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Exposure to HBCDD – a Brominated Flame Retardant – Induces Altered Synaptic Protein Expression in the Hippocampus and the Frontal Cortex of a Male Mouse Model

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Bachelor of Science in Biology
The University of Georgia
2013

Thesis Committee Chair: W. Michael Caudle, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Environmental Health
2016

Abstract

Exposure to HBCDD – a Brominated Flame Retardant – Induces Altered Synaptic Protein Expression in the Hippocampus and the Frontal Cortex of a Male Mouse Model

By: Chad R. Camp

HBCDD, a brominated flame retardant, is rapidly replacing its neurotoxic predecessor, PBDEs, in a myriad of commercial and consumer products. HBCDD is a persistent, bioaccumulative compound with a propensity to deposit in lipid-rich tissues, including the brain. Although HBCDD is structurally similar to PBDEs, its neurotoxic potential is still unclear. Rodent neurobehavioral studies have indicated that exposure to HBCDD impairs the learning and memory process, and causes increased hyperactivity. In addition, HBCDD exposure was shown to alter synaptic protein expression in the striatum of a mouse model. Given these neurobehavioral outcomes, we hypothesized that HBCDD may also be affecting the hippocampus and the frontal cortex. Accordingly, we used an in vivo male mouse model to evaluate HBCDD-mediated synaptic protein alterations in these two brain regions. In the hippocampus, we dosed male mice with 25mg/kg HBCDD for 30 days and evaluated alterations in synaptic protein expression immediately following their last exposure. Here, we found that HBCDD altered synaptic proteins vital to dopaminergic, glutamatergic, and GABAergic neurotransmission including: TH, VMAT2, mGluR1, mGluR2, NMDAR-2B, and GAT-1. Additionally, in the hippocampus, HBCDD exposure caused a decrease in tau expression while increasing several regulatory proteins involved in neurogenesis, synaptogenesis, axonal growth, and synaptic plasticity such as Ube-3A, BDNF, PSA-NCAM, and GAP43. In the frontal cortex, we used a persistent HBCDD dosing paradigm that involved dosing male mice with 25mg/kg HBCDD for 30 days then allowing them to sit for 42 days before synaptic proteins were evaluated. Using this paradigm, we found that HBCDD altered frontal cortex expression of TH and mGluR1, while mGluR2, NMDAR-2B, vGlut, and GAT-1 expression was unchanged. Additionally, HBCDD exposure induced an upregulation of frontal cortex cytoskeletal proteins including β-actin and tau. All of these alterations can produce an additive effect, giving rise to the many neurobehavioral deficits seen following HBCDD exposure, including difficulty with learning and memory, and increased hyperactivity.

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Acknowledgements

First and foremost, I would like to thank Dr. Mike Caudle for taking me under his wing and teaching me the essentials of biomedical research and manuscript writing. Dr. Caudle is more than an academic mentor, extending his positive influence into every facet of my life and for that, I am forever grateful.

Next, I would like to thank Dr. Dana Barr for helping secure this particular thesis topic. She has always been extremely helpful, receptive, and accommodating throughout my stay at Emory. Without her, my progression as a scientist would not be possible.

Lastly, I would like to thank Joshua Bradner and the graduate students of the Miller lab for helping me conduct experiments and welcoming me into their lab.

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Figures and Tables

Introduction

Flame retardants are compounds that are added to commercial and consumer products to help improve fire safety standards. Although there are several different kinds of flame retardants, the vast majority are organohalogens. Organohalogen flame retardants are composed of an organic hydrocarbon backbone and decorated with various halogen atoms (Br, Cl, or F). The use of organohalogen flame retardants came under heavy scrutiny after polychlorinated biphenyls (PCBs) were banned in the United States in 1979[1]. After PCBs were phased out, a new category of organohalogen flame retardants were introduced into the market. Brominated flame retardants (BFRs) are currently added to everyday items such as electronics, building insulation, polyurethane foam, textiles, and wires and cables[2].

Immediately after PCBs were banned, a specific class of BFR, known as polybrominated diphenyl ethers or PBDEs, dominated the scene. The most controversial use of PBDEs was in their addition to polyurethane foam. This foam is used in the construction of couches, chairs, and office furniture. Recent studies have shown that PBDEs have a strong propensity to leech out of the foam and into environment, thus posing a potential public health risk[3]. Ultimately, the use of PBDEs in foam and all other applications underwent a voluntary phase out in the early 2000's as PBDEs have been found to be neurotoxic[4]. Due to the phase out, a new BFR replacement has been rapidly introduced called 1,2,5,6,9,10-hexabromocyclododecane or HBCDD. HBCDD is structurally similar to PBDEs and is now the dominant polyurethane foam additive[5]. Unfortunately, HBCDD has also shown signs of leeching out into the environment[6]. The problem lies in how these BFRs are added to foam products. To help cut costs, BFRs

including both PBDEs and HBCDD, are simply sprayed onto the finished foam construct as opposed to being incorporated within the foam's polymers. This spray-on application technique is what allows these compounds to leech out of the foam. This poor application is exacerbated by constant agitation from sitting, standing, or jumping on furniture, forcing even more of these compounds out into the environment. Now excised from the foam, exposure risk to BFRs, like PBDEs and HBCDD, is drastically increased.

Once released into the environment, BFRs will bind to house dust[7]. After binding to house dust, they can be easily inhaled and penetrate deeply into the lungs[8]. Contaminated house dust can also be ingested, proving to be a major source of exposure for toddlers[9]. A recent study has shown a significant correlation between levels of BFR-contaminated house dust and total BFR body burden of residents[10]. Once in the body, these compounds will bioaccumulate in lipid-rich tissues and organs[11]. This bioaccumulation helps them resist metabolism that would otherwise lead to their excretion. Lipid sequestering also poses a huge health risk as continued BFR exposure will lead to a constantly-increasing body burden.

Since the brain is a lipophilic organ, is it vulnerable to BFR exposure. The neurotoxic potential of PBDEs is well established, with a general consensus that exposure causes a disruption in intracellular Ca^{2+} homeostasis, inducing oxidative stress[12-14]. Several studies have identified that PBDE-induced neurotoxicity elicits deleterious effects on specific regions of the brain including the cerebellum, hippocampus, and frontal cortex[14-16]. In vitro experiments on human and animal model neural stem cells from these brain regions have determined that exposure to various PBDE congeners are cytotoxic[17-19]. At lower doses, PBDEs can suppress stem cell neurite outgrowths and limit their differentiation into mature neurons and supporting oligodendrocytes[16, 20]. In vivo work has demonstrated that

exposure to certain PBDE congeners decreases hippocampal brain mass and alters mRNA and protein expression in the hippocampus, frontal cortex, and cerebellum[12, 15, 16, 21, 22]. In particular, changes in protein expression within these brain regions were related to neural development, brain maturation, synaptic plasticity and remodeling, energy metabolism, and learning and memory[23].

On a molecular level, many of the proteins altered were synaptic proteins. An in vitro study on rat hippocampal neurons found that exposure to PBDEs directly interacted with and antagonized plasma membrane bound voltage-gated sodium channels, in a dose-dependent manner[24]. Additionally, several in vivo studies have shown that synaptic proteins vital to glutamateric, GABAergic, and dopaminergic neurotransmission experienced an alteration in expression following exposure to PBDEs[16, 21, 25, 26]. These altered synaptic proteins span many different functional classes including ionotropic and metabotropic neurotransmitter receptors, reuptake transporters, synaptic vesicle transporters, and neurotransmitter biosynthetic enzymes[16, 26]. Altering the way these three neurotransmitter systems communicate could cause potential neurobehavioral changes. Studies have shown that glutamatergic, GABAergic, and dopaminergic systems work in concert to affect many different processes, including long-term potentiation (LTP) and long-term depression (LTD)[27].

Neurobehavioral animal model studies have supported these molecular neurotoxicity discoveries. Multiple studies have found that dosing perinatal and neonatal mouse and rat animal models with PBDEs caused significant neurodevelopmental toxicity[28-31]. Specifically, these exposed animals showed signs of hyperactivity, diminished attention span, and decreased potential for learning and memory[30-32]. Neurobehavioral changes were not limited to infants and young adults. Several studies have also shown that mature adult rats

and mice dosed with PBDEs exhibit similar declines in attention span and with learning and memory capabilities[21, 33]. These neurobehavioral changes are most likely explained by altered synaptic protein expression in key areas of the brain such as the frontal cortex and the hippocampus[34].

There have also been a handful of longitudinal human epidemiological cohort studies that further corroborate PBDE exposure, neurotoxicity, and changes in neurobehavior. A New York based longitudinal cohort study (n=152) measured PBDE concentration in cord blood and scored neurodevelopmental outcomes in neonates at 12-48 months and at 72 months. This study found that an increase in PBDE concentration was positively associated with lower scores on cognitive performance tests aimed at measuring mental development and general intelligence (IQ)[35]. In 2012, a North Carolina cohort study (n=222) measured PBDE concentration in breast milk at 3 months postpartum and found a positive association between PBDE concentration and elevated scores on the Infant-Toddler Social and Emotional Assessment (ITSEA) test[36]. Above average scores on the ITSEA test indicate an increase in externalizing behavior, particularly in hyperactivity and impulsivity. Finally, a California based cohort study (n=310) found a positive association between prenatal maternal serum PBDE concentration and poorer attention, cognition, and fine motor abilities in children at seven years of age[37].

The summation of the neurotoxic evidence presented eventually led to the phase out of all PBDE-based flame retardants, setting the stage for the emergence of HBCDD. In addition to being present in house dust, HBCDD has been measured in human serum samples and, most importantly, it has been measured in mouse brain tissue following oral administration[12, 38]. The neurotoxic evidence for HBCDD is not as dense due to the newness of the compound. Still, in vitro studies have shown that exposure to HBCDD is

cytotoxic, alters neurite outgrowths, and suppresses oligodendrocyte expression[39-41]. Just like PBDEs, the molecular toxicity of HBCDD may involve dysregulation of intracellular calcium homeostasis[42, 43]. A recent in vitro study has shown that HBCDD exposure inhibits several calcium-modulating proteins, like the sarco/endoplasmic reticulum ATPases (SERCAs) and plasma membrane bound voltage-gated calcium channels[42, 43].

In addition, in vivo animal models have shown signs of HBCDD-induced neurotoxicity. HBCDD exposure alters mRNA and protein expression in the cortex and the hippocampus, altering expression of several synaptic proteins related to excitotoxicity and G-protein coupled receptor signaling[12, 41, 42, 44]. Additionally, HBCDD has been shown to inhibit the functionality of several key synaptic proteins related to glutamate, GABA, and dopamine neurotransmission[41, 44]. Neurobehavioral studies are sparse, limited only to a few animal models. Prenatal and neonatal HBCDD exposure increases hyperactivity, decreases fine motor control, decreases attention span, and decreases learning and memory potential[45, 46]. These neurobehavioral deviations may be explained by HBCDD-induced interference in dopaminergic, glutamatergic, and/or GABAergic neurotransmission.

Given that PBDE exposure can damage the hippocampus and the frontal cortex, we hypothesized that HBCDD exposure may also damage these two brain regions. This hypothesis is based on the structural similarities between the two BFRs and on similar findings in neurobehavioral studies. The hippocampus and the frontal cortex have been implicated with hyperactivity and learning and memory deficits, which may provide important insights into HBCDD-induced neurotoxicity[34, 47]. Additionally, several studies have shown the propensity of HBCDD to interact with synaptic proteins, which may further explain neurobehavioral changes following exposure to the compound[41, 44].

To test this hypothesis, we measured synaptic protein expression within these two

brain regions. Synaptic proteins were chosen as they are essential to neurotransmission and cellular communication. More specifically, we focused our research on dopaminergic, glutamatergic, and GABAergic neurotransmitter systems. These systems, among many other functions, are associated with activity levels and learning and memory, are found in high abundance in the frontal cortex and the hippocampus, and have been shown to be affected by HBCDD exposure. Finding any alterations in synaptic protein expression within these three neurotransmitter systems would lay the molecular framework for HBCDD-mediated neurobehavioral deficits.

Materials and Methods

Chemicals and Reagents

Hexabromocyclododecane (HBCDD) was purchased from Sigma-Aldrich (St. Louis, MO). The BCA protein assay kit was obtained from Pierce (Rockford, IL). Polyclonal rabbit anti-tyrosine hydroxylase (TH) antibodies, rabbit anti-metabotropic glutamate receptor 1 (mGluR1) antibodies, rabbit anti-phosphorylated-metabotropic glutamate receptor 1 (p-mGluR1) antibodies, and rabbit anti-metabotropic glutamate receptor 2 (mGluR2) 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 (GAT-1) antibodies and vesicular glutamate transporter (vGlut) antibodies were purchased from Synaptic Systems (Germany). Mouse anti-NMDAR-2B

receptor subunit antibodies were purchased from BD Transduction (San Jose, CA). Mouse anti-tau antibodies and mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Rabbit anti-brain-derived neurotropic factor (BDNF) antibodies and rabbit anti-ubiquitin-protein ligase E3A (Ube-3A) antibodies were purchased from ThermoFisher Scientific (Waltham, MA). Mouse anti-polysialylated-neural cell adhesion molecule (PSA-NCAM) antibodies and rabbit anti-growth associated protein 43 (GAP43) antibodies were purchased from Abcam (Cambridge, UK). Goat 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). SuperSignal West Dura Extended duration substrate and stripping buffer were obtained from Pierce. 3,3' Diaminobenzidine (DAB) was purchased from Sigma.

Animal Treatment

Eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Three month old male mice were orally gavaged with 25μL of corn oil control (n=4) or 25mg/kg of HBCDD (n=6) dissolved in corn oil vehicle daily for 30 days, as previously described[16, 26]. For hippocampus samples and some frontal cortex samples, mice were sacrificed one day following the last exposure and hippocampal/frontal cortex brain tissue was collected for subsequent analysis. This dosing paradigm was intended to represent one of the primary routes of human exposure, via oral ingestion, to HBCDD. All hippocampus results reported followed this dosing procedure. For clarity, the frontal cortex samples that followed this dosing procedure will be labeled as cortical-1, to reflect the

one day since their last exposure. Next, to evaluate the persistence of HBCDD-induced changes to synaptic proteins, another set three month old male mice were orally gavaged with 25μL of corn oil control (n=6) or 25mg/kg of HBCDD (n=7) dissolved in corn oil vehicle daily for 30 days, as previously described[16, 26]. Then, these mice were sacrificed 42 days following the last exposure and their frontal cortex regions were collected for subsequent analysis. For clarity, the frontal cortex samples corresponding to this persistent dosing paradigm will be labeled as cortical-42, to represent the 42 days since their last exposure. 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.

Western Blot Analysis

Western blots were used to quantify the amount of TH, VMAT2, NMDAR-2B, mGluR1, mGluR2, GAT-1, Ube-3A, GAP43, PSA-NCAM, BDNF, and tau present in samples of hippocampal brain tissue from treated and control mice. Furthermore, Western blots were used to quantify the amount of TH, NMDAR-2B, mGluR1, p-mGluR1, mGluR2, vGlut, GAT-1, β-actin, tau, and GAPDH present in samples of frontal cortex brain tissue from treated and control mice. Analysis was performed as previously described[26]. Briefly, tissue samples were homogenized and 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 primary monoclonal antibodies to the N-terminus of TH. TH antibody binding

was detected using a goat anti-rabbit horseradish peroxidase secondary antibody (1:10,000) and enhanced with chemiluminescence. The luminescence signal was captured on an Alpha Innotech Fluorochem imaging system and stored as a digital image. Hippocampal membrane samples were stripped for 15 minutes at room temperature with Pierce Stripping Buffer and sequentially reprobed with VMAT2 (1:10,000), NMDAR-2B (1:1,000), mGluR1 (1:1,250), mGluR2 (1:1,250), GAT-1 (3:5,000), Ube-3A (1:1,000), GAP43 (1:5,000), PSA-NCAM (1:5,000), BDNF (1:5,000), and tau (1:500). Additionally, frontal cortex membrane samples were stripped for 15 minutes at room temperature with Pierce Stripping Buffer and sequentially reprobed with NMDAR-2B (1:1,000), mGluR1 (1:1,250), p-mGluR1 (1:1,250), mGluR2 (1:1,250), vGlut (1:50,000), GAT-1 (3:5,000), β-actin (3:10,000), tau (1:500), and GAPDH (1:1,250). GAPDH blots were used to ensure equal protein loading across samples.

Immunohistochemistry

Immunohistochemical analysis of tissue was performed as previously described[26]. Briefly, brains were removed and serially sectioned at 25μm on a freezing microtome. Tissue was rinsed in 1× TBS and incubated in 3% H_2O_2 to remove endogenous peroxidases. Sections were blocked in 10% normal goat serum for 1 hour at room temperature before being incubated overnight with primary antibodies (rabbit anti-mGluR1 (1:1,250), mouse anti-PSA-NCAM (1:5,000), and mouse anti-tau (1:500)). The following day, tissue was rinsed in 1× TBS and then incubated for 1 hour in goat anti-rabbit secondary antibodies conjugated to biotin. Tissue was rinsed in 1× TBS and incubated for 1 hour in ABC solution. Tissue was rinsed in 1× TBS and the final product visualized using DAB. Free-

floating slices were mounted onto slides, serially dehydrated in ethanol and coverslipped. Immunostained sections were analyzed using bright-field microscopy and images captured on a Leitz microscope (Leica, Wetzlar, Germany).

Statistical Analysis

Analysis of the effects of HBCDD on dopaminergic, glutamatergic, GABAergic, and various neuronal integrity endpoints by immunoblotting was performed on raw data from each treatment group per each brain region via Student's t-test. Immunohistochemical data was for qualitatively evaluated alterations in protein expression. Significance is reported at the $p=0.05$ level.

Results

Hippocampus Samples

Given previous findings by our group regarding HBCDD-mediated damage to dopaminergic proteins in the striatum, we evaluated the effect of HBCDD on the dopamine system in the hippocampus using an in vivo male mouse model[41]. To accomplish this, 3-month old male mice were orally gavaged with 25mg/kg HBCDD for 30 days, sacrificed one day after their last exposure, and then evaluated for alterations in dopaminergic synaptic protein expression in control and HBCDD-treated mice. Using this dosing method, we found that mice exposed to HBCDD exhibited significant reductions in both TH and VMAT2 (Figure 1). TH is a biosynthetic enzyme responsible for the conversion of tyrosine

into dopamine and is highly expressed in dopaminergic neurons. VMAT2 is a synaptic vesicle protein that loads dopamine neurotransmitters into synaptic vesicles for release into the synapse. The downregulation of both TH and VMAT2 may indicate that HBCDD can impair dopaminergic neurotransmission in the hippocampus.

Next, we evaluated the effect of HBCDD on glutamate and GABA neurotransmitter systems in the hippocampus. We found that mice exposed to HBCDD experienced a decrease in post-synaptic NMDAR-2B expression and an increase in pre-synaptic mGluR2 expression (Figure 2). These results are interesting, as mGluR2 has been shown to negatively regulate NMDA receptor activity[48]. Next, Ube-3A – a ubiquitin ligase that is preferential towards the glutamate neurotransmitter system – was significantly increased in HBCDD-exposed mice (Figure 2). Lastly, GAT-1 – a pre-synaptic GABA reuptake transport protein – showed increased expression in HBCDD-treated mice (Figure 3). These findings show that, outside of the striatum, exposure to HBCDD does not show preference towards dopaminergic endpoints as glutamatergic and GABAergic protein markers also experienced alteration from basal expression.

Furthermore, we evaluated the effect of HBCDD on several neuronal integrity markers, including cytoskeletal proteins, synaptic cell-adhesion proteins, and neuronal growth factors in the hippocampus. Our results showed that HBCDD-treated mice had a decrease in tau expression, a cytoskeletal protein essential for axonal transport (Figure 4). Additionally, mice exposed to HBCDD displayed an increase in several synaptic growth and plasticity proteins including BDNF, GAP43, and PSA-NCAM (Figure 5). These findings demonstrate that HBCDD-induced toxicity might not be limited to neurotransmitter systems but also includes several proteins involved in neuronal integrity and proliferation.

Finally, immunohistochemical staining was performed on HBCDD-treated and

control mouse hippocampal brain tissue to obtain a visual representation of these alterations in synaptic protein expression. First, we stained for tau – a microtubule-associated protein involved in axonal transport. Exposure to HBCDD caused a decrease in tau expression in the hippocampus when compared to an untreated control (Figure 6). Next, we stained for mGluR1 and PSA-NCAM expression. mGluR1 is a post-synaptic metabotropic glutamate receptor while PSA-NCAM is a pre-synaptic cell adhesion protein that plays important roles in neurite outgrowth as well as in learning and memory formation. We found that HBCDD-exposed mice exhibited a reduction in hippocampal mGluR1 expression and an increase in hippocampal PSA-NCAM expression. Then, we merged these two immunohistochemical protein stains together to visual where these alterations where taking place. In Figure 7, HBCDD-treated mice show a reduction in hippocampal mGluR1 expression directly adjacent to showing an increase in hippocampal PSA-NCAM expression. These results indicate a possible interplay between these two synaptic proteins.

Frontal Cortex Samples

To further explore the effect of HBCDD on the dopamine system, we examined dopaminergic protein markers in the frontal cortex using an in vivo male mouse model. Instead of sacrificing the mice one day after exposure to HBCDD, we sacrificed them 42 days after exposure to HBCDD. This would allow us to evaluate the persistence of HBCDD-induced damage in the frontal cortex. This new dosing paradigm included 3-month old male mice that were orally gavaged with 25mg/kg HBCDD for 30 days, allowed to sit for 42 days, and then evaluated for dopaminergic synaptic protein expression in the frontal cortex of control and HBCDD-treated mice. For clarity, these mice will be referred to as

cortical-42 mice. We found that cortical-42 mice exposed to HBCDD exhibited a decrease in TH expression, a necessary enzyme in the dopamine biosynthetic pathway (Figure 8). This finding indicates that not only does HBCDD damage the dopaminergic neurotransmitter system, but its damage is not mitigated by a cessation in exposure.

Next, we evaluated the effect of HBCDD on glutamatergic and GABAergic neurotransmitter systems in the frontal cortex. We found that cortical-42 mice exposed to HBCDD showed a decrease in mGluR1 expression (Figure 9). Interestingly, we found that HBCDD-treated cortical-42 mice experienced no change in p-mGluR1, mGluR2, NMDAR-2B, or vGlut expression (Figure 9). In addition, HBCDD-treated cortical-42 mice showed no change in GAT-1 expression (Figure 10). These results could indicate that HBCDD-mediated damage to glutamatergic and GABAergic synaptic proteins in the frontal cortex can be alleviated by discontinued exposure or that the frontal cortex is less susceptible to glutamatergic and GABAergic synaptic protein damage than the hippocampus.

Furthermore, we evaluated the effect of HBCDD on several cytoskeletal proteins in the frontal cortex. First, we decided to test for tau expression in cortical-1 mice. These mice followed the exact same dosing paradigm as mentioned in our hippocampal results. 3-month old male mice were orally gavaged with 25mg/kg HBCDD for 30 days, sacrificed one day after their last exposure, and examined for tau expression in the frontal cortex. We found that there was no difference in tau expression between HBCDD-treated and control cortical-1 mice (Figure 11). This finding indicates that perhaps cytoskeletal proteins in the frontal cortex are not immediately vulnerable to HBCDD-mediated damage. Next, we examined β-actin and tau in cortical-42 mice. We found that HBCDD-treated cortical-42 mice showed an increase in both β-actin and tau expression (Figure 11). These results were very surprising given that β-actin is usually used as a loading control, to ensure that protein

concentrations have been standardized across all samples. To make sure these results were not due to error, we probed for GAPDH – another loading control. GAPDH is an enzyme involved in glucose metabolism and is highly conserved in all neuronal cells. Our results showed that GAPDH did not exhibit an alteration in expression between HBCDD-treated and control cortical-42 mice (Figure 11). Lastly, we probed for cytoskeletal protein involved in synaptic vesicle fusion called synapsin. We found that there was no difference in synapsin expression between HBCDD-treated and control cortical-42 mice (Figure 11).

Finally, immunohistochemical staining was performed on HBCDD-treated and control cortical-42 mouse frontal cortex brain tissue. We stained for mGluR1 protein expression in two different areas of the frontal cortex – the frontodorsal cortex and the frontolateral cortex. We found that mGluR1 protein expression was decreased in HBCDD-treated cortical-42 mice in both of these frontal cortex regions (Figure 12). These results indicate that the decreased mGluR1 expression following HBCDD exposure is not localized to any particular area of the frontal cortex.

For a summary for all Western blot data, please refer to Table 1.

Discussion

HBCDD is a highly persistent environmental toxicant with a tendency to resist metabolism and bioaccumulate in lipid-rich tissues, such as the brain. Due to the phase-out of PBDEs, HBCDD is seeing a dramatic rise in commercial and consumer use. Still, the neurotoxic potential of HBCDD is limited. Exposure to the compound has been associated with numerous neurobehavioral deficits, including hyperactivity and decreased learning and

memory potential. Previous work by our group has demonstrated that exposure to HBCDD causes specific damage to dopaminergic synaptic proteins using an in vivo male mouse model[41]. To expand upon our work with HBCDD, we decided to examine its effects on synaptic protein expression in the hippocampus and the frontal cortex.

Based on our prior findings, we first examined select dopaminergic endpoints in the hippocampus. In this study, exposure of 3-month old male mice to HBCDD caused significant reductions in both tyrosine hydroxylase (TH) and vesicular monoamine transporter 2 (VMAT2). TH is a necessary biosynthetic enzyme involved in the conversion of tyrosine into dopamine and VMAT2 is responsible for loading cytosolic dopamine into synaptic vesicles for release into the synapse cleft. Separately, reductions in both TH and VMAT2 would decrease the amount of dopamine released from pre-synaptic neurons. When evaluated together, decreases in both TH and VMAT2 work in a synergistic fashion with the end result being a marked decreased dopamine release. Dopaminergic neurotransmission in the hippocampus has many functions, one of which involves acting as a neuromodulator of glutamate and GABA neurotransmission in the processes of long-term potentiation (LTP) and long-term depression (LTD)[49]. Several in vivo mouse studies using the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) confirmed the role of dopamine neurotransmission in hippocampus-dependent LTP and LTD[50, 51]. MPTP is a neurotoxic compound that destroys dopaminergic neurons in the substantia nigra, a brain region implicated in Parkinson's disease. These dopaminergic neurons send axonal projections all throughout the brain, including the hippocampus. Treatment with MPTP is an effective way to evaluate neurobehavioral outcomes due to loss of dopaminergic input. Researchers have reported that mice treated with MPTP experienced significant reductions in hippocampal LTP, while hippocampal LTD was increased[51]. Additionally, treatment with

MPTP caused altered calcium homeostasis in the hippocampus. Specifically, MPTP treatment resulted in significant reductions in the enzyme calcium/calmodulin-dependent protein kinase II (CaMKII), vital enzyme involved in hippocampal LTP induction[50]. Accordingly, decreased dopamine exocytosis from pre-synaptic hippocampal neurons can interfere with LTP and LTD, potentially explaining HBCDD-mediated learning and memory deficits.

Next, synaptic proteins involved in glutamate neurotransmission were evaluated. We found that HBCDD-treated mice showed an increased expression of pre-synaptic MGluR2 glutamate receptor as well as a decreased expression of post-synaptic NMDAR-2B glutamate receptor. Although it is unclear what caused an increase in pre-synaptic MGluR2 expression, the decreased NMDAR-2B expression may be due to enhanced MGluR2 autoregulation[52]. Group II metabotropic glutamate receptors, including MGluR2, are embedded within the pre-synaptic membrane and modulate glutamate release into the synaptic cleft. Studies have shown that an increased MGluR2 expression will decrease pre-synaptic glutamate release, ultimately leading to a downregulation of certain NMDA receptor isoforms. This decreased expression of NMDAR-2B could explain problems with learning and memory following HBCDD exposure as the specific activation of these receptors are critical in hippocampus-dependent LTP and LTD. Additionally, we found that HBCDD-treated mice had a decreased expression of another post-synaptic glutamate receptor, MGluR1. Knockout studies with mice have demonstrated that MGluR1 function is involved in activity-dependent synaptic plasticity and associative learning in the hippocampus[53]. Taken as whole, HBCDD exposure decreases hippocampal glutamate neurotransmission and alters the expression of several vital glutamate synaptic proteins involved in the learning and memory process.

One explanation for the overall decrease in glutamate function as seen in the hippocampus of HBCDD-treated mice may be the upregulation of Ube-3A. Ube-3A is a ubiquitin ligase that serves to tag defective proteins for recycling and/or degradation. This enzyme was significantly increased in the hippocampus of mice exposed to HBCDD and recently has been implicated in the development of autism spectrum disorder (ASD)[54]. The expression of Ube-3A is controlled by a calcium-dependent transcription factor called myocyte enhancer factor-2 (MEF2)[55]. Previous work has confirmed that exposure to HBCDD alters neuronal calcium homeostasis, which may lead to an increased expression of Ube-3A via upregulated MEF2 activity. Ube-3A has many substrates, including the regulatory synaptic protein Arc[56]. Arc regulates neuronal responses to excitatory neurotransmission by modulating the insertion of AMPA glutamate receptors into the post-synaptic membrane[57]. Since Ube-3A is a ubiquitin ligase, increased Ube-3A expression would lead to decreased Arc levels, and thus an increase in post-synaptic AMPA receptor insertion. Dysregulation of AMPA receptor insertion may cause these neurons to alter their expression of other glutamate neurotransmitter receptors, including NMDA receptors and mGluRs. Additionally, the insertion and removal of AMPA glutamate receptors is a tightly controlled component of hippocampus-dependent LTP and LTD[58]. Increased AMPA receptor insertion into post-synaptic membranes would perturb these processes, potentially explaining deficits in learning and memory following exposure to HBCDD. Further research is needed to confirm this hypothesis, but the role of Ube-3A and Arc in HBCDD-mediated neurotoxicity should be examined more closely in the future.

Synaptic proteins involved in GABA neurotransmission were also examined. Just like glutamate, GABA activity in the hippocampus has been linked to LTP and LTD. We found that HBCDD exposure caused a significant increase in hippocampal GAT-1

expression. GAT-1 is a pre-synaptic transport protein that removes GABA from the synaptic cleft, shuttling it back into pre-synaptic neurons. An increase in GAT-1 activity means that less GABA neurotransmitter will be available to bind to post-synaptic receptors, thus leading to an overall decrease in inhibition. A loss of controlled inhibition in the hippocampus may lead to neurons firing out of sync. Precise spatial and temporal control of neuronal firing is crucial for hippocampus-dependent LTP and LTD. Altered hippocampal GAT-1 expression may disrupt this balance, causing problems with learning and memory. Recently, researchers decided the test this hypothesis by generating transgenic mice with increased levels of GAT-1 via microinjection of the GAT-1 transgene directly into the hippocampus. They found that these transgenic mice experienced a significant reduction in associative learning capacity and decreased memory retention[59]. In summation, these results show that in the hippocampus, HBCDD-mediated damage does not show specificity for dopaminergic neurotransmission. Here, we have demonstrated that HBCDD exposure affects hippocampal synaptic proteins involved in dopamine, glutamate, and GABA neurotransmitter systems.

We also decided to test for markers of neuronal integrity, including cytoskeletal proteins and neuronal growth factors. Surprisingly, we found that HBCDD-treated mice had a significantly decreased expression of tau. Tau is microtubule-associated protein (MAP), with several different functions. Mainly, tau is associated with anchoring the microtubule transport system near the axon terminal. This transport system follows a monorail design, with microtubules stretching from the neuronal cell body to the axon terminal. This allows proteins synthesized in the soma to be transported to the opposite end of the neuron, reaching the pre-synaptic membrane. It is difficult to say whether this observed decrease in tau expression caused a disruption in axoplasmic transport.

Researchers have shown certain tau knockout mice did not experience any loss of synaptic protein expression, such as Fyn and GluN2B, when compared to a control[60]. These proteins are synthesized in the soma and then transported to the axonal terminal, indicating that axoplasmic transport is unaffected in these tau knockout mice. Further research has shown that a loss of tau can trigger an increased expression of another MAP protein, MAP1A, which may be evidence of possible compensatory mechanisms inherent in certain MAPs[61]. In contrast, this study also found that the loss of tau caused microtubule destabilization and significantly altered microtubule organizing centers in the axon[61]. This indicates the importance of tau in providing axonal integrity and may leave these hippocampal neurons more vulnerable to future insults. Additionally, it should be noted that our method for detecting tau expression is independent of it's level of phosphorylation. Many tauopathies, including Alzheimer's disease, are propagated by hyperphosphorylated tau. Further research needs to be done to determine how HBCDD affects the phosphorylation state of tau and whether decreased tau expression alters axonal integrity in the hippocampal neurons.

In addition to tau, we also examined several other neuronal integrity markers. Specifically, we measured the expression of a three different neuronal growth factors: brain-derived neurotropic factor (BDNF), growth-associated protein 43 (GAP43), and polysialylated-neural cell adhesion molecule (PSA-NCAM). We found that HBCDD-treated mice showed significant increases in hippocampal expression of all three growth factors. BDNF is a mainly secreted from excitatory neurons, where it binds to an extracellular tyrosine kinase receptor called TrkB[62]. Activation of TrkB sets off a myriad of intracellular signaling cascades that ultimately ends up increased transcription and protein synthesis[63]. Specifically, BDNF has been shown to induce the synthesis of proteins involved in neuronal

growth, proliferation, survival, and synaptic plasticity[64]. Studies have also shown that BDNF can alter GABAergic neurotransmission by increasing inhibitory synapses on excitatory neurons[62]. Additionally, BDNF can upregulate the expression of glutamatergic synaptic proteins like NMDA and AMPA receptors through modulation of the Arc pathway[65]. The direct effect of increased BDNF on hippocampal neurons is unclear, but many studies have implicated hippocampal BDNF in synaptic plasticity formation related to learning and memory[64, 66]. PSA-NCAM is a protein marker for neurogenesis[67]. Since our experiment was conducted in adult mice, that means neurogenesis could only occur in the dentate gyrus region of the hippocampus[68]. Adult neurogenesis within the dentate gyrus produces a single type of neuron a called granule cell[68]. Granule cells are excitatory neurons that have been shown to be upregulated during the learning and memory process, with a particular function in generating hippocampus-dependent LTP[69]. GAP43 is another growth factor protein, many associated with synaptogenesis[70]. Localized in the axon terminals, GAP43 stimulates synapse generating in growing neurons or those undergoing structural plasticity[71]. Studies with GAP43 knock-in mice have shown that an overexpression of GAP43 in hippocampal neurons leads to dramatic increases in learning and memory potential, indicating that GAP43 may also be involved in hippocampal-dependent LTP and LTD[72]. Additionally, several studies have shown that BDNF, GAP43, and PSA-NCAM are all elevated following severe brain injuries[73, 74]. These results indicate that HBCDD exposure causes immense stress in hippocampal neurons, eventually causing them undergo neurogenesis and synaptic reorganization. Perhaps these neurons are attempting to establish new neural connections to compensate for the HBCDD-mediated disruption of dopaminergic, glutamatergic, and GABAergic neurotransmission. Further evidence of hypothesis this is supported by IHC data in FIGURE 7. In this figure, treatment with

HBCDD causes increased hippocampal PSA-NCAM activity directly adjacent to a reduction in mGluR1 expression. In all, we have demonstrated that, in the hippocampus, exposure to HBCDD: 1) alters synaptic protein expression involved in several neurotransmitter systems, 2) potentially weakens axonal integrity, and 3) stimulates the upregulation of multiple growth and repair mechanisms.

To expand our study outside of the forebrain, we also evaluated the frontal cortex for synaptic protein alterations following HBCDD exposure. As an added layer of complexity, we decided to evaluate the persistence of HBCDD-imposed changes in synaptic protein expression. To do this, we dosed 3-month old male mice with 25 mg/kg HBCDD for 30 days and allowed them to sit for 42 days before we collected the frontal cortex. All frontal cortex samples mentioned in this discussion followed this dosing paradigm and will be referred to as cortical-42. We started our investigation in the frontal cortex by examining the dopamine neurotransmitter system. Results showed that cortical-42 mice exposed to HBCDD experienced a significant reduction in TH expression. This indicates that HBCDD may also decrease dopamine synthesis in the frontal cortex, ultimately lowering the amount of dopamine available for exocytosis. Several studies indicate that attention-deficit/hyperactivity disorder (ADHD) can be attributed to aberrant neural activity in the frontal cortex, mainly mediated by dopamine and norepinephrine neurotransmitter systems[75, 76]. Similar to MPTP, administration of 6-hydroxydopamine (6-OHDA) selectively destroys dopaminergic neurons and can be used to test loss of dopamine function within the brain. Researchers found that when 6-OHDA was injected into the frontal cortex of monkeys, they experienced impaired attention spans with increased distractibility[77]. Accordingly, the HBCDD-mediated reduction in dopaminergic neurotransmission in the frontal cortex may explain the hyperactivity seen following HBCDD exposure.

Next, we measured synaptic protein expression in the frontal cortex related to glutamate and GABA neurotransmission. Surprisingly, we found that cortical-42 mice exposed to HBCDD exhibited no change in NMDAR-2B, phosphorylated-mGluR1, mGluR2, vGlut, or GAT-1 expression when compared to a control. These results could be due to our persistent dosing paradigm, in that HBCDD-induced synaptic protein alterations in the frontal cortex can somehow be ameliorated following a cessation in exposure. This explanation seems unlikely, however, as HBCDD tends to bioaccumulate in lipid-rich tissues, resisting metabolism and excretion. Additionally, HBCDD exposure has shown a propensity to alter synaptic protein levels in the frontal cortex as evident by reductions in both TH and mGluR1 expression. Another explanation is that, for some unknown reason, glutamatergic and GABAergic neurotransmitter systems in the frontal cortex are less susceptible to HBCDD-mediated damage than in the hippocampus and the striatum.

Glutamatergic neurotransmission in the frontal cortex was not completely unaltered following HBCDD exposure. We found that cortical-42 mice treated with HBCDD experienced a significant reduction in post-synaptic mGluR1 expression. In the frontal cortex, mGluR1 receptors have a myriad of functions, including involvement in synaptic plasticity and perpetuation of cortex-dependent LTD[78]. Moreover, we found that HBCDD exposure causes decreased expression of mGluR1 in both the hippocampus and in the frontal cortex. This may indicate that HBCDD has a specific inclination to interact with this particular glutamate receptor. Furthermore, mGluR1 expression was the only synaptic protein altered in the frontal cortex related to glutamatergic or GABAergic neurotransmission. Our results demonstrate that HBCDD exposure may cause decreased mGluR1 expression throughout the brain. Although further research needs to be conducted to confirm this hypothesis, one possible explanation for this brain-wide decrease might

involve the synaptic protein Homer1b/c. The Homer1b/c isoform is responsible for coupling to group I metabotropic glutamate receptors, regulating their expression in response to neuronal stressors. Studies have shown that these anchoring proteins serve to monitor intracellular Ca^{2+} homeostasis and can downregulate mGluR1 and mGluR5 levels to protect the neuron from potential glutamate excitotoxicity[79, 80]. Considering that activation of mGluR1 receptors stimulate the release of intracellular calcium stores and that one of the proposed mechanisms of HBCDD neurotoxicity involves altered calcium homeostasis, the interaction between mGluR1-Homer1b/c and HBCDD might provide useful insight into the molecular underpinnings of HBCDD-mediated neuronal damage.

Finally, we decided to examine cytoskeletal protein markers in the frontal cortex. First, we evaluated β-actin and tau expression using a non-persistent HBCDD dosing paradigm. These frontal cortex samples followed the same dosing procedure as described in our hippocampal experiments. By harvesting the frontal cortex immediately following the last dose of HBCDD, we found that β-actin and tau levels were unchanged between treated and control mice. Next, we decided to test for β-actin and tau expression in the frontal cortex using our persistent dosing paradigm. We found that cortical-42 mice exposed to HBCDD experienced significant increases in both β-actin and tau expression. To ensure consistent protein concentration across all samples, we probed for another conserved neuronal protein, GAPDH. We found that GAPDH protein expression was unchanged in treated and control cortical-42 mice. These results indicate that various cytoskeletal proteins in the frontal cortex are not immediately affected by HBCDD exposure. Given that β-actin performs a myriad of different roles in neuronal cells, we decided to perform one last test. We probed for synapsin in cortical-42 mice and found that its expression was unaltered due to HBCDD exposure. One of the roles of β-actin in neurons is to facilitate synaptic vesicle

fusion to the pre-synaptic membrane for neurotransmitter exocytosis. To do this, β-actin must interact with synapsin. If synapsin levels were also increased, then perhaps frontal cortex neurons exposed to HBCDD would be releasing increased amounts of neurotransmitter into the synaptic cleft. Since synapsin was unchanged, however, the altered β-actin levels in HBCDD-treated cortical-42 are most likely disrupting axoplasmic transport. This claim is further substantiated by the increased levels of tau in HBCDD-treated cortical-42 mice. Tau proteins serve as an intermediate link between microtubules, various other neurofilaments, and the actin cytoskeleton backbone. Increased β-actin and tau levels may disrupt axoplasmic transport via steric hindrance, essentially blocking motor protein movement along the microtubule tract. Indeed, researchers have shown that increased levels of tau disrupt kinesin-dependent axoplasmic transport, leading to decreased numbers of mitochondria in the axon terminal[81]. Additionally, recent studies have shown that β-actin mRNA was found to be upregulated following neuronal injury, which is a potential sign for actin cytoskeletal remodeling[82-84]. Diminished axoplasmic transport can contribute to axonal degeneration and eventual neuronal cell death. These are results are in contrast to our hippocampal samples, where tau expression was decreased following HBCDD exposure. These seemingly paradoxical results are just further evidence that different parts of the brain respond differently to neuronal stressors like environmental toxicants and should be a point of further research. Overall, we have shown that, in the frontal cortex, persistent HBCDD exposure: 1) alters dopaminergic neurotransmission, 2) selectively alters glutamatergic neurotransmission, and 3) induces an upregulation of cytoskeletal proteins involved in axoplasmic transport.

On the whole, this project serves to enrich previous work by our group concerning the neurotoxicity of HBCDD. Although prior research has indicated our dosing paradigm

of 25mg/kg HBCDD for 30 days is applicable to human exposure, there are multiple steps we can take to hone in our dosing range and compile a more complete picture of HBCDD toxicokinetics[41]. In future studies, measuring HBCDD concentration in each brain region examined would allow us to determine how much of an oral dose is partitioned to certain areas within the CNS. These measurements could be compared to HBCDD concentration in human post-mortem brain tissue to help researchers dial-in the most accurate dose of HBCDD to simulate a lifetime exposure. Additionally, much of our work has focused on flame retardant exposure in an adult mouse model. Future studies should investigate the effects of HBCDD on the developing nervous system. Children are at a disadvantage is that their neural connections are still forming and they ranked among the highest in terms of exposure concentration. Lastly, effect of multiple co-exposures should be evaluated. Realistically, we are not exposed to just HBCDD, but a multitude of other environmental toxicants simultaneously. In fact, researchers have shown a synergistic interaction between PCBs and PBDEs in a cytotoxicity assay using human neuroblastoma stem cells[19]. Even though PCBs and PBDEs are no longer in use, our body burden of these compounds remains unchanged. This synergism is now exacerbated by the introduction of yet another neurotoxic compound, HBCDD, into the environment. Accordingly, future studies investigating co-exposures should also include HBCDD.

In conclusion, our study should be considered seminal research into the molecular mechanisms of HBCDD neurotoxicity. We have demonstrated that HBCDD exposure alters synaptic protein expression related to dopaminergic and glutamatergic neurotransmission in the hippocampus and in the frontal cortex. Additionally, we have shown that damage to synaptic proteins in the GABAergic neurotransmitter system may be confined to just the hippocampus. Furthermore, HBCDD exposure induces changes in

protein expression implicated in neural network rewiring, synaptic plasticity, and cellular integrity in the hippocampus while potentially altering axoplasmic transport the frontal cortex. All of these alterations can produce an additive effect, giving rise to the many neurobehavioral deficits seen following HBCDD exposure, including difficulty with learning and memory and increased hyperactivity.

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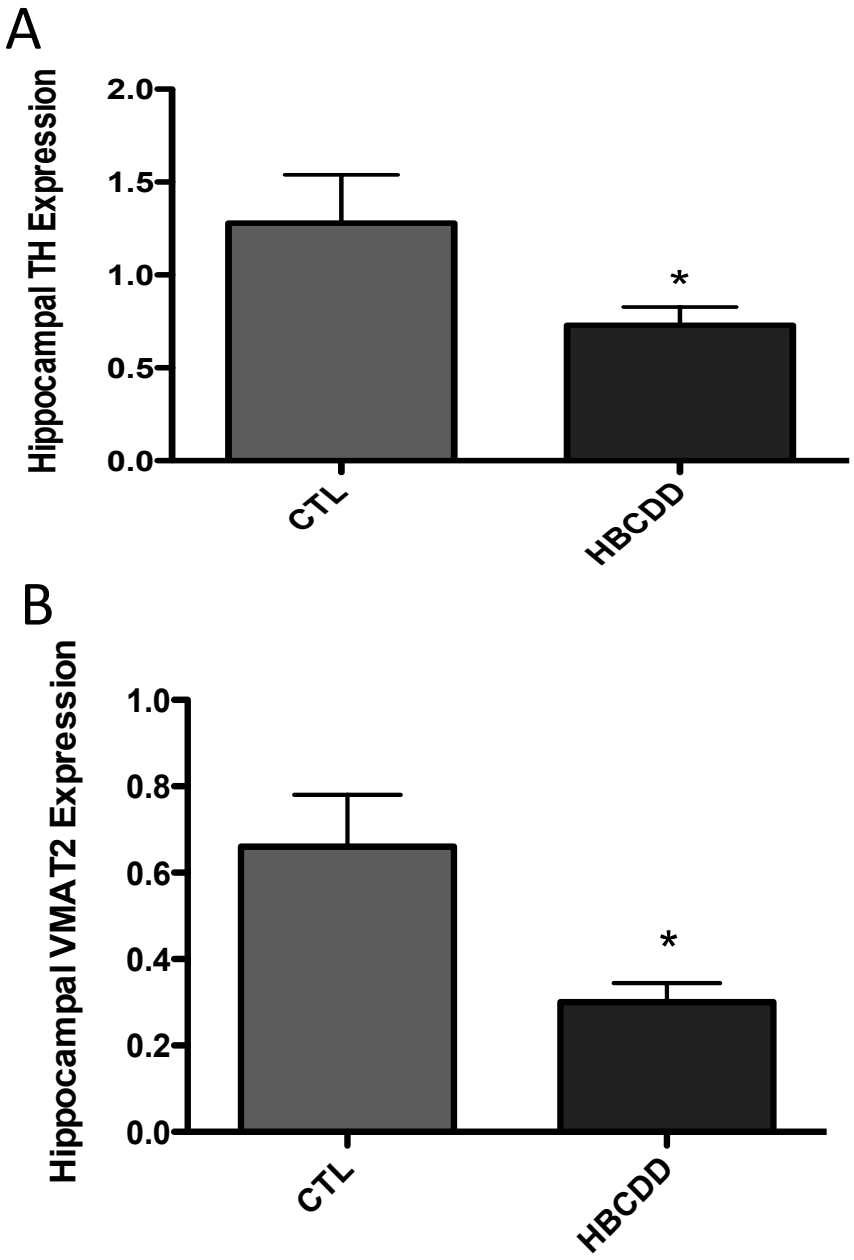


Figure 1. In vivo exposure of mice to HBCDD causes a significant reduction in hippocampal proteins involved in dopamine biosynthesis (TH) and synaptic vesicle loading (VMAT2). Animals received either 0 (control) or 25mg/kg HBCDD for 30 days and then were evaluated for alterations in hippocampal expression of A) TH and B) VMAT2. Data represents mean ± SEM (4 control and 6 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p<0.05$).

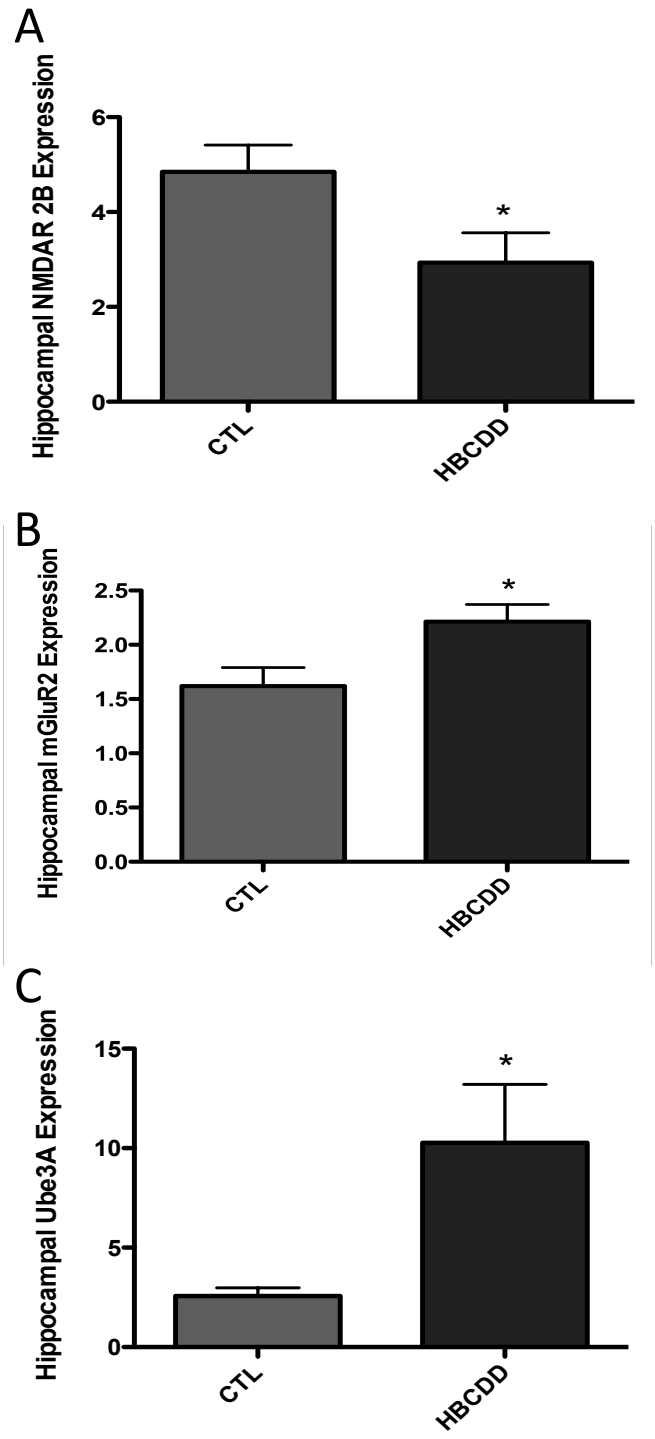


Figure 2. In vivo exposure of mice to HBCDD causes significant alterations in hippocampal proteins involved in glutamatergic neurotransmission. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days and then were evaluated for alterations in hippocampal expression of A) NMDAR-2B, B) mGluR2, and C) Ube-3A. Data represents mean ± SEM (4 control and 6 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p<0.05$).

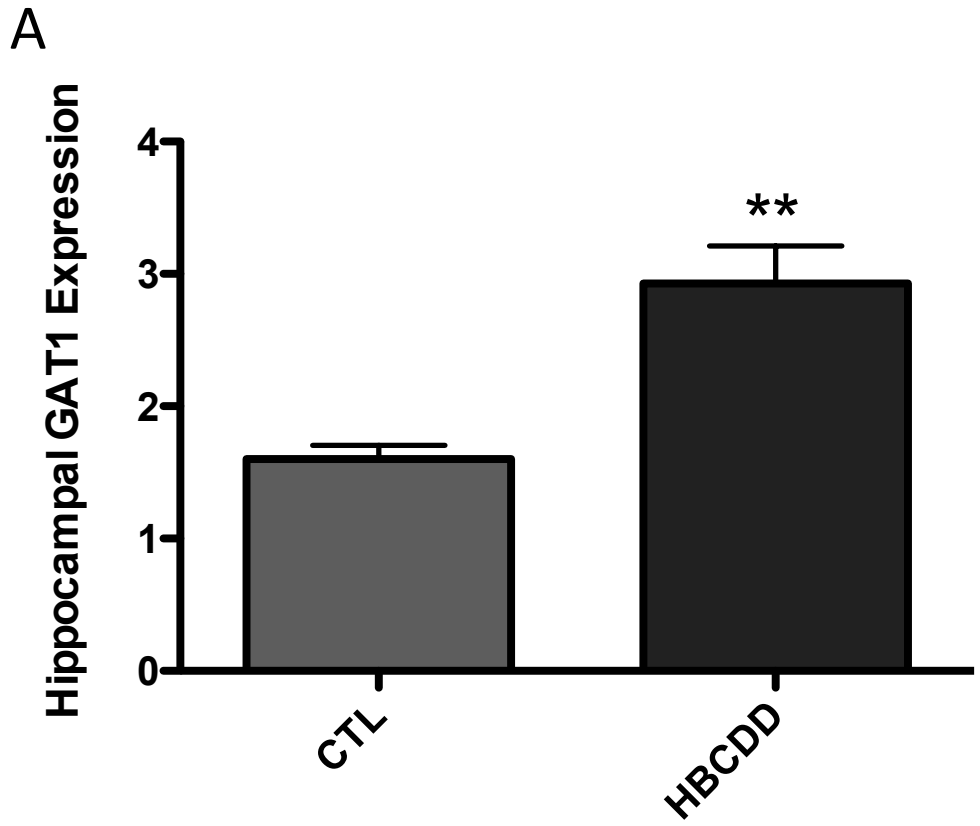


Figure 3. In vivo exposure of mice to HBCDD causes a significant increase of a hippocampal protein responsible for removing the neurotransmitter GABA from synaptic clefts and shuttling it back into pre-synaptic neurons for recycling. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days and then were evaluated for alteration in hippocampal expression of A) GAT-1. Data represents mean ± SEM (4 control and 6 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p = 0.05$). **Values that are significantly different from control ($p<0.01$).

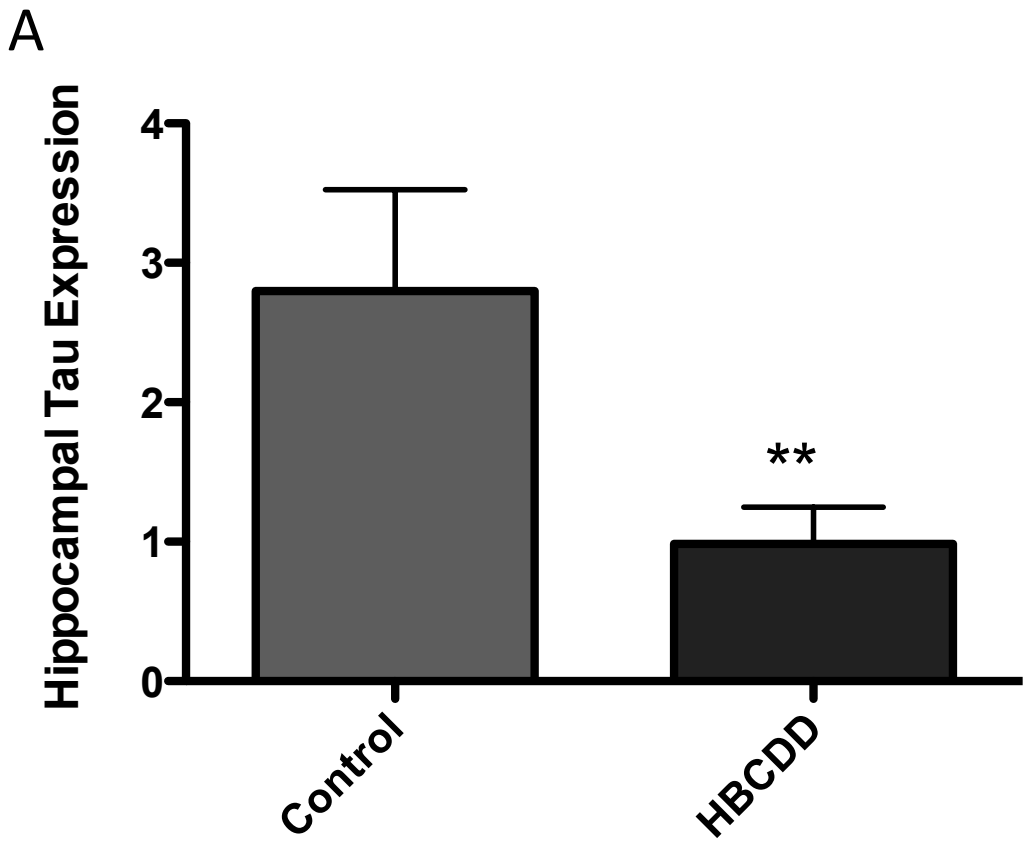


Figure 4. In vivo exposure of mice to HBCDD causes a significant reduction in a hippocampal cytoskeletal protein, tau, involved in neuronal axoplasmic transport. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days and then were evaluated for alteration in hippocampal expression of A) Tau. Data represents mean ± SEM (4 control and 6 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p<0.05$). *Values that are significantly different from control ($p = 0.05$). **Values that are significantly different from control ($p<0.01$).

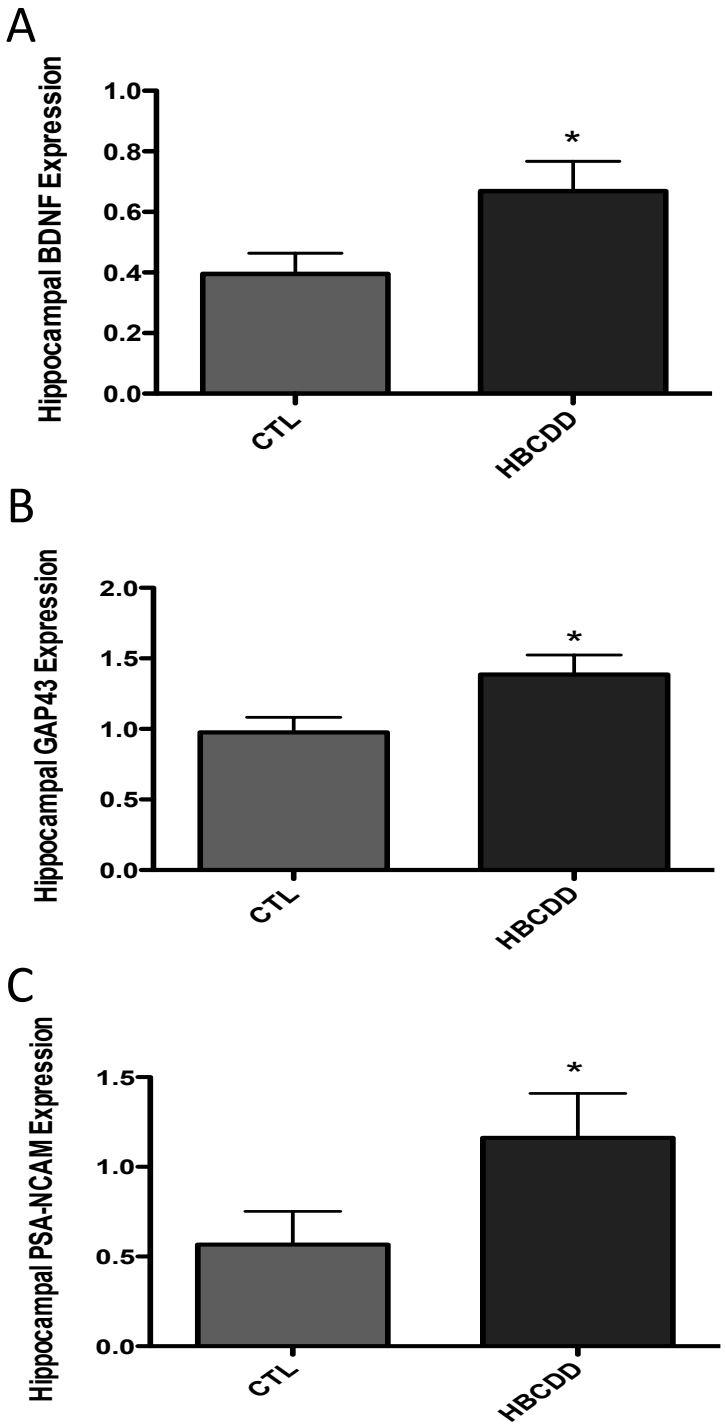
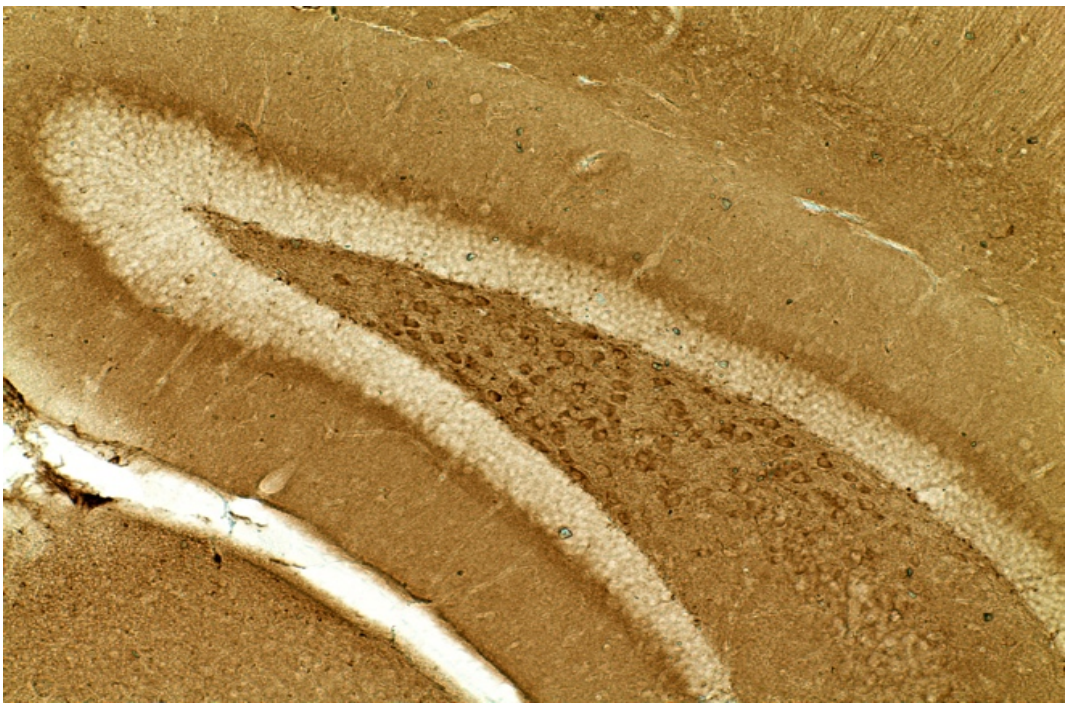


Figure 5. In vivo exposure of mice to HBCDD causes significant increases in several hippocampal neurotropic growth factors involved in axonal growth and synaptic plasticity. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days and then were evaluated for alteration in hippocampal expression of A) BDNF, B) GAP43, and C) PSA-NCAM. Data represents mean ± SEM (4 control and 6 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p<0.05$). *Values that are significantly different from control ($p = 0.05$).

A



B

Figure 6. Immunohistochemical assessment of hippocampal expression of the cytoskeletal protein tau in mice exposed to 25mg/kg HBCDD for 30 days. When compared to a control (A), exposure to HBCDD caused a reduction in hippocampal expression of tau (B).

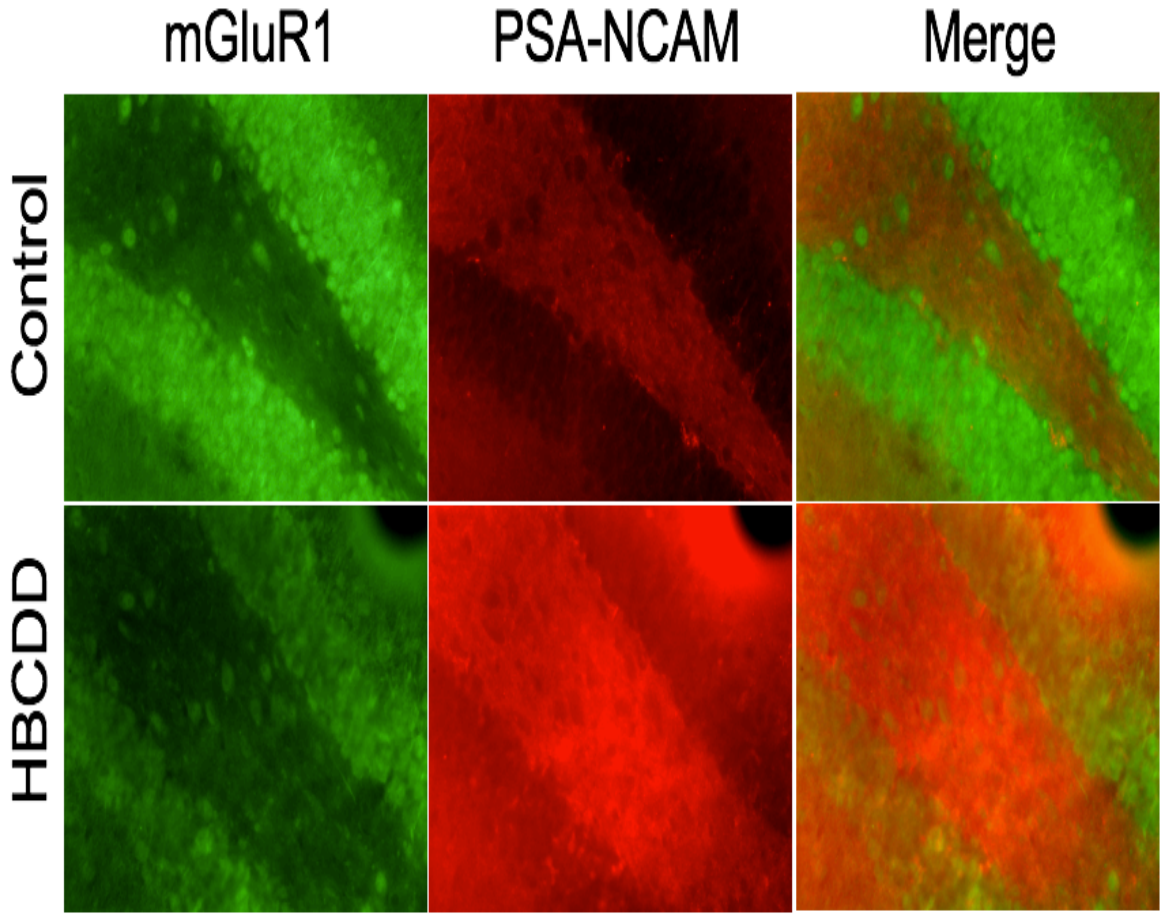


Figure 7. Immunohistochemical assessment of hippocampal expression of a post-synaptic glutamate receptor, mGluR1, and a neurotropic growth factor, PSA-NCAM, in mice that were exposed to 25mg/kg HBCDD for 30 days. When compared to controls, exposure to HBCDD caused a reduction in hippocampal mGluR1 expression while causing an increase in hippocampal PSA-NCAM expression. When these immunohistochemical stains were merged together, exposure to HBCDD caused a reduction in mGluR1 expression directly adjacent to stimulating an increase in PSA-NCAM expression.

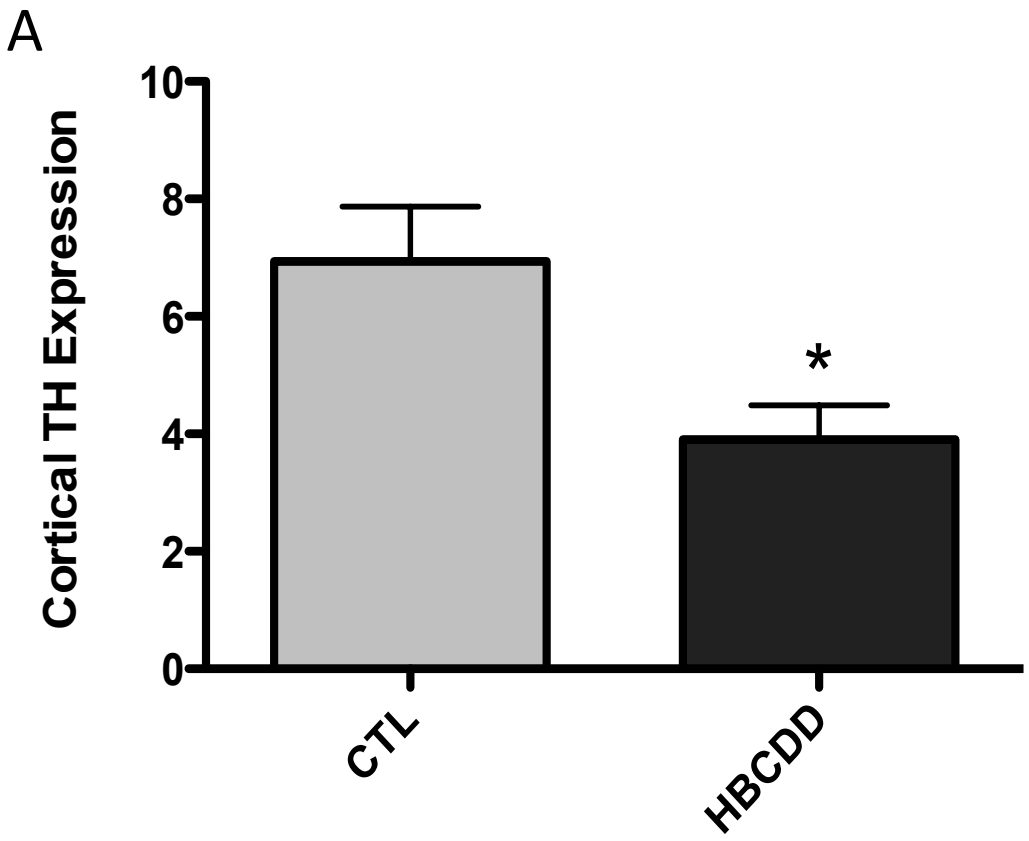


Figure 8. In vivo exposure of mice to HBCDD causes a significant reduction of a frontal cortex protein involved in dopamine biosynthesis. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days, were allowed to sit for 42 days, and then were evaluated for alteration in frontal cortex expression of A) TH. This frontal cortex dosing paradigm allowed us to evaluate the persistence of HBCDD-mediated damage to synaptic proteins. Data represents mean ± SEM (6 control and 7 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p<0.05$).

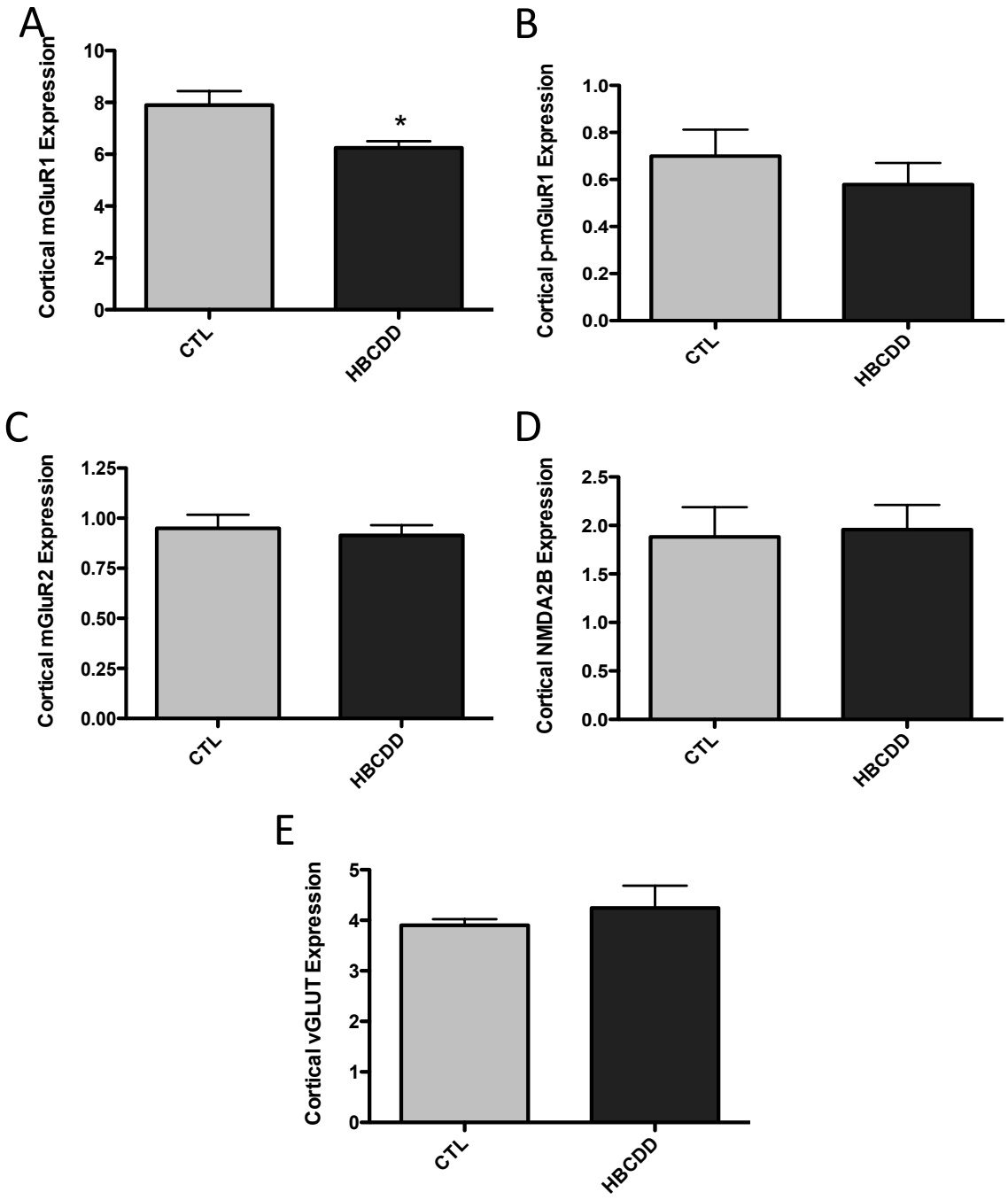


Figure 9. In vivo exposure of mice to HBCDD causes a significant decrease of a frontal cortex glutamate receptor, mGluR1, while all other protein markers for glutamatergic neurotransmission in the frontal cortex were unchanged. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days, were allowed to sit for 42 days, and then were evaluated for alterations in hippocampal expression of A) mGluR1, B) phosphorylated-mGluR1, C) mGluR2, D) NMDAR-2B, and E) vGlut. This frontal cortex dosing paradigm allowed us to evaluate the persistence of HBCDD-mediated damage to synaptic proteins. Data represents mean ± SEM (6 control and 7 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p<0.05$).

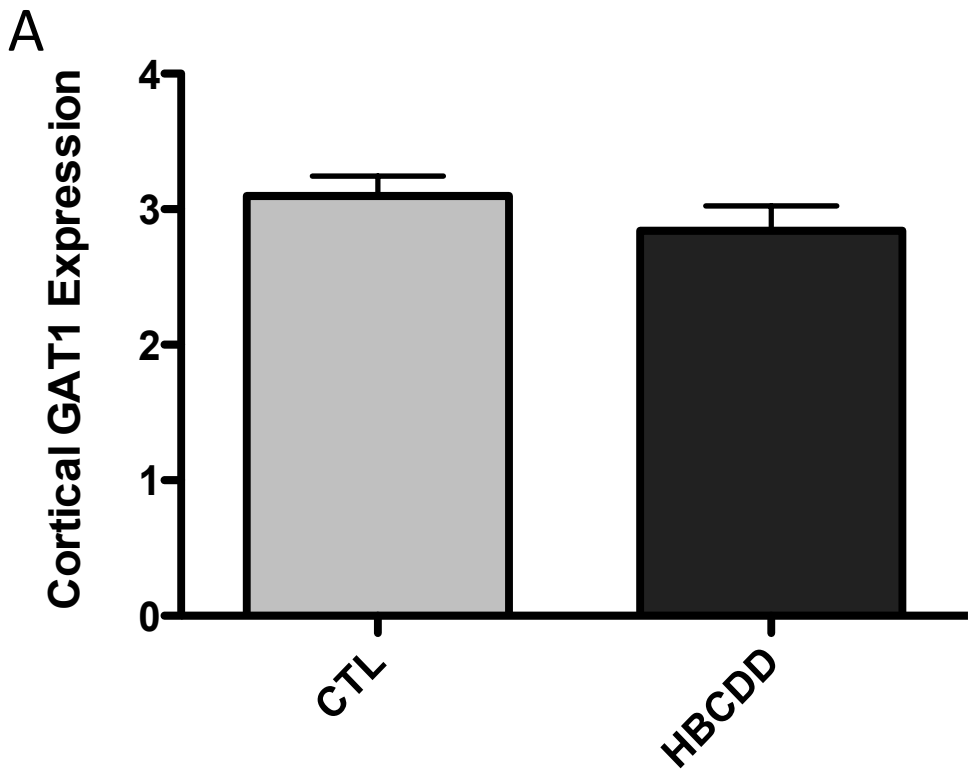


Figure 10. In vivo exposure of mice to HBCDD caused no change in expression of a frontal cortex protein responsible for removing the neurotransmitter GABA from synaptic clefts and shuttling it back into pre-synaptic neurons for recycling. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days, were allowed to sit for 42 days, and then were evaluated for alteration in hippocampal expression of A) GAT-1. This frontal cortex dosing paradigm allowed us to evaluate the persistence of HBCDD-mediated damage to synaptic proteins. Data represents mean ± SEM (6 control and 7 HBCDD treated animals per experimental group).

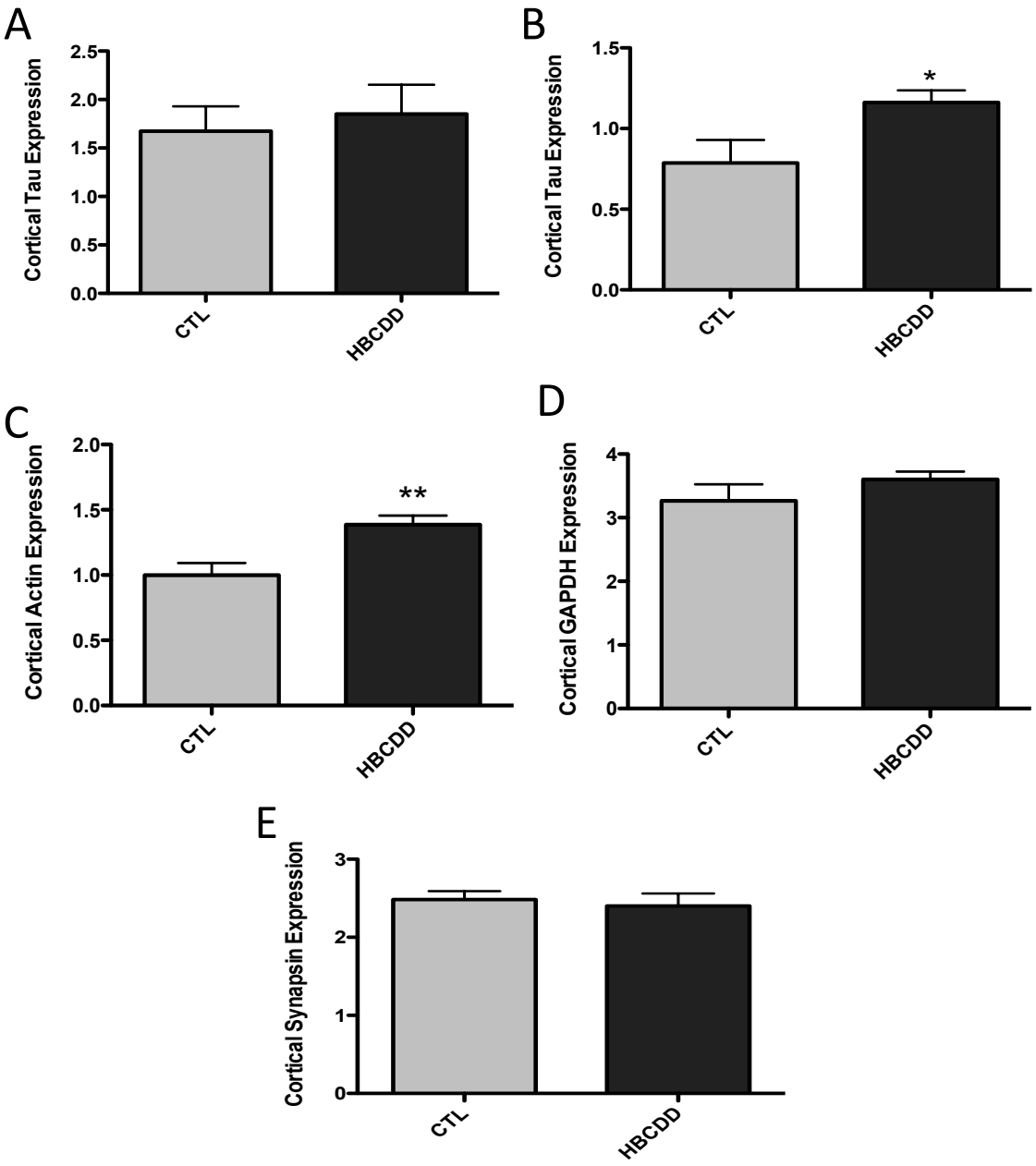


Figure 11. In vivo exposure of mice to HBCDD causes a significant increase in a two frontal cortex cytoskeletal proteins. First, tau was evaluated using a non-persistent HBCDD dosing paradigm. Animals received either 0 (control) or 25mg/kg HBCDD for 30 days and then were evaluated for alteration in frontal cortex expression of A) tau. Data represents mean ± SEM (4 control and 6 HBCDD treated animals per experimental group). Next, we evaluated cytoskeletal protein markers in the frontal cortex using a persistent HBCDD dosing paradigm. Here, animals received either 0 (control) or 25mg/kg HBCDD for 30 days, were allowed to sit for 42 days, and then were evaluated for alterations in frontal cortex expression of B) tau, C) β-actin, D) GAPDH, and E) Synapsin. This frontal cortex dosing paradigm allowed us to evaluate the persistence of HBCDD-mediated damage to synaptic proteins. Data represents mean ± SEM (6 control and 7 HBCDD treated animals per experimental group). *Values that are significantly different from control ($p = 0.05$). **Values that are significantly different from control ($p<0.01$).

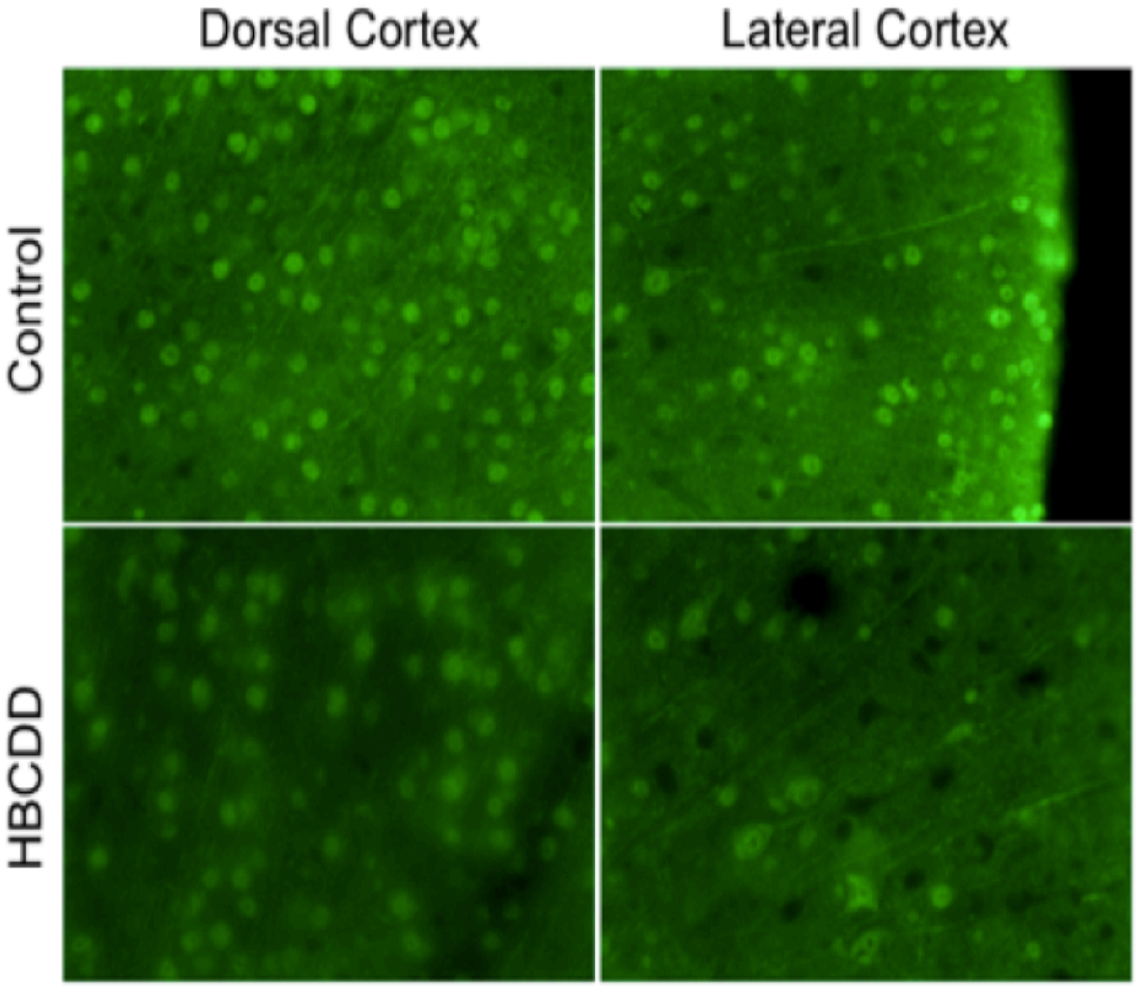


Figure 12. Immunohistochemical assessment of frontal cortex expression of a post-synaptic glutamate receptor, mGluR1, in mice that were exposed to 25mg/kg HBCDD for 30 days and then allowed to sit for 42 days before the frontal cortex was stained. When compared to controls, exposure to HBCDD caused a reduction in mGluR1 expression in both the frontodorsal cortex and the frontolateral cortex.

Table 1. Summary of all synaptic proteins evaluated via Western blot.

	Hippocampus	Cortical-1	Cortical-42
TH	Decreased	Not Determined	Decreased
VMAT2	Decreased	Not Determined	Not Determined
mGluR1	Decreased	Not Determined	Decreased
p-mGluR1	Not Determined	Not Determined	No Change
mGluR2	Increased	Not Determined	No Change
NMDAR-2B	Decreased	Not Determined	No Change
vGlut	Not Determined	Not Determined	No Change
GAT-1	Increased	Not Determined	No Change
β-actin	No Change	No Change	Increased
Tau	Decreased	No Change	Increased
GAPDH	Not Determined	Not Determined	Not Determined
BDNF	Increased	Not Determined	Not Determined
GAP43	Increased	Not Determined	Not Determined
Ube-3A	Increased	Not Determined	Not Determined
PSA-NCAM	Increased	Not Determined	Not Determined
Synapsin	Not Determined	Not Determined	No Change