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Transcriptome analysis of the brain of the sea bream (*Sparus aurata*) after exposure to human pharmaceuticals at realistic environmental concentrations

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ABSTRACT

Human pharmaceuticals such as Acetaminophen, Atenolol and Carbamazepine are pseudo persistent aquatic pollutants with yet unknown sub-lethal effects at environmentally relevant concentrations. Gilthead seabream (*Sparus aurata*) were exposed to Acetaminophen: 31.90 ± 11.07 μg L^{-1}; Atenolol: 0.95 ± 0.38 μg L^{-1} and Carbamazepine: 6.95 ± 0.13 μg L^{-1} in a 28 day flow through experiment to (1) determine whether exposure to low concentrations in the μg·L^{-1} range of the pharmaceuticals alters the brain transcriptome and, (2) identify different expression profiles and treatment specific modes of action and pathways. Despite low exposure concentrations, 411, 7 and 612 differently expressed transcripts were identified in the individual treatments with Acetaminophen, Atenolol and Carbamazepine, respectively. Functional analyses of differentially expressed genes revealed a significant over representation of several biological processes, cellular compartment features and molecular functions for both Acetaminophen and Carbamazepine treatments. Overall, the results obtained in seabream brain suggest similar physiological responses to those observed in humans also at environmental concentrations, as well as the existence of treatment specific processes that may be useful for the development of biomarkers of contamination.

1. Introduction

Pharmaceuticals are being released into the environment in large quantities on a regular basis. Thousands of prescription and nonprescription drugs are purchased and ingested or applied on millions individuals every day. Since medical substances are developed with the intent of performing some sort of biological function, most of them are relatively stable to avoid being biologically inactivated before carrying out their intended pharmaceutical effects in the body. Unfortunately, this stability causes also their persistence outside the human body, which can create environmental problems (European Environmental Agency, 2010). Un-metabolized pharmaceuticals are thus among the most non-biodegradable substances in the environment (Stuer-Lauridsen et al., 2000). Ingested drugs are eventually excreted from individuals through urine or feces but these products are being released not only after usage, but also during manufacturing and disposal of unused or expired drugs (Boxall and Breton, 2003) ultimately winding up in the effluent of sewage treatment plants and aquatic environments. Although some pharmaceuticals present high transformation and removal rates in sewage treatment plants, they may display the same exposure characteristics as persistent pollutants as their high transformation and removal rates are often compensated by their continuous input into the environment. The exact effects that each drug is having on ecosystems, biota, and humans on the long term, however, are still not completely understood. Some pharmaceutical substances have already shown to

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pose potential adverse human and environmental effects from indirect exposure (Halling-Sørensen et al., 1998; Cleuvers, 2004; Harder, 2003; Webb et al., 2003), for others, including most metabolites, long-term effects and non-target organisms, important gaps of knowledge still exist.

Chronic low-dose effects generally do not cause overt toxicities such as mortality or formation of histopathologies, but may cause adverse ecological outcomes through rather subtle changes in the health and physiological traits (e.g. behavior) of the aquatic organisms which can ultimately have ecological relevance (Relyea and Hoverman, 2006). This may be particularly relevant for chemicals with specific modes of action such as pharmaceuticals (e.g. Segner, 2011; Shore et al., 2014). Also, higher level effects such as physiological and pathological changes of the exposed organism are related to underlying changes at the molecular level. To be able to detect and understand such complex effects and the involved causative processes, investigations at the cellular and molecular levels are as essential as studies of ecological processes (Hampel et al., 2015). In this context "omic" techniques have been gaining more and more importance in ecotoxicology to understand how large numbers of genes, proteins or metabolites interact with each other under different conditions displaying characteristic expression profiles which help to decipher the mode of action of a certain (group of) chemicals.

Some of the most important groups of pharmaceuticals that are currently found in aquatic environments are analgesics, anti-epileptics and β-blockers (Buser et al., 1998; Ternes, 1998; Ternes et al., 1999; Kümmerer, 2001; Kolpin et al., 2002). In this study, we have chosen the three most representative pharmaceuticals from the above mentioned groups: Acetaminophen (APAP; analgesic), Carbamazepine (CBZ; anti-epileptic) and Atenolol (AT; β-blocker) as model compounds to assess the effects on the brain transcriptome of the gilthead sea bream (*Sparus aurata* Linnaeus, 1758) after exposure to low concentrations in the $\mu g \cdot L^{-1}$ range, close to environmentally relevant concentrations (Bendz et al., 2005; Gros et al., 2006; Camacho-Muñoz et al., 2010).

The gilthead sea bream is a marine teleost that belongs to the family *Sparidae* with great importance in fisheries and aquaculture. Sea bream have been frequently employed in the effect evaluation of environmental contaminants (Hampel and Blasco, 2002; Gabbianelli et al., 2003; Hampel et al., 2004; Serrano Gallego et al., 2008; Barros et al., 2011) as they are important sentinel species in aquatic ecosystems. The great importance of the gilthead sea bream for marine aquaculture has triggered an important research effort in many different areas improving significantly, in recent years, the genomic toolkit for this species, such as the assembly of reference transcriptomes and custom oligo DNA microarray platforms (Ferraresso et al., 2008; Calduch-Giner et al., 2013; Louro et al., 2016).

Concentrations found in sewage treatment plant influents and effluents generally range between $\mu g \cdot L^{-1}$ and $ng \cdot L^{-1}$ levels in ground and surface waters, being those for AT and CBZ generally one or two orders of magnitude lower than APAP (Gros et al., 2006; Gómez et al., 2007; Choi et al., 2008; Focazio et al., 2008). APAP is a non-steroid anti-inflammatory and analgesic drug. The exact molecular processes of APAP are not known yet, and only a limited number of pathways have been identified (Salminen et al., 1997; Liu et al., 1999). Effects of APAP on gene and protein expression in rodents have been published elsewhere (Reilly et al., 2001; Ruepp et al., 2002; Jaeschke and Bajt, 2006). AT is a selective β-adrenergic receptor antagonist or β-blocker for the treatment of angina, glaucoma, heart failure, high blood pressure and other related conditions (Black and Stephenson, 1962; Bowman and Rand, 1980; Toda, 2003). An extensive review about the comparative physiology, pharmacology and toxicology of β-blockers, including AT, in fish has been published (Owen et al., 2007). Finally, CBZ is a mood-stabilizing treatment for bipolar affective disorder. The molecular mechanisms underlying the actions of CBZ and the illness itself are unknown. However, several molecular mechanisms have been postulated as possible targets of mood stabilizing drugs (Berridge et al., 1989; Klein and Melton, 1996; Lucas and Salinas, 1997; Beutler et al., 2005; Lee et al., 2007).

The purpose of this study is to determine sets of transcripts, in the brain of gilthead sea bream, whose expression is changed in response to environmentally discharged pharmaceuticals. Although brain derived cDNA microarrays for teleost fish are now developed (van der Ven et al., 2005; van der Ven et al., 2006; Martyniuk et al., 2006), there are still few studies that investigate the effects of pharmaceuticals found in the environment on brain function. However, it is important to profile the genomic response in the brain which contains major sites for neuroendocrine control of different processes on which exposure to environmentally released pharmaceuticals may exert effects. Overall, the ontological analysis of the sea bream transcriptome associated with the pharmaceutical treatments will provide an indication of the potential of these compounds for eliciting toxic or other responses.

2. Material and methods

2.1. Exposure

APAP (CAS Nº: 103-90-2), AT (CAS Nº: 29122-68-7) and CBZ (CAS Nº: 298-46-4) were purchased from Sigma. Gilthead sea bream individuals (approximately one year old) were purchased from Cupimar S.A. aquaculture facility (San Fernando, Spain). The fish were maintained under laboratory conditions for acclimatisation for 14 days. After that, 15 fish per treatment were exposed for 28 days to environmentally relevant concentrations of the selected pharmaceutical compounds under continuous flow through conditions. Nominal exposure concentrations were: APAP: 50 $\mu g\ L^{-1}$; AT: 5 $\mu g\ L^{-1}$ and CBZ: 5 $\mu g\ L^{-1}$. Stock solutions of 10 $g\ L^{-1}$ for APAP and 1 $g \cdot L^{-1}$ for AT and CBZ were prepared in DMSO and working stock solutions supplied to the tanks were obtained by dilution of the stock solutions in sea water (SW) maintaining the DMSO concentration in the tanks at 0,0005% (v/v). Control and solvent control trials were run simultaneously. A peristaltic pump supplied the compounds from daily renewed working stock solutions. Water flow through the system was adjusted to 360 $L\ d^{-1}$. Water samples were collected from each tank at days 1, 5, 15 and 21 and stored at 4 °C not longer than 24 h until their pre-treatment for posterior analysis by high performance liquid chromatography (HPLC). After 28 days of exposure, all fish per treatment were sacrificed and brain tissues sampled. Final weights and length were measured. The brain was rapidly submerged into TriReagent (Sigma), homogenised on ice using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, U.K.), and immediately frozen and stored at −80 °C for transcriptome analyses.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish National Council for Scientific Research (CSIC). The protocol was approved by the Sub-Committee of Bioethics on Animal Experiments of the CSIC. All surgery was on ice, and all efforts were made to minimize suffering.

2.2. Exposure concentration analysis

Exposure concentrations were measured as described by Santos et al., 2005. Briefly, after solid phase extraction (OASIS HLB cartridges; 60 mg, 3 mL; Waters, Milford, MA, USA), the analytes were separated under isocratic conditions with acetonitrile and a 50 mM

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