EFFECTS OF SERTRALINE EXPOSURE ON FATHEAD MINNOW (*Pimephales promelas*) STEROIDOGENESIS

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Sertraline is a selective serotonin reuptake inhibitor (SSRI) that is widely used for the treatment of depression and anxiety. Due to the abundant therapeutic use of sertraline, low levels have been detected in municipal wastewater effluents suggesting that aquatic organisms may be exposed. The purpose of this study was to evaluate the steroidogenic effects of sertraline on larval (FHM) and adult female fathead minnows (FFHM), *Pimephales promelas.* Larval FHM were exposed to 0.1, 1, and 10 µg/L sertraline for 28 days and analyzed via RT-qPCR for differential expression of 11β-Hydroxysteroid dehydrogenase (11β-HSD), 20β-Hydroxysteroid dehydrogenase (20β-HSD), aromatase (CYP19), and nuclear thyroid receptor alpha (TRα). FFHM were exposed to 3 or 10 µg/L sertraline for 7 days with the brain and ovary excised at exposure termination. Juvenile FHM exposed to 0.1 µg/L sertraline had a significant upregulation of both 20β-HSD and TRα. FFHM exposed to 10 µg/L sertraline had a significant upregulation of 11β-HSD expression in brain tissue, while no steroidogenic changes were observed in the FFHM ovary. Similarly, in FFHM brain tissue, CYP19 and 20β-HSD expression levels were significantly higher in fish exposed to 10µg/L sertraline compared to control. The significance of these findings with respect to survival, growth and reproduction are currently unknown, but represent future research needs.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

1.1 Statement of Purpose

In 2011, sertraline (Figure 1) was the third most prescribed drug in the United States with well over 37 million prescriptions (Grohol et al., 2012). SSRIs, including sertraline, have been detected in surface waters and non-treated wastewater in North America with measurements between 0.014 µg/L and 0.034 µg/L observed in Canadian wastewater treatment plants (Metcalfe et al., 2010).

Due to the overwhelming prescription of sertraline for depression and anxiety, trace amounts of these pharmaceutically active compounds are currently being measured in drinking water (Metcalfe et al., 2010). Therefore, it is imperative to determine the chronic effects of low-level exposure to sertraline. More importantly, sertraline may be able to cross the placental barrier and/or the fetal blood brain barrier causing teratogenic complications (Weinberger & Klaper, 2014). The effects of sertraline on fish and other aquatic organisms are very important for understanding possible toxic effects in humans (Henry et al., 2004).

Elevated serotonin levels in FHM may have a detrimental effect on steroidogenesis, particularly in the brain due to the highly lipophilic nature of sertraline and the ability for it to cross the blood brain barrier. For example, exposure to fluoxetine, another widely prescribed SSRI, reduced FSH and testosterone concentrations, while estradiol concentrations increased in male goldfish at levels that are normally seen in the environment (Mennigen et al., 2010). The purpose of this study is to determine the effect of sertraline on steroidogenic gene expression.

1.2 Sertraline

Sertraline ((1S-cis)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-N-methyl-1-napthalenamine (M.W. = 342.7)) was first introduced to the pharmaceutics market in 1991 by Pfizer Incorporated to treat social anxiety disorder, but has also been successful in the treatment of major depressive disorder, obsessive-compulsive disorder, panic disorder, posttraumatic stress disorder, and premenstrual dysphoric disorder (Pfizer Inc., 2002). Sertraline is available in dosing tablets of 25, 50, and 100 mg and is administered orally. Sertraline, after oral ingestion, is primarily metabolized via N-demethylation to the less active N-desmethylsertraline. After demethylation has occurred, sertraline and N-desmethylsertraline are metabolized further via oxidative deamination, reduced, hydroxylated, and glucuronide conjugated for elimination. N-desmethylsertraline has a plasma half-life of roughly 62-104 hours. Only roughly 5% of ingested sertraline reaches systemic circulation (Pfizer Inc., 2002). The primary target of sertraline is the serotonin transporter (SERT) which is responsible for the reuptake of serotonin at the presynaptic terminal. According to Pfizer Inc., sertraline has little to no affinity for the following receptors: serotonergic, histaminergic, dopaminergic, GABA, cholinergic, adrenergic, or benzodiazepine in humans (Pfizer Inc., 2002).

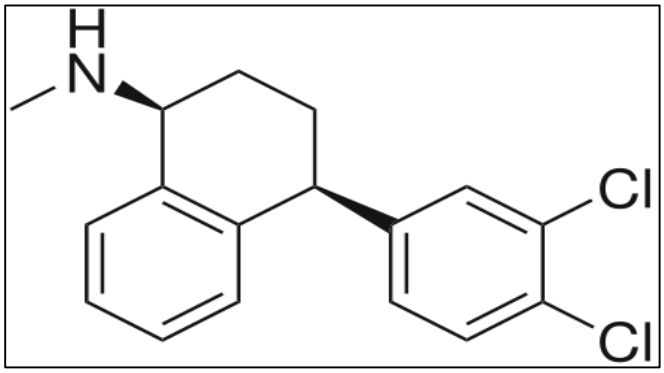


Figure 1: Sertraline was first introduced to the pharmaceutic market to treat drepression in 1991 by the Phizer Inc. (Grohol, 2012).

1.3 Pharmaceuticals in the Environment

As the human population and pharmaceutical use both increase, it is easily understood why a strong focus should be placed on the negative effects of chronic exposure to low concentrations of the most frequently prescribed drugs. Whether a pharmaceutical is passed from a human's digestive tract into wastewater or pills are disposed into wastewater, some pharmaceuticals retain their active biochemical state and can still have an effect at therapeutically low concentrations (Ainter et al. 2009). Additionally, the effect some pharmaceuticals, namely selective serotonin reuptake inhibitors (SSRIs), might have on aquatic life is not well studied and very few data points exist (Brooks et al., 2003).

Recently, studies suggest fluoxetine, another popular SSRI, and sertraline may be present in wastewater treatment plant effluents at much higher levels than previously thought and may even be endocrine active in some aquatic organisms (Ainter et al., 2009; Brooks et al., 2003; Schultz & Furlong, 2008; Schultz et al., 2010; Weinberger & Klaper, 2014). More specifically, selective serotonin reuptake inhibitors (SSRIs), including fluoxetine and sertraline, have been found in aquatic systems at concentrations as high as 70 ng/L (Batt et al., 2008). While most pharmaceuticals may not pose an immediate threat to the health and safety of our society, some may have a large impact on our ability to maintain normal biological processes, i.e., endocrine function (Taylor & Senac, 2014).

This study focuses primarily on the endocrine disruption of one of the most widely prescribed psychotherapeutic antidepressants, sertraline. Little is known about the chronic exposure of sertraline at low concentrations, but it can be postulated that long term neurotransmitter, i.e., serotonin and/or dopamine, alterations may have a negative effect on both aquatic organisms and humans. The importance of studying SSRI presence in the environment

lies on the fact that serotonin regulates many of the most basic biological functions in vertebrates (Ainter et al., 2009). The first line of organismal uptake, occurring in aquatic life, can be detrimental to their survival, endocrine function, immunological function, and neural homeostasis (Ainter et al., 2009). Therefore, one can assume that eventually the presence of SSRIs in the environment might affect humans on the same biological level as our aquatic counterparts.

1.4 Pharmaceuticals in the Environment (North America)

In 2008, a study by the U.S. Environmental Protection Agency tested surface waters from East Fork River in Cincinnati, OH and wastewater treatment plant (WWTP) effluents from seven wastewater treatment plants in New Mexico. All samples were analyzed using an optimized method of solid-phase extraction liquid chromatography tandem mass spectrometry (SPE-LC-MS/MS). The average concentration of sertraline in the seven WWTP effluents was roughly 71.1 ng/L, while the average concentration of sertraline in the single surface water sample was undetectable (Batt et al., 2008). The average concentration of another SSRI, fluoxetine, was near to sertraline at 53.1 ng/L in WWTP effluents and also at undetectable levels in surface water (Batt et al., 2008).

While undetectable levels may cause a reader to question the validity of this study, understanding the reality of our current detection systems should play a significant role in how we proceed. Sulfamethoxazole, a widely used antibacterial in livestock, shows the highest surface water concentrations at 140 ng/L and WWTP effluents at 1104 ng/L (1.1 µg/L), which can easily be caused by factory farm runoff into local streams and rivers (Batt et al., 2008; Duffet al., 2007). Hydrochlorothiazide, a diuretic used to treat high blood pressure, was also found at

high concentrations in WWTP effluents and surface waters: 2062 ng/L (WWTP) and 75 ng/L in surface waters (Batt et al., 2008). The study by the U.S. Environmental Protection Agency to measure levels of active pharmaceutical ingredients in WWTP effluents and surface water was merely a test of methods to assess pharmaceuticals in the environment in a quick and specific manner. The scientific community still has a long way to go in regards to detecting low levels of pharmaceuticals in the environment. Regardless, as more humans are prescribed SSRIs for depression and/or anxiety it is only a matter of time before the current concentrations exceed current levels.

1.5 Steroidogenesis

Steroid hormones are involved in various biological functions including: development, growth, reproduction, and many other chemical responses (Garcia-Reyero et al., 2014). Endocrine sensitivity to SSRIs and/or other pharmaceuticals in the environment may pose a threat to the reproduction and development of aquatic life and possibly even effect humans. In addition, the ability for SSRIs to alter steroid hormone synthesis is a hot topic in toxicology and may prove to be invaluable in correlating serotonin with the catabolism of estrogen, testosterone, aldosterone, and/or cortisol. Therefore, it is important to have a model organism that is easily screened for endocrine disruption.

FHM and other teleost fish, i.e., zebrafish, have been deemed “aquatic mammals” due to their highly conserved steroidogenic pathway with mammals. While FHM may have a highly conserved steroidogenic pathway with mammals, some of the substrates may have altered functions or even no function at all (Tokarz et al. 2013). Regardless, importance can be place on the differential expression of various catalytic enzymes in the steroidogenic pathway and a

possible correlation with human health and aquatic survival/reproduction. In this study, 11β-Hydroxysteroid dehydrogenase (11β-HSD), aromatase enzyme (CYP19), 20β-Hydroxysteroid dehydrogenase (20β-HSD), and thyroid receptors alpha (TRα/β) were screened after FHM exposure to various concentration of sertraline (Overturf et al. 2014).

Thyroid function may also be affected by SSRIs. A recent study shows that patients without depression and with normal thyroid function may have decreased in triiodothyronin (T_3) and thyroxin (T_4) after SSRI treatment, but no effect in individuals with depression (de Carvalho et al., 2009). T_3 plays a significant role in fetal development, as well as possibly increasing serotonin levels in the brain (Brochet et al., 1987). The following gives a brief description and schematic (Figure 2) for each steroidogenic enzyme and substrate screened in this study:

- 11β-HSD is the primary catalytic enzyme for the synthesis of corticosterone from deoxycorticosterone
- 20β-HSD is the primary catalytic enzyme for the synthesis of 17α,20β-dihydroxy-4-pregnen-3-one from 17α-OH-progesterone
- CYP19 is the primary catalytic enzyme for the synthesis of estrone or estradiol
- TRα/TRβ are nuclear thyroid receptors that primarily recognizes triiodothyronine (T_3) in the brain and regulate thyroid hormone function

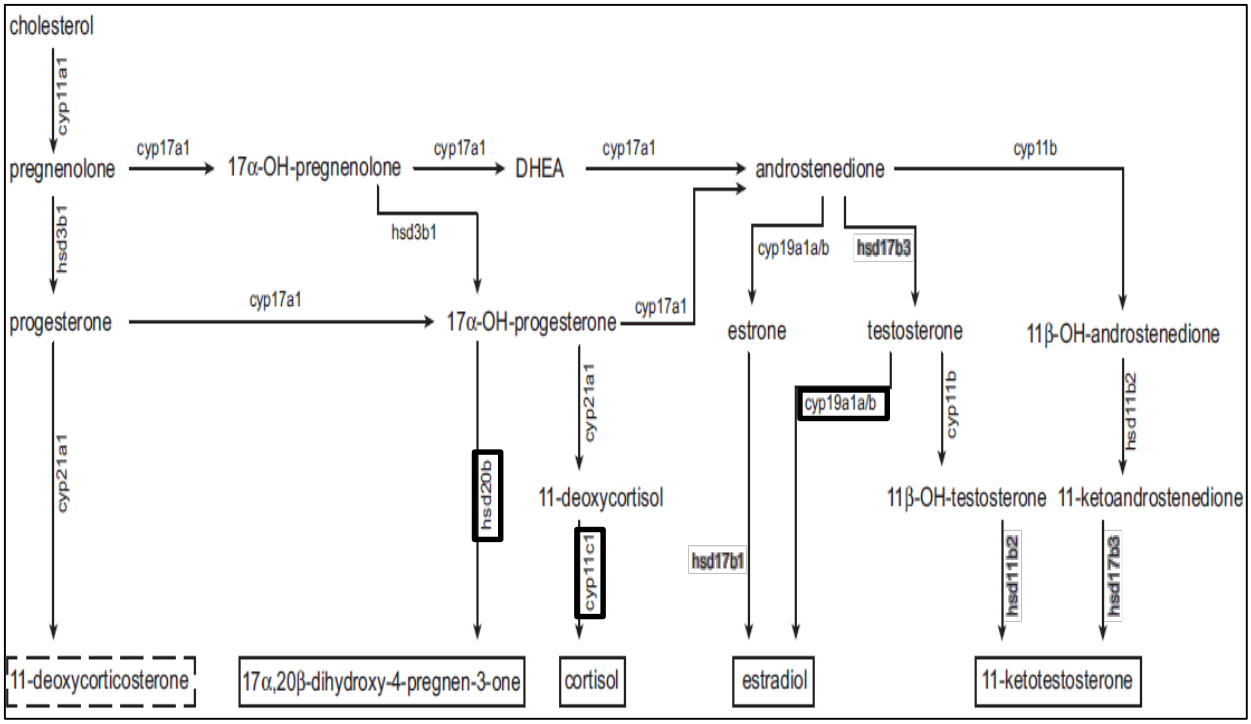


Figure 2: Steroidogenesis pathways in FHM. The above image represents the known pathways of steroidogenesis in teleost fish with both enzymes and substrates depicted. Gene names corresponding to each catalytic enzyme are given on the arrows. Major substrates/hormones synthesized by steroidogenesis are boxed (Tokarz et al., 2013).

1.6 Serotonergic System

In the early 20th century, serotonin (5-HT; 5-hydroxytryptamine) was first discovered in various parts of brain and peripheral tissue. Serotonin is an indoleamine that is directly derived from tryptophan and has a very similar structure to *d*-lysergic acid diethylamide (LSD). LSD is currently known as one of the most potent hallucinogenic drugs (Aghajanian & Liu, 2009). In addition to LSD, serotonin is also structurally similar to *N*,*N*-dimethyltryptame (DMT; found in Ayahuasca or Yagé) and mescaline (peyote)(Figure 3). Late in the mid-1960s, serotonin was found to primary congregate in the raphe nuclei of the brain stem and fate maps of serotonin were soon to follow. Due to the discovery of fate maps and possible psycho-active properties of serotonin, it has been linked to almost every biological function in the human body. In addition,

the serotonergic system in humans compared to teleost fish is thought to be highly conserved therefore, allowing for a model organism such as: zebrafish, Japanese medaka, and/or fathead minnow to be efficiently screened (Gould, Brooks, & Frazer, 2007). Deregulation of serotonin, in humans, has been correlated with depression, anxiety, autism, and schizophrenia.

While the central nervous system (CNS) in humans is thought to only contain 1% serotonergic neurons, these neurons are dispersed throughout the entire CNS. Serotonergic neuronal activity is directly proportional to the state at which an organism is behaving, e.g., high activity during waking hours and low activity during sleeping periods. The regulation of serotonergic neurons occurs primarily via pacemaker activity, various ion currents flux, brain noradrenergic inputs, and hypocretin inputs. SSRIs work by blocking the reuptake of serotonin therefore, allowing more serotonin availability (Aghajanian & Liu, 2009).

The effect of serotonin on steroidogenesis has been widely studied since the 1970s and is now becoming recognized as a key player in regions of the mammalian system. i.e., peripheral cell differentiation and even mammary gland development (Dubé & Amireault, 2007; Matsuda et al., 2004). Additionally, serotonin has been identified in non-neural tissue in mammals, including the liver, mammary glands, and ovaries (Dubé & Amireault, 2007). Serotonin's presence in various tissue types may imply a much more diverse network of serotonin receptors and serotonin activity than first thought; therefore, the goal of this study is to further exam the possible effects of serotonin on fish steroidogenesis.

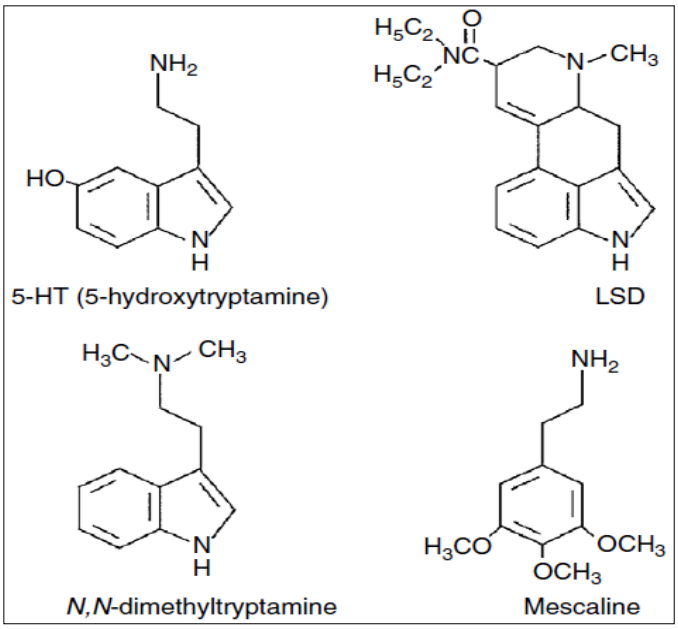


Figure 3: Serotonin (5-HT) shares a similar structure, an indole group, with d-lysergic acid diethylamide (LSD) and N,N-dimethyltryptamine (DMT). Mescaline, while not containing an indole group, shares a phenethylamine group with LSD (Aghajanian & Liu, 2009).

1.7 Serotonin Synthesis

Tryptophan is the primary substrate giving rise to sertraline via two enzymatic alterations. Tryptophan, one of the 22 standard amino acids, is found in regular dietary intake and is considered an essential amino acid for normal biological function. Tryptophan is highly abundant in the brain and can easily cross the blood-brain barrier via large neutral amino acid transporters (LAT1) located in brain capillaries (Boado et al., 1999; Young, 2007). Once tryptophan has crossed the blood-brain barrier, serum tryptophan is transported into synaptic terminals where serotonin synthesis occurs as depicted in Figure 4 (Best et al., 2010). The first step of serotonin synthesis, which is also the rate limiting step, adds a hydroxyl group to the 5' carbon of the indole ring of tryptophan via tryptophan hydroxylase forming 5-hydroxytryptophan (5-HTP) (Best et al., 2010). Next, an aromatic amino acid decarboxylase (AADC) removes the carboxyl group from 5-HTP forming serotonin (5-HT) (Best et al., 2010). Finally, serotonin can

be transformed even further to synthesize melatonin. When serotonergic neurons are firing, 5-HT can then be transported into vesicles via monamine transporter (MAT) and further released in the synapse and recycled by sertraline transporters (SERTs)(Best et al., 2010).

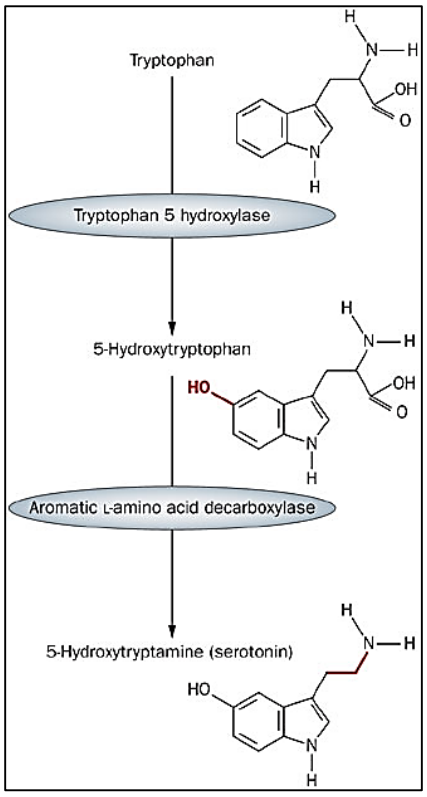


Figure 4: Tryptophan is an essential amino acid that is involved in the primary synthesis of serotonin. Tryptophan 5-hydroxylase is an enzyme involved in the rate-limiting step of serotonin synthesis by adding a hydroxyl group to the indole ring of tryptophan. An aromatic L-amino acid decarboxylase finishes the synthesis by removing a carboxyl group from 5-HTP forming serotonin 5-HT (Best et al., 2010; Druce et al., 2009).

1.8 Serotonin Receptors and Steroidogenesis

5-HT, from the time of discovery in the 1960s, has become known as one of the most widely utilized neurotransmitters in vertebrates. Recently, the number of known serotonin receptors has increased to over seven classes (5-HT_{1-7}) and 14 subtypes, all with different modes of action and exponentially more biological functions in which to regulate (Weinberger &

Klaper, 2014). 5-$HT_{1,2,4,6,7}$ primarily cascade signal transduction via G-protein signaling while 5-HT_3 signals via ligand-gated mediation (Figure 5)(Aghajanian & Liu, 2009).

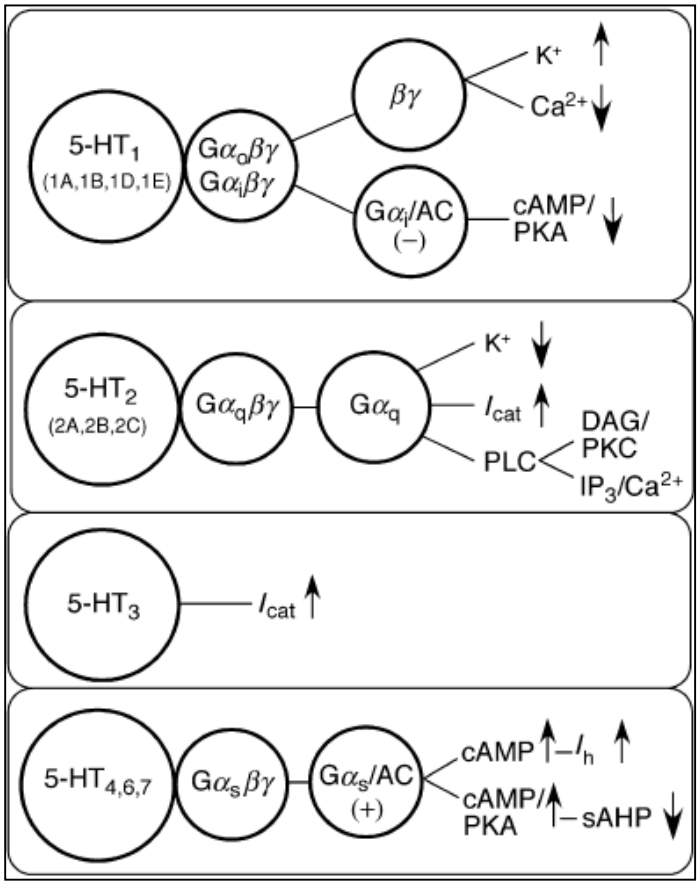


Figure 5: 5-$HT_{1,2,4,6,7}$ receptors produce their modulation to the serotonergic system via G-protein cascading. 5-HT_3 receptors produce their modulation through ligand-gated channel and do not utilize G-protein for their signaling. Up and down arrows signify an inducing or inhibitory effect on the ions/molecules mentioned ((Aghajanian & Liu, 2009).

1.8.1 Serotonin Receptors

While sertraline has an extremely low affinity for serotonin receptors and does not play a role in receptors acquisition, the receptors mediate serotonergic neural firing and recognition of serotonin. 5-HT_1 family of receptors mediates an inhibitory effect via K^+ and Ca^{2+} channel regulation through second-messenger G-protein pathways. Inwardly-rectifying K^+ channels are opened while Ca^{2+} channels are closed, therefore inducing a resting state inhibiting neuronal

action potentials. Within the raphe nuclei, 5-HT_1 receptors are considered somatodendritic autoreceptors that ultimately play a negative feedback role on serotonin neurotransmitter firing forward to the presynaptic cleft (Aghajanian & Liu, 2009). 5-HT_1 receptors are also thought to be located not only in raphe nuclei, but also in the spinal cord, brain stem, and diencephalon (Aghajanian & Liu, 2009).

5-HT_2 family of receptors mediate their effect via K^+ and Ca^{2+} influx/efflux like 5-HT_1 receptors, except 5-HT_2 receptors have an excitatory effect through closing K^+ channels and/or opening nonselective cation channels. While low concentration of 5-HT_2 are generally found in the brain stem and spinal cord, abundant mRNA levels of 5-HT_2 are concentrated in the forebrain, piriform cortex, claustrum, and olfactory tubercle (Aghajanian & Liu, 2009).

5-HT_3 family of receptors is the only family that relies on ligand-gated ion channels instead of second-messenger G-protein signaling. Therefore, the excitatory characteristics of 5-HT_3 are very fast and shortly lived opposed to their slower second-messenger 5-$HT_{1,2,4,6,7}$ counterparts. In addition to 5-HT_3 being the only ligand-gated ion channel serotonin receptor, 5-HT_3 is also a member of the ligand-gated ion channel superfamily and is homologous to the nicotinic acetylcholine receptor and $GABA_A$ β_1 receptor (Aghajanian & Liu, 2009).

5-$HT_{4,6,7}$ family of receptors is quite different from the other 5-HT receptors due to the nature of G-coupling. 5-$HT_{4,6,7}$ receptors couple their serotonin induction to adenylyl cyclase. Serotonin coupling to adenylyl cyclase inherently increases cyclic AMP which can produce a signal cascade through ion channels or PKA pathways. The addition of PKA pathways allows for signal transduction via a number of ion channels, transporters, and additional kinases. Currently, 5-HT_4 receptors are thought to aggregate in the striatum, substantia nigra, olfactory tubercle, and hippocampus. In addition, 5-HT_4 functions postsynaptically inhibiting calcium-activated

potassium channels which leads to an inhibition of the refractory period. 5-HT_7 receptors also couple with adenylate cyclase and utilize the cyclic AMP pathway to regulate action potential firing (Aghajanian & Liu, 2009).

1.8.2 5-HT Transporters and Receptors in Fathead Minnow

The serotonin transporter (SERT), which is the primary target of sertraline and other SSRIs, is primarily responsible for the reuptake of serotonin neurotransmitter at the presynaptic terminal. Sertraline blocks the reuptake of serotonin at SERT allowing for more serotonin availability at the postsynaptic terminal, therefore increasing neuronal action potential firing downstream and hence increasing serotonin release. Not only do FHM contain SERT, but sertraline has a high affinity for binding and targeting SERT in fathead minnow and other aquatic organisms (Valenti et al., 2012). SERT, in fish, has been previously reported to have a ~65% ortholog homology to that of human SERT (Valenti et al., 2012). Additionally, $SERT_a$ and $SERT_b$, two coding genes for SERT in zebrafish, have between 66-75% sequence similarity to the coding genes for SERT in humans (Wang et al., 2006).

In 2007, radiolabeled citalopram, a member of the SSRI family, was used to analyze the binding affinity of citalopram to fathead minnow SERT. Results show a similar K_D value for [^{3}H] citalopram binding to SERT in fathead minnows, zebrafish, and rats (Gould et al., 2007). In addition to SERT, 5-HT receptors have also been characterized as being highly conserved among all vertebrates (Gould et al., 2007).

1.8.3 Serotonin and Steroidogenesis

Within the last 10 years, research has been conducted that correlates estrogen levels with depression severity in females. It has also been shown that females have a greater occurrence of depression than men, especially at a younger age, most likely due to change in various steroid hormone levels (Piccinelli, 2000). Additionally, the hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-thyroid axis (HPT) could possibly play a significant role in the onset of depression and sustainability of depression in both females and males (Piccinelli, 2000). The HPA axis could possibly be influenced by steroid hormone levels in females and males, but with greater prevalence in females according to Weiss et al, (1999). Cortisol levels have been correlated with steroid hormone imbalance particularly in young females (Halbreich & Lumley, 1993). Males and post-menopausal women do not show a correlation between cortisol and age, therefore drawing a direct connection between HPA activity and steroid hormone imbalance (Halbreich & Lumley, 1993). In regards to the HPT axis, thyroid-stimulating hormone levels are increased in over 25% of the depressed patients and seen primarily in females (Piccinelli, 2000).

Interestingly, it has been reported that antidepressant drugs, such as sertraline and/or other SSRIs, have an increased efficacy in pre-menopausal women compared to post-menopausal women (Borrow & Cameron, 2014). For women taking SSRIs, steroid hormone levels, especially estradiol, has been directly related to a positive therapeutic outcome (Holsen et al., 2011). Also, researchers have found SSRI treatment alone is not as effective as SSRI treatment in addition to estradiol therapy (Westlund & Parry, 2003).

1.8.4 Serotonin Transporters and Estradiol

Serotonin transporters (SERT) within the raphe nuclei are primarily responsible for the reuptake of serotonin, but also regulate tryptophan hydroxylase (TPH) which is the primary enzyme in the rate limiting step of serotonin synthesis (MacGillivray et al., 2010). Also located in the raphe nuclei are nuclear estrogen receptors alpha and beta (ERα, ERβ) with ERβ being the most concentrated of the two (Sheng et al., 2004). Due to the congregation of both SERT and ER receptors in the same region of raphe nuclei, it can easily be seen how activation of ER receptors can play an important role in SERT activity.

Serotonin release via 5-HT family of receptors ($5\text{-}HT_1$) in the presynaptic cleft can be regulated through estrogen receptor activation in the nucleus of serotonergic neurons in the raphe nuclei, depicted in Figure 6 (Borrow & Cameron, 2014). A previous study on rodents found an increase in both serotonin and its metabolite 5-hydroxyindoleacetic (5-HIAA) when estradiol levels were much highr than during normal biological function (Di Paolo et al., 1983). Also, when estradiol was administered to rats lacking ovaries, mRNA expression levels of TPH in the raphe nuclei were upregulated, suggesting an increase of serotonin synthesis (Donner & Handa, 2010). In addition to estradiol's effect on 5-HT receptors, estradiol can also regulate brain derived neurotrophic factors (BDNF) via tropomyosin receptor kinase B (TrkB). The influence of estradiol on pTrkB receptors can influence the release and synthesis of serotonin, but less is known about this interaction (Madhav et al., 2001; Siuciak et al., 1998).

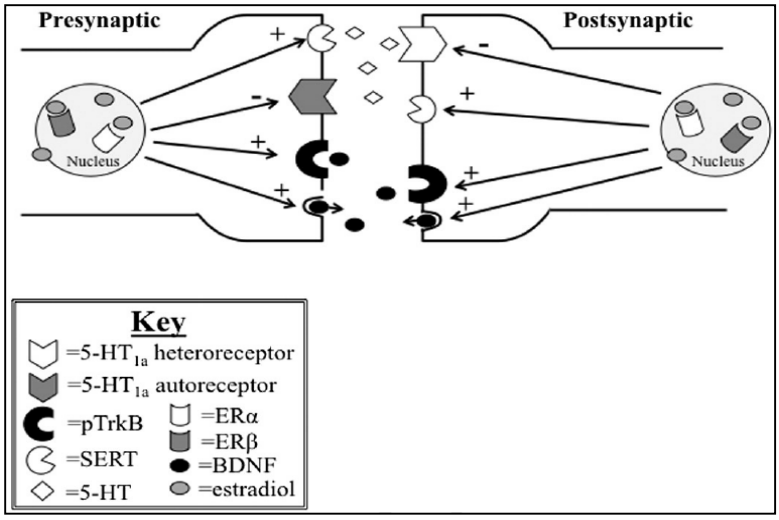


Figure 6: Estradiol receptors can regulate both the 5-HT heteroreceptor and autoreceptor while activating sertraline transporters. Also, estradiol can activate the release of BDNF via pTrkB receptors (Borrow & Cameron, 2014).

1.9 Thyroid System

Normal thyroid function, regulated by thyroid-stimulating hormone (TSH), is important for the synthesis and mobilization of the two main thyroid hormones: triiodothyronine (T_3) and thyroxine (T_4). Almost all of the T_4 produced is eventually converted to T_3, therefore T_3 is considered the primary acting thyroid hormone. In regards to the anatomy of the thyroid gland, it houses a very large number of follicles that are closed off to the surrounding cytoplasm allowing for secretion to the interior portion via the cuboidal epithelial cells. The cuboidal epithelial cells secrete a large amount of colloid, the primary shuttle compound for thyroblobulin, which contains thyroid hormones. The primary mechanism at which the thyroid hormones act is via binding to DNA bound thyroid receptors, therefore inducing gene transcriptional changes that regulate development, metabolism, cardiovascular function, and various other biological systems (Hall & Guyton, 2011). In this study, thyroid nuclear receptors were screened for a significant differential expression of thyroid receptor mRNA. If the nuclear receptors are differentially

regulated in response to sertraline, an assumption can also be made that T_3 and/or T_4 levels and more importantly thyroid function are also affected.

Interestingly, SSRI's can impact thyroid hormone levels, especially in conjunction with other drugs, i.e., levothyroxine. Levothyroxine is a synthetic thyroxine (T_4) often used for thyroid hormone therapy, but also has the ability to lower thyroid-stimulating hormone (TSH). A recent study has shown hypothyroid patients that start taking sertraline have increased TSH levels (Gaitonde et al., 2012). Additionally, another study conducted on patients with hypothyroidism taking sertraline and levothyroxine concluded that serum thyrotropin levels increased in all patients (McCowen et al., 1997). An increase in thyrotropin levels due to sertraline use shows a decreased efficacy in levothyroxine of hypothyroid patients, therefore showing a direct correlation between sertraline and synthetic T_4 levels and may possibly even effect endogenous T_4 in healthy patients. Moreover, researchers at the University of California-Los Angeles School of Medicine studied TSH, T_3, T_4, and free T_4 levels in conjunction with the usage of sertraline and fluoxetine. Results show an increase in TSH after SSRI administration and a significant decrease in T_3 and T_4 after SSRI administration (Gitlin et al., 2004). The mechanisms in which sertraline may act upon the HPT axis are still unclear, but a number of 5-HT receptors are involved in inhibitory processes that can significantly reduce cAMP levels and therefore have a significant influence on the adenohypophysis' release of TSH and possibly even dopamine levels that may affect hypothalamic stimulus.

1.10 Objectives and Hypothesis

The purpose of this study is to determine the effect of sertraline on juvenile and adult female fathead minnow steroidogenesis. The null hypothesis for this study is:

$H_{0:}$ Sertraline, at concentrations ≤ 10 µg/L, has no effect on targeted steroidogenic catalytic enzyme mRNA levels in the fathead minnow.

CHAPTER 2

MATERIALS AND METHODS

2.1 Fathead Minnow

As endocrine disrupting compounds (EDCs) become more prevalent in our environment, it is imperative to have a model organism that can be easily screened, includes similar biological pathways/systems as mammals, cost effective, quick maturation time, and requires little resources for maintenance. More importantly, it is necessary to become familiar with animals that may act as a warning system in regards to environmental contamination, i.e., canaries of water safety (Ankley et al., 1998). Due to their similarity to mammals, teleost fish are an ideal candidate for, not only endocrinology and physiology studies, but also genetic and molecular research. Increasing vitellogenin levels in the plasma of fresh water fish when exposed to wastewater effluents has already been shown in both the United Kingdom and the United States (Folmar et al., 1996; Purdom et al., 1994). In addition to vitellogenin research, there is a lot of data available in regards to FHM and other regulatory systems such as metabolism and steroidogenesis, therefore the use of FHM is well documented and recognized as a standard in toxicology testing (Ankley et al., 1998). All FHM used in this study were purchased from recognized dealers and preserved in normal living conditions. Upon the termination of each exposure period, proper humane euthanization was utilized.

2.2 Adult Female Fathead Minnow (*Pimephales promelas)* Sertraline Exposure

Five month old adult female fathead minnows (FFHM) were obtained from the Tarrant County Regional Water District. Six FFHM were housed in three dechlorinated tap water flow through systems kept at 25°± 1°C with 16:8 light/dark cycles and fed tetramine flakes twice

daily. pH, DO, alkalinity, hardness, ammonia, nitrate, and nitrite were measured in normal ranges throughout the exposure. FFHM were exposed to 3 and 10 µg/L sertraline, as well as a dimethyl-formaldehyde (DMF) solvent control in three 5 liter tanks via a flow through system. Substocks of sertraline were formulated at 8.34 and 27.8 mg/L and pumped into mixing chambers at 5 µl/min along with fresh water at 13.9 ml/min (Mohamed, 2013). Tanks received four turn-overs of test water per day (Mohamed, 2013). DMF levels in the solvent control tank were less than or equal to 0.01%. At the conclusion of seven days, FFHM were euthanized in buffered ethyl 3-aminobenzoate methanesulfonate salt (MS-222, Sigma). After euthanization, ovary and brain tissue were excised and placed into RNA*later,* a RNA stabilization reagent (Qiagen), and stored at -80°C until RT-qPCR analysis.

2.3 Early Life Stage FHM Sertraline Exposure

Fathead minnow (FHM) eggs were purchased from Aquatic Biosystems, Inc. (Fort Collins, CO). FHM larvae were fed 28.1 ± 8.3 mg artemia (brine shrimp) once a day and housed in a contained walk-in unit. Newly spawned FHM eggs (<48 hours) were placed in glass beakers fitted with stainless-steel wire mesh false bottoms that allowed for proper water circulation and inhibition of egg congregation. FHM larvae were housed at 25°C ± 0.5 with 16:8 light/dark cycles. 600 mL glass beakers were separated into 4 groups: solvent control, 0.1, 1, and 10 ug/L sertraline, all in quadruplet. Beakers were filled with 500 mL test water with 300 mL freshly prepared test water replaced daily. Reconstituted hard water prepared according to EPA methods (USEPA, 2002) was used in this study. pH, hardness, alkalinity, dissolved oxygen, and conductivity were measured weekly to insure an acceptable range. pH, dissolved oxygen, and conductivity were measure using a H280G meter once every five days. Alkalinity was measured

by titrating 100 ml study water sample to pH 4.5 at 25°C with 0.1 N H_2SO_4. Alkalinity calculations were assessed using the equation: $CaC0_3 = A \times 50$, where A = volume (mL) of 0.1 N H_2SO_4 titrated (Eaton et al., 2005). Hardness was measured by mixing 25 mL of study water, 25 mL distilled water, 2 mL hardness buffer solution (NH_4Cl, NH_4OH, magnesium salt of EDTA, and DI water), and 9 drops of calmagite indicator (1-(1-hydroxy-4-methyl-2-phenylazo)-2-napthol-4-sulfonic acid). After mixing test water sample with all reagents, the sample was then titrated with 0.01 M (0.02 N) EDTA until sample turned blue. Hardness calculations were assessed using the equation: $CaCO_3 = A \times 40$, where A = volume (mL) of 0.01 M EDTA titrated (Eaton et al., 2005).

Sertraline stocks were prepared in methanol at the previously stated concentrations. Methanol levels in the solvent control beakers were less than or equal to $4x10^{-8}$%. Sertraline concentrations were verified via liquid chromatography-tandem mass spectrometry (LC-MS/MS) on day 0, 14, and 28. Water samples were collected at random from test beakers and spiked with internal standard, sertraline-D_3 (Cerilliant), before analysis. 0.1 µg/L water samples were extracted using hexane ethyl-acetate, dried under nitrogen, and reconstituted in methanol before analytical analysis. At the completion of 28 days, FHM larvae were randomly separated into two groups. Group 1 larvae were placed in buffered MS-222 and dried for weight measurements, while group 2 larvae were pooled into ≈ 15 mg groups, placed in RNA*later* and stored at -80°C until RT-qPCR analysis.

2.4 Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS)

Water samples were collected from exposed beakers at day 0, 14, and 28 then analyzed via LC-MS/MS to insure ideal exposure concentrations of sertraline. Samples, except 0.01 µg/L

sertraline, were spiked with internal standard sertraline-D_3 (Cerilliant) and directly analyzed on the LC-MS/MS. 0.01 µg/L samples, due to LC-MS/MS sensitivity ranges, had to be extracted via liquid extraction method and reconstituted at 1 µg/L before analyzing. The liquid extraction process began with 10 ml of 0.01 µg/L sample water spiked with 10x internal standard. Next, 5 ml of 1:1 hexane ethyl-acetate was mixed with 10 ml of 0.01 µg/L water sample (2x) and extracted. The 10 ml of hexane-ethyl acetate extract was then evaporated using nitrogen. The evaporated hexane ethyl-acetate vial was then reconstituted in 1.5 ml of methanol and evaporated again using nitrogen until dry. Once dry, the 1.5 ml methanol vial was reconstituted in 100 µL methanol to a concentration of 1 µg/L and then analyzed on the LC-MS/MS.

2.5 RNA Extraction, Integrity, and Purity/Concentration

Adult FFHM brain and ovary tissue and pooled larval FHM were removed from RNA*later* and placed directly into TRI Reagent (Sigma). RNA isolation occurred via the TRI-Reagent method according to the manufactures protocol. Initially, all samples were hand homogenized in TRI Reagent until no visible tissue was present. Next, samples were centrifuged at 12,000 g for 10 minutes at 4°C. After centrifugation, the middle layer, between a fatty and organic layer, was removed and placed in a clean microcentrifuge tube. Next, the samples incubated at room temperature for 5 minutes until chloroform was added. After the chloroform was added, the layers were allowed to separate before centrifuging at 12,000 g for 15 minutes at 4°C. The resulting three layers after centrifugation were as follows: top layer (RNA), middle layer (DNA), and bottom layer (protein). The RNA was removed and placed in a clean microcentrifuge tube. Next, isopropanol was added to the RNA samples, vortexed, allowed to incubate at room temperature for 5 minutes, and then centrifuged at 12,000 g for 10 minutes at

4°C. During centrifugation, RNA congregated at the bottom of the microcentrifuge tube forming a pellet. RNA pellets were then resuspended in 75% ethanol, vortexed and centrifuged at 7,600 g for 5 minutes at 4°C. Ethanol supernatant was then removed and RNA pellet was resuspended in either Tris-EDTA buffer (Sigma) or molecular grade RNase/DNase free water. After RNA isolation concluded, all samples were treated for gDNA contamination and analyzed for RNA integrity and purity/concentration.

RNA integrity and purity/concentration was verified/quantified on an Experion RNA analysis system and a BioTek Take3 micro-volume plate, respectively. RNA integrity, using the Experion RNA analysis system, used chromatography and gel analysis software in order to quantify the ratio between 28S rRNA and 18S rRNA within each sample. Depending on the area beneath the curve for each standard ribosomal RNA (rRNA) and the ratio between 28S:18S, an RNA quality indicator (RQI) number was assigned to each sample. All samples used in this study were in the acceptable RQI range for RNA integrity (Figure 7). RNA purity/quantification was measure in duplicate and averaged using a BioTek Take3 micro-volume plate by calculating the ratio of absorbance values of RNA (260 nm) to the absorbance values of protein (280 nm). For RNA, an acceptable $A_{260/280}$ should be 2 ± 0.2. All samples used in this study were in the acceptable $A_{260/280}$ range. After RNA integrity and purity/concentration validation, RNA samples were diluted in molecular grade RNase/DNase free water or Tris-EDTA buffer to 100 ng/uL stocks and stored at -80°C.

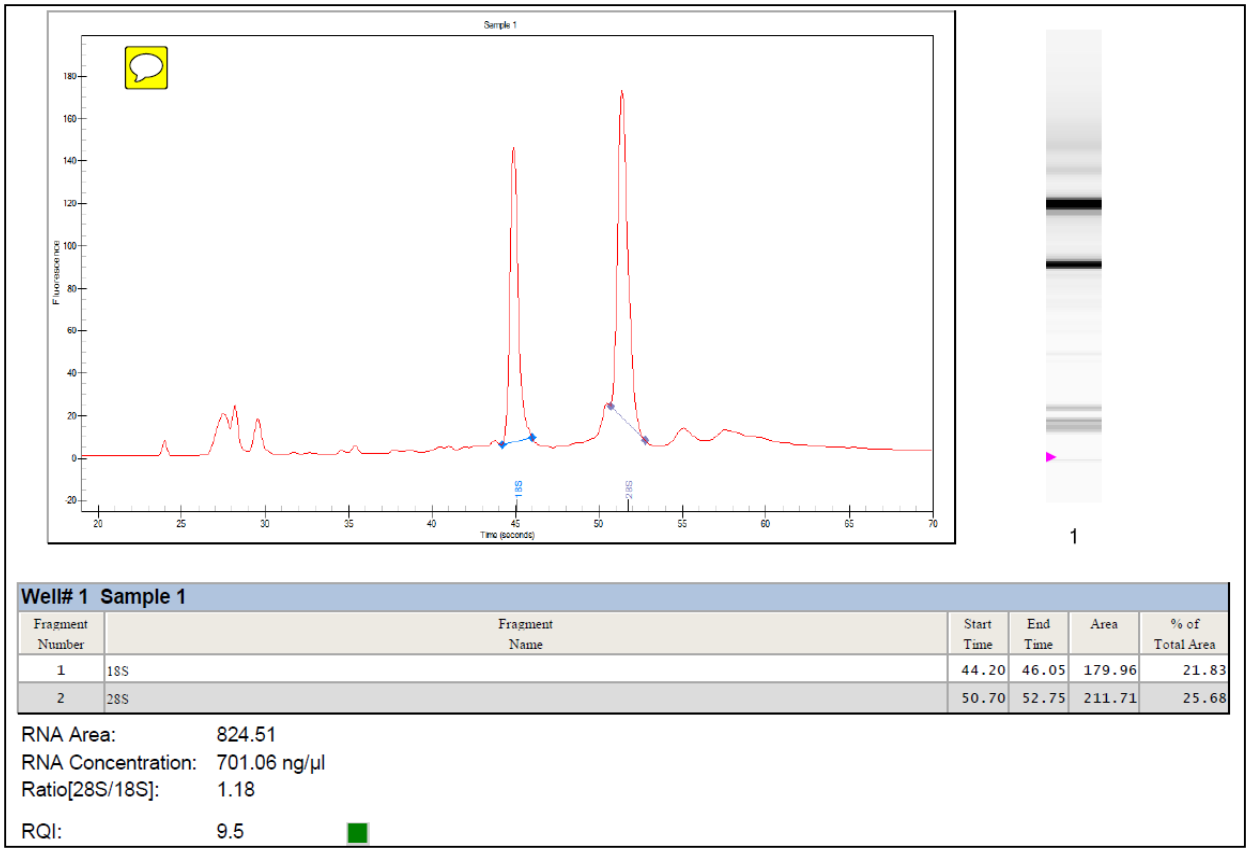


Figure 7: Experion RNA analysis kits were used to measure the ratio between 28S:18S rRNA areas beneath each curve (peak). After ratios were measured, a RQI value was assigned to each sample and either accepted or rejected.

2.6 The Polymerase Chain Reaction

PCR was first utilized in 1986 by Kary Mullis in an attempt to propagate the amplification of DNA sequences with high specificity (Mullis et al., 1986). During a typical PCR assay, DNA polymerases and primers for a specific gene/sequence are added to a mixture along with a target nucleic acid sample, e.g., gDNA from a specific tissue or organism. The sample is then denatured, typically at 95°C, and then cooled, around 60°C, allowing for hybridization of the double stranded molecules followed by an extension step at roughly 70°C. Once hybridization has occurred, the enzymatic activity of DNA polymerase extends the primers synthesizing a direct copy of the target gene/sequence. Cycling between hot and cold

temperatures has an amplification effect, in essence, doubling the amount of PCR products in the sample after each cycle. Once the PCR is complete, samples are generally run on a gel for base pair length analysis and can be later sequenced for sequence recognition.

Table 1: Primers used for RT-qPCR analysis are provided below along with their NCBI accession numbers and forward/reverse sequences.

Primer Name	NCBI Accession	Sequence 5'-3'	R^2	Efficiency (%)
11β-HSD	DT228665	FW-GCATCGGCGAGC	0.996	111
		RV-CTCCTCGCCGTG		
20β-HSD	DT259130	FW-TGTCATGCTCTTC	0.981	117
		RV-CTTGCTAACAAAGC		
CYP19	AF288755	FW-TGCTGACACATGCA	0.96	101
		RV-CAGCTCTCCGTGGC		
TRα	DQ074645	FW-ATGACCCAGAGA	0.984	115
		RV-CATCAGACACCA		
TRβ	AY533142	FW-GCCAACCAGTCAGG	0.99	113
		RV-AGCAACAGAATGAGG		
RP-L8	AY919670	FW-CATACCACAAGTACAAGGCCAAGA	0.99	111
		RV-ACCGAAGGGATGCTCAACAG		

2.7 Reverse Transcription Quantitative Polymerase Chain Reaction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays were assessed using SYBR-green detection chemistry on a Rotor-Gene 6000 cycler (Corbett-Qiagen) (Heid et al., 1996). 11β-HSD, 20β-HSD, TRα/β, CYP19, and RP-L8 primers used in the RT-qPCR assays were previously published (Table 1) and cycler parameter optimization for each primer was achieved in house. Fathead minnow 60s ribosomal protein RP-L8 has previously been recognized as a remarkably stable internal control (reference gene) used for RT-qPCR analysis (Filby & Tyler, 2007). Quantitative real-time PCR follows the exact same principal as PCR except camera and/or laser technology has allowed the quantitation of PCR products in real-time as the amplification of PCR products takes place (Higuchi et al., 1993). As the PCR

products amplify a sigmoidal curve is graphically produced correlating fluorescence (y-axis) and cycle time (Ct) (x-axis). A threshold can then be set at a specific fluorescence value across all samples. After a threshold has been set, the Ct values can then be statistically analyzed via $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

The $2^{-\Delta\Delta CT}$ method statistically analyzes the difference in Ct values, at a specific fluorescence, across a set of exposed and control samples. After the difference in Ct values has been taken between groups of control samples, e.g., the ΔCt between TRα and RP-L8 solvent control groups, the control ΔCt are then compared to the ΔCt between TRα and RP-L8 exposed groups. Finally, the change between the control ΔCt and the exposed ΔCt are calculated and generally presented in a doubling graph (log (2)). Statistical significance was calculated via a student's T-test ($p \leq 0.05$) comparing all control ΔCt values verse all exposed ΔCt values for a particular gene. Standard error of the mean was used in order to identify error within sample groups by taking the standard deviation of all ΔCt samples for a given gene and divided by the square root of the total number of samples.

CHAPTER 3

RESULTS

3.1 Water Quality and Water (LC-MS/MS) Sertraline Concentrations

Water quality parameters were measured for each new batch of hard water. Every new batch of water fell within acceptable ranges for pH (7.92 – 8.6), hardness (168 – 180 mg/L), alkalinity (110 mg/L), dissolved O_2 (8.2 – 9.1 mg/L), and conductivity (0.0563 – 0.0604 S/m) (Table 2). Sertraline concentration in test water was measure before the adult FFHM minnow exposure began and at day 0, 14 and 28 of the ELS study. Additionally, test water was analyzed at hour 0 and 24 for day 14 and 28 of the ELS study. Concentrations of sertraline for the adult FFHM study were roughly 3 and 10 µg/L at the beginning of the 7 day exposure period (Mohamed, 2013). Sertraline concentrations for the FHM ELS study were as follows: solvent control beakers were consistently below the LC-MS/MS limit of detection at non-detectable ranges while 0.1, 1, 10 µg/L beakers contained varying levels of sertraline (Table 3). Average time-weighted sertraline concentrations were calculated via averaging the time 0 and 24 sertraline concentrations for day 0, 14, and 28 for each exposure concentration.

Table 2: Water quality was assessed once every five days from day 0 until day 25. Dissolved O_2 was assessed beginning on day 10 due to instrument failure.

ELS Water Quality					
Day	pH	Hardness (mg/L)	Alkalinity (mg/L)	Dissolved O_2 (mg/L)	Conductivity (S/m)
0	8.08	172	110	-	0.0583
5	7.92	180	110	-	0.0604
10	8.11	176	110	8.2	0.0580
15	8.35	168	110	8.8	0.0571
20	8.6	172	110	8.9	0.0563
25	8.1	180	110	9.1	0.0575

Table 3: Test water was analyzed for sertraline concentrations on day 0 then days 14 and 28 at time 0 hr and 24 hr directly before a water change occurred. Solvent control beakers never reached the LC-MS/M limit of detection (LOD) for sertraline. Test beaker concentrations were within acceptable ranges for time 0 hr and expected ranges at time 24 hr.

	ELS LC-MS/MS Sertraline Concentration (µg/L)					
	Day 0	Day 14		Day 28		
Test Beaker	Time 0	Time 0	Time 24	Time 0	Time 24	Time-weighted Sertraline ($\bar{x}$)
Solvent Control	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
0.1 µg/L	0.13	0.09	0.06	0.1	0.03	0.09
1 µg/L	0.95	1.1	0.48	0.8	<LOD	0.71
10 µg/L	9.95	9.1	2.8	10.95	10.65	8.9

3.2 ELS FHM Aliquoted Brine Shrimp Biomass, Mortalities, and Dry Weight

FHM larvae were fed brine shrimp once a day and their biomass analyzed at least once a week for the duration of the study. Average brine shrimp biomass equaled roughly 28.1 ± 8.3 mg per beaker per day (Table 4). In addition to brine shrimp mass, FHM larvae mortalities were observed and noted.

Table 4: After feedings on day 0, 8, 10, 15, 18, 24, and 28 an additional aliquot of brine shrimp was dried and weighed. Average brine shrimp biomass for the entirety of the 28 day exposure was roughly 28.1 mg per beaker per day.

Aliquoted Brine Shrimp Biomass		
Day	($\bar{x}$) mg	Total ($\bar{x}$) mg
0	27.3	28.1 ± 8.3
8	23	
10	44.83	
15	29.7	
18	20.23	
24	21.77	
28	29.7	

Table 5: Fish mortalities were documented daily for solvent control, 0.1 µg/L, 1 µg/L, and 10 µg/L sertraline. Dead fish were immediately removed from test beakers and checked for obvious physiological abnormalities and/or disease.

	ELS Fish Survival Rate (%)		
	Fish (∑)	Dead (∑)	Survival (%)
Solvent Control	73	5	93.2
0.1 µg/L Sertraline	81	4	95.1
1 µg/L Sertraline	60	5	91.7
10 µg/L Sertraline	72	14	80.6

Solvent control, 0.1, 1, 10 µg/L sertraline beaker survival rates were all greater than 90% except for 10 µg/L sertraline beakers with 80.6% survival rate (Table 5). At the conclusion of the 28 day exposure period dry weights for FHM larvae, not included in the RT-qPCR gene expression assays, were analyzed. Interestingly, a positive correlation was observed between sertraline dosing and FHM larvae dry weight. FHM weight nearly doubled between solvent control (0.42 mg) and 1 µg/L sertraline (0.7 mg), but lacked significance in accordance to a one-way analysis of variance (one-way ANOVA) test ($p = 0.1102$) (Figure 8).

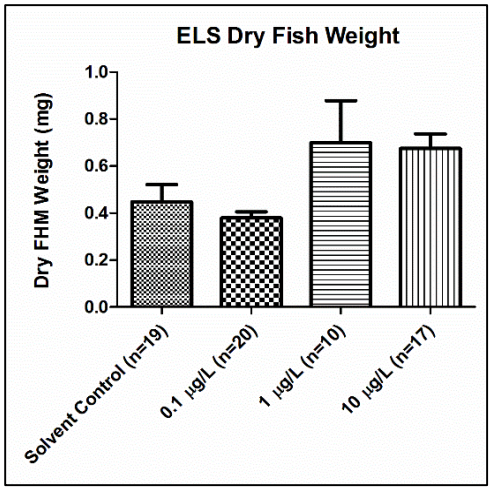
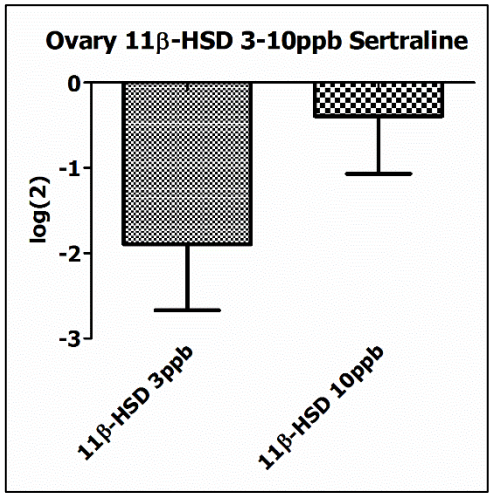


Figure 8: FHM larvae dry weight was assessed at the conclusion of the 28 day exposure period. FHM were pooled per beaker, weighed, and averaged. Total pooled averages per beaker are depicted above. One-way analysis of the variance showed no statistical significance ($p = 0.1102$) between control groups and exposed groups.

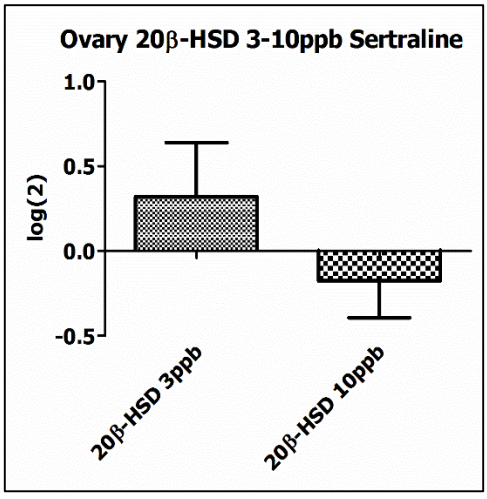
3.3 Adult FFHM Gene Expression Results

FFHM ovary expression of steroidogenic enzymes did not show a significant regulatory change, but imply a down-regulation trend in 11β-HSD at 3 µg/L sertraline (Figure 9-A). Sertraline exposure to brain tissue caused a statistically significant up-regulation of 11β-HSD ($\log(2) = 0.364$, $p = 0.03$) and 20β-HSD ($\log(2) = 0.384$, $p = 0.0002$) at 10ug/L sertraline, but lacked significance at 3 µg/L sertraline (Figure 10-A, B). CYP19 showed relatively no change in either the ovary or brain of FFHM; brain transcripts are increased compared to control groups yet lack significance (Figure 10-C). Thyroid receptors, while showing an increase in expression, did not reach significance due to the variance in samples (Figure 10-D,E). Transcriptional analysis of sertraline exposure at 3 and 10ug/L indicates that steroidogenic enzyme expression levels may be altered, especially in brain tissue, but technical analysis of RT-qPCR results is very sensitive to noise resulting in variation in transcript levels with no statistical significance. Additionally, RP-L8 levels of expression from solvent control groups to exposed groups can have an effect on ΔCt values resulting in a skewed ΔΔCt value that lacks a correlative change in expression of any given gene transcript.

A)



B)



C)

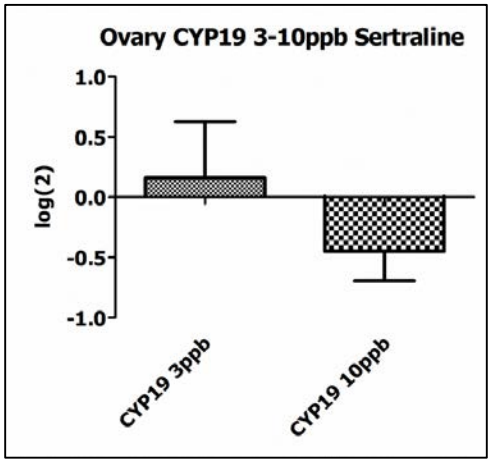
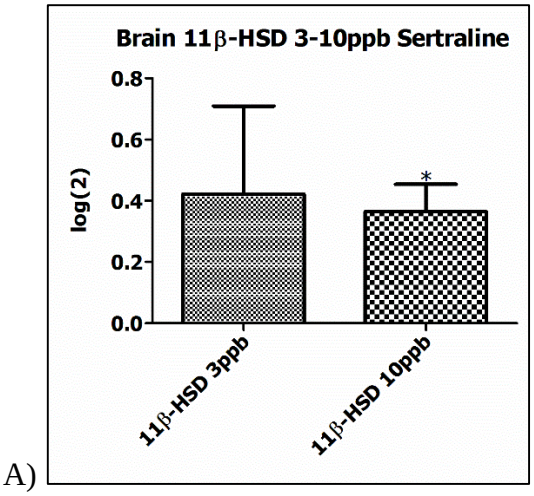


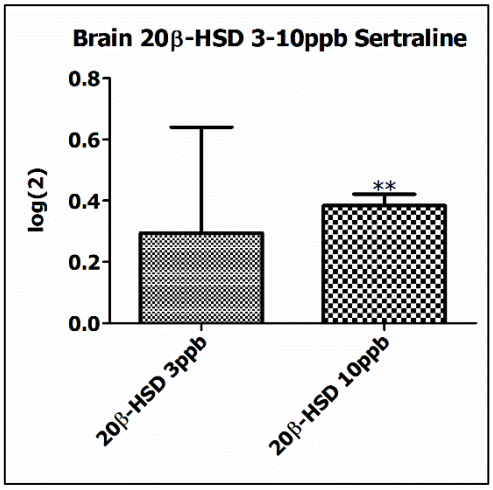
Figure 9: A-C, Ovary steroidogenic gene expressions in FFHM exposed to 3 and 10 µg/L sertraline organized by gene target. No significant changes in transcript abundance was observed in 11β-HSD, 20β-HSD, or CYP19 after exposure to either 3 or 10 µg/L. A) 11β-HSD showed signs of a significant down-regulation of target transcripts, but lacked significance and produced larger than anticipated SEM.

3.4 ELS Study Gene Expression Results

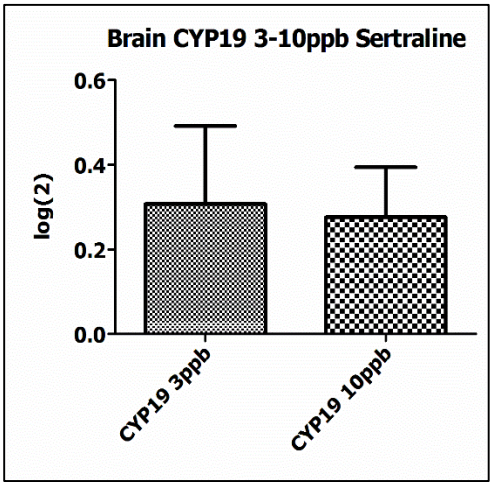
Early life-stage results for 11β-HSD and 20β-HSD somewhat mirror one another with an increase in transcript at 0.1, 1, and 10 µg/L sertraline (Figure 11-A, B). 20β-HSD (Figure 11-B) at 0.1 µg/L is the only significantly up-regulated gene with log(2) = 0.844 and p = 0.01, therefore almost double the amount of 20β-HSD transcripts were present in the 0.1 µg/L samples.



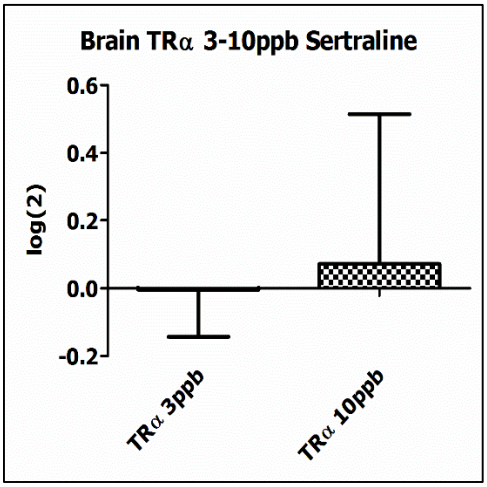
A)



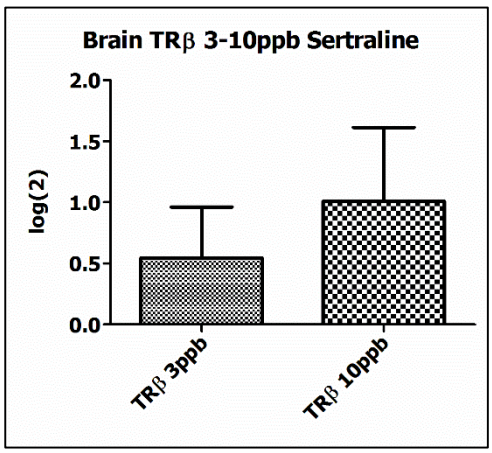
B)



C)



D)



E)

Figure 10: A-E represent the gene expressions of 11β-HSD, 20β-HSD, CYP19, TRα, and TRβ in FFHM brain tissue. A-B) 11β-HSD and 20β-HSD are both up-regulated after exposure levels of 10 µg/L sertraline. C) CYP19 transcripts seemed to be increased, but lacked significance. CYP19 at 10 µg/L ($p = 0.054$). D-E) Both thyroid receptor variants expressed transcripts at relatively normal levels.

Both, 11β-HSD and CYP19 resulted in a variety of transcript numbers, but lacked sufficient significance. TRα transcripts were significantly increased in the 0.1 µg/L exposure beakers with log(2) = 0.406 and p = 0.02 (Figure 11-D). CYP19 and TRα transcript amounts were inversely proportional to sertraline concentrations, but due to variance between samples they lacked significance at lower concentrations of sertraline.

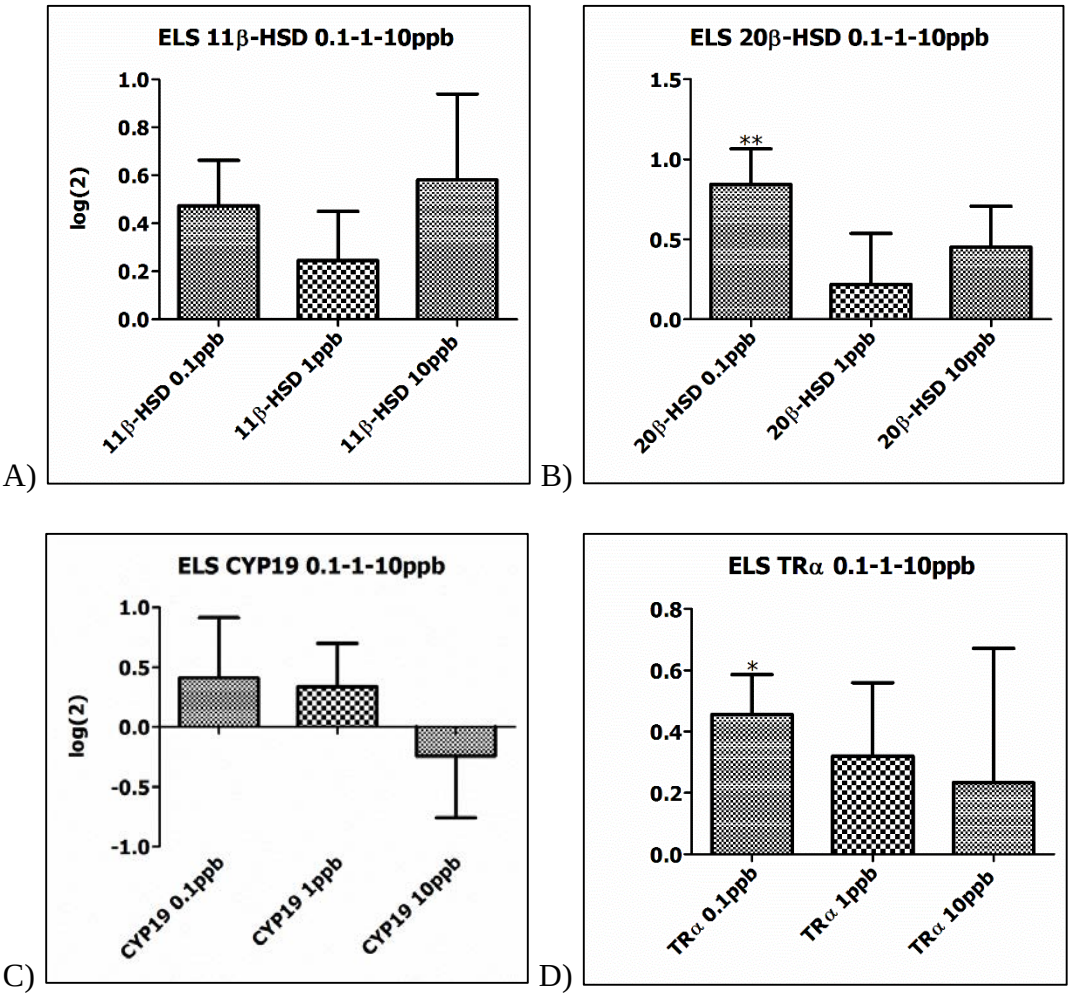


Figure 11: A-D represents the early life stage exposure of fathead minnow larvae for 28 days at 0.1, 1, and 10 µg/L sertraline. A,C) 11β-HSD and CYP19 show varying transcript abundance compared to control, but lack significance. B, D) 20β-HSD and TRα have a significant up-regulation of transcript number at 0.1 µg/L, but lack any significance at higher concentration of sertraline.

CHAPTER 4

DISCUSION AND CONCLUSION

4.1 Discussion

In this study, adult female fathead minnows and larval fathead minnows were exposed to slightly elevated concentrations, compared to environmentally relevant levels, of sertraline and tested for differential genetic expression of various steroidogenic enzymes. Differential neuroendocrine activity in aquatic organism can play an imperative role in our knowledge of chronic exposure to environmental concentrations of antidepressants and give an insight to possible genetic ramifications (Valenti et al., 2012). Due to sertraline targeting SERT transporters in neural tissue most of the differential gene expression effects realized in adult FFHM occurred in the brain. At 10 µg/L sertraline, 11β-HSD, 20β-HSD, and CYP19 transcripts in FFHM brain tissue were increased compared to solvent control.

11β-HSD is the primary catalytic enzyme responsible for the conversion of 11-doxycortisol to cortisol and/or the synthesis of other corticoid proteins, therefore increased cortisol levels should be influenced by an up-regulation of 11β-HSD. Elevated plasma corticoid levels are generally thought to assist in stress and inflammatory responses, but can eventually have a muscle wasting effect when chronically exposed to elevated glucocorticoids (Simmons et al., 1984). 20β-HSD converts 17α-OH-progesterone to 17α,20β-dihydroxy-4-pregnen-3-one (17α,20-DP) during steroidogenesis. 17α,20β-DP is a maturation-inducing hormone responsible for gamete maturation through its affinity to the membrane progesterone receptor (Overturf et al., 2014; Tokumoto et al., 2006).

The results produced by this study suggest that sertraline exposure may induce maturation in FHM as seen by the increase of 20β-HSD transcripts in the early-life stage study

and FFHM study. CYP19 had increased transcripts compared to control groups in the FFHM study but was slightly out of a statically significant range ($p = 0.054$). CYP19 is the primary catalytic enzyme in the synthesis of estradiol from testosterone and estrone via androstenedione. A feminization effect may occur in male FHM with an increase of aromatase and hence estradiol, but more research needs to be conducted on male FHM and sertraline exposures (Kuhl & Brouwer, 2005). In addition, an ELS study can check for genetic sexual change among maturating male FHM to see if sexual differential gene expression is occurring due to sertraline exposure. Significant changes in steroidogenic gene expression did not occur in FFHM ovary tissue most likely due to the short seven day exposure period to sertraline and the time it may take for differential gene expression to occur in down-stream organ tissue. Subsequent studies should analyze TRα,β receptors in ovary tissue due to the fact that TRα expression is most abundant in ovary tissue in zebrafish (Power et al., 2001).

The ELS study also provides evidence for an up-regulation of 20β-HSD transcripts compared to control which may result in an induction of maturation via mPR receptors. 11β-HSD was also increased in all exposure groups buck lacked statistical significance. In regards to thyroid receptor expression, the thyroid receptors were only differentially expressed during the ELS study. The TRα transcripts were increased compared to control at 0.1 µg/L implying a higher binding capacity of thyroid hormones to DNA, therefore inducing synthesis of regulatory mechanism governing growth, differentiation, and metabolism (Power et al., 2001). In the future, instead of whole body homogenization, I propose individual organs should be excised and analyzed due to the importance of the thyroid receptors during embryogenesis and gametogenesis.

Recent research involving zebrafish and an overexpression of TRα caused a loss of midbrain-hindbrain border and rostral hindbrain depletion (Power et al., 2001). In regards to maturation induction, it may be interesting to point out a positive correlation between larval FHM dry weight and sertraline exposure concentrations. Average weight of dried larval FHM at the conclusion of the exposure period for 10 µg/L compared to solvent control were nearly double, but lacked significance when applied to a one-way analysis of variance. Unfortunately, FHM from each beaker were pooled and then weighed which limited individual sample data, therefore excluding individual weight in the final statistics. Instead, statistical analysis was achieved via one-way ANOVA from pooled average weight from each beaker per exposure group. In the future, two sets of beakers should be used to measure true endpoints from an ELS study. The first set of beakers should be used for physiological endpoints, e.g., dry weight, behavior, food intake, etc. A second set of beakers should be utilized for the gene expression/screening endpoints.

As the use of pharmaceuticals increases in the United States, it is imperative that the scientific community play a major role in determining the effects of low concentration chronic exposure to the most widely prescribed drugs. The effect of pharmaceutical contamination in surface waters can not only effect the ecotoxicity of aquatic organisms, but may also contaminate drinking water which may be detrimental to human health. Population awareness of proper pharmaceutical disposal and the lack of pharmaceutical elimination technology in WWTP will continue to compound the accumulation of drugs in our environment.

Risk assessments involving *in vivo* accumulation and *in vitro* toxicity of the most popularly prescribed pharmaceuticals in the U.S. have been in place for many years, but education regarding the outcome of these research areas is far disconnected from the general

human population. A study in 2010 showed that environmentally relevant concentrations of propranolol, a widely prescribed *β*-adrenergic receptor antagonist, had little effect on xenobiotic-metabolizing enzymes, but when increased to human therapeutic levels xenobiotic-metabolizing enzymes were significantly induced (Bartram et al., 2011). While WWTP effluent and surface water concentrations of most anti-depressants are in the ng/L range, proportionality of drug contamination will only increase with drug prescriptions and WWTP lack of urgency for proper filtering technology (Schultz et al., 2010).

4.2 Conclusion

Future studies regarding SSRIs and their accumulation in the environment and how they might affect neuroendocrine function, steroidogenesis, development, and reproduction in aquatic organism is imperative due to the increase of said drugs in both surface waters and WWTP effluents. An important aspect of this study that requires further attention is the consistency of 20β-HSD to be differentially regulated in both adult FFHM and larval FHM. 17α,20β-DP's ability to activate progesterone receptors is an important concept, therefore correlating mPR expression with 20β-HSD expression levels in the presence of SSRIs such as sertraline may incorporate an important piece of the SSRI/steroidogenesis puzzle. Additionally, information regarding estradiol and serotonin neurotransmitter activation may be a unique avenue for future research, i.e., knocking down CYP19 expression along with SSRI exposure and how that may play a role in serotonin levels and/or other effects that may pose to normal development and reproduction.

Results from this study also suggest no detrimental growth or survival endpoints are involved in the chronic low-level exposure of sertraline. While the previous statement may be

true, further research is needed in regards to the generational toxicology of sertraline. Genetic alterations, i.e., methylation and acetylation, may directly influence enzymatic differential expression that can then be passed generation to generation, but more research is necessary to show the possible epigenetic characteristic of low-level sertraline exposure.

An *in vitro* assessment of sertraline is also needed in regards to human cell lineages. Sertraline may affect various cell lines differently and the genetic alterations are easily screened via several *in vitro* methods. Also, as estrone and estradiol levels are induced by sertraline exposure, certain endocrine active cancers may be induced even further into tumor growth and metastasis. Therefore, sertraline may need to be analyzed further in regards to the potentiality of cancer progression due to the increase of aromatase activity and abundance. In addition to *in vitro* assessment, steroid hormone concentrations in conjunction with catalytic enzyme abundance is imperative to future analysis. Enzyme and substrate data will not only back up the transcript abundance data shown in this study, but it will also set the stage for future endeavors into this very complex field of research.

SSRI prescriptions in the United States will continue to rise as long as pharmaceutical companies continue to produce them in mass quantities and physicians will continue to prescribe SSRIs as long as their patients suffer from anxiety, depression, etc. Halting SSRI production and/or prescriptions is not the suggestion of this study, but a better mechanism in which we filter SSRIs and other pharmaceuticals out of WWTP effluents would be a good start. Correlating SSRIs with neuroendocrine disruption is no longer pseudoscience nor are the possible side effects of thyroid function and feminization.

Pharmaceutics persist in our environment at ever increasing levels and it is the responsibility of the science community to have a series of steps to insure safety of not only the

human population, but also our aquatic brethren. The use of model aquatic organisms is imperative for early detection of pharmaceuticals in the environment and other pollutants that continue to plague our natural resources. Whether we are using aquatic organisms as canary analogues or as a form of pharmaceutical toxicity awareness in humans, they offer insight into the many complexities found in our forever evolving ecosystem.

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