LOCALIZATION OF THE SODIUM IODIDE SYMPORTER (NIS) IN ZEBRAFISH

A Thesis

by

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ABSTRACT

Iodine, an essential component of thyroid hormone, can only be obtained through the diet. The sodium iodide symporter (NIS) transports iodide across mammalian intestinal and thyroid epithelia to deliver iodide for thyroid hormone production. To determine whether a homolog of NIS performs a similar function in teleost fish, we confirmed expression of a homolog of mammalian NIS in both sub-pharyngeal thyroid follicles and intestine in multiple teleost species, indicating a conserved mechanism for intestinal-thyroid iodine transport across vertebrates. We then examined expression in these locations during development using in situ hybridization (ISH) staining of zebrafish (Danio rerio) embryos. This revealed expression of nis as early as 2 days post fertilization (dpf) along the dorsal surface of the yolk sac, suggesting a function to import iodine from yolk, potentially placed there by maternal deposition. To evaluate this possibility, RT-PCR and further in situ staining of ovarian tissue in gravid female zebrafish confirmed nis mRNA presence in the ooplasm and granulosa layer of early stage follicles. This suggests NIS can function to transport iodine into the yolk, and that maternally-deposited NIS mRNA may be available for early embryogenesis. Additionally, ISH in embryos revealed nis expression in the central nervous system throughout days 2-5 dpf, with adult whole brain ISH localizing expression in the hypothalamus, cerebellum, and optic tectum. RT-PCR on whole brain tissue from 5 species of adult fish representing 3 taxonomic orders likewise revealed robust expression. These unexpected, non-canonical locations suggest novel, as yet undescribed reproductive and neural functions of NIS in teleost species.

DEDICATION

To the incomparable Madi Kirk, and the City of Pasadena: you've made me.

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Mom and Dad, I love you.

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Contributors

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All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

NIS	Sodium Iodide Symporter
MCT8	Monocarboxylate Transporter Number 8
OATP14	Organic Anion Transport Protein Number 14
RT-PCR	Reverse Transcription – Polymerase Chain Reaction

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1. INTRODUCTION

Iodide is an essential element, vital to bodily functions including reproduction, metabolism, and development in vertebrates (Carrasco 1993, Martillotti 2016, Mahapatra et al. 2017, Davies 2017, Velasco et al. 2018). Iodide in the body is concentrated by the thyroid gland, which utilizes a protein called the sodium iodide symporter (NIS) to transport iodide from the blood into thyroid epithelial cells (Carrasco 1993), where it is then organified to make thyroid hormones. In mammals, NIS is located in thyroid follicle cells, where it transports iodide in an energy dependent manner across the basolateral plasma membrane. (Carrasco 1993)

Because iodine is an element that can only be obtained by dietary means, it is not surprising that aside from its location in the thyroid gland, NIS is expressed in the gastrointestinal tract of mammals (Nicola et al. 2009) where it transports iodine from ingested food into the circulation. NIS distribution has also been characterized in rats (Kotani et al. 1998) and mice (Josefsson et al. 2002) to be prominent in the stomach and anterior digestive tract. In mammals NIS is thus well conserved in its location and function in digestive and thyroidal tissues. However, NIS has also been found in mammary glands where it transports iodide to milk as the sole source for newborns, and in salivary glands where it is suspected to aid in iodide secretion and recycling by the small intestine and possibly contribute antimicrobial activity (Ravera et al. 2017, Portulano et al. 2013). Alternative locations, however, are more cryptic. NIS expression has recently been identified in human and mouse pituitary glands (in mice roughly 15% compared to the mammary tissue; Perron et al. 2001, Zimmerman 2009). Additionally weak NIS expression was noted in the cerebral and cerebellar regions (Kotani et al. 1998), although there has been little follow up to characterize NIS presence or function in the nervous system.

Little characterization of iodine transport exists for non-mammalian species. In one of the first studies in non-mammalian vertebrates (Wright 1974), iodine transport was characterized in the choroid plexus of the American bullfrog (*Rana catesbeiana*), but there was no consideration of a specific transport mechanism in any anatomical location in the brain, nor confirmation of its presence. However, recent follow up studies in bullfrogs have found *nis* expression in locations similar to mammals including the thyroid and gastrointestinal tract (Carr et al. 2008), and in the stomach of *Xenopus laevis* (Carr et al. 2015). Likewise, there has been confirmation of *nis* expression in zebrafish thyroid follicles (Alt et al. 2006, Opitz et al. 2011), although characterization of *nis* expression in the gut of any teleost fish is lacking. Additionally, there has been no identification of NIS activity in marine or euryhaline fish, where it may have an important role in iodine uptake from the environment (Klaren et al 2007).

This study initially set out to identify NIS presence in conserved locations (i.e. the gut and thyroid follicles according to mammalian literature) in a coastal euryhaline teleost fish: red drum (*Sciaenops ocellatus*). Identification of *nis* expression in thyroid follicles in a teleost fish that experiences variations in salinity and environmental iodine, as well as a sampling of fish from diverse orders, was also undertaken to determine whether the basic mechanism of iodine transport in gastrointestinal and thyroid tissue has been broadly conserved phylogenetically and across diverse environments. Among the species chosen, the zebrafish (*Danio rerio*), represents a well-characterized model for teleost developmental biology, thereby enabling characterization of NIS expression during development and thus filling a significant gap in the literature that usually identifies transporter presence but lacks specificity of timing and location. In this study we provide evidence for a conserved presence of NIS in tissues previously reported in mammalian literature across five species of teleost fish. The analytical tools available for

zebrafish then enabled us to identify specific novel locations of *nis* expression in both larvae and adults that suggest new, and as yet uncharacterized, functions for this protein.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Red drum were obtained from the Texas Parks and Wildlife Department Sea Center hatchery in Lake Jackson, Texas. Animals were kept in artificial seawater with temperatures ranging from 24°C-27°C, salinity ranging from 15ppt-30ppt, and with a 12L:12D photoperiod. Seawater was created and maintained using reverse osmosis water, Morton salt (IL, USA), and Fritz Super Salt Concentrate (Fritz Aquatics, TX, USA). The fish were fed a diet of commercial pellets (Rangen, TX, USA) once daily shortly after lights on. Zebrafish were obtained from the Texas A&M University Department of Biology BioAquatics zebrafish breeding colony by transfer from existing stocks maintained by Dr. Bruce Riley. Zebrafish were kept in 5 gallon tanks supplied by a recirculating system filled with reverse osmosis water supplemented with 0.5% Instant Ocean (Instant Ocean, VA, USA) at 27°C,14L:10D photoperiod. Fish were fed a commercial diet (Total Tropical, Wardley, NJ, USA) twice daily and brine shrimp once daily. For tissue collection, wildtype zebrafish were then paired in small tanks with a divider between the males and females, 2 females for every 1 male, and at lights on the following morning the divider was removed. Embryos were then collected after fertilization, and fish were placed back in tanks until tissue collection. Because wild type embryos exposed to common *in situ* depigmentation reagents such as propylthiouracil (PTU) are more likely to suffer from developmental defects due to PTU being a known thyroid hormone inhibitor (Tonyushkina et al. 2017) *Casper* mutants (embryos that are genetically altered to lack pigmentation in their skin) were used instead of pigmented wild type embryos to provide clearer visualization of staining for *in situ* hybridization procedures.

2.2 Tissue Collection For RT-PCR Identification of Location of nis Expression

Tissues were collected from adult animals for comparative RT-PCR localization of *nis* expression and for *in situ* identification of *nis* expression in zebrafish. To obtain adult tissue, red drum were euthanized in an overdose of anesthesia (MS-222), while zebrafish were euthanized by immersion in an ice water bath, until movement ceased. Sub-pharyngeal tissue was collected as a source of thyroid tissue for positive controls. To isolate thyroid tissue, dissections of gill arches 1-2, 3-4, and 5 were also made from the sub-pharyngeal regions of hybrid striped bass, channel catfish, and tilapia using animals euthanized on other protocols as part of a tissue sharing program with the Texas A&M Aquaculture Research and Teaching Facility (ARTF). These fish were kept in outdoor ponds on natural photoperiods in September. Muscle samples were taken from the tails of each of the fish to serve as negative controls along with whole brains. Tissues were immediately placed in 1-1.5mL RNALater (Thermofisher) for 4-6 hours at room temperature, then frozen and stored in RNALater at -80°C for future RNA extraction.

2.3 RNA Extraction

To obtain high quality transcripts of NIS, RNA isolation was performed using the ZR RNA MiniPrep Kit (Zymo Research, CA, USA) following the manufacturer's protocol. Each tissue was snap frozen and mechanically homogenized in liquid nitrogen using a pre-chilled mortar and pestle. The homogenate was added to 300 µL DNA/RNA shield (Zymo Research, CA, USA) followed by 300 µL 2X Digestion Buffer (Zymo Research, CA, USA) and 15 µL of Proteinase K. This solution was mixed and incubated in a 55°C water bath for 30 minutes, and then centrifuged at 14,000 rpm for 2 minutes. Aqueous supernatant was then treated with 300 µL lysis buffer (Zymo Research, CA, USA) and transferred to a Zymo-Spin IIIC column in a collection tube, where it was centrifuged at 8,000rpm for 30 seconds. An aliquot of 320 µL of

pure ethanol was then added to the flow through, transferred to a Zymo-Spin IIC column, and eluted in a collection tube, where it was centrifuged at 12,000rpm for 1 minute. 300 µL of RNA wash buffer was added to the column, and then centrifuged for 30 seconds at 12,000rpm. 90 µL of DNase I cocktail (80 µL of RNA wash buffer, 5 µL of RNase-Free DNase I, and 5 µL of 10X reaction buffer) was added directly to the column and incubated at 27°C for 15 minutes, then centrifuged at 12,000rpm for 30 seconds. Next 400µL of RNA prep buffer was added to the column and centrifuged at 12,000rpm for 1 minute, followed by 700 µL of RNA wash buffer centrifuged at 12,000rpm for 30 seconds. A final wash of 300µL RNA wash buffer was added to the column and centrifuged at 12,000rpm for 30 seconds, followed by an additional 2-minute spin to elute any remaining wash buffer. Finally, 25 µL of double distilled water was added to the column in a 1.5 mL Eppendorf Tube and after 1 minute of incubation at room temperature was centrifuged to elute the RNA product. RNA was then frozen and stored at -80°C until further quantification. Extracted RNA (1.5 µL) was analyzed for quantity (ng/µL) and purity using a NanoDrop instrument (Thermo Scientific, NC, USA), excluding samples that were outside a purity range of 1.5-2.2 A260/A280.

2.4 Reverse Transcription

To obtain a more stable product for future amplification, previously extracted RNA was used as a template to create complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Invitrogen, NY, USA). For each sample of RNA the following reagents were mixed in a microfuge tube: 5 µL of 10X RT buffer, 2 µL of 25X dNTPs, 8 µL of 10X RT random primers, 2.5 µL of Oligo-dt, 5 µL of respective RNA sample, 25 µL of double-distilled and autoclaved water (ddH_2O), and lastly 2.5 µL of reverse transcriptase for a total volume of 50µL in each tube. Tubes were then placed in a thermocycler and incubated at 25°C

for 10 minutes, 37°C for 2 hours, and 85°C for 5 minutes to denature the enzyme. Each sample was then kept at 4°C before being stored at -20°C for further amplification.

2.5 Primer Design

Primers for tilapia (*Oreochromis niloticus*), hybrid striped bass (*Morone saxatilis* x *Morone chrysops*), and channel catfish (*Ictalurus punctatus*) were designed to yield PCR products of approximately 500bp using available sequences, similar in size to primers that had been previously designed in our lab for red drum *nis* expression (St. Clair 2017). Homologous primers for the *nis* gene in zebrafish were also previously designed (Alt et al. 2006), but new ones were made to yield a longer PCR product, close to 500 bp using the available *nis* sequence (https://www.ncbi.nlm.nih.gov/nuccore/BC134942.1).

2.6 PCR

A 2 µL sample of each cDNA sample was mixed with 10 µL of GoTaq Green (Promega, WI, USA), 2 µL of respective *nis* primers, and 6 µL of ddH_2O. For no template controls, ddH_2O was used as a substitute for sample cDNA. The same ratios were used for actin, our reference gene. Each mixture was then placed in the thermocycler and incubated at the following temperatures: 95°C for 2 minutes, followed by 32 cycles of 95°C for 30 seconds, respective annealing temperatures (50°C – 60°C) for 30 seconds, and 72°C for 2 minutes. They were then held at 72°C for an additional 5 minutes and cooled to 4°C before being stored at -20°C until gel electrophoresis.

2.7 DNA Gel Electrophoresis

A 1% gel was made by melting 0.5g of agarose in 49mL of ddH_2O and 1 mL of 50XTAE. After the solution was mixed, 2 µL of ethidium bromide was added for visualization of the DNA under UV light. The mixture was then poured into a sterile 8cm x 10cm tray with a 10-

lane comb in place. The gel was left at room temperature to solidify. Once solid the 10-lane comb was removed carefully so as not to cause tearing of the wells. Afterwards the gel was placed in a gel box with 500 mL of TAE running buffer. A molecular weight DNA ladder was made by mixing 1 μL of 100 base pair ladder (New England BioLabs, MA, USA), 2 μL of 6XDNA loading buffer (New England BioLabs, MA, USA), and 9 μL of ddH2O. Each of the samples from the PCR were thawed by hand and inserted into corresponding wells, and then run at 94V for approximately 75 minutes or until the loading dye reached the bottom of the gel. The gels were removed and visualized under UV light. Finally, each band of DNA was cut out of the gel and eluted through a Zymo-Spin IIIC for sequencing provided by the Laboratory for Genome Technology at Texas A&M to confirm the gene identity via BLAST recognition.

2.8 In Situ Hybridization

To localize *nis* expression, zebrafish embryos were obtained by crossing adults in existing stocks of casper and wild-type fish maintained by Dr. Bruce Riley, and adult tissues were taken from euthanized animals of the same stocks. Anti-sense plasmids were used to hybridize with existing transcripts. The plasmid for the *in situ* hybridization was developed and donated by Dr. Sabine Costagliola's lab in Belgium (Opitz et al. 2011) where *nis* expression was used as a positive control for thyroid follicle identification. Plasmids were linearized with both Spe-I and Not-I, and then transcribed with T7 and T3 enzymes, respectively. In situ hybridization was then performed as in Phillips et al. (2001), with the following modifications according to Thisse et al. (2008): embryos at 24 hours post fertilization (hpf) received a proteinase K treatment for 2 minutes; embryos at 48hpf received 6 minutes of proteinase K treatment; and embryos for 72hpf and 96hpf received 10 minutes of proteinase K treatment; and finally, embryos older than 96hpf received 30-40 minutes of proteinase K treatment. Embryos

greater than 72hpf were allowed to hybridize in anti-sense probe for up to 24 hours to better penetrate the more developed tissue to identify NIS transcript location.

2.9 Tissue Sectioning & Histology

Both non-treated and *in situ* hybridized tissues were embedded in either freezing media or in a water soluble plastic (JB-4 glycol methacrylate, Polysciences Inc, Warrington, PA). Those that were frozen were placed in plastic molds and covered with tissue freezing media (Leica Biosystems) and kept at -20°C until frozen. Tissues were then taken out of the mold and slices were taken at 13µm using a cryostat (Leica Biosystems). Tissues that were embedded in plastic for histology were dehydrated from 75% glycerol to double distilled water, and then for 5 minutes at a time submerged in 30%, 50%, 75%, and 95% ethanol. They were then infiltrated using 0.5g of catalyst mixed with 40mL of monomer A for 5 minutes. The solution was changed three times to ensure infiltration. Tissues were then placed in molds filled with a mixture of 0.5g catalyst, 40mL monomer A and 1.6mL of solution B. They were then topped with a block and left in 4°C overnight to harden. Sections were then taken using a microtome at 10µm. Histological sections were then stained using a polychrome mixture of methylene blue and azure II, followed by basic fuchsin (MacKenzie et al.1989). They were then dried and cleared using 95% ethanol.

2.10 Spawning

To characterize *nis* expression in developing ovaries, thee female zebrafish were paired with two males in small breeding tanks with a divider in between them. At lights on their divider was removed and they were kept together until females had completed oviposition. Females were then removed to individual tanks and fed normally until their ovaries were dissected at 0, 3, 6, and 10 days after spawning. Ovaries were then kept in RNA Later at -20°C for RT-PCR.

3. RESULTS

Initial studies were undertaken to localize NIS expression in tissues of the red drum. Figure 1 shows identification via RT-PCR of *nis* expression in red drum sub-pharyngeal tissue, anterior intestine, medial intestine, posterior intestine, and brain. The robust neural *nis* expression was regionalized to three lobes in the red drum brain: the inferior lobe, the optic lobe, and the cerebellum (Figure 2). Figure 3 indicates that neural *nis* expression is not unique to this euryhaline species, but instead we show its expression across 3 orders of teleost fish, including the popular developmental model species zebrafish.

In situ hybridization revealed broad distribution of *nis* transcript in the anterior region of the embryo with darker staining in the subpharyngeal region and anterior intestine of zebrafish from 2 to 5dpf (Figure 4). Staining in the anterior most portion of the developing gastrointestinal tract is noticeable by day 3, and extends posteriorly by days 4 and 5. Anterior digestive tract expression localizes on the dorsal surface of the yolk sac. Figure 5A and 5B show a higher magnification of *in situ* hybridization results for 6dpf larvae where *nis* transcript is localized in the sub-pharyngeal region and anterior intestine, respectively. In adult zebrafish, *in situ* hybridization reflects the pattern previously observed for red drum *nis* expression, with the *nis* transcript observed in the hypothalamus, optic tectum and cerebellum (Figure 6).

Because *nis* expression localized along the dorsal most surface of the yolk sac, suggesting yolk might contain iodide, *in situ* hybridization was used to examine the possible expression of *nis* in gravid female ovaries. Figure 7A shows that in zebrafish ovarian tissue there is abundant expression of *nis*, comparable to expression in both sub-pharyngeal thyroid region and brain. *nis* ovarian expression is lowest immediately after spawning and then increases within 3 days, remaining elevated until the next spawning (Figure 7B). Whole-mount staining of the ovarian

tissue via *in situ* hybridization (Figure 8A) shows that there is abundant transcript presence in primary growth-staged oocytes compared to our negative control (Figure 8B). Sectioning of *in situ* stained ovaries (Figure 8C) shows a representative picture of mutliple ovarian hybridizations, with transcript localization at a higher resolution in what we confirm as primary growth and pre-vitellogenic follicles based on histological staining (Figure 8D) and previously defined metrics (Selman et al. 1993).

4. DISCUSSION

nis expression in thyroid follicles has been extensively characterized in mammals where it plays an essential role in transporting iodine into epithelial cells for thyroid hormone synthesis (Portulano et al. 2013). As animals only obtain iodine through their diet, NIS also serves to transport iodine across intestinal epithelia where it then travels through the circulation to thyroid follicles (Carrasco et al. 1993, Dohan et al. 2003, Portulano et al. 2013). These processes are less well understood in nonmammalian vertebrates. NIS has been found using RT-PCR in adult bullfrogs and *Xenopus* in the gastrointestinal tract and thyroid, suggesting a conserved mechanism for iodine transport in amphibians (Carr et al. 2008, Carr et al. 2015), but there are few studies that identify *nis* expression in thyroidal or extra-thyroidal tissues in other nonmammalian species (Alt et al. 2006, Opitz et al. 2011, St. Clair et al. 2017).

Our first goal for this study was to determine if locations of *nis* expression in a teleost fish were similar to those that had been previously reported in mammals and amphibians (Portulano et al. 2013, Carr et al. 2008). Teleosts are the most diverse group of vertebrates (Nolte 2020), and can experience a wide range of environmental iodine concentrations in their habitats (Elderfield et al. 1980). While there have been previous reports of *nis* expression in thyroid follicles in stenohaline zebrafish (Alt et al. 2006, Opitz et al. 2011), our investigation of iodine transport served to expand identification of locations of *nis* expression in multiple teleost species representing more diverse life histories and habitats.

NIS in mammalian thyroid follicles is present on the basolateral plasma membrane (Nicola et al. 2009). In teleosts, thyroid follicles are diffusely distributed in the sub-pharyngeal region making individual follicles difficult to isolate (Norris et al. 2013). We therefore first identified *nis* expression in the sub-pharyngeal thyroid tissues, shown to contain thyroid follicles

(MacKenzie, 1988), as well as the gastrointestinal tract, and brains of red drum. We found consistent *nis* expression in each of these locations by RT-PCR, and further showed that it could be localized to specific regions of the gastrointestinal tract (primarily anterior and medial) and brain (inferior lobe, optic lobe, and cerebellum). We also identified *nis* expression in sub-pharyngeal thyroid tissues and brains of striped bass, channel catfish, tilapia, and zebrafish, thus supporting a conserved function of *nis* as an important iodide tranporter in thyroidal tissues across diverse teleosts, amphibians, and mammalian vertebrates.

nis expression has been reported in the anterior intestinal epithelium of mammals (Joseffson et al. 2002, Dohan et al. 2003), and in the intestines of adult amphibians (Carr et al. 2008, Carr et al. 2015). It serves in the intestine to transport iodine from the diet into general circulation, making the gastrointestinal tract another primary location for iodine transport. We have found the first evidence of *nis* expression in the gastrointestinal tract of red drum, similar to *nis* expression in the gastrointestinal tracts of mammals (Nicola et al. 2009) and amphibians (Carr et al. 2015) where it functions to transport iodine from digested food. Our demonstration of *nis* expression in a euryhaline species provides evidence that using NIS as the mechanism for iodine transport from the anterior gastrointestinal tract into general circulation is also broadly conserved.

Inclusion of zebrafish in our analysis enabled us for the first time to characterize expression of *nis* in the developing gastrointestinal tract, providing insight to when in development gastrointestinal *nis* expression first appears in relation to first feeding. *In situ* hybridization results show robust expression of *nis* mRNA in the developing gastrointestinal tract of zebrafish, further supporting this as a primary location of iodine transport in fish. Interestingly, our study shows that *nis* mRNA is present as early as 3dpf in zebrafish when the animal is still resorbing

yolk and not yet feeding on its own. NIS transcript localization adjacent to the dorsal surface of the yolk sac this early in development suggests a possible function to transport iodine from the yolk into the embryo for embryonic thyroid hormone synthesis, in a similar fashion to intestinal iodine transport from food in adults. Embryonic intestinal *nis* expression therefore suggests presence of free iodine in the yolk, and evidence of NIS at the site of yolk resorption during development suggests a mechanism for iodine transport during embryogenesis.

To determine whether there might be a maternal mechanism for iodide transport into yolk, we identified *nis* expression in ovaries of gravid females. The robust expression of *nis* in gravid ovaries suggests a role in packaging of iodine into yolk. Additionally, we found that this expression diminished immediately after spawning, returning to a robust expression 3 days to 10 days later, during which time a new cohort of oocytes is developing (Ribas et al. 2014). Currently there is little evidence of NIS or iodine packaging into oocytes in ovaries of teleosts, amphibians (Carr et al. 2008), or mammals. This is distinguished from what has been previously reported for thyroid hormones where it is well known that oviparous mothers package intact thyroid hormone directly into the yolk (Power et al. 2001, Arukwe et al. 2014). Abundant *nis* expression in gravid ovarian tissue suggests a mechanism for maternal iodine packaging into the yolk for embryonic uptake during yolk resorption. Additionally, the increase in *nis* expression three days after spawning suggests upregulation of *nis* expression during early oocyte maturation around the time of development of late-staged follicles (Selman et al. 2004, Wang et al. 1993).

Whole mount *in situ* hybridization showed staining for *nis* mRNA in primary growth (PG) and pre-vitellogenic (PV) follicles. Plastic embedded sections showed localization of *in situ* staining in the ooplasm and granulosa layer of early-staged follicles, suggesting two functions: 1) direct transport of iodine into primary growth and pre-vitellogenic oocytes by NIS in granulosa

cells, and 2) maternal packaging of *nis* mRNA into oocytes for expression during embryonic intestinal development. Multiple studies have found evidence for maternally derived mRNAs deposited in oocytes, where transcripts remain quiescent until fertilization (Ladomery et al. 1997, Moore 2005). If both of these suggestions are true, and these functions are working in concert, then mothers would package both iodine and *nis* mRNA so that during development embryos could express the transcript into intestinal epithelia (Moore 2005) as a means to import the iodide in the yolk for endogenous thyroid hormone production. To evaluate the significance of early intestinal expression of *nis* it would be of interest to selectively knock out *nis* transcript in embryonic fish to see the resulting effect in both adults and offspring. This should be possible in zebrafish using the powerful tools available for genetic manipulation of this species.

Initially, our objective for including brain tissue in RT-PCR studies was to serve as a negative control, but we surprisingly found a robust expression conserved across all species examined and regionalized in red drum to the cerebellum, optic lobe, and inferior lobe. These locations were conserved in adult zebrafish based on our *in situ* hybridization results which revealed transcript in the cerebellum, optic tectum, and hypothalamus. Our observation of *nis* expression in the brain is novel, as it has not previously been described as a major site of expression in mammals or amphibians. While extensive data support thyroid hormone's critical function in the central nervous system (Bernal 2002, Bernal 2005), especially in areas of endocrine feedback (Nakane et al. 2014), there has been little characterization of NIS function in the brain in vertebrates. Wright (1974) concluded that his observation of iodine accumulation in the brains of American bullfrogs (*Rana catesbaiana*) was not exclusively transporter-mediated, and suggested brain permeability as an alternative mechanism. There has since been little follow up on iodine accumulation in the nervous system. Our results therefore imply a novel, as yet

undescribed, function of NIS in the central nervous system, likely conserved across at least three teleost orders. Because of its location in the hypothalamus, and taking into the account the diverse literature describing deiodinase activity in the hypothalamus of multiple vertebrate species (Bird: Nakane et al. 2014, Frog: Langlois et al. 2011, Fish: Lorgen et al. 2015, Mice: Mayerl et al. 2012), we suggest that NIS in this location may be acting to transport free iodine out of the central nervous system. Active deiodination of thyroid hormone in the brain suggests an accumulation of iodide, and NIS may thus serve the unique function to prevent degradation of neural tissue from increased iodine accumulation (Chandra 2017, Mahapatra et al. 2017).

Previous studies have found *nis* to be regulated by thyroid stimulating hormone (TSH) in mammalian thyroid follicles. In red drum, TSH injection can stimulate *nis* expression in thyroid tissues but not in the gut (St. Clair et al. 2017). But *nis* expression is not solely under hormonal control as it is regulated by iodine concentration as well (Eng et al. 1999). With our new evidence of extra-thyroidal expression of *nis*, identifying its function and physiological regulation in these novel locations becomes of interest. Identification of hormonal regulation of *nis* expression in these extrathyroidal tissues could help elucidate its function and identify crosstalk between centrally regulated hormone axes. Given that we've found strong evidence for NIS presence in primary follicles, gonadotropins would be the most likely candidate to regulate its expression in this location (Dissen et al. 1994, Norris et al. 2013). Follicle stimulating hormone (FSH) is known to regulate early follicle development in vertebrates (Nagahama et al. 2008), and with prominent *nis* expression in PG and PV follicles it would be logical to suggest that FSH regulates *nis* expression therein. Another possibility for ovarian regulation could be prolactin. Prolactin in fish has a variety of functions outside of its classic association with lactation in mammals (Rilemma et al. 2003, Tazebay et al. 2000). However given that we are

suggesting NIS is being used to package maternally derived iodine for embryogenesis in an oviparous species, *nis* expression in the granulosa of ovaries is similar to mammalian expression of *nis* in mammary tissue (Perron et al. 2001, Spitzweg et al. 1998). Mammalian mothers are known to express *nis* in mammary tissue as a means to transport maternally derived iodine into breast milk, where it serves as the infant's sole source of iodine for its own endogenous thyroid hormone production. If we could show that prolactin injection upregulates iodine transport in ovaries as has been shown in mammary tissues in mammals (Tazebay et al. 2000, Rilemma et al. 2003), then this would show both a novel expression for *nis* in an oviparous species' reproductive tissues, as well as identify a conserved mechanism among vertebrates for transporting iodine to offspring in development.

In conclusion, this study has confirmed *nis* expression in thyroid tissue and the gastrointestinal tract in teleost fish, as well as expanded our knowledge of the distribution of NIS in previously unreported extra-thyroidal tissues. We suggest new functions of NIS based on our findings in the brain and the ovaries. These locations may be unique to teleosts, where neural *nis* expression is conserved among fish that vary in natural habitats and life histories. Future studies should focus on the function of NIS, particularly in novel extrathyroidal locations such as the brain and ovaries. Functional experiments using the powerful molecular genetic tools available for zebrafish could help establish a role for NIS in these tissues via selective knock outs, and contribute evidence for cross talk between thyroid and reproductive hormonal axes by quantifying *nis* expression in response to hormone injections.

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