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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.

1. Introduction

Metabolically active pharmaceuticals have been detected in wastewater, 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 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 (Metcalfe 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

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Table 1
Observed values of psychoactive pharmaceuticals in various systems.

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

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 (Metcalfe 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.