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Global gene expression profiles in brain regions reflecting abnormal neuronal and glial functions targeting myelin sheaths after 28-day exposure to cuprizone in rats

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ABSTRACT

Both developmental and postpubertal cuprizone (CPZ) exposure impairs hippocampal neurogenesis in rats. We previously found that developmental CPZ exposure alters the expression of genes related to neurogenesis, myelination, and synaptic transmission in specific brain regions of offspring. Here, we examined neuronal and glial toxicity profiles in response to postpubertal CPZ exposure by using expression microarray analysis in the hippocampal dentate gyrus, corpus callosum, cerebral cortex, and cerebellar vermis of 5-week-old male rats exposed to 0, 120, and 600 mg/kg CPZ for 28 days. Genes showing transcript upregulation were subjected to immunohistochemical analysis. We found transcript expression alterations at 600 mg/kg for genes related to synaptic transmission, *Ache* and *Prima1*, and cell cycle regulation, *Tfap4* and *Cdkn1a*, in the dentate gyrus, which showed aberrant neurogenesis in the subgranular zone. This dose downregulated myelination-related genes in multiple brain regions, whereas KLOTHO$^+$ oligodendrocyte density was decreased only in the corpus callosum. The corpus callosum showed an increase in transcript levels for inflammatory response-related genes and in the number of CD68$^+$ microglia, MT$^+$ astrocytes, and TUNEL$^+$ apoptotic cells. These results suggest that postpubertal CPZ exposure targets synaptic transmission and cell cycle regulation to affect neurogenesis in the dentate gyrus. CPZ suppressed myelination in multiple brain regions and KLOTHO-mediated oligodendrocyte maturation only in the corpus callosum. The increased number of CD68$^+$ microglia, MT$^+$ astrocytes, and TUNEL$^+$ apoptotic cells in the corpus callosum may be involved in the induction of KLOTHO$^+$ oligodendrocyte death and be a protective mechanism against myelin damage following CPZ exposure.

1. Introduction

Cuprizone (CPZ) is a copper chelator that selectively injures oligodendrocytes and induces subsequent demyelination in the mouse central nervous system following dietary exposure (Carlton, 1966, 1967). In rats, we have found that repeated oral doses of CPZ cause mild white matter vacuolation associated with a slight decrease in the levels of myelin basic protein immunoreactivity in white matter tissue (Abe et al., 2015a). These CPZ-exposed rats revealed accumulation of activated microglia immunoreactive for ionized calcium-binding adaptor molecule 1 (IBA1), with foamy macrophage morphology suggestive of phagocytosis of destroyed myelin, and accumulation of astrocytes immunoreactive for glial fibrillary acidic protein (GFAP) showing gliosis in the white matter (Abe et al., 2015a). In CPZ-exposed rats, vacuolated changes are caused by myelin splitting (Kanno et al., 2012). These findings suggest that CPZ exposure induces both myelin splitting and demyelination in rats, but they are modest in severity.

Adult neurogenesis in the subgranular zone (SGZ) of the hippocampal dentate gyrus is known to continue throughout postnatal life (Kempermann et al., 2004). We have previously used immunohistochemistry techniques to examine the effects of CPZ exposure during development or during postpuberty on rat neurogenesis and myelinogenesis (Abe et al., 2015a, 2015b). Maternal dietary CPZ

Abbreviations: ACHE, acetylcholinesterase; CCL2, chemokine (C–C motif) ligand 2; CD68, cluster of differentiation 68; CPZ, cuprizone; C_T, threshold cycle; CNPase, 2′,3′-cyclic nucleotide 3'-phosphodiesterase; ER, endoplasmic reticulum; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; IBA1, ionized calcium-binding adaptor molecule 1; MT, metallothionein; OLIG2, oligodendrocyte lineage transcription factor 2; PCNA, proliferating cell nuclear antigen; PDGFRA, platelet-derived growth factor receptor alpha; RT-PCR, reverse transcription-polymerase chain reaction; SGZ, subgranular zone; TFAP4, transcription factor AP-4; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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exposure at 0.4% causing myelin vacuolation in the white matter of dams induced aberrations in hippocampal neurogenesis due to dysfunction of brain-derived neurotrophic factor and cholinergic signals, as well as decreased density of oligodendrocyte progenitor cells suggestive of suppressed myelinogenesis, in the dentate gyrus of offspring (Abe et al., 2015b). Similar effects on neurogenesis and myelinogenesis were detected in young adult rats after administration of CPZ by gavage at a dose of 600 mg/kg/day in a standard 28-day toxicity study that caused myelin vacuolation (Abe et al., 2015a). These results suggest that CPZ affects functions of various neuronal signals to the SGZ through suppression of myelinogenesis.

Toxicogenomic technology, which has contributed to the knowledge of target gene profiles in toxicity, providing early predictions in many organs in toxicity studies, offers important approaches for estimating the toxicity of new chemicals. In particular, gene expression profiling using cDNA microarray analysis is a standard methodology for investigating the toxicity of a chemical based on the mode of action, providing a global view of molecular changes associated with the mechanisms underlying diseases or toxicity development following chemical exposure.

The central nervous system has an anatomically elaborate architecture with region-specific differences in the distribution of neuronal and glial cell populations. Thus, to examine neurotoxicity, function and differentiation potentials of both neuronal and glial cell populations should be analyzed. We have recently established a high-throughput tissue sampling method that enables the identification of molecular profiles of RNAs and polypeptides simultaneously in anatomically different brain regions by applying a whole-brain fixation method using a methacarn fixative (Akane et al., 2013).

We have also previously performed global gene expression profiling in different brain regions of rats after developmental exposure to CPZ and revealed alterations in the expression of genes related to neurogenesis, myelination, and synaptic transmission in specific brain regions of the offspring (Abe et al., 2016). Additionally, we found upregulation of transcript level for the *Kl* gene that encodes KLOTHO, an *anti*-aging protein, and increased density of KLOTHO^{+} oligodendrocytes in the corpus callosum, suggestive of a protective mechanism against CPZ toxicity in the white matter (Abe et al., 2016). These data suggest that global gene expression profiling in multiple brain regions can be helpful for studying neurotoxicity involving both neuronal and glial cell functions.

In the present study, to capture target gene profiles in response to CPZ toxicity in the brain using a regular 28-day toxicity study framework, global gene expression profiling was performed in specific brain regions, including the hippocampal dentate gyrus, corpus callosum, cerebral cortex, and cerebellar vermis, of young adult rats after repeated oral doses of CPZ using identical study samples that we have revealed aberration of hippocampal neurogenesis (Abe et al., 2015a). Based on the obtained gene expression profiles, the cellular localization of the molecules showing altered expression was immunohistochemically examined to confirm changes in cellular phenotypes in response to CPZ exposure.

2. Materials and methods

2.1. Chemicals and animals

Cuprizone (CPZ; purity: >99.0%; CAS No. 370-81-0) was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). Four week-old male Crl:CD®(SD) rats were purchased from Charles River Japan Inc. (Yokohama, Japan) and maintained in an air-conditioned animal room (temperature: 23 ± 2 °C, relative humidity: 55 ± 15%) with a 12-h light/dark cycle. Animals were housed in plastic cages with paper chip bedding with four animals per cage and were provided a pelleted basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum throughout the experimental period.

2.2. Experimental design

The present experiments were identical to that previously reported (Abe et al., 2015a). In brief, five-week-old male rats were randomly divided into three groups of 20 or 28 animals, which were administered 0, 120, or 600 mg CPZ/kg body weight in corn oil by gavage for 28 days. The doses were based on our results from a preliminary 28-day dose-finding study in five-week-old male rats. At the next day of the last CPZ treatment, all animals were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and subjected to necropsy.

2.3. Tissue sampling of specific brain regions for gene expression analysis

For expression microarray and real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses, whole brains were removed from euthanized young adult male rats (n = 6/group) and fixed with a methacarn solution for 5 h at 4 °C with agitation (Akane et al., 2013). Fixed brains were dehydrated in 99.5% ethanol and subjected to tissue sampling of the hippocampal dentate gyrus, parietal cerebral cortex, corpus callosum and cerebellar vermis using the brain-matrix cast (Muromachi Kikai Co., Ltd., Tokyo, Japan) and punch-biopsy devices (Kai Industries Co., Ltd., Gifu, Japan) according to the previously described method (Akane et al., 2013).

2.4. Gene expression microarray analysis

Isolation of total RNA from each sample was performed using QIAzol (Qiagen, Hilden, Germany) together with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Gene expression analysis was performed using Agilent Rat Oligo arrays with approximately 60,000 probes for known genes and expressed sequence tags (Agilent Technologies Inc., Santa Clara, CA, USA). For sample preparation and array processing, the Agilent protocol "One-Color Microarray-Based Gene Expression Analysis" was used. Briefly, the recommended volume of control RNAs (Agilent One-Color RNA Spike-In Kit) was added to 100 ng of total RNA from the each brain region of the 0 mg/kg controls, 120 mg/kg and 600 mg/kg group (n = 3/group). Thereafter, Cy3-labeled cRNA was produced using the Agilent Low Input Quick Amp Labeling (one-color), purified with the RNeasy Mini Kit, fragmented using the In Situ Hybridization Kit (Agilent Technologies Inc.), and subjected to hybridization by incubation in a hybridization oven (Agilent Technologies Inc.). Hybridized slides were scanned (G4900DA scanner, Agilent Technologies Inc.), and data were obtained using Agilent Feature Extraction software (version 11.7.1.1) with defaults for all parameters. Microarray data analyses were performed using GeneSpring GX (version 11.5.1) software (Agilent Technologies Inc.). Expression values of less than 1 were substituted by 1, and 75th percentile normalization was performed using GeneSpring normalization algorithms. Reliability of each expression value was represented by a flag based on the default setting of GeneSpring (Detected, Marginal and Not Detected).

Gene ontology-based functional annotation clustering of gene expression was performed using the Database for Annotation, Visualization and Integrated Discovery, version 6.7, provided by National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD; Huang et al., 2009a, 2009b), to clarify biological functions of all genes expressed at least 1.50-fold higher or 0.67-fold lower in magnitude in the 600 mg/kg group compared with the 0 mg/kg controls. From the gene clusters that are related to neuronal function and myelination, genes of known functional mechanisms and those altered in multiple brain regions were selected for validation of expression changes, as well as well-known myelination-related genes, by real-time RT-PCR.