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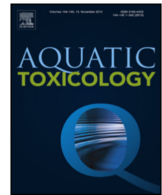

Differential responses of female and male brains to hypoxia in the marine medaka *Oryzias melastigma*

Keng-Po Lai[a,b,*], Jing-Woei Li[c,d], Anna Chung-Kwan Tse[a,b], Simon Yuan Wang[a,b], Ting-Fung Chan[c,d], Rudolf Shiu-Sun Wu[a,b,**]

[a] *School of Biological Sciences, The University of Hong Kong, Hong Kong SAR, China*
[b] *State Key Laboratory in Marine Pollution, Hong Kong SAR, China*
[c] *School of Life Sciences, The Chinese University of Hong Kong, Hong Kong SAR, China*
[d] *State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong SAR, China*

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ABSTRACT

Hypoxia, an endocrine disruptor, affects synthesis and balance of sex steroid hormones, leading to reproductive impairment in both female and male fish. Cumulating reports demonstrated the alternation of hypothalamus-pituitary-gonad axis (HPG-axis) by hypoxia. However, the detail mechanism underlying how hypoxia may alter other brain functions remains largely unknown. In this report, we used marine medaka as a model and conducted a high-throughput RNA sequencing followed by bioinformatics analysis on hypoxia-exposed brain tissues, aiming to determine the change of transcriptional signature and to unravel the pathways that are induced by hypoxia. We found that hypoxia lead to dysregulation of brain functions (including synaptic transmission, axon guidance, potassium ion transport, neuron differentiation, and development of brain and pituitary gland), and also signaling pathways (e.g., gap junction, calcium signaling pathway, and GnRH signaling pathway). Our results further demonstrate gender-specific responses to hypoxia in female and male fish's brains, which provides novel insights into the mechanism underlying the hypoxia induced sex specific brain functions impairments.

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1. Introduction

Hypoxia is an endocrine disruptor and affects hormonal regulation and synthesis along the Hypothalamus-pituitary-gonad axis, leading to reproductive impairment in both female and male fish (Shang et al., 2006; Thomas and Rahman, 2009; Wu et al., 2003). Hormonal disturbance associated with impaired testicular development and reduced sperm production were also found in natural fish populations in the Gulf of Mexico, one of the largest dead zones in the world (Thomas et al., 2007), and decreases in fisheries production over large areas have been reported worldwide (Wu, 2009).

Cumulating evidence demonstrated that production and regulation of various hormones along the fish BPG axis can be altered by hypoxia, and some of the alternations are sex specific while others are common in both sexes (Shang et al., 2006; Thomas et al., 2006). Also, a study in ruffe (*Gymnocephalus cernua*) and flounder (*Platichthys flesus*) revealed that the mRNA expression levels of stress-response genes including members of the anaerobic energy metabolism, oxidative stress and oxygen supply were altered after hypoxic treatment (Tiedke et al., 2014). However, there was hitherto no study which investigated the effect of hypoxia on teleost brain in a transcriptomic level. The present study was therefore carried out to unravel the detailed mechanism underlying how hypoxia may alter brain functions.

Using high-throughput RNA sequencing followed by bioinformatics analysis, this study aims to determine the transcriptional changes induced by hypoxia, and unravel the genes and pathways that are affected by hypoxia which subsequently lead to dysregulation of brain functions (including synaptic transmission, axon guidance, potassium ion transport, neuron differentiation, and development of brain and pituitary gland), and also signaling pathways (e.g., gap junction, calcium signaling pathway, and GnRH signaling pathway). Our results further demonstrate gender-specific responses to hypoxia in female and male fish's brains, which provides novel insights into the mechanism underlying the hypoxia induced sex specific brain functions impairments, not only limited to reproductive axis.

* Corresponding author. Present address: Department of Biology and Chemistry, City University of Hong Kong, Hong Kong SAR, China.
** Corresponding author.
E-mail addresses: balllai@hku.hk, kengplai@cityu.edu.hk (K.-P. Lai), marcowanger@gmail.com (J.-W. Li), annatse@hku.hk (A.C.-K. Tse), bioywang@hku.hk (S.Y. Wang), tf.chan@cuhk.edu.hk (T.-F. Chan), rudolfwu@hku.hk (R.S.-S. Wu).

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2. Materials and methods

2.1. Marine medaka maintenance and hypoxic exposure

All animal research procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR, #2714-12), the University of Hong Kong. The stock of marine medaka *Oryzias melastigma* (*O. melastigma*) used in our experiment was obtained from Interocean Industries (Taiwan) and has been reared in our laboratory for over 10 generations. The marine medaka were maintained under optimal growth and breeding conditions (5.8 mg O_2 L^{-1}, 28 ± 2 °C, pH 7.2 in a 14-h light: 10-h dark cycle) as described in our previous study (Lau et al., 2014). The fish were fed with hormone-free Aquatox Feed (Zeigler Bros., Inc., Gardners, PA) and live artemia. Fifty three-months old sexually matured male and fifty female marine medaka were reared together under normoxia (5.8 ± 0.2 mg O_2/L) or hypoxia (1.5 ± 0.2 mg O_2/L) for one month. There were 6 tank replicates per condition.

2.2. RNA isolation and qualification

After the exposure, the fish were anesthetized in an ice bath. Brain tissues were dissected from randomly selected female fish (*n* = 6 from normoxia and *n* = 6 from hypoxia) and male fish (*n* = 6 from normoxia and *n* = 6 from hypoxia), respectively. Three brains per treatment group were pooled to prepare one RNA sample. Two replicate pooled RNA samples each were prepared from the male and female from the normoxic control and hypoxia treatment. Total RNA from the pooled tissue samples was extracted using the mirVana™ RNA isolation kit (Applied Biosystems). RNA quality was assessed using the Agilent 2100 Bioanalyzer system and samples with a RNA Integrity Number (RIN) greater than 9 were used for RNA library construction. An independent set of tissue samples (*n* = 16) were harvested for qPCR validation.

2.3. RNA library construction and sequencing

Eight RNA (cDNA) libraries of female and male's brains were constructed (2 × 2 for the normoxic group, 2 × 2 for the hypoxic group) using the TruSeq Stranded RNA LT Sample Prep Kit (Illumina), each prepared from 300 ng of total RNA. The cDNA libraries were prepared according to manufacturer's instruction as previously described (Gu et al., 2015). Briefly, Index codes were ligated to identify individual samples. mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads (Illumina, San Diego, USA), and was then fragmented using divalent cations in the Illumina fragmentation buffer under 94 °C for 1 min. First and second strand cDNAs were synthesized using random oligonucleotides and SuperScript II, followed by DNA polymerase I and RNase H. Overhangs were blunted using exonuclease/polymerase and, after 3′ end adenylation, Illumina PE adapter oligonucleotides were ligated. DNA fragments that ligated with adaptor molecules on both ends were enriched using the Illumina PCR Primer Cocktail in a 15-cycle PCR reaction. Products were purified and quantified using the AMPure XP and the Agilent Bioanalyzer 2100 systems, respectively. Then the concentration of libraries was quantified using KAPA Library Quantification Kits. Paired-end reads, each of 150 bp read-length, were sequenced on the Illumina MiSeq sequencer.

2.4. Illumina sequencing data analysis

It included sequencing read trimming and mapping. In order to obtain a high-quality sequence-read, adapters and reads containing poly-N were first trimmed and the sequence-reads were dynamically trimmed according to BWA's—q algorithm. The running sum algorithm aims to find the longest fragment in a sequence where the Phred quality is at least 30. The trimmed sequence cannot be extended at either end without adding segments that have an error rate above the cutoff values. Finally, the trimming requires that the trimmed sequence is at least 35 bases long. Any sequence that is shorter after trimming will be discarded. Technically, a running sum algorithm was executed in which a cumulative area-plot is plotted from 3′-end to the 5′-end of the sequence reads and where positions with a base-calling Phred quality lower than 30 cause an increase of the area and vice versa. Such plot was built for each read individually and each read was trimmed from the 3′-end to the position where the area was greatest. Read-pairs were then synchronized such that all read-pairs with sequence on both sides longer than 35 bp after quality trimming were retained, and any singleton read resulting from read trimming was removed (Lai et al., 2015; Li et al., 2014). All the downstream analyses were based on quality-trimmed reads. In an attempt to identify the transcript, sequencing reads were mapped to the assembled transcripts using Novoalign (v3.00.05) with parameter-r ALL to report all multi-mapped reads (http://www.novocraft.com/). Alignment files were sorted using Samtools (http://samtools.sourceforge.net/) to generate a read-name sorted BAM file. Then, "Samtools view −F 0 × 4" was used to parse the mapped reads from the BAM file and the number of read-pairs mapping to each transcript in each sample were summarized to generate a count table (http://seqanswers.com/forums/showthread.php?t=29745) (Li et al., 2012). Ambiguously mapped read-pairs with each end mapped to different transcripts were discarded.

2.5. Differential gene expression and comparative pathway analysis

Read-count data were then subjected to differential expression analysis using the edgeR package (Robinson et al., 2010). Samples with identical treatments (normoxic or hypoxic) were considered to be biological replicates. Genes with B&H corrected *p*-value < 0.05 and |log2 (fold change)| > 0.4 were considered to show statistically significant differential expression. The differentially expressed genes were subjected to GO functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using GeneCodis tool to determine molecular functions, biological processes and biochemical pathways of these deregulated genes to decipher the molecular interaction networks that are deregulated by hypoxic stress (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009; Tabas-Madrid et al., 2012).

2.6. cDNA synthesis and gene expression analysis

16 each female and male brain tissues were harvested and 1 μg of total RNA was used to synthesize cDNA using SuperScript® VILO™ cDNA Synthesis Kit. The expression of target genes was determined using quantitative polymerase chain reaction (qPCR). The primer sequences are listed in Supplementary Table S1. The emission intensity was detected by StepOne real-time PCR system (Applied Biosystems) under the following steps: initial denaturation step at 95 °C for 20 s, 40 thermal cycling step consisted of 3 s at 95 °C, 30 s at 60 °C. Threshold cycles were averaged from triplicate reactions. To adjust for variations in amount of starting template, gene expression was normalized against 18s rRNA.

3. Results

3.1. Illumina RNA-Seq analysis and differential gene expression

Using RNA sequencing, we obtained 32.5 M and 29.9 M quality-trimmed Illumina reads from the female brain tissues (normoxia and hypoxia, respectively), and 26.7 M and 19.0 M quality-trimmed

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