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Rapid modulation of gene expression profiles in the telencephalon of male goldfish following exposure to waterborne sex pheromones

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ABSTRACT

Sex pheromones rapidly affect endocrine physiology and behaviour, but little is known about their effects on gene expression in the neural tissues that mediate olfactory processing. In this study, we exposed male goldfish for 6 h to waterborne 17,20βP (4.3 nM) and $PGF_{2\alpha}$ (3 nM), the main pre-ovulatory and post-ovulatory pheromones, respectively. Both treatments elevated milt volume ($P = 0.001$). Microarray analysis of male telencephalon following $PGF_{2\alpha}$ treatment identified 71 unique transcripts that were differentially expressed ($q < 5\%$; 67 up, 4 down). Functional annotation of these regulated genes indicates that $PGF_{2\alpha}$ pheromone exposure affects diverse biological processes including nervous system functions, energy metabolism, cholesterol/lipoprotein transport, translational regulation, transcription and chromatin remodelling, protein processing, cytoskeletal organization, and signalling. By using real-time RT-PCR, we further validated three candidate genes, ependymin-II, calmodulin-A and aldolase C, which exhibited 3–5-fold increase in expression following $PGF_{2\alpha}$ exposure. Expression levels of some other genes that are thought to be important for reproduction were also determined using real-time RT-PCR. Expression of sGnRH was increased by $PGF_{2\alpha}$, but not 17,20βP, whereas cGnRH expression was increased by 17,20βP but not $PGF_{2\alpha}$. In contrast, both pheromones increase the expression of glutamate (GluR2a, NR2A) and γ-aminobutyric acid ($GABA_A$ γ2) receptor subunit mRNAs. Milt release and rapid modulation of neuronal transcription are part of the response of males to female sex pheromones.

1. Introduction

Chemical communication is important for intra-species communication. Vertebrates have evolved cryptic pheromonal signalling to help navigate the complex environment of cues while avoiding eavesdropping. One such example of signalling is sex pheromones, which vertebrates have used to signal reproductive status and readiness. This strategy obviates the need for species to spend time and energy inspecting one another for reproductive cues, thereby allowing them to expand their range for feeding while expending their energy on growth, development and predator avoidance. Species-specificity of pheromone signalling is achieved through chemical form, size, polarity and blends depending on biosynthetic and environmental constraints (Brennan and Zufall, 2006; Symonds and Elgar, 2008).

Sex pheromones are important for goldfish reproduction because they are oviparous and live in murky waters where visibility is greatly reduced. Therefore, in order for male and female goldfish to coordinate their reproduction, they have established an elaborate and sophisticated chemical communication system to aid in advertising their reproductive status to conspecifics (Stacey, 2003). The sex pheromones in goldfish are sex hormones that have been co-opted through evolution and given new functionality. For example, 17α,20β dihydroxy-4-pregnene-3-one (17,20βP) – the prevovulatory priming pheromone – is an oocyte maturation-inducing steroid hormone in females, and when it is released in the water acts on males to induce milt production and courtship (Sorensen et al., 1998; Stacey, 2003). In addition, prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) is a female hormone that stimulates female sexual behaviour but when released to the water by the female it becomes a postovulatory releaser pheromone that triggers milt production and male sexual behaviour (Sorensen et al., 1988; Stacey et al., 2003). It is now well established that 17,20βP and $PGF_{2\alpha}$ are the primary sex pheromones controlling spawning in goldfish. Female goldfish, responding to environmental cues, release a priming pheromone (17,20βP and its metabolites) triggering an increase

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in luteinizing hormone (LH), milt production and courtship in males (Kobayashi et al., 1986a). A few hours later, the ovulating females emit the releasing pheromone ($PGF_{2\alpha}$ and its metabolites) which synchronizes the sexual behaviour of both sexes leading to spawning (Kobayashi et al., 2002).

Experimental studies on sexually mature male goldfish indicate that sex pheromones acting via the olfactory epithelium induce LH release from the pituitary gland through a suppression of dopaminergic inhibition of gonadotropin-releasing hormone (GnRH) in the preoptic area (POA) to regulate male courtship and sexual behaviour (Kobayashi et al., 1986b, 2002; Stacey, 1983; Trudeau, 1997). However, how sex pheromones regulate genes important for reproduction is not well understood. Thus, we performed a cDNA microarray analysis to profile gene expression in the telencephalon of male goldfish exposed to waterborne $PGF_{2\alpha}$ for 6 h. We chose to profile the effects of $PGF_{2\alpha}$ because it is the potent post-ovulatory pheromone responsible for induction of spawning behaviour and the final increase in milt production (Sorensen et al., 1988, 1989; Stacey et al., 2003). Importantly, $PGF_{2\alpha}$ exposure modulates neurogenesis in the male goldfish coinciding with behavioural changes during spawning (Chung-Davidson et al., 2008; Kobayashi et al., 2002). Additionally, we used targeted real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) on a series of transcripts so that we could compare the effects of the preovulatory pheromone, 17,20βP, to that of post-tovulatory pheromone, $PGF_{2\alpha}$ These transcriptomic data support the physiological data obtained to offer insights into the molecular mechanism underlying sex pheromone-mediated changes in the brain.

2. Materials and methods

2.1. Animals

In the experiment to measure gene expression, goldfish ($N = 36$) were purchased from Aleong's International Inc., (Mississauga, ON, Canada) in April and sorted according to their sexual maturity based on tubercles on their pectoral fins and opercula. The sexually mature male goldfish (14–30 g body weight) were then acclimated to 18 °C in 70-liter round tanks, filled with dechlorinated tap water (12 per tank; three tanks total), for 3 weeks before commencing experimentation. The fish were fed daily on standard goldfish flaked food (Martin Mills, Elmira, ON, Canada) and maintained under natural photoperiod (L:D – 13:11). For all dissections, fish were anaesthetized with 0.05% 3-aminobenzoic acid ethyl ester (MS222: Aquatic Eco-Systems, Apopka, FL). The study was conducted in early June and all experimental procedures were approved by the University of Ottawa Protocol Review Committee and followed the established Canadian Council on Animal Care guidelines on the ethical treatment of animals in research.

2.2. Chemicals and experimental procedure

Stock solutions of 17,20βP and $PGF_{2\alpha}$ (both from Sigma–Aldrich, Oakville, ON, Canada, purity $\geqslant$99%) were prepared by dissolving 1 mg of each of the respective chemical compounds in 1 ml of 95% ethanol (EtOH: Commercial Alcohols Inc., Brampton, ON, Canada, purity $\geqslant$99%) and stored at -20 °C (Sorensen et al., 1989). The vehicle and pheromones were then applied to the fish tanks so that their final concentrations were 0.28×10^{-6}% (v/v) EtOH, 4.3 nM 17,20βP or 3 nM $PGF_{2\alpha}$. These concentrations are based on other studies showing robust effects at this level (Chung-Davidson et al., 2008; Mennigen et al., 2010; Sorensen et al., 1989). The whole telencephalon was rapidly removed, pooled (2 telencephalon per tube) and frozen on dry ice. Samples were then stored at −80 °C until required for RNA isolation.

2.3. Milt volume

Milting males were selected and randomly assigned to groups of 12 in flow-through 70-liter tanks. The fish were then exposed to waterborne 4.3 nM 17,20βP and 3.0 nM $PGF_{2\alpha}$ with the appropriate vehicle control for 6 h. The exposures were timed to coincide with goldfish spawning in the early morning (Kobayashi et al., 2002). Fish were then anaesthetized, stripped of milt before being sacrificed.

Milt, extracted by gently squeezing the abdomen from the mid-ventral region posterior to the gonopore, was drawn by aspiration into a pre-weighted hematocrit tube which was weighed again to calculate the weight of the milt as described and validated elsewhere (Kyle et al., 1985). Milt density is assumed to be 1.0 and milt data are expressed as volumes (μl) rather than as weight. Milt volume was corrected for body weight and the milt/body weight is presented. Fish were stripped of milt twice. The first stripping was conducted before the start of the experiment to reduce *a priori* the intra-individual variability between fish by ensuring that all milt reserves are empty and they all start from the same basal level. The second stripping was to measure the replenishments of the strippable milt reserves following exposure to either sex pheromone.

2.4. RNA extraction and cDNA synthesis

Homogenization of the telencephalon samples was performed using an MM301 Mixer Mill (Retsch, Newton, PA) at 20 Hz for 2 min. Total RNA was, then, isolated from the telencephalon using Qiagen's RNeasy Plus Mini Kit (Mississauga, ON, Canada). Total RNA was extracted by RNeasy kit (Qaigen, Mississauga, ON, Canada). The quality of the RNA was assessed by an Agilent 2100 Bio-Analyzer (Agilent, Palo Alto, CA), and the quantity determined on a NanoDrop spectrophotometer (Nano-Drop Technologies, Wilmington, DE). For real-time reverse transcriptase polymerase chain reaction (RT-PCR), the RNA (2 ug), from control or treatment groups, was reverse transcribed (in a 20 uL volume) into cDNA using Superscript II RNase H^- reverse transcriptase (SSII) with 200 ng random hexamer primers according to Invitrogen's protocol. A negative control, with RNase-free water instead of SSII enzyme (NRT), was also conducted for the cDNA synthesis at the same time.

2.5. Microarray hybridization

We used version 1.1 of our carp-goldfish cDNA microarray which has been validated for use with neuroendocrine tissues (Marlatt et al., 2008; Martyniuk et al., 2006; Mennigen et al., 2008; Popesku et al., 2008; Zhang et al., 2009a). The data from this experiment has been deposited in the Gene Expression Omnibus database (accession number GPL3735). Amplification and cDNA labelling with Cy3 and Cy5 for microarray hybridization followed the manufacturer's instructions (Genisphere, Hatfield, PA, USA).

A total of four microarray slides were hybridized to study the effects of $PGF_{2\alpha}$ on gene expression in the telencephalon. Control samples ($N = 4$) were pooled to form our reference or technical replicates while treatment samples ($N = 4$), which formed our biological replicates, were ran separately (Allison et al., 2006). In addition, two dye swaps were performed for our biological samples for the treatments. This microarray design has also been extensively validated in our laboratory (Marlatt et al., 2008; Martyniuk et al., 2006; Mennigen et al., 2008; Popesku et al., 2008; Zhang et al., 2009a). The arrays were scanned at full speed 10μ resolution