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Cuprizone short-term exposure: astrocytic IL-6 activation and behavioral changes relevant to psychosis

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Abstract

A growing body of evidence suggests the involvement of inflammatory processes in the pathophysiology of schizophrenia. Four to eight-week exposure to cuprizone, a copper chelator, causes robust demyelination and has been used to build a model for multiple sclerosis. In contrast, we report here the effects of one-week cuprizone exposure in mice. This short-term cuprizone exposure elicits behavioral changes that include augmented responsiveness to methamphetamine and phencyclidine, as well as impaired working memory. The cellular effects of one-week cuprizone exposure differ substantially from the longer-term exposure; perturbation of astrocytes and microglia is induced without any sign of demyelination. Furthermore, the proinflammatory cytokine interleukin-6 was significantly up-regulated in glial fibrillary acidic protein (GFAP)-positive cells. We propose that this cuprizone short-term exposure may offer a model to study some aspects of biology relevant to schizophrenia and related conditions.

Keywords

inflammation; astrocyte; cytokine; interleukin-6; schizophrenia

Introduction

Although we are still far from a complete picture of the cause of schizophrenia, our understanding of this disease has come a long way. A mounting body of epidemiological data suggests that inflammatory dysregulation may underlie the pathology of some cases of schizophrenia (Benros *et al.*, 2011; Yolken and Torrey, 2008). Genome-wide association studies have added weight to the idea of immune involvement in the pathology by revealing an association between schizophrenia susceptibility loci and the major histocompatibility complex (MHC) locus on chromosome 6p, a well-replicated common variant locus (Duan *et al.*, 2010; Purcell *et al.*, 2009; Shi *et al.*, 2009; Stefansson *et al.*, 2009). Immune system-related cytokines, including interleukin 6 (IL-6), are reportedly elevated in serum and cerebrospinal fluid (CSF) of schizophrenia patients (Coughlin *et al.*, 2013; Garver *et al.*, 2003; Lin *et al.*, 1998; Miller *et al.*, 2011; Potvin *et al.*, 2008; van Kammen *et al.*, 1999). Furthermore, recent positron emission tomography studies with radioligands for translocator

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protein (peripheral benzodiazepine receptor) support the presence of excess neuroinflammation in the brains of patients with schizophrenia or early psychosis compared with those of normal controls (Doorduyn *et al.*, 2009; Kreisl *et al.*, 2012).

Here we propose that a short-term (one-week) systemic exposure of mice to a copper chelator, cuprizone, may emulate an important aspect of biology underlying schizophrenia and related disorders. Long-term exposure (4-8 weeks) to cuprizone has been used to generate a model for multiple sclerosis, in which robust demyelination is elicited (Koutsoudaki *et al.*, 2009; Liu *et al.*, 2010; Matsushima and Morell, 2001). In contrast, we demonstrate that short-term (one-week) exposure to this compound leads to a combination of glial activation and behavioral changes without inducing demyelination.

Materials and methods

Animals and cuprizone exposure

All experimental procedures followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, the Johns Hopkins University Animal Care and Use Guidelines, and the guidelines provided by Mitsubishi Tanabe Pharma Corporation. C57BL/6J male mice were obtained from Charles River Japan. They were kept in quiet facilities under controlled temperatures ($23 \pm 3^\circ\text{C}$) and a controlled light schedule (lights were on from 7am to 7pm) with unlimited food and water. Eight-week-old mice were randomly allocated to two groups. For one week, mice were fed either a diet containing 0.2% cuprizone (Sigma-Aldrich), or a control diet consisting of standard mouse chow. The diets of the two groups were identical except for the presence of cuprizone. All experiments described below were performed on the final day of cuprizone exposure.

Behavioral analyses

Each of the behavioral tests described below were performed with a different cohort of mice. Animal behavior and movement were monitored with the SCANET system (Melquest). In testing hyperlocomotion induced by metamphetamine (0.3 mg/kg: from Dainippon Pharma) or phencyclidine (2.5 mg/kg: synthesized by Mitsubishi Tanabe Pharma), animals were habituated in the apparatus for 30 min and then injected subcutaneously with saline, or one of the above drugs. Ambulatory activity, measured as “counts” passing a set of infrared beams in the SCANET system, was measured in 5 min intervals for 60 min after the injection. The Y-maze (22 cm long, 20 cm high and 6.5 cm wide, with arms positioned 120° degrees apart) was used according to a published protocol (Fukumoto *et al.*, 2010). Briefly, each mouse was placed at the end of one arm and allowed to move freely through the maze for 5 min. The sequence of arm entries was recorded manually. A spontaneous alternation was defined as entry into all three arms on consecutive occasions. Therefore, the possible maximum alternation was the total number of arm entries minus two, and the ratio of spontaneous alternation was calculated as actual alternations/maximum alternations. The total number of arms entered during the sessions was also determined. The novel object recognition test was conducted according to a published protocol (Barker *et al.*, 2007). Briefly, mice were habituated to the arena ($45.5 \times 25 \times 35$ cm) without any objects for 5 min. An acquisition phase was conducted 24 hours later, followed by a recognition test 3h after acquisition. The SCANET MV-10 system (Melquest) was used to measure movements. During acquisition, two identical objects were placed in the arena and the animal was allowed to explore for 5 min. Exploratory behavior was defined as the animal biting, sniffing or touching the objects. Other behaviors, such as looking around while sitting on or resting against the object, were not considered as exploration. In the recognition test the animal was presented with a familiar object and a novel object, distinct in color and shape, in the same positions as during acquisition for 5 min. The percentage of exploratory

preference was defined as the ratio of the time spent exploring the novel object (Tn) over the time spent exploring the novel and the familiar objects (Tf), $[Tn/(Tn + Tf)] \times 100$. Prepulse inhibition was assessed using the San Diego Instruments apparatus according to a published protocol (Kopeck *et al.*, 2010). Briefly, mice were habituated for 5 min with background noise levels (70 dB) prior to testing. The 20 min test sessions consisted of 5 trial types: (1) pulse alone (120 dB for 40 ms); a 20 ms prepulse at (2) 10 dB, (3) 15 dB, or (4) 20 dB above background levels, followed 100ms later by the main pulse; or (5) background only (70 dB, no stimulus). The inter-trial interval was 12-18 s (average is 15 s), and each session consisted of 12 sets of each 5 trials, thus a total of 60 trials. Startle amplitude was recorded for each trial except for the first set of trials. The prepulse inhibition ratio at each prepulse sound level was calculated as $100 \times (\text{averaged response amplitude in trials with a prepulse stimulus} / \text{averaged response amplitude in trials with the startle stimulus alone})$. The numbers of animals used for each assay is described in the figure legends.

Black-Gold staining

Mice were perfused with 10% neutral phosphate buffered formalin. The brains were fixed overnight in the same solution, then treated with 25% sucrose solution, and mounted with O.C.T. compound. Sections were cut in the vicinity of bregma -2.0 mm at a thickness of 20 μm on a freezing sliding microtome. Black-Gold staining was performed on control (n=3) and one-week cuprizone exposure mice (n=4). Four sections were stained per mouse (control: 12 sections, cuprizone: 16 sections) using the Black-Gold II myelin staining kit (Millipore) with minor adjustments to the incubation period stated in the manufacturer's protocol. Brain sections were imaged using TE300 (Nikon) and DP controller (Olympus). Four images were analyzed per mouse. The average light intensity within the corpus callosum was quantified using Image J software.

Electron microscopy

Electron microscopy was conducted on control (n=3) and one-week cuprizone exposure mice (n=3). Mice were perfused with 2.5% glutaraldehyde. Sections were postfixed in 2.5% glutaraldehyde and 2% osmium tetroxide solution, then dehydrated and mounted with epoxy resin. Sections of 100 nm thickness were cut using an EM UC7 ultramicrotome (Leica) then stained with uranyl acetate and lead citrate. Quantification was conducted using eight to thirteen sections per mouse imaged with a CCD camera. All fibers within each 3 μm^2 image were digitized. A total of 40-70 digitized fibers were analyzed per mouse. Image J software was used to calculate G-ratios (defined as the diameter of the axon divided by the diameter of the axon and myelin) based on the widest point for each axon.

Molecular histology

Immunohistochemistry was performed according to a published protocol (Kousaka *et al.*, 2009). Briefly, 8 μm thick coronal sections were incubated with antibodies against GFAP (rabbit polyclonal antibody, Dako, 1:50) and Iba1 (rabbit polyclonal, Wako, 1:16,000), followed by secondary antibodies with horseradish peroxidase (Nichirei) and 3, 3'-diaminobenzidine for signal detection. *In situ* hybridization was conducted as published (Kikawada *et al.*, 2007). Briefly, a 500 bp DNA fragment corresponding to nucleotide positions 66-461 of mouse interleukin 6 (NM_031168.1) was subcloned into pGEMT-Easy vector (Promega) and used to generate the RNA probe. Brain tissue was fixed with Tissue Fixative (Genostaff), embedded in paraffin, then sectioned at 6 μm . Hybridization was performed with probes at a concentration of 300 ng/ml in Probe Diluent-1 (Genostaff) at 60°C for 16 h. Digoxigenin (DIG)-labeled probes were detected using NBT/BCIP solution (Sigma-Aldrich) for color reactions. Cell counting was conducted blind to treatment.

Quantitative real-time PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen), which was reverse transcribed to cDNA with ReverTra Ace (Toyobo) with random primer pd(N)9 (TAKARA BIO, Shiga, Japan). Quantitative real-time PCR was performed using Taqman reagents and the ABI 7500 Fast Sequence Detection System (Life technologies). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primer and probe information is shown in Supplementary Table 1.

Copper measurement

Total copper levels in brain were measured in control (n=3) and one-week cuprizone exposure mice (n=3). Brains were homogenized in 0.01 M HCl, centrifuged at 4°C and the supernatant was assessed using the Metallo Assay Low Copper LS (Metallogenics) kit according to the manufacturer's instructions. Briefly, color reagent was added to samples, copper standard solution (40 µg/dL) and distilled water in a 96 well plate and incubated at room temperature for 10 min. Copper concentrations in samples were calculated from the standard by measuring the absorbance at 582 and 675 nm using Spectra Max M2 (Molecular Devices).

Statistical analysis

Behavioral data of two groups were analyzed by Student's *t*-test. Prior to statistical analysis of quantitative RT-PCR data, the mRNA expression level in the cuprizone group was normalized to the control group for each brain region. Analysis was conducted using Student's *t*-tests within each brain region and results were considered significant at a Bonferroni adjusted *p* value of < 0.05. The data are shown as mean ± S.E.M.

Results

Exposure of mice to cuprizone for one week results in abnormal behaviors

To explore the behavioral outcome of short-term (one-week) exposure to cuprizone, we performed several behavioral tests. Mice exposed to the cuprizone-containing diet for one week showed increased locomotor activity in response to a low dose of psychostimulant (0.3 mg/kg methamphetamine) whereas mice fed the control diet showed no increase in locomotion (Fig. 1a). At higher doses of methamphetamine (1.0 and 3.0 mg/kg), both groups responded with similar levels of hyperlocomotion. (Supplementary Fig. 1). The short-term exposure of mice to cuprizone also augmented locomotor response to phencyclidine, a N-methyl-D-aspartate (NMDA)-type glutamate receptor antagonist (Fig. 1b). There was no difference between groups in baseline locomotor activity (Supplementary Fig. 2). Evaluation of working memory using the Y-maze test demonstrated that short-term cuprizone exposure significantly decreased spontaneous alternation without affecting total arm entry (Fig. 1c). The cuprizone mice also displayed decreased preference for the novel object during the retention phase of the novel object recognition test (Fig. 1d). The acoustic startle response and prepulse inhibition were measured, however, no significant difference was observed between control and cuprizone mice (Supplementary Fig. 3).

Pharmacokinetics for PCP and methamphetamine following cuprizone exposure

Methamphetamine and PCP concentrations within the brain and blood plasma were measured to determine whether there was any difference in the pharmacokinetics of drug metabolism following cuprizone exposure. At the dose utilized in the locomotor assay (methamphetamine, 0.3 mg/kg and phencyclidine, 2.5 mg/kg), drug concentrations in the brain, 0.5 and 1 h after administration, did not differ between control and cuprizone mice (Supplementary Fig 4). The plasma concentration of PCP was significantly lower in the

cuprizone mice 1 h after injection. No difference was observed at 0.5 h for PCP or either time point for methamphetamine (data not shown). Therefore, it seems that the observed cuprizone-induced hypersensitivity to psychostimulants does not result from increased exposure to the drugs.

Short-term (one-week) exposure to cuprizone does not cause demyelination

To investigate whether short-term (one-week) exposure to cuprizone induces demyelination, we performed Black-Gold myelin staining and electron microscopy studies in the corpus callosum of control and cuprizone-exposed mice. The Black-Gold stain showed no gross differences in myelination levels between the control and one-week cuprizone-exposed groups (Fig. 2a, b). The G-ratios obtained by measuring individual axons using electron microscopy further support the lack of demyelination after a one-week exposure to cuprizone (Fig. 2c, d). Of note, the level of copper in the brain was significantly augmented following short-term cuprizone-exposure (Supplementary Fig 5). An increased copper level in various tissues, including brain, was previously reported in long-term (3-9 months) cuprizone-exposed mice (Zatta *et al.*, 2005).

Short-term exposure to cuprizone robustly activates astrocytes and microglia

Given the absence of demyelination, we wondered if there were alterations in other types of glia in the mice. Immunohistochemical staining revealed a robust increase in the number of glial fibrillary acidic protein (GFAP)-positive cells in mice exposed to cuprizone, indicating the activation of astrocytes (Fig. 3a). Cells positive for ionized calcium binding adaptor molecule 1 (Iba1), a marker of activated microglia, were also increased in cuprizone-exposed mice (Fig. 3b). Consistent with the immunostaining, GFAP mRNA expression was augmented in the hippocampus, striatum and frontal cortex of the cuprizone mice (Fig. 3c). Likewise, Iba1 mRNA was also up-regulated in the hippocampus and striatum of the cuprizone-exposed mice (Fig. 3d).

Short-term exposure to cuprizone induces interleukin 6 (IL-6)

Glial cell activation is known to induce cytokine and chemokine expression and release, which modulate neural plasticity (Ben Achour and Pascual, 2010; Glass *et al.*, 2010; Singh *et al.*, 2011). Among such molecules, an increase in IL-6 is reported in the blood of patients with schizophrenia (Coughlin *et al.*, 2013; Potvin *et al.*, 2008). Measurement of IL-6 mRNA levels revealed elevation in the hippocampus and striatum of mice exposed to short-term cuprizone (Fig. 4a). Histological co-staining revealed that the majority of IL-6-positive cells were co-labeled with GFAP-, but not with Iba1-immunoreactivity (Fig. 4b).

Discussion

The present study shows that short-term (one-week), systemic exposure of mice to a copper chelator, cuprizone, elicits unique cellular changes that are accompanied by behavioral deficits, such as hypersensitivity to methamphetamine and phencyclidine as well as working memory deficits. The phenotype of this model, especially lack of demyelination, is a clear contrast to mice exposed to cuprizone for longer durations (4-8 weeks), which display robust demyelination and are used as a model for multiple sclerosis (Koutsoudaki *et al.*, 2009; Liu *et al.*, 2010; Matsushima and Morell, 2001). Although elevated expression of mRNA and protein for pro-inflammatory cytokines has been reported in mice displaying robust demyelination following cuprizone exposure (Arnett *et al.*, 2001; Yoshikawa *et al.*, 2011), our study is the first to examine cytokine levels in cuprizone-exposed mice prior to the onset of demyelination. Given that inflammatory cascades are thought to be involved in major mental illnesses, cuprizone short-term (one-week) exposure may offer a promising model to study some aspects of schizophrenia and related conditions.

A possible association between copper and schizophrenia has been historically suggested (Bowman and Lewis, 1982), however no consistent relationship has been established: some studies have reported increased copper in serum or plasma (Herran *et al.*, 2000; Wolf *et al.*, 2006; Yanik *et al.*, 2004), whereas others reported no difference in CSF and postmortem brains (Kornhuber *et al.*, 1994; Shore *et al.*, 1983). Copper acts as a modulator of neuronal transmission, and its release from neurons may regulate N-methyl-D-aspartate receptor activity (Gaier *et al.*, 2013; Schlieff and Gitlin, 2006). Copper is also a component of several metalloenzymes linked to dopamine synthesis, in biochemical pathways involving either antagonism of dopamine production or catalysis of its breakdown. It has been suggested that chronic copper exposure may result in overcompensation for dopamine deficiency, via an increased number or sensitivity of post-synaptic dopamine receptors in certain areas (Bowman and Lewis, 1982; Wolf *et al.*, 2006).

Animal models are useful for understanding some aspects of the pathophysiology of psychiatric disorders and for testing pharmacological interventions based on specific hypotheses. Although drug-induced models may not have etiological validity, their ease of generation and high throughput makes them valuable when focusing on specific endophenotypes or pathophysiology relevant to diseases. Previous works have observed behavioral changes in rodents that may be reminiscent of psychiatric disorders upon long-term cuprizone exposure (Gregg *et al.*, 2009; Xu *et al.*, 2009). Some of these changes were ameliorated by neuroleptic treatment (Xu *et al.*, 2010; Xu *et al.*, 2011; Zhang *et al.*, 2008), which suggests that the long-term cuprizone exposure model may mimic psychiatric manifestations associated with multiple sclerosis and related conditions that involve robust white matter damage. Pioneering work indicates that the atypical antipsychotic, quetiapine, facilitated oligodendrocyte development by directing the differentiation of neural progenitors towards the oligodendrocyte lineage (Xiao *et al.*, 2008). In contrast, the short-term cuprizone exposure model presented here may model psychotic conditions that occur in the absence of demyelination.

The short-term cuprizone exposure model raises at least two major questions. The first issue is the cause of the astroglial changes, including IL-6 activation. Although it is beyond the scope of this study, future experiments will be needed to explore the neurochemical and behavioral consequence of region-specific IL-6 modulation in astrocytes. Region-specific difference in the response to cuprizone may be related to heterogeneity in the age of maturation of white matter tracts (Yang *et al.*, 2009). Indeed, the effects of cuprizone are more severe when mice are exposed at earlier ages (Makinodan *et al.*, 2009; Wang *et al.*, 2013). The second issue is how to reconcile recent reports supporting the involvement of inflammation in schizophrenia (Doorduyn *et al.*, 2009; Kreisl *et al.*, 2012), with classic observations showing no robust gliosis in autopsied brains from patients (Harrison, 1999). Although further studies are needed, one possible working hypothesis is the transient activation of glial cells in a state-dependent fashion, preferentially associated with the onset and several relapse phases during the course of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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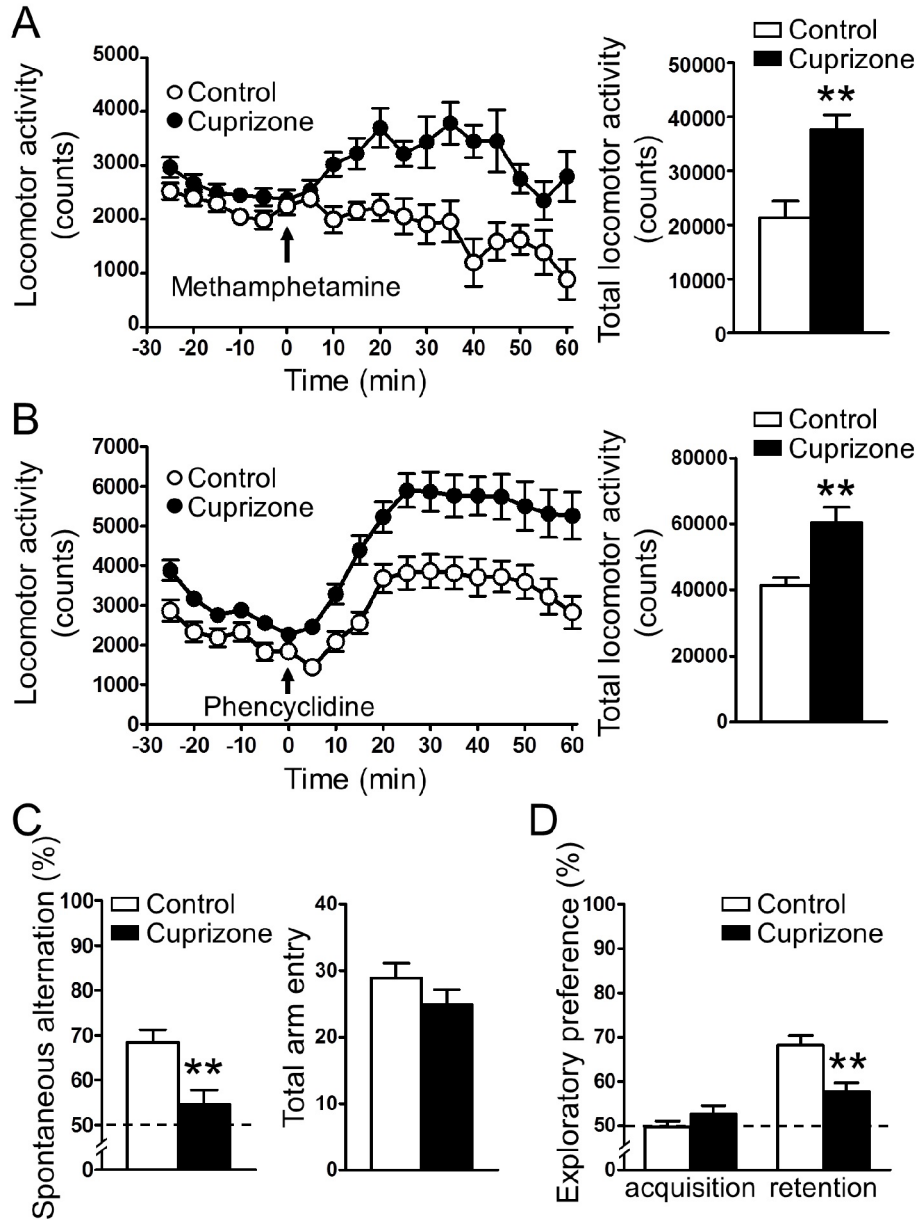


Figure 1. Short-term (one-week) exposure to cuprizone results in abnormal behaviors in mice (A, B) Short-term cuprizone exposure augmented the locomotor response to 0.3 mg/kg methamphetamine (A), and 2.5 mg/kg phencyclidine (B). The left panels depict the time course of locomotion in 5 min blocks. Arrow indicates time of psychostimulant injection. The right panels show total locomotion (cumulative horizontal movement) from $t = 0$ to 60. (C) Short-term cuprizone exposure caused decreased spontaneous alternation in the Y-maze (left), while total arm entry remained unchanged (right). (D) Short-term cuprizone exposure decreased exploratory preference for the novel object in the retention phase of the novel object recognition test. The dashed line represents interaction with the novel object at the level of chance. $n = 10$. $**p < 0.01$: Student's t -test. Data are mean \pm S.E.M.

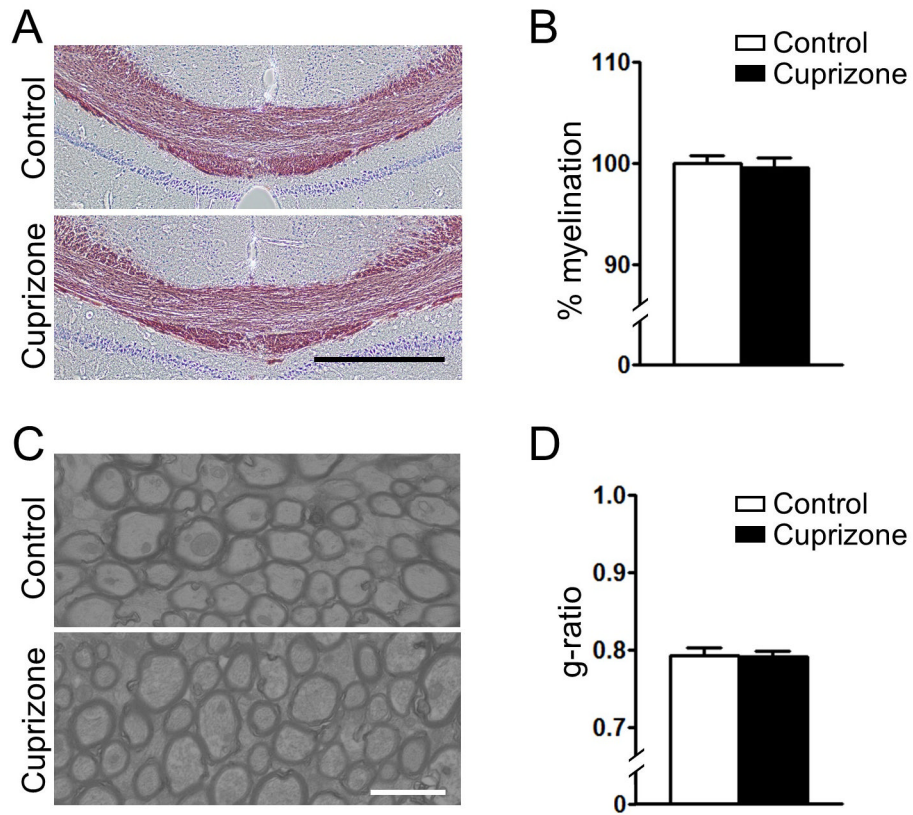


Figure 2. Short-term (one-week) exposure to cuprizone does not induce demyelination (A) Representative black-gold staining of the corpus callosum of control and short-term cuprizone exposed mice. Scale bar: 500 μ m. (B) Quantification of black-gold staining showed no demyelination in the short-term cuprizone-exposed mice. (C) Electron microscopy of axons in the corpus callosum of control and short-term cuprizone-exposed mice (representative image). Scale bar: 2 μ m. (D) Analysis of the g-ratio in the corpus callosum revealed no demyelination in the short-term cuprizone-exposed mice. n = 3.

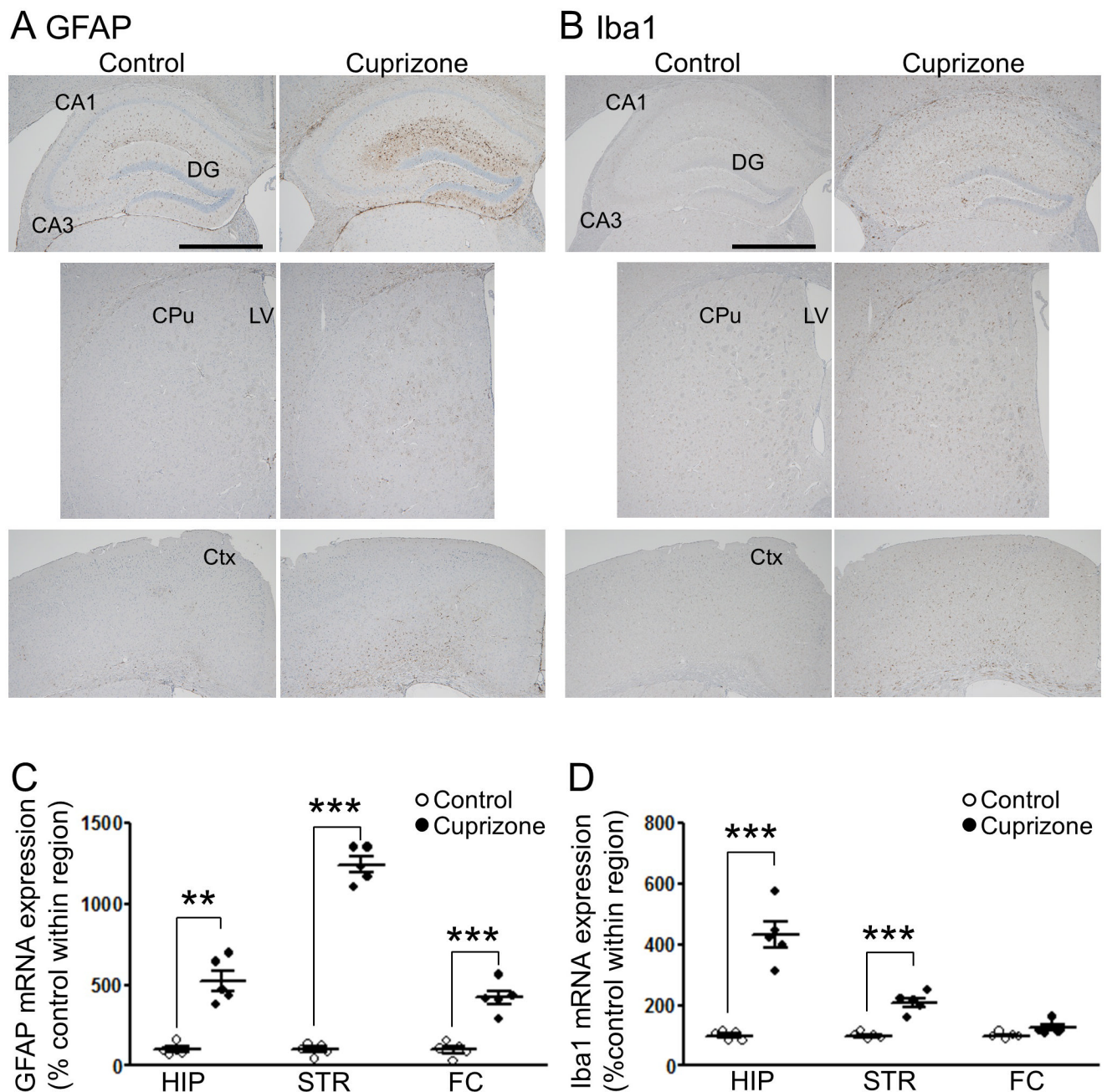


Figure 3. Astrocytes and microglia are activated in short-term (one-week) cuprizone-exposed mice

Representative images of immunohistochemistry for GFAP (**A**) and Iba1 (**B**). Scale bars, 1 mm. DG, dentate gyrus; CPu, caudate putamen; LV, lateral ventricle; and Ctx, cortex. (**C**) GFAP mRNA was significantly up-regulated in the hippocampus, striatum, and frontal cortex of cuprizone-exposed mice compared to control. (**D**) Iba1 mRNA was significantly up-regulated in the hippocampus and striatum of cuprizone-exposed mice. HIP, hippocampus; STR/CPu, striatum; and FC, frontal area of Ctx. $n = 5$. $**p < 0.01$, $***p < 0.001$: Student's t -test, significant at the indicated Bonferroni adjusted p values. Data are mean \pm S.E.M.

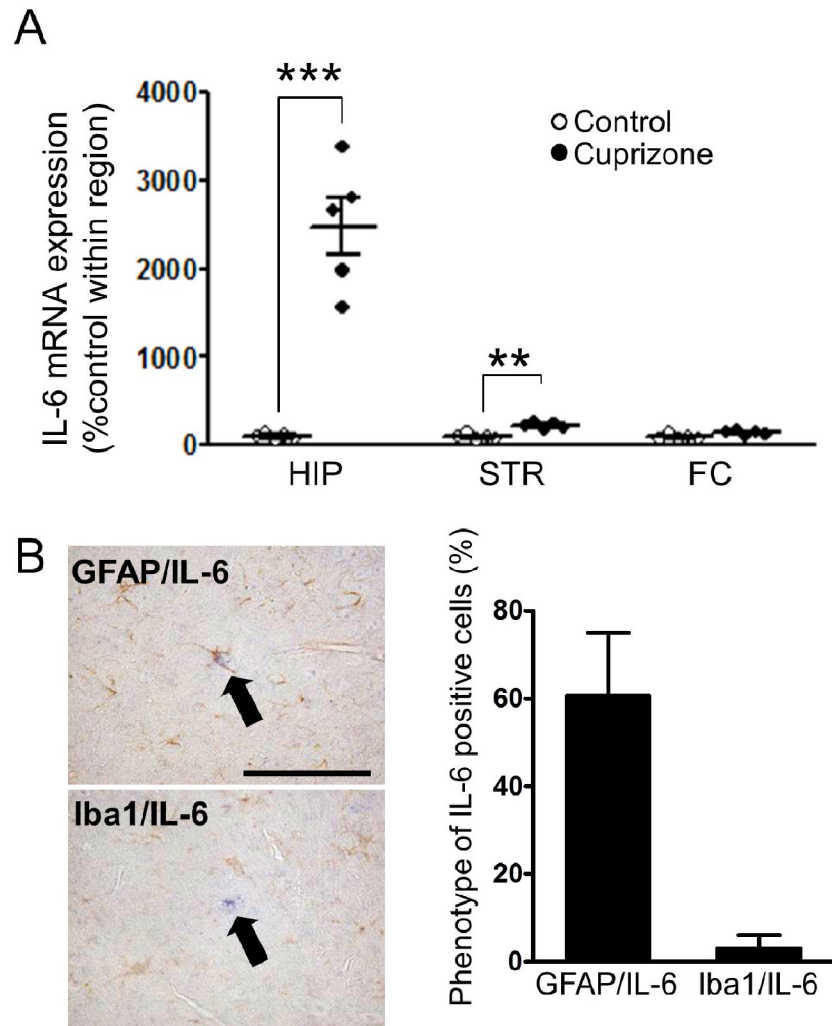


Figure 4. Interleukin 6 is markedly induced by short-term exposure to cuprizone
(A) Cuprizone exposure significantly increased expression of IL-6 mRNA in the hippocampus and striatum. $n = 5$. $**p < 0.01$, $***p < 0.001$: Student's *t*-test, significant at the indicated Bonferroni adjusted *p* values. HIP, hippocampus; STR/CPu, striatum; and FC, frontal area of the cortex. **(B)** Left, representative images of IL-6 *in situ* hybridization (blue) co-stained with GFAP or Iba1 immunohistochemistry (brown) in cuprizone-exposed mice. Right, percentage of IL-6-positive cells co-labeled with GFAP or Iba1 (63 were cells counted). HIP, hippocampus; STR/CPu, striatum; and FC, frontal area of Ctx. Scale bar, 50 μ m. $n = 3$.