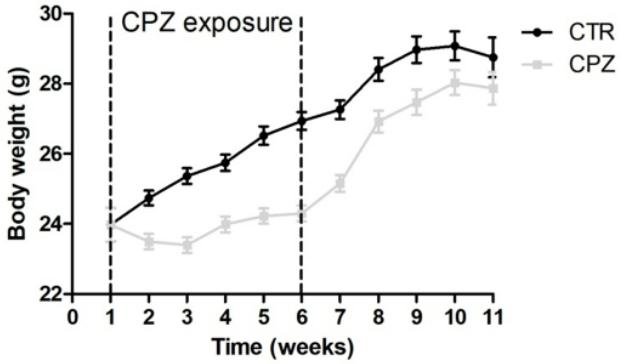
with standard diet (week 8) showed minor number of entries into the light than the controls ($p<0.05$). At ten weeks, treated animals showed greater number of entries into the light ($p<0.05$), spent less time in the darkness ($p<0.01$) and showed a bigger ratio distance travelled in the centre:total distance travelled at the interval of 10-15' ($p<0.05$). The recovery group exhibited persistent behavioural dysfunctions. In particular, these animals lower latency to enter into the light compartment ($p<0.05$), performed more entries into the light ($p<0.05$) and spend less time in the darkness ($p<0.05$). In the open field, we reported that the recovery group showed lower number of entries ($p<0.05$), spent less time ($p<0.05$) and travelled shorter distances in the centre at the interval 0-5' ($p<0.05$). Moreover, these animals showed lower number of rearings in the centre at the interval of 0-5' ($p<0.05$) and smaller ratio distance travelled in the centre:total distance travelled at the interval of 0-5' ($p<0.05$). The remaining comparisons are depicted in the table. Data are expressed as mean ± SEM. N=12 mice/group.

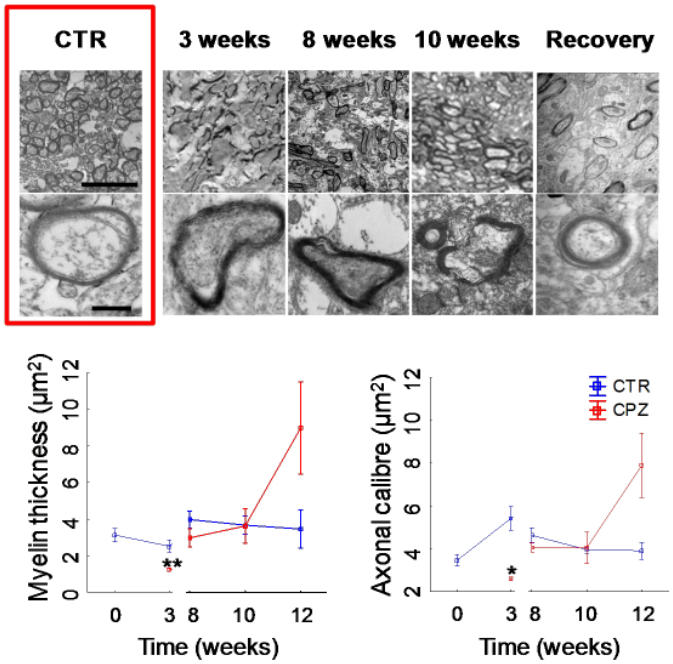
Figure S1. Mice body weight. Body weight was measured in grams and registered weekly throughout the experimental period. It remarkably diminished after 3 week undergoing exposure to CPZ ($p<0.001$) and then restored to control levels after 6 weeks of CPZ withdrawal (NS). Data are expressed as mean ± SEM. N=12 mice/group.

Figure S2. The corpus callosum myelination. Myelinated fibres tracts were analysed in a longitudinal follow-up of CPZ feeding to corroborate both de- and remyelination processes. We reported a clear demyelination following 3 weeks with CPZ which in turn, was associated with smaller axonal calibre ($p<0.05$) and thinner myelin sheath ($p<0.01$). Myelin thickness and axonal area in CPZ-fed mice was restored to control levels during the spontaneous remyelination phase after CPZ withdrawal.


CTR
Normal chow diet
N= 72
CPZ
0.2% Cuprizone diet
Normal chow diet
N= 60
Behavioural testing
Time (weeks)
0
3
6
8
10
12
Demyelination
Remyelination


8
6
4
2
0
2^-DDcq
Aif1
Gfap
Paranodine
***
***
*
10.0
8.5
7.0
5.5
4.0
2.5
1.0
2^-DDcq
CTR Aif1
CPZ Aif1
CTR Gfap
CPZ Gfap
***
***
*
T=0
Demyelination
End CPZ
Remyelination
Recovery


CPZ exposure
CTR
CPZ
Body weight (g)
30
28
26
24
22
0 1 2 3 4 5 6 7 8 9 10 11
Time (weeks)


CTR
3 weeks
8 weeks
10 weeks
Recovery
Myelin thickness (µm²)
0
2
4
6
8
10
12
**
0
3
8
10
12
Time (weeks)
Axonal calibre (µm²)
2
4
6
8
10
12
CTR
CPZ
*
0
3
8
10
12
Time (weeks)

		Group			
		Control	Cuprizone		
Week	**Parameter**				**P value**
3	Grooming home-cage	2.33± 0.65	0.75± 0.25	$t(16) = 2.26$	0.04
6	Touch response	32/12	24/12	$\chi^2(1, 24)=4.80$	0.028
8	Rearings	4.92±1.12	2.08±0.70	$t(22)=2.15$	0.043
10	Touch response	32/12	27/12	$\chi^2(2, 24)=7.06$	0.029
	Invertedscreen	24/12	35/12	$\chi^2(1, 24)=5.04$	0.025
12	Grooming open field	0.67±0.23	1.64±0.28	$t(21)=-2.73$	0.013
	Touch response	32/12	22/12	$\chi^2(1, 23)=4.44$	0.035

Table 1

			Group			
			Control	Cuprizone		
Week	**Test**	**Parameter**				**P value**
3	RR	Latency first trial	30.17±12.50	2.50±0.31	t(22)=2.21	0.049
		Latency second trial	57.42±11.08	21.92±7.67	t(22)=2.63	0.015
		Number of falls first trial	4.75±1.25	10.42±1.36	t(22)= -3.06	0.006
8	RR	Number of falls first trial	16.25±2.89	8.92±1.51	t(22)=2.36	0.027
	BS	Coordination	22/12	15/12	$\chi^2(2, 24)=6.35$	0.042
	FP	Distance between two consecutive footprints	7.09±0.92	6.54±0.12	t(21)=3.62	0.002
10	BS	Grip strength	3.33±0.58	1.83±0.40	t(22)=2.12	0.046
12	BS	Coordination	19/12	10/12	$\chi^2(2,23)=8.02$	0.018
	FP	Distance between two consecutive footprints	3.92±0.06	3.59±0.06	t(21)=3.68	0.001

Table 2

			Group			
			Control	Cuprizone		
Week	Test	Parameter				p value
6	OF	Entries into the center 0-5'	17.17±1.65	10.83±0.69	F(1,23)=12.57	0.002
		Time in center 0-5'	31.13±3.86	18.47±1.31	F(1,23)=9.67	0.005
		Distance travelled in the center 0-5'	567.08±62.31	328.67±26.64	F(1,23)=12.38	0.002
		Total distance travelled 0-5'	3405.42±246.05	2612.17±129.35	F(1,23)=8.14	0.009
		Rearings in center 0-5'	14.17±1.50	8.58±0.62	F(1,23)=11.80	0.002
		Rearings 0-5'	32.92±3.85	20.75±2.25	F(1,23)=7.44	0.012
		Rearings 5-10'	35.50±3.44	26.33±1.77	F(1,23)=5.61	0.027
		Rearings 10-15'	33.75±2.48	25.92±2.57	F(1,23)=4.82	0.039
		Total distance travelled	8973.20±448.09	7476.63±313.69	F(1,23)=7.49	0.012
		Distance travelled in the center	1746.14±169.54	1304.45±104.30	F(1,23)=4.92	0.037
		Rearings	102.17±6.66	73.00±4.98	F(1,23)=12.29	0.002
8	B&W	Entries into the light compartment	6.92±0.50	5.17±0.42	F(1,23)=7.14	0.014
10	B&W	Entries into the light compartment	5.42± 0.50	7.08± 0.53	F(1,23)=5.25	0.032
		Time spend in the dark compartment	197.33± 4.62	175.75± 5.55	F(1,23)=8.93	0.007
	OF	Distance travelled in the center /total 10-15'	0.20±0.013	0.25±0.021	F(1,23)=5.31	0.031
12	B&W	Latency to first entry	64± 11.31	35.08± 5.85	F(1,23)=5.22	0.032
		Entries into the light compartment	5.17± 0.37	7.25± 0.74	F(1,23)=6.37	0.019
		Time spend in the dark compartment	186.50± 6.78	166.83± 3.88	F(1,23)=6.99	0.015
	OF	Entries into the center 0-5'	16.25±1.69	11.00±1.37	F(1,23)=5.81	0.025
		Time in center 0-5'	30.35±3.47	18.78±2.65	F(1,23)=7.01	0.015
		Distance travelled in the center 0-5'	454.09±44.28	312.41±38.33	F(1,23)=5.85	0.024
		Rearings in center 0-5'	12.00±1.31	7.83±1.33	F(1,23)=4.98	0.036
		Distance travelled in the center /total 0-5'	0.16±0.02	0.12±0.01	F(1,23)=4.45	0.047

Table 3