



Transcriptional profiling of rat hypothalamus response to 2,3,7,8-tetrachlorodibenzo- ρ -dioxin

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ABSTRACT

In some mammals, halogenated aromatic hydrocarbon (HAH) exposure causes wasting syndrome, defined as significant weight loss associated with lethal outcomes. The most potent HAH in causing wasting is 2,3,7,8-tetrachlorodibenzo- ρ -dioxin (TCDD), which exerts its toxic effects through the aryl hydrocarbon receptor (AHR). Since TCDD toxicity is thought to predominantly arise from dysregulation of AHR-transcribed genes, it was hypothesized that wasting syndrome is a result of TCDD-induced dysregulation of genes involved in regulation of food-intake. As the hypothalamus is the central nervous systems' regulatory center for food-intake and energy balance. Therefore, mRNA abundances in hypothalamic tissue from two rat strains with widely differing sensitivities to TCDD-induced wasting syndrome: TCDD-sensitive Long-Evans rats and TCDD-resistant Han/Wistar rats, 23 h after exposure to TCDD (100 μ g/kg) or corn oil vehicle. TCDD exposure caused minimal transcriptional dysregulation in the hypothalamus, with only 6 genes significantly altered in Long-Evans rats and 15 genes in Han/Wistar rats. Two of the most dysregulated genes were *Cyp1a1* and *Nqo1*, which are induced by TCDD across a wide range of tissues and are considered sensitive markers of TCDD exposure. The minimal response of the hypothalamic transcriptome to a lethal dose of TCDD at an early time-point suggests that the hypothalamus is not the predominant site of initial events leading to hypophagia and associated wasting. TCDD may affect feeding behaviour via events upstream or downstream of the hypothalamus, and further work is required to evaluate this at the level of individual hypothalamic nuclei and subregions.

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1. Introduction

Halogenated aromatic hydrocarbons (HAHs) are a class of toxic compounds widely present within the environment as a result of plastics incineration, electronics recycling, pesticide application and paper bleaching (Linden et al., 2010; Okey 2007).

2,3,7,8-tetrachlorodibenzo- ρ -dioxin (TCDD) is the best-studied HAH, and exposure to it has been linked to teratogenesis, immunosuppression, acute lethality and many other toxicities (Mimura and Fujii-Kuriyama 2003; Pohjanvirta and Tuomisto 1994). Perhaps most significantly in many mammalian models, TCDD causes wasting syndrome—a potentially fatal dose-dependent reduction in body-weight (Linden et al., 2014; Seefeld et al., 1984). Many laboratory animals exposed to TCDD experience hypophagia and appear to defend a lowered body weight set point (Linden et al., 2010; Seefeld et al., 1984). The role of wasting in TCDD-induced lethality remains obscure: dietary interventions such as force feeding and high-energy diets reduce or prevent weight loss, but have minimal impact on mortality (Seefeld et al., 1984; Tuomisto et al., 1999).

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TCDD toxicity is mediated by the aryl hydrocarbon receptor (AHR), a ligand-responsive transcription factor involved in regulation of cytochrome P450 proteins involved in the phase I xenobiotic metabolism pathway (Mimura and Fujii-Kuriyama 2003; Okey 2007). The AHR is a member of the PAS/basic helix-loop-helix (bHLH) superfamily, with a bHLH domain and two PAS domains within its N-terminus (Dolwick et al., 1993) and a transactivation domain at its C-terminus (Ko et al., 1997). In the absence of ligand, the AHR is cytoplasmic, where it forms a complex with stabilizing chaperone proteins hsp90, ARA9/XAP2 and p23 (Petrulis and Perdew 2002). Activation occurs following ligand binding to the PAS-B domain, which promotes translocation to the nucleus. The AHR then dimerizes with the AHR nuclear translocator (ARNT) (Reyes et al., 1992), and this dimer binds to DNA and regulates transcription. Many lines of evidence indicate that major toxicities from TCDD are caused by dysregulation of AHR target genes (reviewed in Okey 2007).

The hypothalamus is central to regulation of food intake and energy balance, mainly through small peptide signalling molecules. The hypothalamic arcuate nucleus (ARC) translates adipose level signals from peptides (insulin and leptin) to the rest of the CNS by releasing neuropeptides and hormones (Konner et al., 2009). Depending on the stimulus, these peptides may be orexigenic (e.g. agouti-related peptide (AgRP) and neuropeptide Y (NPY)) or anorexigenic (e.g. α -melanocortin-stimulating hormone (α -MSH) and cocaine-and-amphetamine regulated transcript (CART)). Lesions in hypothalamic nuclei that prevent normal responses to signalling peptides can cause either hypophagia or hyperphagia depending on lesion location (Elmqvist et al., 1999; Wang et al., 2013). As such, many groups have hypothesized that TCDD-mediated changes in the hypothalamus underlie the observed wasting syndrome. A previous study discovered a non-additive interaction between ventromedial hypothalamic lesions and TCDD on body weight, suggestive of the involvement of a hypothalamic feed-intake regulation pathway in the wasting syndrome (Tuomisto et al., 1995). TCDD has been previously shown to modulate mRNA abundances of numerous neuropeptides and receptors, particularly orexigenic peptides, in both directions (Linden et al., 2005). Additionally, several components of the AHR-signalling pathway are expressed in the hypothalamus, with three AHR-regulated genes (*Ahr*, *Cyp1a1* and *Cyp1a2*) significantly up-regulated in response to TCDD (Korkalainen et al., 2005).

To evaluate the role of TCDD-induced transcriptomic changes in the hypothalamus on observed toxicities, two rat strains that markedly differ in susceptibility to TCDD-induced toxicities were evaluated: TCDD-sensitive Long–Evans (Turku/AB) rats (L–E; LD₅₀ ~20 μ g/kg for male rats) and TCDD-resistant Han/Wistar (Kuopio) rats (H/W; LD₅₀ >9600 μ g/kg) (Pohjanvirta et al., 1995; Unkila et al., 1994). The great resistance of H/W rats to toxicity is due to a point mutation in the intron/exon boundary that causes alternative splicing (Moffat et al., 2007; Pohjanvirta et al., 1998). This leads to variation within the transactivation domain of AHR and alters some responses to TCDD such as resisting lethality (Moffat et al., 2007; Pohjanvirta et al., 1998). Wasting syndrome is another example of this altered response; a mitigated variety of wasting manifests in H/W rats but only after >100-fold higher doses of TCDD than is required in L–E rats (Linden et al., 2010; Pohjanvirta et al., 1998). The use of H/W rats allows for identification of transcriptomic responses that differ from those in susceptible strains (Boutros et al., 2011; Franc et al., 2008). Such differences may identify the key genes whose dysregulation underlies pathogenesis from TCDD exposure.

2. Methods

2.1. Samples

Sixteen male rats, eight L–E and eight H/W, were examined. Rats were housed singly in stainless-steel wire-mesh cages and subjected to light and dark cycles lasting 12 h each, with lights on from 07:00 to 19:00. Animals were fed pelleted R36 feed (Lactamin, Södertälje, Sweden) and provided with tap water. The temperature within the housing environment was 21 ± 1 °C with relative humidity at $50\% \pm 10\%$. H/W rats were 15–16 weeks of age upon treatment while L–E rats were 16–22 weeks of age (to compensate for the more rapid growth of H/W rats).

2.2. Animal handling

Four rats from each strain were treated with 100 μ g/kg of TCDD dissolved in corn oil vehicle while the remaining four were treated only with corn oil which served as the vehicle control. Animals were distributed such that each group was similarly body weight matched prior to treatment. Treatments were administered by oral gavage. Towards the end of the daily dark phase (between 5:40 and 6:45 a.m.), 23 h post exposure, all rats were euthanized by decapitation. Hypothalamus (incision sites: rostral border of the optic chiasm, caudal border of the mamillary body, ventral border of the anterior commissure and lateral borders of the tuber cinereum and mamillary body complexes) was rapidly removed and snap-frozen in liquid nitrogen. Tissues were stored at -80 °C or lower until processed. All study plans were approved by the Animal Experiment Committee of the University of Kuopio and the Provincial Government of Eastern Finland. All animal handling and reporting comply with ARRIVE guidelines (Kilkenny et al., 2010).

2.3. Sample processing and microarray hybridization

Total mRNA was extracted using Qiagen RNeasy Lipid Tissue Mini kits according to manufacturer's instruction (Qiagen, Mississauga, Canada). Total RNA yield was quantified by UV spectrophotometry and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA from individual rats was hybridized to Affymetrix RAE230-2 arrays at The Centre for Applied Genomics (Toronto, Canada) and RNA abundances were quantified using an Affymetrix GeneChip Scanner 3000.

2.4. Data preparation

Raw microarray data were loaded into the R statistical environment (v3.1.0) using the affy package (v1.42.2) of the BioConductor library and the EntrezGene ID map rat2302rnen-trezgcd (v18.0.0) (Dai et al., 2005; Gautier et al., 2004). Raw data were pre-processed using the RMA algorithm (Irizarry et al., 2003) and quality-control plots were generated using the affy (v1.42.2), lattice (v0.20-29) and latticeExtra (v0.6-26) packages to assess sample homogeneity (Supplementary Fig. S1). Unsupervised pattern recognition used the DIANA agglomerative hierarchical clustering algorithm, as implemented in the cluster package (v1.15.2) and Pearson's correlation was used as a similarity metric. The distribution of the coefficient of variations for each gene was analyzed to ensure low inter-replicate variance. All raw and pre-processed microarray data are available in the GEO repository (accession: GSE61039).

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2014.12.016>.

2.5. Statistical analysis

A linear model was fit to each gene using contrasts to relate TCDD treatment and vehicle controls for each rat strain (i.e. HWT–HWC and LET–LEC). The variance amongst probes was reduced using an empirical Bayes method (Smyth 2004). Moderated *t*-tests compared each coefficient to zero, while F-tests were applied gene-wise to test for significant differences in variation between the two rat strains. All *p*-values were false discovery-rate adjusted for multiple testing to generate *q*-values (Storey and Tibshirani 2003). Linear modeling and hypothesis testing were completed using the limma package (v3.20.4). Genes were analyzed at multiple *q*-value thresholds to ensure results were threshold-independent, but for primary analyses a threshold $q < 0.05$ was used.

2.6. Data visualization

Inter-strain *p*-value variability was quantified and plotted as above. Inter-strain *p*-value variability plots compared the *p*-value

distribution between the two strains when mRNA abundance was both up- and down-regulated. A Venn diagram, created using the VennDiagram R package (v1.6.7), depicted the number of significantly responsive genes in both rat strains at $q < 0.05$ (Chen and Boutros 2011). A heatmap gave a visual representation of the change in gene expression, up- versus down-regulation, for the most variable genes (variance > 0.05 across all samples). Data was mean centred and scaled using the standard deviation for each variable and clustered as described above. Dotmaps were used to depict the magnitude of change in \log_2 -space (M) and significance of TCDD-induced dysregulation for different subsets of genes. Covariates were used to indicate significance of selected genes in other tissues and species (rat liver (Yao et al., 2012) and in mouse tissues (Boutros et al., 2009)).

2.7. Pathway analysis of TCDD-responsive genes

Functional pathways analysis was performed with the GOMiner software (v. 2011–01) (Zeeberg et al., 2003). Genes found to be significantly responsive in either strain examined (q -value < 0.1)

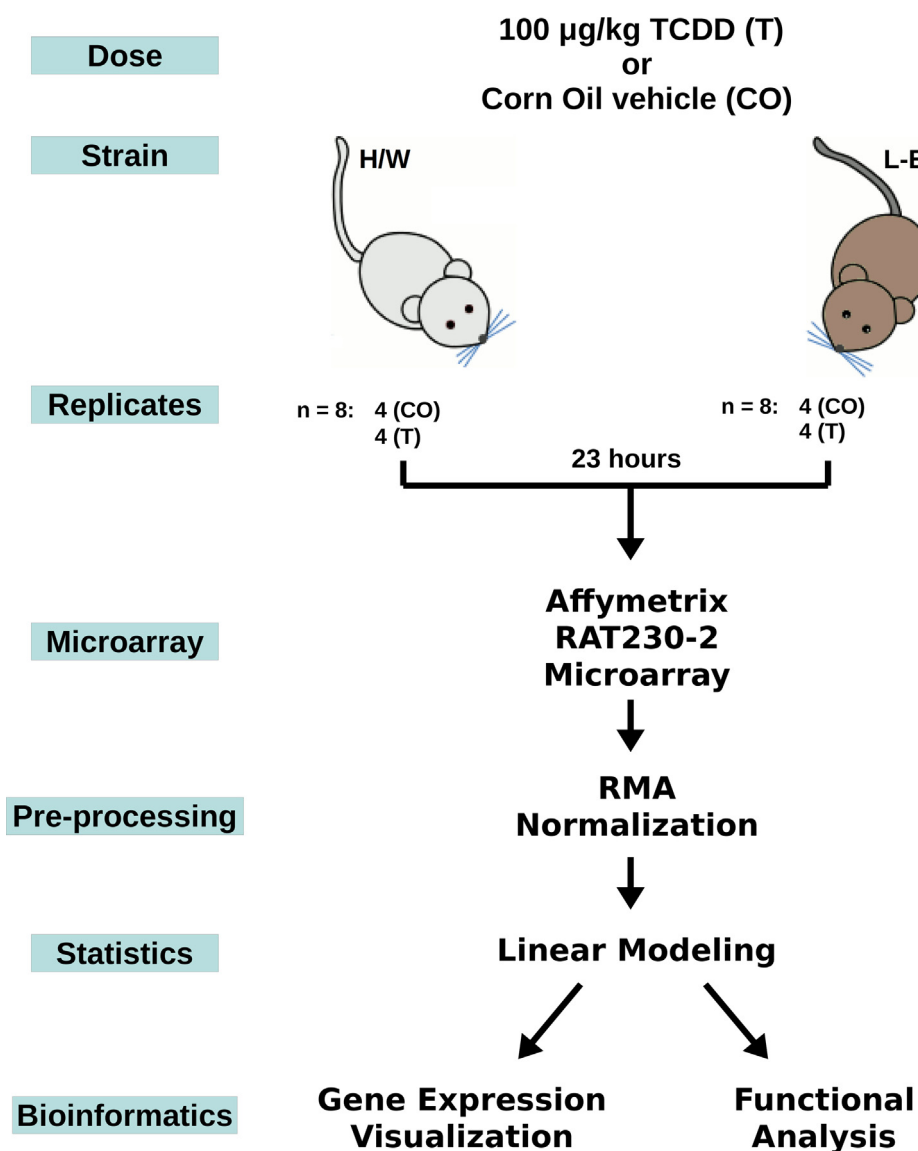


Fig. 1. Experimental design.

Han/Wistar (H/W) and Long-Evans (L-E) rats were evaluated 23 h after treatment with either 100 µg/kg of TCDD (T) or corn oil vehicle (CO). mRNA abundances were measured on Affymetrix Rat Genome 230 2.0 arrays and RMA-normalized prior to downstream analysis.

were analyzed against a list of all genes on the array. A false-discovery rate threshold of 0.1 was applied, with all look-up options and gene ontologies selected and 1000 randomizations used. A minimum category size of five was required to reduce multiple-testing.

2.8. Transcription factor binding site analysis

To further explore the functionality of gene regulation by TCDD in the hypothalamus, we examined each gene for the AHR-associated conserved transcription-factor binding sites: AHRE-I (core), AHRE-I (full) and AHRE-II. These sites contain the sequences GCGTG, [T]G[NGCGTG[A]C][G]C[A and CATG{N6}C[T]A TG, respectively (Denison and Whitlock 1995; Sogawa et al., 2004). Transcription start sites were determined using REFLINK and REFFLAT tables from UCSC genome browser, downloaded on May 9, 2012 (Karolchik et al., 2003). The number of each motif present in each gene was counted and a PhyloHMM conservation score was calculated, ranging from zero to one (Siepel and Haussler 2004). This score measured conservation across species with a score a

zero reflecting minimal conservation and a score of one reflecting complete conservation.

2.9. Validation

A subset of 50 transcripts including the AHR-core and candidate genes (as identified above) was validated using the NanoString system. Hypothalamic RNA (≥ 100 ng) was shipped on dry ice to the Princess Margaret Genomics Centre (Toronto, ON) for analysis. The target gene list was submitted in advance and the required CodeSet (multiplexed set of endogenous and control probes) was developed by NanoString.

Raw data (RCC files consisting of direct molecule counts) were received and normalization performed prior to analysis. Data were read into the R statistical environment (v3.1.2) and normalization performed using the NanoStringNorm package (v1.1.18) (Waggott et al., 2012). Endogenous probes were normalized to the positive control counts using the 'sum' method and to housekeeping genes counts using the 'housekeeping.geo.mean' method in NanoStringNorm. Housekeeping genes (*Hprt1*, *Pgk1* and *Sdha*) had been

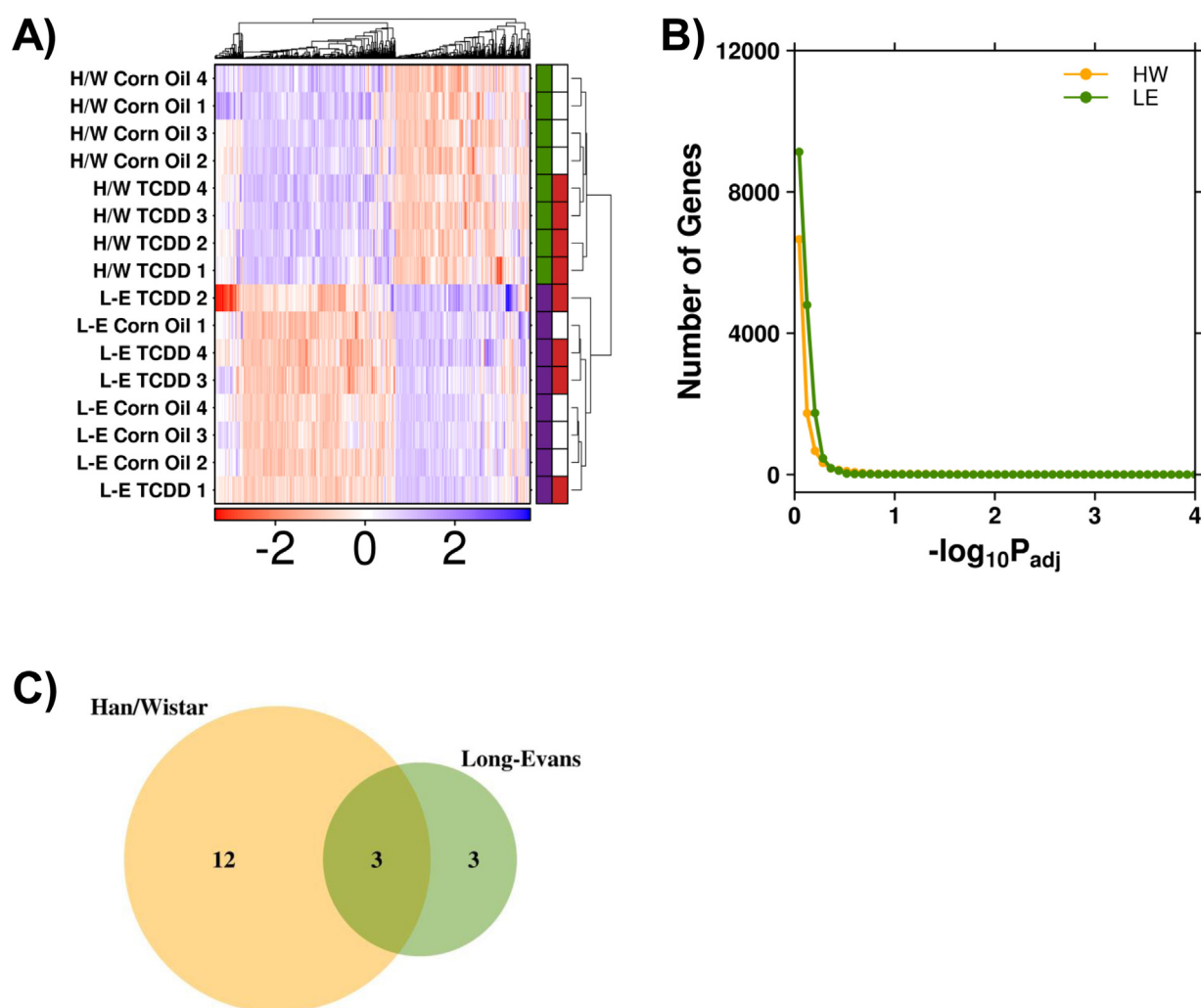


Fig. 2. Strain variability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(A) Transcriptomic expression profiles of a subset of genes with the greatest variance (variance >0.05) across the study were subjected to DIANA agglomerative hierarchical clustering. Covariates specify either rat strain (H/W and L-E; green and purple, respectively) or treatment status (TCDD or corn oil control; red or white, respectively). Clustering indicates greater variability in expression profiles between rat strains than between treatment groups. (B) An analysis of the number of genes significantly altered by TCDD at various q -value thresholds demonstrate a minimal number of significantly altered genes in either strain (both up- and down-regulated). (C) Overlap of significantly altered genes with a 0.05 q -value threshold. Three genes were significantly altered in only L-E rats while 12 genes showed significantly altered expression in only H/W rats. Three genes were found to be significantly differentially expressed in both rat strains.

validated previously (Pohjanvirta et al., 2006) for use in TCDD studies of rat hypothalamus. Similarly, this normalization method had been previously identified as the most accurate (in comparison to qPCR) for use in a similar study with rat hepatic tissue (Prokopec et al., 2013). Normalized data was log₂-transformed and linear modelling and visualizations performed as above [R packages: limma (v3.20.9), lattice (v0.20–29), latticeExtra (v0.6–26)].

3. Results

3.1. Experimental design

Since the hypothalamus is associated with the regulation of food intake and metabolism, the effects of TCDD exposure on the hypothalamic transcriptome were analyzed. Two rat strains were examined: TCDD-sensitive L/E rats and TCDD-resistant H/W rats. Animals were treated with 100 µg/kg TCDD or corn oil vehicle. In L–E rats this dose of leads to an irreversible hypophagia and body weight loss, with food intake plummeting from approximately 20 g/day to 1–2 g/day. this rapid decrease in food intake occurred within 5 days of TCDD exposure and thereafter persisted at that level. In contrast in H/W rats, food intake diminishes from slightly above 20 to about 10 g/day 6 days post-treatment, followed by a rapid recovery to near control levels after 14 days (Lensu et al., 2011a). In L–E rats, the reduction in food consumption reaches statistical significance by 24–48 h (Lensu et al., 2011a). Therefore, hypothalamus tissue was isolated 23 h post exposure to identify primary transcriptional changes preceding hypophagia and body weight loss. Moreover, tissues were collected near the end of the darkness period because this phase coincides with one of the two feeding peaks of L–E rats (Lensu et al., 2011b): we thus aimed to optimize the probability of detecting critical alterations. In contrast, at this time of day the circadian feeding rhythm of H/W rats is approaching its nadir (Lensu et al., 2011b). The experimental approach is summarized in Fig. 1. Animals and arrays used are listed in Supplementary Table 1.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2014.12.016>.

3.2. Response to TCDD Exposure

Normalized microarray data demonstrated very similar mRNA abundances, with hierarchical clustering recognizing greater variability between rat strains than between samples treated with TCDD or corn oil (Fig. 2A). This result suggests that in hypothalamus, unlike liver (Yao et al., 2012), inter-animal variability is larger than the effects of TCDD exposure. Further, the distribution of gene-wise coefficients of variation (CV=ratio of the standard-deviation to mean) shows a strong peak around 0.10 (Supplementary Fig. S2) for each treatment group. There was no evidence of differential variance between the rat strains, with only 15/12,503 genes showing differences (*F*-test; *q* < 0.05). Taken together, these results demonstrate minimal transcriptomic differences between replicates and minimal transcriptomic alterations as a result of TCDD exposure.

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A total of 12,503 individual genes were evaluated (Supplementary Table S2). Surprisingly, a larger number of genes were dysregulated in TCDD-resistant H/W rats than in TCDD-sensitive L–E rats, independent of statistical threshold applied (Fig. 2B). At a standard threshold of *q* < 0.05, we detected 15 genes dysregulated by TCDD in H/W rats and 6 in L–E rats. Only 3 genes, *Cyp1a1*, *Nqo1* and *Stab1*, were altered in both strains (Fig. 2C).

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3.3. AHR-core gene responses

A set of nine genes that are altered by TCDD in an AHR-dependent fashion across a broad range of tissues, species, doses and time-points were assembled (Boutros et al., 2008; Nebert et al., 1993, 2000; Watson et al., 2014; Yeager et al., 2009). These genes (*Ahr*, *Aldh3a1*, *Cyp1a1*, *Cyp1a2*, *Fmo1*, *Inmt*, *Nfe2l2*, *Nqo1* and *Tiparp*) provide a measure of the sensitivity of the hypothalamus to transcriptional dysregulation by TCDD, as their effects in other tissues have been well documented. Of these, *Cyp1a1* was significantly up-regulated in both species (3.9-fold in H/W and 3.6-fold in L–E), as was *Nqo1*, though to a lesser extent (0.8-fold in H/W rats and 0.7-fold in L–E). The remaining genes did not show TCDD-induced alteration, despite *Cyp1a2* having previously shown to be upregulated by TCDD in the hypothalamus (Korkalainen et al., 2005). Note that hepatic studies in rats involving similar conditions (100 µg/kg of TCDD, 19 h post-exposure) in both L–E and H/W rats (Yao et al., 2012) demonstrated significantly more TCDD-mediated changes in this subset of genes (Fig. 3A). Similarly, murine studies of liver and kidney tissue following comparable treatment conditions (1000 µg/kg of TCDD, 19 h) (Boutros et al., 2009), showed considerably more TCDD-mediated transcriptional changes (Fig. 3A).

3.4. Non-core gene responses

A subset of genes determined to be significantly altered by TCDD in either strain was identified and further examined. AHR-core genes were excluded from this subset, resulting in 16 genes. These genes exhibited only moderate magnitude dysregulation following TCDD-exposure (Fig. 3B). *Stab1* showed the largest up-regulation amongst non-core genes (1.0-fold induction in the H/W rat) while *Ero1l* showed the greatest down-regulation (H/W, –0.7-fold change). Intriguingly, none of the genes within this subset showed transcriptional dysregulation by TCDD in rat liver, mouse liver or mouse kidney (Boutros et al., 2009; Yao et al., 2012).

3.5. Validation of TCDD-responsive genes

A full validation experiment was performed using a NanoString custom gene expression assay, including both AHR-core and candidate genes. An increased response to TCDD for both strains was observed across the AHR-core genes in the validation relative to the microarray data (Fig. 4, top panel). Both *Cyp1a1* and *Nqo1* showed similar induction, however additional genes were also determined as significantly differentially abundant, including *Cyp1a2* and *Cyp1b1* in both strains and *Aldh3a1* and *Tiparp* in only H/W rats. Conversely, fewer candidate genes were determined to be altered by TCDD in either strain with only 20/32 cases validating (Fig. 4, bottom panel). Of these, *Stab1*, *LOC100361558*, *Ctgf*, *Tmem63a*, and *Hspb9* could be validated in both strains.

3.6. Functional analysis and hypergeometric testing

Functional analysis of those genes showing a significant response to TCDD was completed with the GOMiner software (Zeeberg et al., 2003). No significantly enriched pathways were detected following FDR correction (*q* < 0.1; Supplementary Table S3). Hypergeometric testing was performed to determine if there was chromosomal bias represented in the significantly responsive genes (Supplementary Table S4). No chromosomes were significantly enriched, indicating no chromosomal bias for responses to TCDD in hypothalamus.

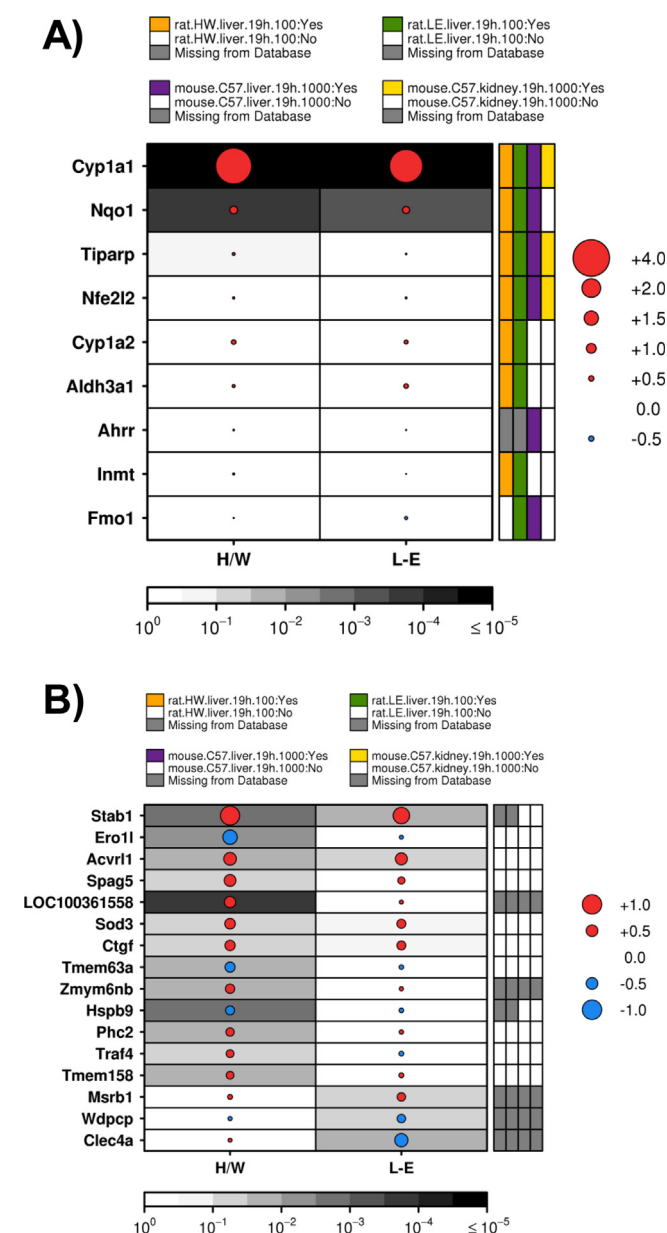


Fig. 3. Gene response to TCDD exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) (A) Nine genes within the AHR-core genes were analyzed for their response to TCDD in hypothalamus. The size of the dot depicts the magnitude of the change (\log_2 fold change) while colour depicts the direction of change – up (red) or down (blue). Background shading reflects the q -value. Covariates convey whether the gene is significantly expressed in H/W or L-E liver and C57BL/6 mouse kidney or liver. (B) 16 genes outside of the AHR-core were significantly altered by TCDD either H/W ($n = 15$) and/or L-E ($n = 6$). The figure shows the \log_2 fold-change values with their corresponding q -value and covariates as described in Fig. 3A. None of these genes were significantly altered in H/W rat liver, L-E rat liver, mouse liver, or mouse kidney.

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3.7. Transcription factor binding site analysis

AHR conserved transcription factor binding site motifs may give further mechanistic insight into the AHR-regulation of genes (Denison and Whitlock 1995; Sogawa et al., 2004). Violin plots (Supplementary Fig. S3) show the occurrence and maximal

conservation score of each motif against the number of strains in which the gene was found to be significantly responsive to TCDD exposure (AHRE-I (core), AHRE-I (full) and AHRE-II). AHRE-I (core) was found to occur more often and with higher conservation in genes significantly responsive in both rat strains (*i.e.* *Cyp1a1* and *Nqo1*). By contrast, AHRE-II was not enriched at either the level of conservation or frequency.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2014.12.016>.

4. Discussion

The hypothalamic arcuate nucleus (ARC) plays a pivotal role in the regulation of energy balance (Linden et al., 2010). The ARC responds to insulin and leptin signals originating from the periphery and acts via production and release of orexigenic or anorexigenic neuropeptides and hormones and therefore could play an important role in wasting syndrome. Previous studies have indicated that the hypothalamus plays a role in cachexia (Ihnatko et al., 2013) and hypothalamic injury can lead to hyperplasia (Elmqvist et al., 1999; Wang et al., 2013). To better understand the role the hypothalamus may play in TCDD-induced wasting syndrome, we analyzed transcriptomic changes occurring in the hypothalamus following TCDD exposure.

mRNA abundance for two of the AHR-core genes, *Cyp1a1* and *Nqo1*, displayed significantly increased abundance following treatment with TCDD. While the magnitude of *Cyp1a1* induction was dramatically lower than that observed in hepatic tissue of similarly treated rats (Yao et al., 2012), levels reached similar abundances as those observed previously in the hypothalamus (Korkalainen et al., 2005). The reduced magnitude of induction of *Nqo1* in hypothalamus relative to liver following TCDD exposure (as observed by both microarray and NanoString) is an interesting finding that has not been reported previously. Alternatively, the absence of significant *Cyp1a2* induction following treatment may be an artifact of the array technology as significant induction resembling that observed previously (Korkalainen et al., 2005) was validated by NanoString in these samples.

With the exception of *Cyp1a1*, altered genes exhibited only modest changes in abundance in response to TCDD, with changes of less than two-fold relative to control animals. This low magnitude of change in candidate genes was similarly observed by NanoString validation. The relatively few alterations to the transcriptome, combined with the small magnitude of these changes may suggest that the hypothalamus is largely refractory to direct local effects of TCDD. However, the specific genes showing significant alterations may still shed evidence on downstream toxicities. A group of 3 genes were significantly altered only in L-E rats and may therefore be involved in the sensitive phenotype. MSRB1 is involved in the protein repair mechanism during oxidative stress (Lee et al., 2009) and has been shown to regulate assembly of actin filaments relating to macrophage activity (Lee et al., 2013). Increased abundance of this mRNA may indicate a potential defense mechanism against TCDD-induced reactive oxygen species. Alternatively, decreased mRNA abundances of *Wdpcp* and *Clec4a* may have etiological roles in TCDD-induced toxicity. *Wdpcp* is required for the stabilization of actin filaments and development of cell polarity (Cui et al., 2013) while *Clec4a* (DCIR) is typically expressed in immune tissues (Bates et al., 1999) and is involved in the immune response.

Of the 11 genes uniquely altered in H/W rats, nine were up-regulated by TCDD while the remaining three were down-regulated. *Ctgf* has been validated as an AHR-target gene coding for CTGF, a regulatory protein that mediates cell division and apoptosis and has been documented to promote tumour growth

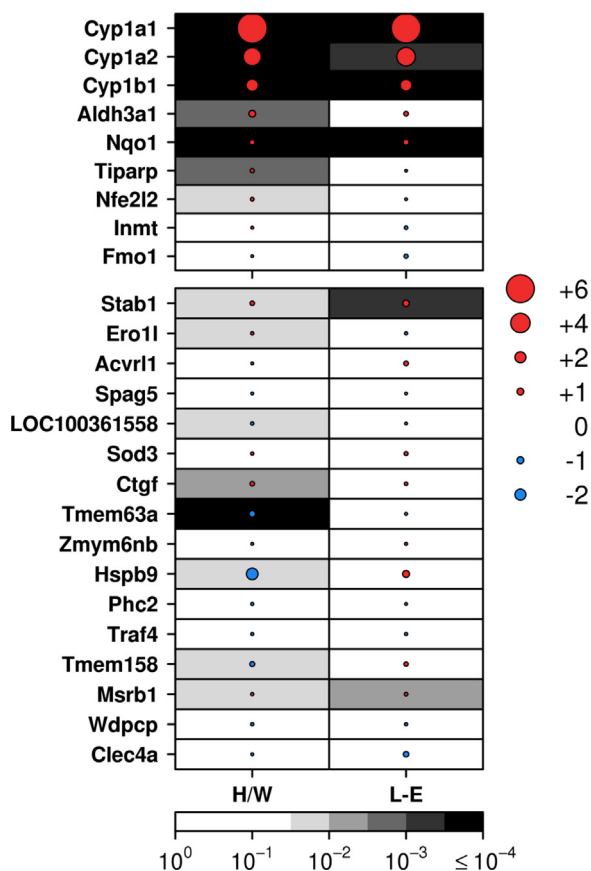


Fig. 4. Validation of candidate genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) Validation was performed on 9 AHR-core genes and 16 candidate genes. The size of the dot depicts the magnitude of the change (\log_2 fold change) while colour depicts the direction of change – up (red) or down (blue). Background shading reflects the q -value.

and progression (Chu et al., 2008; Faust et al., 2013). It also plays an important role in mediating cell adhesion and migration primarily in activation of rat oval cells (Pi et al., 2008). *Sod3* has been shown to act as a tumour suppressor in prostate (Kim et al., 2014) and pancreatic cancer (Sibenaller et al., 2014) by modulation of ROS. Alternatively, induction of *Ero1l* by HIF-1 α within a hypoxic microenvironment leads to angiogenesis and improved tumour survival (May et al., 2005). Within the resistant H/W strain, these findings (overexpression of *Sod3* and reduced expression of *Ero1l*) align with the reduced carcinogenic effects of TCDD. *Traf4* is a common oncogene (Camilleri-Broet et al., 2007), however overexpression has also been shown to be essential for homeostasis of CNS myelination (Blaise et al., 2012).

Two genes, *Stab1* and *Acvrl1*, were overexpressed in both H/W and L-E rats. STAB1 is a scavenger receptor expressed by macrophages and may aid in phagocytic and anti-inflammatory processes (Park et al., 2009). ACVRL1 is a type 1 receptor for TGF-beta proteins and is required for angiogenesis (Oh et al., 2000). Increased mRNA abundance of these genes may represent a common adaptive response to TCDD-induced damages.

Previous studies comparing mRNA abundance changes following TCDD treatment have shown L-E rats have significantly higher numbers of genes dysregulated by TCDD, as compared to H/W rat (Franc et al., 2008; Yao et al., 2012). However, the hypothalamus of H/W rats had more altered transcripts than in L-E rats. Given that all animals from both rat strains had reached sexual maturity at the time of TCDD exposure, it is not likely that the difference in age

affected the transcriptomic response. Similarly, animals experienced identical environmental and handling conditions. Although the magnitude of *Cyp1a1* induction is low in hypothalamus when compared with liver, the observed induction in both L-E and H/W rats indicates that TCDD does in fact reach and effectively activate the AHR in the hypothalamus of both strains, in accordance with previous work (Pohjanvirta et al., 1990). It is important to note that our study was designed to emphasize detection of early transcriptomic responses during the time when the onset of measurable feeding responses occurs in TCDD-treated rats.

A study conducted by Linden et al. (2005) on the effect of TCDD exposure on neuropeptide concentrations further supports our results as it showed that TCDD alters anorexigenic and orexigenic neuropeptides but not consistently (Linden et al., 2005). Attention has now turned to nitric oxide and its combined effects with orexigenic peptides, ghrelin, NPY and orexin-A, in food intake regulation. An alteration in nitric oxide concentration may indirectly affect this regulatory pathway, although results of these studies have not been conclusive (Linden et al., 2010). Ventromedial hypothalamic lesions aggravate TCDD-induced weight loss and, therefore, indicate that TCDD implements toxic effects at some point along the hypothalamic pathways regulating energy homeostasis (Tuomisto et al., 1995). The minimal impact of TCDD on hypothalamic transcriptomic responses in hypothalamus seen in this study, along with studies on TCDD response of mRNAs for hypothalamic neuropeptides and *bHLS/PAS* proteins, suggest that hypothalamic changes alone are not responsible for TCDD-induced hypophagia (Korkalainen et al., 2005; Linden et al., 2005). Analysis of the entire hypothalamic tissue block may mask strictly localized alterations in functionally-specific nuclei.

The hypothalamus is a highly compartmentalized structure consisting of clusters of functionally specialized cells (Schindler et al., 2012), each type of which could have diverse responses to TCDD. The tissue samples we analyzed are derived from the whole hypothalamus. An in depth analysis of specialized cell types might provide more enlightening results. Alternatively, the hypothalamus may be indirectly involved in wasting-syndrome-associated hypophagia as TCDD may directly affect other feed-intake regulatory organs. Analysis of alternate nervous system components in this regulatory pathway (i.e. caudal brainstem and medullary area postrema) as well as factors upstream or downstream (i.e. adipose tissue) may provide more insight into the mechanisms underlying toxic effects of TCDD, as they pertain to wasting syndrome.

Authors' contributions

Animal work: RP, JL, SL; sample preparation: IDM, RP, SDP; bioinformatics analysis: KEH, SDP, PCB; wrote the first draft of the manuscript: KEH; initiated the project: ABO, RP; supervised research: ABO, RP, PCB; generated tools and reagents: SDP; approved the manuscript: all authors.

Conflict of interest

ABO has served as a paid consultant to the Dow Chemical Company as a member of their Dioxin Scientific Advisory Board. All other authors declare that they have no conflicts of interest.

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Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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