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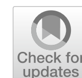

Transgenerational epigenetic and transcriptomic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure in rat

Stephenie D. Prokopec[1] · Matti Viluksela[2,3] · Hanna M. Miettinen[3,4] · Paul C. Boutros[5,6,7,8,9,10,11] · Raimo Pohjanvirta[12]

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Abstract

In rats, direct exposure to TCDD causes myriad toxicities. Exposed rats experience hepatotoxicity, wasting syndrome and immune suppression, amongst others. "Inherited exposure", as occurs in the F3 generation of directly exposed F0 animals, has also been shown to cause toxicity: both male and female F3 rats demonstrate an increased incidence of adult onset disease, females also display reproductive abnormalities and increased incidence of ovarian diseases while males show increased incidence of kidney disease and an altered sperm epigenome. Here, we explore the hepatic transcriptomic profile of male and female F3 Sprague–Dawley rats bred through the paternal germ line from F0 dams exposed to a single dose of TCDD (0, 30, 100, 300 or 1000 ng/kg body weight) by oral gavage. We hypothesize that RNA transcripts with altered abundance in livers of unexposed F3 progeny of treated F0 Sprague–Dawley rats may result from epigenetic modifications to the genome. We further survey patterns of differential methylation within male F3 rat testis. Female F3 rats demonstrated more TCDD-mediated hepatic transcriptomic changes than males, with differences primarily in the lowest dose group. In testis from male F3 rats, multiple olfactory receptors displayed patterns of differential methylation. Hypermethylation of *Egfr* and *Mc5r* among testes from TCDD lineage rats was observed, but without corresponding changes in hepatic mRNA abundance. Further studies examining these differences in other tissue types are warranted.

Keywords 2,3,7,8-Tetrachlorodibenzo-p-dixoin (TCDD) · Sprague–Dawley rat · Transgenerational inheritance · Transcriptome · Differential methylation

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✉ Paul C. Boutros
pboutros@mednet.ucla.edu

✉ Raimo Pohjanvirta
raimo.pohjanvirta@helsinki.fi

[1] Ontario Institute for Cancer Research, Toronto, Canada

[2] Department of Environmental and Biological Sciences, School of Pharmacy (Toxicology), University of Eastern Finland, Kuopio, Finland

[3] Environmental Health Unit, Finnish Institute for Health and Welfare (THL), Kuopio, Finland

[4] A. I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland

[5] Department of Medical Biophysics, University of Toronto, Toronto, Canada

[6] Department of Pharmacology and Toxicology, Universally of Toronto, Toronto, Canada

[7] Department of Human Genetics, University of California, Los Angeles, 12-109 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA

[8] Department of Urology, University of California, Los Angeles, Los Angeles, USA

[9] Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, Los Angeles, USA

[10] Institute for Precision Health, University of California, Los Angeles, Los Angeles, USA

[11] Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles, USA

[12] Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 66, 00014 Helsinki, Finland

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent, highly lipophilic, environmental contaminant produced in herbicide and pesticide manufacture, as well as a by-product of various industrial products (Schecter et al. 2006; Shen et al. 2009; Von Burg 1988). In rats, TCDD has a half-life of ~ 19 days (Geyer et al. 2002) and exposure to even small amounts (less than 50 µg/kg in sensitive strains) can cause a myriad of toxic outcomes including hepatotoxicity, reproductive and developmental toxicity, thymic atrophy, wasting syndrome, immune suppression and acute lethality (Pohjanvirta and Tuomisto 1994). In humans, the half-life of TCDD is considerably longer, approximately 8 years (Geyer et al. 2002). Direct exposure to TCDD in humans can cause chloracne (Baccarelli et al. 2005), tumourigenesis, possibility including prostate cancer in men (Ansbaugh et al. 2013; Leng et al. 2014), and has been implicated in development of chronic diseases (Yi et al. 2014). Much of what is known about TCDD exposure in humans comes primarily from two sources: exposure from Agent Orange used in the Vietnam War and an industrial accident in Seveso, Italy in 1979. In both cases, a wide range of toxic outcomes have been documented in those individuals exposed directly as well as subsequent generations (Baccarelli et al. 2008; Mocarelli et al. 2011).

Due to its long half-life, effects of TCDD that are observed up to and including the F2 generation can be attributed to direct, multigenerational exposure; TCDD exposure of females (F0) can persist in the body leading to direct exposure of any offspring (F1), through the placenta and breast milk (Nau et al. 1986). This further exposes the developing germ cells (F2) within these offspring. In zebrafish, transgenerational effects, such as skeletal and reproductive abnormalities, have been documented up to the F2 generation (equivalent to rodent F3 generation) (Baker et al. 2014). In rats, repeated exposure to TCDD of F0 animals has been shown to alter the sex ratio of the F2 generation (Ikeda et al. 2005). Various TCDD-mediated effects have been documented in the F3 generation that have been linked to heritable changes of the epigenome of exposed individuals. In particular, Manikkam et al. described an increased prevalence of kidney disease (Manikkam et al. 2012b) in male F3 rats following exposure of the F0 generation to TCDD, as well as reduced serum testosterone levels (Manikkam et al. 2012a). In F3 females increased prevalence of ovarian disease, decline in ovarian follicle numbers and early onset of puberty have all been reported previously in relation to ancestral TCDD exposure (Manikkam et al. 2012a, b; Nilsson et al. 2012; Yu et al. 2019).

In rodents, the liver is a primary site of TCDD-mediated toxicities (Pohjanvirta et al. 1990). Numerous studies have identified substantial transcriptomic (Boutros et al. 2011; Boverhof et al. 2006; Fletcher et al. 2005; Franc et al. 2008; Moffat et al. 2010; Nault et al. 2013; Yao et al. 2012) and proteomic (Forgacs et al. 2012; Lee et al. 2005; Pastorelli et al. 2006; Prokopec et al. 2017) hepatic effects following direct exposure to TCDD in various strains of rats, including the TCDD-sensitive Long–Evans (L–E; oral LD_{50} of 9.8–17.7 µg/kg TCDD) and TCDD-resistant Han/Wistar (H/W; oral LD_{50} of > 9600 µg/kg TCDD) strains (Pohjanvirta and Tuomisto 1994). Sprague–Dawley (SD) rats have an oral LD_{50} of 20 µg/kg TCDD (Bickel 1982) and are considered to be sensitive to the effects of TCDD. Here we examine the hepatic transcriptome of male and female SD rats (F3) from TCDD-exposed lineages to identify genes showing altered mRNA abundance profiles. Furthermore, the promoter-region methylation patterns of genes demonstrating altered RNA abundance are assessed to identify candidate mechanisms for these transgenerational effects. Finally, as transgenerational epigenetic effects have been previously detected in sperm (Manikkam et al. 2012a), and an enhanced testicular inflammation phenotype has been described in F3 rats (Bruner-Tran et al. 2014), we additionally contrast genomic methylation patterns within testes of F3 rats from TCDD-exposed and control lineages.

Results

Experimental design

We evaluated the transgenerational hepatic transcriptomic or testicular epigenetic effects caused by TCDD exposure of pregnant Sprague–Dawley (SD) rats through the paternal germline. Twenty-five pregnant SD rats were treated with a single dose of TCDD (0, 30, 100, 300, 1000 ng/kg). Each dam initiated either a control (control lineage) or TCDD-exposed lineage (TCDD lineage), terminating with the F3 generation (Fig. 1). Hepatic tissue from male and female F3 animals from each treatment group ($n = 5$–8 each male and female; Table 1) was collected and transcriptomic profiling performed. A total of 67 F3 animals were initially included in this study; complete lineage information is available in Supplementary Table 1. A single array was identified as an outlier and was removed from downstream analyses (Supplementary Figures 1–2), reducing the total number of animals examined to 66. Concurrently, testicular tissues were collected from male F3 rats from the 1000 ng/kg TCDD lineage ($n = 4$) and control lineage ($n = 4$) groups and targeted bisulfite sequencing performed to identify differentially methylated regions (DMRs).

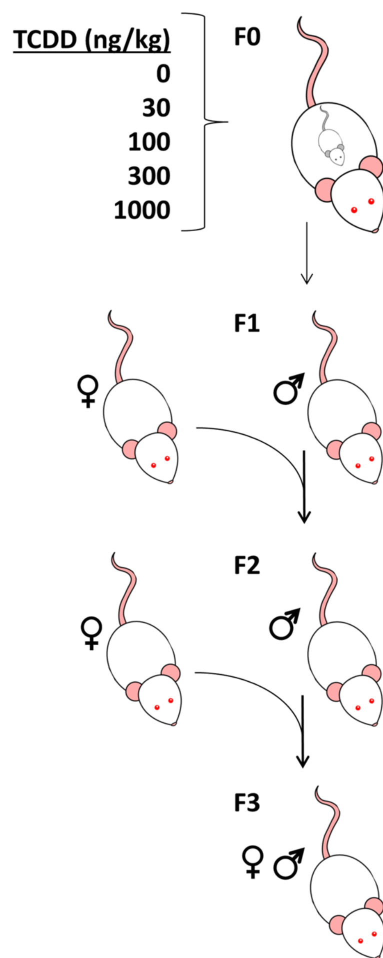

Fig. 1 Experimental design. Pregnant Sprague–Dawley (SD) rats were treated with a single dose of TCDD (0, 30, 100, 300, 1000 ng/kg) dissolved in corn oil on gestational day 11 by oral gavage. Adult F1 males were mated with untreated female rats to produce the F2 generation. Similarly, adult F2 males were mated with untreated females to produce the F3 generation. Tissues were collected from F3 animals from each lineage (liver from male and female animals; testis from males). Hepatic transcriptome was evaluated using Affymetrix Rat Gene 2.0 ST arrays and testicular tissues used for bisulfite sequencing

Table 1 Number of animals per treatment group

	F0 dam dose (ng/kg TCDD)				
	0	30	100	300	1000
F0 dam	6	5	6	4	4
F3 female	6	6	6	8 (7)	8
F3 male	5	6	6	8	8

Groups of F0 dams ($n = 4$–6) bred with untreated male rats were treated with a single dose of TCDD (0, 30, 100, 300, 1000 ng/kg body weight) dissolved in corn oil by oral gavage on gestational day 11. F1 (and subsequently F2) males were mated with untreated females to produce the F3 generation. Hepatic tissue was harvested and profiled from 5–8 F3 animals (each male and female) for each treatment group. A single F3 female (300 ng/kg) was excluded from the downstream analysis (numbers in brackets indicate final number used). Likewise, testicular tissue was collected from 4 F3 males from the control and high dose groups

TCDD has minimal impact on the F3 hepatic transcriptome

The hepatic transcriptome demonstrated minimal differences between the TCDD- and control lineage, regardless of dose or sex (Supplementary Figure 3). After filtering, we identified 280 genes that demonstrated moderate differences, with a variance in normalized intensity values > 0.5 across the cohort (Supplementary Figure 3b). To evaluate these clustering patterns, we employed the Adjusted Rand Index (ARI). Values of the ARI approaching one indicates that the clustering by RNA abundance data exactly matches the variable of interest, while ARI values near zero suggest the variable has no influence on RNA abundance. From this analysis, the greatest influence was attributed to sex ($ARI_{sex} = 1$), rather than treatment ($ARI_{treatment} = -0.008$) or specific dose ($ARI_{dose} = 0.027$). Within each sex, however, moderate associations were observed with treatment ($ARI_{sex:treatment} = 0.567$; green *vs.* white in the first covariate) but not by specific dose ($ARI_{sex:dose} = 0.188$; all levels within this first covariate). This highlights the presence of TCDD-mediated effects on RNA abundance that are not dependent on any specific dose and may in fact differ between the males and females. To confirm this, linear modelling was performed to identify specific changes in each treatment group relative to control animals of the same sex. Consistently, minimal changes were detected in any group, with 23 and one gene respectively altered in livers of female F3 rats descending from dams exposed to the lowest (30 ng/kg) and highest (1000 ng/kg) dose of TCDD ($FDR < 0.1$; Supplementary Figure 3c, Supplementary Table 2). Interestingly, this did not include any typical "AHR-core" response genes (Supplementary Figure 3d). While not statistically significant, we did observe a small increase in RNA abundance for *Ahr* in female rat liver, particularly at the lower doses of TCDD (foldchanges = 0.3 and 0.05 relative to controls, for 30 and 1000 ng/kg dose groups respectively), while the opposite was observed in males (small decrease in RNA abundance for *Ahr*, particularly at the higher doses; foldchanges = 0.08 and − 0.25 for 30 and 1000 ng/kg dose groups respectively, relative to controls). Female rats have been shown to be more sensitive to TCDD-induced toxicities than male rats (Pohjanvirta et al. 1993; Silkworth et al. 2008), however, the larger number of TCDD-responsive genes in females of the lowest dose group is unusual based on previous studies. This may represent hormesis, as proposed previously (Kociba et al. 1978; Tuomisto et al. 2006), albeit with specific reference to tumour promotion rather

than molecular abundance, or be secondary to a female hormonal change with a non-monotonous dose–response relationship (Karman et al. 2012).

Those genes identified as having differentially abundant transcripts ($q < 0.1$) in any treatment group were examined further (Fig. 2). Of these, carboxypeptidase A4 (*Cpa4*), a secreted zinc-dependent metallocarboxypeptidase, showed the largest magnitude change—repressed nearly two-fold ($\log_2$|fold-change|= − 0.99) in female F3 rat liver. Curiously, we observed decreasing effect size with increasing TCDD dose, again consistent with hormesis. In previous studies of liver from rats directly exposed to TCDD, only one (L–E treated with 100 µg/kg for 10 days) (Boutros et al. 2011; Moffat et al. 2010) showed significantly reduced mRNA abundance of *Cpa4*. However, C57BL/6 mouse liver often showed TCDD-mediated effects on mRNA abundance of this gene: in female mice treated with a single dose of 500 µg/kg TCDD for 6 h or treated with 125 or 500 µg/kg TCDD for 4 days, and in male mice treated with 500 µg/kg TCDD for 6 days (Lee et al. 2015; Prokopec et al. 2015). In

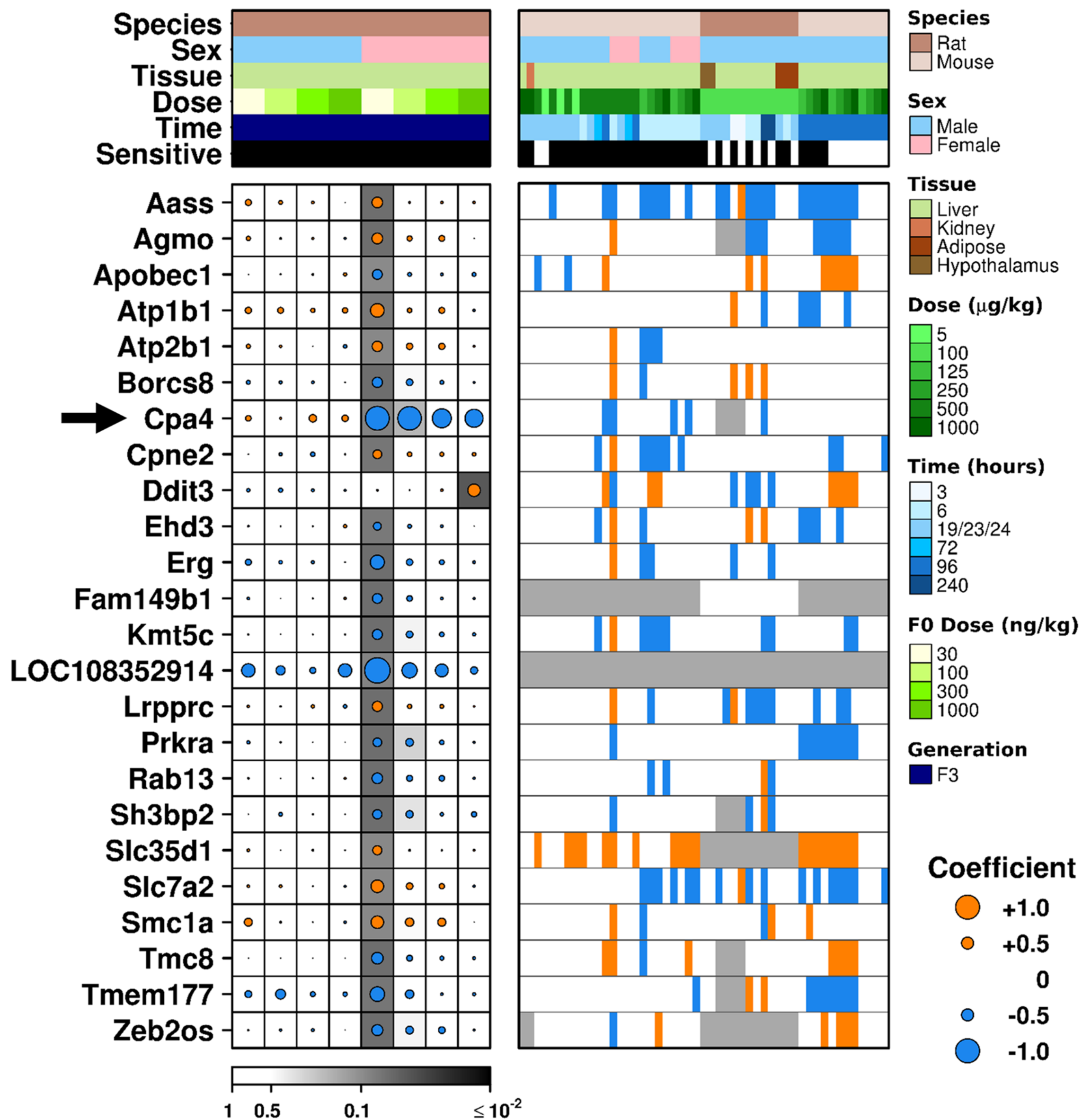

Fig. 2 Hormesis-like response within female F3 hepatic transcriptome. Twenty-four genes demonstrated altered mRNA abundance in TCDD-exposed lineages relative to controls. Dot size indicates magnitude of change ($\log_2$|foldchange|) while dot colour indicates direction. Background shading represents significance level (FDR adjusted *p* values). Right heatmap indicates significant differences at FDR < 0.1 (where orange indicates an increase and blue a decrease in abundance relative to controls) in previous studies of TCDD-mediated transcriptomic changes among various species, strains, sexes and tissues exposed to various doses of TCDD for multiple timepoints (Prokopec et al. 2017); here grey indicates that the gene was not tested

humans, *CPA4* is a maternally imprinted gene (Kayashima et al. 2003) shown to be upregulated in prostate cancer (Huang et al. 1999); it has been suggested to regulate the extracellular environment (Tanco et al. 2010) and adipogenesis (He et al. 2016).

Alternatively, DNA-damage inducible transcript 3 (*Ddit3*) showed increased mRNA abundance in female liver originating from the highest dose lineage. Transgenic mice expressing the DEL variant showed similar increases to mRNA abundance for this gene (Prokopec et al. 2017), as did livers of male C57BL/6 mice (Lee et al. 2015; Prokopec et al. 2015). In contrast, male rat liver often showed reduced expression: L–E (100 µg/kg TCDD for 3 h and 4 days) and H/W (100 µg/kg TCDD for 4 and 10 days) (Boutros et al. 2011; Moffat et al. 2010); female mouse liver also demonstrated significantly reduced mRNA abundance of *Ddit3*; however, this was short lived (6 h following treatment with 500 µg/kg TCDD) (Lee et al. 2015; Prokopec et al. 2015). *Ddit3* has been shown to mediate insulin resistance in adipocytes, with increased expression correlating with systemic insulin resistance (Suzuki et al. 2017).

Physiological transgenerational effects have been previously reported, along with a subset of molecular characteristics (Manikkam et al. 2012a, b). These studies identified 50 significantly DMRs in sperm from F3 progeny of TCDD-exposed F0 rats. We sought to determine whether these genes demonstrated altered transcription in livers of our male F3 rats. However, there was no overlap between these studies, possibly due to differences in sample types. Finally, due to the small number of differentially abundant transcripts observed, we next repeated the above modelling, however pooling the TCDD lineages (Supplementary Figure 3e). We observed clear differences in the mRNA profile between male and female rat livers, however these effects were not associated with exposure to TCDD.

Exposure to TCDD leads to differential methylation patterns in F3 testis

TCDD-exposure of F0 dams did not have any statistically significant influence on global DNA methylation status in the F3 generation, as assessed in the livers of both sexes and in the testes of male rats (Supplementary Table 3).

We next sought to explain the testicular inflammation phenotype previously observed in F3 progeny from F0 mice exposed to TCDD (Bruner-Tran et al. 2014). We performed bisulfite sequencing to identify any DMRs between the TCDD and control lineages. A genome-wide approach was first applied to discover DMRs outside of the gene-body (Supplementary Table 4). Multiple genes contained separate regions of hyper- and hypo-methylation in TCDD lineage when compared to control lineage (for example, *Atrnl1* and *Ehbp1*). Additionally, four separate DMRs were found adjacent (up or downstream) to olfactory receptor genes (Fig. 3a–c). While such receptors may not be expressed in testicular tissue, these results are intriguing if such patterns hold throughout other tissues—aversion to novel foodstuffs is a well-documented response following exposure to AHR agonists (Lensu et al. 2011a, b; Manikkam et al. 2012b).

EGFR binding has previously been shown to be reduced in a sustained manner in various tissues of laboratory animals after TCDD exposure, including testes in Sprague–Dawley rats (el-Sabeawy et al. 1998). Although this phenomenon may largely be attributable to receptor internalization (Campion et al. 2016), the pronounced hyper-methylation of *Egfr* in this study in the TCDD-exposed lineage (62% of reads with methylation *vs.* 0% in the control animals) is of interest and would warrant analyzing its mRNA expression levels (Fig. 3d). This hyper-methylation is particularly interesting as it occurs within the gene body of *Egfr*—a mechanism proposed to contribute to transgenerational plasticity to environmental stimuli (Dixon et al. 2018) which has also been linked to tumourigenesis through elevated oncogene expression in liver cancer (Arechederra et al. 2018).

To improve confidence in our calls, we next limited our search to targeted regions ($n = 1462$; median 4346 reads per region). We identified 43 statistically significant DMRs ($q < 0.01$) within coding regions (Supplementary Table 5). In particular, three genes demonstrated large increases in the proportion of methylated reads between groups: *Mc5r* had 21% more reads with methylation in the TCDD lineage compared to the control lineage; *Ppp1r27* and *Fam109a* also had increases of 11.5% and 10.3% methylated reads. Alternatively, *Hspa8* and *Olr1108* exhibited 10.0 and 8.6 percentage point decreases in methylated reads in testes from the TCDD lineage in comparison to controls. Hyper-methylation of *Mc5r* (melanocortin 5 receptor; Fig. 4a) may lead to repressed expression of the Hspa8 receptor, a deficiency of which has been implicated in reduced pheromone signalling (Morgan et al. 2004) and obesity (Chagnon et al. 1997; Shukla et al. 2012). Two additional genes previously shown to harbour DMRs in promoter regions in sperm, *Pi16* and *Rasal3* (Manikkam et al. 2012b), also contained gene-body DMRs in testes (increased methylation in the TCDD-lineage). No DMRs were detected in or around *Ahr* or any “AHR-core” genes.

Finally, as differences among methylation levels for individual genes were generally small between our treatment and control groups, we next sought to combine genes to identify effects of ancestral TCDD-exposure on broader biological pathways. We performed pathway analysis separately for all of the hyper- and hypo-methylated gene sets using a standard bioinformatic strategy (Reimand et al. 2007). This identified unique sets of enriched pathways depending on DMR direction (hyper- or hypo-methylated in the TCDD-lineage; Fig. 4b). Specifically,

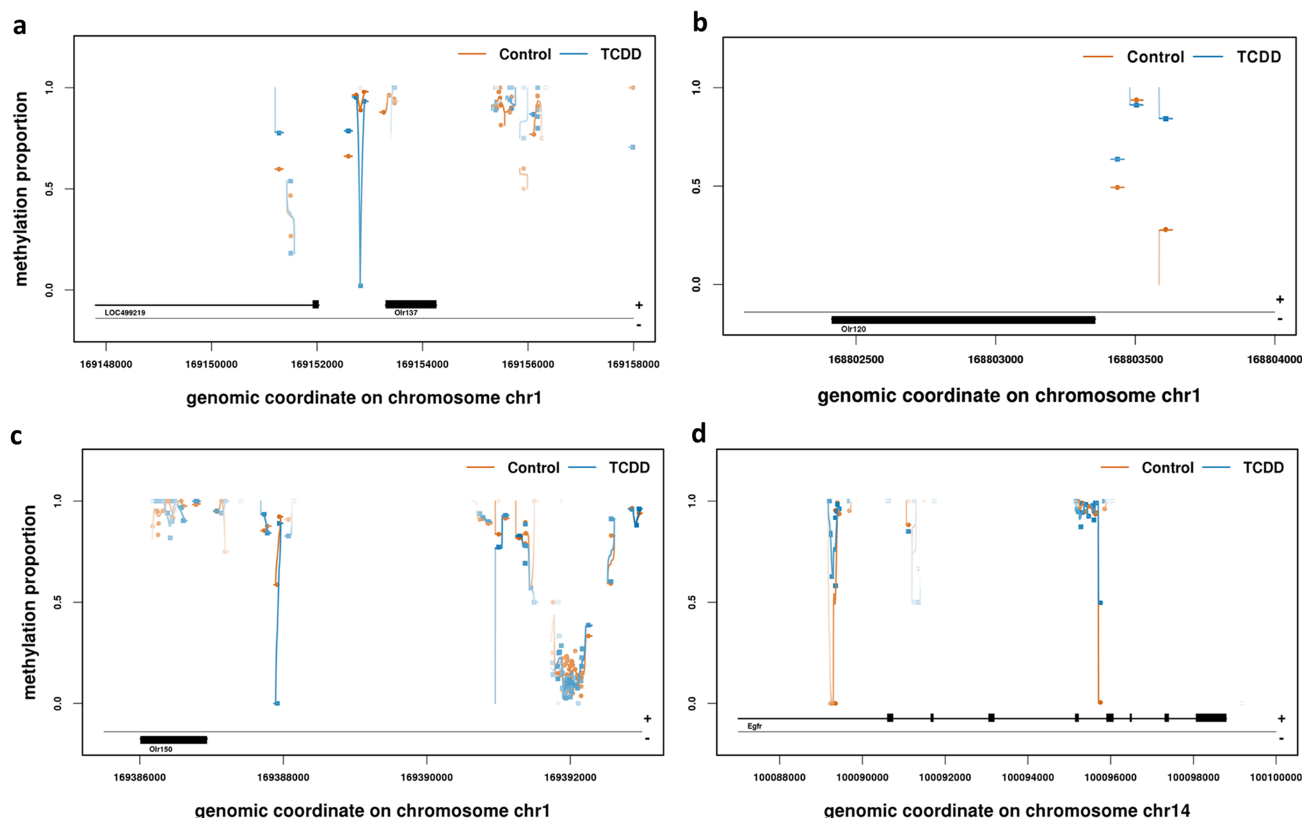

Fig. 3 Signal receptor genes are often differentially methylated. DMRs were identified adjacent to four olfactory receptor genes. DMRs were identified **a** upstream of *Olr137*, and downstream of **b** *Olr120* and **c** *Olr150*. **d** Hyper-methylation within the gene-body of *Egfr* among the TCDD lineage rat testes. Points (and lines) represent group (colour) and coverage (intensity) for each CG site, with position indicating genomic position (*x*-axis) and proportion of reads demonstrating methylation (*y*-axis) (colour figure online)

genes demonstrating hyper-methylation are frequently involved in biosynthetic processes (including 154 unique genes; FDR = 0.07), cell–cell adhesion molecules ($n = 18$ genes including many protocadherins; FDR = 0.002) and cell–cell signalling ($n = 56$ genes; FDR = 0.01). We also detected enrichment of voltage-gated potassium channel activity by a small subset of these hyper-methylated genes ($n = 10$; FDR = 0.06). As hyper-methylation can lead to reduced mRNA levels, these pathways may all have impaired functionality; this may explain some of the physiological effects observed by Manikkam et al. (2012a; b). Interestingly, many of these hyper-methylated genes produce proteins that are enriched within intracellular, membrane-bound organelles (particularly mitochondria; Fig. 4b), suggesting a long-lasting heritable impact on cellular respiration and perhaps ROS homeostasis. Concurrently, no significant pathway enrichment was observed for genes affected by hypo-methylation (Supplementary Table 6).

Discussion

The degree of hepatotoxicity observed following exposure to TCDD differs dramatically between TCDD-sensitive and TCDD-resistant strains of rat (Niittynen et al. 2007; Viluksela et al. 2000) and is further evidenced by the myriad transcriptomic changes observed within the liver between these animals (Boutros et al. 2008; Yao et al. 2012). The liver is the major site of accumulation of TCDD in rats at doses that result in induction of xenobiotic-metabolizing enzymes and this accumulation persists for a considerable duration after exposure (Pohjanvirta et al. 1990). Previous studies on physiological effects of transgenerational exposure to TCDD did not disclose any hepatic toxicities (Manikkam et al. 2012a, b); however it seemed reasonable to assess the transcriptomic effects of transgenerational exposure within this tissue. Here, few transcriptional changes were detected in livers of male

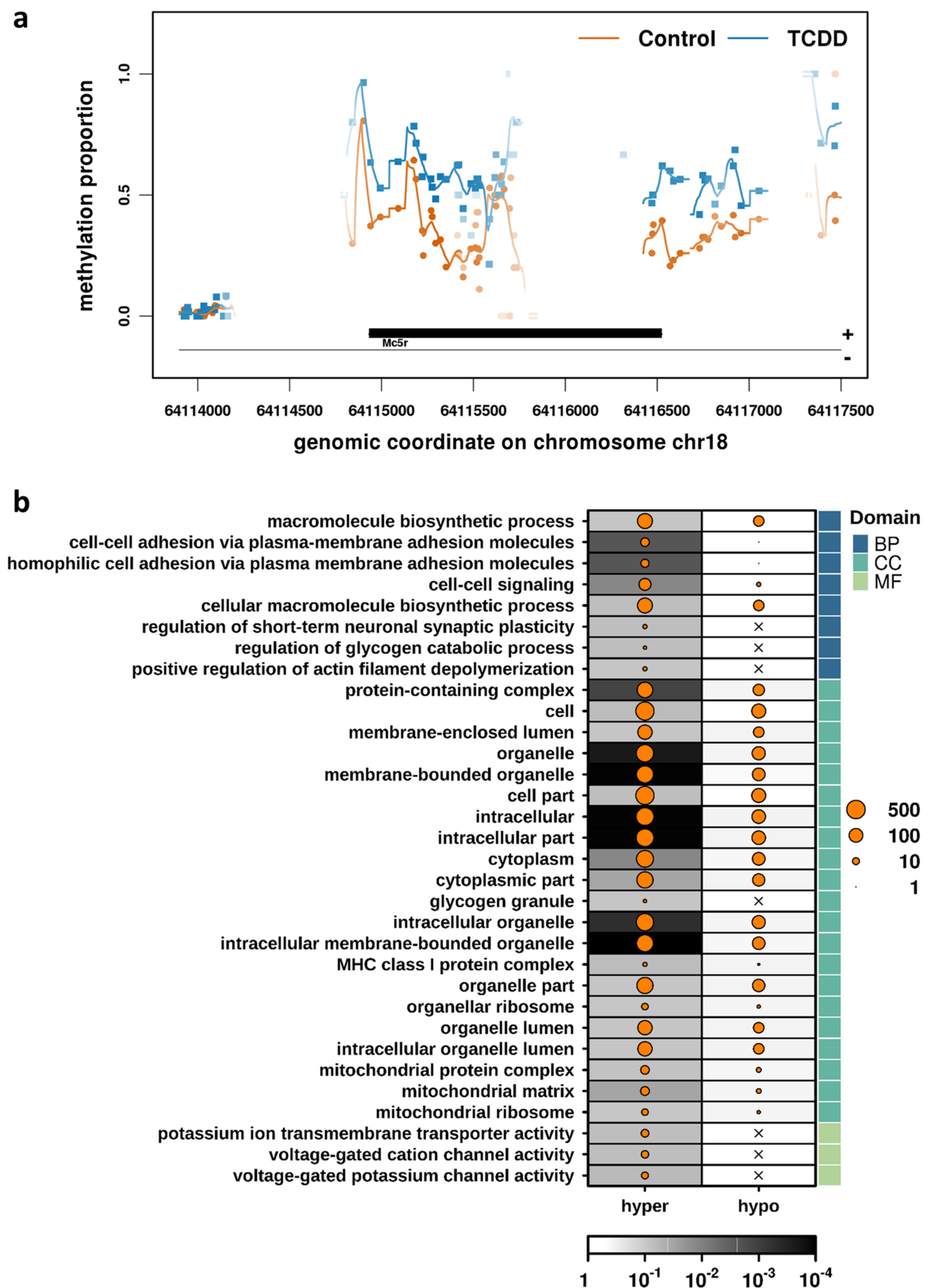

Fig. 4 Gene-body differential (hyper) methylation. **a** *Mc5r* demonstrated differential (hyper) methylation in testis tissue obtained from the TCDD-exposed lineage relative to control samples. Points (and lines) represent group (colour) and coverage (intensity) for each CG site, with position indicating genomic position (*x*-axis) and proportion of reads demonstrating methylation (*y*-axis). **b** Pathway analyses were performed to identify areas of enrichment for hyper- or hypo- methylation. Dot size indicates number of hyper- or hypo- methylated genes overlapping the indicated pathway while background shading indicates statistical significance (FDR). Represented domains include biological processes (BP), cellular components (CC) and molecular function (MF) (colour figure online)

offspring of the F3 generation. In contrast, a number of changes were observed in female rat liver, possibly demonstrating an increased sensitivity, as female rats have been previously shown to be more sensitive to TCDD-induced toxicities than their male counterparts (Pohjanvirta et al. 1993; Silkworth et al. 2008). The heightened response occurring here at such a low dose, and not the higher doses, is intriguing; however, these effects were relatively weak with low statistical significance. Despite this, the alleged impacts of low-dose exposure to endocrine disruptors (such as TCDD) are known to exist (reviewed in Vandenberg et al. 2012). Tuomisto et al. observed an inverse relationship between risk for soft-tissue sarcomas and dioxin exposure (Tuomisto et al. 2004). Since exposure to high levels of dioxin has long been linked to tumour development, they proposed a hormesis-like effect for dioxin: tumorigenic at both high and low doses (Tuomisto et al. 2006). It is possible that a similar pattern would occur at the molecular level, with a large effect induced by both extremely high and low dose. Therefore,

our findings warrant further study. Similarly, as previous studies have revealed an increased rate of kidney disease and pubertal and reproductive abnormalities within ancestrally-exposed animals (Manikkam et al. 2012a, b; Nilsson et al. 2012; Yu et al. 2019), transcriptomic analyses of these tissues may produce more exciting results.

Along these lines, and consistent with these previous studies (Manikkam et al. 2012b), targeted bisulfite sequencing was performed on testicular tissue from F3 rats. Despite the absence of physiological changes in testes from previous studies (Manikkam et al. 2012a), we detected numerous DMRs within this tissue. In our study, a conspicuous finding was that olfactory receptors seemed to be frequently affected by differential methylation; this in itself is quite interesting as a highly sensitive behavioural response to AHR agonists is aversion to novel foodstuffs (Lensu et al. 2011a; Mahiout and Pohjanvirta 2016), and this may involve the sense of smell. Differential methylation of *Olr60* was also reported by Manikkam et al., albeit in a different tissue type (sperm rather than testis) (Manikkam et al. 2012b). Similarly, both studies identified DMRs within regulatory subunits of protein phosphatase 1 (*Ppp1r27* in testis and *Ppp1r14a* in sperm) (Manikkam et al. 2012b); however, these are typically expressed in different tissue types (muscle and lung respectively) (Yu et al. 2014).

In conclusion, a conspicuous sex-based difference was identified in hepatic transcriptomes of unexposed F3 rats derived from TCDD-treated F0 dams through paternal germ line. In female rats, numerous genes exhibited altered expression at the lowest dose tested (30 ng/kg), whereas there were no significant changes at any dose level in male rats. Hyper- and hypo-methylated regions both inside and outside of the gene coding regions were detected in the testes of the TCDD lineage, with genes demonstrating hypermethylation being enriched in pathways of biosynthetic processes, cell adhesion and cell–cell signalling. These findings warrant further transgenerational studies focusing on other tissues, especially in female rats after low TCDD doses.

Methods

Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from the UFA Oil Institute (Ufa, Russia) and was determined to be over 99% pure as analyzed by gas chromatography-mass spectrometry. TCDD was dissolved in corn oil (Sigma Chemicals, St. Louis, MO) and thoroughly mixed using a magnetic stirrer. Solutions were ultra-sonicated for 20 min prior to dosing.

Animal handling

Outbred male and female Sprague–Dawley rats were obtained from Harlan Netherlands (Zeist, The Netherlands). Throughout the study, animals were subjected to regular health surveys to ensure animals were free of typical rodent pathogens (Rehbinder et al. 1996). Animals were acclimatized to the experimental conditions for one week prior to commencing the study. Rats were mated overnight and pregnancy day 0 assigned when the presence of sperm in a vaginal smear was confirmed. On gestational day 11, groups of pregnant females ($n = 6–8$) were treated with a single dose of TCDD (0, 30, 100, 300 and 1000 ng/kg bodyweight) dissolved in corn oil by oral gavage. Litter sizes were adjusted to 4 males and 4 females on postnatal day 1 whenever possible.

Upon weaning, same-sex littermates were housed in stainless steel, wire-mesh bottom cages with pelleted rat feed (R36, Lactamin, Stockholm, Sweden) and tap water available ad libitum. The housing environment was maintained at 21 ± 0.2 °C and relative humidity at $50 \pm 3\%$, with a 12/12 h artificial light/dark cycle. Adult males were mated with untreated female rats to achieve F2 generations. The above procedure was repeated to further achieve F3 generations. At the end of the examination period, rats were euthanized by carbon dioxide exposure and subjected to tissue sampling. Hepatic and testicular tissue was shipped on dry ice to the analytical laboratory for processing. The study protocols were approved by the Finnish National Animal Experiment Board (Eläinkoelautakunta, ELLA; permit code: ESLH-2008–07,223/Ym-23) and all animal handling and reporting comply with ARRIVE guidelines (Kilkenny et al. 2010).

Microarray hybridization

A total of 67 animals from the F3 generation were examined (Table 1; Supplementary Table 1). Total hepatic RNA was isolated for analysis on microarrays. Briefly, 20 mg hepatic tissue was ground to a powder in liquid nitrogen using a mortar and pestle, followed by rapid homogenization using a Brinkmann Polytron (Polytron PT1600E with a PT-DA 1607 generator). RNA was extracted using an RNeasy Mini Plus Kit following the manufacturer's recommended protocol (Qiagen, Mississauga, Canada). Total RNA was quantified using a NanoDrop UV Spectrophotometer (Thermo Scientific, Mississauga, ON) and RNA quality was verified by electrophoresis using RNA 6000 Nano kits on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples demonstrated RNA integrity number (RIN) greater than 8. Aliquots of RNA were transported to The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children (Toronto, Canada) and assayed on

Affymetrix GeneChip Rat Gene 2.0 ST arrays using the manufacturer's protocols.

Statistical analysis of hepatic transcriptome

Raw microarray data (CEL files) were loaded into the R statistical environment (v3.4.0) using the affy package (v1.48.0) of the BioConductor library (Gentleman et al. 2004). All samples were processed together using the RMA algorithm (Irizarry et al. 2003). Mapping of probes to Entrez Gene IDs was performed using the custom cdf ragene20strnentrezgcdf (v21.0.0) package for R (Dai et al. 2005). Distributional and spatial homogeneity was assessed (Supplementary Figure 1) and a single female animal was identified as an outlier. This sample was removed and the remaining arrays were re-processed as described above (Supplementary Figure 2). Filtering was performed using a threshold determined by examining the normalized intensity levels of genes on chromosome Y within the female samples (removed 3007 probesets; Supplementary Figure 3a). Linear modelling was performed to identify those transcripts with altered abundance in rats from TCDD-exposed lineages relative to rats from control lineages. Models were applied to determine coefficients based on the interaction between the sex and dose variables, with familial lineage included as a blocking variable. Contrasts were fit to compare each TCDD-treated lineage with controls. The standard error of the coefficients was adjusted using an empirical Bayes moderation of the standard error (Smyth 2004) and model-based *t*-tests were applied to determine whether a coefficient was significantly different from zero. A false-discovery rate (FDR) adjustment for multiple testing was applied (Storey and Tibshirani 2003). Linear modelling and subsequent adjustments were performed using the limma (v3.32.5) package for R. For downstream analyses, a significance threshold of $p_{adj} < 0.1$ was used to define genes with significantly altered mRNA abundance (Supplementary Figure 3c). Alternate models were applied as above to identify differentially abundant transcripts based on sex and TCDD (alone or in combination; Supplementary Figure 3e). Visualizations were generated using the BPG package (v5.7.6) (P'ng et al. 2017), with the lattice (v0.20-35) and latticeExtra (v0.6-28) packages for R. Results were contrasted with previously published datasets using the TCDD.Transcriptomics (Prokopec et al. 2017) package for R.

Bisulfite sequencing

Library preparation, quality control, target capture and sequencing was performed at the Biomedicum Functional Genomics Unit (FuGU) at the University of Helsinki. Target capture was carried out using the Agilent SureSelect XT Rat Methyl-Seq kit. Sequencing was performed using Illumina NextSeq 500 with the NextSeq 500/550 v2 kit (High Output) to produce single-end directional reads (75 bp read length).

Alignment and data processing

Raw sequence data were aligned with Bismark (v0.15.0) (Krueger and Andrews 2011), using Bowtie2 (v2.2.6), to the *rattus norvegicus* (rn6) reference genome. Bismark was run using default settings, using single-end, unidirectional fastq files. Aligned files were coordinate sorted using samtools (v1.3) and library-level merged with duplicate reads marked using picard tools (v1.141). Finally, Bismark's methylation extractor tool, with –comprehensive, –merge_non_CpG, –cytosine_report, –CX, –split_by_chromosome, and –bedGraph settings, was used to extract the number of methylated and unmethylated reads at each CpG position in each sample.

The resulting data files for each sample were loaded into the R statistical environment (v3.3.1) and pooled across treatment groups using the DMRcaller package (v1.6.0) on a per-chromosome basis (due to computational intensity). Target regions were first converted to rn6 coordinates from rn4 using liftover (v20111127) and target coverage was estimated across these regions for each chromosome using the compute Methylation Profile function for each CX (CG, CHG, CHH) context. The overall methylation profiles for each group were visualized using the DMRcaller function plot Methylation Profile from data using a window size of 1000 bp for each CX context (Supplementary Figures 4–5).

Differentially methylated regions (DMRs) were identified using a chromosome-wide or gene-wise approach. Gene-wise DMRs were identified using the filterDMRs function in DMRcaller, for each chromosome independently. Specifically, regions evaluated were restricted to genes (Rnor 6.0.88) that had some overlap with the Methyl-Seq target regions (following liftOver conversion from rn4 to rn6 coordinates). Two chromosomes (chrY and chrM) had no targeted regions, therefore all genes on these chromosomes were evaluated. Default methods (noise filter method with a window size of 100 bp with a triangular kernel function) were used, with the score test for statistical significance, followed by (default) adjustment for multiple testing using the Benjamini and Hochberg method. Genes were examined if it contained at least 1 CG site, with minimum of 10 reads per treatment group and a minimum difference in methylation of 1% (the proportion of reads with a methylated CG differed between groups by at least 1%). DMRs less than 200 bp apart were then merged, with respect given to direction of change. Statistically significant DMRs ($p_{adj} < 0.01$) were then visualized using the plot Local Methylation Profile function, using a region ± 5 Kbp around the DMR. Again, default methods were used, however with a window size of 1000 for smoothing. Similarly, chromosome-wide DMRs were identified using the compute

DMRs function, for each chromosome independently. Default methods (noise filter method with a window size of 100 bp with a triangular kernel function) were used, again with the score test for statistical significance and correction for multiple testing as above. Statistically significant DMRs ($p_{adj} < 0.001$; DMRs with gene overlap within 1000 bp up/downstream of the DMR) were then visualized as above.

Pathway analysis was performed using those genes with either increased or decreased methylation in the TCDD-exposed lineage relative to the control lineage (up- and down-methylated genes analysed separately). Pathway analysis was performed using gProfileR (v0.6.1) for R, using the *rnorvegicus* 'GO' database, with a minimum overlap of 1 gene, and application of false discovery rate adjustment for multiple testing.

Global DNA methylation status determination

Global DNA methylation status was determined in the liver and testis of F3 generation rats by colorimetric MethylFlash™ Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions.

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Author contributions Sample preparation: MV, HMM. Performed statistical and bioinformatics analyses: SDP. Wrote the first draft of the manuscript: SDP. Initiated the project: MV, RP, PCB. Supervised research: MV, RP, PCB. Generated tools and reagents: SDP. Approved the manuscript: all authors.

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Data availability All data have been deposited in the Gene Expression Omnibus (GSE126216) and are publically available. Raw, normalized, and modelled data are also available in the TCDD Transcriptomics package for R (v2.2.5).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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