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THE POTENTIAL ASSOCIATION OF PSYCHOACTIVE PHARMACEUTICALS AT LOW CONCENTRATIONS WITH AUTISM SPECTRUM DISORDERS (ASD)

By

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DEDICATION

I dedicate this dissertation to my parents (Mr. J.P. Kaushik and Mrs. Rajbala Kaushik) who have always been the motivation and driving force behind my success.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ASD: Autism Spectrum Disorders

DSM: Diagnostic and Statistical Manual of Mental Disorders

PPCP: Pharmaceuticals and Personal Care Products

SSRI: Selective Serotonin Reuptake Inhibitors

SNRI: Selective Norepinephrine Reuptake Inhibitors

WWTP: Wastewater Treatment Plant

GSEA: Gene Set Enrichment Analysis

ADME: Absorption, Distribution, Metabolism and Excretion

RA: Retinoic Acid

FACS: Fluorescence-Activated Cell Sorting

AutDB: Autism Database

HPRD: Human Protein Reference Database

PPI: Protein-Protein Interaction Network

IRMS: Isotope Ratio Mass Spectrometer

ABSTRACT

Autism Spectrum Disorders (ASD) is a complex neurodevelopmental disorder and the prevalence in the U.S. is currently estimated to be 1 in 50 children. A majority of cases of idiopathic autism in children likely result from unknown environmental triggers in genetically susceptible individuals. These triggers may include maternal exposure of a fetus to minute concentrations of pharmaceuticals and personal care products (PPCPs). Unmetabolized psychoactive pharmaceuticals reach drinking water through a variety of routes, including ineffectively processed sewage. Previous studies in our laboratory examined the extent to which gene sets associated with neuronal systems were up- and down-regulated (enriched) in the brains of fathead minnows treated with psychoactive pharmaceuticals at low concentrations. In this thesis, I hypothesized that psychoactive pharmaceuticals from drinking water could cross maternal biological barriers, and alter *in vitro* neuronal protein and gene expression associated with ASD. This study sought to address four independently tested hypotheses using *in vitro, in vivo* approaches as well as bioinformatics tools to determine the extent to which psychoactive pharmaceuticals induced molecular patterns similar to neurological disorders and crossed maternal biological barriers when provided to pregnant female mice at low concentrations. Results from *in vitro* studies showed that psychoactive pharmaceuticals at low concentrations altered the expression of ASD-associated synaptic proteins and gene expression profiles of neuronal functions associated with neurological disorders like ASD. Network analysis showed that genes from gene sets enriched by psychoactive pharmaceuticals at low concentrations in fish brains and human cell cultures were found to be more inter-connected within ASD-associated protein-protein interaction network. Results from *in vivo* mice experiment demonstrated that psychoactive pharmaceutical (i.e.

carbamazepine) crossed maternal barriers from the drinking water and reached developing fetus brains. The significance of the thesis was to consider the ways in which psychoactive pharmaceuticals at low concentrations altered fundamental molecular expressions that may result in abnormal neuronal growth, regulation and development, which are implicated in the etiology of ASD. The thesis is innovative because it considers the propensity of psychoactive pharmaceuticals in the drinking water to cross maternal biological barriers, which might trigger, aggravate, or mimic human ASD.

CHAPTER ONE: INTRODUCTION

I. Summary

This thesis carried out a series of four experiments to investigate the molecular and physiological mechanisms of psychoactive pharmaceuticals at low concentrations in the etiology of neurological disorders. I proposed that psychoactive pharmaceuticals at low concentrations (μg/l) could induce an expression pattern (gene or protein) in human neuronal cells associated with neurological conditions such as autism spectrum disorders (ASD). We used *in vitro, in vivo* approaches as well as bioinformatics tools to determine whether psychoactive pharmaceuticals induced molecular patterns similar to neurological disorders and crossed maternal biological barriers when provided to pregnant female mice at low concentrations.

The contribution of this thesis was significant to the understanding of ASD etiology because I considered the ways in which psychoactive pharmaceuticals at low concentrations altered fundamental molecular expressions that may result in abnormal neuronal growth, regulation and development that are implicated in the etiology of ASD. Our results demonstrate that psychoactive pharmaceuticals can induce molecular patterns that are similar to those associated with ASD. This thesis also shows that some psychoactive pharmaceuticals can cross maternal intestinal and placental barriers and thus reach developing fetus brains from maternal drinking water.

The research is innovative because it demonstrates that psychoactive pharmaceuticals in the drinking water can cross maternal biological barriers, and alter *in vitro* molecular mechanisms that potentially have a key role in the development, growth and regulation of neuronal systems affected by ASD.

II. Background

A. ASD definition and prevalence

Autism spectrum disorders (ASD) is a range of neurodevelopmental disorders that usually persist throughout life (1). The disorder was first observed by Leo Kanner in 1943, when he diagnosed 11 children with autism (2, 3). According to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), ASD used to be defined as a group of conditions that included classical autism, Asperger's syndrome and pervasive developmental disorder-not otherwise specified (PDD-NOS) (4). The three behavioral domains which used to be observed to diagnose ASD were social interaction, language delay and repetitive behaviors (3, 4). The currently revised DSM-V has removed the language delay domain from the diagnostic process of ASD, and now defines ASD as a spectrum of disorders with impairments in social communication and repetitive domains (3, 5).

For the last two decades, the number of children diagnosed with ASD has been increasing dramatically in the United States (6, 7). The prevalence before the 1990s was one in 2500 children (5). According to CDC reports, it was one in 110 children in 2010 (8) and then one in 88 children in 2012 (7). Currently the prevalence is close to one in 50 children (6). ASD is more prevalent in boys than girls, with a ratio of 4:1 (3). Although some of this increase may be due to changes in diagnostics and reporting of this disorder, it is critical to determine whether there is some other factor driving this rapid increase in incidence.

Many researchers have described the etiology of ASD as an enigma (9). Some scientists have argued that there is a genetic predisposition to ASD and some have argued that environmental factors play a role (10). Previous studies with twins have demonstrated a genetic role in ASD because of a higher concordance among

monozygotic (MZ) twins than dizygotic (DZ) twins (11). However, both types of twins (MZ and DZ) share their *in utero* environment. Therefore, recent studies have speculated whether shared *in utero* environments or genetic factors contribute more to the development of ASD (3). These studies examined a large population of twins, and found 25% concordance in DZ twins, suggesting the involvement of similar *in utero* environmental factors (3). It is still unclear, however, whether genetic or environment factors contribute more to the occurrence of ASD (3).

For the last two decades, researchers have been making tremendous efforts to identify candidate ASD genes using linkage and whole genome association approaches (3, 12, 13). Due to heterogeneity in genotypes and phenotypes (14), these studies have categorized candidate genes into two groups: rare and common susceptibility genes (3). Rare (inherited) candidate genes are those that have high penetrance but are reported in very few cases (3, 10). In contrast, common variants (copy number variations, single nucleotide polymorphisms etc.) with low penetrance are found in comparatively larger numbers and are responsible for making an individual highly susceptible to ASD (3, 10). A number of experts have speculated that ASD genetics is mostly due to the interaction of common susceptibility genes with other unknown environmental, epigenetic and stochastic factors (3, 10). Scientists have studied potential ASD genes and, when appropriate, have added them to the autism database (AutDB; www.midspec.org/autdb) (13). Approximately 631 candidate genes have been found and added to AutDB (accessed November 29, 2014) (13).

B. Molecular mechanisms in ASD

1. Altered level of synaptic proteins

Neurons communicate through synapses (15), and altered synaptogenesis (formation of synapses) has been considered a neuropathological mechanism in ASD (16). There are three main regions of a synapse, which are pre- and post-synaptic regions, and cleft. Alterations in regulatory proteins' expression in any of these regions would affect the overall synaptic plasticity and connections (13, 15, 17). Our study is focused on the proteins localized at the synaptic cleft; either pre- or post-synaptic regions of the neurons. Synaptic plasticity and connections are represented by a vast array of synaptic proteins and mutations in those key proteins might be associated with neurological disorders like ASD (13, 18). This array consists of voltage-gated ion channels (*CACNA1A-H, CACNA2, KCNA1-6, KCNMA, SCN2A*), glutamate receptors (*GRIA, GRIN1, GRIN2A, GRMs*), scaffolding proteins (*DLG, DLGAP, SHANK, MPP, PSD, SIPA*) at post-synaptic density, synaptic vesicles (*SV2A*), calcium signaling/kinases (*CAMK2A, CALB, PTK2B, GNAQ, PRKACB*), cytoskeleton proteins (*ARC, ACTB, MAPs*), transporters (*SLCs*), cytosolic signaling proteins (*PRKCG, ITPR, PIPs*), translational factors (*EIFs, EEFs, FMR1*), inhibitory receptors (*GABRAs*) and other receptors (*HTRs, OXTR, EPHA*) (18, 19). Three major regulatory systems control the overall translation of key synaptic proteins (13). First, fragile X mental retardation protein (FMRP) binds to mRNAs of key proteins (mainly glutamate receptors) and brings mRNAs to dendrites for local translation (13). Studies have found that the absence of FMRP resulted in either excess or altered translation processes at the synapse. Second, methyl-CpG-binding protein (MECP2) binds to methylated DNA and controls the expression of neurotrophic factors such as BDNF, thus playing a key role in synaptic plasticity (13, 17). Third, the rapamycin-sensitive

mTOR-raptor complex regulates overall cellular translation of surface receptors and channels including NMDAR, AMPA, 5-HT and OXTR receptors (13, 20). Dysregulation of these regulatory mechanisms may play a key role in the etiology of ASD (13).

2. Altered neuronal circuits formation

Abnormal neuronal circuit formation is one of the key molecular mechanisms in ASD (13, 21, 22). Neuronal circuit formation is mostly controlled by GABAergic (inhibitory) and glutamatergic (excitatory) receptors (13), which also control synaptic plasticity (15). Key regulators of these two receptors are neurologins (NLGN) and neurexins (NRXN). Mutations in the *NLGN4X* gene are strongly associated with ASD (13, 16).

C. Potential role of environmental contaminants in ASD

To date, several studies have found that ASD-associated genetic factors, including candidate genes and copy number variations (CNVs) (2, 13, 23), are responsible for only 1-2% of ASD cases. As a result, many studies have found that the presence of low risk susceptible genes, or common variants (13) are not sufficient causal agents, and that they must interact with other environmental, epigenetic, or stochastic factors to cause ASD (3, 10, 14). This finding suggests that the majority of cases result from the presence of an unknown environmental trigger in genetically susceptible individuals (2, 3, 14, 24). Additional studies suggest that maternal environmental exposures (especially in the first trimester of pregnancy) play a role in the etiology of ASD (2, 24).

Many studies contributed to a list of chemicals that are known to be associated with neurodevelopmental disorders (2). Environmental exposure of a fetus to these chemicals via maternal blood is a potential and poorly considered possibility.

Chemicals associated with neurodevelopmental disorders include a class of contaminants known as pharmaceuticals and personal care products (PPCPs). PPCPs include psychoactive and antiseizure pharmaceuticals, which in the United States, are mostly obtained by prescription. Some PPCPs include sunscreen agents, fragrances, nutraceuticals like huperzine A (used as a food supplement), phthalates (found in cosmetics), and bis-phenol A (BPA) which is added to some plastics (2). Aside from PPCPs, there are other chemicals such as lead, polychlorinated biphenyls (PCBs), manganese, arsenic, methylmercury, DDT, ethyl alcohol and organophosphate insecticides (like chlorpyrifos). A number of teratogens have also been found to be strongly associated to autism. These include thalidomide, the psychoactive pharmaceutical valproate, misoprostol, and insecticides (2).

Approximately 15% of women experience psychiatric disorders during pregnancy (25). Many women experience stress during and post-pregnancy. Thus, they are prescribed psychotropic pharmaceuticals to prevent any health complications in the child due to maternal stress, depression and anxiety. These complications generally include prematurity, low birth weight, lethargy, irritation and other behavioral problems in infants (25). Because of these complications, nearly 3.5% of pregnant women worldwide have been consuming psychoactive pharmaceuticals, especially selective serotonin reuptake inhibitors (SSRIs) during their pregnancies (25). Many clinical studies have reported that approximately 30% of infants exposed to psychoactive pharmaceuticals *in utero* exhibit withdrawal and toxicity symptoms, and poor neonatal adaptation (PNA). Common symptoms of PNA include those that are neurological (tremors, agitation, irritation, sleeping problems and high pitched crying), cardiovascular, respiratory, gastro-intestinal (diarrhea, vomiting, feeding problems), and other behavioral and learning problems in infants. Clinical studies

have observed that the most common psychoactive pharmaceuticals are antidepressants (SSRIs, SNRIs), mood stabilizers (anti-epileptics) and benzodiazepines (like diazepam, nitrazepam etc.). However, it is still unknown how *in utero* exposure to these psychoactive pharmaceuticals induces neurological and other psychological problems in infants (25). Psychoactive pharmaceuticals are highly lipophilic: therefore, some scientists have postulated that these pharmaceuticals cross the placenta and interfere with the early brain development of a fetus by altering neuronal connections within the brain (25-27).

D. Previous studies of the association of psychoactive pharmaceuticals in autism and other neurological disorders

1. Fluoxetine

Clinical studies have reported that approximately 1.8% - 2.8% of women consume SSRIs (mainly fluoxetine and paroxetine) during their pregnancy (25). With the increasing number of psychiatric patients, the consumption of SSRIs has also increased in the general population over the last two decades (25). Many researchers have investigated the increasing consumption rate of SSRIs might be correlated with the increasing prevalence of ASD (28). One clinical study in Sweden, Reis *et al.* (2010) analyzed the largest pool of data thus far including 14821 females and 15017 infants from 1995 to 2007 (29). These researchers found that of the 892 mothers who had consumed SSRIs, mostly fluoxetine, during their pregnancy, 561 gave birth to infants with complications including intracerebral hemorrhages, CNS diagnoses, lower IQ scores, congenital malformations (like cardiovascular defects, cystic kidney etc.), hypoglycemia and hypertension (29). In a more recent study, Harrington *et al.* (2014) examined 966 mother-infant pairs and found that prenatal exposure to SSRIs (fluoxetine) was linked to an increased risk of ASD in infants, especially boys.

Specifically, the researchers identified 492 ASD diagnoses, 320 cases of typical development and 154 development delays. Thus, they proposed that the prenatal exposure to SSRIs could disturb the serotonergic system, disrupting biological processes (like cell division, neuron migration and outgrowth) of early stage fetal brain development (30). These recent studies support the hypothesis that prenatal SSRI (mainly fluoxetine) exposure, especially in the first trimester, could lead to higher risk of ASD in genetically susceptible individuals.

2. Venlafaxine

Compared to SSRIs, only a few studies have been conducted to identify the association of selective norepinephrine reuptake inhibitor (SNRIs) with neurological disorders. However, a SNRI called venlafaxine is one of the most prescribed psychoactive pharmaceuticals among pregnant women. Boucher *et al.* (2009) examined seven mother-infant pairs where venlafaxine (75mg/day) was prescribed to mothers in the third trimester (25). After delivery, they observed that newly born infants were suffering from poor neonatal adaptation (PNA), with respiratory and pulmonary hypertension problems (25). Another study by Reis *et al.* (2010) found that 80 out of 363 women who used venlafaxine during pregnancy delivered infants with congenital deformations (29). An *in vivo* study using rats, Dubovicky *et al.* (2012) found that prenatal exposure to venlafaxine resulted in lower infant body weight than the control group, although this difference was not statistically significant at $\alpha = 0.05$ (31). As venlafaxine is one of the most prescribed drugs in the United States for neurological disorders, many researchers are conducting *in vivo* and clinical studies to identify whether or not venlafaxine and the occurrence of neurological disorders are associated.

3. Carbamazepine

The teratogenicity of anti-epileptic drugs (valproate, carbamazepine) has been well studied and reviewed by many scientists (32). Approximately six out of 1000 women suffer from epilepsy, which makes it one of the most prevalent neurological disorders observed during pregnancy. Many *in vivo* and *in vitro* studies have found that biological processes associated with nervous system development, neuron migration, axon guidance and synaptic transmission are altered during treatment with anti-epileptic drugs (AEDs) (24). In a clinical study by Rasalam *et al.* (2005) in Scotland, 626 children exposed to AEDs (like valproate and carbamazepine) prenatally were examined (32). It was found that 2.5% of those infants exposed prenatally to carbamazepine were diagnosed with deficits in social communication and language development. Another study in the United States, Meader *et al.* (2009) examined 98 infants exposed to carbamazepine during prenatal development, from 1999 to 2004, and found that those children had lower IQ scores and impaired cognitive function compared to non-exposed infants (33).

III. Detection of psychoactive pharmaceuticals in drinking water

Psychoactive pharmaceuticals are one of the mostly widely prescribed classes of drugs in the United States (34, 35) and patient excretions contain active metabolites that have long half-lives (35, 36). Waste-water treatment plants (WWTP) release treated water containing pharmaceuticals (with their metabolites and isoforms) into rivers and lakes, thus introducing contamination into aquatic systems (35, 36). Isoforms of psychoactive pharmaceuticals in WWTP are active for two reasons. First, some parts of the pharmaceuticals are not metabolized completely by the digestive systems and are excreted from the human body in an active form (36). Second, although most of the metabolized pharmaceuticals excreted by humans are

glucuronide conjugates, which is an inactive metabolite (36), some bacteria such as *Escherichia coli* present in excessive amounts in the WWTP reconvert the inactive form into an active one by secreting large amounts of the enzyme β-glucuridase (36). This thesis hypothetically proposed that psychoactive pharmaceuticals at low concentrations in the maternal drinking water cross the placenta and interfere with the early brain development of a fetus by altering neuronal connections within the brain.

IV. Previous studies in our lab

To investigate the effect of these pharmaceuticals in water, researchers in the Thomas lab exposed juvenile fathead minnows to psychoactive pharmaceuticals such as fluoxetine, venlafaxine and carbamazepine individually and in mixtures at concentrations equivalent to what is expected in the environment (37). After 14 days of treatment, fish brains were dissected, homogenized and analyzed with microarray analysis. Gene-class analysis was performed on microarray data using Gene Set Enrichment Analysis (GSEA) (38). It was determined that certain gene sets associated with synapse function and development were enriched or strongly up- or down-regulated. These gene sets include SYNAPSE, SYNAPSE PART, NEUROTRANSMITTER BINDING, NEUROTRANSMITTER SECRETION, NEURAL TUBE DEVELOPMENT, NEUROTRANSMITTER TRANSPORT, ENSHEATHMENT OF NEURONS, SYNAPSE ORGANIZATION and NEURON PROJECTION. From these gene sets, several receptor genes that are strongly up- or down- regulated and are known to be associated with ASD (listed, in Table 1) were selected for this study.

Table 1.1. List of genes that were highly enriched in preliminary studies and known to be associated with ASD.

Up-Regulated (highly expressed) genes in Fish brains		**Down-Regulated (less expressed) genes in Fish brains**	
Receptors	**Gene Symbol**	**Receptors**	**Gene Symbol**
Gamma-Aminobutyric acid receptors	*GABRB3*	Metabotropic Glutamate receptors	*mGLuR1, mGLuR4*
Ligand-gated glutamate receptors (N-methyl-D-aspartate)	*NMDAR1, NMDAR2A, NMDAR2D*	Serotonin	*HTR1A, HTR1B, HTR2C, HTR4*
Oxytocin	*OXTR*	Other Synaptic Proteins	*PSD95, SV2A, NCAM, NeuN*

Preliminary work in the Thomas lab demonstrated that psychoactive pharmaceuticals at low (sub-clinical) concentrations could alter the expression in fish brains of genes that are associated with growth, development and regulation of neurons in humans (34, 35). Moreover, the altered expression patterns in fish brains were found to be similar to human neurological disorders including autism (34, 35). These findings prompted consideration of the potential role of psychoactive pharmaceuticals at environmental concentrations as a cause of neurological disorders in humans.

V. Pharmacology of three drugs used in this study

A. Fluoxetine (FLX)

FLX is marketed in the United States as Prozac (Fluoxetine hydrochloride), an antidepressant (39). Prozac is a selective serotonin reuptake inhibitor (SSRI), mostly prescribed for patients suffering from depression and other psychiatric disorders. The dosage for FLX ranges from 20-40mg per day. FLX is consumed orally because it is completely absorbed through the intestinal barrier. The half-life of FLX is 1-3 days in case of acute dosing and 4-6 days in chronic dosing. The active metabolite, Norfluoxetine, has a half-life of 4-16 days in both acute and chronic dosing and thus takes more time to eliminate from the human body (40-42).

ADME: Bioavailability of FLX (approximately 90%) in the plasma is achieved 6-8 hours after consuming a 40mg dose orally. Because FLX is highly lipophilic in nature, it is absorbed completely through the intestinal barrier into the blood stream. FLX has a 94% protein binding capacity; thus it binds frequently to plasma proteins, especially albumin and glycoprotein. FLX is metabolized in the liver by the N-demethylation process. Metabolism of FLX produces two enantiomers (R-Norfluoxetine and S-Norfluoxetine) by the catalysis of CYP2C9 and CYP2D6 cytochromes. Studies have also found that FLX has the highest volume of distribution (V_d = 14-100 L/kg) among all SSRIs, and so accumulates in many tissues (especially the lungs). The elimination of FLX requires oxidation and the conjugation processes, which produce fluoxetine glucuronide and other metabolites. Elimination of FLX takes place primarily through the kidneys. Studies have found that less than 10% of the excreted metabolites are fluoxetine glucuronide (40-43). This inactive form could be converted into an active form by *Escherichia coli* bacteria, which is highly presented in waste-water treatment plants.

Mechanism: FLX belongs to a class of selective serotonin reuptake inhibitors (SSRI), which provide more serotonin in the synaptic cleft by blocking serotonin transporters (SCL6A4) at the pre-synaptic membrane (44, 45). Clinical patients suffering from depression or other psychiatric disorders release less serotonin at the synaptic cleft. SCL6A4 is an integral membrane carrier protein that terminates the action of serotonin (5-HT) at the synaptic cleft by transporting freely available 5-HT back into the presynaptic membrane (46). Thus, this protein is the major target of fluoxetine.

SSRI mediated signaling: Researchers have studied signaling pathways that might be involved in the mechanism of SSRIs. In the presynaptic neuron, free

tryptophan (TRP) is converted to serotonin (5-HT) with the help of tryptophan hydroxylase (TPH) and aromatic decarboxylase (DDC) enzymes. Once 5-HT is produced, it is transported into presynaptic vesicles by vesicular monoamine transporters (SLC18A2). The remainder of 5-HT is degraded by the monoamine oxidase A (MAOA) enzyme. Calcium dependent exocytosis of 5-HT filled vesicles is mediated by an action potential. To trigger this process, vesicles release 5-HT into the cleft. There are four mechanisms that regulated by freely available 5-HT in the cleft. First, 5-HT controls a feedback loop by binding to presynaptic HTR1 receptors outside the cell, which inhibit calcium dependent exocytosis. Second, 5-HT binds to post-synaptic HTR1 receptors, which are coupled with GNAI ($G_{i/o}$ protein alpha subunit). This interaction decreases the production of cyclic AMP (cAMP) by inhibiting adenylate cyclases (ADCY) enzymes. Third, 5-HT binds to post-synaptic HTR2 receptors, activating phospholipase C (PLCB) enzymes using GNAQ (Gq/11 protein alpha) coupled with these receptors. 5-HT also binds to HTR3 (ligand-gated ion channel) receptors, activating HTR2 receptors by depolarizing through potassium efflux and sodium influx. Activated PLCB then produces IP3 (myoinositol- 1, 4, 5-triphosphate) and DAG (diacylglycerol). Finally, 5-HT activates post-synaptic HTR4, HTR6 and HTR7 receptors, which are coupled with GNAS (G_s protein alpha). This interaction activates ADCY enzymes and produces cAMP. The amplified levels of cAMP further mediate the release of 5-HT from cells into the brain (46). By targeting and blocking re-uptake inhibitors by SSRIs, any of those four pathways can be associated with the dysregulation of synaptic proteins.

B. Carbamazepine (CBZ)

CBZ is an anticonvulsant and mood stabilizer marketed in the United States as Tegretol (47). The drug is used to treat epileptic seizures and bipolar disorders. For

treatment of epilepsy the initial dosage of CBZ is generally 200mg twice a day. Dosages are usually increased to 800 - 1200 mg/day over a matter of weeks. The dosage for bipolar disorder is generally 200mg every 12 hours. CBZ is insoluble in water but soluble in alcohol and other organic solvents. The half-life of CBZ ranges from 1-3 days (40-42).

ADME: CBZ is also highly lipophilic, allowing it to cross the gut through the mucosa membrane. The highest bioavailability (75-85%) of CBZ in plasma is achieved 4-12 hours after consumption. CBZ has a 76% protein binding capacity and thus binds to plasma proteins. CBZ is metabolized in the liver by many isoenzymes such as CYP3A4, CYP2B6, CYP2E1, CYP2C8 and CYP1A2. Primarily CYP3A4 is involved in the biotransformation of CBZ, which produces carbamazepine-10, 11-epoxide, an active metabolite. CBZ-10, 11-epoxide has a 50% protein binding capacity. Studies found that this active metabolite can cross the blood brain barrier by binding with P-glycoprotein, and the placental barrier therefore accumulating in the fetal tissues. The volume of distribution of CBZ-10, 11-epoxide is 0.8-2.0 L/kg. CBZ-10, 11-epoxide is further metabolized into trans-CBZ diol (an inactive form) by epoxide hydrolase. Most elimination of CBZ and its metabolites takes place through the kidneys (72%) and the rest (28%) occurs through bile and feces. It has been reported that 1-5 % of the excreted metabolites in urine remain unchanged, thus might be contaminating the environmental water resources by inefficient filtration by treatment plants (40-42).

Mechanism: CBZ, a member of tricyclic anti-depressant compounds, is used to treat epileptic seizures and mood disorders. CBZ and its active metabolite (CBZ-10, 11-epoxide) block voltage-gated sodium channels, thus preventing the attainment of action potentials in the neurons. CBZ also interacts with other voltage-gated Ca^{2+}

channels and K^+ channels to a lesser extent. Studies on neuroblastoma cell cultures and rat dorsal root ganglia have shown that sodium channels in an inactive state have greater affinity to CBZ than the resting phase state (48). These possible mechanisms could be associated with the alteration of neuronal systems by carbamazepine, however, scientists have been trying to explore more in-depth analysis of signaling pathways.

C. Venlafaxine (VNX)

VNX is a selective norepinephrine reuptake inhibitor (SNRI) antidepressant, marketed in the U.S. as Effexor (venlafaxine hydrochloride) (49). To treat depression, anxiety and other panic disorders, patients are generally prescribed one 75mg tablet of VNX per day. Since this drug is water soluble, it can be consumed orally with a glass of water. The half-life of VNX is 5-7 hours, which is very short compared to other drugs. The half-life of its active metabolite, O-desmethylvenlafaxine is 11 hours (40-42).

ADME: With the octanol:water coefficient of 0.43, venlafaxine is particularly lipophilic. However, VNX is 92% absorbed from the gut after consuming a 75mg dose due to its lesser lipophilicity and the nature of the extended release tablets. Within 5-6 hours, 45% of VNX is bioavailable in the plasma. The protein binding capacity of VNX is 27%, which is lower than FLX and CBZ. Before entering the blood stream, VNX is metabolized into O-desmethylvenlafaxine (ODV) and N-desmethylvenlafaxine (NDV) in the liver primarily by CYP2D6 enzymes. ODV has a 30% protein binding capacity, which is slightly higher than VNX, although much lower than CBZ. The elimination of VNX occurs mainly through the kidneys. Clinical studies have found that nearly 87% of VNX is eliminated after 48 hours in the urine.

The excreted amounts consist of 5% venlafaxine (unchanged), 26% ODV (conjugated), 29% ODV (unconjugated) and 27% other metabolites (40-42).

Mechanism: VNX is a selective norepinephrine reuptake inhibitor (SNRI) that blocks the reuptake of norepinephrine into the pre-synaptic membrane. The mechanism of VNX is very similar to that of SSRIs, except it blocks the SCL6A2 transporter. Moreover, some studies have shown that VNX at low concentrations blocks the reuptake of norepinephrine (NE) and also blocks the reuptake of 5-HT at higher concentrations (45, 50). Scientists have been trying to explore signaling pathways of venlafaxine. However, a few studies have found that venlafaxine may mediate the SSRI signaling at higher concentrations because VNX works as an SSRI at those concentrations (50). Considering those mechanisms from other studies, this thesis investigated the extent to which venlafaxine could alter the expression of synaptic proteins and genes.

VI. Physiological details of biological barriers

A. Transport through membranes

Today, many people are treated with psychoactive pharmaceuticals. Some individuals take these drugs on a daily basis, while others take them only when acutely distressed. Psychoactive pharmaceuticals carry out molecular-receptor interactions within the body. Their mechanism of action is contingent on whether they are an agonist or antagonist to the target receptor. Any pharmaceutical that enters the blood stream must be absorbed in one of three ways: 1) the intestinal mucosal cells inside the gut, 2) through the skin, or 3) by inhaling into the lungs. The rate of absorption depends upon their size and degree of lipophilicity (51, 52).

The process of passive diffusion is defined by Fick's law:

$$\text{Rate of passive diffusion} = D \times \Delta C \times A/d,$$

where, D = Diffusion constant, ΔC = concentration gradient of the pharmaceutical, A = Surface area, d = thickness of the barrier (51, 52). The psychoactive pharmaceuticals used in our study were fluoxetine, venlafaxine and carbamazepine. All three drugs are mostly lipophilic in nature, thus crossing biological barriers by passive diffusion.

B. Intestinal barrier

The intestinal barrier consists of sequential barriers of epithelial cells. Once a drug in solution form reaches the lumen of the intestines, it must pass through these epithelial cells. Permeability and surface area of the epithelial cells affect the degree of drug absorption. Absorption is increased by many structures in the lumen. These structures are villi, microvilli and intestinal folds, which increase the total surface area for absorption. The intestinal epithelium consists of three types of cells: exocrine, endocrine and absorptive cells. There are two types of exocrine cells; goblet cells that secret mucus, and paneth cells that secret antimicrobial peptides. Endocrine cells produce digestive hormonal peptides. Absorptive cells are known as enterocytes, which are simple columnar epithelial cells. These cells occupy 80-90% of the intestinal epithelial system. Most absorption takes place in the crypt-villus axis, which is also called the functional unit of the intestinal epithelial system. The crypt-villus axis consists of three regions: 1) the lower crypt region that contains proliferating cells; 2) the upper crypt region that contains differentiating cells; and 3) the villus tip that contains enterocyte cells for maximum absorption. The absorption of a drug solution occurs mostly along the crypt-villus axis. Studies have shown that the absorption of low permeability drugs takes place in the lower cryptic region and high permeability drugs through the villus (52, 53).

C. Placental barrier

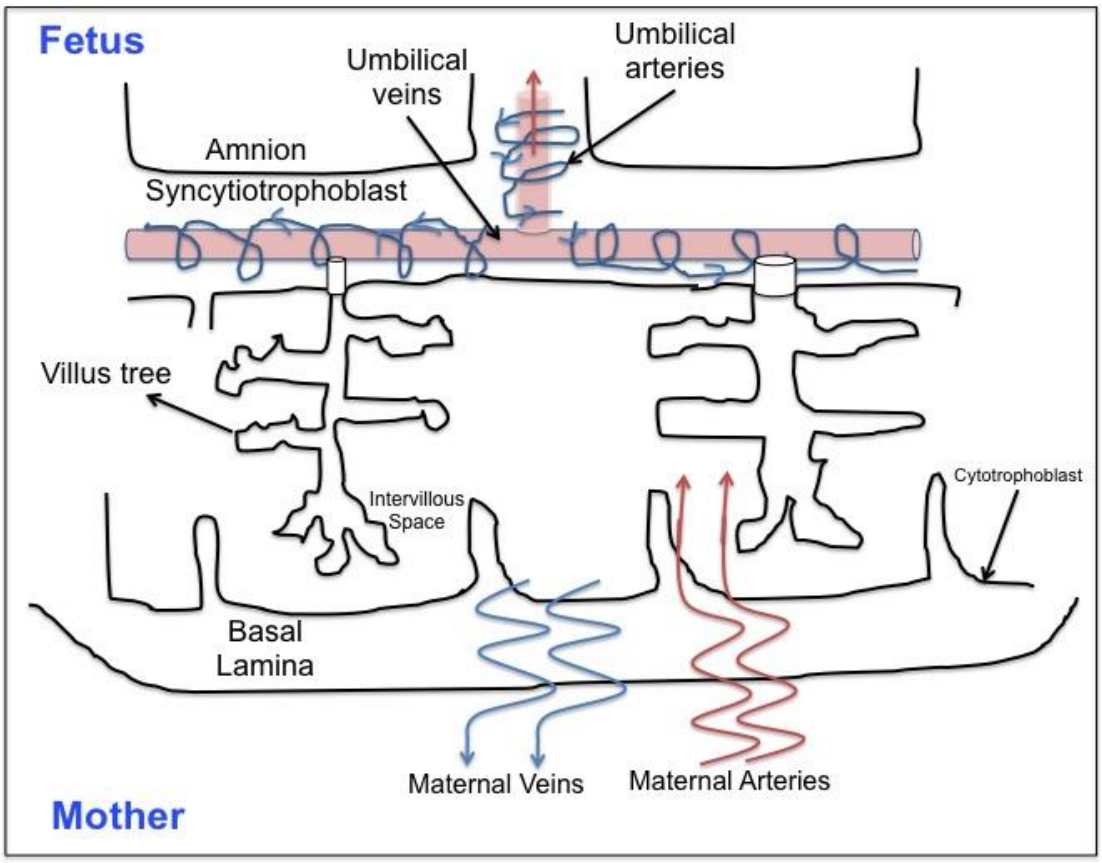

Figure 1.1: Physiological representation of a placental barrier (modified and redrawn from Myren *et al.* 2007) [47].

Once the drug enters the blood stream and the hepatic portal vein, it is converted in the liver into active metabolites. These active metabolites enter systemic circulation and reach the placental barrier, where maternal blood is mixed with fetal blood. The functional unit of a placenta is the villus tree. Most of the absorption takes place through this tree. A placental barrier is made up of five main layers: 1) syncytiotrophoblast (which covers the villi), 2) cytotrophoblast, 3) basal lamina, 4) connective tissue and 5) fetal endothelium (Fig. 1). The formation of the placenta begins at day 6 or 7 following post conception. Endometrial arteries carry oxygen rich blood to the intervillous space. Across the villous tree, the exchange of waste products, nutrients, gases and pharmaceuticals takes place. The villous tree is the

primary absorption site in the placenta. As our drugs are lipophilic, they get into umbilical veins and reach the fetus through passive diffusion (51, 52).

D. Blood-Brain-Barrier

The blood-brain-barrier (BBB) acts as a defense mechanism by stopping most of the blood compounds and other substances from entering the brain. Most psychoactive pharmaceuticals are lipophilic in nature, thus crossing the BBB. BBB is made up of endothelial cells, astrocytes, pericytes and the basement membrane (in Figure 2). Astrocytes play an important role in the development of the BBB. Pericytes regulate the permeability of endothelial cells and vesicle trafficking by inhibiting those molecules that increase vascular permeability and inflammation. The main functional unit of the BBB is a junction between endothelial cells. Each junction consists of tight junctions and adherens junctions (54, 55).

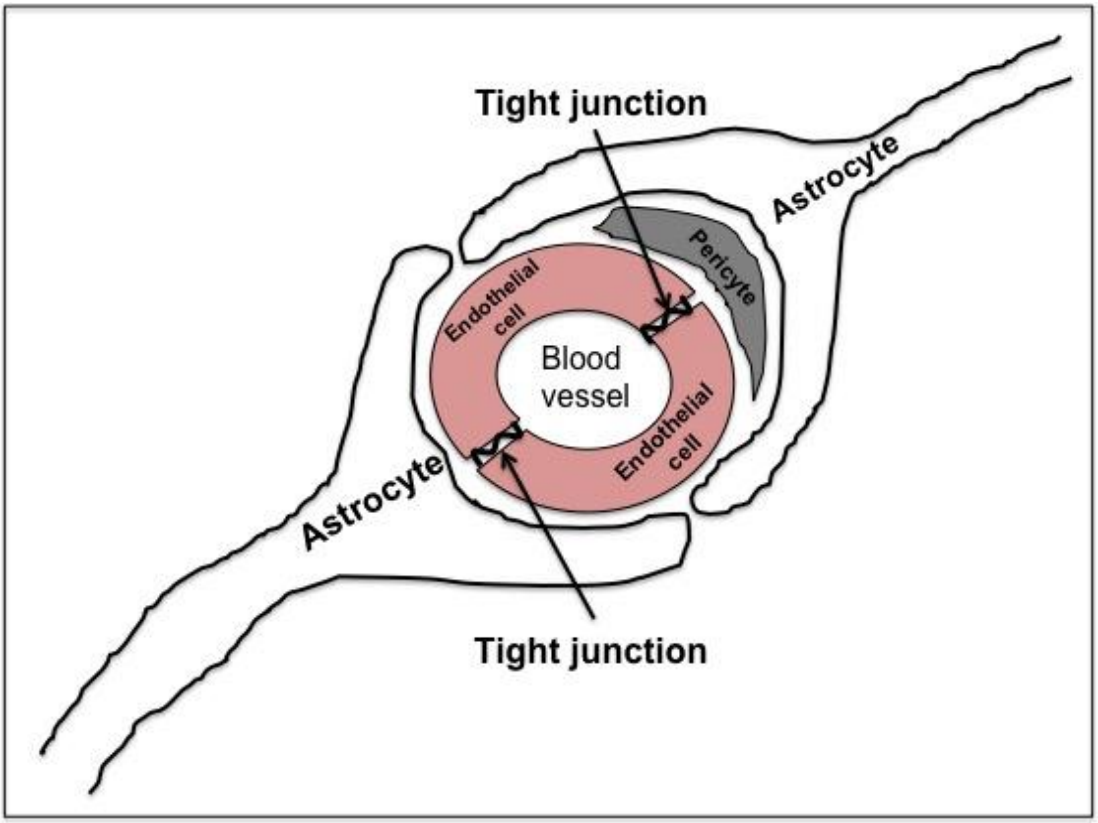

Figure 1.2: Physiological representation of blood-brain-barrier (modified and redrawn from Saunders *et al.* 2012) [48].

Tight junctions are made up of trans-membrane and cytoplasmic proteins. Trans-membrane proteins include occludin protein, members of claudin family (primarily claudin 5 and other smaller molecules such as claudin 3, 10, 12), and other junctional adhesion molecules. Cytoplasmic proteins include PDZ-containing proteins such as zonula occludens (ZO1, ZO2), non-PDZ proteins such as cingulin, and other transmembrane link proteins (54, 55).

Adherens junctions mediate the adhesion of endothelial cells and strengthen these connections. Adherens junctions are primarily composed of vascular endothelial (VE) cadherins. These VE-cadherins are further linked to the cell cytoskeletons with the help of catenins (α, β, γ). Various transporters move molecules to and from the brain. Two types are ABC-transporters (P-glycoprotein, MRP and BCRP) and SLC-transporters (Glut1, OAT and OATP) (54, 55).

Many researchers have sought to identify the onset of development of the BBB in humans and other species and have found that it starts developing in mice and baboons at embryonic stages 12 (56) and 100 respectively (57). Other studies have hypothesized that the development of the BBB in humans might begin during the third trimester (at 28 weeks pregnant) (57).

VII. Primary research questions

A. *In vitro* synaptic proteins analysis

1. Can psychoactive pharmaceuticals alter the expression of synaptic proteins associated with ASD in human neuronal cells?

To determine the extent to which psychoactive pharmaceuticals altered ASD-associated synaptic proteins' expression in humans, this study analyzed their expression profiles in human neuroblastoma cell cultures. After the expression change in ASD-associated synaptic proteins is identified, this *in vitro* system would serve as a model to dissect the mechanisms underlying altered synaptogenesis, which is not well understood in the etiology of idiopathic ASD.

2. Use of SK-N-SH cell line as a model and its differentiation

The use of neuronal cell lines has been increasing dramatically in the field of neuroscience. Researchers can use these primary neuron cells to better understand the biology of cellular processes. There are, however, several problems with this approach. First, most of the primary neurons are matured. They cannot grow more than 1-2 passages. This characteristic limits the types of experiments that can be attempted. Second, when primary neuronal cells are extracted, the extract contains other types of cells such as oligodendrocytes and astrocytes. These non-required cells must be filtered out and this process requires a great deal of experiments and precision. Given those limitations, immortal SK-N-SH cells are chosen for many *in*

vitro cytotoxic studies as an alternative. This neuroblastoma cell line was derived from bone marrow at a metastatic site. The cell line consists of neuron-like cells and epithelial-like cells (which are undifferentiated). Using different reagents that are added to the media, this cell line can be cultured into a specific type of neuronal population. Moreover, this cell line grows quickly which allows us to do multiple experiments. Toxicological studies have been using the SK-N-SH cell line for many years to understand cellular processes of neurons, thus supporting the use of this cell line for this study (58, 59).

For neurological studies, more neuronal cells than epithelial cells are needed. Chemicals such as retinoic acid (RA), phorbol esters and dibutyryl cyclic AMP are used to facilitate the differentiation process (58). Upon differentiation with RA, SK-N-SH cells mature into neuronal-type cells by expressing long neuritic processes. The multiplication rate is also reduced and the differentiation process produces post-mitotic neurons. The formation of functional synapses with the expression of neurotransmitters and their receptors, and neuron-specific enzymes have also been observed during differentiation (58). In 1988, one study quantified the number of neuroblast-type cells after differentiation with RA (60). They found that undifferentiated SK-N-SH cells increased the neuronal population from 14% to 22% in 24 hours. After treating with 10μM RA, the neuronal population increased to 49% in 24 hours (60). In the last decade, many studies have defined the differentiation process for the SK-N-SH cell line. Charting many neuronal markers on this cell line has validated the differentiation into post-mitotic neurons. These neuronal markers are NeuN, GFAP, Tau, NgnI, NeuroD, MAP2, NN18, GAPDH, and MYCN (59, 61-63). Pizzi *et al.* (2002) also detected the expression of functional NMDA receptors and their subunits on differentiated SK-N-SH cells as a mature neuronal marker (61). All

these studies have shown experimentally that the SK-N-SH cell line expresses more neuronal-type cells after differentiation with retinoic acid.

Many neurological studies have used the SK-N-SH cell line to understand the process of synaptic transmission as affected by psychoactive pharmaceuticals. A recent study by Park *et al.* (2013) treated a cultures of SK-N-SH with α2 adrenergic agonists (xylazine and dexmedetomidine) to investigate the feedback mechanism of norepinephrine through its binding with pre-synaptic receptors (64). Another recent study by Asai *et al.* (2013) examined the gene-environment interaction to determine the epigenetic role of neuronal cells in the brain. These researchers used *in vitro* approach by treating SK-N-SH neuronal cells with anti-epileptic drugs (AEDs) such as carbamazepine and analyzing the DNA methylation pattern of the SLC6A4 transporter gene, which is also a target protein for SSRIs. They found that AEDs hypomethylated the promoter region of the SLC6A4 transporter gene, thus demonstrating the epigenetic involvement of pharmaceuticals (65). Researchers have also analyzed the expression of the human brain-derived neurotrophic factor (BDNF) gene in SH-SY5Y cells (derived from SK-N-SH cells) after treating cells with antidepressants such as fluoxetine, desipramine and reboxetine. The neurotrophic factor BDNF modulates synaptic plasticity (13, 17). Desipramine and reboxetine were found to decrease the expression of BDNF mRNA after 6 hours, while increasing the mRNA expression after 48 hours. Researchers did not observe any significant change in BDNF mRNA expression after treating SH-SY5Y cells with fluoxetine (66). Considering previous studies about SK-N-SH cells, it shows that these cells exhibit synaptic properties and therefore, it has been used by many neurotoxicologists to understand the neuronal mechanisms of pharmaceuticals.

3. Instrumentation: Flow Cytometry vs. Western Blot

After differentiating SK-N-SH cells with retinoic acid, immunostaining for synaptic proteins is a critical step. There are some basic techniques, flow cytometry (FACS), fluorescence/confocal microscopy and western blot (WB), which are typically used for immunostaining. There are six reasons that compelled me to use flow cytometry over western blot in this study (67). First, flow cytometry requires fewer cells (5,000 or 10,000 cells) after preparation for staining. In contrast, WB staining requires more cells, which would be more difficult to obtain because the number of SK-N-SH cells is reduced due to the differentiation process. Second, FACS requires less than 12 hours for the sample preparation, while WB takes usually two days. Third, the FACS technique is cheaper than WB because FACS requires fewer reagents for sample preparations. Fourth, FACS protocol is easier to optimize because it involves fewer steps than WB. The optimization of the WB technique is more laborious and troublesome. Fifth, with FACS, I could identify differentiated neuronal cells from other cells and gate them out for my analysis. The differentiation process does not produce a complete neuronal population. However, using the differentiation protocol of Jain *et al.* 2007, I achieved a complete post-mitotic neuronal cells by treating them with mitotic inhibitors (10μM FUdR, 10μM Urd and 10μM araC) (59). In contrast, I could not separate cells with the WB technique. Finally, the FACS technique is more sensitive in terms of detecting signals from a fewer cells than the WB technique, and thus FACS can detect a significant change in the protein expression of partially differentiated neuronal cells (67). FACS seems to be the best option to analyze the synaptic protein expression for this study.

B. *In vitro* gene expression analysis

1. Can psychoactive pharmaceuticals induce the pattern of gene expression in human neuronal cells associated with neurological disorders (including ASD) observed in previous fish study?

To determine the extent to which psychoactive pharmaceuticals altered expression of neuronal genes associated with neurological disorders (including autism) in humans, I analyzed the gene expression profiles with respect to neuronal systems in human neuroblastoma cell cultures. I used valproate as a positive control in this study as it has been found strongly associated with ASD. Identifying altered gene expression profiles in treated cells and finding a pattern similar to valproate-induced gene expressions would reveal the extent to which psychoactive pharmaceuticals at very low concentrations could induce gene expression potentially associated with neurological disorders such as ASD.

2. Use of Valproate to induce ASD like patterns

For this gene expression study, I treated differentiated SK-N-SH cells with psychoactive pharmaceuticals (CBZ, VNX and FLX) and compared their gene expression with the gene expression observed from fish brains in our previous study. A positive control treatment that induces the gene expression associated with neurological disorders like autism would be used for comparison as well. I found some teratogens (valproate, thalidomide etc.) that are strongly associated with autism (2). Of these, I chose Valproic acid. Valproic acid (valproate) is in a class of anti-epileptic drugs (AEDs), and it is used as a mood stabilizer and antiseizure medication (25). Valproate is no longer prescribed during pregnancy as studies discovered that *in utero* exposure to valproate resulted in fetal valproate syndrome in newborn infants (2, 25, 68). Similar to autism spectrum disorders, this syndrome causes defects in neural tube development, and deficits in behavioral and cognitive functions (69).

Many animal studies have observed autism-like phenotypes in newborn infants after prenatal exposure to valproate (68). One rodent study found that prenatal valproate exposure caused repetitive behaviors, social interaction dysfunction, and altered sleep patterns in newborn babies (69). Another study involving rats observed physiological and behavioral problems that were more prevalent in males than females after valproate *in utero* exposure (69). Taken together, these studies have provided adequate evidences that prenatal valproate exposure could induce autism-like phenotypes in newborn infants.

3. Instrumentation: RNA sequencing

In order to evaluate gene set enrichment following treatment, RNAs were extracted from differentiated SK-N-SH cells after treatment with psychoactive pharmaceuticals and valproate. Using Illumina MiSeq, the transcriptome was sequenced to determine whether there were any gene sets enriched that were associated with neuronal growth, regulation and development. Few neurological and molecular studies have used SK-N-SH neuronal cells for transcriptome sequencing. The new ENCODE project extracted small RNAs such as microRNA from SK-N-SH cells to examine their roles at the genomic and cellular levels (70). After sequencing small RNAs and mapping them, the researchers found these RNAs were composed of 53% intronic regions, 34% inter-genic regions and 5% untranslated regions (70). Another recent *in vitro* study sequenced a transcriptome of SK-N-SH cells to determine the role of epigenetic silencing within the NBAT-1 gene and other genes during tumor progression (71). Thus, SK-N-SH cells have been used by several studies for transcriptome analysis and should pose no difficulties in transcriptome analysis.

C. Protein network analysis

1. Do psychoactive pharmaceuticals affect genes with higher network importance in the ASD-associated protein-protein interaction network?

I wished determine the importance of protein products of gene sets enriched by psychoactive pharmaceuticals (individually and in mixtures) in fish brains and human cell cultures within an Autism-associated protein interaction network. To do this, I created a protein-protein interaction network based on AutDB and analyzed the network characteristics of enriched gene sets from both fish brains and human cells. We determined the network importance of gene sets enriched by PPCPs in fish brains and human cell cultures using ten network indices. Of particular interest was the identification of relationships between treatments and highly interconnected gene sets. These relationships more likely to have profound effects on the function of protein networks because of their ripple effects on downstream proteins.

2. Use of network analysis approach to understand the complexity of neurological disorders like ASD

Proteins do not work in isolation. They interact with DNA, RNA, many metabolites (including pharmaceuticals) and other proteins at a molecular level to form a system within a cell. These cellular systems consist of complex protein interactions and signaling cascades, which contribute to one or more biological processes making them phenotypically functional. Using protein networks, we can explore how a change in the expression of one protein can affect the overall network. Protein networks have been used to understand the molecular basis of complex diseases like ASD. Some researchers have argued that ASD is caused by different proteins acting in separate pathways, while others argue that it is a result of complex diseases where different proteins act in common biological pathways (13). However, the mechanisms of protein interactions within ASD and the interaction of ASD with

other diseases are still not understood. Scientists have been using protein networks to understand the complexity of ASD by identifying new candidate genes and biological pathways. A recently published network study by Correia *et al.* (2014) found 14 novel candidate genes in the etiology of ASD by mapping data from two association studies with the human protein-protein interaction network (72). In that study, researchers identified 14 *de novo* common variants which were highly connected in the network (72).

To gain more insights into the molecular basis of ASD, a group of scientists integrated two networks (the protein-protein network and the microRNA-target network) to understand the regulatory role of microRNAs in ASD (73). They created networks by using interactions from the HINT, BioGRID and HPRD databases. Based on mathematical algorithms and network indices, they identified new candidate genes associated with ASD (73). Scientists have also used a network approach to understand the interaction of proteins with drug metabolites as well. For instance, Wu *et al.* (2009) identified an effective combination of drugs for diabetes by using the protein network approach (74). The researchers created an interaction network based on protein-DNA interactions, signaling pathways and protein-protein interactions. They then studied sub-networks affected by drugs individually and in combinations, and compared the interactions using a mathematical model. They used a systems biology approach to design an effective drug cocktail (75). In the present study, I analyzed the interactions of products of genes enriched by psychoactive pharmaceutical treatment in fish brains and human cell cultures.

3. Instrumentation: Cytoscape bioinformatics software

Cytoscape is a freely available Java® software that is primarily used for protein network analysis. The software can be downloaded from www.cytoscape.org.

There are many reasons for using Cytoscape as a bioinformatics tool (76-80). First, Cytoscape allows us to analyze any type of network including protein-protein, protein-DNA, protein-microRNA and other molecular interaction networks. Cytoscape comes with statistical plugins that help users to analyze and understand their networks. These plugins measure network indices like degree, clustering coefficient, closeness centrality, and betweenness. Second, we can access databases such as HRPD, BIND, DIP, OMIM and OPHID from within Cytoscape to explore interactions at any molecular level. We can create a comprehensive network using interactions delineated by these databases. Third, we can identify enriched biological pathways in a network by mapping any gene expression data onto the network. Thus, using plugins, we can separate complexes that have exceptional parameters. Fourth, we can use alternate layouts to understand the network better. For instance, we can change the size of interacting nodes (proteins) based on their importance in the network. Lastly, we can build our own network by searching interactions from other published literature and databases, and annotate the network by comparing highly connected complexes with biological pathways from databases such as KEGG and OMIM (76-80).

Many network studies have used Cytoscape for creating and analyzing networks. For instance, other studies in our lab have used Cytoscape for understanding Alzheimer's disease. In those studies, a comprehensive network of 4,945 nodes and 26,064 interactions was created and mapped with existing gene expression data. After analyzing the network, two key signaling pathways (MAPK/ERK and clathrin-mediated endocytosis) were identified in the etiology of Alzheimer's disease (81).

D. *In vivo* isotope analysis

1. Can psychoactive pharmaceuticals cross biological barriers at low concentrations?

To determine whether psychoactive pharmaceuticals (carbamazepine, venlafaxine and fluoxetine) were capable of crossing biological barriers (intestinal and placental) at very low concentrations, I added ^{2}H isotope labeled pharmaceuticals into the drinking water of female mice for 10 pre- and 10 post-pregnancy days, and measured the $\delta^{2}H$ isotope signal in liver samples of pregnant mice and brains from their fetuses. Detection of psychoactive pharmaceuticals in the developing fetus brain demonstrated that pharmaceuticals from maternal drinking water at environmental concentrations could cross intestinal and placental barriers to the fetus.

2. Use of animal models to understand pharmacokinetics of psychoactive pharmaceuticals

In this study, I was interested in discovering whether psychoactive pharmaceuticals (FLX, VNX, CBZ) could cross maternal biological barriers at low concentrations. Previous studies had looked at the transfer of these pharmaceuticals at clinical doses, but not at lower concentrations. Olivier *et al.* (2011) recently conducted an *in vivo* rat study where the concentration of fluoxetine and norfluoxetine (active metabolite) in the blood of mother rats and newborn rats was measured after administering FLX at 12mg/kg (clinical dose) in the maternal drinking water from gestational day 11. Using HPLC, they detected fluoxetine at 510ng/ml and 430ng/ml concentrations in the blood of mother rats and newborn rats respectively. They also detected norfluoxetine at 736ng/ml and 574ng/ml concentrations in the blood of mother rats and newborn rats respectively (82). Fewer *in vivo* studies have been carried out regarding the pharmacokinetics of VNX during pregnancy. However, a recent study by Aryal *et al.* (2012) measured the plasma concentration of VNX and its metabolite in mice after administering VNX intravenously through the jugular vein.

Researchers injected 13mg/kg (clinical dose) of VNX into the jugular vein and sampled blood from the carotid artery over time. Using the LC-MS technique, they detected VNX and O-desmethylvenlafaxine (ODV) in the blood samples at 33µg/ml and 2µg/ml respectively. They found that the average time for VNX to clear the blood stream in mice was two hours (83). In 1995, a group of scientists carried out a pharmacokinetics study on carbamazepine (CBZ) in rats and found out that CBZ not only crossed the intestinal barrier but also crossed the blood brain barrier (84). The researchers injected CBZ and its metabolite (CBZ-epoxide) I.P. 2.5mg/kg and monitored the concentrations in the blood, cerebellum and hippocampus. Sixty minutes after administering CBZ, they detected it at 1nmol/ml, 0.6nmol/ml and 0.5nmol/ml in the blood, cerebellum and hippocampus of the rat, respectively. Forty minutes after administering CBZ-EPO, they detected it at 1.2nmol/ml, 0.8nmol/ml and 0.7nmol/ml in the blood, cerebellum and hippocampus of the rat, respectively. They also found that the metabolism of CBZ-EPO was slower than CBZ (84). It appears that no pharmacokinetic studies involving the three pharmaceuticals in our study have been done at environmentally relevant concentrations.

3. Instrumentation: Isotope-labeled pharmaceuticals and their detection

We had access to an isotope-ratio mass spectrometer (IRMS) in our department. Therefore, I designed this study around the available equipment I could use to detect pharmaceuticals labeled with stable isotopes. Many studies use commercially available stable isotopes to examine the physiological mechanisms of biological molecules. In an *in vivo* study, McLaren *et al.* (2011), researchers described the mechanism of disposition, synthesis and usage of lipids by feeding fatty acids labeled with ^{13}C-stable isotope orally to C57BL/6 mice. By using LC-MS/MS, they measured the amount of enriched isotope in the plasma collected over time (85). In

another recent study, Berry *et al.* (2012), L-threonine labeled with ^{13}C- and ^{15}N-stable isotopes was used to understand the mechanism of host-degradation compounds and their usage by microbiota within the intestines. They delivered a stable isotope labeled threonine I.V. to C57BL/6 mice, and analyzed its composition in the intestinal lumen, cecum tissue and blood plasma samples that were collected over time (86). These recently published studies provided a common technique to carry out my *in vivo* study by adding isotope-labeled pharmaceuticals to the maternal drinking water and tracing them through the biological barriers.

CHAPTER TWO

Dysregulation of autism-associated synaptic proteins by psychoactive pharmaceuticals at environmental concentrations

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Abstract

Autism Spectrum Disorders (ASD) is a complex neurological disorder for which the prevalence in the U.S. is currently estimated to be 1 in 50 children. A majority of cases of idiopathic autism in children likely result from unknown environmental triggers in genetically susceptible individuals. These triggers may include maternal exposure of a fetus to environmentally relevant minute concentrations of psychoactive pharmaceuticals through ineffectively purified drinking water. Previous studies in our lab examined the extent to which gene sets associated with neuronal development were up- and down-regulated (enriched) in the brains of fathead minnows treated with psychoactive pharmaceuticals at environmental concentrations. The aim of this study was to determine whether similar treatments would alter *in vitro* expression of ASD-associated synaptic proteins on differentiated human neuronal cells. Human SK-N-SH neuroblastoma cells were differentiated for 2 weeks with 10μM retinoic acid (RA) and treated with environmentally relevant concentrations of fluoxetine, carbamazepine or venlafaxine, and flow cytometry used to analyze expression of synaptic proteins found to be associated with ASD in other studies. Data showed that carbamazepine, venlafaxine and mixture treatment at environmental concentrations significantly altered the expression of key synaptic proteins (NMDAR1, PSD95, SV2A, HTR1B, HTR2C and OXTR). Data indicate that psychoactive pharmaceuticals at extremely low concentrations alter the *in vitro* expression of key synaptic proteins that may potentially contribute to neurological disorders like ASD by disrupting neuronal development.

Background

With the prevalence of one in 50 children in the USA (6), autism spectrum disorders (ASD) is a complex neurodevelopmental disorder that lasts throughout a person's life (10). While several studies found that genetic factors such as mutations in candidate genes (high and low susceptibility) and copy number variations (CNV) (13) might impact risk for ASD, these factors are responsible for only 2-3% of ASD cases (3, 87).

Evidence indicates that other unknown factor(s) contribute to the etiology of idiopathic ASD (2, 3, 14, 24). Humans interact with approximately 3000 synthetic chemicals via food, air and water (2). These synthetic chemicals may serve as environmental factors that act as a trigger in genetically susceptible individuals (2, 3, 14, 24, 88). Our lab focused on a specific class of potential toxicants, pharmaceuticals and personal care products (PPCP) (34). This study focused on only psychoactive pharmaceuticals, which have been detected in the drinking water at low concentrations (35, 89).

Psychoactive pharmaceuticals are among the most highly prescribed classes of drug in United States (34, 35, 90) and patient excretions still contain active metabolites that have considerably long half-lives (35, 36). Waste-water treatment plants release PPCP-contaminated water into surface water like rivers and lakes (36). Due to inefficient treatment of sewage water in waste-water treatment plants, the active metabolites reach drinking water in minute concentrations (35). As their metabolites have long half-lives, it is postulated that the environmentally present psychoactive pharmaceuticals might act as a trigger in genetically susceptible individuals by disturbing the process of synaptogenesis or synapse formation, especially during development (2, 13, 90).

To address this hypothesis, our lab previously treated juvenile fathead minnows (*Pimephales promelas*) with psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) individually and in mixtures at environmentally relevant concentrations, and observed that gene sets associated with neuronal development, regulation and growth were altered (34, 35). Based upon our observations, synaptic proteins were identified that were significantly up-and down-regulated in fish brains (Table 1.1). The protein products of these genes are known to play a key role in synaptogenesis, and also found to be associated with ASD (13, 16, 91).

The aim of the present study was to examine whether psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) at environmental concentrations alter *in vitro* expression of ASD-associated synaptic proteins in human neurons. Specifically, we predicted that 1) protein products of genes (Table 1) which were enriched in fish brains would be altered in response to pharmaceuticals treatment at very low concentrations, and 2) other key synaptic protein expression would be more likely to be dysregulated because altered synaptogenesis has been proposed as a pathophysiological mechanism of ASD in other studies. Thus expression profiles in differentiated human SK-N-SH neuroblastoma cell line were determined using flow cytometry.

On identifying the expression change in ASD-associated synaptic proteins, we hope that this *in vitro* system will serve as a model to dissect the mechanisms underlying altered synaptogenesis, which is still considered as a conundrum in the etiology of idiopathic ASD.

Methods

Cell culture and differentiation

Human SK-N-SH cell line was obtained from American Type Culture Collection (ATCC #HTB-11). Cells were cultured in polystyrene tissue culture flasks (Corning) as a monolayer in Eagle's Minimum Essential Medium (EMEM; ATCC). This medium was supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin-neomycin (Sigma). As this cell line is a mixture of different cells, retinoic acid (RA; Sigma) was used for differentiating SK-N-SH cells (59) into more neuronal cells (60). Approximately 30,000 cells (per well in 6-well plates) were cultured in supplemented EMEM media for two days, followed by RA (10μM) treatment for two weeks and old media replaced every three-four days (59). Cultures were monitored visually using light microscopy for morphological changes, and evaluated for neuronal cell markers (NeuN, PSD95 and NCAM) during the differentiation process by flow cytometry and confocal microscopy (*See supplementary information*) (59, 61).

Pharmaceuticals Treatments

Stock solutions (10mM) of fluoxetine (FLX; Sigma F133), venlafaxine (VNX; Sigma D2069) and carbamazepine (CBZ; Sigma C4206) were prepared in dimethyl sulfoxide (DMSO). After differentiation for 2 weeks, cells were treated individually and in mixtures with high (FLX-1mg/l; VNX-5mg/l; CBZ-10mg/l), medium (FLX-10μg/l; VNX-50μg/l; CBZ-100μg/l), low (FLX-1μg/l; VNX-5μg/l; CBZ-10μg/l) concentration ranges. Control cells were treated with DMSO (vehicle) only, and the final concentration of DMSO in the cultures was 0.05%. Cells were treated with pharmaceuticals for 48hr in

EMEM media without FBS to avoid any binding of pharmaceuticals with serum proteins. All of the treatments were shown not to affect overall cell viability with respect to control (no treatment), based on the adherent nature of the monolayers and by CyQuant Viability Assay (Life Technologies) (92). After treatment for 48 hours, cells were collected with Versene solution (Gibco).

Antibodies for synaptic proteins

Following antibodies were purchased from Abcam (Cambridge, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). NMDAR1 (ab109182), NCAM (ab75813), PSD95 (ab2723), SV2A (ab49572), HTR1B (sc1460-R), HTR2C (sc10802), GRM4 (sc99043), OXTR (sc33209), GABRA6 (sc7359), NeuN (mab377), appropriate Alexa488-conjugated secondary antibodies (A11008, A11078).

Flow Cytometer Analysis

Antibody staining was conducted using a protocol modified from Pacheco et al 2004 (93). SK-N-SH cells (1×10^6) were fixed with 500µl 4% paraformaldehyde for 10 min. Cells were washed with ice-cold 500µl PBS and centrifuged to remove supernatant at 3500rpm for 5 min. Cells were then permeablized in 0.1% Tween 20 for 20 min. Cell were again washed and centrifuged 3500rpm for 5 min. The pellet was resuspended in blocking buffer (PBS, 10% BSA, 300mM glycine) and left for 20 min. Cells were incubated with 1µg/200µl of primary antibodies (Anti-NMDAR1, NCAM, PSD95, SV2A, NMDAR2A, HTE2b, HTR2C, GRM4, OXTR, GABRA6, NeuN) for 30 min at room temperature, followed by washing with PBS twice. After re-suspending in 200µl of blocking buffer, 1µg Alexa488-conjugated secondary antibody was added to cells and kept for 30 min in the dark at room temperature. After washing twice with PBS, cells

were analyzed on a FACS Caliber Flow Cytometer using CellQuest software (BD Biosciences). Gates were drawn using a side scatter x forward scatter dot plot to select the major cell population, omitting dead cell debris and cellular clumps. Unstained cells and cells labeled with only secondary antibody were also analyzed to determine autofluorescence and non-specific binding of secondary antibody, and background levels were subtracted from all median fluorescence intensity values reported in the data.

Statistical approach

Flow cytometer data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test to determine if controls were significantly different (significance defined as $P < 0.05$) than pharmaceutical treatments. Experiments were replicated 5 times, and data are presented as mean $\pm$ SEM. Statistical analyses were carried out using R programming (94).

Results

Carbamazepine (CBZ) treatment at environmental concentration

We found that N-methyl-D-aspartate (NMDAR1) and oxytocin (OXTR) protein expression was increased significantly (Figure 2.1.A, 2.1.B) at all concentrations compared to untreated control. In contrast, we found carbamazepine treatment significantly decreased expression of post-synaptic density (PSD95), synaptic vesicle (SV2A), and serotonin (HTR2C and HTR1B) synaptic proteins (Figure 2.1). No significant change was noted in expression of other synaptic proteins (NCAM, GRM4, GABRA6 and NeuN, data not shown). This result demonstrated that carbamazepine at

low concentrations altered *in vitro* expression of a subset of synaptic proteins which was consistent with the fish study.

Venlafaxine (VNX) treatment at environmental concentration

We then sought to determine the effect of venlafaxine on synaptic protein expression at environmental concentrations. To accomplish this, we treated differentiated SK-N-SH neuroblastoma cells with venlafaxine in three concentrations (5μg/l, 50μg/l, 5mg/l). Concentrations were chosen based on observations in our previous fish study (34).

We found that SV2A and OXTR synaptic protein expression was increased significantly at medium and high concentrations compared to control (Figure 2.2). In contrast, we found venlafaxine treatment decreased the expression of PSD95 and HTR1B synaptic proteins significantly. No significant change was noted in other (HTR2C, NMDAR1, NCAM, GRM4, GABRA6 and NeuN, data not shown) synaptic protein expression. This result reiterates the effect of psychoactive pharmaceuticals at very low concentrations on synaptic proteins expression, which was consistent with the fish study.

Fluoxetine (FLX) treatment at environmental concentration

The data summarized above provide evidence in support of the fact that psychoactive pharmaceuticals altered synaptic proteins expression at environmental concentrations. For a more concise comparison, one more psychoactive pharmaceutical, fluoxetine was examined. SK-N-SH cells were incubated with fluoxetine in three concentrations (1μg/l, 10μg/l, 1mg/l) for 48 hr. Concentrations were chosen based on observations in our previous fish study.

Interestingly, no significant change in the expression of synaptic proteins (PSD95, SV2A, OXTR, HTR1B, HTR2C, NMDAR1, NCAM, GRM4, GABRA6 and NeuN, data not shown) was found compared to controls.

Mixture treatment (CBZ, VNX and FLX) at environmental concentration

We then sought to determine the effect of the mixture of 3 psychoactive pharmaceuticals (CBZ, VNX and FLX) on synaptic proteins expression at environmental concentrations. We were interested in analyzing the effect of an interaction of these drugs. To accomplish this, differentiated SK-N-SH neuroblastoma cells were incubated with carbamazepine, venlafaxine and fluoxetine in three concentrations, which were chosen based on observations in our previous fish study.

We found that HTR1B and OXTR synaptic protein expression was increased significantly at low, medium and high concentrations compared to control (Figure 2.3). In contrast, mixture treatment reduced expression of GABRA6, NMDAR1A, PSD95 and SV2A synaptic proteins significantly (Figure 2.3). No significant change in other (HTR2C, NCAM, GRM4 and NeuN, data not shown) synaptic protein expression was found. Data demonstrate the effect of individual psychoactive pharmaceuticals at low concentrations on synaptic proteins expression, but that the mixture resulted in different results compared to either drug alone (Figure 2.3), except in the case of PSD95 and OXTR.

Discussion

In our study, SK-N-SH cell line was selected as our model because this cell line is a fast-growing cell line and differentiation results in a mixture of different neuronal cells.

Using RA, these cells differentiate into more neuronal cells, which was validated by neuronal cell markers (NeuN, PSD95 and NCAM) (59, 61). In the future, it may prove valuable to use primary neurons to gain more insights into the mechanisms underlying psychoactive pharmaceuticals -induced changes in synaptic proteins. Knowing the source of primary neurons from a specific part of brain, localized effects of psychoactive pharmaceuticals on that particular neuronal population might be evaluated. Whole transcriptome analysis could be performed to understand the broad impact of psychoactive pharmaceuticals on a neuronal population. These future experiments may provide more insights regarding psychoactive pharmaceuticals in dysregulating synaptic proteins associated with ASD.

It has been found that waste-water treatment plants (WWTP) are releasing pharmaceuticals into surface water, thus producing contamination into the aquatic systems (35, 36). Most of the isoforms of these psychoactive pharmaceuticals in WWTP are active because of two reasons. First, some parts of the pharmaceuticals are not metabolized completely by clinical patients and are excreted through human liver and kidneys in an active form (36). Second, although most of the metabolized pharmaceuticals excreted by humans are in glucuronide conjugates, which is an inactive form (36), some bacteria like *Escherichia coli* present in feces reconvert the inactive conjugated form into an active one (deconjugated) by secreting large amounts of an enzyme β-glucuronidase (36).

Abnormal levels of synaptic proteins play a critical role during synaptogenesis in the etiology of ASD (13). Due to many genetic factors like CNV deletions, mutations, allelic exclusion, or epigenetic silencing, the expression of synaptic proteins may be

altered. This alteration in the level of synaptic proteins leads to the formation of abnormal neuronal circuits, which is considered as one of the potential mechanisms underlying ASD (13). Could psychoactive pharmaceuticals, at environmentally relevant concentrations, dysregulate the expression of key synaptic proteins that further disturb the process of synaptogenesis? To address this question, the extent to which psychoactive pharmaceuticals altered the expression of specific synaptic proteins (whose expression was found to be altered in our previous study) in a differentiated human neuroblastoma cell line was examined. As predicted, key synaptic proteins exposed to pharmaceutical treatment at environmental concentrations were significantly dysregulated compared to (no treatment) control.

To study a concentration effect of these psychoactive pharmaceuticals in the present study, cell cultures were incubated with 10-fold lower and 100-fold higher than the medium concentration range. Interestingly, it was observed that carbamazepine and venlafaxine reduced the expression of synaptic proteins (NMDAR, OXTR, HTR1B, HTR2C, PSD95, SV2A) significantly at all concentrations, including the lowest. Considering our results *in vitro*, and given existing knowledge of the effects of psychoactive pharmaceuticals on developmental brain, it seems conceivable that psychoactive pharmaceuticals at low concentrations interact with common genetic variants, to produce abnormal levels of synaptic proteins and this might contribute to ASD (3, 10, 13).

Carbamazepine (CBZ) is an anticonvulsant used as an antidepressant to treat bipolar disorders (95). CBZ blocks sodium channels, thus inhibiting the epileptic effects in the brain (95). Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI),

which blocks reuptake of 5-HT (96). VNX (SNRI) inhibits 5-HT reuptake at lower concentrations where as it blocks noradrenaline effects at higher concentrations (96). CBZ, FLX and VNX were detected at low concentrations in surface waters of United States (34), and eventually found in groundwater (35). In our study, CBZ, VNX individually and in mixtures at environmental concentrations lowered *in vitro* expression of synaptic proteins, similar to fathead minnow brains (34).

CBZ, VNX and mixture treatments (CBZ, VNX, FLX) decreased expression of PSD95 and increased OXTR expression significantly, which is consistent with expression patterns observed in fish brain (34). PSD95 protein was found to be associated with ASD in a recent study (91), which suggests that alteration of PSD95 expression due to psychoactive pharmaceuticals treatment could be a possible mechanism in etiology of ASD. It is unclear how psychoactive pharmaceuticals treatment increases OXTR expression, but other studies found that oxytocin might regulate the release of serotonin by activating oxytocin receptors, thus mediating the anxiolytic effect of oxytocin (97).

Both CBZ and VNX individual treatments decreased the expression of HTR1B, similar to their effect in fish brain (34). This might indicate a process of desensitization of post-synaptic receptors noted in other studies (98). Similar to our fish study, HTR2C expression was also decreased by CBZ treatment. Interestingly, increased NMDAR1 expression due to CBZ treatment was also found in fish brain (34). Similar studies also found a significant increase in NR2A and NR2B subunits of NMDA receptors on treatment with valproate, which is also an anticonvulsant like CBZ (99).

However, mixture treatment resulted in a different outcome with respect to HTR1B and NMDAR1 synaptic proteins compared to both the fish study and individual

CBZ, VNX treatments in this study. A significant decrease of inhibitory GABRA6 protein was found after mixture treatment, which is again opposite to our results in fish brain. It is unclear how mixture treatment of these pharmaceuticals produced different results than individual treatments and the fish study, but it is conceivable that psychoactive pharmaceuticals might interact with each other. The interaction between their active metabolites may have altered cell adhesion molecules (key organizers) which further altered the expression of glutaminergic and inhibitory receptors that further changed synaptic plasticity (13).

Conclusions

Psychoactive pharmaceuticals at low concentrations altered the expression of synaptic proteins, which were previously found to be associated with ASD. Data suggest that environmental contaminants like psychoactive pharmaceuticals might change the neuronal connections inside the developing fetus brain by dysregulating the synaptic proteins expression (2, 87). Psychoactive pharmaceuticals altered the expression of synaptic proteins *in vtiro* significantly at low concentrations. These altered proteins play a key role in synapse formation.

Authors' contributions

GK, JP, CC and MAT designed the experiments, and GK performed them. JP, CC and MAT provided the direction and guidance for the research. YX carried out some experiments for this study and GK collected all the data and interpreted it after testing hypotheses. All authors have read and approved the final manuscript.

Acknowledgements

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Figures

Figure 2.1

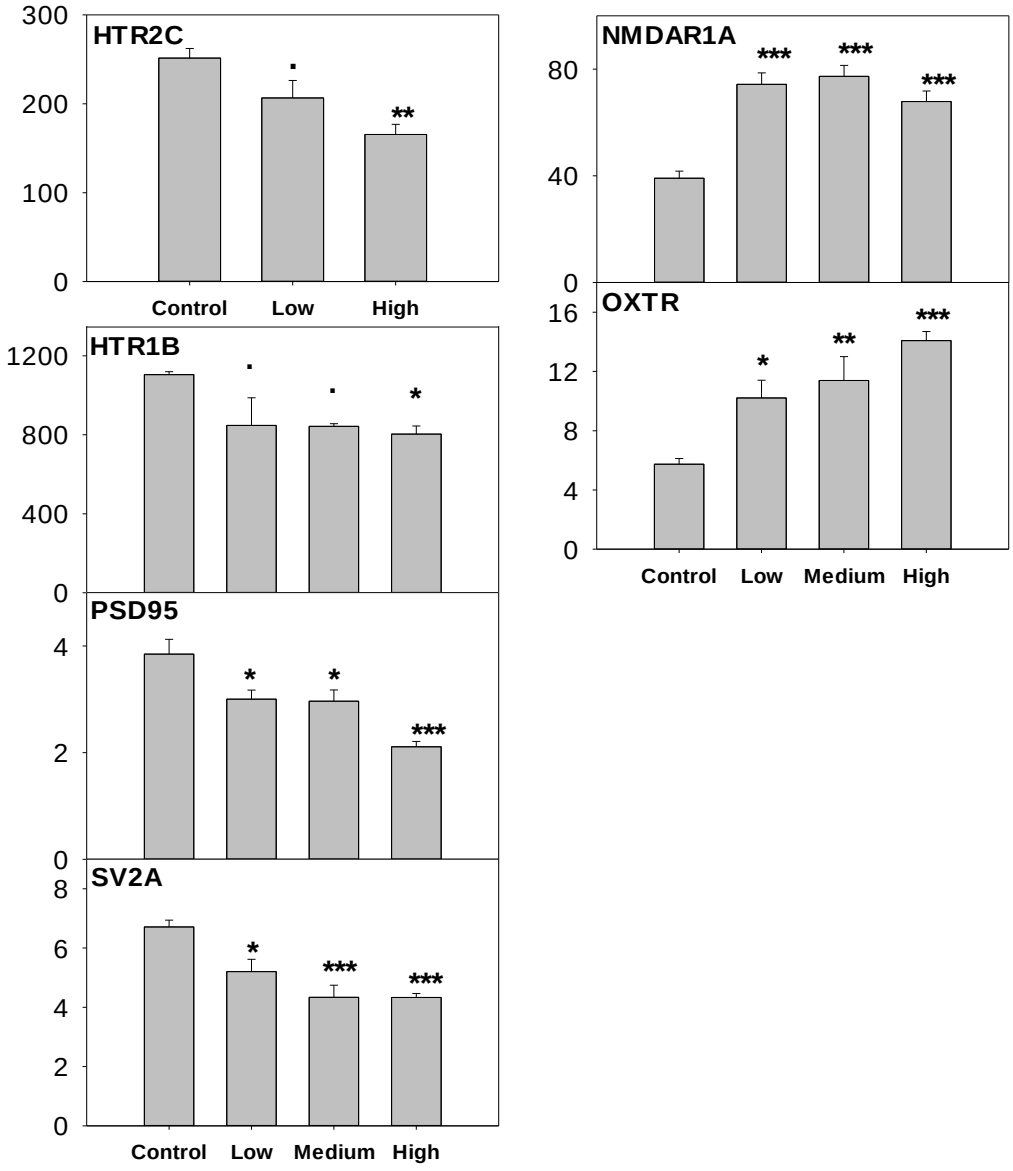


Figure 2.1: Carbamazepine treatment on synaptic proteins expression. Differentiated SK-N-SH cells line were cultured and treated with carbamazepine for 48 hr. After fixing with 4% paraformaldehyde (PFA), cells were incubated for 30 min at RT with primary

antibodies and then incubated with Alexa488-labeled secondary antibody. The median fluorescence intensity was measured by flow cytometry analysis. Data were analyzed using one way ANOVA, followed by Dunnett's post hoc comparisons of treatments to controls. Symbols above bars indicate significant differences from the control after Dunnett's adjustment for family-wise type I error at significance level: "***" = 0.001, "**" = 0.01, "*" = 0.05, "." = 0.1. Error bars represent SEM.

Figure 2.2

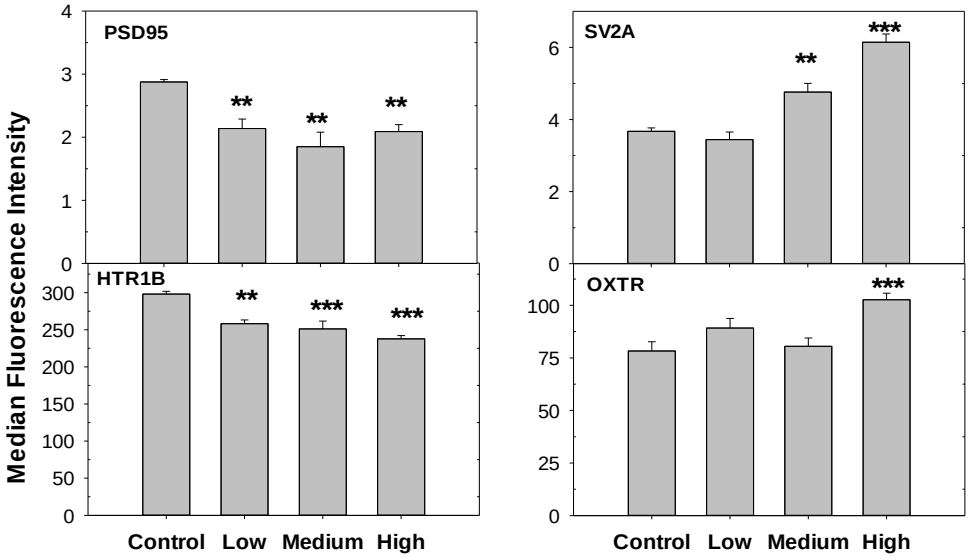


Figure 2.2: Venlafaxine treatment on synaptic proteins expression. Differentiated SK-N-SH cells line were cultured and treated with venlafaxine for 48 hr. After fixing with 4% paraformaldehyde (PFA), cells were incubated for 30 min at RT with primary antibodies and then incubated with Alexa488-labeled secondary antibody. The median fluorescence intensity was measured by flow cytometry analysis. Data were analyzed using one way ANOVA, followed by Dunnett's post hoc comparisons of treatments to

controls. Symbols above bars indicate significant differences from the control after Dunnett's adjustment for family-wise type I error at significance level: "***" = 0.001, "**" = 0.01, "*" = 0.05, "." = 0.1. Error bars represent SEM.

Figure 2.3

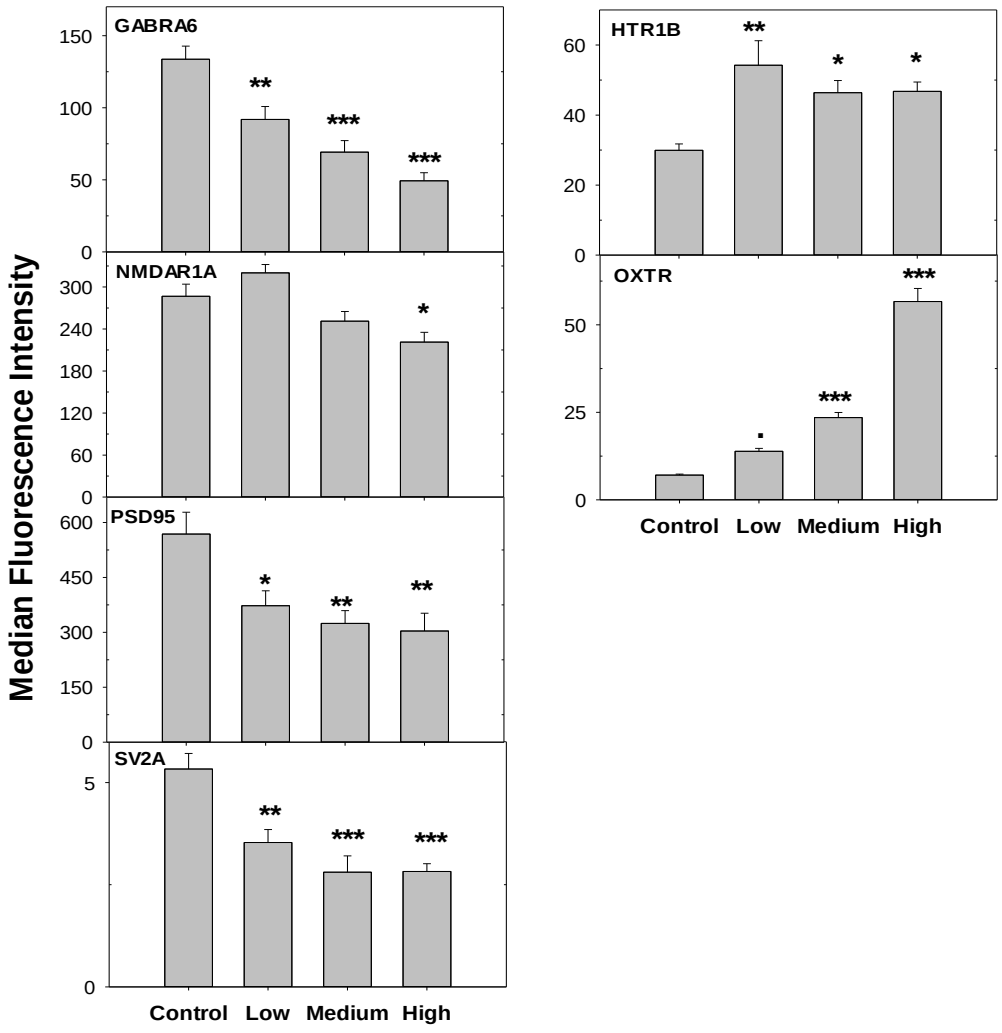


Figure 2.3: Mixture treatment on synaptic proteins expression. Differentiated SK-N-SH cells line were cultured and treated with carbamazepine, venlafaxine and fluoxetine for 48 hr. After fixing with 4% paraformaldehyde (PFA), cells were incubated for 30 min at RT with primary antibodies and then incubated with Alexa488-labeled secondary

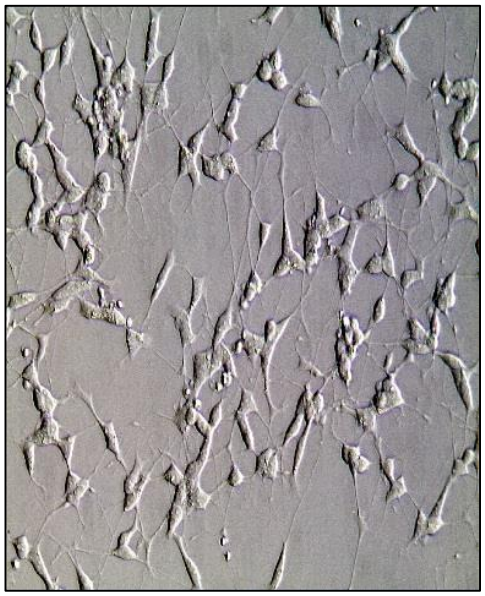
antibody. The median fluorescence intensity was measured by flow cytometry analysis. Data were analyzed using one way ANOVA, followed by Dunnett's post hoc comparisons of treatments to controls. Symbols above bars indicate significant differences from the control after Dunnett's adjustment for family-wise type I error at significance level: "***" = 0.001, "**" = 0.01, "*" = 0.05, "." = 0.1. Error bars represent SEM.

Supplementary Information

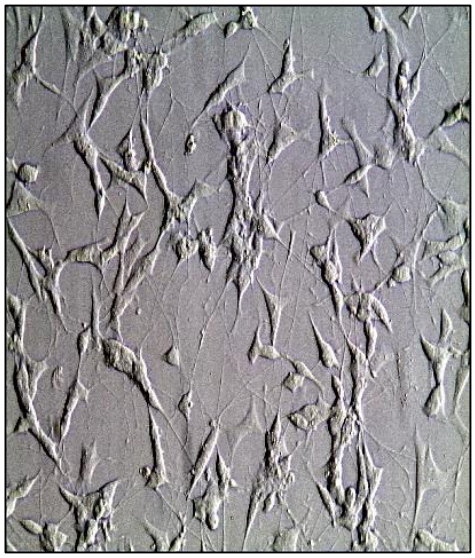
SK-N-SH Differentiation with Retinoic acid (10μM):

A. Undifferentiated cells (Image magnification: 100x)

B. Differentiated cells – 2 days (Image magnification: 100x)



C. Differentiated cells – 2 weeks (Image magnification: 100x)



D. Differentiated cells – 4 weeks

Image magnification: 100x

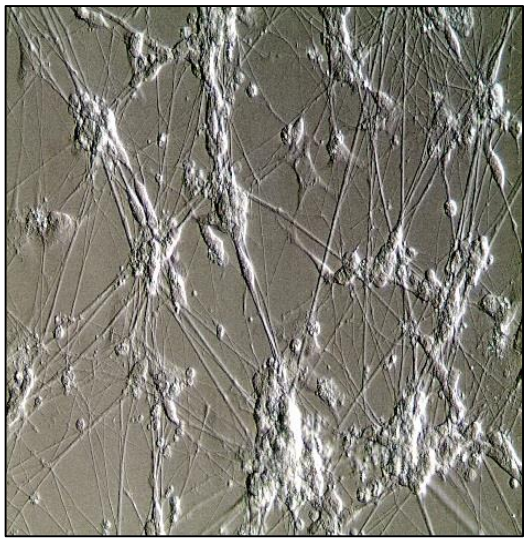
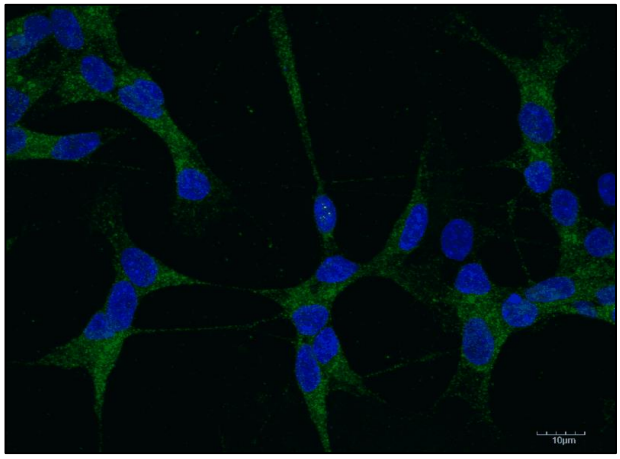


Image magnification: 400x

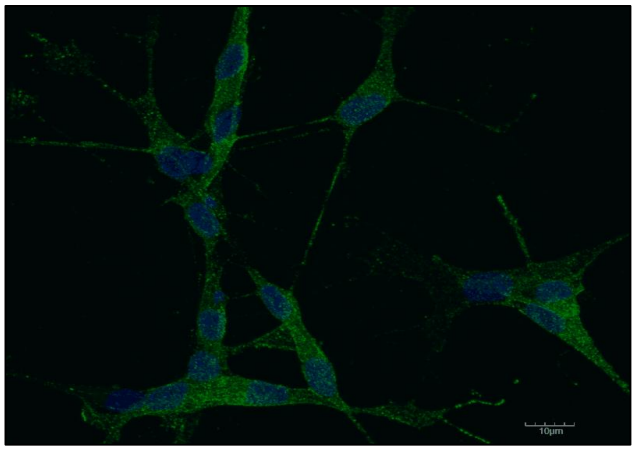
Confocal Imaging: Confocal microscopy analysis of differentiated SK-N-SH cell line after culturing them in media containing 10μM RA for 2 weeks. Cells were fixed with

4% paraformaldehyde (PFA) for 10 mins, followed by the permeablization with 0.1% TWEEN for 15 mins at room temperature (RT). After blocking with 10%BSA, cells were incubated for 30 mins at RT with primary antibodies and then incubated with FITC-labeled secondary Alexa488 antibody. Cell nucleus were stained with DAPI. After staining protocol, cells were visualized and images captured by confocal microscopy technique. Scale bar, 10μm.

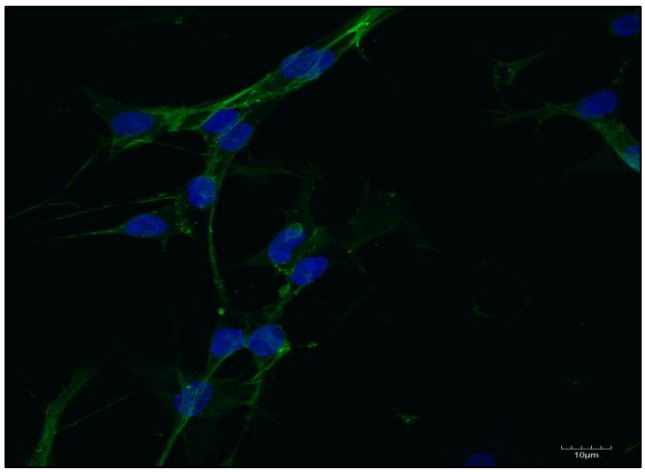
E. NMDAR1



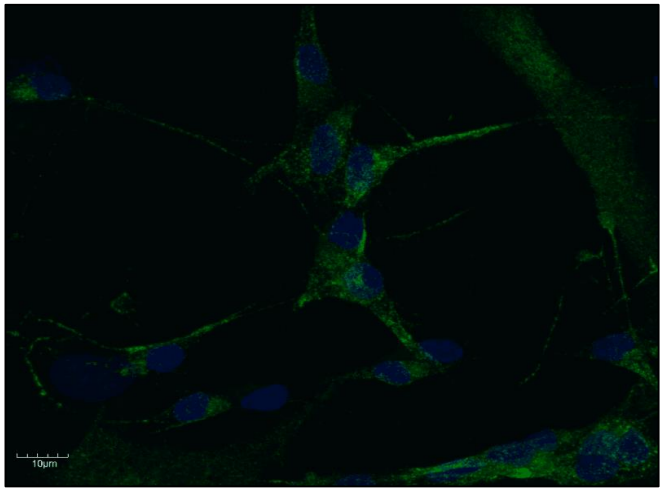
F. PSD95



G. NCAM



H. SV2A



CHAPTER THREE

Psychoactive pharmaceuticals at environmental concentrations induce *in vitro* gene expression associated with neurological disorders

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Abstract

With the current prevalence of 1 in 50 children, neurological disorders like idiopathic autism has increased dramatically in the United States. However, genetic factors are responsible for only 2-3% of all cases, suggesting unknown environmental contaminants play a role in triggering idiopathic autism. Psychoactive pharmaceuticals have been considered as potential environmental contaminants as they are detected in the drinking water at very low concentrations. Preliminary studies in our laboratory identified gene sets associated with neuronal systems and human neurological disorders that were significantly enriched after treating fish brains with psychoactive pharmaceuticals at environmental concentrations. These gene expression inductions were associated with changes in fish behavior. Here, we tested the hypothesis that similar treatments would alter *in vitro* gene expression associated with neurological disorders (including autism) in human neuronal cells. We differentiated and treated human SK-N-SH neuroblastoma cells with a mixture (fluoxetine, carbamazepine and venlafaxine) and valproate (used as a positive control to induce autism-associated profiles), followed by transcriptome analysis with RNA-Seq approach. We found that psychoactive pharmaceuticals and valproate significantly altered neuronal gene sets associated with human neurological disorders (including autism-associated sets). Moreover, we observed that altered expression profiles in human cells were similar to gene expression profiles previously identified in fish brains. Psychoactive pharmaceuticals at environmental concentrations altered *in vitro* gene expression profiles of neuronal growth, development and regulation. These expression patterns were associated with potential neurological disorders including

autism, suggested psychoactive pharmaceuticals might mimic, aggravate, or induce neurological disorders.

Background

There are approximately 3000 synthetic chemicals that are known to interact with humans through air, water, and food (2, 34). These chemicals might serve as environmental contaminants by acting as a trigger for neurological disorders, like autism spectrum disorders (ASD) in genetically susceptible individuals (14, 24). Among this diverse group of environmental contaminants, we focused on pharmaceuticals and personal care products (PPCPs) (34). PPCPs include psychoactive pharmaceuticals that are highly prescribed in the United States, and other chemicals like bis-phenol A (BPA) in plastics, phthalates in cosmetics and teratogenic chemicals (2).

Psychoactive pharmaceuticals like fluoxetine, venlafaxine and carbamazepine, have been detected in the drinking water at very low concentrations (34-36). These pharmaceuticals, which are metabolically active and have relatively long half lives for over a month, reach waste-water treatment plants (WWTP) through excretion by clinical patients (34, 36). Due to the chemical properties of these drugs and inefficient filtration of WWTP, these drugs end up mixing up with the ground water, and thus reach drinking water at low concentrations (34, 36).

We previously hypothesized that psychoactive pharmaceuticals as environmental contaminants, alter neuronal gene expression associated with neurological disorders like ASD. To determine this, our lab treated treating juvenile fathead minnows (*Pimephales promelas*) with psychoactive pharmaceuticals (fluoxetine, venlafaxine and

carbamazepine) individually and in mixtures at environmentally relevant concentrations (34, 35). After treating them for 15 days, we extracted the brains and carried out microarray analysis. Using gene set enrichment analysis (GSEA) (38), we identified enrichment (up- or down-regulation) of gene sets associated with neuronal growth, regulation and development in the juvenile minnow brains in response to psychoactive drug exposure (34, 35). We also identified altered neuronal gene sets associated with neurological disorders, including ASD (35). Moreover, fish exposed to psychoactive pharmaceuticals exposed had an altered behavioral phenotype (34).

In the present study, we hypothesized that psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) at environmental concentrations would alter *in vitro* human neuronal gene expression that is 1) similar to gene expression profile in fathead minnow, and 2) associated with neurodevelopmental disorders. To determine if altered gene expression profile was associated with idiopathic autism, we also treated human neuronal cells with valproic acid, which is known to induce autism-like phenotypes in mice (68). Identifying altered gene expression profiles in treated cells and finding a similar pattern with valproic acid induced gene expression would reveal the extent to which psychoactive pharmaceuticals at very low concentrations could induce gene expression associated with potential neurological disorders like ASD.

Methods

Cell culture and differentiation

Human SK-N-SH cell line was obtained from American Type Culture Collection (ATCC #HTB-11) . Cells were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC). This media was supplemented with 1% penicillin-streptomycin-neomycin (Sigma) and 10% (v/v) fetal bovine serum (FBS). Retinoic acid (RA; Sigma) was used to induce SK-N-SH cells (59) to differentiate into more neuron-like cells (60) because this cell line is a mixture of different cell types. Cells Approximately15,000 cells were cultured in in T-75 flask (Corning) supplemented EMEM media for two days, and then Retinoic acid (10μM) was added. Cells were kept in RA for two weeks and media was replaced every three-four days (59). Cultures were monitored visually using light microscopy for morphological changes, and evaluated for neuronal cell markers (NeuN, PSD95 and NCAM) during the differentiation process (59, 61).

Pharmaceuticals Treatments

Stock solutions (10mM) of fluoxetine (FLX; Sigma F133), venlafaxine (VNX; Sigma D2069) and carbamazepine (CBZ; Sigma C4206), and 1mM stock solution of valproic acid (sc211393; Santa Cruz Biotechnology) were prepared in dimethyl sulfoxide (DMSO). After differentiation for two weeks, cells were treated with a mixture composed of (MIX: FLX 10μg/l; VNX 50μg/l; CBZ 100μg/l), and Valproate (VPA: 0.035mM). Control cells were treated with DMSO (vehicle) only, and the final concentration of DMSO in the cultures was 0.05%. Cells were treated (in 3 replicates) with the pharmaceuticals for 48 hr in EMEM media without FBS to avoid any binding of

pharmaceuticals with the serum proteins. All of the treatments were shown not to affect overall cell viability with respect to control (no treatment), based on the adherent nature of the monolayers and the result from CyQuant Viability Assay (92). After treating cells for 48hr, they were collected with Versene solution (Gibco).

RNA extraction, cDNA synthesis and sequencing

After 48hr of exposure, cells were collect with trypsin, centrifuged, and RNA was extracted using Qiagen RNeasy Plus Mini Kit (74134) according to the manufacturer's protocol. Total RNA concentration was determined using NanoDrop (Thermo Scientific), and the RNA integrity value (RIN) was analyzed on Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA was quantified with Qubit spectrophotometer (Life technologies). Using Illumina Tru-seq stranded total RNA kit, cDNA library was prepared in following steps: RiboZero depletion and fragmentation, first and second strand cDNA synthesis, adenylation of 3' ends, adapter ligation, PCR amplification, library validation using qPCR – Kapa Biosystems Library Quantification Kit, and normalization and pooling in preparation for cluster generation on MiSeq. Samples were then loaded onto flow cell (MiSeq Reagent kit v3 150 cycle) and sequenced on Illumina MiSeq 2 according to the manufacturer's instructions.

Bioinformatics Data Analysis

Quality Control, Alignment, and Read Counting: In total, 3 treatments (mixture, valproate, and control) with 3 replicates each were sequenced with 5 flow cells using Illumina MiSeq, which generated more than 10M paired-end reads for each replicate (Refer Supplementary).The raw sequences in FASTQ files underwent quality control analysis using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). All

the samples were sequenced at 75b read length and they all passed the quality check. We aligned the quality checked reads to human genome hg19 using TopHat version 2.0.11 (100). The reference genome sequence, the bowtie index files for the reference genome sequence, and the gene annotation file were downloaded from Illumina iGenomes project (http://support.illumina.com/sequencing/sequencing_software/igenome.html). The number of reads that map to human known genes were counted by summerizeOverlaps function in GenomicAlignments R package (94). Only the genes that have at least one read for all replicates were retained for downstream analysis.

Analysis of Differential Gene Expression: Differentially expressed genes were identified using DESeq2 version 1.6.1 (101). Differentially expressed genes from 2 groups (Mixture and Valproate) with respect to the control treatment were identified.

Gene Set Enrichment Analysis: We used GAGE version 2.14.4 (102) for gene set enrichment analysis. Gene sets from different groups (Neuronal development, regulation, growth; Neurological Disorders (ND); Autism spectrum disorders (ASD); MSigDB 'C2' and 'C5') were obtained from supplementary data of previous studies and MSigDB. Enrichment analyses were carried out using gage function using non-parametric Kolmogorov-Smirnov tests. We used GAGE package to identify significantly enriched (significantly up- and/or down-regulated; P-value < 0.01 and Q-value < 0.1) gene sets within mixture and valproate treatments.

Results

Patterns of psychoactive pharmaceuticals – induced *in vitro* gene expression in neuronal development, growth and regulation.

We postulated that psychoactive pharmaceuticals at environmental concentrations would alter gene expression of neuronal systems. Support for this hypothesis would suggest that dysregulation of neuronal systems would result in altered neuronal circuits and may result in fewer neuronal connections. To address this question we used differentiated SK-N-SH neuroblastoma cells and treated them with the mixture (MIX: FLX-10µg/l; VNX-50µg/l; CBZ-100µg/l), and valproate (VPA: 0.035mM) in replicates of 3 samples for each treatment.

For the development collection, we observed 6 enriched gene sets (significantly down-regulated, *P*-value < 0.01, *Q*-value < 0.1) by MIX treatment. VPA treatment enriched the expression of 7 gene sets (4 gene sets down- and 3 gene sets up-regulated, Table 3.1a). AXONOGENESIS, REGULATION OF NEUROGENESIS and SYNAPSE PART gene sets were enriched in both VPA and MIX treatments. And, SYNAPSE PART gene set was also enriched (up-regulated) in fish brains.

For the regulation collection, we observed 14 enriched gene sets (13 gene sets down- and 1 gene set up-regulated, *P*-value < 0.01, *Q*-value < 0.1) by MIX treatment. VPA treatment enriched the expression of 8 gene sets (up-regulated, Table 3.1b). All 8 up-regulated gene sets in VPA treatment were down-regulated in MIX treatment. Among

those sets, 2 gene sets (NEUROTRANSMITTER_BINDING and SYNAPSE) were also enriched in fish brains.

For the growth collection, we observed 8 enriched gene sets (all down-regulated, *P*-value < 0.01, *Q*-value < 0.1) by MIX treatment. VPA treatment enriched the expression of 5 gene sets (up-regulated, Table 3.1c). All 5 up-regulated gene sets in VPA treatment were down-regulated in MIX treatment. 2 gene sets (AXON and NEURON_PROJECTION) were also enriched in fish brains.

Patterns of psychoactive pharmaceuticals – induced *in vitro* gene expression in neurological disorders (ND) and ASD groups.

We then sought to determine if altered *in vitro* gene expression was associated with neurological disorders and ASD. To accomplish this, we analyzed and compared the gene expression of already published ND and ASD gene sets in MIX and VPA treatments.

For the ND collection, we observed 6 enriched gene sets (4 down- and 2 up-regulated, *P*-value < 0.01, *Q*-value < 0.1) by MIX treatment. VPA treatment enriched the expression of 3 gene sets (2 gene sets down- and 1 gene sets up-regulated, Table 3.1d). AUTISM_IDIOPATHIC and PARKINSONS gene sets were enriched in both VPA and MIX treatments as well as in fish brains.

For the ASD collection, we observed 2 enriched gene sets (2 up-regulated, *P*-value < 0.01, *Q*-value < 0.1) by MIX treatment. VPA treatment enriched the expression of 5 gene sets (down-regulated, Table 3.1e). ASD_MILD gene set was enriched in both VPA and MIX treatments as well as in fish brains.

Ranked gene lists from the mixture and valproate treatment.

We sorted all genes from gene expression profiles in human neuronal cells treated with the MIX (FLX, VNX, CBZ) and valproate (VPA). In each treatment, we sequenced ~ 17,000 genes and analyzed their expression. We then ranked them based on their fold change expression within each treatment. In Table 3.2 & 3.3, the 50 most strongly up-and down-regulated genes from each treatment were tabulated. We also reported the fold-change score of genes which were presented on the fish microarray chip.

Discussion

Comparison between human MIX treatment and fish gene expression patterns.

The results partially supported our first hypothesis that the MIX treatment on human SKN-SH cell line yield gene sets enrichment patterns similar to that of the fish experiment following mixture treatment, although the degree of similarity is not high.

Among the neural circuit development gene sets, all the six significantly enriched gene sets in human neuronal cells following MIX treatment were enriched in a down-regulated manner; whereas in the fish experiment, the two significantly enriched sets were both up-regulated. We do not know why the direction was the opposite, but we do notice that, one gene set, SYNAPSE PART, was enriched in both treatment (34). Previous studies have found that altered expression of NCAM, IRX3 and NKX6.1 genes in SYNAPSE PART changed the fate and position of neurons generated in the chick neural tube (103, 104). Also within those down-regulated gene sets were gene PSD95

(DLG4) and GABA, which have recently been found to be associated with neurological disorders like autism by altering the synaptic assembly (13, 61, 91).

In the growth group, we observed similar patterns where all enriched sets in MIX treatment were down-regulated and all those in the fish experiment were up-regulated. Among those sets, two were enriched in both treatments, AXON and NEURON PROJECTION. Other studies have found that genes within these 2 gene sets modulate the fate, lineage, and timing of neuronal development by playing a critical role in the formation and maturation of neural circuits (13, 105, 106).

In the regulation group, we observed NEUROTRANSMITTER BINDING gene set was down-regulated in fish brains as well as in human cells (34). This could be possible due to the therapeutic effect of fluoxetine (SSRI) in the mixture treatment (34). Fluoxetine is known to reduce the re-uptake of serotonin by inhibiting monoamine transporters on the pre-synaptic neuronal membrane (43, 107). Due to the longer availability of neurotransmitters in the synaptic cleft, the expression of serotonin receptors is down-regulated, thus a decrease in neurotransmitter binding (108). Another gene set SYNAPSE was significantly up-regulated in fish brains (34), but down-regulated in treated human cells. This gene set in responsible for modulating wiring of neuronal circuits by controlling the number of synapse as well as organization of synaptic assembly and specificity (106, 109). Altered synaptogenesis has been strongly considered as a potential mechanism in ASD pathogenesis (16, 22).

Comparison between human MIX and VPA gene expression patterns.

We used valproate (an anticonvulsant) to treat human neuronal cells as a postive control, because prenatal exposure of valproate has been found to be strongly associated with autism (24) and valproate is also known to induce autism-like phenotypes in mice (68). Similarly, carbamazepine (presented in the mixture treatment) is a mood stabilizer and anticonvulsant (68, 95) and it also inhibits the epileptic effects in the brain by blocking sodium channels (68, 95). By and large, the results support our second hypothesis that the MIX and VPA treatments change the RNA expression profile in similar ways.

In the development group of VPA treatment, we found 4 gene sets to be down-regulated and 3 gene sets significantly up-regulated in human neuronal cells. Three gene sets (AXONOGENESIS, REGULATION OF NEUROGENESIS and SYNAPSE PART) were enriched in both mixture and valproate treatments, but in the opposite direction. Similar to fish gene expression, VPA treatment up-regulated SYNAPSE PART gene set (34). This states that VPA exposure might be associated with disturbed neuronal fate and position (103, 104) as well as synaptic assembly (2, 104, 110).

In the growth group, we observed that 5 gene sets were up-regulated in the VPA treatment. Interestingly, the same five sets were all down-regulated in the MIX treatment. This suggested that both treatments disturbed the human gene expression in similar pathways despite the different directions. One gene set NEURON PROJECTION was up-regulated similar to fish brains (34). In the regulation group, we noticed similar results i.e. 8 gene sets were up-regulated in valproate treatment, but down-regulated in the mixture treatment. From this repeating phenomenon of opposite direction enrichment od

the same gene set, we could deduce a general pattern that the three pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) exert a different response compared to valproate, however the deduction remains yet to be confirmed.

Association of human MIX and VPA gene expression patterns with neurological disorders.

To determine the extent to which altered gene expression in both MIX and VPA treatments were associated with neurological disorders (including ASD), we examined the expression of the already-published gene sets from neurological disorders (ND) and ASD groups in both treatments. We also compared those to their corresponding expression pattern in fish brains. For ND group, MIX and VPA treatments altered PARKINSONS and Autism_Idiopathic gene sets significantly but in different directions. Interestingly, mixture treatment of human cells and fish brains up-regulated Autism_Idiopathic gene set (35). For another ASD group, MIX treatment up-regulated 2 gene sets (ASD_2Class and ASD_Mild) similar to fish brains (35). On the other side, VPA treatment down-regulated 3 gene sets (ASD_2Class, ASD_Mild, ASD_Savant) in a different direction to the expression in fish brains (35). These expression patterns stated that VPA and MIX treatments of human cells exhibited a similar response to neurological disorders (including ASD), suggested a common induction effect.

Insights into the identification of important genes in both MIX and VPA treatments.

We sought to identify important, or novel genes that were significantly up- and down-regulated in human neuronal cells treated with MIX and VPA. We generated ranked lists of genes based on their fold change, and tabulated the most 50 strongly up-

and down-regulated genes (Table 2 & 3). We also compared genes from ranked lists with the ones from fish microarray data. For MIX treatment, we found 4 genes (NUPR1, RTL1, THBS1 and HTR2B) which were considered important and novel. The thrombospondin (THBS1) gene plays an important role in synaptogenesis in the developing brain (111). Recent association studies have found that both rare and common variants of this gene are associated with autism (111). Although this gene was found to be down-regulated by 2-fold, but it was up-regulated in fish brains under similar mixture treatment (34). Another important gene, HTR2B, which codes for serotonin receptor 2B, were down-regulated by ~ 2-fold in human cells. Similar serotonin receptor genes were also found to be down-regulated in fish brains. Moreover, recent protein studies in our lab showed that HTR2B protein was down-regulated in the same mixture (FLX, VNX, CBZ) treatment (112). This mechanism is explained by the drug effect of fluoxetine (SSRI), which provides more neurotransmitter in the synaptic cleft, thus reducing serotonin receptors (108).

In the ranked list of genes by valproate (VPA) treatment, we found 3 genes (VSNL1, PTER and OXTR) of particular importance. VSNL1 gene, which encodes for visinin-like protein 1 in humans, modulates neuronal morphology by controlling the key signaling pathways in CNS. This gene was up-regulated by 3-fold in the valproate treatment. We observed similar up-regulation of this gene in fish brains following mixture (FLX, VNX, CBZ) treatment. Other studies have recently found the association of single-nucleotide polymorphisms (SNPs) in the VSNL1 gene with neurological disorders like schizophrenia (113). Another important gene, oxytocin receptor (OXTR) was found to be down-regulated by 2-fold in human cells treated with valproate (Table

3). However, this gene was up-regulated in fish brains exposed to the mixture of psychoactive pharmaceuticals (34). Moreover, recent protein studies in our lab showed an increased expression of OXTR in the same cells treated with carbamazepine and the mixture (FLX, VNX, CBZ) (112). Other studies have shown that OXTR serves as an anxiolytic agent by modulating serotonin release in serotonergic neurons of the raphe nuclei (97).

Conclusions

As a potential environmental contaminant, we focused on psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) that have been detected in drinking water. We found that psychoactive pharmaceuticals altered the gene expression of neuronal systems *in vitro* at environmental concentrations. These altered gene expression are associated with potential neurological disorders including autism, probably play a key role in the formation, growth and regulation of neurons. Our data suggests that psychoactive pharmaceuticals might mimic, aggravate, or even induce neurological disorders by altering the neuronal gene expression, thus disrupting the neuronal connections.

Authors' contributions

GK, YX and MAT designed the experiments, and GK performed them. MAT provided the direction and guidance for the research. YX and LY carried out analyses using R-programming and GK wrote the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 3.1 Descriptions of gene sets and results of analyses

Source	Gene set name	Size	Function/Description	MIX_UP	MIX_DOWN	VPA_UP	VPA_DOWN
a. *Development collection, containing 20 sets*							
GO:0007409	**AXONOGENESIS**	378	Formation of long process of a neuron, carrying outgoing action potential		**p= 0.00 q=0.00**	**p= 0.00 q=0.05**	**p= 0.00 q=0.04**
GO:0007420	**BRAIN_DEVELOPMENT**	255	Maturation of the brain structure				**p= 0.00 q=0.00**
GO:0021954	CENTRAL NERVOUS SYSTEM NEURON DEVELOPMENT	29	Progression and functional differentiation of a neuron whose cell body lies in CNS				
GO:0007417	**CENTRAL_NERVOUS_SYSTEM_DEVELOPMENT**	378	Progression and maturation of CNS				**p= 0.00 q=0.00**
GO:0021545	CRANIAL NERVE DEVELOPMENT	20	Progression and formation of cranial nerves				
GO:0016358	**DENDRITE_DEVELOPMENT**	58	Development of dendrites		**p= 0.00 q=0.00**		
GO:0007272	*(ENSHEATHMENT OF NEURONS)*	43	Formation of the insulated part of a neuron				
GO:0030900	FOREBRAIN_DEVELOPMENT	142	Progression and formation of forebrain				
GO:0048699	**GENERATION_OF_NEURONS**	72	Production of neuroblasts and differentiation into neurons				**p= 0.00 q=0.04**
GO:0030902	HIND BRAIN DEVELOPMENT	43	Progression and formation of hindbrain				
GO:0050769	NEGATIVE REGULATION OF	47	Reduces the rate of neurogenesis				

8	NEUROGENESIS						
GO:0007399	**NERVOUS_SYSTEM_DEVELOPMENT**	328	Progression and formation of the nervous tissue		**p= 0.00 q=0.00**		
GO:0021915	*(NEURAL_TUBE_DEVELOPMENT)*	68	Progression and formation of the neural tube				
GO:0022008	NEUROGENESIS	17	Generation of new cells				
GO:0048666	NEURON_DEVELOPMENT	25	Progression and formation of the neuron				
GO:0050769	**POSITIVE REGULATION OF NEUROGENESIS**	67	Increases the rate of neurogenesis		**p= 0.00 q=0.00**		
GO:0050772	POSITIVE_REGULATION_OF_AXONOGENESIS	31	Increases the rate of axonogenesis				
GO:0050767	**REGULATION_OF_NEUROGENESIS**	193	Controls the rate of neurogenesis		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:0050808	*(SYNAPSE_ORGANISATION)*	62	Organizes the synaptic assembly or arrangement				
GO:0044456	***(SYNAPSE_PART)***	228	Formation of a junction between a neuron and another neuron, or fiber, or glial cell		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
b. *Regulation collection, containing 22 sets*							
GO:0043679	**AXON_TERMINUS**	33	Formation of the neuron terminal part that controls neurotransmitter secretion		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:0008066	**GLUTAMATE RECEPTOR ACTIVITY**	25	Binding of glutamate to the neuron		**p= 0.00 q=0.00**		
MSigDB C2 CPG	**HSA04080_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION**	45	Interaction of neuro-active ligand-receptor		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
MSigDB C2	LU_AGING_BRAIN_DN	232	Genes found down regulated in the frontal cortex of old subjects				

CPG							
MSigDB C2 CPG	*(LU_AGING_BRAIN_UP)*	132	Genes found up regulated in the frontal cortex of old subjects				
GO:000727 0	NERVE-NERVE SYNAPTIC TRANSMISSION	44	Communicates between neurons				
GO:000683 6	**NEUROTRANSMITTER TRANSPORT**	97	Controls the movement of neurotransmitters		**p= 0.00 q=0.00**		
GO:004216 5	***(NEUROTRANSMITTER_BINDING)***	47	Controls the impulse from a neuron to other cells		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:004213 3	**NEUROTRANSMITTER_METABOLIC_PROCESS**	19	Controls chemical pathways and reactions of neurotransmitters		**p= 0.00 q=0.00**		
GO:003059 4	**NEUROTRANSMITTER_RECEPTOR_ACTIVITY**	40	Controls the binding of neurotransmitter to the cell		**p= 0.00 q=0.00**		
GO:000726 9	*(NEUROTRANSMITTER_SECRETION)*	68	Controls the secretion of neurotransmitters				
GO:005043 3	REGULATION OF CATECHOLAMINE SECRETION	17	Controls the secretion of catecholamines				
GO:004816 8	REGULATION OF NEURONAL SYNAPTIC PLASTICITY	27	Controls the neuronal plasticity				
GO:005196 9	**REGULATION OF TRANSMISSION OF NERVE IMPULSE**	105	Controls the transmission of a neuron impulse		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:001922 8	*(REGULATION_OF ACTIONPOTENTIAL_IN_NEURON)*	53	Controls the action potential of a neuron				
GO:003164 4	**REGULATION_OF_NEUROLOGICAL_SYSTEM_PROCESS**	111	Controls the fate of a neurological process		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:000150	**REGULATION_OF_NEUROTR**	90	Controls the level of neurotransmitters		**p= 0.00 q=0.00**		

5	**ANSMITTER_LEVELS**						
GO:000548 4	**SNAP RECEPTOR ACTIVITY**	19	Modulates the fusion of membranes	**p= 0.00 q=0.00**			
GO:000014 9	*(SNARE BINDING)*	26	Helps in the interaction of SNARE protein				
GO:004520 2	***(SYNAPSE)***	319	Formation of the junction of one neuron to others		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:000726 8	**SYNAPTIC_TRANSMISSION**	288	Mediates the transmission between synapses		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:001922 6	**TRANSMISSION_OF_NERVE_IMPULSE**	336	Mediates the depolarization and repolarization of a nerve impulse		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
c. *Growth collection, containing 16 sets*							
MSigDB C2 CPG	*(APPEL_IMATINIB_RESPONSE)*	28	Up-regulation by imatinib during dendritic cell differentiation				
GO:003042 4	***(AXON)***	158	Mediates the formation of long process of a neuron		**p= 0.00 q=0.00**		
GO:000836 6	AXON_ENSHEATHMENT	43	Formation of the insulated part of a neuron				
GO:004867 5	AXON_EXTENSION	42	Mediates the growth of the long process of a neuron				
GO:000741 1	**AXON_GUIDANCE**	282	Mediates the migration of the long process of a neuron		**p= 0.00 q=0.00**		
GO:003326 7	**AXON_PART**	63	Mediates the cell projection of a neuron		**p= 0.00 q=0.00**	**p= 0.00 q=0.01**	
GO:004866 7	**CELL MORPHOGENESIS INVOLVED IN NEURON DIFFERENTIATION**	403	Mediates the formation of neuron structures		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	

GO:0021953	CENTRAL NERVOUS SYSTEM NEURON DIFFERENTIATION	54	Modulates the formation of differentiation of CNS				
GO:0042551	NEURON MATURATION	18	Mediates the maturation of a neuron				
GO:0048812	**NEURON PROJECTION MORPHOGENESIS**	408	Controls the formation and organization of neuron projection		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:0070997	NEURON_DEATH	93	Mediates the neuron death				
GO:0030182	**NEURON_DIFFERENTIATION**	61	Mediates the differentiation of a neuron		**p= 0.00 q=0.00**		
GO:0043005	***(NEURON_PROJECTION)***	322	Controls the projection of a neuron		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:0043523	*(REGULATION OF NEURON APOPTOSIS)*	76	Mediates the occurrence/rate of neuron death by apoptosis				
GO:0045664	**REGULATION OF NEURON DIFFERENTIATION**	156	Mediates the occurrence/rate of neuron differentiation		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
GO:0007416	SYNAPSE ASSEMBLY	39	Organizes the assembly of a synapse				
d. *Neurological Disorders (ND) collection, containing 12 sets*							
GSE12457	**ADHD_down**	32	Down-regulated genes by molecular alterations in genetic and environmental rat models of ADHD		**p= 0.00 q=0.00**		
GSE12457	**ADHD_up**	44	Up-regulated genes by molecular alterations in genetic and environmental rat models of ADHD		**p= 0.00 q=0.02**		
GSE1297	**ALZHEIMERS**	308	Genes upregulated in the CA1 region of the hippocampus in Alzheimer's disease	**p= 0.00 q=0.02**			
GSE7329	ASD_Secondary	49	Genes expressed in lymphoblastoid cells from individuals with fragile X syndrome				

	(Autism_idiopathic)	439	Combination of Chakrabarti, Hu and ASD_2class; excluding duplicates	**p= 0.00 q=0.00**			**p= 0.00 q=0.00**
	Bipolar	57	Upregulated genes in individuals with bipolar disorder				
GSE12654	Depression	38	Upregulated genes in individuals with depression				
	MS_Bomprezzi	34	Genes expressed in peripheral blood mononuclear cells from individuals with MS				
GSE17393	***(MS_gilli)***	320	Altered genes from non-pregnant MS patients				**p= 0.00 q=0.03**
	(PARKINSONS)	130	Genes associated with Parkinsons		**p= 0.00 q=0.00**	**p= 0.00 q=0.00**	
	RETT	26	Upregulated genes in females with Rett syndrome				
	Schizophrenia	29	Differentially expressed proteins in the brains of SCZ patients		**p= 0.00 q=0.00**		
e. *ASD collection, containing 8 sets*							
GSE15402	***(ASD_2Class)***	354	Differentially expressed genes (significantly) from a SAM 2-class analysis of the data from combined autistic samples and neurotypical controls	**p= 0.00 q=0.00**			**p= 0.00 q=0.00**
GSE15402	***(ASD_Mild)***	306	Differentially expressed genes (significantly) from a SAM 2-class analysis of the data from mild autistic samples and neurotypical controls	**p= 0.00 q=0.00**			**p= 0.00 q=0.00**
	(ASD_Savant)	71	Differentially expressed genes (significantly) from a SAM 2-class analysis				**p= 0.00 q=0.00**
GSE15402	ASD_Severe	176	Differentially expressed genes (significantly) from a SAM 2-class analysis of the data from the group of individuals with severe language impairment and neurotypical controls				
GSE15402	*(ASD_Shared)*	67	Common genes from GSE15402 sets				
	Chakrabarti	54	Genes associated to neural growth, sex steroids and social impairment behavior				**p= 0.00 q=0.00**

			related to autistic traits and Asperger's syndrome; excluding severe language impairment				
	Hu	30	Genes examined by ASD individuals with severe language impairment vs. neurotypical individuals				
	Pinto	81	Genes associated with ASD genetic susceptibility				**p= 0.00 q=0.00**

Table representing the analyses of gene sets within neuronal systems (a. Development, b. Regulation, c. Growth), (d) Neurological Disorders, and (e) ASD groups. Source indicates the database from where the gene set was derived, Gene Ontology (GO) or Molecular Signatures Database (MSigDB). Size represents the number of genes in each gene set. MIX_UP, MIX_DOWN, VPA_UP and VPA_DOWN indicates gene sets which were found up-regulated in the mixture, down-regulated in the mixture, up-regulated in the valproate, and down-regulated in the valproate treatments, respectively. Gene sets with p-value < 0.01 and q-value < 0.1 were considered as statistically significantly enriched (102). Enriched gene sets in human cells and corresponding scores are marked in bolds. Enriched gene sets in fish brains are italicized and marked in parentheses.

Table 3.2 Ranked gene list from the mixture treatment (50 most up- and down-regulated)

Up-regulated Genes					Down-regulated Genes				
Rank	**Genes**	**Fold change**	***P*-value**	**Fold change in Fish brains**	**Rank**	**Genes**	**Fold change**	***P*-value**	**Fold change in Fish brains**
1	FAM129A	3.25	0.000		17350	ANGPTL4	-3.36	0.000	
2	CACNA2D3	1.99	0.000	0.90	17349	THBS1	-2.19	0.000	0.34
3	NUPR1	1.97	0.002	-0.10	17348	STARD13	-2.17	0.000	-0.46
4	GRM8	1.90	0.000		17347	DRP2	-2.15	0.000	
5	PTCHD1	1.89	0.000		17346	TNNT2	-2.11	0.000	-0.23
6	TMEM132C	1.76	0.000		17345	AQP10	-2.10	0.000	
7	DDIT3	1.55	0.000	0.73	17344	STRA6	-1.97	0.000	-0.10
8	XKR4	1.53	0.010		17343	PAPPA2	-1.92	0.000	
9	CHRNA10	1.33	0.002		17342	NEDD9	-1.88	0.000	-1.21
10	LPHN3	1.24	0.004		17341	GGTA1	-1.85	0.001	
11	SERPINF2	1.23	0.008	0.15	17340	SH3TC1	-1.83	0.000	
12	NKX3-1	1.22	0.008		17339	ADAMTSL2	-1.80	0.000	-0.25
13	HRK	1.22	0.051		17338	RGS13	-1.78	0.000	
14	SCARNA20	1.22	0.009		17337	ANO1	-1.77	0.000	
15	PABPC3	1.21	0.006		17336	LOC283731	-1.75	0.000	
16	KIT	1.18	0.001	0.15	17335	CMKLR1	-1.73	0.000	0.86
17	KIAA1045	1.18	0.002	-0.12	17334	VIP	-1.71	0.000	
18	DLL1	1.15	0.013	0.05	17333	HTR2B	-1.69	0.000	
19	C9orf150	1.12	0.000		17332	RAG2	-1.67	0.004	0.20
20	SNORA14A	1.11	0.164		17331	ACVRL1	-1.67	0.000	
21	B3GALT1	1.09	0.134		17330	VCAN	-1.66	0.000	

22	RIMBP3C	1.09	0.096		17329	C6	-1.65	0.006	-1.11
23	NOS1AP	1.06	0.172	0.37	17328	ENO3	-1.64	0.000	-0.00
24	GLYATL2	1.06	0.021		17327	TM4SF1	-1.63	0.008	0.22
25	STAC2	1.04	0.259		17326	PCTK3	-1.62	0.004	
26	CRYBB2	1.04	0.020	0.01	17325	FAM65C	-1.60	0.000	
27	SLC16A10	1.01	0.167	-0.31	17324	FLJ36208	-1.60	0.000	
28	CDH7	1.01	0.031	-0.00	17323	LOC400950	-1.58	0.000	
29	MYB	1.01	0.030	-0.17	17322	RARRES3	-1.58	0.000	
30	LCA5L	1.00	0.176		17321	SLCO4C1	-1.53	0.000	
31	ID4	0.99	0.033	-0.17	17320	PRAP1	-1.52	0.022	
32	MALAT1	0.99	0.004		17319	SLC18A1	-1.51	0.000	
33	SNAR-A3	0.99	0.230		17318	TFPI2	-1.51	0.000	
34	EDN1	0.98	0.033		17317	FBP1	-1.50	0.000	0.43
35	VSTM2A	0.97	0.000		17316	FGL1	-1.49	0.001	
36	HERV-FRD	0.97	0.036		17315	COL9A3	-1.48	0.001	
37	CNTD1	0.97	0.037	-1.03	17314	LRRTM1	-1.48	0.015	0.25
38	ELL2	0.96	0.000	-0.30	17313	BTK	-1.48	0.000	-0.30
39	SLC13A3	0.96	0.027	0.02	17312	SLC24A2	-1.46	0.000	0.11
40	DENND1B	0.96	0.114	0.06	17311	IRF6	-1.45	0.000	0.17
41	CYP2C18	0.95	0.041		17310	LOC392232	-1.45	0.012	
42	AMN	0.95	0.041	-0.33	17309	RD3	-1.45	0.000	
43	NCRNA00087	0.95	0.301		17308	DDC	-1.43	0.000	
44	FUT9	0.94	0.042	-0.19	17307	PRLHR	-1.43	0.008	
45	SCGB1D2	0.92	0.047		17306	NOTUM	-1.42	0.052	-0.31
46	TRIB3	0.92	0.000	0.67	17305	TMIGD2	-1.41	0.008	
47	MYBL1	0.91	0.019		17304	RTL1	-1.40	0.048	-0.40
48	ZNF726	0.91	0.260		17303	P2RX6	-1.40	0.000	

49	DLEU1	0.90	0.173		17302	DKK1	-1.39	0.000	
50	SLC7A11	0.90	0.309	-0.41	17301	TRIM9	-1.38	0.000	

Table representing the list of 50 most up- and down-regulated genes in human neuronal cells treated with the mixture (FLX, VNX, CBZ). Genes were ranked based on their expression in fold-change in human cells. *P*-value represents the significance level of the expression change in the mixture treatment than control. Corresponding fold change of genes in fish brains is also reported in this table, where blank cells represents genes that are not found in the fish microarray chip.

Table 3.3 Ranked gene list from the valproate treatment (50 most up- and down-regulated)

Up-regulated Genes					Down-regulated Genes				
Rank	**Genes**	**Fold change**	***P*-value**	**Fold change in Fish brains**	**Rank**	**Genes**	**Fold change**	***P*-value**	**Fold change in Fish brains**
1	OPRK1	4.03	0.000	-0.29	17673	CMKLR1	-2.81	0.000	0.86
2	NEUROG2	3.55	0.000		17672	ADAMTS2	-2.74	0.000	0.09
3	VSNL1	3.19	0.000	2.84	17671	HIST1H4L	-2.67	0.000	
4	GALNAC4S-6ST	3.09	0.000	0.96	17670	ELFN1	-2.44	0.000	
5	POSTN	2.93	0.000	1.22	17669	COL4A2	-2.39	0.000	-0.42
6	EVX2	2.86	0.000		17668	GREM2	-2.33	0.000	
7	SERPINE1	2.69	0.000	-1.45	17667	FLJ45455	-2.32	0.000	0.86
8	ODZ1	2.64	0.000		17666	VCAN	-2.31	0.000	
9	DRD5	2.34	0.000	-0.23	17665	SOCS3	-2.29	0.000	0.02
10	CSGALNACT1	2.33	0.001		17664	TGFBI	-2.27	0.000	-1.11
11	HOXD13	2.31	0.000		17663	C11orf53	-2.26	0.000	
12	C3orf57	2.29	0.000		17662	SNAI1	-2.17	0.000	-0.82
13	B3GALT1	2.29	0.000		17661	LOC283480	-2.11	0.000	
14	SECTM1	2.28	0.001		17660	ID1	-2.05	0.000	0.23
15	PLAU	2.20	0.001		17659	PPP1R9A	-2.03	0.000	-0.05
16	PDE1A	2.19	0.000		17658	LOC646498	-2.02	0.004	
17	VIM	2.14	0.000	0.25	17657	RPS16P5	-2.02	0.000	
18	MME	2.11	0.000		17656	COL4A1	-1.98	0.000	0.10
19	KCNJ2	2.09	0.000		17655	GLT8D2	-1.98	0.000	
20	HS6ST2	2.09	0.000	-0.15	17654	CYP26A1	-1.96	0.000	0.26
21	SNORA42	2.00	0.003		17653	SLC10A1	-1.95	0.004	
22	RASEF	1.99	0.000	0.31	17652	PART1	-1.94	0.000	

23	APOL6	1.93	0.000		17651	OXTR	-1.89	0.000	1.26
24	NCRNA00164	1.91	0.007		17650	PTPLAD2	-1.84	0.007	
25	COLQ	1.91	0.000		17649	C9orf131	-1.83	0.001	
26	CACNA2D3	1.90	0.000	0.90	17648	FOS	-1.80	0.000	-0.63
27	DEGS2	1.90	0.000		17647	C9orf135	-1.79	0.009	
28	NHS	1.89	0.000		17646	GLP1R	-1.79	0.000	
29	PTER	1.89	0.001	0.11	17645	ETS1	-1.79	0.000	-0.79
30	EDIL3	1.87	0.000	-0.41	17644	NXPH3	-1.79	0.000	
31	INSM2	1.85	0.000		17643	LOC100128505	-1.79	0.013	
32	GFRA3	1.82	0.000		17642	KCTD12	-1.76	0.000	0.49
33	PROKR2	1.82	0.000		17641	ANGPTL4	-1.74	0.000	
34	GRM8	1.81	0.001		17640	FOSB	-1.73	0.000	
35	TLE4	1.79	0.000		17639	MPPED2	-1.72	0.000	-0.62
36	WNT5A	1.77	0.001	-0.38	17638	C11orf92	-1.71	0.003	
37	SMOC2	1.77	0.000	-0.26	17637	WDR38	-1.69	0.001	
38	VCAM1	1.76	0.014	0.53	17636	GALNT14	-1.68	0.000	0.03
39	FRMD4B	1.74	0.000	0.10	17635	LOC283143	-1.67	0.002	
40	PTRF	1.74	0.000		17634	ACCN1	-1.66	0.002	
41	DLK1	1.70	0.000	0.69	17633	C14orf53	-1.66	0.002	
42	PTCHD1	1.67	0.001		17632	TAPBPL	-1.65	0.021	
43	GPR126	1.66	0.022		17631	NRP1	-1.63	0.000	1.12
44	ANKDD1B	1.65	0.000		17630	BFSP2	-1.63	0.002	
45	MOXD1	1.65	0.002	-1.00	17629	SMAD7	-1.63	0.000	0.08
46	FAM111B	1.65	0.000		17628	HOPX	-1.62	0.028	
47	LOC728739	1.63	0.002		17627	ERBB4	-1.62	0.000	
48	CHRNA10	1.62	0.002		17626	PAPPA2	-1.62	0.000	
49	NELL1	1.62	0.000		17625	PCDHGB8P	-1.62	0.011	

50	NELL2	1.62	0.000	-0.76	17624	LOC284454	-1.60	0.000	

Table representing the list of 50 most up- and down-regulated genes in human neuronal cells treated with the valproate. Genes were ranked based on their expression in fold-change in human cells. *P*-value represents the significance level of the expression change in the mixture treatment than control. Corresponding fold change of genes in fish brains is also reported in this table, where blank cells represents genes that are not found in the fish microarray chip.

CHAPTER FOUR

Psychoactive pharmaceuticals as environmental contaminants may disrupt highly inter-connected nodes in an Autism-associated protein-protein interaction network

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Abstract

Most cases of idiopathic autism spectrum disorder (ASD) likely result from unknown environmental triggers in genetically susceptible individuals. These triggers may include maternal exposure of a fetus to minute concentrations of pharmaceuticals, such as carbamazepine (CBZ), venlafaxine (VNX) and fluoxetine (FLX). Unmetabolized pharmaceuticals reach drinking water through a variety of routes, including ineffectively treated sewage. Previous studies in our laboratory examined the extent to which gene sets were enriched in minnow brains treated with pharmaceuticals. Here, we tested the hypothesis that genes in fish brains and human cell cultures, significantly enriched by pharmaceuticals, would have distinct characteristics in an ASD-associated protein interaction network. We accomplished this by comparing these groups using 10 network indices. A network of 7212 proteins and 33,461 interactions was generated. We found that network characteristics for enriched gene sets for particular pharmaceuticals were distinct from each other, and were different from non-enriched ASD gene sets. In particular, genes in fish brains, enriched by CBZ and VNX 1) had higher network importance than that in the overall network, and those enriched by FLX, and 2) were distinct from FLX and non-enriched ASD genes in multivariate network space. Similarly, genes in human cell cultures enriched by pharmaceutical mixtures (at environmental concentrations) and valproate (at clinical dosages) had similar network signatures, and had greater network importance than genes in the overall ASD network. The results indicate that important gene sets in the ASD network are particularly susceptible to perturbation by pharmaceuticals at environmental concentrations.

Background

Autism is a complex neurobiological developmental disorder belonging to a group of conditions known as Autism Spectrum Disorder (ASD) (4, 10). ASD has an overall prevalence of approximately one case in every 50 children in USA (6), notably affecting four times as many males as females (13, 87). To date, several studies have reported ASD−associated genetic factors and have categorized them into 2 groups: rare variants (genes with low susceptibility and high penetrance) and common variants (genes with high susceptibility and low penetrance) (13). These genetic factors, however, are responsible for only 2-3% of identified ASD cases (3, 87).

In most other instances, studies suggest that ASD results from unknown environmental triggers acting on genetically susceptible individuals (2, 3, 14, 24). Susceptibility may be associated with gene variants (14) involved in biological pathways associated with ASD such as cell adhesion, synaptic vessel release, neurotransmission, and synaptic structure (3, 34, 35). These biological pathways are inter-connected in a very complex manner (91). However, it is unclear how environmental contaminants interact with or otherwise perturb ASD-associated biological pathways (91).

Today, genetically susceptible individuals may be exposed to combinations of 3000 synthetic chemicals via air, food and water (2). Synthetic chemicals are generally categorized into two groups: pharmaceuticals and personal care products (PPCPs), and other industrial chemicals, such as organophosphate insecticides and organic solvents (e.g., ethyl alcohol) (2). PPCPs include extensively used psychoactive pharmaceuticals (114), but also include bis-phenyl A in plastics, phthalates in cosmetics and household products, and known teratogenic pharmaceuticals (2). In this study we focus on

psychoactive pharmaceuticals that 1) may find their way to drinking water from clinical dosages excreted by patients, 2) are generally untreated by waste-water treatment plants (115), and 3) have sufficiently long half-lives (35) to eventually emerge in drinking water. Because many PPCPs are known to perturb neurological systems, exposure of a fetus to these contaminants by way of the pregnant mother's water consumption is a plausible environmental risk factor for neurological disorders like ASD (2, 24).

In a previous study, we investigated psychoactive pharmaceuticals presented at very low concentrations in the environment (34, 35). Juvenile fathead minnows (*Pimephales promelas*) were exposed to fluoxetine (FLX), venlafaxine (VNX) and carbamazepine (CBZ) individually and in mixtures at environmentally relevant concentrations (34). Using gene-class analysis (38), gene expression data indicated enrichment (significant up- or down-regulation) of gene sets associated with neuronal growth, regulation, and development in the juvenile minnow brains in response to psychoactive drug exposure (34). Moreover, a significant behavioral change (number of turns, lateralization, distance travelled) was observed in fish exposed to pharmaceuticals. We sought to identify potential pharmaceutical-associated gene expression mechanisms underlying these responses. As a first step, we considered the interaction of pharmaceuticals within the underlying ASD-associated protein network. This approach has been used elsewhere to determine how altering expression of one or a few genes can influence biological pathways, ultimately contributing to complex phenotypes (110).

We found previously that fathead minnows exposed to PPCPs had a significantly altered behavioral phenotype compared to a non-exposed (control) group (34), and that PPCP-enriched gene sets represented biological pathways, which play a major role in

neuronal systems. Thus, we predicted 1) that gene sets enriched by CBZ, VNX, FLX and MIX (all three) contained proteins that would be more interconnected than genes in the overall ASD-network, and 2) that gene sets enhanced by individual pharmaceuticals would have distinct network characteristics from each other.

To address these hypotheses, we constructed a protein-protein interaction (PPI) network of gene products known to be associated with ASD (77, 81), and examined 1) genes enhanced by particular PPCPs in fish brains, and 2) genes enhanced by a PPCP mixture and valproate (VPA) in human neuronal cells VPA is known to induce ASD-like phenotypes in mice (68). We sought to quantify patterns among gene sets enriched by PPCPs in both fish brains and human cell cultures by analyzing their network indices. Of particular interest was the identification of relationships between PCPP treatments and highly interconnected gene sets, as these are more likely to have profound effects on the functioning of the protein network because of their ripple effects on downstream proteins (116).

Methods

Construction of ASD-associated network

A list of 304 ASD-associated genes was retrieved from the Autism Database (AutDB) (13, 117), hereafter defined as the ASD gene set. To construct the network, we used the bioinformatics plugins for the Cytoscape visualization system, freely available from http://www.cytoscape.org/ as an open source java application (77).

We used the MiMi Cytoscape plugin (76) to create the ASD-associated network (80). MiMi searched for protein interactions for query genes in HPRD protein database (80).

We examined all primary and secondary neighbors (i.e. neighbors of neighbors) in HPRD database against the ASD gene set. We then identified all primary and secondary protein interactions for the ASD gene set.

From our previous microarray study on fish brain tissue, we identified all gene sets that were significantly ($\alpha = 0.05$) enriched by the psychoactive pharmaceuticals carbamazepine, venlafaxine and fluoxetine individually. We merged all single gene sets that were enriched by particular pharmaceuticals into separate groups, resulting in an enriched gene set for each of the pharmaceuticals. (See supplementary data).

In a parallel application, we cultured and differentiated human neuronal SK-N-SH cells with retinoic acid. After differentiation, we treated cells with a mixture treatment (CBZ, VNX, FLX) at environmental concentrations (59, 60). As a positive control, we also treated cells with Valproate at a clinical dose, 0.035mM. We then carried out transcriptome analysis on treated cells and identified significantly ($\alpha = 0.05$) enriched gene sets. Thus, we defined two groups: mixture (MIX) and valproate (VPA) containing all gene sets significantly enriched by the corresponding treatment (118) (See supplementary data).

Network analysis with plug-ins and identifying parameters of gene sets

We used the popular Java-based freeware package Cytoscape to map the ASD protein network (77). We used the Cytoscape plug-in, Network Analyzer (76), to summarize the nodes of the generated network for ten network measures (Table 1). To this network we applied a number of statistical approaches relatively novel in the analysis of protein networks including recently developed robust univariate and multivariate null hypothesis testing methods and high dimensional mapping procedures.

Statistical approach

Univariate comparisons: Network data for most indices were characteristically strongly positively skewed, and often contained outliers (extreme values). This necessitated the use of robust procedures for hypothesis testing and estimation. For location (typical value) summaries we felt that the pseudomedian (Hodges-Lehman location estimator) was the most appropriate measure (119). For a single set of observations, the pseudomedian is simply the median of all possible pairwise means. For a difference of two populations the pseudomedian is the median of all possible pairs of differences, and is an unbiased estimator of effect size from a Wilcoxon rank sum test [38].

Characteristics of treatments were considered in two ways. First, nodal network outcomes for particular indices were compared to the pseudomedian values of the complete network using one-sample Wilcoxon rank sum tests with upper tailed alternative hypotheses. Second, nodal network outcomes for particular treatment were compared to each other using Brunner-Dette-Munk (BDM) rank-based permutation tests (120). BDM tests are robust to both non-normality and treatment heteroscedasticity (121). The latter is not true for conventional rank-based permutation procedures (i.e., the Kruskal-Wallis test). In BDM comparisons an ominbus one way layout with three factor levels was used. If the null hypothesis of equal population shifts was rejected, then pairwise BDM tests comparing treatments were applied, and *P*-values for these tests were corrected for family-wise type I error using Holm's procedure (122).

To test the null hypothesis of identical network characteristics for the treatment groups, including the non-enriched portion of the ASD-network, we used permutation multivariate analyses of variance PERMANOVAs (123). Steinhaus dissimilarity was

used for the underlying resemblance matrix (124). *P*-values were calculated using 10,000 permutations of the vector of treatment assignments with respect to the resemblance matrix. To depict patterns in multivariate network space we used nonmetric multidimensional scaling (NMDS; (125)). This method attempts to reduce discrepancies (stress) in a resemblance matrix and a mapping solution defined, in part, by a user-specified dimensional choice. We again used Steinhaus dissimilarity for the underlying resemblance matrix. Following the recommendations of Kruskal and Wish (126), our NMDS solutions were the lowest stress results from 20 randomized starting configurations.

All statistical analyses we conducted using the statistical package R (94). In particular, nodal summary data were from Cytoscape using the package R-Cytoscape (78), and estimation procedures and hypothesis tests were run using R-base packages, and the packages asbio (127) and vegan (128).

Results

We postulated that proteins within PPCP-enriched groups would have both distinct network characteristics from each other and higher levels of importance in the ASD protein-protein network. Support of this hypothesis would suggest that any dysregulation in the expression of PPCP-enriched proteins would result in large impacts to the network due to ripple effects on downstream proteins. To address this question, we generated an ASD protein-protein interaction network. The network consisted of 7212 nodes, with approximately 2000 primary neighbor proteins and 5000 secondary neighbor proteins (Figure 4.1). The average number of adjacent neighbors (average degree centrality; Table

4.1) for proteins in the network was 9.279, while the median degree centrality was 5.000. Additional summary statistics are listed in a supplementary document to this manuscript.

In Fish brains: Carbamazepine- and Venlafaxine- enhanced gene sets were more inter-connected in the ASD-network than both Fluoxetine- enhanced gene sets and the non-enriched portion of the network.

When considering fish brain cells, the VNX and CBZ groups had significantly higher location shifts (median and pseudomedian), compared to the overall network, for network indices widely deemed to be most important in identifying nodal importance (i.e., degree centrality, closeness centrality, betweenness centrality, and stress). The omnibus null hypothesis of equal location shifts for groups was rejected for all four of these measures allowing protected pairwise comparisons of groups (129). In these comparisons the CBZ and VNX groups were statistically equivalent with respect to degree centrality, betweenness centrality and stress. The CBZ and VNX groups, however, had significant location shifts compared to the FLX group, and significantly higher locations than the overall ASD network (Figure 4.2). Strikingly, the FLX group degree centrality, closeness centrality, betweenness centrality, and stress locations were less than or equal to those of the overall ASD network (Figure 4.2).

The collective network characteristics of pharmaceutical groups were considered using both permutation multivariate analysis of variance (PERMANOVA) and non-metric multidimensional scaling (NMDS). The fish brain cell genes of the non-enriched portion of the ASD network and individual pharmaceutical groups were significantly different in network space ($F_{3,1936} = 3.68$, $P = 0.001$). In pairwise PERMANOVA tests

CBZ and VNX groups were indistinguishable from each other ($F_{1,123} = 1.06$, $P = 0.352$). Both of these groups, however, were significantly different from FLX (or essentially so) after adjustment for family-wise type I error (CBZ vs. FLX: $F_{1,48} = 3.08$, $P = 0.052$; VNX vs. FLX: $F_{1,101} = 3.69$, $P = 0.023$). Further, both the CBZ and VNX groups were statistically distinct from the non-enriched portion of the ASD network (CBZ vs. non-enriched: $F_{1,1832} = 4.35$, $P = 0.020$; VNX vs. non-enriched: $F_{1,1885} = 4.70$, $P = 0.012$), whereas the FLX group was not significantly different from the non-enriched fraction of the ASD-network ($F_{1,1811} = 2.12$, $P = 0.136$). The basis for these results is graphically evident in a non-metric multidimensional scaling (NMDS) dimension reduction of network space (Figure 4.3). We note that the CBZ group and VNX groups have similar network characteristics with high degree centrality, betweenness centrality, stress, and radiality. Conversely, the FLX group (more representative of non-enriched gene sets) had particularly small responses for these variables, but high values for eccentricity, average shortest path length and clustering coefficient.

In human neuronal cells: Mixture (CBZ, VNX, FLX) and Valproate - enhanced gene sets were more inter-connected than the overall network.

To extend inference to human tissues, we extracted RNA from human neuronal cells treated with a PCPP mixture (CBZ, VNX, FLX) and valproate (VPA), and carried out transcriptome analysis. We then compared network characteristics of gene set groups, enriched by pharmaceuticals, both to each other, and to the overall network.

We found that both the mixture and VPA group locations were significantly higher than the overall network with respect to degree centrality, betweenness centrality, and

stress. The mixture and VPA groups were, however, not statistically distinguishable from each other with respect to these measures (Figure 4.4).

We rejected the omnibus null hypothesis that collective ASD network characteristics were equal for all groups, including the non-enriched portion of the ASD network ($F_{2,1690}$ = 2.34, P = 0.017). Pairwise differences among groups, however, were not significant after adjustment for family-wise type I error (VPA vs. MIX: $F_{1,255}$ = 2.10, P = 0.133; VPA vs. non-enriched: $F_{1,1876}$ = 2.11, P = 0.132 ; MIX vs. non-enriched: $F_{1,1690}$ = 2.85, P = 0.099). Characteristics of the VPA and mixture groups that may distinguish them from the overall ASD network are evident in Figure 4.5. We note both VPA and MIXTURE groups are split between genes that have higher degree centrality, betweenness centrality, closeness centrality, and radiality, and those --more representative of the non-enriched portion of the ASD network-- that have smaller outcomes for these measures.

Discussion

Researchers have identified many abnormal level of gene expression in the potential etiology of complex psychiatric disorders like ASD (13). Due to many genetic factors like CNV deletions, mutations, allelic exclusion, or epigenetic silencing, the expression of synaptic proteins within the developing brain of a fetus might get altered (13). This alteration in the level of synaptic proteins leads to the formation of abnormal neuronal circuits, which has been considered as one of the potential mechanism in ASD (13). Other linked and association studies have found that these genetic factors are responsible for 2-3% of ASD cases (3, 87), suggested the role to unknown environmental factors. Therefore, environmental factors are likely to be a major determinant because they may

interact in combination with other genetic (pleiotropic and epistatic) factors causing varying ASD disease phenotypes (116, 130).

We believed that psychoactive pharmaceuticals at environmental concentrations may dysregulate the expression of key synaptic proteins that further disturb the process of synaptogenesis. We carried out preliminary studies on juvenile fathead minnow fish exposed with Carbamazepine (100µg/l), Venlafaxine (50µg/l) and Fluoxetine (10µg/l) at concentrations, which were detected in the surface waters in US (34, 35). Interestingly, along with significantly altered gene expression in exposed fish brains, we also observed a significant behavioral change in treated fish (number of turns, lateralization, distance travelled) (34, 35). Similar work has been done in zebrafish, which showed abnormal behavior after exposing them with valproic acid (anticonvulsant like carbamazepine) (110). This suggested that psychoactive pharmaceuticals at environmental concentrations might be altering neuronal circuits by interacting with key genetic factors, thus caused different phenotypes in fish (13, 34).

In the context of fish brain tissues our primary hypothesis was that human homologs of protein products of genes in synaptic cells, perturbed by exposure of pharmaceuticals, would have distinct ASD network parameters. To test this, we determined the extent to which protein products of genes from gene sets enhanced by individual pharmaceuticals in fish brains were interconnected in the ASD-associated protein-protein interaction network. As predicted, PPCP-enhanced sets had higher degree, closeness, betweenness and stress than the overall network. Further, we found that CBZ and VNX groups had higher degree centrality, closeness centrality, stress and betweenness centrality than FLX group, and were distinct from the FLX group and the

non-enriched portion of the ASD-network in multivariate network space (Figure 4.2 & 4.3). Interestingly, these distinctions were reflected by behavioral data in our previous study (34). In this work, fish exposed to environmental concentrations of CBZ and VNX displayed more agitated behavior (e.g., number of turns, lateralization, distance travelled) than fish exposed to environmental concentrations of FLX (34).

To extend these findings to human tissues, we cultured and differentiated human SK-N-SH neuronal cells and carried out transcriptome analysis after exposing them with the mixture (CBZ, VNX, FLX) at environmental concentrations and valproate at a clinical dosage (118). We then identified proteins groups significantly enriched in the presence of these pharmaceuticals, and quantified their importance in the ASD protein-protein network. We chose valproate (an anticonvulsant) as a treatment because prenatal exposure is associated with childhood autism (24). Mixture and valproate groups had greater importance (e.g., higher degree, closeness, stress and betweenness) than the overall network (Figure 4.4 & 4.5). We note that half of the genes from mixture group were also in the valproate group (although overlapping genes were not included in analyses). This suggests that the PCPP mixture would induce gene expression in cell cultures in a similar pattern to valproate.

Carbamazepine is a mood stabilizer and anticonvulsant used in conjunction with valproate to treat bipolar disorders and epilepsy (68, 95). CBZ and VPA block sodium channels, thus inhibit the epileptic effects in the brain (95). CBZ is present at very low concentrations in the surface waters of United States (34, 35), and may diffuse into groundwater (35). Because the mixture group (which included CBZ) and valproate perturbed similar genes with relatively high network importance, we posit that 1) protein

products of genes from both PCPP mixture and VPA serve as important nodes within the ASD-associated network, 2) enrichment effects of clinical doses of VPA are similar to those for environmental concentrations of pharmaceutical mixtures, and 3) gene dysregulation caused by PCPPs will have relatively profound effects on ASD protein network because these genes would effect more downstream proteins when perturbed (116).

Conclusions

Genes connected to a large number of neighbors in the ASD-associated protein-protein interaction network may play an important role in neuronal growth, development, and regulation. We found that protein products from gene sets with enriched expression in fish brains and human neuronal cells, due to an exposure of psychoactive pharmaceuticals, were comparatively more inter-connected to other neighboring proteins than protein products of non-enriched gene sets. Thus, these genes are more likely to experience altered expression upon exposure to PPCPs, causing further dysregulation of the whole interactome due to a ripple effect.

Competing Interests

The authors declare that they have no competing interests.

Authors' contributions

GK, KA and MAT designed and carried out the research. MAT provided the direction and guidance for the research. KA carried out analyses using R-programming and GK

collected all the data and interpreted it after testing hypotheses. GK wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

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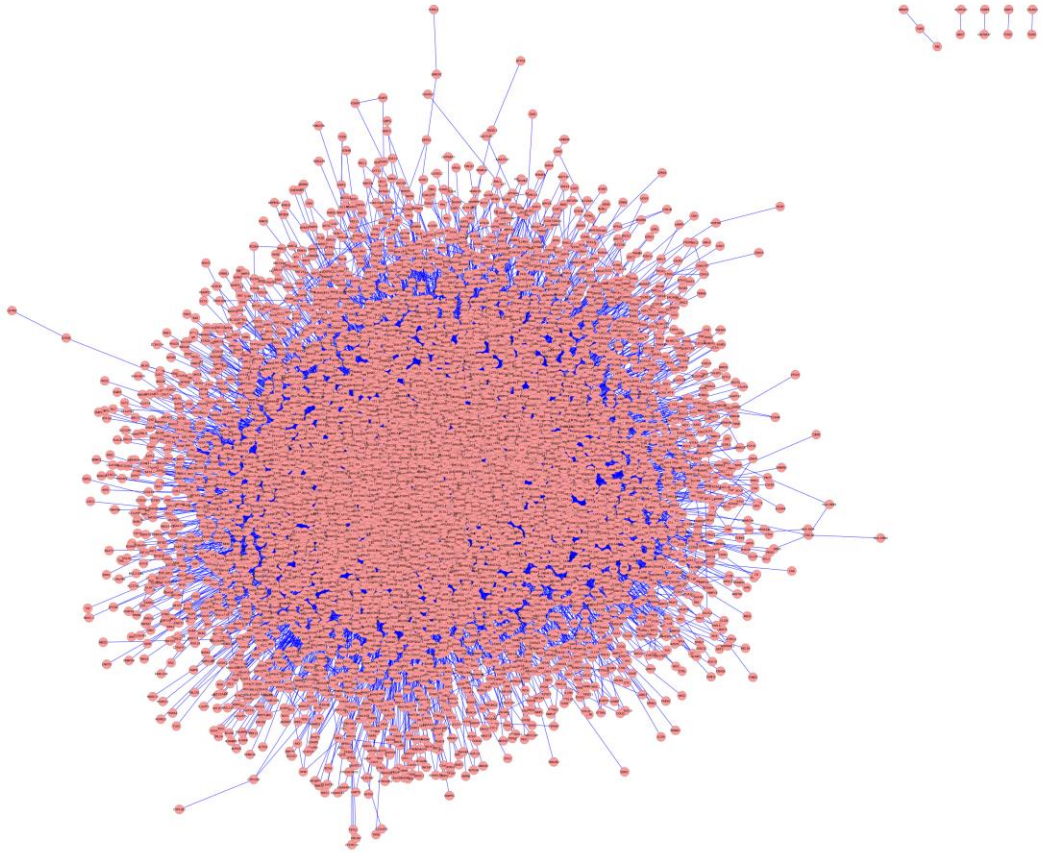
Figures

Figure 4.1 - Protein-protein interaction network associated with ASD



The network is generated and visualized by bioinformatics software, Cytoscape v.2.8.3. Red dots represent nodes (or, proteins) and blue lines represent edges (or, connections among nodes).

Figure 4.2

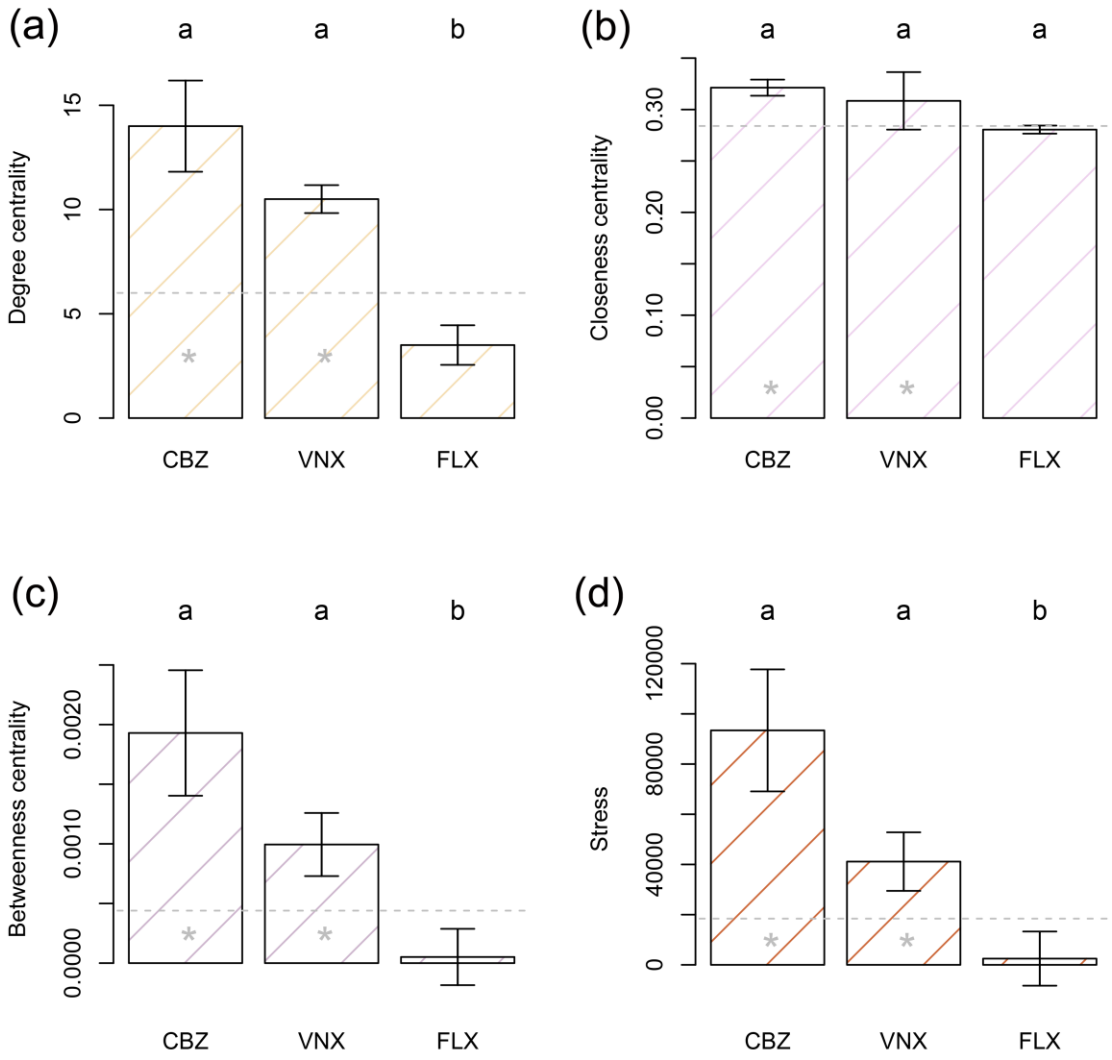


Figure 4.2: Carbamazepine- and Venlafaxine- enriched gene sets were more inter-connected than Fluoxetine- enriched gene sets, and the overall network. This figure shows the comparisons of network characteristics of enhanced gene sets by individual pharmaceuticals (CBZ, VNX, FLX) in fish brain tissue. Bars are pseudomedians (i.e., Hodges-Lehman estimators of location) whiskers are bootstrap standard errors of pseudomedians. Location measures with the same letter are not significantly different,

using the BDM rank-based permutation procedure, after Holm's adjustment for simultaneous inference. Gray dashed horizontal lines are the pseudomedian values for the complete ASD network. Gray stars indicate that the treatment locations are significantly greater than the complete network pseudomedian, using Wilcoxon one-sample rank sum tests.

Figure 4.3

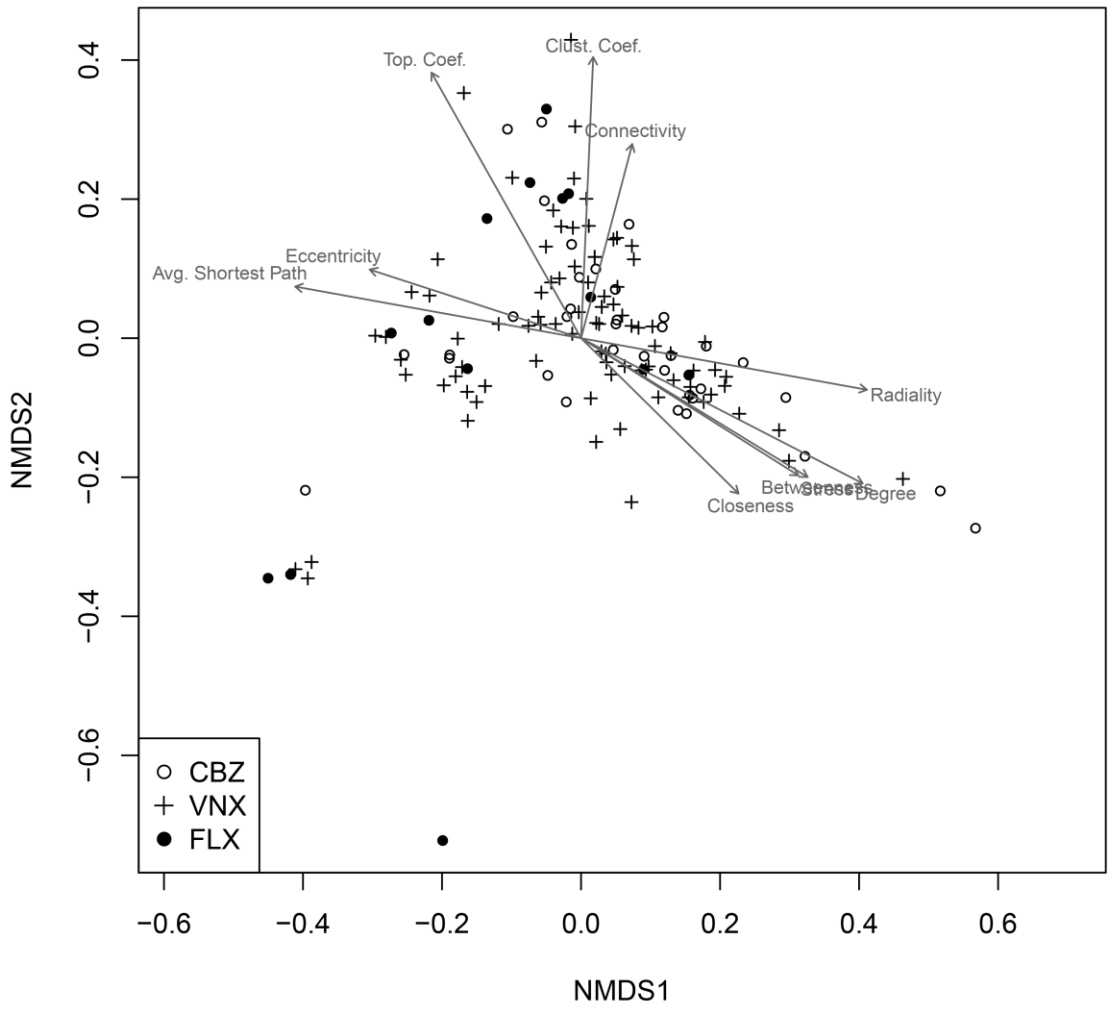


Figure 4.3: Non-metric multidimensional scaling (NMDS) representation of significantly enhanced gene sets in fish brains. This figure represents the comparison of network parameters of enhanced gene sets by individual pharmaceuticals in multivariate network space. Arrows within the scatterplot indicate the direction of most rapid increase for an indicated variable. Arrow length is scaled by the R^2 value of a multiple regression model in which the variable in question is the response and the NMDS axis scores are explanatory variables.

Figure 4.4

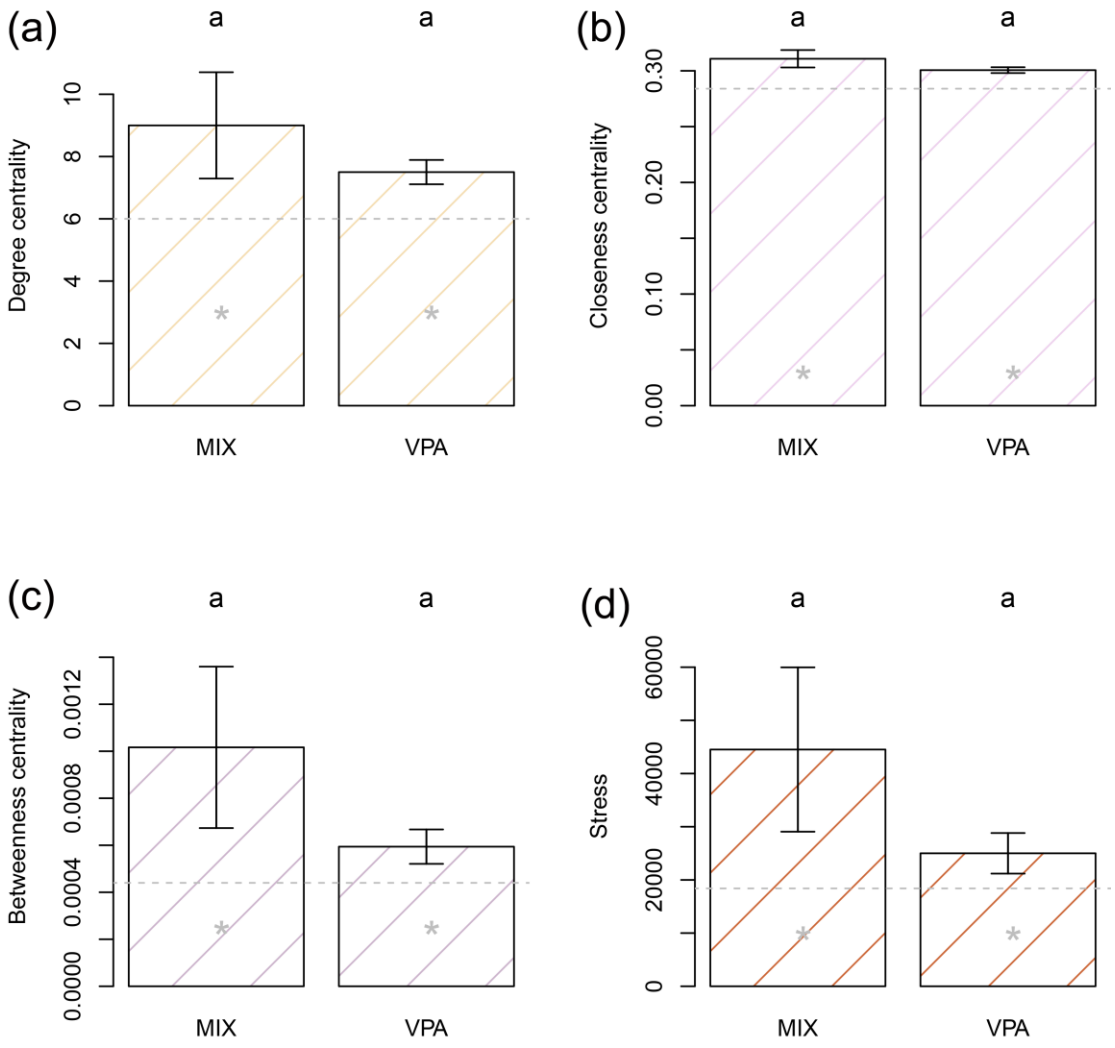


Figure 4.4: PPCP MIX (CBZ, VNX, FLX) and Valproate (VPA) - enriched gene sets were more inter-connected than the overall network. This figure shows comparisons of network characteristics of enhanced gene sets by a mixture of pharmaceuticals (CBZ, VNX, FLX) and valproate (VPA) in human cell cultures. Bars are pseudomedians (i.e., Hodges-Lehman estimators of location) whiskers are bootstrap standard errors of pseudomedians. Location measures with the same letter are not significantly different,

using the BDM rank-based permutation procedure, after Holm's adjustment for simultaneous inference. Gray dashed horizontal lines are the pseudomedian values for the complete ASD network. Gray stars indicate that the treatment locations are significantly greater than the complete network pseudomedian using Wilcoxon one-sample rank sum tests.

Figure 4.5

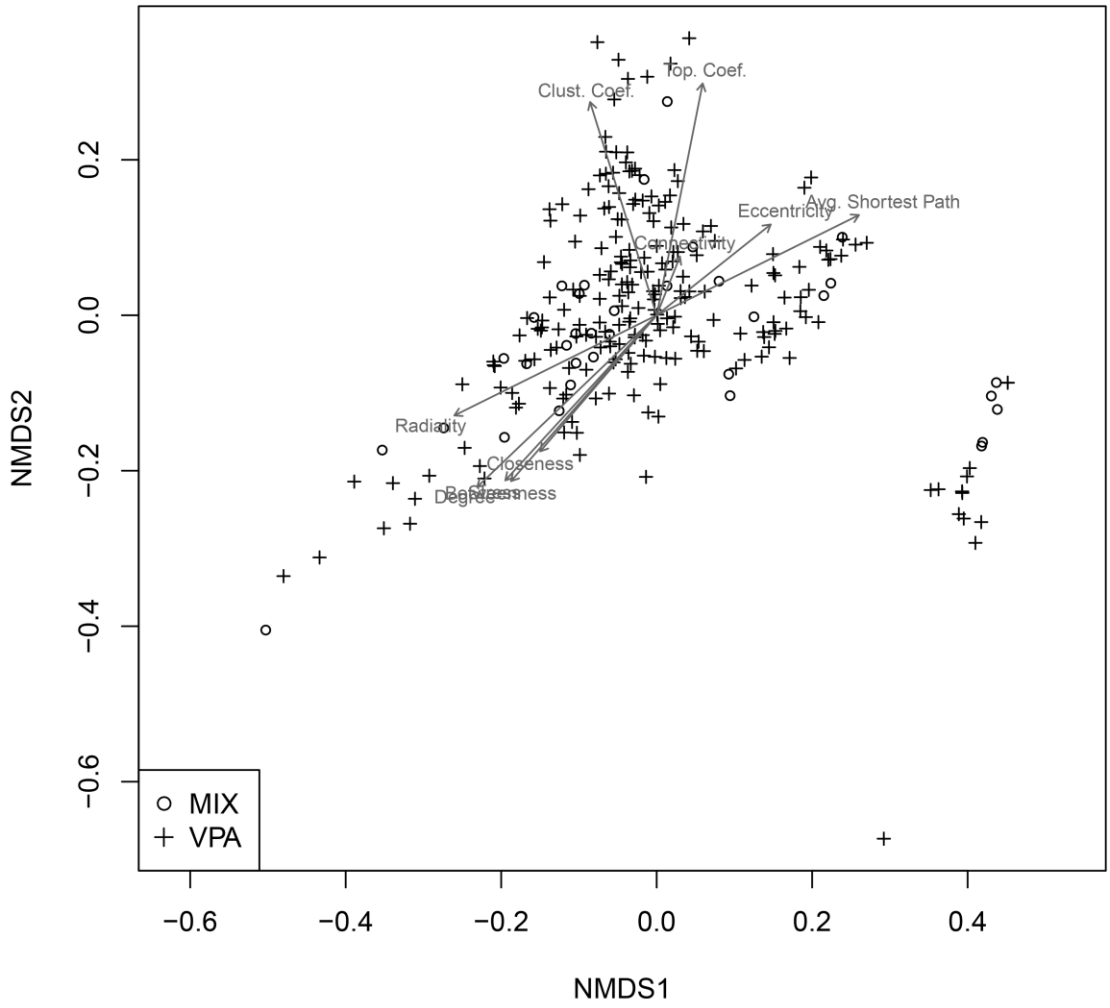
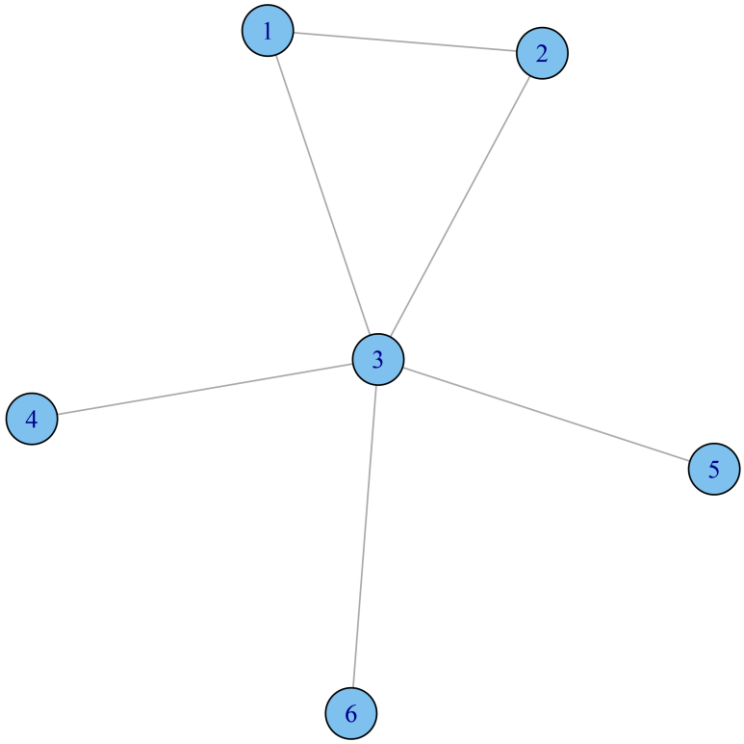


Figure 4.5: Non-metric multidimensional scaling (NMDS) representation of significantly enhanced gene sets in human cell cultures. This figure represents the comparison of network parameters of enhanced gene sets by pharmaceuticals in mixtures and valproate in multivariate network space. Arrows within the scatterplot indicate the direction of most rapid increase for an indicated variable. Arrow length is scaled by the R^2 value of a multiple regression model with the variable in question as the response variable and the NMDS axis scores as explanatory variables.

Tables

Table 4.1 - Description of network indices used in this paper



Example network with 6 nodes.

Degree centrality of the *i*th node is the number of direct attachments of that node to other nodes. Thus node three has degree 5, and node six has degree 1.

Closeness centrality of a node is the inverse of the sum of the geodesic distances of the node to every other node in the network.

$$C_C(n_i) = \left[\sum_{j=1}^{g} d(n_i, n_j) \right]^{-1}$$

where n_i indicates the *i*th node, n_j indicates the *j*th node $j \neq i$, d indicates geodesic distance, and g is the overall number of nodes.

Node three has closeness 1/(1+1+1+1+1)=1/5. Node six (and every other node) has closeness 1/(1 + 2 + 2 + 2 + 2) = 1/9.

Betweenness centrality of a node *i* is the number of "shortest geodesic paths" between node *j* and node *k* ($i \neq j \neq k$) that node *i* resides on.

$$C_B(n_i) = \sum_{j<k} g_{jk}(n_i) / g_{jk}$$

where g_{jk} is the number of shortest geodesic paths connecting node *j* and node *k*, and $g_{jk}(n_i)$ is the number of shortest geodesic paths that node *i* occupies. We note that g_{jk} will always equal $(g^2 - g)/2 - (g - 1)$.

In the figure above, we have $[(6^2 - 6)/2] - 5 = 10$ shortest pairwise paths, excluding those associated with the *i*th node. For node three these are (1,2) = 1, (5,6) = 2, (4,5) = 2, (1,4) = 2, (2,4) = 2, (1,5) = 2, (2,5) = 2, (4,5) = 2, (1,6) = 2, (2,6) = 2, (4,6) = 2, and (5,6) = 2. Node three occupies the shortest path in 9 of these. Thus, its betweenness centrality is 9/10. All other nodes have a betweenness centrality of zero.

The **clustering coefficient** (also called transitivity) of a node is the proportion of connections among its neighboring nodes that are realized compared to the number of all possible connections among the neighboring nodes. In the above Figure, node three has 5 neighbors. Thus, there are $(5^2 - 5)/2 = 10$ possible connections between those neighbors. However only one set of neighbors actually communicate. Therefore the clustering coefficient of node three = 1/10 = 0.1. Nodes one and two each have 2 neighbors with $(2^2 - 2)/2 = 1$ possible connections. In both cases this connection is realized. Thus, the coefficient for nodes one and two is 1. The clustering coefficient of nodes four, five, and six is undefined because these nodes each have only one neighbor, preventing simultaneous consideration of communication between the nominal node and neighboring nodes, and communication among neighboring nodes.

The **topological coefficient** of a node measures the proportion of neighboring nodes shared with other nodes. Nodes with one or no neighbors are typically assigned a topological coefficient of 0. Node three has 5 neighbors, and 2 of these communicate. Therefore the topological coefficient for node three is 2/5 = 0.4.

The **average shortest path** for a node is simply the average of the shortest geodesic distances to every other node in the network. Node three has an average shortest path of (1 + 1 + 1 + 1 + 1)/5 = 1, nodes four, five, and six have an average shortest path of (1 + 2 + 2 + 2 + 2 + 2)/5 = 1.8, and nodes one and two have an average shortest path of (1 + 1 + 2 + 2+ 2)/5 = 1.6.

The **eccentricity** of a node is simply the largest geodesic distance of that node to all other nodes in the network. Node three has an eccentricity of 1, and all other nodes have an eccentricity of 2.

The **stress** of a node is the number of shortest paths passing through it. Thus, a node will have high stress if it is traversed by a large number of shortest paths. Again, we have 10 shortest paths. Node three is on 9 of these, so it has a stress of 9.

The **radiality** of a node describes its capacity to reach into a network and provide novel information. It has the form:

$$R(n_i) = \frac{\sum_{j=1}^{g}[diam(G) + 1 - d(n_i, n_j)]}{(n-1)diam(G)}$$

where *diam*(*G*) is the maximum geodesic distance in the graph. The distance of node three to all other nodes is 1, and the maximum geodesic distance in the graph is 2. Therefore the radiality of node three is [5(2 + 1 - 1)]/5 = 2.

This table represents a brief explanation of the ten social network measures used in this paper. Examples given in the table are with respect to the embedded figure (79).

Additional files

Supplementary file 4.1 – ASD-associated PPI network Cytoscape file

Cytoscape compatible file of Core network is available at

https://sites.google.com/a/isu.edu/aho/

(Cytoscape software can be downloaded for free at www.cytoscape.org).

Supplementary file 4.2 – Complete data of gene sets for fish and human cells

Excel file containing a complete data of all gene sets (CBZ-enriched in fish brains, VNX-enriched in fish brains, FLX-enriched in fish brains, Mixture-enriched in human cells, Valproate-enriched in human cells). This file is available at

https://sites.google.com/a/isu.edu/aho/

Supplementary file 4.3 – Nodal summary statistics for gene set groups

The file contains summary statistics for all nodes in the network.

This file is available at https://sites.google.com/a/isu.edu/aho/

CHAPTER FIVE

In vivo intestinal and placental transfer of carbamazepine at very low concentrations from drinking water to the developing fetus brain

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Abstract

The main objective of this study was to test the hypothesis that psychoactive pharmaceuticals (fluoxetine, venlafaxine, and carbamazepine) at environmental concentrations could reach brain of the developing fetus by crossing intestinal and placental barriers following consumption by the mother in drinking water. Psychoactive pharmaceuticals have been found as teratogens at clinical dosage during pregnancy. These pharmaceuticals have been detected in minute concentrations in drinking water in the US, and are environmental contaminants that may be complicit in triggering neurological disorders (such as autism spectrum disorders, ASD) in genetically susceptible individuals. Previous studies have determined that sets of genes associated with neuronal systems were enriched (significantly up- or down-regulated) in the brains of fathead minnows treated with psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) at environmental concentrations. Altered gene sets were also associated with potential neurological disorders including ASD. *In vitro* studies indicate that psychoactive pharmaceuticals alter ASD-associated synaptic protein expression and gene expression in human neuronal cells. However, it was unknown if very low concentrations of these pharmaceuticals are able to cross biological barriers from mother to fetus. Here, we addressed this question by adding ^{2}H-isotope labeled pharmaceuticals to the drinking water of female mice for 20 days (10 pre-and 10 post-conception days), and quantifying ^{2}H-isotope signals in the liver and brain of developing fetuses using isotope ratio mass spectrometry (IRMS). Significant levels of ^{2}H were measured in the livers and brains of fetuses from carbamazepine-treated pregnant mice but not in those from control mothers. These results provide the first evidence that carbamazepine, even

in minute quantities of environmental contamination, is transmitted from mother to fetus and therefore available to potentially perturb neuronal connections within the developing fetus brain.

Background

Genetic factors that may contribute to neurological disorders (like autism spectrum disorders) have been identified through linked and whole genome association studies (2, 3, 87). Factors such as copy number variation, gene mutations, SNPs, and others account for a few cases (2, 3, 87). There is a relatively smaller literature addressing the role of environmental contaminants in the etiology of neurological disorders (2, 13).

Currently, humans are exposed to nearly 3000 synthetic compounds (2, 34). It remains poorly understood how these contaminants interact with humans through water, air, and/or food (2, 34) and to what extent they may contribute to neurological disorders in susceptible individuals (14, 24). Pharmaceuticals and personal care products (PPCPs) are one of the largest and most widely used classes of synthetic compounds, which includes commonly prescribed pharmaceuticals, phthalates in cosmetics, bis-phenol A (BPA) in plastics and other teratogenic chemicals (2). Among PPCPs, our lab has been studying psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) as potential contaminants that are being detected in the drinking water (34, 35).

Some of these pharmaceuticals are highly prescribed (35), and found in the water at waste-water treatment plants (WWTP) (34, 36) after excretion from patients. The excreted products of psychoactive pharmaceuticals are metabolically active and have half-lives that can be greater than a month (34, 36). These pharmaceuticals reach surface

waters after inefficient filtration at WWTP, and therefore, reach drinking water through ground-water or other supply routes (34, 36).

To determine if these psychoactive pharmaceuticals alter neurophysiology at concentrations found at WWTP, our lab carried out preliminary studies by treating juvenile fathead minnow fish with pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) at environmental concentrations (34, 35). Using microarray analysis of exposed fish brains, those studies found that neuronal growth, development, and regulation gene sets associated with potential neurological disorders (including idiopathic autism) were significantly up-or down-regulated (34, 35).

Recent studies have found that *in utero* exposure to psychoactive pharmaceuticals like antipsychotics, antidepressants and benzodiazepines during pregnancy may result in the development of neurologic, respiratory, gastro-intestinal and autonomic abnormalities in newly born infants (25). Most of these pharmaceuticals are selective serotonin reuptake inhibitors (SSRI) like fluoxetine; selective norepinephrine reuptake inhibitors (SNRI) like venlafaxine; mood stabilizers like valproate and carbamazepine; and benzodiazepines like diazepam, temazepam and nitrazepam (25, 131). Studies from human subjects have reported the placental transfer of antidepressants (fluoxetine, sertraline, nortriptyline and desmethyl clomipramine) to the umbilical cord (27, 132) when taken by the mother at clinical dosage, providing evidence that these antidepressants did cross the intestinal and placental barriers (27, 132). It is, however, unknown if these psychoactive pharmaceuticals cross intestinal and placental barriers when they are ingested at environmental concentrations such as may be present in

drinking water. The answer to this question is relevant to for all pregnancies where contaminated drinking water may be consumed.

In the present study, we hypothesized that psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) consumed by pregnant mothers at environmental concentrations would cross the intestinal and placental barriers, and reach the brain of the developing fetus. To determine this, we dissolved isotope-labeled psychoactive pharmaceuticals in the drinking water of pregnant mice. We then measured the concentration of isotope in the developing fetuses' brain and liver. Identifying psychoactive pharmaceuticals in the developing fetus brain would document that pharmaceuticals from maternal drinking water could cross intestinal and placental barriers when consumed by the mother in environmental concentrations.

Methods

Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Idaho State University and performed in accord with the NIH (National Institutes of Health) *Guide for the Care and Use of Laboratory Animals.*

Animal Experiments

Female and male C57Bl/6 mice (50 days old) were obtained from Jackson laboratory (Sacramento, CA). Mice were placed on a standard chow diet for two weeks prior to the experiment. Each experiment was ~20 days long. On day 1, three-four female mice were fed water containing isotope-labeled pharmaceuticals at environmental

concentrations. The water bottle was protected from light to avoid any photo-degradation of pharmaceuticals and the level of water was monitored everyday. On day 10, each female was housed with one male. As the length of estrous cycle for mice is five days, the vaginal plugs in the females were monitored every morning for five days. Because the female mouse was housed with the male from Day 11- Day 16 in the cage, the day when a plug was observed was marked as day 0 (or, embryonic day 0) of the pregnancy. Pregnant females (similar pharmaceutical treatment) were kept together in one cage and provided water containing isotope-labeled pharmaceuticals at environmental concentrations *ad libitum*. On embryonic day 10 (E10), pregnant mice were euthanized with CO_2 followed by cervical dislocation. It has been found that the formation of the blood-brain barrier (BBB) is initiated at E12 in a developing mouse fetus (133). Thus, pregnant females were sacrificed prior to E12 to avoid any stoppage of pharmaceuticals by BBB within the developing brain and to mimic the same physiology of an early human fetus where the BBB does not develop until 28 weeks (57).

Treatments

For our labeling experiments, all stable isotope-labeled compounds were obtained from Cambridge Isotope Laboratories Inc. (MA). Fluoxetine D6- oxalate (FLX) [# F919; 98 atom (at) % ^{2}H], Carbamazepine (CBZ) [# DLM-2806-1.2; 98 at % ^{2}H], Venlafaxine (VNX) [# V009; 98 at % ^{2}H] were used for pharmaceutical treatments. L-glutamic acid (GLUT) [# DLM-556-0.05; 98 at % ^{2}H] was used as a positive control. Non-labeled fluoxetine (#F132), carbamazepine (C4024) and venlafaxine (V7264) pharmaceuticals were obtained from Sigma. For *high* treatment levels we used 100% isotope-labeled pharmaceuticals; for *low* treatments we used 20% isotope-labeled pharmaceuticals

supplemented with 80% non-isotope labeled pharmaceuticals (Sigma). The over-all experimental design comprised 12 treatments: FLX high (concentration = 10µg/l; 100% isotopic; n=4), FLX low (10µg/l; 20% isotopic/80% non-isotopic; n=4), VNX high (50µg/l; 100% isotopic; n=4), VNX low (50µg/l; 20% isotopic/80% non-isotopic; n=4), CBZ high (100µg/l; 100% isotopic; n=4), CBZ low (100µg/l; 20% isotopic/80% non-isotopic; n=4), GLUT highest (0.3mg/ml; 100% isotopic; n=1), GLUT high (0.03mg/ml; 100% isotopic; n=1), GLUT med (0.003mg/ml; 100% isotopic; n=1), GLUT low (0.0003mg/ml; 100% isotopic; n=1), Control (no treatment; n=4) and negative control (contaminated with isotope-labeled L-glutamine; n=1). To avoid the possibility of contaminating fetal tissue with mother's blood during dissection, we performed control experiments where we intentionally contaminated phosphate buffer saline (PBS) with 100µL of isotope-labeled L-glutamine solution (0.03mg/ml) in which we bathed dissected fetuses. Following this bathing, we washed fetus samples 25 times with fresh PBS solution. These control experiments resulted in no detection of isotope in respective brains and livers of the fetuses, demonstrating that our experimental tissues were not contaminated by the blood of the mother and, therefore, did not contain false positive results.

Collection of samples and preparation

On E10, the pregnant mouse was euthanized with CO_2 followed by cervical dislocation. We started dissection on the ventral side and collected a lobe of the mother's liver which was placed in a cryogenic vial stored in liquid nitrogen. We then carefully opened the mother's uterus and exteriorized the fetuses into a petridish. Each embryo was separated from the placenta and put in the petridish containing ice cold PBS. Using a

dissecting microscope, we removed the brains using a knife and stored them in cryogenic vials in liquid nitrogen. Thus, we collected 1 liver (a lobe) and 5 fetus' brains from each pregnant mouse. All collected tissues were speed-dried using vacuum concentrators (Labconco) for 6 hours, and dried samples were kept inside an oven at 50^{o}C overnight. Dried samples were stored in Eppendorf tubes in a silica gel chamber (moisture-free environment).

Isotopic Analysis

Dried tissue samples (brain and liver) were analyzed for the isotopic composition of H. Samples were weighed (typical weights~ 0.4-0.8 mg) in silver capsules and analyzed using a ThermoFinnigan High Temperature Conversion Elemental analyzer (TC/EA) interfaced to a Delta V Advantage isotope ratio mass spectrometer (IRMS) through the ConFlo IV system. The samples were weighed in silver capsules and loaded into a Zero-Blank Autosampler (Costech Analytical, Valencia, CA, USA). Samples were combusted at 1400°C in a graphite crucible inside a glassy carbon reactor, and resultant gases separated in a GC column at 85°C and subsequently fed into the IRMS for analysis. The resulting hydrogen isotopic ratios, expressed as $\delta^{2}H$, are reported as ‰ values relative to the VSMOW scale. International/certified standards (NBS-22, IAEA-CH-7, methyl ester #Z3) were analyzed concurrent with the samples to normalize the raw data and monitor accuracy. Precision is estimated to be better than 2 ‰.

Statistical Analyses

Each group had 1-4 female mice and each mouse had 5 fetus' brain samples. For each female mouse, we calculated a mean $\delta^{2}H$ VSMOW value from 5 fetus' brain samples, and we used that mean as, *n=1*. We quantified nested effects in the

experimental design (fetus in maternal mouse) using a nested mixed effect Analysis of Variance (ANOVA). Significance in omnibus pharmaceutical fixed treatment effects were followed by Dunnett's post-hoc analyses comparing treatments to the control. Statistical analyses were carried out using R statistical platform (94), with heavy reliance on the packages lmer (134) and asbio (127, 129).

Results

Passage through the intestinal barrier: patterns of detected ^{2}H isotope in liver samples

We hypothesized that psychoactive pharmaceuticals at environmental concentrations would cross intestinal barrier *in vivo* and reach the maternal circulatory system. To address this question, we treated female mice with one of 10 pharmaceutical treatments, 4 positive controls, and 1 control. We collected 1 liver sample from the maternal mouse in each group.

The ^{2}H isotope ratios in the liver samples of Carbamazepine high (100µg/l; 100% isotopic; n = 3; $p < 0.05$) and Carbamazepine low (100µg/l; 20% isotopic/80% non-isotopic; n = 3; $p < 0.1$) were significantly higher than the control mean (Figure 5.1), suggesting that carbamazepine at different isotopic concentrations passed through the intestinal barrier of the pregnant mice. We did not notice a significant change in the livers of other treatments, including positive control (GLUT). None of the FLX low and GLUT high mice got pregnant within those treatments.

Passage through the placental barrier: patterns of detected ^{2}H isotope in grouped brain samples of developing fetuses

Next, we determined the extent to which psychoactive pharmaceuticals at environmental concentrations could cross placental barrier *in vivo* and reach the developing fetus' brains. To accomplish this, we removed and measured ^{2}H isotopic ratios for 5 fetus brains (pseudoreplicates) at E10 from each pregnant mouse (true replicates).

In factor level comparisons at the level of true replicate, ^{2}H isotopic ratios in the brain samples of the carbamazepine high (100μg/l; 100% isotopic strength; $n = 4$,) treatment were significantly higher than the control (no treatment; $n = 2$; $p < 0.05$; Figure 5.2). The carbamazepine low (100μg/l; 20% isotopic/80% non-isotopic strength; $n = 4$) treatment had significantly higher levels of isotope detection than the control at $\alpha = 0.075$. Detection of labeled carbamazepine at two different isotopic concentrations confirms that the pharmaceutical crossed the placental barrier and reached the fetus' brains.

Although unreplicated, the positive control treatments GLUT high (0.03mg/ml; 100% isotopic; $n = 1$) and GLUT low (0.0003mg/ml; 100% isotopic; n = 1) had higher isotope values than control, suggesting the usage by the fetus of L-glutamic acid from mother's blood. However, GLUT med (0.003mg/ml; 100% isotopic; $n = 1$) showed an abnormal pattern relative to the other positive controls. Although also unreplicated, the negative control (no treatment; $n = 1$) $\delta\ ^{2}H$ levels were lower than the control, indicating our efforts to avoid contamination from mother's blood to fetus brains at the time of dissection were successful.

For the FLX high, VNX high and VNX low treatments, we observed no significant change relative to control. None of the FLX low and GLUT high mice were successfully impregnated in the experiment.

Discussion

Consistent with our hypothesis, we detected isotope-labeled carbamazepine in the liver and brain of developing fetuses of female mice given carbamazepine in their drinking water at 100% (100 μg/l) and 20% (20 μg/l) isotopic concentrations (Figure 2). Carbamazepine is an anticonvulsant used to treat bipolar disorders and is known to block sodium channels in order to inhibit epileptic seizures (95). It is also used as a mood stabilizer during pregnancy, and many studies have noticed poor neonatal adaptation in newly born infants due to its consumption by their mothers (25). Other *in vivo* studies have also noticed teratogenic affects of carbamazepine including abnormal embryonic eye development on injecting at clinical doses in pregnant mice (135). These studies suggest that carbamazepine could be a potent teratogen during pregnancy. Recently, carbamazepine has been discovered as an environmental contaminant (detected in the drinking water at 100 μg/l concentration) which may contribute to neurological disorders (34).

To validate the experiment, we used isotope-labeled L-glutamic acid as a positive control (136), because it is an non-essential amino-acid for the development, and a developing fetus would utilize amino acids from maternal blood through placental exchange (137). Interestingly, we did not detect significant higher ^{2}H isotope in fetus brain of pregnant mice treated with L-glutamic acid relative to control mice. However,

after reviewing studies on glutamate metabolism, we found that glutamate does not cross the placenta efficiently (138). This factor likely explains why we did not see significant ^{2}H isotope signals in glutamate-treated mice; glutamate is apparently not appropriate to serve as a positive control.

We also validated our experiment with the negative control by contaminating fetal brains of control mice (no treatment) with 100μL of isotope-labeled glutamic acid in a petri dish. After washing those contaminated brains with PBS, we were unable to detect higher ^{2}H isotope signal relative to control mice. This served as a cross-check for potential contamination of fetal brains with maternal blood (containing isotope-labeled pharmaceuticals) during dissection.

Recent *in vitro* studies have demonstrated that carbamazepine at very low concentrations altered autism-associated synaptic proteins in human neuroblastoma cell cultures (112). The expression of key synaptic proteins (NMDAR1, OXTR, HTRs, PSD95, SV2A) were significantly up- or down-regulated in the human neuronal cells treated with carbamazepine at 10μg/l concentration (10-fold lower than the concentration used in the present study) (112). In other work, network analysis of pharmaceuticals in ASD-associated protein-protein interaction network. On analyzing protein connections of enriched gene sets in fathead minnow fish brains treated with psychoactive pharmaceuticals at environmental concentrations (34), we determined that carbamazepine had more protein interactions than other pharmaceuticals, which suggests that carbamazepine could potentially disrupt protein interactome at environmental concentrations (139). Other *in vitro* transcriptome studies in our lab examined that valproate (an anticonvulsant similar to carbamazepine) at clinical dosage induced gene

expression associated to potential neurological disorders including autism in human neuronal cells (140). Valproate, which is known to have properties similar to carbamazepine (141), has been previously found linked to ASD by inducing autism-like phenotypes in mice (68). Other human studies have found that *in utero* exposure of valproate during pregnancy is associated to higher risk of ASD in the offspring (68). These studies suggest that carbamazepine, even at very low concentrations, after crossing placental barrier, could potentially induce autism-like symptoms in the developing fetus.

There are some limitations of the present study. First, glutamate did not successfully serve as a positive control. Second, we could not conclude that other pharmaceuticals (fluoxetine and venlafaxine) crossed the placental and intestinal barriers. We used carbamazepine at 100μg/l concentration, which is much higher than fluoxetine (10μg/l) and venlafaxine (50μg/l) concentrations. Before carrying out this experiment, we calculated detection levels necessary for these ^{2}H-isotope labeled pharmaceuticals required to be higher than the ^{2}H found in the water, and it was mathematically impossible to detect fluoxetine and venlafaxine. Third, in liver samples (Figure 1), we did not see higher ^{2}H-isotope signal in treatments (other than carbamazepine) than the control. As we used isotope-labeled pharmaceuticals (or, compounds), this implies that the liver did not metabolize these compounds at rates high enough during the short experimental period to increase ^{2}H levels above that of non-labeled mice. Mice may metabolize these drugs differently than carbamazepine. Last, we did not achieve 100% pregnancy in all treatments. Even though we did find some pregnant mice, it is difficult to detect Day 0 of the pregnancy.

Conclusions

In the present study, *in vivo* detection of carbamazepine traces in the liver and brains of pregnant mice fed on similar environmental concentrations (100 μg/l and 20 μg/l) that are found in the drinking water, suggests that carbamazepine could play as an environmental contaminant by triggering ASD like symptoms in the developing fetus during pregnancy.

Authors' contributions

GK, DH, CJC, SB and MT designed the experiments, and GK performed them. DPH assisted GK in designing and carrying out the experiment, and in writing. MAT, CJC, SB and BF provided the direction and guidance for the research. KA carried out analyses using R-programming and generated results, and GK wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

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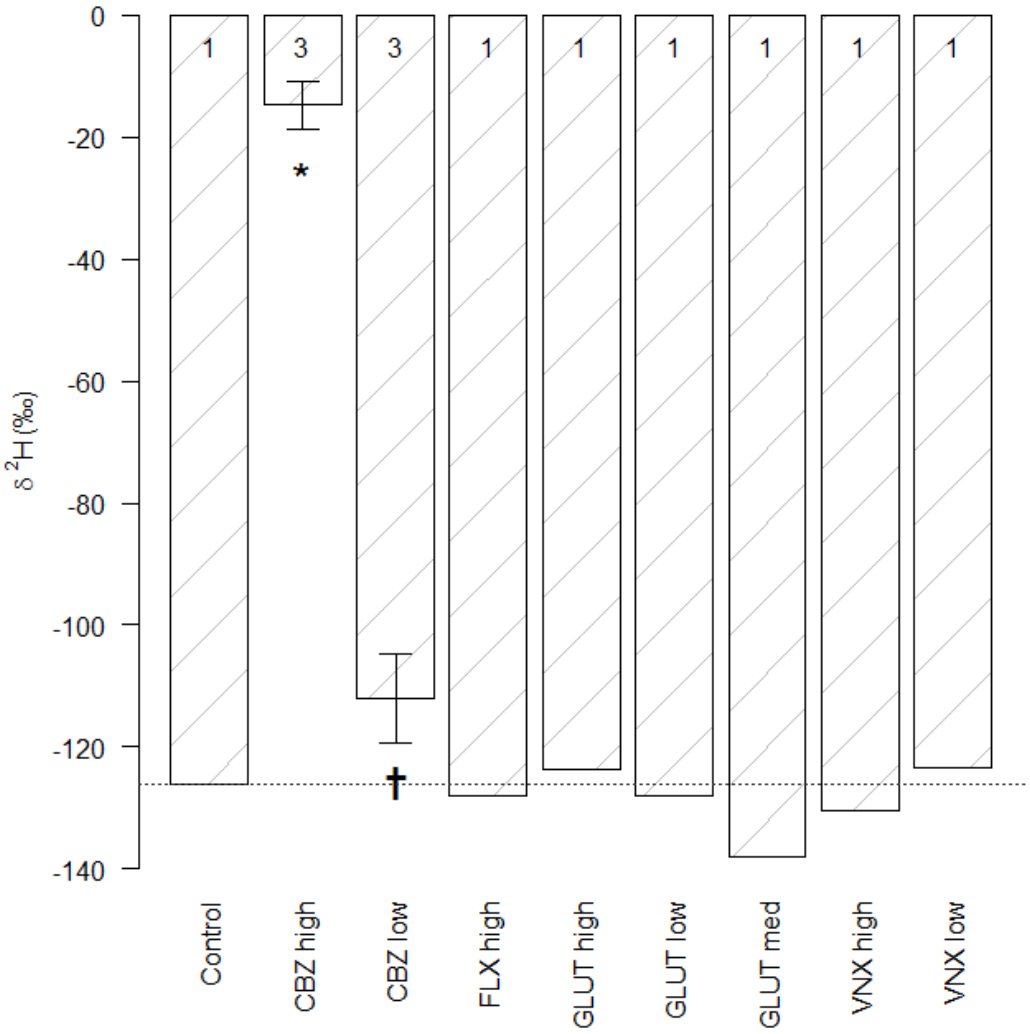
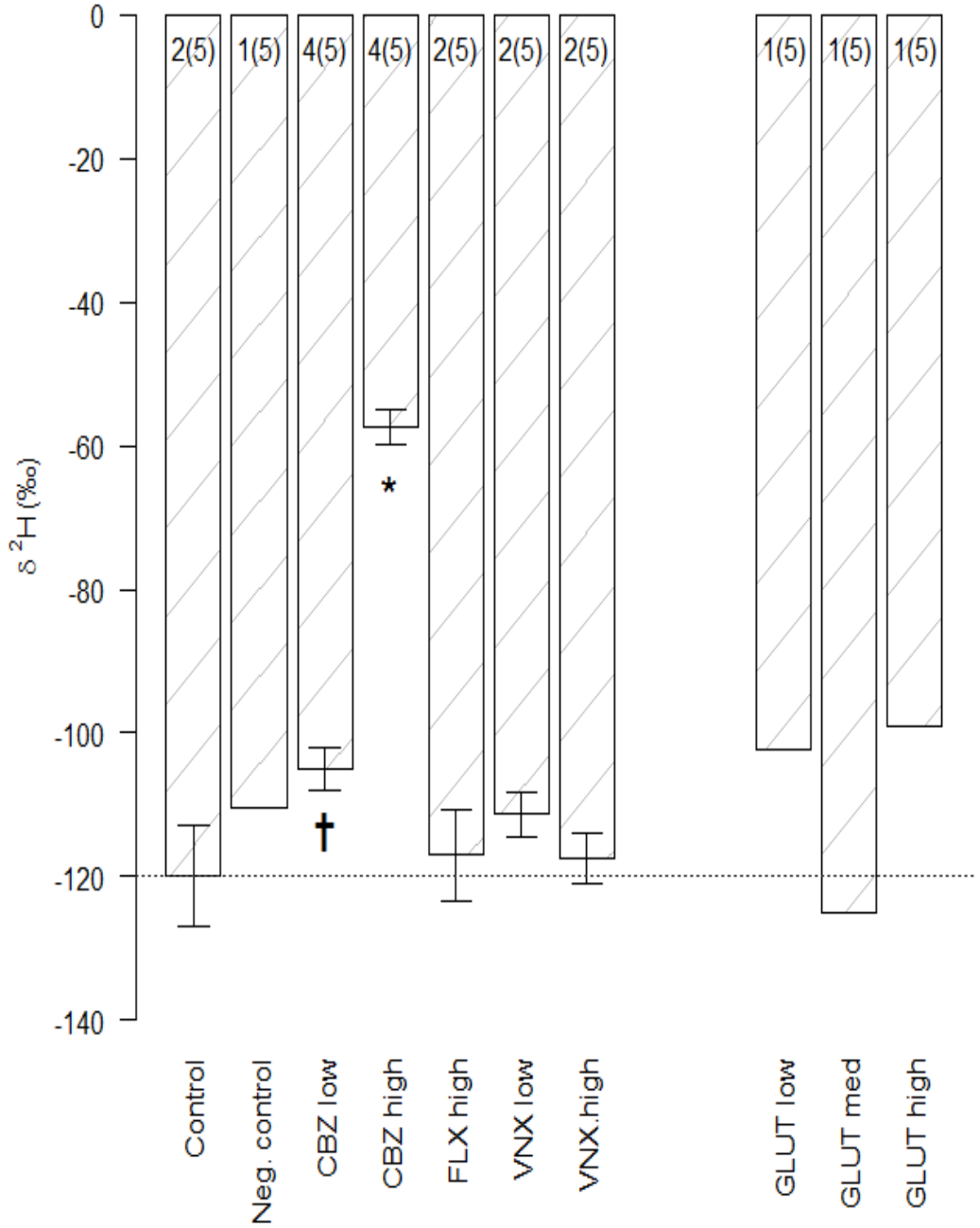
Figures

Figure 5.1



Levels of δ ^{2}H isotope in liver samples: Pregnant mice were treated with CBZ high, CBZ low, FLX high, GLUT high, GLUT med, GLUT low, VNX high, VNX low and control (no treatment; see Methods for treatment descriptions). Liver samples from CBZ high (100µg/l; 100% isotopic; n = 3; $p < 0.05$) and CBZ low (100µg/l; 20% isotopic/80% non-isotopic; n = 3; $p < 0.1$) showed a significantly higher ^{2}H isotope than the control mean. Data were analyzed using ANOVA followed by Error bars represent SEM. Symbols above bars indicate significant differences from the control after Dunnett's adjustment for family-wise type I error at significance level: "*" = 0.05, "†" = 0.075. Numbers at the top of each bar represent the sample size.

Figure 5.2


0
-20
-40
-60
-80
-100
-120
-140
δ 2H (‰)
2(5)
1(5)
4(5)
4(5)
2(5)
2(5)
2(5)
1(5)
1(5)
1(5)
*
†
Control
Neg. control
CBZ low
CBZ high
FLX high
VNX low
VNX.high
GLUT low
GLUT med
GLUT high

Levels of δ ^{2}H in brain samples: In different treatments, female pregnant mice were treated with CBZ high, CBZ low, FLX high, GLUT high, GLUT med, GLUT low, VNX high, VNX low, control (no treatment) and negative control (no treatment but contamination test performed). Brain samples of fetuses from CBZ high (100µg/l; 100% isotopic; n = 4) and CBZ low (100µg/l; 20% isotopic/80% non-isotopic; n = 4) had significantly more (significant level "*" = 0.05; "†" = 0.075) ^{2}H isotope than control. Data were analyzed using ANOVA followed by Dunnett's post-hoc comparisons of treatments to controls. In this analysis,. Error bars represent SEM. Numbers outside parentheses at the top of each bar indicate true replicates, i.e., the number of maternal mice, whereas numbers in parenthesis indicate the number of nested pseudoreplicates; i.e., the number of fetus from each maternal mouse).

CHAPTER SIX: CONCLUSIONS

I. Summary, empirical findings, and significances

In this thesis, we hypothesized that psychoactive pharmaceuticals from drinking water could cross maternal biological barriers, and alter *in vitro* neuronal protein and gene expression associated with autism spectrum disorders (ASD). To address this overarching hypothesis, we determined the extent to which psychoactive pharmaceuticals at low concentrations could induce the gene or protein expression patterns associated with neurological disorders including ASD in human neuronal cells and fish brains. The information presented in previous literature concerning the role of psychoactive pharmaceuticals in the etiology of neurological disorders (like ASD) is very limited. Therefore, this study sought to address four independently tested hypotheses to improve upon limited knowledge of these chemical contaminants present in the environment.

A. Can psychoactive pharmaceuticals alter *in vitro* expression of synaptic proteins associated with ASD in human neuronal cells?

In this study, we determined that psychoactive pharmaceuticals at low concentrations altered *in vitro* expression of ASD-associated synaptic proteins. We differentiated human SK-N-SH neuronal cells, and treated them with psychoactive pharmaceuticals at low concentrations, and analyzed the expression of synaptic proteins using the flow cytometry technique *(Chapter 2)*. Consistent with our hypothesis, we found that the expression of ASD-associated synaptic proteins (HTR1B, HTR2C, OXTR, NMDAR1A, GABRB3, PSD95 and SV2A) were altered significantly in human neuronal cells when they were treated with carbamazepine and venlafaxine individually, and when they were treated with a mixture of carbamazepine, venlafaxine and fluoxetine at very

low concentrations. The corresponding genes of many of these synaptic proteins were also found up- and down-regulated in the same direction in fish brains exposed to psychoactive pharmaceuticals at similar concentrations (34, 35). The synaptic proteins analyzed in this study were recently established as key players in synaptic plasticity and connection formation by Schuman *et al.* (2012) (18). Moreover, similar synaptic proteins were reported in the pathophysiology of ASD by a recent study published in *Nature* (91). Our results suggest that environmental contaminants, like psychoactive pharmaceuticals, might change the neuronal connections inside the developing fetus by altering synaptic protein expression.

B. Can psychoactive pharmaceuticals alter *in vitro* gene expression of neuronal systems associated with neurological disorders (including ASD) in human neuronal cells observed in previous fish studies?

In this study, we treated human SK-N-SH cells with psychoactive pharmaceuticals at low concentrations and analyzed the gene expression of neuronal systems using the RNA-Sequencing approach *(Chapter 3).* The working hypothesis of this study was that psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) at low concentrations would alter *in vitro* human neuronal gene expression in a way that was 1) similar to the gene expression profile of fathead minnows in previous studies, and 2) associated with neurodevelopmental disorders including ASD. After analysis, we found that very low concentrations of psychoactive pharmaceuticals altered the gene expression of neuronal systems *in vitro* similar to altered gene expression in fish brains and gene expressions were also associated with potential neurological disorders including ASD. Our results demonstrate that psychoactive pharmaceuticals at

low concentrations have a tendency to alter the expression of genes that have a role in the formation, growth and regulation of neurons.

C. Do psychoactive pharmaceuticals affect genes with higher network importance in the ASD-associated protein-protein interaction network?

The working hypothesis for this study was that, when significantly enriched by low levels of psychoactive pharmaceuticals, genes in fish brains and human cell cultures would have distinct network characteristics recognized within an ASD-associated human protein interaction network. We created a human protein-protein interaction network based on an autism database (AutDB) and analyzed the network characteristics of enriched gene sets from both fish brains and human cells in the overall ASD-network using network indices such as degree, betweenness, closeness centrality and stress *(Chapter 4)*. We found that genes in fish brains, enriched by carbamazepine and venlafaxine 1) had higher network importance than both the overall network and those genes enriched by fluoxetine, and 2) were distinct from fluoxetine and non-enriched ASD genes in the multivariate network space. Similarly, genes in human cell cultures enriched by pharmaceutical mixtures (at low concentrations) and valproate (at clinical dosages) had similar network signatures and greater network importance than genes in the overall ASD protein-protein interaction network. Our results indicate that important gene sets in the ASD network are particularly susceptible to perturbation by psychoactive pharmaceuticals at very low concentrations.

D. Can psychoactive pharmaceuticals cross biological barriers at low concentrations?

Upon discovering that low concentrations of psychoactive pharmaceuticals altered *in vitro* gene and protein expression associated with neurological disorders and perturbed

highly connected proteins in the protein interaction network, we wanted to determine whether low concentrations of psychoactive pharmaceuticals (fluoxetine, venlafaxine and carbamazepine) could cross maternal biological barriers. To address this question, we added ^{2}H isotope labeled pharmaceuticals into the water source of female mice for 20 (10 pre- and post-conception) days, and determined the ^{2}H isotope signal present in both liver samples of the pregnant mice and fetus brains *(Chapter 5)*. Consistent with our hypothesis, we detected significantly higher levels of ^{2}H isotope in the liver and fetus brains of carbamazepine-treated mice than control mice. Our results suggest that low concentrations of carbamazepine ingested from drinking water can cross maternal biological barriers (intestinal and placental) and can, therefore, potentially disrupt neuronal connections within the developing fetal brain tissue during pregnancy.

II. Challenges, limitations and recommendations

A. In vitro study (Experimental Chapter 1 and 2)

We know that the SK-N-SH cell line can be differentiated, resulting in more neuronal cells, by treatment with retinoic acid. These neuronal cell populations can be made up of serotonergic, glutamanergic or adrenergic neuronal cells. The variability in cell growth made it challenging to produce a similar neuronal cell population consistently, and to optimize the differentiation process of cells.

In this study, we used active metabolites of fluoxetine, carbamazepine and venlafaxine because I found SK-N-SH cells non-responsive to the regular form of these pharmaceuticals, as they weren't binding to synaptic receptors. I recommend using commercially available human hepatosomes in future experiments because these hepatosomes metabolize pharmaceuticals into active metabolites. Using hepatosomes

would simulate the biotransformation process inside the human body. Due to the less availability of hepatosomes and costly methods, I could not use this approach in this study.

B. Protein network analysis (Experimental Chapter 3)

Many versions of Cytoscape software are available and all versions differ with respect to available plugins. The challenging part of this study was to find an appropriate version of Cytoscape compatible with my required plugins to allow creation and quantification of an interaction network. As an extension of our results, I would recommend analyzing differentially expressed genes with commercially available Integrity Pathway Analysis (IPA) software and identifying the enrichment of biological processes in the network.

C. *In vivo* mice study (Experimental Chapter 4)

There were many limitations in our study. If pharmaceuticals labeled with the different isotopes had been available in commercial markets, then our experiment would have been much simpler and cost effective. Since they were not, groups of mice had to be treated with each pharmaceutical separately rather than in combination. This made the experimental design more complex and laborious. Due to the detection limit of our instruments, we could not detect fluoxetine and venlafaxine in our samples as there might be a possibility that the detection of those pharmaceuticals were close to the background deuterium levels. We chose glutamic acid as a positive control but it did not serve as we predicted due to its lesser tendency to cross placental barriers. Pregnancy was not achieved in all of our mice undergoing treatment. Although some mice did become pregnant, it was difficult to detect on day 0 of the pregnancy and because of that we could

not dissect those mice at E10 stage accurately. Thus, this limitation might have brought variations in our data.

Considering these limitations, I recommend using radiolabeled isotopes of pharmaceuticals, rather than the stable hydrogen isotope, for better detection and a larger number of mice to overcome the limitation of pregnancy detection. Instead of glutamic acid, steroids (like dexamethasone) should be used as a positive control as many studies have noticed that steroids cross biological barriers independently and efficiently (142).

III. Future directions

A. *In Vivo* Mice Study:

Our results showed that carbamazepine at low concentrations crossed the placental barrier and was detected inside the fetal brain tissue. Due to experimental limitations, we could not detect isotope-labeled fluoxetine and venlafaxine in maternal liver samples or fetal brain tissue. Therefore, it is still unclear whether psychoactive pharmaceuticals (including fluoxetine and venlafaxine) can cross maternal barriers, reach fetal brain tissue, or induce autism-like phenotypes in newly born mice. Based on our findings, the following protocols can be used in future studies. Pharmaceuticals (fluoxetine, carbamazepine and venlafaxine) at environmental concentrations can be added to the drinking water of female mice. At least five females should be used for each treatment so that at least three females are impregnated. Pharmaceuticals should be supplied in the maternal drinking water until pups are born (gestational day 21) to understand the complete pharmacodynamics and pharmacokinetics of psychoactive pharmaceuticals.

1. Detection of pharmaceuticals and their active metabolites in plasma using HPLC or LC-MS/MS

After adding pharmaceuticals to the maternal drinking water, blood samples can be collected every day from the female mouse through a tail cut, saphenous vein bleed, or orbital bleed. Plasma should be separated by centrifugation (15 min/4000rpm/4^{o}C) and stored at −20^{o}C for future use. Blood and brain samples should be collected from pups at post-natal day 1 (P1). Fetal brains should then be weighed and homogenized. Plasma and brain homogenates should be extracted for the analysis. Using HPLC or LC-MS/MS, the amount of pharmaceuticals and their active metabolites should be detected and analyzed. Using these plasma samples, the pharmacokinetics of psychoactive pharmaceuticals (and their active metabolites) inside the mother, and their tendency to cross the placental barrier can be studied (82).

2. Analyzing newly born pups' behavior

Some behavioral tests can be carried out in pups after PND 65 to examine the ASD-phenotypes. Many scientists have designed behavioral tests, which correlate with the behavior domains (deficits in social interaction, communication, and repetitive behaviors) of ASD in humans. Some of the behavioral tests include (82, 143):

- A three-chambered social approach to identify abnormal social interaction.
- Morris water maze and self-grooming tests to identify repetitive behaviors.
- A forced swim test to examine anxiety-like behavior.
- Social communication of food preference (like a sucrose preference test) to identify non-verbal communications.

With the help of these behavioral tests, ASD-phenotypes in pups resulting from prenatal exposure to psychoactive pharmaceuticals can be detected and quantified.

3. Analyzing gene expression using RNA-Sequencing

Similar to the sequencing study that we carried out in human cell cultures, the brain transcriptome of pups after behavioral analysis can be sequenced. Total RNA can be extracted by a Trizol reagent kit and sequenced according to the manufacturer's instructions (Illumina). GAGE analysis can identify the pattern of neuronal systems by comparing differentially expressed genes in pharmaceutical treatments (FLX, CBZ, VNX) to the control. In this experiment, the enrichment of neuronal gene sets can be compared with their expression in human cell cultures and fish brains. Any correlations of expressions within human *in vitro* cells, mice and fish brains can also be examined.

4. Analyzing network connections in ASD-associated network

Since we have already created an ASD-associated human protein-protein interaction network, the network characteristics of differentially enriched genes in the brains of mice pups from different pharmaceuticals treatment can be studied. With the help of network parameters, the connectedness of one pharmaceutical treatment can be compared with the other treatment and with the overall network. The correlation of network parameters between human *in vitro* cells, mice and fish brains can also be studied.

B. *In vitro* study

1. Exploring signaling pathways of synaptic receptors NMDAr, OXTR and HTRs

Our *in vitro* results demonstrated that the expression of synaptic proteins associated with ASD in SK-N-SH cells exposed to psychoactive pharmaceuticals at low concentrations (ppb) were altered. Considering those results, we used an SK-N-SH cell line as a model to further investigate the molecular mechanisms of psychoactive

pharmaceuticals involved in altering key synaptic receptors (NMDAR, HTRs and OXTR). The next step would be to examine the signaling pathways and other mechanisms underlying those receptors. These experiments can identify the underlying signaling mechanisms of these receptors and determine whether low concentrations of psychoactive pharmaceuticals can alter underlying signaling mechanisms.

NMDAR: During long-term potentiation and synaptic plasticity, calcium enters neuronal cells through NMDA receptors (144). We found that NMDAR protein expression was increased in differentiated SK-N-SH cells after treatment with low concentrations of psychoactive pharmaceuticals. Other studies have found that the over-expression of NMDARs can be associated with neurotoxicity and can induce apoptotic mechanisms within the cell (144, 145). During NMDAR over-expression, more calcium will enter the cell and eventually it will cause calcium overload and form more free radicals. Excess calcium influx will activate calcium-dependent enzymes like DNAases, proteases and lipases, which contribute to apoptosis of the cell. Given this mechanism, differentiated SK-N-SH cells can be treated with excess NMDA (as a positive control), memantine (NMDAR antagonist as a negative control) (146) and psychoactive pharmaceuticals at low concentrations, while the intracellular calcium concentration is measured using dyes such as Fura-2 AM and Fluo-4 AM (147). The apoptotic signals can also be examined to determine the neurotoxicity.

OXTR and HTRs: Oxytocin (OXTR) and serotonin (5-HTR) receptors are G protein-coupled receptors (GPCRs) and the alterations in underlying signaling mechanisms by psychoactive pharmaceuticals can be a possible hypothesis. Therefore, the signaling mechanisms of OXTR and HTRs can be analyzed by different experimental

protocols after treating differentiated SK-N-SH cells with psychoactive pharmaceuticals at low concentrations. The levels of MAP kinase, phosphorylation of MAP kinase, cyclicAMP activity, and other protein kinases can be measured after treatments. OXTR antagonists (like atosiban) (148) and HTR antagonists (like risperidone) (149) can be used as negative controls.

2. Analyzing the expression of regulatory microRNAs

In our study, we observed that treatment with a mixture of psychoactive pharmaceuticals (CBZ, FLX and VNX) increased the protein expression of OXTR but decreased the mRNA expression of the OXTR gene in differentiated SK-N-SH cells. This observation suggested the involvement of a regulatory mechanism such as microRNAs. MicroRNAs are small (22-23 nucleotides) (150, 151), endogenous and act as potent biological regulators of translation (152). Several recent studies have found that microRNAs are involved in neurodegenerative and psychiatric disorders (152-154). A recent study also found that the regulation at synapses inside neurons is more compartmentalized and thus mediated by microRNA more than transcription factors (150). Multiple microRNAs that regulate the expression of key ASD-associated proteins have been discovered (155). To determine the extent to which microRNAs are altered, their expressions in human SK-N-SH cells can be analyzed after treatment with low concentrations of psychoactive pharmaceuticals.

C. Testing Pocatello water with LC-MS/MS

As our hypotheses are based on the amount of psychoactive pharmaceuticals that are present in the environment, we will analyze the amount of these pharmaceuticals

found in water from the greater Pocatello, Idaho area. Samples will include household tap water, ground water from wells, pre- and post-treatment water from the wastewater treatment plant (WWTP) and American Falls Reservoir, Idaho. Part of the study has already been done in collaboration with Dr. Sarah Godsey at Idaho State University. Researchers sampled the Portneuf River and sediments from the WWTP, and detected carbamazepine, venlafaxine and fluoxetine at 22ng/l (in water sample), 9.2ng/l (in water sample) and 29ng/g (in sediments) respectively. It is clear from these data that these psychoactive pharmaceuticals are present in Pocatello's water system, but at very low concentrations.

IV. The Road Ahead

This thesis studied the potential association of psychoactive pharmaceuticals (FLX, CBZ and VNX) at low concentrations with neurological disorders like autism spectrum disorders (ASD). We now know that low concentrations of these pharmaceuticals can induce *in vitro* expression of synaptic proteins and neuronal genes that are associated with neurological disorders like ASD *(Chapter 2&3)*. We have also found that the drug-induced gene expression changes in fish brains and human neuronal cells represent higher connectedness in an ASD-associated protein-protein interaction network *(Chapter 4)*. We have also demonstrated that low concentrations of carbamazepine present in the drinking water can cross maternal intestinal and placental barriers and reach developing fetus brains *(Chapter 5).* As a broader perspective, my thesis suggests that psychoactive pharmaceuticals at low concentrations could be interacting with genetic factors and causing the altered neuronal growth, regulation and

development associated with ASD. This dysregulation might produce a phenotype, which is in the ASD spectrum, thus triggering, mimicking, or aggravating ASD symptoms in genetically susceptible individuals.

Psychoactive pharmaceuticals are excreted by clinical patients and they are being detected in water, although at very low concentrations (in ng/l) (34). We used higher concentrations (in μg/l) of these pharmaceuticals in our experiments because we accounted the concentrations of all possible isoforms of the metabolites derived from psychoactive pharmaceuticals (FLX, CBZ and VNX) (34, 35). Studies have detected the isoforms of these psychoactive pharmaceuticals in the environment and found that they have similar pharmacokinetics and pharmacodynamics properties (156). However, we believe that psychoactive pharmaceuticals contaminants are building up within the water system (especially groundwater) slowly because these pharmaceuticals and their metabolites are stable and have long half-lives in the environment.

Due to higher lipophilicity and longer half-lives than other non-psychoactive pharmaceuticals, these pharmaceuticals once consumed stay inside the human body for a longer time and get accumulated within tissues (157). Several clinical studies have observed the accumulation of psychoactive pharmaceuticals within brain and liver tissues of fetuses after consumption at clinical dosages. They found that the concentration within tissues was ten times higher than the plasma concentration (157-159). Other toxicological studies have also found that psychoactive pharmaceuticals (like fluoxetine) get trapped within cell organelles like lysosomes and mitochondria, thus affecting biological processes like lipids degradation (157, 159). Our results demonstrate that carbamazepine at low concentrations (μg/l) can cross placental barriers, and may alter neuronal

connections inside fetus brains by dysregulating the expression of key neuronal genes and proteins, which may further perturb the expression of their downstream proteins. As a broader perspective, this thesis shows that psychoactive pharmaceuticals at low concentrations (μg/l) may cause cytotoxic effects on neuronal cells in the fetus during pregnancy.

With an expanding knowledge of brain and neuronal function, future studies can be conducted to better understand idiopathic ASD. The greatest challenge is the heterogeneity of ASD phenotypes. To address the range of these phenotypes, scientists are using mechanisms such as gene knock-out to develop animal models that closely represent the behavioral patterns of ASD. To apply insights gained from that research to human subjects, we are also using experimental techniques including transcriptomics, imaging, computational tools and other molecular approaches. The knowledge gained by using these techniques will eventually help solve the puzzle of idiopathic ASD.

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