1521-009X/50/10/1414–1428$35.00
DRUG METABOLISM AND DISPOSITION

dx.doi.org/10.1124/dmd.121.000453
Drug Metab Dispos 50:1414–1428, October 2022

Effect of Chronic Cadmium Exposure on Brain and Liver Transporters and Drug-Metabolizing Enzymes in Male and Female Mice Genetically Predisposed to Alzheimer's Disease[S]

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Received March 9, 2021; accepted June 27, 2022

ABSTRACT

Cadmium (Cd) exposure is associated with increased Alzheimer's disease (AD) risks. The human Apolipoprotein E (*ApoE*) gene encodes a lipid-transporting protein that is critical for brain functions. Compared with *ApoE2* and *E3*, *ApoE4* is associated with increased AD risk. Xenobiotic biotransformation-related genes have been implicated in the pathogenesis of AD. However, little is known about the effects of Cd, *ApoE*, and sex on drug-processing genes. We investigated the Cd-ApoE interaction on the transcriptomic changes in the brains and livers of ApoE3/ApoE4 transgenic mice. Cd disrupts the transcriptomes of transporter and drug-processing genes in brain and liver in a sex- and ApoE-genotype-specific manner. Proinflammation related genes were enriched in livers of Cd-exposed ApoE4 males, whereas circadian rhythm and lipid metabolism related genes were enriched in livers of Cd-exposed ApoE3 females. In brains, Cd up-regulated the arachidonic acid-metabolizing Cyp2j isoforms only in the brains of ApoE3 mice, whereas the dysregulation of cation transporters was male-specific. In livers, several direct target genes of the major xenobiotic-sensing nuclear receptor pregnane X receptor were uniquely upregulated in Cd-exposed ApoE4 males. There was a female-specific hepatic upregulation of the steroid hormone-metabolizing Cyp2 isoforms and the bile acid synthetic enzyme Cyp7a1 by Cd exposure. The dysregulated liver transporters were mostly involved in intermediary metabolism, with the most significant response observed in ApoE3 females. In conclusion, Cd dysregulated the brain and liver drug-processing genes in a sex- and ApoE-genotype specific manner, and this may serve as a contributing factor for the variance in the susceptibility to Cd neurotoxicity.

SIGNIFICANCE STATEMENT

Xenobiotic biotransformation plays an important role in modulating the toxicity of environmental pollutants. The human *ApoE4* allele is the strongest genetic risk factor for AD, and cadmium (Cd) is increasingly recognized as an environmental factor of AD. Very little is known regarding the interactions between Cd exposure, sex, and the genes involved in xenobiotic biotransformation in brain and liver. The present study has addressed this critical knowledge gap.

Introduction

With a growing aging population, Alzheimer's disease (AD) is a significant public health issue, causing grave social and economic burdens. In 2021, AD and other types of dementia are estimated to cost the nation $355 billion, and it could rise to $1.1 trillion by 2050. Unfortunately, therapeutic management remains extremely limited to symptom targeting options, and few drugs have been approved to modify or reverse the disease (Eid et al., 2019).

Increasing evidence has demonstrated that an interaction between susceptible genetic risk factors and toxic environmental exposures is an important mechanistic contributor to the pathogenesis of AD (Eid et al., 2019). Cd is an emerging neurotoxicant with a long biologic half-life in humans (Mendez-Armenta and Rios, 2007; Rafati Rahimzadeh et al., 2017; Branca et al., 2018). Cd ranks No. 7 on the Substance Priority List of Agency for Toxic Substances and Disease Registry (ATSDR, 2019). In the United States, the primary source of Cd exposure for nonsmokers is food (Satarug et al., 2010). Multiple epidemiologic studies have found that Cd exposure is associated with cognitive deficits in humans (Ciesielski et al., 2012; Ciesielski et al., 2013; Min and Min, 2016). Importantly, blood Cd levels are positively associated with AD mortality among seniors in the United States (Min and Min, 2016). Analysis from the 1999–2004 third National Health and Nutrition Examination Survey showed that compared with participants with blood Cd levels below 0.3 μg/l, those with blood Cd levels higher than 0.6 μg/l exhibited a 3.83-fold increased risk of AD mortality (Min and Min, 2016). Regarding the genetic risk factors, the apolipoprotein E4 (*ApoE4*) allele is the strongest known genetic risk factor for AD (Liu et al., 2013). The *ApoE* gene encodes a lipid-transporting protein and is critical for brain functions (Elliott et al., 2010). The human *ApoE* gene has three polymorphic *ApoE* alleles, namely ε2 (*ApoE2,* 8.4%), ε3 (*ApoE3,* 77.9%), and ε4 (*ApoE4,* 13.7%). We have used a novel AD mouse model where the mouse *ApoE* gene was replaced with either the common allele (*ApoE3*) or the susceptible allele (*ApoE4*) of human origins (Xu et al., 1996; Bour et al., 2008) and investigated the molecular mechanisms underlying the pathogenesis of neurodegenerative diseases *in vivo*

This work was supported by National Institutes of Health grants [NIEHS (R01ES025708, R01ES030197, R01ES031098), NIGMS (R01GM111381)] (To J.Y.C), the University of Washington Superfund Research Program [NIEHS (P42ES004696)] (To Z.X), the University of Washington Center for Exposures, Diseases, Genomics, and Environment [P30ES007033] (To J.Y.C), the University of Washington Sheldon Murphy Endowment (To J.Y.C), and the University of Washington Environmental Health and Microbiome Research Center (EHMBRACE, DEOHS) (To J.Y.C).

dx.doi.org/10.1124/dmd.121.000453.

[S] This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: ABC, ATP-binding cassette; AD, Alzheimer's disease; AhR, aryl hydrocarbon receptor; ApoE, apolipoprotein E; Cd, cadmium; Gst, glutathione *S*-transferase; Oatp, organic anion transporting polypeptide; Slc, solute carrier; Sult, sulfotransferase; PXR, pregnane X receptor.

(Engstrom et al., 2017; Zhang et al., 2020). Specifically, we found that the male ApoE4 mice are more susceptible to Cd neurotoxicity, as evidenced by an earlier onset of hippocampus-dependent memory deficit (Zhang et al., 2020). However, little is known regarding the effects of Cd and the *ApoE* genotype on xenobiotic biotransformation-related genes in the brain and other organs. It is important to characterize this because AD patients usually take multiple medications that require drug processing genes for bioactivation and/or detoxification (Cacabelos, 2007; Cacabelos et al., 2012).

Liver is a major organ for xenobiotic biotransformation and involved in AD progression (Estrada et al., 2019). In AD patients, serum-based liver function markers were associated with cognitive performance and AD biomarkers (amyloid, tau, and neurodegeneration). An elevated aspartate aminotransferase to alanine aminotransferase ratio and lower alanine aminotransferase levels were associated with AD diagnosis and poorer cognitive test performance. This has highlighted the involvement of hepatic metabolic disturbances during AD, and these findings have opened avenues for novel diagnostics and therapeutics (Nho et al., 2019). There has been evidence suggesting that the liver is the origin of the brain amyloid-β (Aβ) deposition and is involved in the peripheral clearance of circulating Aβ in the blood (Bassendine et al., 2020). In addition, liver diseases are implicated in the severity of AD; for example, nonalcoholic fatty liver disease produced chronic inflammation and advanced pathologic signs of AD in both wild type and the transgenic AD mouse model (Howlett and Richardson, 2009).

Dysfunction of the liver and other drug-metabolizing organs may impact the xenobiotic biotransformation, which in turn alters the pharmacokinetics of various drugs used in AD patients. Various phase-I and -II drug-metabolizing enzymes, as well as transporters, contribute to the xenobiotic biotransformation in the drug-metabolizing organs (Aleksunes and Klaassen, 2012; Almazroo et al., 2017). It has been shown that in an AD transgenic mouse model overexpressing both Amyloid Precursor Protein and the mutant human presenilin 1 (APP/S1) (De Strooper, 2007), there was an increase in the phase-I cytochrome P450 (Cyp) 2c29 and Cyp51a1 proteins in the livers associated with accumulation of hepatic lipids (Pan et al., 2018). There was also an up-regulation of the phase-II conjugation enzyme UDP-glucuronosyltransferase 2b5 and the ATP-binding cassette (ABC) efflux transporter multidrug resistance-associated protein 2 (Mrp2/Abcc2), but a down-regulation of the monocarboxylate transporter 1 (Mct1) in the APP/S1 mice (Pan et al., 2018). The organic anion transporting polypeptide 1b2 (Slco1b2, also known as Oatp2) is an important xenobiotic uptake transporter, and its expression decreased in the brains but increased in the livers of the APP/S1 AD mice (Wen et al., 2020), suggesting that this transporter may be a target for the pathogenesis and treatment of AD.

Current literature evidence has suggested the importance of xenobiotic biotransformation-related genes in the pathogenesis of AD. However, little is known regarding how the Cd-ApoE4 interaction impacts the regulation of drug processing genes in the liver and brain. To fill this knowledge gap, we investigated the effects of Cd on brain and liver drug-processing genes in ApoE3/E4 mice.

Materials and Methods

Animals. Humanized ApoE3 and ApoE4 knock-in (ApoE3-KI and ApoE4-KI) mice were originally obtained from Dr. Nobuyo Maeda from the University of North Carolina at Chapel Hill and maintained in the laboratory. All mice were housed in standard conditions (12-hour light and dark cycles) with water and food provided ad libitum at University of Washington animal facilities. All animals were housed according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International guidelines; animal care and exposure was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Washington.

Cd Exposure. ApoE3-KI and ApoE4-KI mice were weaned at 28 days, and littermates of the same sex were randomly separated into groups of 3–5 mice in each cage. Starting at 8-weeks of age, mice in the cadmium (Cd) exposure group were switched to drinking water with 0.6 mg/l $CdCl_2$ (Cat. 202908, Millipore-Sigma, Burlington, MA) and were maintained on this contaminated water for 14 weeks. As we previously described, this Cd dose resulted in a peak blood Cd concentration between 0.3–0.4 mg/l in mice, which falls within levels found in the US general population (Min and Min, 2016; Zhang et al., 2020). The Cd-contaminated water was prepared from a stock solution and replaced every week. The Cd-exposed mice were then switched back to regular drinking water for the remaining period of the study. Mice in the control group were provided normal drinking water throughout the entire study. Body weight was recorded every 1–2 weeks throughout the study. Samples were collected when mice reached 75-weeks of age (the overall experimental design is summarized in Fig. 1). The preparation, use, and disposal of hazardous reagents were conducted according to the guidelines set forth by the Environmental Health and Safety Office at the University of Washington.

RNA-Seq of Liver and Brain Transcriptome. Total RNA was isolated from livers and brains of control or Cd-exposed ApoE3-KI and ApoE4-KI mice (n = 3 per exposure per sex) using RNA zol Bee reagent (Tel-Test Inc., Friendswood, TX). The RNA concentration was determined at 260 nm by using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA). In addition, the quality of RNA was determined by visualizing the 28S and 18S rRNA bands under UV light in formaldehyde agarose gel electrophoresis. The RNA integrity value was examined by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and samples with an RNA integrity value above 8.0 were used for RNA sequencing. cDNA libraries were prepared using a Clontech cDNA library prep kit (Clontech Laboratories Inc., Mountain View, CA) and were sequenced by using the NextSEq. 500 sequencing platform (75 bp paired end).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) of Liver and Brain Genes. The mRNA expression of selected liver and brain genes was quantified in livers and brains of vehicle- or Cd-exposed male and female mice of either ApoE3 or ApoE4 genotypes (n = 4-5/sex/genotype/exposure). Total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). The resulting cDNA products were diluted 1:10 and were amplified by qPCR using the SsoAdvanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-

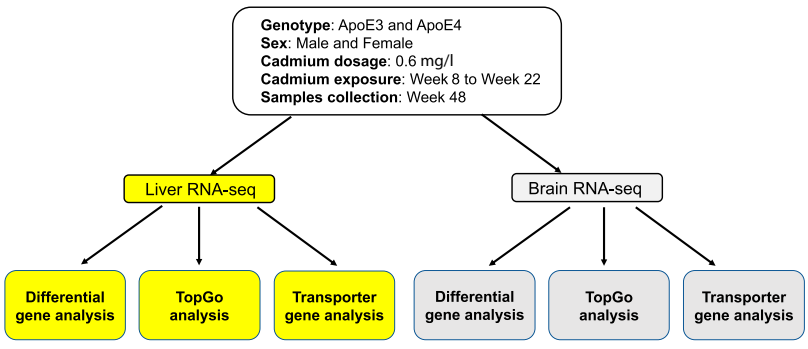

Fig. 1. Experimental design of the study. Eight-week-old humanized ApoE3 and ApoE4 transgenic mice (male and female) were exposed to $CdCl_2$ (0.6 mg/l) via drinking water for 14 weeks (week 8 to week 22), and samples were collected at age 75 weeks. RNA-Seq was conducted in the liver and brain (n = 3 per sex per genotype per exposure). Differential gene analysis, pathway analysis (TopGo), and xenobiotic biotransformation related genes (transporters and drug-metabolizing enzymes) were performed as described in Materials and Methods.

Time PCR Detection System (Bio-Rad, Hercules, CA). The primers for all qPCR reactions were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are shown in Supplemental Table 4. Data are expressed as the percentage of the expression of the housekeeping gene Actin Beta (β-actin). Data were analyzed using three-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($P < 0.05$).

Western Blot Analysis. Western blot analysis was performed as previously described elsewhere with minor modifications (Renaud et al., 2011). Protein concentrations were determined using BCA assay reagents according to the manufacturer's instructions. Samples containing 10 μg protein were separated by gel electrophoresis on a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The primary antibodies and dilutions used were Slc40a1 (1:1000, PA5-22993), Slc38a3 (1:200, sc-398982, Santa Cruz Biotech, Dallas, TX), Slc38a4 (1:200, sc-376664), and GAPDH (1:1000, 2118, Cell Signaling, Beverly, MA). The horseradish peroxidase-conjugated secondary antibodies were purchased from MilliporeSingma. All the primary and secondary antibodies were diluted into the appropriate blocking buffer. Following antibody incubation, the protein of interest was detected with Amersham ECL prime Western Blotting Detection Reagent (Cytiva, Marlborough, MA) by using a ChemiDoc XRS Imaging System. ImageJ (National Institutes of Health, Bethesda, MD) was used for the densitometry analysis.

Data Analysis. For liver and brain transcriptome data, the FASTQ files were demultiplexed and concatenated for each sample. Quality control of the FASTQ files was performed using FastQC (Andrews, S. 2010). Paired-end sequence reads from the FASTQ files were mapped to the mouse reference genome (University of California Santa Cruz mm10) using HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts, version 2.1) (Kim et al., 2019). The resulting SAM (sequence alignment/map) files were then converted to their binary forms (BAM: binary alignment/map) and sorted by using SAMtools (version 1.2) (Li et al., 2009). By using the University of California Santa Cruz mm10 as the reference annotation, transcript abundances were estimated with featureCounts (part of the Subread package, version 1.5.3) (Liao et al., 2019), and EdgeR (version 3.26.8) was used for differential expression analysis, with RUVSeq (version 1.18.0) used to estimate factors of unwanted variation from the residuals of the count data. A gene was considered significantly differentiated if the false discovery rate (FDR) was less than 0.05. Hierarchical clustering was performed using the R package Complex Heatmap (Gu et al., 2016). Differentially up- and down-regulated genes were used as input for gene ontology enrichment using the R package topGO (Alexa and Rahnenfuhrer, 2021), and the list of genes in the unfiltered expression table was used as the background.

The transporter categories include the solute carrier (SLC) superfamily, including the organic anion transporting polypeptide (Oatp/Slco) family, as well as the transporter ATPase superfamily, including the ABC family, which were selected based on the previous review (Klaassen and Aleksunes, 2010). Three-way ANOVA test with Tukey's posthoc multiple comparisons ($\alpha = 0.05$) was used to analyze the transporters data among the eight groups considering the factors of sex, ApoE genotype, and exposure. Differentially regulated transporter genes were plotted using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA).

Results

Cd Exposure Induced the Most Significant Functional Changes in ApoE4 Male Mouse Livers. As shown in Supplemental Fig. 1 and Fig. 2, gene ontology enrichment analysis was performed using topGo to identify transcriptome-wide pathways regulated by Cd exposure in the brains (Supplemental Fig. 1) and livers (Fig. 2) of male and female ApoE3 and ApoE4 mice. None of the downregulated genes by Cd were significantly enriched for any pathways in brain or liver (data not shown), and none of the upregulated genes by Cd were significantly enriched for any pathways in brain. In liver, whereas Cd did not upregulate any pathway in ApoE3 male (Fig. 2A) or ApoE4 female mice (Fig. 2D), there was a significant enrichment of upregulated in ApoE3 female (Fig. 2B) and ApoE4 male mice (Fig. 2C). Specifically, livers of ApoE4 males had the most enriched upregulated gene ontology (GO) terms involved in inflammation, including antigen processing and presentation related pathways, immune response, immunoglobulin mediated immune response, innate immune response, and B cell mediated immunity (Fig. 2C). This finding indicates that the immune system process was activated after Cd exposure uniquely in the livers of ApoE4 male mice and, given our previous report that ApoE4 male mice were also the most susceptible to Cd-induced memory deficit (Zhang et al., 2020), hepatic inflammation may be an important biomarker and/or a contributor to the sex- and genotype-specific Cd neurotoxicity within the "liver-brain axis".

In contrast, in the livers of ApoE3 female mice, which were the least susceptible to Cd-induced memory deficits (Zhang et al., 2020), Cd exposure enriched for upregulated pathways involved in multiple circadian rhythm-related pathways (rhythmic process, circadian regulation of gene expression, circadian rhythm, photoperiodism, entrainment of circadian clock by photoperiod, entrainment of circadian clock, and regulation of circadian rhythm) (Fig. 2B). In addition, cholesterol and fatty acid metabolism related pathways were another class of Cd-upregulated pathways uniquely enriched in the livers of ApoE3 female mice (Fig. 2B). Although a bidirectional link has been suggested between circadian rhythm pathways and AD, it is still unclear which one holds the causative role (Homolak et al., 2018). In addition, very little is known whether the peripheral clock outside the central nervous system (CNS), such as in the liver, contributes to the pathogenesis of AD. However, it is known that circadian clock genes contribute to the lipid metabolism in nonalcoholic liver disease and may help alleviate the metabolic disturbance (Shi et al., 2019), and that nonalcoholic liver disease produces chronic inflammation and advanced pathologic signs of AD in mice (Howlett and Richardson, 2009). Therefore, upregulation of these circadian rhythm related genes may be a beneficial factor in preventing the enrichment in hepatic inflammation pathways (as seen in male ApoE4 mice) and reducing the susceptibility to AD phenotypes in female ApoE3 mice.

Effects of Cd Exposure on the Drug Metabolizing Enzymes in Mouse Brains. To compare the effects of Cd on various drug metabolizing enzymes in the brain and how sex and the ApoE genotype modulate the Cd effects, we compared the mRNA expression of various phase-I and -II drug metabolizing enzymes in the brains of male and female ApoE3 and ApoE4 mice. Interestingly, response to Cd exposure was only observed in the brains of ApoE3 mice but not in ApoE4 mice of both sexes (Fig. 3). Cd upregulated the mRNA of the arachidonic acid metabolizing enzyme Cyp2j12 in the brains of male ApoE3 mice, and the mRNA of Cyp2j6 (another arachidonic acid metabolizing enzyme) in the brains of female ApoE3 mice. Cd exposure also down-regulated the phase-II conjugation enzymes Sult2b1, which is an enzyme that plays a role in cholesterol metabolism and epidermal proliferation and regulation (Heinz et al., 2017), and Sult4a1, which encodes a brain-specific sulfotransferase that is thought to be involved in neurotransmitter metabolism (Allali-Hassani et al., 2007).

Effects of Cd Exposure on the Transporters in Mouse Brains. To compare the effects of Cd on various transporters in brain and how sex and the ApoE genotype modulates the Cd effects, we compared the mRNA expression of transporters in the brains of male and female ApoE3 and ApoE4 mice. The mRNA abundance [Fragments per kilobase of exon per million mapped fragments (FPKM)] of all transporters is shown in Supplemental Table 1, and the differentially regulated mRNA of transporters in brain is shown in Supplemental Table 2. To note, under basal conditions (i.e., without Cd exposure), the cumulative mRNA abundances of Abc and Slco/Oatp transporters were both higher in the livers than brains in all four mouse groups (male ApoE3, female ApoE3, male ApoE4, and female ApoE4 mice); conversely, the cumulative mRNA abundances of Slc (other than Slco) and transport ATPases (other than ABC) were both lower in the livers than brains in all four mouse groups (Supplemental Fig. 2). Because the ABC and Slco/Oatp transporter families are well-known for xenobiotic efflux and uptake respectively, whereas

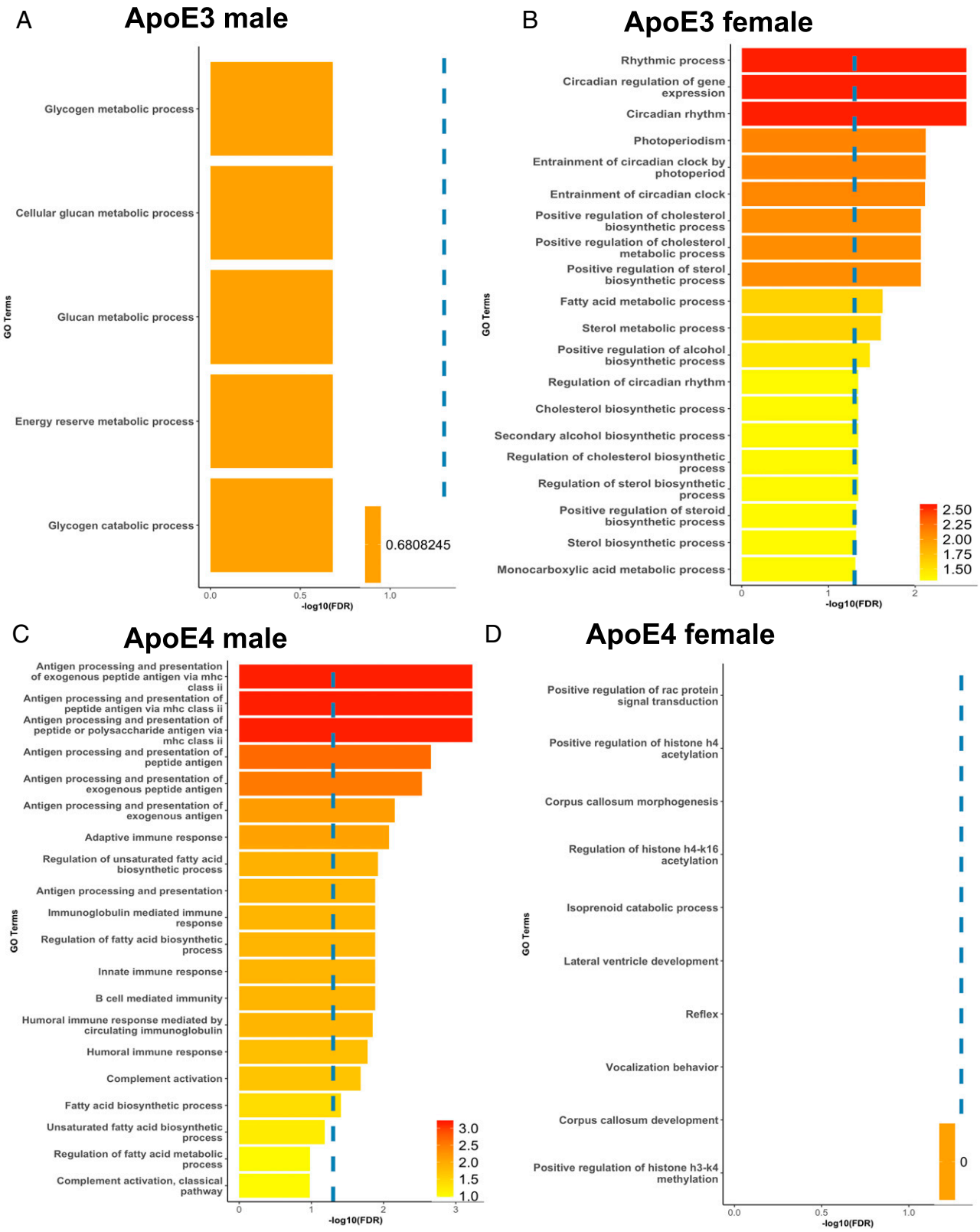

Fig. 2. Gene ontology (GO) enrichment analysis of differentially regulated genes using the R package TopGo. Up-regulated gene ontology enrichment terms in livers for ApoE3 male (A), ApoE3 female (B), ApoE4 male (C), and ApoE4 female mice (D) are shown (n = 3/sex/genotype/exposure). The dotted line represents -log10(FDR) = 1.3.

many other Slc and transport ATPases transport endogenous molecules involved in intermediary metabolism (although some transport xenobiotics as well), the distribution of the basal mRNA abundance among these transporter families further confirmed the role of the liver as a major organ for xenobiotic biotransformation relative to the brain.

In the brain, the effects of Cd exposure on transporters were more prominent in males (Fig. 4). Whereas none of the ABC, Slco, or other

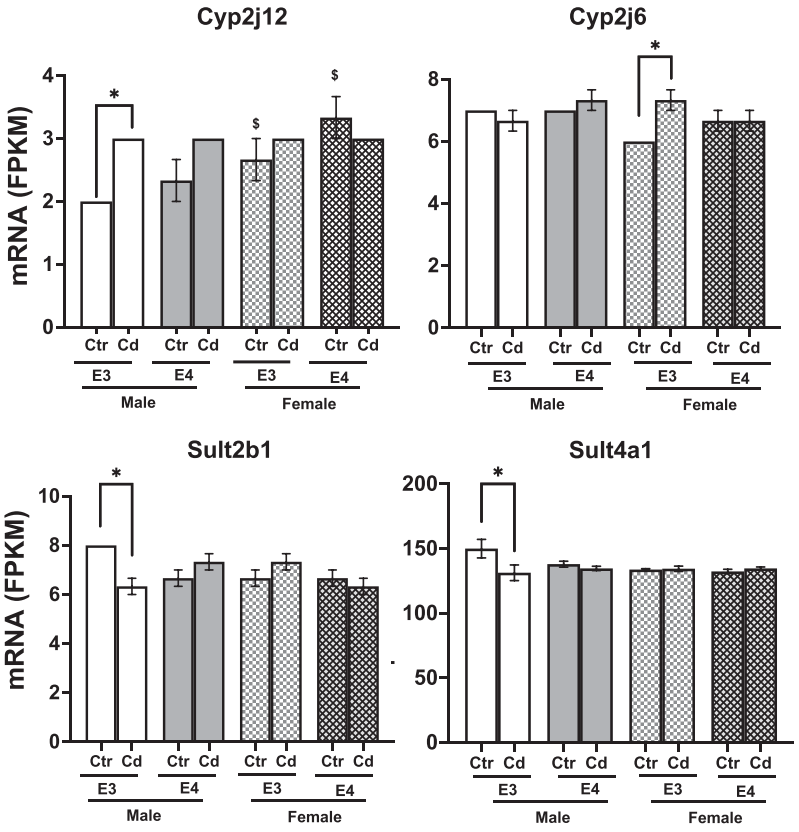

Fig. 3. Cd-mediated differential expression of mRNAs encoding drug-metabolizing enzymes in mouse brain. The mRNA values in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

transport ATPases were differentially regulated in any group, in the brains of male ApoE3 mice, Cd down-regulated Slc3a2, which plays an important role in intracellular calcium levels and the transportation of L-type amino acid (Mastroberardino et al., 1998; Broer et al., 2001; Yanagida et al., 2001), but upregulated Slc44a5, whose human homolog is known to be expressed in brains (Ayka and Sehirli, 2020) and is associated with Choline Deficiency Disease (Michel and Bakovic, 2012; Traiffort et al., 2013). In the brains of male ApoE4 mice, Cd up-regulated the cation-chloride cotransporter Slc12a9 and the mitochondrial dicarboxylate carrier Slc 25a10, but down-regulated the zinc transporter Slc39a10 (Taylor and Nicholson, 2003) (Fig. 4). Furthermore, in the brains of female mice, the basal expression of Slc25a10 was higher in ApoE3 than in ApoE4 mice; whereas in the brains of male mice, the Cd-regulated expression of Slc12a9 was lower, and the Cd-regulated expression of Slc39a10 was higher in ApoE3 than in ApoE4 mice.

Effects of Cd Exposure on the Drug Metabolizing Enzymes in Mouse Livers. To compare the effects of Cd on various drug metabolizing enzymes in liver and how sex and the ApoE genotype modulates the Cd effect, the mRNA expression of various phase-I and -II drug metabolizing enzymes in the livers of male and female ApoE3 and ApoE4 mice were compared (Fig. 5 and 6). Interestingly, uniquely in livers of ApoE4 males, which is the most susceptible group to Cd neurotoxicity, Cd downregulated Cyp1a2 (Fig. 5), a protein that can metabolize some polycyclic aromatic hydrocarbons and it is a prototypical target gene of aryl hydrocarbon receptor (AhR), and upregulated Cyp3a11, which contributes to the metabolism of most drugs that require phase-I biotransformation for elimination and is a target gene of pregnane X receptor (PXR) (Fig. 6) (Cui et al., 2010; Aleksunes et al., 2012). In addition, Cd upregulated the mRNAs of glutathione *S*-Transferase (Gst) a1 and Gstm1 uniquely in livers of male ApoE4 mice (Fig. 6). These Gst enzymes are involved in protecting cells from reactive oxygen species electrophilic compounds and are also target genes of PXR (Cui et al., 2010; Aleksunes et al., 2012). In contrast to the Cd-mediated changes in the mRNAs of these important drug metabolizing enzymes in livers of ApoE4 males, livers of ApoE3 males were generally resistant to Cd-mediated changes in the expression of drug metabolizing enzymes, except for a slight decrease in the mRNA of Cyp2g1 (Fig. 5).

In the livers of female mice, Cyp2a4 mRNA was upregulated by Cd in both genotypes, and this enzyme is highly active in the 15-alpha-hydroxylation of progesterone, androstenedione, and testosterone. In addition, in the livers of female ApoE3 mice, Cd exposure increased the mRNAs of Cyp2a5 (which shows a high coumarin 7-hydroxylase activity; Abu-Bakar et al., 2013), Cyp2g1 (which exhibits high activity with sex steroid hormones, arachidonic acid, and numerous exogenous compounds; Zhuo et al., 2004), and Cyp3a13 (which can activate aflatoxin B1 to a genotoxic product; Deng et al., 2018). Cd also increased the expression of the rate-limiting bile acid synthetic enzyme Cyp7a1, whereas a slight decrease in Cyp2a12 (an enzyme that is actively involved in the 7-alpha-hydroxylation of testosterone; Honkakoski and Negishi, 1997) mRNA was observed in the livers of ApoE4 females. The effects of the ApoE genotype on the expression of these enzymes were also noted, in that the hepatic basal expression levels of Cyp2a4, Cyp2a5, and Cyp2g1 were lower in ApoE3 females than in ApoE4 females. Cyp2a4 mRNA was lower, whereas Cyp3a13 and Cyp7a1 mRNAs were higher in ApoE4 females than in APoE4 females (Fig. 5 and 6). Sex also contributes to the regulation of these enzymes. Cyp2a4 and Cyp2a12 mRNAs were consistently higher, whereas Cyp3a11 mRNA was consistently lower in females than males of the same genotype and exposure. Cyp2a5 and Cyp2g1 mRNAs were higher in Cd-exposed females than Cd-exposed males of the same genotype (although the Cyp2g1 basal expression levels between female ApoE3 and male ApoE3 mice were not statistically significant). Cyp3a13 and Cyp7a1 mRNAs were higher, whereas the Gsta1 and Gstm1 mRNAs

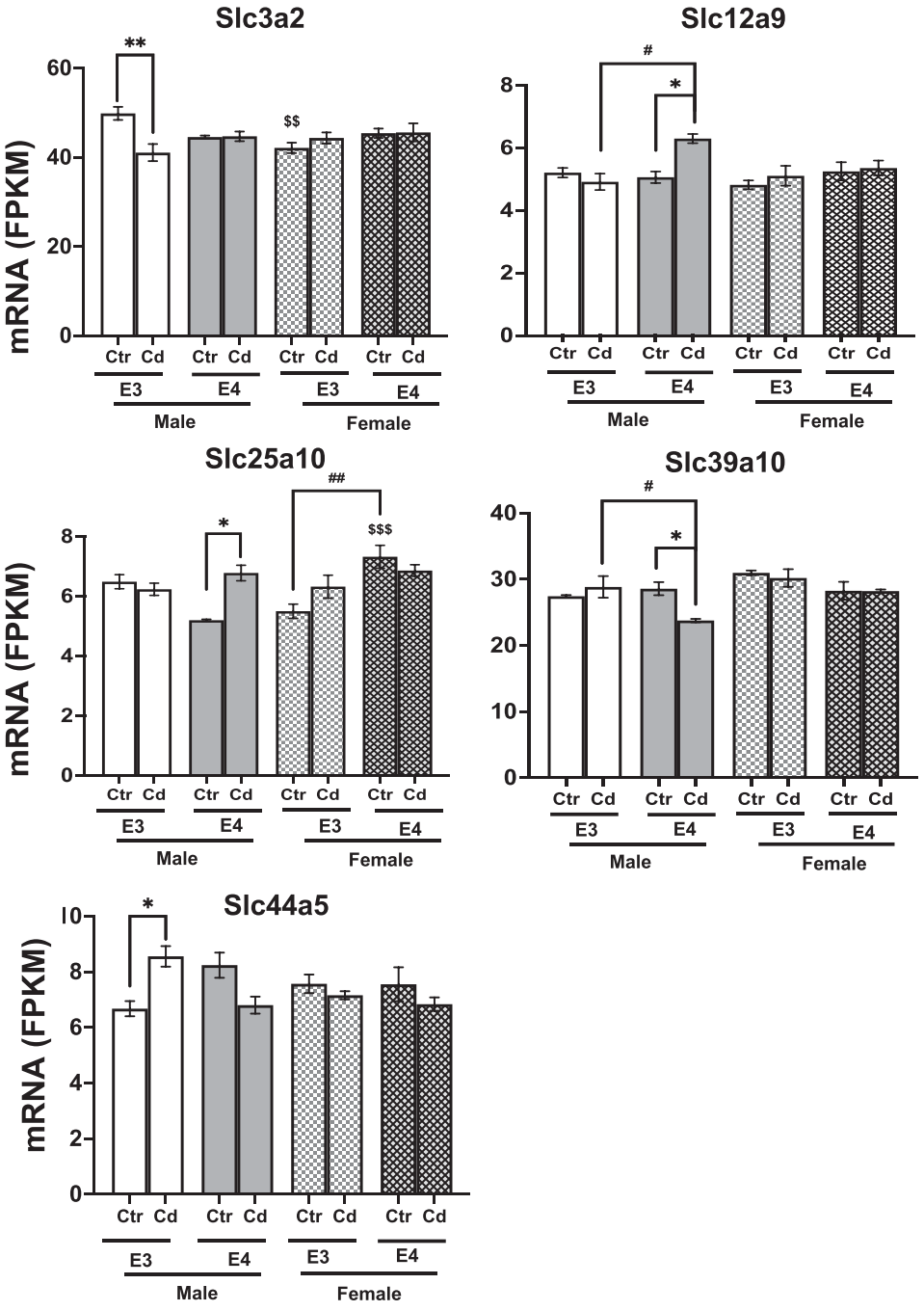

Fig. 4. Cd-mediated differential expression of mRNAs encoding transporters in mouse brain. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

were lower in Cd-exposed ApoE3 females than Cd-exposed ApoE3 males (Fig. 5 and 6).

Effects of Cd Exposure on the Alterations of Transporters in Mouse Livers. To further compare the effects of Cd exposure on hepatic transporters, sex, and genotype effects, we investigated the mRNA expression of transporters in the brains of male and female ApoE3 and ApoE4 mice (Fig. 7–10). The mRNA abundance (FPKM) of all transporters is shown in Supplemental Table 1, and the differentially regulated mRNA of transporters in liver is shown in Supplemental Table 3. Overall, Cd did not induce any effect on any transporter mRNA in the livers of female ApoE4 mice. Regarding the transport ATPases, Cd upregulated Abcg1, Atp5s, and Atp6vIe1 uniquely in the livers of ApoE4 males. To note, Abcg1 is a member involved in macrophage cholesterol and phospholipids transport. Atp5s encodes a subunit of mitochondrial ATP synthase, and the subunit is necessary for the energy transduction activity of the ATP synthase. Atp6v1e1 encodes a component of vacuolar ATPase, which mediates acidification of eukaryotic intracellular organelles. In addition, in livers of ApoE3 females, Cd uniquely upregulated Atp6v0d2, a transporter involved in proton transmembrane transporter activity (Qi et al., 2020).

The Slc transporter family had the largest numbers of liver transporters that were differentially regulated by Cd exposure, with ApoE3 females being the most responsive, followed by ApoE4 males and ApoE3 males (Figs. 8–10). In the livers of male ApoE4 mice, Cd upregulated the monocarboxylate transporter Slc16a1, the ADP/ATP translocase Slc25a4, the putative solute transporter Slc35f5, the sodium-coupled neutral amino acid transporter Slc38a4, and the iron-regulated transporter Slc40a1. Livers of ApoE3 males only had two Cd-regulated Slc transporters, namely Slc30a10 and Slc35d2, and both of which were up-regulated

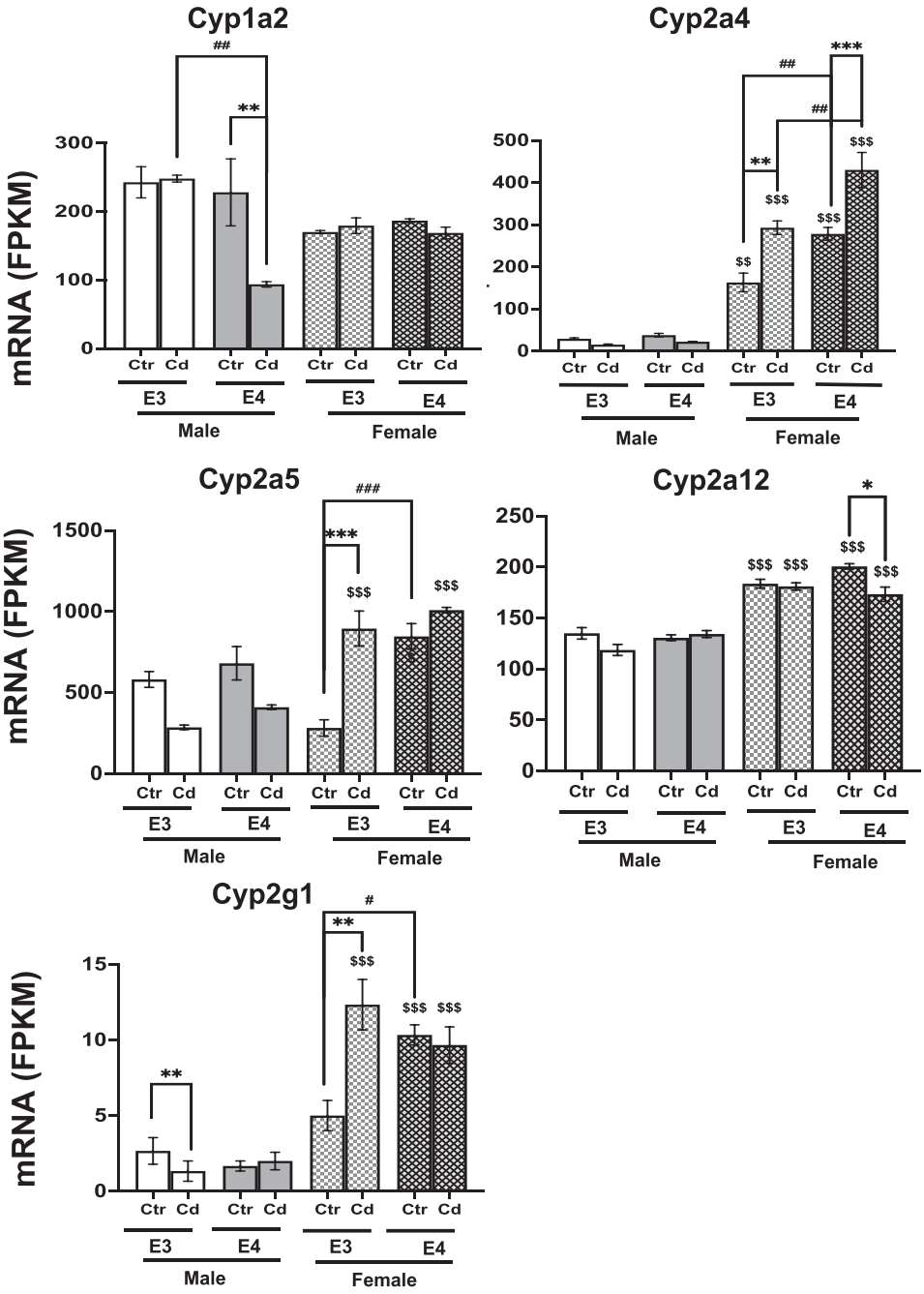

Fig. 5. Cd-mediated differential expression of mRNAs encoding drug-metabolizing enzymes (Cyp1-2) in mouse liver. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

by Cd exposure. Slc35d2 is a nucleotide sugar transporter that mediates the translocation of nucleotide sugars from the cytosol into the lumen compartment (Suda et al., 2004). In the livers of ApoE3 females, Cd upregulated Slco1a4, Slc5a6, Slc38a3, Slc38a4, and Slc39a14, but downregulated Slc16a1, Slc17a4, and Slc30a10. Among these transporters, Slco1a4 is a sodium-independent transporter which can mediate the uptake of organic anions in liver and is known to be important for xenobiotic uptake (Takano et al., 2018). Slc5a6 is a sodium-dependent transporter that is associated with neurodegeneration diseases (Subramanian et al., 2017; Byrne et al., 2019), and Slc38a3 is a transporter that mediates electrogenic cotransporter of glutamine and sodium ions in exchange for protons. In addition, Slc38a4 is predominantly a transporter in the liver and mediates the transport of both cationic and neutral amino acids (Rubio-Aliaga and Wagner, 2016), and Slc39a14 is divalent metal transporter that mediates the uptake of metals, including cadmium, zinc, manganese and iron (Taylor et al., 2005; Girijashanker et al., 2008; Tuschl et al., 2016; Hendrickx et al., 2018). Slc16a1 is proton-linked monocarboxylate transporter that catalyze the monocarboxylates (including lactate, pyruvate) movements (Halestrap, 2013). Slc17a4 is a membrane potential-dependent organic anion transporter, which requires a low concentration of chloride ions (Togawa et al., 2012; Reimer, 2013). Additionally, Slc30a10 is highly expressed in the liver and is involved in maintaining manganese levels and has higher specificity for zinc and manganese (Bosomworth et al., 2012; Patrushev et al., 2012; Chen et al., 2015; Zhao et al., 2016).

Cd Exposure Induced the Changes of mRNA Expression of Selected Liver and Brain Genes in RT-PCR. We further quantified the mRNA expression of selected genes by using RT-PCR to confirm our findings in the RNA sequencing data (Supplemental

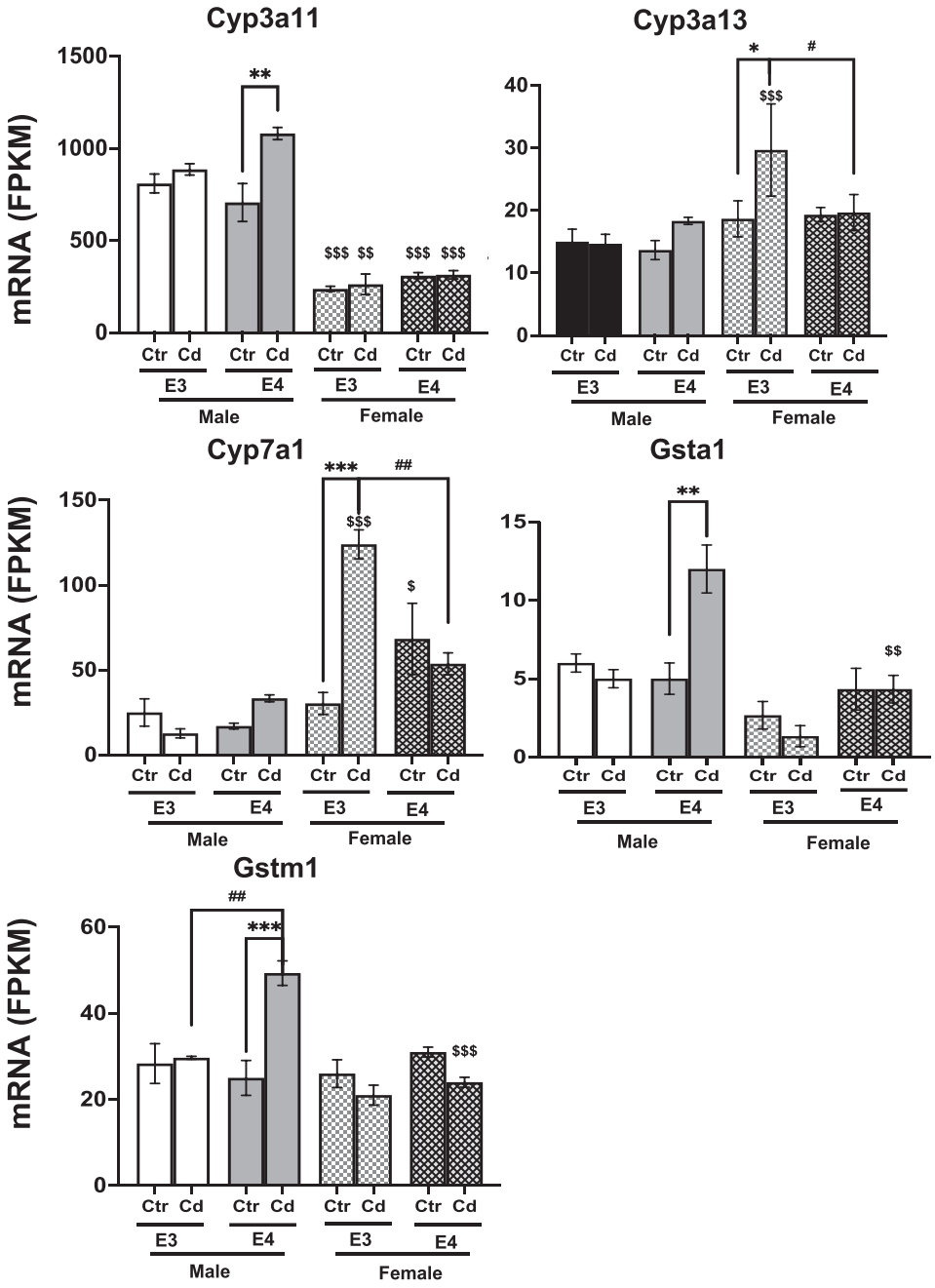

Fig. 6. Cd-mediated differential expression rof mRNAs encoding drug-metabolizing enzymes (Cyp3a11-Gstm1) in mouse liver. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

Figs. 3 and 4). A similar regulatory trend was observed between RT-qPCR and RNA-Seq. For example, in liver, we found a trend of increase of the sodium-coupled neutral amino acid transporter Slc38a4, the iron-regulated transporter Slc40a1, and the cholesterol efflux transporter Abcg1 in Cd-treated ApoE4 male mice. In the brains, Cd exposure reduced the expression of Slc3a2, which is important for the regulation of intracellular calcium levels and L-type amino acid transport, in ApoE3 males. However, a discrepancy in the regulatory trend of the Slc35f5, Slc25a4, Atp6v1e1, and ATP5s in liver, as well as Slc12a9, Slc25a10, Slc39a10, and Slc44a5 in brain was also observed. For example, the RNA-Seq results showed that Cd exposure significantly upregulated the Slc25a4 and Slc35f5 in ApoE4 males (Fig. 9), whereas we did not observe the same trend in the RT-PCR data (Supplemental Fig. 3).

Cd Exposure Did Not Induce Significant Changes in the Protein Levels of Slc38a3, Slc38a4, and Slc40a1 in Livers. We also performed western blot to investigate the protein expression levels of Slc38a3, Slc38a4, and Slc40a1 in the livers (Supplemental Figs. 5 and 6). We found an increasing trend in these three protein levels in Cd-treated female ApoE4 mice, although not statistically significant. These results are different from the RNA sequencing data (Figs. 9 and 10), suggesting that certain post-transcriptional modification may play a role in leading to the differences between mRNA levels of protein expression levels of these selected transporters.

Discussion

As summarized in Fig. 11, we previously demonstrated that male ApoE4 mice were the most susceptible to Cd neurotoxicity (Zhang

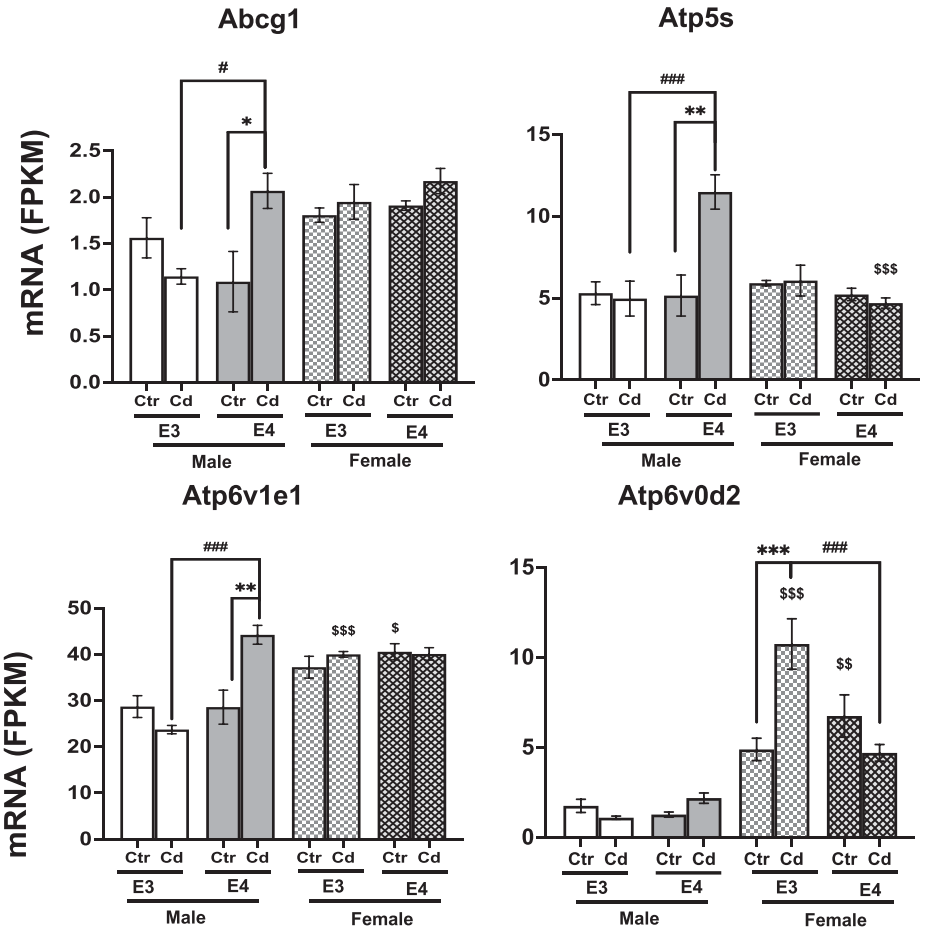

Fig. 7. Cd-mediated differential expression rof mRNAs encoding transport ATPases in mouse liver. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

et al., 2020). In addition, the memory deficits manifested earlier in ApoE4 mice within the same sex, and earlier in males within the same genotype (Zhang et al., 2020). Accompanied by the sex- and genotype-dependent Cd neurotoxicity, the present study showed that Cd-induced transcriptomic changes in the brain and liver are also sex- and ApoE-genotype specific. In general, corresponding to the highest susceptibility to Cd neurotoxicity (Zhang et al., 2020), Cd-induced dys-regulation of genes involved in proinflammation was also uniquely up-regulated in the livers of ApoE4 males, although pathways in brain were not altered in any other Cd-exposed groups. Genes involved in drug metabolism and transport were differentially regulated by Cd in both brain and liver in a sex- and ApoE-genotype specific manner, with the hepatic effect more prominent.

In the brain, the drug-metabolizing enzymes were regulated by Cd in an ApoE-genotype specific manner; the Cd effect was only observed in ApoE3 mice of both sexes. Cd upregulated the Cyp2j12 mRNA in ApoE3 males, and the Cyp2j6 mRNA in ApoE3 females (Zhang et al., 2020). The Cyp2j family is important in the metabolism of arachidonic acid to epoxyeicosatrienoic acid. Arachidonic acid is a critical mediator in amyloid-beta induced pathogenesis, leading to spatial memory impairments in AD mouse models (Sanchez-Mejia and Mucke, 2010). Cyp2j6 is upregulated in the white matter of brain during aging in female mice associated with a build-up of arachidonic acid (Klosinski et al., 2015), and arachidonic acid consumption is elevated in AD patients (Rapoport, 2008). Furthermore, Cd exposure can increase arachidonic concentrations in immortalized monocytes differentiated into macrophages to promote inflammation (Olszowski et al., 2018). Together, these observations indicate that the up-regulation in Cyp2j isoforms in the brains of ApoE3 mice may provide a compensatory mechanism to reduce Cd neurotoxicity and render protection during AD. Thus, the lack of up-regulation of Cyp2j by Cd in ApoE4 mice may be a contributing mechanism for increased susceptibility to Cd neurotoxicity. However, Cd exposure down-regulated the phase-II conjugation enzymes Sult2b1 and Sult4a1, which are sulfotransferases involved in detoxification, uniquely in the brains of ApoE3 mice. Sult4a1 expression is known to be increased in mouse mature neurons (Hashiguchi et al., 2018). Although little is known about the involvement of Sult2b1 in AD, SULT2A1 activity is reduced in AD patients (Vaňková et al., 2015). Therefore, the downregulation of sulfotransferases may contribute to Cd neurotoxicity in the ApoE3 carriers.

The Cd effect on the brain transporters was only observed in males but not females of both ApoE genotypes (Fig. 11). In the brains of ApoE4 males, Cd up-regulated the cation-chloride symporter Slc12a9 and the mitochondrial dicarboxylate carrier Slc25a10, but down-regulated the zinc transporter Slc39a10. In a mouse model of anxiety and depression-like disorder, most Slc25 genes were up-regulated in hypothalamus (Babenko et al., 2018), and our findings suggest that the up-regulation of Slc25 may contribute to Cd-neurotoxicity. The human Slc39 family (e.g., Slc39A8 and Slc39A14) are known to transport Cd (Dalton et al., 2005; Liuzzi et al., 2006; Liu et al., 2008). Because zinc and Cd are both cationic metals, the downregulation of Slc39 by Cd may be a compensatory response to Cd insult. In the brains of ApoE3 males, Cd downregulated Slc3a2, which is involved in regulating intracellular calcium levels and L-type amino acid transport. The downregulation of Slc3a2 may limit the

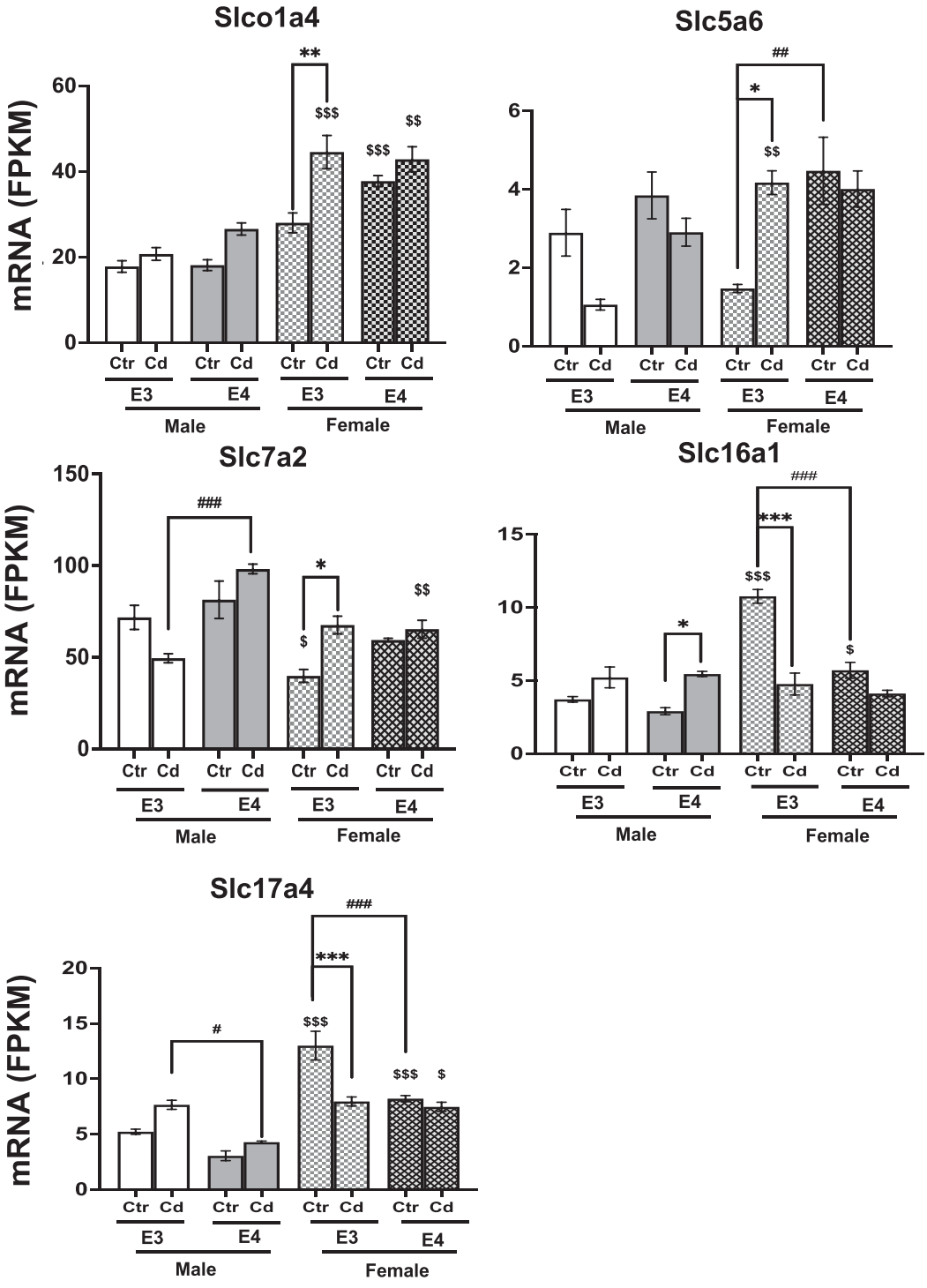

Fig. 8. Cd-mediated differential expression rof mRNAs encoding solute carriers (Slco1a4-Slc17a4) in mouse liver. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

accumulation of Cd as a compensatory mechanism, but in term may reduce cellular uptake of amino acids such as phenylalanine, tyrosine, L-DOPA, and tryptophan (Mastroberardino et al., 1998; Broer et al., 2001; Friesema et al., 2001; Yanagida et al., 2001; Kim et al., 2002; Simmons-Willis et al., 2002; Milkereit et al., 2015; Yan et al., 2019), which are essential for brain functions. Cd up-regulated Slc44a5 in ApoE3 males; whereas this transporter is also expressed in human brains, its role in neurodegenerative diseases needs further characterization (Ayka and Sehirli, 2020).

In the liver, the mRNA of Cyp3a11, the major phase-I oxidation enzyme and its human ortholog Cyp3A4 can metabolize over 50% of drugs in the market, was up-regulated by Cd uniquely in ApoE4 males (Fig. 11). The phase-II glutathione *S*-transferase (Gst) a1 and m3, which detoxify electrophiles, were up-regulated by Cd uniquely in ApoE4 males (Fig. 11). To note, all three genes (Cyp3a11, Gsta1, and Gstm3) are bona fide target genes of the major xenobiotic-sensing nuclear receptor pregnane X receptor (PXR/Nri12). The hepatic

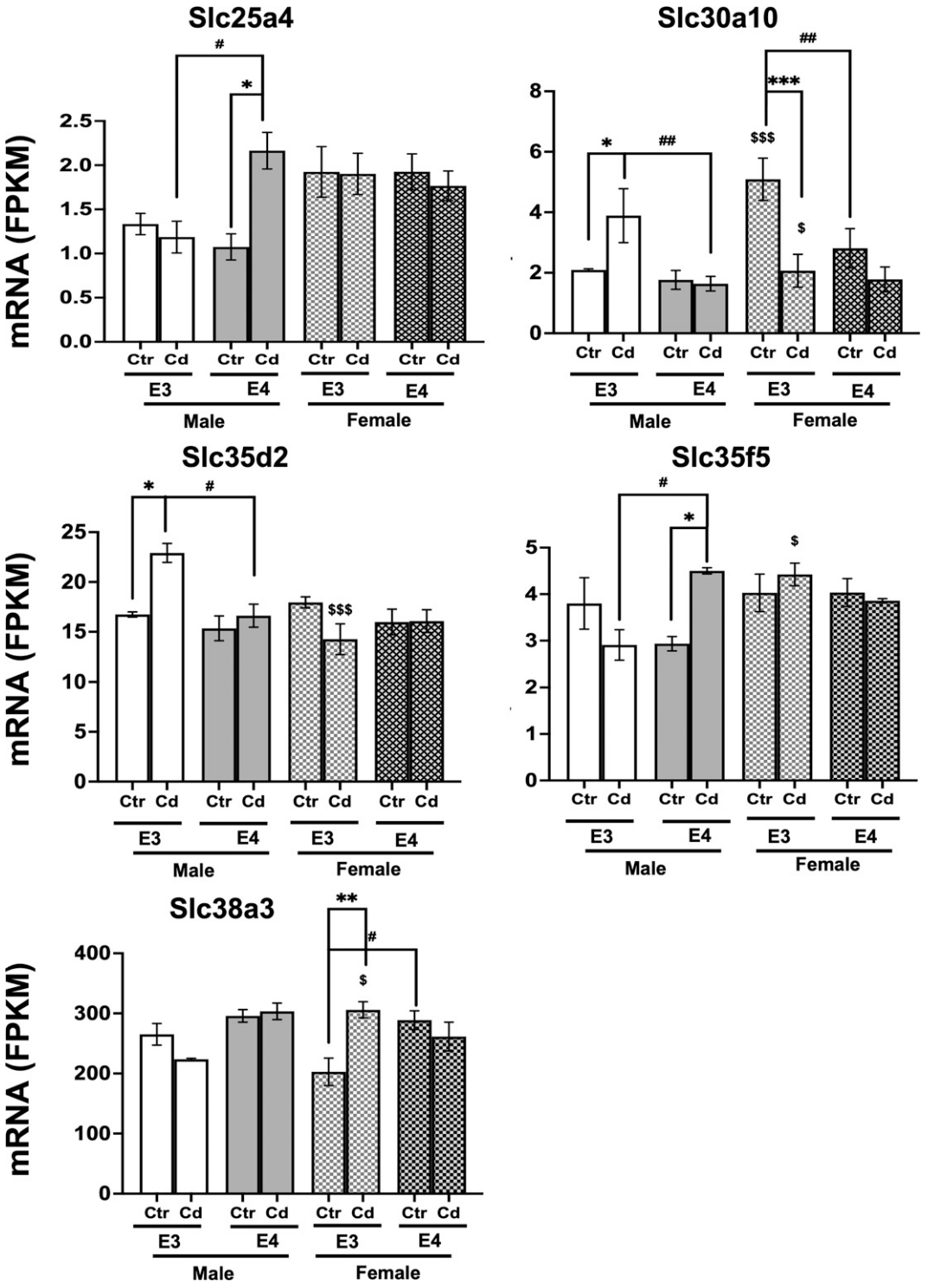

Fig. 9. Cd-mediated differential expression rof mRNAs encoding solute carriers (Slc25a4-Slc38a3) in mouse liver. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

up-regulation of these genes is dependent on the presence of PXR (Cui et al., 2010; Aleksunes and Klaassen, 2012). The unique up-regulation of these genes suggests an activation of the PXR signaling in ApoE4 males. Because PXR activation is well-known for drug-drug interactions (Willson and Kliewer, 2002; Hogle et al., 2018; Staudinger, 2019), and that AD patients usually take multiple types of medications for AD management and other complex diseases, our study suggests that drug-drug interactions in AD patients should be examined in a sex- and ApoE genotype specific manner. Cyp1a2 mRNA was the only downregulated P450 in the livers of ApoE4 males (Fig. 5). It is a prototypical target gene of AhR (Vrzal et al., 2004; Mandal, 2005), suggesting that AhR signaling may be suppressed in the livers of Cd-exposed ApoE4 males.

In the livers of females, Cyp2a4 mRNA was upregulated by Cd in both genotypes. In addition, in the livers of ApoE3 females, several other Cyp2 isoforms were upregulated by Cd, namely Cyp2a5, 2a13,

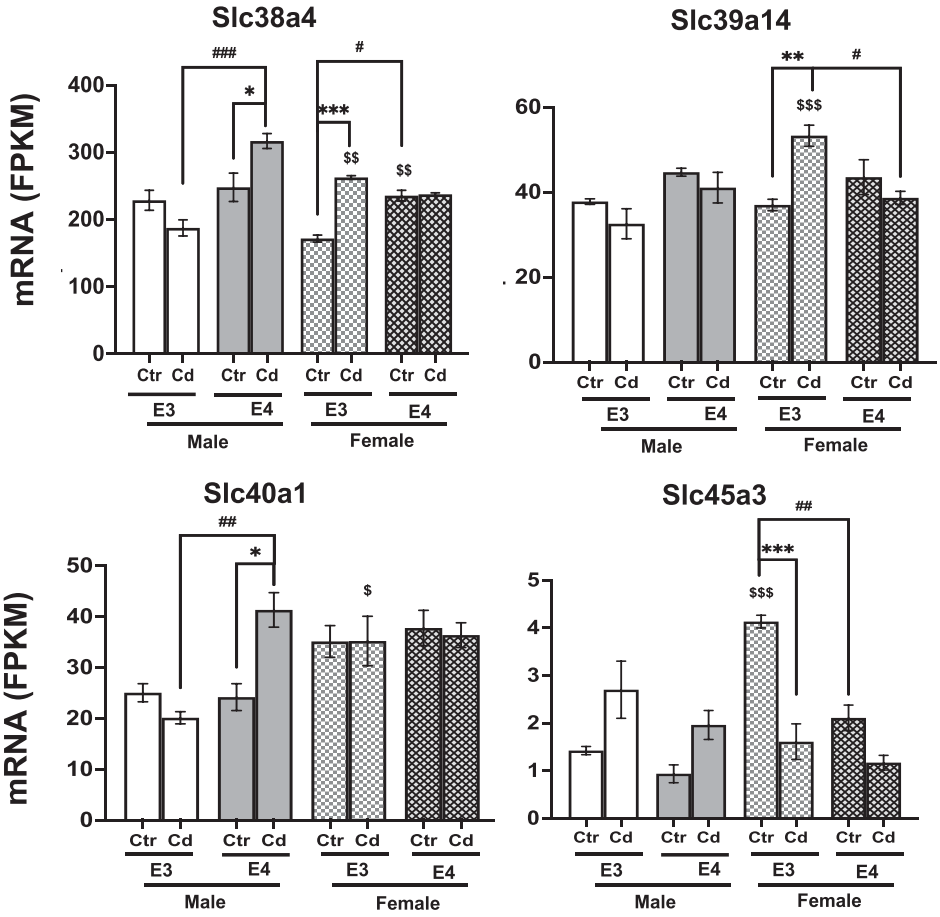

Fig. 10. Cd-mediated differential expression of mRNAs encoding solute carriers (Slc38a3-Slc45a3) in mouse liver. The mRNA values in FPKM in each group (n = 3/sex/genotype/exposure) were analyzed using three-way ANOVA considering the effects of sex ($), genotype (#), and exposure (*). Data are presented as mean ± S.E.M. $^{*/\$/\#}P < 0.05$; $^{**/\$\$/\#\#}P < 0.005$; $^{***/\$\$\$/\#\#\#}P < 0.001$.

and 2g1 (Fig. 11). These enzymes and their human orthologs have a broad substrate specificity, including steroid hormones, lipids, and xenobiotics. Cyp2a4 and Cyp2a5 are known to display circadian rhythmic expression patterns in mouse liver, as regulated by the transcription factor D-Box Binding PAR BZIP Transcription Factor (Lavery et al., 1999). Cyp2g1 is not a liver enriched P450 isoform, but its expression is necessary in maintaining the constitutive expression of Cyp2a4 in liver (Zhuo et al., 2004). Both Cyp2g1 and the rate-limiting BA-synthetic enzyme Cyp7a1 are known to display circadian rhythmic expression patterns in mouse liver (Zhang et al., 2009; Li et al., 2020). Together, these findings partly explained the Cd-mediated unique enrichment of the circadian rhythm pathways in livers of ApoE3 females (Fig. 2B). Circadian rhythm desynchronization has been observed during AD and is implicated in neuroinflammation and neurodegeneration within CNS (Homolak et al., 2018). Cd is also identified as a disruptor of circadian rhythm in zebrafish (Xiao et al., 2016). It is increasingly recognized that hepatic circadian gene regulation is important in liver injuries (Tahara and Shibata, 2016; Zhou et al., 2016; Zheng et al., 2021). Our study showed that the liver is also a target of Cd-mediated disruption of circadian rhythm-related genes, and such regulation is specific to female ApoE3 carriers.

Most of the differentially regulated liver transporters by Cd exposure were involved in intermediary metabolism. For example, in ApoE4 males, Cd up-regulated the cholesterol efflux transporter Abcg1, the monocarboxylic acid transporter Slc16a1, the mitochondrial adenine nucleotide translocator Slc25a4, the sodium-coupled amino acid transporter Slc38a4, and the proton-coupled divalent metal ion transporter Slc40a1. The upregulation of Slc40a1 may facilitate Cd export as a compensatory mechanism to reduce Cd levels in the liver of ApoE4 males. A couple of transport ATPase and Slc35f5 were differentially regulated by Cd in this group. In ApoE3 males, Cd upregulated the manganese transporter Slc30a10, likely also a compensatory response. In addition, Cd up-regulated UDP-GlcNAc/UDP-glucose transporter (Slc35d2) in ApoE3 males. Cd differentially regulated the most transporters in livers of ApoE3 females; these transporters are all involved in intermediary metabolism, including an up-regulation of the sodium/multivitamin and iodide cotransporter Slc5a6, the cationic amino acid transporter Slc7a2, the sodium-coupled amino acid transporters Slc38a3 and Slc38a4, the metal ion transporter Slc39a14, as well as a downregulation of the monocarboxylic acid transporter Slc16a1, the sodium phosphate transporter Slc17a1, the manganese transporter Slc30a10, and Slc45a3, which is associated with glycosaminoglycan metabolism. To note, Cd-mediated regulatory patterns of and Slc16a1 and Slc30a10 are completely reversed between ApoE3 females and the males, suggesting the critical role of sex as a modifying factor in Cd-mediated regulation of transporters.

To further confirm the findings in the RNA-Seq data, we selected several important genes and quantified their mRNA expression by using RT-PCR (Supplemental Figs. 3 and 4). A similar regulatory trend was observed for the Slc40a1, Slc38a4, and Abcg1 in liver, as well as Slc3a2 in brain. However, a discrepancy trend of the Slc35f5, Slc25a4, Atp6v1e1, and ATP5s in liver, as well as Slc12a9, Slc25a10, Slc39a10, and Slc44a5 in brain was also observed. For example, the RNA-Seq results showed that Cd exposure significantly upregulated the

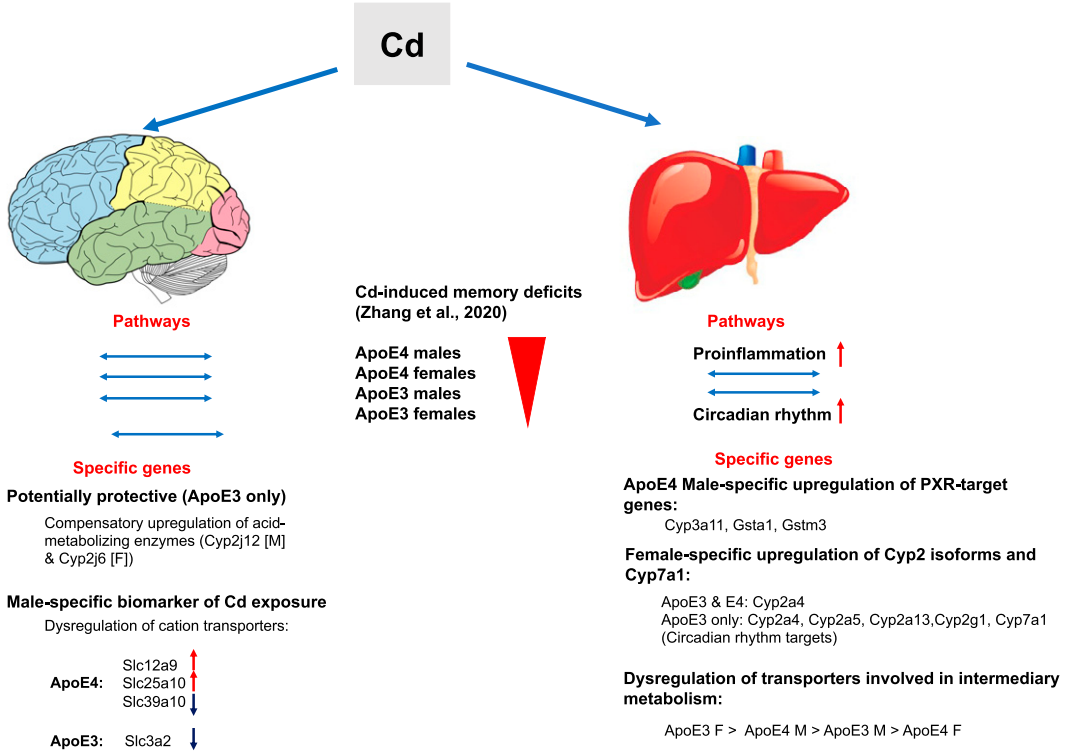

Fig. 11. Summary diagram regarding the sex- and ApoE-genotype specific regulation of brain and liver transcriptomes associated with the reported Cd-induced memory deficit in these mice (Zhang et al., 2020).

Slc25a4 and Slc35f5 in ApoE4 males, whereas the RT-PCR data showed that they were downregulated or unchanged. The discrepancy may be caused by the detection of distinct transcript variant using primer-based method versus total fragment counts. We also quantified the protein expression of Slc38a3, 38a4, and 40a1 in livers (Supplemental Figs. 5 and 6). The data showed that there is an increasing trend in these protein levels in Cd-treated ApoE4 females, which is not observed in the mRNA data. These results suggest that post-transcriptional modification may play a role and cautions need to be made while interpreting transcriptomic results.

Taken together, our study showed that in addition to neurotoxicity, Cd also disrupted the transcriptomes in brain and liver, with more prominent effects observed in liver. The Cd-induced proinflammation pathways were mostly enriched in the livers of ApoE4 males, whereas the circadian rhythm pathways were mostly enriched in the livers of ApoE3 females. The dysregulation of brain and hepatic transporters, and drug metabolizing enzymes, were also in sex- and ApoE genotype specific manner. It is important to note that most differentially regulated transporters by Cd exposure are involved in intermediary metabolism, suggesting that environmental exposures to toxicants may contribute to complex metabolic diseases. However, this study has limitation, including lack of investigation on the protein functions and the underlying mechanisms. In the future, research is needed to investigate the effects of Cd on the transporter function levels and underlying regulatory mechanisms.

Acknowledgements

The authors would like to thank members of the Cui Laboratory for editing the manuscript.

Authorship Contributions

Participated in research design: Wang, Zhang, Xia, and Cui.
Conducted experiments: Wang, Zhang, and Cui.
Performed data analysis: Wang, Xia, and Cui.
Wrote or contributed to the writing of the manuscript: Wang, Xia, and Cui.

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