

**ORIGINAL ARTICLE**

Cognitive disturbances in the cuprizone model of multiple sclerosis

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Abstract

Cognitive problems frequently accompany neurological manifestations of multiple sclerosis (MS). However, during screening of preclinical candidates, assessments of behaviour in mouse models of MS typically focus on locomotor activity. In the present study, we analysed cognitive behaviour of 9 to 10-week-old female C57Bl/6J mice orally administered with the toxin cuprizone that induces demyelination, a characteristic feature of MS. Animals received 400 mg/kg cuprizone daily for 2 or 4 weeks, and their performance was compared with that of vehicle-treated mice. Cuprizone-treated animals showed multiple deficits in short touchscreen-based operant tasks: they responded more slowly to visual stimuli, rewards and made more errors in a simple rule-learning task. In contextual/cued fear conditioning experiments, cuprizone-treated mice showed significantly lower levels of contextual freezing than vehicle-treated mice. Diffusion tensor imaging showed treatment-dependent changes in fractional anisotropy as well as in axial and mean diffusivities in different white matter areas. Lower values of fractional anisotropy and axial diffusivity in cuprizone-treated mice indicated developing demyelination and/or axonal damage. Several diffusion tensor imaging measurements correlated with learning parameters. Our results show that translational touchscreen operant tests and fear conditioning paradigms can reliably detect cognitive consequences of cuprizone treatment. The suggested experimental approach enables screening novel MS drug candidates in longitudinal experiments for their ability to improve pathological changes in brain structure and reverse cognitive deficits.

KEYWORDS

diffusion tensor imaging, fear conditioning, learning, MRI, multiple sclerosis, touchscreen

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1 | INTRODUCTION

Multiple sclerosis (MS) is triggered by dysregulated activity of T and B lymphocytes, which causes demyelination, loss of axons and pathological proliferation of glia.¹⁻⁴ Consequently, preclinical studies of novel drugs for MS tend to focus on the effects of lead compounds on inflammation parameters,^{5,6} extent of remyelination^{7,8} and neuroprotective properties,⁹ in line with the main current therapeutic strategies for MS.^{10,11}

MS symptoms such as spasticity, dizziness, fatigue, tremor and optic neuritis, are primarily in the neurological domain, however cognitive and behavioural problems are also a considerable burden to individuals with MS.¹²⁻¹⁴ The effectiveness of current disease-modifying therapies in alleviating cognitive impairments is meagre,¹⁵ therefore preclinical tests of MS drug candidates should ideally include assessment of cognitive performance in animal MS models. However, the translation of the results of learning and attention tasks in animals to the human is not straightforward, as preclinical and clinical tasks are often quite dissimilar. The use of neuropsychological test batteries in cognitive assessments of individuals with MS has been increasingly advocated.¹⁶⁻¹⁹ Some of these batteries involve touchscreen-based testing,^{20,21} an approach that is becoming popular for the studies of memory and attention in rodents.^{22,23} Touchscreen testing in both humans and rodents utilises similar setting (images on a screen) and comparable reactions (finger touches/nose pokes), therefore it is reasonable to expect that the mechanisms of disturbances revealed by touchscreen tests in animal models of MS and the ways of their alleviation will have direct clinical relevance. Here, by using the cuprizone mouse model of MS, we sought to: (a) establish for the first time whether a short sequence of simple touchscreen tasks can detect behavioural disturbances in cuprizone-treated mice; (b) compare the sensitivity of touchscreen-based testing with that of fear conditioning, a more widespread operant approach, to show cognitive phenotype and (iii) analyse whether changes in cognitive parameters correlate with structural alterations reported by *in vivo* diffusion tensor imaging (DTI). We chose the cuprizone model of MS because this toxin relatively rapidly disrupts the metabolism of oligodendrocytes and causes their subsequent degeneration.²⁴⁻²⁷ Although the cuprizone model does not feature strong inflammation-mediated insult to the myelin sheet, such as that observed in the classical experimental autoimmune encephalitis model of MS, certain aspects of cuprizone-induced pathology, for example, cortical deep grey matter demyelination, are reminiscent of structural changes seen in the brain of MS patients,²⁸⁻³⁰ in particular those with pattern III lesions.³¹

2 | MATERIALS AND METHODS

2.1 | Animals

All animal experiments were performed as specified in the licence authorised by the National Animal Experiment Board of Finland (Eläinkoelautakunta, ELLA) and according to the National Institutes of

Health (Bethesda, Maryland) guidelines for the care and use of laboratory animals. In total, 67 C57BL/6J female mice (Charles River Laboratories, Sulzfeld, Germany) were used, which were 9 to 10 weeks old at the start of the experiment. All mice were housed in groups of five per cage at a standard temperature ($22 \pm 1^\circ\text{C}$) and in a light-controlled environment (lights on at 07:00 AM and off at 8:00 PM). Cages (IVC type II, Allentown, Inc., Allentown, New Jersey) were kept at negative pressure and furnished with corn cob-derived bedding (Scanbur, Karlslunde, Denmark), nesting material (aspen wool, Tapvei Oy, Kortteinen, Finland) and a tinted polycarbonate tunnel (Datesand, Manchester, UK). Behavioural testing was performed during the light phase of the cycle, between 11:00 and 14:00. Five days prior to the experiments in touchscreen chambers, mice received restricted amount of their usual diet (Teklad Global 16%-protein rodent diet, Envigo, Huntington, UK), so that their weight remained at 85%-90% of their free-feeding weight in order to maintain motivation for touchscreen tasks, with water *ad libitum* (Figure 1A,B). Although cuprizone treatment by itself caused some weight loss, we found that cuprizone-treated animals in our study tolerated food restriction relatively well. After touchscreen experiments were completed, the animals were returned to *ad libitum* access to food.

2.2 | Cuprizone treatment

Despite in the majority of published studies cuprizone was admixed to dry food, we chose to administer it by oral gavage.³² Although oral gavage requires more handling and is therefore more stressful for the animals, it also ensures that mice receive an exact daily dose of the toxin. In the free-feeding regime, chow consumption between different animals varies and not necessarily in direct relation to the initial body weight. Furthermore, cuprizone may change the taste of food and as we also used food restriction for touchscreen tests, this could lead to undernourishment of cuprizone-treated animals, which would complicate the interpretation of behavioural testing data. Cuprizone (#14690, Sigma) was suspended in .5% methyl cellulose (#M0430, Sigma) in water and given by oral gavage at a daily dose of 400 mg/kg, which was split into two equal doses a day with ~12-hour interval administered at ~8 AM and 8 PM. Cuprizone suspension (20 mg/mL) was prepared fresh daily.

2.3 | Experimental groups

Animals were divided in the experimental cohorts that received cuprizone or vehicle for 18 days or 31 days in total. These cohorts are referred to as '2-week' and '4-week' cohorts, respectively. In the '2-week' cohort, 19 mice were treated with cuprizone, and 16 mice received vehicle. In this cohort, mice in the same cage received the same treatment except for one cage where four mice received cuprizone, whereas the fifth mouse received vehicle. In the '4-week' cohort, 17 mice were treated with cuprizone, and 15 mice received vehicle. In this cohort, every cage initially had both cuprizone-treated

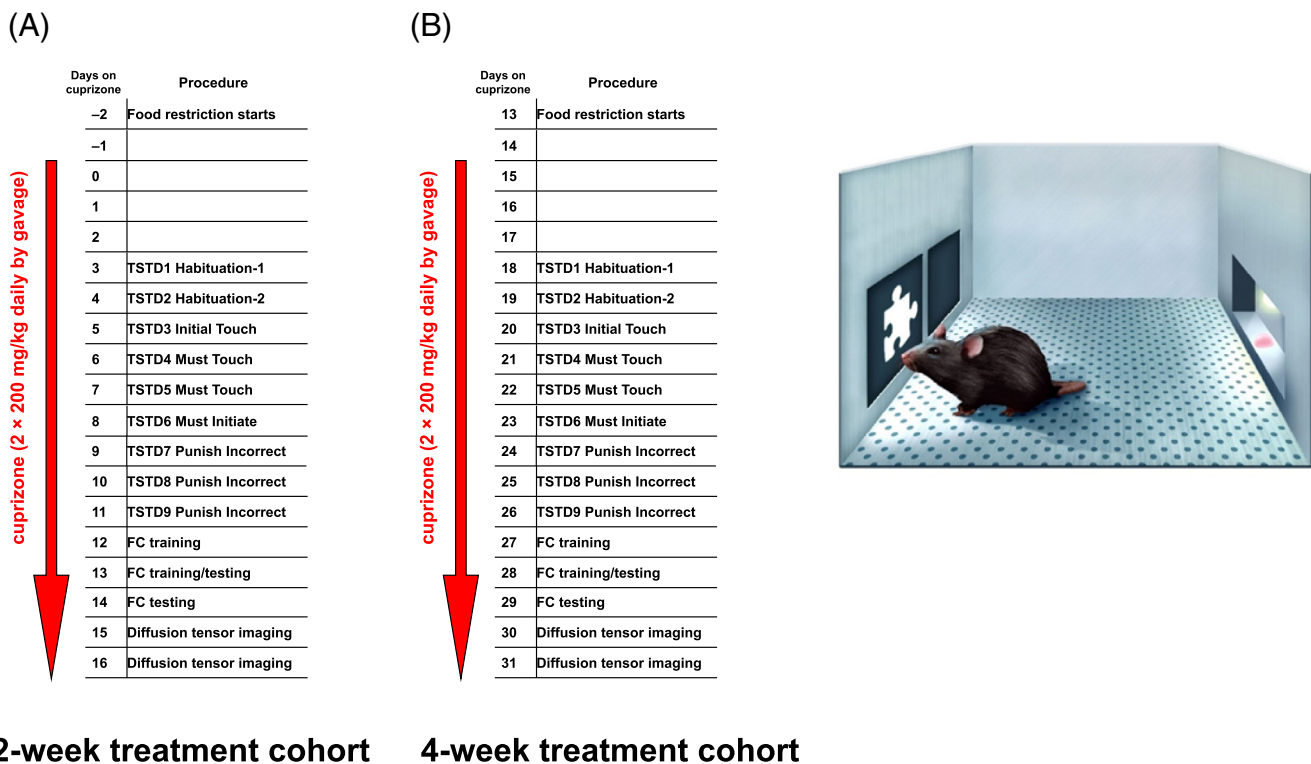


FIGURE 1 Experimental schedules. A,B, Sequence of experimental steps in cohorts treated with cuprizone or vehicle for 2 or 4 weeks, respectively. Numbers in the left column indicate days relative to the start of the treatment with cuprizone or vehicle (gavage, twice daily), administered for the first time on day 0 and continued for the whole duration of the experiments. FC, fear conditioning; TSTD, touchscreen testing day. C, Illustration of the typical touchscreen chamber experimental set-up. A single image appears in one of the two windows on the touch-sensitive screen. Nose poke into the image is rewarded by a drop of liquid nutritional reward in the food tray on the back of the chamber

and vehicle-treated mice. However, when food restriction was initiated prior to touchscreen tests, we noticed that mice receiving cuprizone tended to lose weight more quickly. Therefore, for the duration of touchscreen testing, mice from the '4-week' treatment cohort were additionally split into different cages according to the treatment group, so that every cage housed 2 to 3 animals. Animals were again re-united with their cage mates after the last touchscreen session.

Two cuprizone-treated mice developed severe clinical signs during the experiment (one in the '2-week' group and one—in the '4-week' group) and were euthanised. Data from these animals were excluded from the final analysis.

2.4 | Test schedules

Schedules of experiments in the two cohorts are illustrated in Figure 1A,B. Mice began to receive cuprizone or vehicle and underwent sequential experimental steps, starting from the 5-day gradual food restriction, followed by touchscreen tests of increasing difficulty for 9 consecutive days, contextual/cued fear conditioning for 2 days and DTI (Figure 1A,B). Cuprizone/vehicle administration and touchscreen testing were performed for the whole cohort simultaneously, however, it was not possible for animals of the same cohort

to complete fear conditioning and imaging steps all in the same days for logistical reasons. For example, whereas all mice in the '2-week' cohort finished touchscreen tests on day 11, only half of them completed fear conditioning test on days 12 and 13, whereas another half was tested on days 13 and 14 (Figure 1A,B). Therefore, contextual/cued fear conditioning and imaging data were pooled from animals that differed in the duration of cuprizone treatment by 1 or 2 days.

2.5 | Touchscreen testing

In these experiments, we used Bussey-Saksida touchscreen operant chambers (Campden Instruments, Loughborough, UK) and tasks commonly utilised for mouse pretraining before the touchscreen test of pairwise visual discrimination.^{33,34} We decided to use these pre-training tasks and not more complex paradigms, such as pairwise visual discrimination or 5-choice serial reaction time, for simplicity and relative shortness. It should be noted that normally mice are advanced to the next, more complicated touchscreen testing stage only after having attained certain performance criteria,³⁴ and the time required for that may vary substantially even in animals of the same group.³³ In the context of our experiment, this would mean that mice would start some test stages after different periods of the exposure to cuprizone,

a circumstance that we wished to avoid. Therefore, we followed a fixed routine of touchscreen testing steps (Figure 1A,B), so that all mice were advanced to subsequent stages simultaneously, even though some of the animals failed to achieve the criteria for several tasks.

Two-window masks for Pairwise Visual Discrimination tests were used in front of the touch-sensitive screens (Figure 1C). Mice received one 60-minute long training session daily for 9 touchscreen testing days (TSTDs). Five days before the start of touchscreen testing, mild food rationing was introduced to increase the motivation to emit operant responses for nutritional reward. Animals were gently handled and weighed. Access to food was restricted, so that each animal received only 3 to 4 g on average of standard lab pellets daily. In addition, a small quantity (100-200 μ L per each mouse) of Valio Profeel strawberry-flavoured milk drink (Valio, Helsinki, Finland) was provided to accustom the animals with the taste and flavour of the reward to be used during testing. The amount of food was restricted, so that by the start of touchscreen testing, animals were within 85%-90% of their free feeding weight.

Touchscreen tasks administered on TSTDs 1 and 3 to 9, namely 'Habituation-1/Activity', 'Initial Touch' and 'Must Touch' (for 2 days), 'Must Initiate' and 'Punish Incorrect' (for 3 days) were identical to pre-training stages 1 to 5 described in the methodological paper on this subject.³⁴ On TSTD2, however, we used 'Habituation-2' task, during which mice learned that the chamber tray might be the source of food. Mice were left in the chamber for a 30-minute session, with the tray light turned on. A tone was played, and the food tray was primed with reward delivery (150 μ L). The programme waited for the mouse to pop its head into the food tray and when the mouse left it, the reward tray light was turned off. There was a 10-second delay before the tray light was turned on again, a tone was played and liquid reward was then delivered for 280 ms (7 μ L). If the mouse was in the reward tray at the end of the 10-second delay, an extra 1 second was added to the delay. The procedure was repeated until the session finished. The number of trials (ie, the number of rewards collected) was recorded.

Spontaneous activity parameters, such as beam breaks, screen touches and reward tray entries were recorded in unlit chamber on TSTD1 during 'Habituation 1/Activity' stage. The number of collected rewards was counted in TSTD2 during 'Habituation 2' stage. During 'Initial Touch', 'Must Touch', 'Must Initiate' and 'Punish Incorrect' stages, mice could receive rewards for touching screen images upon correct interpretation of increasingly complex rules. To analyse goal-oriented cognitive behaviour across these stages, we determined image touch rate, blank screen touch rate as well as latency to touch the image after it appeared and latency to collect the reward. Finally, during the 'Punish Incorrect' stage, mice were discouraged to touch the blank part of the screen as doing so switched off the house light and delayed the acquisition of the reward.³⁴ Thus, we also analysed the accuracy of responding, measured as the fraction of touches to the image in the total number of first touches to the screen after the image had appeared (% of correct responses).

2.6 | Contextual/cued fear conditioning

Fear conditioning (FC) training and testing were conducted on two consecutive days using a Coulbourn FreezeFrame system (Coulbourn, Whitehall, PA). On FC day 1 (day 10 of the overall behavioural testing), the mouse was placed into the chamber with illuminating stimulus and house lights on and allowed to explore for 2 minutes. Afterward, an auditory cue (conditioned stimulus [CS]) was presented for 15 seconds. A 2-second electric foot shock (1.5 mA; unconditioned stimulus [US]) was administered for the final 2 seconds of the CS. This procedure was repeated, and the mouse was removed from the chamber 30 seconds later. In 20 hours after training, on FC day 2, the mouse was returned to the same chamber in which the training occurred, and its freezing behaviour was recorded by the software (memory for context). Freezing was defined as episodes of the lack of movement, except for that required for respiration, which lasted for at least 2 seconds. At the end of the 5-minute context test, the mouse was returned to its home cage. Eighty minutes later, the mouse was placed in another chamber that had structural features different from those of the initial chamber, for example, red plastic side walls, black Plexiglas floor, and decreased illumination. Freezing behaviour in this novel environment (altered context) was recorded for 3 minutes. The auditory cue was then presented for 3 minutes and freezing behaviour was analysed again (memory for cue).

2.7 | Diffusion tensor imaging

Magnetic resonance imaging was performed following FC experiments, at the end of the 2- or 4-week cuprizone treatment periods. In both cohorts, 10 cuprizone-treated and 10 vehicle-treated mice were examined in a horizontal 11.7 T magnet with a bore size of 160 mm, equipped with a gradient set capable of maximum gradient strength of 750 mT/m and interfaced to a Bruker Avance III console (Bruker Biospin GmbH, Ettlingen, Germany). A volume coil (Bruker Biospin) was used for transmission, and a surface phased array coil (Rapid Biomedical GmbH, Rimpar, Germany) was used for receiving.

Mice were anaesthetised using isoflurane (5% for induction, 1.5% for maintenance in 300 mL/min N₂/O₂), fixed to a head holder and positioned in the magnet bore in a standard orientation relative to gradient coils. During the measurements, animal temperature was monitored and kept at 36 to 37 °C with a warm water circulation heating blanket. After acquisition of the fast localizer images, DTI was performed using a 4-segment echo-planar imaging sequence with 30 diffusion directions (*b*-values 0 and 900 seconds/mm²), time-to-repeat of 6000 ms and echo time of 28 ms. A field-of-view of 20 × 10 mm² was used with the matrix of 256 × 128 resulting in 78- μ m in-plane resolution. Twenty-five 0.5-mm slices were acquired with four averages. Preprocessing of DTI data consisted of eddy current correction and brain masking. Diffusion tensor was calculated by using dtifit-programme within the FSL software (<https://www.fmrib.ox.ac.uk/fsl>) on the basis of the default linear regression model. Outputs of the dtifit-program, fractional anisotropy (FA) and mean

diffusivity (MD) were used as such. The first eigenvalue (L1) represented axial diffusivity (AD), whereas second and third eigenvalues (L2 and L3, respectively) were averaged to provide radial diffusivity (RD) values. FA maps were used in manual region-of-interest analysis for anterior part of the anterior commissure, the forceps minor of the corpus callosum, genu of the corpus callosum, body of the corpus callosum, external capsule, internal capsule, splenium of the corpus callosum, optic tract and cerebral peduncles. Manually determined individual regions of interests from FA maps were transferred to MD, AD and RD maps to match the anatomy and obtain the corresponding values.

2.8 | Data analysis

In this study, we analysed three distinct types of experimental parameters, namely, (a) spontaneous mouse behaviour, (b) learning behaviour and (c) brain structural features in mice treated with vehicle or cuprizone for two different periods. Spontaneous behaviour parameters (beam breaks, screen touches, reward tray entries during 'Habituation 1' and spontaneous freezing in altered context during FC), imaging data and several learning parameters (number of collected rewards during 'Habituation 2', fraction of correct responses during 'Punish Incorrect' stage, FC freezing to context, and FC freezing to cue) were analysed by two-way analysis of variance (ANOVA). We looked at the main effects of treatment (cuprizone or vehicle), treatment duration (2 or 4 weeks) and interaction of these factors. The remaining learning behaviour parameters (image touch rate, blank screen touch rate, latency to touch the image and latency to collect the reward) were measured across several touchscreen testing stages and therefore analysed by three-way ANOVA. Test stage was analysed as a repeated measure and in addition to the main effects of treatment, treatment duration and their interaction effect, we also looked at the test stage \times treatment, test stage \times duration and test stage \times treatment \times duration interactions. The use of several variables and multiple statistical analyses to infer the effects of cuprizone on the parameters of spontaneous activity, learning behaviour and DTI necessitated controlling for a family-wise error rate. To this end, we grouped all ANOVA P -values for each of the three experimental categories and applied the Bonferroni-Dunn correction. Individual tests were deemed statistically significant only if unadjusted P -values were $<.0042$ for spontaneous activity data (four variables, three two-way ANOVA effects each), $<.0014$ for learning behaviour data (four variables analysed by three-way ANOVA with six effects each and four variables analysed by the two-way ANOVA with three effects each), and $<.00046$ for DTI data (four variables measured in nine different areas and analysed by the two-way ANOVA with three effects each).

Two-way ANOVA of FA, RD, AD or MD values showed that cuprizone treatment, duration and/or treatment \times duration interaction significantly affected at least one of these DTI parameters in six out of nine brain myelinated areas studied. We then explored whether FA, RD, AD or MD values in these six regions correlated

with the parameters of learning behaviour that had been found to be significantly affected by cuprizone treatment. According to the applied Bonferroni-Dunn correction to control for family-wise error rate, such correlations were considered significant only if unadjusted P for Pearson coefficient r was $<.000556$ (four DTI variables measured in six brain areas correlated with five learning behaviour variables).

To compare values of treatment effect size for learning variables, we calculated generalised eta squared (η_G^2)³⁵ because this measure allows for the comparison of effects assessed in experiments with different designs (in our case two-way and three-way ANOVA).³⁶ For variables analysed by the three-way ANOVA, we used the following equation^{36,37}:

$$\eta_G^2 = \frac{SS_{\text{treatment}}}{SS_{\text{treatment}} + SS_{\text{subject}} + SS_{\text{residual}}},$$

where $SS_{\text{treatment}}$, SS_{subject} and SS_{residual} are respective sums of squares from the three-way ANOVA output. For CFC variables and '% correct' of the 'Punish Incorrect' touchscreen stage variables, which lacked the repeated measures factor of test stage and were analysed by the two-way ANOVA, treatment η_G^2 is equivalent to partial eta squared, η_p^2 , and can be calculated as follows^{36,37}:

$$\eta_G^2 = \eta_p^2 = \frac{F \times df_{\text{treatment}}}{F \times df_{\text{treatment}} + df_{\text{residual}}}.$$

using F ratio for the treatment effect and respective values of the degrees of freedom.

Data are presented as the mean \pm SD in the text and as Tukey boxplots in the Figures (middle line: median; box: 25th and 75th percentile; whiskers/inner fences: smallest and largest values within the intervals of the 25th percentile minus $1.5 \times$ IQR and the 75th percentile plus $1.5 \times$ IQR, respectively). Values that were beyond the upper and lower inner fences were plotted individually as outliers. All statistical analyses were conducted by using Prism 8 (GraphPad Software, Inc., La Jolla, California).

3 | RESULTS

The schedule of experimental steps relative to the duration of treatment with cuprizone is presented in Figure 1A,B.

3.1 | Body weight

Before the start of gavage treatments, mice that were scheduled to receive either cuprizone or vehicle did not differ significantly in their mean weight. In the '2-week' cohort, the initial weights were $18.6 \pm .6$ g and $18.2 \pm .6$ g, respectively ($t_{(32)} = 1.849$; $P = .074$, Student's t -test). In the '4-week' cohort, the initial weights comprised $19.1 \pm .8$ g and 18.7 ± 1.0 g, respectively ($t_{(32)} = 1.367$; $P = .181$).

Following gradual 5-day food restriction, by the first day of touchscreen testing, the weight of cuprizone- and vehicle-treated mice dropped to $17.6 \pm .9$ g and $17.5 \pm .6$ g in the “2-week” cohort, who were on the fourth day of gavage treatment by then. In the “4-week” cohort, the rate of weight drop was faster in cuprizone-treated mice, so by the first day of touchscreen testing, they were slightly, but significantly lighter than vehicle-treated mice (17.0 ± 1.0 g and $18.0 \pm .7$ g; $t_{(32)} = 3.112$; $P = .004$). The weight difference in this cohort is likely explained by the longer treatment with cuprizone, which is known to cause some weight loss. Nonetheless, by carefully adjusting daily food rations, we managed to correct this difference, so the weights of cuprizone- and vehicle-treated mice were similar again on TSTD 9 ($18.3 \pm .8$ g vs $18.0 \pm .7$ g; $t_{(32)} = .99$; $P = .328$).

3.2 | Spontaneous behaviour in touchscreen chambers

Total number of infrared beam breaks during the first 30-minutes exposure to the touchscreen chamber (Habituation-1 stage) was not affected by cuprizone treatment (Figure 2A; $F_{1,61} = .069$; unadjusted $P = .794$). However, cuprizone-treated animals overall made more touches to the unlit screen than control mice (Figure 2B; $F_{1,61} = 15.33$; Bonferroni-Dunn adjusted $P = .0024$). In addition, the number of screen touches was overall significantly different between the 2-week and 4-week cohorts (treatment duration effect: $F_{1,61} = 15.33$; Bonferroni-Dunn adjusted $P = .000408$). Furthermore, cuprizone-treated mice attended empty reward tray nominally more frequently than vehicle-treated mice (Figure 2C; $F_{1,61} = 5.751$; unadjusted $P = .0196$), but the effect did not survive correction for multiple testing of spontaneous behaviour variables.

3.3 | Operant behaviour in touchscreen chambers

On TSTD2, during ‘Habituation-2’ stage mice learned that the reward tray can be a source of food. The programme required the mouse to attend the tray before the next drop of the reward was released at a fixed time interval after exiting the food tray. All mice in both cohorts readily consumed milk drink drops, which indicated that none of them had any particular aversion to the reward. The number of reward dispensations (or trials) during the fixed 60-minute period of this test stage varied between animals, depending on the pace with which mice collected the reward. Cuprizone-treated mice overall earned fewer reward drops at this stage (Figure 3A; $F_{1,61} = 13.93$; Bonferroni-Dunn adjusted $P = .0144$). We believe that this result was unlikely associated with the lower attraction of the reward after long-term exposure to cuprizone, because all but two cuprizone-treated animals from the 4-week cohort consumed 30 rewards within 60 minutes during the next ‘Initial Touch’ stage.

During the next touchscreen testing stages, all mice were nutritionally motivated to touch random images appearing in one of the two screen windows. During the ‘Initial Touch’ stage on TSTD 3, animals received reward automatically, when successively shown random images disappeared after being shown for 30 seconds. Touching an image at that stage triggered a triple amount of reward. At subsequent stages, reward dispensation was conditioned by the rules that required touching randomly appearing image (‘Must Touch’, TSTDs 4, 5), initiating image appearance by a poke into empty reward tray (‘Must Initiate’, TSTD 6) and withholding touches to blank window without the image (‘Punish Incorrect’, TSTDs 7-9). We have analysed several parameters across these stages that characterised the ability of the mice to learn the rules.

Image touch rate (Figure 3B) increased in the course of training in vehicle-treated animals but stayed largely unchanged in cuprizone-

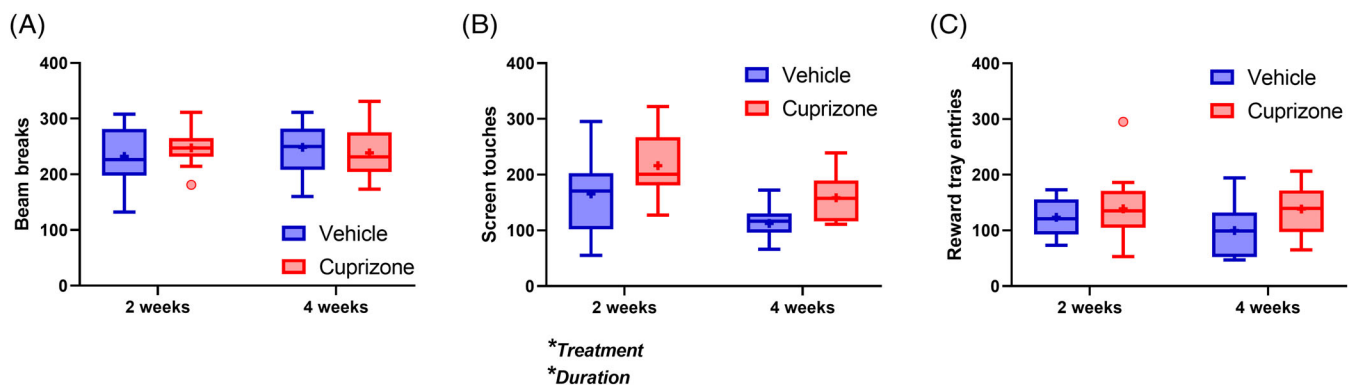


FIGURE 2 Spontaneous behaviour of cuprizone- and vehicle-treated mice during the first 30-minutes exposure to the touchscreen chamber (Habituation-1 stage). Experiments were performed on days 3 and 18 after the start of cuprizone treatment in the ‘2-week’ and ‘4-week’ cohorts, respectively. Infrared beam breaks, A, screen touches, B and visits to the reward tray, C were counted. Here and in other similar Figures, data are presented as Tukey boxplots (middle line: median; box: 25th and 75th percentile; whiskers/inner fences: smallest and largest values within the intervals of the 25th percentile minus $1.5 \times$ IQR and the 75th percentile plus $1.5 \times$ IQR, respectively). Values that were beyond upper or lower inner fences are plotted individually as outliers. In the 2-week cohort, $N_{\text{cuprizone}} = 18$; $N_{\text{vehicle}} = 16$; in the 4-week cohort, $N_{\text{cuprizone}} = 16$; $N_{\text{vehicle}} = 15$. Statistical significance of the main effects of treatment and treatment duration derived by two-way analysis of variance is indicated under the respective plots as follows: $*P < .05$ (Bonferroni-Dunn adjusted)

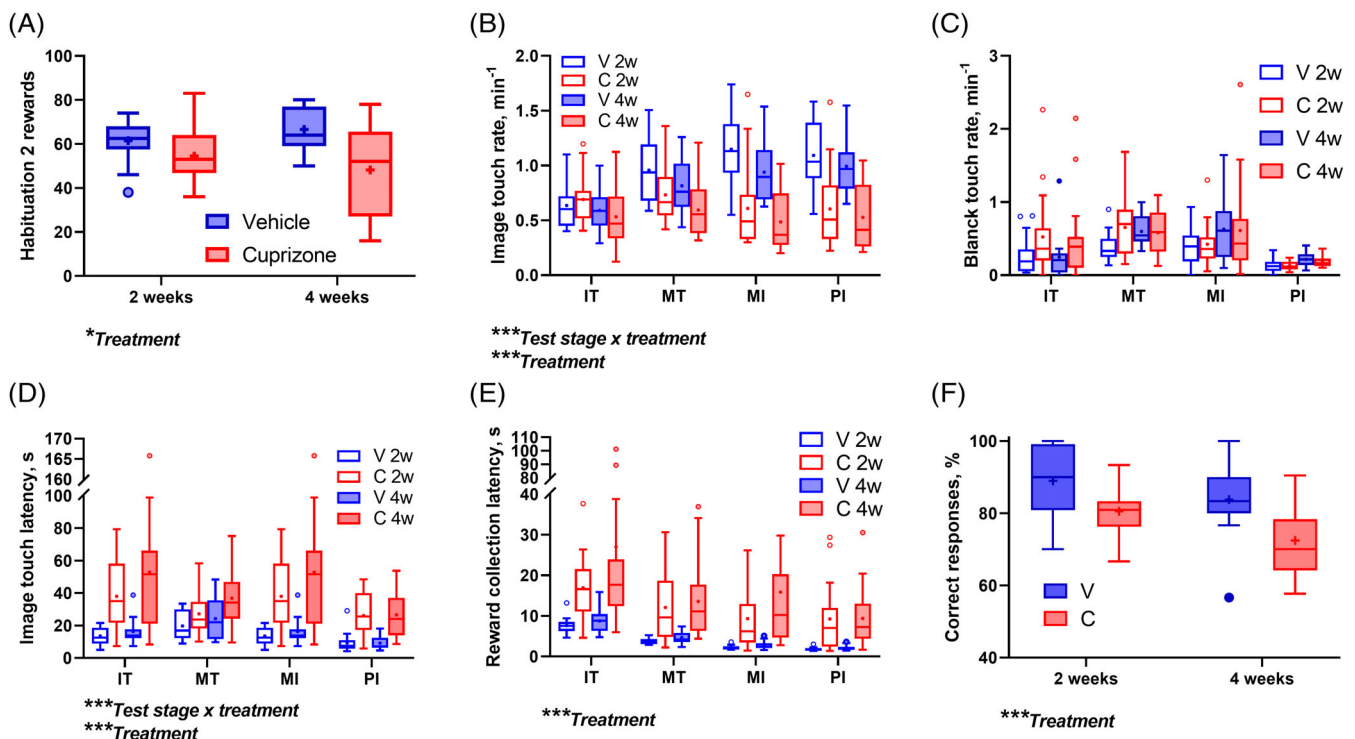


FIGURE 3 Effect of cuprizone on parameters of instrumental learning. A, Reward consumption during Habituation-2 stage of touchscreen testing. B, Image touch rate in the course of the 'Initial Touch' (IT), 'Must Touch' (MT), 'Must Initiate' (MI) and 'Punish Incorrect' (PI) stages of touchscreen testing. C, Blank touch rate. D, Image touch latency. E, Reward collection latency. F, Percentage of correct responses during the 'Punish Incorrect' stage. Statistical significance of the main effect of treatment and treatment \times duration interaction derived by two-way analysis of variance is indicated under respective plots as follows: * $P < .05$; *** $P < .001$ (Bonferroni-Dunn adjusted). C, cuprizone; V, vehicle; 2w, 2-week; 4w, 4-week

treated mice (main effect of treatment: $F_{1,61} = 25.80$, Bonferroni-Dunn adjusted $P = .000138$; test stage \times treatment interaction: $F_{3,183} = 29.47$, Bonferroni-Dunn adjusted $P < .00001$). Blank touch rate (Figure 3C) was nominally affected by the test stage \times treatment interaction (unadjusted $P = .0461$), but this effect did not survive correction for multiple testing. The slower image touch rate on the background of cuprizone administration may be caused by the procrastination to respond to the visual stimulus after it appears on the screen and/or to collect the reward thereafter. We therefore analysed the median latencies to touch the image after its appearance and to attend the reward tray for reward collection across different session (Figure 3D,E). We found that both latencies were significantly prolonged by cuprizone treatment (image touch latency, main effect of treatment: $F_{1,61} = 36.26$, Bonferroni-Dunn adjusted $P = .00000385$; reward collection latency, main effect of treatment: $F_{1,61} = 25.33$, Bonferroni-Dunn adjusted $P = .000164$). In addition, image touch latency was significantly influenced by the test stage \times treatment interaction ($F_{3,183} = 10.67$, Bonferroni-Dunn adjusted $P = .0000608$). In addition, we found that treatment had a significant negative effect on the percentage of correct responses during the 'Punish Incorrect' stage ($F_{1,61} = 18.72$, Bonferroni-Dunn adjusted $P = .0021$).

The strength of the cuprizone action on behavioural parameters was comparable in the 2-week and 4-week cohorts: no significant effects of the duration \times treatment, duration \times test stage or test

stage \times treatment \times duration interactions were observed, even at nominally significant levels.

It could be argued that because many cuprizone-treated mice did not pass the usual criteria at the preceding test stages, this circumstance could largely account for the impaired accuracy in the 'Punish Incorrect' task. In particular, during the 'Must Initiate' stage, when the mouse had to start every subsequent image presentation by making a nose poke into and exiting the reward tray, a significant fraction of cuprizone-treated animals exhibited impaired performance. Whereas all control animals completed 30 trials at this stage within 60 minutes, which is the usual required level of performance before advancement to the 'Punish Incorrect' stage,^{34,38} only 9 out of 18 mice from the 2-week cohort and 6 out of 16 mice from the 4-week cohort attained this criterion. Conventionally, mice would be kept trained at the earlier tasks until they reached those criteria, however this could result in large discrepancies in the duration of exposure to the toxin in different individuals. To verify if the compromised performance in whole cuprizone-treated cohorts was explained exclusively by the deficits in animals that did not pass the criteria for preceding tasks, in a separate analysis, we compared the accuracy of responding during the 'Punish Incorrect' task only in those mice that had passed criteria of the 'Must Initiate' stage performance (Figure S1). We found that treatment still had a significant overall effect on the percentage of correct responses ($F_{1,42} = 4.464$, unadjusted $P = .0369$), albeit at an uncorrected level of significance, which would not survive correction for multiple testing

of learning variables if their analysis was restricted only to the animals that had passed the criterion of the preceding stage.

3.4 | Fear conditioning

In 1 or 2 days following the completion of touchscreen tests, mice underwent FC, and on the next day, their freezing responses to context (same chamber) and cue (same sound, new chamber) were measured. Cuprizone-treated mice exhibited significantly decreased freezing to context compared with freezing level observed in vehicle-treated mice ($F_{1,61} = 24.72$, Bonferroni-Dunn adjusted $P = .000205$; Figure 4A). There was a nominal overall difference between the levels of freezing to context between 2-week and 4-week cohorts (duration effect: $F_{1,61} = 5.229$, unadjusted $P = .0257$), but it did not remain significant following the correction for multiple testing of learning variables. When animals were placed in the new chamber, the majority of them displayed minimal or no spontaneous freezing, and this was not affected by the treatment ($F_{1,61} = .416$, unadjusted $P = .521$; Figure 4B). Animals that were administered cuprizone displayed nominally lower levels of freezing to cue (Figure 4C), particularly in the 2-week cohort (treatment effect: $F_{1,61} = 10.01$, unadjusted $P = .0024$; treatment \times duration effect: $F_{1,61} = 4.435$, unadjusted $P = .0393$). However, as testing the memory for cue was part of exploring a wider hypothesis about cognitive deficits because of cuprizone treatment, those treatment and treatment \times duration interaction effects did not remain significant after the correction for multiple testing.

3.5 | Treatment effect size for learning variables

Next, we sought to compare the values of treatment effect size for learning variables that were significantly affected by cuprizone. We found that η_G^2 for freezing to context (Figure 4A) was the highest (0.288), and η_G^2 values for the effect of cuprizone on touchscreen parameters 'image touch latency', 'image touch rate' and 'reward collection latency', '% correct' during 'Punish Incorrect' (Figure 3B,D-F)

were slightly lower: 0.278, 0.231, 0.233, 0.213 and 0.235, respectively. In comparison, cuprizone treatment η_G^2 values for freezing to cue and number of rewards during Habituation-2 stage were much lower: 0.141 and 0.186, respectively.

3.6 | Diffusion tensor imaging

After FC experiments, on days 15 to 17 (2-week cohort) and days 30, 31 (4-week cohort), 10 mice in each subgroup underwent DTI in a Bruker 11.7 T magnet and maps of FA, RD, AD and MD were determined (Figures 5A, S2, and S3). Decreases in FA values have been detected in normal-appearing white matter in MS patients^{39,40} as well as in white matter of mice treated with cuprizone.⁴¹⁻⁴⁴ In line with these observations, cuprizone treatment had an overall negative effect on FA in the forceps minor ($F_{1,30} = 22.35$, Bonferroni-Dunn adjusted $P = .0054$), body ($F_{1,36} = 41.56$, Bonferroni-Dunn adjusted $P < .0001$) and splenium ($F_{1,32} = 48.43$, Bonferroni-Dunn adjusted $P < .0001$) of the corpus callosum, as well as in the external capsule ($F_{1,36} = 17.09$, Bonferroni-Dunn adjusted $P = .022$; Figures 5B, S3 and S4B,D,F). Furthermore, we observed a significant treatment \times duration interaction effect on FA in the splenium ($F_{1,32} = 23.14$, Bonferroni-Dunn adjusted $P = .0037$; Figure 5B) and body of the corpus callosum ($F_{1,36} = 21.75$, Bonferroni-Dunn adjusted $P = .0045$; Figure S4D). Post hoc tests showed that in both locations, FA was significantly lower in cuprizone-treated mice than in vehicle-treated animals in the 4-week cohort ($P < .0001$, Holm-Šidák test), but not in the 2-week cohort ($P > .05$, Holm-Šidák test). Finally, we observed minor overall differences in FA values between the 2- and 4-week cohorts with a statistically significant difference in the internal capsule ($F_{1,36} = 24.93$, Bonferroni-Dunn adjusted $P = .0017$; Figure S4E).

Although decreases in FA values have been frequently suggested to be direct evidence of demyelination, it has been pointed out also that water diffusion anisotropy is not exclusively defined by myelin integrity but also may be affected by axonal pathology.^{45,46} In addition, it was also suggested that MD and RD could be better metrics differentiating white matter condition in control and cuprizone-treated animals than FA.⁴⁷ To this end, we also analysed RD, AD and

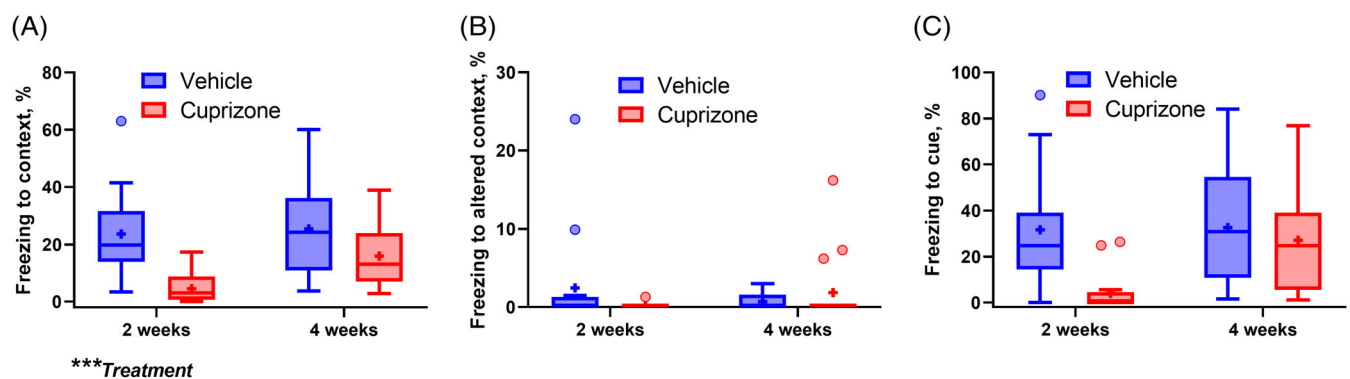
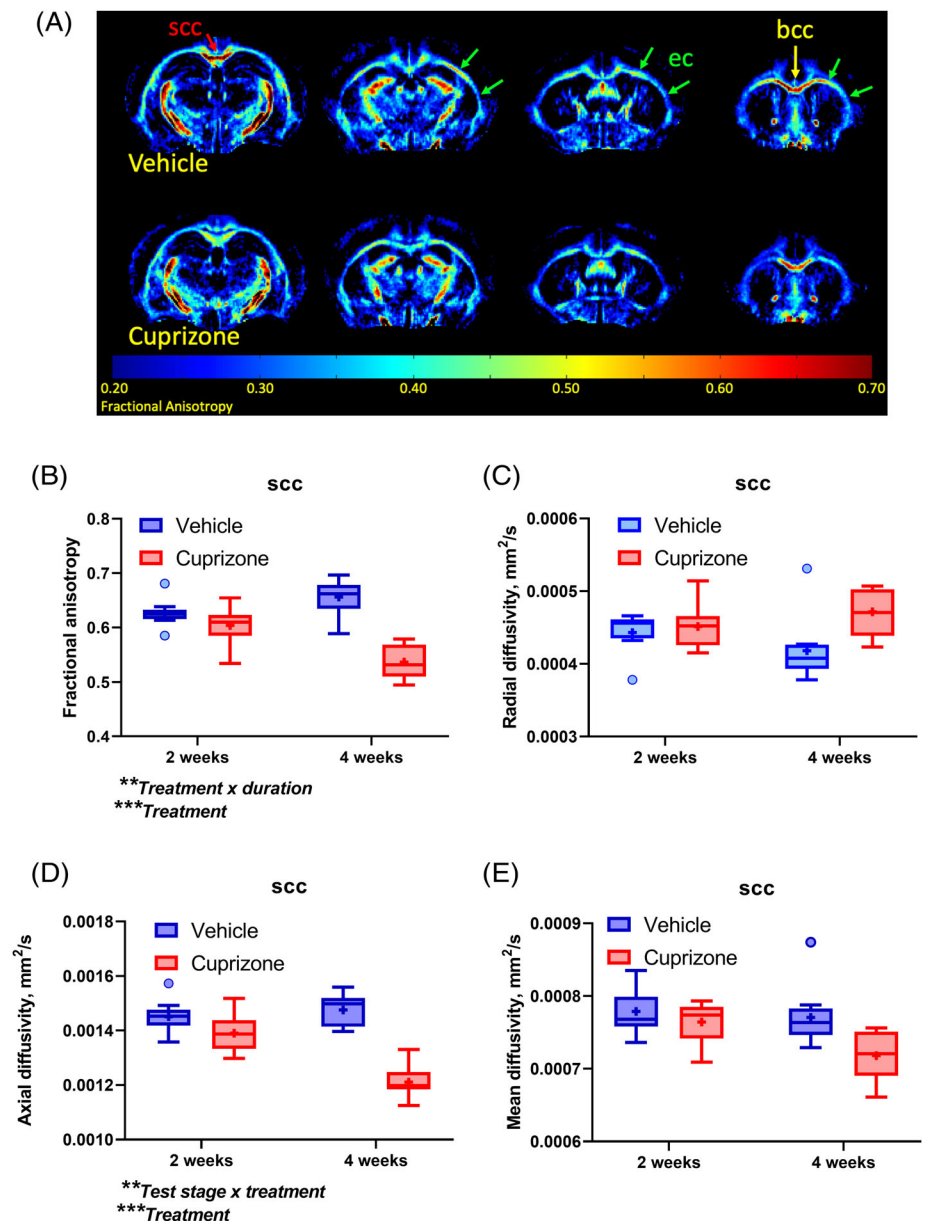


FIGURE 4 Effect of cuprizone on contextual fear conditioning learning. Fractions of time spent freezing to context, A, in altered context, B and to cue in altered context, C are shown. Statistical significance of the main effects of treatment derived by two-way analysis of variance is indicated under the respective plot as follows: *** $P < .001$ (Bonferroni-Dunn adjusted)

FIGURE 5 Diffusion tensor imaging in cuprizone- and vehicle-treated mice. Experiments were performed on days 15 to 16 and 30 to 31 after the start of cuprizone treatment in the '2-week' and '4-week' cohorts, respectively. A, Representative fractional anisotropy (FA) images obtained from mice that received vehicle (top panel) and cuprizone (bottom panel) treatment for 4 weeks (bcc, body of corpus callosum; scc, splenium of corpus callosum; ec, external capsule). Effects of cuprizone on diffusion tensor imaging data are shown on the example of the splenium of the corpus callosum (scc): FA, B; radial diffusivity, RD, C; axial diffusivity, AD, D; mean diffusivity, MD, E. Statistical significance of the main effect of treatment and treatment \times duration interaction derived by two-way analysis of variance is indicated under respective plots as follows: * $P < .05$; *** $P < .001$ (Bonferroni-Dunn adjusted)



MD parameters in our cohorts. RD values were nominally increased in cuprizone-treated mice in the forceps minor ($F_{1,31} = 6.861$, unadjusted $P = .0135$; Figure S5B) and splenium of the corpus callosum ($F_{1,32} = 7.366$, unadjusted $P = .011$; Figure 5C). However, these differences did not survive the correction for multiple testing. In contrast, we observed prominent and widespread decreases in AD following cuprizone treatment in all studied regions except for the optic tract and cerebral peduncles (Figures 5D and S6). Following the correction for multiple testing, statistically significant treatment effect was present in the anterior part of the anterior commissure ($F_{1,35} = 32.61$, Bonferroni-Dunn adjusted $P = .0002$), splenium ($F_{1,32} = 61.40$, Bonferroni-Dunn adjusted $P < .0001$) and body of the corpus callosum ($F_{1,36} = 43.99$, Bonferroni-Dunn adjusted $P = .000018$), internal capsule ($F_{1,36} = 20.08$, Bonferroni-Dunn adjusted $P = .0078$) and external capsule ($F_{1,36} = 15.64$, Bonferroni-Dunn adjusted $P = .037$). In addition, there was a significant treatment \times duration effect on AD in the

splenium of the corpus callosum ($F_{1,32} = 24.03$, Bonferroni-Dunn adjusted $P = .00285$; Figure 5D). Post hoc Holm-Šidák tests showed that cuprizone-treated animals had lower AD in both the 2-week ($P = .0455$) and 4-week cohorts ($P < .0001$). As in the case with AD, nominal decreases in MD following cuprizone administration were observed in several of the studied myelinated areas (Figures 5E and S6), but following the correction for multiple testing, the significant treatment effect was noted only in the body of the corpus callosum ($F_{1,36} = 19.31$, Bonferroni-Dunn adjusted $P = .01$; Figure 5D).

3.7 | Correlation of DTI indices with learning parameters

To establish whether changes in learning behaviour following cuprizone treatment correlated with structural alterations shown by

DTI, we normalised all FA, AD, RD and MD indices to the respective mean values of the 2- and 4-week control groups and focused our analysis on the six brain regions where DTI indices were significantly altered by cuprizone administration (anterior part of the anterior commissure, forceps minor of the corpus callosum, external capsule, internal capsule, body of the corpus callosum and the splenium of the corpus callosum) (Figures S4-S7). Analysed learning variables found to be sensitive to cuprizone included CFC freezing to context as well as % of correct responses, image touch rate, image touch latency and reward collection latency (all determined during the 'Punish Incorrect' stage). We showed that those learning behaviour parameters nominally (unadjusted $P < .05$) correlated with 2, 8, 8, 13 and 8 DTI measurements in individual brain regions (Figures S8-S12). Notably, learning behaviour data correlated with FA, AD, and MD but never with RD measurements. Given that all these individual comparisons were analysed to test a general hypothesis about the correlation of changes in DTI and cognitive parameters following cuprizone treatment, we applied a correction for multiple testing and found that the only relationships that remained statistically significant were correlations of the % of correct responses during the 'Punish Incorrect' stage with normalised FA and AD values in the body of the corpus callosum (Figure 6).

4 | DISCUSSION

Cognitive impairments in rodent models of MS have received less attention than neurological manifestations despite the former often have a considerable impact on the quality of life of individuals with MS. In this study, we used the relatively novel touchscreen-based approach as well as more conventional CFC testing and showed robust changes of cognitive behaviour in the cuprizone mouse model of MS. The relatively short duration of the used touchscreen testing routine (9 days in total) and the correlation of the detected cognitive deficits with changes in DTI parameters make touchscreen tests suitable for potential multiple longitudinal assessments in models of recurring/relapsing MS.

The current consensus regarding cuprizone effects is that it acts as a copper chelator and thereby influences the activity of multiple copper-dependent enzymes, in particular that of cytochrome oxidase involved in cellular respiration in mitochondria.^{25,29,30} In the brain, cuprizone treatment elevates the levels of oxidative stress and endoplasmic reticulum stress, which both have a relatively selective detrimental effect on oligodendrocytes.^{48,49} As a result, the chain of cellular events comprising oligodendrocyte apoptosis, microglia activation, astrocytosis and demyelination in cuprizone-treated mice leads to multiple physiological and behavioural changes resembling MS manifestations.^{25,29,30}

In our experiments, we showed multiple alterations of spontaneous behaviour and learning parameters in mice treated with cuprizone. One of the characteristic behavioural changes in the cuprizone model of MS is hyperactive locomotion reflected in more frequent crosses of infrared beams, total distances travelled, and higher speeds in the open field,⁵⁰⁻⁵⁶ or in the total number of Y-maze arm entrances.⁵⁷⁻⁵⁹ Nonetheless, in other studies, no clear hyperactivity in the open field was seen,⁶⁰⁻⁶⁴ and one study reported reduced total distance travelled in mice that received cuprizone with chow for 5 weeks.⁶⁵ Our observations in mice that were on cuprizone for 3 or 18 days showed that cuprizone- and vehicle-treated mice made similar number of infrared beam crosses (Figure 2A). Perhaps the relatively small size of the touchscreen chamber compared with the dimensions of typical open field arenas and short duration of treatment precluded detection of potential hyperactivity manifestations in cuprizone-treated mice in our experiments. The higher number of screen touches in cuprizone-treated mice potentially indicated accentuated willingness to interact with novel objects (Figure 2B). It has been reported that mice treated with cuprizone for 3 weeks spent longer time exploring the objects in the novel object recognition test.⁵⁷ However, in other studies, either no effect or an opposite result (diminished interaction) was reported in that test after 1 week on cuprizone.^{52,59} Furthermore, cuprizone-treated mice spent longer time in the open arms of the elevated plus maze,^{53,60,63,66} which likely indicated their decreased anxiety and/or increased tendency to explore novel locations, although the absence

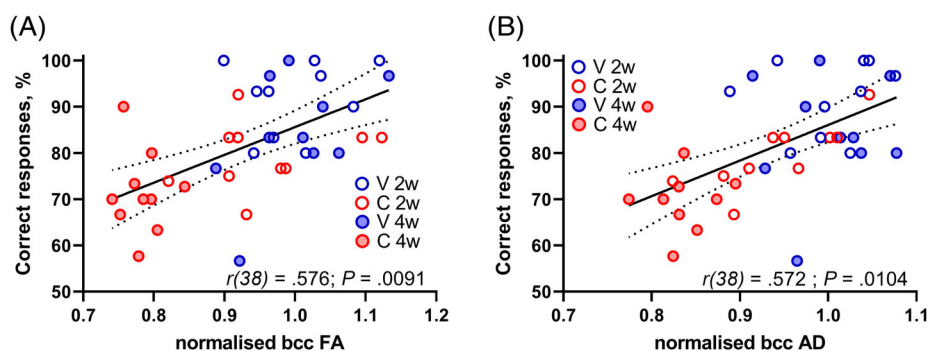


FIGURE 6 Correlations between diffusion tensor imaging parameters and accuracy of performance in the 'Punish Incorrect' task. A, Fractional anisotropy (FA) and B, axial diffusivity (AD) in the body of the corpus callosum (bcc) were normalised by the mean FA and AD values in vehicle-treated mice in the 2-week or 4-week cohorts and compared with the cumulative percentage of correct responses during the 3 days of the 'Punish Incorrect' touchscreen testing stage. Statistical significance of the correlations is indicated after Bonferroni-Dunn correction for multiple testing

of such a phenotype has also been reported.⁶⁷ Discrepant evidence regarding anxiety and propensity to explore in cuprizone-treated mice was obtained from the analysis of mouse presence in the central area of the open field: whereas Xiu et al⁶³ reported increased distance travelled in the central area in cuprizone-treated animals, others observed an opposite result.^{62,65,68}

It has been recently suggested that cellular and behavioural changes following short (<4 weeks) exposures to cuprizone are relevant to the biology of schizophrenia and other affective disorders.^{61,69} Observations that were compatible with this notion included impaired spatial working memory in the Y-maze test, decreased paired-pulse inhibition and lower intensity of social interactions observed in mice that received 0.2% to 0.4% cuprizone in the diet for short periods.^{59,60,67,70} Furthermore, the sensitivity of these phenotypes to antipsychotic drugs such as quetiapine or clozapine,^{52,54,55,66,70,71} as well as increased levels of brain dopamine in cuprizone-treated mice^{57,60,70} also supported this view. Our touchscreen-based tests showed several additional behavioural changes that further extend the range of putative schizophrenia-related symptoms in cuprizone-treated mice. The extremely long latencies to collect the reward observed in cuprizone-treated mice across different phases of touchscreen testing (Figure 3E) may be a sign of impaired reward processing, which is a known comorbidity in schizophrenia.⁷² In particular, delayed reward collection along with the slower rates of image touches (Figure 3B) are in agreement with decreased reward anticipation, reduced learning from rewards and psychomotor slowing in individuals with schizophrenia.⁷³ At the same time, these signs are also in accord with decreased responsiveness to rewards, slower reaction time and reduced processing speed in decision-making tasks in individuals with MS.^{21,74-76} Touchscreen CANTAB tasks 'Match to Sample', 'Paired Associates Learning' and 'Spatial Working Memory' have revealed deficits in multiple cognitive domains in MS patients, including impairments in processing speed, reaction time, attention and executive function.²⁰ Thus, suboptimal performance of cuprizone-treated mice in a series of simple touchscreen-based tasks may be translationally relevant to those manifestations. Furthermore, the extent of myelination affects reaction time and processing speed^{77,78} and correlates with disturbances of these parameters in MS and schizophrenia.^{79,80}

It should be noted that the ability to emit vigorous and accurate responding in touchscreen tasks depends on good eyesight: to be able to discriminate windows with and without visual stimuli and to differentiate between stimuli in discrimination paradigms.³⁴ Good sense of hearing is also essential as sound is used as a reinforcing signal of reward dispensation in touchscreen tests. Thus, the inferior performance of cuprizone-treated animals might be partially explained by deficits in visual and auditory circuitries described for this model,⁸¹⁻⁸³ although those impairments were usually observed after longer cuprizone administration regimens. Impaired visual memory of cuprizone-treated mice in Morris water maze has been shown by several laboratories,^{68,84-87} although some reports indicated preserved performance^{63,88,89} or very mild alterations.⁹⁰

The impaired accuracy in the last stage of our touchscreen testing routine, the 'Punish Incorrect' task (Figure 3F), could have occurred because many cuprizone-treated mice were moved to that stage without having attained the usual criteria for the 'Must Touch' and/or 'Must Initiate' tasks. The need for longer duration of these tasks may be a characteristic phenotype by itself, as has been reported for several genetically altered mice during similar routines.^{91,92} Because our initial experimental design was based on equal exposure of all treated animals to cuprizone, we could not ensure that all animals attained the respective criteria. However, even when the analysis was restricted to 'criteria-attaining' animals, the accuracy was lower in cuprizone-treated mice, although the effect did not reach significance threshold because of significantly smaller sample size (Figure S1). In future experiments, it will be useful to keep testing all cuprizone-treated animals until they reach the criteria for all pretraining stages, to exclude the possibility that the detrimental effect of the treatment on response accuracy and various reaction times was solely to under-training. Our prediction would be that when properly powered, such an experiment would show both a longer period needed to attain the "Punish Incorrect" and lower accuracy during the last 2 days at that stage.

In our CFC experiments, memory of context was significantly weaker in cuprizone-treated mice (Figure 4A), which indicated their deficient associative learning ability. Freezing to cue was also nominally lower in cuprizone-treated animals, particularly in the 2-week cohort but the effect did not reach statistical significance. To the best of our knowledge, there was only one published study of CFC in cuprizone-treated animals, in which no differences were found in the memories of context and cue after 6 to 7 days of cuprizone treatment.⁸⁹ It was of interest to compare the sensitivity with which touchscreen testing and CFC detected abnormalities in cuprizone-treated mice. Overall treatment effect size η^2_G was the largest for CFC freezing to context, but η^2_G values for several touchscreen parameters were only 4% to 35% lower. Thus, we could cautiously conclude that sensitivity of short touchscreen testing to detect cognitive phenotypes was close to that of CFC.

It is unlikely that the observed changes in behaviour of cuprizone-treated mice were caused by extensive demyelination, as the latter is thought to occur only after many weeks of treatment with cuprizone.^{27,93} In the original paper describing the consequences of cuprizone treatment by gavage, substantial demyelination was detected in mice treated for 5 weeks not only with the same dose as the one used in our study (400 mg/kg), but also in animals that received 200 or 300 mg/kg.³² These observations and similar outcomes after 4-week treatment with cuprizone admixed in food^{42,43} suggest that at least some demyelination should be present in our experiments after 4 weeks of treatment with 400 mg/kg. The conclusion about the presence of a certain degree of demyelination is also in accord with relatively large changes in FA at 4 weeks (Figures 5B and S4B,D,F). Furthermore, in support of the presence of demyelination, nominally higher RD values were seen in the 4-week group in the splenium of corpus callosum (Figure 5C) and forceps minor of the corpus callosum (Figure S5B), however the very stringent statistical

treatment did not detect a significant effect. The literature data regarding the presence and degree of demyelination after shorter (<3 weeks) periods of treatment with cuprizone are less conclusive. On the one hand, no obvious demyelination was seen at 3 weeks in two studies, in which cuprizone was given by gavage⁹⁴ or with food.⁹⁵ On the other hand, other reports detected demyelination at 2 to 3 weeks on cuprizone given with food.^{93,96,97} Accordingly, in line with the time-dependent effect of the toxin, we observed a significant interaction of the treatment and its duration with regards to FA values in the body and splenium of the corpus callosum, with stronger decreases in the 4-week cohort. Overall, our DTI data were broadly in agreement with previous *in vivo* imaging studies that reported a lack of significant alterations in RD and FA after short (≤ 4 weeks) exposure to cuprizone and lower FA following cuprizone treatment for over 5 weeks.^{41-44,47} The fact that we detected lower FA already after 4 weeks and, in some areas, even after 2 weeks of treatment might be potentially explained by a more reliable mode of cuprizone administration (gavage).

Another characteristic observation in our imaging experiments was lower AD in cuprizone-treated animals (Figures S5 and S6). Similar decrease in AD has been reported previously in this model,^{41,44} and it could be a consequence of accumulating myelin debris and developing axonal damage.^{42,43} The time course of axonal damage likely closely followed the development of the demyelination: extensive axonal damage was seen after 4 to 5 weeks of administration with food^{42,43,98} and, therefore, was likely manifest after 4 weeks of treatment in our experiment, whereas treatment for 1 week did not show significant axonal damage.⁹⁹ The lack of detailed histological experiments^{46,100} was a limitation of our study, which precluded a definitive answer to the question whether cuprizone-induced decrease in FA values was principally because of demyelination or changes in axonal characteristics in our setting. Histological analyses will be needed in future to provide ultimate validation of the gavage mode of cuprizone administration, particularly at earlier periods of treatment with the toxin. Changes in some behavioural and DTI parameters seen already in the 2-week group suggest that cuprizone treatment by gavage may be more effective than administration with food.

Although statistically significant correlations with learning parameters were only confirmed for FA and AD in the body of the corpus callosum (Figure 6), a large number of nominally significant correlations of FA, AD and MD across six brain regions with unadjusted $P < .05$ (Figures S8-S12) suggests that larger and more focused studies would readily uncover robust relationships between structural and functional data. Interestingly, despite cuprizone effect size was the maximal for freezing to context, % freezing data nominally correlated with only two out of 24 analysed DTI measurements, whereas the four touchscreen learning parameters correlated with 8 to 13 DTI measurements each.

Notwithstanding several possible interpretations of the obtained imaging data, altered behaviours in our experiments were likely triggered by oligodendrocyte dysfunction and downregulation of expression levels of myelin genes, which occur already in the first week of

treatment with cuprizone, preceding pronounced demyelination.^{95,96,101} Furthermore, although extensive axonal damage because of demyelination in cuprizone-treated mice^{98,102} was unlikely prominent in our experiments because of relatively short exposures to the toxin, axonal conduction has been shown to become slower after just 7 days on cuprizone-containing diet,¹⁰³ in line with observed AD changes. Other neuronal consequences of cuprizone intoxication that could impact behaviour include redistribution of voltage-dependent sodium and potassium channels,^{104,105} dysregulated expression of synaptic receptors and neurotransmitter transporters,^{106,107} and altered activity of enzymes that break down neurotransmitters.^{60,108}

In summary, in view of the popularity of the preclinical cuprizone model of demyelination, we sought to enhance its face validity by assessing the effects of cuprizone treatment on operant learning in translational touchscreen-based tasks and associative memory in FC test. We showed that cognitive disturbances in treated animals are evident already after several days of cuprizone administration and thus are not contingent on extensive demyelination typically seen only after prolonged administration regimens (>4 weeks). Nevertheless, in several cases, imaging data were found to correlate significantly with learning variables. Given that many behavioural assessments of cuprizone-treated mice have so far yielded discrepant results, sensitive touchscreen and CFC tests may be promising techniques to screening novel MS drug candidates for their ability to reverse cognitive deficits in longitudinal experiments.

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CONFLICT OF INTEREST

All research was conceptualised, planned and directed by scientific staff at Charles River Discovery and Teva Pharmaceutical Industries Ltd. Charles River Discovery Services Finland Oy is a contract research organisation that conducted part of the study through a fee-for-service agreement with Teva Pharmaceuticals Industries Ltd. At the time of the study, Maksym V. Kopanitsa, Kimmo K. Lehtimäki, Markku Forsman, Ari Suhonen, Juho Koponen, Tuukka O. Piipponiemi, Artem Shatillo, Anna-Mari Kärkkäinen, Patrick J. Sweeney and Antti Nurmi were employed by Charles River Discovery Services Finland Oy, whereas Joel Kaye, Aric Orbach and Avia Merenlender-Wagner were employed by Teva Pharmaceuticals Industries Ltd.

AUTHOR CONTRIBUTIONS

Maksym V. Kopanitsa, Juho Koponen, Aric Orbach and Antti Nurmi designed the study. Maksym V. Kopanitsa, Markku Forsman, Ari Suhonen, Juho Koponen and Tuukka O. Piipponniemi carried out the experiments. Maksym V. Kopanitsa, Kimmo K. Lehtimäki, Markku Forsman, Ari Suhonen and Pavlina Pavlidi analysed the data. Patrick J. Sweeney and Antti Nurmi provided the resources. Artem Shatillo, Anna-Mari Kärkkäinen and Avia Merenlender-Wagner advised on experimental design and data interpretation. Maksym V. Kopanitsa and Kimmo K. Lehtimäki wrote the manuscript.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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