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AHR Activation Affects Enteric Neuronal Survival by Inducing Apoptosis

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ABSTRACT

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent organic pollutant (POP) and a potent activator of the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor. There has been considerable progress in understanding how AHR activation by TCDD affects the central nervous system (CNS) while TCDD effects on the enteric nervous system (ENS) remains relatively underexplored. This study sought to better understand the effects of TCDD toxicity via AHR activation in the ENS with both *in vivo* and *in vitro* methods. The upregulation of ceramide synthesizing genes and apoptosis were explored as possible routes of TCDD toxicity in the ENS. In both *in vivo* and *in vitro* studies, AHR activation was confirmed with elevated cytochrome P4501A1 (*Cyp1a1*) gene expression levels with TCDD treatment through quantitative polymerase chain reaction (qPCR). *Cyp1a1* is a known AHR target gene involved in xenobiotic metabolism. For the *in vivo* study, the longitudinal muscle myenteric plexus (LMMP) was isolated from male C57BL/6J mice and used for immunostaining and qPCR analysis. A stool frequency test was done before sacrificing the mice for gut motility observations. For the *in vitro* study, immorto fetal enteric neuronal (IM-FEN) cells were used for qPCR analysis (neuronal marker genes and ceramide genes), LDH cytotoxicity assay, Caspase-Glo 3/7 assay, cleaved caspase-3 western blot analysis, and neutral sphingomyelinase (N-Smase) activity assay. Results showed that TCDD reduced the total number of neurons (TUJ1), specifically nitrergic neurons (nNOS) with no difference in cholinergic neurons (ChAT) in TCDD-treated IM-FEN cells and primary cells isolated from WT mice but not AHR global knockout ($Ahr^{-/-}$). Mice treated with TCDD exhibited a reduced interstitial motility (decreased stool frequency). TCDD also elicited cytotoxicity in IM-FEN cells with increased caspase 3/7 levels and activity indicating apoptosis as a consequence of TCDD cytotoxicity. Increased expression of ceramide synthesizing genes and increased N-Smase activity suggests that ceramides may have a significant role in the intrinsic apoptotic process of TCDD-induced cytotoxicity. The results indicate that AHR signaling within the ENS could potentially have an impact on the gastrointestinal pathophysiology caused by TCDD and protective measures should be taken with regard to the GI tract in industrial places prone to TCDD exposure.

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Chapter 1

Introduction

Aryl Hydrocarbon Receptor Activation via Persistent Organic Pollutants

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent organic pollutant (POP) and the most potent model for a family of structurally-related dioxin compounds known as halogenated aromatic hydrocarbons (HAHs)[1]. TCDD can be generated as a byproduct from industrial processes involving 2,4,5-trichlorophenol (TCP) production[2]. TCP has been mainly used as a key component in the production of phenoxy herbicides 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), silvex, and hexachlorophene (bactericide and fungicide) resulting in these products to also be contaminated with TCDD during the production process[2]. Additionally, the further combustion of 2,4,5-T has been known to release TCDD as a stable by-product. TCDD is an occupational biohazard, especially at TCP production and cleanup sites after fires involving polychlorinated aromatics[1,2]. Excess TCDD production has also been noted during high temperature reactions involving 2,4,5-T production[2]. Due to its persistent and carcinogenic nature, TCDD has adverse health effects on vertebrates, such as reproductive and developmental abnormalities, liver scarring, impaired immune system, tumor formation, and neurotoxicity[3-7]. Incidents of human exposure to TCDD have been marked by endocrine disruption and increased cancer cases decades after exposure[8,9].

TCDD is a potent activator of the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor associated with the basic helix–loop–helix/Per-ARNT-single-minded protein (bHLH-PAS) family of proteins[9]. The bHLH-PAS family consists of a class of transcriptional regulators with a highly conserved bHLH domain and variable C-terminal regions; they play a pivotal role in in regulating foreign intracellular and extracellular signals[10]. AHR, a ligand-

activated bHLH-PAS protein, regulates physiological functions including xenobiotic metabolism and immunotoxicity[11]. The toxic biological response caused by TCDD is known to be mediated by AHR, which has a high binding affinity for TCDD. TCDD-mediated AHR activation begins with signal transduction followed by genomic and nongenomic downstream effects.

The genomic pathway is initiated by the binding of TCDD to AHR inducing the cytosolic AHR-TCDD complex to translocate into the nucleus where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This dimer binds dioxin responsive elements (DREs) located in the promoter region of AHR-responsive genes involving drug metabolism, immune response, and cell proliferation. Target genes such as *Cyp1a1*, the gene encoding cytochrome P4501A1 (CYP1A1), is transcriptionally upregulated. CYP1A1 is involved in the metabolism of several xenobiotics (such as HAHs) through oxidation which increases the solubility of xenobiotic compounds to make excretion from the body easier[12]. CYP1A1 induction is associated with an increased transcription of AHR repressor genes causing a negative feedback loop with ARNT interaction to result in AHR degradation[13]. Treating samples with an antagonist before adding TCDD is a method of confirming that AHR activation is occurring through TCDD binding since the antagonist strongly binds to AHR thereby significantly decreasing subsequent toxicant binding. CH-223191 is one such ligand-selective AHR antagonist which selectively inhibits dioxin agonists such as TCDD by preventing the translocation of the AHR-TCDD complex from the cytoplasm into the nucleus[14]. Thus, AHR is unable to dimerize with ARNT to bind to and interact with DREs and the downstream effects of AHR activation would not be significantly observed (such as an increase in CYP1A1)[14].

AHR activation also includes nongenomic pathways which stimulate an immune response and induce inflammation. Examples of nongenomic pathways associated with AHR activation are

PI3K/AKT-pathway-dependent macrophage activation[15] and focal adhesion kinase (FAK) activation[16]. In the brain, studies show AHR activation regulates target neuronal genes involved in neurogenesis and survival[17]. Low-dose gestational exposure to TCDD has demonstrated prolonged effects of brain development and function[18]. Williamson et al., established that cerebellar granule neuroblasts were specific targets of AHR-mediated TCDD neurotoxicity and granule cell neurogenesis may be further disrupted[19]. Additionally, Miyazaki et al., demonstrated that TCDD exposure suppresses glial cell line-derived neurotrophic factor (GDNF) function thereby obstructing adequate fetal blood-brain barrier development in an in vitro model derived from rat cells[20]. Studies analyzing the effects of TCDD have been predominantly performed on the central nervous system, while their effects on enteric nervous system are relatively underexplored.

The Enteric Nervous System

The enteric nervous system (ENS) falls under the umbrella of the autonomic nervous system, which controls involuntary bodily processes such as digestion, immune secretions, blood flow, and gut motility (movement of food through the gastrointestinal [GI] system). Nicknamed the "second brain", the ENS works both independently and in conjunction with the central nervous system (CNS). The ENS is comprised of two main bundles of intersecting nerves: the myenteric plexus and the submucosal (Meissner's plexus) plexus. The myenteric plexus is situated between the longitudinal and circular muscle layers of the gut and stimulates the muscularis to relax or contract the intestinal wall (gut motility). The submucosal plexus is found between the muscularis mucosae and the submucosa and regulates GI blood flow and intestinal gland secretions. Both of these major ENS plexi control GI functions[21].

The myenteric plexus encompasses nitrergic (inhibitory) neurons in the descending nerve fibers and cholinergic (excitatory; most abundant) neurons in the ascending nerve fibers which innervate the GI tract[22]. Class III beta-tubulin (TUJ1) is the overarching neuronal marker responsible for microtubule stability and axonal transport. TUJ1 includes subtypes neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT). nNOS (encoded by the gene *Nos1*) is associated with nitrergic neurons involved in muscle relaxation. ChAT (encoded by the gene *Chat*) is associated with cholinergic neurons involved in muscle contraction. Together, nNOS and ChAT expressions modulate the contractive and relaxatory state of the GI tract[21].

ENS studies can be done *in vivo* or *in vitro*. *In vivo* methods are done by sacrificing treated mice to isolate the longitudinal smooth muscle-myenteric plexus (LMMP) and analyze the enteric neurons. *In vitro* methods involve culturing primary enteric neurons. Gastroenterology research promotes primary enteric neurons for *in vitro* experiments because they preserve the original physiological characteristics of the tissue. A study by Emory University developed the immorto fetal enteric neuronal (IM-FEN) cell line using the myenteric plexus as it possesses a greater connection to gut motility[23].

Ceramides, the ENS, and AHR

Ceramides serve as the foundation for all sphingolipids. Their general structure is comprised of a sphingosine backbone and a variable fatty acid chain connected through N-acyl linkage. A well known physiological role of ceramides is to maintain the water permeability barrier in the stratum corneum of the epidermis[24.] Ceramides also play a regulatory role in mediating apoptosis. An essential step in the intrinsic apoptotic pathway involves the expulsion of

mitochondrial proapoptotic intermembrane space proteins which trigger the activation of caspases and DNases associated with the downstream steps of apoptosis. One of the mechanisms through which the proapoptotic intermembrane space proteins can be released from the mitochondria is by large, ceramide-formed protein-permeable channels in the outer mitochondrial membrane[25]. Additionally, inhibiting ceramide synthase before apoptosis-inducing treatments (TNF-α, phorbol ester, ultraviolet radiation) has been shown to reduce vulnerability of cells to apoptosis[26].

There are three main pathways contributing to ceramide synthesis: de novo synthesis pathway (endoplasmic reticulum), sphingomyelin hydrolysis pathway (plasma membrane), and the salvage pathway (lysosome). The de novo synthesis pathway begins with the rate-limiting step involving serine palmitoyltransferase (encoded by *Sptlc 1* and *Sptlc 2* [functional subunit]), followed by ceramide synthase (encoded by *CerS*), and degenerative spermatocyte homolog 1 (encoded by *Degs1*). Ceramides mainly act as the substrate for complex sphingolipids via the de novo synthesis pathway. The sphingomyelin hydrolysis pathway involves the conversion of sphingomyelin to ceramide through neutral sphingomyelinase (encoded by *Smpd2/3/4*) as well as the conversion of sphingosine to ceramide through ceramide synthase (*CerS*). The salvage pathway involves the conversion of sphingomyelin to ceramide through acidic sphingomyelinase (encoded by *Smpd1*). The level of contribution of each pathway to propel apoptosis is debated[25,26].

The aim of this thesis was to investigate the effects of TCDD toxicity via AHR activation in the ENS through both *in vivo* and *in vitro* methods. Upregulation of ceramide synthesizing genes and apoptosis were explored as possible routes of TCDD toxicity. In both *in vivo* and *in vitro* studies, AHR activation was confirmed with *Cyp1a1* gene expression levels through quantitative polymerase chain reaction (qPCR). AHR mediated TCDD effect on enteric neurons was confirmed *in vivo* with AHR global knockout ($Ahr^{-/-}$) mice and *in vitro* CH-223191 treatment. For the *in vivo*

study, the LMMP was isolated from mice and used for immunostaining and RNA extraction for qPCR analysis. The stool frequency test was done before sacrificing the mice for gut motility observations. For the *in vitro* study, IM-FEN cells were used for RNA extraction for qPCR analysis (neuronal marker genes and ceramide genes), LDH cytotoxicity assay, Caspase-Glo 3/7 assay, cleaved caspase-3 western blot analysis, and neutral sphingomyelinase activity assay.

Chapter 2

Materials and Methods

Animal Model and Diet

Male C57BL/6J mice (Strain # 000664) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were grouped into short-term (ST, 5 days) and long-term (LT, 8 weeks) studies and trained to eat dough pills (Transgenic bacon-flavored dough diet from Bio-Serv, Flemington, NJ) prepared with a tablet mold. ST group received one dough pill with TCDD at a concentration of 2.4 μg/kg body weight or a control dough pill on day one and were observed until day 5. LT group received one dough pill with TCDD at a concentration of 2.4 μg/kg body weight or a control dough pill once per week for 8 weeks. As shown by Foxx et al., this dosage has been used in orally-administered TCDD experiments with mouse animal models and is known as "an AHR activating dose"[27,28]. In addition, TCDD-treated mice did not exhibit significant reproductive consequences related to dosage for doses up to 2.4 μg/kg; higher doses induced fetotoxicity in male mice which could have interfered with this study[29,30]. TCDD was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). All mice experiments were ethically performed in accordance with Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) protocols.

Stool Frequency

Excreted stool pellets were promptly counted and collected from individual mice in separate cages for one hour from both short-term and long-term studies (TCDD treatment and control).

Whole Mount Immunostaining

The longitudinal muscle myenteric plexus (LMMP) was isolated from the distal colon along with the myenteric ganglia and fixed with 4% paraformaldehyde. Immunostaining protocol was performed and analyzed for TUJ1, nNOS and ChAT as previously published[31] using the antibodies listed in Table 1. Ten randomly selected fields were counted to evaluate the number of neurons per area unit for each marker.

Table 1. Antibodies Used in Immunostaining

Antibody/host	Company	Cat. No.	Dilution
TUJ1/Mouse	abcam (Waltham, MA)	ab78078	1:500
nNOS/Rabbit	abcam (Waltham, MA)	ab76067	1:500
ChAT/Goat	MilliporeSigma (Burlington, MA)	AB144P	1:100
Donkey anti-Rabbit, Alexa Fluor 594	Invitrogen (Waltham, MA)	A21207	1:200
Donkey anti-Goat, Alexa Fluor 594	Invitrogen (Waltham, MA)	A32758	1:200
Donkey anti-Mouse, Alexa Fluor 594	Invitrogen (Waltham, MA)	A21203	1:200

Cell Culture and TCDD Treatment

All in vitro experiments were done using IM-FEN cells. Cells were cultivated in N2 medium supplemented with 10% fetal bovine serum (FBS), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF) (Shenandoah Biotechnology, Inc. Warminster, PA), and 20 U/mL recombinant mouse interferon-γ (MilliporeSigma, Burlington, MA) and incubated in 10% CO_2 at 33°C for 1 to 2 days to allow for proliferation and optimal cell expansion[23]. After cells reached approximately 70% confluency, the medium was then replaced with complete neurobasal-A medium (NBM) containing B-27 serum-free supplement (Thermo Fisher Scientific, Waltham, MA, USA), 1 mmol/L glutamine, 1% FBS, and 10 ng/mL GDNF, and incubated in 5% CO_2 at 39°C to allow for differentiation [23]. Differentiated cells were treated with three, ten-fold increasing doses of TCDD (0.1 nM, 1 nM, and 10 nM) and incubated in 5% CO_2 at 39°C for 24 hours. To show that the TCDD effect is mediated by AHR, cells were treated with CH-223191 (10 µM), a potent and specific AHR antagonist for 1 hour at 39°C before adding 1 nM TCDD and incubating in 5% CO_2 at 39°C for 24 hours. Cells were passaged for further experimentation when the appropriate confluency was observed. 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) was used to lift cells for subculturing and passaging. Cells were frozen in 90% FBS and 10% DMSO in Cryovial tubes and stored at -80°C. The IM-FEN cell line was maintained in complete N2 medium as previously described[23].

LDH-Glo Cytotoxicity Assay

IM-FEN cells were cultured in 96-well white plates and treated with varying doses of TCDD (0.1 nM, 1 nM, and 10 nM) in NBM in 5% CO_2 at 39°C for 24 hours. TCDD cytotoxicity

was measured (amount of LDH released into the culture medium) using the LDH-Glo Cytotoxicity Assay kit from Promega (Madison, WI, USA) based on the manufacturer's protocol.

Caspase-Glo 3/7 Assay

IM-FEN cells were cultured in 96-well white plates and treated with varying doses of TCDD (0.1 nM, 1 nM, and 10 nM) and 10 μM CH-223191 in NBM in 5% CO_2 at 39°C for 24 hours. Caspase-Glo 3/7 reagent was made with luminogenic Caspase-Glo 3/7 substrate and buffer from the fluorescence-based Caspase-Glo 3/7 Assay kit from Promega (Madison, WI, USA). The reagent was added to treated cells and incubated for 1 h at room temperature, protected from light. Caspase cleavage of substrate and subsequent luminescence (by thermostable Ultra-Glo Recombinant Luciferase) was measured using a GloMax luminometer.

RNA Extraction and qPCR

RNA was extracted from snap frozen LMMP tissue from ileum and colon of ST and LT treated mice, and from vehicle and TCDD (0.1, 1, and 10 nM) treated IM-FEN cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA samples were resuspended in nuclease-free diethyl pyrocarbonate (DEPC) treated water and stored in -80°C until use. Concentration and purity were measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Quantabio 5X qScript cDNA SuperMix (Beverly, MA, USA) was used to synthesize cDNA from 1 μg of RNA according to the manufacturer's protocol. Subsequently, qPCR was done using the PowerUp SYBR Green Master Mix (Applied Biosystems, Waltham, MA) using primers

listed in Table 2. Results were assessed using the ΔΔCt method to analyze fold change normalized to a housekeeping gene, *Gapdh* for *in vivo* studies and *B2M* for *in vitro* studies.

Table 2. Oligonucleotide Primer Sequences Used for Real-time PCR

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
Cyp1a1	CTCTTCCCTGGATGCCTTGAA	GGATGTGGCCCTTCTCAAATG
Tubb3	AACCATGGACAGTGTTCGGTCT	TATAGTGCCCTTTGGCCCAGTT
Nos1	AGGAGGATGCTGGTGTGTTC	CTCAGATCTAAGGCGGTTGG
Chat	CCCTCCAGCTGGCTTACTAC	CAGGAGTGGCCGATCTGATG
Syp	TTGGCTTCGTGAAGGTGCTGCA	ACTCTCCGTCTTGTTGGCACAC
B2M	CATGGCTCGCTCGGTGAC	CAGTTCAGTATGTTCGGCTTCC
Gapdh	TTGTGATGGGTGTGAACCACGA	TCTTCTGGGTGGCAGTGATGG
Sptlc1	CGAGGGTTCTATGGCACATT	GGTGGAGAAGCCATACGAGT
Sptlc2	TCACCTCCATGAAGTGCATC	CAGGCGTCTCCTGAAATACC
Cers2	AAGTGGGAAACGGAGTAGCG	ACAGGCAGCCATAGTCGTTC
Cers5	CTTCTCCGTGAGGATGCTGT	GTGTCATTGGGTTCCACCTT
Cers6	AAGCCAATGGACCACAAACT	TGCTTGGAGAGCCCTTCTAAT
Degs1	AATGGGTCTACACGGACCAG	TGGTCAGGTTTCATCAAGGAC
Smpd1	GTTACCAGCTGATGCCCTTC	AGCAGGATCTGTGGAGTTG
Smpd2	GACATCCCCTACCTGAGCAA	CCAGGAGAGCCAGATCAAAG
Smpd3	CCTGACCAGTGCCATTCTTT	AGAAACCCGGTCCTCGTACT

Smpd4	ACCTGGCCCTCAATCCATTTG	ATAGGCACAGTCCGAAGTACG

Sphingomyelinase Assay

Neutral sphingomyelinase (N-SMase) activity was measured in IM-FEN cells treated with vehicle and 1 nM TCDD in complete NBM for 24 h using colorimetric sphingomyelinase assay kit from Sigma (Burlington, MA) according to the manufacturer's protocol. The readings taken at 655 nm using spectrophotometer is proportional to the SMase activity present in the samples.

Total Protein Extraction

IM-FEN cells were cultured in 6-well Poly-D-Lysine/Laminin coated plates and treated with varying doses of TCDD (0.1 nM, 1 nM, and 10 nM) in NBM in 5% CO_2 at 39°C for 24 hours. For total protein extraction, cell lysis buffer was prepared with cell lysis buffer stock (Cell Signaling Technology, Danvers, MA), protease inhibitor cocktail (MilliporeSigma, Burlington, MA), and PhosSTOP **(**phosphatase inhibitor) (MilliporeSigma, Burlington, MA). Protein concentrations were measured by performing a colorimetric bicinchoninic acid (BCA) assay with reagents and bovine serum albumin (BSA) standards from Thermo Fisher Scientific (Waltham, MA, USA) using a 96-well microplate according to the manufacturer's protocol. Protein concentrations were calculated based on the BSA standard curve absorbances at 562 nm using a spectrophotometer. Total protein extraction samples were stored at -80°C.

Western Blot

Protein samples were obtained from IM-FEN cells treated with or without TCDD (0.1, 1 and 10 nM) for 24 h. 15 μg of prepared total protein extractions/lane were loaded onto a 4–20% Criterion TGX Precast Gel (Bio-Rad, Hercules, CA) and run for 1h at 150V. Gel was transferred to a PVDF membrane (Bio-Rad, Hercules, CA) using the Trans-blot Turbo (Bio-Rad, Hercules, CA). 5% nonfat dry milk in tris-buffered saline with 0.1% Tween 20 Detergent (TBS-T) was used as the blocking solution. As listed in Table 3, the appropriate antibodies were used to probe for cleaved caspase-3 and GAPDH (internal loading control). Dilutions of primary and secondary antibodies were prepared with blocking solution. Scion Image computer software (Scion Corporation, Frederick, MD) was used to semi quantitatively measure and express band intensity as a ratio with respect to the internal loading control.

Table 3. Antibodies Used in Western Blot

Antibody/Host	Company	Catalog #	Dilution
Cleaved caspase-3/Rabbit, Primary Antibody	Cell Signaling Technology (Danvers, MA)	9661S	1:1000
HRP-Conjugated Anti-Rabbit Secondary Antibody (IgG)	Cell Signaling Technology (Danvers, MA)	7074S	1:2000-5000
GAPDH (14C10)/Rabbit, Primary antibody	Cell Signaling Technology (Danvers, MA)	2118S	1:2500

Statistical Analysis

GraphPad Prism 7 software was utilized for data analysis. Student's t-test was used for statistical analysis of two treatment groups. One-way analysis of variance (ANOVA) was used for statistical analysis of multiple data sets, followed by Tukey's multiple-comparison test. Results are presented as the mean ± standard error of the mean (SEM). Statistical significance was given to P values less than or equal to 0.05.

Chapter 3

Results

In Vivo and *In Vitro* Neuronal Marker Genes qPCR Analysis

The *in vivo* study involved RNA extraction from the LMMP of both the distal ileum and proximal colon from vehicle and TCDD-treated (2.4 µg/kg of mouse body weight) mice. qPCR was done to assess the neuronal marker *Tubb3* (gene symbol for TUJ1, neuronal marker beta Tubulin 3), its subtypes, *Nos1* (gene symbol for nNOS) and *Chat* (gene symbol for ChAT), and *Cyp1a1* gene expression. ST treatment was for 5 days and LT was carried out for 8 weeks. *In vivo* qPCR gene expression was normalized to *Gapdh* (housekeeping gene). In both ST and LT studies, *Cyp1a1* expression substantially increased ($P < 0.0001$) in both TCDD-treated ileum and colon samples compared to their respective vehicle samples (Figure 1a). *Tubb3* expression in TCDD-treated colon samples in the LT study presented a significant decrease at $P < 0.01$ (Figure 1b). *Nos1* expression in TCDD-treated colon samples in the LT study presented a significant decrease at $P < 0.05$ (Figure 1c). *Chat* expression was not affected by TCDD in both ST and LT colon and ileum myenteric plexi (Figure 1d).

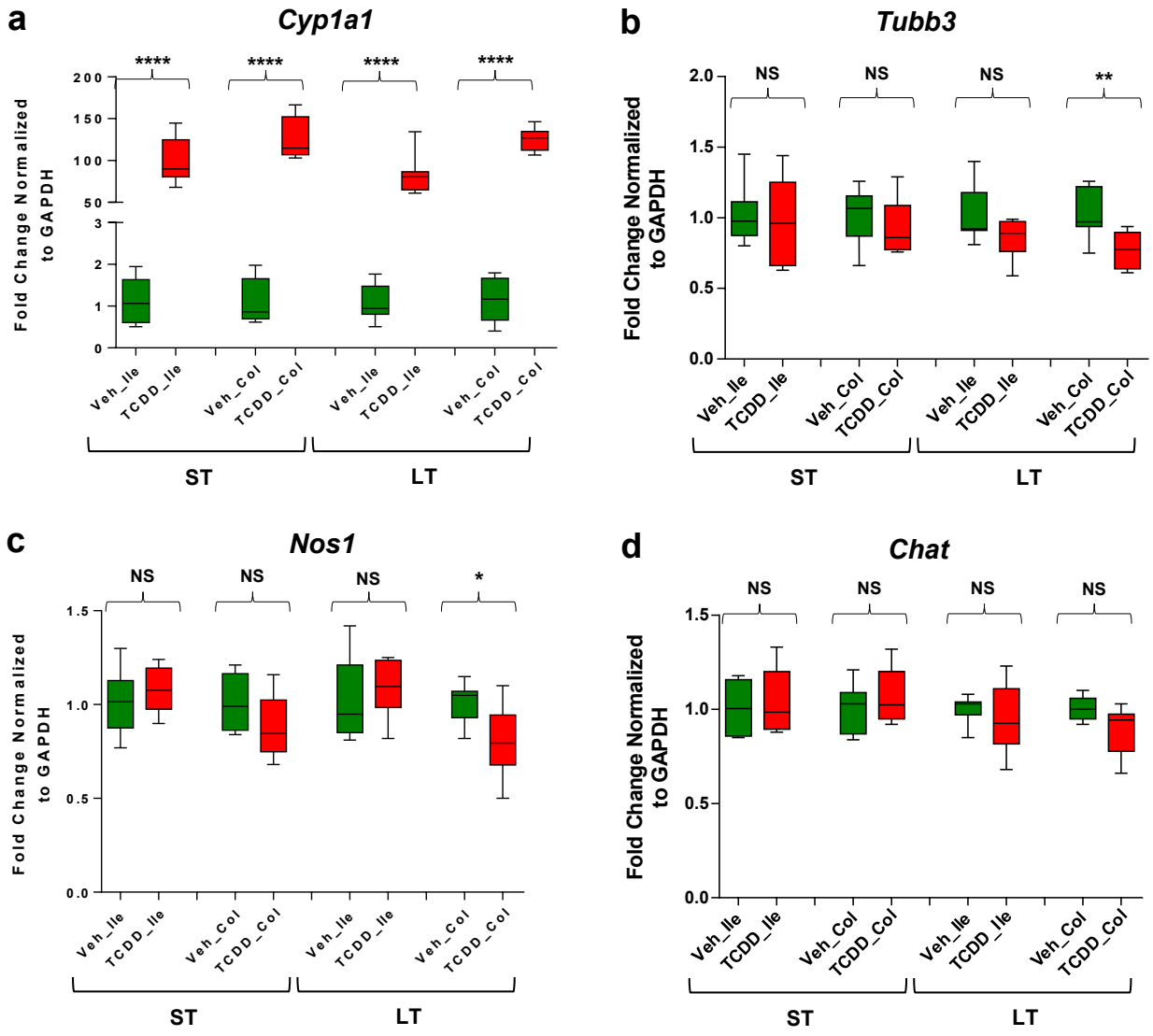

Figure 1. ***In vivo*** **qPCR analysis from LMMP of ileum and colon of mice treated with vehicle and TCDD for 5 days (ST) and 8 weeks (LT). (a)** ***Cyp1a1*** **(b) Neuronal marker class III beta-tubulin TUJ1 (*Tubb3)* (c) Neuronal nitric oxide synthase nNOS (*Nos1)* (d) Choline acetyltransferase ChAT (*Chat)*. Results are mean ± SEM, n=7-8 mice; *P < 0.05, **P < 0.01, ****P < 0.0001, ns (not significant).**

In vivo (mice ileum and colon LMMP) qPCR data was verified with *in vitro* (IM-FEN cells) qPCR data.

The *in vitro* study involved RNA extraction from vehicle (dimethyl sulfoxide [DMSO]) and 24h TCDD-treated IM-FEN cells at three increasing concentrations: 0.1 nM, 1 nM, and 10 nM. qPCR was also done with the *in vitro* samples to assess *Cyp1a1*, synaptic marker Synaptophysin (*Syp*), neuronal marker *Tubb3*, and its subtypes, *Nos1* and *Chat* gene expression. *In vitro* qPCR gene expression was normalized to *B2M* (housekeeping gene). All three doses of TCDD treatment resulted in a highly statistically significant increase in fold change ($P < 0.0001$, approximately 3000-fold) in *Cyp1a1* expression (Figure 2a). *Syp* expression exhibited a significant decrease in fold change (more significant decrease seen in 10 nM TCDD treatment) in TCDD-treated cells when compared to vehicle (Figure 2b). *Tubb3* expression showed a significant decrease across all three TCDD treatment concentrations at $P < 0.01$ (Figure 2c). *Nos1* expression also decreased in a dose-dependent manner with TCDD treatment (Figure 2d) while *Chat* expression was unchanged across all three TCDD concentrations when compared to vehicle (Figure 2e).

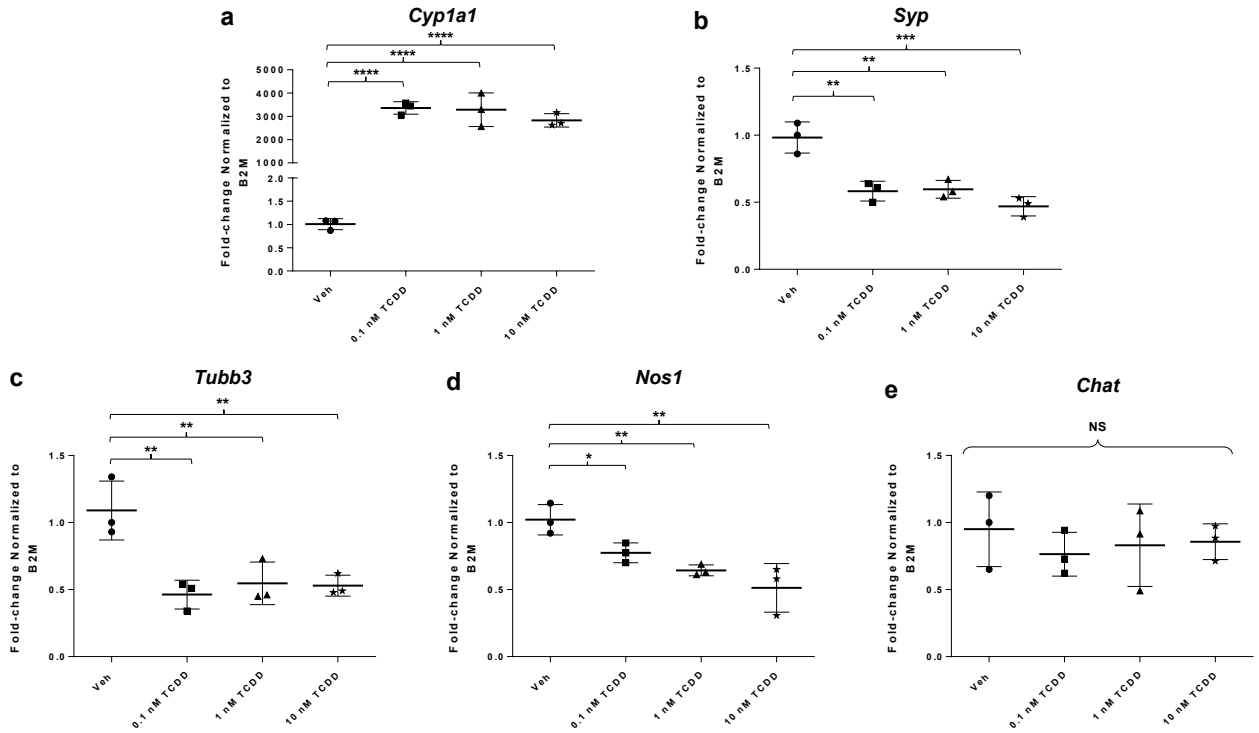

Figure 2. *In vitro* qPCR analysis from IM-FEN Cells: Vehicle and 0.1 nM, 1 nM, and 10 nM TCDD treatment. (a) *Cyp1a1* (b) Synaptic marker, synaptophysin (*Syp)* (c) *Tubb3* (d) *Nos1* (e) *Chat.* Results are mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns (not significant).

The *in vitro* study also involved RNA extraction from enteric neuronal primary culture cells isolated from wildtype (WT) and AHR global knockout (*Ahr*$^{-/-}$) mice. Cells were treated with vehicle, 0.1 nM, and 1 nM TCDD. qPCR was done to assess *Cyp1a1*, neuronal marker *Tubb3*, its subtypes, *Nos1* and *Chat* gene expression. *In vitro* qPCR gene expression was normalized to *Gapdh*. There was no significant fold change observed in vehicle, 0.1 nM, or 1 nM TCDD-treated samples across all four gene expressions in *Ahr*$^{-/-}$ mice (Figures 3a-d). *Cyp1a1* expression showed a statistically significant increase in fold change as TCDD treatment concentrations increased in WT samples at a significance of $P < 0.0001$ (Figure 3a). In WT samples, both *Tubb3* (Figure 3b) and *Nos1* (Figure 3c) expression showed a statistically significant decrease in fold change as

TCDD treatment concentrations increased at a significance of $P < 0.05$. *Chat* expression showed no statistically significant fold change associated with TCDD treatment for WT samples (Figure 3d).

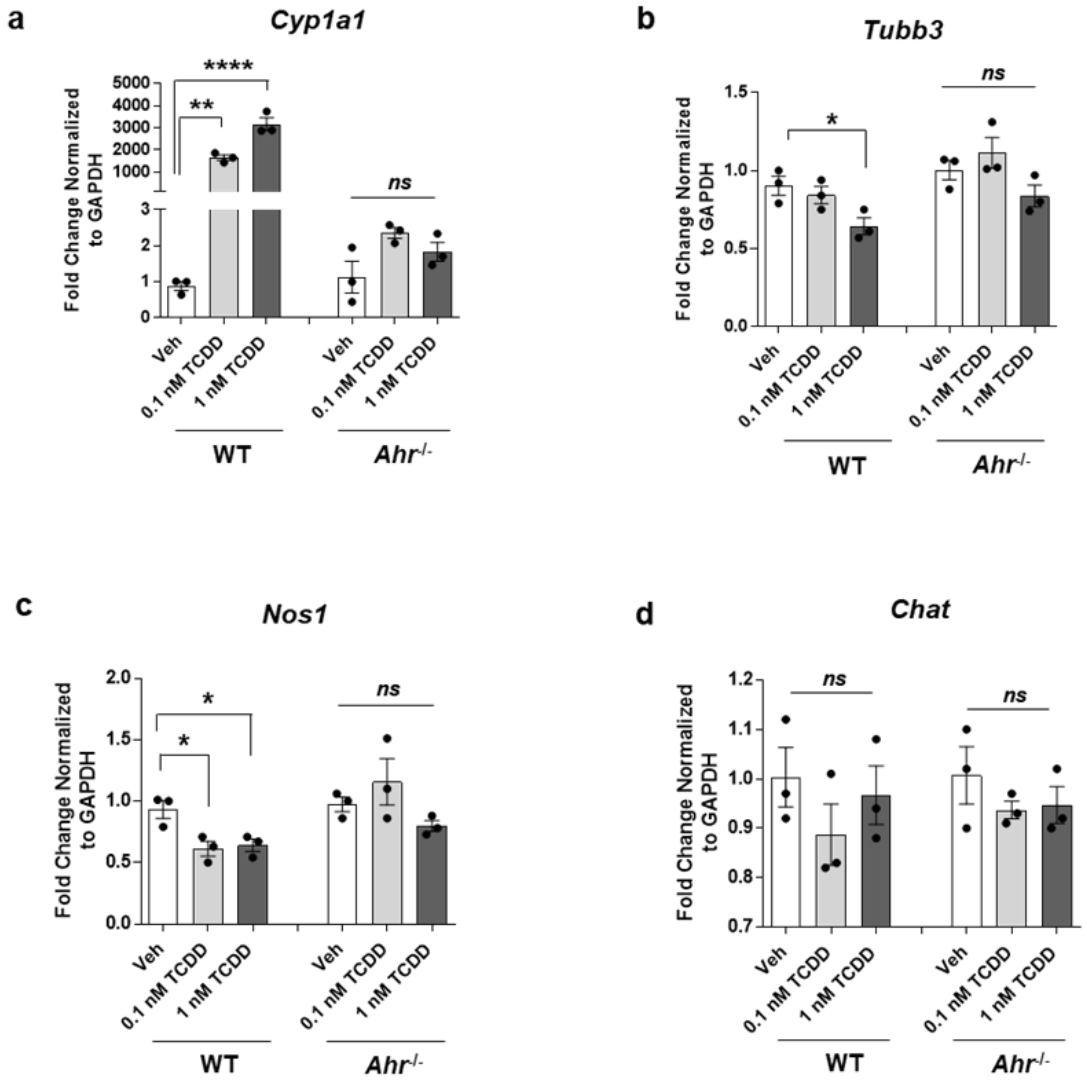

Figure 3. *In vitro* qPCR analysis from primary enteric neuronal culture cells isolated from wildtype (WT) and global AHR knockout (*Ahr*$^{-/-}$) mice myenteric plexi with vehicle, 0.1 nM, and 1 nM TCDD Treatment. (a) *Cyp1a1* (b) *Tubb3* (c) *Nos1* (d) *Chat.* Results are mean ± SEM, n=3; *P < 0.05, **P < 0.01, ****P < 0.0001, ns (not significant).

Further, the *in vitro* study also involved RNA extraction from IM-FEN cells treated with vehicle and 1 nM TCDD with or without AHR antagonist 10 µM CH-223191. qPCR was done to assess synaptic marker Synaptophysin (*Syp*), neuronal marker *Tubb3* and its subtypes, *Nos1* and *Chat*. *In vitro* qPCR gene expression was normalized to *B2M*. TCDD reduced *Tubb3, Nos1* and *Syp* expression significantly ($P < 0.01$) and had no effect on the expression of cholinergic neurons, *Chat*. CH-223191 reversed the inhibition of expression of *Tubb3, Nos1 and Syp* induced by TCDD (Figure 4) indicating that AHR was involved in the TCDD-induced effect.

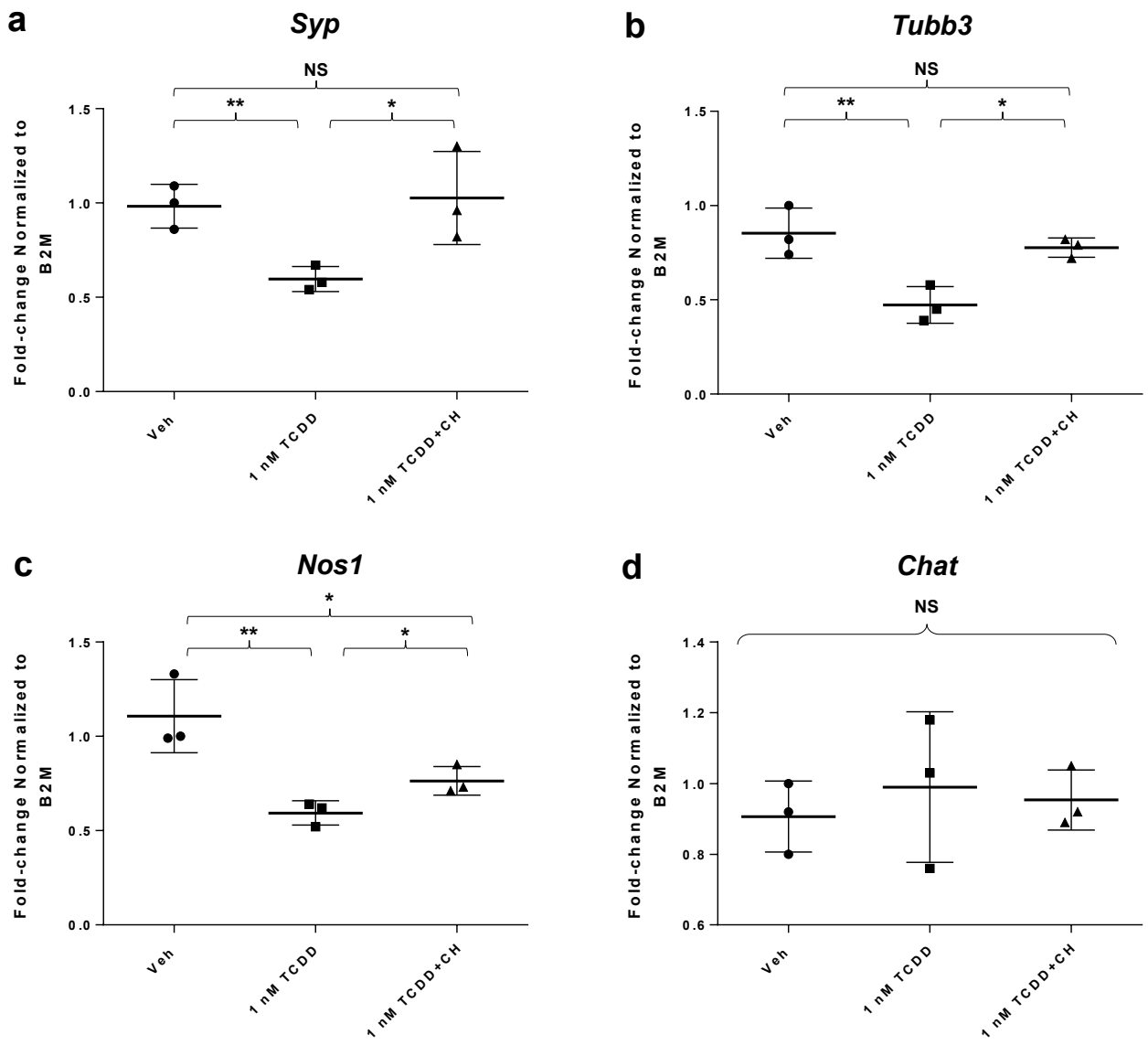

Figure 4. *In vitro* qPCR analysis from IM-FEN Cells: Vehicle and 1 nM TCDD with or without an AHR antagonist, CH-223191 (10 µM) treatment. (a) *Syp* (b) *Tubb3* (c) *Nos1* (d) *Chat.* Results are mean ± SEM, *P < 0.05, **P < 0.01, ns (not significant).

Whole Mount Immunostaining

The effect of TCDD on enteric neurons and subtypes was also verified by whole mount immunostaining of colon LMMP from vehicle and TCDD treated mice (LT). There was a reduction in the total number of neurons (neuronal marker, TUJ1) and nNOS neurons with no difference in ChAT neurons (Figure 5).

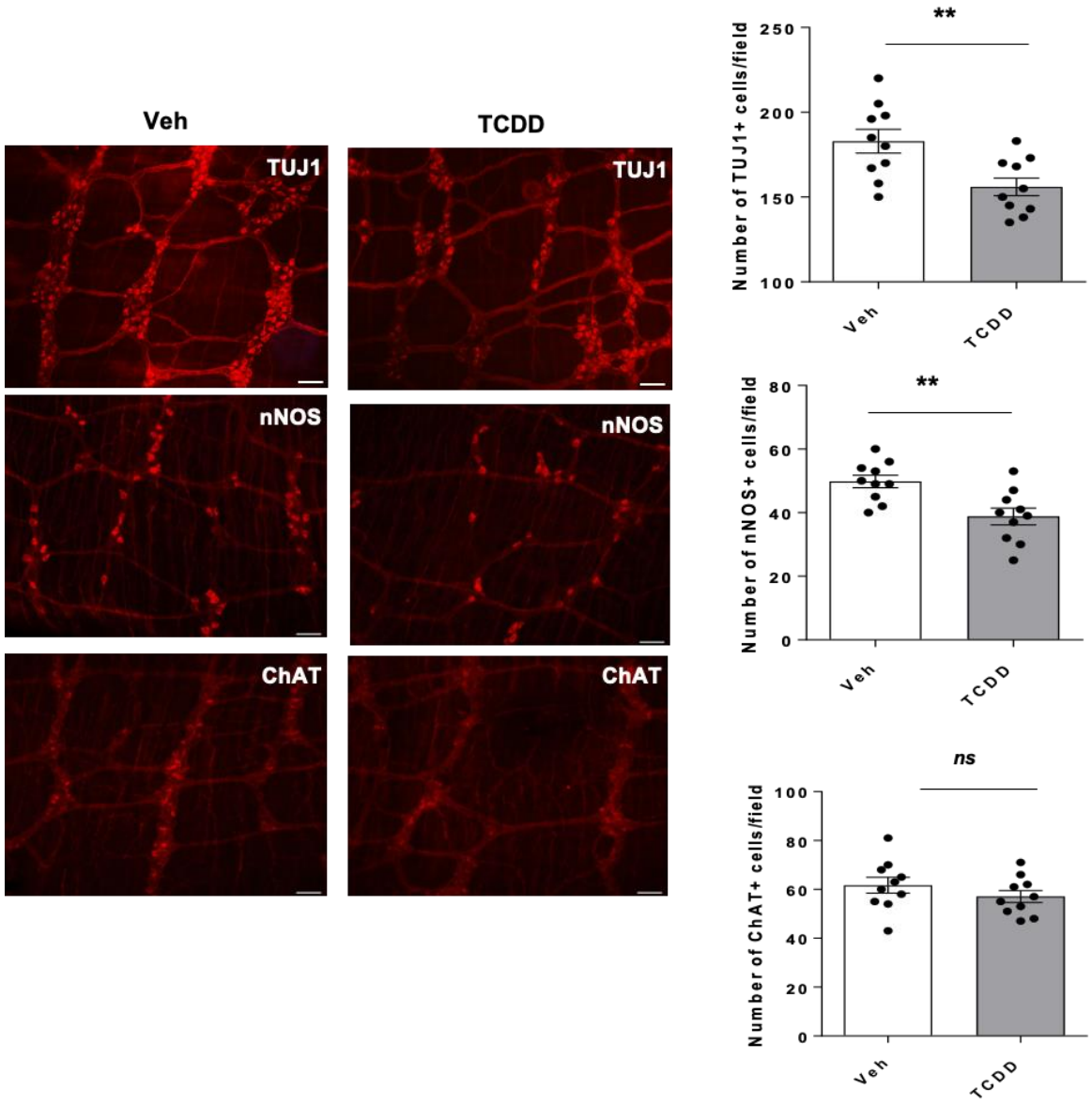

Figure 5. Whole mount immunostaining of distal colon LMMP of vehicle and TCDD-

treated LT mice. Representative images and graphical representation of neuronal marker TUJ1, nNOS, and ChAT. Scale bar, 50 µm. Results are mean ± SEM, **P < 0.01.

Stool Frequency Analysis

The physiological effect of TCDD on enteric neurons was examined by assessing gut motility. Excreted stool pellets were counted for one hour for the short-term (ST) study and biweekly for the long-term (LT) study. The ST study did not have significant differences in stool frequency between vehicle and TCDD-treated mice (Figure 6a). The LT study had significant decreases in stool frequency when comparing TCDD-treated mice to vehicle with the most significant decrease observed in week 6 and 8 (Figure 6b).

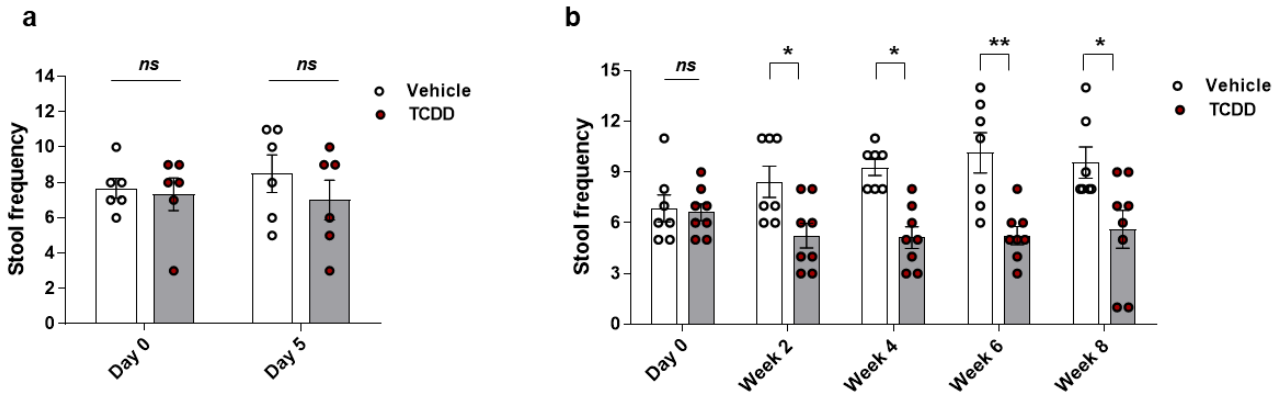

Figure 6. Stool pellet frequency for vehicle and TCDD-treated mice for (a) 5 days (ST) and (b) 8 weeks (LT). Results are mean ± SEM, n=7-8 mice; *P < 0.05, **P < 0.01, ns (not significant).

LDH Cytotoxicity Assay Analysis

TCDD cytotoxicity was assessed in IM-FEN cells treated with three concentrations of TCDD (0.1 nM, 1 nM, and 10 nM) for 24h by the lactate dehydrogenase (LDH) release assay, a well-established assay for cell viability. All three TCDD concentrations resulted in equally

statistically significant increases in cytotoxicity (higher amount of LDH released into the culture medium) when compared to the vehicle at a significance of $P < 0.01$ (Figure 7).

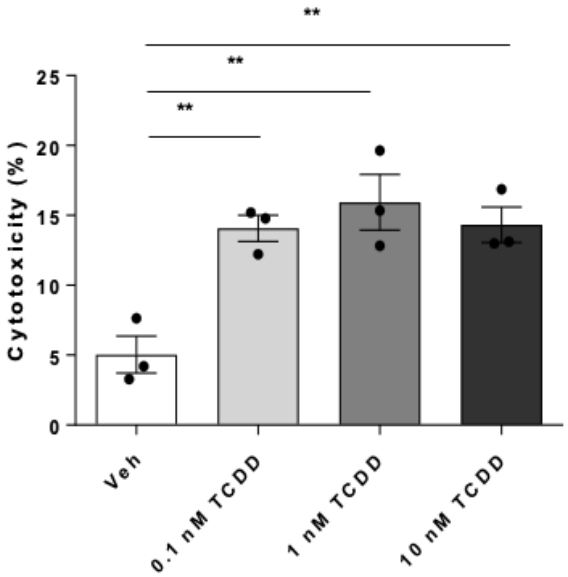

Figure 7. IM-FEN cells percent cytotoxicity from vehicle and 0.1 nM, 1 nM, and 10 nM TCDD treatment based on LDH cytotoxicity assay. Results are mean ± SEM, **P < 0.01.

Caspase 3/7 Assay Analysis

Having observed the cytotoxic effect of TCDD (Figure 5), caspase 3/7 activity, an important executioner of the apoptosis process, was measured in IM-FEN cells to assess apoptosis upon exposure to TCDD. TCDD (1 and 10 nM) caused a significant increase ($P < 0.0001$) in caspase 3/7 activity compared to the vehicle treated cells (Figure 8). 1 nM TCDD treatment increased caspase 3/7 activity by ~10% and 10 nM TCDD treatment increased caspase 3/7 activity by ~25% when compared to vehicle. This data suggests that TCDD can affect cell survival by inducing apoptosis in enteric neurons.

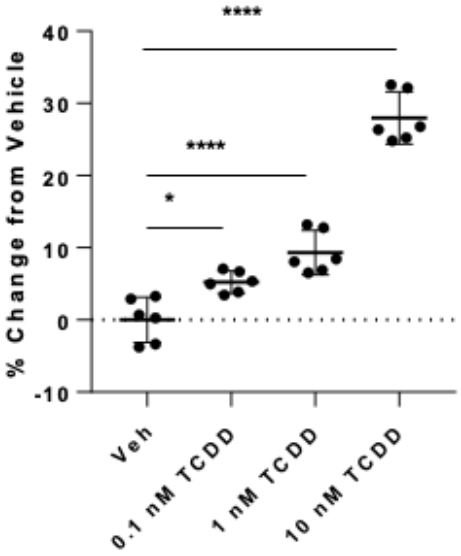

Figure 8. Cell death by apoptosis assessed by measuring caspase 3/7 activity 1 hour after adding the Caspase-Glo 3/7 reagent. Results are mean ± SEM, *P < 0.05; ****P < 0.0001.

Cleaved Caspase-3 Western Blot Analysis

Western blotting was performed with protein samples (15 μg of total protein extractions) from vehicle and 0.1 nM, 1 nM, and 10 nM TCDD-treated IM-FEN cells probing for the apoptotic marker, cleaved caspase-3 (17 kD). GAPDH (37 kDa) was used as the internal loading control. The antibodies and dilutions were used as listed in Table 3. All three doses of TCDD treatment resulted in a highly statistically significant increase in cleaved caspase-3 band density ($P < 0.0001$) normalized to GAPDH (Figure 9b). 1 nM TCDD treatment observed the highest increase in caspase 3/7 density when compared to vehicle.

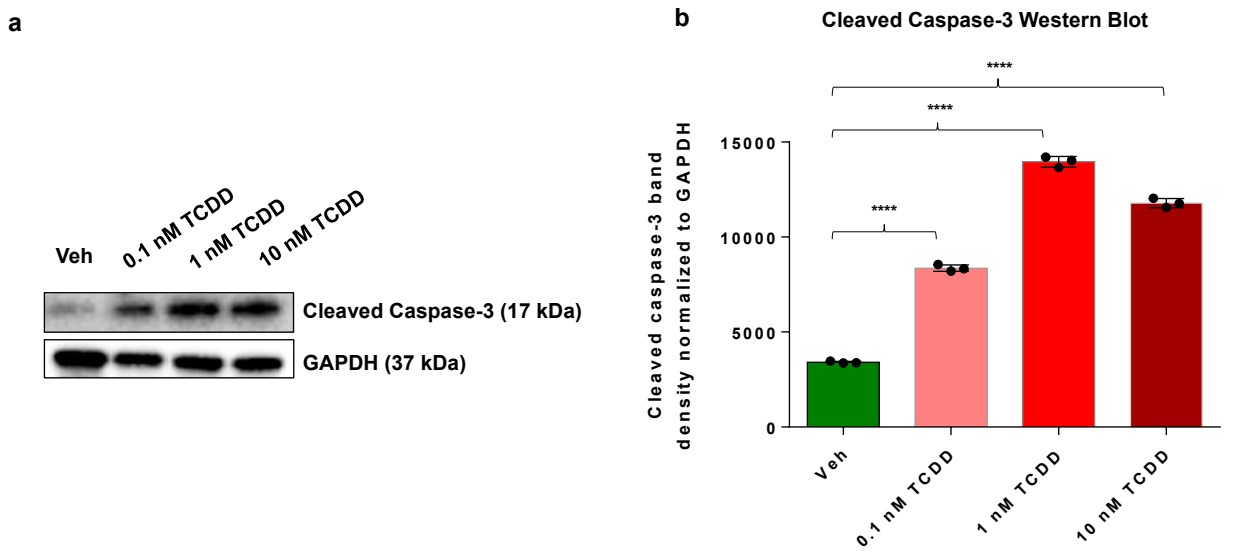

Figure 9. Western blot for (a) Cleaved caspase-3 (17 kDa) and GAPDH, an internal loading control (37 kDa). (b) Graphical representation of cleaved caspase-3 band density normalized to GAPDH. Results are mean ± SEM, ****P < 0.0001.

Ceramide Genes qPCR and Sphingomyelinase Assay Analysis

To examine if TCDD induces increased ceramide synthesis, qPCR was performed with IM-FEN cells treated with vehicle and 1 nM TCDD. The expression of genes encoding key enzymes involved in the ceramide synthesis pathway (*Sptlc1*, *Sptlc2*, *Cers2*, *Cers5*, *Cers6*, *Degs1*, *Smpd1*, *Smpd2*, *Smpd3 and Smpd4*) was assessed. Ceramide synthesis-related mRNAs such as serine palmitoyltransferase long-chain base subunits (*Sptlc1* and *Sptlc2*), ceramide synthases (*Cers2*, *Cers5* and *Cers6*), degenerative spermatocyte homolog 1 (*Degs 1*), and sphingomyelin phosphodiesterase (*Smpd1*, *Smpd2*, *Smpd3*, *Smpd4*) were significantly up-regulated ($P < 0.05$; $P < 0.01$) by TCDD compared to vehicle (Figure 10a). In addition, N-Smase activity ($P < 0.001$) was significantly increased in the IM-FEN cells treated with TCDD compared to vehicle (Figure 10b).

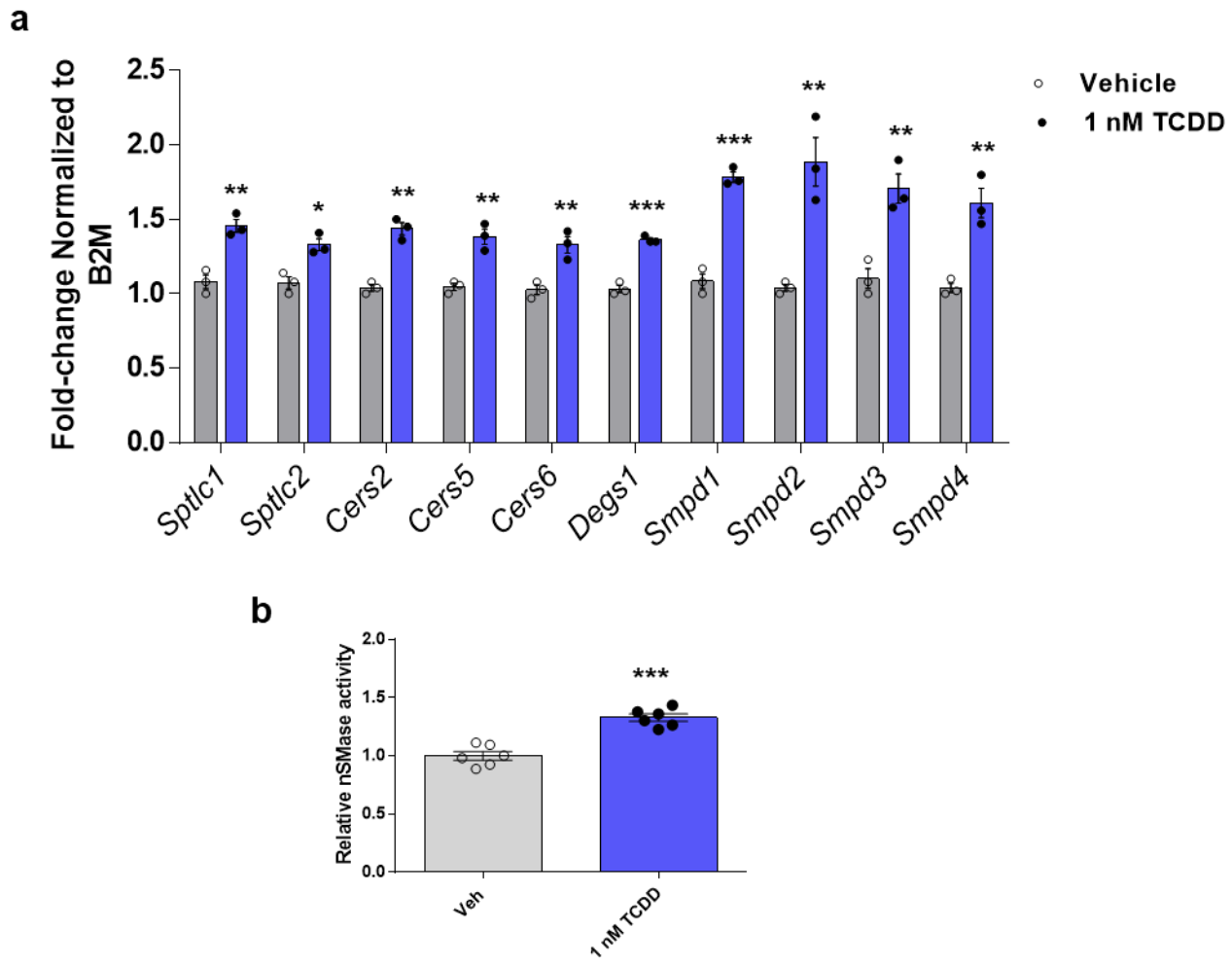

Figure 10. qPCR for key ceramide-synthesizing genes. (a) mRNA levels of ceramide biosynthesis-related genes (*Sptlc1, Sptlc2, Cers2, Cers5, Cers6, Degs1, Smpd1, Smpd2, Smpd3 and Smpd4*) expression in vehicle and 1 nM TCDD-treated IM-FEN cells for 24 h. (b) N-SMase activity of vehicle and 1 nM TCDD-treated IM-FEN cells. Results are mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Chapter 4

Discussion

AHR signaling in IM-FEN cells (*in vitro*) and the ileum and colon myenteric plexi of both ST and LT treated mice (*in vivo*) was examined by assessing *Cyp1a1* expression by qPCR. The qPCR data was consistent in both in vivo and in vitro studies: *Cyp1a1* expression was significantly increased with TCDD treatment as shown in Figures 1 and 2. Elevated *Cyp1a1* expression is indicative of AHR activation in mice and IM-FEN cells as *Cyp1a1* is a target gene that is transcribed when the AHR-ARNT dimer binds the proper DRE. Thus, CYP1A1 is highly expressed in TCDD-treated samples as it plays a major role in xenobiotic toxin metabolism[12]. As shown in Figure 3, the TCDD-treated LMMP samples from the *Ahr*$^{-/-}$ mice did not display any fold change in *Cyp1a1* expression when compared to vehicle whereas WT exhibited a dose-dependent increase in *Cyp1a1* expression. Similarly, in IM-FEN cells, CH-223191 treatment before TCDD treatment showed a significant increase in *Syp*, *Tubb3,* and *Nos1* gene expression when compared to their respective TCDD-treated samples as seen in Figure 4. The comparative lack of *Cyp1a1* expression reflected in *Ahr*$^{-/-}$ mice and increase in neuronal marker expression with CH-223191 antagonist treatment signify that AHR activation is occurring through TCDD binding.

AHR activation by TCDD led to a decrease in enteric neuronal marker expression and specifically decreased nNOS neurons as indicated by qPCR data and whole mount immunostaining results. The beta-tubulin protein (TUJ1) is a positive neuronal marker used to represent total neurons. nNOS (nitrergic neurons) and ChAT (cholinergic neurons) were assessed to determine the type of neuron affected. ChAT expression consistently showed no statistically significant fold change with TCDD treatment in both *in vivo* and *in vitro* qPCR data (Figures 1-4). In IM-FEN cells, TCDD induced a significant reduction in *Tubb3* and *Nos1* gene expression across all three

TCDD dosages (Figures 2c and 2d). The same results were observed in the LT colonic myenteric plexi samples (Figures 1b and 1c). Additionally, TCDD-treated primary enteric neuronal culture cells isolated from WT mice exhibited a significant decrease in *Tubb3* and *Nos1* expression compared to vehicle treated cells and cells isolated from *Ahr*$^{-/-}$ mice (Figures 3b and 3c). However, there was no change in *Tubb3* and *Nos1* expression in ST colon and ileum and LT ileum in TCDD treated mice compared to vehicle. This suggests that long-term TCDD exposure predominantly impacts colonic nitrergic neurons responsible for muscle relaxation. qPCR data was validated by the immunostaining results of LT colon LMMP from vehicle and TCDD treated mice in Figure 5: TCDD induced a reduction in the total number of neurons (neuronal marker, TUJ1) and nNOS with no difference in ChAT. These results confirmed AHR's role in reducing nitrergic neurons upon activation by TCDD.

The physiological effect of AHR in the ENS following TCDD exposure was briefly explored by examining stool frequency. GI motility was tested via a stool frequency test where the frequency of bowel movements was measured as an average number of fecal pellets passed per hour in both ST and LT groups. No significant reduction in the pellet frequency was observed in ST treated mice (Figure 6a). However, in LT treated mice there was a moderate reduction in stool number at week 2 and a significant reduction by week 6 and 8 in TCDD treated mice compared to control mice (Figure 6b). The data indicates that TCDD induces reduced intestinal motility in mice. These results correspond with the decrease in colonic nNOS neurons responsible for muscle relaxation. As nNOS neurons decrease, colonic muscle relaxation is more difficult to achieve, thereby leading to increased colonic contraction and withholding of stool (constipation) in TCDD-treated mice. Thus, AHR signaling within the ENS could potentially have an impact on the GI pathophysiology caused by TCDD.

After establishing the reduction of enteric neurons via AHR activation by TCDD, TCDD cytotoxicity in IM-FEN cells was assessed for 24h by LDH release assay to observe cell viability post TCDD treatment. LDH is a key cytoplasmic enzyme involved in the conversion of lactate to pyruvate which is released upon damage to the cell or cell death. All three TCDD concentrations (0.1 nM, 1 nM, and 10 nM) resulted in equally significant increases in cytotoxicity (more LDH released) when compared to vehicle (Figure 7). Coupled with the decrease in nitrergic neurons, the increased cytotoxicity signifies the prospective damage TCDD inflicts on enteric neuron viability.

IM-FEN cell vulnerability to apoptosis (programmed cell death) was evaluated as a cytotoxic effect by looking at caspase 3/7 activity and levels after TCDD exposure. TCDD (1 and 10 nM) treatment caused a significant increase in caspase 3/7 activity compared to vehicle (Figure 8). Similarly, western blot results showed that all three doses of TCDD treatment (0.1 nM, 1 nM, and 10 nM) resulted in a statistically significant increase in cleaved caspase-3 band density normalized to GAPDH (Figure 9). Caspase 3/7 is a group of executioner proteases with a crucial role in cleaving specific proteins to trigger apoptosis. Thus, the higher caspase 3/7 activity observed represents an increase in apoptotic signaling due to TCDD exposure. Taken together, the data suggest that apoptosis may be a consequence of TCDD cytotoxicity.

Because ceramides are known to play a regulatory role in mediating apoptosis, IM-FEN cell ceramide synthesis was observed as it may be an apoptotic route caused by TCDD binding to AHR. An essential step in the intrinsic apoptotic pathway involves the expulsion of mitochondrial proapoptotic intermembrane space proteins by large, ceramide-formed protein-permeable channels in the outer mitochondrial membrane which trigger the activation of caspases such as caspase 3/7[25]. Additionally, inhibiting ceramide synthesis before apoptosis-inducing treatments

has been shown to reduce cell susceptibility to apoptosis[26]. Neutral sphingomyelinase assay and qPCR was performed with IM-FEN cells treated with vehicle and 1 nM TCDD and assessed the expression of genes encoding key enzymes involved in the ceramide synthesis pathway (*Sptlc1, Sptlc2, Cers2, Cers5, Cers6, Degs1, Smpd1, Smpd2, Smpd3 and Smpd4*). All ceramide synthesizing genes exhibited a significant increase in expression (Figure 10a) as did N-Smase activity (Figure 10b) in IM-FEN cells treated with 1 nM TCDD when compared to vehicle. N-Smase is an essential enzyme involved in ceramide generation; ceramide production through N-Smase is known to induce apoptosis[25]. The data supports an increase in ceramide production caused by TCDD treatment as shown by the increase in ceramide synthesizing gene expression and increased N-Smase activity. This suggests ceramides may have a significant role in the intrinsic apoptotic process of TCDD-induced cytotoxicity.

A future consideration to further the research done in this thesis is to investigate the conversion of dihydroceramide to ceramide in mitochondria by looking specifically at dihydroceramide desaturase 1 (DEGS1) expression. Healthy mitochondria are known to have higher dihydroceramide (precursor for ceramide) levels than ceramide levels[25]. Thus, TCDD-treated cells should theoretically have lower dihydroceramide: ceramide levels (reduced dihydroceramide desaturase 1 activity) since apoptosis would be induced. This can be done through lipid extraction after cell fragmentation to analyze the ratio of dihydroceramide to ceramide levels. This would further show that the mitochondrial ceramide synthesis upregulation could be one of the TCDD-induced apoptotic pathway in IM-FEN cells.

Appendix A

List of Abbreviations with Full Description

ABBREVIATIONS	FULL DESCRIPTION
2,4,5-T	2,4,5-Trichlorophenoxyacetic Acid
AHR	Aryl Hydrocarbon Receptor
ANOVA	Analysis of Variance (one-way was used)
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BCA	Bicinchoninic Acid
bHLH-PAS	Basic Helix–Loop–Helix/Per-ARNT-Single-Minded Protein
BSA	Bovine Serum Albumin
CNS	Central Nervous System
DEPC	Diethyl Pyrocarbonate
DMSO	Dimethyl Sulfoxide
DRE	Dioxin Responsive Element
ENS	Enteric Nervous System
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GDNF	Glial Cell Line-Derived Neurotrophic Factor

GI	Gastrointestinal
HAH	Halogenated Aromatic Hydrocarbons
IACUC	Institutional Animal Care and Use Committee
IM-FEN	Immorto Fetal Enteric Neuronal (Cell Line)
LMMP	Longitudinal Muscle Myenteric Plexus
LT	Long-Term
N-SMase	Neutral Sphingomyelinase
NBM	Neurobasal-A Medium
NS	Not Significant
POP	Persistent Organic Pollutant
qPCR	Quantitative Polymerase Chain Reaction
SEM	Standard Error of the Mean
ST	Short-Term
TBS-T	Tris-Buffered Saline with 0.1% Tween 20 Detergent
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin
TCP	2,4,5-Trichlorophenol
WT	Wildtype

Appendix B

List of Genes and Proteins

GENE	PROTEIN
Cers 2/5/6	Ceramide Synthase (CerS)
Chat	Choline Acetyltransferase (ChAT)
Cyp1a1	Cytochrome P4501A1 (CYP1A1)
Degs1	Degenerative Spermatocyte Homolog 1 (Des)
Nos1	Neuronal Nitric Oxide Synthase (nNOS)
Smpd1	Acidic Sphingomyelinase (A-SMase)
Smpd2/3/4	Neutral Sphingomyelinase (N-SMase)
Sptlc 1	Serine Palmitoyltransferase (SPT)
Syp	Synaptophysin
Tubb3	Class III Beta-Tubulin (TUJ1)

BIBLIOGRAPHY

1) White SS, Birnbaum LS. An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2009 Oct;27(4):197-211. doi: 10.1080/10590500903310047. PMID: 19953395; PMCID: PMC2788749.
2) Hay, Alastair. 1979. "Accidents in Trichlorophenol Plants: A Need for Realistic Surveys to Ascertain Risks to Health." Special Collections, USDA National Agricultural Library. Accessed February 17, 2023.
3) Gray E, Ostby S, and Kelce R. A dose-response analysis of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin in male Long Evans Hooded rat offspring. *Toxicol. Appl. Pharmacol*. 1997;146:11-20
4) Gray E, Wolf C, Mann P. and Ostby S. In utero exposure to low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive development of female Long Evans hooded rat offspring. *Toxicol. Appl. Pharmacol*. 1997;146:237-244
5) Zhang T, Zhou X, Ren X, Zhang X, Wu J, Wang S, Wang Z. Animal Toxicology Studies on the Male Reproductive Effects of 2,3,7,8- Tetrachlorodibenzo-p-Dioxin: Data Analysis and Health Effects Evaluation. *Front. Endocrinol*. 2021;12:696106
6) Knerr S, Schrenk D. Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in experimental models. *Mol Nutr Food Res*. 2006;10:897-907
7) Duval C, Teixeira-Clerc F, Leblanc AF, Touch S, Emond C, Guerre-Millo M, Lotersztajn S, Barouki R, Aggerbeck M, Coumoul X. Chronic Exposure to Low Doses of Dioxin Promotes Liver Fibrosis Development in the C57BL/6J Diet-Induced Obesity Mouse Model. *Environ Health Perspect*. 2017;125(3):428-436
8) Eskenazi B, Warner M, Brambilla P, Signorini S, Ames J, Mocarelli P. The Seveso accident: A look at 40 years of health research and beyond. Environ Int. 2018 Dec;121(Pt

1):71-84. doi: 10.1016/j.envint.2018.08.051. Epub 2018 Sep 1. PMID: 30179766; PMCID: PMC6221983.

9) D. W. Nebert, Aryl hydrocarbon receptor (AHR): “pioneer member” of the basichelix/loop/helix per-Arnt-sim (bHLH/PAS) family of “sensors” of foreign and endogenous signals. *Prog. Lipid Res*. 2017;67:38–57

10) Kolonko M, Greb-Markiewicz B. bHLH-PAS Proteins: Their Structure and Intrinsic Disorder. Int J Mol Sci. 2019 Jul 26;20(15):3653. doi: 10.3390/ijms20153653. PMID: 31357385; PMCID: PMC6695611.

11) S.W. Yi, J.S. Hong, H. Ohrr, J.J. Yi. Agent orange exposure and disease prevalence in korean vietnam veterans: the korean veterans health study.

12) Ma Q. Induction of CYP1A1. The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles. Curr Drug Metab. 2001 Jun;2(2):149-64. doi: 10.2174/1389200013338603. PMID: 11469723.

13) Larigot, L., Juricek, L., Dairou, J., and Coumoul, X. (2018). AhR Signaling Pathways and Regulatory Functions. Biochemie Open, 7, 1-9.

14) Zhao B, Degroot DE, Hayashi A, He G, Denison MS. CH223191 is a ligand-selective antagonist of the Ah (Dioxin) receptor. Toxicol Sci. 2010 Oct;117(2):393-403. doi: 10.1093/toxsci/kfq217. Epub 2010 Jul 15. PMID: 20634293; PMCID: PMC2940411.

15) Großkopf H, Walter K, Karkossa I, von Bergen M, Schubert K. Non-Genomic AhR-Signaling Modulates the Immune Response in Endotoxin-Activated Macrophages After Activation by the Environmental Stressor BaP. Front Immunol. 2021 Mar 31;12:620270. doi: 10.3389/fimmu.2021.620270. PMID: 33868237; PMCID: PMC8045971.

16) Tomkiewicz, C., Herry, L., Bui, L.C., Metayer, C., Bourdeloux, M., Barouki, R., and Coumoul, X. (2013). The Aryl Hydrocarbon Receptor Regulates Focal Adhesion Sites Through a Non-genomic FAK/Src Pathway. Oncogene, 32, 1811-1820.

17) Juricek L, Coumoul X. The Aryl Hydrocarbon Receptor and the Nervous System. *Int J Mol Sci.* 2018;19(9):2504

18) Gileadi TE, Swamy AK, Hore Z, Horswell S, Ellegood J, Mohan C, Mizuno K, Lundebye AK, Giese KP, Stockinger B, Hogstrand C, Lerch JP, Fernandes C, Basson MA. Effects of Low-Dose Gestational TCDD Exposure on Behavior and on Hippocampal Neuron Morphology and Gene Expression in Mice. Environ Health Perspect. 2021 May;129(5):57002.

19) Williamson MA, Gasiewicz TA, Opanashuk LA. Aryl Hydrocarbon Receptor Expression and Activity in Cerebellar Granule Neuroblasts: Implications for Development and Dioxin Neurotoxicity. *Toxicological Sciences*, 2005(83), 340–348

20) Miyazaki W, Fujiwara Y, Katoh T. The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the development and function of the blood-brain barrier. Neurotoxicology. 2016 Jan;52:64-71. doi: 10.1016/j.neuro.2015.11.003. Epub 2015 Nov 12. PMID: 26582458.

21) Goyal, R.K. and Hirano, I. (1996). The Enteric Nervous System. New England Journal of Medicine, 334 (17), 1106-1115.

22) Bódi N, Szalai Z, Bagyánszki M. Nitrergic Enteric Neurons in Health and Disease-Focus on Animal Models. Int J Mol Sci. 2019 Apr 24;20(8):2003. doi: 10.3390/ijms20082003. PMID: 31022832; PMCID: PMC6515552.

23) Anitha M, Joseph I, Ding X, Torre ER, Sawchuk MA, Mwangi S, Hochman S, Sitaraman SV, Anania F, Srinivasan S. Characterization of fetal and postnatal enteric neuronal cell lines with improvement in intestinal neural function. Gastroenterology. 2008 May;134(5):1424-35. doi: 10.1053/j.gastro.2008.02.018. Epub 2008 Feb 14. PMID: 18471518; PMCID: PMC2612783.

24) Uchida Y, Park K. Ceramides in Skin Health and Disease: An Update. Am J Clin Dermatol. 2021 Nov;22(6):853-866. doi: 10.1007/s40257-021-00619-2. Epub 2021 Jul 20. PMID: 34283373.

25) Siskind LJ. Mitochondrial ceramide and the induction of apoptosis. J Bioenerg Biomembr. 2005 Jun;37(3):143-53. doi: 10.1007/s10863-005-6567-7. PMID: 16167171; PMCID: PMC2246044.

26) Kitatani K, Idkowiak-Baldys J, Hannun YA. The sphingolipid salvage pathway in ceramide metabolism and signaling. Cell Signal. 2008 Jun;20(6):1010-8. doi: 10.1016/j.cellsig.2007.12.006. Epub 2007 Dec 14. PMID: 18191382; PMCID: PMC2422835.

27) Foxx CL, Nagy MR, King AE, Albin D, DeKrey GK. TCDD exposure alters fecal IgA concentrations in male and female mice. BMC Pharmacol Toxicol. 2022 Apr 21;23(1):25. doi: 10.1186/s40360-022-00563-9. PMID: 35449084; PMCID: PMC9026712.

28) Lamb JC 4th, Marks TA, McConnell EE, Abeywickrama K, Moore JA. Toxicity of chlorinated phenoxy acids in combination with 2,3,7,8-tetrachlorodibenzo-p-dioxin in C57BL/6 male mice. J Toxicol Environ Health. 1981 Nov-Dec;8(5-6):815-24. doi: 10.1080/15287398109530117. PMID: 7338943.

29) Lamb JC 4th, Moore JA, Marks TA, Haseman JK. Development and viability of offspring of male mice treated with chlorinated phenoxy acids and 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Toxicol Environ Health. 1981 Nov-Dec;8(5-6):835-44. doi: 10.1080/15287398109530119. PMID: 7338945.

30) Lamb JC 4th, Marks TA, Gladen BC, Allen JW, Moore JA. Male fertility, sister chromatid exchange, and germ cell toxicity following exposure to mixtures of chlorinated phenoxy acids containing 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Toxicol Environ Health. 1981 Nov-Dec;8(5-6):825-34. doi: 10.1080/15287398109530118. PMID: 7338944.

31) Anitha M., Vijay-Kumar M., Sitaraman S. V., Gewirtz A. T., Srinivasan S. Gut Microbial Products Regulate Murine Gastrointestinal Motility via Toll-Like Receptor 4 Signaling. Gastroenterology. 2012 (143): 1006-1016.e4.

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- Streamlined sample documentation by implementing new data organizational strategies.

PUBLICATION

Anitha Vijay, Nina R. Boyle, **Supriya M. Kumar**, Gary H. Perdew, Shanthi Srinivasan, Andrew D. Patterson (2023). Aryl Hydrocarbon Receptor Activation Affects Nitrergic Neuronal Survival and Delays Intestinal Motility in Mice. *Toxicological sciences: an official journal of the Society of Toxicology*, 1-12.

CLINICAL EXPERIENCE

Volunteer **State College, PA**
Mount Nittany Medical Center—310+ hours *January 2016-August 2023*

- Serve 25 patients per shift in the Women and Children's Services Unit by instigating regular wellness checks, aiding nurses with equipment transport, and safely discharging patients.
- Train new volunteers on hospital volunteering rules, HIPAA confidentiality, and placenta samples protocols.
- Honored with Suzanne C. DeTuerk Teen Volunteer Scholarship Award (2019) for distinguished service.

Certified NHA Phlebotomy Technician *December 2022-Present*

LEADERSHIP EXPERIENCE

Resident Assistant **University Park, PA**
Eastview Terrace (Living Learning Community), Penn State Residence Life *September 2021-May 2023*

- Counseled and supported residents, mediated roommate conflicts, and served as a resource for 85 residents.
- Coordinated with staff to organize engaging community events that fostered a safe and inclusive environment.
- Strongly exercised ethical decisions following protocols for time-sensitive situations pertaining to residents' needs.

Fundraising Chair **University Park, PA**
Penn State Natya (Indian Classical Dance Team) *September 2020-January 2023*

- Collaborate on Indian classical dance (*Bharatanatyam*) pieces and perform at campus events.
- Spearhead annual fundraising opportunities to raise $3,000 for dance competitions through community events.