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An exploratory investigation of various modes of action and potential adverse outcomes of fluoxetine in marine mussels

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ABSTRACT

The present study investigated possible adverse outcome pathways (AOPs) of the antidepressant fluoxetine (FX) in the marine mussel Mytilus *galloprovincialis*. An evaluation of molecular endpoints involved in modes of action (MOAs) of FX and biomarkers for sub-lethal toxicity were explored in mussels after a 7-day administration of nominal FX concentrations encompassing a range of environmentally relevant values (0.03–300 ng/L). FX bioaccumulated in mussel tissues after treatment with 30 and 300 ng/L FX, resulting in bioconcentration factor (BCF) values ranging from 200 to 800, which were higher than expected based solely on hydrophobic partitioning models. Because FX acts as a selective serotonin (5-HT) re-uptake inhibitor increasing serotonergic neurotransmission at mammalian synapses, cell signaling alterations triggered by 5-HT receptor occupations were assessed. cAMP levels and PKA activities were decreased in digestive gland and mantle/gonads of FX-treated mussels, consistent with an increased occupation of 5-HT1 receptors negatively coupled to the cAMP/PKA pathway. mRNA levels of a ABCB gene encoding the P-glycoprotein were also significantly down-regulated. This membrane transporter acts in detoxification towards xenobiotics and in altering pharmacokinetics of antidepressants; moreover, it is under a cAMP/PKA transcriptional regulation in mussels. Potential stress effects of FX were investigated using a battery of biomarkers for mussel health status that included lysosomal parameters, antioxidant enzyme activities, lipid peroxidation, and acetylcholinesterase activity. FX reduced the health status of mussels and induced lysosomal alterations, as suggested by reduction of lysosomal membrane stability in haemocytes and by lysosomal accumulation of neutral lipids in digestive gland. No clear antioxidant responses to FX were detected in digestive gland, while gills displayed significant increases of catalase and glutathione-*s*-transferase activities and a significant decrease of acetylcholinesterase activity. Though AOPs associated with mammalian therapeutic MOAs remain important during assessments of pharmaceutical hazards in the environment, this study highlights the importance of considering additional MOAs and AOPs for FX, particularly in marine mussels.

1. Introduction

A substantial amount of work has been done on the occurrence and fate of pharmaceuticals in surface waters, including some regulatory developments (WHO, 2012). Though the majority of studies on pharmaceutical fate and effects have occurred in inland waters, coastal marine environments have been rather neglected despite the high number of human population living on the coasts and the consequent highly relevant impact. Much has still to be learned on the effects of pharmaceuticals on aquatic organisms, and debate has arisen on whether the standard ecotoxicological methods are able to identify ecologically relevant effects (Boxall et al., 2012; Brausch et al., 2012; Brooks et al., 2009; Schmitt et al., 2010).

Pharmaceuticals behave quite differently from conventional pollutants, being designed to interact with specific cellular targets (e.g., receptors, enzymes) in humans, while they can be pharmacologically active in organisms in which the drug targets are expressed and functional. Therefore, the evolutionary conservation of molecular targets in a given species could potentially increase the risk of eco-toxicological effects (Gunnarsson et al., 2008; Huggett et al., 2003). Further effects classified as secondary and irrelevant for therapeutic activity in humans may play an important role in

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non-target organisms (Seiler, 2002), and unknown toxic actions cannot be excluded, depending on the pathways of interactions and potentially unknown cross-talk triggered in aquatic animals (Berninger and Brooks, 2010).

Recent reports outlined that adverse outcomes may be observed following pharmaceutical exposures at concentrations approaching environmental levels. For example, Brooks et al. (2012) recent predicted that 10% of pharmaceuticals may present hazards to aquatic life below 29 ng/L. Therefore, identifying biological endpoints related to therapeutic modes/mechanisms of action (MOA) or molecular initiation events, and to sub-lethal toxicity outcomes, becomes a priority task in the framework of pharmaceutical risk assessment (Ankley et al., 2007, 2010), particularly for antidepressants (Brooks et al., 2003a). A recent study by Valenti et al. (2012) employed an Adverse Outcome Pathway (AOP; Ankley et al., 2010) approach to link a therapeutic molecular initiation event response (serotonin reuptake transporter, 5-HTT) to sublethal behavioral adverse outcomes in the fathead minnow following exposure to sertraline (ST). Similar studies in marine bivalves are lacking. This data gap appears of particular concern given previous observations of bioaccumulation of the antidepressants fluoxetine (FX) and ST by fish (Brooks et al., 2005; Chu and Metcalfe, 2007; Ramirez et al., 2007, 2009) and bivalves from inland waters (Bringolf et al., 2010; reviewed by Daughton and Brooks, 2011). More recently, a work by Klosterhaus et al. (2013) identified accumulation of ST in marine bivalves from the San Francisco Bay, California, USA. Clearly, an understanding of FX and other antidepressant influences on marine bivalves is needed. Though assessment of therapeutic MOAs and related AOPs remain critically important for future studies (Boxall et al., 2012), it also remains important to consider that many pharmaceuticals elicit responses through multiple targets, and thus a number of AOPs may be triggered by a single pharmaceutical. For example, the US EPA Tox21 program (http://epa.gov/ncct/Tox21/) is examining biological activities of numerous compounds with hundreds of *in vitro* assays in an attempt to identify various toxicological targets of industrial chemicals, including a number of pharmaceuticals. In the case of diphenhydramine, a first generation antihistamine with a primary therapeutic activity of blocking the H1 histamine receptor (Brown and Roberts, 2001), its ability to block 5-HT reuptake stimulated development of FX and other selective serotonin reuptake inhibitor antidepressants (Wong et al., 2005). Further, diphenhydramine blocks the acetylcholine receptor (Brown and Roberts, 2001), which may appears to explain comparative sensitivity of aquatic invertebrates in a recent study by Berninger et al. (2011). For FX in particular, additional MOAs and potential AOPs appear important. For example, Brooks et al. (2003b) observed green algae to be more sensitive to FX than invertebrate and fish models; this effect was not expected because algae do not possess the mammalian therapeutic target for FX. Here again, an advanced understanding of FX interaction with various targets in marine bivalves seems necessary.

In the present study, our primary objective was to explore several MOA relevant endpoints and biomarkers for sub-lethal toxicity responses to investigate possible adverse outcome pathways of FX on a marine invertebrate species, the Mediterranean mussel (*Mytilus galloprovincialis*), which was used as a indicator species to assess pharmaceutical impacts on coastal environments. Specifically, the approach employed is related (i) to specific effects of FX on parameters coupled to the 5-HT_1 signaling pathway in mammals, since 5-HT_1 is the sole 5-HT receptor documented in mussels (Cubero-Leon et al., 2010), and (ii) to non-specific effects induced by FX due to its chemical properties. cAMP levels, PKA activity and mRNA expression for the mussel 5-HT_1 receptor were assessed as molecular endpoints involved in 5-HT_1 signaling pathway and as potential targets for FX specific effects in mussels. mRNA expression of the ABCB gene encoding the MXR-related ABC transporter P-glycoprotein was assessed to find out whether impairment of regulatory pathways may affect the ability of animals to elaborate strategies of defense or adaptation toward further stress factors. Finally, non-specific effects induced by FX were investigated through biomarker analysis. Biomarkers represent the first early-warning signals of environmental disturbance, even in the absence of acute toxic responses observed in organisms (Viarengo et al., 2007). We carried out a multi-biomarker approach that reflects the general health status of the mollusks and the counteracting responses developed by mussels. The simultaneous measurement of FX bioaccumulation provided further insights, since only a few papers report on both aspects.

2. Materials and methods

2.1. Experimental animals and holding conditions

Adult specimens of *M. galloprovincialis* (5–7 cm in length) were collected from the northwestern Adriatic Sea coast by fisherman of the "Cooperativa Copr.al.mo" (Cesenatico, Italy), and transferred to the laboratory in seawater tanks with continuous aeration. Animals (30 per aquarium) were acclimated for 3 days in aquaria containing 60 L of aerated artificial 35 psu seawater at 16 °C, under a natural photoperiod. Mussels were fed once a day with an algal slurry (Koral filtrator, Xaqua, Italy). Fifteen mussels were sampled at zero time to assess parameters at the onset of each experiment.

2.2. Experimental design

At the onset of the experiment, mussels were transferred to 6 $440 \times 375 \times h$ 190 mm plastic vessels containing 20 L of water. Each vessel contained 20 mussels. One liter of seawater for each mussel proved to be the suitable volume of water to avoid overloading and prevent the onset of unfavorable health conditions for mussels. For each experimental treatment level, 6 vessels containing a total of 120 mussels represented the 6 replicates.

Mussels were exposed for 7 days to nominal 0.03, 0.3, 3, 30 and 300 ng/L fluoxetine ((±)-N-Methyl-γ-[4-(trifluoromethyl) phenoxy]benzenepropanamine), which was obtained from Sigma Aldrich (Milan, Italy) (purity ≥ 98%) as fluoxetine hydrochloride (FX). Nominal concentrations of exposure were chosen within the environmental range for the compound (Fig. 1). A group of unexposed (control) mussels were maintained in parallel to the treatment groups.

As the vessels used in this study are made up of a plastic material for use with foodstuff, vessels walls should neither absorb nor release chemicals. Although we cannot exclude that some interaction of FX with plastic could have occurred, our observations in previously published studies with FX (e.g., Brooks et al., 2003b; Stanley et al., 2007) suggest it is minimal, also considering the concentrations used for present treatments.

FX hydrochloride is water soluble up to 4 mg/mL according to the manufacture's datasheet, so that we avoided using further (organic) solvents, and a stock solution was prepared in distilled milliQ-grade water at a 0.1 mg/mL concentration, aliquoted and stored at −20 °C. At each FX administration, 1 aliquot of stock solution was employed and serially diluted (in distilled milliQ-grade water) to achieve the suitable volume to be spilled in each vessel (max 1 mL depending on the final concentration to be achieved in each vessel). Half-life for FX in water solutions ranged between 277 and 102 days at pH 7 and 9, respectively (Kwon and Armbrust, 2006), whereas in this study pharmaceutical administration was on a daily base along with mussel feeding and after water changes.

At the end of the experiment, haemolymph samples were taken from the abductor muscle of individual mussels. The gills, digestive