Gender specific changes in key regulators of neurodevelopment and autistic behavioral pathology in mice exposed to water chlorination byproducts

by

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Abstract

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Autism is a heterogeneous group of disorders with no definitive etiology. Out of concern for higher than expected prevalence, the Agency for Toxic Substances and Disease Registry (ATSDR) investigated the municipal water supply in Brick Township, New Jersey. The ATSDR found that two trihalomethanes (THMs), specifically chloroform and bromoform, as well as tetrachloroethylene (perchlorethylene; PCE), were present in concentrations that exceeded allowable maximum contaminant (MCL) values. In a related study, it was found that THMs and PCE act synergistically to increase the level of catalytic Protein Kinase A (PKA) in neurons of clam embryos. PKA is a key regulator of neurodevelopment, and it is hypothesized that abnormalities in PKA

activity could induce both histopathological and biochemical manifestations that are found in autism. Based upon these findings, we hypothesized that THM/PCE exposure induces changes in key regulators of neurodevelopment and behavioral pathology similar to that which is found in autism. In our experiments we found that exposure to THM/PCE induces an increase in the level of catalytically active PKA in zebrafish neurons and increases PKA activity in microglia cell culture. In a mouse model, we found that exposure to THM/PCE via drinking water induces an increase in the activity of PKA in the cerebral cortex of male animals at postnatal day 4 (P4) and postnatal day 10 (P10). Females cortical PKA activity was unaffected by THM/PCE exposure. By P15, male cortical PKA activity is no longer affected by THM/PCE exposure and female cortical PKA activity remains unaffected. Behaviorally, we found that the THM/PCE exposed males develop autistic like behavioral pathology as they evidence deficits in communication and social behavior and demonstrate both perseverance behavior and anxiety. Again, this finding is gender specific, as female behavior is unaffected by THM/PCE exposure. These findings suggest that these chemicals may be involved in the etiology of autism and that males are more susceptible to this set of insults.

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List of Abbreviations

5HT1a	5-hydroxytryptamine 1a receptor
AD	Autistic Disorder
AKAP	A Kinase Binding Protein
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorder
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BCl	B-cell lymphoma
BDNF	Brain Derived Neurotrophic Factor
BTMUA	Brick Township Municipal Utilities Authority
C/EBP	CCAAT-enhancer-binding proteins
cAMP	Cyclic adenosine monophosphate
CDC	Center for Disease Control
CDD	Childhood Disintegrative Disorder
CRE	cAMP Response Element
CREB	cAMP response element binding
D2	Dopamine 2 recptor
DCC	Deleted in Colorectal Cancer
DMEM	Dulbecco's Modified Eagle Medium

DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
EM	Electron Microscope
FBS	Fetal Bovine Serum
FXS	Fragile X syndrome
GABA	Gamma Aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GTP	Guanosine-5'-triphosphate
ICD	International Classification of Disease
MAPK	Mitogen Activated Kinase
MCL	Maximum Contaminant Level
MTHFR	Methylenetetrahydrofolate reductase
NGS	Normal Goat Serum
NIH	National Institutes of Health
P10	Postnatal Day 10
P11	Postnatal Day 11
P15	Postnatal Day 15
P21	Postnatal Day 21
P30	Postnatal Day 30
P4	Postnatal Day 4
P60	Postnatal Day 60

P85	Postnatal Day 85
PBS	Phosphate Buffered Saline
PCE or PERC	Perchloroethylene or tetrachloroethylene
pCREB	Phosphorylated cAMP response element binding
PDD	Pervasive Developmental Disorder
PDD-NOS	Pervasive Developmental Disorder - Not Otherwise Specified
PKA	Protein Kinase A
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
THM(s)	Trihalomethane(s)
TrkB	Tyrosine receptor kinase B
TSC	Tuberous Sclerosis
US EPA	United States Environmental Protection Agency

1. Introduction and Background

1.1 What is autism?

Pervasive Developmental Disorders (PDD) or Autism Spectrum Disorders (ASD) refers to a group of five disorders that are characterized by delays in the development of multiple functions, which include socialization and communication. There are five PDDs, which include Autistic Disorder (AD), Rett's syndrome, Childhood Disintegrative Disorder (CDD), Asperger's syndrome and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS; DSM-IV-TR, 2000). Diagnosis of these conditions is done based upon psychological evaluation, and to date, no biochemical or morphological tests are available for diagnostic use, with the exception of Rett's syndrome (Lord and Bishop, 2009).

1.2 Symptoms and Diagnosis of each of the PDDs

Each of these disorders is characterized by a specific set of behavioral criteria. In the case of AD, a diagnosis is made if the person shows a qualitative impairment in social interaction. Impairment in social interaction includes marked impairment in the use of multiple nonverbal behaviors, failure to develop peer relationships appropriate to their developmental level, a lack of spontaneous seeking to share enjoyment with other people and a lack of social reciprocity.

Two of these behaviors concerning social interaction must be present for autism diagnosis. A second criterion for behavior that must found for autism diagnosis is the presence of qualitative impairments in communication. This category includes a delay or total lack of spoken language and no attempt to compensate, a lack of spontaneous make believe play or social imitative play appropriate to the developmental level, stereotyped and repetitive use of language or idiosyncratic language, and in the case that language exists, an inability to initiate or sustain conversation with others. One of the impairments regarding communication must be met in order to receive a diagnosis of autism. The third and final criterion for autism diagnosis is the presence of restricted, repetitive and stereotyped behavior patterns. This includes an encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal in intensity or focus. This also includes an inflexible adherence to specific, nonfunctional routines and rituals, stereotyped and repetitive motor mannerisms (hand flapping) or persistent preoccupation with parts of objects. One of the above behaviors concerning restricted and repetitive behavior patterns must be found in order to achieve a diagnosis of autism. In addition, these behavioral abnormalities must be present before three years of age, and must not be caused by Rett's disorder or childhood disintegrative disorder (DSM-IV-TR, 2000).

Diagnosis for Rett's disorder is made if a child has apparently normal prenatal and perinatal development, apparently normal psychomotor development through the first five months after birth and normal head

circumference at birth. If these criteria are met and after the period of normal development deceleration of head growth occurs between five years to six years of age, as well as a loss of previously purposeful hand skills, social engagement and expressive language development with severe psychomotor retardation, the child is diagnosed with Rett's Disorder (DSM-IV-TR, 2000).

A diagnosis of Childhood Disintegrative Disorder is made when a child develops normally for at least the first two years after birth, but then shows deficits in age appropriate verbal and non-verbal communication, social relationships, play and adaptive behavior. Additionally, there must be a loss of two previously acquired skills before ten years of age. These skills include expressive or receptive language, social skills and adaptive behavior, bowel or bladder control, play and motor skills. Finally, the child must show abnormalities in at least two of the following areas, which include a qualitative impairment in social interaction, qualitative impairments in communication and restricted, repetitive and stereotyped patterns of behavior. Additionally, any of the diagnostic criteria used to diagnose a child with Childhood Disintegrative Disorder must not be caused by another specific PDD or schizophrenia (DSM-IV-TR, 2000).

The fourth PDD is Asperger's Disorder. In order to receive a diagnosis of Asperger's Disorder, a child has to show a qualitative impairment in social interaction. This includes a marked impairment in the use of multiple non-verbal behaviors as well as a failure to develop peer relationships appropriate to developmental level and a lack of spontaneous sharing of enjoyment, interests or

achievements with other people along with a lack of social or emotional reciprocity. In addition to impaired social interaction, a person must also evidence restricted, repetitive and stereotypic patterns of behavior, which include an encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal in either intensity or focus, or an inflexible adherence to specific nonfunctional routines or rituals and persistent preoccupation with parts of objects. These disturbances must cause significant impairment in social, occupational or other important areas of functioning and do there must be no significant delay in language, self help skills or curiosity about the environment in childhood. None of the behaviors used for Asperger's diagnosis may be caused by another PDD or Schizophrenia (DSM-IV-TR, 2000).

The final PDD is PDD-NOS. This diagnosis is given when there is a severe impairment in the development of reciprocal social interaction associated with impairment in either verbal or non-verbal communication skills or with the presence of stereotyped behavior, interests or activities, but the criteria are not met for a specific PDD, Schizophrenia, Schizotypical Personality Disorder, or Avoidant Personality Disorder (DSM-IV-TR, 2000).

1.3 Prevalence of ASDs and AD

In recent years, reports show that there has been an apparent rise in the prevalence of ASDs and AD (Fombonne, 2005), which has sparked much debate, as the diagnostic criteria for ASD and AD have been revised multiple

times and epidemiological studies use a wide variety of methodologies, both of which confound prevalence estimates.

In regard to changing criteria, cases of PDDs reported in the 1960s were primarily assessed using Kanner's criteria (Kanner and Eisenberg, 1957). Lotter's and Rutter's definitions and the ICD-9, which was used in the 1970s were replaced by the DSM-III (1980) and the DSM-III-R (1987) in the 1980s. Over the past 20 years, cases have been defined by the DSM-IV (1994) and the ICD-10 (1992). Changing the criteria for diagnosis is a very important factor that affects epidemiological studies of AD and the ASDs. For example, in one study, 38 adults diagnosed with developmental language disorders as children in 1980 were evaluated and if diagnosed by criteria today, 25% of them would be diagnosed with an ASD (Bishop, 2008). This is just one example that demonstrates that growing prevalence estimates confound comparisons of current versus past prevalence.

In addition, factors such as population size and survey methodology are likely to result in an inability to make an accurate comparison of prevalence estimates to determine if prevalence is really increasing. For example, in two studies that used educational services to determine ASD prevalence in populations in Texas and Missouri, ASD was found to be drastically different. The Texas survey estimates ASD prevalence of 16 per 10,000, and the Missouri survey estimates 4.8 per 10,000 (Sturmey, 2001; Hillman, 2000). It must be - noted that even though the same methodological strategy for identification of persons affected with ASD was used for both surveys, the age group surveyed

was different (6-18 years of age in the Texas study and 5-9 years of age in the Missouri study), which would obviously result in different prevalence rates, as the Missouri study was much more limiting. This also brings to light the point that the population size used will also affect the results of prevalence estimates. In a study done in California, using educational services and 3,564,577 persons between 4-9 years of age, prevalence estimates for ASD are 15 per 10,000 (Department of Developmental Services, 1999). In a prevalence study done in England and Wales, using 10,438 people and a household survey of psychiatric disorders, it was shown that the prevalence rate was 26.1 per 10,000 (Powell, 2000). This is again just one more demonstration of how different variables such as age group, identification strategy and total number of persons studied could affect prevalence estimates.

In 2005, taking confounding factors into account, it was estimated that 13 per 10,000 children are affected by AD (Fombonne, 2005). The authors of this study warn that this estimate is conservative, as a true estimate would be higher because some children will be missed.

Other ASDs, such as Childhood Disintegrative Disorder, proved to be exceedingly rare. Only six surveys have reported children meeting diagnostic criteria for Childhood Disintegrative Disorder (Fombonne, 2005). Based upon these surveys, and adjusting for different methodological strategies used for estimation, the prevalence of Childhood Disintegrative Disorder is 2.0 cases per 100,000. In the case of Asperger's disorder, which was introduced into the ICD-10 in 1992 and into the DSM-IV in 1994, prevalence of was estimated to be

approximately 2.6 cases per 10,000, or about one-fourth of the prevalence of AD. PDD-NOS was estimated to have a prevalence of 20.8 per 10,000, which exceeds that of AD. Taken together, the prevalence of all ASDs is estimated to be 36.6 per 10,000, which is again conservative, as children are likely to be left out of diagnosis. The authors of this paper suggest that a less conservative prevalence estimate of 60 per 10,000 people in the United States are affected by an ASD or at least 300,000 people under the age of 20 are affected by one of the ASDs in the US.

More than thirty studies have been done concerning the prevalence of autism in both genders. The mean male to female ratio has been determined to be 4:1 (Fombonne, 2005). However, among children with ASD and mental retardation, the ratio is less pronounced. The trend shows that the more profound the retardation, the lower the sex ratio, as it is thought that the sex ratio in this population is two males to every one female (Fombonne, 1997; Fombonne, 2001). In the reverse direction, meaning the higher functioning children with AD, the ratio of males to females is six to eight boys for every one girl (Fombonne, 2001b).

1.4 Pathological Findings in autism

Given the heterogeneity of AD and ASDs, there has been no consistency in the pathological findings. This is partially due to the dynamics of brain

development, small sample sizes, and uncontrolled biases that result from histological studies.

1.4.1 Brain size

Macrocephaly, which is defined as a head circumference above the 97th percentile, was found in approximately 20% of subjects with AD aged 1 year or above (Woodhouse, 1996). Consistent with these findings, brain weight of autistic individuals is increased (Bailey, 1998; Kemper and Bauman, 1998; Courchene, 1999; Casanova, 2002). Brain size is also elevated, as found in neuroimaging studies (Courchene, 2003; Herbert, 2003). However, conflicting data concerning brain size exists for adolescents and adults with AD, as normal controls appear to have larger brains (Piven, 1995, 1996; Hardan, 2001). Additionally, some studies have found that there is no difference in brain volume of adults with AD as compared with controls (Courchene, 2002; Awlward, 2002).

1.4.2 Limbic system

Bauman and Kemper were the first to investigate abnormalities in the limbic system in autistic cases. After two studies, they compiled their data and found that in six autistic cases, as compared with six matched controls, there was increased cell packing and reduced cell size in the hippocampus, subiculum and amygdala, and to a lesser extent, in the entorhinal cortex, mamillary bodies and

septal nuclei. Five of the brains under investigation were from males with mental retardation, four of whom had epilepsy. However, in a later study, only one of five autistic cases presented with smaller more densely packed neurons in the hippocampus (Piven, 1998). Unfortunately, limbic system pathology has not yielded uniform results, which complicates our understanding of AD and ASD.

1.4.3 Cerebellum

Several reports have concluded that there is a reduction in Purkinje cell number in the cerebellum of persons with AD (Kemper and Bauman, 1993; Williams, 1980). Many case studies have substantiated these findings, as Purkinje cell number was reduced as well (Fehlow, 1993; Bailey, 1998, Whitney et al., 2008, Whitney et al., 2009). However, again, there is conflicting data, as some have not found a reduction in Purkinje cell number in AD (Guerin et al., 1996; Bailey et al., 1998) and one group found no difference in cell number, but a difference in Purkinje cell size (Lee et al., 2002). Therefore, again, there is no consistency in the pathological findings even regarding cerebellar Purkinje cells.

1.4.4 Neocortex

Abnormalities in the neocortex of individuals with autism are even less concordant among AD cases than those that are found in limbic regions and in the cerebellum. In one study, 42 comparisons were made between a 21-year-old

autistic female, with probable mental retardation, and two female controls aged 18 and 25. Only six of the 42 comparisons yielded any differences between the autistic and control brains. Additionally, the two control brains were more different in comparison to each other than in comparison to the autistic brain (Coleman et al., 1985). The only differences that were found in the autistic case included a decreased number of glial cells in the left primary auditory cortex and a decreased number of pyramidal neurons in the right auditory association cortex in the autistic case. In another study, the brain of a 24-year-old female with autism, mental retardation and self injurious behavior showed neurofibrillary tangles in layers II and III of the cerebral cortex of the temporal region, and some tangles in the amygdala (Hof et al., 1991). These findings are similar to those that are found in boxers, which may have resulted from the woman's self-injurious behavior, rather than autism (Hof et al., 1992). Kemper's investigation of six autistic cases in 1993 showed evidence of a poorly laminated anterior cingulate cortex in five of the six cases. In other studies, no abnormalities of the cerebral cortex were found, and there were no abnormalities in cell counts in the superior frontal cortex of autistic cases (Guerin et al., 1996; Bailey et al., 1998).

1.4.5 Cortical dysgenesis and migration abnormalities

Cortical dysgenesis, which included thickened cortices, high neuronal density, presence of neurons in the molecular layer, irregular laminar patterns, heterotopias and poor grey-white matter boundaries was found in four of six

autistic brains (Bailey et al., 1998, Bailey et al., 1993). In addition to these abnormalities, more numerous, but less compact cortical minicolumns were found in eight autistic subjects, of which seven had profound mental retardation (Casanova et al., 2002). Related to these studies, levels of the protein Reelin, which is regarded as being critical in regulation of neuronal migration and correct lamination and BCl-2, which is a member of the anti-apoptotic family of BCl proteins, were found to be significantly reduced in the cerebellum of five autistic cases (Fatemi et al., 2001). Additionally, the pro-apoptotic transcription factor p53 was elevated by 130% in the parietal and 68% in the superior frontal cortex of autistic cases (Fatemi et al., 2001). It must be noted that increases in apoptosis in the brain should result in smaller brain volumes, which is not consistent with that found in many studies. However, it has been proposed that a decrease in brain volume would be associated with more severe AD cases, and increased brain volume associated with high functioning subjects (Akshoomoff, 2002; Frietag et al., 2009).

1.4.6 Alterations in the GABAergic system

The GABAergic system has an important role in early development, as it serves as the principal source of excitation in the early neonatal period, opposed to its primary function as an inhibition system as it is found in adults (Cherubini et al., 1991; Barker et al., 1998). In the hippocampus of four male autistic cases, GABA was the only neurotransmitter significantly reduced. Later e12xperiments

revealed that the levels of glutamic acid decarboxylase (GAD) the rate limiting enzyme responsible for the conversion of glutamate to GABA in the brain were reduced by 50% in the cerebellum and by 48% in the parietal cortex of autistic cases (Fatemi et al., 2009). It must be noted that sample sizes used for these studies were small, so more evidence is needed to support the involvement of the GABAergic system in autistic disorders.

1.5 Genetics of autism

The idea that autism and other ASDs is a genetic condition that is heritable is widely accepted. There is strong evidence that autism is a heritable disorder, as the rate of recurrence in siblings of affected individuals is 2% to 8%, which is much greater than the prevalence rate in the general population (Gillberg, 2000; Chakarabarti and Fombonne, 2001; Chudley, 1998). Supporting these findings, studies using monozygotic twins report a rate of concordance to be greater than 60% for autism disorder, and no concordance between dizygotic twins (Bailey et al., 1995; Steffenburg et al., 1989). Additionally, when the unaffected monozygotic twin was evaluated for other ASDs, the concordance among the twins rose from 60% to 92%. In the dizygotic twins, the concordance rate rose from 0% to 10% (Bailey et al., 1995; LeCouteur et al., 1996). Other similar studies have yielded similar results, evidencing a 36% to 96% concordance of ASD for monozygotic twins and concordance of 0% to 30% for dizygotic twins (Bailey et al., 1995; Folstein and Rutter, 1977; Ritvo et al., 1985;

Steffenburg et al., 1989). Disparity regarding the subset of ASD in the monozygotic twins, who share the same genetic complement, evidence that phenotype could be largely different, and that both pre and perinatal environmental factors and stressors could result in epigenetic modulation, which can account for the differences found in the twins (Petronis et al., 2006).

Family studies also suggest that ASD and related psychoses may be highly heritable. A case control study compared families with an autistic child versus families with a Down syndrome child. 5.8% of the siblings of autistic children also had autism. This was not found in the Down syndrome group (Bolton et al., 1994). In a more convincing study, siblings of autistic children were found to have greater incidence of impairments in communication abilities (Bishop et al., 2006), but overall language abilities were not found to be impaired in siblings (Pilowsky et al., 2003). In studies concerning stereotypy, social impairments and nonverbal communication, there was a high concordance rate for siblings of autistic children (Silverman et al., 2002; MacLean et al., 1999; Spiker et al., 1994). This is further evidence that a genetic component that undermines the behaviors evidenced to be aberrant in autism is largely heritable.

Cytogenetic analysis shows that abnormalities of chromosome 15 and the sex chromosomes are frequently found in ASDs, in particular cases that are associated with mental retardation and seizures (Oudim et al., 2007; Wassink et al., 2001; Wolpert et al., 2000). These abnormalities often are supernumerary isodicentric chromosome 15, or less frequently a maternally derived interstitial duplication of the 15q11-q13 region (Wang et al., 2008; Wang et al., 2004; Boyar

et al., 2001). The predominant phenotype of people with this supernumerary chromosome 15 is AD, developmental delay, mental retardation, behavioral disturbances, and absence of speech or abnormalities of speech. Individuals have also been found to be clumsy, seizure prone, aggressive, and hyperactive, have short attention spans, self-mutilating and have stereotypical behaviors (Boyar et al., 2001, Oudim et al., 2007). Additionally, in one study, a microscopic genomic deletion in this region has been found in comparison with control chromosomes (Sebat et al., 2007). These findings may prove that this is a potential genetic marker in this population. This same region is also deleted in the Prader-Willi and Angelman Syndromes (Jiang et al., 2008; Camprubi-Sanchez et al., 2006), with Angelman syndrome most frequently occurring from a de novo maternal microdeletion of the 15q1-q13 region (Horsthemke and Wagstaff et al., 2007). In autism, maternally derived chromosomal aberrations are found more frequently (Schrorer et al. 1996). Chromosome 15 is considered one of the most genomically unstable regions, as it shows a high frequency of deletion events, accounting for ~50% of supernumerary marker chromosomes observed (Karaman et al., 2006).

There are many candidate genes in ASDs (Basu et al., 2009), some of which are found on chromosome 15. The $GABA_A$ receptor cluster, which encodes genes for three of the receptor's subunits, namely a5, B3 and y3 is strongly implicated in the pathogenesis of autism, given its involvement in controlling both excitatory pathways in early development and inhibitory pathways in later development (Owens and Kreigstein, 2002). Mice deficient in

the GABA B3 subunit display autistic like behaviors (Huntsman et al., 1999; Delorey et al., 1998).

Chromosomal translocations have also implicated the 7q22-33 region (Ashley-Koch et al., 1999; Yan et al., 2000; Scherrer et al., 2003). The reelin gene, whose expression results in a large secretory glycoprotein which is critical to neural migration during development (Hong et al., 2000; Bailey et al., 1998), has been found to have a long trinucleotide repeat polymorphism in the 5' region in individuals with AD (Persico et al., 2001). Western blot analysis of protein expression has found this protein to be reduced by 44% in autistic subjects (Fatemi et al., 2001).

Luckily, the autism gene database has been developed (www.mindspec.org/autdb.html). This is an extensive database, which provides a list of genes, broken down by chromosomal location, that are candidate in the etiology of autism (Basu et al., 2009). There are currently 100 candidate genes that may be involved in the etiology of AD or ASD, which can be found on the previously mentioned web site, along with references that implicate them in the etiology of AD or ASDs. Also, there are references that provide conflicting data as well. This is a very valuable tool. The majority of listed genes are found as rare single gene mutations in persons with AD or ASDs. Some have been implicated in more than one study. Some of these genes include the $GABA_A$ B3 receptor subunit (15q11.2-q12; Mao et al., 2000; Ma et al., 2005), the serotonin transporter gene (17q11.1-q12; Cook et al., 1997; Sutcliffe et al., 2005; Mulder, 2005; Coutinho et al., 2004; Kim et al., 2002), neuroligin-3 (Xq13.1; Ylisaukko-oja

et al., 2005; Jamain et al., 2003; Tabuchi et al., 2007) and X-linked neuroligin 4 (Xp22.32-p22.31; Jamain et al., 2003; Ylisaukko-oja et al., 2005; Lawson-Yuen, 2008 et al.; Jamain et al., 2008).

Other single gene disorders are associated with an increased risk for AD. The most prevalent are tuberous sclerosis (TSC) and Fragile X syndrome (FXS). TSC is an autosomal dominant neurocutaneous disorder, characterized by facial angiofibromas and cortical and cerebral tubers. This disorder is caused by various mutations that occur on the TSC1 (9q34) and TSC2 (16p13.3) genes, and such individuals are much more likely to have AD or ASDs (Smalley, 1998; de Vries et al., 2007). In one study, it was shown that children with TSC are 100 times more likely to develop ASDs (Bolton and Griffiths, 1997). Similar to the association of the TSC genes to AD and ASDs, individuals with FXS are much more likely to have AD or ADS FMR1 (Xq27.3) than those found in the normal population (Hatton et al., 2006; Bailey et al., 2000).

The main point of this section is to reveal that though there has been progress made regarding the genetics of autism, it is highly unlikely that it is a monogenic disorder, or even a specific combination of genes. However, it does seem that mutation in genes critical to brain patterning or in early brain development, induces AD or ASDs.

1.6 The Agency for Toxic Substances and Disease Registry (ATSDR) Report: High Autism Prevalence and Chlorination Byproducts in Brick Township, New Jersey

In 2000, the ATSDR released a report based on a series of investigations that took place during 1998 in Brick Township, New Jersey (ATSDR, 2000). These investigations were done in response to parents of autistic children and to Senator Robert Torricelli urging the government to investigate a qualitative observation of an increased prevalence of autism in that community. The ATSDR did find that prevalence in this community was higher than national estimates. Corroborating ATSDR's findings, a group from the NIH, the CDC, the ATSDR and Robert Wood Johnson published a paper revealing the methodologies used to determine the ATSDR's estimates. This group confirmed the estimated prevalence of AD in this community to be 4.0 cases per 1000 children. They also confirmed that total ASD prevalence was 6.7 cases per 1000 children and that prevalence of Asperger's Disorder is 2.7 cases per 1000 children. All of these estimates exceeded national prevalence estimates, even estimates that used a similar number of persons. 8,896 children were evaluated in this particular study.

1.6.1 Autism Prevalence according to the ATSDR report

The first ATSDR investigation was that of autism prevalence in children between 3 and ten years of age in the township. This investigation took place in three phases. In the first phase, school records and doctor's records were used to identify possible cases of autism. In phase II, clinicians from the ATSDR diagnosed cases of autism using the Autism Diagnostic Observation Schedule (ADOS; Lord et al. 2000) and the DSM-IV-R. Children included in the study were either born conceived in Brick, or immigrated to Brick. The ATSDR reported that the prevalence of Autism Spectrum Disorders (ASD) was 6.7 per 1000 children and prevalence of Autistic Disorder (AD) was 4.0 per 1000 children in Brick. These prevalence estimates were identical to those found by Bertrand and colleagues (Bertrand et al., 2001). Sixty-six percent of the children diagnosed with ASD were either born or conceived in Brick. The report did not include if the mothers of children who were born in Brick resided in Brick throughout the duration of the pregnancy. Also, the report did not contain data concerning prevalence of ASD in other areas but did report that prevalence of AD in other areas has been reported as high as 3.1 per 1000 children in other areas. However, due to inclusion of children who were not conceived or born in Brick Township in the ASD/AD prevalence estimates, this report of elevated autism prevalence is strongly disputed by autism investigators as being biased, with major criticism resulting from the idea that parents of children with AD are immigrate to this area for medical and school services that are readily available

for their children. In response to this criticism, the ATSDR did report that 66% of the children with ASDs were born in Brick. Therefore, if the 33% of children not born in Brick are left out of the survey, prevalence estimates become of 4.5 per 1000, which is still higher than ASD prevalence that was found in other studies by which similar methodology was used to identify cases (Yeargin-Allsopp et al., 2003).

1.6.2 Trihalomethanes and Tetrachloroethylene in drinking water in Brick Township

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In addition to the ASD and AD prevalence study, the ATSDR reported that two trihalomethanes (THMs), specifically chloroform and bromoform, and tetrachloroethylene (perchloroethylene; PCE or PERC) were found in municipal drinking water during the years 1987-1995. There was an increase in these byproducts over the study period due to change of source water supply. Before 1987, the source of water in Brick Township was ground water, which is devoid of algae and organic matter. By 1995, 2/3 of the source water was from the Metedeconk River, which was rich in organic matter. Water chlorination resulted in excessive formation of these byproducts. Additionally, the Brick Township Municipal Utilities Authority (BTMUA) added a second water chlorination treatment during the summer months, when organic contaminants in source water were elevated.

The ATSDR reported that chloroform was found to be elevated in 356 samples taken in during the years 1987- 1995. The total number of samples taken for analysis was not reported. 320 of these 356 samples exceeded the ATSDR Maximum Contaminant Level (MCL) value of 6 ppb. MCLs have been established by the United States Environmental Protection Agency (USEPA) for public water supplies to reduce the chances of adverse health effects from contaminated drinking water. Bromoform was detected in 14 samples, and exceeded the ATSDR comparison value of 5 ppb only one time. PCE was detected in 12 samples, and exceeded the ATSDR comparison value of 0.7 ppb in five samples. The total number of samples taken for analysis was not reported.

1.6.3 Autism prevalence and THM/PCE exposure correlation study

In the next phase of the investigation, parents of 43 children who participated in the clinical exams who were diagnosed with ASD were contacted to obtain information concerning place of birth and residence during pregnancy. Twenty-eight of these children were born in Brick Township, twelve were born outside Brick Township and information could not be obtained on one child. ATSDR then plotted residence of these children and residence of the mothers of children during pregnancy with places where water was found to have a total THM level of above 80 ppb or above 60 ppb, which is above the accepted MCL. The exact levels of chloroform and bromoform were not given (note: 80 ppb is

more than 12 times higher than the allowable MCLs of chloroform and bromoform according to the US EPA). Fourteen of these children were found to live within 1 mile of where the samples exceeded over 80 ppb and five children were born to mothers who lived within this area during pregnancy (18%). All 28 children were found to live within 1 mile of where THM levels were above 60 ppb. Eight of these children were born to mothers who lived within this area (29%). It must be noted here that the ATSDR did not explicitly state whom they used in this phase of the investigation, as the number of individuals living in the 80 ppb area and the number of individuals living in the 60 ppb area exceed the number of subjects (41) that were originally named in the investigation. It is possible that they used only the 28 born in Brick Township, and counted the persons living in the 80 ppb group in the 60 ppb group as well. However, this is conjecture and the report does not explicitly state which participants were included.

ATSDR then calculated the month and year of the first trimester for the aforementioned children who participated in the clinical exams and were diagnosed with ASD. For approximately 40% of the children who participated in the clinical exams, the first trimester was in 1991 or 1992. ATSDR reviewed THM data for the years prior to 1991 and 1992. The peak THM level (251 ppb) was in March 1988 and there were several THM samples that exceeded 100 ppb in September 1990 and September 1992. These levels do not correspond with the birth or conception periods of children with autism or PDD. In 1988 when the peak THM level occurred in March one child in the study was in the first trimester. In 1990 several THM levels exceeded 100 ppb in September and there

were four children in the study in their first trimester. In 1992 several THM levels exceeded 100 ppb in September and there were five children in the study in their first trimester at or about this time. In 1991 the year with the highest number of children in the study with ASD total THM levels did not exceed 100 ppb. It must be noted once again that the report does not explicitly state if participants were included or excluded from the study. It is also noteworthy to mention that it is very likely that all 41 participants (which included children born of mothers pregnant while residing in Brick and children born of mothers who immigrated to Brick) from the clinical exams were used since the report states that 41 was used as a denominator

On the basis of these data, the ATSDR concluded that there is no evidence that the increased prevalence of autism in Brick was due to chemical exposure. The methods used for this investigation do provide evidence that it is unlikely that these chemicals were involved in the etiology of the prevalence the autistic cases found in the township, since the majority of persons with autism in this community were born to mothers who were not exposed to the high THM levels during pregnancy.

However, the ATSDR report did not provide evidence that in the 66% of cases, or in the 28 subjects, that were born in the township were independent of THM and PCE exposure, because those that immigrated into the community were counted in demographic studies that linked exposure and therefore diluted the prevalence of exposed individuals in the autism population. Prevalence estimates of exposed individuals were never made nor compared to prevalence

estimates of persons not exposed to the toxic insult. Therefore, the question of THM/PCE exposure as possible etiological agents in ASD and AD could not be answered by the methods used in the ATSDR investigation.

1.7 Toxicological profiles of chemicals found in Brick Township

1.7.1 Chloroform

Chloroform is a colorless, volatile non-flammable liquid with a molecular formula of $CHCl_3$ and a molecular weight of 119.38. It is a byproduct of chlorine disinfection of water, which is readily absorbed via inhalation or oral exposure. Chloroform's solubility in water is 7.95 g/L at 25˚C, and it is readily miscible with other organic solvents. Dermal exposure requires contact with liquid, rather than vapor. Absorbed chloroform distributes widely throughout the body, with substantial uptake and storage in adipose tissue. Chloroform can cross the placenta, and appears very high in human colostrums and mature breast milk (ATSDR, 1995).

The cytochrome P450 pathway metabolizes chloroform predominantly by oxidative metabolism to trichloromethanol, which spontaneously dehydrochlorinates to form phosgene. Phosgene will be discussed in detail later. Reductive metabolism also results in a highly reactive dichloromethyl free radical. Excretion of non-metabolized chloroform occurs primarily through exhalation (Gemma et al., 2004).

In long-term studies, chloroform induces renal and hepatic cancer in rats and in mice. Long-term occupational exposure of humans to chloroform has been linked to various neurological effects. However, the most common exposure to chloroform is via ingesting contaminated drinking water, bathing or inhalation, which can occur while bathing in hot water containing chloroform (Lee et al., 2004).

Chloroform ends up in drinking water supplies as a consequence of residual chlorine in water distribution systems. The residual chlorine, which exists as hypochlorous acid and hypochlorite in water reacts with naturally occurring organic matter to form a wide range of organic compounds (Rock, 1970). These organic compounds include trihalomethanes (THMs), haloacetonitriles, haloketones, chloropicrin and haloacetic acids. The most common THMs are chloroform, bromodichloromethane, chlorodibromomethane and bromoform. The US EPA has regulated total THM content at a MCL of 80 ug/L, which is below values that are known based on the potential for an increased risk of cancer and other health effects (US EPA, 1983).

Chloroform is the most prevalent byproduct formed when drinking water is chlorinated, although brominated THMs can occur at high concentrations when water with high bromide contents is chlorinated (Weisel and Chen, 1994). Various factors can affect the formation of chloroform in treated drinking waters, such as temperature, pH, concentration of residual chlorine, reaction time, transit time within the system and total organic compounds present.

In reference to exposure, gastrointestinal absorption of chloroform in humans and in animals appears to be both rapid and extensive (ATSDR, 1997). Absorption through the skin requires contact with chloroform in liquid form (Davidson et al., 1982; Gordon et al., 1998). Temperature is also key in dermal exposure as it was demonstrated that subjects bathing in 40˚C water exhaled about 30 times more chloroform than the same subjects bathing in 30˚C water (Corley et al., 2000).

Absorbed chloroform distributes widely throughout the body, with human and animal studies identifying concentrations of chloroform in fat, kidney, liver, brain, and blood (ATSDR, 1997). Chloroform storage can be substantial, especially in obese persons. Distribution may be influenced by route of exposure. It has been previously demonstrated that inhalation and dermal exposure results in higher levels of chloroform circulating throughout the body and to the bladder, whereas ingestion results in higher levels of chloroform being delivered to the liver (Blancato, 1993).

In reference to the metabolism of chloroform, CYP2E1 a member of the cytochrome P450 family is responsible for metabolism of chloroform at lower concentrations. The locus of CYP2E1 is 10q24.3. If high chloroform concentration exposure occurs, the cytochrome primarily responsible for metabolism is CYP2B1/2 (Testai et al., 1996). Almost all tissues in the body are capable of metabolizing chloroform (ATSDR, 1997) and males show greater CYP2E1 activity than females, as they have elevated expression of the enzyme (Bebia et al., 2004). This allows males to produce more highly reactive

metabolites, such as phosgene and dichloromethyl radical. These two compounds will be discussed in greater detail later, in reference to their effects on the body. It must also be noted that low doses of chloroform are almost completely metabolized, whereas capacity to metabolize chloroform appears to reach saturation at higher doses. This study was conducted in humans. Rate of chloroform metabolism was determined by the level of pulmonary excretion of 13-C labeled chloroform (Davidson et al.,, 1982). Also of interest, leaner subjects released more chloroform than those with more adipose tissue.

Since chloroform-contaminated water was found in an area where there is a possible elevation of AD and ASD prevalence, it is imperative to know the types of interaction that occur with it and the nervous system. Chloroform was once used as an anesthetic, until it was discovered that it is considered a potential carcinogen, as exposure has resulted in tumors in bladder, liver and rectum in rats and mice. It is difficult to determine its potential as a carcinogen in humans because other THMs are usually found in water supplies where chloroform exceeds maximum contaminant levels. Regardless of this, if chloroform exposure occurs, it produces anesthetic effects by interaction with the $GABA_A$ receptor, as it has been demonstrated that chloroform enhances chlorine currents using patch clamp recordings (Jenkins et al.,, 2008). The specific site for chloroform binding is likely to be an amphipathic cavity on the alpha subunit of the $GABA_A$ receptor, as chloroform evoked Cl^- potentials have been abolished in mutants lacking this region (Jenkins et al., 2001). Other known anesthetics, such as isoflurane and halothane, as well as bromoform, another byproduct of water

chlorination that was found in the Brick Township municipal water supply, appear to bind to this region to exert their anesthetic action as well (Kash et al., 2001).

1.7.2 Bromoform

Bromoform is a nonflammable liquid at 25˚C, with a chloroform-like smell. It has a molecular weight of 252.8. It has moderate solubility in water, 3.2 g/L at 25˚C. Bromoform exposure generally occurs through exposure to contaminated drinking water, as it is a byproduct of the water chlorination process. Bromoform will be found in water normally, as plants release small amounts of this chemical naturally. However, chlorination of water that contains plant material leads to elevations of brominated byproducts in water (Richardson et al.,, 2003).

The principal route of human exposure to bromoform is by drinking contaminated drinking water (ATSDR, 1990). Bromoform is readily absorbed into the gastrointestinal tract (Mink et al., 1986) and is absorbed by the respiratory tract. It is distributed into various tissues, with highest levels being found in adipose tissues and the blood (Chu et al.,1982). Bromoform, like chloroform, is metabolized by the cytochrome P450 CYP2E1, with a brominated form of phosgene, carbon monoxide and carbon dioxide as the primary metabolites (Stevens and Anders, 1979; Ahmed et al.,1977). Bromoform and its metabolites are excreted primarily through the lungs, and to some extent in the urine (Mink et al.,1986).

In animals, it has been shown that the liver, kidneys, and central nervous system are the primary target organs for bromoform toxicity. Overdose causes central nervous system depression that leads to death (Chu et al., 1980). Unfortunately, there is no published evidence that bromoform is able to cross the placenta, or is present in breast milk. However, based upon data from related compounds, it is very likely that bromoform, like chloroform, will cross the placenta readily and ends up in milk.

As for the effect of bromoform on the nervous system, it has been proven that bromoform potentiates $GABA_A$ receptor-elicited Cl^- currents. Like chloroform, bromoform binds to a particular pocket on the alpha subunit of the $GABA_A$ receptor, which allows it to potentiate Cl^- currents (Kash et al., 2003).

1.7.3. Tetrachloroethylene

Tetrachloroethylene is a halogenated aliphatic hydrocarbon, which occurs as a colorless non-flammable liquid at 25˚C. The chemical formula for tetrachloroethylene is C_2Cl_4, and the molecular weight is 165.83. It is negligibly soluble in water, as 0.015% is soluble at 20˚C. It is primarily used as a solvent in industry and in commercial dry cleaning operations (ATSDR, 1993).

Absorption of tetrachloroethylene occurs through the respiratory tract (ATSDR, 1993), and is rapidly absorbed (Hake and Stewart, 1977). The gastrointestinal tract also absorbs Tetrachloroethylene, and only trace amounts are absorbed through the skin (Koppel et al., 1985). Tetrachloroethylene

accumulated in tissues with high lipid content, and the half-life is considered to be 55 hours (Stewart, 1969). Other sites of tetrachloroethylene storage include perirenal fat, brain, liver, placentofetal tissue and amniotic fluid (Savolainen et al., 1977). It also is found in the milk of mothers who are exposed to tetrachloroethylene (ATSDR, 1993). The first step in metabolism of this chemical is the conversion of tetrachloroethylene into an epoxide, which is thought to be responsible for the carcinogenicity of the chemical (Phillip et al., 2007).

Tetrachloroethylene is excreted mainly unchanged through exhalation, regardless of route of administration (NTP, 1986). Urine and feces comprise secondary routes of excretion (Ohtsuki et al., 1983). The major metabolite of tetrachloroethylene is trichloroacetic acid, which is formed via the cytochrome P450 system, namely CYP2E1, just like the previously mentioned THMs (ATSDR, 1993). Trichloroacetic acid is known to be a serious skin irritant, but has not been shown to have any neurological effects (ATSDR, 1997). Excretion of tetrachloroethylene does not appear to be different based on exposure route, but is different with varying levels of exposure. Increased exposure of the chemical leads to a reduction in production of metabolites (Daniel, 1963 Schumann et al., 1980).;

Tetrachloroethylene, unlike chloroform and bromoform, is most likely not a byproduct of water chlorination and instead is found in water supplies through leakage of underground stores of the toxic chemical or as runoff from waste (ATSDR, 1993). This is why the major route of exposure for tetrachloroethylene is inhalation exposure, whether it is from occupational hazards or from

recreational drug abuse, as it is common for teenagers to sniff whiteout, which contains tetrachloroethylene. Many studies show that inhalation exposure depresses the nervous system and has other serious consequences, and limited experiments have shown that exposure to tetrachloroethylene by oral means is equally as dangerous. Many of these studies were conducted because in tetrachloroethylene was once used as a de-worming agent in humans.

Tetrachloroethylene has no longer been used as an anesthetic after it was shown to be a potent carcinogen. It has been shown that tetrachloroethylene reduces calcium currents in vivo systems (Shafer et al., 2005), and potentiates GABA-elicited Cl^- currents (Briving et al., 1986). Due to its interactions with altering ionic currents of ion channels in the nervous system, it is not surprising that exposure to tetrachloroethylene has been found to induce irritability, impaired coordination, lightheadedness, headache, slurred speech, malaise, nausea, ataxia, sedation, coma and death. Sub-lethal central nervous system effects generally resolve quickly, but may be delayed due to fat uptake or by ingestion exposure.

1.8. THM and PCE metabolites

1.8.1 Dichloromethane

Dichloromethane is produced in the body following chloroform exposure as the result of the reductive pathway of chloroform metabolism. Although

dichloromethane is considered to be one of the less toxic THMs, it has been implicated in causing severe optic neuropathy (Kobayashi et al., 2008). It must be noted that the subject who was exposed to dichloromethane was treated with vitamin B12. In other work, chloroform is thought to inhibit the activity of vitamin B12, which leads to disruption of the MTHFR enzyme (Alston, 1991).

1.8.2. Phosgene

Phosgene is a chemical compound with the formula $COCl_2$. It was used as a chemical weapon during World War I, as it induces pulmonary edema after exposure to high concentrations. Information concerning phosgene via an oral route is not available, as the common route for phosgene exposure is through inhalation (US EPA, 1986). Formation of phosgene in the body occurs as the result of the oxidative metabolism of chloroform, however, phosgene levels resulting from such exposure have not been directly measured, perhaps due to the rapid reaction of phosgene with tissue molecules, as it is very electrophillic and undergoes attack by a variety of nucleophiles. It is known to covalently bond to enzymes, proteins and polar head groups of phospholipids, which may interfere with cellular function (US EPA, 1986). To date, the only known damage to the central nervous system that occurs due to phosgene exposure is phosgene-induced hypoxia, which may cause anoxic injury to the brain (Borak and Diller, 2001).

These chemicals are not known to interact with ion channels, but have been described because both are known to be highly reactive and may bond to various cellular components, including the polar head groups of phospholipids. It must be reiterated that at low doses, CYP2E1 is likely to metabolize these THMs and PCE into these chemicals (see references above), and at high doses, CYP2B1/2 becomes active, but is far less efficient at metabolizing these compounds, so they remain unmetabolized until excreted. Additionally, males have more active CYP2E1 than females, so they metabolize these THMs and PCE into dichloromethane and phosgene more efficiently than do females. This is of utmost importance in understanding possible mechanisms concerning behavioral results resulting from different exposure concentrations.

1.9 How can the question of the possible role THMs and PCE in the development of autism be answered?

Autism diagnosis is determined on the basis of psychological criteria. Therefore, in order to link THM/PCE as environmental toxins that may lead to an autistic phenotype, it must be proven that autistic-like behaviors are present after THM and PCE exposure.

Many behavioral tests for rodents that exploit the presence of autistic like behaviors are available (Crawley, 2004; Moy et al., 2007; Crawley, 2007; Moy et al., 2008). Therefore, rodents exposed to THMs and PCE, via their mother's drinking water during gestation and during weaning and subsequent direct

ingestion of such water in early development can be subjected to a battery of behavioral tasks relevant to autism, may be useful in determining if THMs and PCE are involved in the development of autism.

1.9.1 Ultrasonic Vocalization Tasks

The first behavioral criterion for autism diagnosis is the presence of impaired communication. In mice, ultrasonic vocalization analysis can be used to determine if there are deficits in communication. There are many different ways to use ultrasonic vocalization to assess communication, but the maternal separation task is most widely accepted when screening for deficits in communication (Jamain et al, 2008). The idea behind the maternal separation task is the fact that pups will call to their mothers if they are distressed, which usually occurs as a result of being separated from the mother and from their nest. If an animal has deficits in communication, it is thought that they will make fewer calls. However, the problem in this experimental design that makes interpretation of results difficult is the animal's perception of stress. If an animal is not stressed by being separated from its mother and nest, it may not vocalize. This may be indicative of maternal attachment disorder (Moles et al., 2004). Additionally, if the mother of a particular animal is not attentive, the animal may not vocalize to her, even if stressed, as they are habituated to receiving no maternal care in response to calls. If such a deficit in maternal care exists, the animal may appear as if it has deficits in communication.

In order to parse out the experimental confounds of the maternal separation task, and to determine if there is truly a deficit in communication, variant maternal separation tasks can be performed. One variant separation task involves the placement of a pup into a dry beaker that was submerged into a container of cold water. This task is done to determine if the animal has vocal ability. The idea here is that the animal would be highly stressed by the cold, and should vocalize if capable. If the animal could vocalize, reasons for deficits in vocalization could not be attributed to crude abnormalities in structures necessary for vocalization. Additionally, and more importantly, cold exposure reveals an animal's stress response. If an animal performs comparably on the maternal behavior task and cold exposure task, it shows that the stress of maternal separation is comparable to the stress of being cold. If an animal were to be considered as having deficits in communication, ideally, it would make fewer calls than control animals on both of these tasks. If the animal makes more vocalizations during cold exposure, it shows that the animal perceives cold exposure as being more stressful than maternal separation, which shows that any deficits in communication may be the result of a maternal attachment deficit disorder, and not necessarily a deficit in communication (Sternberg et al., 2005).

A more benign variation of the cold test can also be done to rule out sensitivity to cold, and to see if other socially relevant stimuli could induce changes in communication. This test involves separation from the mother and nest, and instead of exposure to cold, animals are exposed to soiled stranger male bedding. The idea behind this variation of the maternal separation task is

to determine if the animal will communicate to its mother in response to a social stressor, rather than a physical stressor. If an animal responds with an increased number of calls in response to this task, it shows that the animal does not necessarily have a deficit in communication, and may instead have social anxiety (Moles et al., 2004). It is thought that pups could identify stranger scent by P4 (Hoefer et al., 2002).

Finally, in an attempt to determine if any changes in communication are truly communication deficits, a maternal retrieval test can be done on all mothers of pups who were subjected to any of these vocalization tasks. Since the mothers themselves are exposed to THM/PCE water, which was necessary to expose the developing pups, it is possible that the THM/PCE water could have had some effect in the mother that altered maternal behaviors.

Even with trying to parse out confounds of vocalization testing, communication in mice is still very difficult to determine. Perception of stress can confound these results. On a positive note, pups that are not stressed by maternal separation may have social deficits, which is another criterion necessary for autism diagnosis.

Figure 1: The ultrasonic vocalization apparatus. Animals are placed into a sound proof chamber with an ultrasonic frequency detector above. The number of vocalizations made by the animal is sent to a computer equipped with Ultravox software, which allows for quantitation of the number of vocalizations emitted by the animal.

1.9.2 Anxiety testing

Autism is often accompanied by mood disorders, including anxiety and stress, depression and obsessive-compulsive behaviors (Howard et al., 2000; Boddaert et al., 2004). Studies show that 84% of autistic children meet criteria for an anxiety disorder (Boddaert and Zilbovicius, 2002). Presence of anxiety disorders varies with the ASD subtype, as those with Asperger's disorder exceed those with PDD-NOS, and both Asperger's disorder and PDD-NOS exceed those with autistic disorder (Welchew et al, 2005). In histological studies, the amygdala, which is critical in the regulation of anxiety behaviors, exhibits increased neuronal packing density, which is indicative of structural changes that

may result in changes in behavior like those with autism (Kemper and Bauman, 2002).

Therefore to determine if THM/PCE exposure induces anxiety, the elevated plus maze can be used. The elevated plus maze rests on the naturalistic conflict between the tendency for mice to explore a novel environment and the aversive properties of an open area (Gonzalez and File, 1997). The elevated plus maze is regarded as a behavioral task that is relevant to autism (Crawley, 2004; Crawley 2007), and is frequently used in studies that attempt to determine the validity of autism animal models (Moy et al., 2007).

The elevated plus maze consists of an elevated cross shaped maze, in which two arms of the maze are closed off by clear Plexiglas and two arms remain open. An animal is placed on the maze, facing an open platform, and is left there for 5 minutes to explore. Mice tend to be exploratory creatures, therefore it is expected that they should explore all four arms of the maze. Animals that have higher levels of anxiety, tend to spend less time exploring the open arms of the maze and make fewer entries onto the open arms, as anxiety sequesters their tendencies for exploration as they are more fearful of being on an open, high platform. The reverse is true for animals with lower levels of anxiety. These animals will spend more time exploring the open arms of the platform and make more entries onto the open arms.

Figure 2: The elevated plus maze. The elevated plus maze is used to detect anxiety in mice. Animals who spend more time in the closed arms or the maze are more anxious than animals that spend more time on the open arms of the maze.

1.9.3 Stereopathy and perseverance behavior

Another behavioral pathology that is necessary for a patient to receive an autism diagnosis is the presence of stereotypical behaviors or insistence on sameness (DSM IV-TR, 2000). This behavior is often referred to as perseverance behavior. Since the question at hand is to determine if exposure to THM/PCE contributes to the development of autistic behavioral phenotypes, the water-T- maze, a rodent behavioral test used to test for the presence of perseverance behavior, was used to accomplish the aim of this investigation. In order to utilize the water-T-maze to investigate the presence of perseverance behavior, an animal is trained to swim to a submerged platform in one arm of the T. The animal is trained daily until it reaches learning criteria, or proves that it has learned a routine. Once it learns the routine, the submerged platform is switched to the other arm of the T, and the number of times that the animal

attempts to go to the original location is recorded. Animals that perform poorly when the platform is switched are thought to have perseverance behavior, as they prove that they rigidly adhere to the previous routine, rather than evidencing flexibility in learning the new location of the platform.

Learning ability can also assessed in these animals, as the number of days needed to reach learning criteria is indicative of an animal's spatial learning ability. This could therefore used to determine if differences in learning are different between control and THM/PCE treated groups.

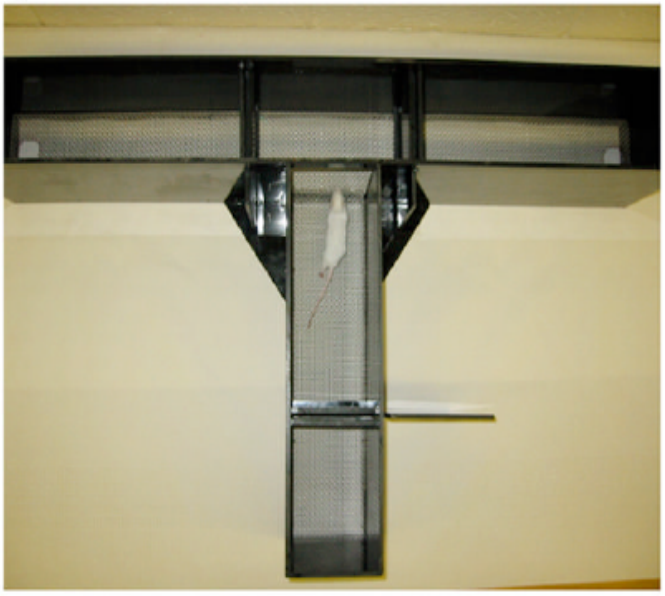


Figure 3: The water-T-maze. The water-T-maze can be used to assess spatial learning behavior as well as perseverance behavior. Animals are placed at the bottom of the T and are expected to find a submerged platform in one of the arms of the T to facilitate their escape. Once an animal learns the task, the submerged platform is placed in the opposite arm of the T, to determine if perseverance behavior exists.

1.9.4 Social Behavior

The third and final criterion for a person to be diagnosed with autism is the presence of impairments in socialization (DSM IV-TR, 2000). If THM/PCE exposure is to be considered a possible causal agent in the development of

autism, animals exposed to THM/PCE must be assessed for social ability. The test for assessing social ability in rodents is the three-chambered social box apparatus, which allows the experimenter to observe how the animal behaves in response to the introduction of a novel animal.

In this test, an animal is allowed to habituate to a three-chambered apparatus, which contains an empty container in two chambers that flank a center empty chamber. Once habituation is complete, a novel gender matched animal is placed in one of the containers in the flanking side chambers. If the animal under investigation is social, it should spend more time in the chamber of the novel animal, make more entries into that chamber and spend time sniffing the novel animal. An animal that is not social will try to avoid the novel animal, which is evidenced by a decrease in the amount of time spent in the chamber of the novel animal, as well as fewer entries into the chamber and less sniffing interactions.

Social novelty can also be assessed using this apparatus. This can be tested by placing a second novel animal in the empty chamber, while leaving the original novel animal in its original location. If the animal under investigation spends more time in the chamber of the second novel animal and sniffs the second novel animal more than the original, it is thought that the experimental animal has a proclivity for social novelty.

In this experiment, the THM/PCE treated animals were tested for social behavior and social novelty behavior. If the THM/PCE treated animals are found

to be less social in response to treatment, they display another characteristic behavior present in autism.

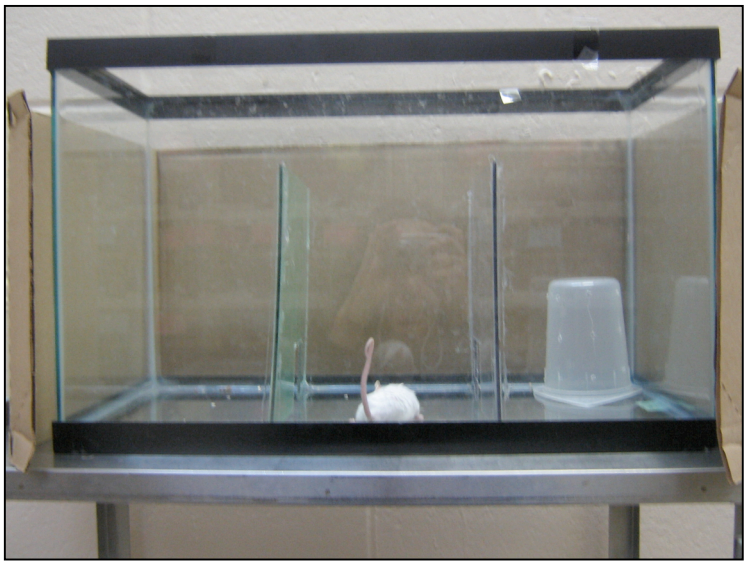


Figure 4: The social behavior apparatus. Social behavior can be examined with use of the social behavior apparatus. An animal that spends more time in the chamber of a novel animal is thought to be more social than an animal that does not spend time in the chamber of the novel animal. A test of social novelty can also be done by placing another animal in the opposite chamber, and studying the amount of time spent by the animal under investigation with the original novel animal or the "new" novel animal.

1.10 The effect of THM/PCE on signal transduction in developing neurons: The Kreiling Report

In response to the ATSDR investigation, a team from Woods Hole, MA investigated the effects of these chemicals on neurodevelopment of clam embryos (Kreiling et al., 2005). In this investigation, clam embryos were exposed to varying concentrations of the chemical triad, which included a 10, 100 and 1000 times the maximum observed concentrations of chloroform, bromoform and PCE found by the ATSDR in Brick Township. Embryos were exposed to the chemical triad, pairs of chemicals, and individual comp

onents three to four hours post fertilization and remained on treatment until analysis at 24, 48, 72 and 96 h post fertilization time points.

Levels of cAMP dependent protein kinase A (PKA) were tested in these embryos at the time points and exposure levels noted above, using measurements of fluorescence intensity observed under confocal microscopy. The antibody used for detection was to the RII isoform of the regulatory subunit of PKA, which remains detached from the catalytic subunits when PKA is activated. Therefore, increases in the level of free RII subunit reflect the amount of free active catalytic subunit. Results from these experiments show that there are no changes in RII levels at 24 hours in any combination or concentration of chemicals, but at 72 and 96 hours the 100 and 1000 fold concentrations of the ternary mixture evidences a statistically significant and equivalent increase in free RII in comparison to controls in neurons of the embryo innervating the gill and ciliated velar epithelial regions. Also, addition of the chemicals after onset of neurodevelopment resulted in no observable changes. These observations were confirmed using SDS-PAGE and Western blotting. Embryos treated with the ternary mixture showed enhanced RII labeling over controls at the 96 and 120-hour time points. However, data concerning PKA activity of the treated embryos showed no significant difference with use of the Promega non-radioactive PKA assay kit. It was concluded by Kreiling that this was due changes in PKA activity that were specific to neurons, and no global changes in PKA activity.

In addition to the RII investigation, cilliary beat frequency and swimming behavior was examined. Embryos exposed to the 100 or 1000 fold ternary

mixture evidenced an early hyperkinetic effect that was significantly different from control embryos at 96 and 110 hours post fertilization. There were no observable differences in kinetic activity between control and treated embryos ay 120 hours. Since cilliary movement is controlled by the serotonergic and dopaminergic systems, investigation of levels of serotonin receptor (5HT1a), the dopamine 2 receptor (D2), serotonin, and tyrosine hydroxylase were examined using western blotting and confocal microscopy. No significant differences were noted between control and treatment groups. The results of these data show that the chemical triad does have an effect on PKA that is specific to neurons during neurodevelopment. It must be noted that the concentrations used to induce such differences are drastically higher than the chemical concentrations that were found in Brick. However, there is no bioaccumulation of these chemical compounds in aquatic organisms, which may account for the reason why only high concentrations resulted in an observable effect (McCulloch et al., 2003).

1.11 PKA and its role in neurodevelopment

Abnormalities of PKA activity may lead to aberrant neurodevelopment, as it is a key regulator of cellular mechanisms fundamental to establishment of proper neuronal connectivity and communication at synapses.

1.11.1 PKA

PKA is a second messenger dependent enzyme that has been implicated in a wide variety of cellular processes. PKA is a tetrameric enzyme that contains two regulatory subunits (R) and two catalytic subunits (C). The R subunits occur in two forms, RI and RII, and each of the R units can occur as an alpha or beta isoform. There are three isoforms of the C subunits, which are alpha, beta and gamma. When in the holoenzyme configuration, the C subunits remain bound to homo- or heterodimers of either RI or RII subunits. The two C subunits do not interact with each other. Additionally, when in this configuration, the enzyme possesses no catalytic activity. However, cooperative binding of two molecules of cAMP bind to two sites on each subunit results in a conformational shift that releases the bound catalytic subunits. The C subunit will transfer the gamma phosphate from ATP to a variety of protein substrates that contain the consensus sequence K/R-R-X-S/TY and less stringently R-X-X-S/TY, where Y tends to be a hydrophobic residue (Park et al., 2008).

PKA is classified as a Type I or Type II enzyme depending on the associated R subunit. Type I PKA is located primarily in the cytoplasm and Type II is associated with cellular structures by means of A Kinase Binding Proteins (AKAPs), which prevents indiscriminate phosphorylation of residues. Anchored PKA modulates the activity of many cellular proteins, such as AMPA/Kainate channels and glutamate receptor gated ion channels (Appert-Collin et al., 2006).

1.11.2. PKA activation

The binding of transmitter to receptors linked to the cAMP cascade leads to a conformational shift in the receptor that allows binding of a trimeric G protein, which is made of Ga, Gb and Gg subunits. Ga normally has a GDP bound to it at rest. Upon association with a receptor, Ga exchanges GDP for GTP, thereby allowing for disassociation of the Ga subunit from the trimeric complex and exposing a binding site for adenylate cyclase on the alpha subunit. The alpha subunit then stimulates adenylate cyclase, an integral membrane protein, which catalyzes the conversion of ATP to cAMP. Ga also acts as a GTPase, and hydrolyzes its GTP to GDP. As a result adenylate cyclase will stop conversion of ATP to cAMP. In the continued presence of transmitter, the cycle is able to begin all over again (Nugent et al., 2009).

1.11.3 PKA and Brain Derived Neurotrophic Factor

Once PKA can recruit Mitogen Activated Kinase (MAPK), and together they can translocate to the nucleus. A target for PKA phosphorylation is CREB-1, on the serine 133 residue. PKA cannot phosphorylate this transcription factor until CREB-2, which is bound to CREB-1, is removed. This is accomplished by MAPK. Once this occurs, phosphorylation of CREB-1 on the serine 133 residue ensues. CREB-1 will then bind to cAMP response elements (CRE), which are located upstream of two types of cAMP-inducible genes. One gene activated by

CREB encodes ubiquitin hydroxylase, a component of a specific ubiquitin protease that leads to the regulated proteolysis of the regulatory subunit of PKA, resulting in persistent PKA activity. The other gene activated by CREB encodes another transcription factor C/EBP. C/EBP binds to the DNA response element CAAT, which encodes a variety of genes that are important for synaptic connectivity. One such gene is that which encodes Brain Derived Neurotrophic Factor (BDNF). BDNF will be secreted from cells and will activate the TrkB receptor on nearby cells. Activation of TrkB by BDNF is thought to cause neuronal processes to increase contact with BDNF-releasing neurons, resulting in enhanced synaptic connectivity (Shelton et al., 2007).

1.11.4. PKA and Netrin Receptors

It has been proven that PKA potentiates the insertion of the Deleted in Colorectal Cancer (DCC) receptor into the axonal growth cone membrane (Bouchard et al., 2004). The DCC receptor uses the diffusible guidance cue, Netrin-1 as a ligand. Depending on the dimerization state, DCC may serve to cause filipodia formation or filipodia retraction. It appears that if DCC is dimerized with the UNC5 receptor, the axon will exhibit a chemorepulsive response to Netrin-1. If DCC remains alone, it will result in an axon that displays a chemoattractive response to Netrin-1. Therefore, changes in PKA activity effect the levels of receptor that are expressed on the axonal membrane. Enhanced PKA activity will result in a higher number of free DCC receptors on

the axonal membrane, resulting in a chemoattractive response, and attenuated PKA activity will result in less DCC receptors on the axonal membrane, which can result in an increased prevalence of dimerized receptors, thus causing a chemorepulsive response to Netrin-1.

Additional data shows that neurons with elevations of cAMP will show a chemoattractive response to netrins, via activation of the Rho-like GTPases Cdc42 and Rac1, which ultimately activate N-Waap and Pak, resulting in filipodia formation. Data also shows that low levels of cAMP result in the activation of RhoA, which in turn activates collapsen and causes the retraction of filipodia (Shekarabi et al., 2005).

1.12 Are the effects of THM/PCE exposure unique to clam embryos, or do they have the same effect in more complex models?

Kreiling found the regulatory subunit of PKA, specifically the regulatory II isoform (RII), to be significantly elevated in the neurons of developing clam embryos. An increase of RII subunit is thought to be directly proportional to the level of free catalytic subunit. Since free catalytic subunit is unbound, or not found in the quiescent holoenzyme PKA complex, it has the potential to phosphorylate a variety of cellular intermediates, some of which are responsible for events critical to proper neurodevelopment.

1.12.1 THM/PCE in zebrafish

Since zebrafish undergo external development, it is possible to expose them to THMs and PCE directly. If they do undergo the same changes that were observed in the clam embryos, Kreiling's results can be replicated in a more complex system, which provides evidence that the observed effect translate across different species. Therefore, to replicate Kreiling's study, and to expand upon her findings, determination of the effect of THM/PCE exposure on the level of catalytically active PKA, as well as proteins that are phosphorylated by active PKA activity, such as CREB can be done. Additionally BDNF expression could be assessed, as BDNF expression increases in response to the level of phosphorylated CREB (pCREB). Increased levels of pCREB and increased expression of BDNF are indicative of heightened PKA activity.

Unlike Kreiling's experiments, electron microscopy (EM) can be used instead of confocal microscopy, which was used in Kreiling's work. EM with immunogold labeling of protein provides a more detailed and specific localization of proteins in the tissues, by counting labeled proteins of interest in a randomly determined area. This approach was selected over luminescence intensity measurements, because standardization between samples is difficult, as many variables can affect fluorescence intensity. The disadvantage to using EM is the time spent on specimen preparation protocols.

1.12.2 Activity of PKA

To confirm changes found in PKA levels translate to activity, cell cultures could be used, as they can be directly exposed to THM/PCE, and PKA activity can be examined. The PKA assay could not be accomplished on the larval zebrafish brains, because it is almost impossible to dissect the brain, which is approximately 200 um, from such tiny organisms. Additionally, even if dissection were possible, the amount of synchronous material resulting from such dissections would be sparse. Therefore, an easy to maintain immortalized murine microglia cell culture can be used to directly test the activity of PKA before proceeding to a more complex mouse model.

1.12.3 Activity of PKA in the mouse brain

To determine if THM/PCE induces changes in PKA activity in mammals, which undergo internal development, unlike clams and zebrafish, PKA activity assays can be done in the brain tissue of animals that are exposed to THMs and PCE.

Developing animals can be exposed to THM/PCE throughout the entire duration of development by addition of THM/PCE to the mother's drinking water after onset of breeding. Exposure through drinking water is the least benign route of THM/PCE exposure, as dermal exposure or inhalation exposure, lead to much higher levels of THM/PCE in the bloodstream (Levesque et al., 1994; Jo et

al., 2005). Additionally, exposing the animals to THM/PCE via drinking water seems to be the best method to deliver the chemical dose, since wetting the mother or placing her in a humidifying chamber for the dermal or inhalation methods of exposure would have caused a great deal of distress, which could cause detrimental effects to the offspring that would confound results of the experiments.

1.13 Would THM/PCE treatment cause different effects in males and females that could correlate to the preponderance of males with autism?

In the general population of those with normal function affected with ASDs, the ratio of males to females is 4:1. The reason for this gender bias in males has not yet been found, as the mechanisms that result in ASD pathology still remain very unclear. However, such differences are likely to be the result of intrinsic differences in development that are found between males and females.

Estradiol is the most biologically prevalent and active compound of the estrogens, as it exerts potent effects on the developing brain (McEwen, 1987). Experimentation has proven unequivocally that estradiol and the activity of P-450 aromatase as well as estrogen receptors are at their highest levels in the brain both prenatally and during the first few weeks of life. They ultimately diminish and decline to adult levels (Vito et al., 1985; Lephart, 1997).

Estradiol is derived from testosterone following aromatization of the A ring via p-450. It has been shown that newborn males have 2-3 times more estradiol

in the hypothalamus (Amateau et al., 2004). However, in regions outside the hypothalamus, this does not apply, and it has been shown that the brain is capable of making its own estradiol de novo from cholesterol (Hojo et al., 2004; Schlinger and Arnold, 1992). However, available estradiol in the brain is greater in males.

Higher levels of estradiol in the male brain are important, especially regarding the action of estradiol on GABAergic neurotransmission. GABA was once considered the primary source of inhibitory neurotransmission, but is now known to the principle source of excitation via depolarization induced calcium influx through voltage gated calcium channels. This is predominant during development, and appears to be present throughout the brain. The excitatory effects of GABA are mediated through the $GABA_A$ receptor, and the relative transmembrane Cl^- gradient. Whether $GABA_A$ elicits an inward or outward Cl^- current is dependent upon the Cl^- concentration gradient, which is in turn determined by Cl^- cotransporters (Delpire, 2000). During the neonatal period, the reverse potential (Ecl) is positive relative to the resting membrane potential (Barna et al., 2001), resulting in a net outward driving force on Cl^- when $GABA_A$ receptors are activated. This results in sufficient depolarization that allows for the opening of L-type Ca^{2+} channels (Leinekugel et al., 1995). As development progressed, the Ecl becomes negative with respect to the resting membrane potential, resulting in a net inward driving force on Cl^-, which induces the classic GABAergic hyperpolarization, which is the primary source of inhibition in the mature brain.

This developmental shift is controlled by changes in Cl^- cotransporter expression or activity, namely from the NKCC1 and KCC2, along with Cl^- channels such as ClC2 (Schwartz-Bloom et al., 2001). NKCC1 promotes Cl^- transport into the cell along with Na+ and K+. Expression is high in the neonatal brain, but declines with age. KCC promotes Cl^- efflux from the cell. This transporter has low expression in the neonate, but it increases as development proceeds. By the end of the second week, KCC expression exceeds that of NKCC1 (Plotkin et al., 1997a; Plotkin et al., 1997b) causing the developmental shift from GABA-mediated depolarization to GABA-mediated hyperpolarization.

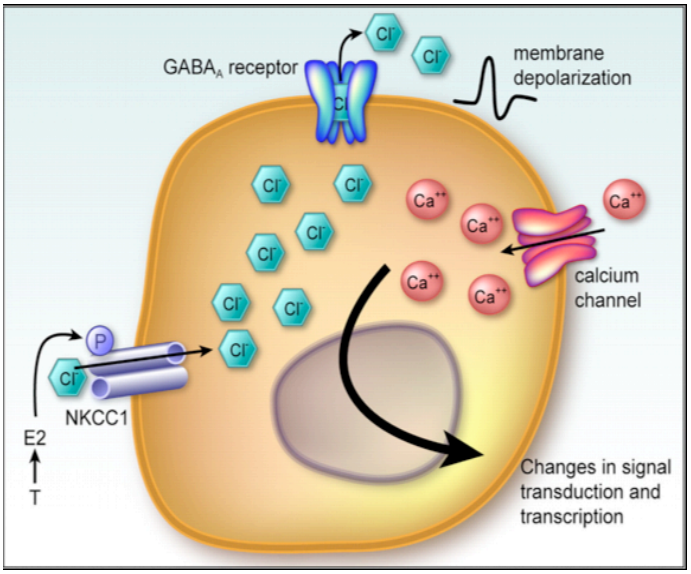


Figure 5: GABA$_A$ mediated membrane depolarization. Estradiol increases the expression and activity of NKCC1 that maintains high intracellular Cl-, which allows GABA to induce membrane depolarization. Membrane depolarization induced by GABA is sufficient to activate voltage-gated calcium channels and promote calcium influx. NKCC1 transporter is expressed at high levels in developing neurons but gradually declines as development proceeds and is superceded by another transporter, KCC2, which transports Cl- out of the cell. Once this shift occurs, activation of the GABA$_A$ receptor induces membrane hyperpolarization.

It has been demonstrated that the hyperpolarizing GABA evoked potentials appear earlier in the female than they do in the male. Specifically, female rats show evidence of hyperpolarizing current beginning at P4, whereas

males evidence hyperpolarization at P14 (Galanopoulou, 2008). This fact will later prove to be of utmost importance in later discussions.

Estradiol, which is elevated in males, enhances the depolarizing action of GABA in developing hypothalamic neurons by increasing the magnitude of the Ca^{2+} transient with each depolarization, increasing the number of neurons that respond to $GABA_A$ receptor activation with a calcium transient, and extending the developmental duration of depolarizing GABA action (Perrot-Sinal et al., 2007). Similar findings of estradiol enhancement of depolarizing GABA have been made for hippocampal neurons (Nunez et al., 2005). In both cases, estradiol appears to upregulate the activity of NKCC1, thus maintaining a favorable depolarizing gradient upon activation by GABA. Estradiol has also been shown to down-regulate KCC2, which sends Cl^- out of cells, which again helps to maintain a favorable depolarization gradient.

Additionally, it has been demonstrated that there is a higher level of pCREB in the ER-rich regions of the male neonatal rat brain. There is also a dramatic difference in the induction of pCREB following depolarizing GABA activity on the day of birth (Auger et al., 2001a; Auger et al., 2001b). Administration of the $GABA_A$ agonist muscimol to neonatal males induces a massive induction of pCREB in response to the influx of Ca^{2+} via the voltage gated Ca^{2+} L-type channels. Female littermates, exhibited the exact opposite, showing that a divergence in signal transduction pathways occurs in response to the same treatments which outcome is gender-specific.

1.14 Summary

THMs and PCE were found in an area of high autism prevalence and was been proven to increase the level of catalytically active PKA during early development. Increased activity of PKA can potentially cause aberrant neural development, some of which may lead to pathological manifestations found in autism. Therefore, it seems that a direct investigation of THM/PCE on behaviors found to be pathological in autism is needed to determine if these chemicals are involved in the high prevalence rate found in Brick.

2. Specific Aims

THMs and PCE were found in an area of high autism prevalence and was been proven to increase the level of catalytically active PKA during early development. Increased activity of PKA can potentially cause aberrant neural development, some of which may lead to pathological manifestations found in autism. Based upon these findings we hypothesize that THM/PCE exposure induces changes in key regulators of neurodevelopment and behavioral pathology similar to that, which is found in autism.

To test out hypothesis we propose to:

1. Determine if THM/PCE exposure induces a change in the level of catalytically active PKA in a different model, the zebra fish
2. Determine if THM/PCE exposure induces a change in the activity of PKA in vitro and in vivo
2. Determine if THM/PCE exposure induces a behavioral phenotype that is similar to that which is found in autism

3. Materials and Methods

3.1 Zebrafish immunohistochemical experiments

3.1.1 Housing of zebrafish

One hundred adult zebrafish (~90 days old) were housed in a ten-gallon tank of water containing 0.6 g of instant ocean salt at 28 ºC. Zebrafish were fed Tetra brand food twice daily and kept on a 14-hour light to ten-hour dark cycle.

3.1.2 Breeding of zebrafish

A breeding container, which was six inches long, 4 inches wide and 4 inches tall, was suspended by a magnet and placed in the corner of the housing tank. A 4-inch by 2-inch rectangular hole on the breeding container allowed for entry of zebrafish into the breeding container. A grate was placed at the bottom of the breeding container, so that eggs and embryos could pass through and be trapped underneath. The grate was necessary to prevent the zebrafish from eating their embryos. Above the grate were artificial aquatic plants, which provided a place for the animals to hide while breeding. Animals were fed three times a day before onset of breeding. The breeding tank was left in the housing tank overnight during the dark cycle. Embryos were collected within 1 hour of returning to the light cycle.

3.1.3 Chemical treatment of zebrafish

Embryos were removed and checked for viability under a light microscope and were subsequently placed in one of four 250 mL beakers. The control beaker contained 100 mL of egg water (60 ug of Instant Ocean salts per mL of tap water). The second beaker contained 100 mL of 10x THM/PCE concentration in egg water. The third beaker contained 100 mL of 100x THM/PCE concentration in egg water. The fourth beaker contained 100 mL of 1000x THM/PCE concentration in egg water. Beakers containing the embryos were then placed in a 28ºC water bath and allowed to incubate until harvesting at specific time points. Water was changed daily to prevent fungal accumulation.

PKA catalytic subunit, pCREB and BDNF were examined at day 7 of development. A total of 12 animals were used in these experiments. Three animals were used in the control group, three animals were used in the 10x group, three animals were used in the 100x group and three animals were used in the 1000x group. PKA levels were also examined in preliminary experiments by labeling the RII subunit, as was done in the Kreiling experiments. RII subunit was examined at three time points of development: days 5, 8 and 14. For each time point, a total of four animals were used. These groups consisted of a control animal, a 10x THM/PCE treated animal, a 100x THM/PCE animal and a 1000x THM/PCE treated animal. Immunogold labeled proteins were counted in five different neurons.

3.1.4 Preparation of zebrafish brain for EM

Larval zebrafish were removed from their beaker and placed in 0.1M PBS containing 1% Paraformaldehyde/1% Glutaraldehyde fixative for 2 hours. Fixed specimens were washed in 0.1M PBS for 30 min and placed in 1% Osmium Tetroxide for 90 minutes. Specimens were dehydrated in 50% ethanol for 20 minutes, followed by 70% ethanol for 20 minutes. Specimens were cleared of alcohol with a 1:1 mixture of London Resin White (medium grade) and 100% ethanol for 60 minutes. Specimens were placed in the center of a gelatin capsule, with heads facing down, and were embedded in pure London Resin White. The resin polymerized for 3 days at 54 ºC. Gelatin capsules were removed by soaking in warm water while spinning on a stir plate. Following embedding, 65 nm coronal sections of the head were made using a Sorvall 5000 Ultramicrotome equipped with a 2.4 mm Diatome diamond knife. Sections were placed on 200 mesh nickel grids after being stretched with chloroform vapors.

3.1.5 Immunogold labeling of zebrafish brain for EM

Saturated sodium metaperiodate in H_2O was centrifuged at 14000 rpm in a microcentrifuge and supernatant was placed on each of the grids for 10 minutes. Grids were rinsed in Millipore filtered H_2O and placed in a blocking solution containing 20% Normal Goat Serum (NGS) in 0.1 M PBS for 30 minutes. Grids were incubated in a blocking solution, which contained primary antibody for

either PKA catalytic subunit (1:50), pCREB (1:50) or BDNF (1:100) for day 7 studies or PKA RII subunit (1:20) for days 5, 8 and 14 studies. All primary antibodies used were polyclonal and raised in rabbit. After incubation in primary antibody, each grid was rinsed in 0.1M PBS four times for 5 minutes. Grids were then incubated in a secondary antibody solution, which contained 0.1 M PBS and a 1:50 dilution of Goat anti Rabbit antibody, which was bound to a 10 nm gold particle. Following incubation in secondary antibody, grids were jet washed with blocking solution three times and immersed in the blocking solution three times for 5 minutes. Grids were then stained with lead acetate and jet washed with H_2O. Specimens were examined on a Hitachi 7500 Transmission Electron Microscope operated at 75 kV, equipped with an Advanced Microscopical Technology (AMT) digital camera. Images were analyzed using Image J software.

3.1.6 Zebrafish study result analysis

In the day 7 studies of catalytic PKA and pCREB levels and BDNF expression, the number of particles per square micron in neurons was counted. Regions were selected randomly. For days 5, 8 and 14 studies, the numbers of particles found in entire neurons were counted. Data was analyzed for significant differences using Kruskal-Wallis non-parametric ANOVA, as the sample size was too small to used ordinary ANOVA, on GraphPad Instat software. Dunn's post

hoc analysis was done in cases where significant differences were noted between groups. Results were considered significant if $p < 0.05$.

3.2 PKA activity in microglia

3.2.1 Microglia cell culture conditions

A Microglia cell culture (C8-B4) was allowed to become confluent in a 75 mL flask, containing 20 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS with 1% Penstrep, and was kept in an incubator at 37 ºC in 5% CO_2. Once confluent, media was removed and 2 ml of 0.25% trypsin was added to the flask, and drawn off after 2 minutes. 3 ml of 0.25% trypsin was added to the flask, and the flask was placed in a 37ºC incubator for 7 minutes. Once cells were no longer adherent, 10 ml of DMEM was added to the flask, drawn off and then placed in a 15ml centrifuge tube. Cells were centrifuged for 5 minutes at 800 RPM. Supernatant was drawn off and the pellet was resuspended in 3 ml of DMEM supplemented with 10% FBS and 1% penstrep. ~430 ul aliquots of suspended cells were added to seven new 75 ml cell culture flasks, which contained 20 ml of the DMEM/10% FBS/1% penstrep media. Flasks were left in the incubator at 37 ºC in 5% CO_2 for 3 days.

After 3 days, the media was removed and seven experimental conditions were set. In flask one, media was replaced. In flask two, the media was supplemented with 0.001% DMSO. In flask three, 10 nm of forskolin suspended

in 0.001% DMSO was added to the media. In flask four, 1X THM/PCE suspended in 0.001% DMSO was added to the media. In flask five, 10x THM//PCE suspended in 0.001% DMSO was added to the media. In flask six, 100x THM/PCE suspended in 0.001% DMSO was added to the media. In flask seven, 1000x THM/PCE suspended in 0.001% DMSO was added to the media. Cells were incubated for 48 hours under all experimental conditions. The experiment was carried out in triplicate.

3.2.2 PKA activity assay

After incubation, media was removed, and cells removed from the flask using the trypsinization protocol previously described. The pellet was washed in 4 ºC, 0.1M PBS and was suspended in 300 ul of PKA extraction buffer (25mM Tris-HCl [ph 7.4], 0.5 mM EDTA, 0.5 mM EGTA, 10mM B-mercapatoethanol, 1ug/mL leupeptin and 1 ug/ml aprotinin). Cells were homogenized with an electric homogenizer on ice. Lysate was centrifuged for 5 minutes at 4 ºC at 14,000 rpm in a microcentrifuge. A Bradford protein assay was done using the lysate. ~9 ul of supernatant per sample, which contained ~10ug of protein, was added to 5ul of Peptag PKA reaction buffer, 5 ul PKA activator 5x solution, 1 ul of peptide protection solution and placed on ice. Samples were then placed in a 30°C water bath for 1 min. 5ul Peptag A1 peptide was added to each sample and then was allowed to incubate for 30 minutes at room temperature. After incubation, the samples were placed in a 95ºC water bath for 10 minutes to stop

the reaction.1 ul of 80% glycerol was added to the samples and 15 ul of each sample was loaded into a 0.8% agarose gel prepared with 50 mM Tris-HCl, ph 7.4. The gel was run for 18 minutes at 100V and viewed after placement onto an UV transilluminator. Pictures of each gel were taken using a Polaroid GelCam.

3.2.3 Microglia activity assay result analysis

Pixel intensity of phosphorylated peptide in each lane was measured using Image J software. The average pixel intensity for each treatment group was compared for significant differences using Kruskal-Wallis non-parametric ANOVA. Dunn's multiple comparison test was used to determine significant differences between groups. Results were considered to be significant if $p < 0.05$. All statistical analyses were accomplished using GraphPad Instat software.

3.3 General methods for experiments using mice

3.3.1 Housing of mice

CD-1 mice were purchased from Charles River Laboratories. Animals were provided with Purina chow and tap water ad libitum. Tap water used contained no PCE and less than 60 ppb THMs. The housing room was maintained at 23°C on a 12-h light/dark cycle (lights off at 7 PM). All procedures

were conducted in compliance with protocol 364 approved by the Institute for Basic Research Institutional Animal Care and Use Committee (IBR IACUC).

3.3.2 Breeding of CD-1 mice

Six 50-day old non-sibling female CD-1 mice were placed in individual breeding cages. A non-sibling 50-day old male CD-1 mouse was placed in each of the breeding cages with the female to generate enough animals for experiments. Twenty-four female offspring (four siblings from each mother) were then placed in their own breeding cages. Twenty-four males were then placed in each of the breeding cages (four male siblings from each mother). Breeding pairs were all non-sibling pairs.

3.3.3 Chemical exposure groups

Six of the 24 breeding pairs were used as a control group, and were referred to as Control a-f. They were given tap water without THM/PCE added. Concentration of total THMs in the tap water were below 60 ppb, and no PCE was found (NYS DEP, 2008). Six of the twenty-four breeding pairs were given the exact concentration of the Brick Township chemical triad in their drinking water. These groups were referred to as the 1x a-f groups. Six of the twenty-four breeding pairs were given a ten-fold concentration of the Brick Township chemical triad in their drinking water. These groups were referred to as the 10x

a-f groups. Six of the 24 breeding pairs were given a 100-fold concentration of the Brick Township chemical triad in their drinking water. These groups were referred to as the 100x a-f groups. This process of exposure from onset of breeding to time of a particular study was repeated after initial postnatal (P) day 11 vocalization studies, P21 anxiety studies, P60 perseverance studies and P85 socialization studies, as experiments new experiments were conducted at different time points. Additionally, 1x THM/PCE treatment was not repeated in post preliminary studies, as results from this group in preliminary studies were not significant.

3.3.4 Chemical treatment

Animals in the 1x THM/PCE treatment groups were given tap water to which 200 ng/L chloroform, 6 ng/L PCE and 5 ng/L of bromoform were added at onset of breeding. Tap water had less than 60 ppb of all trihalomethanes (NYC DEP, 2008). Animals in the 10x THM/PCE treatment groups were given water containing 2.51 ug/L chloroform, 60 ng/L PCE and 50 ng/L bromoform at onset of breeding. Animals in the 100x THM/PCE group were given water containing 25.1 ug/L chloroform, 500ng/L PCE and 500 ng/L bromoform. A 10^6 fold stock of these chemicals was made using ethanol as a co-solvent for mixing with water. Final concentration of ethanol in the 100x THM/PCE group was 0.000001%, which is well below the concentration of ethanol needed to induce any neurological effects. The stock solution was kept covered to prevent conversion

of chloroform into phosgene by light. The stock was diluted by addition of 500 ul of stock to 5 L of water for the 100x THM/PCE group. For the 10x THM/PCE exposure group, 50 ul of stock was diluted in 5 L of standard tap water. For the 1x THM/PCE exposure group, 5 ul of stock was diluted into 5 L of standard tap water. Water was distributed into the drinking water bottles of all animals from the appropriate 5L dilution stock. Cautions were taken to ensure that THM/PCE was completely dissolved in water, as it was usually left standing for 12 hours to ensure that THM/PCE did not form a precipitate. Water was changed on a daily basis once breeding pairs were placed together until resulting offspring reached PD 30. Water then was changes three times weekly for animals used in P60 experiments.

3.3.5 Identification of pups

Animals will have a toe corresponding to a number amputated at P8 for identification. Anesthesia will not be used as isoflurane, the inhalation anesthetic of choice available in our laboratory exerts influence on the same neurological pathways as THM/PCE does.

3.4 PKA activity in mouse brain

3.4.1 Housing, Breeding and Chemical Treatment of Mice

Please refer to General Method sections 3.3.1 through 3.3.4.

3.4.2 Animals

PKA activity was determined at three different time points during development. Eight four-day old males were used in the first PKA activity experiments. This group consisted of two control males, two 1x THM/PCE treated males, two 10x THM/PCE treated males and two 100x THM/PCE treated males. Eight four-day old females were also used in these PKA activity experiments. This group consisted of two control females, two 1x THM/PCE treated females, two 10x THM/PCE treated females and two 100x THM/PCE treated females.

In the second PKA activity experiments, nine ten-day old males were used in the PKA activity experiments. This group consisted of three control males, three 10x THM/PCE treated males and three 100x THM/PCE treated males. Nine ten-day old females were also used in these PKA activity experiments. This group consisted of three control females, three 10x THM/PCE treated females and three 100x THM/PCE treated females.

In the last set of PKA activity experiments, nine 15-day old males were used in the PKA activity experiments. This group consisted of three control males, three 10x THM/PCE treated males and three 100x THM/PCE treated males. Nine 15-day old females were also used in these PKA activity experiments. This group consisted of three control females, three 10x THM/PCE treated females and three 100x THM/PCE treated females.

3.4.3 Preparation of sample

Animals were decapitated live and heads were snap frozen using liquid nitrogen. Brains were dissected from the skull, and the cortex was removed. Cortex was homogenized using an electric homogenizer, in ice-cold PKA extraction buffer (25mM Tris-HCl (ph 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10mM B-mercapatoethanol, 1ug/mL leupeptin and 1 ug/ml aprotinin). Homogenate was centrifuged and protein concentration of supernatant was determined using Bradford estimation.

3.4.4 PKA activity assay

Supernatant containing equal amounts of protein (10 ug) was added to 5ul of Peptag PKA reaction buffer, 5 ul of PKA activator 5x solution and 1 ul of peptide protection solution in a microcentrifuge tube on ice. Tubes containing sample were subsequently placed in a 30ºC water bath for 1 min. 5ul Peptag A1

peptide was added and the reaction was allowed to incubate for 30 minutes at room temperature. After incubation, the samples were placed in a 95ºC water bath for 10 minutes to stop the reaction.1 ul of 80% glycerol was added to each of the samples. 15 ul of sample was loaded into a 0.8% agarose gel prepared with 50mM Tris-HCl, pH 7.4. The gel was run for 18 minutes at 100V and viewed after placement on top of an UV transilluminator. Photographs of the gels were obtained using a Polaroid GelCam.

3.4.5 Brain PKA activity assay result analysis

Pixel intensity of phosphorylated peptide in each lane was measured using Image J software. The average pixel intensity for each treatment group was compared for significant differences using Kruskal-Wallis non-parametric ANOVA. Dunn's multiple comparison test was used to determine significant differences between groups. Results were considered to be significant if $p < 0.05$. All statistical analyses were accomplished using GraphPad Instat software.

3.5 Communication and maternal behavior

3.5.1 Housing, Breeding and Chemical Treatment of Mice

Please refer to General Method sections 3.3.1 through 3.3.4

3.5.2 Animals

For maternal separation experiments, 184 animals were used. This group consisted of 17 control females and 36 control males, 18 1x THM/PCE treated females and 14 1x THM/PCE treated males, 19 10x THM/PCE treated females and 32 10x THM/PCE treated males, and 24 100x THM/PCE treated females and 24 100x THM/PCE treated males. For the cold exposure and male bedding experiments, 73 animals were used. This group consisted of eight control females and 14 control males, ten 10x THM/PCE treated females and 11 10x THM/PCE treated males and 12 100x THM/PCE treated females and 17 100x THM/PCE treated males. The 1x treatment was left out of later vocalization experiments because results from this group were not significantly different from controls.

3.5.3 Ultrasonic vocalization experiments

Individual P11 pups were placed in a 200 ml beaker cleaned with 10% ethanol. The beaker that contained the animal was placed inside of a Styrofoam box, which also contained an ultrasonic frequency detector. The box was closed to prevent detectors from detecting sounds that were not derived from the animal inside the testing box. The frequency detectors were set to detect frequencies of

60 kHz, which is within the vocalization range of the pups. The frequency detectors were attached to a computer equipped with Ultravox software, which was able to analyze the number and duration of calls, as determined by the parameters set by the user. Criterion used to count the number of calls made by the animal was a necessary minimum call duration of 10 ms. Additionally, 5 ms of silence was needed between vocalizations for each of them to be considered independent calls.

This test was done under three separate conditions. In the first set of conditions, animals were separated from their mothers and littermates for 15 minutes. Vocalizations data was obtained for the entire period of separation. In the second set of conditions, animals were placed in a beaker containing 2 grams of unrelated, virile male home cage bedding. The bedding was soiled. Vocalizations data was collected for 5 minutes. In the third set of conditions, animals were placed in a clean beaker, and the beaker that contained the animal was then placed in a small glass box that contained 20 ml of 8ºC water. This apparatus was then placed in the vocalization box, and vocalization data was collected for 5 minutes. Parameters for call criteria were the same for all three variations of the test.

3.5.4 Maternal retrieval tests

Mothers were removed from the home cage temporarily while pups were removed from the home nest and placed in a corner of the cage diagonally

opposite to the nest. Mothers were then placed back into the cage. The cage cover was placed on, and retrieval behavior was viewed from the side of the cage. The amount of time that had elapsed between the onset of placement of the mother back into the home cage and retrieval of each pup was recorded. Additionally, if the retrieval was direct, meaning that the mother picked up the pup and brought it directly to the nest, or if the mother picked up the pup and dropped it in a location outside the nest was also recorded.

3.5.5 Communication and maternal behavior result analysis

For all of the vocalization tasks, the number of vocalizations was compared between the groups using ANOVA or the nonparametric Kruskal Wallis test. Appropriate post hoc analyses were done to determine significant differences between groups. In the maternal retrieval tests, the amount of time that was needed for all pups to be retrieved back to the home nest was compared using ANOVA and a post hoc Bonferroni's Multiple Comparison test. Results were considered significant for all tests if $p < 0.05$. All analyses were performed using GraphPad Instat software.

3.6 Anxiety Behavior

3.6.1 Housing, Breeding and Chemical Treatment of Mice

Please refer to General Method sections 3.3.1 through 3.3.4

3.6.2 Animals

Eighty-two males were used for anxiety determination at the P21 time point. This group consisted of 37 control animals, 23 10x THM/PCE treated animals, and 22 100x THM/PCE treated males. 75 females were used for anxiety determination at the P21 time point. In this group there were 20 control animals, 32 10x THM/PCE treated animals and 23 100x THM/PCE treated animals. Eighty-males were used for the P60 anxiety study. This group contained ten control animals, ten 10x THM/PCE treated animals and ten 100x THM/PCE treated animals. 31 females were used for the P60 anxiety study. In this group there were 11 control animals, ten 10x THM/PCE treated animals and ten 100x THM/PCE treated animals.

3.6.3 Elevated Plus Maze

The elevated plus maze is cross-shaped. Plexiglas walls that are 40 cm in height close two arms of the plus. Two arms of the plus remain open. All arms are 30 cm long and 5 cm wide. The maze stands 1m from the surface of the floor. Animals were placed in the center of the maze facing an open arm and were left on the maze to explore for 5 minutes. The total amount of time spent in each arm was recorded as well as the total number of entries into each of the arms. Criterion for an animal to have entered a particular arm was counted only

if all four paws were placed in the arm of interest (Gonzalez and File 2004; Andersen and Teicher, 1999).

The number of entries into each of the arms was recorded, as well as the amount of time that was spent in each of the arms. The amount of time spent on the open arms was compared between groups, as well as the number of entries onto the open arms and into the closed arms. Inadvertently, kinetic behavior was also determined, by comparison of total entries into all arms between groups. This analysis provides a built in control measure for general hyperactivity or sedation, therefore preventing a need for a second test for general locomotor activity (Crawley, 2007).

3.6.4 Anxiety behavior result analysis

The amount of time spent on the open arms of the maze was compared between groups as well as the number of entries onto the open arms of the maze for anxiety determination. The number of entries into all arms of the maze was compared between groups for kinetic activity determination. All comparisons were done using ANOVA or Kruskal-Wallis non-parametric ANOVA with appropriate post hoc analyses. Groups were considered significantly different if $p < 0.05$. All analyses were performed using Graph Pad Instat software.

3.7 Spatial Learning and Perseverance Behavior

3.7.1 Housing, Breeding and Chemical Treatment of Mice

Please refer to General Method sections 3.3.1 through 3.3.4

3.7.2 Animals

Thirty male animals were used in the P30 perseverance study. This group consisted of ten control males, ten 10x THM/PCE treated males and ten 100x THM/PCE treated males. Thirty female animals were used in the P30 perseverance study. This group consisted of ten control females, ten 10x THM/PCE treated females and ten 100x THM/PCE treated females. Thirty-six male animals were used in the P60 perseverance study. This group consisted of 12 control males, 12 10x THM/PCE treated males and 12 100x THM/PCE treated males. Thirty-six females were used in the P60 perseverance study. This group consisted of 12 control females, 12 10x THM/PCE treated females and 12 100x THM/PCE treated females. Animals used in P60 trials were not the same animals used in P30 trials, to prevent prior exposure to the behavioral test. Animals were bred and chemically treated by methods described in the general methods section.

3.7.3 Water T-Maze

The Water T-Maze was used to assess the presence of perseverance behaviors and inadvertently used to test differences in spatial learning ability. The T-maze was constructed from a plastic reservoir. It contained a start arm, which was 30 cm long and 15 cm wide. The exit of the start arm is located at the center of a perpendicular corridor that is 80 cm long and 15 cm wide. This construction allowed for a left arm and a right arm that were each 32.5 cm long. The start arm and the perpendicular corridor were filled with water that was 25 ºC and made opaque with white Crayola non-toxic white powder paint. The depth of the water was 15 cm. The entire T-maze was placed inside a box, to reduce the number of distant visual cues.

Animals were trained to learn a particular routine, so that presence of perseverance behavior could be determined. On the first day of training, individual animals were placed in the start arm of the water T-maze, so upon reaching the end of the start arm, the animal had to make a decision to either go left or right. The choice of the animal was noted. The animal was allowed to swim for 60 seconds to habituate. In the second trial, a platform was placed in the arm opposite to the arm that was selected during the preceding habituation trial. Above the platform was an obvious marker that was placed there for the animal to use as a spatial cue. This platform was clear and located 1 cm below the surface of the water. The animal had to find the location of the submerged platform and sit on it for 10s, before being rescued. If the animal took longer than 60 seconds to complete the task, it was gently guided to the platform and guided to sit on the platform for 10s before rescue. Eight more trials identical to

the second trial were done, to train the animal to complete the task it before it could be rescued. After the training day, an animal was considered to have learned the task if it was able to complete eight out of ten trials per day without error for four consecutive days. The criterion for learning this routine was intentionally determined to be rigid, so that there was no question that the animal had learned a specific routine. If an animal swam directly to the platform and waited for rescue, no errors were charged to the animal's performance. Errors were charged if the animal left the entry arm and all four paws of the animal entered the arm of the t-maze where the platform was not located, or if the animal turned back around and re-entered the entry arm. The number of days needed for each animal to reach learning criteria was recorded and compared to assess learning ability.

3.7.4 Perseverance behavior experiments

The perseverance test was done on the day following the animal's achievement of reaching learning criteria. In the perseverance tests, the submerged platform was moved into the opposite arm to test if the animals would adhere to the previously learned routine or if they would be flexible and learn the new location of the submerged platform. The animal was given ten trials under the new experimental conditions. The number of errors was recorded for each of the trials.

3.7.5 Spatial learning and perseverance behavior result analysis

The number of days necessary for the animal to reach learning criteria was compared between groups using ANOVA or non-parametric Kruskal-Wallis and appropriate post hoc analysis. In the perseverance behavior tests, the number of trials done without error was compared between groups, as well as the number of total errors made on all trials and the number of errors made on the first reverse probe trial were compared between groups using ANOVA or non parametric Kruskal-Wallis and appropriate post hoc analysis. Results were considered to be significant if $p < 0.05$.

3.8 Sociality and social novelty behavior

3.8.1 Housing, Breeding and Chemical Treatment of Mice

Please refer to General Method sections 3.3.1 through 3.3.4

3.8.2 Animals

Thirty-males were used for the P85 time point. This group consisted of ten control animals, ten10x THM/PCE treated animals and ten 100x THM/PCE treated animals. Thirty females were used for the P85 time point. This group consisted of ten control animals, ten 10x THM/PCE treated animals and ten 100x

THM/CPE treated animals. A total of 20 animals were used for the P30 socialization tests. There were ten control males and ten 100x THM/PCE treated males in this group. The reason for the testing of only the 100x THM/PCE males is due to the time needed to test each animal, as the test takes a minimum of 20 minutes. Additionally, 100x THM/PCE treated males were the only group to test positive in all behavioral assessments that are critical in the diagnosis of autism. Breeding and chemical treatment of animals was done by methods described in the general methods section.

3.8.3 Social behavior experiments

The social behavior box consisted of three chambers that were separated by partitions. Partitions had a small 8cm high and 8cm wide opening, which allowed the animal to traverse all chambers. The center chamber of the apparatus was empty. The left and right chambers contained an inverted disposable 250 ml beaker, with holes drilled 1.5 cm apart on the entire perimeter of the surface. The beakers were placed in the back corner of the apparatus. A Petri dish containing pennies was taped closed and placed on the top of the inverted beakers, to prevent mice placed under the inverted beaker from escaping.

The first trial in the social behavior apparatus was the habituation trial, which lasted for 10m. In this trial, the animal under investigation was placed in the empty center chamber and was allowed to explore. The number of entries

into the left and right chambers was recorded as well as the amount of time spent in the empty left and right chambers. Once it was determined that the animal did not significantly favor the left or right empty chamber, the second socialization trial commenced.

In the socialization trial, a gender-matched animal was placed under one of the empty inverted beakers located in either the left or right chamber. The animal under investigation was placed in the center chamber and again allowed to explore for 10m. The location of the novel animal was recorded, as well as the number of times the animal under investigation entered the chamber of the novel animal and the empty chamber. Time spent by the animal in each of the chambers was also recorded. Additionally, the amount of time the animal under investigation spent sniffing the novel animal was recorded.

In the third trial, the animal's affinity for social novelty was assessed. In this trial, the novel animal from the second socialization trial remained under the beaker and in the chamber where it was originally placed, and a new novel gender matched animal was placed under the empty inverted beaker in the chamber on the opposite side of the apparatus. The animal under investigation was allowed to explore for 10m. The number of times that the animal under investigation entered each chamber, as well as the amount of time spent by the animal in each chamber and the amount of time spent sniffing the old novel animal and the new novel animal was recorded.

3.8.4 Social behavior result analysis

To determine if a group of animals showed preference for a novel animal, the amount of time spent in the chamber of the novel animal, as well as the amount of time spent in the empty chamber was compared using a student's T test. The same was done for the number of entries into each chamber for each group under investigation. ANOVA or nonparametric Kruskal-Wallis with appropriate post hoc analyses were done to determine if there were any significant differences in the amount of time spent by the different treatment groups sniffing the novel animal. All analyses were done using GraphPad Instat software.

4. Results

To test our hypotheses that that THM/PCE exposure induces changes in key regulators of neurodevelopment and behavioral pathology similar to that which is found in autism, we:

1. Determined if THM/PCE exposure induces a change in the level of catalytically active PKA in a different model, the zebra fish;
2. Determined if THM/PCE exposure induces a change in the activity of PKA in vitro and in vivo;
2. Determined if THM/PCE exposure induces a behavioral phenotype that is similar to that which is found in autism.

4.1 THM/PCE exposure induces changes in the levels of catalytic PKA, pCREB and BDNF in developing neurons of the zebrafish brain

In order to investigate whether THM/PCE exposure induces an increase in the level of catalytic PKA and the possible effects of such increases, zebrafish were exposed to varying concentrations of the THM/PCE mixture. At specific time points, these zebrafish were harvested and prepared for immunohistochemical examination of catalytic PKA levels, pCREB and BDNF using EM. Immunogold particles were quantified in the tectal neurons of the zebrafish brain, and the number of particles was compared between groups.

4.1.1 Day 7 study

PKA catalytic subunit level was significantly increased in the tectal neurons of zebrafish exposed to both the 10x and 100x concentrations of THM/PCE ($p < 0.001$). PKA catalytic subunit was increased, though not significantly, in the tectal neurons of the 1000x THM/PCE treatment group. There were no significant differences in catalytic PKA levels found between the 10x and 100x groups. The 10x and 100x THM/PCE treated groups showed a significant increase of PKA level in comparison to the 1000x THM/PCE treated group ($p < 0.01$; Figure 6, Table 1).

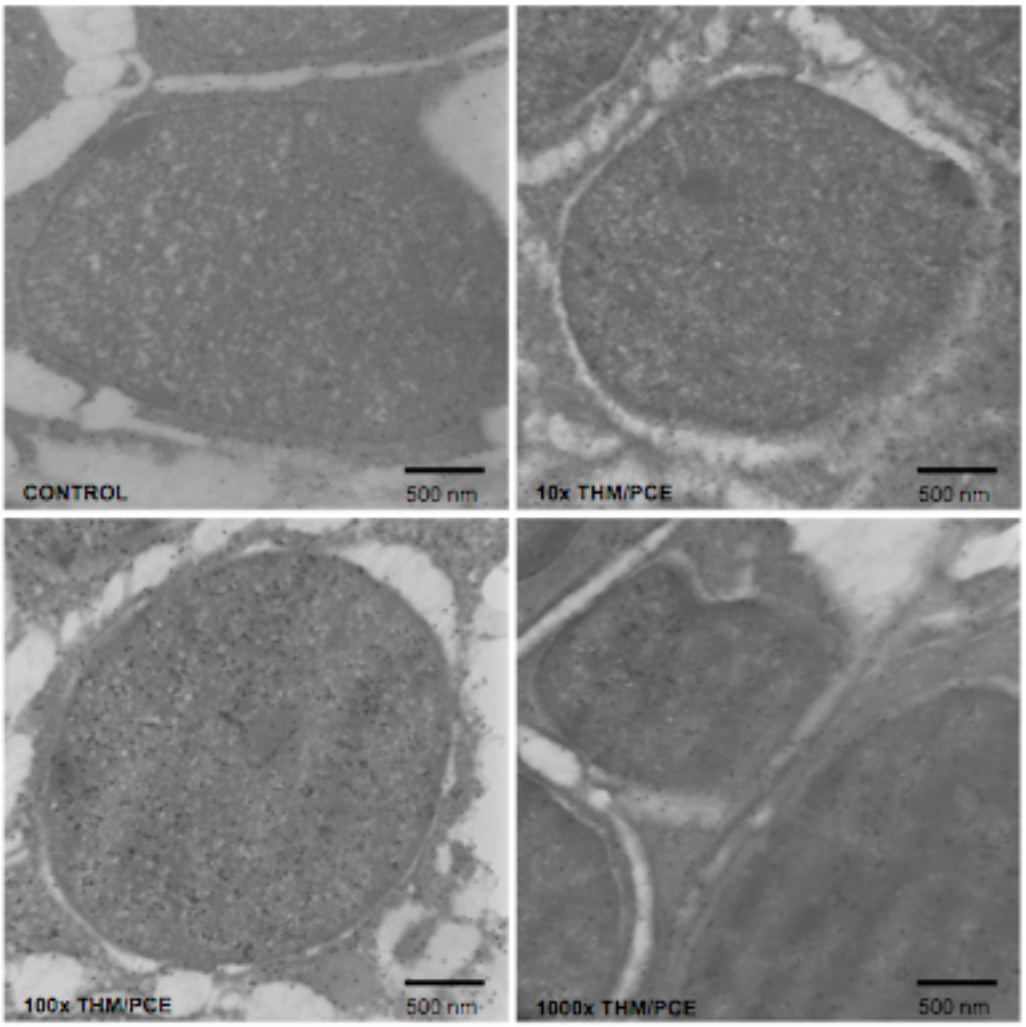


Figure 6: Catalytic PKA in zebrafish neurons at day 7 of development. There is a significant increase in the amount of catalytic PKA present in neurons of zebrafish exposed to a 10x and 100x THM/PCE concentration. A non-significant increase of PKA was found in the neurons of the 1000x THM/PCE treatment group. Three control animals, three 10x THM/PCE treated, three 100x THM/PCE treated and three 1000x THM/PCE treated animals were used (n=12). A one um-square area from 5 different neurons per animal was examined and used in analyses.

The only group to show a significant increase in the level of pCREB in the tectal neurons was the 1000x group ($p < 0.05$). pCREB in both the 10x and the 100x groups was increased, though not significantly. There were no significant differences in the levels of pCREB between the THM/PCE treatment groups. Labeling of pCREB was sparse therefore making the validity of these results

questionable, as it was expected that there would be heavier labeling of pCREB, since this is a ubiquitous transcription factor. The primary antibody used for pCREB in this experiment was not tested for use in zebrafish for this application (Figure 7; Table 1).

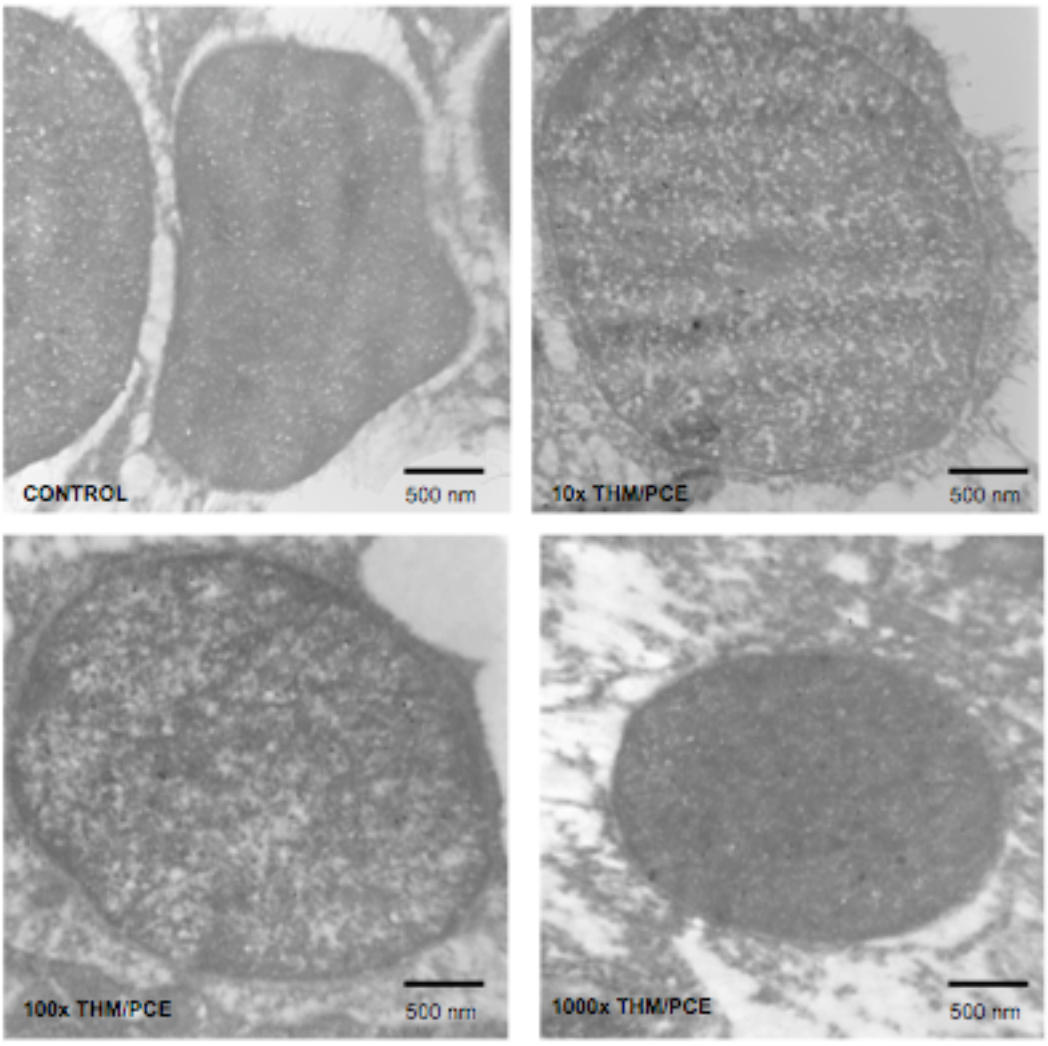


Figure 7: pCREB in zebrafish neurons at day 7 of development. There is a significant increase in the amount of pCREB present in neurons of zebrafish exposed to a 1000x THM/PCE concentration. A non-significant increase of pCREB was found in the neurons of the 10x and 100x THM/PCE treatment groups. Three control animals, three 10x THM/PCE treated, three 100x THM/PCE treated and three 1000x THM/PCE treated animals were used (n=12). A one um-square area from 5 different neurons per animal was examined and used in analyses.

There were significant increases in the levels of BDNF in the tectal neurons of zebrafish in both the 100x and 1000x groups, in comparison to the control group ($p < 0.05$). The 10x THM/PCE group also showed an increase, which was not statistically significant. There were no significant differences found in the level of BDNF among any of the THM/PCE treatment groups (Figure 8, Table 1).

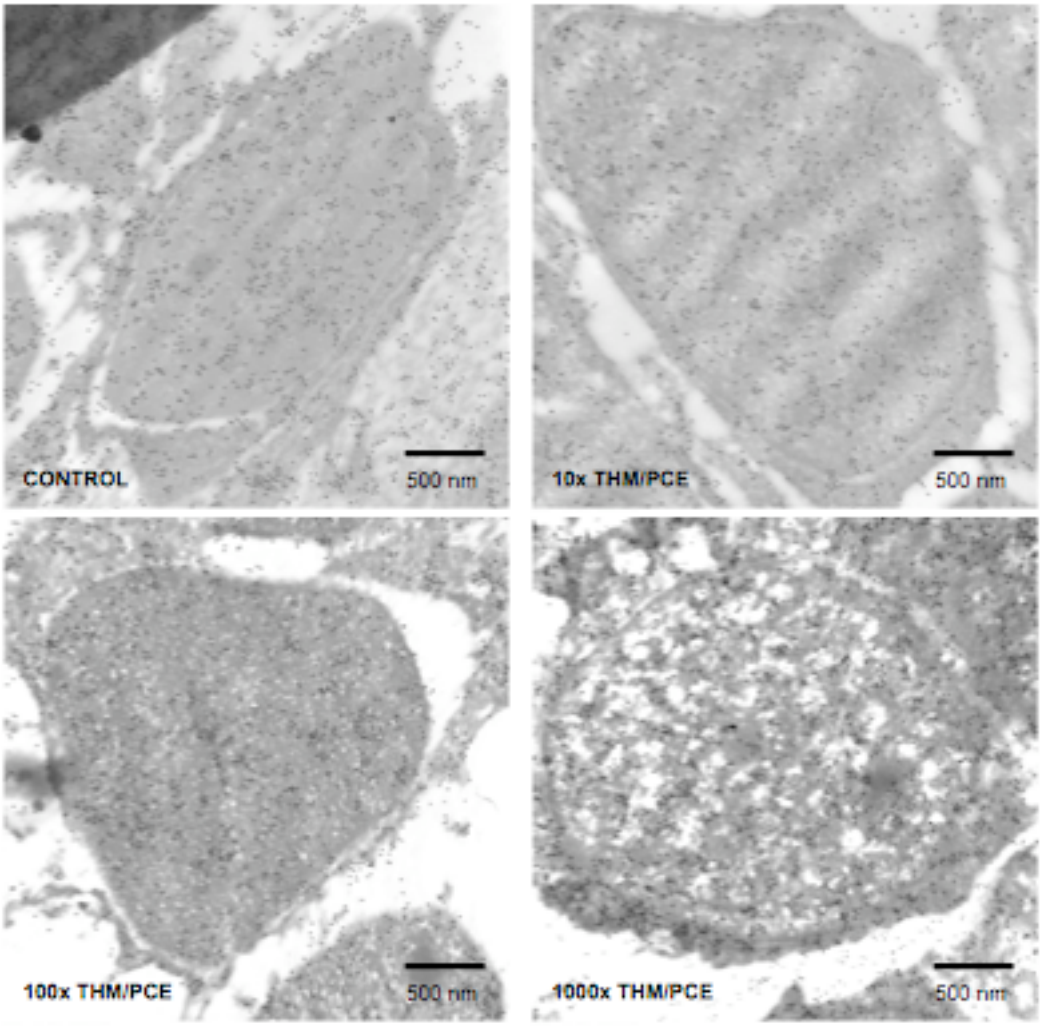


Figure 8: BDNF in zebrafish neurons at day 7 of development. There is a significant increase in the amount of catalytic PKA present in neurons of zebrafish exposed to a 10x and 100x THM/PCE concentration. A non-significant increase of PKA was found in the neurons of the 1000x THM/PCE treatment group. Three control animals, three 10x THM/PCE treated, three 100x THM/PCE treated and three 1000x THM/PCE treated animals were used (n=12). A one um-square area from 5 different neurons per animal was examined and used in analyses.

	Control (n=3)	10x THM/PCE (n=3)	100x THM/PCE (n=3)	1000x THM/PCE (n=3)	Comments
PKA	31.27	75.18	92.04	58.87	PKA was significantly elevated in the 10x and 100x groups in comparison to controls ($p < 0.001$). There were no significant differences in the levels of PKA between any of the THM/PCE treatment groups ($p > 0.05$).
pCREB	1.67	4.18	3.24	4.75	pCREB was significantly elevated in the 1000x THM/PCE treatment group in comparison to the control ($p < 0.05$). There were no significant differences in the levels of pCREB between any of the THM/PCE treatment groups ($p > 0.05$).
BDNF	45.71	70.11	106.81	93.28	BDNF was significantly elevated in the 100x and 1000x THM/PCE treatment groups ($p < 0.01$). There were no significant differences in the levels of BDNF between any of the THM/PCE treatment groups ($p > 0.05$).

Table 1: Mean number of particles of catalytic PKA, pCREB and BDNF per square micrometer in 7-day-old Zebrafish neurons. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test. Results were considered significant if $p < 0.05$.

4.1.2 Day 5, 8 and 14 PKA RII subunit study

At the day 5 time point, there was a significant elevation of PKA in the 10x and 100x THM/PCE treatment groups in comparison to the control group ($p < 0.001$). The 100x THM/PCE treatment group evidenced a significant PKA elevation in comparison to both the 10x and 1000x THM/PCE treatment groups ($p < 0.05$). These findings follow the same trend of increase in PKA levels (Table 2).

Interestingly, at the second time point examined, which was day 8, there was a significant decrease in PKA in all of the THM/PCE treatment groups in comparison to the controls ($p < 0.001$). There was significantly less PKA in the 100x group in comparison to the 1000x group ($p < 0.05$; Table 2).

At day 14, there was a significant decrease in PKA in all of the THM/PCE treatment groups in comparison to the control group ($p < 0.001$). There was a significant decrease in the 1000x THM/PCE group when compared to both the 10x and 100x THM/PCE treatment groups ($p < 0.01$ and $p < 0.05$, respectively; Table 2).

	Control (n=1)	10x THM/PCE (n=1)	100x THM/PCE (n=1)	1000x THM/PCE (n=1)	Comments
5 day old neurons	118	212	330	153	There is a significant elevation of PKA in the 10x and 100x THM/PCE treatment groups in comparison to control ($p < 0.001$). The 100x group shows a significant PKA elevation in comparison to both the 10x and 1000x treatment groups.
8 day old neurons	268	20	8	39	There is a significant decrease in PKA in all of the THM/PCE treatment groups in comparison to the controls ($p < 0.001$). There was significantly less PKA in the 100x group in comparison to the 1000x group ($p < 0.05$).
14 day old neurons	290	112	100	58	There is a significant decrease in PKA in all of the THM/PCE treatment groups in comparison to control ($p < 0.001$). There is a significant decrease in the 1000x group when compared to both the 10x and 100x groups ($p < 0.01$ and $p < 0.05$, respectively).

Table 2: Mean number of particles of RII subunit of PKA in 5, 8 and 14 day old zebrafish neurons. Particles were counted in 5 different neurons of similar size from each animal under investigation. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test. Results were considered significant if $p < 0.05$.

4.2 Microglia evidence enhanced PKA activity in response to THM/PCE exposure

In order to investigate the activity of PKA in cells, a microglia cell culture was used, as cells could be exposed to THM/PCE directly and large amounts of synchronous material could be obtained to do the appropriate PKA activity assay. The PKA activity assay yielded results that were in concordance with the previous immunohistological findings from the 7 and 5 day old larval zebrafish. Microglia that were exposed to all concentrations of THM/PCE showed a significant increase in activity when compared to the positive control forskolin group ($p < 0.001$). Forskolin activates adenylate cyclase, which is the enzyme responsible for conversion of ATP to cAMP. cAMP is necessary for PKA activation (Schneyer et al., 1983). The 1x and 10x THM/PCE treated groups also showed significantly increased activity in comparison to the untreated control ($p <0.01$) and in comparison to the DMSO sham group ($p < 0.01$). The DMSO group was used to determine if it had an effect of PKA activity, since THM/PCE was dissolved in it to increase solubility in the microglia growth media.

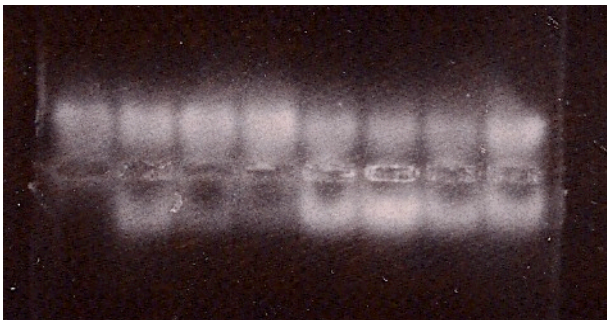
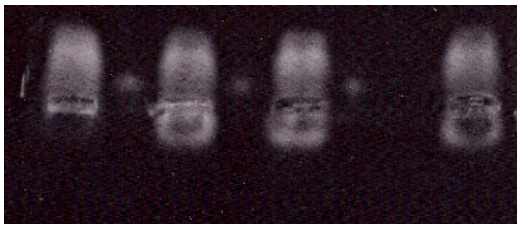


Figure 9: PKA activity assay of microglia. Bands located toward the bottom of each lane represent peptide phosphorylated by PKA. Bands located toward the top of each lane represent unphosphorylated peptide. Lane 1 (far left) represents the negative control. No cellular protein or PKA added was added to the negative control. Lane 2 represents untreated microglia (control). Lane 3 represents microglia treated with 0.001% DMSO (sham). Lane 4 represents microglia treated with 100 nm forskolin suspended in 0.001% DMSO (positive control). Lane 5 represents microglia treated with 1x THM/PCE suspended in 0.001% DMSO. Lane 6 represents microglia treated with 10x THM/PCE suspended in 0.001% DMSO. Lane 7 represents microglia treated with 100x THM/PCE suspended in 0.001% DMSO. Lane 8 (far right) represents microglia treated with 1000x THM/PCE suspended in 0.001% DMSO. Intensity of lower bands in each lane is indicative of elevated PKA activity. Lanes 5 (1x THM/PCE), lane 6 (10x THM/PCE), lane 7 (100x THM/PCE) and lane 8 (1000x THM/PCE) evidence significant elevations in PKA activity in comparison to Lane 2 (control), Lane 3 (sham control) and lane 4 (positive control; $p < 0.01$ for all comparisons).

4.3 PKA activity is enhanced in the cortex of P4 and P10 male mice, but not in female littermates. PKA activity increases diminish by P15 in male mice.

4.3.1 PKA activity is enhanced in the cortex of P4 males, but not in females

THM/PCE exposure induced an increase in PKA activity in the cortex in the males of all treatment groups, evidenced by a statistically significant increase the amount of phosphorylated peptide ($p = 0.0051$), which is a target for PKA phosphorylation (n=8). Results are shown in Figure 10a. Female littermates evidenced no statistically significant changes in the activity of PKA (n=8). Results are shown in Figure 10b.



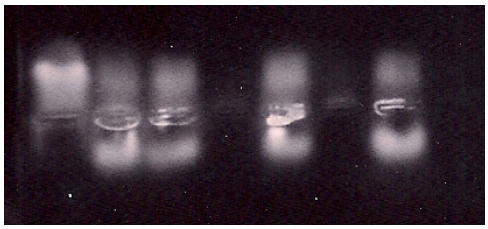


Figure 10a (left panel): PKA activity assay of P4 male cerebral cortex. Bands located toward the bottom of each lane represent peptide phosphorylated by PKA. Bands located toward the top of each lane represent unphosphorylated peptide. Lane 1 (far left) represents control or untreated male cerebral cortex. Lane 2 represents 1x THM/PCE treated male cerebral cortex. Lane 3 represents 10x THM/PCE treated male cerebral cortex. Lane 4 (far right) represents 100x THM/PCE treated male cerebral cortex. Lane 2 (1x THM/PCE), lane 3 (10x THM/PCE) and lane 4 (100x THM/PCE) show significant increases in the intensity of lower bands, which is indicative of enhanced PKA activity in response to all levels of THM/PCE treatment ($p < 0.005$).

Figure 10b (right panel): PKA activity assay of P4 female cerebral cortex. Bands located toward the bottom of each lane represent peptide phosphorylated by PKA. Bands located toward the top of each lane represent unphosphorylated peptide. Lane 1 (far left) represents a negative control to which no protein of PKA was added. Lane 2 represents control or untreated female cerebral cortex. Lane 3 represents 1x THM/PCE treated female cerebral cortex. Lane 4 represents 10x THM/PCE treated female cerebral cortex. Lane 5 (far right) represents 100x THM/PCE treated female cerebral cortex. There are no significant increases in the intensity of lower bands, which is indicative of no enhancement of PKA activity in response to all levels of THM/PCE treatment.

4.3.2 PKA activity is enhanced in the cortex of P10 males, but not in females

THM/PCE exposure induced an increase in PKA activity in the cortex in the males of all treatment groups, evidenced by a statistically significant increase the amount of phosphorylated peptide ($p = 0.0088$), which is a target for PKA phosphorylation (n=9). Female littermates evidenced no statistically significant changes in the activity of PKA (n=9). Results are shown in Figure 11.

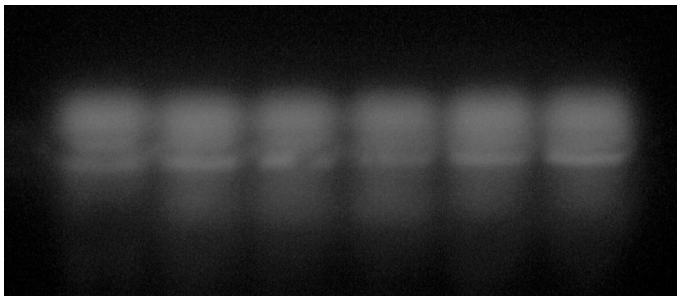


Figure 11: PKA activity assay of P10 male cerebral cortex and P10 female cerebral cortex. Bands located toward the bottom of each lane represent peptide phosphorylated by PKA. Bands located toward the top of each lane represent unphosphorylated peptide. Lane 1 (far left) represents control or untreated male cerebral cortex. Lane 2 represents control or untreated female cerebral cortex. Lane 3 represents 10x THM/PCE treated male cerebral cortex. Lane 4 represents 10x THM/PCE treated female cerebral cortex. Lane 5 represents 100x THM/PCE treated male cerebral cortex. Lane 6 (far right) represents 100x THM/PCE treated female cerebral cortex. Lane 3 (10x THM/PCE treated male) and lane 5 (100x THM/PCE treated male) evidence significant increases in the intensity of lower bands in comparison to lane 1 (control male), which is indicative of enhanced PKA activity in response to both levels of THM/PCE treatment ($p = 0.0088$). There are no significant differences in the intensity between lower bands of lane 2 (control female), lane 4 (10x THM/PCE treated female) and lane 6 (100x THM/PCE treated female), which is indicative of no changes of PKA activity in response to both levels of THM/PCE treatment.

4.3.3 PKA activity is not affected by THM/PCE treatment in P15 cortex of both genders

THM/PCE exposure did not induce an increase in PKA activity in the cortex in the males of all treatment groups, evidenced by a non-statistically significant increase the amount of phosphorylated peptide, which is a target for PKA phosphorylation (n=9). Female littermates evidenced no statistically significant changes in the activity of PKA (n=9). Results are shown in Figure 12.

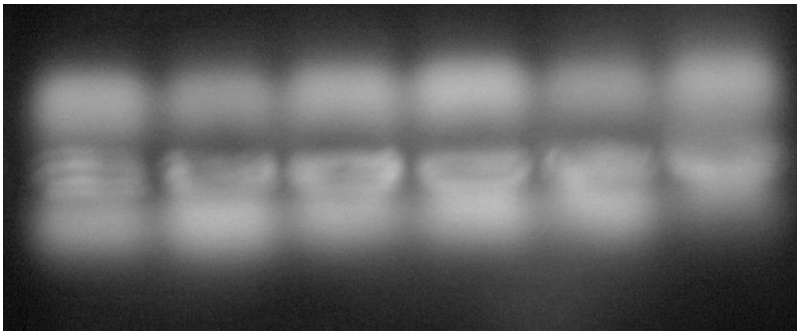


Figure 12: PKA activity assay of P15 male cerebral cortex and P15 female cerebral cortex. Bands located toward the bottom of each lane represent peptide phosphorylated by PKA. Bands located toward the top of each lane represent unphosphorylated peptide. Lane 1 (far left) represents control or untreated male cerebral cortex. Lane 2 represents control or untreated female cerebral cortex. Lane 3 represents 10x THM/PCE treated male cerebral cortex. Lane 4 represents 10x THM/PCE treated female cerebral cortex. Lane 5 represents 100x THM/PCE treated male cerebral cortex. Lane 6 (far right) represents 100x THM/PCE treated female cerebral cortex. There are no significant differences in the intensity between lower bands of lane 1 (control male), lane 4 (10x THM/PCE treated male) and lane 6 (100x THM/PCE treated male), which is indicative of no changes of PKA activity in response to both levels of THM/PCE treatment. There are no significant differences in the intensity between lower bands of lane 2 (control female), lane 4 (10x THM/PCE treated female) and lane 6 (100x THM/PCE treated female), which is indicative of no changes of PKA activity in response to both levels of THM/PCE treatment.

4.4 THM/PCE exposure induces deficits in communication in males, but not in females

Since deficits in communication are one of the pathological manifestations of autism, the communication abilities of animals exposed to THM/PCE were studied under a variety of experimental conditions. The number of calls as well as the call duration was compared between groups, to determine any differences in communication.

4.4.1 Ultrasonic vocalizations in response to maternal separation

Results from these experiments show that there is a significant difference in the mean number of vocalizations made by males of different groups ($p = 0.0094$). The mean number of vocalizations in each THM/PCE treatment group decreased with increasing concentration of THM/PCE treatment. Males in the 100x treatment group exhibited a significant reduction in the mean number of vocalizations in comparison to controls ($p < 0.05$; Figure 13). No significant differences were found in the mean duration of call length between any groups of males.

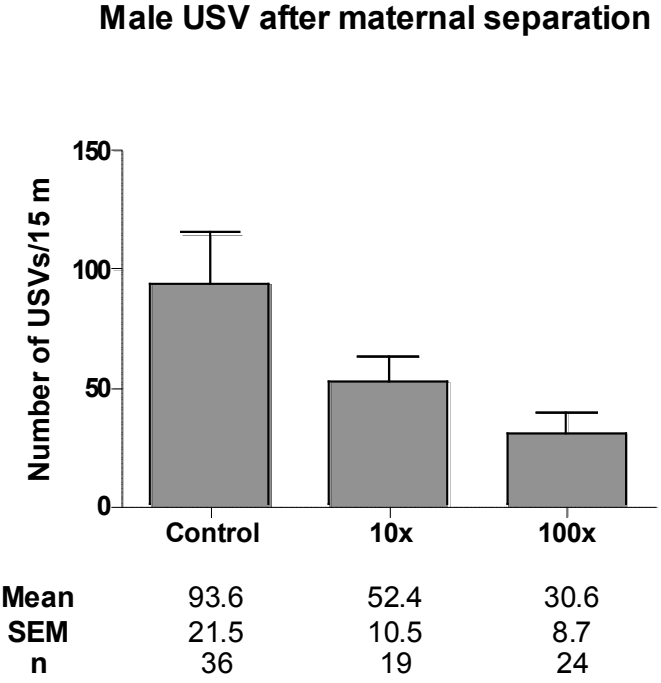


Figure 13: Mean number of ultrasonic vocalizations emitted by P11 males in response to maternal separation. There is a significant difference in the number of vocalizations emitted by the males of the treatment groups. The males of the 100x THM/PCE group showed a significant reduction in the number of vocalizations emitted in response to maternal separation, as compared to the controls ($p < 0.05$). The 10x THM/PCE treated group evidenced a non-significant decline in the number of vocalizations. Reductions of the number vocalizations are indicative of lower anxiety in response to separation or maternal attachment deficits. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

The mean number of vocalizations made by females was not significantly different between groups (Table 3). The mean duration of call length made by females was not significantly different (Table 4).

Group	Control (n=17)	1x (n=18)	10x (n=19)	100x (n=24)
Mean number of vocalizations	76.8	109.5	82.4	114.8

Table 3: Mean number of vocalizations made by P11 females in response to maternal separation. There are no significant differences in the number of calls made by females, regardless of treatment ($p = 0.8955$). Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test. Results were considered significant if $p < 0.05$.

Group	Control (n=17)	1x (n=18)	10x (n=19)	100x (n=24)
Average duration of call	0.13	0.15	0.17	0.14

Table 4: Mean duration of vocalizations made by females in response to maternal separation. There are no significant differences in the duration of the calls made by females in response to maternal separation (p=0.4941). Statistics were done using ANOVA and Bonferroni's post-hoc test. Results were considered significant if p <0.05.

4.4.2 Ultrasonic vocalizations in response to cold

Results from these experiments show that there is a significant difference in the mean number of vocalizations made by males in response to cold (p= 0.0013). The males of the 100x THM/PCE treatment group show a significant increase in the mean number of vocalizations made in response to cold when compared to both the control and to the 10x THM/PCE treatment group ($p<0.05$ and $p<0.01$, respectively; Figure 14). Differences in mean call duration of males were not significantly different among any groups (Table 5).

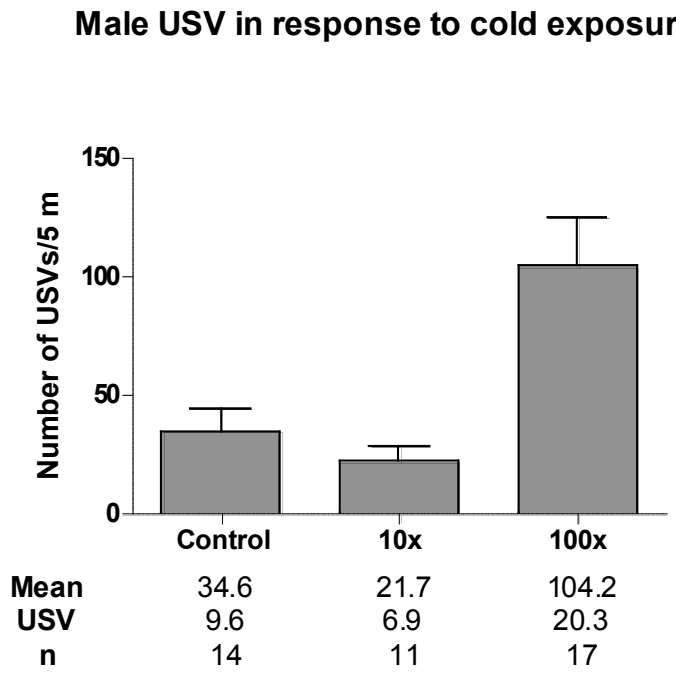


Figure 14: The mean number of vocalizations made by P11 males in response to cold. There was a significant difference in the number of vocalizations emitted by males in the groups in response to cold ($p=0.0013$). The males of the 100x THM/PCE treatment group vocalized significantly more than males of the control group ($p < 0.05$) and males of the 10x THM/PCE group ($p<0.01$). An increase in the number of vocalizations is indicative of heightened stress in response to cold exposure. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

Group	Control (n=14)	10x (n=11)	100x (n=17)
Average duration of call	0.14	0.16	0.15

Table 5: Mean duration of vocalizations made by P11 males in response to cold exposure. There are no significant differences in the duration of the calls made by males in response to maternal separation ($p=0.4891$). Statistics were done using ANOVA and Bonferroni's post-hoc test. Results were considered significant if $p < 0.05$.

Females of the 100x THM/PCE group showed a non-significant increase in the mean number of vocalizations in comparison to the control group (Table 6), nor were the duration of vocalizations (Table 7).

	Control (n=9)	10x (n=10)	100x (n=12)
Mean number of vocalizations	55.89	60.3	94.92

Table 6: Mean number of vocalizations emitted by P11 females in response to cold exposure. There are no significant differences in the number of vocalizations emitted by females in response to cold exposure (p= 0.2791). Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test. Results were considered significant if $p < 0.05$.

Group	Control (n=9)	10x (n=10)	100x (n=12)
Average duration of call	0.14	0.13	0.14

Table 7: Mean duration of vocalizations made by P11 females in response to cold exposure. There are no significant differences in the duration of the calls made by females in response to maternal separation (p=0.5220). Statistics were done using ANOVA and Bonferroni's post-hoc test. Results were considered significant if $p < 0.05$.

4.4.3 Ultrasonic vocalizations in response to male scent

Results from these experiments show that there is a non-significant reduction in the mean number of calls made by the males of the 100x THM/PCE treatment group in response to stranger male bedding (Table 8). There were no significant differences in mean call duration between any groups of males (p = Table 9).

	Control (n=14)	10x (n=11)	100x (n=17)
Mean number of vocalizations	49.4	59.6	32.53

Table 8: Mean number of vocalizations emitted by P11 males in response to placement in stranger male bedding. There are no significant differences in the number of vocalizations emitted by males in response to stranger male bedding (p= 0.2542). Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

Group	Control (n=14)	10x (n=11)	100x (n=17)
Average duration of call	0.12	0.14	0.13

Table 9: Mean duration of vocalizations made by P11 males in response to stranger male bedding. There are no significant differences in the duration of the calls made by males in response to stranger male bedding (p=0.3820). Statistics were done ANOVA and Bonferroni's post-hoc test.

Results from these experiments show that there are no significant differences in the mean number of calls made by females of any group in response to stranger male bedding (Table 10). There were no significant differences in the mean duration of the calls between any of the groups (Table 11).

	Control (n=9)	10x (n=10)	100x (n=12)
Mean number of vocalizations	60.25	81.4	102.8

Table 10: Mean number of vocalizations emitted by P11 females in response to placement in stranger male bedding. There are no significant differences in the number of vocalizations emitted by males in response to stranger male bedding (p= 0.2542). Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

Group	Control (n=9)	10x (n=10)	100x (n=12)
Average duration of call	0.15	0.16	0.13

Table 11: Mean duration of vocalizations emitted by P11 females in response to placement in stranger male bedding. There are no significant differences in the number of vocalizations emitted by females in response to stranger male bedding (p= 0.5262). Statistics were done using ANOVA and Bonferroni's post-hoc test.

4.4.4 Maternal retrieval results

Results from these experiments show that there are no significant differences in the amount of time taken for mothers to retrieve all pups when placed in a new location. Additionally, all mothers performed direct retrieval, as pups that were picked up were placed back in the nesting area.

Female	Control (n=6)	10x (n=6)	10x (n=6)
Average time spent to retrieve all pups	175.2s	180.0	177.1

Table 12: Mean amount of time needed to retrieve all pups by mothers of pups in vocalization study. There are no significant differences in the amount of time needed to retrieve all pups to the nest from which they were moved (p=0.8640). Statistics were done using ANOVA and Bonferroni's post-hoc test. Results were considered significant if $p < 0.05$.

4.5 THM/PCE exposure induces anxiogenic behavior in juvenile males and anxiolytic behavior in both sexes if treatment is prolonged

Heightened anxiety is present in a large percentage of children diagnosed with autism. Therefore, in these experiments, THM/PCE treated animals were tested for anxiety using an elevated plus maze. The effect of prolonged treatment was also determined, to see if persistent exposure was necessary to maintain any behavioral phenotypes that were found earlier in development.

4.5.1 Anxiety in P21 animals

Males of the 10x THM/PCE treated group as well as males of the 100X THM/PCE treated groups spent significantly less time on the open arms of the elevated plus maze in comparison to control males ($p= 0.0020$; Figure 15, Table 13). In addition, there was a significant difference in the average number of entries onto the open arms of the maze ($p=0.0331$), but post hoc analyses show that there significant differences do not exist between any groups specifically (Figure 16, Table 13). No significant differences were found in the number of entries into the closed arms between any groups (Table 13).

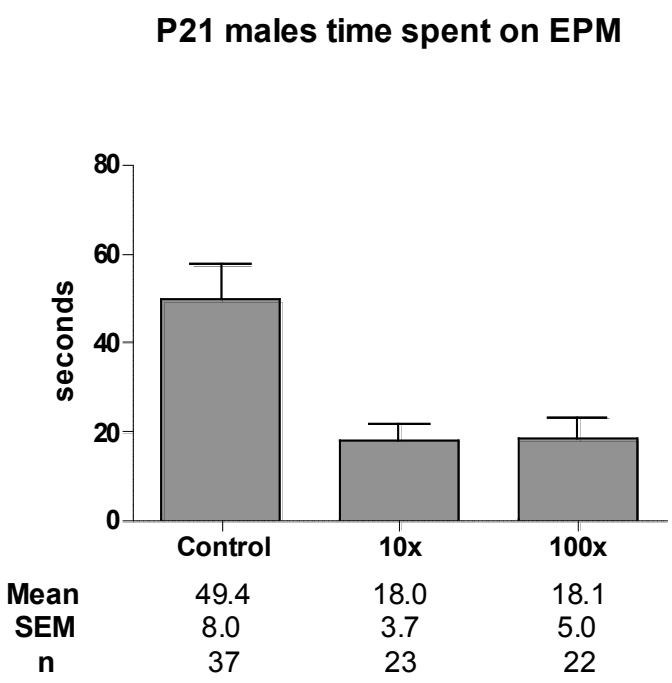
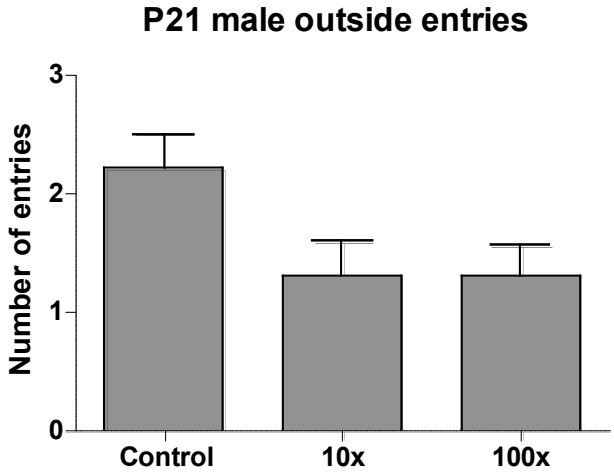


Figure 15: Mean time spent on outside platforms of the elevated plus maze by P21 males. There is a significant difference in the time spent on outside platforms by the treatment groups ($p=0.0020$). There was a significant reduction in the time spent outside by the 10x and 100x groups in comparison to the control group ($p < 0.05$, $p < 0.01$, respectively). Reduction in the time spent outside is indicative of increased anxiety in response to exposure to both the 10x and 100x THM/PCE concentrations. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.



	Control	10x	100x
Mean	2.2	1.3	1.3
SEM	0.29	0.29	0.26
n	37	23	22

Figure 16: Mean number of entries made by P21 males onto outside arms of the elevated plus maze. There is a significant difference in the mean number of entries made onto the outside platforms of the elevated plus maze by the treatment groups ($p=0.0331$). However, there were no significant differences found in the number of entries outside between specific treatment groups ($p > 0.05$). A decrease in the number of entries is indicative of anxiety in response to exposure to both the 10x and 100x THM/PCE concentrations. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

	Control (n=37)	10x THM/PCE (n=23)	100x THM/PCE (n=22)	P value
Average time spent on open arms of elevated plus maze	49.4s	18.0s	10.4s	p= 0.0020 p< 0.05 difference between control and 10x groups; p<0.01 difference between control and 100x group (KW/Dunn)
Average number of entries onto open arms of elevated plus maze	2.2	1.4	1.4	p= 0.0331 There are no significant differences in pair wise comparisons (KW/Dunn)
Average number of entries into closed arms of elevated plus maze	9.2	10.4	9.7	0.2128 Not significant (ANOVA/Bonferroni)

Table 13: Summary of P21 male performance on elevated plus maze.

Females in the THM/PCE treatment groups showed no differences in the average time spent outside in comparison to controls, nor do did they differ significantly in the average number of entries onto the open arms of the maze (Table 14). No significant differences were found in the number of entries into the closed arms between any groups (Table 14).

	Control P21 (n=20)	10x THM/PCE (n=32)	100x THM/PCE (n=23)	P value
Average time spent on open arms of elevated plus maze	42.4s	26.7s	27.1s	0.5671 Not significant (KW/Dunn)
Average number of entries onto open arms of elevated plus maze	1.6	2.0	1.7	p= 0.9587 Not Significant (KW/Dunn)
Average number of entries into closed arms of elevated plus maze	8.9	10.0	9.4	p=0.5109 Not significant (ANOVA/Bonferroni)

Table 14: Summary of P21 female performance on elevated plus maze.

4.5.2 Anxiety in P60 animals

Males of the 100x THM/PCE treatment group evidenced a significant increase in the average amount of time spent on the outside arms of the elevated plus maze ($p < 0.0001$). Pair wise group comparisons showed that they spent significantly longer amounts of time outside than both control males ($p < 0.001$) and males of the 10x THM/PCE treatment groups ($p < 0.001$; Figure 17, Table 15). Males of the 100x THM/PCE treatment group also showed a significant increase in the average number of entries onto the open arms in comparison to males of both the control ($p < 0.001$) and 10x treatment groups ($p < 0.001$; Figure

18, Table 15). No significant differences were found in the number of entries into the closed arms of the maze by any of the treatment groups when compared with controls or with each other (Table 15).

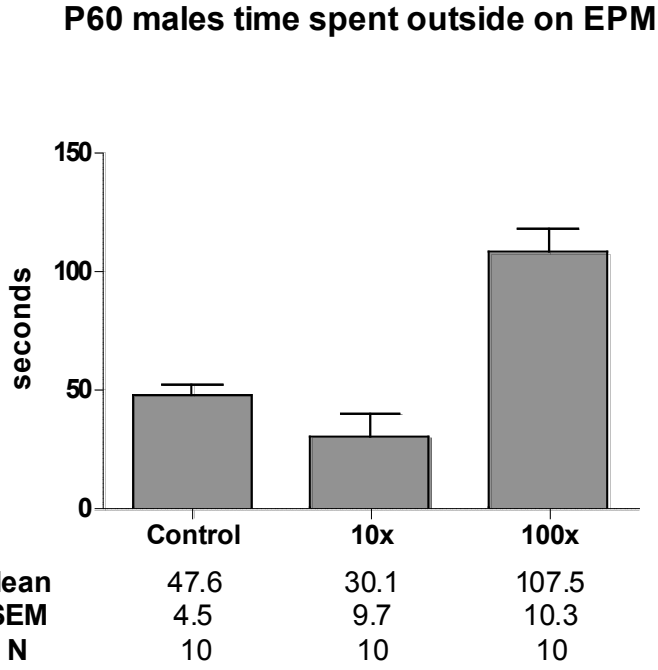
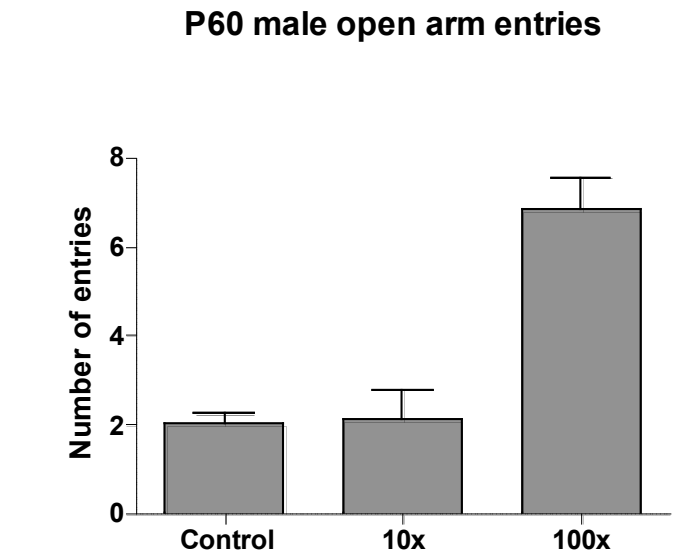


Figure 17: Mean time spent on outside platform of the elevated plus maze by P60 males. There is a significant difference in the time spent on outside platforms by the treatment groups ($p < 0.0001$). There is a significant increase in the time spent outside by the 100x group in comparison to the control group ($p < 0.01$) and a significant increase in time spent outside by the 100x group in comparison to the 10x group ($p < 0.01$). An increase in time spent outside is indicative of an anxiolytic effect of prolonged 100x THM/PCE treatment. Statistics were done using ANOVA and Turkey's post-hoc test.



	Control	10x	100x
Mean	2.0	2.1	6.8
SEM	0.24	0.68	0.74
n	10	10	10

Figure 18: Mean number of entries made by P60 males onto outside arms of the elevated plus maze. There is a significant difference in the mean number of entries made onto the outside platforms of the elevated plus maze by the treatment groups ($p=0.0003$). There is a significant increase in the number of entries made onto outside platforms by the 100x group in comparison to the control ($p < 0.01$) and a significant increase in the number of entries made onto outside platforms by the 100x group in comparison to the 10x group ($p < 0.01$). An increase in the number of entries onto outside is indicative of an anxiolytic effect of prolonged 100x THM/PCE treatment. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

	Control (n=10)	10x THM/PCE (n=10)	100x THM/PCE (n=10)	p value
Average time spent on open arms of elevated plus maze	47.6s	30.1	107.5	p<0.0001 p<0.001 between animals of control and 100x groups; p<0.001 between animals of 10x and 100x groups (ANOVA/Bonferroni)
Average number of entries onto open arms of elevated plus maze	2	2.1	6.8	p<0.0003 p<0.001 between animals of control and 100x groups,; p<0.001 between animals of 10x and 100x groups (KW/Dunn)
Average number of entries into closed arms of elevated plus maze	12	10.3	9.2	p= 0.9223 Not significant (ANOVA/Bonferroni)

Table 15: Summary of P60 male performance on elevated plus maze.

Females of the 100x THM/PCE treatment group also evidenced a significant increase in the average amount of time spent on the outside arms of the elevated plus maze (p=0.0022). These females spend a significantly greater amount of time outside than both the control and the 10x THM/PCE treatment groups (p <0.01 and p <0.01, respectively; Figure 19, Table 16). The 100x THM/PCE treated females also showed a significant increase in the number of entries onto the outside arms of the elevated plus maze, as they entered the

open arms a significantly greater number of times than the control group ($p < 0.01$; Figure 20, Table 16). No significant differences were found in the number of entries into the closed arms between the control and THM/PCE treated groups (Table 16).

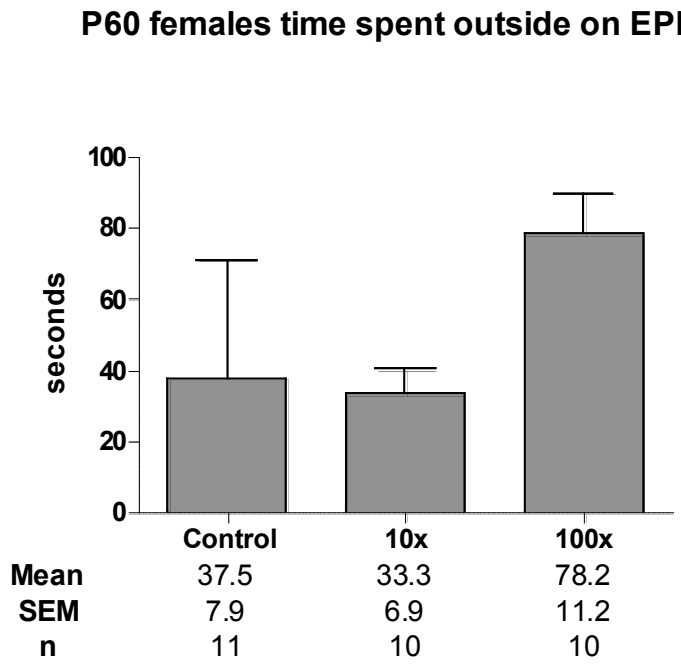
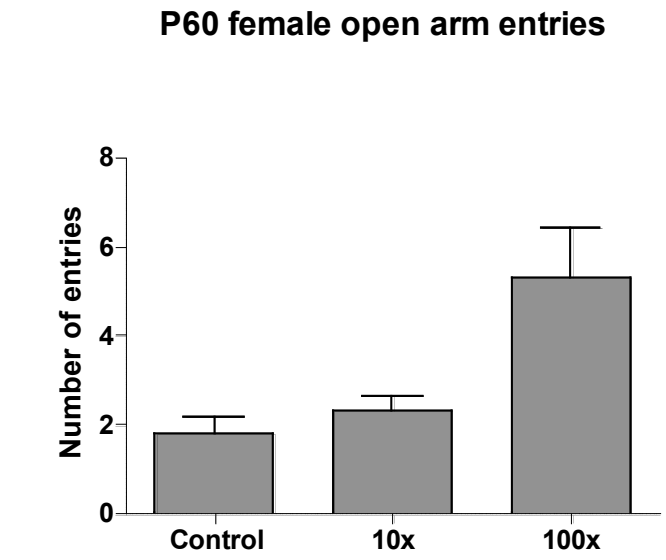


Figure 19: Mean time spent on outside platform of the elevated plus maze by P60 females. There is a significant difference in the time spent on outside platforms by the 100x THM/PCE treatment group ($p < 0.0022$). There is a significant increase in the time spent outside by the 100x group in comparison to the control group ($p < 0.01$) and a significant increase in time spent outside by the 100x group in comparison to the 10x group ($p < 0.01$). An increase in time spent outside is indicative of an anxiolytic effect of prolonged 100x THM/PCE treatment. Statistics were done using ANOVA and Turkey's post-hoc test.



	Control	10x	100x
Mean	1.8	2.3	5.3
SEM	0.38	0.34	1.1
n	11	10	10

Figure 20: Mean number of entries made by P60 females onto outside arms of the elevated plus maze. There is a significant difference in the mean number of entries made onto the outside platforms of the elevated plus maze by the treatment groups (p=0.0039). There is a significant increase in the number of entries made onto outside platforms by the 100x group in comparison to the control ($p < 0.01$). An increase in the number of entries onto outside is indicative of an anxiolytic effect of prolonged 100x THM/PCE treatment. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

	Control (n=11)	10x THM/PCE (n=10)	100x THM/PCE (n=10)	p value
Average time spent on open arms of elevated plus maze	37.5s	33.3s	78.3s	p= 0.0022 p <0.01 between control and 100x groups; p <0.01 between 10x and 100x groups (ANOVA/Bonferoni)
Average number of entries onto open arms of elevated plus maze	1.8	2.3	5.2	p= 0.0039 p <0.01 between control and 100x groups (KW/Dunn)
Average number of entries into closed arms of elevated plus maze	10.1	10.7	10.9	p= 0.7699 Not significant (ANOVA/Bonferroni)

Table 16: Summary of P60 female performance on elevated plus maze.

4.5.3 Comparison of anxiety in P21 and P60 animals

There were no significant differences found in the amount of time spent outside, the number of entries onto open arms, or in the number of entries into the closed arms when control P21 males and 10x THM/PCE treated males were compared with P60 control and 10x THM/PCE treated males (Tables 17 and 18). There was a significant increase in the amount of time spent outside and the number of entries onto open arms of the maze by P60 100x THM/PCE treated males when compared with P21 100x THM/PCE treated males ($p < 0.0001$;

Table 19). There were no significant differences in the number of entries into closed arms (Table 19).

	P21 (n=37)	P60 (n=10)	p value
Average time spent on open arms of elevated plus maze	49.4s	47.6s	p= 0.2612 (T-test)
Average number of entries onto open arms of elevated plus maze	2.2	2	p=0.8897 (T-test)
Average number of entries into closed arms of elevated plus maze	9.2	12	p= 0.5331 (T-test)

Table 17: Control P21 male performance vs. control P60 male performance on elevated plus maze.

	P21 (n=23)	P60 (n=10)	p value
Average time spent on open arms of elevated plus maze	18.0s	30.1	0.2708 (T-test)
Average number of entries onto open arms of elevated plus maze	1.4	2.1	0.4623 (T-test)
Average number of entries into closed arms of elevated plus maze	10.4	10.3	0.9741 (T-test)

Table 18: 10x THM/PCE P21 male performance vs. 10x THM/PCE P60 male performance on elevated plus maze.

	P21 (n=32)	P60 (n=10)	p value
Average time spent on open arms of elevated plus maze	10.4s	107.5	$p < 0.0001$ (T-test)
Average number of entries onto open arms of elevated plus maze	1.4	6.8	$p < 0.0001$ (T-test)
Average number of entries into closed arms of elevated plus maze	9.7	9.2	$p = 0.5786$ (T-test)

Table 19: 100x THM/PCE P21 male performance vs. 10x THM/PCE P60 male performance on elevated plus maze.

Females of the P21 control group showed no significant differences in the amount of time spent outside, as well as the number of entries onto the open arms or into the closed arms of the maze when compared with the P60 control group (Table 20). Females of the P21 10x THM/PCE treatment group showed no significant differences in the amount of time spent outside, as well as the number of entries onto the open arms or into the closed arms of the maze when compared with the P60 10x THM/PCE treatment group (Table 21). Females of the P60 100x THM/PCE treatment group spent a significantly greater amount of time on the open arms of the maze ($p = 0.0012$) , as well as a significant increase in the number of entries onto the open arms ($p = 0.004$; Table 22). There were no differences between P21 and P60 females in the number of entries into the closed arms of the maze (Table 22).

	P21 (n=20)	P60 (n=11)	p value
Average time spent on open arms of elevated plus maze	42.4s	37.5s	p= 0.6348 (T-test)
Average number of entries onto open arms of elevated plus maze	1.6	1.8	p = 0.6331 (T-test)
Average number of entries into closed arms of elevated plus maze	8.9	10.1	p = 0.3455 (T-test)

Table 20: Control P21 female performance vs. control P60 female performance on elevated plus maze.

	P21 (n=32)	P60 (n=10)	p value
Average time spent on open arms of elevated plus maze	26.7s	33.3s	p= 0.4019 (T-test)
Average number of entries onto open arms of elevated plus maze	2.0	2.3	p= 0.2613 (T-test)
Average number of entries into closed arms of elevated plus maze	10.0	10.7	p= 0.8476 (T-test)

Table 21: 10x THM/PCE treated P21 female performance vs. 10x THM/PCE treated P60 female performance on elevated plus maze.

	P21 (n=23)	P60 (n=10)	p value
Average time spent on open arms of elevated plus maze	27.1s	78.3s	p= 0.0012 (T-test)
Average number of entries onto open arms of elevated plus maze	1.7	5.2	p= 0.004 (T-test)
Average number of entries into closed arms of elevated plus maze	9.4	10.9	p= 0.1580 (T-test)

Table 22: 100x THM/PCE treated P21 female performance vs. 100x THM/PCE treated P60 female performance on elevated plus maze.

4.6 Prolonged THM/PCE treatment induces spatial learning deficits in both sexes and perseverance behavior in juvenile males

Perseverance behaviors are one of the behavioral pathologies found in persons with autism. Therefore, to determine if THM/PCE treatment induces such behaviors, animals were trained to learn how to escape from the water T-maze. Once learning criteria was reached, the escape route was moved to a different location and the flexibility of the animal to learn the new routine was determined.

4.6.1 Learning criterion of P30 mice

Males of the 10x and 100x THM/PCE treatment groups did not differ significantly from controls in the number of days that were needed to reach learning criteria (Table 23). Females of the 10x and 100x THM/PCE treatment also did not differ significantly from controls in the number of days that were needed to reach learning criteria (Table 24).

Test	Control (n=10)	10x THM/PCE (n=10)	100x THM/PCE (n=10)	P value
Average number of Days to reach learning criterion	4.8	5.4	4.6	0.9674 Not significant (ANOVA/Bonferroni)
Average number of trials completed without error on reversal learning set	8.0	6.5	6.0	0.0283 Significant *p <0.05 between the 10x and 100x THM/PCE groups (KW/Dunn)
Average number of errors made on 1st reversal learning probe	1.6	2.9	3.0	0.2939 Not significant (KW/Dunn)
Average number of errors made on all trials	4.5	4.0	5.9	0.3858 Not significant (KW/Dunn)

Table 23: Summary of P30 male performance in the water-T-maze.

Test	Control (n=10)	10x THM/PCE (n=10)	100x THM/PCE (n=10)	P value
Average number of Days to reach learning criterion	4.6	4.4	5.4	0.3355 Not significant (KW/Dunn)
Average number of trials completed without error on reversal learning set	5.8	6.8	7.4	0.1162 Not significant (KW/Dunn)
Average number of errors made on 1st reversal learning probe	2.1	2.1	3.6	0.8495 Not significant (KW/Dunn)
Average number of errors made on all trials	5.3	5.8	5.9	0.2524 Not significant (KW/Dunn)

Table 24: Summary of P30 female performance in the water-T-maze.

4.6.2 Perseverance behavior in P30 mice

As compared to the control animals, males of the 100x THM/PCE treatment group did show a significant increase in perseverance behavior, demonstrated by the average number of trials completed without error on the reverse learning trial day (p= 0.0295; Figure 21; Table 23). Females did not vary significantly in their performance on the perseverance behavioral task (Table 24).

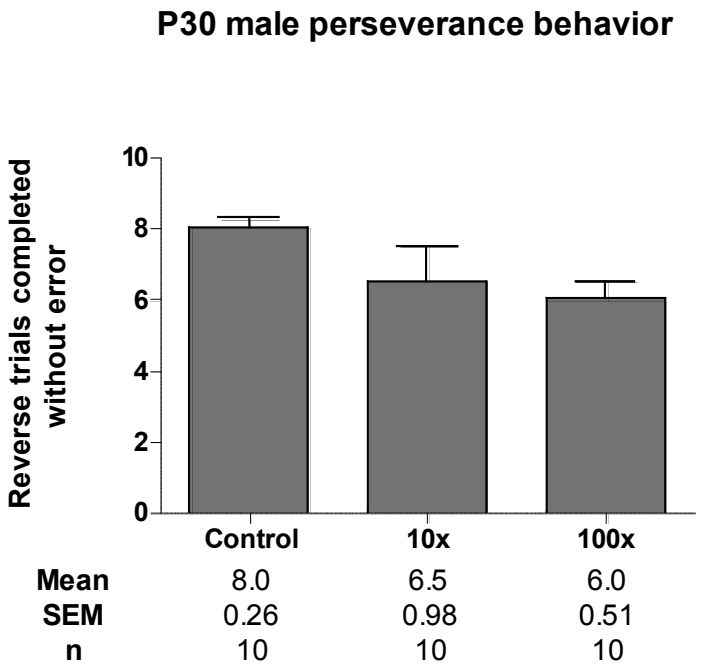


Figure 21: The mean number of trials completed without error on the reverse learning probe by P30 males. There was a significant difference between treatment groups in the number of trials completed without error ($p = 0.0295$). The 100x animals showed a significantly reduced number of trials completed in comparison to the control group ($p < 0.05$). This reduction in the number of trials done without error by the 100x THM/PCE treatment group is indicative of perseverance behavior. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

Males and females of the THM/PCE treatment groups did not show any significant differences in comparison to control animals in the number of errors made on the first reversal-learning probe, or in the number of total errors made in the ten perseverance behavior trials (Tables 23 and Table 24).

4.6.3 Learning criterion of P60 mice

Significant differences in the average number of days males needed to reach criterion in the treatment groups were found ($p=0.0498$). The 10x

THM/PCE treated males needed more days than all other groups of males to reach learning criterion ($p < 0.05$; Figure 22; Table 25). Females also showed a significant difference in the average number of days needed to reach criterion between treatment groups ($p = 0.0080$). Similar to the results in males, the 10x THM/PCE treatment group needed more days than all other groups of females to reach learning criterion ($p < 0.01$; Figure 23; Table 26).

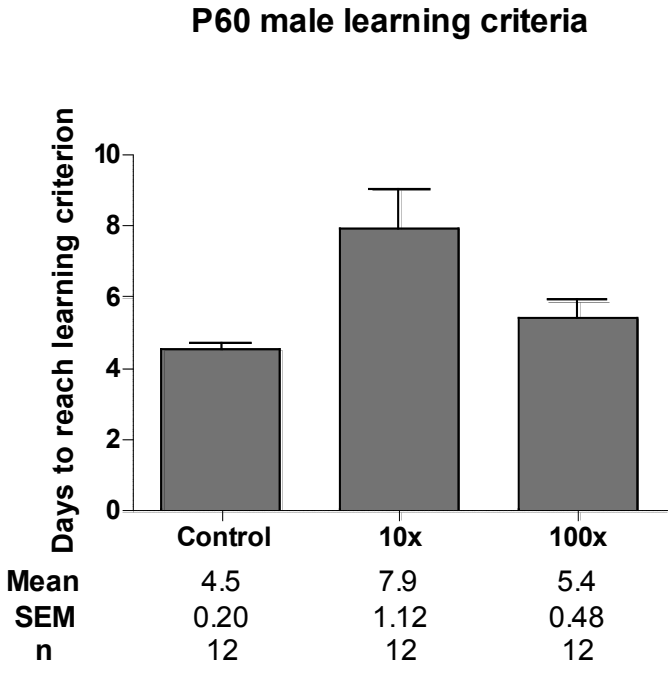
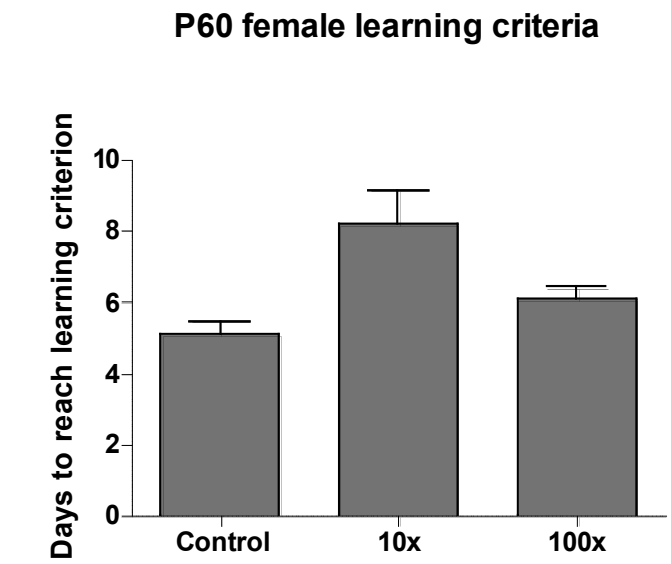


Figure 22: Mean number of days needed by P60 males to reach learning criteria for the water T-maze task. There was a significant difference in the number of days needed to reach learning criteria by the treatment groups ($p = 0.0498$). The 10x group took significantly longer to reach learning criteria than did the animals of the control group ($p < 0.05$). This is evidence of impairment in the spatial learning ability of animals treated with the 10x THM/PCE concentration. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.



	Control	10x	100x
Mean	5.08	8.17	6.08
SEM	0.36	0.94	0.38
n	12	12	12

Figure 23: Mean number of days needed by P60 females to reach learning criteria for the water T-maze task. There was a significant difference in the number of days needed to reach learning criteria by the treatment groups ($p= 0.0080$). The 10x group took significantly longer to reach learning criteria than did the animals of the control group ($p <0.01$). This is evidence of impairment in the spatial learning ability of animals treated with the 10x THM/PCE concentration. Statistics were done using Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test.

Test	Control P60 (n=12)	10x THM/PCE P60 (n=12)	100x THM/PCE P60 (n=12)	P value
Average number of Days to reach learning criterion	4.5	7.9	5.4	0.0498 *p <0.05 between animals of the control and 10x groups (KW/Dunn)
Average number of trials completed without error on reversal learning set	5.8	5.2	6.1	0.5743 Not significant (KW/Dunn)
Average number of errors made on 1st reversal learning probe	3.4	4.2	3.5	0.5817 Not significant (KW/Dunn)
Average number of errors made on all trials	7.9	9.7	7.1	0.4122 Not significant (KW/Dunn)

Table 25: Summary of P60 male performance in the water-T-maze.

Test	Control P60 (n=12)	10x THM/PCE P60 (n=10)	100x THM/PCE P60 (n=10)	P value
Average number of Days to reach learning criterion	5.1	8.2	6.1	0.0080 $p<0.01$ between animals of the control and the 10x groups (KW/Dunn)
Average number of trials completed without error on reversal learning set	6.7	5.4	4.6	0.0670 Not significant (KW/Dunn)
Average number of errors made on 1st reversal learning probe	3.7	4.75	2.8	0.2296 Not significant (KW/Dunn)
Average number of errors made on all trials	6.5	10.7	7.6	0.6805 Not significant (KW/Dunn)

Table 26: Summary of P60 female performance in the water-T-maze.

4.6.4 Perseverance behavior at P60

Both males and females of the THM/PCE treatment groups showed no differences in perseverance behavior in comparison to the controls. The average number of trials completed without error in the reversal learning probe series was

not significant, nor was the number of errors made on the first reversal learning probe, nor was the total number of errors on all probes (Table 25 and Table 26).

4.6.5 Comparison of P30 and P60 learning

Males of the control and of both THM/PCE treated groups showed no significant differences in the number of days needed to reach learning criterion when comparing the performance of the animals at P30 and P60 (Table 27; Table 28 and Table 29).

Test	Control P30 (n=10)	Control P60 (n=12)	P value
Average number of Days to reach learning criterion	4.8	4.5	0.8579 (T-test)
Average number of trials completed without error on reversal learning set	6.5	5.8	0.2038 Not significant (T-test)
Average number of errors made on 1st reversal learning probe	1.6	3.4	0.6429 Not significant (T-test)
Average number of errors made on all trials	4.5	7.9	0.0678 Not significant (T-test)

Table 27: Control P30 male performance vs. control P60 male performance in the water-T-maze.

Test	10x THM/PCE P30 (n=10)	10x THM/PCE P60 (n=12)	P value
Average number of Days to reach learning criterion	5.4	7.9	0.1269 Not significant (T-test)
Average number of trials completed without error on reversal learning set	8.0	5.2	0.0007 Significant (T-test)
Average number of errors made on 1st reversal learning probe	2.9	4.2	0.4467 Not significant (T-test)
Average number of errors made on all trials	4.0	9.7	0.0066 Significant (T-test)

Table 28: 10x THM/PCE treated P30 male performance vs. 10x THM/PCE treated P60 male performance in the water-T-maze.

Test	100x THM/PCE P30 (n=10)	100x THM/PCE P60 (n=12)	P value
Average number of Days to reach learning criterion	4.6	5.4	0.2294 (T-test)
Average number of trials completed without error on reversal learning set	6.0	6.1	0.9153 Not significant (T-test)
Average number of errors made on 1st reversal learning probe	3.0	3.5	0.6191 Not significant (T-test)
Average number of errors made on all trials	5.9	7.1	0.4248 Not significant (T-test)

Table 29: 100x THM/PCE treated P30 male performance vs. 100x THM/PCE treated P60 male performance in the water-T-maze.

Females of the control and 100x THM/PCE treatment groups also showed no significant differences in the number of days needed to reach learning criterion when comparing performance of the groups at P30 and P60. Females of the 10x THM/PCE treatment group did show significant differences in the number of days needed to reach learning criterion, as it takes the P60 females

significantly longer to reach learning criterion than it takes for the females tested at P30 (p=0.0012; Table 30; Table 31 and Table 32).

Test	Control P30 (n=10)	Control P60 (n=12)	P value
Average number of Days to reach learning criterion	4.8	5.1	0.2849 Not significant (T-test)
Average number of trials completed without error on reversal learning set	6.5	6.7	0.0794 Not significant (T-test)
Average number of errors made on 1st reversal learning probe	1.6	3.7	0.0682 Not significant (T-test)
Average number of errors made on all trials	4.5	6.5	0.3570 Not significant (T-test)

Table 30: Control P30 female performance vs. control P60 female performance in the water-T-maze.

Test	10x THM/PCE P30 (n=10)	10x THM/PCE P60 (n=12)	P value
Average number of Days to reach learning criterion	4.4	8.2	0.0012 Significant (T-test)
Average number of trials completed without error on reversal learning set	6.8	5.4	0.1281 Not significant (T-test)
Average number of errors made on 1st reversal learning probe	2.1	4.75	0.2459 Not significant (T-test)
Average number of errors made on all trials	5.8	10.7	0.1034 Not significant (T-test)

Table 31: 10x THM/PCE treated P30 female performance vs. 10x THM/PCE treated P60 female performance in the water-T-maze.

Test	100x THM/PCE P30 (n=10)	100x THM/PCE P60 (n=12)	P value
Average number of Days to reach learning criterion	5.4	6.1	0.1280 (T-test)
Average number of trials completed without error on reversal learning set	7.4	4.6	0.0118 Significant (T-test)
Average number of errors made on 1st reversal learning probe	3.6	2.8	0.8669 Not significant (T-test)
Average number of errors made on all trials	5.9	7.6	0.0696 Not significant (T-test)

Table 32: 100x THM/PCE treated P30 female performance vs. 100x THM/PCE treated P60 female performance in the water-T-maze.

4.6.6 Comparison of P30 and P60 perseverance behaviors

Males of the control and 100x THM/PCE treatment groups showed no significant differences in the number of reverse learning trials completed without error when comparing the performance at P30 and P60 (Table 27; Table 29). Males of the 10x THM/PCE treatment groups do showed significant differences in the number of trials completed without error in the reverse learning series, as

males of the P60 group complete significantly less trials without error than males of the P30 group (p=0.0007; Table 28).

Females of the control and 10x THM/PCE treatment groups show no differences in the number of trials completed without error in the reversal learning series when comparing performance between P30 and P60 groups (Table 30; Table 31). P60 100x THM/PCE treated females do perform significantly worse on the reversal learning probes than females of the P30 group, as the P60 females complete significantly fewer trials without error than the females of the P30 group (p= 0.0118; Table 32).

Males and females of the control and THM/PCE treatment groups do not show significant differences in the number of errors made on the first reverse learning probe when comparing performance at P30 and P60 (Tables 27-32). Additionally, males and females of the control groups, the 100x THM/PCE treatment groups and 10x THM/PCE treated females showed no significant differences in the total number of errors made on the reversal leaning series when comparing P30 and P60 performance (Tables 27-32). However, males of the 10x THM/PCE treatment groups did show significant difference in the total number of errors made during the reversal learning probe series, as the males of the P60 group make far more errors than the males of the P30 group (p=0.0066; Table 31).

4.7 THM/PCE exposed males show deficits in sociality

The third and final criterion for a person to be diagnosed with autism is the presence of impairments in socialization (DSM IV-TR, 2000). If THM/PCE exposure is to be considered a possible causal agent in the development of autism, animals exposed to THM/PCE must be assessed for social ability. The test for assessing social ability in rodents is the three-chambered social box apparatus, which allows the experimenter to observe how the animal behaves in response to the introduction of a novel animal.

4.7.1 P85 habituation

All groups and genders of animals did not significantly prefer one chamber over the other, evidenced by the amount of time spent in each chamber and the number of entries into each chamber (Table 33; Table 34; Table 35; Table 36). Animals were allowed to proceed to the socialization trials.

	Left	**Right**	**p value**
Control (n=10)	230.1s	274.1s	0.5457
10x THM/PCE (n=10)	256.0s	236.6s	0.3023
100x THM/PCE (n=10)	252.1s	232.3	0.4384

Table 33: Mean amount of time spent in left and right chambers by males during social habituation trials. Statistics were done using a T-test with Welch correction.

	Left	Right	p value
Control (n=10)	217.4s	299.0s	0.0705
10x THM/PCE (n=10)	235.5s	255.3s	0.3678
100x THM/PCE (n=10)	233.8s	241.1	0.7960

Table 34: Mean amount of time spent in left and right chambers by females during social habituation trials. Statistics were done using a T-test with Welch correction.

	Left entries	Right entries	p value
Control (n=10)	9.4	8.8	0.7830
10x THM/PCE (n=10)	10.3	9.8	0.8069
100x THM/PCE (n=10)	8.1	8.6	0.6839

Table 35: Mean number of entries by males into right and left chambers during social habituation trials. Statistics were done using a T-test with Welch correction.

	Left entries	Right entries	p value
Control (n=10)	6.25	7.25	0.6985
10x THM/PCE (n=10)	11.3	10.6	0.7643
100x THM/PCE (n=10)	9.1	8.8	0.8214

Table 36: Mean number of entries by females into right and left chambers during social habituation trials. Statistics were done using a T-test with Welch correction.

4.7.2 P85 socialization

Females of the control, 10x and 100x THM/PCE treatment groups spent a significantly greater amount of time in the chamber of the novel animal (p=0.0122, p=0.0010 and p=0.0001, respectively; Table 37). However, entries into the chamber of the novel animal and entries into the empty chamber did not

vary significantly (Table 38). Even though all females spent a significantly greater amount of time in the chamber of the novel animal, the females of both the 10x and 100x groups did show a significant decline in entries into the chamber that contained the novel animal when compared to entries into that chamber when it was empty during habituation trials (p=0.0201 and p= 0.0092, respectively; Table 39). The time spent sniffing the novel animal did not vary significantly between groups (Table 40).

	Empty chamber	**Novel mouse**	**p value**
Control (n=10)	178.4s	310.1s	0.0122
10x THM/PCE (n=10)	174.2s	325.6s	0.0010
100x THM/PCE (n=10)	175.9s	310.0s	0.0001

Table 37: Mean amount of time spent in chamber by females with novel mouse during socialization trials. Statistics were done using a Mann-Whitney non-parametric T-test.

	Empty chamber	**Novel mouse**	**p value**
Control (n=10)	6	6.4	0.7719
10x THM/PCE (n=10)	7.3	6.5	0.6010
100x THM/PCE (n=10)	6.3	5.5	0.5505

Table 38: Mean number of entries into chamber of with novel mouse by females during socialization trials. Statistics were done using a T-test with Welch correction.

	Without mouse (habituation)	Same chamber with novel mouse	p value
Control (n=10)	6.5	6.4	0.7127
10x THM/PCE (n=10)	16.1	6.5	0.0201
100x THM/PCE (n=10)	13.3	5.5	0.0092

Table 39: Mean number of entries into the chamber during habituation trial and after placement of the novel mouse during the socialization trial by females. Statistics were done using a T-test with Welch correction.

	Control (males: n=10; females n=10)	10x THM/PCE (males: n=10; females n=10)	100x THM/PCE (males: n=10; females n=10)	p value
Male average time spent sniffing	89.1s	68.9s	94.4s	0.5649
Female average time spent sniffing	104.0s	86.3s	113.5s	0.1528

Table 40: Mean amount time spent sniffing novel mouse during socialization trials. Statistics were done using a Mann-Whitney non-parametric T-test.

Males of the 10x THM/PCE group spent a significantly greater amount of time with the novel animal (p=0.0276). Males of the control group spent less time in the chamber of the novel animal, but the difference was not significant. 100x THM/PCE treated males spent less time in the chamber of the novel animal, but again results were not significant. Out of all groups, control animals spent the least amount of time in the chamber of the novel animal (Table 41). The number of entries into the chamber of the novel animal and the empty chamber did not

differ significantly in any of the treatment groups (Table 42). Additionally, the time spent sniffing the novel animal did not vary significantly between the groups (Table 40).

	Empty chamber	Novel mouse	p value
Control (n=10)	308.1s	220.3	0.1615
10x THM/PCE (n=10)	202.4s	268.0s	0.0276
100x THM/PCE (n=10)	257.6s	293.6s	0.2202

Table 41: Mean amount of time spent in chamber by males with novel mouse during socialization trials. Statistics were done using a T-test with Welch correction.

	Empty chamber	Novel Mouse	p value
Control (n=10)	9.22	7.22	0.2610
10x THM/PCE (n=10)	10.0	6.3	0.0514
100x THM/PCE (n=10)	8.75	8.25	0.7278

Table 42: Mean number of entries into chamber of with novel mouse by males during socialization trials. Statistics were done using a Mann-Whitney non-parametric T-test.

4.7.3 P85 social novelty

Both the males and the females of the 10x THM/PCE group showed preference for social novelty, based upon the amount of time spent in the chamber of the second novel animal and their time spent in the chamber of the first novel animal (p=0.0032 and p=0.0440, respectively; Table 43; Table 44). However, this preference was not supported by the number of chamber entries, as there were no significant differences found in the number of entries into the

chamber of the original novel animal or the second novel animal (Table 45; Table 46). Additionally, there were no significant differences in the amount of time spent sniffing the original or the second novel animal (Table 47; Table 48; Table 49).

	Original mouse	Novel mouse	p value
Control (n=10)	215.1s	288.6	0.1750
10x THM/PCE (n=10)	182.6s	305.3s	*0.0032
100x THM/PCE (n=10)	215.6s	256.0s	0.2701

Table 43: Mean amount of time spent in chamber by males with original and novel mouse during social novelty trials. Statistics were done using a T-test with Welch correction.

	Original mouse	Novel mouse	p value
Control (n=10)	249.0s	237.9s	0.8289
10x THM/PCE (n=10)	163.0	302.0s	*0.0440
100x THM/PCE (n=10)	229.1s	247.8s	0.7209

Table 44: Mean amount of time spent in chamber by females with original and novel mouse during social novelty trials. Statistics were done using a T-test with Welch correction.

	Original mouse	Novel mouse	p value
Control (n=10)	5.6	6.6	0.4097
10x THM/PCE (n=10)	6.1	7.1	0.4387
100x THM/PCE (n=10)	7.6	7.0	0.6749

Table 45: Mean number of entries into chamber of original mouse and novel mouse by males during social novelty trials by males. Statistics were done using a T-test with Welch correction.

	Original mouse	Novel mouse	p value
Control (n=10)	6	6	>0.9999
10x THM/PCE (n=10)	5.1	5.5	0.8294
100x THM/PCE (n=10)	6.3	6.1	0.8439

Table 46: Mean number of entries into chamber of original mouse and novel mouse by females during social novelty trials. Statistics were done using a T-test with Welch correction.

	Original Mouse	Novel Mouse	p value
Control (n=10)	47.1s	54.1s	0.6556
10x THM/PCE (n=10)	36.1s	47.1s	0.5264
100x THM/PCE (n=10)	55.3s	66.0s	0.4515

Table 47: Mean time spent sniffing original mouse and novel mouse during social novelty trials by males. Statistics were done using a T-test with Welch correction.

	Original Mouse	Novel Mouse	p value
Control (n=10)	59.8s	56.6s	0.8475
10x THM/PCE (n=10)	46.1s	50.6s	0.3823
100x THM/PCE (n=10)	52.1s	60.0s	0.5328

Table 48: Mean time spent sniffing original mouse and novel mouse during social novelty trials by females. Statistics were done using a T-test with Welch correction.

	Control (males: n=10; females n=10)	10x THM/PCE (males: n=10; females n=10)	100x THM/PCE (males: n=10; females n=10)	p value
Male average time spent sniffing "new" novel mouse	54.1s	47.1s	66.0s	0.4399
Female average time spent sniffing "new" novel mouse	56.6s	50.6s	60.0s	0.7650

Table 49: Mean time spent sniffing novel mouse during social novelty trials. Statistics were done using ANOVA and Bonferroni's post-hoc test. Results were considered significant if $p < 0.05$.

4.7.4 P30 Habituation

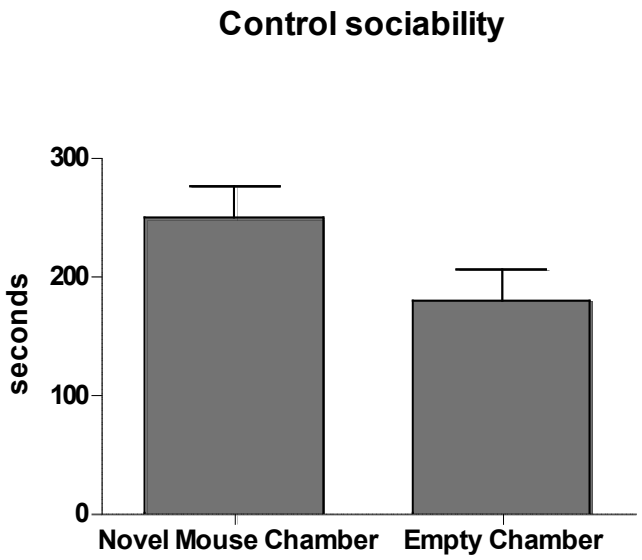
There were no significant differences in the number of entries into the either of the chambers by the control or 100x groups (Table 50).

	Left chamber	Right Chamber	p value
Control (n=10)	8.14	9	0.6784
100x THM/PCE (n=10)	9	7.5	0.4951

Table 50: Mean number of entries into chamber of novel mouse by P30 males during socialization trials. Statistics were done using a T-test with Welch correction.

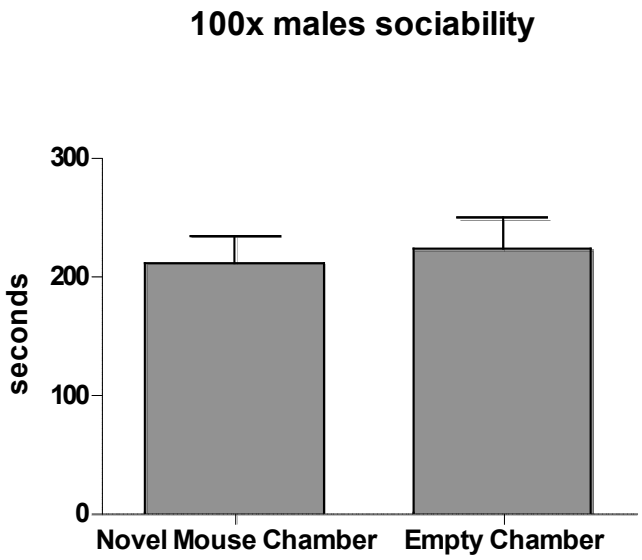
4.7.5 P30 socialization

The control males spent a significantly increased amount of time in the chamber of the novel animal versus the time spent in the empty chamber ($p=0.0281$; Figure 24). The 100x THM/PCE treated males showed a slight increase in the amount of time spent in the empty chamber, but time spent in the empty chamber was not significantly elevated (Figure 25). The control males spent a significantly greater amount of time in the chamber of the novel animal that did the males of the 100x THM/PCE treatment group ($p=0.0049$; Figure 26). The control males evidenced a significant increase in the amount of time spent sniffing the novel animal in comparison to the 100x THM/PCE treated group ($p=0.0459$; Figure 27).



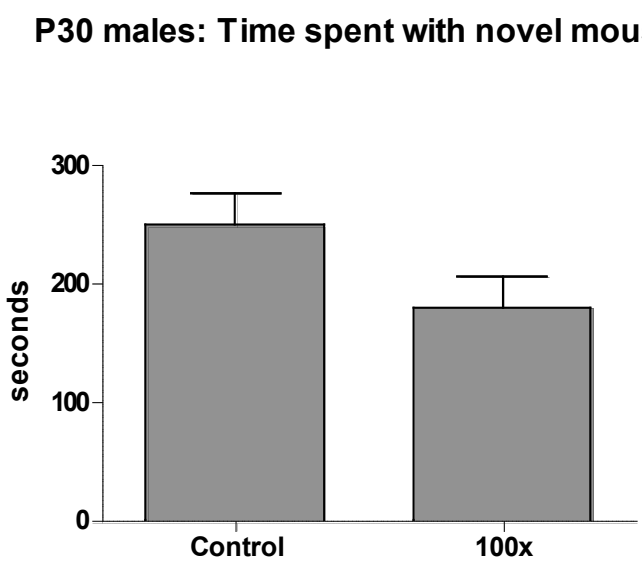
	Novel Mouse Chamber	Empty Chamber
Mean	248.6	179.5
SEM	27.1	26.4
n	10	10

Figure 24: Mean time spent by P30 males in chambers of the social apparatus. The control males spend a significantly greater amount of time in the chamber of the novel mouse ($p=0.0281$). Spending time with the stranger mouse is indicative of social behavior. Statistics were done using Mann-Whitney non-parametric T-test.



	Novel Mouse Chamber	Empty Chamber
Mean	210.3	222.0
SEM	23.3	26.1
n	10	10

Figure 25: Mean time spent by 100x THM/PCE P30 males in chambers of the social apparatus. The 100x males show no preference to the novel mouse or to the empty chamber, evidenced relatively equal amount of time spent in both chambers (p=0.0281). Statistics were done using a T-test with Welch correction.



	Control	100x
Mean	248.6	179.5
SEM	27.1	26.4
n	10	10

Figure 26: Time spent in the chamber of the novel mouse by P30 control and 100x THM/PCE male mice. The control animals spend a significantly greater amount of time in the chamber of the novel animal (p=0.0049). The 100x THM/PCE treated males spent less time with the novel animal, which is indicative of deficits in social behavior. Statistics were done using a T-test with Welch correction.

	Control	100x
Mean	23.6	12.2
SEM	4.4	2.2
n	10	10

Figure 27: Mean time spent sniffing the novel animal by P30 control and 100x groups. The control animals spend a significantly greater amount of time sniffing the novel animal than animals of the 100x THM/PCE treatment group (p=0.0459). The 100x THM/PCE treated males spend less time sniffing the novel animal, which is indicative of deficits in social behavior. Statistics were done using a T-test with Welch correction.

5. Discussion

These experiments were conducted in response to the ATSDR report that was released in 2000 which found that Brick Township, New Jersey, a region that was found to have an elevated prevalence of autism and elevated concentrations of THM/PCE in the drinking water supply. As stated in the introduction, ATSDR concluded that it was unlikely that the presence of the water chlorination byproducts played a role in the high prevalence of autism found in that community. The first evidence of the fact that the ATSDR report may have fallen short in their conclusion was the Kreiling study, which indicated that the chemicals induce changes in the levels of catalytically active PKA, a key regulator of neurodevelopment, in the neurons of clam embryos.

Based upon Kreiling's findings and the flaws in the experimental design used by the ATSDR, it seemed imperative to investigate the effect of these chemicals on neurodevelopment, using an animal model, so that abnormalities in brain regions critical to the regulation of behaviors aberrant in autism could be identified. Before moving into a time invasive behavioral model, Kreiling's results were replicated in the zebrafish model, in a microglia cell culture model, and in P4 cortex of THM/PCE treated animals.

In regard to the zebrafish model, results support Kreiling's findings, as they prove that THM/PCE exposure during early development does indeed increase the level of catalytically active PKA. These results also expand upon her findings, as there were increases in BDNF, which is likely the result of enhanced PKA activity. Therefore these results not only provide the idea that

THM/PCE exposure induces PKA to be in a catalytically active state, but also provide evidence that it is highly catalytically active. Statistically non-significant results that were found at the 1000x THM/PCE exposure are likely to be the result of compensatory mechanisms in response to the high exposure to these toxins. Additionally, the sample sizes are somewhat small, so this evidence is not conclusive.

These findings are important for two reasons. First, they support the idea that THM/PCE exposure does activate a key regulator of neural development. Hypothetically, the consequences of such activation can induce aberrant neural development. Secondly, since Ca^{2+}-dependent adenylate cyclases dominate during early development, which are responsible for the production of cAMP and necessary for activation of catalytically active PKA (Krishnan et al., 2008), a proposed pharmacological mechanism of THM/PCE action on developing neurons can be proposed.

Since it is known that THM/PCE exerts its action by binding to the volatile anesthetic binding site on the $GABA_A$ receptor, it would be assumed that such binding would induce Cl- influx and subsequent hyperpolarization, which should in theory reduce the number of L-type Ca^{2+} channels open, thereby decreasing the activity of PKA. However, at early developmental time points, the exact opposite occurs, as was demonstrated in this study and in the Kreiling study. Since it is known that $GABA_A$ mediated currents are depolarizing during early development, THM/PCE exposure would induce an outward Cl^- current, which would induce activated L-type Ca^{2+}channels, resulting in Ca^{2+}influx and

subsequent PKA activation. More interestingly, if this is the case, the $GABA_A$ receptor in zebrafish is still depolarizing at day 7 of development, evidenced by the subsequent rise in catalytic PKA levels and expression of Ca^{2+} dependent genes, such as BDNF.

Luckily, the arbitrary decision to examine day 8 in development, led to the discovery of a possible time point for the $GABA_A$ depolarizing to hyperpolarizing switch, in zebrafish neurons, evidenced by the preliminary results which show a switch in PKA configuration from dominantly catalytic to dominantly quiescent. Reduced RII subunit is indicative of inactive PKA, which is the configuration of PKA expected in adult neurons after THM/PCE exposure. Again, $GABA_A$ mediated Cl^- currents should reduce PKA activity in adult neurons. A decrease in active PKA was found in neurons taken at the later day 14 time point, although it was less pronounced than that found at day 8. This is important, because it is known that $GABA_A$ receptor expression declines during development. Therefore, at day 14, the levels of PKA are possibly less affected than they are at day 8, because there are less $GABA_A$ receptors expressed. Interestingly, the 1000x THM/PCE treatment group showed the most significant decline in PKA at day 14. Following the same line of logic that $GABA_A$ receptor expression decreases as development proceeds, it is possible that the 1000x THM/PCE treatment was potent enough to effect cells with lower $GABA_A$ expression.

In regard to the microglia cell culture model, results show unequivocally that exposure to THM/PCE increases PKA activity, which again is a key regulator

of neurodevelopment. These results are consistent with immunohistochemical data presented in the previous results from the zebrafish experiments.

Again, THM/PCE acts upon the $GABA_A$ receptor and potentiate inward Cl^- currents. This microglia cell culture produces glutamate and was grown in media that was supplemented with glutamate. Glutamate can be taken up by these cells and converted to GABA via GAD, which could account for activation of the $GABA_A$ receptor. Enhancement of GABAergic neurotransmission may have occurred due to potentiation of the $GABA_A$ receptor by THM/PCE exposure, which could account for the increase in PKA activity, since the cell culture is derived from an immortalized line of microglia from P8 male cerebellum (Butovsky et al., 2007).

In any event, these experiments and Kreiling's experiments prove that THM/PCE do induce changes in PKA levels and activity in neural tissues. Kreiling's experiments prove that the effect is not ubiquitous in all tissue types, and is specific to neurons. However, if THM/PCE is to be linked as etiological factors of autism, demonstration of similar physiological effects must be demonstrated in the cells of an organism by which the chemicals do not come in physical contact. Therefore, all subsequent aims of this experiment were moved into a mammalian model.

Results obtained in PKA activity assays in P4 animals provide evidence that THM/PCE induces increased activity of PKA, in a mammalian system, as was proven in the clam, zebrafish models and microglia cell culture models. More interestingly, activity of PKA was only increased in males exposed to

THM/PCE. Additionally, control males evidenced less PKA activity than control females.

Since THM/PCE is a $GABA_A$ current potentiator, gender-specific differences regarding the development of the GABAergic system may provide insight into understanding the pharmacological mechanisms that caused the observed results. As previously stated, $GABA_A$ receptor currents change from inducing depolarization to inducing hyperpolarization. Interestingly, there is a gender specific difference in the timing of this developmental switch. Males undergo the switch at a later point in time, as it has been reported that GABA currents result in depolarization at P14. It is thought that this extension of $GABA_A$ mediated depolarization is the result of increased levels of estradiol in the neonatal male brain. Females undergo the $GABA_A$ developmental switch much earlier, as GABA currents are found to result in hyperpolarization at P4, which is ten days earlier than that which is observed in males.

Since the females undergo this switch at P4, it is possible that the THM/PCE treatment did not show any effect, because the $GABA_A$ mediated currents are in the process of switching from depolarizing to hyperpolarizing. In the case of the males, since they have a delay in $GABA_A$ current switch, THM/PCE binding to this receptor may have induced depolarization in cells, thereby increasing the chances of L-type Ca^{2+} channels opening and subsequent activation of PKA.

In regard to behavioral testing, it was found that the males of 100x THM/PCE treatment group evidence a significant decrease in vocalizations in

response to maternal separation. This decrease in vocalizations is not due to defects in structures critical to vocal ability, as they were capable of emitting calls during the separation and were shown to have a significant increase in the number of vocalizations emitted in response to the cold. Additionally, these animals did not come from mothers who evidenced lessened maternal care; therefore, maternal care deficits were not the cause of the fewer number of calls.

Since these animals did show significantly decreased vocalizations in response to separation from the mother and littermates, and a significant increase in vocalizations in response to cold, the vocalization results reveal the animal's perception of stress. Their heightened number of vocalizations in response to cold proved that the 100x THM/PCE treated males perceived the cold stimulus as being more stressful than the control and 10x THM/PCE group. The control and 10x THM/PCE treatment groups did not differ significantly in their perception of cold, evidenced by the number of vocalizations that they emitted. Therefore, any hypotheses that suggest that the 100x THM/PCE males make fewer calls during maternal separation because THM/PCE treatment is anxiolytic, can be rejected, since they made a significantly greater number of calls than all other groups in response to cold and that fewer calls during maternal separation is indeed some form of maternal attachment deficit.

It must also be noted here that the females in all THM/PCE treatment groups did not vary significantly from controls in any of the experimental parameters. This is also evidence that supports the hypothesis that the maternal care of the pups did not vary between groups, even before evaluating the results

of the maternal retrieval tests. If maternal care were a factor influencing the male's responses to the maternal separation task, female littermates would be expected to have results more similar to their male siblings.

The idea that the males of the 100x THM/PCE group vocalized less specifically under the condition of maternal separation brings up a controversial point, as these results point to the idea that there are deficits in maternal attachment behavior. Similar results in animals lacking the mu-opiod receptor and the suggestion that such a model may be useful in autism research has been met with a firestorm of controversy (Moles et al, 2004). In response to this controversy, it has never been proven that autistic individuals have impairments in expressing physical discomfort, and it is proven that they do not communicate effectively, even with their mothers. Therefore, disregard of an animal model that displays these exact behaviors seems to be unsubstantiated.

Although results were not significant, the 100x males did vocalize less in response to male bedding, which is also suggestive that these animals are somewhat indifferent to social interactions, yet are highly stressed, which again, resembles autistic behavior.

Importantly, the gender specific results found in these experiments bolsters the idea that the differences found in the PKA activity assays in response to THM/PCE, may be involved in the development of the pathological behaviors found in these experiments.

In regard to anxiety testing, results prove that exposure to THM/PCE at both the 10x and 100x THM/PCE concentrations results in anxiety that is gender

specific, as anxiety at p21 is exclusive to males. Since 84% of persons diagnosed with autistic disorders have anxiety, these results support the idea that early exposures to these chemicals induce similar behaviors that are found in persons with autism.

Again, as was found in the communication experiments, females were unaffected by THM/PCE treatment. This supports the idea that pathological differences found during early development are likely linked to gender specific differences in the ways by which neurons of males and females respond to THM/PCE treatment. Again, since THM/PCE interacts with the $GABA_A$ receptor, it is possible that gender specific differences in the development of the GABAergic system, are involved in the sexually dimorphic development of pathological behaviors.

The results at P60 can also be used to support this hypothesis. At P60, both the males and females displayed the same anxiolytic types of behaviors. The P60 100x THM/PCE treatment group was the only group of animals that evidenced significant differences, or a reversal in behavior, when compared to behavior at the earlier P21 time point. It was expected that exposure to THM/PCE throughout adult development would result in animals displaying lowered anxiety since these chemicals are known to potentiate $GABA_A$ currents. In addition, at the P60 time point, gender specific response to THM/PCE had disappeared, which is probably due to the fact that the male animals have caught up to the females in reference to GABAergic development. Since the target receptor of THM/PCE is no longer different between males and females, in

reference to its pharmacological action, it was not surprising that prolonged exposure to THM/PCE induced a similar behavioral phenotype in both genders.

This finding, yet again, points to the idea that these chemicals are indeed mediating behavioral effects through the GABAergic system, and that behavioral differences found at earlier time points are the result of gender specific differences in GABAergic development.

In regard to spatial learning, experiments were originally performed at P60. P60 results show that both males and females of the 10x THM/PCE treatment groups have deficits in learning, as they took significantly longer to reach learning criterion than the control and 100x THM/PCE treatment groups. It is known that there are abnormalities in neuronal cell packing in the hippocampus of some individuals with autism (Bauman and Kemper, 2005). However, since autism is characteristically diagnosed during early development, and the same hippocampal deficits are not shown by any of the THM/PCE treated animals at P30, it is unlikely that damage to the hippocampal region occurred during early development, and is more likely that the damage found here is the result of prolonged chemical exposure. Additionally, this damage is not gender specific, so it points to the idea that persistent exposure to these chemicals, regardless of gender induces changes in the hippocampal region. Such changes may be the result of apoptotic cell death.

Strangely, the animals of the 100x THM/PCE treatment group do not show any deficits in learning. However, considering data presented in the anxiety tests that prove that the 100x THM/PCE animals have significantly reduced anxiety in

response to prolonged treatment; it is possible that performance is better on the learning task because their spatial learning ability is not hindered by stress. Since the animals of the 10x THM/PCE treatment group had increased anxiety in comparison to the 100x THM/PCE group, it is possible that the 10x THM/PCE group displayed impaired spatial learning ability because they are more anxious than both the controls and 100x THM/PCE treatment group.

No animals in any of the P60 treatment groups displayed perseverance behavior, which is characteristic of the autism phenotype. It is possible that perseverance behavior was ameliorated in this group of animals because of the anxiolytic effects of prolonged THM/PCE treatment, which was demonstrated by performance on the elevated plus maze. However, comparison of results obtained at different time points concerning the 10x THM/PCE treated males and the 100x THM/PCE treated females do not help to substantiate the anxiolytic explanation for reversal of perseverance behavior.

10x THM/PCE treated males did make significantly fewer errors on perseverance behavior trials at P60. They did show a reduction in anxiety at P60 in comparison to P21, yet this reduction was not significant. Still it is possible that even a small reduction in overall anxiety alleviates the presence of perseverance behaviors. Interestingly, if comparing the total number of trials completed without error, the P6010x THM/PCE treatment group had a significant increase in the total number of errors made on all reverse learning trials. It is possible that once the animal makes a mistake when searching for the platform, it becomes extremely stressed. Therefore, the increased number of errors made

on the total number of trials may be more indicative of an anxiety response and not perseverance behavior, which supports the idea that prolonged treatment of THM/PCE does specifically alleviate perseverance behaviors at low concentrations, but not overall levels of anxiety.

However, a major confound in the interpretation of this data exists in response to the 100x THM/PCE treated females performance on the perseverance behavior task. These animals perform significantly worse at P60. Previous experiments show that the P60 females have a statistically significant reduction in anxiety in response to 100x THM/PCE treatment when compared to the control and 10x THM/PCE treatment groups, but reduction of anxiety is not significant when compared to anxiety levels determined at earlier time points. Still at earlier time points, these females performed better on the perseverance behavior task, when anxiety levels were relatively similar. This data shows that it may be preemptive to explain amelioration of perseverance behavior in males based upon reduction of anxiety, as it is not supported by results obtained in females.

In regard to social behavior, tests of socialization were originally conducted on the P85 group of animals. These results were difficult to interpret, as the animals do not show clear preference for socialization. In cases such as all female groups and the 10x THM/PCE treated males, it would appear that they are more social, based upon the amount of time they spent in the chamber of the novel animal. However, this preference was not supported by the number of entries into the chamber of the novel animal versus the empty chamber, as the

number of entries was not significantly different. Additionally, the idea that the animals had a preference for socializing was not supported by data that showed that there was actually a significant reduction in the number of entries into the chamber of the novel animal in comparison to entries into that same chamber during habituation trials. Finally, further proving that the preference for social interactions between all groups was equivocal was the fact that there were no significant differences found in the amount of time spent sniffing the novel animal.

In reference to the social novelty tests, both the male and the female 10x THM/PCE treatment groups spent more time in the chamber of the second novel animal. However, their preference for social novelty was not supported by the number of entries into the chamber of the second novel animal, nor was it supported by the amount of time they spent sniffing the second novel animal, when compared with the time spent sniffing the first novel animal. Finally, preference for social novelty was not supported by any significant increase in time spent sniffing the novel animal, evidenced by no significant differences in sniff time of the novel animal between the treatment groups.

Therefore, P85 results do not definitively show that there are differences in social behavior between animals of the control and experimental groups, nor do they show a definitive preference for social novelty. In any event, prolonged THM/PCE exposure proved to have an anxiolytic effect, so it is possible that prolonged treatment makes the animals behave more like their control

counterparts, as they have significantly reduced levels of anxiety to influence their social behavior.

Performing this assessment of social ability at P85 can not prove if there are developmental effects on the animals, as prolonged treatment has been shown to ameliorate some of the pathological behaviors found at earlier time points, such as perseverance behavior and anxiety. Therefore, in order to determine if the 100x THM/PCE treated males, who have met all of the behavioral criteria for an autistic mouse model, did display the final behavior necessary for use as a valid autism model, P30 animals were subjected to the socialization tasks.

Results from these tests are supportive that 100x THM/PCE treatment does induce social indifference, as control animals preferred the novel animal and 100x THM/PCE animals did not. This was evidenced by the significantly increased amount of time spent in the chamber of the novel animal by the controls, and by the significantly increased amount of time spent sniffing the novel animal by the controls. 100x THM/PCE treated animals did not prefer either chamber, thereby showing indifference to the presence of the novel animal. Additionally, they did spend significantly less time sniffing the novel animal than did the controls.

Taking all findings into account, the gender specific finding of enhanced PKA activity in the neurons of male animals, carried over into the behavioral model, which helps to explain what pharmacological mechanisms may be at work to induce gender specific behavioral differences in response to THM/PCE

treatment. There are many gender specific differences in neurodevelopment and in the metabolism of THM/PCE. However, based on results in these experiments, it is difficult to say which set of differences are culprit in the etiology of the pathological behaviors found in the mice.

In the case of the gender specific metabolism of the chemicals, males metabolize THM/PCE faster than females. This is not dynamic, as males will always metabolize these chemicals faster than females. Two possibilities may lead to the gender specific behavioral pathologies. One possibility is that the metabolic byproducts, phosgene and dichloromethane, induce more cellular damage than the unmetabolized THM/PCE. If this is the case, the males will be more affected at earlier time points. However, it would be assumed that once enough of these metabolized chemicals accumulated in the brains of the females, they would develop the same behavioral pathology as the males. This was not observed, but could have been missed, as repeat tests were often done 30 days apart. However, it does seem unlikely that this is the culprit leading to the pathological behaviors shown. The reason for this is the fact that animals that tested to be anxious at P21 were relieved of their anxiety after prolonged chemical treatment. If the chemicals were inducing cellular damage to the amygdala by P21, it is likely that there would be no reversal of anxiety by P60, as more cells in the amygdala would be destroyed, further exacerbating the anxiety shown at P21. However, the possibility can not be ruled out until tests of cell death, such as Hoescht or DAPI staining, in the amygdala are done at varying time points during development.

As for the alternative hypothesis, it is known that THM/PCE exert anesthetic effects by binding to the anesthetic binding site on the $GABA_A$ receptor. Because of this, it is possible that any gender differences in the expression of $GABA_A$ receptor, or $GABA_A$ receptor function, could cause the gender specific behavioral pathologies observed. Literature searches regarding gender specific differences in the GABAergic system revealed that males have a prolonged period of depolarizing $GABA_A$ current, in comparison to females, as was previously discussed throughout this paper. Eventually, around day 14 of postnatal development, male GABAergic development catches up to the female, and currents become hyperpolarizing. However, this ten-day lag in GABAergic neurodevelopment may be enough to have long lasting consequences that persist throughout development. Again, if the $GABA_A$ current is depolarizing, and PKA is activated, as was shown in the P4 males, heightened PKA activity could lead to a variety of aberrant molecular cascades that could cause all kinds of histological manifestations that could be culprit in the pathological behaviors observed. Since the males hypothetically would have ten extra days of heightened activity, it is possible that the increased activity of this enzyme resulted in abnormal neurodevelopment, which could cause the behavioral abnormalities observed.

To reiterate, heightened activity of PKA induces an increase in BDNF expression, which could cause aberrant synaptic connectivity. If connectivity in regions critical to the regulation of the behaviors tested is aberrant, behavioral manifestations may result, such as those shown by the THM/PCE treated males.

Additionally, long-range connectivity that is regulated by the netrin-DCC system could also be affected by heightened activity of PKA, thus resulting in abnormal long-range connectivity, which again could account for the pathological behaviors found in the males. Since the females undergo the developmental switch earlier, and therefore reduction in the expression of the $GABA_A$ receptors earlier than males, they are not as affected by THM/PCE exposure. It is the possible mechanism that is responsible for the pathological behaviors that were observed in the males and not in the females. However, data obtained from these experiments cannot be used to prove the validity of this hypothesis, so additional tests are needed. It is suggested here that electrophysiological recordings of Ca^{2+} current in various brain regions bathed in THM/PCE are done in males and females at P4 and P10. This would support or disprove the idea that $GABA_A$ elicited depolarization in males could induce Ca^{2+}dependent gene expression.

The attempt to prove this hypothesis using protein expression level comparisons as determined by Western Blot has come up negative. However, the experimental approach to prove this idea may be inappropriate. First, it is possible that to detect such subtle differences in protein expression is difficult with this method. Immunohistochemistry would likely be a better approach, but has flaws as well. If using confocal microscopy, a quantitative comparison of GAD levels between samples could be nearly impossible to accomplish, as processing of each sample under investigation could vary, as could microscope settings, which would influence luminescence intensity measurements.

In any event, results from these experiments prove unequivocally that 100x THM/PCE treatment in males does induce all behavioral abnormalities that are needed for one to be diagnosed with autism. However, it must be first be noted that even though this is a much higher concentration of THM/PCE that was found in the Brick Township Municipal Water supply, mice are known to metabolize these chemicals 78x faster than humans (Delic et al., 2000). Additionally, developing humans exposed to the Brick Township water had additional mechanisms of exposure than did the mice used in this study. It has been shown that the most effective mechanism for THM/PCE exposure is inhalation exposure, followed by dermal exposure, with gastrointestinal absorption being the most benign route of exposure. The primary source of inhalation exposure is showering in warm water. Making this route of exposure even more dangerous is the temperature of the water that is used during the shower. A 10ºC difference in water induces a 100-fold increase in THM/PCE found in the bloodstream (Yang et al,. 2009). Therefore, women taking warm showers while pregnant or during breastfeeding, end up with very elevated concentrations of THM/PCE in their blood, which is delivered to the developing fetus or to the infant via milk.

Therefore, a 100x THM/PCE concentration that was introduced to the mice in the study is probably comparable to the levels of THM/PCE that would be present in the blood of a human exposed to a 1x exposure. Therefore, behavioral manifestations that were found in these experiments should not be dismissed because on the basis of the higher concentration used.

The 100x THM/PCE males show that these chemicals are not benign and do cause behavioral abnormalities. The 100x THM/PCE animals were found to have communication deficits, as evidenced by a significantly lower number of vocalizations in response to maternal separation. The animals were not stressed by separation, and did not communicate distress to their mothers, as did the other THM/PCE treatment groups and the controls. Interestingly, they were more stressed by cold than the other THM/PCE treatment groups, and did make a significantly greater number of vocalizations in response to cold exposure. These findings support the idea that the animals have a maternal attachment deficit and a communication deficit with their mothers. The animals response to the cold shows that they are more stressed by situations that do not have social components, which is supported by the results on the elevated plus maze and the P30 social behavior tests. The 100x animals were highly stressed by the elevated plus maze, a nonsocial stressor, and were indifferent to the presence of the novel animal during the social behavior tests, which could be considered a social stressor. The combination of these tests shows a classic autistic phenotype, as autistic individuals are not antisocial, they are indifferent to socialization.

Interestingly, the 100x THM/PCE treated males do show elevated perseverance behavior as well, which supports the idea that these chemicals are involved in autistic behavioral pathology.

The 10x THM/PCE treatment group evidenced some autistic behaviors, but not all, as did the 100x THM/PCE GROUP. This groups showed elevated

anxiety, but no significant deficits in communication or increased perseverance behavior. These findings point to the idea that the amygdala is damaged more easily than other brain regions those are responsible for the more complex behaviors that are found in autism.

The females do not demonstrate pathological behaviors during early development, and even during late development do not evidence any pathological behaviors that are used in autism diagnostics. These results point to the idea that there is a gender specific susceptibility to toxins that can be involved in the etiology of autism. This idea corresponds with the known gender ratios of autism, as in persons within normal IQ range the prevalence is 4:1.

As for the late development results, it seems that anxiety plays a critical role in regulating more complex behaviors. The 10x both the males and the females of the THM/PCE treatment group evidenced a significant deficit in spatial learning ability, yet the animals of the 100x THM/PCE treatment groups did not evidence such deficits. Anxiety testing at this same time point showed that both males and females of the 100x THM/PCE treatment group had significant reductions in anxiety. Also, there were no deficits in learning at P30, even though males of both the 10x and 100x THM/PCE treatment groups evidenced anxiety just eight days earlier at P21. It is proposed here that the hippocampus of the animals in both the 10x and 100x THM/PCE treatment groups are not affected by P30, and since anxiety between groups was not significantly different, this had no influence on learning behavior. However, by P60, hippocampal deficits were found in the 10x THM/PCE groups and not the 100x THM/PCE

treatment groups. It is possible that deficits do exist in 100x THM/PCE groups, but they were not exacerbated by anxiety. There may be other factors involved, but there is no explaination at the present time. Since the 100x THM/PCE group appeared less stressed, they may have been more apt to learn, unlike their 10x THM/PCE counterparts who did not have the same anxiolytic effect, as their treatment was not as concentrated. Therefore, it is possible that the defects noted in the 10x THM/PCE treatment group were found because there was no alleviation of anxiety as there was in the 100x THM/PCE groups. Animals treated with 100x THM/PCE performed similarly to control animals, which are thought to have normal hippocampal development, but more anxiety than the 100x THM/PCE treatment. Therefore, there it is proposed that spatial learning ability can be enhanced, if stress is reduced.

In reference to the reversal in anxiety found in the 100x THM/PCE treated males, and the reduction of anxiety in the 100x THM/PCE treated females, support for the idea that the chemicals do indeed interact with the $GABA_A$ receptors of the amygdala. This also bolsters evidence that THM/PCE exert their influence pharmacologically through the $GABA_A$ receptor, and that differences in behavior may have been mediated through differential development of the GABAergic system all along.

Taking all of these results into account, results suggest that GABAergic gender related differences in neurotransmission might be involved in the etiology of autism. If this is the case, that the pharmacological mechanisms that induced such behavioral abnormalities were mediated through differential development of

the GABAergic system, other chemicals, such as anesthetics and alcohol may induce similar effects and may contribute to the autistic phenotype in males. Studies of rodent behavior using these behavioral tests would ascertain the validity of such a relationship.

THM/PCE, chemicals found in Brick Township, an area found to have high autism prevalence, do induce all three of the behavioral pathologies that are used in autism diagnosis. This study demonstrates for the first time that environmental pollutants or toxins do induce autistic like behaviors in mice. Data obtained in this study may provide some answers as to why there is high autism prevalence in Brick Township, New Jersey.

6. Conclusions

An increase in autism prevalence was found in a region where there was an excess concentration of THMs and PCE in the drinking water supply. THMs and PCE are potentiators of $GABA_A$ receptor currents. Previous investigations provided evidence that THM/PCE induced an increase in the amount of catalytically active PKA in neurons of clam embryos. PKA is a key regulator of neurodevelopment. In this study, we systematically investigated the effect of THMs and PCE on PKA levels and activity in multiple model systems and investigated the behavior of animals exposed to these chemicals. We have found that:

1. Zebrafish exposed to THM/PCE have a higher level of catalytically active PKA and higher BDNF expression. Prolonged treatment induces a reduction of catalytically active PKA, which suggest that the PKA response may be due to a developmental switch in $GABA_A$ receptor currents.

2. THM/PCE induces an increase in the activity of PKA in microglia cell cultures and in the cortex of P4 and P10 male mice. Since findings were gender specific, this suggests that differences in $GABA_A$ receptor development between males and females may be involved in the mechanism that induces gender specific effects on PKA activity.

3. THM/PCE induces autistic behavioral pathology that is specific to males during early and juvenile development. These findings prove that males are particularly susceptible to toxins that act upon the $GABA_A$ receptor and that such interactions may contribute to the development of the autistic behavioral phenotype.

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