A COMPARATIVE INVESTIGATION OF NEURAL SODIUM IODIDE SYMPORTER (NIS) EXPRESSION IN TELEOST FISH

An Undergraduate Research Scholars Thesis

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ABSTRACT

A Comparative Investigation of Neural Sodium Iodide Symporter (NIS) Expression In Teleost Fish

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Thyroid hormones regulate essential physiological processes, including metabolism, reproduction, and growth. A key constituent of all thyroid hormones is iodine. To obtain and concentrate iodine, vertebrates utilize a protein called the sodium iodide symporter (NIS). While most commonly associated with the thyroid and digestive tract, I have confirmed preliminary evidence from a single fish species, red drum (*Sciaenops ocellatus*), for a novel location of NIS expression: the brain. The objective of this study was to examine whether this expression exists in other teleost fish species and to more precisely identify the anatomical locations of neural NIS expression. Brains from several species of marine and freshwater fish, (tilapia; *Oreochromis niloticus*, channel catfish; *Ictalurus punctatus*, zebrafish; *Danio rerio*, and hybrid striped bass; *Morone saxatilis*) spanning three taxonomic orders (Cypriniformes, Perciformes, and Siluriformes), were collected and subjected to RT-PCR to identify NIS expression. This study found NIS to be present in the brains of all 5 experimental species, but only present in the previously confirmed sub-pharyngeal areas of red drum and zebrafish. Its uniform distribution across these species suggests that NIS may perform a novel, as yet undescribed role in iodine transport in the central nervous system. Localization of NIS expression within the brain is, therefore, a critical next step in elucidating its function.

DEDICATION

For Paul and Sandy Holloway: I hope this serves as a foundation to prove that I am not only proud to call you my parents, but am more-so that you call me your son. I could not have accomplished anything without you.

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Thank you, to my research advisor who is responsible for fostering my drive and passion to achieve great things in endocrinology, Dr. Duncan MacKenzie. Thank you, to my mentors and life coaches, Allison Wilkes and Brie Myre, for treating me as both an equal and a friend. And thank you, to Dr. Richard Jones and Dr. Bill Cohn, for their support and advice for the duration of this project.

CHAPTER I

INTRODUCTION

Iodine is an essential element, vital to bodily functions like metabolism, reproduction, and proper development in vertebrate organisms [5, 10]. The gland that concentrates iodine in the body is the thyroid, which utilizes a protein called the sodium iodide symporter (NIS) to transport iodide from the blood into thyroid epithelial cells [5]. Because iodine is an element that can only be obtained by dietary means, it has been found that aside from its location in the thyroid, NIS is active in the gastrointestinal tract of mammalian species [12], as well as perciform and cyprinid fish [4, 10], where it likely serves to transport iodine from ingested food into the circulation. Interestingly, early experiments showed that injecting fish with radioiodine resulted in a slow increase in iodine absorption in the brain [16]. These results of radioiodine uptake [16] in the brain were inconsistent, however. The presence of radiolabeled iodine in the brain was largely attributed to brain vascularity and permeability, rather than an active import or export, and there has since been little subsequent research on a potential role for iodine uptake in the brain in fish species. However, during early development thyroid hormones have important roles in brain differentiation as well as many post-natal effects on central nervous system function [3]. One study found a thyroid hormone transport protein, MCT8, which has a high affinity for triiodothyronine (T_3), while another suggested a similar thyroid hormone transporter, OATP14, which functions in the brain capillaries as well as the choroid plexus of the brain [3, 8, 16]. These transporters allow thyroid hormone to aid in proper neuronal cell migration and glial cell differentiation in the cerebral and cerebellar areas of the brain. It has also been shown that in brain development, an absence of thyroid hormone leads to a lack of myelination in neurons,

decreased cell migration and proliferation, along with increased cell death [3]. Thus, thyroid hormone transport and activation is vital to developing central nervous systems.

While these transport proteins have been researched extensively, there have only been some indications of the presence of NIS in the brain, most of which are relegated to mammalian species [15]. Although NIS expression has recently been characterized in humans with a presence in the pituitary gland, little characterization exists for marine species. In one of the first experiments characterizing neural iodine transport in nonmammalian vertebrates [19], iodine transport was studied mathematically and biochemically in the brains of the American bullfrog (*Rana catesbeiana*) but there was no discussion or consideration of a specific channel or protein in any anatomical location in the brain. Experimenters believed the process of iodine moving across the choroid plexus to be carrier-free [19], but then showed a mechanism of inhibition using now known NIS antagonists like perchlorate (ClO_4). The only indication of neural iodine transport in teleost fish was discovered in an exploratory experiment to characterize locations of NIS expression in red drum (*Sciaenops ocellatus)*, where the brain was originally meant to serve as a negative control [4]. However, it was found that the brain had a robust expression of the *nis* gene, almost identical to that of the positive control. As NIS is known to concentrate iodine, it was unexpected that it would be expressed in an organ that has no involvement in dietary iodine absorption or thyroid hormone production. These results should therefore be replicated, to confirm its presence in the brains of red drum. Additionally, it is presently unknown whether this is a unique property of red drum, a freely euryhaline species which may have unusual ionoregulatory characteristics, or a broadly distributed characteristic across teleost species.

This research aims to expand the finding of NIS expression in the red drum brain to other euryhaline species (tilapia; *Oreochromis niloticus*, and hybrid striped bass; *Morone saxatilis*), as

well as two intensively cultured stenohaline species (zebrafish; *Danio rerio*, and channel catfish; *Ictalurus punctatus*). These species were chosen because they are representative of fish found in a variety of environments, ranging from coastal habitats of the southeastern United States to fresh water and brackish habitats throughout the world. Therefore if NIS is more broadly distributed than in a single species, it would suggest that it is performing novel, as yet undescribed roles in iodine transport in the central nervous systems of aquatic animals. These could include developmentally essential functions, as well as functions in maintenance of differentiated neural tissue. For example, Nakane et al. [11] studied the effects of thyroid hormone and seasonal reproduction and gametogenesis in Japanese quail. Based on their research model, they suggested a role of thyroid hormone activating and inactivating enzymes (deiodinase 2 and deiodinase 3, respectively) in glial cells located in the medial basal hypothalamus of the brain. As thyroid hormone is activated by the removal of iodine from the outer ring of thyroxine (T_4), to make triiodothyronine (T_3), deiodination could account for the production of free iodine in the brain. Because thyroid hormone plays an important role in cell migration in the brain (in the cerebral cortex during fetal development and in the cerebellum in the postnatal period) products of the deiodinases (inorganic iodine) likely build up intracerebrally. These inorganic anions would need to be exported from the brain, potentially providing a unique function of NIS in the central nervous system.

The present study aims to confirm and compare the expression of *nis,* the gene coding for the NIS protein, in the brains of multiple teleost fish species across diverse taxonomic orders, and ultimately localize it to a specific area of the brain. If *nis* is indeed expressed in the brain of aquatic teleost fish across multiple lineages, and its expression is confined to a specific

anatomical location, this study should provide an important first step in the discovery of its potentially novel functions in the central nervous system.

CHAPTER II

MATERIALS AND METHODS

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 BSBE surplus breeding stock as part of a tissue-sharing program following euthanasia. Zebrafish were kept in recirculating tanks, filled with reverse osmosis water and 0.5% Instant Ocean (Instant Ocean, VA, USA) at 27°C. Their photoperiod consisted of 14L:10D and their diet consisted of commercial feed (Wardley, NJ, USA) twice daily supplemented with brine shrimp once daily.

Catfish, hybrid striped bass, and tilapia were all obtained from the Texas A&M Aquaculture Research and Teaching Facility (ARTF) as part of a tissue-sharing program after previous use in various nutritional studies. Catfish were kept in outdoor flow-through freshwater tanks exposed to ambient light. Tilapia and hybrid striped bass were kept indoors in recirculating tanks of reverse osmosis water of salinities between 3ppt-7ppt at 27°C on 12L:12D photoperiod. All three species were fed commercial diets, ranging from 32% protein and 8% lipid, to 45% protein and 10% lipid.

Tissue Collection

All animals were euthanized according to approved animal use protocols. A bone saw was used to extract the brains from catfish due to the size and density of their skulls. Incisions were made on the dorsal side of the skull in a 1-inch width by 2-inch length, beginning at the anterior portion of the head near the orbital sockets and reaching posterior to the spinal cord. Once the cuts were made the section of skull was removed. The brain cavity was opened using dissection scissors cutting on the ventral side along the spinal cord towards the anterior, following the optic nerves to expose the brain tissue. For red drum, tilapia, hybrid striped bass, and zebrafish a horizontal cut was made along the posterior dorsal surface of the head and neck. Cuts to the right and left of the exposed spinal cord were made, followed by a third cut just above the spinal cord towards the optic nerves. Sub-pharyngeal tissue was collected as a source of thyroid tissue for positive controls. As thyroid follicles were known to be between gill arches 2-4 in red drum and gill arches 1-3 in zebrafish [4, 10] similar dissections were made in the sub-pharyngeal regions of the remaining species between gill arches 2-5. Finally, small samples of muscle were taken from the tails of each of the fish as negative controls. Tissues were immediately placed in 1-1.5mL RNALater (Ambion, Vilnius, Lithuania) for 4-6 hours at room temperature, then were frozen and stored in RNALater at -80°C for future RNA extraction.

RNA Extraction

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-cooled 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 -80°C until further quantification

RNA Quantification

Using a NanoDrop instrument (Thermo Scientific, NC, USA) 1.65 µL of the extracted RNA was analyzed for quantity (ng/µL) and purity. Purity was based on an A260/A280 ratio of between 1.5 and 2.2 with concentration being optimum between 200-1000 ng/µL. Concentrations outside of this range were still considered acceptable, but those under 200 ng/µL received a longer PCR amplification process after reverse transcription.

Reverse Transcription

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$_2$O), 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.

Primer Design

Red drum

Homologous primers for the *nis* gene in red drum were previously designed [4]. These primers from Butler (2013) were found to work using similar RT-PCR methods and gel electrophoresis confirmation, and were therefore used in these experiments as well. The forward primer was 5'- GCT GTG ATC TGG ACT GAT GTG TTC CA -3' and the reverse primer was 5'- TCC CGC TGT ATG CAC AGG CAA GAA -3' [4].

Zebrafish

Homologous primers for the *nis* gene in zebrafish were also previously designed [2], 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). The forward primer was 5' –

ACA GGC CTG AAC ATG TGG GCG TCT C – 3' and the reverse primer was 5' – ACG GCA TGT ACT GGT CAG GTG C – 3'.

Catfish

Primers for the *nis* gene in channel catfish had not yet been reported in the literature. Therefore the sequence of the entire *nis* gene was found (https://www.ncbi.nlm.nih.gov/nuccore/375703826) and used to create primers for amplification. FastPCR software (FastPCR Helsinki, Finland) was used by inputting the entire gene sequence, and then generating a spreadsheet of possible primers. Primers that gave a quality of above 90%, would not dimerize, and would produce a PCR product between 300-500 base pairs were chosen. The forward primer that was designed was 5'- TCG ACC AGC ATT AAC GCC ATG GCT G-3' and the reverse primer was 5'- TGA GAT CCC ACC ACA GCA AAC CAG G -3'.

Tilapia

Similarly, the *nis* gene for tilapia is fully sequenced and available on GenBank (https://www.ncbi.nlm.nih.gov/nuccore/XM_005453978.3), but homologous primers have not yet been published for this gene. Using FastPCR, the entire gene sequence was input into the software and a list of primers was generated. Primers that gave a quality of above 90%, would not dimerize, and would produce a PCR product between 300-500 base pairs were chosen. The forward and reverse primers for tilapia were 5'- GCC AGC TTC ATG TCA GCA GTC CAG G - 3' and 5'- CGG CAG GAG ATG TAT CGC TGG ACT TG -3' respectively.

Hybrid striped bass

Hybrid striped bass is a hybrid of two closely related perciform species: *Morone saxatilis* (striped bass) and *Morone chrysops* (white bass). The hybrid genome is currently being characterized by a team at North Carolina State University (https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=JTCL01#contigs) and the full *nis* sequence has yet to be listed. However, North Carolina State has released a spreadsheet detailing the percent identity with other known genes indicating there is a 92% identity when compared to *Gasterosteus aculeatus* (stickleback). Using this sequence for the *nis* gene, primers were made using FastPCR software (https://www.ncbi.nlm.nih.gov/nuccore/224925979). Primers that gave a quality of above 90%, would not dimerize with each other, and would produce a PCR product between 300-500 base pairs were chosen. The forward and reverse primers were 5'- ATG TAC GGG GTG AAC CAA GCT -3' and 5'- CAG ATC CTC CAT CGT TAC CGC A -3' respectively.

PCR

Red drum

A 2 µL sample of each red drum cDNA was mixed with 10 µL of GoTaq Green (Promega, WI, USA), 2 µL of *nis* primers, and 6 µL of ddH_2O. For no template controls, ddH_2O was used as a substitute for sample cDNA. 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, 50°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.

Zebrafish

6 μL of cDNA from each sample were mixed with 10 μL of GoTaq Green (Promega, WI, USA), 2 μL of *nis* primers, and 2 μL of ddH_2O. For no template controls, ddH_2O was used as a substitute for sample cDNA. Samples were then placed in the thermocycler and incubated at the following temperatures: 95°C for 2 minutes, followed by 33 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 2 minutes. The samples 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. Annealing temperatures were lowered 2°C below those that were used in previous work [10].

Catfish

4 μL of cDNA from each sample were mixed with 10 μL of GoTaq Green (Promega, WI, USA), 2 μL of *nis* primers, and 4 μL of ddH_2O. For no template controls, ddH_2O was used as a substitute for sample cDNA. Samples were 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, 58°C for 30 seconds, and 72°C for 2 minutes. The samples 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. Annealing temperatures were started at 5°C below suggested values.

Tilapia

4 μL of cDNA from each sample were mixed with 10 μL of GoTaq Green (Promega, WI, USA), 2 μL of *nis* primers, and 4 μL of ddH_2O. For no template controls, ddH_2O was used as a substitute for sample cDNA. Samples were then placed in the thermocycler and incubated at the following temperatures: 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 2 minutes. The samples 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. Annealing

temperatures were started at 5°C below suggested values, and were lowered based on a lack of amplification, or raised if multiple bands were present.

Hybrid striped bass

2μL of cDNA from each sample were mixed with 10μL of GoTaq Green (Promega, WI, USA), 2μL of *nis* primers, and 6 μL of ddH_2O. For no template controls, ddH_2O was used as a substitute for sample cDNA. Samples were then placed in the thermocycler and incubated at the following temperatures: 95°C for 2 minutes, followed by 45 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 2 minutes. The samples 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. Annealing temperatures were started at 5°C below suggested values and were lowered based on a lack of amplification, or raised if multiple bands were present.

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 were 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 the 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, and 9 μL of ddH2O (New England BioLabs, MA, USA). Each of the samples from the PCR were thawed by hand and inserted into corresponding wells, then were then run at 94V until for approximately 75 minutes or until the loading dye reached the bottom

of the gel. The gels were then 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 to confirm the gene identity via BLAST recognition.

CHAPTER III

RESULTS

RNA Yields

RNA yields and absorbance values from tissue samples are shown in Tables 1-5, with species' common name followed by its taxonomic name. 260nm/280nm ratios of between 1.8-2.2 were considered acceptable. All other ratios outside of this range were excluded from further analysis. RNA extracted in previous studies in our lab was used for both red drum (lower jaw and muscle) and zebrafish (muscle) [10].

Table 1. RNA Results for Red Drum (*Sciaenops ocellatus*)

Tissue	RNA Yield (ng/µL)	260/280 Ratio	260/230 Ratio	Absorbance	A-280	A-260
Brain 1	692.2	2.11	2.29	7.567	17.304	8.21
Brain 2	341.8	2.03	2.31	3.702	8.545	4.204

Table 2. RNA Results for Zebrafish (*Danio rerio*)

Tissue	RNA Yield (ng/µL)	260/280 Ratio	260/230 Ratio	Absorbance	A-280	A-260
Brain 1	344.2	1.97	1.85	4.653	4.368	8.606
Brain 2	95.4	2.03	2.26	1.054	2.385	1.173
Brain 3	75.3	2.09	2.17	0.866	1.881	0.9
Sub-Pharyngeal 1	529.9	1.98	1.93	6.85	13.247	6.675
Sub-Pharyngeal 2	670.1	2.02	2.14	7.822	16.753	8.286
Sub-Pharyngeal 3	510	1.98	1.63	7.828	12.75	6.434
Sub-Pharyngeal 4	558.1	2.01	1.96	7.137	13.953	6.95

Table 3. RNA Results for Tilapia (*Oreochromis niloticus*)

Tissue	RNA Yield (ng/µL)	260/280 Ratio	260/230 Ratio	Absorbance	A-280	A-260
Brain 1	833.6	2	2.3	9.058	10.44	20.841
Brain 2	177.7	2.01	2.26	1.96	2.209	4.42

Table 3. RNA Results for Tilapia (*Oreochromis niloticus*) Continued

Brain 3	877.4	1.98	2.35	9.353	11.094	21.935
Brain 4	1068.9	2.01	2.37	11.296	13.309	26.724
Brain 5	24.5	1.51	0.49	1.25	0.613	0.405
Sub-Pharyngeal 1	1250.3	2	2.36	13.27	15.622	31.257
Sub-Pharyngeal 2	1866.1	2	2.3	20.26	23.304	46.653
Sub-Pharyngeal 3	409.6	1.99	2.25	4.541	5.148	10.24
Sub-Pharyngeal 4	1472.8	1.99	2.31	15.944	18.484	36.821
Sub-Pharyngeal 5	58.7	1.54	0.69	2.112	1.467	0.953
Muscle 1	200.5	2.07	2.13	2.348	2.425	5.011
Muscle 2	170.7	2.06	1.98	7.153	2.071	4.268
Muscle 3	706.7	2.12	2.19	8.082	8.352	17.669
Muscle 4	238.7	2.08	1.95	3.059	2.873	5.969
Muscle 5	6.6	1.66	0.33	0.505	0.1	0.166

Table 4. RNA Results for Channel Catfish (*Ictalurus punctatus*)

Tissue	RNA Yield (ng/µL)	260/280 Ratio	260/230 Ratio	Absorbance	A-280	A-260
Brain 1	155.1	2.05	1.87	2.07	3.877	1.887
Brain 2	328.2	2.04	2.1	3.9	4.01	8.206
Brain 3	139.4	2.06	1.85	1.88	1.689	3.484
Brain 4	249.3	2.04	2	3.12	3.053	6.231
Brain 5	95.8	2.1	1.76	1.35	1.143	2.396
Sub-Pharyngeal 1	208.7	2.05	1.85	2.83	2.545	5.217
Sub-Pharyngeal 2	181.7	2	1.86	2.44	2.202	4.527
Sub-Pharyngeal 3	778.4	2.04	2.09	9.3	9.557	19.46
Sub-Pharyngeal 4	335	2.06	2.03	4.12	4.063	8.374
Muscle 1	405.3	2.01	1.84	5.52	5.036	10.131
Muscle 2	674.8	2.2	2.15	7.86	7.677	16.871
Muscle 3	481.4	2.03	1.68	7.16	5.977	12.038
Muscle 4	602.3	2.03	1.96	7.69	7.434	15.059
Muscle 5	554.4	2.23	2.5	5.54	6.206	13.86

Table 5. RNA Results for Hybrid Striped Bass (*Morone saxatilis*)

Tissue	RNA Yield (ng/µL)	260/280 Ratio	260/230 Ratio	Absorbance	A-280	A-260
Brain 1	826.2	2.09	2.09	9.891	9.871	20.656
Brain 2	456.1	1.99	1.69	6.76	5.727	11.402
Brain 3	704.1	2.13	2.25	7.833	8.263	17.601

Table 5. RNA Results for Hybrid Striped Bass (*Morone saxatilis*) Continued

Brain 4	371.2	2.08	1.99	4.66	4.472	9.28
Brain 5	233.8	2.04	1.87	3.123	2.87	5.844
Sub-Pharyngeal 1	5.6	1.82	1.11	0.127	0.077	0.141
Sub-Pharyngeal 2	4.3	1.74	0.59	0.182	0.062	0.108
Sub-Pharyngeal 3	101.9	2.14	2.51	1.104	1.188	2.547
Sub-Pharyngeal 4	7.3	2.11	0.41	0.438	0.086	0.181
Sub-Pharyngeal 5	2.3	3.41	0.8	0.079	0.017	0.059
Muscle 1	7.1	2.11	0.79	0.223	0.084	0.177
Muscle 2	2	6.38	0.92	0.054	0.005	0.049
Muscle 3	8.8	1.78	1.31	0.169	0.124	0.221
Muscle 4	7.6	2.16	0.68	0.277	0.088	0.19

Figure 1. Gel Electrophoresis of Amplified *nis* cDNA DNA gels from each tested species showing NIS amplification. Lanes: La: molecular weight ladder, B: brain tissue, SP: sub-pharyngeal tissue, M: muscle tissue, NT: no template negative control.

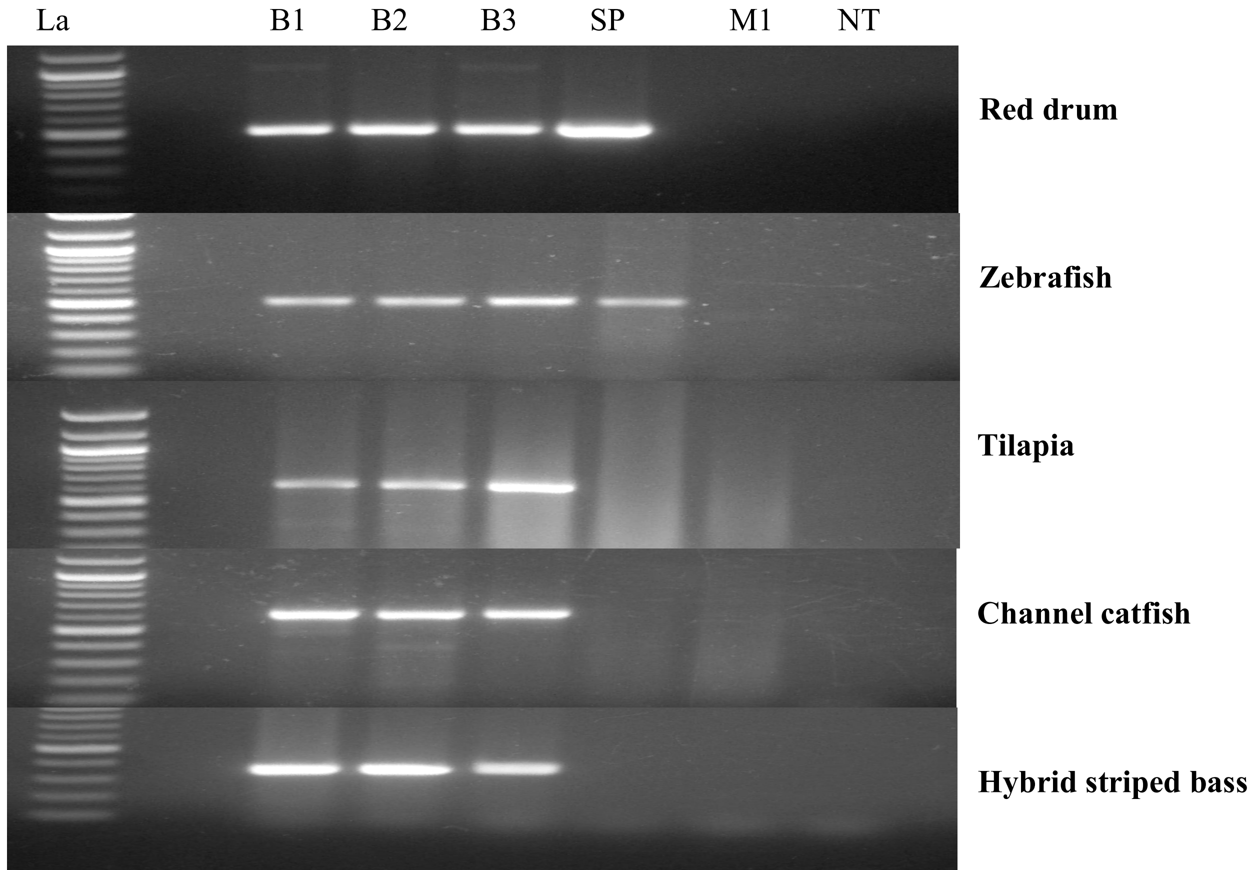

Table 6. Species-Specific Annealing Temperatures for PCR Amplification of *nis*

Species	Annealing Temperature (T_m)
Sciaenops ocellatus	50°C
Danio rerio	59°C
Ictalurus punctatus	58°C
Niloticus oreochromis	53°C
Morone saxatilis	53°C

Table 6. Species-specific annealing temperatures, optimum for PCR protocol and amplification of respective *nis* genes.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

This research investigated the expression of *nis* in the brains of five teleost fish that spanned three orders (Perciformes, Cyprinformes, and Siluriforms). After tissue collection, RT-PCR, and DNA gel electrophoresis, *nis* was found to be expressed in the brains of all five test species. This not only supports my hypothesis that *nis* is present in the brains of adult teleost fish, but more-so suggests that with such robust expression *nis* could indeed be performing an important physiological function. This was achieved through appropriate optimization of PCR protocols for each experimental species. Using suggestions from the primer generator (fastPCR), based on *nis* sequence input and respective A-T and G-C content, annealing temperatures were initially set five degrees below maximum temperature. Based on initial gel electrophoresis, annealing temperatures were increased if more than one band was present on the gel, and decreased if there was little to no binding. Subsequently, gel band sequencing revealed similar to exact matches based on BLAST sequence comparisons. This further confirmed that each band present was indeed NIS, in both brain tissues and sub-pharyngeal tissues.

While this supports my hypothesis that NIS is present in the brains of teleost fish, many authors have discussed a conflicting general anatomical location for neural *nis* expression [5, 15, 18, 19]. Wolff [1] believed that with so little iodine present in the brain it should not be considered as an important tissue participating in iodine transport. Moreover, NIS was later shown to be present by histological staining, but was noted in the pituitary instead of the choroid plexus [18]. While iodine transport was originally observed in the choroid plexus in frogs [18], NIS histological [15] staining was performed on normal human tissues. While some would

consider this an inconsistency, I believe that this, along with current proof of broad distribution of *nis* expression across multiple orders, underscores the importance of neural NIS presence, and more-so the necessity of discovering a more accurate and definite location of NIS in the brain.

Similarly, without an established location in the brain, NIS neural function has been undercharacterized. The study performed by the Spitzweg group [15] used similar RT-PCR techniques to mine to characterize neural NIS presence, but included other areas of expression such as the parotid and the submandibular glands, lacking a focus on iodine transport in the brain. As stimulating hormones like thyrotropin do not regulate iodine transport in extrathyroidal tissues [15], *nis* expression in these tissues might be constitutive or substrate-regulated. Because non-thyroidal iodine transporting tissues do not have the ability to organify accumulated iodine [15] it is most likely that iodine transport in the brain is not for thyroid hormone production.

Because thyroid hormone action is dependent on deiodination for activation or inactivation [11] I propose that the neural actions of deiodinase II and deiodinase III cause an accumulation of inorganic iodine in the brain extracellular or cerebrospinal fluid. While some iodide accumulation may be desirable for antioxidant effects [13] I believe this accumulation of inorganic iodine helps explain the necessity of *nis* expression in the brain. NIS could provide a means to reduce high concentrations of intracerebral iodide, thus returning iodide to the circulation for recycling by the thyroid. In early studies of neural iodine transport it was found the net directional flow of iodide was from the ventricular surface of the choroid plexus to the surrounding serous fluids [18]. This suggests a more unidirectional flow of iodine out of the brain rather than an importation of iodine into neural tissues. I therefore suggest that iodine transport in the brain is not only mediated by NIS, but is also necessary as a means of iodine export, possibly out of the cerebrospinal fluid.

Confirmation of authentic *nis* expression was established through the use of positive and negative controls. Muscle tissue was taken as a negative control. As *nis* expression has been reported to be absent in muscle tissues [4, 10], this was successfully used to confirm that positive bands were not an amplification of something other than *nis*. For positive controls, thyroid follicles expressing *nis* are known to exist in the sub-pharyngeal regions of both red drum and zebrafish, but have not been confirmed in tilapia, channel catfish, or hybrid striped bass. The sub-pharyngeal positive controls for *nis* expression served to confirm that the RT-PCR worked properly, but were only successful in two of the five species: red drum and zebrafish. As the other three test species contained a robust expression of *nis* in the brain, but not in the sub-pharyngeal regions, further investigation is warranted into the location of a more accurate positive control in these species.

With clear amplification of *nis* in the sub-pharyngeal regions of both red drum and zebrafish, the lack of any *nis* expression in the sub-pharyngeal regions of channel catfish, tilapia, and hybrid striped bass is suspect. With similar RNA yields obtained in each of the isolations, it is unlikely that the lack of *nis* expression on the respective DNA gels was due to low concentrations of cDNA. It could be explained by the non-uniform location and clustering of thyroid follicles. While it has been found that sub-pharyngeal regions are good positive controls for *nis* expression in red drum and zebrafish, other species of teleost fish have been known to cluster thyroid follicles in places such as the head kidney [6]. The lack of a positive control amplification from channel catfish, tilapia, and hybrid striped bass sub-pharyngeal tissue could therefore be due to a different anatomical location of their thyroid follicles and consequent lack of *nis* expression in the sub-pharyngeal region collected. Intestine seems to have a more consistent positive expression of *nis* across teleost species [4, 10] and should therefore be

examined as a more reliable anatomical location for a positive control in future experiments with these same species

The robust expression of nis in the brain across these five teleost species reemphasizes the need for a more precise identification of where *nis* is expressed. Using the sophisticated genetic tools available for zebrafish it should be possible to locate a more specific anatomical location for NIS expression. *In situ* hybridization using an *slc5a5* RNA probe, as was used in a recent study monitoring TSH receptor function [15], could precisely identify locations in the central nervous system where nis is expressed. Moreover, to further elucidate its function in the brain, *nis* knockouts using CRISPR-Cas9 techniques could be utilized. This might also help identify physiological functions of NIS during specific developmental stages or in response to changes in environmental iodine availability.

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