Effects of bisphenol-A on oxidative stress, mitochondrial dysfunction and behavior: lymphoblasts and Drosophila melanogaster studies - Potential implications in autism

By

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Abstract

Effects of bisphenol-A on oxidative stress, mitochondrial dysfunction and behavior: lymphoblasts and Drosophila melanogaster studies - Potential implications in autism

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Autism is a behaviorally defined neurodevelopmental disorder characterized by impairments in three main areas of social interaction, communication, and repetitive, restricted interests and behaviors. There has been an increase in the prevalence of autism with recent estimation of 1 in every 50 children diagnosed with autism. Though there is no single identifiable cause for autism, several studies have shown an increase in oxidative stress and decrease in antioxidants in autism. The role of environmental factors has also been implicated in autism. Bisphenol A (BPA) is a widely used chemical in the manufacturing of plastics, and its exposure has raised concerns in a variety of conditions. The present study with lymphoblastoid cells and Drosophila melanogaster identifies BPA as an environmental risk factor for the increased oxidative stress, mitochondrial dysfunction and behavioral impairments in lymphoblasts and Drosophila melanogaster. When lymphoblastoid cells were exposed to BPA, there was an increase in lipid peroxidation and free radicals (reactive oxygen species) and decrease in mitochondrial membrane potential generation suggesting BPA induced oxidative stress and

mitochondrial dysfunction. The study also illustrates an increase in the mitochondrial DNA (mtDNA) copy number in the lymphoblasts in response to the BPA exposure. In neurodevelopmental disorders such as autism, behavior is an important component of the condition. We therefore attempted to detect behavioral modifications in Drosophila melanogaster following exposure to BPA. In this study, we used an open field assay to help identify disturbances in locomotion along with repetitive behavior in BPA-exposed flies. We also observed an abnormal social interaction between the BPA-exposed flies in a social setting. Along with the behavioral modifications, there was also an increase in the lipid peroxidation in the brains of the BPA-exposed flies. Furthermore there was also a delay in the development of the Drosophila embryos, although we did not detect any gross morphological changes in the peripheral nervous system of the embryos following BPA exposure. We have therefore demonstrated that Drosophila may be used as an animal model for complex neurodevelopmetal disorders, which have a poorly understood etiology.

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Abbreviations

ADI-R - Autism Diagnostic Interview-Revised

AGRE - Autism Genetics Resource Exchange

AIF - Apoptosis inducing factor

APAF1- Apoptosis protease activating factor 1

ASD - Autism spectrum disorder

ATP - Adenosine triphosphate

BPA - Bisphenol A

CDC - Center for Disease Control and Prevention

DSM-IV - Diagnostic and Statistical Manual

ETC - Electron transport chain

EPA - Environment protection agency

$FADH_2$ - flavin adenine dinucleotide

GPx - Glutathione peroxidase

GSH - Glutathione

H_2O_2 - Hydrogen peroxide

IL - Interleukin

MAPK - Mitogen activated protein kinase

JC-1 - 5,5′,6,6′-tetrachloro1,1′,3,3′-tetramethylbenzimidazolyl - carbocyanine iodide

LCL - Lymphoblastoid cell lines

LOAEL - Lowest observed adverse effect level

MAO - Monoamine oxidase

MDA - Malonyldialdehyde

MeOH - Methanol

MMP - Mitochondrial Membrane Potential

NADH - Nicotiamide Adenine Dineucleotide

NGS - Normal Goat Serum

NO - Nitric Oxide

PDD-NOS - Pervasive developmental disorder-not otherwise specified

PNS - Peripheral nervous system

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SOD - Superoxide dismutase

TBA - Thiobarbituric acid

VNC - Ventral nerve cord

VPA - Valproic acid

XO - Xanthine oxidase

8-iso-PGF2α - 8-isoprostane-F2α

8-OHdG - 8-hydroxy-2-deoxyguanosin

CHAPTER I

INTRODUCTION

I. Autism

Autism is a behaviorally defined neurodevelopmental disorder with an increase in prevalence in the past few years (1). The disorder was first formally described by Leo Kanner in 1943 (2), and it was considered a rare disorder for a very long time. The Centers for Disease Control and Prevention (CDC) has recently estimated that 1 in every 50 children is diagnosed with autism (1). Autism is grouped under an umbrella of autism spectrum disorders (ASD), which also includes Aspergers syndrome and Pervasive developmental disorder-not otherwise specified (PDD-NOS). According to the Diagnostic and Statistical Manual (DSM-IV) by the American Psychiatric Association (3), autism is defined by an onset in the first three years of life and characterized by impairments in three main areas – social interaction, communication and restricted, repetitive interests and behaviors. Impairments in social interactions are manifested as difficulties in establishing and maintaining eye contact, failure to develop peer relationship, and lack of social or emotional reciprocity. In the communication paradigm, there is delay in speech, stereotyped and repetitive use of words known as echolalia as well as limitation in spontaneous or imaginative play. Restricted and repetitive behaviors are manifested as obsessive desire for sameness of routines and rituals, stereotyped and repetitive motor movements. Autism is considered a heterogeneous disorder with no two children having the same manifestations. Research into early development of children that were later diagnosed with autism (4), by

analyzing their earlier home videos illustrates that these children had an indication of early developmental abnormalities as early as the first years of their life (5-7). In some children that are later diagnosed with regressive autism, there is initial normal development of social and speech skills that are lost later. Regression can occur as a loss of either language (8, 9) or social skills (10) with a smaller impact on their other acquired skills.

There is no single cause linked to autism, although the roles of genetic and environmental factors, oxidative stress, inflammation and immune abnormalities have been suggested in autism (11-14). There is an increase in the concordance rate of approximately 1-10% in dizygotic twins and a 82-92% in monozygotic twins, which suggests that interactions between multiple genes is likely the cause for autism (15, 16). There are over 103 known disease genes that have either been mutated, deleted, duplicated or disrupted in individuals with ASD, and 44 genomic loci in subjects diagnosed with ASD (17). Thus autism has a strong genetic component but with so much heterogeneity, there is no single compelling genetic rationale for the phenotypes in ASD.

II. Oxidative stress

For the production of energy i.e. adenosine triphosphate (ATP) by the cells, oxygen is transported into the mitochondria via the electron transport chain (ETC). During the transfer of oxygen via the ETC, electrons may escape and result in reduction of the molecular oxygen to form a superoxide anion ($O_2^{\cdot -}$) or free radical. Along with the superoxide ion, there is also the production of other oxygen-derived pro-oxidants - hydroxyl, peroxyl, alkoxy and nitric oxide (NO) free radicals. Superoxide ion can lead to the production of hydrogen peroxide (H_2O_2) and other harmful free radicals. During the fenton reaction, H_2O_2 reacts with iron to produce highly reactive hyroxyl radical which initiates lipid peroxidation. Apart from these reactions, there is

also production of reactive oxygen species (ROS) internally by endogenous enzymes such as xanthine oxidase (XO), NO synthase and monoamine oxidase (MAO). The free radicals can also be produced by the oxidation of catecholamines and the activation of arachidonic acid. Usually these free radicals are removed by the body's anti- oxidant system. The elimination of the ROS is carried by the primary enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) along with secondary enzymes – glutathione reductase and glucose-6-phosphate dehydrogenase, which help in maintaining a steady concentration of glutathione and NADPH necessary for optimal functioning of the primary antioxidant enzymes. These enzymes necessitate the need of cofactors such as selenium, iron, copper, zinc and manganese for their optimal activity. Other proteins necessary for ROS elimination are glutathione (GSH), iron-binding transferrin, copper-binding ceruloplasmin, α- tocopherol, carotenoids and ascorbic acid. When the increase in the pro-oxidants overpowers the body's antioxidant capacity, oxidative stress occurs leading to lipid peroxidation, protein oxidation and DNA damage thus causing cellular damage. The free radicals can also alter methylation levels thereby affecting the gene expression.

Brain has a demand for higher energy that is provided by the mitochondria (18). It is highly vulnerable to oxidative stress because of high amount of unsaturated lipids along with relatively inadequate antioxidants (19, 20). Several studies have implicated oxidative stress in neurodegenerative disorders (21) such as Huntington's disease (22), Alzheimer's disease (23, 24) and Parkinson's disease (25) and amyotrophic lateral sclerosis (26). Infants have a low glutathione levels from conception through infancy, and there is an increased need for antioxidants in the early critical period of neuronal development.

III. Increased oxidative stress in autism

Metabolic studies in autism have shown that there is an increased oxidative stress and decreased antioxidants in autism. Study on the markers of lipid peroxidation has reported an increase that may be linked to the pathophysiology in autism. Malonyldialdehyde (MDA) a marker for lipid peroxidation showed an increase in the plasma of 87% of the autistic subjects (27). There is also an increase in the excretion of 8-hydroxy-2-deoxyguanosine (8-OHdG) a product of DNA oxidation, and of 8-isoprostane-F2α (8-iso-PGF2α) - a product of fatty acid oxidation, in the urine of children with autism (28). In the erythrocytes from subjects with autism when compared to normal subjects, there was an increase in the thiobarbituric acid (TBA) reactive substances in the erythrocytes (29). In the cortical brain regions of autistic subjects, an increase in the density of lipofuscin which forms as a result of oxidative damage to cell membrane lipids has been reported (30). The important part of their findings is that the regions that were identified in the cortical brain regions are involved in language and communication. In addition to increased oxidative damage in autism, alteration in the levels or activities of antioxidants have also been reported in autism. Glutathione peroxidase (GPx) was reduced in the plasma (31) and erythrocytes (32) in subjects with autism. Chauhan et al. (27) have shown a correlation between the decrease in the antioxidant proteins - ceruplasmin and transferrin in the serum, with regression in autistic children where there was loss of previously acquired skills. Ceruplasmin and transferrin are important antioxidants in the tissues, where ceruplasmin inhibits lipid peroxidaton by iron and copper, and transferin reduces the concentration of free iron. The ceruplasmin levels were reduced in 84% of the autistic children while the transferrin was reduced in 68% of the autistic children. Another important finding for oxidative stress in autism is the presence of lower glutathione levels and increased oxidized glutathione in the plasma (33) and

brain samples of subjects with autism (34). There was also a decrease in the catalase (29) and SOD (31) activity in autism. In addition, there is an increase in the free radical production in autism. NO is a toxic free radical which upon reacting with superoxide anion generates peroxynitrate anions that are cytotoxic. Nitric oxide can be stimulated by cytokines i.e. interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-1β. NO is short lived and is measured as a stable derivative - nitrate and nitrate. Increased levels of NO in red blood cells (35) as well as an increase in the plasma levels of total nitrate-nitrite (36, 37) have been reported in children with autism. XO is a pro-oxidant that results during the production of uric acid from xanithine (38), and is increased in the erythrocytes from children with autism (29).

IV. Mitochondrial dysfunction in autism

a. Structure and function of mitochondria

Mitochondria is composed of an outer and an inner membrane; the later contains a large surface area molded into cristae that contains enzymes for oxidative phosphorylation. ROS and reactive nitrogen species (RNS) are formed endogenously by the mitochondria during oxidative metabolism and energy production. The major role of mitochondria is the generation of ATP by the oxidative phosphorylation. Acetyl-CoA is formed from the oxidation of glucose and fatty acids, which enters the tricarboxylic acid (TCA) cycle in the mitochondrial matrix. Mitochondria produces ATP by the movement of the electrons through ETC. The electrons are generated from the intermediates of the TCA cycle such as nicotiamide adenine dineucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The ETC is made of five enzyme complexes – complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc 1),

complex IV (cytochrome c oxidase) and complex V (ATP synthase) (39, 40). Complex I,III and IV help generate the proton gradient that is used by the ATP synthase to catalyze the phosphorylation of adenine diphosphate (ADP) to ATP. The complex V transports protons from the intermembrane space of the mitochondria into the mitochondrial matrix. The ETC is the main source of the ROS and the RNS, where the complex I and III produce the O_2^-. These superoxide radicals are neutralized and converted by manganese superoxide dismutase (Mn SOD) and copper/zinc SOD into H_2O_2.

The mitochondria from the human cells encode fifteen copies of small genome of 16,569 base pairs. The mtDNA contains 37 genes that encode for subunits of the complexes I, III, IV and V with the other subunits of the ETC encoded by 850 nuclear DNA (nDNA) genes. Therefore, mtDNA is a critical region for oxidative damage, mitochondrial dysfunction and ATP generation.

b. Autism and mitochondrial dysfunction

There are reports showing a disruption in the energy metabolism of the brain in autistic individuals (41-43). In a case study by Zimmerman et.al (44), there was regression of the learned skills and speech that had atypical creatinine kinase, aminotransferase and bicarbonate levels in the serum as well as reduced activities of complex I and III. The initial report that associated mitochondrial dysfunction to autism was by Lombard et.al (43), who hypothesized the role of mitochondrial dysfunction and defects in neuronal oxidative phosphorylation as the basis of autism. Many case studies of ASD subjects showed an increase in the levels of lactate, increased lactate to pyruvate ratio, increased alanine and low carnitine. Altered activities of ETC complexes have also been reported in ASD with a decrease in the complexes I–IV (45). There is not only a decrease in the activities of the ETC as evidenced by the muscle biopsies but also a

decrease in the expression levels of the ETC complexes in the postmortem brain samples of ASD subjects (42). These decreases were seen in specific brain regions. Complex I was shown to be significantly decreased in the frontal lobe, the temporal lobe had a decrease in the complexes II, III and V, and the cerebellum showed a decrease in complexes III and IV, while no change was detected in the parietal and occipital lobes when compared to age-matched controls. These changes were observed in autistic children between the ages of 4-10 years but not in older subjects (14-39). Changes in the complexes III and IV were also reported in the brain of a 3 year old child as measured by positron emission tomography (PET) (46). Another study showed an increase in the levels of brain lactate by cranial magnetic resonance spectroscopy (MRS) (47). In addition, studies of the lymphoblast cells also suggest ETC abnormalities in autism (48-50).

The mitochondrial membrane potential (MMP) is an indicator of the energy status of the cells, and there was a decrease in the MMP of lymphoblasts and an increase in the ROS production in autism (48). A higher mitochondrial respiratory rate was observed in 40% - 50% in the lymphoblast mitochondria (51) along with inhibition of the complexes I and IV when compared to controls, suggesting the increase in respiratory rate as a compensatory response to the inhibition of ATP synthesis. In the CHARGE study (50), a decrease in the activity of complex I in 60% of the autistic subjects and 40% decrease in the complex V activity was recently reported in autism.

V. Genetic abnormalities and association with oxidative stress in autism

Family and twin studies have shown an increased concordance rate in monozygotic twin (82%-92%) when compared to dizygotic twins (1-10%) and with a heritability rate for ASD at >

90% (15, 16). Abnormal gene expression, deletions and copy number variations are all linked to autism. There is general consensus that a genetically susceptible population may be vulnerable to oxidative stress in autism. Autism cannot be defined as simple monogenic disorder because many genetic mutations have been identified in autism. Genomic studies of the autism susceptible loci have identified 1q, 2q, 5q, 6q, 7q, 13q, 15q, 17q, 22q, Xq, and Xp to be involved in autism (52). Not only the loci, many neurodevelopmemtal genes as the MECP2 (53), FMRP (54), cAMP-GEF (55) , SHANK3 (56), NrCAM (57), RELN (58), NLGN4 (59, 60) have been suggested to be involved in ASD. Many genes indicated in autism are functionally involved in chromatin remodeling, actin cytoskeleton, scaffolding proteins, neurotransmitter transporters, apoptosis and cell adhesion molecules. Though there is no known single cause for autism, etiological role of environmental factors has been suggested in ASD.

The mitochondrial dysfunction in autism could also be due to the abnormal gene expression or the mtDNA mutations or deletion. 15q11-q13 duplication is one of the most frequent genetic abnormality in autism occurring in 1-5% of the autistic population (61, 62). Studies in subjects with this duplication showed mitochondrial hyperphosphorylation and deficiency of complex III (63). The deletion of 5q14.3 region in a subject with autism showed a significant decrease in complex IV activity and a slight decrease of complex I activity levels (64). Another study by Pons et al. (65) described five subjects with either mtDNA mutations or deletions. In two of the cases, there was the mutation in A33243 of the mtDNA. In a separate study, G8363A mutation in the mtDNA $tRNA^{lys}$ was reported along with complex IV defect in blood and skeletal muscle from an autistic boy (66). In a case controlled study, significant association was reported between NADH-ubiquinone oxidoreductase 1 alpha subcomplex 5

(NDUFA5) gene and autism (67). However in another study, there was no difference in the expression level of the complex I mRNA levels between the control and the ASD groups (68).

VI. Environmental factors in ASD

Environmental agents as risk factors for ASD have been widely discussed in recent years (69). Genetic susceptibility or the environment alone cannot account for the majority of autism cases (70-72). During the critical periods of development such as prenatal and perinatal, exposure to environmental factors such as viral infections – rubella and cytomegalovirus or to chemicals – thalidomide and valproic acid (VPA) have gained attention in the search for the etiology of autism. Increase in cases of autism in certain regions has shown that there is strong link between the geography and the genetic predisposition (73-75). Vulnerable individuals when exposed to these risk factors during critical period in neurodevelopment would suffer more than those individuals with no genetic vulnerability in the same environment. The environmental factors are usually non heritable, but heritable epigenetic factors can increase the susceptibility for autism as well as the variable phenotype. The link between environmental chemicals and autism susceptibility should be looked at in regions where there are increased cases of autism. Autism though behaviorally defined has recently been linked to many biochemical and immunological abnormalities. When looking at the gene x environment link, chemical exposures at low levels can cause individuals with genetic vulnerabilities to react differently than typically developing individuals. Prenatal and postnatal exposure to environmental pro-oxidants may trigger increased oxidative stress observed in children with autism (76, 77). The environmental factors may thus explain, in part, the variability that is seen in autism

a. Infections

In looking for the etiology of autism, many studies have attempted to associate autism with prenatal exposure to infections. Epidemiological studies have suggested that there is an increased incidence of disorders such as schizophrenia and autism in infants that were exposed to maternal infections in the prenatal period (78, 79). Viral infections such as the rubella have been widely implicated in the etiology of autism (80).

b. Metals

Metals are ubiquitously present in the environment with increasing exposure to humans due to the rising industrialization. There have been many studies in autism that have shown toxic levels of heavy metals in children (81-83). There is a decrease in the levels of transferring (iron-transport protein) and ceruloplasmin (copper-transport protein) in the sera of subjects with autism, and this decrease was related to the regression that was seen in these subjects (27). Phosphatidylethanolamine (PE), a phospholipid on the membrane of the erythrocytes, is reduced in autism (84). Copper oxidizes PE in a concentration and time-dependent manner and thereby reducing its levels in autism. Increased levels of metal ions such as copper along with a decrease in zinc and copper was observed in the plasma in autism. These altered levels in the metals were also correlated with the severity of autism seen in these subjects (85). In a study by Holmes et al. (82), a decrease in the levels of mercury was reported in the hair of children with autism compared to the controls, though the children with autism had a higher exposure of mercury. Ecological study in Texas showed a 61% increase in the rate of autism with an increase in the environmental mercury (74, 86). Heavy metal exposure causes a decrease in the GSH levels of the cells, thereby sequestering the metals in the brain leading to an oxidative response (87, 88)

c. Drugs

Maternal consumption of certain drugs especially during the first trimester of pregnancy can be teratogenic to the fetus. Thalidomide was a sedative drug used to treat morning sickness in pregnant women that was withdrawn later due to its teratogenicity and neuropathic effects. Research into its deleterious effect pointed to a critical period of exposure between the 20^{th} to 24^{th} days of gestation, which coincides with the closure of the neural tube. Clinical research into the behavioral effects of thalidomide exposure showed a 33% rate of autism in the exposed population (89). The molecular mechanism for the action of thalidomide has been identified as oxidative stress as a result of increased development of toxic hydroxyl radicals (90, 91).

Another drug prescribed of concern is VPA used as an anticonvulsant in the treatment of epilepsy and bipolar disorder. Epidemiological studies (92) have implicated VPA in 8.9% of the exposed population diagnosed with autism. In another study, there was a seven-fold increase in the incidence of ASD in children exposed in utero to VPA (93). Rats prenatally exposed to VPA also showed behavioral alterations that are similar to children with autism (94), many of which were reversed by environmental enrichment (95). Treatment of the mice with VPA both prenatally and postnatally also resulted in behavioral alterations (96). VPA exposure increased ROS production and induced apoptosis in post-implantation embryos (97). In human subjects, exposure to VPA also caused increase in oxidative stress (98).

d. Endocrine disrupting chemicals (EDCs)

Chemicals such as bisphenol A (BPA), polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and pesticides such as endosulfan interfere with the

functioning of the endocrine system, and hence are widely known as EDCs. The exposure to these chemicals is mainly through widely available consumer products. Many studies have looked at the developmental defects due to exposure to the chemicals even in low doses (99-102). These chemicals can cross the placental barrier to have an effect on the growing fetus, and are found in the breast milk. Exposure to EDCs may lead to disturbances in neurodevelopmental processes, which may be central to cognition, memory and attention (103-105). Many of these deficits were linked to early prenatal exposure indicating a critical period in neurodevelopment. The use of pesticides is on the rise, which has raised concerns of increased exposure to human population. Many studies have shown an increase in the presence of these pesticides and their metabolites in populations in close proximity to its application (106-108). Children are more vulnerable to this exposure, many through secondary exposure from their parents (109). These pesticides affects the developing neural system and may be the risk agents for neurodevelopmental disabilities and increased neurobehavioral disorders (110-113). Epidemiological study into the prevalence of ASD in areas near pesticide application found an increase in the prevalence in the cases when the application of pesticide was during the early embryogenesis stages of nervous system (114). Increase markers of oxidative stress such as lipid peroxidation, protein carbonyl, 8-deoyguanosine are identified in response to pesticide exposure (115-118)

- ***Bisphenol A (BPA)***

BPA (4,4'-dihydroxy-2,2-diphenylpropane) is a monomer used in the hardening of plastics. It is widely utilized in the production of polycarbonate plastics such as drinking bottles, food containers, toys and dental sealants. It is also used in the production of epoxy resins used in the production of beverage metal cans and the lining of food cans. Production of BPA has been

on the rise in recent years, in part due to the increased demand of plastics and the ease of use of these products. Exposure to BPA is mainly through the consumption of the contaminated food and beverages as a result of leaching of BPA from the polycarbonate bottles. This phenomenon was first noted in a study by Krishna et al. (119) when plastic bottles were placed in a hot water bath. An increase in the temperature and pH in the use of the bottles and containers frequently causes hydrolysis of the ester bonds, and as a result, BPA leaches out (120-122). The Chapel Hill BPA expert panel held a meeting to discuss the growing concerns of the BPA levels and the damaging effects on the human health such as attention deficit hyperactivity disorder (ADHD) and autism (123). The exposure to humans is variable depending on the dietary consumption habits and regions, though there is consensus on the exposure of BPA (124). BPA and its metabolites are found in majority of biological fluids including blood and urine. BPA is known to cross the placenta (125) and is found in the amniotic fluid, placental tissue, umbilical cord and fetal serum (126). Prenatal exposure studies have shown a five-fold increase in the BPA concentration in the amniotic fluid. Growing concerns on the exposure has led to FDA-mandated BPA-free baby bottles and other objects that are closely related to infant use.

The concerns surrounding BPA is due to its activity as an endocrine disruptor (ED). Structurally, BPA resembles estradiol and has an affinity for estrogen receptors - ERα and ERβ (127-129). Expert testimony from FDA revealed that there is an increase in the consumption of BPA in infants when they were bottle-fed, (13 μg -14.7 μg/kg body weight/day) (130) whereas the infants who were breast fed had a much lower estimated intake of about 0.2 μg-1 μg/kg body weight. The US Environment Protection Agency (EPA) suggests a lowest observed adverse effect level (LOAEL) of BPA to be 50 mg/kg bodyweight / day. Consumption of canned soup

alone can increase the levels of BPA by 77% (131). Behavioral abnormalities following BPA exposure have been reported in ADHD and schizophrenia (132-134).

The exposure to BPA causes an increase in the oxidative stress in animal models. Exposure to BPA in the prenatal period caused an increase in the lipid peroxidation (135), and a decrease in the antioxidant activity of glutathione peroxidase (135, 136). There is also decrease in the proliferation of the neural progenitor cells exposed to BPA (137). Many studies have shown that there is induction of ROS and mitogen activated protein kinase (MAPK) in response to oxidative stress due to BPA exposure (138). Vitamin C (an antioxidant) administration has shown to bring about a reversal of the oxidative damage due to BPA (139, 140).

The major aim of the study was to study whether BPA is a risk factor for inducing oxidative stress and mitochondrial dysfunction similar to that seen in autism. To analyze this we used lymphoblast cells as an in-vitro model for the study. The project also attempted to study behavioral and embryonic alterations following BPA exposure. For this, we used Drosophila melanogaster as an in-vivo model for the study.

CHAPTER II

STUDIES IN LYMPHOBLASTS

Materials and Methods

I. Subjects and Cell Culture

Lymphoblastoid cell lines (LCL) from autistic (n=10) and age matched sibling controls (n=10) were obtained from Autism Genetics Resource Exchange (AGRE), a publically available biomaterials repository (Los Angeles, CA). Sibling (controls) are from families who have at least one another child diagnosed with autism. Sample details are summarized in Table 1. The diagnosis of autism was based on Autism Diagnostic Interview-Revised (ADI-R) criteria (141). The age (Mean±S.E.) for the autistic subjects and control subjects was 5.17±0.845y and 5.13±0.89y respectively (Table 1). Diagnostic assessments and scores were obtained on each subject from the AGRE, and are shown in Table 2. LCLs were grown under continuous culture in RPMI-1640 media supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Invitrogen USA), 5% L-glutamine (Invitrogen USA) and 5% penicillin/streptomycin (Invitrogen, USA). LCLs were incubated at 37°C in a humidified atmosphere under 5% CO_2 with the cells fed every 2-3 days and split into new cultures to maintain 90% confluency.

II. Treatment of the cells with BPA or with vehicle (control)

BPA (> 99% purity; Sigma-Aldrich, St. Louis, MO, USA) was solubilized in dimethylsulfoxide (DMSO), and a filter-sterilized stock solution (200 mM) was freshly prepared for every experiment. Serial dilutions of BPA were made from which the required amount was

added to the cells. The cells were counted by automated cell counter (Countess, Invitrogen, USA), $1x10^6$ cells were used from each group for the BPA treatments. The BPA treatment of the LCLs consisted of an overnight treatment with different concentrations of BPA (25, 50, or 100 μM) in a serum and antibiotic free, and phenol red free RPMI media (treatment media) (138). Care was taken to keep the DMSO in the cells at a concentration less than 0.1%. A 0.1% DMSO dissolved in the treatment media without any BPA was used as vehicle control (0 μM BPA).

III. Effect of BPA on the cell viability

Cell viability following treatment with different concentrations of BPA was measured in the control lymphoblast cells to check for dose-dependent viability. Cell viability was measured using (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye, which is reduced to blue formazan product by mitochondrial respiratory enzyme succinate-dehydrogenase (142). LCLs ($1x10^6$) were incubated overnight with different concentrations of BPA (25, 50, 100, 200 and 300 μM). Following overnight treatment, the cells were washed and incubated at 37°C with 0.25 mg/ml MTT for 4 h. The formazan crystals that formed following the addition of MTT were solubilized by DMSO. The fluorescence of the samples was read at 600 nm on the Spectramax M5 fluorescence plate reader (Molecular Devices, USA). The data was calculated as the percentage viability of the control. The optimal BPA concentration was selected as 25 μM -100 μM for further experiments.

IV. Effect of BPA on the mitochondrial membrane potential (MMP) in the lymphoblasts.

MMP is an index of the energy status of the cell. The MMP was assayed using the fluorescent mitochondrial dye 5,5′,6,6′-tetrachloro1,1′,3,3′-tetramethylbenzimidazolyl - carbocyanine iodide (JC-1) following previously described protocol (143) with some modifications. JC-1 is a lipophilic dye that selectively enters the mitochondria. Oxidative phosphorylation across the mitochondria membrane due to the ETC complexes generates a transmembrane electrochemical potential or proton gradient, which helps drive the proton pumps for the ATP synthesis. JC-1 changes its color reversibly from green to orange to red as the membrane potential increases. This property is due to formation of J-aggregates with intense red color at high MMP upon membrane polarization that causes the shift in the emitted light from 530 nm (emission at monomeric form) to 590 nm (emission at J-aggregates) when excited at 485 nm. The color of the dye changes from green to orange to red as the mitochondrial membrane becomes more polarized.

Briefly, for each concentration of BPA, $1x10^6$ cells were first washed with serum-free media and treated overnight with BPA (25 μM, 50 μM, 100 μM) or vehicle control (0 μM BPA). Following treatment, the cells were centrifuged for 5 mins and washed with the treatment media to remove any BPA or DMSO. Cells were then stained with JC-1 (5 μg/ml) in treatment media for 30 min at 37°C in an incubator with 5% CO_2. After staining, the cells were collected at room temperature, and washed twice with treatment media. The cells were then resuspended in phosphate buffered saline (PBS), and the JC-1 fluorescence was quantified using Spectramax M5 fluorescence plate reader. The fluorescence of the JC-1 aggregate was measured at 590 excitation / 645 emission, and of JC-1 monomers was measured at 485 excitation / 530 wavelengths

emission. For each treatment, a ratio of the aggregate/monomer i.e. red/green fluorescence was calculated to measure the changes in the MMP.

V. Effect of BPA on the generation of ROS i.e. free radicals.

The effect of BPA on the levels of ROS in the lymphoblasts from control and autistic samples was determined using 2′,7′- dichlorofluorescin diacetate (DCFH-DA) (144, 145), the fluorescent dye. DCFH-DA is a non-polar, nonionic dye that crosses the cell membrane where it is deacetylated to fluorescent 2′,7′- dichlorofluorescin (DCFH) by the intracellular estrases. In the presence of ROS, DCFH converts to a highly fluorescent 2′,7′-dichlorofluorescin (DCF). The DCF fluorescence intensity is proportional to the amount of ROS. For the assay, $1x10^5$ cells were washed with the treatment media, and incubated for an hour with 100 μM DCFH-DA dye in BPA-free treatment media. After an hour, the cells were spun down and washed with the BPA-free treatment media to remove the extracellular DCFH-DA. Following this treatment, the cells were incubated overnight with BPA (100 μM) or vehicle control (0 μM BPA). The cells were then washed and resuspended in the BPA-free treatment media. The fluorescence intensity of the cells was measured using the Spectramax M5 fluorescence plate reader at the excitation wavelength of 495 nm and emission wavelength of 530 nm.

VI. Analysis of mtDNA copy number in the cells following BPA exposure by real-time polymerase chain reaction (PCR)

The mtDNA copy number was evaluated by the ratio of mtDNA to the nuclear DNA. Cytochrome b (Cyt B), NADH dehydrogenase 1 (ND1) and NADH dehydrogenase 4 (ND4)

were used to represent mtDNA, and pyruvate kinase (PK) was used to represent nuclear DNA. LCLs (n=10) from control subjects were treated overnight with either 100 μM BPA or 0.1% DMSO (vehicle control). Total genomic DNA was extracted from the treated LCLs using the DNeasy kit (Qiagen, CA, USA); and the DNA concentration was measured using Nano Drop (Nano Drop, USA). 10 ng of the DNA from each sample was used as a template for a single reaction. For the quantification of the samples, custom-made primers were purchased from Sigma (Sigma life sciences, USA). Human primers for CYTB were - forward 5'-CAC GAT TCT TTA CCT TTC ACT TCA TC-3'; reverse 5'- TGA TCC CGT TTC GTG CAA G-3'. Human primers for ND1 were - forward 5'-CCC TAA AAC CCG CCA CATCT-3'; reverse 5' CCC TAA AAC CCG CCA CAT CT -3'. Human primers for ND4 were - forward 5'-CCA TTC TCC TCC TAT CCC TCA AC-3', reverse, 5'-CAC AAT CTG ATG TTT TGG TTA AAC TAT ATT T-3'. Human primers used for PK were - forward 5'-AGC CCA AAT GGC CTT GAA G-3'; reverse 5'-AGA GAC AGA ATG CCA GTG AGC TT-3'. The reaction was performed using the SYBR green fluor qPCR Mastermix (Bio-rad, USA) in 96-well plate by combining 10 ng of the template, 320 nM of primers; and each sample was analyzed in triplicates. The amplification program of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C was used on the Bio-Rad iCycler IQ system instrument (Bio Rad, USA). Relative mtDNA copy number to the nuclear DNA was calculated by the comparative Ct method using the following equation: $mtDNA/nDNA = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{mitochondrial} - Ct_{nuclear}$.

VII. Statistical analysis

Statistical analysis of the data was performed by Graphpad Prism 6.0 software for Mac. The difference between varying BPA concentrations in the treatment groups and vehicle control

groups was evaluated by ANOVA. p-value < 0.05 was considered significant. To evaluate the correlation between BPA concentration and the different test parameters, the data was analyzed by linear regression analysis and the Spearman correlation coefficient (r) was calculated.

Results

a. Dose-dependent decrease in the cell viability following BPA treatment.

To evaluate the optimum dose of BPA for the treatment of cells, we examined cell viability using MTT assay. Following overnight treatment with BPA, the cells showed a dose-dependent decrease in their viability (fig 1). From the dose-dependent curve, we selected optimal BPA concentration to be between 25 μM and 100 μM for overnight treatment of the cells. Treatment above 100 μM decreased the cell viability by more than 50%, and therefore was not used for further experiments.

b. BPA induced decrease in MMP in a dose-dependent manner in both autism and control lymphoblasts.

The MMP of the cells from autism and control subjects was measured following BPA treatment using the JC1 dye. Comparison between different BPA and control (0 μM BPA) treatments was done by one-way ANOVA in the same diagnostic group i.e autism or control group. In the autism group (Fig 2A), there was significant decrease in the MMP ($p<0.05$) of the cells treated with 50 μM BPA (mean±S.E.=0.549±0.066) and the 100 μM BPA

(mean±S.E.=0.394±0.072) in comparison to the vehicle control samples with no BPA treatment (mean±S.E.=0.782±0.089), while there was no significant effect at 25 μM BPA treatment (mean±S.E.=0.749±0.086). There was also a significant decrease in the MMP in the cells treated with 50 μM BPA ($p<0.01$) and in the cells treated with 100 μM BPA ($p<0.05$) when compared to 25 μM BPA-treated cells. LCLs from control subjects also showed a dose dependent decrease in MMP (Fig 2A). In control group, a significant decrease in the MMP ($p<0.05$) was also observed with 25 μM BPA (mean±S.E.=0.645±0.076) when compared to cells without BPA treatment (mean±S.E.=0.749±0.081). A one-way ANOVA between the 0 μM and the 50 μM BPA treatment (mean±S.E.=0.488±0.063) showed a highly significant decrease ($p<0.001$) in the MMP of the cells but a significant decrease ($p<0.05$) when treated with 100 μM BPA (mean±S.E.=0.334±0.096). Linear regression analysis showed a significant negative relationship between BPA concentrations and MMP in both the autism ($r=-0.540$, $p=0.0003$) (Fig 2B) and control ($r=-0.543$, $p=0.0003$) groups (Fig 2C).

c. Increase in the ROS levels in response to BPA exposure of the lymphoblasts

Exposure to environmental agents may increase the ROS levels that can cause oxidative stress. A measure of the ROS generation when exposed to BPA or vehicle control (without BPA treatment) was calculated by the DCF fluorescence. In the samples from subjects with autism (Fig 3A) when exposed to an overnight dosage of 100 μM BPA (mean±S.E.=1798±91.7), there was a significant ($p<0.01$) increase by 32.7% (Fig 3A) in the levels of ROS production as compared by one-way ANOVA to the vehicle control (0 μM BPA) (mean±S.E.=1355±55.6). In

the control LCLs exposed to 100 μM BPA (mean±S.E.=1515±117.5), there was a highly significant ($p<0.001$) increase by 27.4% in the ROS levels compared to vehicle-treated (0 μM BPA) (mean±S.E.=1189±113.4). Linear regression analysis showed a significant positive relationship between the BPA concentration and ROS generation in both the autism ($r=0.698$, $p=0.0006$) and control ($r = 0.425$, $p=0.0615$) groups as illustrated in Fig 3B and 3C respectively.

d. Increase in the mtDNA copy number in response to BPA exposure in the lymphoblasts

The effect of BPA (100 μM) on the relative mtDNA copy number was evaluated in the lymphoblast cells from the control subjects. The copy numbers for ND1, ND4 and CYTB were measured when normalized to nuclear gene PK (Table 3). Complex I (NADH dehydrogenase) and III (cytochrome bc_1 complex) of the ETC are the major source of free radicals and hence the genes corresponding to these complexes were used to analyze the mtDNA copy number. The mtDNA copy number for ND1 was significantly increased ($p=0.0075$) by 235.1% when exposed to 100 μM BPA (mean±S.E=3194±735.8) as compared to the vehicle-treated (0 μM BPA) group (mean±S.E=953.1±110.9) as seen in Fig 4A. There was also a significant increase ($p=0.0102$) by 272.6% in the mtDNA copy number in the ND4/PK ratio when the cells were exposed to 100 μM BPA (mean±S.E=3240±819.2) as compared to the vehicle-treated (0 μM BPA) (mean±S.E=869.5±103.2) as illustrated in Fig 4B. Another mtDNA gene analyzed was the Cyt B, which also showed significant ($p=0.0061$) increase by 250% (Fig 4C) in the copy number of the gene when exposed to the BPA treatment (mean±S.E=4439±1012) compared to the vehicle-treated group (mean±S.E=1266±141.4). When 99% CI was taken as reference range, the mtDNA

in seven of the ten cases (70 %) had copy number increase in all the three genes affected, while one case had only ND4 and Cyt B gene affected upon BPA treatment of the cells (Table 3).

Discussion

There has been an increase in the incidence of autism in past few years, with recent reports of prevalence of autism to be 1 in every 50 children (1). This sharp increase in autism could be partly attributed to better diagnosis, owing to improved diagnostic criteria and increased awareness, but it cannot account for the exponential increase in ASD. There is an urgent need in identifying the etiology of autism as to understand and curb the rise (146-148). Genetic factors such as gene deletion, gene mutations and copy number variations all play important role in the pathophysiology of autism in relatively small number of cases. Increased incidence of autism is seen in families with earlier cases of autism and also in twin pairs, along with autistic traits in other family members (149). In a recent study of identical twins, it was shown that genetics accounted for 38% of the risk of autism while environmental factors accounted for the remaining 62% of the cases (150). Research studies into prenatal exposure to environmental contaminants such as pollution, proximity to highways, maternal occupation, have acquired heightened interest as a possible role in the etiology of autism (151-156). This trend could be due to confluence of multiple factors - interaction between the environment and the genes.

Autism as a disorder is diverse in both its etiology as well its phenotypical characteristic. With the etiology still indefinable, one feature that is common in most of the cases is the increase in the oxidative stress that is observed in autism (70, 157). There is a growing concern regarding exposure to BPA and increase in the incidence of neurobehavioral problems such as ADHD and autism along with childhood diabetes and hormonal-mediated cancers (123). The US EPA

determined 50 mg/kg/day as LOAEL of BPA, from which a safe level or maximum concentration of 50 μg/kg/day was chosen, but many studies have shown higher levels of BPA in the human samples (158, 159).

Biomarkers of increased oxidative stress and altered mitochondrial dysfunction are identified in autism (44, 63, 160-162). In this study, the effects of BPA exposure on oxidative stress markers and mitochondrial function were analyzed.

Exposure to BPA decreased the MMP of the cells in a dose dependent manner. The decrease observed was similar in LCLs from both the autism and the age-matched sibling control subjects. There was no significant difference between the vehicle-treated autism and the control group as previously shown in other studies. This discrepancy may be due to the reason that siblings from families with autism cases were used as controls in the present study and may have an inherent genetic susceptibility to damage by external factors. Studies in animals have shown that following BPA exposure, there is an increase in oxidative stress which may effect the development of the nervous system (135, 136). The MMP is an important indicator of the energy status of the cell, and depolarization of the MMP as seen due to the BPA exposure can cause an imbalance in the proper functioning and activity of the cells. The decrease in the MMP is the initial step to apoptosis and a continuous exposure as that occurs in the environment can lead to cell death.

There was also an increase in the ROS generation following exposure in both the groups (autism and age-matched sibling control). ROS are regularly produced by the cell as a byproduct of normal oxygen metabolism and may damage the cells if not removed by the antioxidants (163). This increase in the ROS production, similar to that seen in autism produces loss of membrane integrity and fluidity (49, 164)

Copy number variations (CNV) are the most common form of structural deviation in the human genome (165, 166), accounting for the heterogeneity observed in humans and also their susceptibility diseases such as schizophrenia and autism. Research of the genes necessary for mitochondrial oxidative phosphorylation show that CNV (167) and oxidative stress may be indicated in the changes in the copy number of the mtDNA (168). An interesting finding in this study is the increase in the mtDNA copy number in all the three genes evaluated (ND1, ND4 and Cyt B), following BPA exposure of the control samples. Exposure to environmental agents such as BPA results in the altered copy number similar to that seen in a significant number of subjects with autism (50). The increased ROS that is observed following the BPA exposure may result in the compensatory increase in the mtDNA (169) and mtDNA damage (170). Defects in the mitochondrial ETC complexes I and III are common in autism, the complexes encoded by the ND1, ND4 and Cyt B genes that are altered in our study. The mtDNA is highly susceptible to wide-ranging damage following exposure to ROS due to the absence of introns and histones along with decreased level of repair following injury (171). Damage to the mtDNA may last longer than damage to the nDNA, as there may be formation of consequent ROS due to lipid peroxidation, and the mtDNA is present near the mitochondria membrane closer to the mitochondrial ETC where there is an increase in the lipid peroxidation products (172-175). The mtDNA copy number was increased in 70%, 80% and 80% of the ND1, ND4 and Cyt B genes respectively following BPA exposure, though one of the control samples did show an increase even with no BPA treatment.

Previous studies have shown that there is dose-dependent increase in the intracellular calcium levels following BPA exposure, which may then induce an increase in the ROS generation (138, 176, 177). The ROS produced can affect the functioning of the mitochondria

and a decrease in the activity of the mitochondrial complex 1 activity (178). Dysfunction of mitochondria ETC is observed in individuals with autism. Research has shown that BPA inhibits the function of the GABAergic system (179), which is excitatory during the embryonic stage of neurodevelopment by triggering the influx of calcium. There is deficit in the GABAergic system in ASD (180) and the effect of other EDC such as PCBs on the GABA system may be the reason for the deficit seen in autism (181)

Prenatal exposure to BPA has an effect on the executive functioning and behavior in children (182, 183) Exposure to BPA may cause an increased oxidative stress that is seen in autism and the persistent exposure may cause the production of vicious cycle by damaging the mitochondrial function and further increase ROS levels. Taken together, these findings suggest that exposure to environmental agents such as BPA during the critical period of development may bring about increased oxidative damage and mitochondrial dysfunction similar to that seen in autism.

Figures

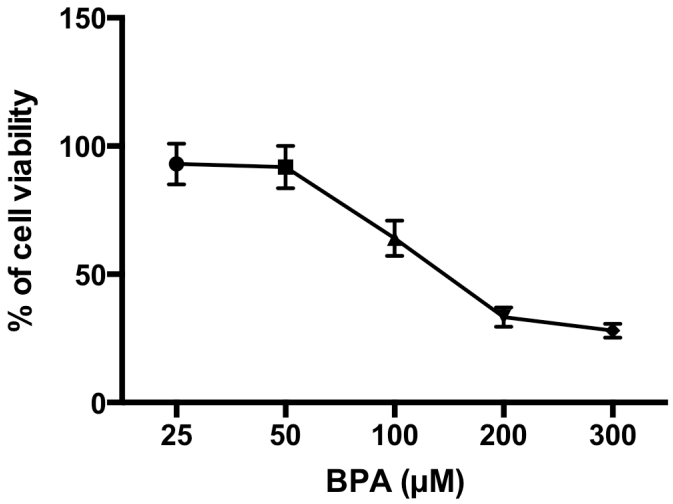


Figure 1: Effect of BPA concentration on cell viability. The viability of the lymphoblastoid cells from control subjects following overnight treatment with BPA was evaluated with MTT. The data is represented as mean±S.E. of cell viability at different concentrations of BPA calculated as a percentage of the vehicle control (0 μM BPA) treatment.

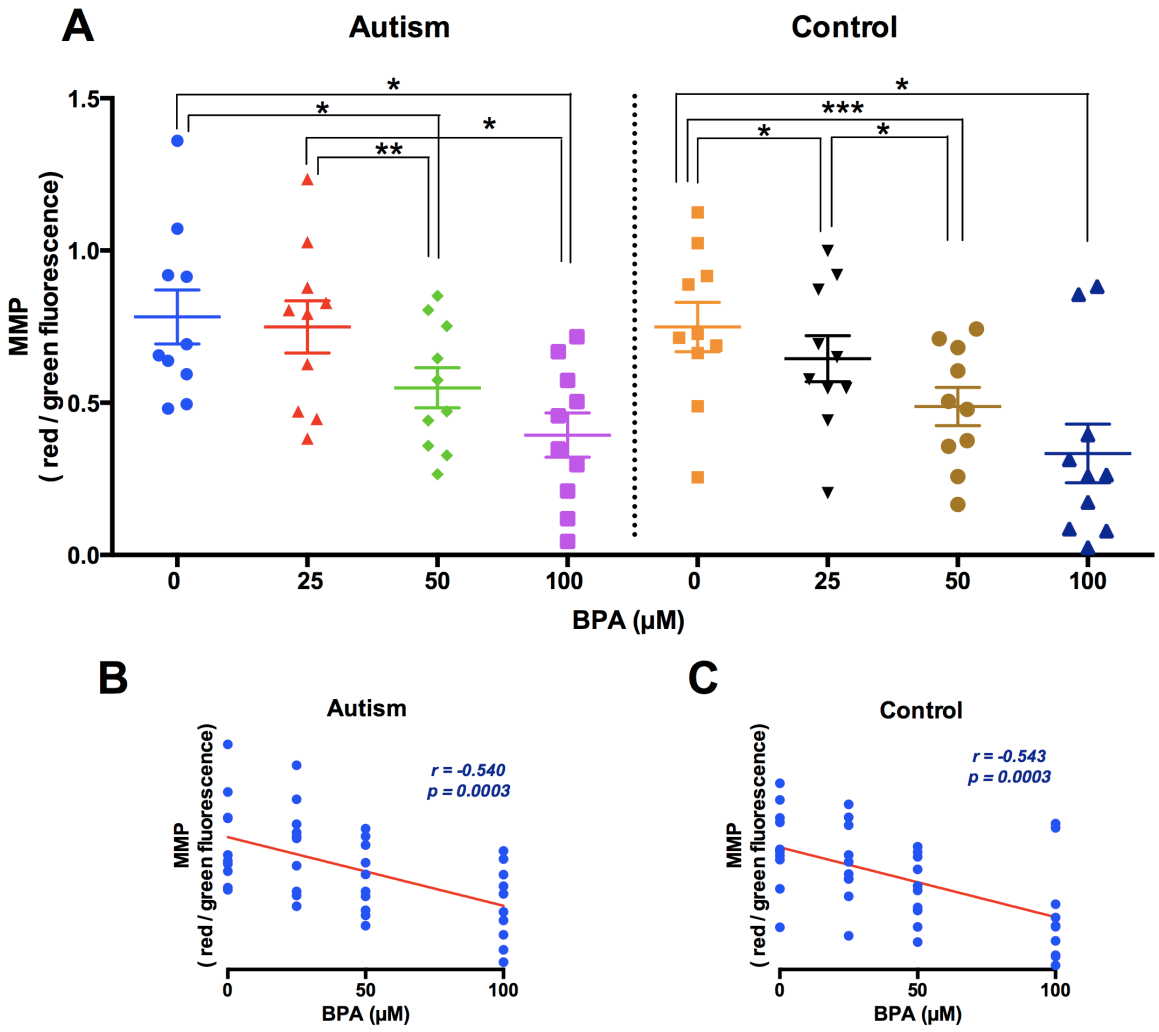


Figure 2: BPA decreases MMP in autism and control lymphoblasts. The data represents the ratio of the red to the green fluorescence of JC1 as a measure of the MMP. Fig 2A and 2B represent scattered plot of the data with the lines at the mean±S.E of the autism and control lymphoblasts, respectively. One-way ANOVA was used to measure the difference between the BPA-treated groups separately for the autism and the control lymphoblast samples. To evaluate the relationship between BPA concentration and MMP in autism and control groups, linear regression analysis was used, where r is the Spearman correlation coefficient

For statistical analysis, *p<0.05, ** p<0.01, ***p<0.001.

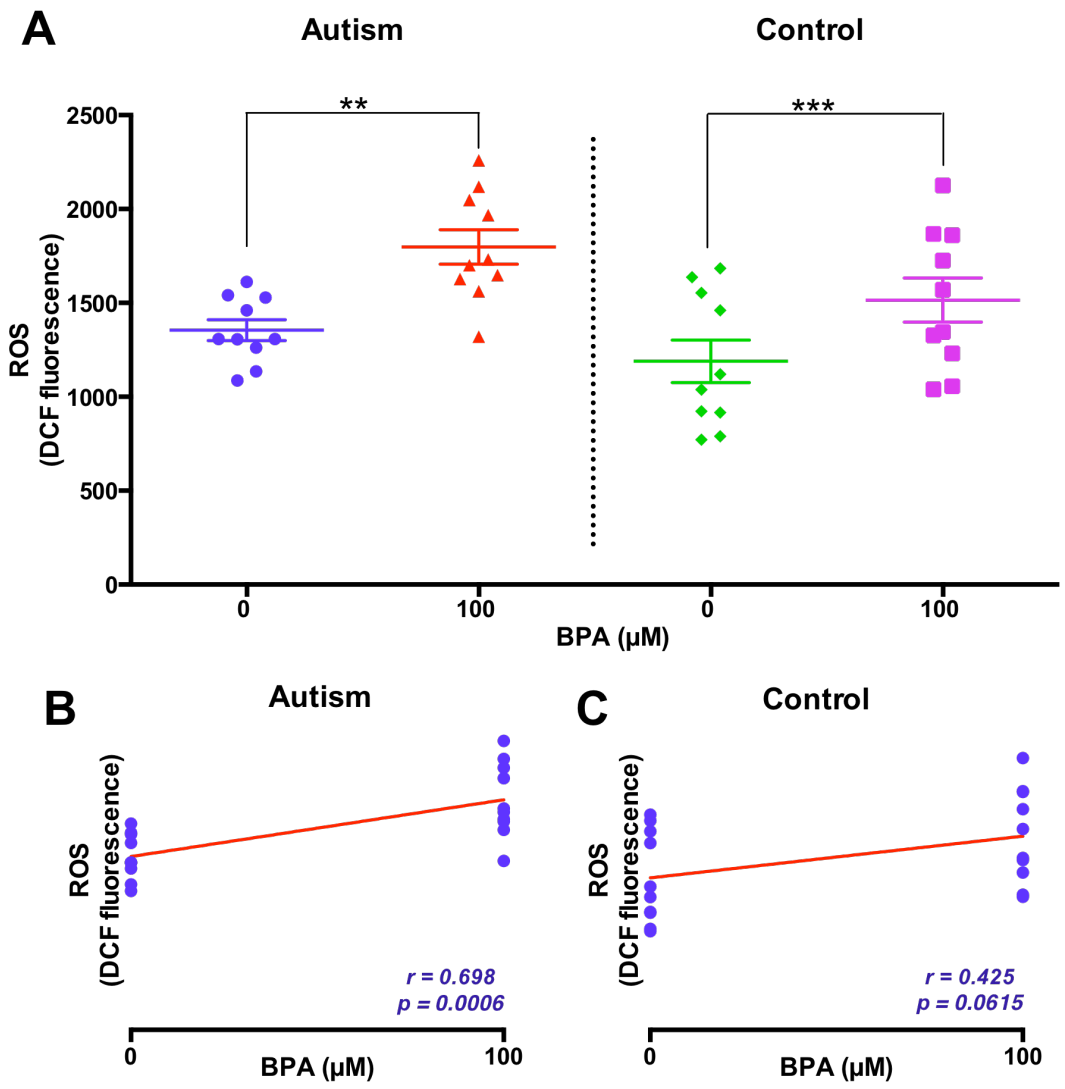
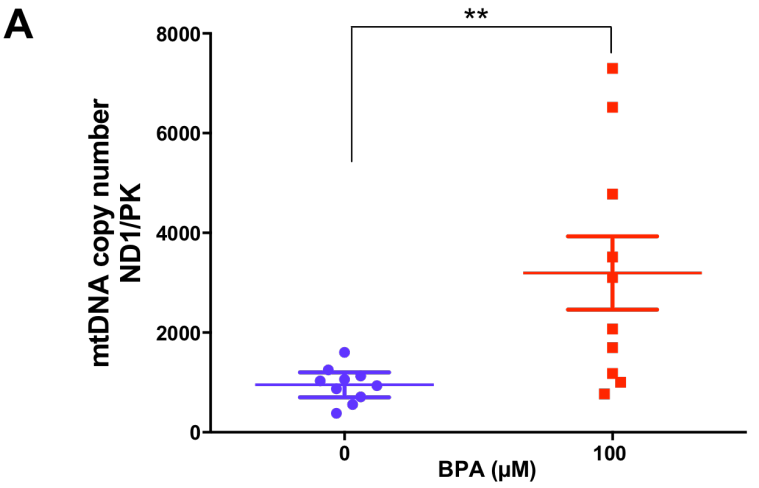


Figure 3: Increased BPA mediated ROS generation in autism and control lymphoblasts. ROS levels were measured by DCF fluorescence. Scattered plot of the data with the line at mean±S.E. is shown in fig 3A. Student t-test was used to analyze the difference between the BPA-treated groups separately for the autism and the control samples. Linear regression analysis was done to evaluate the relationship between BPA concentration and ROS levels in autism (fig 3B) and control (fig 3C) lymphoblasts, where (r) represents the spearman correlation coefficient.

For statistical analysis ** $p<0.01$, ***$p<0.001$.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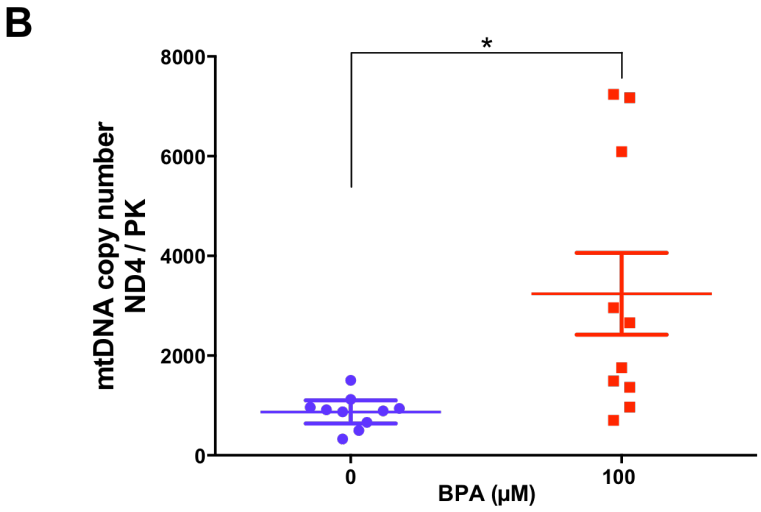
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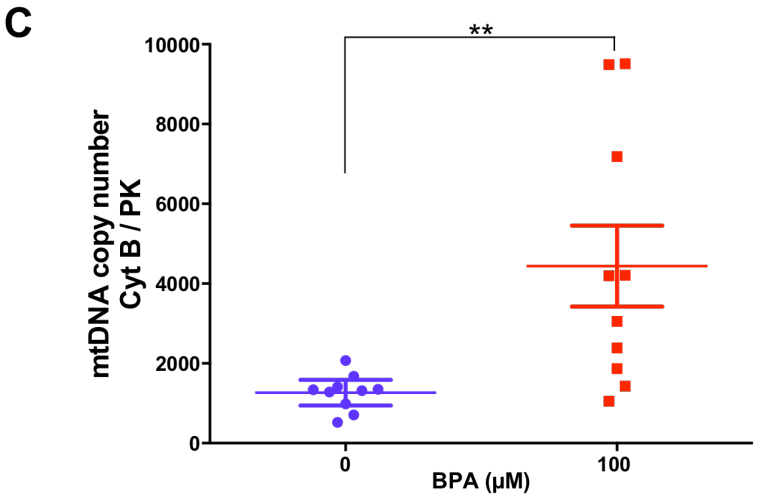
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mtDNA copy number
Cyt B / PK
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100
BPA (μM)

Figure 4

Figure 4: Increase in the mtDNA copy number following BPA exposure in lymphoblasts from control subjects. Relative copy number was assessed after ND1 (fig 4A), ND4 (4B) and Cyt B (4C) were normalized by single-copy nuclear gene PK. The data represents scattered plot with lines at mean±S.E.

For statistical analysis *p<0.05, ** p<0.01.

Table 1: Sample details of lymphoblast cell lines from autism and age-matched sibling control subjects (from affected families). All the cell lines were obtained from Autism Genetics Resource Exchange (AGRE). Mean age of the autistic subjects was 5.17±0.845y and control subjects was 5.13±0.89y

Family ID	*Blood ID*	*Age (y)*	*Diagnosis*	*Sex*
AU016705	HI0372	2.1	Autism	F
AU003304	HI0391	2.5	Autism	F
AU1359304	HI3816	2.8	Autism	F
AU067208	HI1683	3.9	Autism	M
AU038304	HI0299	4.4	Autism	M
AU004903	HI0322	5.5	Autism	F
AU054303	HI1624	6	Autism	M
AU024104	HI1076	6.5	Autism	M
AU055303	HI0752	7	Autism	M
AU052003	HI2139	11	Autism	M
AU080805	HI2019	1.9	Not Met	M
AU024005	HI0455	2	Not Met	F
AU082504	HI2255	3.1	Not Met	M
AU1231304	HI3055	3.8	Not Met	M
AU010806	HI0735	4.2	Not Met	M
AU1911302	HI5339	5.5	Not Met	F
AU1915302	HI5354	6	Not Met	F
AU1943302	HI5808	6.5	Not Met	F
AU1620302	HI4778	7	Not Met	F
AU068303	HI5030	11.3	Not Met	F

Table 2. Autism Diagnostic Interview – Revised (ADI-R) test scores in lymphoblasts from autistic subjects

Blood ID	*Abnormalities in social interaction (0-30)*	*Abnormalities in communication*		*Restricted, repetitive and stereotyped patterns of behavior (0-12)*
		Verbal (0-26)	*Non-verbal (0-14)*	
HI0372	23	-	12	4
HI0391	23	-	13	4
HI3816	25	-	12	5
HI1683	22	-	14	6
HI0299	26	-	14	6
HI0322	24	-	16	6
HI1624	19	14	-	7
HI1076	21	13	-	9
HI0752	30	-	14	7
HI2139	28	26	-	11
Cut off score for autism	***10***	***8***	***7***	***3***

Table 3: Mitochondrial DNA (mtDNA) copy number in lymphoblasts upon treatment with BPA

Blood ID	***ND1/PK***		***ND4/PK***		***Cyt B/PK***	
	No BPA	*BPA*	*No BPA*	*BPA*	*No BPA*	*BPA*
HI2019	1029.96	***3513.69****	912.58	***2658.33****	1351.66	***4209.43****
HI0455	556.35	769.30	497.84	699.94	707.41	1053.57
HI2255	381.56	***2071.85****	328.57	***1757.45****	521.49	***3055.06****
HI3055	1251.62	1005.32	1121.78	966.25	1674.97	1427.87
HI0735	1060.09	***3105.77****	891.62	***2961.86****	1317.59	***4199.05****
HI5339	1128.35	***6517.03****	964.17	***7170.54****	1414.75	***9510.50****
HI5354	1601.27*	***1698.45****	1505.23*	***1489.60****	2071.13	***2385.00****
HI5808	711.64	***7298.24****	660.59	***7239.26****	985.00	***9492.04****
HI4778	936.85	1180.35	939.67	***1363.25****	1339.76	***1871.47****
HI5030	873.10	***4776.26****	873.24	***6091.56****	1279.86	***7186.62****
Mean±S.E.	953.1±110.9	3194±753.8	869.5±103.2	3240±819.2	1266±141.4	4439±1012
99% CI	(592.7-1313)	(802.4-5585)	(534.1-1205)	(577.4-5902)	(806.9-1726)	(1150-7728)
% Increase with BPA		235.1%		272.6%		250.6%
Affected subjects (%)		70%		80%		80%

The lymphoblasts from the control group were exposed to 100 μM BPA. Relative mtDNA copy numbers of ND1, ND4 and CytB were assessed by normalization with the single-copy nuclear gene PK.
* Values were higher than the upper limit of 99% CI in the control group (no BPA) for all the genes evaluated.
# Values were higher than the upper limit of 99% CI in the control group (no BPA) for only two of the genes evaluated.

Studies with Drosophila to analyze the effect of BPA on behavior and neuronal development

CHAPTER III

DROSOPHILA MELANOGASTER

INTRODUCTION

I. Drosophila as a model to study human diseases

Drosophila melanogaster commonly known as the fruitfly, is a widely used model for the studies of neurodegenerative (184, 185) and neuropsychiatric disorders such as Parkinson's disease(186), Alzheimer's disease (187, 188), Huntington's disease (189), Fragile X syndrome (190) and Angelmans syndrome (191). Recent studies on sleep, alcoholism, and addiction have also used Drosophila as a model (192-195). The genetic makeup of the Drosophila is comparatively simple consisting of four chromosomes encoding approximately 14,000 genes (196, 197), half of (198, 199) predicted 25,000-30,000 genes in humans. Only three of the four chromosomes carry 95% of the Drosophila's genetic component. Approximately 50% of the proteins identified in the Drosophila have a similar sequence as the mammalian proteins (197, 200). Sequencing of the fly genome illustrates the interrelatedness of the fly to the human genome. Of the 289 human disease genes examined (200, 201), 177 genes (>60%) had orthologs in the Drosophila genome (202). Among 59 of the human neurological genes examined, 38 show as orthologs in the Drosophila genome (200). Neurological disorders, cancer, malformation disorders and metabolism disorders in human have a higher chance of Drosophila orthologs (202, 203). A single Drosophila gene may serve the same function as multiple related genes of the mammals, thus decreasing the redundancy seen in other vertebrate models. Thus there is a high degree of conservation of the basic cellular and molecular pathways between the Drosophila and

the mammalian system, providing many advantages to understanding the fundamental processes of a disease. The availability of wide range of genetic tools such as forward genetic screens, reverse genetic screens, markers such as lacZ, green fluorescent proteins and RNAi Drosophila has been successfully used as a model for genetically manipulated experimental models of genetic disease pathogenesis.

The advantage of studying neurobehavioral disorders in Drosophila is the presence of genes that are similar to human for normal cognitive functions, due to phlyogenetic conservation of these genes (204, 205). Though the fly and the human are distinctly different from each other, many of the molecular processes are conserved between them. Cell cycle regulators such as the cell cycle cyclins of the vertebrates share orthologs to the Drosophila genome. Cell adhesion molecules have an important role in development of the organism. The fly genome consists of three cadherin sequences, two of which are similar to the human. The Toll-dorsal pathway in Drosophila and the interleukin (IL) in the mammals have the same conserved orthologs (206). The basic process of apoptosis is similar in both the Drosophila and mammals. Drosophila has the counterparts to apoptosis protease activating factor 1 (APAF1), apoptosis inducing factor (AIF), caspase-activated DNAse as well as it encodes for two BCl2 family members (200). The neuronal system shows some of the best-conserved components between the Drosophila and the vertebrates e.g. both generate sodium-dependent action potentials. Components for both exocytosis and endocytosis for the vesicular trafficking are well conserved between the species, in both the domain structure and amino acid identities (50%-90%) (200). The Drosophila has the enzymes for the synthesis of neurotransmitters – GABA, dopamine, glutamate, serotonin and histamine. Both Drosophila and mammals respond to infection with the activation of Toll and nuclear factor kappa B (NF-κB) (207). There is significant structural and functional similarities

between signaling of the gene induced by injury in the Drosophila and mammalian response to cytokine-induced mammalian proteins (207).

Above studies recommend using fly as a model in studies mainly due to its compact genome that has been fully sequenced and the availability of sophisticated genetic approaches. The Drosophila as a model is also attractive due to its quicker generation time, large number of progeny for better selection, and easy maintenance of the animal model.

II. Life cycle of the Drosophila melanogaster

The life cycle of the Drosophila melanogaster (208) as illustrated in fig 5, lasts on an average of 40-60 days. The adult female is capable of laying hundreds of eggs within a few days, and is capable of mating 12 hrs after ecolosion. From the newly laid egg to the adult fly, there are four stages – embryo, larva, pupa and the adult fly. Once the egg is laid, it takes approximately 24 hours at 25°C to undergo embryogenesis and hatch as a 1st instar larva. The development of the embryo to the larva (1st instar larva) involves neurogenesis and differentiations in the various regions of the ventral neuroectoderm. This leads to a fully functioning larva that is capable of foraging and moving based on motor and chemosensory modalities. There are 30 individual neuroblasts in each hemisegment of the ventral nerve cord, whose birth timing and positions have already been illustrated (209-211). The larva maintains an exponential growth due to increased food intake and it also molts twice (to 2nd and 3rd instar larva) during the larva to the pupa stage. In the pupa stage, there is no more feeding and the larva metamorphosis into an adult fly in 5-6 days. During the pupa stage, the larva undergoes changes that help in tissue reorganization making a dramatic physical change as it emerges from the

pupal case. The newly emerged fly is capable of characteristic behaviors of an adult fly, such as foraging, flight, chemotaxis, phototaxis, geotaxis and mating in few hours.

III. Conserved behavior in Drosophila

One reason to study diseases in organisms is to model not only the pathology of the disease but also to study the behavioral changes that are associated with the pathology. Though the Drosophila and humans are very different from each other, with humans having more complex behaviors than the simple Drosophila; they do exhibit some behaviors that are relevant to the behavior of mammals and other higher organisms. These include courtship, circadian rhythms, learning and memory, aggression, grooming and flight navigation. Many of the molecular mechanisms for complex behaviors were first explained in the Drosophila. The analysis of behavior in experimental models not only validates them but it is also used in validity of therapeutic treatments.

Studying autism in Drosophila can help identify for two autism phenotypes; stereotyped repetitive behavior and impaired social interaction. One of the important contributions of the Drosophila system in autism research is in the field of gene x environment interaction. The fly system is also an important tool in drug discovery for neurological conditions owing to their behavior repertoire.

IV. Nervous system of Drosophila

The adult fly brain is composed of the same basic building structures – neurons and glia, as in the vertebrates. There are approximately 200,000 neurons in the fly's brain, which form distinctive functional areas, each of which are mapped in great detail (212). The Drosophila brain

consists of three parts: the central brain and a pair of optic lobes on either side. The central brain has three morphologically or molecularly defined transient parts of the early developing brain – protocerebrum, deutocerebrum and the tritocerebrum (213); this division is evolutionary homolog to the forebrain, midbrain and the hindbrain in vertebrates. Drosophila brain is bilaterally symmetrical, with innervations to the thorax and the abdomen. Neurons in the Drosophila brain show similar functional and molecular features with the presence of axons and channels similar to the mammalian system. Propagation of the action potentials is facilitated by the ion pumps and the voltage gated channels in the axons. There is the presence of presynaptic terminals with synaptic vesicles and postsynaptic receptor field. The functional circuits in the Drosophila brain mediate fundamental processes such as vision and olfaction as well as complex behaviors such as aggression, circadian rhythms, learning and memory, mating, feeding and flight navigation. The protocerebrum is the most important part of the Drosophila brain, as it contains the functional unit for higher order behaviors. The functional unit of the protocerebrum is the mushroom bodies that mediate olfactory, learning and memory processes. Many of the neurotransmitters – dopamine, glutamate, GABA and acetylcholine are conserved between the fly and the humans. In comparison to the humans, the Drosophila has a smaller amount of neurons and their synapses but is still capable of many complex behaviors that have analogs in humans. Neuronal signaling proteins are highly conserved throughout evolution. When compared to mammals, Drosophila have relatively few duplicated genes. Therefore, any pathway to be studied can be effectively modeled in Drosophila.

The capability to model a human disease in the fly has many advantages to understanding the basic cellular and molecular processes of the disease. The basic biological pathways are conserved between the fly and the human genome. Drosophila gene has orthologs to 177 of the

289 human disease genes examined (200). Another study shows that 548 of the Drosophila genes are associated with human disease genes (203).

V. Comparisons of the developmental processes between the Drosophila and the mammals

In order to study developmental disorders such as autism we need a model that not only has parallels to the nervous system of the humans, but also has a strong ability to study the development. Weisschaus and Nusslein in the 1980s identified genes involved in Drosophila embryo that had functional orthologs in humans and were useful in mammalian developmental processes. The genes that were initially identified in the Drosophila such as the HOX gene has also functional implications in the mammalian embryo. Important embryonic developmental processes for the formation of fully functional nervous system such as the dorsoventral axis patterning of the embryo (214) have similar genes, such as the bone morphogentic proteins (BMP). There is also the conservation of the pathway for the formation of the ectopic eye in the Drosophila eye absent gene (215) and the vertebrate eye gene. Members of the orthodendicle gene family responsible for embryonic brain development are conserved between the insects and mammals (216), and cephalic defects in the Drosophila can be rescued by human OXT genes (217). Thus mammalian and Drosophila genes share biological functions that can be used interchangeably for the studies of human conditions in Drosophila.

CHAPTER IV

STUDIES IN DROSOPHILA MELANOGASTER

Materials and Methods

I. Drosophila stocks

Wild type Oregon-R Drosophila stocks (a kind gift from Dr Heather Cook, Wagner College, New York) were maintained at 25°C on a standard cornmeal diet (Jazz mix Drosophila food, Fisher Scientific, USA) under 12h / 12h light and dark cycle. Newly eclosed flies were anesthetized on ice and sorted to collect virgin flies. The males and females were housed in separate vials on a standard diet prior to the treatments.

II. BPA treatment of the Drosophila

To estimate the exposure dose for the Drosophila, a previously used drug dosing protocol was followed (218). Briefly, we assumed that a 1 mg fly would consume 5% of its body weight as food per day. In the Drosophila study, the dose (1 mM) corresponds to the approximate human LOAEL of 50 mg/kg body weight/ day. A stock solution 1M of BPA (> 99% purity; Sigma-Aldrich, St. Louis, MO, USA) was prepared in dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA). For the oral administration of the BPA to the Drosophila, BPA was mixed with recently cooked and cooled standard fly food. Different concentrations of BPA (0.001, 0.01, 0.025, 0.05, 0.1,0.1 and 1 mM) were used for the social interaction assay, while the higher doses (0.5 and 1

mM) were used for all the other experiments. For all the treatments, the amount of DMSO was kept below 0.1% of the volume added. 0.1% DMSO was used as a vehicle control (0 μM BPA). Five virgin female and three male flies (3-5 day old) that were grown on standard diet were mated in vials with the BPA treated food. The vials were placed in the incubator at 25°C under 12h /12h light and dark cycle. The flies were allowed to feed and lay eggs in the treatment vials for 3-4 days after which the flies were discarded. The vials were placed back in the incubator. Newly eclosed flies (F1) were anesthetized on ice, and separated according to their gender. The F1 progeny were transferred into fresh vials containing their respective BPA-treated or vehicle control food.

III. Behavioral assays on the BPA and vehicle-fed flies

a. Social Interaction

This experiment was performed in collaboration with Dr Simon of York College, CUNY, with a technique specifically designed by her (219). Drosophilas from each treatment group (n=30-40) were separated by gender on a day prior to the experiment. On the day of the experiment, the flies were placed in a special room dedicated to behavior for an hour before initiating the experiment. The male and female flies were kept separate for each treatment to avoid interference from courtship behavior. The experiments were performed between 12 pm-3 pm to avoid interferences due to circadian rhythms, and under adequate daylight. For the social interaction behavior, a special test chamber was prepared. The test chamber (fig 6) was composed of two square glass plates with dimension of 18 cm x 18 cm. Between the two glass plates were two acrylic right angled triangles so placed as to make an internal isosceles triangle chamber with a height of 15.2 cm and base of 15.2 cm. At the bottom of the triangles were two

acrylic spacers that could be moved apart to introduce the flies. The triangles and the spacers had a width of 0.47 cm, so that the flies do not crowd/hide behind each other. Clamps held the triangle plates and the spacers between the square plates together, thus forming a chamber (the internal isosceles triangle) to enclose the flies. For the experiment, the spacers were moved slightly apart to introduce all the flies in the group by a mouth aspirator. After the introduction of the flies, the spacers were moved back to close the opening and the chamber tapped vigorously on the bench three times so that all the flies were knocked to the bottom of the chamber and were at the same starting position in the group. The chamber was placed upright for 20 minutes without disturbance. The 20-minute time period was given so that the flies have enough of time to explore and settle in the new chamber. A digital image (Panasonic camera) of the chamber with the flies was taken after 20-minutes, and exported into iphoto (Macintosh computer). Image J (NIH software) was then used to process the image into an 8 bit binary image (used for converting the flies against the light back-ground into a dark dot). The binary image was then used in the Lispix (NIH image analysis software) program, which calculates the distance between the nearest neighboring fly. The data was exported into Microsoft Excel to calculate the distance between the flies in relation to the size of the chamber.

b. Open field assay - locomotion

This assay was performed as described previously with a few modifications (220, 221). The chamber for the assay was a circular arena of 8 mm in diameter and height of 0.1 mm, open on both sides and placed on a white background and covered with a clear glass slide that could be moved for the placement of the fly and then moved back to prevent the fly from escaping. For this assay, 3-6 day old male flies were used. On the day of the experiment, the flies were

removed from the treatment vials into clean empty vials and placed on the bench in the behavior room for two hours before the experiment. All the experiments were performed at the same time every day (12pm-3pm) to prevent any behavioral changes due to the circadian rhythm. For the experiment, a single naïve male fly was aspirated into the arena and allowed to walk freely. The short height of the arena allowed for the observation of the walking behavior of the fly, and reduced the vertical movement of the fly (flying), making it easier to track the fly. The chamber was placed directly under a mounted video camera (Panasonic full HD SD-90 digital handy camera) for live video recording. The fly was allowed to acclimate to the chamber for 1 min followed by video recording for a 5 min observation period. At the end of this observation period, the fly was aspirated out of the chamber. The arena was cleaned and the glass slide changed to prevent olfactory disturbances between the subjects. The video recordings were converted to mpeg format for analysis using the Ethovision software (Noldus Information Technology, Netherlands)

Analysis of the video recording by ethovision

The Ethovision software was used to analyze several parameters of interest from the video recordings. For the software to analyze the video it divides each 5-minute video into image frames that are few milliseconds apart. Each of these frames is known as a sample, thereby creating many samples for an individual subject. Ethovision uses differencing method to analyze the animal in each video. In this method, the software compares the pixels of the current image to the reference image (previous image), thereby making a statistical (probabilistic) comparison. The statistical comparison uses the variance in the contrast of the current image to the reference image to calculate the probability that is each pixel is the subject. On the other hand, the

differencing method is used for the differences in the samples, the animal in the video is identified from the background using the image subtraction method where a dark subject (fly) is identified from a light (white) background. The different variables that were analyzed by this software were as follows:

1. ***Distance travelled -*** The total distance that the fly travelled during the 5 min observation period was analyzed. The distance moved is used as an estimate of the general activity of the fly.

2. ***Velocity -*** The velocity of the movement of the fly was measured as the walking speed with which the fly moved in the arena during the 5 min observation period. The velocity is also used as a measure of the activity of the subject.

3. ***Movement -*** Spatial movement of the fly was determined as an indicator of the activity and inactivity period of the fly. The movement of the fly was calculated by applying a threshold to the analysis; this was done to remove small wobbling movements due to the capture rate of the video by the camera. Moving vs. not moving (s) was characterized according to the previously used rates (221, 222). The fly was considered as moving when its velocity went above 4 mm/s, and was stated as not moving when its velocity was below 2 mm/s.

4. ***Mobility -*** Mobility was defined based on the changes in the pixels of the sample independent of the spatial displacement of the center of the body point. Mobility was calculated independent of movement of the center point. A threshold of 10% was used as defining immobility, and 80% as highly mobile (the percentage is the change in the pixels), thereby estimating when the fly was mobile or non- mobile, when it was at one position not moving spatially.

As the fly tracking was done in two dimensions, the trajectory could also be calculated by the analysis of the vectors formed and to predict additional data about the orientation of the fly.

5. ***Turn angle -*** It is the angle formed by the change in the direction of the movement of the fly, based on the change in the center point between two consecutive samples.

6. ***Angular velocity -*** It is the change in the moving direction of the fly per unit of time or speed of change of direction. It is computed as the ratio between turn angle and sample interval.

7. ***Meander -*** It is the change in the direction of movement of a subject relative to the distance moved by the subject, and is predicted using the ratio of the turn angle and the distance moved. The meander gives the level of tortuosity.

c. Repetitive behavior - Grooming

The grooming assay was performed as previously described (220) with some modifications. The videos that were recorded for the locomotor assay were examined using imovie for Macintosh computers. Grooming was defined as an activity in which the fly rubs its leg together or uses its legs to rub its wings, head or abdomen. Individual "grooming" episodes were scored manually for all 5 min videos. The data was entered into Microsoft Excel spreadsheet for statistical analysis.

IV. Lipid peroxidtion assay for oxidative stress

Malonyldialdehyde (MDA) is an end product of lipid peroxidation and a marker for oxidative stress in the tissues. MDA reacts with TBA to form a colored complex. Following BPA

treatment, 3-6 day old F1 flies were frozen in liquid nitrogen. For the assay, the vials of the frozen flies (40-50) were shaken vigorously to separate the head from the body. The heads were collected and homogenized in 200 µl RIPA buffer (Millipore, USA) on ice. Samples were centrifuged and the supernatant collected for the assay. To measure lipid peroxidation we used a previously described method (27). The supernatant was mixed with 300 µl 0.37% (w/v) TBA - 15% w/v trichloroacetic acid (TCA)-0.25 M HCl. The samples were heated in boiling water bath for 15 min, and cooled to room temperature. The samples were centrifuged for 10 minutes and the absorbance measured at 535 nm. The MDA content in the samples was calculated using the molecular coefficient for MDA at 1.56×10^5.

V. Immunostaining

In order to study if there is any effect of the BPA on the development, immunostaining of the Drosophila embryos was performed. The development of the Drosophila goes through a series of stages and these stages are identified according to the events or processes taking place at specific times following fertilization (223) . The stages are known as Bownes stages (224) and are illustrated in table 4.

Drosophila embryos were carefully collected at particular stages in their development.

1. Collection and BPA treatment of the embryos

For the collection of the embryos, 3-5 day old virgin female and male flies were placed in vials with BPA treated or vehicle control food. They were then transferred to embryo collection cages containing apple agar plates for embryo deposition, with yeast paste mixed with the

respective concentration of BPA or the vehicle. The cages were placed at 25°C in the incubator, and the agar plates were changed regularly. After two days of the flies getting acclimated to each other and the cage, the agar plate was set for embryo collection. On the day of the collection, the flies were allowed to lay eggs on the plate for two hours after which the plate was removed and kept in the incubator for an additional two hours to reach the blastoderm stage (stage 5). The embryos were then collected in a mesh basket and washed with tap water. Embryos were then dechorinated in 100% bleach for 2 min, followed by numerous washes with water. The dechorionated embryos were then placed in citrasolv (1:9) solution for 5 min and then carefully washed with embryo wash solution (0.9 % Nacl, 0.1% triton) followed by washes with tap water. The dechorionation by bleach followed by citrasolv, which was used for the better penetration of the treatment solution into the embryos. For the BPA treatment, the mesh basket with the embryos was immersed in Robbs medium containing the BPA (50 μM, 100 μM, 500 μM and 1 mM) or vehicle-control (0.1% DMSO). The embryos were dispersed in the mesh basket so that they form a monolayer and immersed in the treatment solution. Care was taken to allow for exposure of the top surface of the embryos for the exchange of air and development of the embryo. The embryos were incubated at 25°C for an additional 9.5 hrs to develop to **stage 14** (table 4). Stage 14 was chosen as the end point for the staining because the germ band has retracted at this stage and there is differentiation of the central and peripheral nervous system (CNS and PNS). After the embryos had been incubated for the additional time, they were collected and washed with tap water. The embryos were then fixed in 1.5 ml fixation buffer (1.3x PBS, 67 mM EGTA, pH 8.0) with 0.5 ml of 37 % formaldehyde and 2 ml heptane (Sigma - Aldrich, USA) in a glass scintillation vial. The vial was shaken vigorously for 25 min on a shaker, which allowed the vitelline membrane to fall to the bottom of the vial. When the vial was

removed from the shaker there was clear bottom layer of the heptane with the membrane that was aspirated. Then 4 ml of methanol (J.T.Baker, USA) was added to the embryos, and the vials were again placed on the shaker for 1 min. The top layer was removed and the devitellinized embryos that had sunk to the bottom were carefully tranferred into an eppendorf tube. The embryos were washed twice with methanol and stored.

2. Immunostaining of the BPA and control treated embryos

Fixed embryos that were stored in methanol were permeabilised in PBS with 0.1% triton X-100 (PBST). The embryos were then incubated in blocking buffer [1% normal goat serum (NGS) in PBST] for an hour at room temperature. Following the blocking, the embryos were incubated in primary antibodies in blocking buffer overnight at 4°C, washed three times with PBST, and incubated with fluorescent labeled secondary antibodies. The embryos were mounted on a glass slide with Flurogel (Electron Microscopy Services, PA, USA) and covered with a coverslip in the dark. To evaluate if there was overall developmental delay following BPA exposure, we stained the staged (stage 14) embryos with Fluorescin (FITC) conjugated AffiniPure Goat anti-Horseradish peroxidase (1:100) (Jackson immunoresearch). During development of the embryo in insect, the embryo is divided along its anteroposterior axis into a series of segments. The anterior portion of this segment forms the head and the brain. Each of the segments consists of a neuromere (molecularly / morphologically defined transient segment of the early brain). The brain neuromeres can be identified by labeling them with antibodies to horseradish peroxidase (HRP) (225). Anti-HRP antibodies are used to stain the surfaces of all insect neurons. The anti-HRP antibody recognizes a neural specific carbohydrate moiety of the neural glycoproteins (226). Although a specific epitope of the anti-HRP antibody has not been identified, the anti-HRP antibody may recognize a set of neural component glycoprotein called

"nervana" (nerve antigen) (227). The treated embryos were also analyzed for neural phenotype of the PNS with following primary antibodies - mouse monoclonal 22C10 (1:10) and rat monoclonal Elav (1:10) (Developmental Studies Hybridoma Bank, Univeristy of Iowa). The 22c10 antibody was used to stain the sensory neurons, and it labels the dendrites axons and cell soma. The elav antibody stains elav a nuclear specific protein to identify cell positioning of the PNS. Goat anti-rat or mouse secondary antibody conjugated with Alexa fluro 488 at 1:5000 dilution was used (Molecular Probes, Carlsbad, CA, USA). The mounted embryos were visualized with Nikon 90i fluorescent microscope equipped with Nikon C1 three-laser confocal system and Nikon DS U1 digital camera.

VI. Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. One-way ANOVA was used to examine the differences between the BPA and vehicle-treated flies. Linear regression analysis was done and correlation coefficient (r) was calculated to analyze the relationship between the behavioral parameters and BPA concentrations. To analyze the social interaction data, we used the non-parametric Kruskal Wallis test. For statistical analysis of the data, $p<0.05$ was considered significant.

Results

I. Behavioral assays

a. Social Interaction

BPA-treated flies were significantly closer than the vehicle-treated control flies

Measuring the distance between individual fly and its closest neighboring flies was used as a measure of the social interaction within the group. In a social setting, the Drosophila maintains atleast a two-body length distance between them and the neighboring fly (> 0.5 cm). The normality of the data was determined using Kolmonogrov Smirnov and Shapiro normality tests. As the distance data in our study did not follow a Gaussian distribution, we used non-parametric Kruskal Wallis analysis to test for statistical significance between the groups. Kruskal Wallis arranges the data from the lowest to highest value and then determines rank order to individual data. The box and whiskers in the fig 7, depict the box as the 1st quartile and the 3rd quartile (25th percent and the 50th percent) with the whiskers representing the maximum and minimum order ranks. The test compares and calculates the p-value for the mean of the rank sum for each group. The BPA treatment showed a significant effect on the distance maintained between the flies in a group. The BPA treated flies settled at distance less than 0.5 cm from their neighboring flies while the vehicle-treated control flies maintained distance of more than 0.5 cm. In the vehicle-treated control (0 μM) flies the upper whisker illustrates the increased values

(number of flies) in the group. Furthermore, the range of distance between BPA-treated flies was smaller when compared to the vehicle-treated control flies.

b. Open field assay

In order to study the behavioral changes due to the exposure of BPA during the prenatal and postnatal period the locomotor activity of the flies was analyzed. Locomotion is a major behavior in animals, and is linked to not only the need for exploration, foraging, mating and to escape predators but also for social interaction. Drosophila is known to display a strong thigmotaxis (affinity to the boundary wall), which was seen in different groups.

1. Exposure to BPA decreases the mean distance moved by the flies in the open field arena.

The first variable analyzed was the total distance (cm) that the fly moved during the 5 min observation period. The results are represented in fig 8A. Flies treated with 1 mM BPA treated flies (mean±SE=0.0096±0.008) travelled a significantly lesser distance (one way ANOVA, $p<0.05$) of the 0.8 cm circular arena when compared to the vehicle- treated (0 mM BPA) control subjects (mean±SE=0.012±0.0008) during the 5 min trial. There was no significant decrease in the distance moved by the 0.5 mM BPA treated flies (mean±SE=0.010±0.0006) when compared to the vehicle-treated control group. One-way ANOVA for trend analysis showed a significant ($p<0.05$) change (fig 8B), for BPA-mediated decrease in the distance travelled ($r=0.434$, $p=0.019$).

2. No significant effect of BPA on the velocity or walking speed of the flies.

There was no significant difference in the velocity or the walking speed between the BPA-treated groups (0.5 mM or 1mM) and vehicle - control treated (0 mM BPA) group as shown in fig 9A. The 0.5 mM BPA-treated (mean±SE=0.2977±0.0203) and 1 mM BPA-treated (mean±SE=0.2803±0.0228) flies did not walk at speeds significantly different from the vehicle-control treated (mean±SE=0.3304±0.0156) flies. Although there was no significant difference between the groups, a pattern towards a slower walking speed in the BPA-treated (0.5 mM or 1mM) was observed compared to vehicle-control group. There was a 10% and 15.2% decrease in the walking speed of the 0.5 mM and 1 mM BPA treated flies respectively when compared to the vehicle-treated control flies.

3. No significant effect of BPA on the movement of flies (dependent on the spatial displacement of the fly in the arena)

During their movement in the circular arena, the Drosophila had periods of activity with intervals of inactivity. For the software to identify if the sample is moving versus not moving, we applied a threshold for spatial displacement to act as a marker of activity (center point moving) and inactivity (center point not moving). One-way ANOVA analysis showed no significant difference in the activity between the 0.5 mM BPA-treated (mean±SE=0.695±0.0865) and the 1 mM BPA-treated (mean±SE=0.6227±0.0576) flies when compared to the vehicle-treated control (mean±SE=0.7113±0.0635) flies during the 5 min observation period. The results are represented in the fig 10A. For the periods of inactivity also there was no significant difference between the

0.5 mM BPA-treated (mean±SE=0.9496±0.1250) and the 1 mM BPA-treated (mean±SE=0.9253±0.1313) flies when compared to the vehicle-treated control (mean±SE=0.6809±0.0607) flies (fig 10B). However a pattern of decrease in the activity (r=0.177, p=0.3565) (fig 10C) and an increase in the inactivity (r=0.2978, p=0.1207) (fig 10D) though not significant was observed between the BPA-treated and the vehicle-treated control flies, which is comparable to the decrease in the distance moved.

4. BPA exposure increases mobility in the flies (independent of the spatial displacement of the fly in the arena)

Significant increase ($p<0.05$) was observed in the mobility of the 1 mM BPA-treated flies (mean±SE=0.1919±0.02604)) when compared to vehicle-treated control flies (mean±SE=0.1267±0.01168) as seen in fig 11A. The 0.5 mM BPA-treated flies (mean±SE=0.1684±0.0169) did not show any significant increase in the mobility. There was a significant trend with a positive slope (0.03260) showing an increase in the mobility of the flies following BPA treatment (r=0.1763, p=0.0233) (11C). When the data was examined for the immobility of the subjects, there was also a highly significant increase ($p<0.01$) in the immobility of the 0.5 mM BPA-treated flies when compared to the vehicle-treated control flies (fig 11B). Comparison of the 1 mM BPA (mean±SE=0.1676±0.0129) treated flies to the vehicle treated (mean±SE=0.1177±0.0156) flies showed a significant increase in immobility ($p<0.05$) with a linear trend of a positive slope (0.02495) and (r=0.1694, p=0.0367) as shown in fig 11D.

5. Increase in the angular velocity in the BPA-treated flies

In the environment, the fly constantly changes direction in response to external clues, food or predator. In the closed arena, the BPA-treated flies showed an increase in the angular velocity when compared to the vehicle-treated control flies, with the results represented in the fig 12A. One-way ANOVA analysis showed that the 1 mM BPA-treated flies (mean±SE=598.9±32.43) showed a significant increase ($p<0.05$) in the angular velocity when compared to the vehicle-treated control flies (mean±SE=493.6±17.14). A significant increase was also observed between the 0.5 mM BPA treated (mean±SE=584.5±24.16) in comparison to the vehicle treated control flies ($p<0.05$). Posttest for linear trend analysis showed a positive slope (52.67) indicating a highly significant trend of increase in the angular velocity ($r=0.494$, $p=0.0065$) between the BPA treated-flies and the vehicle-treated control flies (fig 12B).

6. No effect of BPA treatment on turn angle in the flies

The turn angle gives an estimation of the angle at which the fly moves, as it re-orients its trajectory when it turns. The BPA-treated flies (0.5 mM and 1 mM) turned 19.87° and 20.33° respectively while the vehicle-treated control flies turned at a mean angle of 18°. There was no significant difference between the 1 mM BPA treated (mean±SE=20.33±1.054), 0.5 mM BPA-treated (mean±SE=19.87±0.7192) flies as compared to the vehicle-treated control flies (mean±SE=18±0.5155) as seen in fig 13A. Although individual difference of BPA treatment was not significant, linear regression analysis showed a positive correlation slope ($r=0.376$, $p=0.0438$) between the turn angle and the BPA concentration (fig 13B).

7. BPA significantly increases the meander of the flies

The change in the direction of the fly movement was calculated as the meander. It showed a similar pattern as the angular velocity and the turn angle. 1 mM BPA (mean±SE=5326±7649) treated flies showed a significant increase ($p<0.05$) in the meander compared to the vehicle-treated control flies (mean±SE=3262±4327) as seen in fig 14A. There was no significant increase in the meander of the 0.5 mM BPA-treated flies (mean±SE=4831±4080) when compared to the vehicle-treated control flies. The meander showed a significant positive correlation ($r=0.456$, $p=0.013$) in the linear regression analysis between the BPA concentration and the degree of meander in the Drosophila (fig 14B).

c. Grooming

Increased frequency of grooming in the BPA-treated flies

Individual male BPA-treated flies showed a significantly increased number of grooming episodes when compared to the vehicle-treated control flies. Figure 15A shows the scattered plot data with mean±SEM of the 3 groups (0 mM, 0.5 mM and 1 mM BPA treatment). A significant ($p<0.01$) increase in the grooming of the 1 mM BPA-treated flies (mean±SE=26.7±3.367) and 0.5 mM BPA-treated flies (mean±SE=25.7±1.795) was seen when compared to the vehicle-treated control flies (mean±SE=13.6±2.083). Post-test for linear trend analysis illustrated a trend towards increase in the grooming with an increase in the BPA concentration ($r= 0.557$, $p=0.0014$) illustrated in fig 15B.

II. Increased lipid peroxidation in the brains of BPA- treated flies

Lipid peroxidation was assayed by measuring the formation of MDA, an end product and marker of lipid peroxidation and oxidative stress. There was an increase in the MDA content in the brains of the flies exposed to BPA (fig 16). A significant ($p<0.05$) increase in the MDA formation in the brains of the 1 mM BPA treated flies (mean±SE=1.854±0.131) and 0.5 mM BPA-treated flies show (mean±SE=1.776±0.051) was seen when compared to the vehicle-treated control flies (mean±SE=1.294±0.021).

III. Effect of BPA on the neurodevelopment of Drosophila embryos

Exposure to BPA prenatally and during development may lead to a delay in the development. To help identify the effect of BPA exposure on the development of the Drosophila, staged (stage 14) embryos were collected following BPA treatment and stained for pan-neural anti-HRP antibody. Development of the Drosophila embryo was ascertained by the position of the ventral nerve cord (VNC) tip in the stained embryos. During embryogenesis there is retraction of the VNC at stage 14, which can be visualized by staining with the anti-HRP antibody. We noticed that the BPA exposed embryos had a delay in this retraction compared to the vehicle-treated control embryos, though a further quantification may be needed. To further assess if exposure to BPA had an effect on the development of the peripheral nervous system (PNS) we also stained for cell positioning and neurite outgrowth with anti-elav and 22c10 antibodies respectively. There was no change in the staining pattern between the BPA-treated and vehicle-treated Drosophila embryos, suggesting that the PNS was not affected by the BPA treatment.

Discussion

Autism is a multifactorial disorder and there is no single unified mechanism identified in all of the cases. Drosophila melanogaster is recognized as a valuable model to study gene-environment interaction, and to understand the effect of the exposure of environmental toxicants. Our study is a novel attempt to identify behavioral disturbances in Drosophila following exposure to environmental toxicants such as BPA. While many studies have focused on looking either at the environment or the genetic component separately in autism, our behavioral study may help establish the Drosophila as the model to further look at the genetic disturbances following exposure to environmental toxin. The fly has many of the basic behavioral functions that can be related to the more sophisticated repertoire of the human behavior.

One of the core features of autism and other neurodevelopmental disorders such as autism, Williams and Angelman syndrome is the inability of the individual to interact socially with other individuals (54, 228-230) and it is a widely used feature in mouse models for the study (231). In a social setting, the individual maintains a personal space or a distance from another individual (personal space boundary), but also a spatial proximity to another individual for effective communication (232). When placed in a social setting, Drosophila tend to arrange themselves uniformly rather than in aggregates or randomly (233), and this social interaction in the group leads to learning of higher behaviors from their conspecifics (234). There have been studies which show that social isolation in the Drosophila reduces the fiber number in the mushroom bodies, the functional equivalent of hippocampus (235). Drosophila when placed in settings where they are allowed to interact with other flies usually maintain about a 2-body length (1-5 mm) distance among themselves, similar to other animals (233). This allows for the fly to orient itself to interact with each other. In our study, we found that when the flies exposed

to BPA were placed in a social setting, there was a decrease in the distance between them. This decrease in the distance between the neighboring flies may be due to aberrant social interaction where the flies do not maintain optimum distance that would allow them to interact with each other.

Locomotion is the change in the position of the animal in relation to its surroundings. It is a characteristic behavior of all animals, and is associated either directly or indirectly with other behaviors that the animal performs such as foraging, courtship, avoiding predators, social interaction, as well as learning and memory processes. Exploratory activity allows the animal to gather information about the novel environment, and open field assay is an important test to measure the exploratory activity in animal models of neurological disorders such as depression (236) autism (231), and is widely used to measure for activity following drug administration (237, 238). Since the locomotor activity is the result of the animal's ability to react to its immediate environment such as avoiding predator or mating, it can be regarded as the decision-making process of the current state of the animal, as a motivation to either produce the action or not. This activity can be divided into either spontaneous activity or reactivity suggesting that locomotion might be influenced by genetic and environmental factors (239). Many of the individuals diagnosed with autism are also diagnosed with ADHD (240, 241), though there is no co-diagnosis of the disorders according to the DSMV-IV criteria. Rodent models of autism show a deficit in the exploratory activity as studied by the open field test (242-244)

For the open field assay, we chose two different concentrations of BPA (0.5 and 1 mM), which reflected the federally regulated safe levels in humans (50 mg/kg body weight). The open field activity was used to evaluate the locomotor and the exploratory behavior of the animal. Pre- and postnatal exposure to BPA had an effect on the exploratory activity of the flies. There was a

decrease in the distance travelled by the BPA-exposed flies, though there was no change in the walking speed that may alter the distance that they travelled during the course of the assay. A smaller arena (8 mm) and duration for the test (5 min) ensured that the results were not confounded by other external factors such as fatigue and starvation respectively. Although previous evaluation of exploratory behavior in the Drosophila demonstrated higher initial spontaneous activity levels that decreased over time (239, 245, 246), it was not observed in our experiment as we had chosen a smaller duration (5 min) for the assay.

The BPA-exposed animals not travelling the arena stayed at the same place, and did repetitive movements such as grooming which was manually observed, and correlated with the software observation of the increase in the mobility of the animal while remaining at the same place. The legs of the Drosophila have a high density of tactile sensory organs known as the sensila trichodea (247), which the fly preens back into position after it physically comes in contact with another fly. The increased grooming that we observed in the isolated flies may be due to the need for increased sensory stimulation similar to individuals with autism that show an increase in episodes of self sensory stimulation - auditory or tactile. Restricted repetitive behaviors form a major core of the diagnosis criteria for autism according to the DSM-IV criteria (3). Children with autism manifest these repetitive behaviors in many forms such as motor stereotypies (248), which may increase during times of increased social and emotional demand (249). For the Drosophila, the open field arena is a novel environment that may be stressful and the increased grooming can be seen as a strereotypic behavior, similar to that observed in individuals with autism. A similar significant increase in the grooming behavior was observed in the Drosophila model of fragile X syndrome (220). Cleaning behavior of the Drosophila is a complex activity with organized sets of repeated movements, that disappears when the animal is

left undisturbed (250), but the animal continues to do the behavior (grooming) through out the duration of the experiment in our study.

In circular arenas, the Drosophila moves with smaller turn angles of about 12.6° (246), which is increased following BPA exposure. There is an innate desire to walk in a straight trajectory either towards an object or relevance such as another fly or food, or away from a predator (251, 252), a smaller turn angle may be the result of immediate decision. There is an increase in the turn angle that mirrors the increase in the speed or the angular velocity of movement with an increase in the BPA concentration, and may relate to the inability to process changes in the environment and react immediately. Individuals with autism have difficulty in executive decision-making (253, 254). There is also a tendency for the fly following BPA exposure to meander from the path; they show an increase in the changes made to the path of movement. The BPA-treated animals constantly reorient themselves with an increase in the turn angle and the increase in the angular velocity, thereby making a zigzag pattern of movement or navigation. In the environment, movement follows a purpose either towards food, mate or to escape from a predator. This purpose makes the animal follow a path that changes when there is a change in the purpose of the movement. When there is a change in the motivation of the animal there is a change in the trajectory of the path. Motivation for movement in the novel arena should be foraging, escape and searching for a mate for the males. The open field assay illustrates the impaired exploratory activity, which may be affecting their social approach.

Furthermore, we observed an increase in MDA, a marker for lipid peroxidation suggesting increased oxidative stress in the brains of Drosophila exposed to BPA. We also observed a delay in the retraction of the VNC at stage 14 following BPA exposure, which may propose an overall developmental delay. There was no apparent gross disturbance in the cell

positioning and neurite outgrowth during the specific stage of development following the BPA treatment as evidenced by the elav and 22c10 staining patterns.

Behavior is genetically influenced by the activation of molecules in functional regions of the brain as well as by the environment. The environment influences the genome by affecting the development and the activation of the molecules that influence behavior. Thus the environment can have an effect on the social behavior by influencing the genome, thereby a gene-environment interaction in behavioral disorders (255). The Drosophila model that we employed could be further used to study genetic influences that BPA may execute to provide for the disturbances in the behavior as seen in autism. In the social interaction assay, we see that BPA treated Drosophila are closer to each other than the vehicle treated flies, which may indicate that BPA can induce behavioral disturbances.

One of the most important challenges in studying neurodevelopmental disorders such as autism in animal models is the ability to evaluate behavioral phenotypes that reflect their core behavioral symptoms. In this study, we were able to demonstrate the behavioral deficits in Drosophila when exposed to BPA that are major features of autism. The Drosophila model following BPA exposure showed aberrant social interaction, repetitive behavior and also increased oxidative stress markers. Our model may provide valuable approach to analyze the effects of environmental agents on the behavior, and also provide a preliminary study for the role of genes x environment interaction in the neurodevelopmental and neurobehavioral disorders such as autism. Many disorders with neurobehavioral impairments have a complex etiology along with broad spectrum of symptoms among the affected individuals. Since autism is comorbid with other neurodevelopental disorders such as Fragile X, Retts and Angelmans syndrome, the models for autism need to address behavioral task relevant to them.

FIGURES

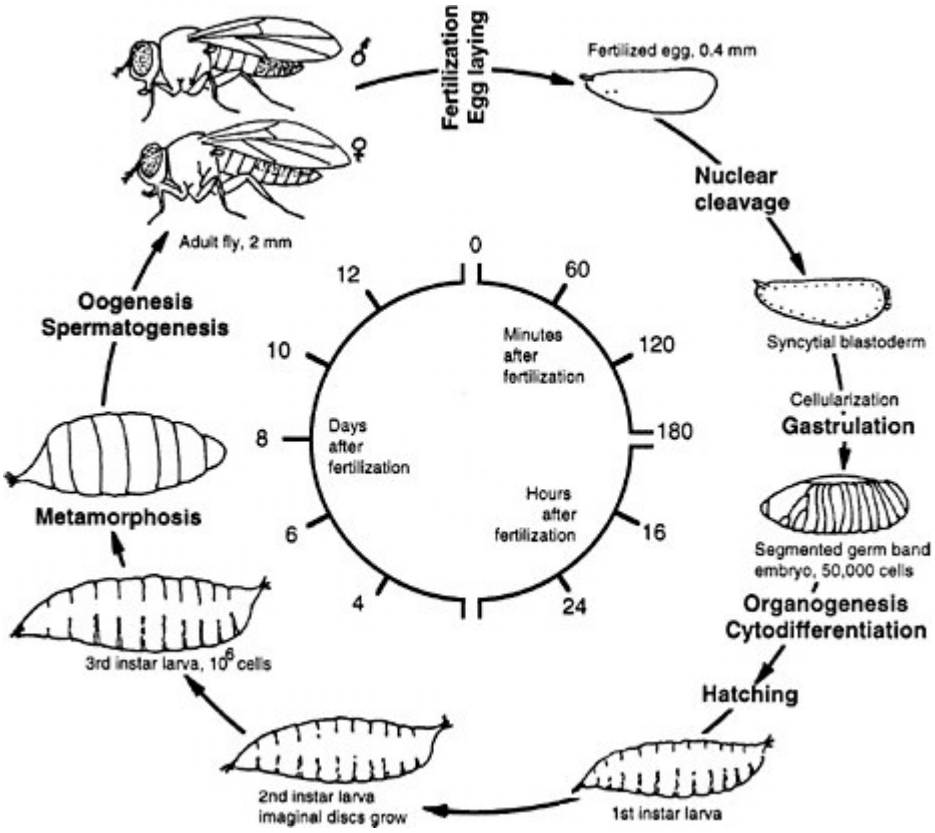

Figure 5 Life cycle of Drosophila Melanogaster (adapted from Wolpert et al.)

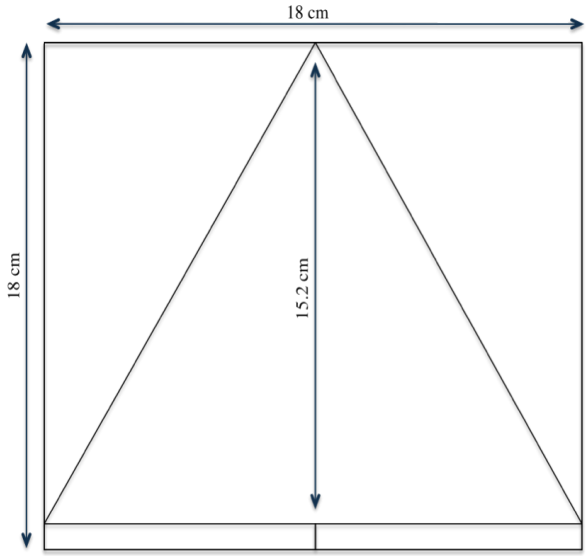

Figure 6 Schematic representation of the triangle chamber used for the social interaction assay. The chamber for the flies with an internal area of 15.2 cm was composed of two triangles between rectangular glass plates with removable glass bars at the bottom for the easy introduction of the flies.

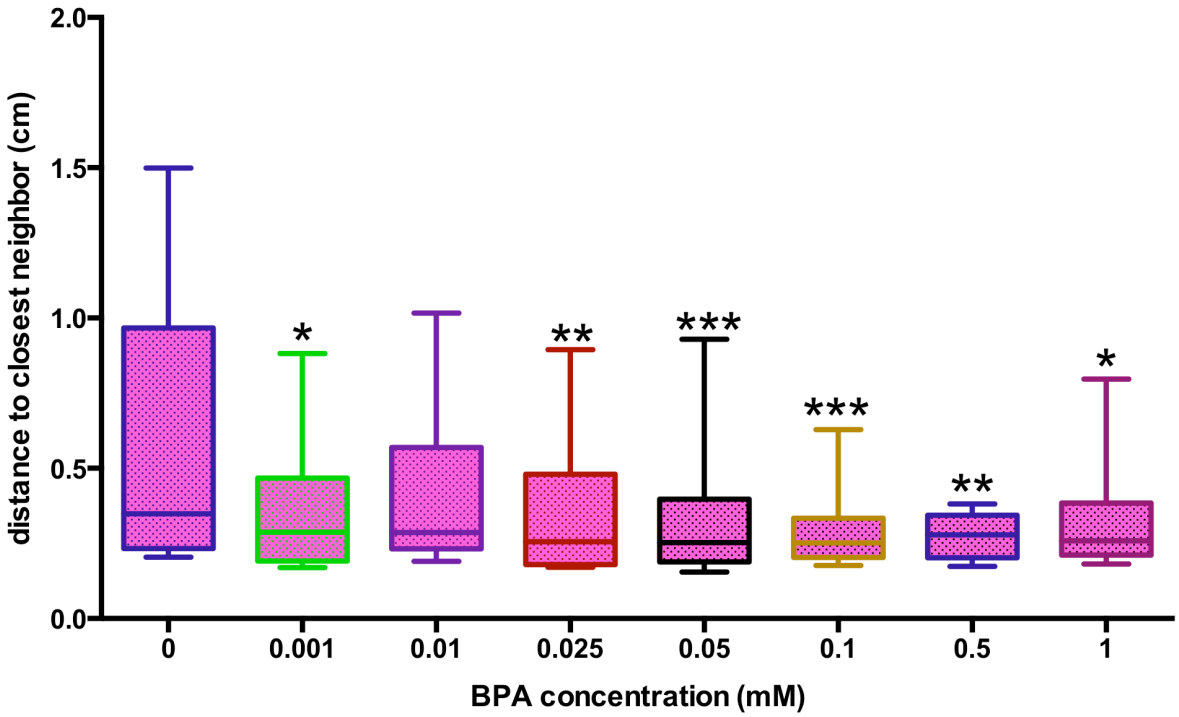

Figure 7. Effect of BPA exposure on the social interaction in the Drosophila. The box and whisker plot of the data shown here with the line inside the box representing the median and the whiskers representing the range of the distance occupied between the Drosophila in a social group. Non parametric-Kruskal wallis test was applied to analyze the data. There was a significant decrease in the distance between the neighboring BPA-treated flies when compared to the vehicle treated - control (0 μM BPA)

*For statistical analysis *p<0.05, ** p<0.01, ***p<0.001*

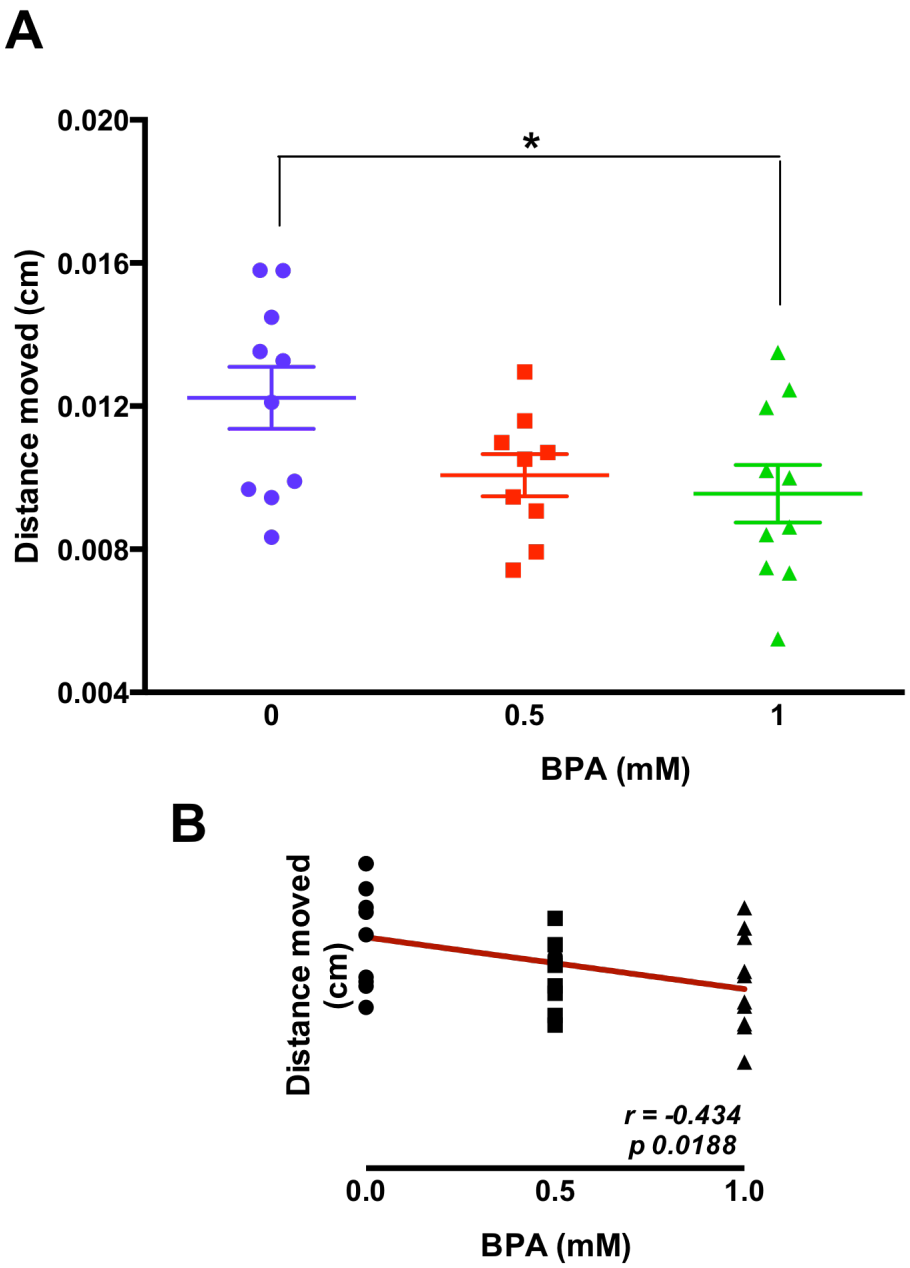

Figure 8. Effect of BPA on the average distance travelled by the Drosophila during the 5 min observation period. Fig 8A represents scattered plot of the data and the lines shown are mean±S.E. The 1 mM BPA-treated flies travelled significantly lesser distance ($p<0.05$). Fig 8B represents linear regression analysis and correlation coefficient (r) between the BPA concentration and the distance travelled by the flies.

*For statistical analysis *p<0.05*

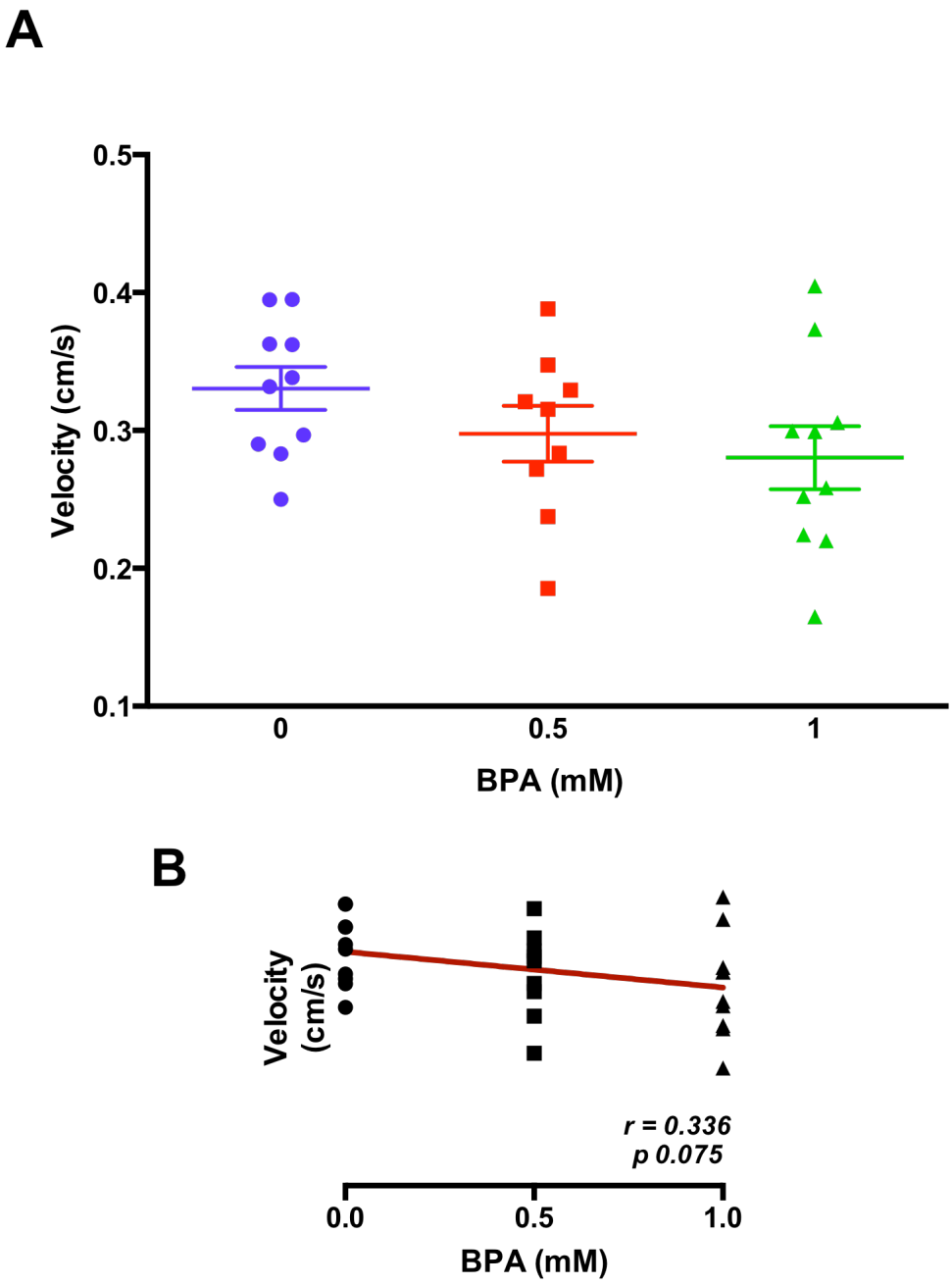

Figure 9. Effect of BPA on the velocity travelled by the Drosophila during the 5 min observation period.
Fig 9A represents the scattered plot of the data with the lines at mean±S.E. There was no significant difference in the velocity between the BPA-treatment and the groups. Fig 9B represents the linear regression analysis and the correlation coefficient (r) between the BPA concentration and the velocity at which the Drosophila travelled.

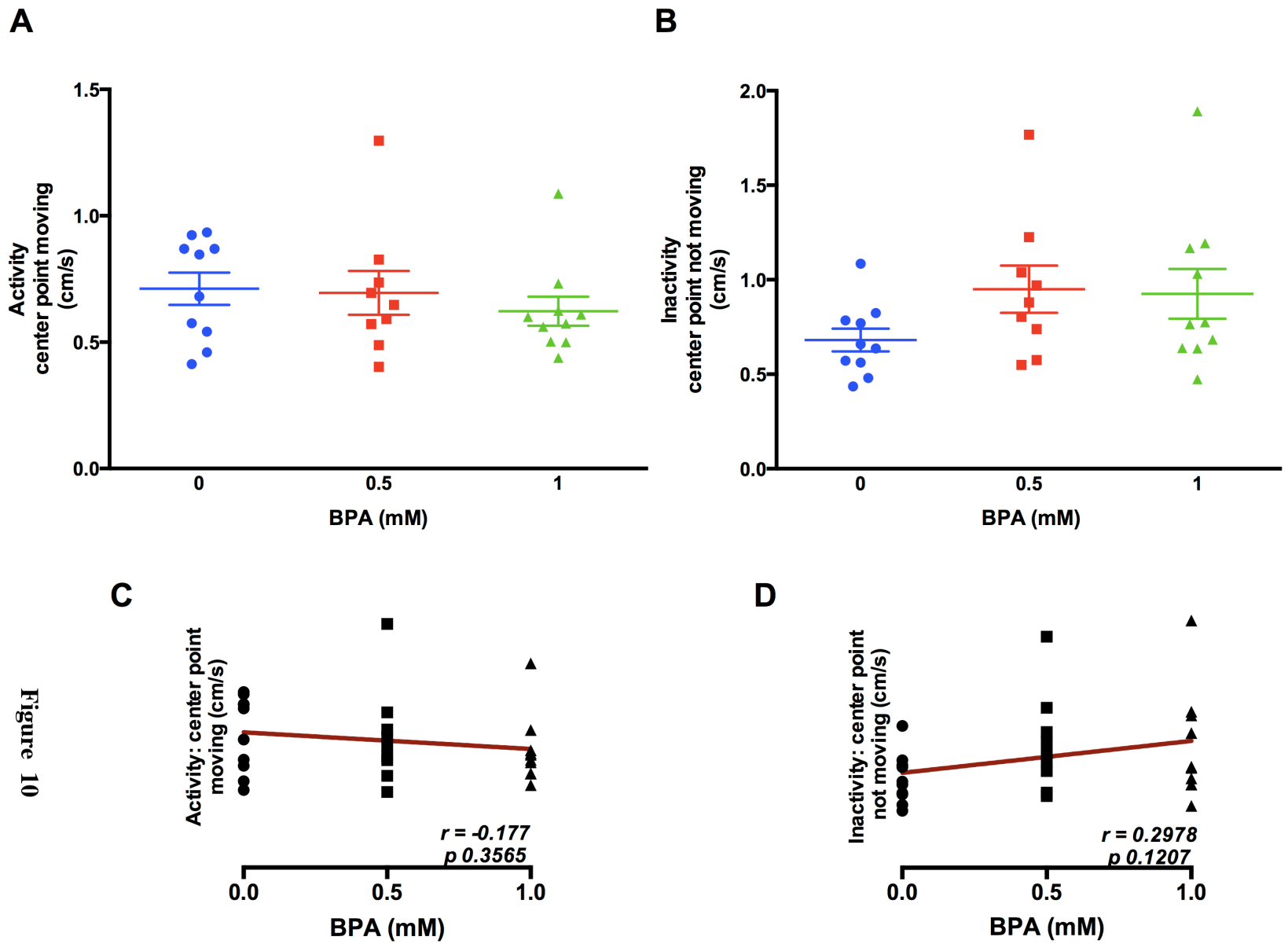
A
Activity
center point moving
(cm/s)
1.5
1.0
0.5
0.0
0
0.5
1
BPA (mM)
B
Inactivity
center point not moving
(cm/s)
2.0
1.5
1.0
0.5
0.0
0
0.5
1
BPA (mM)
C
Activity: center point
moving (cm/s)
r = -0.177
p 0.3565
0.0
0.5
1.0
BPA (mM)
D
Inactivity: center point
not moving (cm/s)
r = 0.2978
p 0.1207
0.0
0.5
1.0
BPA (mM)

Figure 10

Figure 10. Effect of BPA on the movement of the Drosophila during the 5 min observation period.
Fig 10A and 10B represents the scattered plot of the activity and no activity data respectively, with the lines at mean±S.E. There is no significant difference in the movement of the Drosophila between the groups. Linear regression analysis and the correlation coefficient (r) between the BPA concentration and the movement of the Drosophila is presented in Fig 10C and 10D.

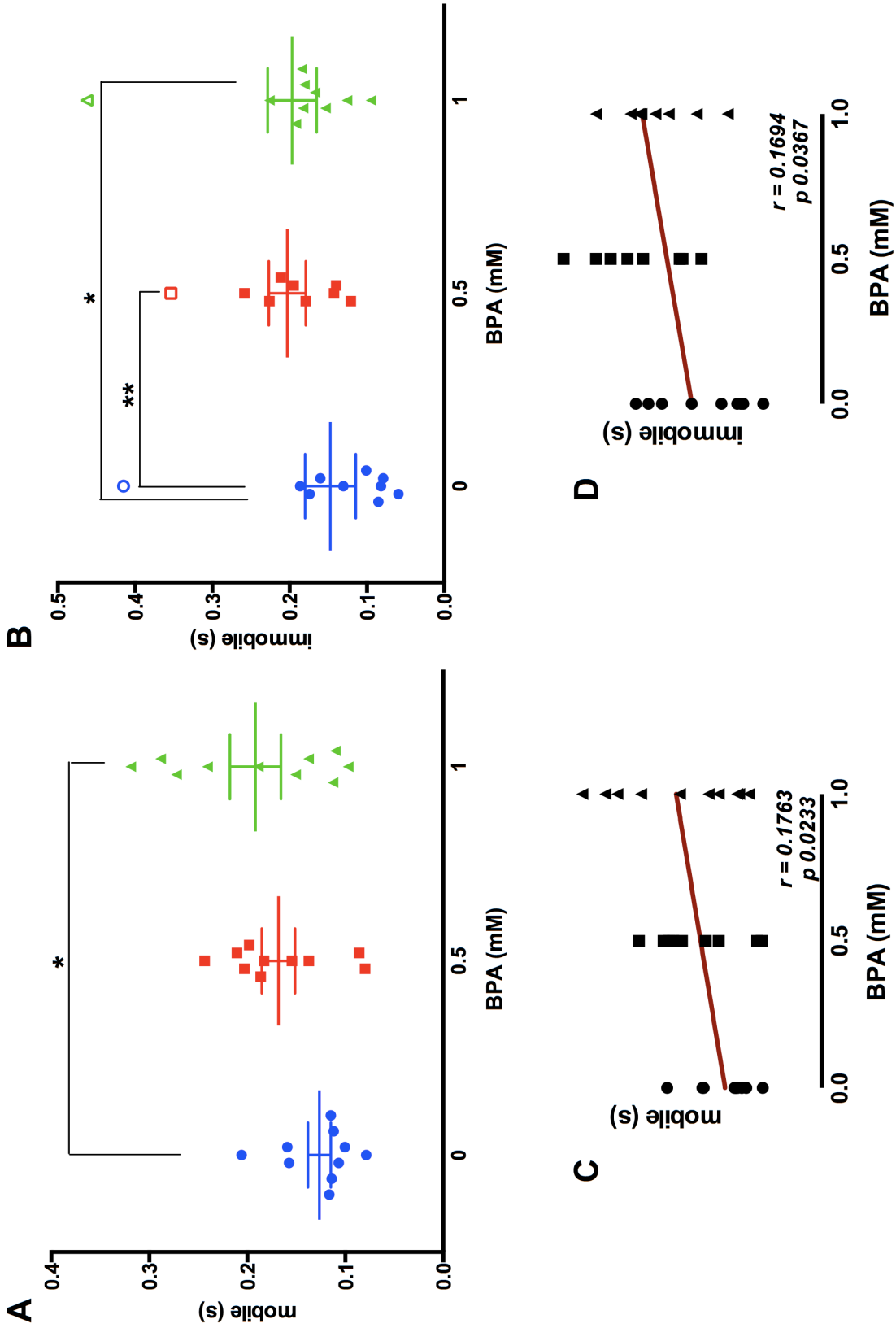
A
mobile (s)
0.4
0.3
0.2
0.1
0.0
*
0
0.5
1
BPA (mM)
B
immobile (s)
0.5
0.4
0.3
0.2
0.1
0.0
**
*
0
0.5
1
BPA (mM)
C
mobile (s)
r = 0.1763
p 0.0233
0.0
0.5
1.0
BPA (mM)
D
immobile (s)
r = 0.1694
p 0.0367
0.0
0.5
1.0
BPA (mM)

Figure 11

Figure 11. Effect of BPA exposure on the mobility of the Drosophila during the 5 min observation period.
Fig 11A and 11B represents the scattered plot of the mobility and the immobility data respectively with the line at mean±S.E. In fig 11B, the open data points represents biological outliers that were not considered for statistical analysis. Fig 11C and 11D represents linear regression analysis and the correlation coefficient (r) between the BPA exposure and the mobility of the three groups.

*For statistical analysis *p<0.05, ** p<0.01*

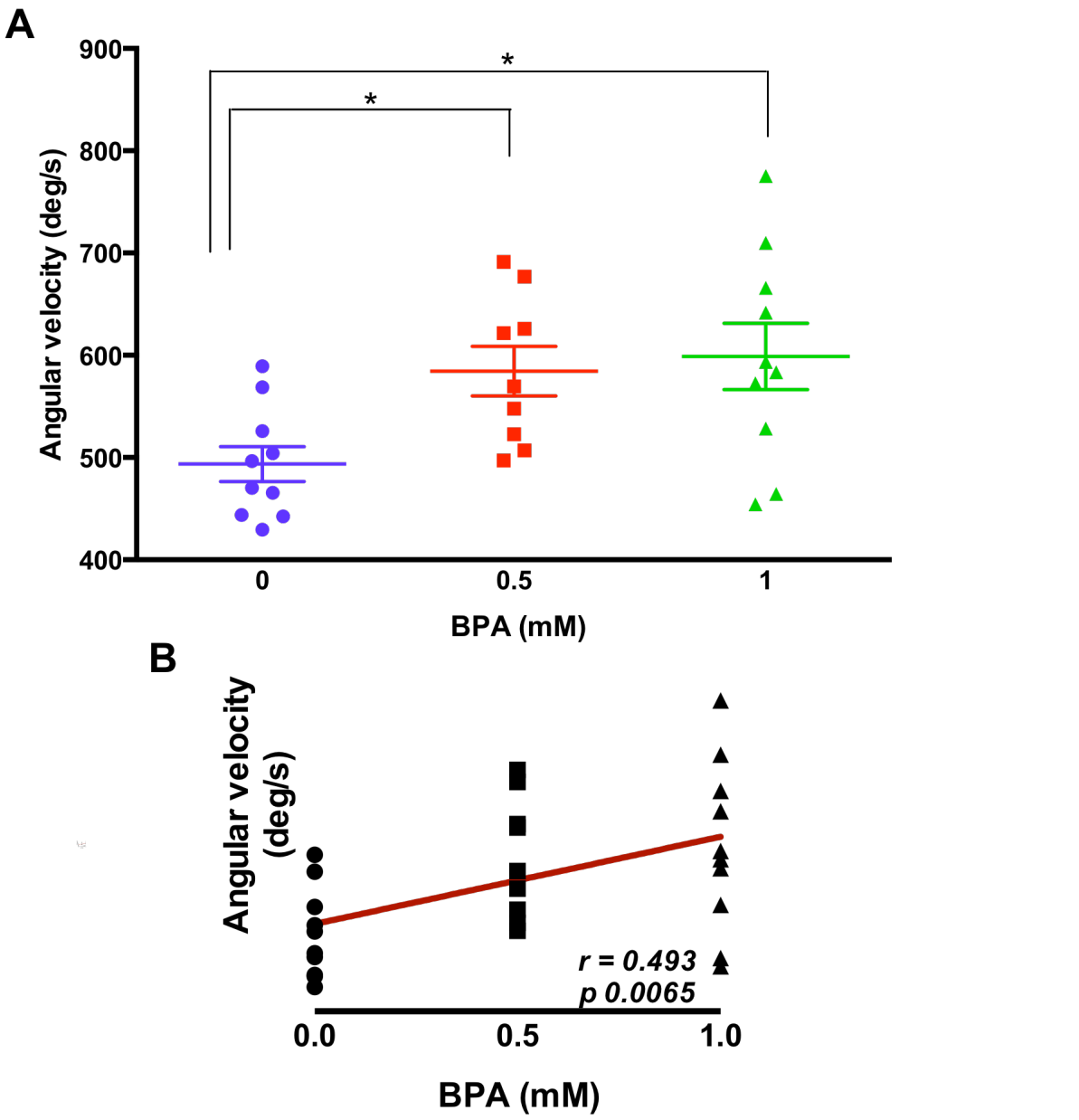

Figure 12. Effect of BPA exposure on the angular velocity of the Drosophila during the 5 min observation period. Fig 12A represents the scattered plot of the data with the line at mean±S.E. There was significant increase in the angular velocity that the Drosophila travelled at with increasing exposure to BPA. Fig 12B represents the linear regression analysis and the correlation coefficient (r) between the BPA exposure and the angular velocity.

For statistical analysis *p<0.05

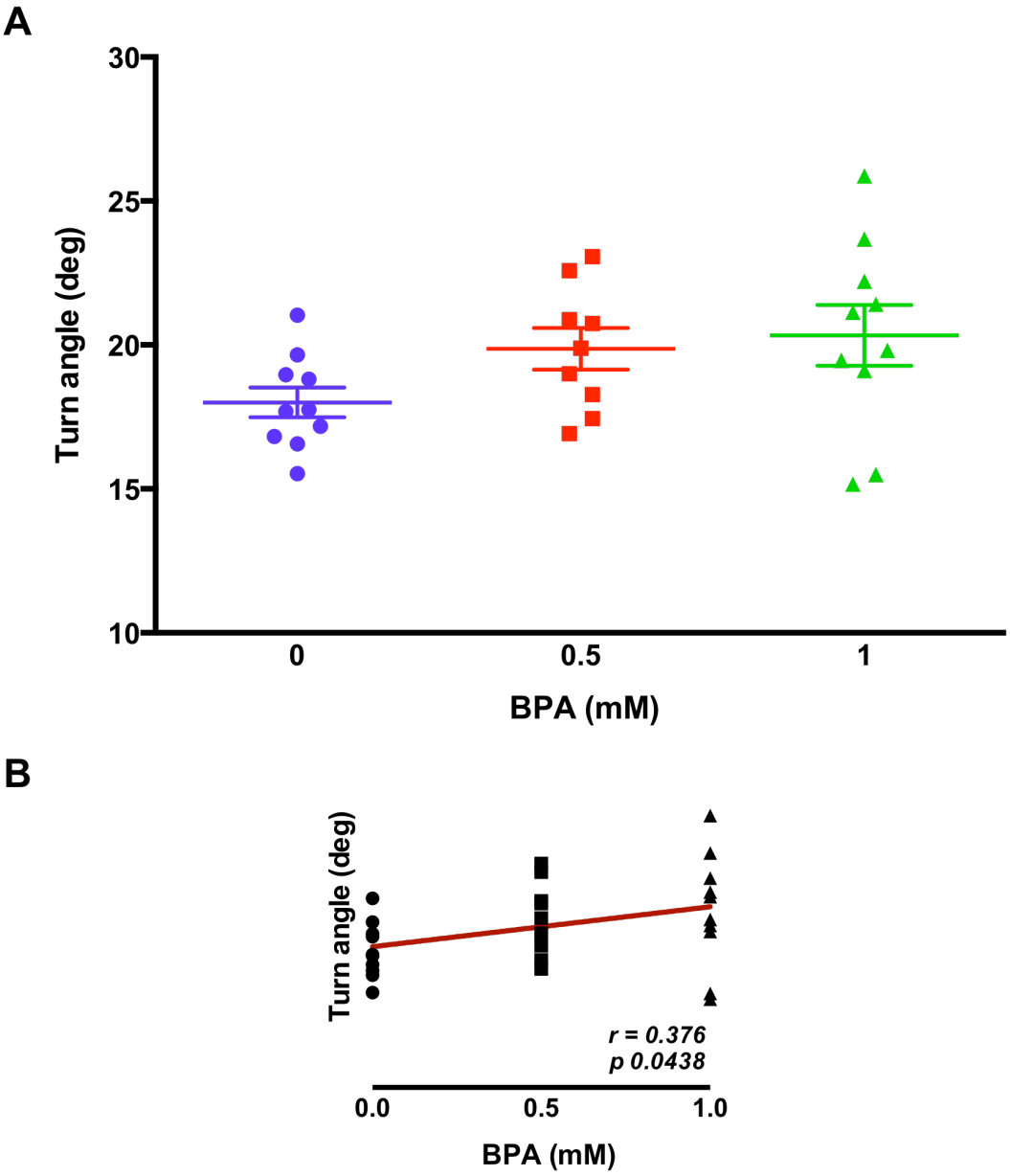

Figure 13. Effect of BPA on the turn angle of the Drosophila during the 5 min observation period. Fig 13A represents the scattered plot of the data with the line at mean±S.E. There was no significant difference in the turn angles executed by the Drosophila in the different groups. Linear regression analysis and the correlation coefficient (r) are represented in fig 13B.

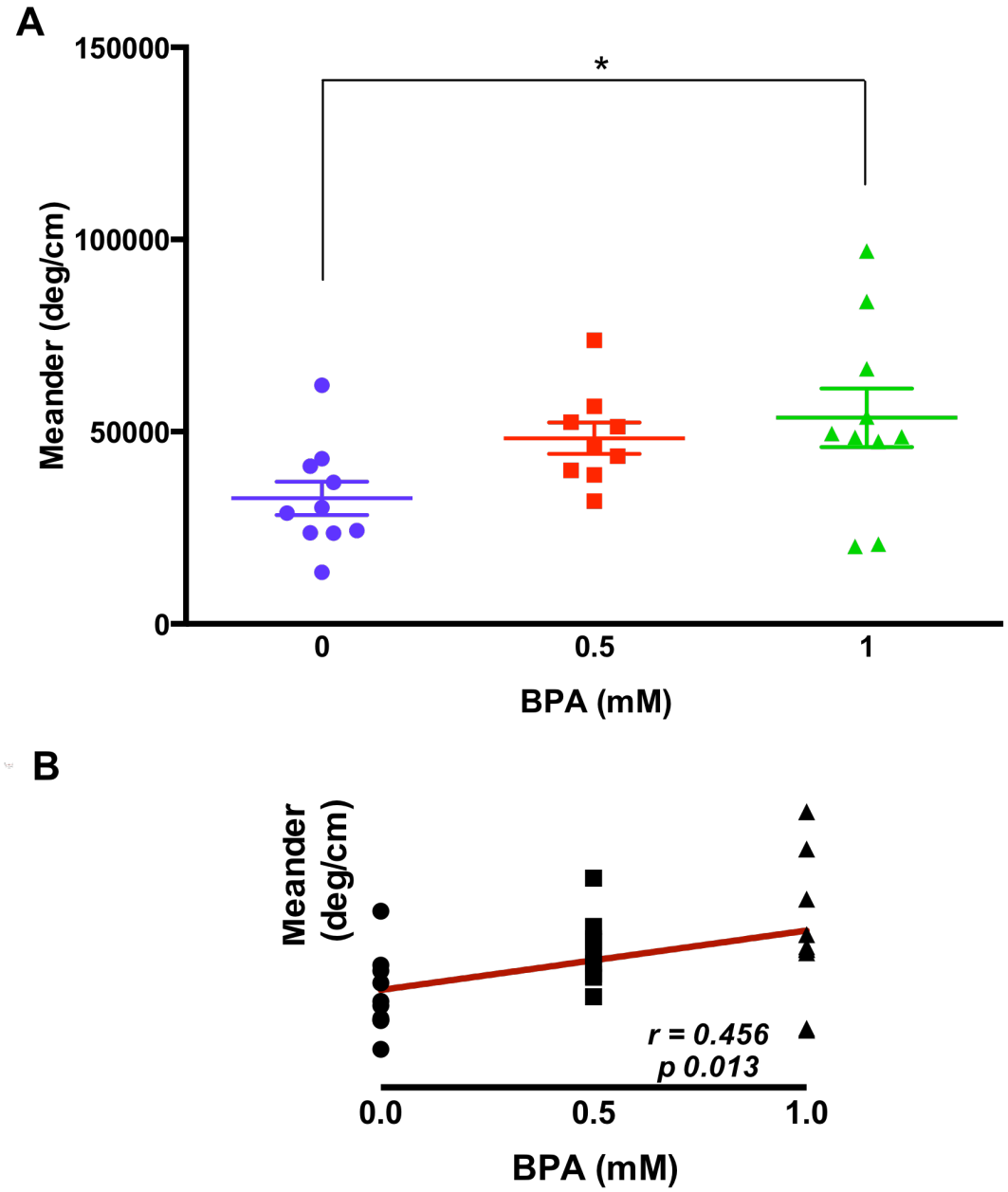

Figure 14. Effect of BPA exposures on the meander of the Drosophila during the 5 min observation period. Fig 14A represents the scattered plot of the data with the line at mean±S.E. There was a significant increase in the meander of the Drosophila exposed to 1mM BPA when compared to the vehicle-treated control Drosophila. Fig 14B represents the linear regression analysis and the correlation coefficient (r) between the BPA exposure and the meander of the Drosophila.

For statistical analysis *p<0.05

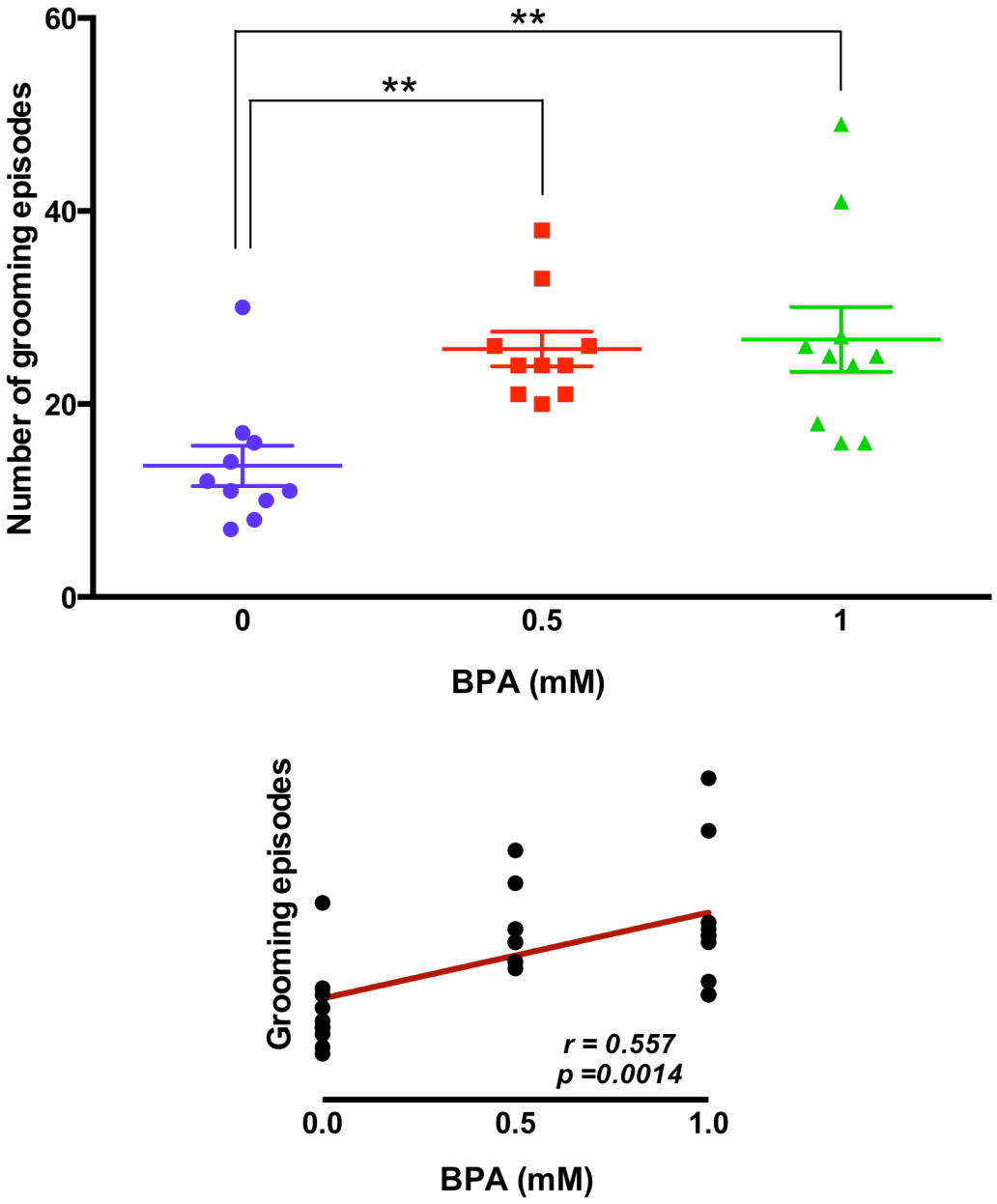

Figure 15. Effect of BPA exposure on the grooming episodes during the 5 min observation period. Fig 15A represents the scattered plot data with the line at mean±S.E. There was significant increase in the grooming by the BPA-exposed Drosophila. Fig 15B represents the linear regression analysis and the correlation coefficient (r) between the BPA exposure and the grooming by the Drosophila.

For statistical analysis *p<0.05

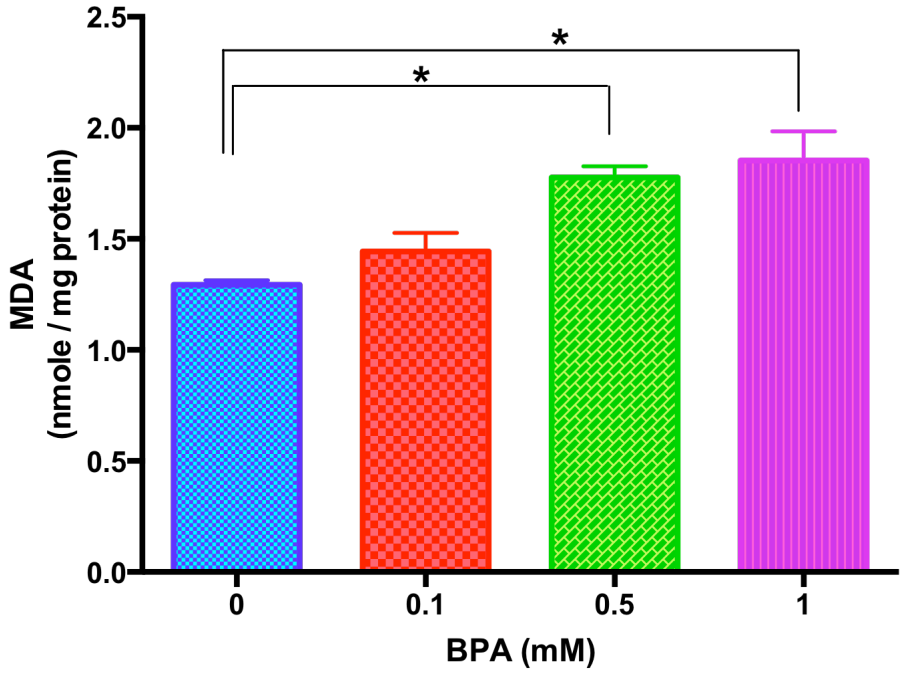

Figure 16. Effect of BPA exposure on the MDA formation in the brain. Fig 16 represents the bar plot of the data with line at mean±S.E. There was a significant increase in the MDA following BPA exposure**.**

Figure 17

Figure 17 Effects of BPA on the embryonic development. Embryos exposed to BPA prenatally and postnatally were analyzed for embryonic development at stage 14. Stage 14 embryos were identified by the features of the CNS and PNS as shown by the staining with the pan neural anti-HRP antibody. Anterior is to left in all the images. Similarly staged embryos were identified by the position of the posterior terminus of the VNC marked by (*). Vehicle treated control embryos show normal development as evidenced by the retraction of the VNC. BPA exposure caused a delay in the retraction of the VNC.

Figure 18

Figure 18. Effects of BPA on the cell positioning of the PNS. There was no disturbance in the pattern of the staining of the PNS cells during at stage 14 following BPA treatment as revealed by the staining with anti-elav antibody.

Figure 19

Figure 19 Effect of BPA on the neurite outgrowth of the PNS. The dorsal (d), lateral (l) and ventral (v) cluster of sensory neurons form a stereotypical pattern. There was no disturbance in the pattern of the staining of the neurite outgrowth / axon projections during at stage 14 following BPA treatment as revealed by staining with 22c10 antibody.

Table 4: Bownes stages for the development of the Drosophila embryo

Stage	Minutes after fertilization	Developmental activity
1	0-15	Pronuclear fusion
2	15-70	Preblastoderm (mitotic cycles 1-9) - early cell division - start of cleavage
3	70-90	Pole bud formation - nuclear division 9
4	90-130	Syncytial blastoderm (mitotic cycles 10-13) - end of cleavage divisions
5	130-180	Cellularization of the blastoderm
6	180-195	Gastrulation to form mesoderm and endoderm - pole cells included in posterior midgut primordium
7	195-200	Germ band elongation - lengthening of the ventral epidermis
8	200-230	Rapid germ band elongation - start of first postblastoderm mitosis - ends with mesodermal parasegmentation
9	230-260	Slow germ band elongation - segmentation of neuroblasts - end of first and start of second postblastoderm mitosis - cephalic furrow formation
10	260-320	Gnathal and clypeolabral lobe formation (head features) - stomodeal invagination - end of second and start of third postblastoderm mitosis
11	320-440	Epidermal parasegmentation evident - tracheal pits invaginate - mesectodermal cell ingress - end of third postblastoderm mitosis - end of neuroblast formation
12	440-580	Germ band retraction - optic lobe invagination - ventral closure - segment formation - fusion of anterior and posterior midgut
13	560-620	End of germ band retraction - CNS and PNS differentation
14	620-680	Dorsal closure of midgut and epidermis - head involution begins
15	680-800	End of dorsal closure - head involution - discs invaginate - cuticle deposition begins - dorsal epidermal segmentation
16	800-900	Advanced denticles visible - Shortening of the ventral nerve cord
17	Lasts until hatching	The tracheal tree fills with air - Retraction of the ventral cord continues
Hatch	21-22 hours	Hatch to first instar larva

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Neurodevelopmental disorders such as ASD, mental retardation, ADHD are on the rise and put a greater strain on the family and also on the society for the additional sources in terms of the services provided. Therefore, there is a greater need to identify the etiological and risk factors for these developmental disorders. The exponential rise in neurodevelopmental disorders such as ASD, could be attributed to the increased utilization of environmental toxins than previously used. An increase in the use of some of the major toxins such as drugs, BPA, mercury, also leads to their accumulation and an overload on the developing system. The results of this study in lymphoblasts and Drosophila indicate the role of BPA in oxidative stress, mitochondrial dysfunction and behavioral alterations, which are the most prevalent abnormalities in autism. Therefore, exposure to BPA may act as a risk factor for the development of neurodvelopmental disorders such as autism.

The increased numbers of toxins that are prevalent in the environment and the exposure during the early stages of development need to be addressed. In order to address this increased burden on the development, there is a need for a model that be easily manipulated to study the gene x environment interaction leading to the neurodevelopmental disorders. To address this question, we attempted to use Drosophila melanogaster as a new model for the study. Following exposure to BPA, we have tried to identify behavioral modifications that may be relevant to neurodevelopmental disorders. We have identified novel behavioral paradigms to study these

modifications in the Drosophila. One of the greatest challenges in studying neurodevelopmental disorders in animals is modeling relevant behaviors to the disorder in the model.

As a preliminary study, this model has shown to be beneficial in the study for behavioral modifications following toxin exposure. A further study and use of the model could help identify the genetic modifications owing to toxin exposure, and help look at gene x environment interactions. The ease of use of this model could also be utilized to the advantage of studying the earlier embryonic alterations and its correlation with the future behavioral and molecular changes following toxin exposure. This model could also be used for the studies looking at effect of more than a single risk agent and hence model the toxin overload model in development.

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