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Selective damage to dopaminergic transporters following exposure to the brominated flame retardant, HBCD

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B.S.
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An abstract of
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Abstract

Selective damage to dopaminergic transporters following exposure to the brominated flame retardant, HBCD

By Kelly Genskow

Over the last several decades, the use of halogenated organic compounds has become the cause of environmental and human health concerns. Of particular notoriety has been the establishment of the neurotoxicity of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). The subsequent banning of PBDEs has led to greatly increased use of hexabromocyclododecane (HBCD) as a flame retardant in consumer products. The physiochemical similarities between HBCD and PBDEs suggest that HBCD may also be neurotoxic to the dopamine system. The purpose of this study was to assess the neurotoxicity of HBCD on the nigrostriatal dopamine system using an *in vitro* and *in vivo* approach. We demonstrate that HBCD causes significant cell death in a dopaminergic cell line, as well as reductions in the growth and viability of TH+ primary cultured neurons. Assessment of the *in vivo* neurotoxicity of HBCD resulted in significant reductions in the expression of the striatal dopamine transporter (DAT) and VMAT2, both of which are integral in mediating dopamine homeostasis and neurotransmission in the dopamine circuit. However, no changes were seen in the expression of TH in the dopamine terminal, or striatal levels of dopamine. To date, these are the first data to demonstrate that exposure to HBCD disrupts the nigrostriatal dopamine system. Given these results and the ubiquitous nature of HBCD in the environment, its possible role as an environmental risk factor for Parkinson disease should be further investigated.

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Thank you to Josh Bradner for working with me on this research, spending hours in a little culture room teaching me all about SK-N-SH cell and primary neuron culture techniques, and listening to my farm stories. Thank you as well to the other members of the Caudle and Miller labs, for providing help when needed and always making it a fun and exciting place to work.

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1.0 Introduction

Over the last several decades, the use of halogenated organic compounds has become the cause of environmental and human health concerns. Particular attention has been given to polychlorinated biphenyls (PCBs), which were widely used for their insulating and flame retarding properties. As the toxicity of PCBs became known and they were phased out of use, a new class of compounds, the brominated flame retardants (BFRs), many of which possess similar physiochemical properties as PCBs, saw a precipitous influx in manufacture and use (Covaci et al., 2011). BFRs are commonly used in household and commercial products such as electronics equipment, plastics, paints, and textiles in order to reduce the flammability of these consumer products (de Wit, 2002). Until recently, the most dominant class of BFRs has been polybrominated diphenyl ethers (PBDEs). PBDEs are persistent, bioaccumulative, and have become distributed globally throughout the environment and population, much as has been the case for PCBs (Law et al., 2003, Covaci et al., 2011). However, investigations into the toxicity of PBDEs revealed adverse effects on learning and memory, neurodevelopment, and have recently been shown by our group to damage the nigrostriatal dopamine system in mice orally exposed to the PBDE mixture DE-71 (Bradner et al., 2013b, Herbstman and Mall, 2014). As a result of toxicity studies, the use of all PBDEs was banned in the European Union in 2004 and most congeners of PBDEs were banned or voluntarily removed in the United States.

As manufacturers reduced the use of PBDEs, hexabromocyclododecane (HBCD) has become a common flame retardant in consumer products. HBCD is a brominated flame retardant that is used predominantly in expanded polystryrene foam for thermal

insulation in buildings, as well as in upholstered furniture, automobile textiles and cushions, and electronics (Covaci et al., 2006). Similar to PBDEs, HBCD is persistent, lipophilic, and bioaccumulative within the environment. Additionally, like PBDEs, the integration of HBCD into products is considered additive rather than being chemically bound to materials, which makes it easy for HBCD to leach from products and deposit in the environment. Because of this, HBCD has been observed ubiquitously in the environment and is also commonly detected in human blood, adipose tissue, and breast milk (Covaci et al., 2006). Over the past decade, concerns regarding the toxicity of HBCD to humans, and corresponding regulatory considerations, have increased. In May 2013, HBCD production and use was listed to be banned under the Stockholm Convention on Persistent Organic Pollutants, and there is now a 5 year phase-out period. In light of these actions, the persistence of HBCD in the environment and the body, similar to that seen for other halogenated chemicals including PCBs and PBDEs, presents a scenario of continual exposure and health effects that could endure for many decades.

Studies evaluating the potential neurotoxicity of HBCD have begun to delineate specific neurodevelopmental and behavioral effects following exposure to the compound (Lilienthal et al., 2009, Saegusa et al., 2009, Saegusa et al., 2012, Miller-Rhodes et al., 2014). Additionally, a few studies have been performed that begin to address the effects of HBCD on the function of the dopamine system. Most notably, work by Dingemans et al. (2009) demonstrated a reduction in neurotransmitter release from PC12 cells following treatment with HBCD, while Mariussen and Fonnum (2003) identified HBCD as a potent inhibitor of dopamine uptake through the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2). These studies provide valuable insight into

the possible molecular targets of HBCD neurotoxicity in the nigrostriatal dopamine system, which is specifically damaged in Parkinson disease (PD). Parkinson disease is defined by pathological damage to dopaminergic neurons and loss of dopamine in the nigrostriatal dopamine system, resulting in the hallmark clinical features of the disease, such as slowness of movement, postural instability, and resting tremor (Blandini et al., 2000). Previous work from our group has shown the nigrostriatal dopamine system to be uniquely sensitive to exposure to halogenated compounds, including PCBs and PBDEs (Caudle et al., 2006, Bradner et al., 2013b). As evidence for the contribution of exposure to environmental toxicants and the risk for developing PD increases, it is imperative to identify and characterize emerging or current environmental hazards that could elicit damage to the dopamine system. Thus, this study sought to further address the potential neurotoxic effects of HBCD on the nigrostriatal dopamine system by coupling *in vitro* and *in vivo* models. With these approaches we found HBCD to be neurotoxic to dopaminergic neurons and their neuronal outgrowths in an *in vitro* model. These findings were further addressed *in vivo*, where oral exposure to HBCD resulted in significant reductions in the expression of several synaptic proteins in the striatal dopamine nerve terminal. These findings suggest that exposure to HBCD is neurotoxic to select dopaminergic targets in the striatum, which are involved in mediating criticial functions in dopamine handling and maintaining the integrity of the nigrostriatal dopamine system.

2.0 Materials and Methods

2.1 Chemicals and Reagents

Hexabromocyclododecane (HBCD) was purchased from Sigma-Aldrich (St. Louis, MO). SK-N-SH cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). WST-1 Cytotoxicity Assay Kit was purchased from Roche (Nutley, NJ). 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Pierce (Rockford, IL). Hibernate A and Hibernate A − Calcium were purchased from BrainBits (Springfield, IL). B27, DNaseI, and Neurobasal A were purchased from Life Technologies (Carlsbad, CA). Papain was obtained from Sigma-Aldrich (St. Louis, MO). Dispase II was purchased from Roche (Nutley, NJ). Aphidicolin was purchased from A.G Scientific (San Diego, CA). The BCA protein assay kit was obtained from Pierce (Rockford, IL). Monoclonal anti-rat dopamine transporter (DAT) and polyclonal rabbit anti-tyrosine hydroxylase (TH) antibodies were purchased from EMD Millipore (Billerica, MA). 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 antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-GABA transporter 1 (GAT1), vesicular GABA transporter (vGAT), and vesicular glutamate transporter (vGlut) antibodies were purchased from Synaptic Systems (Germany). Mouse anti-microtubule associated protein 2 (MAP2) antibodies were purchased from Abcam (San Francisco, CA). Secondary antibodies conjugated to horseradish peroxidase were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Secondary antibodies conjugated to fluorescent tags were obtained from Life Technologies

(Carlsbad, CA). Monoamine standards for dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Culturing of SK-N-SH Cells

Cells were cultured in DMEM F12 media supplemented with 100 units/ml penicillin, 100 units/ml streptomycin and 10% fetal bovine serum. Cells were cultured at 37˚C in a humidified atmosphere with 5% CO2 and propagated according to the protocol provided by the supplier. When cells were confluent they were passaged to working concentrations in the appropriate culture plate for treatment with HBCD.

2.3 WST-1 Cytotoxicity Assay

When cells were confluent, they were passaged to 75,000 cells per well in 96-well plates at 100 µl for treatment with HBCD. Cell death was assessed using the WST-1 Cell Proliferation Assay, as previously described (Wilson et al., 2014b). Following treatment for 24 hours with 0, 5, 10, 15, 20, or 25 µM of HBCD dissolved in DMSO, 10 µl/well of Cell Proliferation Reagent was added to cells and incubated for 3 hours at 37˚C and 5% CO_2. Cytotoxicity was then measured by enzymatic cleavage of the tetrazolium salt WST-1 to a water-soluble formazan dye detected by spectral absorbance. Viable cells form more formazan than less viable cells. Spectral absorbance was measured at 450 nanometers on an Epoch BioTek microplate spectrophotometer and analyzed using Gen5 software (2.0) and GraphPad software.

2.4 DCF Assessment of Oxidative Stress

Intracellular generation of reactive oxygen species (ROS) was evaluated using the carboxy-derivative of fluorescein, (5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA). Cells were plated at 100,000 cells per well in a 96-well plate and grown for 24 hours before treatment. Culture media was removed and cells were exposed to 100 μM DCFDA and incubated at 37˚C for 45 minutes. Cells were then washed with PBS, and media containing 0, 5, 10, 20, or 30 μM of HBCD was added to the cells. Treatment with H_2O_2 was included as a positive control for the assay. Cells were immediately analyzed for 120 minutes at 488 nm excitation and 530 nm emission using a fluorescent microplate reader.

2.5 Primary Culture of Mesencephalic Neurons

Briefly, ventral mesencephalic neuron cultures were prepared from postnatal mice (postnatal day 1-3) as previously described (Bradner et al., 2013b, Wilson et al., 2014b). Brains were dissected in ice cold Hibernate A supplemented with B27. Following isolation of the relevant region and the removal of meninges, tissue pieces were chemically treated with dissociation solution, containing Papain (1 mg/ml), Dispase II (1.2U/ml), and DNase I (1μl/ml) dissolved in Hibernate A- Calcium for 20 minutes at 37°C and gently agitated every 5 minutes. Tissue was then rinsed in plating media, containing Neurobasal-A and 10% heat inactivated fetal bovine serum and mechanically dissociated using gentle trituration. Cells were plated on poly-d-lysine pre-coated 96 well plates at 40,000 cells per well. Plating media was removed and immediately switched to Neurobasal-A based culture media containing B27, 1% L-glutamine and 1% penicillin-

streptomycin after 2 hours of incubation, *in vitro*. The following day culture media containing aphidicolin (1ug/ml) was added to reduce the proliferation of glial cells in culture. Primary cultures were treated for 72 hours *in vitro* with 0, 1.75, 2.5, 5, 7.5, or 10 μM concentrations of HBCD dissolved in DMSO. After 72 hours, cells were fixed in 4% PFA for 20 minutes and incubated overnight in rabbit anti-TH and mouse anti-MAP2 at 4°C. The following day cultures were incubated with fluorescent secondary antibodies, goat anti-rabbit 488 and goat anti-mouse 572 for 1 hour at room temperature. After staining with DAPI, cells were rinsed and stored in PBS. Images of treated cultures were taken using an Array Scan VTI HCS (Cellomics; Pittsburgh, PA). Forty-nine contiguous fields were taken per well, DAPI+ nuclei were counted, TH+ cell bodies were identified, and neurite length and branch point were recorded for all fields containing at least one TH+ cell body. Objects were identified and measured using the neuronal profiling bioapplication from Thermo Scientific. Statistical significance between the control and treatment groups for neuron count, neurite length, and neurite branch point of TH+ neurons was determined using GraphPad analysis software.

2.6 Animals and Treatment

Eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Three month old mice were orally gavaged with 25 μl of 25 mg/kg of HBCD dissolved in corn oil vehicle daily for 30 days, as previously described (Caudle et al., 2006, Bradner et al., 2013b). This dosing paradigm was intended to represent the primary route of human exposure, via oral ingestion, to HBCD. Mice were sacrificed one day following the last exposure, and bilateral striatum was collected for subsequent

analysis. 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.

2.7 High Performance Liquid Chromatography (HPLC) of Striatal Neurochemistry

HPLC analysis of neurochemistry was performed as previously described (Caudle et al., 2007). Briefly, dissected striata were sonicated in 0.1 M perchloric acid. Homogenates were centrifuged at 15,000 ×g and the supernatant filtered through a 0.22 μm filter by centrifugation at 15,000 ×g. The supernatants were analyzed for levels of DA, DOPAC, and HVA. Quantification was made by reference to calibration curves made with individual standards.

2.8 Western Blot Analysis

Western blots were used to quantify the amount of DAT, TH, VMAT2, GAT1, vGAT, vGlut, and β-actin present in samples of striatal tissue from treated and control mice. Analysis was performed as previously described (Caudle et al., 2006). Briefly, striatum 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 in a monoclonal antibody to the N-terminus of DAT. DAT antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody (1:10,000) and enhanced chemiluminescence. The

luminescence signal was captured on an Alpha Innotech Fluorochem imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to coblotted dilutional standards of pooled striata from all control samples. Membranes were stripped for 15 minutes at room temperature with Pierce Stripping Buffer and sequentially reprobed with β-actin (1:1000), TH (1:1000), VMAT2 (1:10,000), GAT1 (1:1,000), vGAT (1:1,000), and vGlut (1:50,000) antibodies. β-Actin blots were used to ensure equal protein loading across samples.

2.9 Statistical analysis

All analysis was performed on raw data for each treatment group by one-way or two-way ANOVA. Post hoc analysis was performed using Tukey's post hoc test. Significance is reported at the $p < 0.05$ level.

3.0 Results

In order to initially establish the neurotoxic potential of HBCD on dopaminergic cells we performed our first assessement using the SK-N-SH neuroblastoma cell line, which is known to exhibit dopaminergic properties (Richards and Sadee, 1986, Kidd and Schneider, 2010). Cytotoxicity was assessed using the WST-1 cell viability assay following exposure to DMSO or increasing concentrations of HBCD (0-25 μM) for 24 hours. Cell viability was reduced in a dose dependent manner beginning at 10 μM and continuing to 25 μM, which resulted in approximately 70% reduction in cell viability (Figure 1A). These findings demonstrate the general neurotoxic potential of HBCD on a dopaminergic cell line.

The generation of reactive oxygen species (ROS) in the nigrostriatal dopamine system has been shown to be a major contributor to PD pathogenesis (Dias et al., 2013). In order to gain insight into a potential mechanism mediating dopaminergic cell loss we assessed the formation of ROS using the well defined DCFDA assay. Cells treated with increasing concentrations of HBCD (0-30 μM) demonstrated an increase in fluorescence, indicative of an elevation in ROS production. These elevations were not dose dependent, instead demonstrating an initial increase with 5 μM that was sustained over increasing concentrations (Figure 1B). Samples treated with 400 μM H_2O_2 were included in each run in order to ensure the reliability of our assay.

We next sought to extend our interrogation of the neurotoxic effects of HBCD on dopaminergic neurons in a more biologically complex *in vitro* model system by exposing dopamine-rich primary cultured neurons isolated from the ventral mesencephalon and assessing these effects using high content analysis. This experimental platform allows for the efficient and sensitive evaluation of alterations to neuronal morphology that extends beyond simple quantification of cell loss and provides insight into the potential neuronal targets altered by HBCD treatment. Using this approach HBCD resulted in a significant decrease in the total number of TH+ neurons in culture at concentrations ranging from 5-10 μM HBCD (Figure 2). In addition to loss of TH+ neurons, we also observed a reduction in neurite branching and neurite length of the TH+ neurons treated with HBCD. These data demonstrate an elaborated neurotoxic profile of HBCD on dopaminergic neurons *in vitro.*

The effect of HBCD on the dopamine system was next assessed using an *in vivo* model. For this approach we orally gavaged 3-month old male mice with 25 mg/kg

HBCD for 30 days and then evaluated alterations to select dopaminergic proteins in the striatum of control and HBCD treated mice. Using this dosing paradigm we found mice exposed to HBCD exhibited a significant reduction in the expression of the plasmalemmal DAT, which functions to terminate the dopaminergic signal by recycling dopamine from the synapse (Figure 3). Additionally, similar reductions were observed for VMAT2, which resides on synaptic vesicles and functions to sequester dopamine from the cytosol and into synaptic vesicles for neurotransmitter release in the striatum. In contrast, we did not observe a change in the expression of the dopamine synthesis enzyme, TH, which is a marker of terminal integrity in the striatal dopamine neurons. To further assess the effects of HBCD on the dopamine system, levels of dopamine and its metabolites were analyzed in the striatum. As shown in Figure 4, exposure to HBCD for 30 days did not affect the concentrations of dopamine, DOPAC, or HVA in this region.

Given our findings related to alterations in the dopamine system we were interested in determining the selectivity of these changes for dopamine neurons. The striatum is heavily innervated with GABAergic and glutamatergic projections and interneurons, which further refine the motor output of the basal ganglia (Obeso et al., 2008). Evaluation of transporter proteins involved in GABAergic and glutamatergic signaling in the striatum demonstrated no change in expression of GAT1, vGAT, or vGlut following exposure to HBCD (Figure 5). These data suggest that the neurotoxic effects of HBCD are preferential for the dopamine neurons in the striatum.

4.0 Discussion

Exposure to a variety of environmental chemicals, including pesticides and industrial compounds, has been shown to damage the nigrostriatal dopamine system and be a significant contributor in the etiopathogenesis of Parkinson disease (PD) (Hatcher et al., 2008, Wirdefeldt et al., 2011, Caudle et al., 2012). Work from our group has specifically demonstrated alterations to this circuit following exposure to PCBs, as well as PBDEs (Caudle et al., 2006, Bradner et al., 2013b). As the manufacture and use of these compounds has been or is currently being reduced, the manufacture of chemically similar compounds, such as HBCD, has been increased and HBCD has thus become a prominent flame retardant in many consumer products. While some studies have addressed the potential neurotoxicity of HBCD on the dopamine system, many questions still remain regarding the explicit targets and mechanisms involved in neurotoxicity. Thus our current study sought to interrogate the impact of HBCD exposure on dopamine neurons using an *in vitro* and *in vivo* approach. Resulting significant reductions in cell viability and levels of important dopaminergic proteins, similar to what has previously been demonstrated for PBDEs as well as PCBs, implicate HBCD as yet another environmental neurotoxin that can target the dopamine system.

Initial assessments to better understand the general neurotoxicity of HBCD focused on acute exposures of dopaminergic neuroblastoma SK-N-SH cells, resulting in significant decreases in cell viability even at low micromolar concentrations. These reductions in cell viability align very well with those previously observed by our group with another brominated flame retardant compound, DE-71, a commercially available

mixture of PBDEs (Bradner et al., 2013b). These results provided initial evidence that HBCD exerts generalized neurotoxicity in a dopaminergic cell line.

As the generation of oxidative stress is considered a major contributor to dopaminergic neuron toxicity we next evaluated if the significant reductions in cell viability in the presence of HBCD could be a function of ROS generation by the compound. Our finding shows that short-term exposure to HBCD results in a significant increase in ROS production in the SK-N-SH cells. Given that elevations of ROS were identified within 2 hours of HBCD treatment while dramatic reductions in cell viability were observed following 24 hours of treatment with HBCD, it can be speculated that generation of ROS could initiate and underlie the loss of cell viability in our culture conditions. Indeed, previous work has suggested that HBCD-mediated reductions in cell viability are dependent upon ROS. While treatment of cerebellar granule cells with HBCD elicited a substantial reduction in cell viability, pretreatment of these cultures with the antioxidant vitamin E attenuated these losses (Reistad et al., 2006).

We expanded upon the SK-N-SH data with *in vitro* HBCD exposure of dopaminergic primary cultured ventral mesencephalic neurons. In our assay, TH+ dopamine neurons also showed sensitivity to HBCD, with significant reductions in a number of different neuronal metrics, including the number of TH+ neurons as well as the length and arborization of the neurite outgrowths. These results are interesting as they begin to address specific aspects of the dopaminergic morphology, beyond cell number, that are sensitive to damage by HBCD. While HBCD treatment resulted in a significant loss of TH+ neurons, HBCD also caused a reduction in the length and complexity of the TH+ neurite outgrowths that arise from the cell body and are involved in mediating

synapse formation and communication with adjacent neurons. Like our SK-N-SH data, these findings are congruent with our previously published observations of PBDE-mediated neurotoxicity in TH+ neurons and follow with additional studies in our lab that have paired primary cultured neurons with high content analysis to delineate specific alterations in neuronal morphology (Bradner et al., 2013a, Bradner et al., 2013b, Wilson et al., 2014a, Wilson et al., 2014b). It is critical to note that analysis of neurite morphology in this HBCD study was not dependent upon loss of TH+ neurons. Our evaluative parameters for these endpoints are performed on intact and viable TH+ neurons, which means a reduction in TH+ neuron number does not underlie a reduction in other morphological endpoints, such as TH+ neurite length or branching. These findings are interesting as they suggest that HBCD-mediated neurotoxicity may be affecting multiple targets on the dopamine neurons. Alterations in neurite length and branching are especially relevant to PD pathogenesis as these dopaminergic processes that innervate the striatum are severely damaged prior to a substantial loss of dopaminergic cell bodies in the substantia nigra pars compacta. This suggests that dysfunction of the presynaptic dopaminergic terminal may be a significant component of PD pathogenesis.

Our findings in TH+ primary cultured neurons served to guide our *in vivo* experiments, which focused on interrogating the effects of HBCD on select presynaptic dopaminergic proteins in the striatum. While prenatal and neonatal HBCD exposures have been used to analyze dopamine-related behavioral effects of HBCD in rodents, there have been no adult exposures or ensuing laboratory measurements of the dopamine circuit *in vivo* (Lilienthal et al., 2009, Miller-Rhodes et al., 2014). In our study, exposure

of 3-month old mice to HBCD resulted in a precipitous reduction in the expression of DAT and VMAT2, both of which are integral in mediating dopamine homeostasis and neurotransmission in the dopamine circuit. In contrast to these reductions, we did not observe a change in TH expression or levels of dopamine and its major metabolites in the striatum. Similar alterations to presynaptic proteins were also seen by our group in mice that were exposed to the PCB mixtures Aroclor 1254 and 1260, as well as in mice exposed to the PBDE mixture DE-71 (Caudle et al., 2006, Bradner et al., 2013b). Based on our previous work these data suggest that HBCD selectively targets the expression of DAT and VMAT2 and these alterations occur prior to explicit damage or loss of the dopamine terminal, as assessed by expression of the TH protein and dopamine concentrations. Of these alterations, the reductions in VMAT2 raise specific questions regarding the potential effects HBCD exposure may have on dopamine handling in the cytosolic compartment of the dopamine terminal. Reductions in the expression and function of VMAT2 have demonstrated the importance of dopamine sequestration by VMAT2 (Caudle et al., 2008). Animals that have a 95% reduction in VMAT2 show an age dependent neurodegeneration in the nigrostriatal dopamine circuit that is defined by accumulation of reactive oxygen species as well as a differential loss of DAT and TH expression and function in the striatum (Caudle et al., 2007). Related effects of HBCD on the function of VMAT2 have also been identified by Mariussen and Fonnum (2003), who found HBCD to be a potent inhibitor of VMAT2 function in an acute synaptic vesicle preparation. The impact of HBCD-mediated alterations to DAT and VMAT2 on dopaminergic neurotransmission in our model remains to be evaluated. However, findings from Dingemans et al., (2009) has found treatment of PC12 cells with HBCD, as

well as its single isomers, causes a reduction in depolarization-induced monoamine neurotransmission. While their findings point to alterations in calcium handling related to transmitter release, dysfunction of other aspects of this process including dopamine handling could be involved.

Finally, the alterations observed in the striatum appear to be specific for the dopamine circuit as no change in GABAergic or glutamatergic transporters was recorded. The preferential damage to the dopamine system in the striatum following exposure to halogenated compounds was also observed by our group following treatment with PCBs and PBDEs and is further supported by a lack of effect of HBCD on GABAergic and glutamatergic transport in isolated synaptosomes and synaptic vesicles (Mariussen and Fonnum, 2003, Caudle et al., 2006, Bradner et al., 2013b). The reason for the selective vulnerability of the dopamine terminals to HBCD-mediated neurotoxicity is unclear but may be related to inherent functional properties of dopamine neurons that predispose them to toxicological insult, including an elevated potential for oxidative stress from metabolism of cytosolic dopamine and a concomitant reduction in antioxidant capacity to attenuate oxidative stress (Dias et al., 2013). Indeed, our results suggest that HBCD has elicited dysfunction in dopamine handling in the striatum, especially via reduction of VMAT2 expression. As discussed above, if cytosolic dopamine is not properly packaged and released, this may lead to increased ROS generation within the dopamine terminals (Caudle et al., 2007, Caudle et al., 2008).

The *in vitro* and *in vivo* studies of the nigrostriatal dopamine system presented here demonstrate the neurotoxic effects of HBCD on dopamine homeostasis. In particular, proteins involved with dopamine handling are significantly reduced in mice

dosed orally with HBCD. This is concerning given the chemical similarities between HBCD and PBDEs, which have also been demonstrated as dopaminergic neurotoxicants, and the ubiquitous nature of HBCD in the environment. Future implications of HBCD exposure to humans should continue to be investigated, starting with its possible role as an environmental risk factor for PD.

5.0 Figures

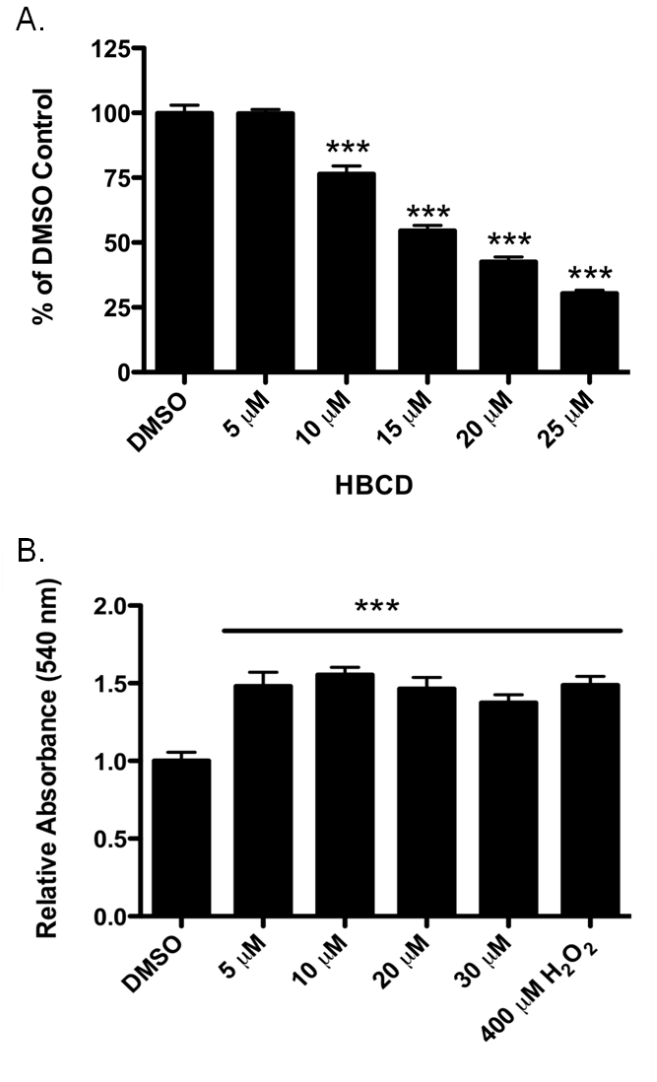

Figure 1: Exposure of SK-N-SH cells to HBCD causes a reduction in cell viability and an increased production of reactive oxygen species. (A) Exposure of the dopaminergic neuroblastoma cell line, SK-N-SH to HBCD caused a dose-dependent decrease in cell viability beginning at 10 μM HBCD. (B) All concentrations of HBCD tested, (5-30 μM), yielded significant increases in reactive oxygen species production compared to DMSO-treated cells. 400 μM H_2O_2 was used as the positive control for ROS generation. Columns represent percent change from DMSO control. Data represent the mean $\pm$ SEM of 8 experimental replicates performed over 3 separate experiments. ***Values that are significantly different from control ($p<0.001$).

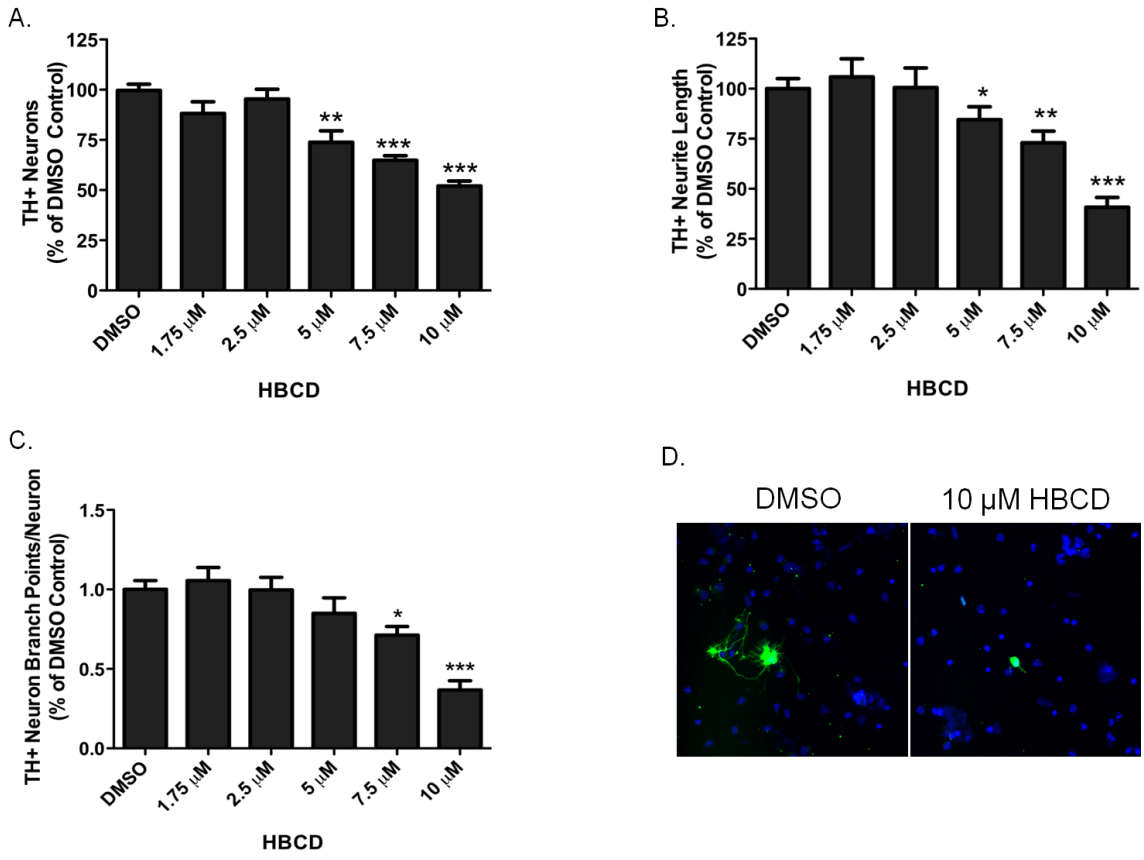

Figure 2: Exposure of TH+ ventral mesencephalic primary cultured neurons to HBCD caused dose-dependent alterations of neuron characteristics. (A) Treatment of primary cultures caused significant decreases in the number of TH+ neurons at 5, 7.5, and 10 μM HBCD. (B) Treatment also caused significant reduction in TH+ neurite length at 5, 7.5, and 10 μM HBCD and (C) a reduction in the number of TH+ neuron branch points per TH+ neuron at 7.5, and 10 μM HBCD. Columns represent percent change from DMSO control. Data represent the mean $\pm$ SEM of 4 experimental replicates across 3 separate experiments. *Values that are significantly different from control ($p<0.05$). **Values that are significantly different from control ($p<0.01$). ***Values that are significantly different from control ($p<0.001$).

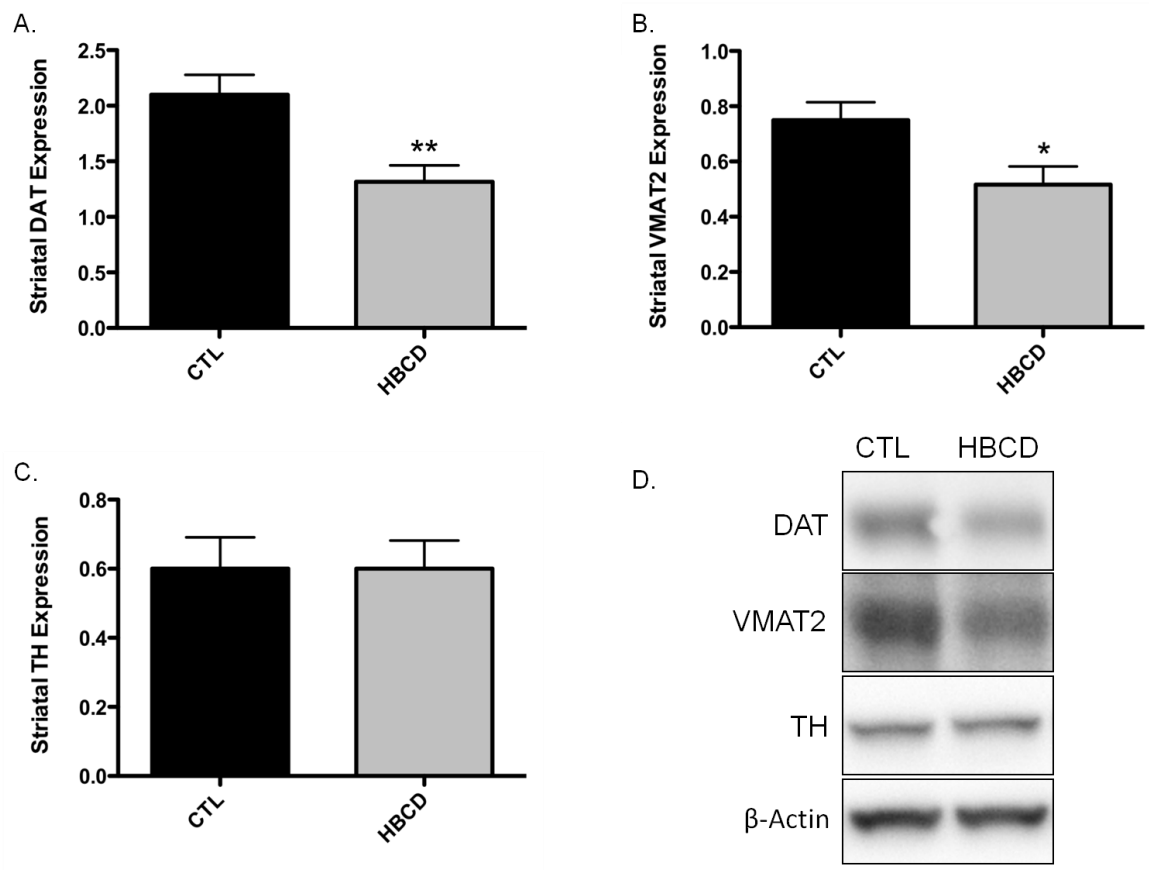

Figure 3: *In vivo* exposure of mice to HBCD causes a reduction in proteins involved in dopamine handling. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations in striatal expression of (A) DAT, (B) VMAT2, and (C) TH. (D) Representative immunoblot bands for each marker. Data represents mean $\pm$ SEM (4-6 animals per experimental group). *Values that are significantly different from control ($p<0.05$). **Values that are significantly different from control ($p<0.01$).

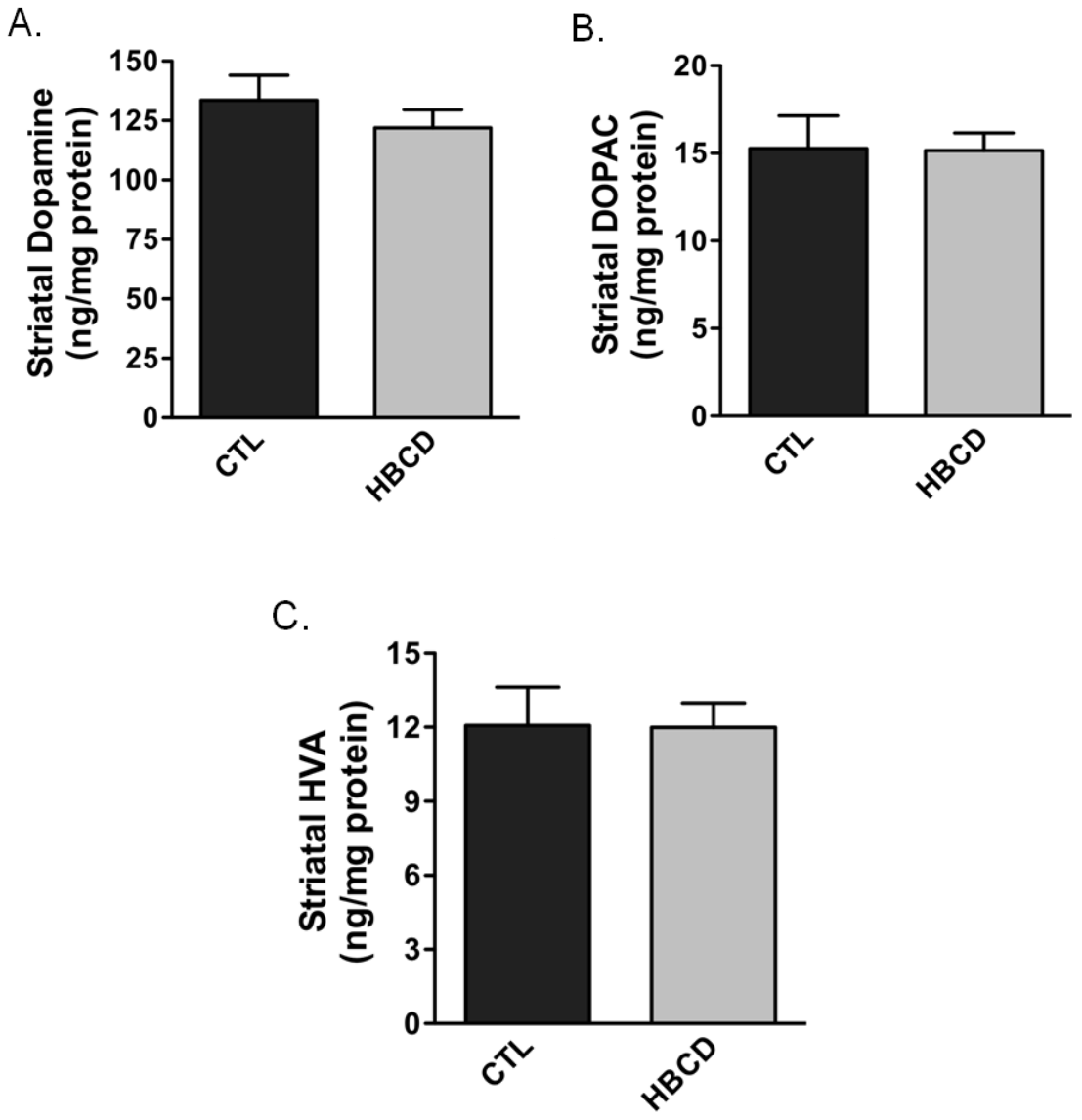

Figure 4: *In vivo* exposure to HBCD does not alter the concentration of dopamine or its metabolites in the striatum. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations to (A) dopamine, (B) DOPAC, or (C) HVA in the striatum. Columns represent the mean $\pm$ SEM of raw values (ng/mg protein) of 4-6 animals per experimental group.

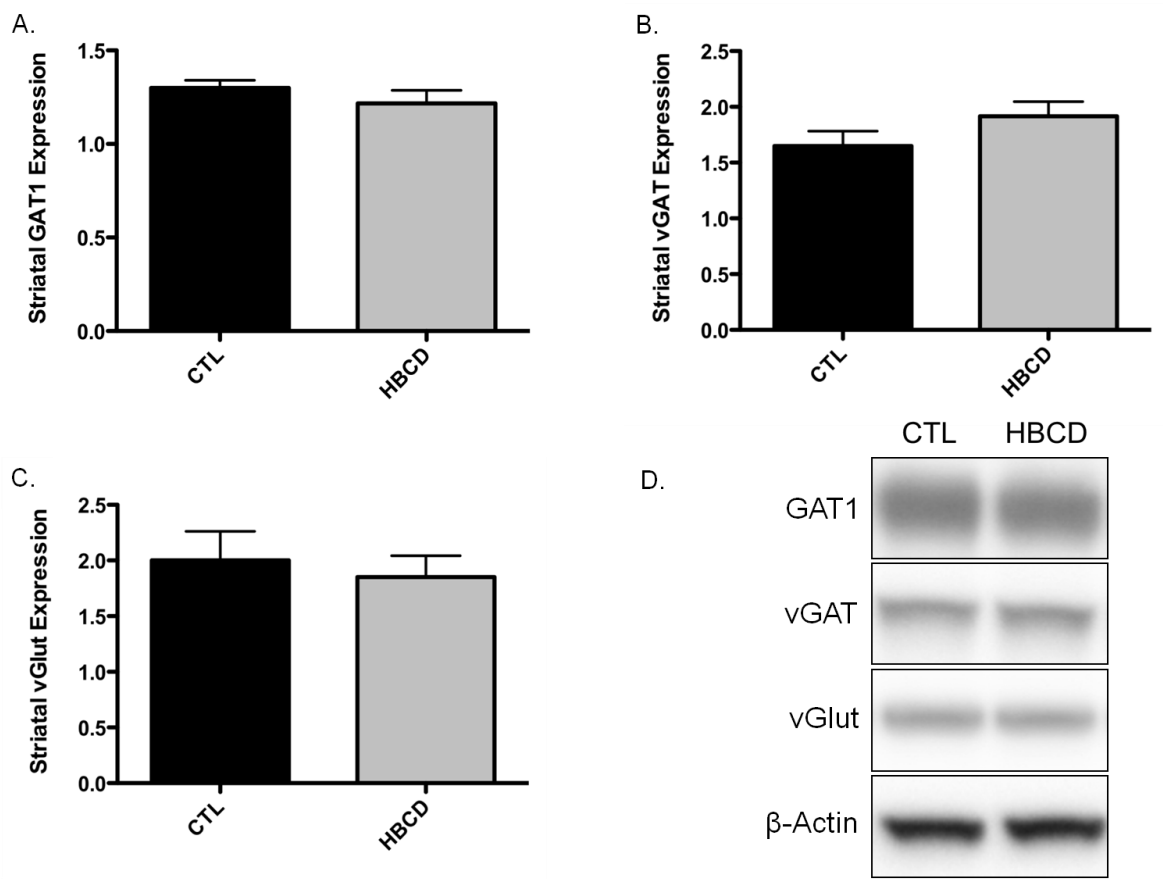

Figure 5: *In vivo* exposure to HBCD does not affect the expression of GABAergic or glutamatergic markers in the striatum. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations to (A) GAT1, (B) vGAT, and (C) vGlut in the striatum. (D) Representative immunoblot bands for each marker. Data represents mean ± SEM (4-6 animals per experimental group).

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Appendix

The effects of HBCD on systems beyond the striatum were also investigated in our *in vivo* mouse models. Experimental results are presented here from further immunoblot interrogations of the same dopaminergic and synaptic proteins as investigated within the straitum, but now within the cortex. Proteomic and metabolomic profiles were created following analysis of brain tissue or blood plasma and liver tissue, respectively. Pathways which demonstrated significant alterations for HBCD-treated mice in comparison to the control mice are presented. Proteomics and metabolomics provide a large-scale, comprehensive exploration of the levels of all proteins or chemical metabolites in an organism, and thus provide an unprejudiced perspective of alterations that may be occurring.

Additionally, data from a novel approach to measuring striatal dopamine release in mouse striatum following exposure to HBCD is also included. This technique is called fast-scan cyclic voltammetry, which allows direct measurement of dopamine release under physiologic conditions. To do so, a microelectrode is inserted into the striatal region of a brain slice that has just been removed from a mouse, and is used to apply a potential. The resulting current is recorded and directly relates how much dopamine was released upon stimulation.

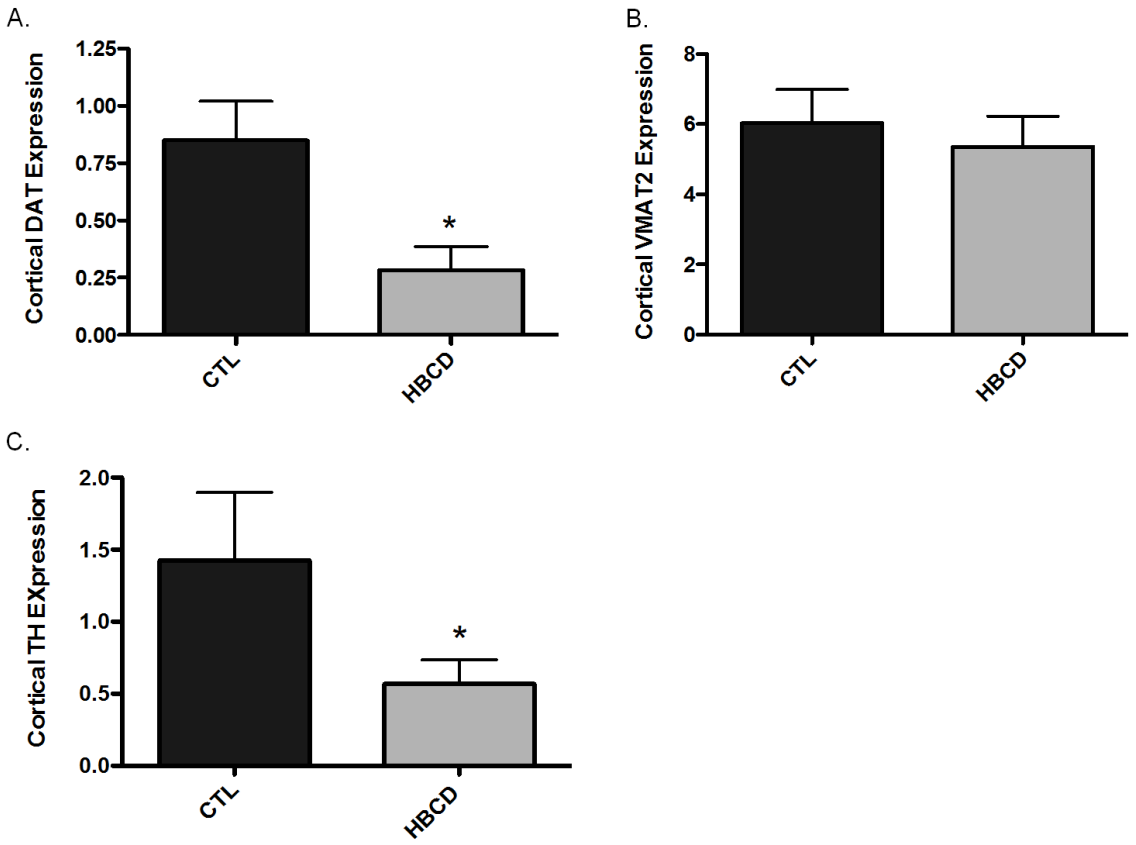


Figure S1: *In vivo* exposure of mice to HBCD causes cortical reductions in proteins involved in dopamine handling. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations to expression of (A) DAT, (B) VMAT2, and (C) TH. Data represents mean $\pm$ SEM (4-6 animals per experimental group). *Values that are significantly different from control ($p<0.05$).

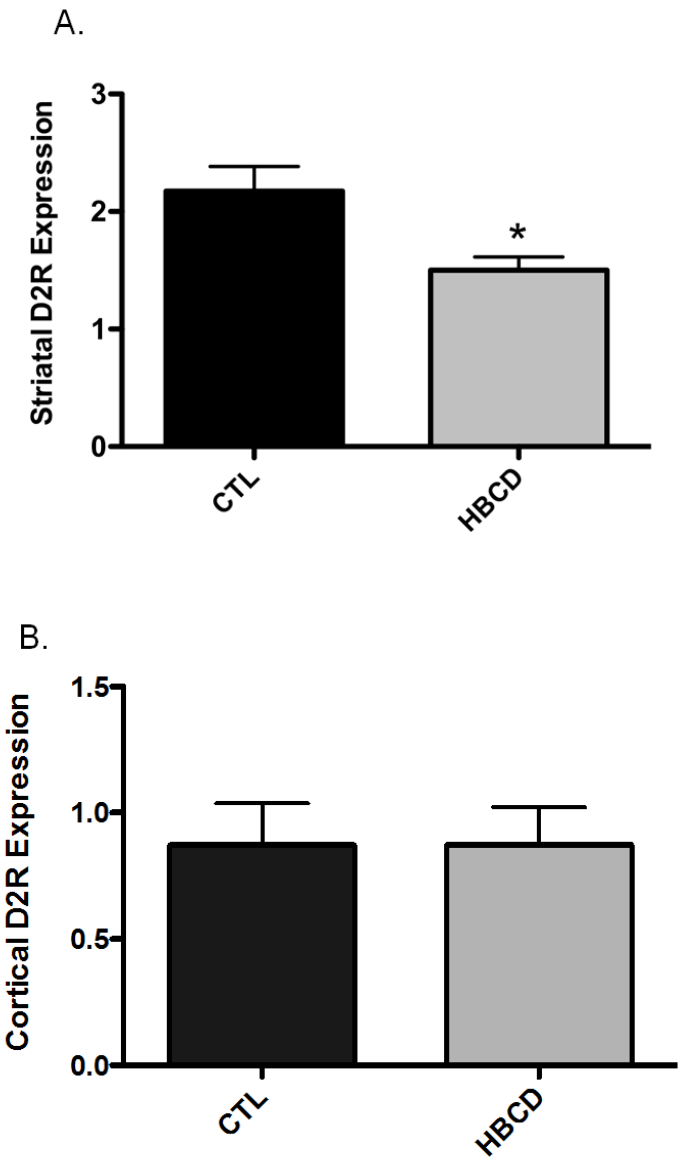


Figure S2: *In vivo* exposure of mice to HBCD causes reductions in D2R, the post-synaptic dopamine receptor, within the (A) striatum but not in the (B) cortex. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations to expression of D2R. Data represents mean $\pm$ SEM (4-6 animals per experimental group). *Values that are significantly different from control ($p<0.05$).

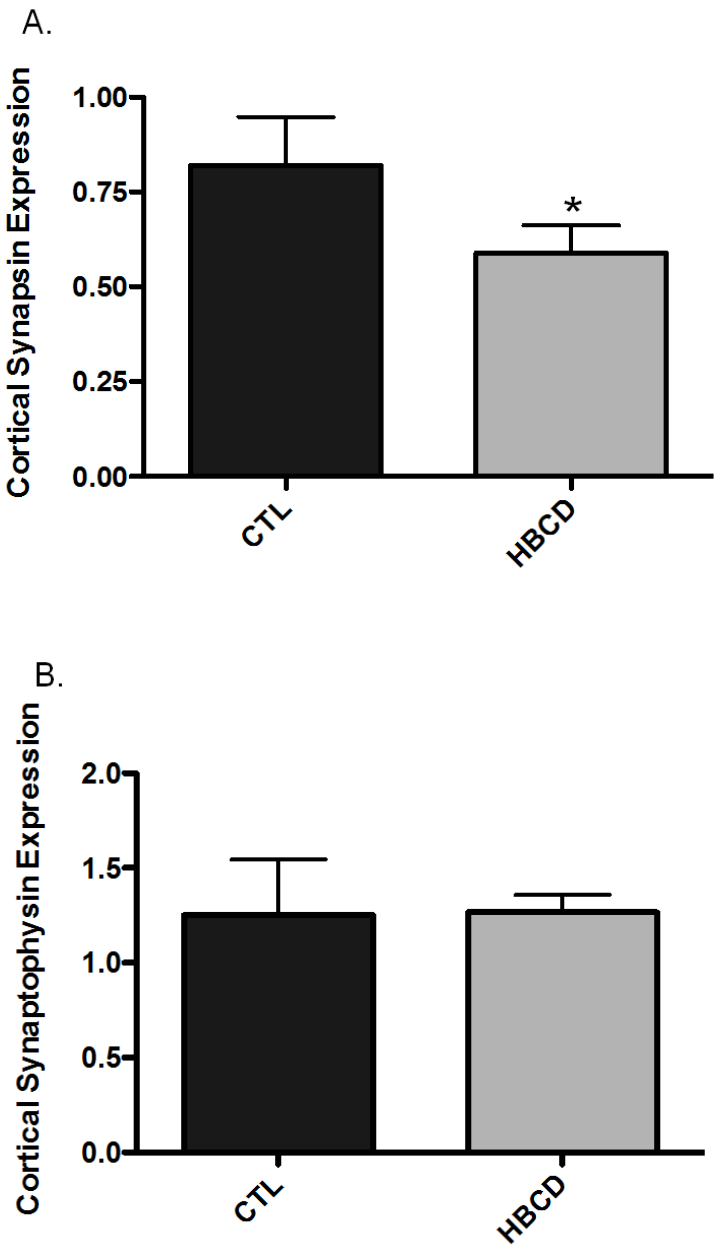


Figure S3: *In vivo* exposure of mice to HBCD causes cortical reductions in major synaptic vesicle proteins. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations to expression of (A) synapsin or (B) synaptophysin. Data represents mean $\pm$ SEM (4-6 animals per experimental group). *Values that are significantly different from control ($p<0.05$).

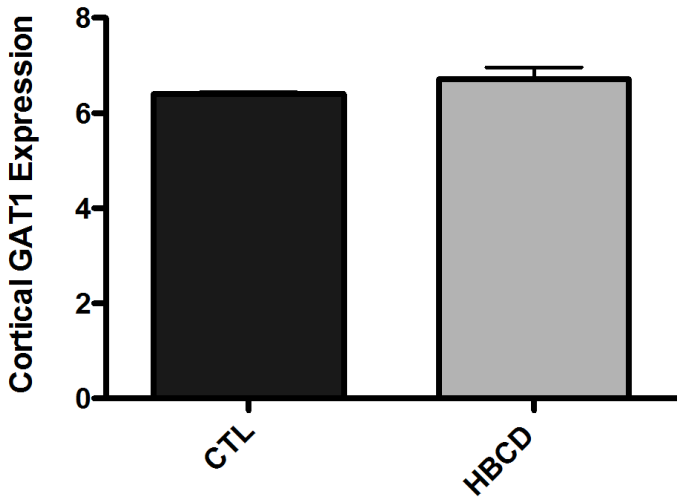


Figure S4: *In vivo* exposure to HBCD does not affect the expression of the GABAergic receptor GAT1 in the cortex. Animals received either 0 (control) or 25 mg/kg HBCD for 30 days and were then evaluated for alterations to GAT1. Data represents mean ± SEM (4-6 animals per experimental group).

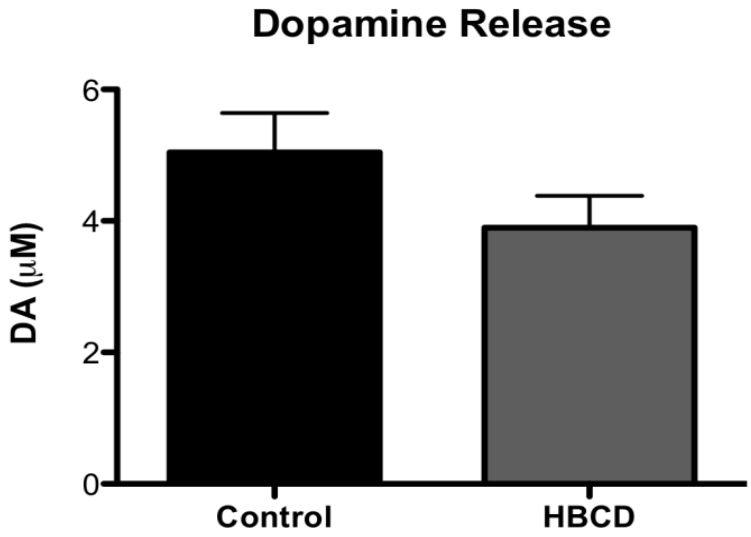


Figure S5: Levels of dopamine release within the striatum as measured using fast-scan cyclic voltammetry. A downward trend for dopamine release in HBCD-treated mice was observed but results did not reach significance ($p<0.05$). A five-recording survey of four different dorsal striatal release sites was taken for each animal with a 5 minute rest interval between each synaptic stimulation (5 animals per experimental group).

Table S1: Alterations in proteomic pathways following *in vivo* exposure to HBCD. All pathways in which HBCD-treated mice had a significant ($p<0.05$) increase or decrease compared to control mice are shown.

Gene	Protein	Primary Function	Magnitude Change (HBCD: Control)	p-value
Cytoskeletal Assembly and Cellular Structure				
Fmnl1	Formin	Regulation of cell morphology and cytoskeletal organization	3.4	0.03
Arfip2	Arfaptin	Involved in membrane ruffling/ cytoskeleton organization	2.5	0.003
Pitpnm1	Membrane-associated phosphatidylinositol transfer protein 1	Plays a role in cytoskeletal remodeling and is needed for brain development	1.7	0.03
Surf4	Surfeit locus protein 4	Maintenance of organelle organization	1.7	0.04
Rhot2	Mitochondrial Rho GTPase 2	Involved in mitochondrial trafficking	1.6	0.01
Sestd1	SEC14 domain and spectrin repeat-containing protein 1	Binds phospholipids in the membrane, may act as primary docking protein during membrane turnover	1.6	0.04
Syne1	Nesprin-1	Forms a linking network between organelles and the cytoskeleton	1.6	0.04
Anln	Anillin	Required for cytokinesis	0.7	0.04
Lamc1	Laminin subunit gamma-1	Mediates the attachment, migration, and organization of cells into tissues; involved in neuron projection development	0.7	0.05
Jam3	Junctional adhesion molecule C	Participates in cell-cell adhesion; associated with hemorrhagic destruction of the brain parenchyma	0.6	0.04
Lcp1	Plastin	Involved in actin filament bundle assembly and intracellular protein transport	0.6	0.01
Mobp	Myelin-associated oligodendrocyte basic protein	Structural constituent of the myelin sheath	0.6	0.02
Tjp2	Tight junction protein ZO-2	Plays a role in tight junctions, protein binding, and absorption	0.6	0.02
Dmd	Dystrophin	Anchors the extracellular matrix to the cytoskeleton; implicated in signaling and synaptic transmission	0.5	0.007
Tns1	Tensin	Involved in fibrillar adhesion formation and cell migration	0.4	0.04
Synaptic Function				
Cnksr2	Connector enhancer of kinase suppressor of ras 2	Regulation of signal transduction	2.7	0.02

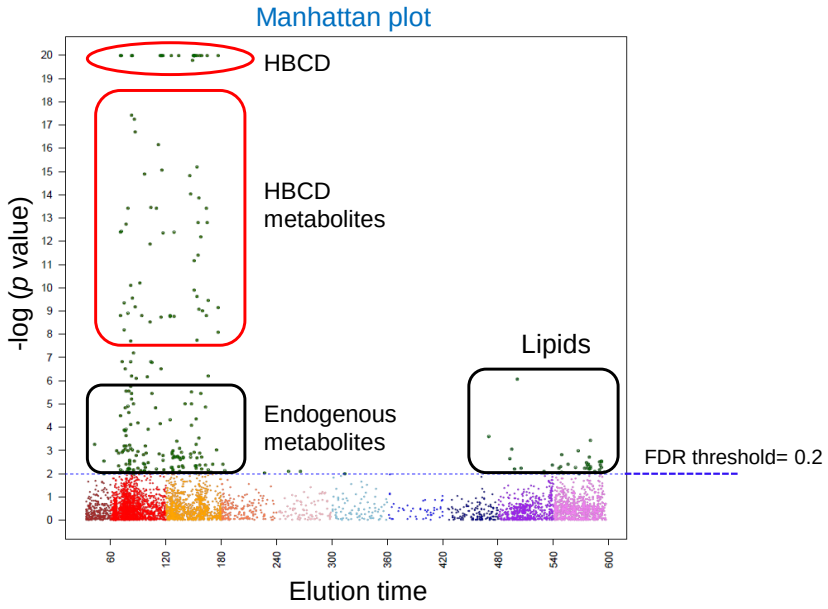
Gene	Protein	Primary Function	Magnitude Change (HBCD: Control)	p-value
Vac14	Protein Vac14 homolog	Receptor activity/ vesicle synthesis	2.7	0.03
Nck1	Cytoplasmic protein NCK1	Plays a role in receptor binding and signaling	2.3	0.008
Rasgrf2	Ras-specific guanine nucleotide-releasing factor 2	Functions in synaptic plasticity by contributing to the induction of long term potentiation.	2.1	0.01
Lrrc7	Densin 180	Required for normal synaptic structure and function; associated with postsynaptic density	1.9	0.03
Trappc11	Trafficking protein particle complex subunit 11	Involved in vesicle-mediated transport	1.7	0.04
Gria1	Glutamate receptor 1	Ligand-gated glutamate ion channel	1.6	0.02
Protein Degradation				
Uchl3	Ubiquitin carboxyl-terminal hydrolyse isozyme L3	Controls levels of cellular ubiquitin; may be involved in memory	1.9	0.0005
Clpp	ATP-dependent Clp protease proteolytic subunit	General hydrolysis of proteins	1.8	0.05
Stub1	E3 ubiquitin-protein ligase CHIP	Targets misfolded chaperone proteins for proteasomal degradation	1.8	0.04
Sumo2	Small ubiquitin-related modifier 2	Can be covalently attached to proteins to act as a signal for proteasomal degradation of modified proteins	1.7	0.03
Trim28	Transcription intermediary factor 1-beta	Involved in sumoylation and proteasomal degradation	1.7	0.002
Atg9a	Autophagy-related protein 9A	Involved in autophagic vacuole assembly	1.6	0.04
Cst3	Cystatin-3	Regulates local enzyme activity; inhibits cysteine proteinases	1.6	0.01
Hnmt	Histamine N-methyltransferase	Plays an important role in degrading histamine and is involved with brain development	1.5	0.04
Psmd9	26S proteasome non-ATPase regulatory subunit 9	Acts as a chaperone during the assembly of the 26S proteasome	0.7	0.02
Ube3a	Ubiquitin-protein ligase E3A	Involved with regulation of the circadian clock by marking component proteins for degradation; associated with Angelman neurodegenerative disorder	0.5	0.03
Cell Signaling				
Arhgap26	Rho-GTPase-activating protein 26	Cytoskeleton/plasma membrane organization; GTPase mediated signal transduction	3.0	0.03

Gene	Protein	Primary Function	Magnitude Change (HBCD: Control)	p-value
Gucy1a2	Guanylate cyclase soluble subunit alpha-2	Intracellular signal transduction	3.0	0.01
Rgs14	Regulator of G-protein signaling 14	Functions either as GTPase activating protein or as a GDP-dissociation inhibitor to regulate G protein signaling	2.5	0.006
Pip4k2c	Phosphatidlyinositol 5-phosphate 4-kinase type-2 gamma	Production of phosphatidylinositol biphosphate, necessary for cell signaling, in the endoplasmic reticulum	2.1	0.04
Rapgef4	Rap guanine nucleotide exchange factor 4	Involved in regulation of cAMP-dependent exocytosis	2.1	0.04
RyR2	Ryanodine receptor 2	Calcium-induced calcium channel; required for cellular calcium ion homeostasis and cardiac muscle contraction	2.1	0.03
Vwa8	von Willebrand factor A domain-containing protein 8	ATPase activity	0.4	0.03
Tinagl1	Tubulointerstitial nephritis antigen-like	Involved in immune response	0.3	0.03
Regulation of cell differentiation and/or apoptosis				
Rbm25	RNA-binding protein 25	Regulation of apoptotic cell death	4.3	0.002
Tsnax	Translin-associated protein X	Cell differentiation and protein transport; associations with schizophrenia and memory	3.0	$2.7x10^{-5}$
Hmgb2	High mobility group protein B2	Binds DNA and is involved in cell differentiation and apoptosis	2.0	0.006
Hnrnpll	Heterogeneous nuclear ribonucleoprotein L-like	Involved in mRNA processing and splicing	2.0	0.03
Npm1	Nucleophosmin	Involved in regulation of cell proliferation and apoptosis	1.9	0.04
Cirbp	Cold-inducible RNA-binding protein	Plays an essential role in cold-induced suppression of cell proliferation	1.8	0.0007
Opcml	Opioid-binding protein/cell adhesion molecule	Involved in neuron recognition and regulation of neurogenesis	1.8	0.04
Khdrbs3	KH domain-containing, RNA-binding signal, transduction-associated protein 3	Inhibits cell proliferation and involved in regulation of transcription	1.7	0.04
Rnps1	RNA-binding protein with serine-rich domain 1	Positive regulation of apoptosis	1.7	0.004

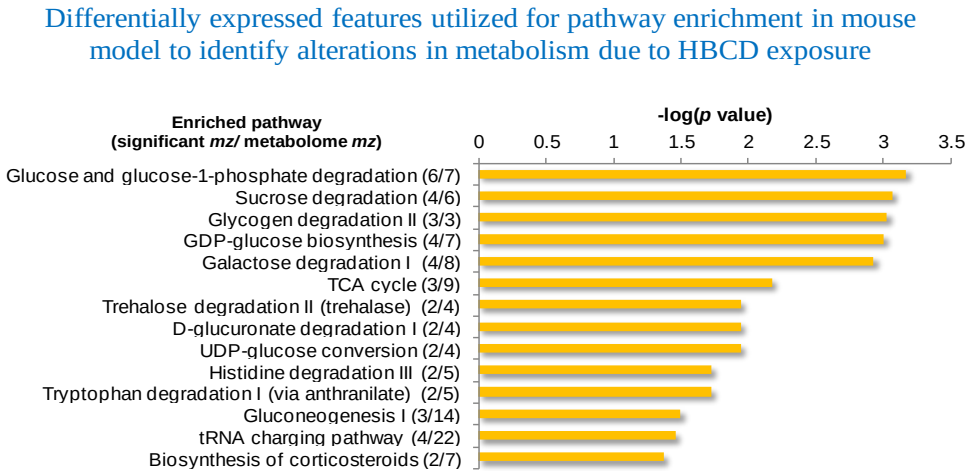
Gene	Protein	Primary Function	Magnitude Change (HBCD: Control)	p-value
Celf2	CUGBP Elav-like family member 2	Involved in mRNA splicing, translation, stability, and regulation of post-translational events	1.6	0.02
Gas7	Growth-arrest specific protein 7	Promotes maturation and morphological differentiation of cerebellar neurons	1.6	0.0007
Lrrc47	Leucine-rich repeat-containing protein 47	Involved in tRNA ligase activity	1.6	0.03
Pgam5	Serine/threonine-protein phosphatase PGAM5	Acts as a central mediator for programmed necrosis induced by reactive oxygen species	1.5	0.01
Fxr1	Fragile X mental retardation syndrome-related protein-1	Required for embryonic and postnatal development of muscle tissue	0.7	0.004
Gart	Trifunctional purine biosynthetic protein adenosine-3	Involved in cerebral cortex, cerebellum, and brainstem development	0.6	0.02
Phf20l1	PHD finger protein 20-like protein 1	Zinc finger involved in zinc ion binding	0.6	0.003
Ist1	IST1 homolog	Involved in cell division and protein localization	0.5	0.0009
Mtfp1	Mitochondrial fission process protein 1	Involved in mitochondrial division; loss leads to apoptosis	0.5	0.009
Tspyl3	Putative testis-specific Y-encoded-like protein 3	Involved in nucleosome assembly	0.5	0.002
Vwa5a	von Willebrand factor A domain-containing protein 5A	Involved in tumorigenesis as a tumor suppressor	0.5	0.04
Smarca5	SWI/SNF matrix-associated actin-dependent regulator of chromatin subfamily A member 5	Helicase involved in regulation of gene expression	0.4	0.04
Mbnl2	Muscleblind-like protein 2	Involved in mRNA processing and regulation of RNA splicing	0.4	0.003
Biosynthesis and metabolic processes				
Mpdu1	Mannose-P-dolichol utilization defect 1 protein	Normal utilization of mannose dolichol phosphate in synthesis of oligosaccharides	3.1	0.005
Rbm3	RNA-binding protein 3	Enhances global protein synthesis at physiologic and mild hypothermic temperatures	2.3	0.002
Acsf3	Acyl-coA synthetase family member 3	Catalyzes initial fatty acid biosynthetic process	1.9	0.04
Arl1	ADP-ribosylation factor-like protein	Involved in toxin metabolism	1.6	0.02
Mpst	3-mercaptopyruvate sulfurtransferase	Acts as an antioxidant and responds to toxic substances	1.6	0.004
Echdc1	Ethylmalonyl Co-A decarboxylase	May be involved in metabolite proofreading as a decarboxylase	0.7	0.02

Gene	Protein	Primary Function	Magnitude Change (HBCD: Control)	p-value
Ckm	Creatine kinase M-type	Plays a central role in energy transduction in the brain and other tissues with large energy demands	0.6	0.02
Mtap	S-methyl-5'-thioadenosine phosphorylase	Involved in amino acid metabolic process	0.6	0.03
Tmx1	Thioredoxin-related transmembrane protein 1	Involved in cell redox homeostasis	0.6	0.0003
Asns	Asparagine synthetase [glutamine hydrolyzing]	Asparagine biosynthesis; ASNS deficiency is characterized by progressive encephalopathy and cortical atrophy	0.5	0.03
Dcxr	L-xylulose reductase	Involved in glucose metabolism	0.5	0.007
Nucb	Nucleobindin-1	Important calcium binding protein; role in calcium homeostasis	0.5	0.003
Slc16a1	Monocarboxylate transporter 1	Required for normal nutrient assimilation and glucose homeostasis	0.5	0.02
Tsta3	GDP-L-fucose synthase	Involved in sugar metabolism	0.5	0.03
Galk1	Galactokinase	Major enzyme for galactose metabolism	0.4	0.002

Protein identification and GO analysis obtained using UniProt, UniProt Consortium



Plasma metabolome wide association study (MWAS) identified HBCD parent compound, metabolites and altered expression of bioactive metabolites



Enriched pathways indicated alteration in key liver metabolic pathways, including glycolysis and glycogen metabolism. Alterations in histidine degradation suggest additional HBCD changes in liver enzymatic processes

Figure S6: Metabolomics profiling for identifying exposure signature and metabolic alteration

As was observed in the striatum, exposure to HBCD caused significant reductions in DAT within the cortex (Figure S1A). However, in the cortex reductions were observed for levels of TH but not VMAT2, which is in direct contrast to results observed in the striatum (Figure S1B, C). Contrasts were also seen between the alterations to striatal and cortical expression of the dopamine receptor D2R (Figure S2). Reasons for these differences in effect based on brain region following exposure to HBCD have not yet been identified. Additionally, HBCD-treated mice had a decrease in expression of the important synaptic protein synapsin, but not synaptophysin (Figure S3). Finally, no alterations to the GABAergic receptor, GAT1, were observed in cortical samples from treated mice (Figure S5), as was also observed within the striatum. These results again imply the possibility that HBCD specifically targets the dopaminergic system but not other neurotransmitters.

Notably, the proteomics screen identifies a number of proteins that were either increased or decreased significantly in HBCD-treated mice compared to the control mice (Table S1). These proteins may broadly be stratified by function into categories affecting cytoskeletal assembly and cellular structure, synaptic function, protein degradation, cell signaling, regulation of cell differentiation and/or apoptosis, or biosynthesis and metabolic processes. Many of these have important implications for brain development and function, and of particular importance may be those proteins involved in synapse formation and function. These findings, combined with the striatal alterations presented above, parallel what has recently been proposed for the progression of PD in humans, which is focused on initial damage to select dopaminergic proteins located in the synapse.

The results from this proteomics screen, as well as the metabolomics (Figure S6, of which a number of the pathways match what was seen within the protein alterations), provide interesting targets to be further interrogated in future studies. Further analysis of the interactions implied by the proteomic and metabolomic screens must also be investigated. Additionally, exploration into the mechanisms through which HBCD acts and reasons why it affects the same proteins differently in the striatum and cortex are planned, as well as further fast-scan cyclic voltammetry experiments and behavioral studies. As a whole, the implications of HBCD exposure for the health of the environment and people, particularly with regard to Parkinson disease, must continue to be investigated, along with considerations for the policies necessary to provide protection from these effects.