# Title: Comparative transcriptomics implicate mitochondrial and neurodevelopmental impairments in larval zebrafish (*Danio rerio*) exposed to two selective serotonin reuptake inhibitors (SSRIs)

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## Abstract

Pharmaceuticals and personal care products are emerging contaminants that are increasingly detected in the environment worldwide. Certain classes of pharmaceuticals, such as selective serotonin reuptake inhibitors (SSRIs), are a major environmental concern due to their widespread use and the fact that these compounds are designed to have biological effects at low doses. A complication in predicting toxic effects of SSRIs in nontarget organisms is that their mechanism of action is not fully understood. To better understand the potential toxic effects of SSRIs, we employed an ultra-low input RNA-sequencing method to identify potential pathways that are affected by early exposure to two SSRIs (fluoxetine and paroxetine). We exposed wildtype zebrafish (*Danio rerio*) embryos to 100 µg/L of either fluoxetine or paroxetine for 6 days before extracting and sequencing mRNA from individual larval brains. Differential gene expression analysis identified 1550 genes that were significantly affected by SSRI exposure with a core set of 138 genes altered by both SSRIs. Weighted gene co-expression network analysis identified 7 modules of genes whose expression patterns were significantly correlated with SSRI exposure. Functional enrichment analysis of differentially expressed genes as well as network module genes repeatedly identified various terms associated with mitochondrial and neuronal structures, mitochondrial respiration, and neurodevelopmental processes. The enrichment of these terms indicates

that toxic effects of SSRI exposure are likely caused by mitochondrial dysfunction and subsequent neurodevelopmental effects. To our knowledge, this is the first effort to study the tissue-specific transcriptomic effects of SSRIs in developing zebrafish, providing specific, high resolution molecular data regarding the sublethal effects of SSRI exposure.

Keywords: selective serotonin reuptake inhibitors; fluoxetine; paroxetine; toxicogenomics; SMART-seq; zebrafish; neurodevelopment

## 1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a diverse and widely used group of emerging contaminants. Environmental researchers are increasingly detecting PPCPs, such as selective serotonin reuptake inhibitor (SSRI) antidepressants, in surface waters, sediments, and fish tissues, with body burdens ranging from parts per trillion to parts per billion levels (Metcalfe et al. 2010; Lara-Martin et al. 2015; Fick et al. 2009). While manufacturing and improper disposal are potential sources of environmental contamination, excretion of ingested pharmaceuticals into municipal wastewater systems is the predominant source of pharmaceuticals released into the environment (Williams 2008). The widespread use of these pharmaceuticals, coupled with typically low removal efficiencies of wastewater treatment plants, has resulted in continuous environmental contamination (Ternes et al. 2004; Kolpin et al. 2002; Lajeunesse et al. 2011).

The underlying mechanism of action SSRIs is still unclear despite widespread human use. Following the monoamine theory of depression, SSRIs and other antidepressants were thought to increase the free concentration of neurotransmitters like serotonin, norepinephrine, or dopamine at the synaptic cleft (Fuller and Beasley 1991). However, the delayed timing of therapeutic effect, low response rate, and high incidence of relapse has thrown into question whether this is truly their mechanism of action (Kirsch 2019). Current alternative hypotheses of SSRI mechanisms of action

include potential anti-inflammatory properties, effects on neural circuitry, and altered neurogenesis, but this still remains an active area of research (Rahmani et al. 2013; Walker 2013; McAvoy et al. 2015). Therefore, the sublethal effects of these compounds on non-target organisms is unpredictable.

SSRIs are specifically designed to induce biological effects at low doses with chronic exposure (Ankley et al. 2007). Thus, even though SSRIs are typically present in the environment at much lower concentrations than other classes of contaminants, the environmental concern is real. Moreover, experimental studies have shown that fish repeatedly exposed to modest levels of SSRIs bioaccumulate the pharmaceuticals to levels that exceed therapeutic concentrations in humans. The potential effects of this bioaccumulation still remain unclear (Valenti et al. 2012). Previous studies have shown that sublethal exposure to SSRIs can significantly alter fish behavior, physiology, and gene expression. For example, short term sertraline, citalopram, and fluoxetine exposure can decrease prey capture ability, predator escape, and reproductive behaviors in a number of fish species (Hedgespeth, Nilsson, and Berglund 2014; Kellner et al. 2015; Gaworecki and Klaine 2008; Bisesi et al. 2016; Painter et al. 2009; Pelli and Connaughton 2015; Perreault, Semsar, and Godwin 2003; Weinberger and Klaper 2014). Other studies found that fluoxetine exposure is associated with altered ovarian gene expression and decreased egg production in zebrafish (*Danio rerio*) as well as increased vitellogenin expression in male fathead minnows (*Pimephales promelas*), indicating possible endocrine disrupting properties (Schultz et al. 2010; Lister et al. 2009).

Transcriptomics has become an integral tool in toxicology and pharmacology as the ability to broadly assay whole transcriptomes allows for the discovery of targeted or key pathways without *a priori* information on the effected genes. Additionally, recent improvements in sequencing technologies have allowed for single-cell and low input sequencing methods like SMART-seq, CEL-seq, Drop-seq, etc., providing high cellular resolution and more detailed information at increasingly minute scales (Hwang, Lee, and Bang 2018; Baran-Gale, Chandra, and Kirschner 2018). These high resolution

sequencing methods have proven to be invaluable in studying complex biological problems like reconstructing cell lineages, characterizing risk factors and identifying candidate genes of mental disorders like schizophrenia, or elucidating mechanisms of toxicity (Raj, Gagnon, and Schier 2018; Fromer et al. 2016; Thyme et al. 2019; Zhang et al. 2019).

In addition to the ever-improving technological aspects of transcriptomics, growing public resources, such as gene ontology databases, further empower scientists to study complex systems by contextualizing sequencing data with up-to-date biological insight (Alexander-Dann et al. 2018). Coupling the data generated by sequencing experiments with systems biology approaches like network analyses has proven to be a powerful method for revealing gene expression patterns, which can greatly facilitate molecular mechanism identification (Wang and Wang 2019; Alexander-Dann et al. 2018). Network-based approaches incorporate gene expression patterns to infer correlation and presume biological interaction among genes, including those that did not differ significantly from controls (Fromer et al. 2016; Wang and Wang 2019). For example, network analyses allowed Maertens et al. (2015) to identify biological pathways impacted by toxic MPTP (methyl-4-phenyl-1,2,3,6-tetrahydropyridine) exposure. Network analyses were also successfully used to develop biologically motivated candidate biomarker genes of sexually dimorphic patterns in zebrafish (Huang et al. 2018; Wong, McLeod, and Godwin 2014). Systems biology approaches like weighted gene co-expression network analysis (WGCNA) can therefore be used to both broaden the scope of genes considered for further study and focus on biologically relevant pathways (Zhang and Horvath 2005).

We have previously shown that repeated exposure to SSRIs can significantly alter the spontaneous swimming behavior of larval zebrafish during the visual motor response, causing a consistent hypoactive response relative to control siblings (Huang, Sirotkin, and McElroy 2019). However, the mechanism causing these behavioral effects is unknown. In this study, we assessed the molecular determinants of the behavioral effects of sublethal exposure to SSRIs by leveraging a low

input RNA-sequencing toxicogenomics approach. Zebrafish embryos were exposed to the SSRIs fluoxetine and paroxetine at doses that consistently alter larval zebrafish swimming behavior during the visual motor response (Huang, Sirotkin, and McElroy 2019). Individual brains were dissected out and sequenced using SMART-seq v.4 technology. In addition to identifying transcriptome-wide changes by differential gene expression analysis, we used WGCNA to identify modules of genes significantly correlated with SSRI exposure. Functional analysis identified multiple terms related to mitochondrial function and neurodevelopment, implying that SSRI toxicity in developing fish involves mitochondrially mediated neurodevelopmental abnormalities.

## 2. Materials and Methods

### *2.1 Animal exposure and tissue collection*

Adult wildtype zebrafish, a hybrid of Tubigen Longfin/Brian's wild-type strain (TLB), were maintained at 28.5°C under 13/11 hours light/dark cycle and fed Gemma micropellets (2 days a week) or newly hatched brine shrimp (5 days a week) following protocols approved by Stony Brook University's Institutional Animal Care and Use Committee.

Sibling embryos from a single pair of TLB adults were dechorionated using a dilute protease solution (1 mg/ml Pronase, Sigma Aldrich) and exposed in groups of 25 at sphere stage (4 hours post fertilization) to 100 µg/L fluoxetine or paroxetine or control embryo media in 100 mm diameter plastic petri dishes lined with 1 % agarose. Eighty percent of the exposure solution was renewed daily until 6 days post fertilization. We have previously shown that repeated exposure to SSRIs at this dose significantly alters the spontaneous swimming behavior of larval zebrafish during the visual motor response resulting in hypoactivity (Huang, Sirotkin, and McElroy 2019). We screened treated larvae for survival and morphological deformities daily and found no effects of fluoxetine or paroxetine on larvae development or survival.

Individual larval brain tissue was collected for RNA-sequencing at 6 days post fertilization following the dissection process outlined by Vargas et al. (2011). Briefly, larvae were anesthetized with ice water and dorsally mounted in 2 % low melting agarose in a 60 mm plastic petri dish and were bathed with artificial cerebrospinal fluid (131 mM NaCl, 2 mM KCl, 1.25 mM $KH_2PO_4$, 2 mM $MgSO_4$, 10 mM glucose, 2.5 mM $CaCl_2$, 20 mM $NaHCO_3$). Whole brains were removed with insect pins (size 00), transferred to individual 1.5 ml plastic capped tubes, and homogenized in 200 µl Trizol reagent by vigorous vortexing before storing at -80°C.

*2.2 RNA-sequencing*

For each brain tissue sample, total mRNA was extracted using the Direct-zol RNA extraction kit (Zymo) and treated with DNase (TurboDNase, Invitrogen) to remove gDNA contamination. RNA quantification and quality were evaluated using a Qubit fluorometer (Invitrogen) and Bioanalyzer (Agilent). Only samples with RIN scores greater than 7 were kept for further analyses. cDNA libraries were prepared using the Clontech Ultra Low v4 kit (Clontech) in accordance with the manufacturer's instructions at the New York Genome Center. cDNA was ligated to Illumina Nextera XT sequencing adapters and amplified by PCR (using 13 cycles). Final libraries were evaluated using Qubit Fluorometer (Invitrogen) and Fragment Analyzer (Advanced Analytics) before sequencing on an Illumina HiSeq2500 sequencer (v4 chemistry) using 2 x 50bp cycles. Reads were aligned to the Ensembl GRCz10 reference using STAR aligner v2.4.2a (PMID:23104886). Quantification of genes annotated in Ensembl v81 was performed using featureCounts (from Subread v1.4.3-p1) (doi:10.1093/bioinformatics/btt656). QC were collected with Picard (v1.77) and RSeQC (2.6.1) (PMID: 22743226) (http://broadinstitute.github.io/picard/). Normalization and differential expression analysis of featureCounts was done using DESeq2 package (v1.14.1). p-values were adjusted using the Benjamini-Hochberg algorithm to control the false discovery rate (FDR). A heatmap of the expression patterns of all differentially expressed genes was generated using the 'pheatmap' package in R (Kolde 2019) to visually

express the similarities/dissimilarities in gene expression patterns between the individual brains sampled.

*2.3 RNA-seq validation and replication using RT-qPCR*

Housekeeping genes for validation RT-qPCR analyses were selected out of the transcriptomic dataset following the methods outlined in Dheilly et al. (2015). Briefly, we filtered for genes that had: (i) no differential expression with fluoxetine or paroxetine exposure, (ii) a coefficient of variation less than 0.05, (iii) a log fold change of 0 (±0.05), and (iv) a mean Transcripts Per Million (TPM) greater than 100. We randomly selected from the pool of genes that satisfied these constraints and used the genes Myc associated factor X (*max*), transmembrane p24 trafficking protein 10 (*tmed10*) and dihydrolipoamide (*dldh*) as housekeeping genes for RT-qPCR assays. Reference gene stability was controlled by plotting the log ratio of the mean Ct divided by the sample Ct (Supplemental Figure 1).

Genes to validate RNA-seq results were considered good candidates if they were: (i) significantly upregulated relative to control samples in both fluoxetine and paroxetine treatment groups, (ii) had $\log_2$(fold change) > 1, and (iii) were highly expressed (mean TPM>1500). The validation genes were randomly selected from the pool of genes that satisfied the outlined criteria and included uncoupling protein 2 (*ucp2*), Kruppel-like factor 9 (*klf9*), FK506 binding protein 5 (*fkbp5*), forkhead box k1 (*foxk1*), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4b (*pfkfb4b*), and serine/threonine kinas 35 (*stk35*).

We validated RNA-sequencing results by assaying candidate gene expression in the same samples sent for RNA-sequencing using RT-qPCR. Breifly, we synthesized cDNA (SuperScript II reverse transcriptase, Invitrogen) and performed RT-qPCR with a QuantStudio 6K Flex using Absolute qPCR SYBR Green (Thermo Scientific). We normalized target gene expression to the geometric mean of all three housekeeping genes for each sample, and used the ΔΔCt method (Livak and Schmittgen 2001) to

determine the relative fold change of target genes and analyzed using a one-way ANOVA followed by a Tukey's Honest Significance post-hoc test.

*2.4 Weighted gene co-expression network analysis (WGCNA)*

A weighted gene co-expression network analysis was conducted using the WGCNA package in R (Zhang and Horvath 2005). Briefly, the transcriptome dataset was filtered to retain the 17,387 transcripts that had detectable expression in all biological replicates. The network was derived using a soft threshold of 9 as a weight function based on a plateau in the generated scale independence curve. This threshold was used to create an adjacency matrix based on the correlation of expression between any pair of two genes among all the samples. This correlational adjacency matrix was used to generate a topological overlap matrix. Using this topological overlap matrix, a complete linkage clustering function organized genes into modules of highly connected genes into a network that was then cut using the dynamic tree cut algorithm with a deep split of 2 and a minimum module size of 30. Modules of genes whose overall expression correlate to exposure to fluoxetine only, paroxetine only, or to both SSRIs were identified by correlating module eigengene values (i.e. the first principle component of the module) to SSRI treatment.

*2.5 Functional enrichment analysis*

Functional groups and biological pathways over-enriched in our genes of interest were identified using gProfiler with the custom g:SCS significance threshold to control for multiple comparisons and a corrected p-value cutoff of 0.05 (Raudvere et al. 2019). We leveraged biological pathway databases, including Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome (REAC), and the Human Phenotype Ontology (HPO) databases to assign known functions, associated pathways, and associated human disease conditions to our genes of interest. Functional analyses were run on the list of all differentially expressed genes identified from DESeq2 (henceforth referred to as the

DE gene list), and genes from WGCNA modules that correlated to SSRI exposure (henceforth referred to as the module gene list).

## 3. Results

### *3.1 RNA-seq results*

In total, we measured the expression of over 27,000 genes in individual larval zebrafish brains. Differential expression analysis using DESeq2 found 1518 genes that were significantly differentially expressed from control brains with paroxetine treatment. Of these differentially expressed genes, 58 % were significantly downregulated with 885 out of the 1518 genes showing decreased expression compared to controls and 633 genes significantly upregulated with paroxetine exposure. Differential expression analysis identified 170 genes that were significantly different from controls in the fluoxetine treatment group. Like paroxetine exposure, 60 % of the fluoxetine responsive transcripts were downregulated, with 102 genes showing decreased expression and 68 genes with increased expression with fluoxetine exposure. Between the two SSRI treatments, there were 1550 unique genes that were differentially expressed, including 138 genes in common between fluoxetine and paroxetine treatment. All 138 of these common genes were consistent in the direction of expression change regardless of the drug to which larvae were exposed (Figure 1).

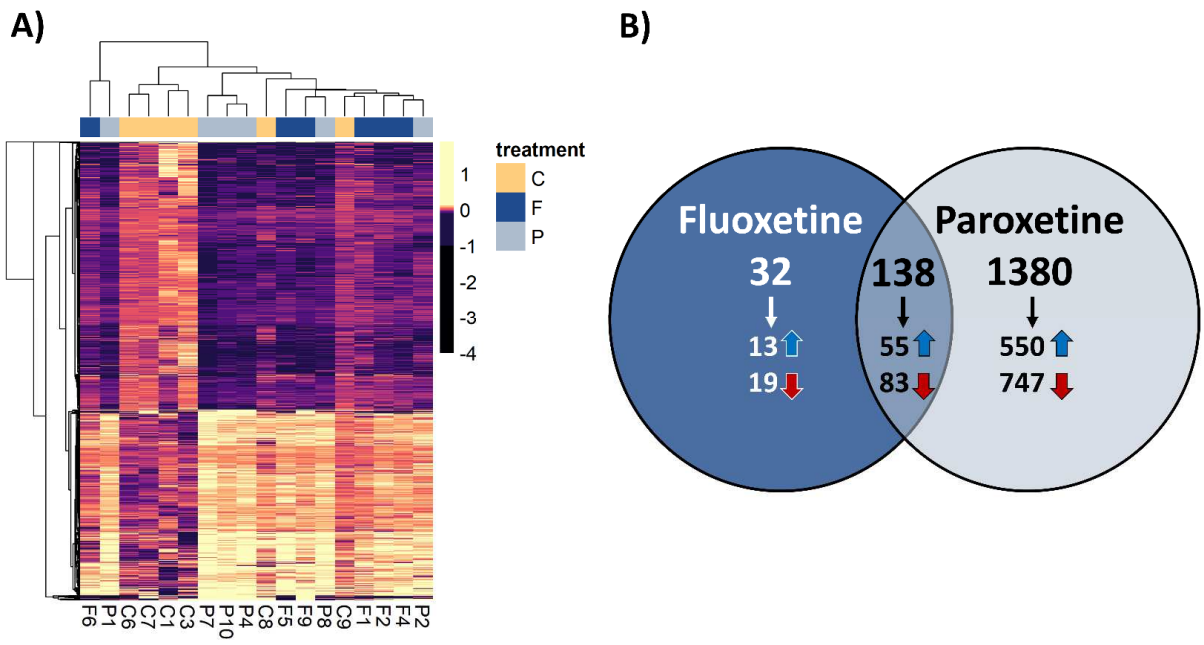


Figure 1. A) Heatmap of the 1550 genes significantly differentially expressed with SSRI treatment. Columns correspond to individual brains, indicated by the serial numbers at the bottom, consisting of a letter code (C = Control, F= Fluoxetine-treated, and P = Paroxetine-treated) and an identifying number. Rows correspond to the relative expression (log2 fold change relative to control larvae) of individual genes with warm colors representing significant upregulation and dark colors representing significant downregulation. B) Over 1500 genes that were differentially expressed with fluoxetine or paroxetine exposure, 138 of which were shared between the two drugs.

*3.2 RNA-seq validation using RT-qPCR*

TPM values of all 6 selected genes from the RNA-seq data show significant increases relative to control samples with fold changes ranging from 1.5 to 2.7, which closely mirrors those observed in the sequencing data and verifies our sequencing results (Figure 2). RT-qPCR assays confirmed that paroxetine treatment resulted in a significant ($p < 0.001$) increase in expression of all selected genes except for *klf9,* which still showed a highly upregulated pattern (2.46-fold increase) despite lack of significance. In contrast, RT-qPCR was not successful at detecting a significantly altered expression following fluoxetine treatment, though a consistent upregulated trend was observed for all selected genes with fold changes ranging from 1.64 to 3.44.

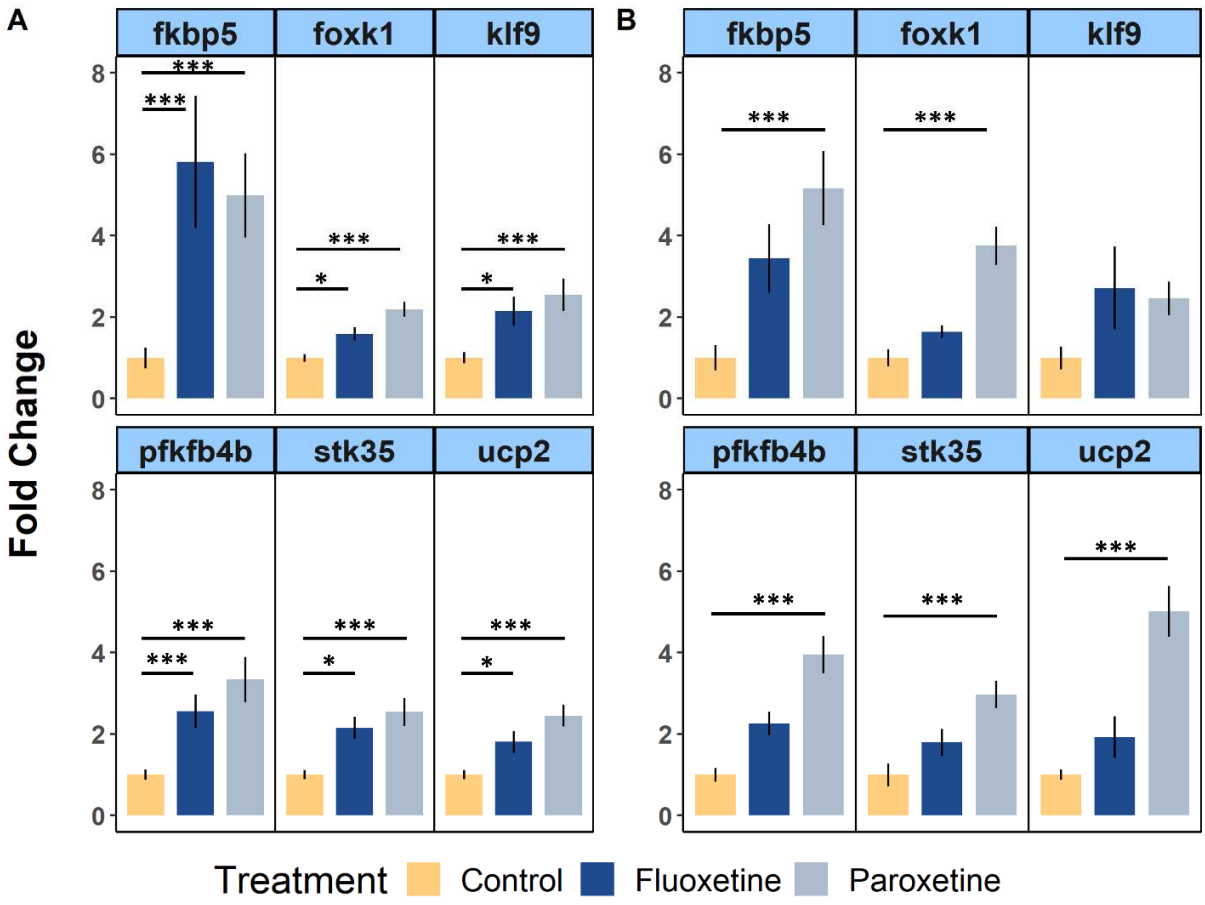


Figure 2. A) fold change of selected validation genes (calculated from TPM values) from RNA-seq data (n=6) shows significant upregulation of all 6 validation genes with both fluoxetine and paroxetine exposure. B) Relative fold change generated from RT-qPCR assays on the same samples used for RNA-seq show consistent upregulation trends in the validation genes. Significance code is as follows: * < 0.05, ** < 0.01, *** < 0.001.

*3.3 Weighted gene co-expression network analysis (WGCNA)*

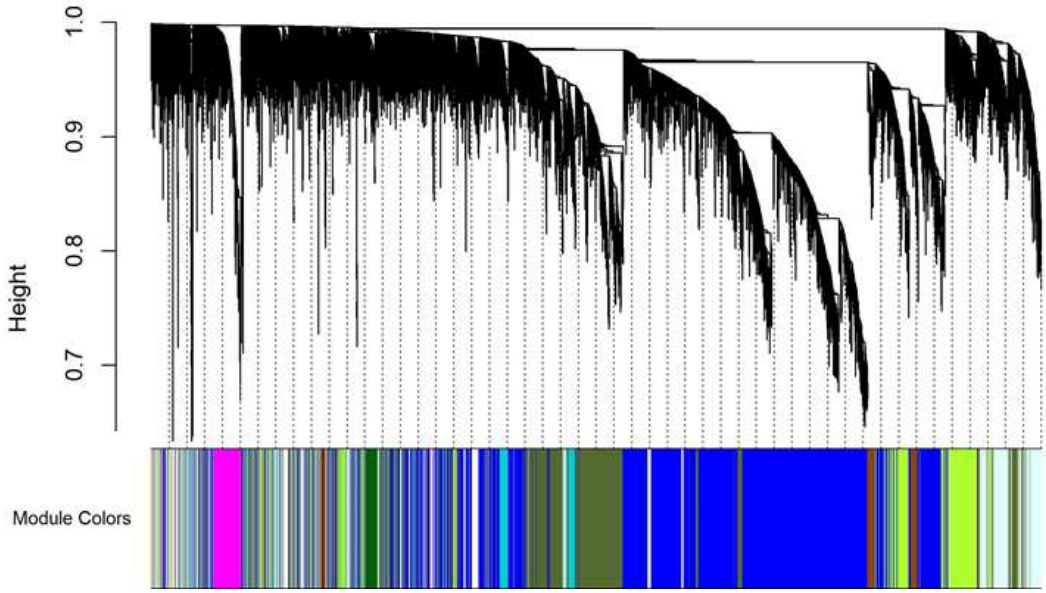


Figure 3. Gene co-expression network generated by our network analysis, which groups genes based on expression similarity using a pair-wise weighted correlation metric, and clusters according to a topological overlap metric into modules (colored bars on bottom).

WGCNA analysis generated a network containing 19 modules of genes (Figure 3; color coded bars on bottom), with modules ranging in size from 51 to 7133 genes per module (Table 1). Seven modules were significantly correlated with SSRI treatment (Table 1, bolded). Modules 5, 6, 11, 14, and 15, were found to be significantly correlated with both fluoxetine and paroxetine exposure whereas module 17 was significantly correlated with fluoxetine treatment only and module 12 was significantly correlated with paroxetine exposure only. Modules 5 and 15 collectively contained over 96 % of the module genes that were significantly correlated with SSRI treatment.

Table 1. WGCNA module sizes and their Pearson Correlation Coefficient between module gene expression patterns and exposure to fluoxetine, paroxetine, or both SSRIs. Modules that are significantly correlated with pharmaceutical exposure are bolded. Colored boxes next to the Module number corresponds to the color bar at the bottom of Figure 3.

| | | | Fluoxetine | | Paroxetine | | Both SSRIs | |
|---|---|---|---|---|---|---|---|---|
| Module | | # genes | Corr. | p-value | Corr. | p-value | Corr. | p-value |
| | 1 | 61 | -0.11 | 0.7 | -0.2 | 0.4 | -0.3 | 0.2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 91 | -0.087 | 0.7 | -0.32 | 0.2 | -0.4 | 0.1 |
| | 3 | 53 | -0.14 | 0.6 | -0.076 | 0.8 | -0.22 | 0.4 |
| | 4 | 400 | -0.017 | 0.9 | -0.25 | 0.3 | -0.27 | 0.3 |
| | **5** | 7133 | -0.23 | 0.4 | -0.36 | 0.1 | -0.59 | **0.01** |
| | **6** | 112 | -0.028 | 0.9 | -0.79 | **9.00E-05** | -0.82 | **3.00E-05** |
| | 7 | 94 | -0.34 | 0.2 | 0.44 | 0.07 | 0.1 | 0.7 |
| | 8 | 53 | -0.27 | 0.3 | 0.03 | 30.9 | -0.24 | 0.3 |
| | 9 | 542 | -0.22 | 0.4 | 0.38 | 0.1 | 0.16 | 0.5 |
| | 10 | 174 | -0.22 | 0.4 | 0.08 | 0.8 | -0.14 | 0.6 |
| | **11** | 57 | -0.52 | **0.03** | 0.47 | **0.05** | -0.05 | 0.8 |
| | **12** | 51 | -0.099 | 0.7 | 0.48 | **0.05** | 0.38 | 0.1 |
| | 13 | 547 | 0.034 | 0.9 | 0.23 | 0.4 | 0.26 | 0.3 |
| | **14** | 80 | 0.14 | 0.6 | 0.61 | **0.008** | 0.74 | **4.00E-04** |
| | **15** | 2908 | 0.13 | 0.6 | 0.48 | **0.05** | 0.61 | **0.007** |
| | 16 | 1090 | 0.026 | 0.9 | 0.26 | 0.3 | 0.28 | 0.3 |
| | **17** | 68 | 0.52 | **0.03** | -0.23 | 0.4 | 0.3 | 0.2 |
| | 18 | 1760 | 0.13 | 0.6 | 0.077 | 0.8 | 0.21 | 0.4 |
| | 19 | 296 | 0.17 | 0.5 | 0.079 | 0.8 | 0.25 | 0.3 |

### *3.4 Functional profiling of differentially expressed and WGCNA module genes*

Functional enrichment analysis of the DE gene list resulted in 64 GO terms that were significantly enriched (Figure 4; full list of results in Supplemental Table 1). Only three molecular function GO terms were enriched in the DE gene list: neurexin family protein binding, catalytic activity acting on RNA, and transcription coregulatory activity. Enriched biological processes included relatively broad terms such as RNA processing, protein folding, and various compound-specific metabolic processes. A large portion of the cellular component GO terms that were enriched in these groups

involved many aspects of the mitochondria; for example, the mitochondrial matrix, mitochondrial protein complex, mitochondrial ribosome, or the inner mitochondrial membrane protein complex.

In addition to GO terms, functional enrichment analysis identified 16 different pathway terms associated with the DE gene list (Table 2), including one KEGG pathway (fatty acid elongation) and multiple Reactome pathways, most of which involved mitochondrial or electron transport chain processes. HPO enrichment found multiple human disease phenotypes related to this gene list, including neurodevelopmental delay and abnormality and muscle physiology.

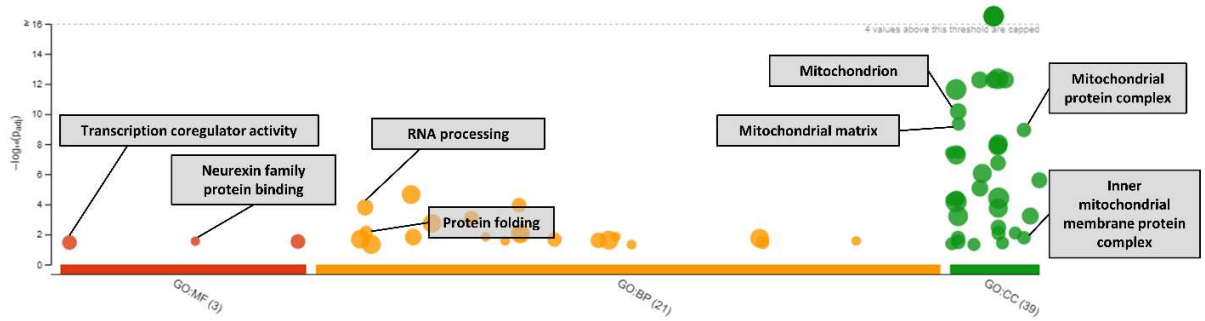


Figure 4. Manhattan plot of all significantly enriched Gene Ontology (GO) terms in the DE gene list. Functional terms (colored circles) are arbitrarily distributed along the unitless x-axis, which is divided into different GO categories (GO:MF = molecular function, GO:BP = biological process, GO:CC = cellular component). Functional term positions are fixed and terms from the same GO subtree are located closer to each other. Y-axis represents the corrected p-value associated with term enrichment on a -log10 scale and circle sizes reflect the size of the term (i.e. terms with more genes known to be associated with them are larger). Functional enrichment analysis found 3 MF terms, 21 BP terms, and 39 CC terms significantly enriched in DE genes.

We also conducted a functional enrichment analysis of the seven modules that were significantly correlated with SSRI exposure. Modules 5 and 15 were the only modules that had significant enrichment of GO terms, as well as various KEGG, Reactome, and HPO terms. Despite its much smaller size, Module 11 had several KEGG and Reactome terms that were significantly enriched (Table 2 and Supplemental Table 4). All remaining modules did not return any GO or pathway terms that were significantly enriched and are not included in subsequent discussions. Between the three retained modules, functional enrichment analysis produced a combined list of 399 GO terms (Figure 5 and

Supplemental Tables 2 and 3), along with 9 KEGG pathways, 48 Reactome pathways, and 130 HP ontology terms that were significantly enriched between the three modules (Table 2).

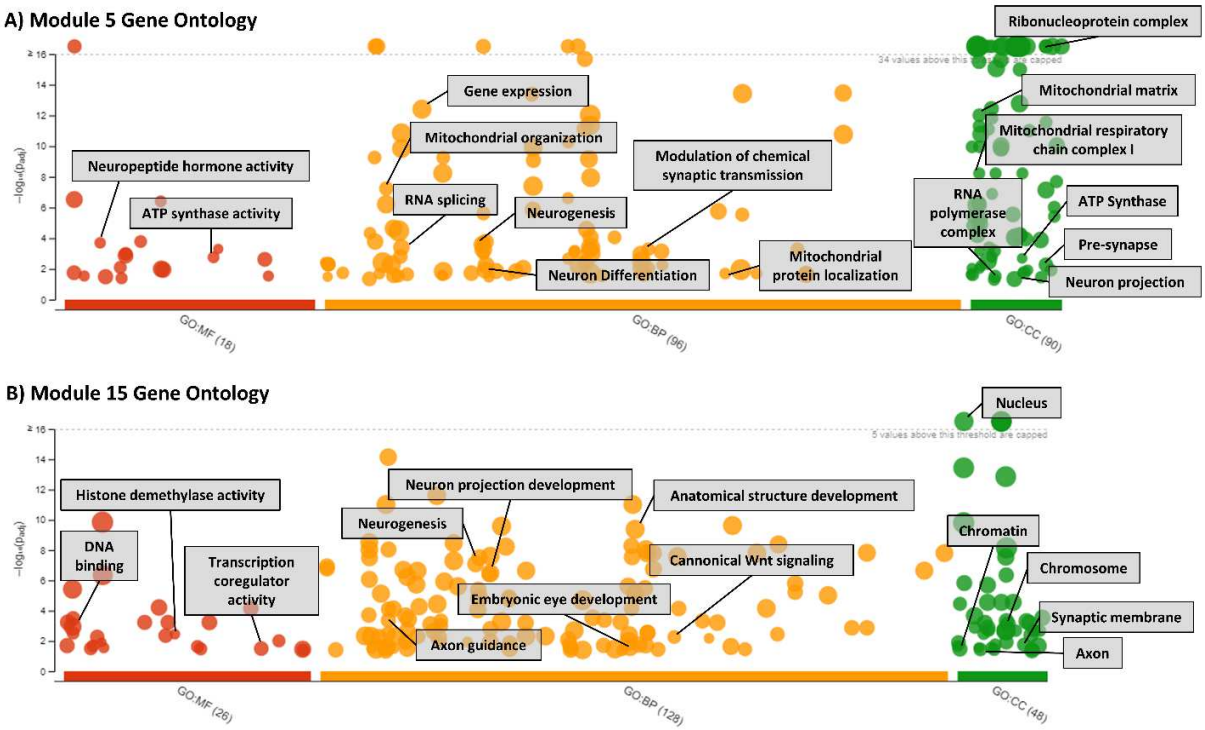


Figure 5. Manhattan plot of all significantly enriched Gene Ontology (GO) terms in WGCNA modules 5 (A) and 11 (B), which were the only 2 modules that were significantly enriched in GO terms. Functional terms (colored circles) are arbitrarily distributed along the unitless x-axis, which is divided into the different GO categories (GO:MF = molecular function, GO:BP = biological process, GO:CC = cellular component). Term positions are fixed and terms from the same GO subtree are located closer to each other. Y-axis represents the corrected p-value associated with term enrichment on a -log10 scale and circle sizes reflect the size of the term (i.e. terms with more genes known to be associated with them are larger). Functional enrichment analysis found 18 MF terms, 96 BP terms, and 90 CC terms that were significantly enriched in Module 5 genes, and 26 MF terms, 128 BP terms, and 48 CC terms that were significantly enriched in Module 15 genes.

Module 5 was significantly enriched in a total of 204 GO terms (Figure 5a). Enriched molecular function terms include various enzyme activities like ATP synthesis, as well as RNA metabolism (e.g. RNA binding and transcription coregulator activity) and neural related functions like neuropeptide hormone activity. There were many enriched biological process terms involved with RNA processing (e.g. mRNA processing, RNA splicing, and gene expression), mitochondria and energy production (e.g. mitochondrial organization, mitochondrial ATP synthesis coupled electron transport, and mitochondrial protein localization), and neurobiology (e.g. neurogenesis, regulation of synaptic transmission, and neuron

differentiation). Similarly, there were a number of cellular component terms associated with RNA metabolism (e.g. ribosome components, ribonucleoprotein complex, and RNA polymerase complex), mitochondria (e.g. the mitochondrial matrix, mitochondrial respiratory chain complex I, or ATP synthase complex), and neurons (e.g. synapses and neural projections).

Module 15 was significantly enriched in 202 GO terms and shared many similar enriched terms as Module 5 (particularly neurodevelopmental processes), however there was no prominent enrichment of mitochondrial or energy metabolism terms (Figure 5b). Molecular function terms that were significantly enriched included nucleic acid binding, transcription coregulator/corepressor activity, and histone demethylase activity, among others. Enriched biological processes included many neurodevelopmental terms (such as neurogenesis, axon guidance, and neuron projection development) but also included terms involved in general embryonic development such as canonical Wnt signaling, embryonic eye development, and anatomical structure development. Similarly, enriched cell component terms included various different neural components like axons or synaptic membrane as well as nuclear structures like chromatin, chromosomes, and the nucleus.

Table 2. Selected results from functional analyses of interested gene sets, including the DE gene list and WGCNA modules significantly correlated to SSRI exposure, that were significantly enriched in pathways related to neurodevelopment or mitochondrial function when searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, and Human Phenotype Ontology (HP) databases. Full results of functional analyses are available in Supplemental Tables 1-4

| Gene List | # genes | Database | Pathway | ID | Corrected p-value |
|---|---|---|---|---|---|
| All DE genes | 29 | REAC | Mitochondrial translation | REAC:R-DRE-5368287 | 2.09E-09 |
| | 16 | REAC | Respiratory electron transport | REAC:R-DRE-611105 | 0.042 |
| | 178 | HP | Neurodevelopmental abnormality | HP:0012759 | 0.012 |
| | 143 | HP | Neurodevelopmental delay | HP:0012758 | 0.013 |
| Module 5 | 60 | REAC | Respiratory electron transport | REAC:R-DRE-611105 | 1.53E-10 |
| | 870 | HP | Neurodevelopmental abnormality | HP:0012759 | 2.21E-08 |
| | 38 | HP | Abnormal activity of mitochondrial respiratory chain | HP:0011922 | 7.34E-06 |
| | 1122 | HP | Abnormality of nervous system physiology | HP:0012638 | 1.02E-05 |
| | 37 | HP | Decreased activity of mitochondrial respiratory chain | HP:0008972 | 1.66E-05 |
| | 789 | HP | Abnormality of brain morphology | HP:0012443 | 1.77E-05 |
| | 81 | HP | Abnormality of mitochondrial metabolism | HP:0003287 | 0.0001 |
| Module 11 | 4 | KEGG | Phototransduction | KEGG:04744 | 6.08E-06 |
| | 5 | REAC | Inactivation, recovery and regulation of the phototransduction cascade | REAC:R-DRE-2514859 | 3.14E-05 |
| | 2 | REAC | Serotonin and melatonin biosynthesis | REAC:R-DRE-209931 | 0.039 |
| Module 15 | 75 | REAC | Axon guidance | REAC:R-DRE-422475 | 5.65E-05 |
| | 416 | HP | Neurodevelopmental abnormality | HP:0012759 | 7.27E-09 |
| | 439 | HP | Abnormality of nervous system morphology | HP:0012639 | 7.94E-07 |
| | 517 | HP | Abnormality of nervous system physiology | HP:0012638 | 8.53E-06 |

## 4. Discussion

### *4.1 Comparative transcriptomics between 2 SSRIs*

We identified 138 genes differentially expressed by both drug treatments, all of which agree in the direction of effect (i.e. no drug caused an up regulation while the other caused down regulation) (Figure 1b). This core demographic of shared genes may represent the common targets of general SSRI treatments while the difference in the number of genes affected may reflect drug specific parameters, such as biological half-lives, binding affinities, metabolite activities, etc. (Vandenberg 1995). While there is a large discrepancy between the number of genes that are differentially expressed with fluoxetine and paroxetine exposure, our results are not atypical from other comparisons of SSRI effects in zebrafish. A study in adult zebrafish exposed to similar concentrations of fluoxetine found a comparable number of differentially expressed genes as our study (Wong, Oxendine, and Godwin 2013). Similarly, a comparative study between 2 SSRIs (fluoxetine and sertraline) in whole body larval zebrafish found a similar number of differentially expressed genes with fluoxetine treatment and showed a large discrepancy in the number of genes affected by fluoxetine and sertraline, another SSRI antidepressant (Park et al. 2012). These studies, in addition to ours, may further support the idea that individual drugs, despite being the same class with presumably the same mechanism of action, can have variable effects on the same system (Vandenberg 1995).

RT-qPCR assays in the same RNA samples used for RNA-sequencing generally showed comparable expression patterns to the RNA-sequencing results, validating our sequencing dataset. Paroxetine exposure caused a significant upregulation in 5 out of 6 selected biomarker genes with similar fold changes relative to control samples regardless of molecular technique used to assay their expression (Figure 2). *Klf9* was the only gene that was not significantly upregulated with paroxetine exposure when assayed by RT-qPCR; however, the upregulation patterns were still present at a similar

fold changes as observed in the sequencing data. Similarly, RT-qPCR detected a general upregulation pattern with fluoxetine exposure; however, none of the genes were significantly different from controls. This difference in statistical significance is likely a result of variable levels of sensitivities between the two methods, which could arise from a multitude of sources including differences in reaction chemistry, detection limits, and data analysis pipelines (Alexander-Dann et al. 2018).

*4.2 Functional profiling identifies mitochondrial and neurogenic pathways involved with SSRI exposure*

The DE gene list was particularly enriched in mitochondrial related GO terms and Reactome pathways, indicating that mitochondria might have a large role in the zebrafish's response to SSRI exposure (Figure 4, Table 2). Additionally, functional analysis identified neuropeptide hormone activity as well as several human neurological disease conditions that were associated with this gene list. Likewise, functional enrichment analysis of Module 5 and Module 15 genes identified many mitochondrial and neurodevelopment related GO terms and pathways (Figure 5, Table 2) and terms related to general early development like anatomical structure development and canonical Wnt signaling. Previous studies have found that SSRI exposure in isolated mitochondria disrupts cellular energy metabolism by uncoupling oxidative phosphorylation and inhibiting mitochondrial complexes I and V, causing a depletion in cellular ATP (Li et al. 2012). Disruption of mitochondrial energy supplies can alter key neurodevelopmental processes like cellular remodeling, neurogenesis, synaptogenesis, and circuit formation (Chen et al. 2013). All together, these functional profiling results suggest that developmental exposure to SSRIs might cause mitochondrial dysfunction, resulting in altered energy production within brain tissue cells and subsequent changes in neurodevelopment and neural morphology. A study using rats as model organisms lead to similar conclusions. Perinatal exposure to paroxetine alters the expression of genes involved in cellular metabolism and neurogenesis, which increases the risk of developing depressive like symptoms and other neurological disorders as adults (Glover et al. 2015).

While we did not select our validation genes based on published research, our selection criteria resulted in the identification of key genes that have been implicated in SSRI response and/or neurodevelopment in other studies. *Fkbp5,* encoding the glucocorticoid receptor chaperone FK506 binding protein 51, has been identified in previous studies as a promising target to assess the risk of developing neurological disorders and for treating depression and was highly upregulated with SSRI exposure in our dataset (Pohlmann et al. 2018; Ellsworth et al. 2013; Park et al. 2012; Wong, Oxendine, and Godwin 2013). In humans, overexpressed genetic variants of *fkbp5* are associated with a decrease in glucocorticoid receptor sensitivity to cortisol and prolonged stress hormone circulation. This ultimately over-activates immune responses, increasing oxidative stress in key brain regions, and contributes to neurological disease pathogenesis (Sadoul et al. 2018; Nold et al. 2019; Zhang et al. 2016). The role of *fkbp5* regulators was functionally tested in mice and demonstrated the importance of this gene in neurogenesis and neuronal development (Zheng et al. 2016). We speculate that the upregulation of *fkbp5* we observed in developing zebrafish in response to paroxetine and fluoxetine could result in higher cortisol levels or oxidative stress. This could lead to mitochondrial damage and subsequent effects on neurodevelopment that would explain the enrichment in GO terms associated with various aspects of neurodevelopment.

*Ucp2* was highly upregulated with SSRI exposure in our dataset. This could be an indication of mitochondrial dysfunction and altered neurodevelopment. *Ucp2* encodes mitochondrial uncoupling protein 2, an important regulator of mitochondrial metabolism that controls ATP and reactive oxygen species production (Ji et al. 2017). In mammalian systems, *Ucp2*'s antioxidant activity has been directly linked to neurogenesis and mitochondrial respiration. *Ucp2* knockout mice show more severe depressive symptoms in response to chronic mild stress and have decreased neurogenesis and enhanced loss of astrocytes and dendritic spines, which are partially rescued by transiently expressing *Ucp2* (Dietrich, Andrews, and Horvath 2008; Du et al. 2016). While the neurodevelopmental role of *ucp2*

has not yet been established in zebrafish, fish *ucp2* is highly similar to its mammalian orthologs (76-78 % similarity) and likely performs similar functions (Wen et al. 2015). Upregulation of *ucp2* in response to SSRI exposure, together with the enrichment in mitochondrial and neural cell components, as well as mitochondrial, respiratory, and neurodevelopmental biological processes is in accordance with the known? effect of SSRIs on neurogenesis and mitochondrial respiration. These results might provide mechanistic insight into the behavioral effects of SSRI exposure on fish observed in previous studies. While our results agree with previous research on the impact of SSRI on *fkbp5* and *ucp2*, and on their role in mitochondrial respiration and neurodevelopment, experimental manipulations are needed to functionally confirm the role of these genes as well as the overall mechanism of action.

*4.3 Technical considerations*

Previous studies have tested the effect of SSRIs on zebrafish. A brain tissue-specific RNA-sequencing study in adult zebrafish found that fluoxetine predominantly affected metabolic processes (lipid metabolism in particular) in their functional enrichment analysis (Wong, Oxendine, and Godwin 2013). Differences in pathways identified between our studies may be attributed to differences in the life-stages studied. Early development is a dynamic period of cellular activity and growth, and even subtle changes to key processes like neurodevelopment or neural plasticity, which very well may be the source of SSRIs' therapeutic effects in affected adults, will be more easily observed in a developing embryo (Glover et al. 2015). In whole larval zebrafish, fluoxetine was found to impact mostly molecular regulation of transcription and translation, and only a few general developmental processes were impacted (Park et al. 2012). But, recent research that has shown that sequencing composite structures (e.g. gross anatomical structures like the head or abdomen that contain a variety of tissue types) can result in high noise to signal ratios as they are accumulating gene expression signals over many tissues types that could have a variety of expression patterns (Johnson, Atallah, and Plachetzki 2013), which led us to study brain tissue only. However, while we reduced background noise and identified pathways

involved in neurodevelopment, the brain is highly heterogenous in expression patterns and different brain regions may exhibit divergent patterns of expression. Therefore, our use of whole brain tissue may have masked more nuanced expression patterns in more specific brain regions (Wang and Wang 2019). Focusing on specific brain regions or performing single cell analysis would likely yield different results and should be pursued in future studies.

In order to further enhance the resolution of this study, we capitalized on current low-input sequencing methods to sequence individual larval brains instead of pooling biological replicates. Pooling across biological replicates is a common strategy for comparative transcriptomics studies when RNA quantities are limited and has been used previously for testing the effect of SSRIs on zebrafish (Wong, Oxendine, and Godwin 2013; Huang et al. 2017; Wu et al. 2017). However, pooling does not encompass potential population variability, and by averaging the signal, pooling can lead to lists of differentially expressed genes that have low positive predictive value (Rajkumar et al. 2015; Mary-Huard et al. 2007; Shih et al. 2004). While zebrafish are common model species, there is still considerable genetic diversity within laboratory strains that encouraged us to attempt to fully capture the extent of individual variation (Guryev et al. 2006). As seen in Figure 1, controls and exposed individuals mostly clustered separately, which allowed us to identify genes differentially impacted by SSRI treatments. However, some controls appeared more similar to SSRI-exposed individuals, and two SSRI-exposed individuals clustered more closely to controls, suggesting that inter-individual variations could indeed alter the effect of SSRIs. That being said, the observed variations could alternatively result from technical variability. Indeed, biological and technical variability are confounded in our study design. Technical variation from low-input sequencing methods like SMART-seq can be quite high – particularly for amplification based sequencing methods (Marinov et al. 2014; McIntyre et al. 2011; Tung et al. 2017). Even though brain dissections were conducted under the same laboratory conditions, by the same individuals, the precision required for the dissection increases the chance of technical variation. While it

would be impossible to remove all potential sources of technical variations, the impact of SSRIs on the expression of key target genes should be further tested in a greater number of individuals in order to even better account for potential inter-individual variation in responses.

Finally it is important to note, we used 100 µg/L of SSRIs with daily renewal, a dosage that causes robust and consistent behavioral effects (Huang, Sirotkin, and McElroy 2019), but is rarely seen in surface water measurements and thus is not representative of average environmental exposures (typically, in pg-ng/L)(Fick et al. 2009). The high dose was chosen for this study to facilitate signal detection and pathway identification, but future work should now focus on key target genes to test fish sensitivity and dose responsiveness in a more environmentally realistic context.

## 5. Conclusions

Herein, we have leveraged modern molecular and data analysis techniques to provide the first tissue-specific transcriptomic study of the effects of SSRIs in developing zebrafish. By focusing on tissues known to be transcriptionally active during early development and maintaining the independence of biological replicates, we were able to conduct high resolution toxicogenomic evaluation of a popular class of drug, SSRI antidepressants, whose mechanism of action still evades us. Our study identified 1550 genes differentially expressed with SSRI treatment in larval zebrafish brain tissue and revealed the possible role of mitochondrial dysfunction and neurodevelopment in SSRI mechanism of action, which was confirmed by the use of network-based approaches. Now, complementary studies can be designed to test further the role of identified genes and pathways, to compare inter-individual responses and assess dose responsiveness.

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