Comparative Biochemistry and Physiology, Part C 155 (2012) 128–135

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Identification of differentially expressed thyroid hormone responsive genes from the brain of the Mexican Axolotl (*Ambystoma mexicanum*)☆

P. Huggins [a,1], C.K. Johnson [b,1], A. Schoergendorfer [a,1], S. Putta [b], A.C. Bathke [a], A.J. Stromberg [a], S.R. Voss [b,*]

[a] Department of Statistics, University of Kentucky, Lexington, KY 40506, USA
[b] Department of Biology, University of Kentucky, Lexington, KY 40506, USA

ARTICLE INFO

Article history:
Received 2 February 2011
Received in revised form 23 March 2011
Accepted 23 March 2011
Available online 30 March 2011

Keywords:
Metamorphosis
Salamander
Microarray

ABSTRACT

The Mexican axolotl (*Ambystoma mexicanum*) presents an excellent model to investigate mechanisms of brain development that are conserved among vertebrates. In particular, metamorphic changes of the brain can be induced in free-living aquatic juveniles and adults by simply adding thyroid hormone (T4) to rearing water. Whole brains were sampled from juvenile *A. mexicanum* that were exposed to 0, 8, and 18 days of 50 nM T4, and these were used to isolate RNA and make normalized cDNA libraries for 454 DNA sequencing. A total of 1,875,732 high quality cDNA reads were assembled with existing ESTs to obtain 5884 new contigs for human RefSeq protein models, and to develop a custom Affymetrix gene expression array (Amby_002) with approximately 20,000 probe sets. The Amby_002 array was used to identify 303 transcripts that differed statistically ($p<0.05$, fold change >1.5) as a function of days of T4 treatment. Further statistical analyses showed that Amby_002 performed concordantly in comparison to an existing, small format expression array. This study introduces a new *A. mexicanum* microarray resource for the community and the first lists of T4-responsive genes from the brain of a salamander amphibian.

1. Introduction

Thyroid hormone (TH) is essential for normal development of the mammalian brain. Maternal, fetal, and neonatal TH levels are closely regulated in temporal and spatial contexts to affect proper cell migration, proliferation, and differentiation, and to orchestrate synaptogenesis and myelination of neurons (Anderson et al., 2003; Koibuchi, 2008; Patel et al., 2011). Later in life, thyroid hormone affects the activity of neuroendocrine axes that regulate reproduction, appetite, behavior, stress response, and longevity (Ooka and Shinkai, 1986; Shi et al., 1994; Gussekloo et al., 2004; Kong et al., 2004; Brambilla et al., 2006; Leggio et al., 2008; Duval et al., 2010; Krassas et al., 2010). That so many fundamental biological processes are associated with the action of a single molecule reflects in part the molecular mechanisms through which TH operates. It has been known for some time that TH interacts with nuclear receptors (TRα or TRβ) and cofactors to regulate transcription directly (Oppenheimer et al., 1974; Samuels et al., 1974; Sap et al., 1986; Weinberger et al., 1986), and recent studies are beginning to use unbiased approaches to identify TH-regulated genes in the brain (e.g. Royland et al., 2008; Das et al., 2009). Much less is known about the way TH signals via "non-genomic" mechanisms, including membrane receptors, transporter molecules, and cytoplasmic receptors that elicit changes in cells through signaling pathways (Caria et al., 2009; reviewed by Davis et al., 2008; Furuya et al., 2009; Cheng et al., 2010). Identification of additional TH-regulated genes and molecular mechanisms will require continued studies of traditional models and development of new models.

Many mechanisms that are associated with TH synthesis, activation, and transcriptional regulation are evolutionarily conserved among vertebrates, and these presumably function during homologous stages of development (Tata, 1993; 2006). Thus, it is possible to investigate mechanisms of mammalian brain development in more experimentally tractable organisms with free-living embryonic and juvenile phases. Anuran amphibians in particular have been extensively studied because it is straightforward to induce metamorphosis in tadpoles with TH and investigate how TH affects developmental changes in vivo through interactions with TRs, accessory co-factors, and changes in chromatin state (e.g. Shi, 2000; Das et al., 2010; Bilesimo et al., 2011). TH levels increase precipitously during anuran metamorphosis to regulate the development of adult tissue and organ systems from pre-existing larval structures and progenitor cell populations. In humans, TH levels similarly increase as the brain matures during the perinatal stage of early development (Brown et al., 2005). Thus, the study of anuran metamorphosis may identify TH-dependent mechanisms that are critical for normal human brain

☆ This paper is based on a presentation given at the *5th Aquatic Annual Models of Human Disease* conference: Hosted by Oregon State University and Texas State University-San Marcos, and convened at Corvallis, OR, USA September 20–22, 2010.

* Corresponding author. Tel.: +1 859 257 9888; fax: +1 859 257 1717.
E-mail addresses: peter.huggins@uky.edu (P. Huggins), carlena.johnson@uky.edu (C.K. Johnson), angela.sch@uky.edu (A. Schoergendorfer), sputt2@email.uky.edu (S. Putta), arne@email.uky.edu (A.C. Bathke), astro11@email.uky.edu (A.J. Stromberg), srvoss@uky.edu (S.R. Voss).

1532-0456/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2011.03.006

development. However, there are limitations in using anuran models to study the actions of TH. Although it is straightforward to administer TH, dosing regimes are generally administered at developmental stages when TH levels are relatively low in tadpoles. Because genes maybe differentially responsive to TH as a function of age, precocious administration of TH may activate or repress genes that are not typical of normal development and metamorphosis. Also, when tadpoles of spontaneously metamorphosing anurans are exposed to TH, they are already developing toward a metamorphic endpoint; thus anurans do not provide a true negative control for evaluating TH signaling.

The Mexican axolotl (*Ambystoma mexicanum*) provides an alternative amphibian model to investigate TH signaling (Page et al., 2007, 2008, 2009). While many amphibians undergo an obligate metamorphosis, *A. mexicanum* juveniles fail to produce enough TH to induce metamorphosis (Kuhn and Jacobs, 1989; Galton, 1991). As a result, *A. mexicanum* retain juvenile traits into the adult stage of life, an adaptation that has been termed pedomorphosis. Importantly, metamorphosis can be induced in *A. mexicanum* by simply adding the thyroxine form of TH (T4) to the water (Page and Voss, 2009). Thus, developmental events can be induced within the context of a natural, hypothyroid condition at juvenile or adult stages of life. In *A. mexicanum*, paedomorphosis is associated with an unidentified genetic factor that affects developmental timing, response to T4, and hypothalamic-pituitary-thyroid function (Voss and Smith, 2005; Galton, 1991; Rosenkilde and Ussing, 1996; Kuhn et al., 2005).

In previous studies, we used microarray analysis to show that metamorphosis is precisely and reliably induced in *A. mexicanum* using 5 or 50 nM T4 (Page et al., 2007, 2008). We also reported an integrative model of epidermal gene expression and whole-animal anatomical metamorphosis (Page et al., 2009). Here, we report on a study that used highly parallel 454 DNA sequencing to discover genes from the *A. mexicanum* brain. The resulting sequence reads were assembled with pre-existing ESTs from Sal-Site (www.ambystoma.org) to design a 2nd generation custom Affymetrix expression array (Amby_002). This new microarray platform was then used to identify genes that are differentially expressed in the *A. mexicanum* brain after treatment with T4. Our study enhances the *A. mexicanum* model by providing a new microarray resource and new EST contigs that increase the total number of *Ambystoma*-human non-redundant orthologous sequences to >15,000. Also, our study provides the first lists of T4-responsive genes from a salamander amphibian, including genes that are predicted to function in neural developmental and physiological processes within the brain.

2. Materials and methods

2.1. Animals and tissue sampling

Nine *A. mexicanum* juvenile siblings were obtained from the *Ambystoma* Genetic Stock Center at the University of Kentucky and reared under the same laboratory conditions to approximately 130 days post hatching. At this age, individuals are immature with respect to gonad maturation and have surpassed the time that metamorphosis typically occurs in related species. Three individuals were anesthetized in 0.02% benzocaine and the brains and pituitaries of each were removed, flash frozen in ethanol and liquid nitrogen, and stored separately at −80 C until the time of RNA isolation. The other 6 individuals were reared individually in a 50 nM T4 (Sigma, St. Louis, MO; T2376) prepared according to the method of Page and Voss (2009). When juveniles are reared in 50 nM T4, morphological metamorphosis initiates approximately 8 days after treatment, morphology rapidly changes between days 15 and 20, and metamorphosis is completed after approximately 28–32 days (Page et al., 2009). We observed early signs of anatomical metamorphosis at Day 8 and changes indicative of metamorphic climax at D18. After 8 and 18 days of treatment, 3 individuals were sacrificed and brain/pituitary tissue was removed and stored as described above. This sampling design yielded three replicates of brain tissue for three groups: no T4 (D0), early metamorphosis (D8), and metamorphic climax (D18). Animal care and use were carried out under University of Kentucky IACUC protocols #01087L2006 and #00907L2005.

2.2. RNA isolation and 454 DNA sequencing

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and RNA samples were further purified using Qiagen RNeasy mini-columns. RNA samples were quantified using a NanoDrop ND-1000 and Agilent BioAnalyzer. RNA from one replicate of each treatment was used to make cDNA libraries for 454 DNA sequencing. We refer to these as 454_D0, 454_D8, and 454_D18. cDNA libraries were generated using standard methods of MINT cDNA synthesis and TRIMMER cDNA normalization kits from EVROGEN, Inc; sequences were size-selected according to manufacturer's instructions. cDNAs were sequenced by the University of Iowa Biology Department Sequencing Core using the Genome Sequencer FLX System with Titanium Chemistry (Roche Applied Science, Indianapolis, IN). SeqClean (http://www.tigr.org/tdb/tgi/software) was used for vector/poor quality trimming, bacterial contaminant screening, and identification of *A. mexicanum* mitochondrial DNA and rDNA sequences. Retained sequences were assembled using Newbler (Version 2.0.01.14) from 454 Life Sciences. Contigs (including singletons) were searched using BLAST algorithms against the *Ambystoma* ESTdb at Sal-Site (Smith et al., 2005), and NCBI protein and nucleotide databases. Queries that returned significant BLAST hits were assigned the gene identifier of the best-matching subject sequence. 454 DNA sequence reads were assembled with previous EST data and the overall assembly is available at Sal-Site. Assembled contigs were submitted to Affymetrix to design approximately 20,000 perfect-match probe sets for a custom gene expression array: Amby_002 (Part Number 520748).

2.3. Microarray analysis

Genome-level expression profiling was conducted using Amby_002 and Amby_001, a small format array (~4500 probe sets) that was designed in 2005 from approximately 60,000 ESTs (Smith et al., 2005). Amby_001 has been used to quantify gene expression in several experiments (Page et al., 2007, 2008; Monaghan et al., 2007, 2009; Cotter et al., 2008), including an experiment that examined brain tissues from *A. mexicanum* and *A. t. tigrinum* (Page et al., 2010). RNA from all 9 of the brain samples was labeled, hybridized to separate Amby_002 arrays, and scanned by the University of Kentucky Microarray Core Facility according to standard Affymetrix protocols. We also hybridized the three D0 and D18 replicates to six Amby_001 arrays to investigate concordance and discordance between the Affy platforms. Background correction, normalization, and expression summaries were obtained using the robust multi-array average (RMA) algorithm (Irizarry et al., 2003).

2.4. Identification of differentially expressed genes (DEGs) using microarrays

The expression data from Amby_002 were examined using standard *F-tests* to identify genes that were differentially expressed as a function of T4 treatment. Then *t-tests* were applied to this list of DEGs to identify the direction of gene expression change between the D0, D8, and D18 time points. For example, between D0 and D8 an expression change can be described as up, down, or unchanged. See Supplementary File 1 for details on how F-tests and t-tests were performed. All statistical analyses were performed using SAS 9.2 (SAS Institute Inc, Cary, NC, USA) and R 2.11.0 (www.r-project.org).