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Elizabeth Aronoff April 8, 2016

Molecular Targets of HBCDD in the Hippocampus

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

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Abstract
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Over the years both environmental and health effects have been discovered surrounding the many halogenated organic compounds. Although the use of some compounds including polychlorinated biphenyls (PCBs) has been banned, many still remain in use including the brominated flame-retardant (BFRs) compounds. However, while polybrominated diphenyl ethers (PBDEs) have been discontinued, this has led to an increase in the use of hexabromocyclododecane (HBCDD) and other similar flame retardant compounds. While few studies have evaluated the neurotoxicity of HBCDD there have been many studies assessing the neurological effects of PBDE including the detrimental effects of exposure to PBDE on the hippocampus. The toxicity in the hippocampus has been associated with deficits in learning and memory and could also then result from exposure to HBCDD. Through both an in vitro and in vivo model this study will help to evaluate the neurotoxic effects of HBCDD in the hippocampus. We have demonstrated the general neurotoxicity using a neuroblastoma cell-line, SK-N-SH, by showing a significant increase in cell death following 24 h exposure to varying concentrations of HBCDD (0-25μM). Significant differences of levels of Ube3a expression were also found when exposing primary hippocampal cultures to HBCDD (0-2.5μM) for 8 days. A mouse model was used to demonstrate in vivo neurotoxicity of HBCDD (25mg/kg for 30 days). This showed a significant decrease in expression of hippocampal NMDAR 2B, Tau, CNPase, TH, and VMAT2 all crucial in multiple neurotransmitter systems. There was also a significant increase in Ube3a, mGluR2, GAP43, BDNF, PSA-NCAM, GAT1, and the D2 receptor in the hippocampus. This wide range of effects shows a significant disruption in the network of the hippocampus, which could lead to behavioral deficits including deficits in learning and memory comparable to those seen with similar compounds. These are the first data that begin to assess the toxicity of HBCDD in the hippocampus. The increasing exposure in the environment to HBCDD provides further support for future studies to continue evaluating its neurological effects.

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Acknowledgements

I would like to thank my mentor Dr. W. Michael Caudle for his assistance, insight, and guidance throughout this project. I would also like to thank Joshua Bradner for his time and assistance with this project. In addition, I would like to give special thanks to my committee members, Dr. Leah Roesch and Dr. Yoland Smith, who helped me to guide the thesis, think critically, and provide sound advice.

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Introduction:

History

Although flame-retardants have been used for over a century, there has been a recent and significant increase in their use, particularly with brominated flame-retardants (BFRs), without accompanying research and analysis of the health risks. In addition to little research into their toxicity, these flame-retardants have been shown to only provide minimal benefit from a fire safety standpoint, delaying the start of a fire by only a few seconds at most (Toxic Hot Seat). The majority of research to this point on flame-retardants involved study of polychlorinated biphenyls (PCBs). PCBs are a class of 209 chemicals that, while possessing a similar biphenyl ring structure, are formed from varying numbers of chlorine substitutions on the rings. PCBs were outlawed in the 1970s and years later, there are still environmental exposures to these toxic chemicals. A 2009 review of serum PCB levels demonstrated that 40 years after banning the use of these chemicals, even those who had only low levels of PCBs in their blood still have not eliminated it from their bodies (Hopf et. al. 2009). PCB levels remain persistently present in the human population for several reasons. Ineffective disposal methods have resulted in continued exposure as PCB remains present in our environment and food supply. More significantly, though, its lipophilic nature makes it difficult to breakdown and metabolize. This characteristic holds true for most highly halogenated compounds, including many pesticides, PCBs polybrominated diphenyl ethers (PBDE), and hexabromocyclododecane (HBCDD) (Lyche et. al. 2015).

Since the manufacture and use of PCBs was outlawed in the 1970s, the chemical industry moved to produce BFRs, a new set of flame-retardants designed to replace the loss of PCBs. These chemicals include (PBDEs), tetrabromobisphenol A (TBBPA), and HBCDD, among others. There has been minimal research evaluating their neurotoxicity. Although these distinct chemicals differ from PCBs, they maintain a similar organic structure and function, which could indicate a similar level of toxicity and inability to expel them from the environment (Ezichiás 2014). Most brominated flame-retardants maintain similar physiochemcial properties, as most are lipophilic making them difficult to metabolize in the body. Unfortunately, while a 2010 study indicates that HBCDD has been found at higher

concentrations than most PBDEs, only minimal neurotoxicology research has been conducted surrounding this class of BFRs (Hermanson 2010).

General Health Impacts of BFRs

A range of health problems are associated with exposure to BFR compounds. The BFRs are usually fat soluble and stored in the tissues of humans and other organisms affecting hormone levels as well as behavior (Kim et al. 2014, Fromme et al. 2016). In chemicals related to HBCDD, including PBDEs, researchers have been able to show many neurotoxicological effects including protein alterations and differences in synaptic plasticity (Dingemans et al. 2011, Kodavanti et al. 2015). In addition to altering molecular pathways, PBDEs can cause apoptosis in brain cells (Costa et al. 2015).

Since PBDE is stored in fat and in breast milk, children are exposed to the toxicant not only in the environment but also both in utero and through nursing (Costa and Giordano 2007). In recent years, epidemiological studies have highlighted intellectual deficits following exposure to PBDE. Prenatal exposure to PBDE has been associated with overall decreased cognitive ability throughout early development (Herbstman et al. 2010). Another study evaluated the cognitive capabilities of 5-year-old children. They found that children exposed to PBDE were correlated with lower full scale intelligence quotient FSIQ scores and increased hyperactivity associated with attention deficit/hyperactivity disorder (ADHD) (Chen et al. 2014). These human studies provide additional support for findings that PBDE and other BFRs possess detrimental neurotoxic characteristics.

Given these findings, it is important to evaluate specific brain regions to understand functionally how the neurotoxic effects could lead to cognitive deficits in an animal model. One critical function affected is learning and memory due to a disruption in the hippocampus. Researchers have found an increase in cell death and a decrease in cell viability in the hippocampus of mice exposed to PBDEs, in vitro (Chen et al. 2014). Therefore it can then be assumed that the degeneration of the hippocampus would create cognitive deficits. PBDE has been linked to detrimental effects on learning and memory in mice (Daubié et al. 2011, Buratovic et al. 2014). Further studies have shown that through the glutamatergic system, PBDE might interfere with the proper function of the hippocampus by upregulating the N-methyl-D-aspartate (NMDA) receptors, leading to a

disruption in the ability to form memories (Yan et al. 2011, Verma et al. 2015).

HBCDD and the Research Gap

Over the past 30 years, "new" BFRs have been developed and put into a wide variety of commercial and consumer products. HBCDD is one of the most prevalent. As the BFR with the greatest presence, it is also crucial to study this specific compound's potential neurotoxicity. The urgency of such an undertaking is heightened due to the Environmental Protection Agency's (EPA) recent actions regarding its continued use. In 2010 the EPA created an action plan due to the widespread use of HBCDD and its concentrations in human population and the environment (EPA 2010). HBCDD is used extensively in building and construction materials and over the years has been absorbed into the dust in houses and surrounding soil. On September 16, 2015, the EPA issued its final statement on the use of this chemical. It is now necessary to apply to use HBCDD far enough in advance to permit the EPA to evaluate the intended use and, if appropriate, to prohibit or limit the activity before it begins (EPA 2015). If large institutions and agencies like the EPA are concerned about these compounds, it is important for the scientific community to begin to understand the health risks associated with the persistent use of these chemicals. The full effects of this toxicant are relatively unknown to scientists and more research needs to be done to begin to understand the their health impacts (Ezichiás 2014).

If chemicals related to HBCDD have toxic and neurological effects in animals and humans, it would be reasonable to assume that exposure to HBCDD could also result in neurological problems. It is critical to be able to understand the underlying mechanisms of how exposure to these chemicals leads to neurological deficits to fully know what kind of neurological problems may result from increased exposure to HBCDD and other similar BFR compounds. Currently, there is minimal research addressing the neurological effects of many BFRs. As a consequence, there is little or no research evaluating the neurotoxicity of HBCDD. Therefore, although this chemical has been in widespread use throughout the United States and other countries for the past 30 years, there is little known about the ultimate effects from exposure to such a compound.

There exists a large gap in the knowledge surrounding the neurological toxicity of HBCDD even though the use of this compound has only recently been restricted. Only this

year has research been published addressing the neuronal effects of HBCDD. This study specifically focused on the striatal dopamine system, and its possible role as a risk factor for Parkinson's disease (PD) (Genskow et. al. 2015). Statistically significant changes in proteins specifically involved in maintaining dopaminergic synaptic function were observed. These changes in the function of the dopamine synapse could result in a decrease in dopamine transmission. However, additional studies assessing the impact of this compound on other brain regions have not been performed.

After seeing these changes in the presynaptic dopamine protein expression in the striatum, a whole brain proteomics study was undertaken to identify other synaptic proteins that may be altered by HBCDD. In this proteomics study, mice were exposed to 25mg/kg of HBCDD for 30 days. Following the exposure, a single hemisphere was isolated from both control and exposed mice and assessed by global proteomics. Preliminary data from this study has shown that exposure of mice to HBCDD results in a large number of protein changes in the brain, specifically related to synaptic function and structural integrity of the synaptic terminal. These proteins are crucial to study because in addition to pure neuronal structure, the synapse has also been identified as a pathological intersection in many neurological diseases and is a target of damage for many environmental chemicals. Moreover, many of the proteins found to be altered could play a large role in hippocampal function, which has been previously demonstrated to be a target for other BFRs.

Because of the critical importance of the hippocampus in the brain's function, it is imperative to study the potential neurotoxic effects of HBCDD in this region. Given the paucity of data on HBCDD, it is important to extend our current understanding of BFR research to include HBCDD. Because of some structural similarities to other BFR neurotoxic compounds, establishing base understandings of the mechanics of HBCDD's potential adverse effects would advance the overall human and environmental health concerns about the dangers of this class of chemicals. Learning and memory effects have been widely noted following exposure to PBDE both in mice and in human epidemiological studies. Therefore, since the hippocampus plays a large role in learning and memory, and since other BFR compounds have been shown to impact this crucial cognitive function, study of the neurotoxicity effects of HBCDD specifically in the hippocampus is both warranted and needed.

Focus of HBCDD Study

Because damage to the hippocampus can result in memory impairments and inability to learn new information, this research principally focuses on the hippocampus. In addition to the critical nature of this region, research has shown that other BFR compounds affect the hippocampus, which in turn could have a significant impact on proper neurological function (Dingemans et. al. 2007). Since the hippocampus is interconnected with other brain regions, many neurotransmitter systems are involved in its function. For example, long-term potentiation (LTP), which is the considered to be the molecular mechanism of learning, is characterized by the glutamate pathway. LTP is the formation of stronger and persistent connections based on associative activation of two neurons, which is why it has been associated as a potential mechanism for memory formation (Escobar and Derrick 2007). One of the main components in this molecular mechanism is the NMDA receptor that helps potentiate connections that are formed. NMDA receptors are blocked by a magnesium ion (Mg2+) so that even in the presence of glutamate, the channel will still not allow ions to pass. Following depolarization, the Mg2+ leaves the NMDA receptor, and in the presence of glutamate, the NMDA receptor will open and allow further depolarization of the neuron. NMDA receptors allow calcium to enter the neuron, which not only depolarizes the cell further, but also activates intracellular signaling mechanisms. These cascades lead to the strengthening of synapses by changing the properties of the neurons and potentially through the increase of AMPA receptors present on the membrane of the neurons (Gazzaniga et al. 2014). Therefore, should this neurotransmitter system be affected by HBCDD, then, in turn, the hippocampus would not be able to function normally and LTP would be abnormal. In addition to glutamate, other neurotransmitter systems including dopamine, GABA and monoamines have also been implicated in the modulation of learning and memory (Myhrer 2003, Rocchetti et al. 2015).

GABA is necessary for the proper formation of memories and its prefrontal cortical modulation of the hippocampus has been found to significantly affect many kinds of memory ranging from spatial to short-term memory (Whissell et al. 2013, Auger and Floresco 2015). GABA is a neuromodulator that interacts with many networks in the brain to help modulate the interactions between different neurons. Release of GABA into the

synapse can inhibit the following neuron and hyperpolarize the postsynaptic neurons by opening chloride channels. Hyperpolarization of the neurons could lead to an inhibition of excitatory activity in the brain (Hasselmo et al. 1996). Therefore, in relation to LTP, the GABA modulation could prevent the firing of a neuron in the circuit, weakening the connection between two neurons.

In addition to GABA, dopamine also contributes significantly to the development of memory, including playing a large role in the formation of spatial memories (da Silva et al. 2012, Thurm et al. 2016). In addition to having a direct role in the hippocampus in the formation of these spatial memories, dopamine can also have modulatory effects on learning and memory. Dopamine is a neurotransmitter that modulates the system through either excitation or inhibition depending on the receptor. Therefore, dopamine can play a role in LTP by helping the cells to fire in sync by further depolarizing the glutamate cell leading to the ability to activate the NMDA receptors on the postsynaptic neuron or vice versa.

Rational and Approach

Given our current understanding of the neurotoxicological effects of previously used BFR compounds, it is critical to develop a more extensive understanding of new generation BFRs, such as HBCDD. As the hippocampus has been a major toxicological target for other BFRs, we focused our investigation on this brain region and further refined our assessment to evaluate proteins involved in synaptic structure and function. Elaborating upon our preliminary proteomic findings we combined in vitro and in vivo approaches to delineate the neurotoxicity of HBCDD. Our initial evaluation utilized a neuroblatoma cell line to establish the general neurotoxicity of HBCDD. We then extended these findings to primary cultured hippocampal neurons to interrogate alterations to specific proteins identified in the proteomic screen. Finally, a mouse model of neurotoxicity was developed to further assess HBCDD-mediated alterations to synaptic circuitry that underlies normal hippocampal function. This approach allowed for critical data to be collected that identifies several molecular targets in the hippocampus that are affected by exposure to HBCDD.

Methods:

Aim1: In Vitro

1. SK-N-SH cells:

SK-N-SH cells were cultured in DMEM with 10% FBS. When the cells were about 90% to 100% confluent, they were passaged at 60,000 cells per well in a 96-well plate. After 24 hours these cells were treated with HBCDD diluted in DMSO to concentrations of 0, 5, 10, 15, 20, 25 μM per column. Each concentration was run in 8 replicates and the experiment was repeated 3 times. After 24 hours of treating these cells, WST-1 was added to the cells, which then incubated an additional 3 hours. The toxicity of HBCDD was then measured using WST-1 to assess the absorbance values at the respective concentrations of HBCDD using a Gen5 software recording at 450nm wavelength (Genskow et al. 2015).

2. Primary culture of hippocampal neurons:

Hippocampal tissue was dissected and prepared from postnatal day (PND) 1-3 mice. Mouse 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 a dissociation solution containing Papain (1 mg/ml), Dispase II (1.2U/ml), and DNase 1 (1ul/ml) dissolved in Hibernate A- Calcium for 20 min at 37°C and gently agitated every 5 min. Tissue was then rinsed in plating media containing Neurobasal-A, 10% heat inactivated fetal bovine serum, pen-strep, and mechanically dissociated using gentle trituration. Cells were plated on poly-d-lysine pre-coated 96 well plates at 80,000 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 hrs, *in vitro*. The following day, culture media containing aphidicolin (1ug/ml) was added to reduce the proliferation of glial cells in culture. These cells were treated at 6 days in vitro (DIV) with 0, 0.6125, 1.25, and 2.5μM HBCDD dissolved in DMSO for 8 continuous days. There were 3 replicates per plate and the experiment was performed twice.

3. Immunocytochemistry:

The primary hippocampal cultures were fixed in 4% Paraformaldehyde (PFA). The cells

were incubated overnight in MAP2 (1:1000) and Ube3a (1:500) at 4ºC and then the respective secondary antibodies were applied the second day. Images of these 96 well plates were taken for each well using an Array Scan VTI HCS. The assay was gaited to set a parameter for the machine to evaluate 400 cells per well to avoid pure cell number affecting intensities. Cells were determined by identifying nuclei from the Hoescht staining and then various size and intensity markers were used to determine what was a cell and what was not. This was accomplished using the Array Scan software to first set both size and intensity specifications for the "circle" area around the nucleus. Second, the settings of the "ring", composing the cytoplasm, were selected by intensity and area. Both the circle and ring settings were chosen to include the most number of live cells possible while limiting to not include dead cells or the glia cells. These parameters were held constant throughout all images and trials. A representative image demonstrating this gating technique can be seen in Figure 2C and Figure 2D. One-way ANOVA statistical tests were used to determine significance for intensity using GraphPad analysis software. A post-hoc Dunnet test was run to assess between group significance and to help visualize the dose-dependent response as a result of increased exposure to HBCDD. This analysis was also completed using the GraphPad analysis software.

Aim 2: In Vivo

4. Animals and Treatment:

Eight week old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Three month old mice were orally exposed to HBCDD through the corn oil. The mice were exposed daily for 30 days to 25μL of corn oil control (4 mice) or 25mg/kg of HBCDD dissolved in a corn oil vehicle (6 mice). This protocol has been previously described and followed based on other experiments (Genskow et al. 2015, Caudle et al. 2006, Bradner et al. 2013). This dosing paradigm was intended to represent the primary route of human exposure to HBCDD based on oral ingestion. Mice were sacrificed the day after the last exposure to the corn oil or HBCDD; following which the hippocampi of the mice were removed and used for further 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.

5. Western blot analysis:

Western blots were used to quantify the expression of several synaptic proteins in both primary cultured hippocampal neurons as well as hippocampal tissue isolated from control and HBCDD-treated mice. Hippocampal tissue from both treated and control animals were used for the western blot analysis. 10μg protein was added per lane for the tissue, while 20μg protein was added for the primary cultures, 5 lanes of control protein and 7 lanes of exposed protein. Gel electrophoresis on 10% Bis-Tris gels was run with a ladder and transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked using nonfat dry milk in TTBS. The luminescence signal was analyzed on an Alpha Innotech Fluorochem imaging system. Ube3a (1:500), Arc (1:500), mGluR2 (1:1500), mGluR4 (1:1,000) NMDAR 2B (1:500), TH (1:1,000), GAT1 (1:1,000), vAchT (1:1,000), VMAT2 (1:10,000), vGAT(1:1,500), D2 Receptor (1:500), Tau (1:500), CNPase (1:2,000), GAP43 (1:1,000), BDNF (1:500), PSA-NCAM (1:500), and Calmodulin Kinase II (1:10,000) were analyzed.

6. Immunohistochemistry (IHC):

The brains from HBCDD-treated and control mice were fixed in 4% PFA for 1 week and then transferred to 30% sucrose before slicing and staining. Fixed brains were sliced on a freezing-sliding microtome to 40 μm. Immunoreactivity to the antibody Tau (1:100) was visualized by the avidin-biotin-peroxidase method and analyzed by light microscopy. Visualization was performed using 0.03% 3,3′-diaminobenzidine (DAB) for 1 min at room temperature.

7. Immunofluorescence (IF)

To demonstrate the colocalization of the PSA-NCAM receptor subunit with mGluR1 located in the hippocampus, treated and untreated male mice were processed as described previously (Caudle et al., 2007). Briefly, whole brains were immersion fixed in 4% PFA and serially sectioned at 40 μm. Sections were incubated with mouse anti-PSA-NCAM (1:100), and rabbit anti-mGluR1 (1:100) overnight and then incubated in fluorescently conjugated

secondary antibodies against mouse and rabbit IgG, raised in goats for 1h at room temperature. Images were captured at 20x magnification using a Zeiss Axio Imager M2 microscope with fluorescent capabilities and merged using Image J software available from NIH.

8. Statistical analysis:
In all cases, statistical analysis of the effects of HBCDD were performed using the raw data for each treatment group and using one-way ANOVA in comparison to the DMSO treatment group. Post-hoc Dunnet test followed the one-way ANOVA to test between group significance. Significance was reported to a $p < 0.05$.

Results:

Our first experiment was designed to show that HBCDD was in fact toxic to neuronal cells. In order to determine this, we used SK-N-SH, a neuroblastoma cell line, to evaluate the toxicity of HBCDD on these cells. There was a dose dependent effect experienced starting at a low dose of 5μM and seen through 25μM (Figure 1). A significant decrease in absorbance evaluated using WST-1 was found when starting at the lowest dose. There was a 50% reduction in viable cells compared to the DMSO control at only 5μM HBCDD. Ultimately, there was a 75% reduction in cell viability experienced at the highest concentration, 25μM HBCDD.

After showing that there was a significant neurotoxic effect of HBCDD, it was necessary to move to hippocampal primary cultures in order to begin evaluating protein expression and changes as a result of HBCDD. From the proteomics data, we found multiple potential synaptic targets that are involved in mediating proper hippocampal function. One of the proteins found to be affected by HBCDD in the proteomics study was Ube3a, which has been shown to modulate the glutamate system. As the glutamate system is a major constituent of the hippocampus, we directed our initial assessment towards synaptic proteins involved in glutamatergic neurotransmission. Since Ube3a regulates the synaptic plasticity of mGluR, changes in Ube3a in the hippocampus could then result in changes to mGluR signaling and the overall glutamate pathway. Using an ArrayScan, we evaluated the

expression of Ube3a by measuring the intensity of Ube3a immunofluorescence in these cells, while controlling for the total number of cells to be 400 per well, to ensure that cell death would not impact these intensities. Circle intensity signifies the area of and around the nucleus. Even at a low dose of HBCDD, 0.6125μM, there was a significant ($p<0.05$) reduction in Ube3a in these neurons showing statistically significant effects of the chemical at low doses (Figure 2A). The second part of this data was used to analyze the ring intensity. The ring signifies the region surrounding the nucleus that would be equivalent to the rest of the soma outside the nucleus (Figure 2C). Similar to the circle intensity, the ring intensity also showed a significant ($p<0.05$) decrease in Ube3a (Figure 2B). These results, taken together, would suggest that it is a decrease in the whole cell and not just in the nucleus or the cell body.

The changes found in the Ube3a in the primary culture, along with the preliminary proteomics data, provided enough support to proceed with an in vivo model to assess protein disruptions in an animal model. This is important to evaluate in addition to the primary culture because cells in a dish do not provide a comprehensive representation of neuronal system. For example, the toxicant is being placed directly on the cells with no tissues or organs to process the chemical. Thus, in order to address this concern we utilized hippocampal tissue isolated from adult male mice previously exposed to HBCDD.

Since Ube3a is involved in maintenance and regulation of the glutamatergic system, the first proteins evaluated were those that make up and are involved in glutamatergic synapses. Ube3a expression in the hippocampus of the in vivo model was found to be significantly ($p<0.05$) increased, providing further support for what was observed in the proteomics data (Figure 3A). One way in which the Ube3a mediates the glutamate system is through the ubiquination of Arc (Kühnle et al. 2013). The Ube3a protein tags Arc for breakdown thereby reducing the concentration of Arc in the cell. Since one role of Arc is to pull mGluR receptors off of the membrane it would therefore, be important to evaluate Arc to see if it was this was altered as well. There was no change in expression found in the Arc protein in the hippocampus of HBCDD exposed mice (Figure 3B). However, while there was no change in the expression of Arc in the hippocampus of the treated mice, there was a differential expression of some of the glutamatergic receptors. The mGluR2 receptor (Figure 3C), which is located presynaptically, was significantly upregulated ($p<0.05$) while

the expression of the NMDAR 2B subunit was significantly decreased ($p<0.05$) (Figure 3E). The mGluR4 receptor, another presynaptic metabotropic glutamate receptor, was found to have no change in expression in the treated mice (Figure 3D).

In order to evaluate if PSA-NCAM was potentially involved in the changes seen in the glutamate system we used IF to assess if there was a colocalization between mGluR1 and PSA-NCAM. Though it was difficult to identify explicit regions of colocalization at a cellular level, the two proteins did appear to demonstrate differences in expression as a result of HBCDD exposure. While mGluR1 (green) appeared to qualitatively decrease following exposure to HBCDD (Figure 4), PSA-NCAM (red), showed a significant increase in expression in the dentate gyrus of the hippocampus after treatment with HBCDD (Figure 4). The apparent changes in PSA-NCAM followed a similar trend demonstrated quantitatively in the immunoblot data (Figure 5E)

Other proteins crucial for maintaining the cell structure and the synaptic junction between the two neurons were also found to be altered in the proteomics data. Based upon those findings, we evaluated synaptic maintenance and structural proteins. First, CNPase, a protein involved in myelination in the central nervous system, was found to significantly decrease ($p<0.05$), potentially leading to problems in the level of myelination of neurons (Figure 5B). Tau protein, involved in stabilizing microtubules, was also found to significantly decrease ($p<0.05$) in HBCDD treated mice (Figure 5A). In addition to immunoblot, this protein was evaluated using IHC with DAB staining. Though qualitative, these images appeared to show a similar decrease in tau expression as seen in the quantitative immunoblot data (Figure 5G). Some proteins significant to LTP are those involved in developing and maintaining the synapse. GAP43 is a growth factor that supports axonal growth, which was found to significantly ($p<0.05$) increase in the HBCDD group (Figure 5C). Brain derived neurotropic factor, BDNF, maintains neuron integrity and is necessary for proper synaptic functioning. This protein, critical for the maintenance of the neuronal circuitry, significantly increased when the mice were exposed to HBCDD (Figure 5D). PSA-NCAM, important for cell migration, which is crucial for LTP to occur, was found to be significantly increased as a result of HBCDD exposure. This disruption could lead to irregular LTP (Figure 5E). The final protein affecting LTP, calmodulin-dependent protein kinase II (CAMKII), is involved in an important signaling cascade that has been

found to mediate and be critical for normal LTP. There was no change found in this protein as a result of exposure to HBCDD (Figure 5F).

It was important to test protein markers of other neurotransmitter systems in the hippocampus to determine if HBCDD is simply targeting the glutamate system or whether it is targeting other neurotransmitter systems as well. Even though there was no change found in vAchT expression following HBCDD exposure, other systems did show changes in exposure (Figure 6F). The GABA transporter, GAT1, showed a significant increase in the exposed mice (Figure 6C). While not significant, vGAT also involved in the GABA transporter, showed a trend towards a decreased level in the hippocampus of these exposed mice (Figure 6D). TH (Figure 6A) and VMAT2 (Figure 6B), both proteins involved in the monoamine neurotransmitter systems, followed a similar trend showing significant decreases in expression after exposure to HBCDD. In order to determine which specific monoaminergic systems were affected, we chose selective protein markers including the D2 receptor. This is a dopamine receptor that showed a significant increase in expression as a result of exposure to HBCDD (Figure 6E).

Discussion:

Flame-retardants have been shown to lead to health effects including deficits in learning and memory formation. Although there is little research on HBCDD, because of its similar characteristics and physical properties as those BFRs that came before it, it is necessary to evaluate the effects of this compound. A preliminary proteomics evaluation was run prior to this experiment to evaluate the global effects of HBCDD in the brain. This comprehensive overview identified specific proteins that were altered following HBCDD treatment and allowed us to refine the focus of our investigation towards synaptic structure and function in the hippocampus.

We confirmed the neurotoxicity of this compound using a standard cell-line. From our use of the SK-N-SH cells, a neuroblastoma cell line, we were able to replicate the findings of the Genskow et al. research showing the general neurotoxicity of the HBCDD compound in a dose-dependent response. We were thus able to move to more complex in vitro and in vivo studies to evaluate both synaptic and structural protein differences as a result of exposure to HBCDD.

HBCDD-Induced Changes in Synaptic Proteins:

The Ube3a protein is involved in the regulation and modulation of the glutamate neurotransmitter system. This protein was also found to increase significantly following exposure to HBCDD compared to control in the proteomics data. Although this was found to increase in the proteomics data, the hippocampal primary cultures showed a significant decrease in the expression of Ube3a protein both in the nuclear region and in the surrounding cell body. The primary culture data was derived from an in vitro model that drastically differs from the in vivo model used in collecting the proteomics data. The system in the in vitro model lacks crucial elements of a whole organism system to handle toxic elements, including the liver. In addition to the lack of the liver, another crucial filtering and support system missing are the support cells in the brain including glial cells and astrocytes. These cells provide important support to the neurons, but their growth is limited in cell cultures to prevent cell overgrowth in the dish. This lack of support cells could also contribute to many other differences between the in vitro and in vivo models of the experiment. The Ube3a protein is involved in maintaining homeostasis in the glutamate system. It contributes by helping to regulate molecular pathways including, for example, the down regulation of the expression of Arc. Arc pulls mGluR off of the membrane and into the cytoplasm. This would decrease the expression of glutamate receptors, which would change the neurons' sensitivity to the release of glutamate into the synapse thereby making it either easier or harder for the neuron to generate an action potential depending on which mGluR was removed.

The decrease in Ube3a expression found in these hippocampal cultures, through the association of Ube3a and Arc, could lead to a potential decrease in the level of glutamate receptors expressed on the membrane as less Ube3a may result in an increased level of Arc. This increase in Arc expression could increase the number of glutamate receptors being pulled off the membrane. A decreased expression of Ube3a has been associated with the development of memory deficits, including Angleman's Syndrome, Autism Spectrum Disorders and Huntington's Disease, which are marked by a significant dysfunction of Ube3a throughout the brain (Gustine et al. 2011, Maheshwari et al. 2012, Ey et al. 2011, Samaco et al. 2005). Angleman's syndrome, characterized by intellectual deficits and difficulty learning, has been associated with a dysfunction between the interaction of

mGluR5 and Ube3a specifically in the hippocampus (Pignatelli et al. 2014). This data helped to support the hypothesis that exposure to HBCDD leads to significant effects in the molecular pathways of neuronal function. However, since cells in a dish lack a number of the key physiological mechanisms that help to process toxins in the body, once changes in protein expression were seen in cell data, the experiment could move to an in vivo model. This model permitted the evaluation of toxicity and effects of HBCDD in a more complex neuronal environment.

The in vivo data showed a considerable change in multiple parts of the glutamate system. In this approach, we were able to confirm the increase in Ube3a in HBCDD-treated mice, previously seen in the proteomic data. Since one function of Ube3a is modulating the level of glutamate receptors, the next proteins evaluated were NMDAR2B, mGluR2 and mGluR4. The increase found in the Ube3a would lead to an increase in mGluR expressed due to the supposed decrease in Arc, even though no difference was found in the expression of Arc in the hippocampus of exposed mice. Although no increase was found in mGluR4, there was a statistically significant increase in expression of the mGluR2 receptor. An increase in mGluR2 has been implicated in the pathology of both epilepsy and Alzheimer's disease (Lee et al. 2004, Tang et al. 2004). While there are many neurological components that have been associated with these disorders, it is very possible that increased exposure to HBCDD can lead to the development of neurological disorders, including these, over time, indicating the need for further study. The decreased NMDAR 2B subunit in the hippocampus found in this study can be representative of impairments in several behaviors including a deficit in learning and memory (Sun et al. 2009).

The results of the in vivo glutamate system data alone are enough to show a serious consequence of exposure to HBCDD. However, more behavioral studies and in depth studies should be completed to fully understand the full effects of HBCDD on this neurotransmitter system. In addition to animal model studies to evaluate behavioral effects of HBCDD exposure, human epidemiological data should be compiled to begin to identify the true effects being observed in the human population.

However, in addition to just glutamate being affected, in drosophila, Ube3a expression has been directly correlated with both dopamine levels and behavior associated with dopamine expression including locomotion (Ferdousy et al. 2011). Studies have

shown that an over-expression of Ube3a leads to an increase in dopamine levels in addition to an increase in motion. This increase in dopamine can be characteristic of disorders including attention-deficit and hyperactivity disorder (ADHD) (Pilszka 2005). In addition to an increase in dopamine, ADHD is marked by an increase in locomotion. Therefore one implication of exposure to HBCDD and the increase in Ube3a could be an increase in the prevalence of ADHD in the population. This is a likely possibility based on previous epidemiological studies of the similar compounds including PBDE (Gascon et al. 2011).

Since the BFR compounds are non-specific and highly toxic it can be assumed that it is not a single neurotransmitter system affected by the compound. Multiple other systems were evaluated in part. The hippocampus is highly innervated by almost every neurotransmitter system, therefore to fully evaluate HBCDD's effect on the hippocampus, it would be necessary to assess other systems in addition to the glutamate system.

Dopamine is greatly involved in the formation of spatial memory and therefore would be important to evaluate in relation to HBCDD exposure. TH levels significantly decreased which could be evident of a decreased production of dopamine in addition to other monoaminergic neurotransmitters. Another protein involved in the monoamine system is VMAT2. VMAT2 is involved in packaging monoamines, including dopamine, into synaptic vesicles to be released following an action potential. However, this protein, following exposure to HBCDD, was significantly decreased. These two proteins together could then be evident of less monoamines being produced, due to a decrease in TH expression, in addition to a decrease in monoamines being packaged into synaptic vesicles to be released. This reduction in monoamine levels and release could indicate difficulty in maintaining LTP and strengthening connections necessary for forming memories since the signal of these systems would not be strong enough. Therefore, if the overall effects of dopamine in the hippocampus are reduced, a decrease in the formation and ability to modulate spatial memory could result .

However, since both TH and VMAT2 are related to more general monoaminergic activity, to evaluate if dopamine specifically shows an effect, we evaluated levels of the D2 dopamine receptor. This protein was found to increase in expression in the hippocampus of the HBCDD-exposed mice. Some studies have shown that an increase in D2 receptor activity can lead to difficulty in memory formation (França et al. 2015). More research

needs to be done into the differences on this system following exposure to HBCDD to show a greater effect of this compound in its effects on dopamine in the hippocampus.

The GABA neurotransmitter system is a significant modulator in the CNS adding an important inhibitory control over neurons. This modulator system would be crucial for proper maintenance of function of the hippocampus. Losing a sense of equilibrium and homeostasis in this system in the hippocampus could lead to learning and memory effects through differences in inhibition of neurons. To begin to evaluate changes in the GABA system we evaluated GAT1, a GABA transporter involved in removing the released GABA from the synaptic cleft. The in vivo data showed a dramatic increase in hippocampal levels of GAT1. This could result in great changes in the inhibitory modulation by GABA on memory formation. Up regulation of this protein has been associated with learning capability by creating difficulties in associative learning abilities (Ma et al. 2001, Hu et al. 2004, Tellez et al. 2012, Tellez et al. 2012). This effect of the GABA system alone would be enough to raise concerns about this chemical's impact on the ability of developing children to learn and properly retain information. However, it is likely that other components of the GABA neurotransmitter system are affected by exposure to HBCDD and need to be studied for a greater understanding of the full impact of this compound on the hippocampus.

HBCDD Induced Changes in Neuronal Integrity Proteins:

Even though differences in expression in the individual neurotransmitter systems were evaluated, it is possible that other sets of proteins would be affected by exposure to HBCDD. The next set of proteins evaluated were those critical for the integrity of both the neuron itself in addition to the structure and functionality of the synapses. Differences in these proteins can lead to deficits in neuronal structure and synaptic stability in many systems throughout the brain including the hippocampus. The tau protein is crucial for maintaining stability of neurons in the CNS. When unregulated and/or dysfunctional, this protein can lead to severe problems between the connections of the neurons since its presence is mainly in the axons on the neurons. Therefore, this protein is crucial for normal neuronal function and when it is not working properly it can lead to detrimental effects in brain structure in addition to resulting behavior (Zilka et al. 2009). Tau, following exposure to HBCDD in vivo, was significantly down regulated in the hippocampus.

If a protein critical for the maintenance and structural support of connections in the brain has gone awry in the hippocampus, significant deficits in learning and memory abilities could result. Since the hippocampus is one of the main structures for LTP and the development of connections, a decrease in tau this could interrupt these processes.
A reduction in tau could decrease the stability of the neurons and the synapses and connections they form. A decrease in functional tau has been found to be associated with many behaviors characteristic of neurodegenerative phenotypes including Alzheimer's disease (Lei et al. 2014, Sydow et al. 2016). Importantly, though, tau was only one of many structural proteins affected by exposure to HBCDD.

PSA-NCAM was another protein involved in synaptic integrity that was significantly affected following HBCDD exposure. However, unlike tau, PSA-NCAM was up regulated following the treatment with HBCDD. Too much protein and too many connections forming is not a positive result. In fact an elevation of PSA-NCAM has been associated with neurological problems including Alzheimer's disease (Jin et al. 2004). This result has been consistently shown in our in vivo experiment both through immunoblotting as well as immunofluorescence. The immunofluorescence allows us to visualize the localization of this protein in the hippocampus, in this case, in the dentate gyrus. It also permits the visualization of the significant increase of PSA-NCAM shown in the immunoblotting results.

Another protein found to increase in the hippocampus of mice exposed to HBCDD was the neurotropic factor BDNF, which helps to maintain and support the functioning of neurons. While it would be clear that a decrease in this protein would be potentially detrimental to the brain as not enough protein would be present to support the developing synapses in the hippocampus, too much of this protein is also not good for the brain. A proper homeostasis of protein interactions is necessary for normal neuronal function. One study has shown an increased expression of BDNF in the hippocampus present in human schizophrenic patients (Iritani et al. 2003, Dong et al. 2015). Since BDNF is crucial in the formation of memories, any alteration from normal levels of protein level could severely inhibit or create significant dysfunction to this important process. (Bekinschtein et al. 2014).

Conclusions and Future Directions:

Our findings are consistent with our original hypothesis that HBCDD exposure would create significant changes in the molecular pathways of the hippocampus. Since there is very little knowledge surrounding the effects of this compound, and even less on its effects in the hippocampus, these findings can provide the basis for future experiments. It is evident by our results that the effects of exposure are not narrow, but rather a large and significant impact can be seen in many structural and integrity proteins for neurons in addition to dramatic effects in multiple neurotransmitter systems. These changes can create significant concerns surrounding the behavioral effects of HBCDD.

There was a focus in this study on the glutamate system and structural proteins due to the involvement of these proteins in the hippocampus and their importance in proper LTP and memory formation. Many of the changes found in this study are consistent with some of the studies surrounding other BFR compounds including the one that has been greatly studied, PBDE. The changes in the glutamate proteins have been associated with learning deficits and lower IQs in addition to longer-term consequences including the progression to Alzheimer's disease. Similar to the effects of the glutamatergic system, the effects found in the synaptic integrity proteins, and other neurotransmitter proteins in the hippocampus have been found to lead to similar deficits and pathologies. In addition, other neurological problems have been associated with these changes including ADHD, schizophrenia, and depression.

Future studies are necessary in order to evaluate the behavioral effects of this compound to see if the molecular changes found in this study in fact lead to clinical deficits in the population. Therefore it is necessary to complete multiple behavioral assays of the mice to evaluate these cognitive impairments both in learning and memory and social behaviors. However, more research is also necessary to further comprehend the molecular changes to the other neurotransmitter systems. While this study does provide evidence that neurotransmitter systems other than the glutamate change, the extent of these changes is not well understood. Some of these would include further evaluation of both the dopaminergic and glutamatergic systems. In addition to these molecular and behavioral studies, human epidemiological evidence would also help to focus these studies to validate the effects seen in humans in addition to the typical human level of exposure. However, this

study provides the first comprehensive overview of the extensive and widespread effects of HBCDD in the hippocampus.

Figures:

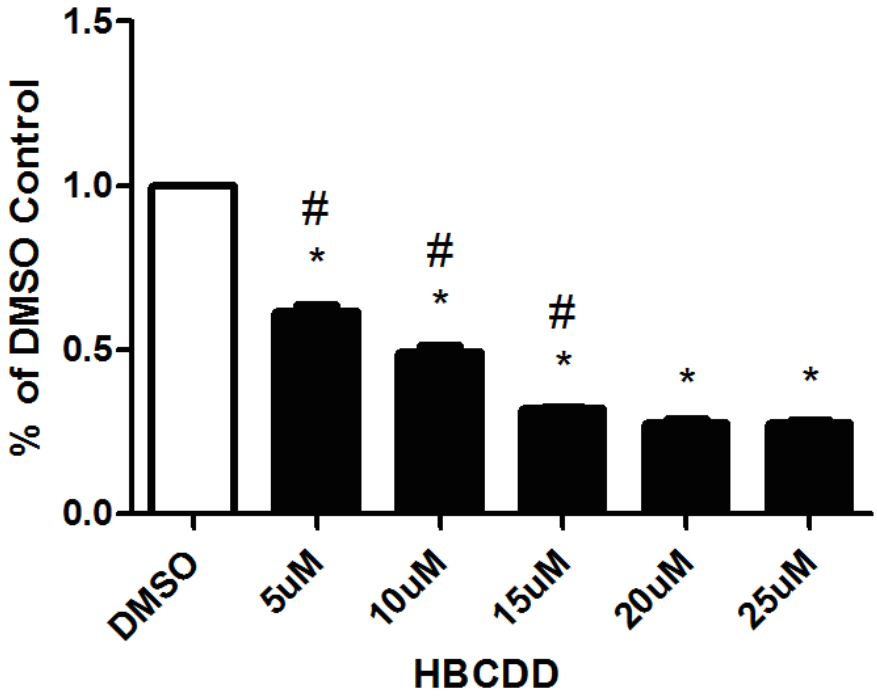


Figure 1: When SK-N-SH cells are exposed to HBCDD causes a concentration-dependent reduction in cell viability. Effects of the toxin were experienced beginning at a dose of 5μM HBCDD. Columns represent the percent of DMSO absorbance measured after WST1. Data represent the mean ± SE of 5 experimental replicates. *indicates significantly different in comparison to DMSO control ($p<0.05$), #indicates $p<0.05$ compared to previous treatment group($p<0.05$)

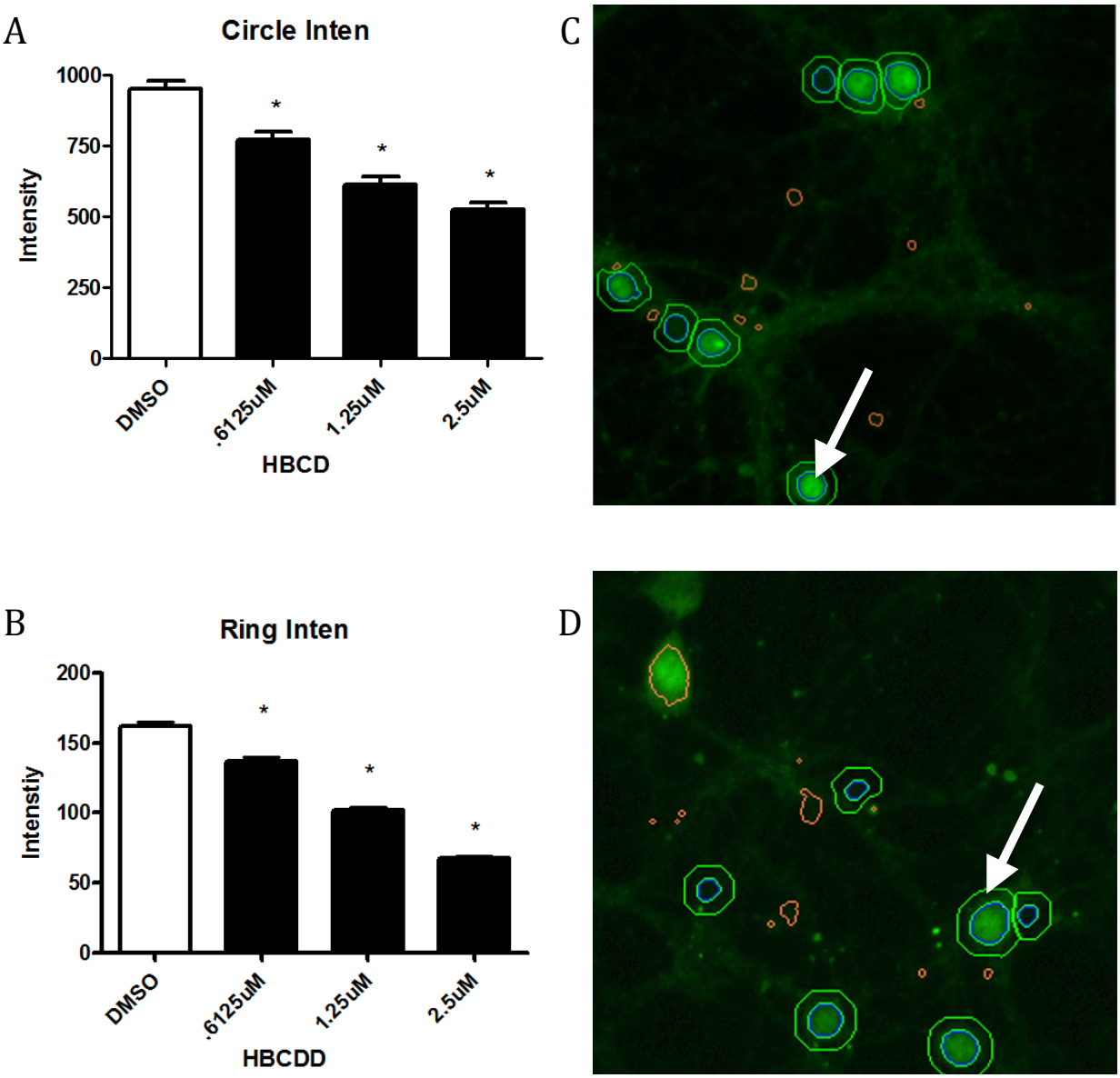


Figure 2: This indicates a significant decrease in Ube3a expression in hippocampal primary cultures starting at doses as low as 0.6125μM. A similar trend is experienced both in the circle (A), around the nucleus, in addition to the ring (B), the cell body outside the nucleus (* indicates $p<0.05$). Figures C (DMSO) and D (2.5μM HBCDD) both represent a field of view and the gaiting used by the Array Scan. In figure C, an arrow denotes the circle measurement and in figure D, an arrow marks the ring area.

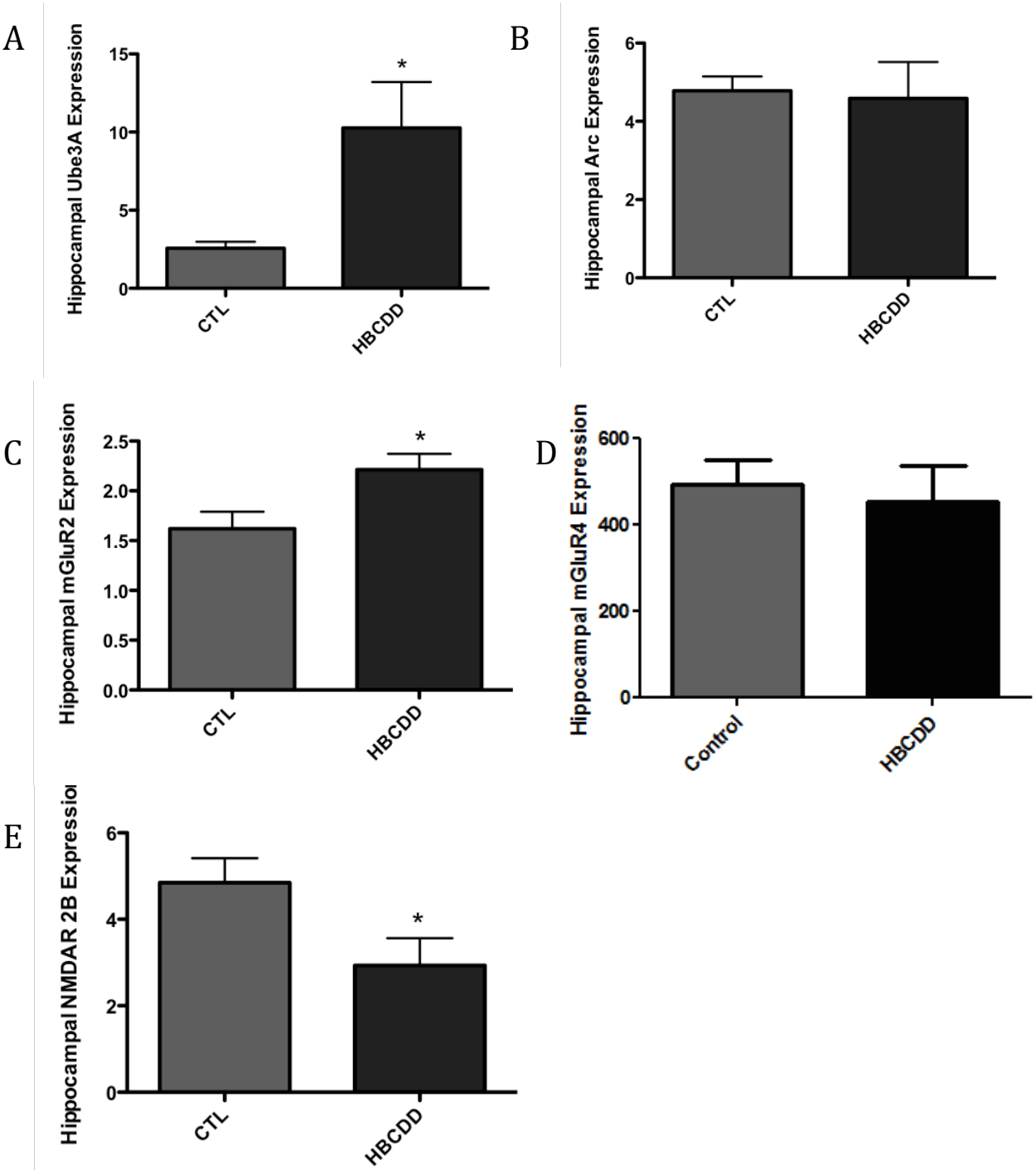


Figure 3: In vivo exposure of mice to HBCDD causes significant changes in proteins involved in the glutamatergic system in the hippocampus. Mice were exposed to either 0 mg/kg (control) or 25 mg/kg HBCDD for 30 days and were then analyzed for differences in (A) Ube3a (B) Arc (C) mGluR2 (D) mGluR4, and (E) NMDAR 2B in the hippocampus. Data represent mean ± SEM. *Values that are significantly different from control ($p<0.05$).

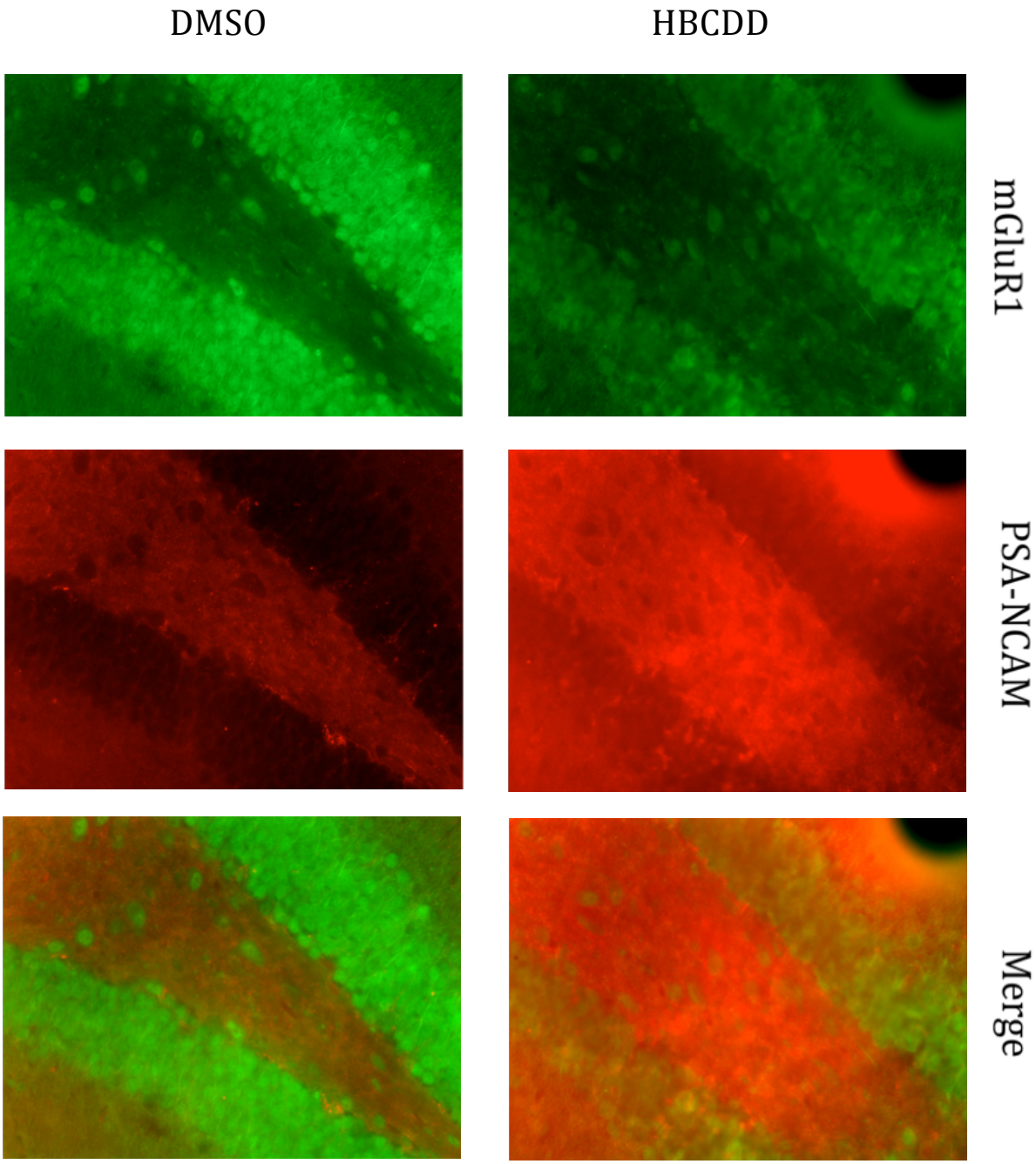
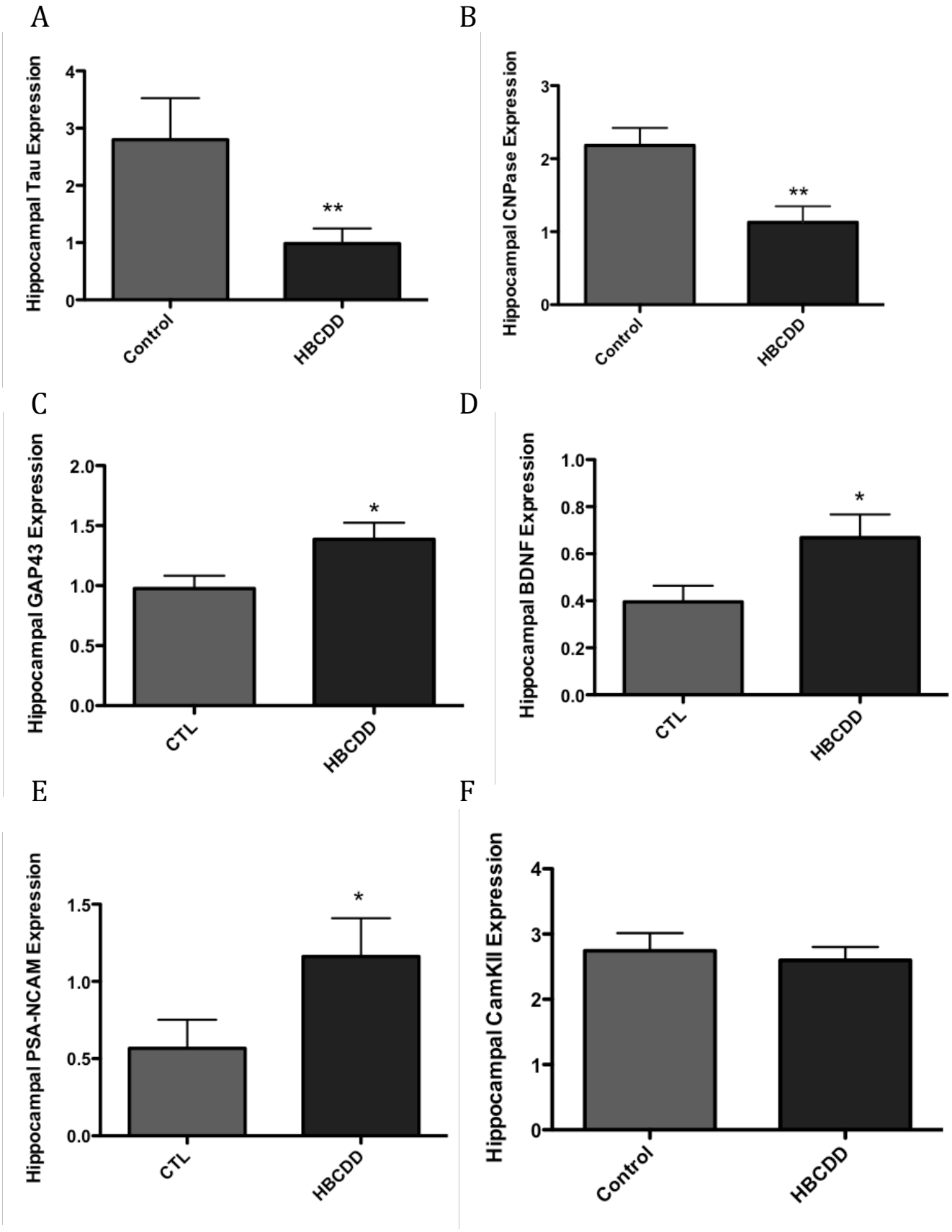


Figure 4: In vivo exposure of mice to HBCDD causes qualitative changes in mGluR1 and PSA-NCAM expression imaged by immunofluoresence. From these images there is an apparent decrease in mGluR1 expression but an increase in PSA-NCAM expression to very defined regions of the hippocampus. The PSA-NCAM expression matches the data from the immunoblot (Figure 5E). Green = mGluR1. Red = PSA-NCAM. Images were taken at a 20x magnification.


A
Hippocampal Tau Expression
4
3
2
1
0
**
Control
HBCDD
B
Hippocampal CNPase Expression
3
2
1
0
**
Control
HBCDD
C
Hippocampal GAP43 Expression
2.0
1.5
1.0
0.5
0.0
*
CTL
HBCDD
D
Hippocampal BDNF Expression
1.0
0.8
0.6
0.4
0.2
0.0
*
CTL
HBCDD
E
Hippocampal PSA-NCAM Expression
1.5
1.0
0.5
0.0
*
CTL
HBCDD
F
Hippocampal CamKII Expression
4
3
2
1
0
Control
HBCDD

G

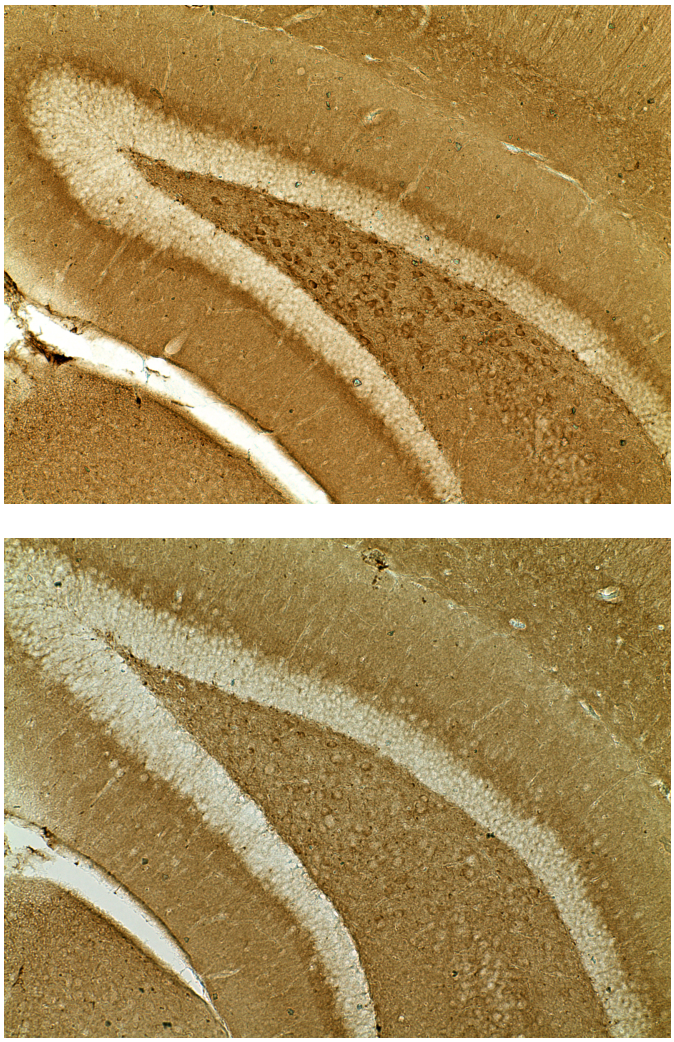


Figure 5: In vivo exposure of mice to HBCDD causes significant changes in proteins involved in neuronal stability and synaptic integrity in the hippocampus. Mice were exposed to either 0 mg/kg (control) or 25 mg/kg HBCDD for 30 days and were then analyzed for differences in (A) Tau (B) CNPase (C) GAP43 (D) BDNF, (E) PSA-NCAM, and (F) CamKII in the hippocampus. (G) Images for Tau in the dentate gyrus of the hippocampus are included. Data represent mean ± SEM. *Values that are significantly different from control ($p<0.05$). Images were taken at 5x magnification.

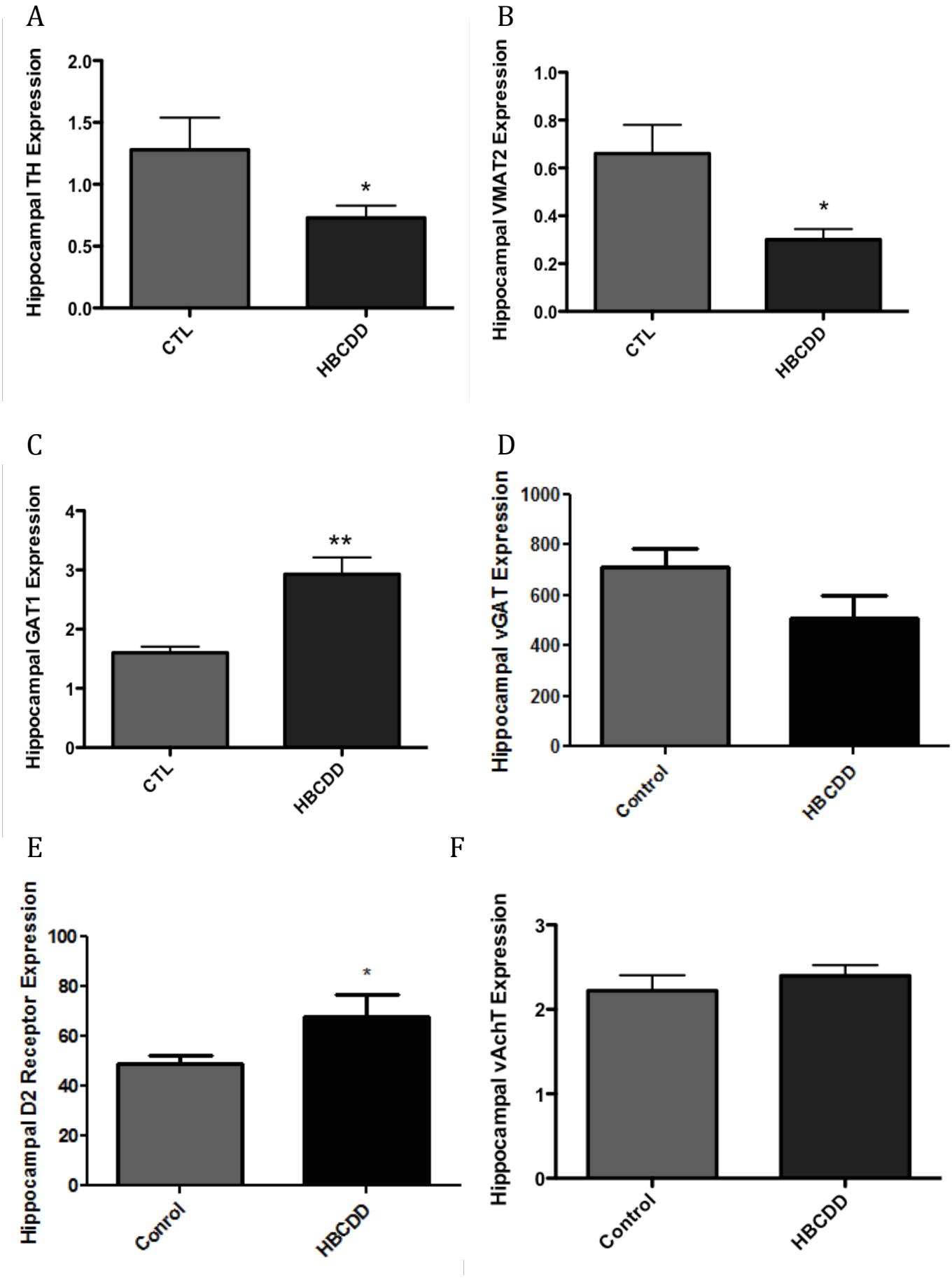

A
Hippocampal TH Expression
2.0
1.5
1.0
0.5
0.0
*
CTL
HBCDD
B
Hippocampal VMAT2 Expression
1.0
0.8
0.6
0.4
0.2
0.0
*
CTL
HBCDD
C
Hippocampal GAT1 Expression
4
3
2
1
0
**
CTL
HBCDD
D
Hippocampal vGAT Expression
1000
800
600
400
200
0
Control
HBCDD
E
Hippocampal D2 Receptor Expression
100
80
60
40
20
0
*
Control
HBCDD
F
Hippocampal vAchT Expression
3
2
1
0
Control
HBCDD

Figure 6: In vivo exposure of mice to HBCDD causes significant changes in proteins involved in the other neurotransmitter systems in the hippocampus. Mice were exposed to either 0 mg/kg (control) or 25 mg/kg HBCDD for 30 days and were then analyzed for differences in (A) TH (B) VMAT2 (C) GAT1 (D) vGAT, (E) D2 Receptor, and (F)vAchT in the hippocampus. Data represent mean ± SEM. *Values that are significantly different from control ($p<0.05$).

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