POLYHALOGENATED ORGANOPHOSPHATE FLAME RETARDANTS AND DEVELOPMENTAL NEUROTOXICITY: A 21ST CENTURY PERSPECTIVE

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Abstract

Ubiquitous exposure to environmental chemicals has come to define contemporary life, although the role of these exposures has only recently been appreciated in the etiology of neurodevelopmental diseases. Organophosphate flame retardants are one such implicated group of chemicals, and as such have faced renewed regulatory scrutiny over the magnitude of risk they pose to the developing human brain. Large epidemiological studies of maternal exposure as well as direct exposures in school-aged children have consistently associated organophosphate flame retardants with clinical indicators of abnormal neurodevelopment. However, the dual challenges of incomplete understanding of these disorders and discordant data in conventional toxicological research has complicated efforts to determine a causal role for flame retardants in the etiology of neurodevelopmental disease.

This investigation aims to determine whether organophosphate flame retardants classified by shared functional, structural, physicochemical, and biological properties exhibit sufficient concordance across a battery of developmental neurotoxicity (DNT) assays in the human induced Pluripotent Stem Cell (iPSC)-derived microphysiological brain model (BrainSpheres) to justify a single assessment of human neurodevelopmental hazards for the entire subclass. The subclass-based approach recommended by the National Academies of Sciences, Engineering, and Medicine was used to design the exposure experiments. At 4 weeks of differentiation, BrainSpheres were exposed for 7 days to three flame retardants in the Polyhalogenated Organophosphate subclass (tris(2-chloroethyl) phosphate—TCEP, tris(1,3-dichloro-2-propyl)phosphate—TDCPP, and tris(2-chloropropyl) phosphate—Tris) and one phased out brominated flame retardant (2,2',4,4'-Tetrabromodiphenyl ether—BDE-

47) at concentrations found in human serum (10 and 20 μM). The results indicate that these compounds impair neurite quality and show a tendency toward minor upregulation in gene expression of microtubule-associated (*TUBB3*, *MAP2*) and functional (*PSD-95*, *GRIN1*, GRIN2a). At 10 and 20 μM exposure, polyhalogenated organophosphate flame retardants do not appear to elicit as strong a toxic effect as BDE-47, the primary congener of polybrominated diphenyl ether (PBDE), which has been removed from the market mainly due to its toxicity to the developing brain.

While these findings should still be considered preliminary, evidence suggests that constituents of the PHOP subclass are both capable of perturbing key events in neurodevelopment and do so with a sufficiently similar magnitude and directionality to justify regulatory consideration of co-exposures in a cumulative human risk assessment.

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Abbreviations

AChE	Acetylcholinesterase
ADHD	Attention deficit / hyperactivity disorder
AOP	Adverse Outcome Pathway
ASD	Autism spectrum diseases
BDE-47	2,2',4,4'-Tetrabromodiphenyl ether
CPO	Chlorpyrifos oxon
CPSC	U.S. Consumer Product Safety Commission
DNT	Developmental neurotoxicity
EPA	U.S. Environmental Protection Agency
NASEM	National Academies of Science, Engineering and Medicine
NTP	U.S. National Toxicology Program
OP	Organophosphate
OPFR	Organophosphate flame retardant
PHOP	Polyhalogenated organophosphate flame retardant
TCEP	Tris(2-chloroethyl) phosphate
TDCPP	Tris(1,3-dichloro-2-propyl)phosphate
Tris	Tris(2-chloropropyl) phosphate

Chapter 1

Introduction

Over the last half-century, the role of chemical exposure in the etiology of human diseases, including neurodevelopmental disorders, has become increasingly clear (Landrigan et al. 2017). The developing human brain is remarkably vulnerable to environmental perturbation *in utero* and throughout childhood and adolescence, with windows of elevated susceptibility occurring at different times, in different cell types, and across different regions of the brain (Grandjean and Landrigan 2014; Lanphear 2015). Perturbations to the proper sequence of neurodevelopmental events can permanently alter neurobehavioral, neurocognitive, and neuromotor outcomes for the affected individual (Landrigan et al. 2017). This is especially relevant because epidemiological studies have identified an increasing prevalence of developmental disabilities, with worrisome consequences for individual and public health (Zablotsky et al. 2019).

Evidence implicates exposure to industrial chemicals, pesticides, and additives in consumer goods as risk factors for neurodevelopmental disorders including autism spectrum diseases, attention deficit/hyperactivity disorder, intellectual disability, communication disorders, learning disorders, and motor disorders (Landrigan et al. 2017). Organophosphate flame retardants are one such implicated class of chemicals. Initial concerns about potential hazards have been raised due to similarities between the compounds' molecular structure and that of organophosphate insecticides, which are known developmental neurotoxins (Hogberg et al. 2021). Organophosphate flame retardant metabolites have also been associated with reductions in intelligence and working memory in large cohort studies (Castorina et al. 2017). Epidemiological data indicates near universal human exposure in

developed countries (NASEM 2019). However, the dual challenges of incomplete understanding of neurodevelopmental disorders and discordant data in toxicological research complicates efforts to determine a causal role of flame retardants in elevating neurodevelopmental disorders (Ibid).

Conventional methods of assessing human neurodevelopmental toxicity are particularly limited in their ability to predict adverse human health effects at a corresponding dose, due in part to limitations in modeling the complexity of higher-order cognition and behavior (Smirnova et al. 2014). Animal testing also tends to be prohibitively expensive in terms of time, resources, and animals sacrificed in a single study, which has contributed to developmental neurotoxicity's position as one of the least-evaluated human health endpoints (Ibid). Consequently, fewer than 1% of chemicals have been assessed for DNT (EPA 2018). Thus, there is a demonstrated need to modernize the assessment of commercially important chemicals for potential hazards to human health, including neurodevelopmental health (NASEM 2019; Landrigan et al. 2017).

New Approach Methodologies (NAMs), including *in silico* modeling and human stem cell-based microphysiological systems, may help advance scientific understanding around the potential neurodevelopmental hazards of flame retardants and other chemicals (Smirnova et al. 2014). Utilizing a class-based approach is one valid method of evaluating human health hazards across structurally, biologically, and functionally related chemicals, and may be a particularly useful way to assess the thousands of chemicals on the market with yet-uncharacterized toxicity (NASEM 2019). Specifically, the National Academies of Science, Engineering, and Medicine has proposed fourteen subclasses of related organohalogen flame retardants, including the polyhalogenated organophosphate subclass, which may be reliably evaluated for shared human health hazards (Ibid). Employing 21st century

toxicological tools that are directly relevant to human health outcomes will aid in determining whether data supports regulating the 22-member PHOP subclass on the basis of developmental neurotoxicity.

Chapter 2

Developmental Neurotoxicity

NEURODEVELOPMENTAL DISORDERS

The fifth edition of the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders* classifies neurodevelopmental disorders as a group of related conditions in which delayed or insufficient achievement of neurobehavioral, neurocognitive and neuromotor milestones impair functionality in personal, social, academic, or workplace settings (American Psychiatric Association, 2013).

These deficits can range in severity from mild, isolated delays in acquiring specific skills to profound, gross functional impairments (Ibid). Importantly, diagnostic criteria require symptom onset during the early developmental years, most often before the child reaches school-age (Ibid). DSM-V recognizes six broad families of conditions that fall under the umbrella of neurodevelopmental disorders: Autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), communication disorders, intellectual disorders, motor disorders, and specific learning disorder (Ibid). The clinical signs and symptoms of these disorders are detailed in Table 1. Evidence in support of the relatedness of the neurodevelopmental cluster of disorders points to the collective occurrence of disorder-specific genetic risk factors, a similar clinical phenotype of abnormal neurodevelopment with early onset and limited remission, and a high co-occurrence of within-cluster diagnoses (Ibid).

Table 1: General signs and symptoms of neurodevelopmental diseases. [a] CDC 2019; [b] American Psychiatric Association 2013.

Neurodevelopmental Disorder	Signs and Symptoms
Autism Spectrum Disorder	• Persistent deficiencies in social, emotional, and communication skills,[a] which impede the individual's capacity for nonverbal communication, social reciprocity, relationship building, and other vital social interactions, accompanied by restrictive, repetitive behaviors, an elevated preference for maintaining routine, and sensory sensitivity.[b]
Attention-Deficit/Hyperactivity Disorder Attention-Deficit/Hyperactivity Disorder Other Specified Attention-Deficit/Hyperactivity Disorder Unspecified Attention-Deficit/ Hyperactivity Disorder	• A degree of inattention, disorganization, hyperactivity and impulsivity that is inappropriate for the individual's age, complicating efforts to stay on task, keep track of items, and exercise patience. [b]
Communication Disorders Language Disorder Speech Sound Disorder Childhood-Onset Fluency Disorder (Stuttering) Social (Pragmatic) Communication Disorder Unspecified Communication Disorder	• Persistent and significant linguistic challenges, including a limited vocabulary, reduced ability to apply proper grammar and sentence structure or connect sentences to convey meaning. [b] • Cannot be explained by a co-occurring neurological, intellectual, or sensory disability or environmental deficit, such as the absence of age-appropriate education. [b]
Intellectual Disabilities Intellectual Disability Global Developmental Delay Unspecified Intellectual Disability	• Impaired intellectual and adaptive functioning, which can include difficulty with abstract reasoning, learning, planning, judgement, social competency, and self-management. [b] • Scoring two or more standard deviations below the population mean on a standardized intelligence test, equating to an IQ of roughly 65 to 75 or below. [b]
Motor Disorders Developmental Coordination Disorder Stereotypic Movement Disorder Tic Disorders	• Persistent and significant challenges acquiring and performing acts of coordination and fine motor movements, [b] or; • Repetitive, purposeless movement, such as rocking or hand waving, [b] or; • Persistent random movement or vocalizations which cannot be explained by a co-occurring disease, such as Parkinson's Disease, or substance use or abuse. [b] • Cannot be explained by a co-occurring neurological, intellectual, or sensory disability. [b]
Specific Learning Disorder	• Persistent and significant challenges acquiring and applying academic skills, such as reading, spelling, grammar, or mathematics. [b] • Cannot be explained by a co-occurring neurological, intellectual, or sensory disability or environmental deficit, such as the absence of age-appropriate education. [b]

While the etiology of many neurodevelopmental disorders is poorly understood, studies indicate a strong genetic component for ASD and ADHD. Several hundred candidate genes and chromosomal regions have been identified as contributing to the risk of ASD (Landrigan et al. 2012; Rylaarsdam and Guemez-Gamboa 2019) and are largely involved in

metabolism, chromatin remodeling, mRNA regulation, protein synthesis, and synapse formation and function (Gilbert and Man 2017). However, individuals with similar pathogenic variants can have drastically different symptom presentation and degree of functionality, and no genetic factor has been consistently observed in all autistic individuals (Rylaarsdam and Guemez-Gamboa 2019). It has been estimated that a combination of genetic factors accounts for 30% to 40% of ASD risk (Landrigan et al. 2012). Similarly, genome-wide association studies indicate that numerous genetic variants have a marginal contribution to ADHD risk, although only one third of the observed heritability can be attributed to genetics alone (Faraone and Larsson 2019). More generally, 3% of developmental disorders can be attributed to environmental exposures, while a further 25% may be attributable to the interaction of genetic and environmental risk factors (National Research Council 2000).

The prevalence of these disorders, particularly ASD and ADHD, is increasing at a rate that suggests expanded diagnostic criteria and increased disease awareness cannot account for the surge in case numbers (Tran and Miyake 2017). The U.S. Environmental Protection Agency (EPA) reports that 6.3% of children had an ADHD diagnosis in 1997, compared to 10.7% in 2017, amounting to a 70% increase over two decades (EPA 2019a). This trend is more pronounced for children with ASD diagnoses, which increased more than twenty-fold over the same timeframe, from 0.1% in 1997 to 2.3% in 2017 (EPA 2019a). Together, ASD and ADHD now account for 64% of childhood developmental disabilities in the United States (Zablotsky et al. 2019).

The precise source of this increase remains elusive. A Danish study estimated that changes in diagnostic criteria and reporting together account for 60% of the observed increase in ASD diagnoses (Hansen et al. 2015). The remaining fraction likely constitutes a true increase in prevalence. Hypothesized sources for this remaining increase include complex

interactions between genetics or epigenetics and environmental, cultural, and socioeconomic factors such as advancing paternal age and greater survival of premature infants (Tran and Miyake 2017; Bishop 2014; Hultman et al. 2011; Leavey et al. 2013).

CLINICAL AND EPIDEMIOLOGICAL INDICATORS OF TOXICITY

While numerous genetic, nutritional, infectious, and chemical teratogens were described by the nineteenth or mid-twentieth century, the impact of environmental toxicants on cognitive and behavioral functionality remained relatively unstudied until the 1970s (National Research Council 2000). Often, early discoveries of developmental toxicity followed environmental disasters involving acute high-dose maternal exposure (Lanphear 2015; Grandjean and Landrigan 2014).

For example, after industrial wastewater was released into Minamata Bay, Japan between 1932 and 1968, contaminated seafood exposed pregnant mothers to high levels of methylmercury and children in the region were born with severe intellectual impairment and motor disfunction (Lanphear 2015). In separate incidents in Japan and Taiwan in 1968 and 1979, children exposed *in utero* to cooking oil contaminated by polychlorinated biphenyls (PCBs) exhibited hyperpigmentation, nail and dental abnormalities, and poor cognitive development, as measured by 5 point score reductions on both the Stanford-Binet test and the revised Wechsler Intelligence Scale for Children (Lanphear 2015; Cheng et al. 1992; Miller 2004). Clinicians had long described learning and behavioral disorders in children who survived acute lead poisoning (Byers and Lord 1943), but studies in the 1970s and 1980s began identifying behavioral, cognitive, concentration, and memory deficits in children with elevated blood-lead levels that did not reach contemporary definitions for acute poisoning

(Gorini et al. 2014). It is now understood that no lower threshold for a safe level of lead exposure exists for children (Grandjean and Landrigan 2014).

More recently, information arising from large prospective birth cohort studies have enabled epidemiologists to detect neurotoxicants that elicit more subtle impacts (Grandjean and Landrigan 2014; Luo et al. 2010). Although the breadth of these findings cannot be completely explored within this paper, the following examples provide a brief overview of recent advancements in scientific understanding of the link between early life environmental exposure and neurodevelopmental disease.

A 2014 review of epidemiological and experimental literature conclusively identified eleven human developmental neurotoxins: lead, methylmercury, polychlorinated biphenyls, arsenic, toluene, manganese, fluoride, chlorpyrifos, tetrachloroethylene, dichlorodiphenyltrichloroethane, and polybrominated diphenyl ethers (PBDEs) (Grandjean and Landrigan 2014). More suspected developmental neurotoxins have been investigated in the years since.

A longitudinal study of 265 children found associations between prenatal exposure to the organophosphate (OP) pesticide chlorpyrifos, as measured through umbilical cord blood concentrations at delivery, and reductions in IQ, and working memory (Rauh et al. 2011). Subsequent investigations using cohorts from the United States and Europe have corroborated these findings (Grandjean and Landrigan 2014).

A systematic review of the association between early-life exposure to environmental toxicants and ASD found strong evidence for general air pollution and pesticides as ASD risk factors, and moderate evidence for phthalates, PCBs, solvents, and toxic waste sites as

ASD risk factors (Rossignol et al. 2014). However, the authors note that the conclusions of many of these studies may be biased by limited sample size and retrospective design (Ibid).

A recent review of 19 large prospective cohort studies indicates that prenatal and childhood exposure to PBDEs is associated with hyperactivity and misconduct, inattention, and impaired executive function (Vuong et al. 2018). Evidence supporting an association with ASD, impaired social behavior, adaptive skills, and internalizing disorders was inconclusive (Ibid). An editorial co-authored by Dr. Linda Birnbaum, former Director of the National Institute for Environmental Health Sciences, notes that prospective studies have indicated associations between IQ reduction, dyslexia, and ADHD and lead, methylmercury, OP insecticides, organochlorine insecticides, polycyclic aromatic hydrocarbons, bisphenol A (BPA), brominated flame retardants, and perfluorinated compounds (Landrigan et al. 2012).

Even in spite of mounting evidence of environmental risk factors for neurodevelopmental disorders, epidemiological studies alone are insufficient to establish a causal role in disordered development (Smirnova et al. 2014).

EARLY LIFE SUSCEPTIBILITY

It is well established that the developing brain is exceptionally sensitive to environmental perturbation, including perturbation due to exogenous exposures (Landrigan et al. 2012; Grandjean and Landrigan 2014). Fetuses and young children are at greatest risk of harm from exposure to exogenous chemicals due to several factors, including permeability of anatomical barriers, immature metabolism and excretion, small body weight relative to exposure dose, and unique windows of susceptibility that accompany early development (Smirnova et al. 2014; Landrigan et al. 2012; Grandjean and Landrigan 2014).

The placenta does not prevent toxic compounds from reaching the developing fetus, despite earlier hypotheses to the contrary (Lanphear 2015; National Research Council 2000). All lipid-soluble compounds are believed to be capable of crossing the placenta, while other physicochemical properties including molecular weight below 500 g/mol, relationships between blood pH and chemical dissociation, and a low degree of protein binding facilitate movement across the placenta (National Research Council 2000; EPA 2015a; Griffiths and Campbell 2014). Additionally, some compounds have been observed to accumulate in fetal blood (Griffiths and Campbell 2014) and the fetal portion of the placenta (Ruis et al. 2019) in concentrations greater than those observed in the mother.

Formation of tight junctions in the endothelial layer of cerebral vasculature, which impart functionality to the blood-brain barrier, occurs contemporaneously with angiogenesis (Moretti et al. 2015; Malaeb et al. 2012; Møllgård and Saunders 1986) as early as gestational week 8 in humans (Moretti et al. 2015). However, either neuroblast, pericyte, or astrocyte differentiation is believed to induce blood-brain barrier formation, so early molecular events preceding this induction may occur in absence of protection (Ibid). Additionally, the blood-brain barrier exhibits increased permeability through gestation, likely due to enhanced protein- and receptor-mediated transportation across cells (Møllgård and Saunders 1986; Moretti et al. 2015; Lanphear 2015).

Given similar exposure scenarios, reduced metabolic capacity and small body size equates to a larger internal dose reaching molecular targets. Biotransformation enzymes including cytochrome P450s are differentially expressed in the fetus compared to the adult, and enzymes involved in glucuronidation are absent before birth (Blumenfeld et al. 2009). Studies also indicate that infants have limited activity of the enzyme paraoxonase-1 (PON1), which plays a role in the breakdown of OPs (Chen et al. 2003).

Together, these factors may render the developing brain particularly susceptible to neurotoxic metabolites and exogenous chemicals during key growth and developmental processes, including neuronal proliferation, migration, differentiation, neurite outgrowth, synaptogenesis, myelination, network formation, brain structure formation, and synaptic pruning (Fritsche et al. 2018; Lanphear 2015). As neurodevelopmental disorders are currently understood, disability arises through disruption to one or more of these molecular processes (Andrews et al. 2009; Fritsche et al. 2018). These key events occur during different times, in different cell types, and in different regions of the brain, yielding distinct windows of susceptibility to environmental insult (Lanphear 2015).

ENVIRONMENTAL DYSREGULATION OF EARLY BRAIN DEVELOPMENT

No single molecular mechanism is responsible for developmental neurotoxicity, and in fact a single toxicant can produce toxicity through numerous pathways. Lead is a classic example of such a compound. Lead increases the spontaneous release of neurotransmitters and blocks the induced release of depolarization-induced neurotransmitters by inhibiting and mimicking calcium (Bressler and Goldstein 1991). This can have deleterious effects on synaptogenesis and synaptic pruning, the latter of which is heavily dependent on neural activity patterns and may alter cognitive ability and behavior (Ibid). Calcium mimicry also disrupts intracellular signaling cascades controlling proliferation, differentiation, and the apical endpoints of memory and learning (Ibid). Lead has also been shown to reduce myelination by interfering with 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase), an essential enzyme for myelin synthesis, and causing gross morphological abnormalities in oligodendroglia, which produce myelin in the central nervous system (Lidsky and Schneider 2003). Lead can accumulate in neuronal mitochondria, inducing apoptosis or making cells

more susceptible to excitotoxicity from overstimulation (Ibid). Moreover, as described in adverse outcome pathway (AOP) 13 in the AOP-Wiki, lead can act as an antagonist for the glutamatergic NMDA-receptor and can lead to decreased synaptogenesis, decreased neuronal network formation and ultimately impairment of learning and memory (Sachana et al. 2018).

There is evidence that ASD pathogenesis may involve changes in neural network functionality that are attributable to alterations in pre-or postsynaptic proteins and receptors in both excitatory and inhibitory neurons (Gilbert and Man 2017). Both macrocephaly and microcephaly have been observed in substantial portions of ASD patients, which may be due to increased neurite outgrowth, deficient neurite pruning, increased neuronal proliferation or reduced apoptosis (Ibid). Abnormal lamination of cortical projection neurons has also been observed. Both lamination and neuronal proliferation are dependent on proper neural progenitor proliferation and pruning, which begin *in utero* and continue through early childhood (Ibid). Finally, cerebral dysplasia observed in multiple brain regions of ASD patients implicates abnormal neurogenesis, neuronal migration, and neuronal interaction with radial glia (Ibid).

Disruption of dopaminergic neuron activity and dysregulation of catecholamine signaling in the prefrontal cortex have both been hypothesized as contributing factors to impaired attention and behavior in ADHD (Brennan and Arnsten 2010). Brain imaging studies have also identified a reduction in white matter volume and alterations to certain brain structures, which may be attributable to some of the same cellular and molecular processes hypothesized to increase ASD risk (Curatolo et al. 2010).

Developmental neurotoxicants can also elicit toxicity by disrupting estrogenic and thyroid hormone signaling, which can have consequences for the developing fetus (Lanphear 2015). Dichlorodiphenyl trichloroethane (DDT), PCBs, PBDEs, phthalates, and BPA have been found to act in this way (Ibid). It is well established that maternal thyroid dysregulation, whether through hypothyroxinemia, hypothyroidism, thyroid autoimmunity, or iodine deficiency, can have negative impacts on child health, including reduced IQ, slowed reaction time, and verbal communication delays (Moog et al. 2015). Environmental exposures that interfere with the fetal or maternal hypothalamic-pituitary-thyroid (HPT) axis are anticipated to have similar outcomes (Ibid). For instance, environmental inhibition of the thyroperoxidase enzyme activity impairs the synthesis of thyroid hormone T4, which is essential to normal development of the hippocampus (Crofton et al. 2019). Reductions in TH-dependent gene expression leads to abnormal neuronal activity, synaptic integrity, neuronal plasticity in the hippocampal region, which plays a major role in memory and learning (Ibid).

Several exogenous compounds have also been observed to induce epigenetic modification through the alteration of methylation patterns, as is the case for air pollution, arsenic, lead, diethylstilbestrol, tobacco, and BPA (Lanphear 2015). These changes are heritable when they occur in the germline, which may pose generational effects if an inappropriately methylated gene is silenced, or a temporally expressed gene is persistently demethylated (Tran and Miyake 2017). Consequently, a compound that does not directly act as a neurotoxicant itself may still be capable of indirectly altering key developmental processes in offspring or grandchildren. However, few studies have explored the transgenerational effects of environmental chemicals on neurodevelopmental disorders and more remains to be learned (Ibid).

PUBLIC HEALTH CONSEQUENCES

Neurodevelopmental disorders are enduring morbidities that can severely limit quality of life and have profound societal effects. While a mild reduction in IQ on the order of that described for early life exposure to lead (Gilbert and Weiss 2006) or PBDEs (Lam et al. 2017) may be indiscernible on the individual level, population-wide effects are profound. A 5-point reduction in IQ as a result of a ubiquitous environmental contaminant results in a 57% increase in the number of individuals incapable of independently functioning in society (Gilbert and Weiss 2006).

Individuals with impaired cognitive ability have reduced academic achievement and truncated lifetime earning potential, which is not confined to those who fall below the 70 IQ point threshold for intellectual disability (Lanphear 2015). After adjusting for years of schooling, loss of one IQ point has been estimated to reduce lifetime earnings by 2.1% for males and 3.6% for females (Salkever 1995). In the United States, the annual economic burden of ASD alone is estimated to reach $461 billion in medical costs, special education and care, and lost parental productivity by 2025 (Leigh and Du 2015). A recent study estimates a $124.5 billion annual economic burden for ADHD (Zhao et al. 2019). Costs increase for individuals with co-occurrence of intellectual disability (Buescher et al. 2014).

The U.S Centers for Disease Control and Prevention's (CDC) Autism and Developmental Disabilities Monitoring (ADDM) Network reports that white children are 10% to 20% more likely to receive an ASD diagnosis by age 8 than black or Hispanic children, although targeted outreach in minority communities and school-centric policies for universal screening for ASD have narrowed this disparity in recent years (Baio et al. 2018). Socioeconomic status likely plays a role in access to diagnostic tools. Children from households in the

highest tertile of socioeconomic factors – comprised by regional educational attainment, income, and low poverty indication – were diagnosed with ASD 70% more frequently than children from households in the lowest tertile of socioeconomic factors (Durkin et al. 2017). Interestingly, this trend does not appear to hold in European countries where universal access to healthcare eliminates barriers to ASD diagnosis and care (Ibid). In the U.S., these factors may limit the opportunity for early intervention, including speech, behavioral, and physical therapies, which have been proven to improve long-term social, academic, and economic functionality (Reichow and Wolery 2009).

At its core, the environmental contribution to neurodevelopmental disorder burden represents a preventable risk factor for a permanent disability. As data mounts in favor of one or several causal relationships between exogenous chemicals and disordered development, evidence-based prevention programs will be imperative to protecting public health.

CONVENTIONAL DNT ASSESSMENTS

The field of toxicological sciences is in the midst of a paradigm shift away from empirically based animal testing, and towards mechanistically based *in vivo*, *in vitro*, and *in silico* approaches that more directly predict human relevance in less time and with fewer resource demands (Smirnova et al. 2014; Bal-Price et al. 2012). This shift is particularly relevant to assessing developmental neurotoxicity.

For regulatory harmonization, DNT testing traditionally followed three guidance studies from the Organisation for Economic Co-operation and Development (OECD) and EPA. The Developmental Neurotoxicity Study guidelines (OECD TG 426) recommend a rat study that

evaluates the effect of chemical exposure in utero and through the postnatal period on brain histopathology, neuropathology, sexual maturation, cognition, behavior, and motor function, none of which provide clear mechanistic evidence (OECD 2018a). The Extended One-Generation Reproductive Toxicity Study (OECD TG 443) considers a neurodevelopmental cohort of pups under similar exposure conditions and evaluated for changes in behavior and motor function, response to auditory stimulus, and brain histopathology (OECD 2018b). The protocol for the EPA Developmental Neurotoxicity Study (OPPTS 8706300, EPA 712-C-98-239) was adopted in 1998 and has not been updated in subsequent years (EPA 1998). It similarly considers pre- and post-natal maternal exposure and the observed effect on pup sexual maturation, motor activity, response to auditory stimulus, learning and memory, neuropathology, and brain morphometry (Ibid). It is estimated that each completed DNT test requires at minimum 1,000 rat pups and 140 mated females, takes 3 months to conduct, and costs approximately $1.4 million (OECD 2018a; Smirnova et al. 2014). In the absence of regulatory requirements for developmental neurotoxicity testing, discretionary evaluation becomes prohibitively costly and developmental neurotoxicity testing is routinely foregone (Smirnova et al. 2014; Bal-Price et al. 2012).

Even when guideline animal tests are performed, they have limited human relevance. Characterization of behavioral endpoints are subjective and can vary from observer to observer even when operating under the same protocol (EPA 1998; Smirnova et al. 2014). Consequently, reproducibility of *in vivo* developmental neurotoxicity assays is low, even among control groups (Smirnova et al. 2014). Dosing is routinely done at high concentrations not representative of the low-dose chronic exposure scenarios by which most of the population comes in contact with environmental chemicals (Ibid). Additionally, in the absence of mechanistic data generation, the biological plausibility of any observed toxicity remains unknown (Ibid). Measuring so many endpoints in a single study also

presents a problem for inferential statistical analyses, which must be corrected to account for multiple testing scenarios (Ibid). Thus, economics and applicability have driven new enthusiasm for alternative means of assessing toxicity.

NEW APPROACH METHODOLOGIES

Several NAMs have been developed in the past 15 years that facilitate the paradigm shift toward a faster, more human-relevant understanding of toxicity. These methods prioritize the assessment of biological perturbations occurring further upstream, such as altered transcriptional or translational output, protein modifications or cell signaling, allowing researchers to evaluate the toxic effects of chemicals that act on multiple targets or through multiple modes of action (Aschner et al. 2017). Importantly, the resultant advancements in cell culture technology and computational modeling have the dual advantage of reducing dependence on animal models with poor translatability to the human population, and capturing toxicity across levels of biological complexity as it occurs in the target species (Ibid).

The AOP approach has recently gained favor as a simplified organizational framework through which a molecular initiating event is linked to an adverse organism-level or population-level response through a series of causal key events at increasing levels of biological organization (Bal-Price and Meek 2017). Key events in neurodevelopment include cell proliferation, differentiation, maturation, myelination, migration, neurite outgrowth, apoptosis, synaptogenesis, synaptic pruning, signal transduction, and neuronal network formation (Smirnova et al. 2014; Fritsche et al. 2018). In accordance with OECD guidelines, AOPs are not toxicant-specific, but rather describe the plausible biological progression to a pathological state (OECD 2018c). Due to the complexity of neurodevelopmental diseases,

this pathological endpoint is symptom-specific, rather than encompassing the full range of diagnostic criteria of a disease (Bal-Price and Meek 2017). Of the 15 AOPs so far reviewed and endorsed by OECD, six cover perturbed neurodevelopment (OECD 2019).
For example, Sachana and colleagues describe how inhibition of the neuronal N-methyl-D-aspartate (NMDAR) glutamate receptor during synaptogenesis in the hippocampus can yield reduced intracellular calcium levels (Sachana et al. 2016). This impairs the release of brain derived neurotrophic factor (BDNF), which subsequently reduces the presynaptic release of glutamate, promotes cell death, and yields abnormal dendritic morphology (Ibid). Together, these actions reduce synaptogenesis, which impairs synaptic strength, plasticity and ultimately learning and memory formation (Ibid).

Other strategies, including the Pathway of Toxicity model proposed by the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) emphasize a systems approach to hazard identification (Kleesang et al. 2014). The PoT approach abandons the linearity of an AOP in favor of a granular description of cellular and molecular perturbation in the context of whole molecular networks, with the ultimate goal of characterizing an integrated pathway that is both necessary and sufficient to the development of an adverse outcome (Ibid).

Among the *in silico* tools, read-across methodologies utilize data from well-characterized chemicals to predict the activity of metabolically or structural similar chemicals that lack toxicity data (Berggren 2014). Similarity is often established using the Tanimoto coefficient, which evaluates chemical structure's similarity based on the presence or absence in both molecular species to be compared (Chung et al. 2019). The principle that similar chemicals have similar toxicological properties supports calls for class-based chemical regulation, whereby chemicals with similar use scenarios that are anticipated to act on the same molecular targets are treated as interchangeable for risk assessment purposes (NASEM

2019). Several tools exist in the public domain to enable reproducibility and systematic read-across, including the OCED QSAR Toolbox, which draws information about the physicochemical properties, environmental fate and transport, ecotoxicity, and human health hazards of more than 92,000 chemicals from 57 chemical databases (OECD 2020).

Quantitative structure-activity relationship (QSAR) models are computational tools that predict biological effect based on a chemical's structural properties (Heo et al. 2019). Unlike traditional toxicology testing, a QSAR has the benefit of being cost effective, consuming fewer resources, and being high-throughput so many chemicals can be tested against a particular biological endpoint (Heo et al. 2019). The models are based on quantitative output, and so are less susceptible to reporter bias than *in vivo* tests of rodent behavior and cognition, and less susceptible to variability in expert opinion, which complicates read-across (Maertens 2019). QSARs may play an integral role in identifying existing chemicals for further screening in *in vivo* or *in vitro* models, or facilitating the development of less harmful alternatives with Green Chemistry (Maertens 2019; Maertens et. Al 2014).

A novel approach called a read-across-based structure activity relationship (RASAR) that integrates human health hazard endpoints with structural descriptors and nearest chemical analog recently demonstrated a superior ability to predict human toxicity for the nine most common toxicological endpoints than OECD guideline animal testing (Luechtefeld et al. 2018). Efforts are underway to expand such models to other human health endpoints, although more must be understood about neurodevelopmental disease pathology before RASAR models can contribute to this field. (Maertens 2019).

Human cell models have also shown promise as a means of detecting developmental neurotoxicity AOP perturbation (Smirnova et al. 2014; Leist 2017). For example, the UKN1

and UNK2 assays identify early changes in neurodevelopment by gauging altered gene expression that occurs during the maturation of human neuroectodermal progenitor (NEP) cells and neural crest cells, respectively (Leist 2017). The NeuriTox assay uses dopaminergic Lund Human Mesencephalic (LUHMES) cells to characterize altered neurite outgrowth, and the test has the advantage of being employed as a medium- or high-throughput screen (Delp et al. 2018).

Utilizing human cell-based systems has the advantage of eliminating species barriers in developmental timing, toxicokinetics, and toxicodynamics (Fritsche et al. 2018). Obtaining cells from donors with neurodevelopmental disorders additionally creates the possibility of modeling gene-environment interactions in individuals with different genetic makeup (Smirnova et al. 2014).

Of particular relevance to neurodevelopment is the application of organotypic three-dimensional cell cultures, which can be comprised of numerous different cell types and directly model cellular progression through key molecular events in brain development (Zhong et al. 2020; Pamies et al. 2017). For example, the BrainSphere iPSC-derived microphysiological system developed by researchers at Johns Hopkins University includes populations of neurons, astrocytes and oligodendrocytes and is capable of modeling complex processes involved in neuronal differentiation, proliferation, synaptogenesis, spontaneous electrical activity, and neuronal-glial interactions including myelination (Pamies et al. 2017). Using both primary rat cultures and human iPSCs, this model has been used to demonstrate the developmental neurotoxicity of the pesticide rotenone (Pamies et al. 2018), antidepressant paroxetine (Zhong et al. 2020), and most recently, organophosphorus flame retardants (Hogberg et al. 2021).

As a product of the National Toxicology Program's (NTP's) involvement in Tox21, the agency orchestrated the assessment of 91 chemicals with known, suspected, or negative developmental neurotoxicity activity in a battery of *in vitro* and lower-order animal assays (Behl et al. 2019). The data, made publicly available in the Developmental NeuroToxicity Data Integration and Visualization Enabling Resource (DNT-DIVER) tool, facilitates easy comparison across different NAMs evaluating neurodevelopmental endpoints at varying levels of biological complexity (Behl et al. 2019; NTP 2018).

Though federal regulatory agencies have played a major role in both the development and adoption of human health-relevant tools for predictive toxicology and identifying pathways of toxicity through the Toxicology in the 21st Century (Tox21) collaboration, (Behl et al. 2019), the march toward a 21st century understanding of toxicology is dependent upon input from academia, industry, clinicians, and regulatory bodies (Bal-Price et al. 2012). Ultimately, understanding of developmental neurotoxicity will require the strategic integration of *in silico*, *in vitro*, and *in vivo* methods that capture the breadth of molecular, cellular, organ, and organism-level perturbations that precede neurodevelopmental disorder manifestation.

Chapter 3

Flame Retardants

OVERVIEW

Flame retardants are a diverse category of chemicals that slow or prevent the spread of fire by interfering with one or more components in the combustion process (Camino et al. 1991). Flame retardant compounds are united by this common functionality rather than by a single shared chemical structure or molecular composition (NASEM 2019).

The heterogeneity of flame retardant compounds arises from the need to mitigate fire hazards posed by synthetic polymers with distinct chemical and physical properties that operate under dissimilar use scenarios (Levchik 2007; Yang et al. 2019). Thus, regulatory and voluntary industry standards implemented to safeguard life and property against the inherent risks posed by flammable polymers have catalyzed the near ubiquitous use of flame retardants in upholstered furniture, textiles, electronics, building components, vehicles, and many other goods and materials (Lyon and Janssens 2005; NASEM 2019).

Despite their variability, flame retardants are still beholden to the maxim that structure begets function. This disparate group of chemicals can be categorized and subcategorized by their role within the flammable material, mode of achieving flame retardancy, and general chemistry in a manner that makes groupings successively less distinct (NASEM 2019).

GENERAL MECHANISM OF ACTION

At the most general level, flame retardants can be classified as either additive or reactive. In additive form, flame retardants are physically integrated into a product's polymer matrix during or after its manufacture (Morgan 2015). They are not chemically bound to the product, which can pose challenges such as modifying polymer strength, manipulability (Lu and Hamerton 2002) and viscosity (Matzen et al. 2015). Substantial evidence indicates that additive flame retardants of lower molecular weight can escape the product matrix, settling on dust in the indoor environment (Meeker and Stapleton 2010; Yang et al. 2019) and polluting the outdoor environment (Iqbal et al. 2017). In contrast, non-additive, or reactive, flame retardants tend to remain confined within the material matrix for the duration of the product's life (Lu and Hamerton 2002; Morgan 2015). Reactive flame retardants involve either covalently attaching the flame retardant unit to the material it is designed to protect, or engineering wholly new, inherently flame resistant polymers (Lu and Hamerton 2002). As a function of their permanency, both of these means of achieving reactive fire retardancy result in reduced human exposure to the flame retardant chemical and heightened fire-resistant capacity as compared to additive varieties (Morgan 2015). However, inherently flame-resistant polymers also tend to be one to three orders of magnitude more expensive than their flammable counterparts (Lyon and Janssens 2005) and are thus less favored than either the additive or grafted reactive approach (Lu and Hamerton 2002).

Flame retardants are often further classified by one of several general modes of action through which they inhibit the combustion process (Camino et al. 1991). Briefly, under sufficient heat from an ignition source, flammable polymers will degrade into combustible gasses and charred byproducts (Camino et al. 1991; Matzen et al. 2015). If thermal degradation is sustained long enough for the gasses to reach a critical concentration at a

temperature exceeding their ignition temperature, the combustible products will undergo rapid oxidation, resulting in flame, heat, and smoke (Camino et al. 1991). A fire becomes self-sustaining when the thermal oxidation of the polymer and its volatile combustion products produces enough heat to sustain the continued degradation of the flammable material, and thus feed additional fuel to the flame (Ibid). Flame retardants inhibit this process through one or a combination of chemical or physical actions:

1. Free Radical Scavenging: Reaction with the flame retardant creates reducing agents that bind hydrogen and hydroxyl radicals in lower-energy reactions, interrupting the thermal oxidation process (Camino et al. 1991; Levchik 2007).
2. Cooling: The flame retardant undergoes endothermic decomposition, which cools the flammable material enough to interrupt the combustion process (Camino et al. 1991; Morgan 2015).
3. Dilution: The flame retardant releases inert gasses, which dilute oxygen and fuel availability (Levchik 2007; Salmeia et al. 2015).
4. Barrier-Forming: The flame retardant reacts to form a protective barrier – either a carbon-based solid, or an inert gas – which physically prevents the polymer from volatilizing and provides insulation from the heat source or propagation (Levchik 2007; Morgan 2015).
5. Melting: The flame retardant increases the ease with which polymers can flow past one another, removing potential fuel and dispersing heat away from the ignition source (Camino et al. 1991; Matzen et al. 2015).

For example, flame retardants containing the halogens chlorine and bromine act as free radical scavengers in the vapor phase (Chen and Wang 2009). Although halogenated compounds are often used in additive form, they tend to readily mix with the host polymer without significantly altering its physical or mechanical functionality and have the added

benefit of being inexpensive (Ibid). Thus, many common flame retardants contain halogens even though the general structure of the compound may differ (Ibid).

HISTORIC EXPOSURE AND IMPACTS ON HUMAN HEALTH

Concern over human exposure to flame retardants emerged in the 1960s with the discovery of polychlorinated biphenyls (PCBs) in human biosamples (NOAA 2020; Eriksson et al. 2006). PCBs are a class of organic chemicals that had widespread applications as industrial coolants, lubricants, plasticizers and flame retardants between 1929 and 1979 (ATSDR 2014; Eriksson et al. 2006; Oregon DEQ 2003). They are composed of a biphenyl backbone saturated with between one and ten chlorine and hydrogen atoms in 209 possible congeners (Mongillo et al. 2016). While the physical and toxicological properties of each PCB is dependent on its number and position of chlorines (Ibid), PCBs were sold commercially as complex mixtures of all congeners to a varying degree (EPA 2019b; Schulz et al. 1989). Thus, human exposure to PCBs involved exposure to numerous congeners at once (EPA 2019b).

A robust body of evidence has linked PCBs exposure with adverse impacts to the neurodevelopmental, reproductive, endocrine, and immune systems (Korrick and Sagiv 2014; EPA 2019b). In humans and other primates, PCBs have been associated with reductions in immune responses to infection, a smaller thymus gland, a reduction in the rate of conception and live births, lower birthweight, thyroid hormone dysregulation, and impaired visual recognition, memory, and learning (EPA 2019b). Planar PCBs, or those without an ortho-chlorine substitution (Mongillo et al. 2016), are capable of eliciting aryl hydrocarbon receptor-mediated toxicity (Siddiqi et al. 2003). Additionally, the NTP classifies PCBs as “reasonably anticipated to be human carcinogens” (NTP 2016).

Prior to the 1979 ban on manufacturing PCBs in the U.S. (EPA 2019b), the most important routes of exposure were occupational and dietary, with consumers of contaminated wild game and nursing infants exposed to PCBs in breastmilk comprising some of the most heavily exposed populations (EPA 1996). Due to their longevity in the environment and tendency to bioaccumulate, PCBs were later designated as persistent organic pollutants under the 2001 Stockholm Convention (UNEP 2018).

As PCB usage first began to fall out of favor in the 1970s, PBDEs began to dominate the flame retardant market (Eriksson et al. 2006). Statewide furniture flammability standards adopted in California in 1975 helped catalyze the widespread incorporation of additive PBDEs into polyurethane foam found in upholstered furniture (Ibid), although PBDE mixtures were also routinely used in electronics, including televisions and personal computers, building materials, airplanes, vehicles, paints, and plastics (ATSDR 2017; Siddiqi et al. 2003). Studies have found that PBDEs alone comprise 5 to 30% of the final weight of some of these products (Siddiqi et al. 2003). Ingestion of PBDE-contaminated dust in the home and workplace became the primary route of exposure for the general population, accounting for 80 to 90% of total PDBE exposure, while contaminated food comprised the remainder of exposure (ATSDR 2017).

The chemical structure of PBDEs is similar to that of PCBs, with the exception of a single ester bond linking the two phenol groups (Mongillo et al. 2016; Siddiqi et al. 2003) and substitution with bromine instead of chlorine, and so the substances were assumed early on to exhibit similar toxicological effects in humans and the environment (Hardy 2002; Madia et al. 2004). Indeed, rodent studies have found associations between PBDEs and neurodevelopmental harm (ATSDR 2017) including impaired memory and learning (Eriksson

et al. 2006), and endocrine disruption including thyroid and estrogen hormone dysregulation (Siddiqi et al. 2003). Although PBDEs and PCBs are associated with many of the same neurodevelopmental endpoints, *in vitro* studies of human astroglial cells indicate that these two classes of compounds may elicit developmental neurotoxicity through different biochemical pathways (Madia et al. 2004). In the mid-2000s, growing concern over adverse human health effects prompted manufacturers and importers to voluntarily phase the three most widely used PBDE mixtures, penta-, octa- and decaBDE, out of commercial use in the United States (ATSDR 2017; Mongillo et al. 2016).

OPFR use was common by 1940, but market share surged as the class became a common substitute for pentaBDE (Yang et al. 2019). By 2011, global market share reached 15%, and relative production has remained stable over the past decade (Ibid). Studies now indicate that OPFRs are found in indoor environments in higher concentrations than their predecessors, due to the chemical class' preferred use in construction, insulation, refrigeration, electronics, mattresses, furniture and fabrics (Ibid).

Unlike PCBs and PBDEs, OPFRs take the general structure of a phosphoric acid bound to three variable alkyl chains or aryl groups (Blum et al. 2019; Quin 2000). However, like the earlier flame retardants, some of these variable groups can contain halogens (Blum et al. 2019). OPFRs share this structural similarity with OP insecticides, which are known to elicit acute toxicity by irreversibly binding acetylcholinesterase (AChE) and preventing the breakdown of acetylcholine in nerve synapses (Yang et al. 2019). During vulnerable periods early in life, low-dose exposure to OPs, including those used as flame retardants, can permanently alter brain chemistry and behavior (Glazer et al. 2018), although neurodevelopmental toxicity likely acts through a different mechanism than acute AChE toxicity (Yang et al. 2019; Castorina et al. 2017). Human epidemiologic data has thus far tied

OPFR metabolites in prenatal urine with reduced cognition, fine motor skills, expressive language, working memory, and IQ (NASEM 2019).

Clearly, the flame retardant market has been characterized by a history of regrettable substitution in which relatively unstudied materials are substituted for those with known capability of harming human health. The substitutes perform a similar function, often adopt a similar chemical structure, and are later discovered to carry similar risks (Blum et al. 2019). This pattern is not unique to the flame retardant market, and its prevalence has catalyzed support for regulating chemicals on the basis of class and similar use, rather than as individual substances (Ibid).

POLYHALOGENATED ORGANOPHOSPHATE FLAME RETARDANTS

In 2015, roughly a dozen public interest groups petitioned the U.S. Consumer Product Safety Commission (CPSC) to utilize the authority granted to it under the Federal Hazardous Substances Act to prohibit the use of "any non-polymeric, additive organohalogen flame retardant" in four categories of consumer products anticipated to contribute substantially to exposure in the indoor environment: children's products, residential upholstered furniture, mattresses, and electronics (American Academy of Pediatrics et al. 2015). In 2017, CPSC granted the petition and solicited the advice of the National Academies of Sciences, Engineering, and Medicine (the National Academies) in assessing the validity of a class-based hazard assessment (NASEM 2019). The National Academies committee endorsed a subclass approach in which human health hazard assessments could be reliably performed for 14 organohalogen flame retardant subclasses categorized by similar structural,

physicochemical, and biological properties (Ibid). The 22 polyhalogenated organophosphates (PHOPs), listed in Table 2, comprise one such subclass (Ibid).

At present, limited public knowledge exists to inform a risk assessment of the subclass. As part of the mock scoping plan illustrated in the National Academies report, an unrestricted query of the PubChem Database returned no results for 9 PHOPs and 10 or fewer results for 5 PHOPs, though these records were not specifically related to developmental neurotoxicity (NASEM 2019). As part of the present investigation into developmental neurotoxicity, a PubMed query utilizing a search strategy that included chemical name, CAS Registry Number, validated synonyms from ToxCast, and key words specific to developmental neurotoxicity returned no results for 14 PHOPS and 10 or fewer results for 3 PHOPs. The most well-characterized members of this subclass are TDCPP, TCEP, TCPP, and TDBPP (NASEM 2019). All PHOPs except TNP1 and TNP2 were accounted for in the latest version of the OECD QSAR toolbox, but data on physicochemical properties, environmental fate and transport, and human health hazards are absent for roughly three fourths of the subclass members (QSAR Toolbox 2020). Only two PHOPs, TCPP and TCEP, have been evaluated as a part of the DNT-DIVER programming (NTP 2018). As demonstrated in Figure 1, the pair exhibited concordant results in 10 of the 16 assays in which both chemicals were tested (Ibid). Both TCPP and TCEP were positive in two independent dechorionated zebrafish embryo behavioral assays measuring larval movement patterns, and negative in eight assays measuring protein accumulation, reduced viability, increased mortality, and gross structural malformations (Ibid). Subsequently, most of the scientific understanding around the human exposure to and health risks of all PHOPs stem from these four chemicals (Figure 1).

Table 2: Physicochemical properties of the Polyhalogenated Organophosphate Flame Retardant subclass

CAS RN[a]	Chemical Name[a]	Molecular Weight (g/mol)b	LogP Octanol-Water [b]	Water Solubility (mol/L) [b]
13674-87-8	Tris(1,3-dichloropropan-2-yl) phosphate (TDCPP)	430.89	3.65	$1.62*10^{-5}$
115-96-8	Tris(2-chloroethyl) phosphate (TCEP)	285.48	1.44	$2.45*10^{-2}$
13674-84-5	Tris(1-chloropropan-2-yl) phosphate (TCPP)	327.56	2.59	3.66*10-^{3}
126-72-7	Tris(2,3-dibromopropyl) phosphate (TDBPP)	697.613	4.29	$1.15*10^{-5}$
66108-37-0	Bis(1,3-dichloro-2-propyl)-3-chloro-2,2-dibromomethyl-1-propyl phosphate	582.29	4.35 †	7.39*10-^{5} †
19186-97-1	Tris(tribromo-neopentyl) phosphate	1018.46	7.63 †	$1.13*10^{-7}$ †
38051-10-4	2,2-Bis(chloromethyl)-1,3-propanediyl bis(bis(2-chloroethyl) phosphate)	582.97	3.36 †	$1.18*10^{-3}$ †
78-43-3	Tris(2,3-dichloropropyl) phosphate	430.89	3.12 †	7.12*10-^{5} †
6145-73-9	Tris(2-chloropropyl) phosphate	327.56	2.55 †	$1.03*10^{-3}$ †
33125-86-9	Tetrakis(2-chloroethyl) ethane-1,2-diyl bis(phosphate)	472.01	1.65 †	$1.30*10^{-1}$ †
115-98-0	Bis(2-chloroethyl) vinylphosphonate	233.02	1.70 †	$1.47*10^{-2}$ †
5412-25-9	Bis(2,3-dibromopropyl) phosphate	497.74	2.56 †	$1.12*10^{-2}$ †
140-08-9	Tris(2-chloroethyl) phosphite	269.48	1.46 †	$1.93*10^{-2}$ †
4351-70-6	Phosphonic acid, (1-(((2-chloroethoxy)(2-chloroethyl) phosphinyl)oxy)ethyl)-, 1-(bis(2-chloroethoxy)phosphinyl)ethyl 2-chloroethyl ester	610.54	2.48 †	$2.08*10^{-4}$†
6294-34-4	Bis(2-chloroethyl) (2-chloroethyl)phosphonate	269.48	1.48 †	$8.82*10^{-3}$ †
6749-73-1	Tris(1,3-dichloropropan-2-yl) phosphite	414.9 † †	-	-
53461-82-8	Oxydiethylene tetrakis(2-chloroethyl) bisphosphate	516.06	1.51 †	$3.24*10^{-2}$ †
61090-89-9	2,4,8,10-Tetraoxa-3,9-diphosphaspiro[5.5]undecane, 3,9-bis[3-bromo-2,2-bis(bromomethyl)propoxy]-, 3,9-dioxide	873.72	4.85 †	$3.64*10^{-6}$ †
49690-63-3	Tris(2,4-dibromophenyl) phosphate	799.66	7.93	$9.46*10^{-9}$
7046-64-2	Phenol, 2,4,6-tribromo-, phosphate	428.795	4.09	$2.17*10^{-3}$
114955-21-4	Polyhalogenated OP + triazines (phosphonic acid, P-[[(4,6-dichloro-1,3,5-triazin-2-yl)oxy]methyl]-, dimethyl ester) (TPN1)	-	-	-
1373346-90-7	Polyhalogenated OP + triazines (phosphonic acid, (4,6-dichloro-1,3,5-triazin-2-yl)-, diethyl ester (9Cl)) (TNP2)	-	-	-

a NASEM 2019; b EPA ToxCast 2020; † Predicted; † † PubChem 2020.

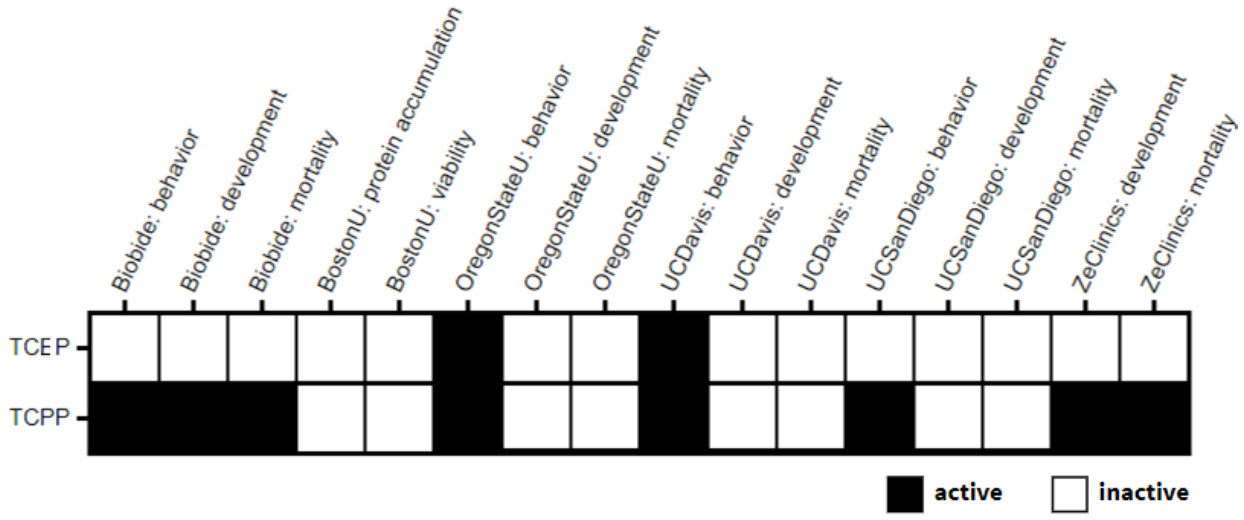

Figure 1: Polyhalogenated Organophosphate Flame Retardants Evaluated in DNT-DIVER exhibit concordance across 10 of 16 assays. Adapted from NTP 2018.

CURRENT PATTERNS OF HUMAN EXPOSURE

PHOPs are incorporated as additive flame retardants and are thus capable of escaping the product matrix (Abou-Donia et al. 2016). EPA's most recent update to the Chemical Data Reporting database identifies eight PHOPs with a diversity of commercial applications, including in construction materials, plastics and rubbers, wood, fabric, textile, leather, adhesive, sealant, foam seating and bedding (EPA 2016). PHOP subclass members have also been identified in smartphone screens (Zhang et al. 2019), and baby products such as changing table pads, nursing pillows, and car seats (EPA 2015b). Notably, several manufacturers declined to provide anticipated use scenarios, even when competitors provided them for the same chemical (EPA 2016). Studies also show that three chlorinated subclass members, TCEP, TCPP, and TDCPP, have been present in wastewater at concentrations over 6,000 µg/L and drinking water in concentrations up to 0.72 µg/L (EPA 2015b).

Several members of the subclass are semi-volatile, and environmental monitoring indicates that PHOPs are present in substantial quantities in household, office, school, and vehicle dust and ambient air samples (EPA 2015b). However, exposure in the indoor environment can vary dramatically. A 2017 study in the United States reported median TCEP, TCPP, and TDCPP concentrations in indoor air at 6.81, 26.3, and 0.372 ng/m^3, respectively, although the total range of exposure sometimes differed by three orders of magnitude (Vykoukalová et al. 2017). A Swedish ambient air study measured TCIPP concentrations as high as 2.2 ng/m^3 in offices and TCEP concentrations up to 0.26 ng m^{-3} in homes, with total airborne OPFR concentrations similar to those found throughout Europe and Asia (Sha et al. 2018).

A negative linear relationship exists between flame retardant log k_{ow} values and skin penetration, with TCEP and TCPP being the most readily absorbed OPFRs (Frederiksen et al. 2018; Abou-Elwafa Abdallah et al. 2016) and dermal absorption ranging from 28% for TCEP to 16% for TDCPP applied to human skin (Abou-Elwafa Abdallah et al. 2016). Unintentional exposure to flame retardants via inhalation, ingestion, and dermal absorption is ubiquitous in the general population (EPA 2015a).

Large biomonitoring studies, including a 2018 evaluation from nationally representative National Health and Nutrition Examination Survey (NHANES) data, have detected OPFR metabolite exposure in the majority of the population (Ospina et al. 2018). Bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) and diphenyl phosphate (DPHP) were detected in approximately 92% of urine samples, bis-2-chloroethyl phosphate (BCEP) was detected in 89% of samples, di-n-butyl phosphate (DNBP) in 81%, bis-(1-chloro-2-propyl) phosphate (BCIPP) in 61%, and di-p-cresylphosphate (DpCP) in 13% of samples, with concentrations ranging up to 4 orders of magnitude (Ibid). Research of exposure in the general population

over the past decade has produced similar findings, with BDCIPP and DPHP as the most ubiquitous metabolites (Ibid).

Of particular concern for neurodevelopmental outcomes is the observation of PHOPs in breast milk (Ma et al. 2019; He et al. 2018; EPA 2015a) and urine samples of pregnant women (Hoffman et al. 2014). Ma and colleagues detected TCEP and TCPP in the breastmilk of U.S. mothers at an average concentration of 0.036 and 0.221 ng/ml, though concentrations reached as high as 0.800 ng/ml TCEP, and 2.51 ng/ml TCPP (Ma et al. 2019). A study of exposure in Australian infants estimated the daily intake of TCEP as 4.6 ng/kg/day from breastmilk (He et al. 2018). A recent investigation into internal exposure of several organophosphate ester flame retardants, including PHOP members TCEP and TDCPP, found plasma concentrations range from below 10^{-6} µM to as high as 31.9 µM (Blum et al. 2019).

No public physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model yet exists for any PHOP subclass member, although in vitro studies have indicated that TCEP is resistant to metabolism in an in vivo salmon model (Arukwe et al. 2018), and that food intake limits oral bioavailability of TCEP and TCPP in an in vitro human gastrointestinal tract model (Quintana et al. 2017).

HUMAN HEALTH HAZARD OF PHOPs

The National Academies committee notes that as a subclass, PHOP hazard data is discordant both within and across species when evaluating chronic toxicity, reproductive and developmental toxicity, mutagenicity, and cancer. While there appears to be a higher degree

of concordance when it comes to neurodevelopmental endpoints alone, data is not entirely in agreement (NASEM 2019).

An increase in degenerative brain lesions after exposure to TCEP was observed in a rat study from the NTP, but not replicated in a subsequent study of TDCPP exposure (EPA 2015a). Reduced cell number and altered differentiation has been found following *in vitro* exposure of rat neuronal cells to all four of the well-characterized PHOP subclass members, while TDCPP was also found to inhibit DNA synthesis and promote oxidative stress (Dishaw et al. 2011). A similar study found that DCIPP and TCEP reduced cell growth, increased apoptosis, reduced neurite outgrowth, and altered both gene expression and production of proteins involved in neuronal development, signal transduction and cytoskeleton formation (Ta et al. 2014). Out of nineteen independent studies reviewed in the National Academies report which evaluated developmental neurotoxicity or altered locomotion in zebrafish exposed to TDCPP, TCEP, TCPP, or TDBPP, only one reported negative results (NASEM 2019).

A battery of *in vivo* and *in vitro* analyses found evidence of reduced neuron firing in rat cortical cell cultures, reduced neurite outgrowth in human hN2 cells but not rat cortical cells, reduced motor activity in larval zebrafish, and no behavioral changes in rat pups exposed to TDCPP (Moser et al. 2014). These conflicting results may be due to poorly conserved developmental biology, inadequate methods of capturing higher cognitive or behavioral function in animals, or differing timing or duration of exposure. EPA does conclusively consider TCEP, TCPP, and TDCPP to be weak AChE inhibitors, a characteristic they share with organophosphate pesticides (EPA 2015a).

Obtaining internal dose-dependent human health data specific to the PHOP subclass is complicated by the fact that PHOPs are hydrolyzed to some of the same diester metabolites as nonhalogenated OPFRs (NASEM 2019). One cross-sectional study instead used silicone passive sampling bracelets to measure 72 preschoolers' exposure to TDCPP, TCPP, TCEP, and one non-PHOP, triphenylphosphate (TPP), and found that cumulative weekly exposure to these compounds was associated with aggression, defiance, hyperactivity, inattention, bullying, and poor responsibility (Lipscomb et al. 2017). Remarkably, the effect size of this association mirrored the effect size of well-established predictors of behavioral problems, such as gender and family context (Ibid). Only early adverse experience, such as parental substance abuse, was a better predictor of behavior than PHOP exposure (Ibid).

A separate EPA assessment of flame retardants designed to replace pentaBDA in furniture foam evaluated the hazard profile of TCEP, TDBPP, TCPP and its isomer, and 2,2-Bis(chloromethyl)-1,3-propanediyl bis(bis(2-chloroethyl) phosphate), and found that for the four human health endpoints relevant to neurodevelopmental outcomes (neurological, developmental, reproductive, and repeated dose toxicity), all PHOPs were classified as a "high" or "moderate" hazard in every endpoint, with the exception of TDCPP and 2,2-Bis(chloromethyl)-1,3-propanediyl bis(bis(2-chloroethyl) phosphate), which were classified as a "low" hazard for neurological toxicity only (EPA 2015a). Subsequently, the 2015 EPA TSCA Work Plan Chemical Problem Formulation and Initial Assessment for TCEP, TCPP, and TDCPP named neurotoxicity and developmental toxicity among "the most significant hazards" from exposure to these flame retardants (EPA 2015b).

In August 2019, EPA designated TCEP as a high-priority substance for hazard assessment, along with two other halogenated flame retardants outside the PHOP subclass (EPA 2019c). As part of the agency's justification for this action, EPA identified 7 reviews from global

health and regulatory authorities, including the World Health Organization (WHO) and the European Chemicals Agency (ECHA), that contained convincing evidence of TCEP neurotoxicity (EPA 2019d).

An underexplored aspect of reconciling discordant human health hazard data is the potential influence of financial interest. Allegations of scientific misconduct have dogged flame retardant industry stakeholders in recent years, following evidence of falsified clinical reports and financial conflicts of interests uncovered during the unravelling of the sector's "Citizens for Fire Safety" campaign (Callahan et al. 2012). The continued potential for bias is not imagined. An analysis of 373 environmental and occupational health studies found that research funded by entities with an interest in the manufacturing, use, or disposal of the compound under study were 4.31 times more likely to report negative findings, and studies funded or undertaken by the military were 9.15 times more likely to report negative findings (Friedman and Friedman 2016). These findings complicate the risk assessment process, which is often reliant on industry-sponsored research as the only available data to inform early appraisal of chemical safety (Hardy et al. 2003).

Ultimately, a comprehensive subclass-based assessment of PHOP hazards to the general public should include evaluations of numerous endpoints relevant to human health, conducted by a variety of stakeholders. It is critical that developmental neurotoxicity data be characterized adequately enough to be considered.

Chapter 4

Modeling PHOP toxicity in human BrainSpheres

Human neurodevelopment begins at gestational week 3 with differentiation of epiblast cells into neural progenitor cells (NPCs), and extends through infancy, childhood, and adolescence (Stiles and Jernigan 2010). Each key point in neurodevelopment – proliferation, differentiation, migration, maturation, myelination, neurite outgrowth, apoptosis, synaptogenesis, synaptic pruning, signal transduction, and neuronal network formation – is governed by a complex array of signal cascades that involve different cell types, occur at different times, and in different regions throughout the developing brain (Stiles and Jernigan 2010; Smirnova et al. 2014; Fritsche et al. 2018). This complexity generates windows of susceptibility throughout the developmental period, which, if perturbed, can result in permanent alterations in brain morphology and physiology (Landrigan et al. 2017).

In vitro studies show that the two most studied members of the PHOP subclass, TCEP and TDCPP have been associated with diminished neurite outgrowth, neuronal differentiation, signal transduction, and increased apoptosis (Dishaw et al. 2011; Moser et al. 2014; Ta et al. 2014). Both are believed to be weak acetylcholinesterase inhibitors (EPA 2015a). Little, if any, public data explores the neurotoxicological profile of the remaining subclass members in any capacity (NASEM 2019). Similarly, few studies have evaluated the association between PHOP subclass members or their metabolites and apical manifestations of neurobehavioral, neurocognitive and neuromotor impairment in humans, but those that have, link subclass members to externalizing behaviors such as aggression and defiance as well as inattention and hyperactivity (Lipscomb et al. 2017). As additional indication of potential neurodevelopmental harm, the subclass shares structural similarity with

organophosphate pesticides, which are neurotoxic at high concentrations and believed to impair neurodevelopment at low concentrations (EPA 2015a).

In light of the permanence and potential severity of poor neurodevelopmental outcomes on both the individual and population levels, it is critical that the scientific community better understand the role that PHOPs may play in the etiology of neurodevelopmental diseases. The extraordinary investment of money, time, and animal life that would be required to perform guideline developmental neurotoxicity studies on each of these poorly described compounds necessitates both the establishment and validation of high-throughput alternative methods to elucidate potential human health hazards, and more confidence in regulating structurally, functionally, and toxicologically related compounds as a group.

The goal of this investigation is to determine whether PHOPs classified by shared functional, structural, physicochemical, and biological properties exhibit sufficient concordance across a battery of developmental neurotoxicity assays to justify a single assessment of human neurodevelopmental hazards for the entire subclass. It was hypothesized that chronic exposure to PHOPs at concentrations currently found in human biosamples is sufficient to perturb neurodevelopment in a human BrainSphere model, and that shared properties among subclass members will result in similar patterns of perturbation of key events in the neurodevelopmental process.

SIGNIFICANCE

The National Academies Committee to Develop a Scoping Plan to Assess the Hazards of Organohalogen Flame Retardants already identified the 22-member polyhalogenated organophosphate subclass as close analogues based on the integration of predicted

structure-activity relationships, cheminformatics, and predicted biological targets (NASEM 2019). As specified in the Committee's report, this subclass is intended to serve as the starting point for subclass-based human health hazard assessments (Ibid). In its 2019 report, the committee did not perform a comprehensive literature review of endpoints specific to human developmental neurotoxicity (Ibid). Additionally, the mode of action by which even the most commonly studied subclass members perturb neurodevelopment remains uncertain. Little, if any, public data explores the developmental neurotoxicity profile of the remaining eighteen subclass members (Ibid). In light of evidence that highly vulnerable populations are chronically and ubiquitously exposed to the PHOPs that have been measured in the environment, and that these exposures may be linked to poor neurodevelopmental outcomes, it is critical that the regulatory community have access to sound scientific data that facilitates a thorough assessment of the human health risks posed by these chemicals.

Biomonitoring data from developed nations consistently indicates that BDCPP and BCEP are among the most ubiquitous OPFR metabolites found in human biosamples (Ospina et al. 2018). While these metabolites can originate from a number of polyhalogenated and unhalogenated parent compounds, they are also the respective products of TDCPP and TCEP metabolism (Abdallah et al. 2015). Moreover, air, dust, and surface samples indicate that TDCPP and TCEP are commonly found in indoor environments and on consumer products where the likelihood of exposure to neonates and pregnant and lactating women is high (EPA 2015b). Robust toxicological and biomonitoring data does not exist for Tris(2-chloropropyl) phosphate (CAS RN 6145-73-9) (TOXCAST 2020; QSAR Toolbox 2020). The predicted LogP octanol-water partition coefficient and water solubility properties falls between the upper and lower bounds of TCEP and TDCPP, enabling the interpolation of related biological behavior (Schultz et al. 2015). This chlorinates Tris also has an average

molecular weight below 500 g/mol (TOXCAST 2020). These physicochemical factors all increase the likelihood that these chemicals can cross the placenta, lending biological plausibility to any observed perturbation of neurodevelopment (Griffiths and Campbell 2014). Thus, this compound was selected as a representative member of the poorly-characterized PHOP members.

INNOVATION

The mechanisms by which PHOPs elicit developmental neurotoxicity remain poorly understood. While this deficiency is detrimental to regulatory efforts to protect the health of vulnerable populations, it also presents an opportunity to employ the NAMs that are facilitating the paradigm shift of toxicology into the 21st century.

This research will focus specifically on toxicological data with immediate human relevance, namely through a human iPSC-derived microphysiological brain model (BrainSpheres). Briefly, the BrainSphere model captures the differentiation of iPSCs into mature glutamatergic, dopaminergic, and GABAergic neurons, astrocytes, and oligodendrocytes in a 3-dimensional environment (Pamies et al. 2017). Developing organoids are capable of recapitulating functional endpoints including axonal myelination, cell-cell interactions, and spontaneous electrical activity that have been largely absent from prior *in vitro* models of human brain development (Ibid).

Although recently published research supported by the Center for Alternatives for Animal Testing (CAAT) laboratory has explored flame retardant toxicity in a rat BrainSphere model, to date, none of the PHOP compounds have been evaluated using the human BrainSphere model (Hogberg et al. 2021). Morphological and functional analysis of BrainSpheres exposed to each of the four PHOP compounds may identify novel evidence in support of a

consistent neurodevelopmental hazard posed by structurally, functionally, and biologically related PHOPs.

METHODS

NPC Culture and Expansion

The BrainSpheres were derived from the NIBSC8-iPSC (N8)-derived neuroprogenitor cells (NPCs). NPCs were cultured and expanded according to a modified Gibco protocol (Gibco 2013). Briefly, cryopreserved NPC stocks were thawed in a 37 °C water bath, resuspended in KnockOut™ DMEM/F-12 (Gibco), and centrifuged for 3 minutes at 3000 x g. Supernatant was discarded and cells were resuspended in complete neural expansion medium (49 mL Neurobasal® Medium (Gibco), 49 mL Advanced™ DMDM/F-12 (Gibco), 2 mL Neural Induction Supplement (Gibco) before being seeded into a 25 cm^2 flask (Thermo Fisher Scientific) coated with Matrigel (Corning) and maintained at 37 °C in 5% CO_2. Culture medium was exchanged every 2 to 3 days.

At 80-100% confluence, NPCs were washed with PBS and treated with Gentle Cell Dissociation Solution (GCDS) (STEMCELL Technologies) for 5 min at room temperature. Before visible detachment of the cells, GCDS was aspirated and replaced with fresh complete neural expansion medium and cells were mechanically detached by scraping and clumps separated by gentle pipetting. NPCs were split into Matrigel-coated 75 cm^2 or 125 cm^2 flasks (Thermo Fisher Scientific) at a 1:3 to 1:5 ratio. Cell number was determined using one-to-one 0.4% Trypan Blue stain (Invitrogen) and a Countess™ Automated Cell Counter. Culture medium continued to be exchanged every 2 to 3 days after splitting.

Differentiation and BrainSphere Formation

Neural differentiation followed the protocol set forth in Pamies et al. 2017. Briefly, at passage 8, NPCs were detached as previously described and plated as a single cell suspension in 3 mL complete neural expansion medium in clear TC-treated 6 well plates (Falcon) at a density of $2.0x10^6$ cells per well. Cells were maintained at 37 °C in 5% CO_2 under constant gyratory shaking (88 rpm, 50 mm orbit diameter) to allow for aggregate formation. After 48 hours, medium was changed to differentiation medium (Neurobasal™ Electro (Gibco), 1x Penicillin-Streptomycin-Glutamine (Thermo Fisher), 1x GlutaMAX Supplement (Thermo Fisher), 1x B-27 Electro Supplement (Gibco), 5 µg GDNF (GeminiBio), 5 µg BDNF (GeminiBio). Half of the culture medium was exchanged every 2 to 3 days, taking care not to expose spheres to air. Each experimental run is comprised of BrainSpheres differentiated at the same time, from the same passage of NPCs. The data presented here are derived from assays performed on runs 21 and 23 (R21 and R23).

Exposure

Stock solutions of experimental compounds were prepared in 0.1% DMSO, a concentration known to be nontoxic in the human BrainSphere model. In the initial range finding experiment, flame retardant concentrations of 5, 10, or 20 µM were selected for evaluation based on recent estimates of PHOP plasma concentrations in the general population reaching as high as 31.9 µM (Blum et al. 2019). As anticipated, no internal exposure estimates could be located for the poorly characterized PHOP, Tris. BDE-47, the most abundant congener of the flame retardant pentaBDE – which has been banned under the Stockholm Convention for bioaccumulation and evidence of neurodevelopmental toxicity – was used as a comparator (UNEP 2006). Following the results of a range-finding

experiment, two non-cytotoxic concentrations, 10 µM and 20 µM, were selected for the main experiment.

At two weeks (range finding) or four weeks (main experiment) after induction of differentiation, healthy aggregates were distributed across 6-well plates, to generate 4 biological replicates per experimental condition with roughly 100 BrainSpheres in 2 mL differentiation medium containing the chemical of interest at 0, 5, 10, or 20 µM. By two weeks post differentiation, markers for GABAergic, dopaminergic, and glutamatergic neurons are positive in the model, and neurons show indications of synaptic activity (Pamies et al. 2017). By four weeks, unpublished CAAT laboratory investigations into receptor kinetics indicate the presence of the glutamate NMDA receptor subunits GRIN1, GRIN2A.

Exposure medium was completely exchanged every 2 to 3 days until collection, and BrainSpheres were gently pipette up and down in new medium to ensure no BrainSpheres fused together while briefly exposed to air. BrainSpheres were collected following one week of exposure (Figure 2).

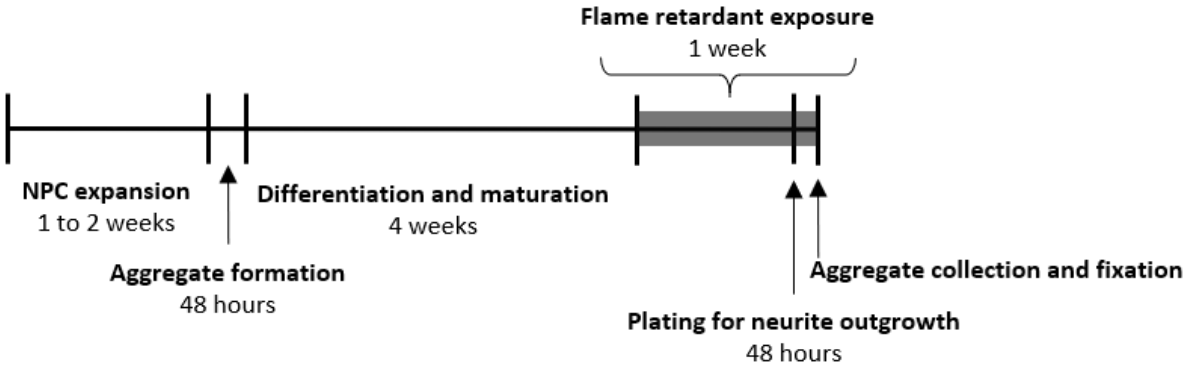

Figure 2: Experimental timeline depicting NPC expansion, BrainSphere aggregate formation, differentiation of neural and glial cells, exposure to experimental compounds, and sample collection.

Viability

The resazurin assay was performed for the range finding experiment only (R21, 2 week of differentiation). On exposure day 7, 10 ± 4 spheres were transferred to a clear, untreated 96 well plate (Corning) in 100 µL exposure medium, with 3 replicate wells per condition. 10 µL of 1 mg/mL Resazurin dye (Signa-Aldrich) was added to each well. The plate was incubated in the dark for 3 hours at 37 °C in 5% CO_2. Fluorescence was measured at 590 nm with a CytoFluor® Series 4000 Multi-Well fluorescence microplate reader (PerSeptive Biosystems) and adjusted based on the number of aggregates in each well. Data was analyzed using Prism 9.0.0 (GraphPad). Data are presented as percentage of mean condition fluorescence versus control. One-way ANOVA test with Bonferroni's multiple comparisons test was performed to determine statistical significance ($p \leq 0.05$).

Acetylcholinesterase Inhibition Assay

On day 7 of exposure, half of the BrainSpheres from each well were collected in labelled 1.5 ml tubes and lysed in lysis buffer (0.3 g NaCl, 1 mL of 1 M Tris, PH 7.5, 1 mL 10% NP-40, 0.2 mL of 0.5 M EDTA at pH 8.0, 17.8 mL ddH2O (standard reagents from Thermo Fisher and Sigma-Aldrich)) for 30 min at room temperature, then centrifuged at 600 g for 5 min. Positive control aggregates exposed for 24 hours to 100 µM chlorpyrifos oxon (CPO), a known AChE inhibitor, were processed in the same manner. The acetylcholinesterase (AChE) assay (Abcam) was performed in a 96 well plate according to manufacturer protocol. Briefly, 50 µL of AChE assay mixture was added to each well containing either 50 µL of lysate, AChE standards, or blank controls. The plate was covered with foil to protect from light and incubated for 20 minutes at room temperature. Fluorescence was measured with an Epoch Microplate Spectrophotometer at ex/em 540/590 nm. Data was analyzed using Prism 9.0.0. AChE activity was calculated from a simple linear regression of the standard

curve and adjusted against sample protein content. Data are presented as the fold change of mean RFU ± SEM. One-way ANOVA test with Bonferroni's multiple comparisons test was performed to determine statistical significance ($p \leq 0.05$). Data presented are from R21.

Total Protein Quantification

The total protein Pierce™ BCA Protein assay (ThermoFisher) was performed according to manufacturer protocol. Briefly, 200 µL of working reagent was added to 10 µL of lysed samples or bovine serum albumin (BSA) standard in a clear 96 well plate (Corning). All samples and standards were measured in duplicate wells. The plate was covered with foil to protect from light and placed on a shaker for 30 seconds before incubating for 30 minutes at 37 °C. Fluorescence was measured with an Epoch Microplate Spectrophotometer at 560 nm. Duplicate sample fluorescence was averaged, and protein concentration was calculated from the standard curve. Data presented are from R21.

Immunocytochemistry

All washing and staining steps were performed in 24 well plates. On day 7 of exposure, BrainSpheres were collected and washed once with cold PBS, fixed in 4% paraformaldehyde for 45 minutes, and washed twice with washing solution I (1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS) before incubating for 1 hour in blocking solution (10% normal goat serum (Rockland), 1% BSA, 0.15% saponin (Millipore) in PBS) on a shaker. BrainSpheres were stained for 48 hours at 4 °C (or 4 hours at room temperature for neurite outgrowth experiment) with primary antibodies diluted in blocking solution. BrainSpheres were washed three times with washing solution II (1% BSA, 0.15% saponin in PBS) for 15 to 30 minutes on a shaker at room temperature before staining with secondary antibodies and 1:10,000 Hoechst 33342 (Thermo Fisher) diluted in blocking

solution for 4 to 6 hours at room temperature (1 hour at room temperature for neurite outgrowth experiment) on a shaker in the dark. Table 3 depicts the combination of primary and secondary antibodies utilized in this investigation. Following incubation, spheres were washed three times with washing solution II for 15 to 30 minutes on a shaker at room temperature, once with washing solution I, and once with PBS. Roughly 5 BrainSpheres per condition were mounted on glass slides with coverslips using Shandon Immu-mount (Thermo Scientific) and stored at 4 °C until imaging.

Table 3: Primary and secondary antibodies used for immunocytochemistry. β-Tubulin Isotype III was utilized in neurite outgrowth experiment, while all other primary antibodies were used to visualize synaptogenesis in aggregates.

Primary Antibodies						
Antigen	**Target Species**	**Host Species**	**Clonality**	**Conjugation**	**Manufacturer**	**Dilution**
β-Tubulin III	Human	Mouse	Monoclonal	None	Sigma-Aldrich	1:1,500
Synaptophysin	Human	Mouse	Monoclonal	None	EMD Millipore	1:200
PSD-95	Human	Rabbit	Monoclonal	None	Invitrogen	1:500
MAP2	Human	Mouse	Monoclonal	None	Sigma-Aldrich	1:200
Secondary Antibodies						
Antigen	**Target Species**	**Host Species**	**Clonality**	**Conjugation**	**Vendor**	**Dilution**
IgG(H+L)	Mouse	Goat	Polyclonal	AF 488 (G)	Invitrogen	1:500
IgG(H+L)	Rabbit	Goat	Polyclonal	AF 568 (R)	Invitrogen	1:500

Synaptic Quantification

Biological replicates were pooled and stained for dendritic (MAP2) and postsynaptic (PSD-95) markers and imaged as described above. Images were taken using a Zeiss LSM700 confocal microscope at 63x magnification with oil immersion. Three organoids per condition were imaged on the leftmost edge in order to reduce bias. Within each image, Z stacks were converted to a maximum intensity projection (MIP) using the open-source ImageJ2

Software. Synaptic puncta density per dendritic unit length was automatically calculated using the SynQuant 1.2.8 plugin (Wang et al. 2020). One-way ANOVA test with Bonferroni's multiple comparisons test was performed to determine statistical significance ($p \leq 0.05$). Data are presented as mean ± SEM.

Neurite Outgrowth

On day 6 of exposure, 5 to 10 spheres in 4 replicate wells per condition were seeded onto a poly-L-ornithine and laminin-coated 24-well black plate in 450 ml fresh exposure medium and incubated for 48 hours without shaking to allow the aggregates to attach and neurites to grow out from the spheres. Samples were fixed with 4% PFA and neurites were visualized by immunostaining against β-III-Tubulin as described above and imaged with an Echo Revolve G-124 fluorescence microscope. Every spheroid with intact neurites that could be visualized without interference from neighboring neural projections was imaged. Sholl analysis was performed ImageJ2 as described previously (Harris et al. 2018). Neurite length and density was quantified as the number of intersections per distance from BrainSphere edge and presented as area under the curve (AUC). The Shapiro-Wilk test was performed to confirm normality of the distribution and one-way ANOVA test with Bonferroni's multiple comparisons test was performed to determine statistical significance ($p \leq 0.05$).

RNA Extraction and cDNA Synthesis

On day 7 of exposure, samples of 10 to 15 spherers per well were washed once with PBS, snap frozen in liquid nitrogen, and stored at -80 °C until ready for RNA extraction.
Total RNA extraction and DNAse I treatment was performed using the Quick-RNA Micro Prep Kit (Zymo Research) according to manufacturer protocol. RNA concentration and quality was measured using a NanoDrop 2000c (Thermo Scientific). For cDNA synthesis,

labelled 0.2 mL PCR tubes (Eppendorf), Master Mix 1 (RHEX primers (Promega), 10 mM dNTP (Promega), M-MLV 5xbuffer (Promega), RNAse inhibitor (40 U/ul)(Thermo Fisher), ddH_2O) was added to 150 ng RNA diluted in ddH_2O. The reaction was incubated for 5 minutes at 70 °C, 15 minutes at 4 °C, 90 minutes at 37 °C, and 5 minutes at 85 °C. Master Mix 2 (M-MLV reverse transcriptase, RNAse inhibitor (40 U/ul)) was added during the 15 minute 4 °C cooling cycle. RNA was reverse transcribed in a 2720 ThermoCycler (Applied Biosystems). cDNA was stored at -20 °C until ready to be used for RT-qPCR.

Quantitative RT-PCR

Gene expression was evaluated with the fast Taqman™ Gene Expression Assay (Applied Biosystems) using a 7500 Fast Real-Time PCR System (Applied Biosystems). Briefly, Master Mix (5 µL Taqman™ Fast Advanced Master Mix, 0.5 µL specific primer, 3.5 µL ddH_2O) was added to 1 µL cDNA sample in a MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems). Primers for all genes of interest (*GAPDH*, *TUBB3*, *MAP2*, *PSD-95*, *SYN1*, *AChE*, *GRIN1*, and *GRIN2a*) were purchased from Applied Biosystems. Gene expression fold change was calculated using the $2^{-\Delta\Delta Ct}$ method with *GAPDH* used as a housekeeping gene (Livak and Schmittgen 2001). Data presented are from R23.

RESULTS

Estimated serum concentrations of selected PHOP compounds are not cytotoxic.

PHOP compounds have not been previously evaluated in the human BrainSphere model. In order to determine an appropriate experimental concentration that coincided with plausible human PHOP serum estimates, 5, 10, and 20 µM flame retardant compounds and vehicle control were evaluated for evidence of cytotoxicity two weeks after initiating differentiation. One way ANOVA with Bonferroni correction for multiple comparisons indicated that no

compound elicited a statistically significant reduction in viability (Table 4 and Figure 3). Of note, BrainSpheres treated with 10 μM BDE-47 showed a 110% increase in resaruzin reduction capacity (adjusted p-value 0.0071) compared to control samples, but significance was not sustained in the 20 μM dose group. This observation is addressed in greater detail in the discussion. Due to the absence of clear cytotoxic effects, the two highest concentrations (10 and 20 μM) were selected for further study.

Table 4: Percent of cell viability of BrainSpheres exposed selected flame retardant compounds for one week compared to control. Exposure occurred two weeks after differentiation of NPCs was initiated. n = 3 biological replicates (wells) for all conditions. Data is presented as mean ± SEM. Data is from R21 only. * indicates $p < 0.05$; ** indicates $p < 0.01$ compared to the vehicle control.

Condition	Concentration (μM)	Percent Viability vs. control
Control	**0**	100 ± 0.0
TCEP	**5**	61.7 ± 12.2
	10	91.0 ± 21.9
	20	66.3 ± 25.1
TDCPP	**5**	76.0 ± 13.6
	10	85.3 ± 34.4
	20	83.0 ± 23.6
Tris	**5**	128.0 ± 37.0
	10	121.3 ± 33.1
	20	135.7 ± 28.8
BDE-47	**5**	163.0 ± 27.0
	10	210.0 ± 18.8 **
	20	180.0 ± 9.5

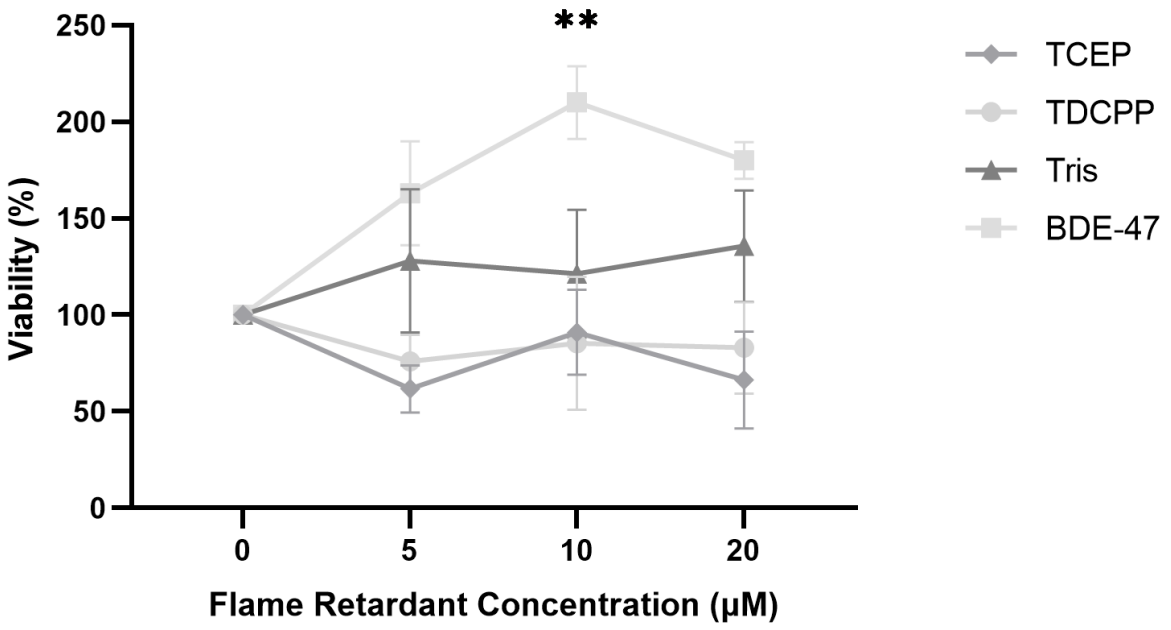

Figure 3: Percent of cell viability of BrainSpheres exposed to 5, 10, or 20 µM flame retardant compounds for one week compared to control. Exposure occurred two weeks after differentiation of NPCs was initiated. n = 3 biological replicates (wells) for all conditions. Data is presented as mean ± SEM. Data is from R21 only. ** indicates $p < 0.01$ compared to the vehicle control.

Selected PHOP compounds do not inhibit AChE activity.

The AChE assay was performed in order to establish that any potential indices of selected PHOP toxicity occur at concentrations below that which inhibit AChE activity in the human BrainSphere model. In the AChE assay, fluorescence intensity is proportional to AChE activity (mU/mL). Prior unpublished work in the CAAT laboratory has shown that exposure to 100 µM CPO for 24 hours inhibits AChE activity in the human BrainSphere model, thus this concentration and duration of exposure were selected as a positive control (Modafferi et al., revised). As expected, AChE activity in 4-week old BrainSpheres exposed to 10 or 20 µM PHOP compounds for one week was not inhibited (Figure 4). A small but insignificant dose-dependent increase in enzymatic activity was observed for TCEP and Tris- exposed BrainSpheres, while the observed 41% increase in AChE activity in BrainSpheres exposed

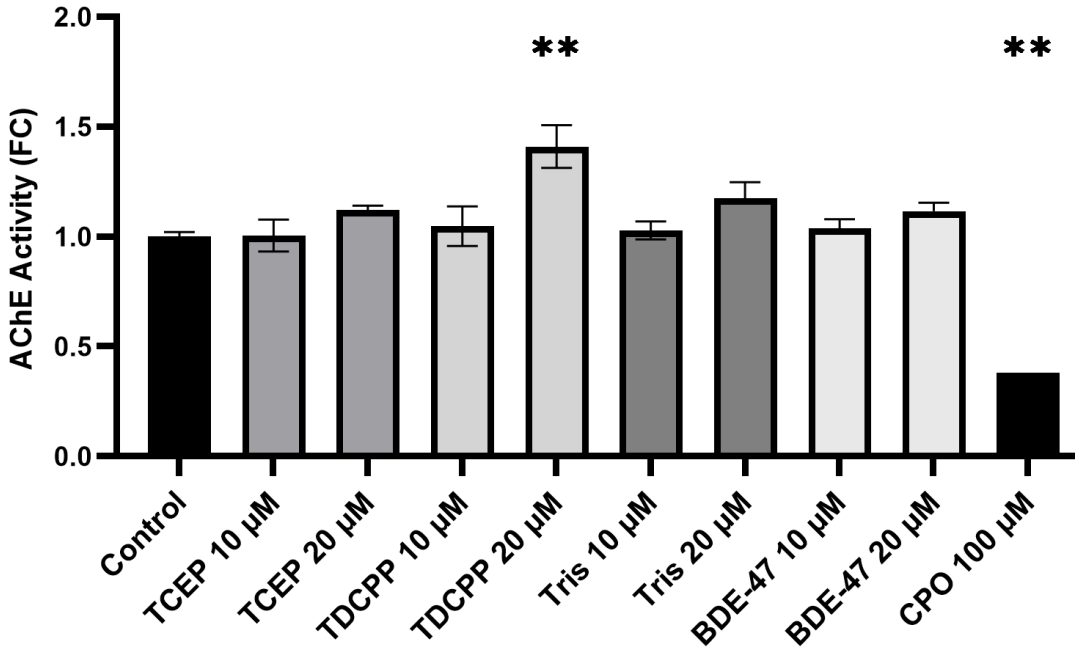

Figure 4: Fold change of AChE activity in 4 week old BrainSpheres. Exposure to 10 or 20 µM flame retardant compounds for one week or 100 µM CPO for 24 hours. Exposure occurred four weeks after differentiation of NPCs was initiated. Mean RFU of triplicate wells from each condition were averaged and AChE activity (mU/mL) calculated from linear regression of the standard curve. Data is presented as fold change of total protein adjusted AChE activity compared to vehicle control ± SEM. Data is from R21 only. * indicates $p < 0.05$; ** indicates $p < 0.01$ compared to the vehicle control.

to 20 µM TDCPP did reach statistical significance compared to the control (adjusted p-value = 0.0063) (Table 5). This increase is likely an anomaly, because OP compounds are known inhibitors of AChE (COT 2019; Boublik et al. 2002). The same effect was observed in BrainSpheres exposed to BDE-47, which is not an organophosphate ester and thus not expected to inhibit AChE activity. The CPO positive control group exhibited a statistically significant 62% reduction in AChE activity (adjusted p-value = 0.0021). Furthermore, the relative expression of *AChE* was evaluated with RT-PCR. No significant altered expression was observed by flame retardant exposure, although a trend towards upregulation in samples exposed to both concentrations of Tris was noted (Tris 10 µM = 0.399, p = 0.3638; Tris 20 µM = 0.358, p = 0.5650) (Figure 7).

Table 5: AChE enzyme activity in 4 weeks old BrainSpheres after exposure to flame retardants for one week or 100 µM CPO for 24 hours. Mean RFU of triplicate wells from each condition were averaged and AChE activity (mU/mL) calculated from linear regression of the standard curve. Data is presented as mean total protein-adjusted AChE activity ± SEM. Data is from R21. n = 1-3 biological replicates (wells) per condition. ** indicates compared to the vehicle control.

Condition	Concentration (µM)	mU/mL
Control	**0**	2.87 ± 0.06
TCEP	**10**	2.89 ± 0.21
	20	3.22 ± 0.05
TDCPP	**10**	3.01 ± 0.26
	20	4.05 ± 0.28 **
Tris	**10**	2.95 ± 0.11
	20	3.37 ± 0.21
BDE-47	**10**	2.98 ± 0.12
	20	3.20 ± 0.11
CPO	**100**	1.09 **

PHOP exposure impairs neurite quality and induces compensatory outgrowth

The neurite outgrowth assay is a functional evaluation of neurons capacity to extend projections from the cell body (Harris et al. 2018). Four-week old BrainSpheres exposed to 10 or 20 µM PHOP compounds for 7 days and plated on a Matrigel®-coated well for 48 hours all exhibited a slight reduction in neurite coverage compared to vehicle control, though no single condition reached statistical significance (percent reduction from control: TCEP 10 µM = 9.5%, $p > 0.9999$; TCEP 20 µM = 11.3%, $p > 0.9999$; TDCPP 10 µM = 28.1%, $p = 0.3131$; TDCPP 20 µM = 11.5%, $p > 0.9999$; Tris 10 µM = 35.7%, $p = 0.1270$; Tris 20 µM = 10.2%, $p > 0.9999$;) (Figure 5B, C).

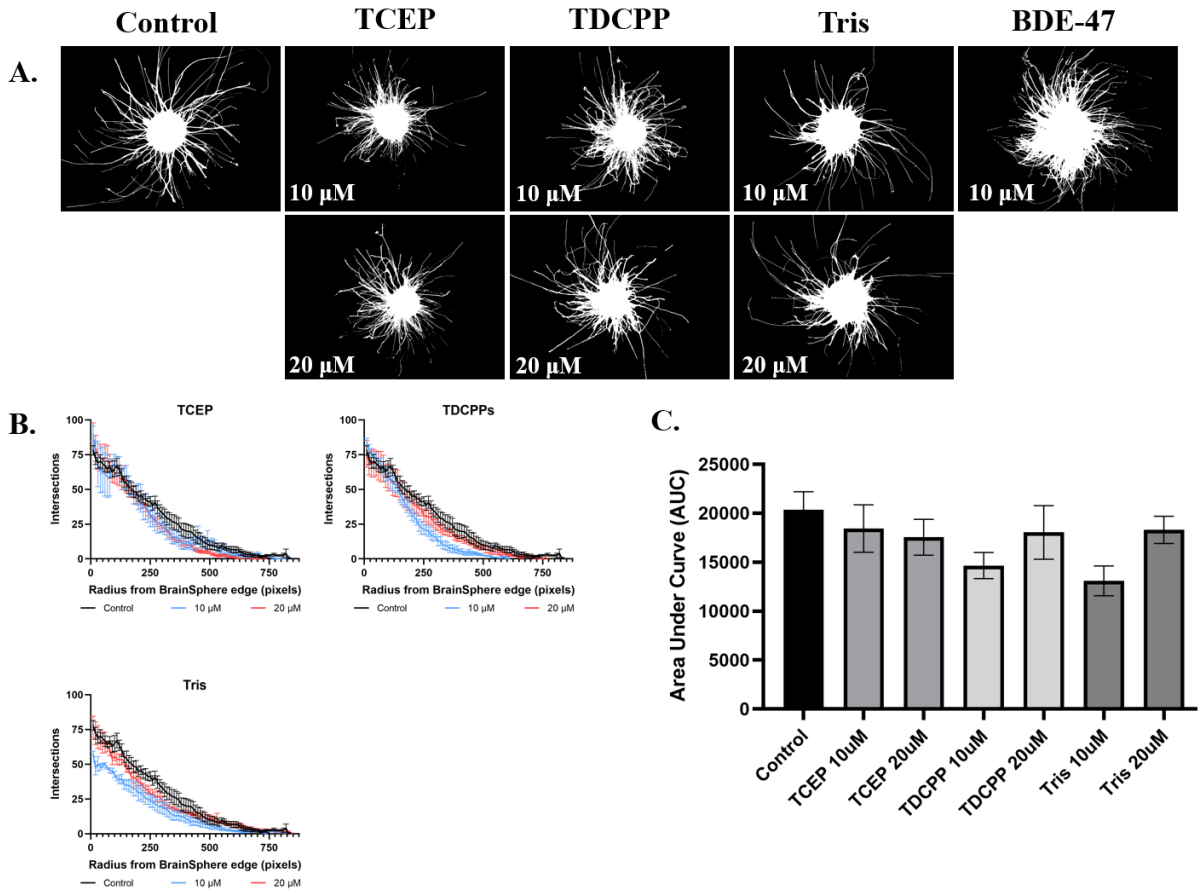

Figure 5: Neurite outgrowth in 4 week old BrainSpheres exposed to 10 or 20 µM flame retardant compounds for one week. Data is from R21 only. **(A)** Representative images of β-Tubulin III staining of neural projections at 4x magnification indicates that individual neurite morphology appears less robust in PHOP exposure groups compared to vehicle control. **(B)** Plot of average number of BrainSphere intersections from the outer edge of each aggregate. **(C)** Area under the curve (AUC) analysis of neurite density.

The absence of statistical significance in AUC analysis may be a consequence of having few experimental units. Twenty to forty BrainSpheres per condition were plated for the neurite outgrowth experiment, but substantial exclusion is expected through the normal course of the assay. Spheres with neural projections in close proximity to surrounding neurites are excluded from analysis because it has been observed in the BrainSphere model that neighboring projections promote neurite growth independent of experimental condition. BrainSpheres adhering adjacent to the edge of the well were also excluded from analysis, because the physical barrier impedes radial outgrowth. Floating BrainSpheres and

BrainSpheres with neurites that have partially detached as a result of washing steps during immunostaining are also excluded (Table 6).

Due to the low numbers of aggregates retained for analysis, a post hoc one-tailed binomial test with Bonferroni correction was performed comparing the observed number of floating BrainSpheres with the expected count based on what was seen in the control. Spheroids with interrupted neurites were not included in the analysis, because positioning near another aggregate or near the well wall occurs as a matter of chance, and mechanical disturbance of neurites may have been a reflection of repeated wash steps rather than a reflection of neurite health. The analysis revealed that fluctuations in the number of floating neurites did not progress in a dose-dependent manner or reached statistical significance for any of the three PHOP compounds. However, the 10 and 20 µM BDE-47 groups significantly deviated from the control samples (adjusted p-values = <0.0001). Indeed, BDE-47 has been shown to reduce axonal length in primary rat neuron-glia cocultures (Chen et al. 2016) and is widely accepted as neurotoxic in the developing fetus (COT 2019). While the 48 hour collection timepoint precedes the maturation of the dominant neurite into a single axon, BDE-47 was clearly toxic in this assay, with neurite outgrowth quantification not possible due to only a single viable BrainSphere remaining in the 10 µM BDE-47 group.

Visually, PHOP exposure appears to impact neurite morphology (Figure 5A). Projections, visualized by anti β-III-tubulin immunostaining, are generally thinner and display erratic directionality in PHOP exposed groups. In addition, Moreover, the relative expression of genes involved in neurite morphology were evaluated. One-way ANOVA with Bonferroni correction for multiple comparisons of relative *TUBB3* gene expression showed a very slight statistically insignificant upregulation in both concentrations of TCEP and Tris and the lowest

Table 6: Floating, interrupted, and viable BrainSpheres imaged in neurite outgrowth assay. Data is presented as counts and proportion of the total number of imaged organoids. Data is from R21 only. ** indicates p < 0.01 compared to the vehicle control.

Condition	Total BrainSpheres Imaged	Floating (No Neurites)		Interrupted (Detached or Well Wall Adjacent)		Used in Analysis	
Control	**11**	**1**	*0.09*	**4**	*0.36*	**6**	*0.55*
TCEP 10 µM	**17**	**2**	*0.12*	**10**	*0.59*	**5**	*0.29*
TCEP 20 µM	**17**	**2**	*0.12*	**9**	*0.53*	**6**	*0.35*
TDCPP 10 µM	**14**	**1**	*0.07*	**8**	*0.57*	**5**	*0.36*
TDCPP 20 µM	**11**	**2**	*0.18*	**4**	*0.36*	**5**	*0.45*
Tris 10 µM	**10**	**4**	*0.40*	**2**	*0.20*	**4**	*0.40*
Tris 20 µM	**10**	**3**	*0.30*	**3**	*0.30*	**4**	*0.40*
BDE-47 10 µM	**9**	**7****	*0.78*	**1**	*0.00*	**1**	*0.22*
BDE-47 20 µM	**7**	**7****	*1.00*	**0**	*0.00*	**0**	*0.00*

concentration of TDCPP compared to control (TCEP 10 µM = 0.341, p = 0.6660; TCEP 20 µM = 0.161, p > 0.9999; TDCPP 10 µM = 0.264, p = >0.9999; Tris 10 µM = 0.408, p = 0.3258; Tris 20 µM = 0.377, p = 0.4545) (Figure 7). In mammals, β-III-tubulin functions in microtubule organization, and plays a role in extending neural projections out from the cell body (NCBI 2021a). This pattern was also present for *MAP2* expression in all PHOPs (TCEP 10 µM = 0.371, p > 0.9999; TCEP 20 µM = 0.440, p > 0.9999; TDCPP 10 µM = 0.453, p > 0.9999; TDCPP 20 µM = 0.462, p > 0.9999; Tris 10 µM = 0.349, p > 0.9999; Tris 20 µM = 0.110, p > 0.9999) (Figure 7). The MAP2 gene family is involved in stabilizing microtubules and are essential to proper neurogenesis (NCBI 2021b). Together, poor neurite quality and upregulated *TUBB3* and *MAP2* indicate that PHOP exposed BrainSpheres may be compensating for poor neurite quality by producing additional neurites.

PHOP compounds do not alter synaptic morphology but may alter the expression of genes involved in synaptic function

In order to investigate the impacts of PHOP exposure on synaptogenesis, BrainSpheres were stained for postsynaptic (*PSD-95*) and dendritic (*MAP2)* markers. Synaptic features were quantified using the automated SynQuant plugin for ImageJ2 (Wang et al. 2020). Flame retardant exposure did not appreciably alter the density of postsynaptic puncta (Figure 6B).

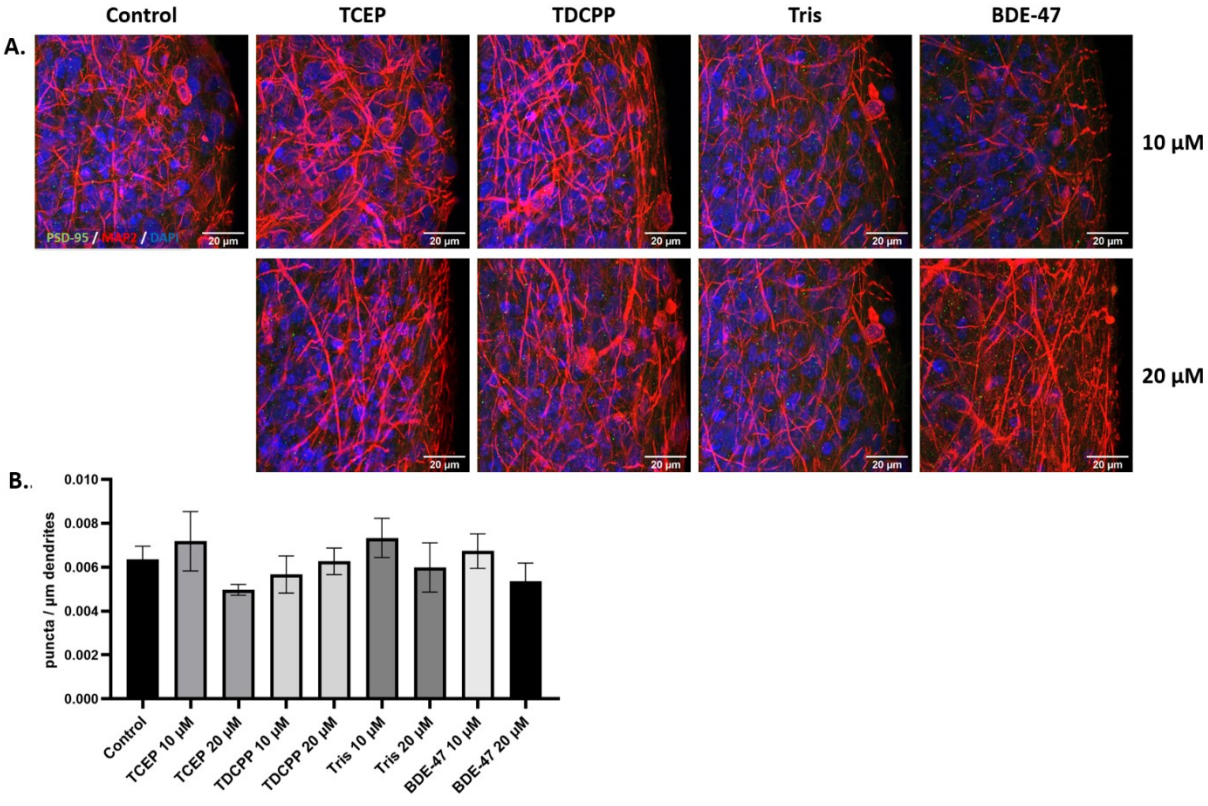

Figure 6:.PHOP exposure does not alter synaptic features. **A)** Representative images of postsynaptic staining for dendrites (MAP2, red), postsynaptic puncta (PSD-95, green), and nuclei (Hoechst staining, blue). Images are taken from R21. **B)** Postsynaptic puncta density. Data is presented as mean number of puncta per µm dendrite ± SEM. Data obtained from 4-week BrainSpheres from R21 and R23.

As discussed previously, *MAP2* gene expression was slightly but not significantly upregulated by exposure to PHOPs and BDE-47 (Figure 7). In contrast to the synaptic quantification, relative gene expression of *PSD-95* was consistently higher in flame retardant-exposed BrainSpheres, although this upregulation again did not reach statistical significance after analyzing with One-way ANOVA with Bonferroni correction (TCEP 10 μM = 0.108, p > 0.9999; TCEP 20 μM = 0.243, p > 0.9999; TDCPP 10 μM = 0.448, p > 0.9999; TDCPP 20 μM = 0.596, p > 0.9999; Tris 10 μM = 0.680, p = 0.8079; Tris 20 μM = 0.459, p > 0.9999). PHOP exposure did not alter presynaptic *SYN1* expression.

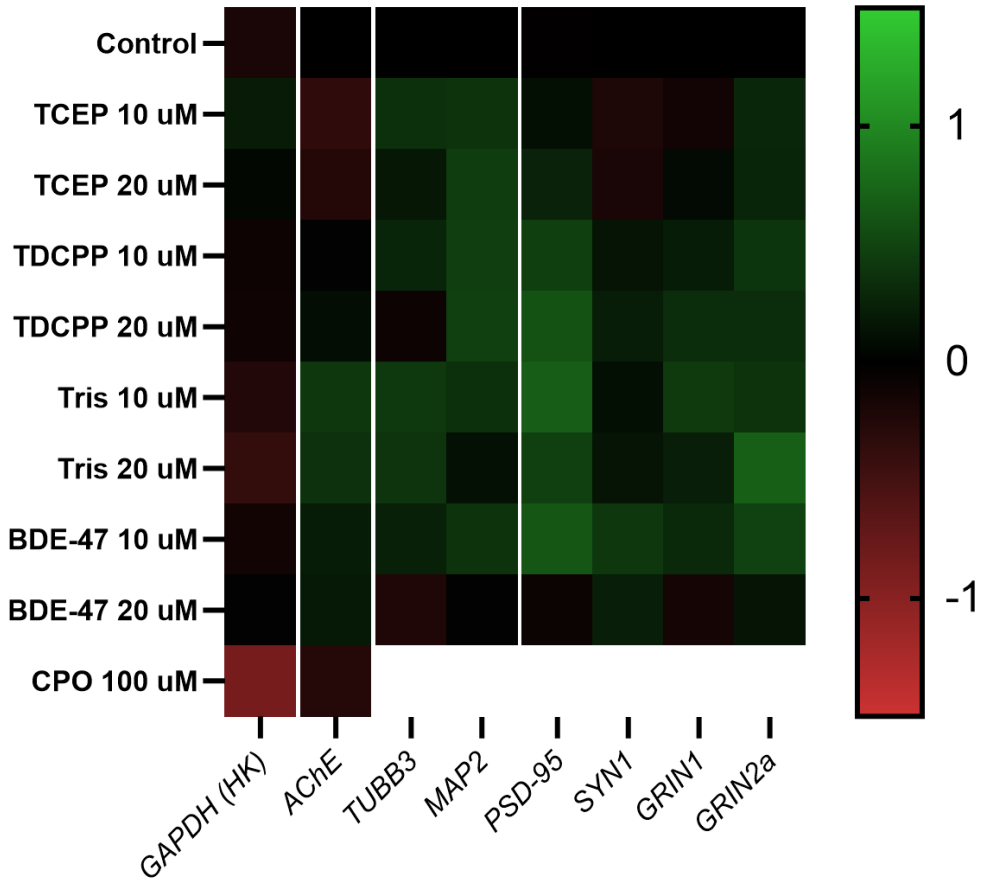

Figure 7: Relative gene expression of selected structural and synaptic proteins. 4 week old BrainSpheres exposed to 10 or 20 μM flame retardant compounds for one week. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method with *GAPDH* as the housekeeping gene. Heat map was constructed in Prism version 9.1.0.221 using mean $\log_2$ fold change compared to untreated controls. Data presented are from R23.

Prior investigations in the CAAT laboratory found that non-PHOP organophosphate flame retardants downregulate the expression of glutamate NDMA receptor subunits *GRIN1* and *GRIN2a* following a two week exposure in a primary rat BrainSphere model (Hogberg et al. 2021). Moreover, it is well documented that the NMDA receptor is a potential toxicant target during brain development. Therefore, these genes were selected to determine whether the observed pattern of alterations persists in PHOP-exposed human BrainSpheres. To the contrary, relative expression of *GRIN2a* appeared slightly but insignificantly upregulated across all PHOP exposure groups (Figure 7). To a lesser degree, relative expression of *GRIN1* was also slightly but insignificantly upregulated in both TDCPP and Tris samples.

DISCUSSION

The class-based assessment of structurally related compounds has a promising application in regulatory toxicology. Among the closely related PHOP flame retardant subclass – which is currently under consideration for collective evaluation of human health risk by CPSC– only seven of twenty two compounds have been evaluated for developmental neurotoxicity. Metabolites of OPFRs in pregnant women have been associated with reduced IQ and working memory in their offspring, while direct exposure in children has been associated with dose-dependent reductions in responsible behavior and increases in aggressive or disruptive behavior (Castorina et al. 2017; Lipscomb et al. 2017). In vitro assays indicate that like their polybrominated predecessors, OP esters like TCEP and TDCPP exhibit toxicity in neuronal proliferation, neurite outgrowth, synaptogenesis, and the establishment of neuronal circuitry (Blum et al. 2019). In vivo assessments in zebrafish, *c. elegans*, and flatworms have also linked these compounds with altered development and motor activity (Blum et al. 2019).

This investigation aimed to provide novel evidence about whether PHOP flame retardants so classified by their shared functional, structural, physicochemical, and biological properties would exhibit sufficient concordance across a battery of assays performed in a human iPSC-derived brain microphysiological model to support their consideration as a single group.

Three-dimensional BrainSphere models have been used to investigate the effects of prenatal exposure to pharmaceuticals and environmental contaminants including pesticides and non-PHOP organophosphorus flame retardants (Zhong et al. 2020; Pamies et al. 2018; Hogberg et al. 2021). At 8 week maturity, the model contains a co-culture of functional neuronal and glial cell populations, and is capable of recapitulating several key events and functional milestones that occur in the developing brain (Pamies et al. 2014). By week 2 post-differentiation, the organoids stain positively for GABAergic, dopaminergic, and glutamatergic neuronal populations and are capable of spontaneous electrical activity, and synaptic receptor architecture is observed by 4 weeks (Pamies et al. 2017). In this study, BrainSpheres were used to investigate morphological and functional endpoints including cytotoxicity, AChE activity, synaptogenesis, and gene expression.

A benefit of the BrainSphere model is its resistance to variability in size, cell composition, functional output between differentiation runs (Pamies et al. 2017). Early attempts to generate reproducible iPSC-derived microphysiological brain systems have widely documented necrotic centers in large organoids as a consequence of poor oxygen and nutrient diffusion (Pamies et al. 2017). Although uncharacteristically large aggregates were removed prior to distributing the organoids among experimental groups, size heterogeneity persisted among small aggregates as well. Whether size acted as a confounding variable in R21 is unknown, and additional runs are necessary to determine the reproducibility of the results presented here.

While resorufin fluorescence was adjusted for the number of BrainSpheres per well, fluorescence was not adjusted for total protein. This transformation likely would have provided a better proxy for cell mass within the aggregates and may have accounted for the large, dose independent fluctuations in viability compared to the vehicle control. Since the resazurin assay correlates the reduction of resazurin by NADH or NADPH with viable cell count, it is also possible that cell death accompanied by a compensatory increase in metabolic activity in living cells could have interfered with viability estimates (Hall et al. 2016; Riss et al. 2013).

Like OP insecticides, the studied PHOPs contain an ester of phosphoric acid that confers an affinity for the AChE active site (COT 2019). The common mechanism and shared cholinergic toxicity of OP insecticides provided the basis for EPA's decision to conduct a cumulative risk assessment for these chemicals, although they are also known to exhibit non-cholinergic toxicity including cognitive deficits following prenatal exposure (EPA 2006; Burke et al. 2017). In contrast, industry and regulatory stakeholders have historically resisted calls for a cumulative OPFR risk assessment over the presumption of safety stemming from the group's weak cholinergic response (COT 2019). In this investigation, the AChE assay was performed in order to establish that any potential toxicity indeed occurs at concentrations lower than that which inhibit AChE activity. As anticipated, neither the PHOP subclass members nor negative flame retardant control BDE-47 inhibited AChE activity at the 10 or 20 μM exposure, while positive control CPO did inhibit the enzyme at 100 μM. The anomalous enzyme activity increase in the TDCPP 20 μM group is likely an artifact of the previously mentioned obstacle in BrainSphere differentiation.

Neurite outgrowth is the preeminent process allowing for neuronal migration and the establishment of circuitry essential for neuron-neuron communication (Khodosevich and

Monyer 2010; Smit et al. 2003). The extension of neuronal processes from the cell body is governed by the complex integration of positive and negative signaling from neurotrophins and neurite growth inhibitors, which differ by brain region and neuron subtype (Ibid). Dysregulation of proper neurite outgrowth and maturation can have wide reaching impacts on synaptogenesis, synaptic pruning, plasticity, and other key processes in CNS development (Ibid). Thus, neurite outgrowth assays have emerged as a common means of assessing developmental neurotoxicity in vitro.

PHOP exposure may impair the quality of neurite outgrowth and is coupled with slight but statistically insignificant upregulated gene expression of microtubule associated structural proteins *TUBB3* and *MAP2.* These findings align with the results of an NTP *in vitro* alternatives testing battery, in which low concentrations (1 to 10 μM) of TCEP did not alter neurite outgrowth in human neuroprogenitor nor rat cells nor rat primary cortical cultures above a prespecified 15% background noise threshold (Behl et al. 2015). TDCPP also appears not to alter neurite outgrowth in the rat PC12 cell line (Dishaw et al. 2012).

Similarly, PHOP flame retardants elicited menial upregulation in gene expression of postsynaptic marker *PSD-95* and NMDA receptor subunits *GRIN1* and *GRIN2a,* but did not impair overall neurite length or density of postsynaptic puncta. These findings are at odds with earlier investigations of OPFR toxicity in the rat BrainSphere model, but underscore NASEM recommendations for subclass-based hazard assessments (Hogberg et al. 2021). As members of the PHOP subclass, TCEP, TDCPP, and Tris are more closely related to one another than they are to OPFRs more generally, and thus evidence of specific toxicity should not be considered emblematic for all OPFRs.

Chapter 5

CONCLUSION

At estimated human serum concentrations, TCEP, TDCPP, and Tris may impair neurite quality and elicit a slight upregulation of microtubule-associated and synaptic proteins. Though these findings should still be considered preliminary, evidence suggests that constituents of the PHOP subclass are both capable of perturbing key events in neurodevelopment and do so with a sufficiently similar magnitude and directionality to justify regulatory consideration of co-exposures in a cumulative human risk assessment. Importantly, the effects of TCEP and TDCPP exposure seemed to predict that of Tris, a poorly characterized compound for which developmental neurotoxicity data is not widely available. This promising relationship lends additional support for ongoing efforts to regulate chemicals with shared structural, functional, and biological properties as one group, even under scenarios where the toxic profile of individual subclass members is unknown.

EPA has historically declined to regulate chemicals as a group without both a common use scenario and substantial evidence that the group's constituent members produce an adverse effect through a shared mechanism of action (i.e. OP insecticides exhibiting cholinergic toxicity). While the preliminary data presented here do not yet rise to that second standard of proof, the consistent directionality and magnitude of effect in the *in vitro* human BrainSphere model provide positive evidence that such a mechanistic relationship may exist. The brain dysregulation behind poor emotional self-regulation and social behavior is incredibly complex, and adverse outcome pathways charting the relationship between chemical exposure to altered neurodevelopmental phenotype are unknown.

Additionally, epidemiological investigations point to a clear association between OPFR exposure in utero and throughout early childhood and poorer executive function. Given the disparate relationships between molecular and epidemiological data, it is entirely possible that cumulative exposure to PHOP flame retardants is a better predictor of DNT than any single compound. Such a scenario further justifies the need to consider exposure to all PHOP subclass members in order to accurately characterize the risks and hazards posed to millions of individuals who are inadvertently exposed to these compounds on a daily basis.

Understanding the burden of environmental chemicals on the developing human brain is an evolving challenge. Ultimately, the unavoidable early life exposure to flame retardant compounds represents a preventable risk factor for a permanent disability. As data mounts in favor of one or several causal relationships between PHOP compounds and disordered development, evidence-based prevention will be imperative to protecting public health. In order for these efforts to be successful, human hazard assessments must consider the effect of cumulative exposure to these closely related compounds, rather than continuing the trend of regulatory whack-a-mole.

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