Environmental Toxicology and Pharmacology 50 (2017) 167–174

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Impairment in the mesohippocampal dopamine circuit following exposure to the brominated flame retardant, HBCDD

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ARTICLE INFO

Article history:
Received 19 January 2017
Accepted 3 February 2017
Available online 4 February 2017

Keywords:
Dopamine transporter
Hexabromocyclododecane
Hippocampus
Substantia nigra pars compacta
Tyrosine hydroxylase
Vesicular monoamine transporter 2

ABSTRACT

Many chemicals have been used to increase the safety of consumer products by reducing their flammability and risk for ignition. Recent focus on brominated flame retardants, such as polybrominated diphenyl ethers (PBDEs) has shown them to contribute to neurobehavioral deficits in children, including learning and memory. As the manufacture and use of PBDEs have been reduced, replacement chemicals, such as hexabromocyclododecane (HBCDD) have been substituted. Our current study evaluated the neurotoxicity of HBCDD, concentrating on dopaminergic innervation to the hippocampus. Using an *in vivo* model, we exposed male mice to HBCDD and then assessed alterations to the dopamine synapse 6 weeks later. These exposures elicited significant reductions in presynaptic dopaminergic proteins, including TH, COMT, MAO-B, DAT, VMAT2, and alpha-synuclein. In contrast, postsynaptic dopamine receptors were not impaired. These findings suggest that the mesohippocampal dopamine circuit is vulnerable to HBCDD and the dopamine terminal may be a selective target for alteration.

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1. Introduction

The manufacture and use of brominated flame-retardants has seen a precipitous increase in the last several decades, given their perceived ability to inhibit or reduce the flammability of consumer products, such as electronics, plastics, furniture, and other textiles (de Wit, 2002). Unfortunately, the increased demand for these products has also led to an elevated and ubiquitous presence of these compounds in our environment that is exacerbated by their ability to leach out of many of these products and bioaccumulate. Significant levels of flame retardant compounds are found throughout the environment, which may underlie an increase in body burdens of these chemicals in the human population (Covaci et al., 2011). Of particular interest are findings that demonstrate the presence of brominated flame retardants (BFRs) in human brain tissue and the association between exposure to these compounds and neurological disruption. Indeed, one of the more recently studied class of BFRs, polybrominated diphenyl ethers (PBDE) have been found to be associated with a variety of neurobehavioral impairments, including deficits in motor and sensory development, as well as several domains associated with cognitive development (Herbstman et al., 2010; Eskenazi et al., 2013; Chen et al., 2014; Cowell et al., 2015; Chevrier et al., 2016). Most concerning is the impact these compounds seem to have on aspects of learning and memory in these exposed populations. Within this context, particular interest has been focused towards the hippocampus, which plays a critical role in mediating a variety of aspects associated with memory. Multiple laboratory-based studies have found exposure to PBDE to significantly impact the function of the hippocampus, leading to deficits in memory function (Viberg et al., 2003; Ta et al., 2011; Yan et al., 2012). From a cellular and molecular perspective, further studies have shown that exposure to PBDE impairs LTP and synaptic plasticity, critical elements associated with learning and memory processes (Dingemans et al., 2007; Xing et al., 2009). Additionally, alterations in the cholinergic and glutamatergic neurotransmitter systems have been suggested to play a role in these behavioral deficits (Viberg et al., 2003; Dingemans et al., 2007; Yan et al., 2012).

Abbreviations: Asyn, alpha-synuclein; BFR, brominated flame retardants; COMT, catechol-*O*-methyltransferase; DAB, 3,3′-diaminobenzidine; DAT, dopamine transporter; HBCDD, hexabromocyclododecane; MAO-B, monoamine oxidase-B; NET, norepinephrine transporter; PBDE, polybrominated diphenyl ethers; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VTA, ventral tegmental area; VMAT2, vesicular monoamine transporter 2.

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http://dx.doi.org/10.1016/j.etap.2017.02.003
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As the neurotoxic properties of PBDEs became better appreciated, their manufacture and use was discontinued or limited throughout the world, allowing similar BFR products to be introduced as potential replacement compounds. One such chemical is hexabromocyclododecane (HBCDD), which appears to embody many of the same physiochemical properties as PBDEs, being lipophilic, difficult to breakdown, leading to bioaccumulation and persistence in the environment (Covaci et al., 2006). Although not as extensively studied as PBDEs, HBCDD has been shown to have neurotoxic properties similar to PBDEs. In animal studies, HBCDD exposure appears to elicit impairments in neurodevelopment and neurobehaviors. Most interestingly, the hippocampus appears to be a target of HBCDD neurotoxicity as groups have shown disruption of neuronal migration in the hippocampus as well as deficits in spatial memory following a single exposure in the developing mouse (Eriksson et al., 2006; Saegusa et al., 2012). While these findings provide valuable evidence for the neurotoxic effects of HBCDD, the underlying cellular and molecular targets that underlie these deficits in learning and memory are unclear.

Evaluation of neurotoxicological effects on the hippocampus is critical, given the importance of this structure in a variety of elements associated with learning and memory. The hippocampus is extensively interconnected with other brain regions that are known to modulate memory (Eichenbaum et al., 1996). Although not always appreciated as a substantial contributor to hippocampal function, dopaminergic projections from the midbrain, specifically the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc), have been found to have a significant impact on modulating/regulating memory processes (Frey and Morris, 1998; Jay, 2003; Lisman and Grace, 2005; Shohamy and Adcock, 2010; McNamara et al., 2014; Rosen et al., 2015). Dopaminergic projections have been found to terminate throughout the hippocampus, having more explicit localizations to cellular layers in the CA1, CA3, and dentate gyrus (Gasbarri et al., 1994, 1997). These projections are generally found to synapse onto dendrites and cell bodies of dentate granule and pyramidal cells, where D1 and D2 receptor groups modulate long-term potentiation (LTP) through their coordinated interaction with glutamatergic receptors and intracellular cascades that underlie LTP (Boyson et al., 1986; Frey and Morris, 1998). Over the last several decades, extensive work has better delineated the role of dopamine in hippocampal function. *In vitro* and *in vivo* studies have found manipulation of dopamine signaling at the pre- and postsynaptic terminal to have significant effects on learning and memory in the hippocampus. Indeed, blockade of D1 or D2 receptors with selective antagonists manifests in memory impairments, which have been replicated in animals lacking the D1 receptor (Ortiz et al., 2010). Moreover, dopamine signaling that has been impaired following damage to dopamine terminals has also been shown to result in deficits in learning and memory. Utilization of the selective dopaminergic neurotoxins, MPTP or 6-OHDA has found reductions in dopaminergic terminals and dopamine in the hippocampus mediate impairments in LTP and memory in the hippocampus (Gasbarri et al., 1996; Zhu et al., 2011; Costa et al., 2012; Bonito-Oliva et al., 2014). These deficits were ameliorated following treatment with dopamine replacement, including L-DOPA.

In light of the importance of dopamine signaling in the hippocampus in mediating learning and memory, we sought to further characterize the potential neurotoxic effects of HBCDD on the mesohippocampal dopamine circuit. As the dopaminergic synapse appears to be uniquely vulnerable to HBCDD, we directed our focus towards the evaluation of proteins known to be critical to dopamine signaling. Indeed, following exposure to HBCDD in adult male mice, significant damage to presynaptic dopamine proteins was observed. These findings highlight the fact that the mesohippocampal dopamine circuit is vulnerable to HBCDD exposure and identifies potential cellular and molecular targets that underlie learning and memory impairments.

2. Materials and methods

2.1. Chemicals and reagents

Hexabromocyclododecane (HBCDD) was purchased from Sigma-Aldrich (St. Louis, MO). The BCA protein assay kit was obtained from Pierce (Rockford, IL). Monoclonal anti-rat dopamine transporter (DAT) and polyclonal rabbit anti-tyrosine hydroxylase (TH) and rabbit anti-Catechol-*O*-Methyltransferase (COMT) antibodies were purchased from EMD Millipore (Billerica, MA). Polyclonal rabbit anti-dopamine D2 receptor antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Monoclonal mouse anti-norepinephrine transporter (NET) was a kind gift from Craig Heilman at Emory University. Polyclonal rabbit anti-vesicular monoamine transporter 2 (VMAT2) antibodies were generated by Covance to the C-terminal sequence in mouse (CTQNNVQPYPVGDDEESESD). Monoclonal mouse anti-β-actin and anti-dopamine D1 receptor antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal mouse anti-alpha-synuclein antibody was purchased from BD Transduction (Franklin Lakes, NJ). Polyclonal rabbit anti-monoamine oxidase B antibody was purchased from Abcam (Cambridge, MA). Secondary antibodies conjugated to horseradish peroxidase or biotin were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). SuperSignal West Dura Extended duration substrate and stripping buffer were obtained from Pierce. 3,3′ Diaminobenzidine (DAB) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals and treatment

Eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Two month old mice were orally gavaged with 25 μl of HBCDD made up to 25 mg/kg body weight (25,000 μg/kg body weight) and dissolved in corn oil vehicle. Animals were exposed daily for 6-weeks, using a protocol similar to that previously described (n = 6 for control and n = 6 for treated groups) (Caudle et al., 2006; Bradner et al., 2013; Genskow et al., 2015). This dosing paradigm was intended to represent the primary route of human exposure to HBCDD. Mice were sacrificed 6-weeks following the last exposure, and unilateral hippocampi were collected for subsequent analysis. While previous studies have investigated the impact of HBCDD exposure on the hippocampus (Eriksson et al., 2006; Saegusa et al., 2012), our study was the first to assess alterations to the dopamine circuit. As the focus of our study was on the dopaminergic synapse, we relied upon our previously published and ongoing studies with HBCDD to inform our dosing paradigm (Genskow et al., 2015). Standard rodent chow and tap water were available ad libitum. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and have been approved by the Institutional Animal Care and Use Committee at Emory University.

2.3. Western blot analysis

Western blots were used to quantify the amount of DAT, TH, VMAT2, D1R, D2R, NET, COMT, MAO-B, and β-actin present in samples of hippocampal tissue from treated and control mice. Analysis was performed as previously described (Caudle et al., 2006; Genskow et al., 2015). Briefly, hippocampal samples were homogenized and samples subjected to polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. Nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline and then membranes incubated overnight

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