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Evidence of neuroendocrine disruption in freshwater mussels exposed to municipal wastewaters

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ABSTRACT

The purpose of this study was to test the hypothesis that exposure to municipal effluents can disrupt the neuroendocrine system in *Elliptio complanata* freshwater mussels. The capacity of ozonation to mitigate these effects was also examined. Mussels were exposed for 14 days to a continuous flow of increasing concentrations of the effluent before and after ozonation. Neuroendocrinal effects were examined by tracking changes in acetylcholinesterase, glutamate, gamma-aminobutyrate, serotonin, dopamine and their respective adenylcyclase activities in synapse membranes, monoamine oxidase and vitellogenin-like proteins. Oxidative stress and damage were examined by superoxide dismutase and lipid peroxidation, respectively, in the visceral tissues. The results revealed that the exposure of freshwater mussels increased the levels of vitellogenin-like proteins in both the primary-treated and ozonated effluents, dopamine and glutamate, and decreased the turnover of the neurostimulant acetylcholine. Moreover, these endpoints were significantly correlated with oxidative stress and damage. A canonical analysis of the responses revealed that dopamine and the neuroexcitatory neuromediators-acetylcholesterase and glutamate-were the endpoints more strongly related with oxidative stress and damage. Mussel morphology and estrogenic biomarkers (vitellogenin-like proteins, gonad lipid stores) were also significantly related, albeit to a lesser extent, to oxidative stress and damage. In general, ozone treatment was not sufficient to mitigate the observed neuroendocrinal effects in freshwater mussels. We conclude that the continuous exposure of freshwater mussels to municipal wastewater effluents leads to neuroendrocinal alterations and to oxidative stress.

1. Introduction

Urban effluents are major sources of a complex array of pollutants in the aquatic environment. Many of the chemicals identified as potent endocrine disruptors include 17α-ethynyl-estradiol-17β (used in birth control drugs), nonylphenol (a breakdown product of alkylphenol polyethoxylate surfactants), and estradiol-17β (Sumpter and Jobling, 1995; Sabik et al., 2003; Verthaak et al., 2005). Moreover, these wastewaters harbor many pharmaceutical and personal care products (PPCPs; Kummerer, 2001) which can not only act as potential endocrine disruptors but disrupt the neuroendocrine pathways in organisms. For example, final maturation of gametes and spawning depends on the balance of the neurotransmitters serotonin and dopamine, which can influence the contraction of smooth muscle to assist egg release in mussels and fish (Gibbons and Castagna, 1984; Fong et al., 1993). This process is further assisted by the production of prostaglandins such as cyclooxygenase, which is the rate-limiting enzyme for the production of prostaglandins (Matsumani and Nomura, 1987; Flippin et al., 2007). Hence, the presence of commonly consumed serotonergic drugs (fluoxetine, tricyclic antidepressants), non-steroidal anti-inflammatory drugs (cyclooxygenase inhibitors) and estrogens (ethynylestradiol) could act as neuroendocrine disruptors of reproduction in aquatic invertebrates and fish. Indeed, tricyclic antidepressants were shown to suppress spawning and fertilization in zebra mussels (Hardedge et al., 1997). A recent study revealed that a physico-chemical treated effluent contains significant amounts of selective serotonin-reuptake inhibitors which were bioavailable to brook trout (Lajeunesse et al., 2011). This study also revealed that sertraline liver tissue level could reach concentrations up to 10 ng/g and was negatively correlated with serotonin-dependent Na/K-ATPase in synapse membrane preparations.

Freshwater mussels are key representatives of the benthic community in many lakes and rivers in countries worldwide. These organisms are particularly at risk of contamination by neuroendocrine disruptors because they are sedentary, long-lived, and filter high volumes of water and suspended matter during respiration and feeding. In searching for markers of neuroendocrine disruption beyond estrogenic chemicals,

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biomarkers that target the mode of action of PPCPs are of value. At a more fundamental level, PPCPs will likely be oxidatively metabolized to assist in their elimination. However, this process could lead to the production of more toxic intermediates. Compounds such as nicotine, cocaine and ethanol could stimulate the release of morphine in mussels and dopamine turnover in nerve cells (Zhu et al., 2006). Dopamine is a neurotransmitter involved in gametogenesis, when vitellogenesis takes place in the scallop (Martinez and Rivera, 1994).

The freshwater unionid *Elliptio complanata* is a sexually dimorphic organism in which females produce eggs containing egg-yolk proteins that are rich in vitellogenin (Vtg). The production of Vtg is controlled, in part at least, by the steroid estradiol-17β (Li et al., 1998; Gagné et al., 2001). The neuromediators dopamine and serotonin are also involved in gamete maturation and spawning, the latter involving serotonin and prostaglandins (Matsumani and Nomura, 1987). These biogenic amines are degraded by a mitochondrial monoamine oxidase (MAO) enzyme complex whose activity can be modulated by various environmental cues (Egashira et al., 2003). Acetylcholine levels are regulated by acetylcholinesterase, which inactivates this neuromuscular excitant (Garcia-Lavanvdeira et al., 2005). The accumulation of glutamate, another neuroexcitant, was involved in excitotoxicity syndromes leading to overstimulation, oxidative stress and inflammation of nerve cells (Olney, 1990; Gagné et al., 2007).

As mentioned above, municipal effluents are well known to contain numerous xenoestrogenic compounds (nonylphenol, ethynylestradiol etc.) including many neuroactive pharmaceutical compounds. The purpose of this study was therefore to test the hypothesis whether municipal effluents could alter the neuroendocrine activity in freshwater mussels. A second hypothesis consisted of verifying whether the application of ozone treatment to a municipal effluent can reduce the observed neuroendocrine disrupting effects of urban effluents from a large, highly populated city to freshwater mussels.

2. Methods

2.1. Mussel handling and exposure to municipal effluents

Freshwater *E. complanata* mussels were collected during the first week of June 2006 in the St. Lawrence River. They were left to stand in 300-L tanks containing UV-treated and charcoal-filtered tap water at 15 °C for 2 to 3 months (which corresponds to the post-spawning stage of the mussels) before initiation of the exposure to the municipal wastewater effluents of a city of 1.5 million inhabitants. The mussels were fed three times a week with *Pseudokirchneriella subcapitata* algal suspensions (100 million cells per feed). For the exposure experiment, mussels (n = 30 individuals) were placed in separate 60-L tanks that received a continuous flow (0.1 L/h) of a physical and chemical-treated effluent before and after ozonation. Hence the mussels were exposed in real-time to the treated municipal effluents. The physical- and chemical-treated effluent consisted of reducing the amount of suspended matter to the mg/L range. The exposure concentrations were 3, 10, and 20% effluent v/v with dechlorinated (charcoal filter) and UV-treated tap water from the City of Montreal (i.e., a number of 7 separate tanks were used). The ozone treatment consisted of passing ozone (generated by electric arcs) through the effluent by a fine bubble diffuser. The ozone concentration ranged between 10 to 20 mg/L in the effluent. Both the physico-chemical-treated and the ozone-treated effluents were maintained for 2 h in the dark before exposure to the mussels to allow removal of gaseous ozone and equilibrate with the atmosphere. The exposure period was terminated after two weeks. Mortality events (18%) were recorded at the highest concentration for both effluents i.e., before and after ozone treatment.

At the end of the exposure period, each individual mussel was allowed to depurate in clean aquarium water overnight in 60-L aquariums at 15 °C. Morphological measurements (mussel weight, shell length, soft tissues weight) were taken and the visceral mass containing the gonad and ganglia were dissected out on ice and homogenized using a Teflon pestle tissue grinder. The homogenization buffer consisted of 125 mM NaCl containing 25 mM Hepes-NaOH, pH 7.4, 1 mM dithiothreitol and 10 μg/mL apoprotinin protease inhibitor. A subsample of the homogenate was centrifuged at 3000 × *g* for 20 min at 2 °C and the supernatant (S3) collected for subsequent isolation of nerve button membranes (synaptosomes) as described below. Another portion of the homogenate was centrifuged at 15 000 × *g* for 30 min at 2 °C and the supernatant (S15) carefully collected from the upper lipid layer. The homogenates, S3 and S15 were stored at −85 °C until analysis. Total proteins were determined using the principle of protein–dye binding with serum bovine albumin for calibration (Bradford, 1976). Synaptosomes were prepared using the sucrose density method (Sherman, 1989). Briefly, the S3 fraction was overlaid on 0.8 M sucrose containing 10 mM Hepes-NaOH, pH 7.4, and 1 mM EDTA. The discontinuous gradient was centrifuged at 10 000 × *g* for 20 min at 2–4 °C to yield the mitochondrial pellet and the synaptosome suspension in the sucrose phase. The sucrose was quickly diluted 1:4 with 10 mM Hepes-NaOH buffer at pH 7.4 and kept on ice before performing the assays.

2.2. Neuroendocrine assessments

Tissue levels of GABA were determined in the S3 fraction using a multi-enzymatic assay (Woff and Klemish, 1991). The assay uses GABA transaminase and succinate semialdehyde dehydrogenase, which catalyzes the formation of the fluorescent cofactor NADPH from α-ketoglutarate and succinate semialdehyde. A volume of 50 μL of the S3 fraction was mixed with 150 μL of 0.2 N HCl and heated at 100 °C for 5 min to remove endogenous NADPH. The levels of GABA were detected by mixing with one volume of 6 mM α-ketoglutarate, 0.1 mM NADP, 3 mM dithiothreitol, 10 units/mL GABA transaminase/succinate semialdehyde dehydrogenase in 0.3 M Tris–HCl pH 8.4. The reaction was allowed to proceed for 30 min at 37 °C and the formation of NAPDH was tracked at 360 nm excitation and 460 nm emission wavelengths (Chameleon-II, Bioscan, USA). External solutions of GABA (and glutamate) were used for calibration. The levels of glutamate were determined using the glutamate oxidase methodology developed by Invitrogen Inc. (A12216). The assay kit consists of the reagents and glutamate oxidase enzyme complex and detection of hydrogen peroxide by the Amplex® Red reagent. The fluorescence of resorufin was tracked at 535 nm excitation and 630 nm emission wavelengths. The data for glutamate and GABA were both expressed as μmol/mg proteins. Acetylcholinesterase (ACHE) activity was determined in the S3 fraction using acetylthiocholine as the substrate and Ellman's reagent for detection (Bonacci et al., 2004). A standard solution of freshly prepared reduced glutathione was used for calibration. Data were expressed as the formation of thiols in μmol/min/mg proteins.

The levels of serotonin and dopamine in gonad homogenates were determined in the S15 fraction using a luminescent enzyme-linked immunoassay procedure (Gagné et al., 2010a). Briefly, 96-well luminescence plates (Microlite 2, Thermo Fisher Scientific, Ontario, Canada) were coated with 0.5 μg of BSA-conjugated dopamine (U.S. Biological, Boston, USA) or 1 μg of BSA-conjugated serotonin (Advanced Targeting Systems, San Diego, CA) in 100 μL of 50 mM Tris–HCl, (pH 8.5) at 4 °C overnight. The wells were washed in PBS and 50 μL of standard, prediluted samples or dilution buffer were added to the wells, followed by the addition of 50 μL of primary antibody 1:5000 (rabbit polyclonal to dopamine ab888; Abcam, MA, USA or rabbit polyclonal to serotonin AB-T03; Advanced Targeting Systems, San Diego, CA). After 90 min of incubation at room temperature, with constant shaking, plates were washed three times with PBS and incubated with 100 μL of HRP-conjugated goat anti-rabbit IgG (1:10 000; Stressgen, MI, USA) for 1 h. Wells were washed three times with PBS and HRP enzyme activity was determined using a chemiluminescent