Psychoactive Pharmaceuticals Induce Fish Gene Expression Profiles Associated with Human Idiopathic Autism

Michael A. Thomas¹*, Rebecca D. Klaper²

¹ Department of Biological Sciences, Idaho State University School, Pocatello, Idaho, United States of America, ² School of Freshwater Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, United States of America

Abstract

Idiopathic autism, caused by genetic susceptibility interacting with unknown environmental triggers, has increased dramatically in the past 25 years. Identifying environmental triggers has been difficult due to poorly understood pathophysiology and subjective definitions of autism. The use of antidepressants by pregnant women has been associated with autism. These and other unmetabolized psychoactive pharmaceuticals (UPPs) have also been found in drinking water from surface sources, providing another possible exposure route and raising questions about human health consequences. Here, we examined gene expression patterns of fathead minnows treated with a mixture of three psychoactive pharmaceuticals (fluoxetine, venlafaxine & carbamazepine) in dosages intended to be similar to the highest observed conservative estimates of environmental concentrations. We conducted microarray experiments examining brain tissue of fish exposed to individual pharmaceuticals and a mixture of all three. We used gene-class analysis to test for enrichment of gene sets involved with ten human neurological disorders. Only sets associated with idiopathic autism were unambiguously enriched. We found that UPPs induce autism-like gene expression patterns in fish. Our findings suggest a new potential trigger for idiopathic autism in genetically susceptible individuals involving an overlooked source of environmental contamination.

Introduction

Autism spectrum disorders (ASD) are characterized by stereotyped behaviors and impaired social skills, typically diagnosed by three years of age [1–3]. Idiopathic ASD, caused by genetic susceptibility factors [4–6] interacting with unknown environmental triggers [7,8], has increased dramatically in the past 25 years [9,10]. Identifying environmental triggers has been difficult due to the poorly understood pathophysiology of ASD and broad, subjective case definitions.

In order to serve as such a trigger, a candidate teratogen must have a biologically plausible etiological mechanism, exist in sufficient environmental concentrations, be capable of passing from mother to fetus and across the fetal blood-brain barrier (if one assumes prenatal exposure), and have experienced historical increases in environmental concentration parallel with observed increases in ASD prevalence.

Coincident with the observed increase in ASD prevalence is the introduction of modern rationally designed psychoactive pharmaceuticals, beginning with selective serotonin re-uptake inhibitors (SSRIs) in 1987 (initially fluoxetine, FLX; now 9+ versions), and serotonin–norepinephrine reuptake inhibitors (SNRIs) in 1994 (initially venlafaxine, VNX; now 8+ versions). These pharmaceuticals result in an increase in the neurotransmitter serotonin, which is responsible for the regulation of neural activity and other physiological functions [11]. The leaky blood brain barrier of the fetus and infants is permeable to many compounds, making this population particularly vulnerable to the effects of serotonin. Maternal exposure to SSRIs result in elevated fetal plasma serotonin levels, which has been associated with autism [12]. In other work, rat pups exposed prenatally to SSRIs exhibited behaviors associated with ASD [13].

The use of SSRIs and SNRIs by pregnant women to treat depression and other psychological disorders has been associated with low APGAR (Appearance, Pulse, Grimace, Activity, Respiration) scores [14], increased risk of spontaneous abortion [15], several other consequences for children [16–20], and, recently, ASD [21]. Several other classes of psychoactive pharmaceuticals are also known autism risk factors when taken prenatally [22,23], including valproic acid used to induce the autism rat model [24,25]. Since the 1960s, for example, pregnant women have used carbamazepine (CBZ) for control of seizures, despite its potential association with developmental issues [26]. Studies of these drugs considered only effects of maternal usage of clinical dosages [21,27]; while widespread, clinical usage of antidepressants is insufficient to account for recent increases in autism prevalence.

There is an alternative source of exposure to antidepressants: Unmetabolized psychoactive pharmaceuticals (UPPs) found in raw

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* E-mail: mthomas@isu.edu
sewage, effluent from sewer treatment facilities, rivers downstream of such facilities, and, ultimately, drinking water \[28,29\]. Because concentrations are so minute (typically ng to \(\mu\)g L\(^{-1}\)), human health consequences of UPPs remain controversial. While the highest observed concentrations of UPPs have biological effects in fish \[30\], these concentrations are many orders of magnitude below human clinical dosages (Table 1). While fetuses and infants may have been exposed to UPPs through maternal water consumption, it has been assumed that UPPs have no measurable and enduring effects on human health \[31,32\]. However, multiple related formulations and active metabolites of UPPs present in the environment exist in complex mixtures \[33,34\] that together constitute much higher dosages, especially in contamination hotspots \[35\]. Therefore, we describe the experimental concentrations used here as similar to the highest observed environmental concentrations, despite the fact that experimental concentrations are an order of magnitude higher than the most recent (and probably conservative) estimates of environmental concentrations (Table 1).

Here, we describe results of an experiment that explores a potential association between UPPs and idiopathic ASD. We tested whether chronic exposure to a mixture of UPPs induced autism-like gene expression profiles in a model organism using gene-class analysis. We used treatments involving a mixture of UPPs similar to that observed in aquatic systems and examined expression of genes expressed by individuals with various forms of idiopathic ASD. For comparison, we examined sets of genes expressed by individuals with other neurological disorders.

**Gene Expression Analysis**

We exposed fathead minnows, *Pimephales promelas*, to FLX, VNX, and CBZ in a 3-component mixture. FLX, VNX, and CBZ were chosen because they represent modern pharmaceutical classes that are highly prescribed and are among the UPPs with the highest observed environmental concentrations (Table 1). We conducted gene-class analysis of expression patterns induced by the pharmaceutical treatments using Gene Set Enrichment Analysis (GSEA) \[36\] and an enhanced annotation of the fathead microarray platform \[37\]. The gene-class analysis approach tests if a set of genes, described *a priori*, is enriched by a given treatment. The data for the present study were derived from a previous analysis of this system \[38\]. In that study, we found enrichment of gene sets associated with neurological development, growth and regulation by the mixture of UPPs. These gene sets were not enriched by treatments of the pharmaceuticals administered separately, and were associated with the formation and regulation of neural circuits, which may indicate formation of altered and imprecise synaptic connections and presage a failure to form typical mature neural circuits.

In the present study, we first tested the prediction that UPPs would induce fish gene expression profiles that mimic human expression profiles observed in individuals diagnosed with various neurological disorders ("ND" sets, described in Table 2). We tested 12 sets of genes associated with idiopathic ASD (broadly defined), autism secondary to known genetic defects (involving fragile X and Rett syndromes), Alzheimer’s disease, Parkinson’s disease, schizophrenia, multiple sclerosis, major depression, bipolar disorder and ADHD.

Second, we tested the prediction that UPPs would induce fish gene expression profiles that mimic human expression profiles observed in individuals diagnosed with autism of various degrees of severity. The idiopathic autism set used above (described in Table 2) was derived from several independent gene expression studies with minimal overlap of gene constituents, each of which identified genes enriched in individuals diagnosed with idiopathic autism. One of these sets \[39\] was deconstructed into specific populations classified by severity of autism symptoms. We created a second collection consisting of 10 autism sets ("ASD" sets; Table 3); nine sets associated with gene expression in individuals diagnosed with some form of autism plus a set of susceptibility genes not associated with any known increase in gene expression but, rather, known to have either mutations (e.g., single nucleotide polymorphisms) or structural variations (e.g., copy number variation) associated with some form of autism \[4\].

**Results**

In the analysis of the ND sets, seven of 12 sets were up-regulated, with significant enrichment of the set associated with idiopathic autism (Table 4). We also observed enrichment of two disorders with adult onset: a set associated with Parkinson’s and one set associated with MS (but not a second, independent MS set). There was no overlap between the autism set and the Parkinson’s & MS sets; 4 genes occurred in both MS and Parkinson’s sets. No other set associated with human neurological disorders was enriched, including secondary autism sets.

In the analysis of ASD sets, all 10 sets were up-regulated, with significant enrichment of five of the nine expression-based sets (Table 5). Very few genes are shared among enriched sets. The susceptibility set was not enriched; this is significant because one would not necessarily expect genes that underlie susceptibility to also experience differential expression. Interestingly, one of the strongly enriched sets (Voineagu\_Down) was identified by the study authors as genes *down*-regulated in individuals with autism.

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**Table 1. Observed values of psychoactive pharmaceuticals in various systems.**

| Source                     | FLX     | VNX     | CBZ     |
|----------------------------|---------|---------|---------|
| **Experimental**           |         |         |         |
| Raw sewage                 | 10 \(\mu\)g L\(^{-1}\) | 50 \(\mu\)g L\(^{-1}\) | 100 \(\mu\)g L\(^{-1}\) |
| Wastewater treatment plant | 0.073 \(\mu\)g L\(^{-1}\) \[50\] | 2.19 \(\mu\)g L\(^{-1}\) \[51\] | 6.3 \(\mu\)g L\(^{-1}\) \[52\] |
| Downstream from WWTP       | 0.509 \(\mu\)g L\(^{-1}\) \[53\] | 1.115 \(\mu\)g L\(^{-1}\) \[47\] | 17.3–22.0 \(\mu\)g L\(^{-1}\) \[54,55\] |
| Effluent from WWTP         | 0.841 \(\mu\)g L\(^{-1}\) \[56\] | No information | 1.16 \(\mu\)g L\(^{-1}\) \[57\] |
| Drinking water             | 0.014 \(\mu\)g L\(^{-1}\) \[59,60\] | No information | 0.25 \(\mu\)g L\(^{-1}\) \[60,61\] |

FLX, VNX and CBZ are fluoxetine, venlafaxine and carbamazepine, respectively. Values reported indicate the highest observed concentrations from various systems. Experimental treatment dosage was selected to reflect combined dosages of multiple active metabolites for each pharmaceutical.

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Table 2. "ND": Sets of genes associated with various human neurological disorders.

| Set name             | Description                                                                 | Number of genes (set) | Number of genes (FH microarray) | GEO link | Reference |
|----------------------|-----------------------------------------------------------------------------|-----------------------|---------------------------------|----------|-----------|
| ASD_Idiopathic       | Combination of Chakrabarti, Hu & ASD_2Class; duplicates were removed (see Table S3 for details). | –                     | 324                             |          |           |
| ADHD_up              | A comparison of molecular alterations in environmental and genetic rat models of ADHD (up-regulated genes) | 50                    | 30                              | GSE12457 | [62]      |
| ADHD_down            | A comparison of molecular alterations in environmental and genetic rat models of ADHD (down-regulated genes) | 34                    | 20                              | GSE12457 | [62]      |
| Rett                 | Genes found to be up-regulated in females with Rett Syndrome                | 39                    | 25                              |          | [63]      |
| ASD_Secondary        | Gene expression profiles of lymphoblastoid cells from individuals with fragile X syndrome and dup(15q). | 67                    | 39                              | GSE7329  | [64]      |
| Schizophrenia        | Proteins consistently differentially expressed in the brains of SCZ patients. | 30                    | 23                              |          | [65]      |
| Alzheimers           | Up-regulated in correlation with incipient Alzheimer’s Disease, in the CA1 region of the hippocampus | 345                   | 237                             | GSE1297  | [66]      |
| Parkinsons           | Genes associated with Parkinson’s Disease.                                  | 162                   | 94                              | KEGG     | HSAO5012  |
| Depression           | Genes upregulated in major depressive disorder (p<0.05, fold change >1.4, mean average difference >150 in at least one of the groups, called present in greater than 20% of all samples) | 45                    | 23                              | GSE12654 | [67]      |
| Bipolar              | Genes found to be up-regulated in individuals with bipolar disorder.         | 71                    | 41                              |          | [68]      |
| MS_Bomprezzi         | In an attempt to identify molecular markers indicative of disease status rather than susceptibility genes for MS, the authors show that gene expression profiling of peripheral blood mononuclear cells by cDNA microarrays can distinguish MS patients from healthy controls. | 45                    | 28                              |          | [69]      |
| MS_Gilli             | Results showed an altered expression of 347 transcripts in non-pregnant MS patients with respect to non-pregnant healthy controls. | 348                   | 216                             | GSE17393 | [70]      |

Discussion

We found enrichment of gene sets associated with idiopathic ASD but not of sets involving autism diagnoses secondary to other disorders (Rett and fragile X syndromes) known to be caused by specific mutations. This is significant, because it indicates that enrichment in our treatments involve only idiopathic forms of ASD.

There was no enrichment of other neurological disorders except MS (in one of two sets) and Parkinson’s. This is significant because it indicates that enrichment of the idiopathic ASD set was not simply associated with general neurological processes, pathways or systems generally common to neurological disorders. The MS set has a low NES (<1.40), and a second MS set (see Table 4) is not enriched; therefore, we are not exceedingly confident in describing that set as enriched. The other enriched non-ASD set, Parkinson’s, is more interesting, with a convincing NES and an intriguing potential connection to ASD involving similar phenomenology involving brain dysfunction [41].

A number of the genes contributing to enrichment of ASD gene sets have been implicated in other recent studies not included in our analysis. For example, Suda et al. [42] found that relative expression levels of EFNB3, PLXNA4 and ROBO2 were significantly different in individuals with autism than in neurotypical individuals; protein levels of PLXNA4 and ROBO2, but not for EFNB3, were significantly reduced in brains of individuals with autism compared to control brains. In the present study, we found 3 plexin genes (PLXNB1, PLXND1 & PLXNA3; PLXNA4 was not on the array) and ROBO2 to be strongly down-regulated in response to the MIX treatment, while EFNB3 and five related genes (EFNB1, EFNA1, EFNA2, EFNA3 & EFNA5) were up-regulated. These and other genes contributing to gene set enrichment are associated with the formation of synapses, perturbation of which may indicate an altered and imprecise synaptic connections or a failure to form mature neural circuits.

The results presented here are consistent with several recent lines of inquiry: First, the hypothesis that hyperserotonemia plays a
role in autism, affecting the developing fetus and potentially involving SSRIs [27]. In that work, Hadjikhani explored a potential role of elevated serotonin levels perturbing brain development during pregnancy (in which he assumes that maternal serotonin ultimately passes the fetal blood brain barrier). The author speculated that elevated levels could be increased by maternal use of serotonin elevating pharmaceuticals (like SSRIs) or consumption of serotonin-rich foods. In the present study, serotonin levels were not measured. However, all six serotonin receptor genes on the array (HTR1A, HTR1B, HTR2C, HTR4, HTR7 & SLC6A4) were strongly down-regulated in response to the MIX treatment. If this implies a consequential elevation of serotonin levels, our results would seem to be consistent with the Hadjikhani hypothesis [27] and with other recent experimental work using model organisms [13].

Second, recent evidence supports an association of antidepressants, including SSRIs, with autism [21]. In that study, Croen and colleagues found a 2-fold increase in ASD risk associated with SSRIs, with the strongest effect occurring in the first trimester. The results of the present study are consistent with this finding. However, maternal SSRI use is not sufficient to explain the increase in prevalence of ASD.

Third, there is evidence for an unambiguous environmental component involved in the etiology of autism [7]. In that study, Hallmayer and colleagues provide robust evidence that, while having a moderate genetic component, ASD also clearly involves an environmental trigger. The results of the present study are consistent with this finding, as is the assumption that the environmental trigger acts in concert with genetic susceptibility.

Fourth, there is evidence of demographic changes that may have increased the proportion of genetically susceptible individuals

| Set name | Description | Number of genes (set) | Number of genes (FH microarray) | GEO link | Reference |
|----------|-------------|-----------------------|---------------------------------|----------|-----------|
| Pinto    | Genes associated with genetic susceptibility to ASD but not known to be up- or down-regulated in the disorder. | 104 | 63 | | [4] |
| Chakrabarti | Genes related to sex steroids, neural growth, and social-emotional behavior associated with autistic traits, empathy, and Asperger's syndrome. Included only mild cases (no severe language impairment). | 66 | 43 | | [71] |
| Hu | Gene identified by comparisons of neurotypical vs. ASD individuals with severe language impairment (with individuals having specific genetic and chromosomal abnormalities and co-morbid disorders excluded from study). | 34 | 22 | | [72] |

**Table 3. “ASD”: Collection of sets of genes associated with idiopathic autism.**

| Set name | Description | Number of genes (set) | Number of genes (set) | GEO link | Reference |
|----------|-------------|-----------------------|----------------------|----------|-----------|
| ASD,2Class | Significantly differentially expressed genes from a 2-class SAM analysis of data from combined autistic samples and neurotypical controls, with FDR <5% | | | GSE15402 | [39] |
| ASD,Mild | Significantly differentially expressed genes from a 2-class SAM analysis of data from the group with mild ASD (M) and neurotypical controls (C), with FDR <5% | 360 | 241 | GSE15402 | [39] |
| ASD,Severe | Significantly differentially expressed genes from a 2-class SAM analysis of data from the group with severe language impairment (L) and neurotypical controls (C), with FDR <0.0001% | 191 | 121 | GSE15402 | [39] |
| ASD,Shared | Common genes to all GSE15402 sets. | 70 | 48 | GSE15402 | [39] |
| ASD,Savant | Significantly differentially expressed genes from a 2-class SAM analysis of data< |

**Table 4. Sets associated with human neurological disorders.**

| Set name             | Size | NES | p-value | FDR q-value |
|----------------------|------|-----|---------|-------------|
| AUTISM_IDIOPATHIC    | 324  | 1.621 | 0.000 | 0.064 |
| PARKINSONS           | 94   | 1.560 | 0.007 | 0.055 |
| MS,GILLI             | 216  | 1.375 | 0.011 | 0.137 |
| SCHIZOPHRENIA        | 23   | 1.232 | 0.181 | 0.364 |
| MS,BOMPREZZI         | 28   | 1.199 | 0.201 | 0.326 |
| ADHD_UP              | 30   | 1.187 | 0.222 | 0.275 |
| DEPRESSION           | 23   | 1.137 | 0.307 | 0.293 |
| ADHD_DOWN            | 20   | −0.684 | 0.894 | 0.924 |
| RETT                 | 25   | −0.784 | 0.798 | 1.000 |
| ALZHEIMERS           | 237  | −0.967 | 0.549 | 0.859 |
| ASD,SECONDARY       | 39   | −1.083 | 0.332 | 0.764 |
| BIPOLAR              | 41   | −1.172 | 0.217 | 1.000 |

**Table 5. Analysis of sets associated with human autism.**

| Set name             | Size | NES | p-value | FDR q-value |
|----------------------|------|-----|---------|-------------|
| ASD,MILD             | 241  | 1.537 | 0.0000 | 0.1261 |
| ASD,2CLASS           | 240  | 1.519 | 0.0000 | 0.0742 |
| VOINEAGU_DOWN        | 121  | 1.514 | 0.0017 | 0.0511 |
| ASD,SAVANT           | 60   | 1.474 | 0.0298 | 0.0537 |
| ASD,SHARED           | 48   | 1.459 | 0.0391 | 0.0489 |
| CHAKRABARTI          | 43   | 1.358 | 0.0769 | 0.0864 |
| HU                   | 22   | 1.352 | 0.1168 | 0.0777 |
| ASD,SEVERE           | 121  | 1.261 | 0.0781 | 0.1233 |
| VOINEAGU_UP          | 132  | 1.117 | 0.2092 | 0.2749 |
| PINTO                | 63   | 1.050 | 0.3558 | 0.3558 |

| Set name | Size | NES | p-value | FDR q-value |
|----------|------|-----|---------|-------------|
| ASD,MILD | 241  | 1.537 | 0.0000 | 0.1261 |
| ASD,2CLASS | 240  | 1.519 | 0.0000 | 0.0742 |
| VOINEAGU_DOWN | 121  | 1.514 | 0.0017 | 0.0511 |
| ASD,SAVANT | 60   | 1.474 | 0.0298 | 0.0537 |
| ASD,SHARED | 48   | 1.459 | 0.0391 | 0.0489 |
| CHAKRABARTI | 43   | 1.358 | 0.0769 | 0.0864 |
| HU | 22   | 1.352 | 0.1168 | 0.0777 |
| ASD,SEVERE | 121  | 1.261 | 0.0781 | 0.1233 |
| VOINEAGU_UP | 132  | 1.117 | 0.2092 | 0.2749 |
| PINTO | 63   | 1.050 | 0.3558 | 0.3558 |

Sets are described in Table 2; size refers to the number of genes in the set; NES is the normalized enrichment scores for the set; p-value is the nominal p-value associated with the NES; FDR q-value is the false discovery rate ratio. doi:10.1371/journal.pone.0032917.t004

Sets are described in Table 2; size refers to the number of genes in the set; NES is the normalized enrichment scores for the set; p-value is the nominal p-value associated with the NES; FDR q-value is the false discovery rate ratio. doi:10.1371/journal.pone.0032917.t005
in contemporary populations [43]. In that study, Baron-Cohen proposed that assortative mating among genetically susceptible individuals has increased the proportion of susceptible individuals in human populations since the 1970s. Especially when coupled with increased levels of an environmental trigger, this would create circumstances in which one would expect an increase in ASD prevalence. Given that SSRIs were introduced in the mid-1980s and SNRIs in the mid-1990s, coincident with increases in ASD prevalence [10], the assortative mating hypothesis provides a framework for understanding why such a trigger is able to induce such a large effect. The results of the present study provide a potential source of exposure to psychoactive pharmaceuticals that does not involve maternal clinical usage of SSRIs.

Given the conserved nature (i.e., sequence and function) of the genes involved in the observed expression profiles, and given that the genes on the Fathead array are homologous to highly conserved human genes, it is reasonable to expect induction of humans gene expression profiles similar to the Fathead profiles. This sort of approach has been effectively used for other models of human disorders [44] and in previous investigations involving the Fathead microarray platform [37]. Here, many of the enriched sets involve genes associated with neuronal development and growth [40,45].

The concentrations used in this study were higher than observed environmental concentrations in order to account for conservative concentration estimates and the presence of related formulations and active metabolites [29,34,46,47]. Future work needs to be conducted to measure the concentrations of all UPP constituents present in aquatic systems and drinking water (with appropriate temporal and geographic sampling) in order to accurately assess human exposure and health consequences.

Conclusions

These results provide a new perspective on the etiology of idiopathic ASD and suggest new directions for research into

### Table 6. Single drug treatments & ASD sets.

| Set                  | FLX  | FLX  | FLX  | VNX  | VNX  | VNX  | CBZ  | CBZ  |
|----------------------|------|------|------|------|------|------|------|------|
|                      | NES  | p-value | FDR q-value | NES  | p-value | FDR q-value | NES  | p-value | FDR q-value |
| ASD_SAVANT           | 1.443 | 0.036 | 0.144 | 1.344 | 0.075 | 0.174 | 1.287 | 0.103 | 0.138 |
| ASD_MILD             | 1.429 | 0.003 | 0.081 | 1.370 | 0.018 | 0.215 | 1.531 | 0.000 | 0.099 |
| ASD_SHARED           | 1.362 | 0.066 | 0.089 | 1.331 | 0.088 | 0.143 | 1.115 | 0.244 | 0.322 |
| ASD_2CLASS           | 1.290 | 0.022 | 0.118 | 1.411 | 0.005 | 0.289 | 1.490 | 0.002 | 0.076 |
| ASD_SEVERE           | 1.199 | 0.115 | 0.172 | 0.979 | 0.514 | 0.634 | 1.342 | 0.039 | 0.155 |
| CHAKRABARTI          | 1.144 | 0.246 | 0.206 | 0.887 | 0.662 | 0.695 | 1.017 | 0.432 | 0.475 |
| VOINEAGU_DOWN        | −0.837 | 0.831 | 0.809 | 1.116 | 0.220 | 0.443 | 1.288 | 0.054 | 0.172 |
| VOINEAGU_UP          | −0.899 | 0.701 | 0.906 | −0.909 | 0.716 | 0.670 | −1.018 | 0.404 | 0.415 |
| PINTO                | −1.061 | 0.339 | 0.677 | 1.018 | 0.420 | 0.627 | −1.065 | 0.337 | 0.659 |
| HU                   | −1.378 | 0.080 | 0.150 | 0.901 | 0.582 | 0.747 | 0.893 | 0.591 | 0.697 |

FLX, VNX and CBZ are fluoxetine, venlafaxine and carbamazepine, respectively. Column labeled as in Table 4. Sets are described in Table 3.

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### Table 7. Single drug treatments & ND sets.

| Set                  | FLX  | FLX  | FLX  | VNX  | VNX  | VNX  | CBZ  | CBZ  |
|----------------------|------|------|------|------|------|------|------|------|
|                      | NES  | p-value | FDR q-value | NES  | p-value | FDR q-value | NES  | p-value | FDR q-value |
| PARKINSONS           | 1.650 | 0.000 | 0.058 | 1.780 | 0.000 | 0.011 | 2.110 | 0.000 | 0.000 |
| AUTISM_IDIOPATHIC    | 1.505 | 0.000 | 0.219 | 1.412 | 0.004 | 0.390 | 1.510 | 0.000 | 0.1930 |
| RETT                 | 1.164 | 0.237 | 0.738 | 1.079 | 0.338 | 0.452 | 0.7529 | 0.8097 | 0.8795 |
| SCHIZOPHRENIA        | 1.104 | 0.316 | 0.661 | 1.164 | 0.251 | 0.497 | 1.2040 | 0.2079 | 0.2905 |
| ADHD_UP              | 1.102 | 0.327 | 0.501 | 1.155 | 0.243 | 0.390 | 1.5102 | 0.0397 | 0.0965 |
| MS_GILLI             | 0.997 | 0.500 | 0.639 | 1.364 | 0.017 | 0.265 | 1.0616 | 0.2827 | 0.4790 |
| MS_BOMPREZZI         | 0.904 | 0.589 | 0.755 | 0.857 | 0.687 | 0.732 | 0.9935 | 0.4295 | 0.5343 |
| ASD_SECONDARY       | 0.631 | 0.974 | 0.965 | −0.749 | 0.896 | 0.870 | −0.622 | 0.970 | 0.968 |
| BIPOLAR             | −0.774 | 0.832 | 0.856 | −1.401 | 0.047 | 0.300 | −0.812 | 0.773 | 1.000 |
| ALZHEIMERS           | −1.118 | 0.204 | 0.447 | −0.847 | 0.906 | 0.928 | −0.934 | 0.640 | 0.969 |
| DEPRESSION           | −1.170 | 0.248 | 0.523 | 0.919 | 0.583 | 0.717 | 1.2313 | 0.1871 | 0.3349 |
| ADHD_DOWN            | −1.715 | 0.004 | 0.019 | −1.375 | 0.110 | 0.177 | −1.322 | 0.152 | 0.465 |

FLX, VNX and CBZ are fluoxetine, venlafaxine and carbamazepine, respectively. Column labeled as in Table 4. Sets are described in Table 2.

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autism’s environmental “exposome” [48]. The results of the gene expression study indicate that a mixture of UPPs can induce an ASD-like gene expression profile in a model organism. Using a low-cost model system like fathead minnows, researchers can rapidly screen potential teratogens for their ability to induce ASD-like gene expression patterns in developing brains. In order to clearly determine if UPPs are associated with idiopathic ASD in humans, future work needs to examine a wider palette of UPPs (and other potential teratogens) and results need to be validated by demonstrating treatment response in another model systems. This could involve using a mouse model, with which one could measure fetal brain expression patterns, UPP concentration in fetal blood, and concentrations of fetal neurohypophyseal hormones, following maternal treatment. Further, epidemiological studies at the individual patient level should be conducted to confirm and specify the relationship between environmental contaminants and ASDs. The mimicry of ASD-like gene expression profiles in fish, described above, does not conclusively indicate UPP induction of ASD in humans. It does, however, serve as the basis for new hypotheses regarding the etiology of idiopathic ASD.

Materials and Methods

Ethics Statement

All fish handling and treatments were performed at the Great Lakes WATER Institute (School of Freshwater Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin) using appropriate UWM Institutional Animal Care and Use Committee (IACUC) approved protocols (approval number 0708#14).

Fish Treatments

Full details of the fish treatments are described in a previous report [38]. Briefly, three 2-gallon tanks were used for each pharmaceutical treatment along with three tanks for a mixture treatment (containing all three pharmaceuticals in the concentrations listed in Table 1) and three tanks for control (containing no pharmaceuticals). Each tank housed five juvenile fathead minnows. Dosages of pharmaceuticals were re-administered with each change of the tank water (every 2 days). Fish were exposed to treatments for eighteen days.

Gene Set Enrichment Analysis

Fish mRNA was pooled within a tank for microarray work (for 3 replicates per treatment) but not for qPCR validation (for 15 replicates per treatment). Details of microarray experiments, including validation by qPCR analysis of 9 genes with high rank correlation and all data files, are described in a previous report [38]. Microarray experiments conformed to MIAME guidelines and results were deposited in GEO (GSE22261). The previous study [38] also described an altered phenotype associated with pharmaceutical treatment that involved measuring fish behavior in response to a startle stimulus modeled after predator avoidance behavior used elsewhere [49]. We found that fish behavior was indicative of a neurologically relevant phenotype: following a “startle,” the distance traveled and number of direction changes both significantly increased for treated fish [38].

Here, two groups of gene sets gene-class analyses were conducted: ND (”neurological disorder”) and ASD (”autism spectrum disorder”). The gene sets in the ND collection, known to be associated with a variety of human neurological disorders, are described in Table 2. The gene sets in the ASD collection, associated with enriched gene expression in autism, are described in Table 3. Both gene sets are provided in Supporting Information (Table S1, “ND gene list,” and Table S2, “ASD gene list”). Each group consisted of a collection of gene sets, with each set tested against the ranked list of genes reflecting signal-to-noise ratio of MIX treatment (combining FLX, VNX & CBZ) relative to control. Additional comparisons between the control and treatments consisting of the three pharmaceuticals considered individually were included for comparison (Tables 6, 7).

Gene-class analyses used GSEA release 2.06 and MSigDB release 2.5. Weighted enrichment scores were calculated using gene expression lists ranked by signal-to-noise ratio. The genes on the array were ranked by correlation between the MIX and CTL treatments (those genes with the strongest up-regulation in treatment relative to control were ranked highest; those with strongest down-regulation were ranked lowest). (See Table S3, “Ranked gene list,” for these data.) 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. Permutations were conducted by gene set (rather than by phenotype). For details of GSEA parameter usage, see Subramanian et al. [36].

Gene sets were examined to ensure they contained only GSEA-recognized primary HUGO symbols, rather than aliases or unapproved symbols. This was accomplished through the use of a custom script that compared each gene in a given set to the GENE_SYMBOLS.chip file (from GSEA) containing a list of HUGO symbols with accepted aliases. Gene set components listed as aliases in this file were replaced with the appropriate HUGO symbol. For additional details of the annotation and GSEA implementation using the EcoArray 15k Fathead Minnow arrays, see Thomas et al. [37].

Supporting Information

Table S1 ND gene list. A list of the neurological disorders gene sets, in the GSEA gene matrix (.GMX) file format. (GMX)

Table S2 ASD gene list. A list of the autism spectrum disorders gene sets, in the GSEA gene matrix (.GMX) file format. (GMX)

Table S3 Ranked gene list. A Microsoft Excel spreadsheet containing all annotated array gene elements sorted by signal-to-noise ratio for the mixture v. control comparison. (XLS)

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Author Contributions

Conceived and designed the experiments: MAT RDK. Performed the experiments: MAT. Analyzed the data: MAT. Contributed reagents/materials/analysis tools: MAT RDK. Wrote the paper: MAT RDK.

References

1. Landrigan PJ (2010) What causes autism? Exploring the environmental contribution. Current Opinion in Pediatrics 22: 219–225.

2. Currenti SA (2010) Understanding and Determining the Etiology of Autism. Cellular and Molecular Neurobiology 30: 161–171.
31. Cunningham VL, Binks SP, Olson MJ (2009) Human health risk assessment from the presence of human pharmaceuticals in the aquatic environment. Regulatory Toxicology and Pharmacology 53: 39–45.
32. Schwab BW, Hayes EP, Fount JM, Mastrocco JJ, Rodem SM, et al. (2005) Human pharmaceuticals in surface water: A human health risk assessment. Regulatory Toxicology and Pharmacology 42: 296–312.
33. Metcalfe CD, Chu SG, Jud C, Li HK, Oakes KD, et al. (2010) Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environmental Toxicology and Chemistry 29: 79–89.
34. Gelz MD, Tso J, Aagaard DS (2009) Pharmaceutical metabolites in the environment: Analytical challenges and ecological risks. Environmental Toxicology and Chemistry 28: 2473–2484.
35. Madureira TV, Barreiro JC, Rocha MJ, Rocha E, Cas QB, et al. (2010) Spatial-temporal distribution of pharmaceuticals in the Douro River estuary (Portugal). Science of the Total Environment 445: 5513–5520.
36. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America 102: 15440–15445.
37. Thomas MA, Yang LB, Carter B, Klaper RD (2011) Gene set enrichment analysis of microarray data from Pimephales promelas (Rafinesque), a non-mammalian model organism. BMC Genomics 12: 60.

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6. Hughes JR (2009) Update on autism: A review of 1300 reports published in 2008. Epidemiol & Behavior 16: 369–389.
7. Pinto D, Pagamonta AT, Klei L, Ameye R, Merico D, et al. (2010) Functional impact of global rare copy number variation in autism spectrum disorders. Nature 466: 368–372.
8. Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, et al. (2007) Strong association of de novo copy number mutations with autism. Science 316: 445–449.
9. Loisler JR, Wang K, Cai GQ, Korvatska O, Kim CE, et al. (2009) Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. Nature 459: 569–573.
10. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, et al. (2011) Genetic heterogeneity shapes Environmental Factors Among Twin Pairs With Autism. Arch Gen Psychiatry: arxiv:2011.10761.
11. Johnson CP, Myers SM, Coad Children D (2007) Identification and evaluation of children with autism spectrum disorders. Pediatrics 120: 1183–1215.
12. Rice C, Nicholas J, Ruiz J, Petrygrove S, Lee LG, et al. (2016) Changes in autism spectrum disorder incidence in 4 areas of the United States. Disability and Health Journal 3: 186–201.
13. McDonald ME, Paul JF (2010) Timing of Increased Autistic Disorder Cumulative Incidence. Environmental Science & Technology 44: 2112–2118.
14. Ulrich ML, Gray JA, Roth BL (2009) The Expanded Biology of Serotonin: Annual Review of Medicine 60: 355–366.
15. McNama IM, Borella AW, Bialows LA, Whaiter-Armist PM (2008) Further studies in the developmental hyperperoxonemia model (DHP of autism): behavioral and behavioral changes. Brain Research 1189: 203–214.
16. Simpson KL, Weaver KJ, de Villers-Sidani E, Lu JYF, Cai ZW, et al. (2011) Characteristics of fetal anticonvulsant syndrome associated autistic disorder. Developmental Medicine and Child Neurology 47: 551–555.
17. Groc CN, Grether JK, Yoshida CK, Oechli R, Hendrick V (2011) Antidepressant Use During Pregnancy and Normal Milestone Development at 6 and 19 Months of Age. Pediatrics 125: E600–E608.
18. Faveliere S, Nourission A, Jafarri N, Pochat MCP (2010) Treatment of depression during pregnancy and the risk of spontaneous abortion. Canadian Medical Association Journal 182: 1031–1037.
19. Pedersen LH, Heruksen TB, Olsen J (2010) Fetal Exposure to Antidepressants and Normal Milestone Development at 6 and 19 Months of Age. Pediatrics 125: E600–E608.
20. Gentile S (2010) Neurodevelopmental effects of prenatal exposure to psychotropic medications. Depression and Anxiety 27: 675–686.
21. Oberlander TF, Gingrich JA, Ansezie MS (2009) Sustained Neurobehavioral Effects of Exposure to SSRI Antidepressants During Development: Molecular to Clinical Evidence. Clinical Pharmacology & Therapeutics 86: 672–677.
22. Oberlander TF, Papdies M, Brain U, Misri S, Ross C, et al. (2010) Prenatal Effects of Selective Serotonin Reuptake Inhibitor Antidepressants, Serotonin Transporter Promoter Genotype, SLC6A4, and Maternal Mood on Child Behavior at 3 Years of Age. Archives of Pediatrics & Adolescent Medicine 164: 444–451.
23. Croen LA, Grether JK, Yoshida CK, Oechli R, Hendrick V (2011) Antidepressant Use During Pregnancy and Normal Milestone Development at 6 and 19 Months of Age. Pediatrics 125: E600–E608.
24. Bromley RL, Baker GA, Meador KJ (2009) Cognitive abilities and behaviour of children exposed to antiepileptic drugs in utero. Current Opinion in Neurology 22: 162–166.
25. Doiron-Ramfay D, Vousch P, Le Guiguet AM, Garreau L, Ternant D, et al. (2010) Behavior and serotonergic disorders in rats exposed in prenatal to valproate: A model for autism. Neuroscience Letters 470: 35–39.
26. Schneider T, Roman A, Basta-Kaim A, Kubera M, Budziszewska B, et al. (2008) Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 33: 728–740.
27. Bromley RL, Mawer G, Clayton-Smith J, Baker GA, Liverpool, et al. (2008) Autism spectrum disorders following in utero exposure to antiepileptic drugs. Nature 451: 1993–1993.
28. Hadjiikani N (2010) Serotonin, pregnancy and increased autism prevalence: Is there a link? Medical Hypotheses 74: 800–803.
29. Jemba PK (2006) Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. Ecotoxicology and Environmental Safety 63: 115–130.
30. Santos L, Araujo AN, Fachini A, Pena A, Delerue-Matos C, et al. (2010) Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. Journal of Hazardous Materials 175: 45–95.
31. Mennigen JA, Sasse J, Theodore VL, Moon TW (2010) Waterborne fluoxetine disrupts feeding and energy metabolism in the goldfish Carassius auratus. Aquatic Toxicology 100: 126–137.
57. Alonso SG, Catala M, Maroto RR, Gil JLR, de Miguel AG, et al. (2010) Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain). Environment International 36: 195–201.
58. Gros M, Petrovic M, Ginrebreda A, Barcelo D (2010) Removal of pharmaceuti- cals during wastewater treatment and environmental risk assessment using hazard indexes. Environment International 36: 15–26.
59. Stackelberg PE, Gibs J, Furlong ET, Meyer MT, Zaugg SD, et al. (2007) Efficiency of conventional drinking-water-treatment processes in removal of pharmaceuticals and other organic compounds. Science of the Total Environment 377: 255–272.
60. Bruce GM, Pleus RC, Snyder SA (2010) Toxicological Relevance of Pharmaceuticals in Drinking Water. Environmental Science & Technology 44: 5619–5626.
61. Stackelberg PE, Furlong ET, Meyer MT, Zaugg SD, Henderson AK, et al. (2004) Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water treatment plant. Science of the Total Environment 329: 99–113.
62. DasBanerjee T, Middleton FA, Berger DF, Lombardo JP, Sagsvolden T, et al. (2008) A Comparison of Molecular Alterations in Environmental and Genetic Rat Models of ADHD: A Pilot Study. American Journal of Medical Genetics Part B-Neuropsychiatric Genetics 147B: 1354–1363.
63. Traynor J, Agarwal P, Lazzeroni L, Francke U (2002) Gene expression patterns vary in clonal cell cultures from Rett syndrome females with eight different MECP2 mutations. BMC Medical Genetics 3: 12.
64. Nishimura Y, Martin GL, Lopez AV, Spence SJ, Alvarez-Retuerto AI, et al. (2007) Genome-wide expression profiling of lymphoblastoid cell lines distinguishes different forms of autism and reveals shared pathways. Human Molecular Genetics 16: 1682–1690.
65. Martins-De-Souza D, Dias-Neto E, Schmitt A, Falkai P, Gormanna P, et al. (2010) Proteome analysis of schizophrenia brain tissue. World Journal of Biological Psychiatry 11: 110–120.
66. Blalock EM, Geddes JW, Chen KC, Porter NM, Marksberry WR, et al. (2004) Incipient Alzheimer’s disease: Microarray correlation analyses reveal major transcriptional and tumor suppressor responses. Proceedings of the National Academy of Sciences of the United States of America 101: 2173-2178.
67. Iwamoto K, Kakinouchi C, Bundo M, Ikeda K, Kato T (2004) Molecular characterization of bipolar disorder by comparing gene expression profiles of postmortem brains of major mental disorders. Molecular Psychiatry 9: 406–416.
68. Matigian N, Windus L, Smith H, Filipovich C, Pantelis C, et al. (2007) Expression profiling in monozygotic twins discordant for bipolar disorder reveals dysregulation of the WNT signalling pathway. Molecular Psychiatry 12: 815–825.
69. Bumperazzi R, Ringuer M, Kim S, Bittner ML, Khan J, et al. (2003) Gene expression profile in multiple sclerosis patients and healthy controls: identifying pathways relevant to disease. Human Molecular Genetics 12: 2191–2199.
70. Gilli F, Lindberg RLP, Valentino P, Marnetto F, Malacchi S, et al. (2010) Learning from Nature: Pregnancy Changes the Expression of Inflammation-Related Genes in Patients with Multiple Sclerosis. Plos One 5.
71. Chakrabarti B, Dufbridge F, Kent L, Wheelwright S, Hil-Cassolle G, et al. (2009) Genes Related to Sex Steroids, Neural Growth, and Social-Emotional Behavior are Associated with Autistic Traits, Empathy, and Asperger Syndrome. Autism Research 2: 157–177.
72. Hu VW, Nguyen A, Kim KS, Steinberg ME, Sarachana T, et al. (2009) Gene Expression Profiling of Lymphoblasts from Autistic and Nonaffected Sib Pairs: Altered Pathways in Neuronal Development and Steroid Biosynthesis. Plos One 4: 13.