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Gene-class analysis of expression patterns induced by psychoactive pharmaceutical exposure in fathead minnow (*Pimephales promelas*) indicates induction of neuronal systems[☆]

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Abstract

Psychoactive pharmaceuticals are among the most frequently prescribed drugs, contributing to persistent measurable concentrations in aquatic systems. Typically, it is assumed that such contaminants have no human health implications because they exist in extremely low concentrations. We exposed juvenile fathead minnows (*Pimephales promelas*) to three pharmaceuticals, fluoxetine, venlafaxine and carbamazepine, individually and in a mixture, and measured their effect on the induction of gene expression in fish brains using microarray analysis. Gene expression changes were accompanied by behavioral changes and validated by qPCR analysis. Gene Set Enrichment Analysis was used to perform gene-class analysis of gene expression, testing for enrichment of gene sets known to be involved in human neuronal development, regulation and growth. We found significant enrichment of gene sets for each of the treatments, with the largest induction of expression by the mixture treatment. These results suggest that the psychoactive pharmaceuticals are able to alter expression of fish genes associated with development, regulation and differentiation of synapses, neurons and neurotransmitters. The results provide a new perspective for the consideration of potential consequence for human health due to environmental exposure to unmetabolized psychoactive pharmaceuticals.

Keywords

GSEA; Fluoxetine; Venlafaxine; Carbamazepine; Neurological development; Autism

1. Introduction

Metabolically active pharmaceuticals have been detected in waste-water, streams and drinking water (Halling-Sorensen et al., 1998; Kolpin et al., 2002; Bruce et al., 2010). These pharmaceuticals primarily enter the environment through the excretion in unmetabolized form by patients taking clinical doses (Kummerer et al., 2000; Besse and Garric, 2008). In the environment, unmetabolized pharmaceuticals are detected in a wide range of

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concentrations, due in part to stable half-lives (Ternes, 1998; Jjemba, 2006; Kwon and Armbrust, 2006), and consistent rate of input (Wong et al., 1995; Corcoran et al., 2010; Fernandez et al., 2010). Psychoactive pharmaceuticals, including fluoxetine (FLX, a selective serotonin reuptake inhibitor, SSRI), venlafaxine (VNX, a serotonin norepinephrine reuptake inhibitor, SNRI) and carbamazepine (CBZ, an anticonvulsant used in the treatment of epilepsy and certain neurological disorders) have half-lives ranging from hours to days and have been detected in wastewater, wastewater treatment plant effluent, rivers and drinking water (Table 1). Psychoactive pharmaceuticals in the environment are usually detected in mixtures composed of constituents with varying metabolically active formulations (Metcalf et al., 2003; la Farre et al., 2008; Celiz et al., 2009). However, it is generally assumed that these pharmaceuticals are in concentrations too low to be of concern for human health (Schwab et al., 2005; Cunningham et al., 2009).

Exposure to psychoactive pharmaceuticals has been shown to alter production and regulation of the neurotransmitters serotonin, dopamine and norepinephrine (Duman et al., 1997; van der Ven et al., 2006; Miller et al., 2008), and result in altered behavior (Airhart et al., 2007; Martinovic et al., 2007; Gaworecki and Klaine, 2008; Painter et al., 2009). Studies of infants exposed to therapeutic levels of SSRIs during pregnancy reported lower APGAR scores (Appearance, Pulse, Grimace, Respiration) and psychomotor and behavioral tests, with the third trimester of pregnancy the most detrimental period for SSRI exposure (Casper et al., 2003). For human development, early exposure to psychoactive pharmaceuticals causes a potentially permanent problem in motor circuitry and psychomotor movement and controls, relative to later exposure (Chambers et al., 1996; Kallen, 2004a,b; Louik et al., 2007).

Perturbation of neuronal systems can be assessed using cDNA microarray-based expression profiling using an aquatic organism as a model (Wong et al., 1996; Gibson, 2002; Snell et al., 2003), which is a useful approach for assessing the potential human health consequences of psychoactive pharmaceuticals in aquatic systems due to the conserved nature of gene interactions (McGary et al., 2010). In particular, there is a growing appreciation of the use of animal models for studies of neurological disorders involving prenatal environmental exposure to psychoactive pharmaceuticals (Dufour-Rainfray et al., 2011), such as the role of valproate in autism (Dufour-Rainfray et al., 2010). Psychoactive pharmaceuticals are therefore especially of interest due to their potential to mimic, aggravate or even induce neurological disorders.

Here, we treated fathead minnows (*Pimephales promelas* Rafinesque) with psychoactive pharmaceuticals (FLX, VNX and CBZ) to determine how gene expression was perturbed in the brains of a developing organism, specifically testing for enrichment of sets of genes known to be associated with human neuronal development and regulation. Identification of such sets can then be used for future comparisons to known gene expression patterns associated with specific human neurological disorders known to be triggered by environmental contaminants, e.g., autism triggered by first trimester fetal exposure to certain teratogens (Dufour-Rainfray et al., 2011).

We tested FLX, VNX and CBZ because they are among the most widely used pharmaceuticals and most abundantly detected in the environment. Our experimental dosages were 6–10× the highest observed environmental concentrations for the three pharmaceuticals (Table 1), which was intended to account for conservative concentration estimates and the presence of related formulations and active metabolites of the target pharmaceuticals (Metcalf et al., 2003; Olver et al., 2004; Celiz et al., 2009; Santos et al., 2010). The three pharmaceuticals have many related formulations (e.g., there are 9 other SSRIs) and numerous metabolites that exert similar effects and are present in the

environment as a mixture (Celiz et al., 2009). Studies of cumulative concentrations reveal that the loads of pharmaceuticals that pass through a single point at a site per day are substantial, e.g., 213 g d⁻¹ for CBZ (Moldovan et al., 2009).

We used a microarray platform for fathead minnow (Klaper et al., 2010) that was enhanced by the annotation of human gene homologs for array elements (Thomas et al., 2011), allowing gene-class analysis, which tests for enrichment of specific human neuronal systems and processes (Subramanian et al., 2005; Metzger et al., 2011). We hypothesized that FLX, VNX and CBZ would induce altered gene expression enrichment and that a mixture of these three compounds (MIX) would cause a different gene expression profile than the effects of the pharmaceuticals when tested individually. We focused the analysis on gene sets specific to biological processes involved with development, regulation and growth of the human central nervous system. By this approach, we hoped to relate the results from the animal model to potential human health consequences known to be associated with these biological processes.

In order to confirm that the observed gene expression patterns are associated with a behavioral phenotype, we performed predator escape tests based on similar experiments conducted elsewhere (Painter et al., 2009; Maximino et al., 2010). Tests involving predator escape by fish are used to assess *in vivo* effects of environmental toxins like psychoactive pharmaceuticals (Weber, 2006; Airhart et al., 2007) because they offer a quantifiable behavior with a clear biological significance linked to fitness (Gerlai et al., 2000; Gerlai, 2010) and to human health (Painter et al., 2009). The fast-start is an innate reflex predator avoidance response that is conserved across the teleost lineage (Eaton et al., 2001). One of the most studied fast-starts is the C-start, a prominent bending of the body into a “C” shape. This reflex behavior starts with a very short latency phase during which the predator is perceived by the fish, followed by a “C” turn and ending with an explosive burst of high-velocity swimming away from the predator (Domenici and Batty, 1997). C-start behaviors are regulated by an integrated sensory-motor axis activated via Mauthner cells, neurons that originate in the hindbrain and excite motor neurons and interneurons (Liu and Fetcho, 1999), stimulating the lateral muscles fibers (Eaton et al., 2001).

We tested whether treated and control fish differed in behavior lateralization, the favored direction of the first turn taken after being startled, would differ between treated and control fish. Behavior lateralization has been observed in vertebrates (Walker and Davis, 1997; MacNeilage et al., 2009) and fish (Vallortigara, 2000). Next, we determined if treated and control fish swam different distances, due to a perturbation of C-start behavior. Last, we determined if treated and control fish differed in number of direction changes after being startled, since frequent coordinated turns are a predator avoidance characteristic of schooling fish like fathead minnows (De Santi et al., 2002).

2. Material and methods

Experimental design—The experiment was conducted in 2-gallon tanks filled with 6 L of filtered, dechlorinated water. Three tanks were used for each pharmaceutical, along with three tanks for a mixture treatment (containing all three pharmaceuticals in the concentrations listed below) and three tanks for control (CTL, containing no pharmaceuticals). Each tank housed five juvenile fathead minnows 75 days old with indeterminate sex at the beginning of the dosing period (mean length 3.6 cm, mean mass 0.62 g), purchased from Aquatic BioSystems Inc. (Fort Collins, Colo.). Fish were fed Tetramin Tropical Flakes twice daily and water was changed every two days (100%). An air stone was placed in each tank for oxygenation and a full-spectrum bulb provided a 16:8 hour light:dark cycle. All fish handling and treatments were performed by PJ at the Great Lakes

WATER Institute (School of Freshwater Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin) using appropriate IACUC approved protocols.

Treatment—Pharmaceuticals were purchased for Sigma Aldrich (FLX: CAS 56296-78-7, VNX: 99300-78-4 and CBZ: 298-46-4) and administered at $10 \mu\text{g L}^{-1}$, $50 \mu\text{g L}^{-1}$, and $100 \mu\text{g L}^{-1}$, for FLX, VNX and CBZ, respectively. CBZ, due to its non-polar nature, was dissolved in $5 \mu\text{L L}^{-1}$ ethanol, resulting in tank concentrations lower than observed no-effect concentrations (Yokota et al., 2001); alcohol controls were maintained but not included in the analysis presented below (no differences were detected). Dosages of pharmaceuticals were re-administered with each change of the tank water. Concentration of pharmaceuticals in the tanks was not measured over the course of the experiment (to determine if concentrations reflected intended dosages or degraded between water changes); other work has shown this to be of little concern for similar experimental designs involving daily changes of 50% of water in the experimental vessel (Painter et al., 2009). Fish were exposed to treatments for eighteen days.

Sample handling—Fish were stunned with a blow to the cranium and blood was extracted and put into vials containing anticoagulant. The fish were then placed sideways and decapitated. The cranium was cut open, the brain was extracted and immediately put into an RNase-free vial, flash frozen in liquid Nitrogen and stored at -80°C .

Nucleic acid extraction—Trizol RNA extraction were performed by PJ at the Great Lakes Water Institute in Milwaukee, Wisconsin, using TRI Reagent LS (Sigma) with chloroform, precipitated with isopropanol according to the manufacturer's protocol. Purity (A260/A280) and yield were quantified using a NanoDrop spectrophotometer using manufacturer protocol. No additional assessment of contamination, RNA integrity or inhibition testing was performed at that time.

2.1. Gene expression system

Array design—The microarray experiment was designed to include 3 replicates for each treatment (representing the three treatment tanks); each replicate consisted of a pooled sample of RNA from three fish per tank, for a total of 15 arrayed samples (4 treatments+1 control, with 3 replicates each). Therefore, RNA of three fish from each tank was combined to make up a concentration of $\sim 300 \text{ ng } \mu\text{L}^{-1}$; each pooled sample contained equal amounts of RNA from the three fish. Pooling was conducted in order to maximize power, efficiency and cost effectiveness in the study (Peng et al., 2003). Others have expressed concerns about loss of information regarding variation among true biological replicates (Shih et al., 2004). Our qPCR validation was performed on every biological replicate (not pooled samples; see below), which allowed us the efficiency of pooling for the microarray experiment with the precision of the qPCR validation.

Sample processing—Pooled samples of DNase treated RNA were sent to EcoArray (Gainesville, FL) for microarray analysis. The 260/280 ratios for the individual samples were between 1.81 and 1.96, and total RNA quality was assessed by running a $1.0 \mu\text{L}$ aliquot (concentration $< 500 \text{ ng } \mu\text{L}^{-1}$) on a 2100 Agilent Bioanalyzer. The RNA Integrity Numbers for all samples were similar to each other and indicated a high background (RIN < 5.0) but the samples were labeled successfully, demonstrated by the specific activity for each sample.

One aliquot of 900 ng L^{-1} of total RNA was removed from each experimental sample to be labeled with Cy5 for the microarrays. The RNA samples were labeled using Agilent's Quick-AMP 2-Color Labeling kit and the Two-Color RNA Spike-In Kit. One-half of the

resulting cRNA (10 μ L) was denatured and immediately used for one round of amplification by T7 in an *in vitro* transcription reaction. The amplified and labeled cRNA probes were purified with RNeasy purification columns (Qiagen, Valencia, CA). Amplification yields and dye incorporation efficiency of the cRNA probes were verified using a NanoDrop ND-1000 spectrophotometer (NanoDrop). Samples that contained >8 pmol of incorporated dye per μ g of cRNA, and that had a concentration >40 ng μ L⁻¹ were determined to be acceptable for use on the arrays.

Each experimental array sample was prepared by combining 300 ng of Cy5 labeled sample with 300 ng of the Cy3 labeled reference sample with blocking agent. The reference sample for these arrays, provided by EcoArray, was a combination of DNase-treated total RNA from liver, brain and gonad of control (untreated) male and female fathead minnows used in another experiment. An equal volume of 2 $\times$ GEx Hybridization Buffer Hi-RPM was added (Agilent Two-Color Microarray-Based Gene Expression Analysis) for a final volume of 50 μ L. The microarrays were hybridized in Agilent hybridization chambers and incubated overnight in a rotisserie oven (Model G2545A, Agilent) at 65 °C. The following day, the slides were washed and dried. Slides were scanned with an Agilent DNA microarray scanner, and raw images were processed using Agilent's Feature Extraction Software, Version 10.1.1.1.

Data analysis—The data were analyzed using Gene Spring (version GX10). First, a simple thresholding substitution converted all expression values below 1.0 to 1.0 to remove very small or negative expression values before log transformation to avoid large negative or missing values in the transformed data. We did not perform a baseline transformation. Second, data were normalized using LOWESS (Locally Weighted Least Squares Regression), fitting a smoothing curve to a dataset. This technique takes into account the possibility of intensity-dependent variation in dye bias introducing spurious variations in the collected data. Data were deposited in the GEO database (GSE22261).

Array annotation—The Fathead 15 k microarrays (GEO: GPL7351) used in this experiment were developed by EcoArray and manufactured by Agilent Technologies, Inc (Klaper et al., 2010). The annotation of about 67% of the array genes (10,069 of 15,000) was enhanced by the addition of HUGO gene symbols (Thomas et al., 2011), allowing GSEA-based gene class analyses. The enhanced annotation involved searching translated full-length EST sequences against NCBI RefSeq protein database (using BLAST and HMM approaches) and validating result with searches against the zebrafish genome; see the re-annotation project report (Thomas et al., 2011) for details. The annotated genes represent a wide variety of biological systems, pathways and processes.

2.2. Validation of gene expression

Microarray results were validated using qPCR analysis of nine genes with high rank correlation in the MIX vs. CTL comparison (Table 2). The genes were not selected based on inclusion in any given gene set in the present study.

Experimental design—All RNA samples were shipped to Idaho State University (Pocatello, Idaho), where they were stored at -80 °C. The experimental group was composed of 15 fish (5 from each of 3 tanks) from the MIX treatment; the control group was composed of 15 fish (5 each from 3 tanks) from the CTL treatment.

Reverse transcription—DNase treatment (Promega PR M6101) and reverse transcription cDNA synthesis (Promega PR A3800) was performed by MT at Idaho State

University following manufacturer protocols with 500 ng of mRNA in a total volume of 7 μ L water.

qPCR target information—Target gene names and access IDs are listed in Table 2, along with primer sequences, amplicon lengths and locations within the genes. Primers were designed by MT using NCBI Primer-BLAST, ZFIN-BLAST and the full-length sequence of the fathead minnow EST for each given gene. Briefly, the sequence of a potential target fathead gene was searched against the ZFIN database to identify the zebrafish homolog. The corresponding region of the zebrafish homolog was used by Primer-BLAST to identify primer pairs within acceptable parameters for qPCR (including the requirement that at least one of the primers spanned an exon–intron boundary). The primers were back-checked to ensure the sequences were conserved in the fathead EST. Primers were produced by IDT (Coralville, Iowa).

qPCR protocol—The qPCR assays were performed by MT in his research lab and the Molecular Research Core Facility (MRCF) at Idaho State University, using an MJ Chromo4 running Opticon Monitor v. 3.1.32. Each 20 μ L reaction contained 100 ng cDNA, 500 nM primers, 0.4 mM dNTPs, 4.0 mM Mg⁺⁺ and 0.45 U HotMaster Taq DNA polymerase. We used the 5-Prime RealMasterMix SYBR ROX kit (catalog #2200800) SYBR Green I fluorescent assay. Reaction conditions used the 3-step approach (1 $\times$: 94 C for 1 min; 45 $\times$: 95 C 10s, 58 C 10s, 68 C 10s). cDNA from each of the 15 control and 15 experimental fish were run in triplicate (15 biological replicates $\times$ 3 PCR replicates). Melting curves (52 $^{\circ}$ C to 99 $^{\circ}$ C, read every 1 $^{\circ}$ C) were performed to ensure only the desired product was amplified. The PCR efficiency values are listed in Table 2.

Data analysis—The qPCR Miner program (6) was used to determine Cq values and verify reaction efficiencies. Outliers were identified by examining Cq values for PCR replicates and melting curves for each biological and PCR replicate; outliers were removed from the analysis. NTCs generally had no reaction; occasionally, a NTC PCR reaction produced a product with an unexpected melting temperature and Cq value. In these cases, the NTC product could be used to further identify outliers in the treatment and control reactions. Several reference genes were considered, but only TUBB2c and RPLP1 were invariant in both microarray and qPCR assays. We used an efficiency-normalized comparative quantification algorithm to calculate fold change that used RPLP1 as a reference gene. Repeatability (intra-assay variation determined by PCR and biological replicates) was compared using Cq values; generally, signal-to-noise ratio was high, but the biological replication allowed meaningful comparisons to be made. A *t*-test was used to determine if there was a significant difference between treatment and control.

2.3. Gene set enrichment analysis of gene expression

GSEA settings—Analyses used GSEA release 2.07, with gene sets from MSigDB release 3.0 (Subramanian et al., 2005) and Gene Ontology (Berardini et al., 2010). For each of the four treatment vs. control comparisons, genes on the array were ranked by signal-to-noise ratio (those genes with the strongest up-regulation in treatment relative to control were ranked highest; those with strongest down-regulation were ranked lowest). The maximum gene set size was set to 500 genes; the minimum gene set size was set to 10 genes; the number of permutations was set to 1000. Permutation in GSEA is conducted to assess statistical significance of the Normalized Enrichment Scores (NES). Permutation involves swapping phenotype labels among all 15 samples (three pooled replicates in each treatment and the control) in a way that preserves gene–gene correlations. We also conducted gene set permutation, which involved creation of random gene sets, size matched to the gene sets of

interest. Both permutation approaches identified the same statistically significant sets; only phenotype permutation results are reported.

GSEA procedures—First, a GSEA analysis using all gene sets from the MSigDB ‘C2’ collection was performed for MIX vs. CTL. The C2 collection contains 3272 curated gene sets that represent genes known to be involved in specific pathways, systems and processes (given the set size limitations imposed, only 2092 C2 sets were tested). The goal of this test was to determine whether there was a directional bias in our expression data (e.g., more up-regulated than down) and to provide a richer context for interpreting the magnitude of the significant NES obtained in the analysis of our gene sets of interest. We also used significant C2 sets as additional information regarding the significance of the experiment.

Next, Gene Ontology was searched for sets of genes associated with the theme of three separate collections of gene sets: Development, Regulation and Growth of neuronal systems. The Development collection contained 20 gene sets containing genes associated with human neuronal development (Table 3a). The Regulation collection contained 22 gene sets containing genes associated with the regulation of human brain function (Table 3b). The Growth collection contained 16 gene sets containing genes associated with differentiation and maturation of human neurons and axons (Table 3c).

Gene sets were processed to ensure they contained only GSEA-recognized primary HUGO symbols, rather than aliases or unapproved gene symbols. This was accomplished through a custom script that compared each gene symbol to the GENE_SYMBOLS.chip file (from GSEA) containing a list of approved HUGO symbols with accepted aliases. Gene set components identified as aliases based on this file were replaced with the appropriate HUGO symbol.

Data analysis—Separate analyses were conducted using each of the three collections of gene sets in order to correct for overlap (genes that might appear in more than one set within a given collection) using False Discovery Rate (FDR). FDR is a conservative test (Kang and Chun, 2011) but useful in this application since many genes have multiple neuronal functions. Enrichment of gene sets from the three collections (Development, Regulation and Growth) was tested using the four treatments (FLX, VNX, CBZ and MIX; each examined relative to control), for a total of 12 GSEA analyses. Gene sets with an NES associated with both $p < 0.05$ and $FDR\ q < 0.25$ were considered to be significantly enriched. For each gene set, the NES, p-value and q-value were recorded for the sets identified as enriched by the analysis (Table 3).

Raw data availability—The phenotypes file (in .CLS format) and the three gene sets (in .GMX format) are available as supplemental data. The GSEA-annotated array file (.CHIP) is available from (Thomas et al., 2011). The microarray data is available from the GEO database (GSE22261).

2.4. Behavior phenotype associated with treatments

Behavioral test—Using the same fish from the gene expression work, a behavioral test was performed following 14 days of pharmaceutical exposure. In previous studies involving FLX exposure (Klaper et al., submitted for publication, Aquatic Toxicology, in review) concentrations similar to the present study indicated that a behavior response was induced at these concentrations and after this period. The test, modeled on similar analyses (Painter et al., 2009; Maximino et al., 2010), involved a rectangular tank with long sides covered by black paper and a video camera (Panasonic SDR-H200 digital video recorder) mounted below for recording fish behavior. A single fish was placed in the tank and allowed to

acclimate for 15 min. Following acclimation, when the fish approached either short side of the tank it was startled with a black circular spot, 8 cm in diameter and printed on a piece of white cardboard. The spot was swung towards the fish without colliding with the tank (to avoid any vibrations). The fish was started by the spot and swam in the opposite direction.

Three measures were recorded by watching the recorded video in slow motion: the proportion of right turns during the startle period was recorded for each fish (to calculate the behavior lateralization), the total number of turns a fish took while swimming was recorded until it stabilized (when the fish did not swim with the initial burst it started off with), and the distance a fish swam due to the startle response measured from the start position (side of the tank) until it stabilized, modeled after other work (Liu and Fetcho, 1999; Eaton et al., 2001). Each of these tests was repeated five times for each fish.

Data analysis—To test for a treatment effect, we used Restricted Maximum Likelihood (ReML) with a Mixed Model Analysis (MMA) and 2-tailed p-values for testing the null hypothesis (that there were no differences among the treatments) using SAS/STAT® software. Since treatments were applied at the level of the tank, the subject level effect was the tank within each treatment. The ReML method was appropriate for this experiment since 6 out of 75 fish were excluded (1 of them was hyperactive, 4 did not move and 1 expired), and missing data were accounted for by this test. Post hoc comparisons were calculated with a Bonferroni adjustment correcting for four comparisons, with each treatment mean compared to the control mean by a *t*-test.

3. Results

3.1. Patterns of pharmaceutical-induced gene expression

In the comparison analysis of the MSigDB ‘C2’ collection of 2092 curated sets for the MIX vs. CTL comparison, the highest observed NES was 1.84 (in our study of psychoactive pharmaceuticals, the highest observed NES value was 1.82). Only 1 of 2092 gene sets in the C2 analysis (<0.05%) was enriched ($p < 0.05$ and FDR $q < 0.25$), “SEMA4D-induced cell migration and growth-cone collapse,” an up-regulated set derived from the Reactome database (Croft et al., 2011), which included 24 genes involved in guiding the growth of axons in the central nervous system. This pathway is especially important in neural system development (Vodrazka et al., 2009). The gene SEMA4D, a member of that set, has obvious implications for our study, given the focus on sets of genes with neurological significance, and was included in eight of the gene sets we examined (4 development and 4 growth sets). SEMA4D is placed by Gene Ontology (Berardini et al., 2010) into biological processes associated with axon guidance, nervous system development and regulation of axonogenesis; it is also recognized to have an immune system function (Nkyimbeng-Takwi and Chapoval, 2011).

Of the 2092 C2 sets examined, 1298 (62%) were up-regulated, potentially indicating a bias towards up-regulation in our expression data set. In our study, nearly 80% of the enriched sets (11 of 14) were up-regulated; given the number of sets involved, it is difficult to conclude that there is a general induction of expression of genes by the psychoactive pharmaceuticals examined.

For the Development collection, we observed 2 up-regulated sets in each of the VNX and MIX treatments (Table 3a). Several other sets have significant NES values but failed the False Discovery Rate (FDR) test (i.e., $q > 0.25$). Key enriched sets (discussed below) are associated with development of neural ensheathment and the neural tube (in FLX) and synapse organization and constituent parts (in MIX). These enriched sets of genes involve

the development of neuron cellular components that are essential for the proper construction of neural networks (see Discussion).

For the Regulation collection, we observed 1 down-regulated set in the FLX treatment and up-regulation of 3, 1 and 4 sets in the VNX, CBZ and MIX treatments, respectively (Table 3b). Several other sets have significant NES values but failed the FDR test (i.e., $q > 0.25$). Key enriched sets (discussed below) are associated with regulation of neurotransmitter binding (in FLX), neurotransmitter secretion and synapse function (in MIX), action potential (in VNX), and SNARE binding associated with synapse vesicle formation (in VNX, CBZ and MIX). These enriched sets of genes involve the propagation of action potentials and the release and binding of neurotransmitters required for nerve impulse transmission (see Discussion).

For the Growth collection, we observed 2 down-regulated sets in the CBZ treatment and 2 up-regulated sets in the MIX treatment (Table 3c). Several other sets has significant NES values but failed the FDR test (i.e., $q > 0.25$). Key enriched sets (discussed below) are associated with growth of axons and other neural projections (in MIX). These enriched sets of genes involve the growth and death of neurons involving in the remodeling of neural networks (see Discussion).

3.2. Validation of gene expression data

The qPCR analysis was consistent with the microarray experiment finding of induction of expression by the MIX treatment, of nine genes selected for validation (Table 2). While qPCR efficiency was lower than optimal, leading to an underestimate of qPCR fold change, the MIX v. CTL difference was statistically significant for 7 of 9 genes. Note that the qPCR validation examined all 15 individuals for each group (MIX and CTL), rather than pooled samples (as in the microarray).

3.3. Behavior phenotype associated with treatments

The mixed model indicated that there was a significant treatment effects for number of turns ($F_{4,10} = 10.47$, $p = 0.0013$), distance traveled ($F_{4,10} = 8.36$, $p = 0.0031$) and lateralization, the direction fish turned after being startled ($F_{4,10} = 4.12$, $p = 0.0317$). These significant treatment effects justified post-hoc pairwise comparisons between each of the four treatments and the control.

Number of turns—All four treatments led to significant increase in the number of turns before fish came to a halt (3.8, 4.0, 4.0 and 4.2 turns for MIX, FLX, CBZ & VNX, respectively), relative to control fish (2.8 turns). Pairwise t-tests of treatment vs. control comparisons were all significant (Bonferroni-adjusted $p < 0.01$).

Distance traveled—Three of the four treatments caused fish to travel significantly longer distances after being startled (22.7, 23.3 and 26.6 cm for VNX, MIX and CBZ, respectively, with 19.4 cm for FLX), relative to the control fish (15.8 cm). Pairwise t-tests of treatment vs. control comparisons involving CBZ, MIX and VNX treatments were significant (Bonferroni-adjusted $p < 0.01$ for CBZ, MIX; $p < 0.05$ for VNX); the comparison involving the FLX treatment was not significant (Bonferroni-adjusted $p > 0.05$).

Lateralization—Two of the four treatments caused significant changes in lateralization, the direction fish turned after being startled (61.4 and 62.9% right turns for MIX and VNX, respectively, with 43.8 and 60.0% right turns for CBZ and FLX, respectively), relative to the control fish (35.7% right turns). The control and CBZ-treated fish showed a left lateralized bias, while the other three treatments (each involving an SSRI, SNRI or both) showed right

lateralization biases. Pairwise t-tests of treatment vs. control comparisons involving VNX and MIX treatments were significant (Bonferroni-adjusted $p < 0.05$); the comparisons involving the FLX and CBZ treatments were not significant (Bonferroni-adjusted $p > 0.05$). It is noteworthy that the FLX treatment had a proportion of right turns very similar to VNX and MIX treatments, with a non-adjusted $p = 0.017$ (Bonferroni adjusted $p = 0.068$).

4. Discussion

4.1. Enrichment of gene expression by psychoactive pharmaceuticals

We found that treatments involving psychoactive pharmaceuticals altered patterns of gene expression of sets of genes representing human biological processes involving neuronal development, growth and regulation.

FLX treatment—Only a single gene set was enriched by FLX treatment. The down-regulation of the gene set NEUROTRANSMITTER BINDING by FLX treatment could be due to a decrease in brain concentration of serotonin receptor demonstrated in previous studies (Lopez-Figueroa et al., 2004; Airhart et al., 2007). FLX is thought to exert its therapeutic effect by inhibiting monoamine transporters, thus reducing the reuptake of the serotonin and activity of serotonin transporter protein (SERT) at presynaptic neuronal membranes (Hiemke and Hartter, 2000). SERT transports serotonin from the synaptic cleft into the nerve terminal; the primary effect of SSRIs is to increase synaptic serotonin levels by selectively inhibiting SERT, elevating concentration of serotonin in the synaptic gap.

VNX treatment—Five sets were enriched by VNX treatment. We observed up-regulation of the gene sets NEURAL TUBE DEVELOPMENT, ENSHEATHMENT OF NEURONS and REGULATION OF ACTIONPOTENTIAL IN NEURON by VNX treatment. Aberrant ensheathment might influence the rate of nerve impulse transmission leading to axonal degeneration (Sherman and Brophy, 2005). The neural tube becomes patterned along the dorsal–ventral axis to establish defined compartments of neural progenitor cells that give rise to distinct classes of neurons (Jessell, 2000). Altered expression of individual homeodomain genes NKX6.1, NKX2.2, IRX3 and NCAM (found in gene sets AXONOGENESIS, NERVOUS SYSTEM DEVELOPMENT and SYNAPSE PART) in the chick neural tube changes the fate and position at which individual classes of neurons are generated by the normal profile of homeodomain protein expression (Briscoe et al., 2000; Jessell, 2000). Neural tube defects, a group of disorders that arise early in fetal development and cause severe congenital malformations, involve multiple genes interacting with environmental factors (Greene et al., 2009). Experiments with rats treated with VNX reported a modulation in action potential (Szabo et al., 1999) and an increase of neuronal firing was observed in aged monkeys (Chang et al., 2005).

CBZ treatment—Three sets were enriched by CBZ treatment. We observed down-regulation of gene sets APPEL IMATINIB RESPONSE and REGULATION OF NEURON APOPTOSIS associated with neural growth. The first set (APPEL IMATINIB RESPONSE) is composed of genes associated with the effect of imatinib on differentiation of dendritic cells (Appel et al., 2005). One of the key genes in this set, cathepsin (CTSD), is associated with Alzheimer's disease, and studies reveal that polymorphism in this gene could cause sporadic Alzheimer's disease (Capurso et al., 2005; Davidson et al., 2006; Mariani et al., 2006). The second set (REGULATION OF NEURON APOPTOSIS) includes the gene Neurofibromin I (NF-1), a negative regulator of Ras kinase (Feldkamp et al., 1999) that is associated with cognitive and learning disability (Hyman et al., 2005; Vedrine et al., 2011) including potentially autism (Mbarek et al., 1999); this gene is strongly down-regulated in our array.

MIX treatment—Eight sets were enriched by MIX treatment. We observed up-regulation of regulatory gene sets NEUROTRANSMITTER SECRETION and NEUROTRANSMITTER TRANSPORT (although the latter set failed the FDR test) in response to the MIX treatment, suggesting that psychoactive pharmaceuticals potentially perturb activity-dependent neurotransmitter deployment. Depending on the severity and pattern of disruption of neurotransmitters and their release into the synaptic cleft, such perturbations could be associated with a spectrum of neurological conditions (Swoboda and Hyland, 2002), since insufficient or excess release of neurotransmitter might alter neurochemical function and neurotransmission (Lohoff, 2010).

We also observed up-regulation of the SNARE BINDING gene set observed in treatments with VNX, CBZ and MIX. The SNARE (Soluble *N*-ethylmaleimide-sensitive-factor Attachment protein REceptor) gene superfamily is one of the more intensively studied elements of the protein machinery involved in cell signaling (Sollner et al., 1993). SNARE proteins mediate vesicle fusion, and are best known for their role in mediating docking of synaptic vesicles in the presynaptic membrane. This gene set contains a large number syntaxin genes, including STX1A, which plays a role in neurodevelopmental disorders (Nkyimbeng-Takwi and Chapoval, 2011). The formation of various SNARE motifs between neurons facilitates synaptic transmission that occurs when synaptic vesicles containing neurotransmitters fuse with the plasma membrane of a neuron, causing the neurotransmitters to be released onto the downstream neuron and other target cells (Schweizer and Augustine, 1998; Sakisaka et al., 2008). The surface of the SNARE complex contains many distinctive sub-surfaces (e.g., grooves, charged patches) that are putative binding sites for other proteins (Sutton et al., 1998; Antonin et al., 2002). These SNARE binding proteins regulate membrane fusion (Sollner et al., 1993; Kweon et al., 2003), and up-regulation in the expression of these genes would alter neurotransmitter-releasing vesicles at synapses. Perturbed regulation of this particular gene set is interesting because it has implications for the regulation of synapses, neural tubes and levels of neurotransmitters. In particular, others have been exploring the role of the SNARE gene STX1A in autism (Nakamura et al., 2011).

Also in response to MIX treatment, we observed enrichment of five gene sets (AXON, SYNAPSE, SYNAPSE ORGANISATION, SYNAPSE PART and NEURON PROJECTION) associated with the formation, growth and regulation of neural circuits. The wiring of neural circuitry requires vast numbers of synapses with high connection specificity; predictably, neuronal synapse organizational specificity is strictly regulated (Goda and Davis, 2003; Margeta and Shen, 2010). Furthermore, lineage, fate, and timing of development can also play critical roles in shaping synaptic organization, resulting in a culmination of many sequential developmental events (Benson et al., 2001). The stabilization, strengthening or elimination of synaptic contacts leads to the formation of mature neural circuits (Eaton and Davis, 2003; Margeta and Shen, 2010). Enrichment of genes sets associated with the formation (or reorganization) of synapses may indicate an altered and imprecise synaptic connections or a failure to form mature neural circuits, both of which may play important roles in the etiology of neurological disorders.

The MIX treatment, combining all three psychoactive pharmaceuticals, induced a different gene expression profile than pharmaceuticals administered individually (Table 3). Overall, the MIX treatment enriched expression of 8 gene sets ($p < 0.05$ and $q < 0.25$), compared to 9 sets for the other three treatments combined. Six of the sets enriched by the MIX treatment were not enriched by any other treatments, consistent with the possibility that the MIX treatment involved interactions among FLX, VNX and CBZ, which have different modes of activity. In cases where a given set was enriched by both MIX and another treatment, MIX always resulted in a higher NES. Several drug interactions are known for these three psychoactive pharmaceuticals that might have contributed to enrichment of gene sets due to

MIX treatment. First, serotonin syndrome may result from sufficient combined concentrations of FLX and VNX (Boyer and Shannon, 2005). Serotonin syndrome is considered by the FDA to be a drug interaction of major concern, and can result in serious side effects in humans, including anxiety, akathisia, hyperreflexia and death (Boyer and Shannon, 2005). Second, FLX can inhibit the hepatic metabolism of CBZ (Lee et al., 2006), which may allow CBZ to accumulate to the degree that it induces gene expression changes and behavioral changes (Borowicz et al., 2006). Third, VNX and CBZ are known to have additive and synergistic effects that can be observed in VEN-resistant patients (Ciusani et al., 2004) and can potentially be generalized.

The MIX treatment is of greatest interest, given observations of large numbers of pharmaceuticals that co-occur in aquatic systems (Camacho-Munoz et al., 2010; Santos et al., 2010). It is logical to assume that dosage effects (e.g., multiple SSRIs) and interactions (such as those described above) would lead to substantially greater enrichment of gene expression in developing fish exposed to unmetabolized pharmaceuticals in natural systems. And, given deeply conserved phenotypes and genes interaction networks (McGary et al., 2010), it is also logical to assume that developing human might have similar enrichment of expression, potentially accompanied by lasting health consequences.

4.2. Behavior phenotype induced by psychoactive pharmaceuticals

We observed that psychoactive pharmaceuticals altered the predator avoidance behavioral phenotype of juvenile fathead minnows. Other studies used the predator avoidance response in fathead minnow and zebrafish to study stress response, the ability to perceive an approaching predator and elicit an appropriate start response, following treatment by estrogen (Martinovic et al., 2007; McGee et al., 2009), mercury (Weber, 2006), alcohol (Gerlai et al., 2000, 2009) and antidepressants (Airhart et al., 2007; Painter et al., 2009). Predator escape behavior occurs when visual sensory information is converted to lateral muscle movement in a species-specific escape response. Alterations in brain function that impair predator avoidance behavior are caused by changes in levels of neurotransmitters, enzyme function, or electrophysiological properties of the brain (Scott and Sloman, 2004).

In the lateralization test, we observed that MIX-, FLX- and VNX-treated fish had a right-skewed directional turn while control and CBZ-treated fish turned more to the left. Other studies on psychoactive pharmaceuticals have shown an effect on cerebral hemisphere lateralization (Ivanov, 2003), in which specific pharmaceuticals activated either the left or the right hemisphere in patients. For example, patients have shown higher activity in the left hemisphere on administration of SSRIs (Bruder et al., 2001; Bochkarev et al., 2004). Lateralization bias, as in our untreated CTL fish, has been observed in several species of minnow (Stennett and Strauss, 2010). Given the strong lateralization bias in our untreated controls, the alteration caused by SSRI and SNRI treatment was especially notable.

We also observed that the dosed fish swam longer distances (in every treatment but FLX) and had a higher number of direction changes (in every treatment) than control fish. This may have been due to an anxiogenic effect that has also been observed with zebrafish and other vertebrates (Walker and Davis, 1997; Gerlai, 2010; Maximino et al., 2010). These movements are regulated by an integrated sensory-motor axis activated via Mauthner cells composed of hindbrain neurons, which excite motor neurons and inter-neurons by a continuous firing of signals to flee from the predator (Liu and Fetcho, 1999; Preuss et al., 2006). Since serotonin propagates signals that guide motor neuron pathways in all vertebrates, alterations in concentrations of serotonin (and other neurotransmitters) would alter the escape response phenotype. Thus, observed alterations in behavior are likely due to exposure of antidepressants in the brain rather than altered reflex response.

The ability of the fish to escape a predator depends on how fast the fish can swim, its lateralization pattern to outmaneuver the predator and the distance it swims (Walker et al., 2005); all of these components of escape behavior appear to be susceptible to the activity of the environmental contaminants examined. Studies on striped bass treated with FLX (23.2–100.9 $\mu\text{g L}^{-1}$ over 6 days) caused a decrease in ability to capture live prey in a concentration-dependent manner (Gaworecki and Klaine, 2008); fish treated with lower concentrations were more successful predators than fish treated with higher concentrations. FLX exposure decreased feeding rates in fathead minnow, with a lowest observed effect concentration (51 $\mu\text{g L}^{-1}$) associated with the highest rate of feeding, and higher concentration associated with lower rates of feeding and growth (Stanley et al., 2007; Corcoran et al., 2010). Similar results have been described in mouse (Griebel et al., 1995; Salchner and Singewald, 2002) and rat models (Myoga et al., 1995; Nakajima et al., 1998). Others have shown that estrogen levels also had an effect on predator escape response of larva fathead minnows (McGee et al., 2009).

Other studies that examined exposure to FLX and VNX using fathead minnows had difficulty identifying unambiguous behavioral effects as a result of those treatments (Painter et al., 2009). That study applied treatments to embryonic and larval minnows, and used concentrations (0.025–0.250 $\mu\text{g L}^{-1}$ FLX, 0.5–5.0 $\mu\text{g L}^{-1}$ VNX) similar to observed environmental concentrations of FLX and VNX described at the time their experiment was planned. It is noteworthy that some more recent observed environmental concentrations of FLX and VNX (Table 1) exceed the experimental dosages used by that study (Painter et al., 2009). We chose to use slightly older fish (though still juvenile, sexually undifferentiated) and higher pharmaceutical dosages (10 and 50 $\mu\text{g L}^{-1}$ for FLX and VNX, respectively), in order to improve chances of obtaining consistent treatment effects associated with differences in gene expression. Other studies (van der Ven et al., 2006; Gaworecki and Klaine, 2008) observed behavioral changes using concentrations similar to those employed by our experiment.

Results of the present study indicate a behavior phenotype altered in response to psychoactive pharmaceuticals and similar to the anxiety response reported in other studies (Maximino et al., 2010; Maximino et al., 2011).

5. Conclusions

This study identifies gene expression changes associated with treatment by psychoactive pharmaceuticals at concentrations slightly higher than observed environmental concentrations (but many orders of magnitude lower than clinical dosages). These expression patterns indicate enrichment of human biological processes involving neuronal differentiation and organization, against which comparisons can be made to biological processes (or other gene expression profiles) associated with specific neurological disorders that have known or suspected environmental etiological components, e.g., ADHD (DasBanerjee et al., 2008), bipolar disorder (Holmans et al., 2009), major depression (Klempan et al., 2009), autism (Dufour-Rainfray et al., 2011), and schizophrenia (Katsel et al., 2005). Such comparisons will potentially generate novel hypotheses about the human health implications of psychoactive pharmaceuticals in the environment that may mimic, aggravate or even trigger these disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Observed values of psychoactive pharmaceuticals in various systems.

	FLX	VNX	CBZ
Experimental	10 $\mu\text{g L}^{-1}$	50 $\mu\text{g L}^{-1}$	100 $\mu\text{g L}^{-1}$
Raw sewage	0.073 $\mu\text{g L}^{-1}$ (Batt et al., 2008)	2.19 $\mu\text{g L}^{-1}$ (Schultzt and Furlong, 2008)	6.3 $\mu\text{g L}^{-1}$ (Ternes, 1998)
Wastewater treatment plant (WWTP)	0.509 $\mu\text{g L}^{-1}$ (Chen et al., 2006)	1.115 $\mu\text{g L}^{-1}$ (Metcalf et al., 2003)	17.3–22.0 $\mu\text{g L}^{-1}$ (Christensen et al., 2009; Camacho-Munoz et al., 2010)
Effluent from WWTP	0.841 $\mu\text{g L}^{-1}$ (Corcoran et al., 2010)	No information	1.16 $\mu\text{g L}^{-1}$ (Alonso et al., 2010)
Downstream from WWTP	0.93 $\mu\text{g L}^{-1}$ (Christensen et al., 2009)	0.387 $\mu\text{g L}^{-1}$ (Alonso et al., 2010)	2.3 $\mu\text{g L}^{-1}$ (Metcalf et al., 2003)
River system	0.12 $\mu\text{g L}^{-1}$ (Gros et al., 2010)	1.31 $\mu\text{g L}^{-1}$ (Schultzt and Furlong, 2008)	1.283 $\mu\text{g L}^{-1}$ (Ternes, 1998)
Drinking water	0.014 $\mu\text{g L}^{-1}$ (Stackelberg et al., 2007; Bruce et al., 2010)	No information	0.25 $\mu\text{g L}^{-1}$ (Stackelberg et al., 2004; Bruce et al., 2010)

FLX, VNX and CBZ are fluoxetine, venlafaxine and carbamazepine, respectively. Values reported indicate the highest observed concentrations from various systems.

qPCR results.

Table 2

Gene symbol	EcoArray ID	Primer sequences (primer 1, primer 2)	Amplicon length	Amplicon location (exons)	qPCR efficiency	qPCR fold change	p-value	Array fold change	Array rank
KLHL4	EA_Pp_66036a	AGGTTTCGACCACCGGGGAACA, CGGAGCCAGAATGCTGTGCACA	103	4–5	0.89	2.12	0.007	3.49 (4.02)	5
ARL3	EA_Pp_69820a	TCCGAGACCGAGTGTGGCAGAT, ATGTGCGTCCAGGGTGAAAGCC	139	5–6	0.84	1.63	0.03	3.08 (2.39)	7
WHSC2	EA_Pp_56161a	AATCATTTTCCAGAGACCGGC, AGATACTGACACTCCAAAGGC	139	2–3	0.9	1.54	0.07	2.64 (2.34)	18
SFRS10	EA_Pp_53169a	GCGCTCCAGGGGTTTTGCTTTC, AGGTGTGTGAGGCCCTCTTTGTA	139	4–5	0.86	1.71	0.02	2.44 (3.30)	28
ZNF292	EA_Pp_52304a	GAGTCGATGCTAGACGCCCTCCAAT, AGCGCAGCACATTATAGCTACCGT	138	1–2	0.82	1.88	0.0001	2.35 (3.52)	36
ZC3H15	EA_Pp_62695a	AGCCAGTGGTGGCAGCTCAGAA, ACCTTTGGTACACTGGCCTTGC	93	3–4	0.89	2.00	0.001	1.52 (4.58)	209
TM9SF2	EA_Pp_63481a	TCACCTGTCTCTGAGGCCACCAT, CGCTGGTCAAGGAAGGAACGCCA	90	15–16	0.9	2.01	0.0008	1.48 (1.45)	235
NAT13	EA_Pp_52097a	TGAAGTAGGAGAGCTCGCCAAAG, CACACGGCACCAACTGCAATGT	60	2–3	0.86	1.73	0.01	1.39 (1.42)	275
PPP2R5C	EA_Pp_67680a	ATCGACCTGTGGCTGTCCAAAGC, ATCTCGATGAGCTTCA TGAGCGG	149	2–3	0.77	1.50	0.06	1.32 (2.95)	321

The gene symbol refers to the HUGO approved symbol from the enhanced annotation of the microarray platform (Thomas et al., 2011). The EcoArray ID refers to the probe name on the array platform (GEO: GPL7351). The amplicon location is referenced to the location on the zebrafish homolog (fathead minnow may vary; see Methods). The p-value is the result of the *t*-test used to test the difference between treatment and control C_q values from the qPCR experiment. Array fold change is the signal:noise ratio ($\mu\text{T} - \mu\text{C}/\sigma\text{T} - \sigma\text{C}$) from the array experiment for the MIX vs. CTL comparison; in parentheses is the untransformed fold change ($\mu\text{T} - \mu\text{C}$). The array rank refers to the relative rank of that gene based on the signal:noise ratio, out of a total of 10,069 ranked genes.

Table 3

Descriptions of genes sets and results of analyses.

Source	Set description	Size	Description	FLX	VNX	CBZ	MIX
<i>a. Development collection, containing 20 sets</i>							
GO:0007409	AXONOGENESIS	437/281	Genes assoc. with generation of a long process of a neuron that carries efferent (outgoing) action potentials from cell body towards target cells		NES=1.23 p=0.00 q=0.430		
GO:0007420	BRAIN DEVELOPMENT	329/200	Genes assoc. with process whose specific outcome is the progression of the brain over time, from formation to the mature structure				
GO:0007417	CENTRAL NERVOUS SYSTEM DEVELOPMENT	480/289	Genes assoc. with process whose specific outcome is the progression of the central nervous system over time, from its formation to the mature structure; the CNS consists of the brain, spinal cord and spinal nerves				
GO:0021954	CENTRAL NERVOUS SYSTEM NEURON DEVELOPMENT	31/20	Genes assoc. with process whose specific outcome is the progression of a neuron whose cell body is located in the CNS, from initial commitment of the cell to a neuronal fate, to the fully functional differentiated neuron				
GO:0021545	CRANIAL NERVE DEVELOPMENT	29/14	Genes assoc. with process whose specific outcome is the progression of the cranial nerves over time, from its formation to the mature structure				
GO:0016358	DENDRITE DEVELOPMENT	63/38	Genes assoc. with dendrite development				
GO:0007272	ENSHEATHMENT OF NEURONS	51/31	Genes assoc. with process by which the axon of a neuron is insulated, and that insulation maintained		NES=1.39 p=0.00 q=0.201	NES=1.32 p=0.00 q=0.359	
GO:0030900	FOREBRAIN DEVELOPMENT	182/114	Genes assoc. with process whose specific outcome is the progression of the forebrain over time, from its formation to the mature structure				
GO:0048699	GENERATION OF NEURONS	82/52	Genes assoc. with process by which nerve cells are generated; includes production of neuroblasts and their differentiation into neurons				
GO:0030902	HIND BRAIN DEVELOPMENT	63/36	Genes assoc. with process whose specific outcome is the progression of the hindbrain over time, from its formation to the mature structure				
GO:0007399	NERVOUS SYSTEM DEVELOPMENT	381/229	Genes assoc. with process whose specific outcome is the progression of nervous tissue over time, from its formation to its mature state				NES=1.21 p=0.00 q=0.325

Source	Set description	Size	Description	FLX	VNX	CBZ	MIX
GO:0021915	NEURAL TUBE DEVELOPMENT	81/48	Genes assoc. with process whose specific outcome is the progression of the neural tube over time, from its formation to the mature structure		NES=1.58 p=0.00 q=0.159		
GO:0022008	NEUROGENESIS	20/12	Genes assoc. with generation of cells within the nervous system				
GO:0048666	NEURON DEVELOPMENT	27/14	Genes assoc. with process whose specific outcome is the progression of a neuron over time, from initial commitment of the cell to a specific fate, to the fully functional differentiated cell				
GO:0050767	REGULATION OF NEUROGENESIS	230/155	Genes assoc. with processes that modulates the frequency, rate or extent of neurogenesis				
GO:0050768	NEGATIVE REGULATION OF NEUROGENESIS	57/41	Genes assoc. with process that stops, prevents, or reduces the frequency, rate or extent of neurogenesis				
GO:0050769	POSITIVE REGULATION OF NEUROGENESIS	74/56	Genes assoc. with process that activates or increases the frequency, rate or extent of neurogenesis				
GO:0050772	POSITIVE REGULATION OF AXONOGENESIS	30/25	Genes assoc. with process that activates or increases the frequency, rate or extent of axonogenesis				
GO:0050808	SYNAPSE ORGANISATION	73/41	Genes assoc. with assembly, arrangement of constituent parts, or disassembly of a synapse				NES=1.61 p=0.00 q=0.128
GO:0044456	SYNAPSE PART	273/159	Genes assoc. with junction between a nerve fiber of one neuron and another neuron or muscle fiber or glial cell		NES=1.33 p=0.00 q=0.649		NES=1.64 p=0.00 q=0.094
<i>b. Regulation collection, containing 22 sets</i>							
GO:0043679	AXON TERMINUS	37/23	Genes assoc. with terminal inflated portion of the axon, containing the specialized apparatus necessary to release neurotransmitters				
GO:0008066	GLUTAMATE RECEPTOR ACTIVITY	28/14	Genes assoc. with combining with glutamate to initiate a change in cell activity				
MSigDB C2 CPG	HSA04080 NEUROACTIVE LIGAND RECEPTOR INTERACTION	67/31	Genes assoc. with neuro-active ligand-receptor interaction				
MSigDB C2 CPG	LU AGING BRAIN DN	260/169	Genes down-regulated in postmortem brain frontal cortex samples from old subjects relative to young ones				
MSigDB C2 CPG	LU AGING BRAIN UP	155/107	Genes up-regulated in postmortem brain frontal cortex samples from old subjects relative to young ones		NES=1.33 p=0.00 q=0.234		NES=1.44 p=0.00 q=0.161

Source	Set description	Size	Description	FLX	VNX	CBZ	MIX
GO:0007270	NERVE-NERVE SYNAPTIC TRANSMISSION	58/37	Genes assoc. with process of communication from one neuron to another across a synapse				
GO:0042165	NEUROTRANSMITTER BINDING	75/31	Gene products capable of transmitting (or inhibiting the transmission of) a nerve impulse from a neuron to another cell	NES= -1.51 p=0.00 q=0.235			
GO:0042133	NEUROTRANSMITTER METABOLIC PROCESS	24/18	Genes assoc. with chemical reactions and pathways involving neurotransmitters				
GO:0030594	NEUROTRANSMITTER RECEPTOR ACTIVITY	65/24	Genes assoc. with combining with a neurotransmitter to initiate a change in cell activity				
GO:0007269	NEUROTRANSMITTER SECRETION	79/53	Genes associated with regulated release of neurotransmitter into the synaptic cleft				NES=1.58 p=0.00 q=0.081
GO:0006836	NEUROTRANSMITTER TRANSPORT	121/75	Genes associated with the directed movement of a neurotransmitter into, out of, within or between cells				NES=1.36 p=0.00 q=0.302
GO:0019228	REGULATION OF ACTIONPOTENTIAL IN NEURON	63/37	Genes assoc. modulating membrane potential involved in the propagation of a neural signal	NES=1.53 p=0.00 q=0.073	NES=1.32 p=0.00 q=0.284		
GO:0050433	REGULATION OF CATECHOLAMINE SECRETION	25/14	Genes assoc. with any process modulating frequency, rate or extent of regulated release of catecholamines	NES=-1.32 p=0.00 q=0.790			
GO:0048168	REGULATION OF NEURONAL SYNAPTIC PLASTICITY	31/21	Genes assoc. with process that modulates neuronal synaptic plasticity, the ability of neuronal synapses to change as circumstances require				
GO:0001505	REGULATION OF NEUROTRANSMITTER LEVELS	109/73	Genes assoc. with process modulating neurotransmitter level				
GO:0051969	REGULATION OF TRANSMISSION OF NERVE IMPULSE	136/82	Genes assoc. with modulating frequency, rate or extent of transmission of a nerve impulse				
GO:0031644	REGULATION OF NEUROLOGICAL SYSTEM PROCESS	146/87	Genes assoc. with process that modulates frequency, rate or extent of a neurophysiological process, an organ system process carried out by any organ or tissue of neurological system				
GO:0005484	SNAP RECEPTOR ACTIVITY	19/14	Genes assoc. with acting as a marker to identify a membrane and interacting selectively with one or more SNAREs on another membrane to mediate membrane fusion				
GO:0000149	SNARE BINDING	30/24	Genes assoc. with interacting selectively and non-covalently with SNARE (soluble N-ethylmaleimide-sensitive factor attached protein receptor) protein	NES=1.47 p=0.00 q=0.109	NES=1.70 p=0.00 q=0.053	NES=1.82 p=0.00 q=0.044	

Source	Set description	Size	Description	FLX	VNX	CBZ	MIX
GO:0045202	SYNAPSE	370/227	Genes assoc. with junction between a nerve fiber of one neuron and another neuron or muscle fiber or glial cell			NES=1.24 p=0.00 q=0.342	NES=1.63 p=0.00 q=0.100
GO:0007268	SYNAPTIC TRANSMISSION	361/200	Genes assoc. with process of communication from a neuron to a target (neuron, muscle, or secretory cell) across a synapse				
GO:0019226	TRANSMISSION OF NERVE IMPULSE	420/235	Genes assoc. with sequential electrochemical polarization and depolarization that travels across the membrane of a neuron in response to stimulation				NES=1.31 p=0.00 q=0.379
<i>c. Growth collection, containing 16 sets</i>							
MSigDB C2 CPG	APPEL IMATINIB RESPONSE	32/20	Genes up-regulated by imatinib during dendritic cell differentiation			NES=-1.46 p=0.00 q=0.065	
GO:0030424	AXON	180/127	Genes assoc. with long process of a neuron that conducts nerve impulses, usually away from the cell body to terminals and varicosities				NES=1.19 p=0.00 q=0.217
GO:0008366	AXON ENSHEATHMENT	51/31	Genes assoc. with processes in which the axon of a neuron is insulated, and that insulation maintained		NES=1.38 p=0.00 q=0.551	NES=1.32 p=0.00 q=0.707	
GO:0048675	AXON EXTENSION	47/32	Genes assoc. with long distance growth of a single process				
GO:0007411	AXON GUIDANCE	325/208	Genes assoc. with process by which migration of an axon growth cone is directed to a specific target site in response to a combination of attractive and repulsive cues				
GO:0033267	AXON PART	71/47	Genes assoc. with part of an axon, a cell projection of a neuron				
GO:0048667	CELL MORPHOGENESIS INVOLVED IN NEURON DIFFERENTIATION	465/297	Genes assoc. with processes in which structures of a neuron are generated and organized		NES=1.22 p=0.00 q=0.614		
GO:0021953	CENTRAL NERVOUS SYSTEM NEURON DIFFERENTIATION	80/45	Genes assoc. with process in which a relatively unspecialized cell acquires specialized features of a neuron whose cell body resides in the central nervous system				
GO:0070997	NEURON DEATH	105/78	Genes assoc. with process of neuron cell death				
GO:0030182	NEURON DIFFERENTIATION	74/43	Genes assoc. with process whereby a relatively unspecialized cell acquires specialized features of a neuron				
GO:0042551	NEURON MATURATION	19/12	Genes assoc. with process, independent of morphogenetic change, required for a neuron to attain its fully functional state				

Source	Set description	Size	Description	FLX	VNX	CBZ	MIX
GO:0043005	NEURON PROJECTION	384/246	Genes assoc. with prolongation or process extending from a nerve cell, e.g. an axon or dendrite				NES=1.41 p=0.00 q=0.240
GO:0048812	NEURON PROJECTION MORPHOGENESIS	468/302	Genes assoc. with process in which anatomical structures of a neuron projection are generated and organized		NES=1.21 p=0.00 q=0.493		
GO:0043523	REGULATION OF NEURON APOPTOSIS	87/64	Genes assoc. with process that modulates the occurrence or rate of cell death by apoptosis in neurons			NES=-1.44 p=0.00 q=0.057	
GO:0045664	REGULATION OF NEURON DIFFERENTIATION	183/126	Genes assoc. with process that modulates the frequency, rate or extent of neuron differentiation				
GO:0007416	SYNAPSE ASSEMBLY	46/23	Genes assoc. with aggregation, arrangement & bonding together of set of components to form synapse				

Source indicates the database from which the gene set was derived, either GO (Gene Ontology) or MSigDB (Molecular Signatures Database). Size indicates the number of genes in the set and the number of those genes that were found on the array. FLX, VNX, CBZ and MIX refer to fluoxetine, venlafaxine, carbamazepine and mixture treatments, respectively. NES, p and q refer to the GSEA Net Enrichment Score, p-value and False Discover Rate q-value, respectively. Positive NES values refer to gene sets up-regulated in the treatment (relative to control) while negative NES values refer to gene sets down-regulated in the treatment. Gene sets with p<0.05 and q<0.25 were considered to be statistically significantly enriched (see Subramanian et al., 2005 for details), enriched sets and corresponding scores are marked in bold to highlight this. Sets with high NES scores but failing the FDR test (p<0.05 and q>0.25) are listed for comparison only.