DIFFERENTIAL EXPRESSION OF FOUR DISTINCT GAD GENES IN THE DEVELOPING ZEBRAFISH EMBRYO

by

CHELSEA E. GUNDERSON

(Under the Direction of James D. Lauderdale)

ABSTRACT

Glutamic acid decarboxylase (GAD) is the enzyme responsible for the synthesis of GABA, the main inhibitory neurotransmitter of the central nervous system. Most vertebrates have two GAD genes, *GAD1* and *GAD2*, each of which encodes their respective GAD enzyme and are thought to be expressed all inhibitory neurons of the mammalian spinal cord. Our lab has shown that there are three GAD genes in zebrafish – *gad1a*, *gad1b*, and *gad2* – which appear to be differentially expressed in the zebrafish spinal cord at 1 day post-fertilization (dpf), and recent evidence has shown the presence of a fourth zebrafish GAD gene, termed *gad3.* We identified differential expression of each GAD gene by *in situ* hybridization. We have determined which interneuron cell types express *gad1a, gad1b,* and *gad2* in the spinal cord at 1dpf and have shown the expression pattern of *gad3* through the first day of development in the zebrafish embryo.

INDEX WORDS: GABA, *gad1a, gad1b, gad2, gad3,* zebrafish, interneurons, EVL, neural development

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DEDICATION

I dedicate this work to my parents, Roni C. and Mark A. Gunderson; my sister, Kalea B. Gunderson; and my grandparents, Charlotte M. Brooks and Alva V. Brooks, Jr. Thank you for your constant support and encouragement over the last three years. I also dedicate this work to my aunt and uncle, Joan M. Gunderson Lyon and Dr. Jeffrey A. Lyon, for their contribution to my education and for always encouraging my ambition in pursuing a career in science. I would not be where I am today without their continuous support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to GABA and GAD in vertebrates

γ-aminobutyric acid (GABA) is the most prominent inhibitory neurotransmitter of the central nervous system (CNS) and is therefore critical in proper nervous system functioning. In addition to its primary function as a neurotransmitter, GABA has been shown to serve in cell metabolism, as well as play a role in early development.

In the mature CNS, inhibition by GABA occurs by interaction with three possible receptors. Binding to the ionotropic $GABA_A$ or $GABA_{A-\rho}$ (previously termed $GABA_C$) receptors located on the post-synaptic neuron results in a direct influx of Cl^- which leads to hyperpolarization and therefore inhibition of an action potential [1–6]. $GABA_A$ receptors are located throughout the CNS, and ligand-binding to this receptor is the primary source of prompt synaptic inhibition. $GABA_{A-\rho}$ receptors are mostly localized to the retina but can also be found elsewhere in the CNS [2, 4, 7, 8]. The metabotropic $GABA_B$ receptor is a G-protein-coupled receptor, and thus binding to GABA induces a signaling cascade for downstream inhibition, which occurs more slowly in comparison to synaptic inhibition via $GABA_A$ receptors [9–12]. $GABA_B$ receptors have been found in pre- and post-synaptic locations and thus mediate inhibition locally [10, 11]. Pre-synaptic ligand-binding by $GABA_B$ receptors results in the closing of Ca^{2+} channels in the pre-synaptic neuron, thereby inhibiting neurotransmission [11], while post-synaptic ligand-binding eventually leads to depolarization by the opening of K^+ channels in the post-synaptic neuron [10].

Within the neuron, GABA is mostly localized to the axon terminal where it is loaded into vesicles for synaptic transmission, but a portion of the total available GABA also localizes to the cell body to be used as an energy source. GABA localized to the cell body is metabolized such that it can enter the Tricarboxylic Acid (TCA) cycle via the GABA shunt pathway [13, 14]. GABA is catabolized by GABA-transaminase (GABA-T) to produce succinic semialdehyde (SSA). Oxidation of SSA produces succinate, which is then able to enter the TCA cycle [13–16]. This process is not restricted to GABAergic neurons, as local glia, astrocytes, and nonneural tissues can metabolize GABA by partial use of the GABA shunt pathway (see Figure 1.1) [14, 15].

GABA is synthesized via an enzymatic reaction, by which glutamic acid decarboxylase (GAD) converts excitatory glutamate into GABA by α-decarboxylation [17–21]. Two isoforms of GAD exist in all vertebrates, each of which is responsible for the conversion of glutamate into GABA and is encoded by an individual gene. These genes, *GAD1* and *GAD2*, encode the enzymes GAD67 and GAD65, respectively, and each isoform is named appropriately for its weight in kilodaltons (weighing 67 and 65 kD) [16, 22–27]. Both GAD67 and GAD65 are composed of an N-terminal domain, which is responsible for proper localization of the enzyme, and the C-terminal or catalytic domain. The latter domain is highly conserved and contains the binding site for its required cofactor, pyridoxal 5'-phosphate (PLP) [28]. When bound to PLP, GAD is a functional holoenzyme and is therefore able to properly convert glutamate into GABA. It has been shown that GAD65 exists in an inactive state *in vivo* as an apoenzyme, becoming active upon binding to PLP, while GAD67 is always found as a holoenzyme [29, 30].

While both isoforms of GAD are active in GABA synthesis, they are differentially distributed throughout the cell body and are thought to contribute to total GABA pools within inhibitory neurons in different capacities. GAD67 is generally localized to the cell body, but also

to axon terminals, and has been hypothesized to provide GABA for metabolic purposes as well as maintaining the cell's GABA resource; GAD65 is localized to the axon terminals and contributes GABA for synaptic transmission[16, 22, 29, 31–33]. These findings are in agreement with evidence for subcellular GABA pools as detailed previously (see Figure 1.1) [13–16]. Despite this apparent delegation for GABA production, studies have shown that GAD67 can compensate for GAD65 deficits, while loss of GAD67 activity is detrimental [34, 35]. Therefore, GAD67 must be providing GABA for neurotransmission as well as cell metabolism [22].

While both GAD genes have generally overlapping expression in the CNS, some differential expression has been found in neural tissues. High levels of *GAD1* expression has been reported in most regions of the murine brain and spinal cord, including the cerebellar cortex and basal ganglia, and in neurons which co-express both GAD isoforms, GAD67 is usually the most prevalent [27, 31, 33, 36]. However, there are exceptions in which GAD65 expression is predominant in regions involved in visual processing, as well as several hypothalamic nuclei [27]. Likewise, differential expression of GAD67 and GAD65 has been shown in the primate visual cortex, with most stellate neurons containing GAD67 [37].

In addition to its presence in the CNS, components of GABAergic signaling (GAD, GABA, and GABA receptors) have been found in adult mammalian nonneural tissues such as the pancreatic islets [38–42], kidney [43–45], testis [46–49], oviduct and ovary [48, 50, 51]. *GAD* mRNA expression was found in rat spermatocytes and spermatids [47], and there is evidence for the presence of $GABA_A$ receptors in human spermatozoa [52]. Both GAD enzymes have been found in the rat oviduct, with GAD65 being the predominant isoform present [48, 50]. GABAergic signaling has also been implicated in estrous cycling in rats as well as pregnancy in humans [48, 53, 54]. Evidence for the importance of GABAergic signaling in endocrine functioning has

likewise been shown in the pancreas, gastrointestinal tract, and adrenal glands [55–58]. *GAD* expression has been found in murine embryonic nonneural tissues such as the lens [59], pharyngeal arches, limb buds, tail bud, and vibrissae placodes [60, 61]. Thus, GABA, and therefore GAD, is essential for proper functioning of many bodily systems in vertebrates.

1.2 GABA and GAD in vertebrate development

Despite GABA's importance for maintaining balance in the mature nervous system via inhibition, GABA performs an excitatory role in early development [62–64]. Upon binding to the $GABA_A$ receptor located on an immature post-synaptic neuron, depolarization – rather than hyperpolarization – occurs due to an efflux of Cl^- from the post-synaptic neuron. In the mature neuron, maintenance of the intraneuronal Cl^- concentration prior to binding of synaptic GABA is controlled by the Cl^- channel, NKCC2 [65–67]. Expression of the gene encoding this channel is upregulated in normal neural functioning, but is downregulated in early development. Another Cl^- channel, NKCC1 is upregulated at this point in time and allows for an increase in Cl^- concentration, which triggers intraneuronal Cl^- to follow its concentration gradient out of the post-synaptic neuron. Thus, the shift from regular inhibitory GABA to an excitatory GABA is the result of fluctuating mRNA levels which results in fluctuation of cellular Cl^- concentration [63, 66–68].

GABA is one of the earliest neurotransmitters present in vertebrate development, and thus has been found to be essential for proper neural development. Interactions of GABA and the $GABA_A$ receptors present in neural stem cells results in a slowing down of the cell cycle, suggesting that it is involved in the regulation of the timing of neurogenesis [69]. Conversely, GABA has also been associated with promoting some sites of cortical progenitor proliferation over others [69, 70]. This seems to be part of a mechanism employed by GABA for maintaining stem

cell pools [71]. In addition to this, GABA has been shown to have a role in guiding synaptogenesis and neural differentiation [16, 72, 73].

GAD expression has also been implicated in vertebrate development. In addition to its involvement in some of the processes mentioned above, evidence from murine literature has shown that transcripts resulting from the alternative splicing of *GAD1* are present only in development [60, 74, 75], and also only found in some tissues [59, 76–78]. Many studies have also investigated the importance of GAD and GABAergic signaling in murine craniofacial development [35, 61, 79–82], and research from our lab has pointed to the importance of GAD in craniofacial and neural development in zebrafish [83–85].

1.3 The zebrafish GAD genes

While it is generally accepted that most vertebrates have only two GAD genes, and thus two isoforms of the GAD enzyme, zebrafish have been found to have four GAD genes: *gad1a*, *gad1b*, *gad2*, and the recently recognized *gad3* [23, 85–88]. *gad1b*, previously referred to as *gad1* [23, 86], and *gad2* are orthologs of the mammalian *GAD1* and *GAD2* genes, which also encode 67kD and 65kD GAD enzymes [23, 89]. However, recent studies and work from our lab has shown that a *GAD1* paralog exists in the zebrafish genome and is expressed in early development, termed *gad1a* [85, 87, 88]. These *GAD1* paralogs likely arose from the teleost-specific genome duplication event, which occurred after the divergence of teleosts from other vertebrates [90–99]. Copies of genes resulting from a whole genome duplication event that are not degraded may undergo neofunctionalization, in which one of the duplicates takes on a new function entirely, or may undergo subfunctionalization, such that a splitting of the original expression pattern occurs and both genes may complement one another [100]. The evidence for functionality of *gad1a* presented by our lab points to the latter being true [85]. We have found that *gad1a* is expressed early in

zebrafish development, prior to the presence of a recognizable nervous system at ~16hpf, and is the most highly expressed *gad* gene in the spinal cord once dorsally positioned neurons begin to appear. Likewise, we have shown what appears to be differential expression of *gad1a*, *gad1b,* and *gad2* in the developing zebrafish brain and spinal cord from ~16hpf to 3dpf (see Figure 1.3) [85]. This points to a complex regulation of GABAergic signaling in the developing zebrafish embryo.

GABA-positive cells are first detected in the zebrafish CNS around 16hpf, with emergence in nuclei of the telencephalon, post-optic commissure, medial longitudinal fascicle of the midbrain, as well as in the ventral diencephalon and in clusters within the hindbrain and spinal cord [101]. Clusters of GABAergic cells begin to increase between 18 – 24hpf, and four of the six types neurons which emerge in the spinal cord are GABAergic interneurons [102, 103]. These GABAergic interneuron subtypes were in defined in the zebrafish spinal cord by their axonal trajectory and position along the dorsoventral axis (see Figure 1.2) [104]. The most dorsally located interneurons are called dorsal lateral descending (DoLA) and commissural secondary ascending (CoSA) neurons. The more medially located interneurons are ventral longitudinal descending (VeLD) neurons, and the most ventrally located interneurons are called Kolmer-Agduhr (KA) neurons (see Figure 1.3) [86, 102, 104]. These GABAergic interneurons were previously thought to mostly co-express *gad1* and *gad2*, as in mammals [86]. Studies in 1 – 3dpf zebrafish have found GABA to be localized in various regions throughout the brain [105, 106], and that the previously termed "*gad1*" and *gad2* are mostly co-expressed in the brain and spinal cord at 24hpf [86, 107].

Prior to the discovery of the *gad1a* paralog, research in other teleost fishes had presented evidence for another GAD gene. RT-PCR experiments of adult brain cDNA from a type of deep sea teleost, the abyssal grenadier (*Coryphaenoides armatus*), revealed *GAD1* and *GAD2* vertebrate

orthologs, as well as another GAD gene termed *GAD3* [23, 108]. This GAD3 was found to be only 64% identical to GAD67 and GAD65. Phylogenetic analysis determined that the abyssal grenadier *GAD3* was equally divergent from both *GAD1* and *GAD2* and has evolved at a similar rate. Thus, it was hypothesized that GAD3 must have emerged prior to the divergence of teleosts from other vertebrates, possibly from an ancient gene duplication [23]. Further experiments determined that GAD3 was co-expressed with GAD67 and GAD65 in the grenadier cerebellum, telencephalon, and hypothalamus, and that GAD3 expression was highest in the cerebellum compared to other brain regions [108]. Later work found an orthologous GAD3 in goldfish (*Carassius auratus*), which was 86% identical to the grenadier GAD3, and was also expressed in the telencephalon and hypothalamus [109, 110].

Recent bioinformatic evidence has shown that highly conserved but uncharacterized GAD3 sequences exist in many vertebrate genomes, including one in zebrafish identified as *zgc:163121* [87], which we refer to as *gad3*. The zebrafish *gad3* transcript is truncated in comparison to *gad1a, gad1b,* or *gad2* transcripts, but the PLP-binding domain is conserved and therefore likely yields a functional GAD enzyme [87, 111, 112]. However, no studies have been published which define an expression pattern for *gad3* or its potential role in GABAergic signaling. Taking this information into account with what we have learned about the expression of the other zebrafish GAD genes implies another layer of complexity and potential for future insights into the roles of GAD in neural development.

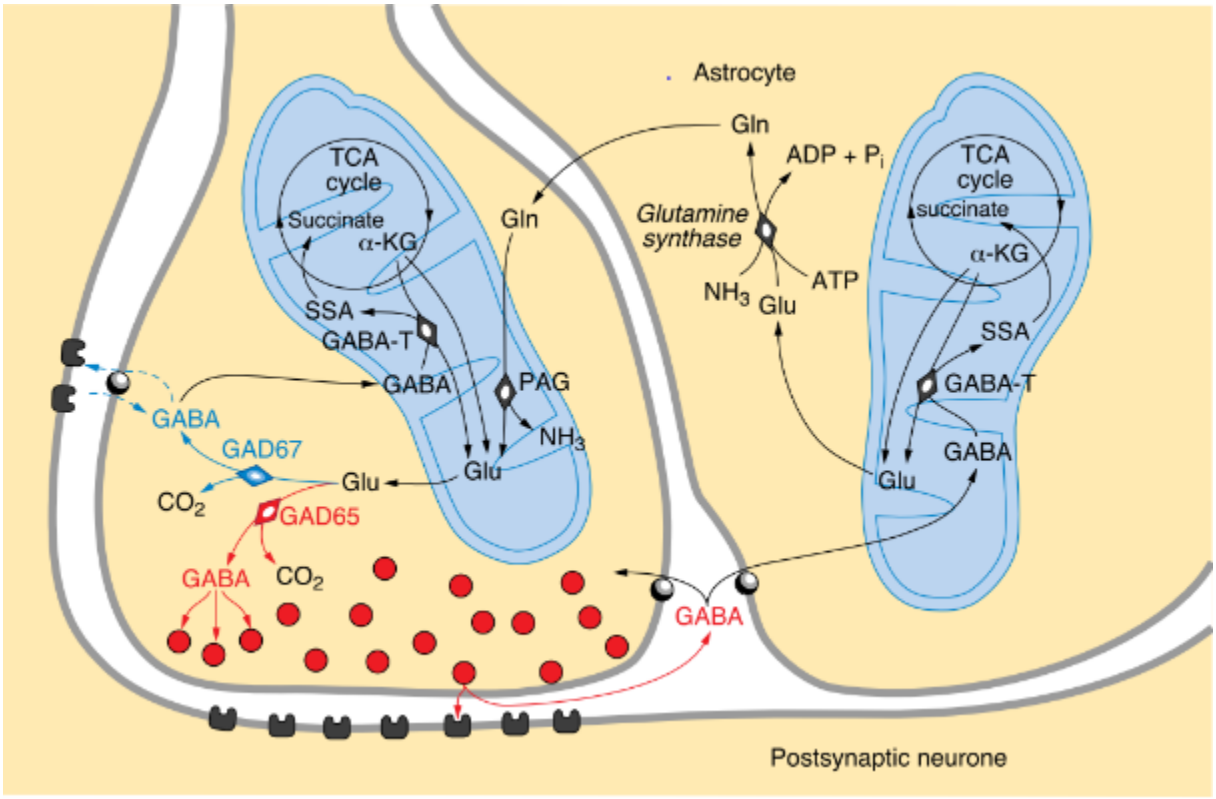

Figure 1.1 GABA production for cell metabolism and neurotransmission. Illustration adapted from [15]. Representative distributions of GAD67 and GAD65 and their function within the pre-synaptic neuron. GAD67 is shown to be mostly involved in the production of GABA for cell metabolism, but some synaptic transmission occurs. GAD65 is shown to be mostly localized to the axon terminal for production of GABA for synaptic transmission. The GABA shunt pathway in the pre-synaptic neuron and partial use of the pathway in a local astrocyte are detailed. Glutamine (Gln) is processed into glutamate (Glu). Glu is then converted to GABA by GAD67 and GAD65. GABA synthesized by GAD67 is diverted into the GABA shunt pathway and is catabolized by GABA-T to make SSA, which following oxidation enters the TCA cycle.

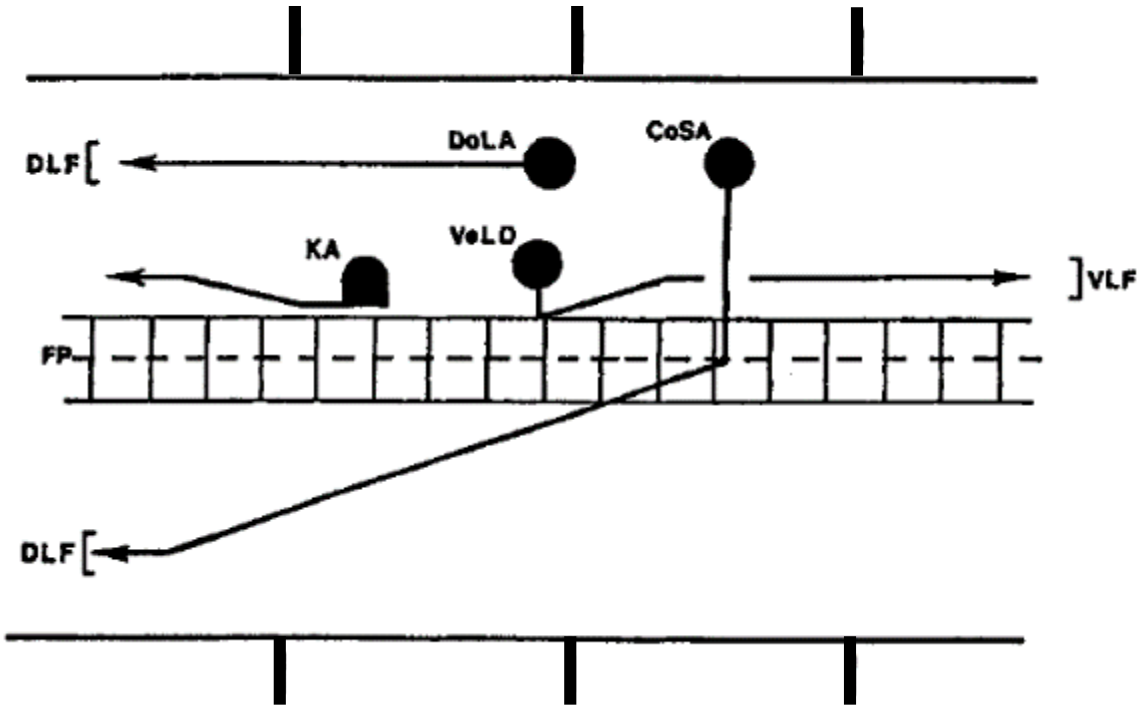

Figure 1.2 GABAergic interneuron subtypes of the developing zebrafish spinal cord. Illustration adapted from [104] showing the positions and axonal trajectory of GABAergic spinal interneurons at ~22 hpf, displayed in an open-book format. Floor plate (FP) is shown in the center with the ventral midline marked by the dashed line. Vertical black lines at the top and bottom indicate segmentation. Dorsal lateral ascending (DoLA), commissural secondary ascending (CoSA), ventral longitudinal descending (VeLD), and Kolmer-Agduhr (KA) interneurons shown, with their respective contralateral or ipsilateral axon trajectories. Longitudinal tracts of GABAergic neurons at this stage are the dorsal longitudinal fasciculus (DLF) and ventral longitudinal fasciculus (VLF).

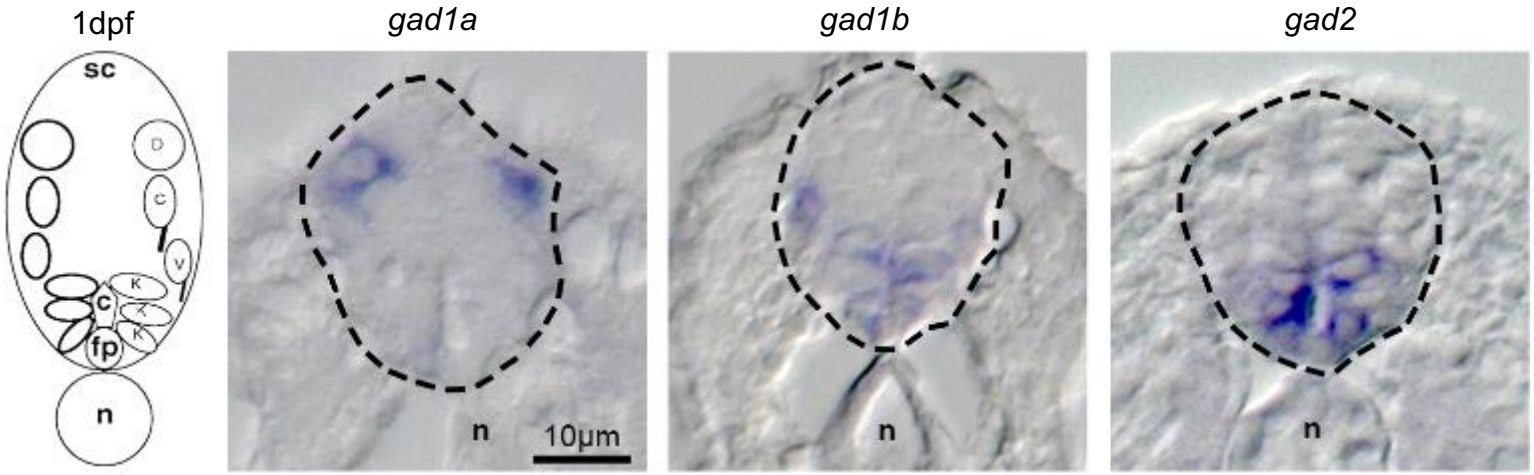

Figure 1.3 Expression of the GAD genes in 1dpf zebrafish. Illustration and images are adapted from [85]. Schematic of the positioning of each GABAergic interneuron subclass along the dorsoventral axis in the spinal cord at 1dpf [86]. Colorimetric *in situ* hybridization was performed to visualizer *gad1a, gad1b,* and *gad2* expression in transverse sections of the spinal cord at 1dpf. Dashed lines indicate the outline the spinal cord. sc, spinal cord; c; central canal, fp, floor plate; n, notochord; D, DoLA neurons; C, CoSA neurons; V, VeLD neurons; K, KA neurons.

CHAPTER 2:

DIFFERENTIAL EXPRESSION OF THE GAD GENES IN THE DEVELOPING ZEBRAFISH SPINAL CORD

2.1 Introduction

γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter of the central nervous system, is synthesized from glutamate via the enzyme glutamic acid decarboxylase (GAD). While most vertebrates have only two GAD genes, and thus two isoforms of the GAD enzyme, zebrafish have been found to have at least three GAD genes: *gad1a*, *gad1b*, and *gad2*. *gad1b*, previously referred to as *gad1*, and *gad2* are orthologs of the mammalian *GAD1* and *GAD2* genes and also encode 67kD and 65kD GAD enzymes [23, 86, 89]. However, recent work from our lab has shown that a *GAD1* paralog exists in the zebrafish genome and is expressed in early development, termed *gad1a* [85]. These paralogs likely arose from the teleost-specific genome duplication event, which occurred after the divergence of teleosts from other vertebrates [90–99].

The inhibitory actions of GABA are mediated by GABAergic interneurons in the spinal cord, which express the genes encoding the GAD enzymes. In zebrafish, there are four subclasses of GABAergic interneurons established at 1dpf in the spinal cord, which are generally defined by their axonal trajectory and position along the dorsoventral axis [102, 104]. The most dorsally located interneurons are called dorsal lateral descending (DoLA) and commissural secondary ascending (CoSA) neurons. The more medially located interneurons are ventral longitudinal descending (VeLD) neurons, which are sometimes referred to as V2b neurons for the

subpopulation of V2 neurons from which they arise [113, 114]. Lastly, the most ventrally located interneurons are called Kolmer-Agduhr (KA) neurons, or cerebrospinal fluid-contacting neurons (CSF-cN) [115, 116]. Previous research appears to show that *gad1* and *gad2* may be differentially expressed in these interneurons at 1dpf [63, 84]. We now recognize this "*gad1*" as *gad1b* and have provided additional evidence for differential expression of *gad1a, gad1b,* and *gad2* in the developing zebrafish [85], but the differential expression patterns in the developing spinal cord have not yet been conclusively determined. In this study, we have sought to determine the identities of the interneuron subtypes which express *gad1a, gad1b,* and *gad2*. To this end, we have identified definitive markers for each interneuron subtype which could be used for determining co-expression with each GAD gene, based on available expression data. The marker *tbx16* was chosen for DoLA interneurons [117, 118]; *pax2a* was chosen as the marker for CoSA interneurons [119, 120]; and *pkd2l1* was chosen as the marker for KA interneurons [115, 116, 121]. Minimal expression information was available in the literature for a definitive marker of VeLD interneurons at 1dpf, so we chose *mnx1*, which is expressed in both VeLD interneurons and motorneurons [122, 123]. Studies have shown that *pkd2l1* is also expressed in VeLD interneurons at 1dpf [115, 116, 121], so we have drawn on this information to accurately determine any co-expression with GAD in VeLD interneurons. Determining which interneuron subtypes express each GAD gene at 1dpf will provide further insight into the importance of the GAD genes in early neural development and will serve as a basis for continued research in order to understand the coordination and potential regulatory actions of these genes in development.

2.2 Methods

2.2.1 Animal Care and Maintenance

Adult zebrafish (*Danio rerio*) of the WIK strain were cared for and maintained in accordance with standard procedures [124, 125]. All experimental procedures were conducted in accordance with the National Institutes of Health guidelines for use of zebrafish in research under protocols approved by the University of Georgia Institutional Animal Care and Use Committee.

2.2.2 Staging and Fixation of Zebrafish Embryos:

Zebrafish used in all experiments were staged appropriately according to standard protocols [125]. Animals were anesthetized with 0.4% tricaine in PBS and then transferred to a 1.5 mL microfuge tube. Anesthetized animals were treated with 4% PFA in PBS for 2 hours at room temperature (RT) or overnight at 4 ºC, followed by washes in PBS and then dehydration in 100% methanol for storage at -20ºC.

2.2.3 Probe Synthesis:

Probes for *gad1a*, *gad2*, and each interneuron marker were constructed from gBlock Gene Fragment (Integrated DNA Technologies, Coralville, IA) templates. gBlocks were designed to contain a sequence of ~ 600 – 900 bp matching the target message, the Sp6 promoter sequence on the 5' end, the T7 promoter sequence on the 3' end, and random stabilizing sequences on each end (see Table 2.S.1 for sequences). Each gBlock was amplified using the Sp6 Fwd primer (5'-TGCATCGCGTGCTGCTGGCCTGGCACTGATTTAGGTGACACTATAG-3') and T7 Rev primer (5'-CTGACTCGACGGCGTCACCGTCCGCTACTCTGGACTAATACGACTCACTATAGGG -3'). PCR was performed with 1 ng of gBlock template under the following conditions: initial denaturation at 95 ºC for 5 minutes, followed by 32 cycles of denaturation at 95 ºC for 30 seconds, annealing at 72 ºC for 30 seconds, and extension at 72 ºC for 1 minute, and then final

extension at 72 ºC for 5 minutes preceding termination. The *gad1b* probe was constructed from 1dpf whole organism cDNA template, which was amplified by the following primers: *gad1b* Sp6 Fwd (5'-ATTTAGGTGACACTATAGTTCTTCCGAATGGTGGTCTC-3') and *gad1b* T7 Rev (5'-TAATACGACTCACTATAGGGTTCACCTCACAAAGGTGCTG-3'). PCR was performed with 100 ng of cDNA template under the following conditions: initial denaturation at 95 ºC for 2 minutes, followed by 32 cycles of denaturation at 95 ºC for 30 seconds, annealing at 55 ºC (T_m *gad1b* primer pairs) for 30 seconds, and extension at 72 ºC for 1 minute, and then final extension at 72 ºC for 2 minutes preceding termination. All amplicons were stored at 4 ºC and then purified with the GeneClean II Glassmilk Purification Kit (MP Biomedicals LLC, Solon, OH), according to the manufacturer's protocol.

Sense and antisense riboprobes for each GAD probe were synthesized by *in vitro* transcription with the DIG RNA Labeling Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Interneuron marker probes were labeled with fluorescein (FITC) by substitution of the DIG-labeling mix with the Fluorescein RNA Labeling Mix (Roche). Sp6 and T7 RNA polymerases were used to transcribe the sense and antisense probes, respectively, from each purified template. Riboprobes were purified by LiCl precipitation with glycogen overnight, followed by ethanol washes and resuspension in nuclease-free water. The concentration for each probe was estimated by UV spectrophotometry with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA dot blot analysis was performed to determine the relative strength of each probe when stained with NBT/BCIP (Roche). Probes were then diluted 1:10 in Hybridization buffer [126] or 10mM Tris (pH 8) and stored at -80 ºC.

2.2.4 Double Fluorescent in situ Hybridization (FISH)

Whole mount double fluorescent *in situ* hybridization was performed with adaptations from published FISH protocols [127–129]. Prior to rehydration from storage, fixed embryos were incubated in 6% H_2O_2 in methanol for 30 minutes at RT to quench endogenous peroxidases. Following rehydration, permeabilization, and post-fixation, specimens were incubated in Hybridization (Hyb) buffer for ≥ 2 hours at 70 ºC. A combination of one DIG-labeled GAD gene probe and one FITC-labeled interneuron marker probe were diluted 1:100 into Hyb buffer and incubated with specimens overnight at 70 ºC. After probe removal and stringency washes, specimens were washed in TNT (0.1M Tris-HCl pH 7.5; 0.15M NaCl; 0.5% Tween-20), and incubated in FISH block (0.5% Roche Blocking Reagent + 5% horse serum in TNT) for ≥ 2 hours at RT. Specimens were incubated in 1:5000 anti-FITC-POD (Roche) antibody overnight at 4 ºC. Following the first antibody removal and subsequent washes in TNT, the first target gene expression was detected by Tyramide Signal Amplification (TSA) with the TSA Plus Cyanine 3 and Fluorescein system (Perkin Elmer), with the addition of the Alexa Fluor 488 Tyramide Reagent (Life Technologies). Specimens were incubated in 1:50 Alexa Fluor 488-tyramide conjugate in 1X Amplification Diluent for 30 minutes – 1 hour at RT in order to detect the FITC-labeled probe. TSA reaction was stopped by washes in TNT followed by incubation for 1 hour in 2% H_2O_2 in TNT to quench the antibody-conjugated peroxidase activity. Specimens were washed again in TNT and then incubated again in FISH block, followed by an overnight antibody incubation in 1:2000 anti-DIG-POD in FISH block at 4 ºC. Following antibody removal and washing, the DIG-labeled probe was detected by incubation in 1:50 Tyr-Cy3 in 1X Amplification Diluent for 30 minutes – 1 hour at RT. The TSA reaction was stopped by washes in TNT, and then specimens were transferred into PBST and treated with 1 µg/mL DAPI in PBST for 10 minutes at

RT. After subsequent washes in PBST, embryos were cleared in 70% antifade glycerol (4% n-propyl gallate in PBST) overnight at 4 ºC. Whole mount specimens were mounted onto slides in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and confocal microscopy was performed using a Zeiss LSM 880 Confocal Microscope, with image processing performed in Zeiss ZEN Black software (Carl Zeiss Microscopy LLC, White Plains, NY).

2.2.5 O.C.T. Embedding and Cryosectioning

Specimens processed for double fluorescent *in situ* hybridization were rehydrated from 70% antifade glycerol into PBST and then incubated in 10% sucrose in PBS for 1 hour at RT, followed by overnight incubation in 30% sucrose in PBS at 4 ºC. Specimens were then mounted in TissueTek O.C.T. compound (Sakura Finetek USA Inc.) and frozen at -80 ºC. Blocks of FISH specimens were then cryosectioned in 8 μm sections and collected on charged slides. Slides were held at -20 ºC until O.C.T. removal. O.C.T. removal was performed by incubating slides in PBS at 37 ºC for ~10 minutes, followed by washes in PBS. Slides were then mounted with FluoroGel mounting medium (GeneTex) and coverslipped. Sections were imaged by confocal microscopy as described above.

2.3 Results

2.3.1 gad1a *is expressed in all GABAergic interneuron subtypes at 1dpf*

The results from our double fluorescent *in situ* hybridization experiments show that *gad1a* is co-expressed with each interneuron marker, *pax2a, tbx16, mnx1,* and *pkd2l1* in 1dpf zebrafish (see Figures 2.1 – 2.4). *gad1a* was found to be present in all DoLA and VeLD interneurons imaged, as shown by co-expression with *tbx16* and *mnx1*, as well as co-expression in VeLD interneurons expressing *pkd2l1* (Figures 2.3 – 2.4). Interestingly, *gad1a* was also found to be co-expressed in some but not all of the more dorsally located *pkdl21*-expressing KA interneurons (Figure 2.4).

Likewise, *gad1a* was co-expressed in most, if not all, *pax2a*-expressing CoSA interneurons at 1dpf (Figure 2.2).

2.3.2 gad1b *is expressed in CoSA, VeLD, and KA interneurons at 1dpf*

Our results have shown that *gad1b* is highly expressed in GABAergic interneurons in the 1dpf zebrafish spinal cord and is co-expressed with *pax2a, mnx1,* and *pdk2l1*. *gad1b* is co-expressed with most, if not all, *pkd2l1*-expressing cells, including both KA and VeLD interneurons (Figure 2.4) as well as *mnx1*-expressing VeLD interneurons (Figure 2.3). Similarly, *gad1b* was shown to be co-expressed with most, but not all, *pax2a*-expressing CoSA interneurons (Figure 2.2). No evidence of co-expression of *gad1b* with *tbx16* was found from these experiments (Figure 2.1).

2.3.3 gad2 *is expressed only in KA interneurons at 1dpf*

We only observed *gad2* co-expression with *pkd2l1*, as a marker for KA interneurons (Figure 2.4). No *gad2* expression was shown in *pkd2l1*-expressing VeLD interneurons at 1dpf. We also did not observe any co-expression of *gad2* and *mnx1,* but we currently consider these data to be inconclusive due to the poor quality of the specimens (Figure 2.3).

2.4 Discussion

The results of our fluorescent *in situ* hybridization experiments have shown that *gad1a, gad1b,* and *gad2* are differentially expressed in GABAergic interneurons of the zebrafish spinal cord at 1dpf. *gad1a* was found to be expressed in all DoLA and VeLD interneurons and in a subset of the CoSA and KA interneurons observed. Similarly, *gad1b* was found to be expressed in most GABAergic interneurons observed, with the exception of DoLA interneurons. Lastly, *gad2* expression was only observed in KA interneurons at 1dpf. As expected, we observed differential expression along the dorsoventral axis of the spinal cord with *gad1a* expression appearing in more dorsally located cells and *gad2* expression in ventrally located cells, while *gad1b* expression was

visualized from ventrally located cells up to the dorsal position of CoSA interneurons. It is interesting that DoLA interneurons only express *gad1a* at 1dpf while all other interneuron cell types observed appear to express at least two GAD genes at this stage. Likewise, it is interesting that while the KA interneuron subtype appears to express all three GAD genes at 1dpf in the more dorsally positioned cell bodies, the more ventrally located cells appear to only express *gad1b* and *gad2.* Perhaps *gad1a* is somehow involved in the regulation of the dorsoventral patterning of GABAergic interneurons in the spinal cord or is necessary for proper formation of dorsal interneurons. We have previously observed that *gad1a* turns on early in neural development [85], which could be indicative of a regulatory function. One report has shown that *gad1a* is lowly expressed in adult zebrafish [88], which may be consistent with the hypothesis that *gad1a* serves a more regulatory purpose in development. Further studies of co-expression at different developmental timepoints, as well as analysis of spinal cord development in GAD mutants will need to be conducted in order to determine the importance of *gad1a* in proper spinal cord development and dorsoventral axial patterning of GABAergic interneurons.

Table 2.S.1 gBlock sequences for *in situ* hybridization probes. Sequences below do not contain Sp6/T7 promoter sequences or random stabilizing ends.

GENE	GBLOCK SEQUENCE (5' – 3')
gad1a	TGTCTGGAGCTGTCGGAGTATCTCTACCACAAGATCAAGAACAGAGAAGGATATGAGATGGTGTTTCAAGGGGAGCCACAGCACACAAATGTATGTT TCTGGTACATTCCTCCAAGCCTGCGGCTTCTGCCAGATGGAGAGGAGAAACGACATCGGCTTCATAAGGTCGCCCCAAAGATCAAGGCACTGATGAT GGAGTGCGGGACAACAATGGTGGGCTACCAGCCTCAGGGTGAGAAGGTTAACTTCTTCAGGATGGTGGTCTCCAATCCGGCGGTTACCAGGTCTGAC ATTGACTTCCTGATCGATGAGATAGAAAGACTGGGACAGGATTTATAGAGAACGCAGAACAAGTTCAGTAGATGTAAATCATCTGGAAATGGAGGA GGCAATCAGACGTGTTGTATCAGCTCGATGTTCCAGAGAATTAGGCCCTGTCCACAGAAACACAGCTA
gad2	TCTACAACAAGATTAAGGACAGGGAAGGATATCAGATGGTGTTTGATGGAAAGCCGCAGCATACCAATGTGTGTTTCTGGTACCTTCCACCGGGCGT GCGCTACCTGGAGGACAAAGTGGAGAGGATGAAGCGTCTGCACAAGGTTGCCCCTGTAATCAAAGCCAGAATGATGGAGTACGGCACGACCATGGT GAGCTACCAGCCACAGGGAGACAAGGTCAACTTCTTCCGCATGGTCATCTCCAATCCAGCCGCTACCTTTGAAGACATTGACTTCCTCATTGAAGAG ATCGAGCGACTGGGGCAGGATCTTTAAAACTTACCGCACCAAAACCTGTTACTCCCGTGTCCCTGGATGGATTGCATATTTGTTGTGAATGTAACGGT AAATCTCTGATTCCTCTTCTCCAAAGTCACATTTAAAC
mnx1	TCCACCGCCGGAATGATGCTGCCTAAAATGGCAGATTTCAATGGCCAGGCGCAGTCGAACTTACTCGGCAAGTGCAGAAGACCAAGAACTGCATTCA CAAGCCAGCAGCTCCTTGAACTTGAGCATCAGTTTAAGCTGAATAAATATCTATCCAGACCAAAACGCTTTGAAGTGGCCACGTCATTGATGCTAAC AGAGACGCAGGTGAAAATCTGGTTTCAGAACAGGCGCATGAAATGGAAGCGCAGTAAAAAGGCCAAAGAACAAGCCGCTCAAGATGCCGAGAAGC AAAAGGGAAAAGGAAACCACGACAAAATGGACGGACTGGAAAAGGACTACCAGAAGGTAGATTCAGGGAAAAGTAACAGAATACGGGACTTTAGG GACAGTGACGACGAAGAAGGAGATAACTATATGCTGAATTCATCTGATTGTTCCTCTGAGGATGAACGAACCAATGACATAAGTCCACAACCATGAG ACCTTATAAAAACGAAAGACTATGAGTGAATTATGTGATTTGTATGTATCATCATCTTGCAGTCAATATGGAAATGCAGAGGTGACATCCACATGAG CTGTTTGTACACATACAAACATC
pax2a	ACTCAACAGCAGCTGGAGGCTCTGGATCGGGTGTTTGAGCGGCCGTCATACCCCGACGTCTTCCCCACGTCAGAACACATCAAGCCAGAGCAGGCTA ATGAGTACTCGCTACCAGCACTGAACCCTGGACTGGACGAAGTCAAGCCCAGTCTGTCAACCAGCGTCAGCTCAGATTTGGGCTCCAGCGTGTCACA GAGCTACCCAGTAGTGACAGGTCGAGAGATGGCGAGCACGACCCTACCAGGATATCCACCTCACGTTCCCCCTACTGGGCAGGGCAGCTACCCCACC TCTACACTTGCTGGAATGGTCCCTGGAAGCGACTTTTCAGGAAATCCCTACTCTCACCCGCAGTACACAACTTACAATGAAGCTTGGCGGTTCAGCAA CCCCGCGTTATTAAGTTCCCCTTATTATTATAGTGCCGCATCCCGGGGCTCCGGGCCTCCCACTGCTGCCACTGCCTATGACCGCCACTAGTTACCATC GCAGCCCAGTCAAACTGCAGGACCACGGCCGCGGCCTCCATATCGTACCCGTCTGAATGGTCAGAGGGATTGAAGATGGATACGCCATCTTCACTTT CATGGAGCCGAAAT
pkd2l1	TAGTGGTGATACTGCTTGCTGTGGTGGCAATTGTTTTCAGTGCATTTCGGACCATCAAAGTGGATGGATTACTCGGAAACCTTCTGAAACAACCAGAC ATCTATGCTGATTTTGAATTTTTGGCATTTTGGCAAACCCAGTACAACAACATGAATGCAGTCAATTTGTTTTTTGCTTGGATTAAGATCTTCAAGTAC ATCAGCTTCAATAAGACGATGACTCAGCTGACGTCCACACTGGCTCGCTGTGCTCTGGACATTTTTGGATTTGCCATCATGTTCTTCATTGTGTTTTTT GCGTATGCTCAGCTTGGGTACCTGCTCTTTGGGACAGAGGTGGAAACTTTCAGCACGTTTAACAAATGCATTTTCACACAGTTTCGAATCATCCTTGG AGATTTCGATTATGATGCCATTGACAGAGCAAATCGAGTGCTTGGACCCATTTACTTCTTCTCCTATGTATTCTTTGTCTTCTTTGTGTTACTGAACATG TTTCTGGCCATCATCAATGACACATACTCTGAAGTGAAGTCAGAGCTCGCATCTCAGAAAGACGAGTTCCAGATTGCAGATCTCATCAAGCAGAGTT ATGCTAAGACTTTCATGAAGCTGAAGCTTAAAAAGGAAAAGATCTCTGATGTTCAGAAAGCTCTGGATTCAGGCGCAAGTGAGCTGGAGTTTAAAGA TTTCAGGAACGCGTTGAAAGAGATGGGTCACAGTGACCGAGAGATCTCAGCCGCTTTCTCCAAATTCGACCAGGATGGAAACCAGACTTTAGACAAG CAGGAGCAGGAGAACATGAAGCAGGAGCTGGAGGAGAAAAGGGATGCTCTCAGTGCAGAGCTGCGTGACCTCGAGAACAGTGTGGAACCA
tbx16	CACATACCAGCCGTACAGATTTCACGAATACGGCAAATCCCCGTCTCCATCTTCATCTTCCAGCGTTGGCGGCAGCAGTGCATGTGGCAGCGCGGGA CGTCCCAGCTTTGAGTCCCGAGTCTTGGACGTGGCCACCGTGCCCGACACAGACAGCTCCAGCAAGCCCTCCTCCGCTCCAGAGTTCAGCCTCCCTCC GCATCCCTCTGCAGGACACCAGGAGTACGCTGGAGTGCTAAACATGGCCATCACCCAGGCCAAACCAGGCATGCTGGGAACTCATCCGCTTTACAGC CACTACAGCACAGAACAGTCTCTGGGGCAGTGGAGCGGGGCAGCAGCATCCCAATACCCGCCTCCACCTCCGCCCCATCATCACCTTCCCACCGAAT ACAGCAGCCAAGCTGTCCATCATGGCTATCACCATGGAAACGTTGGCGATTGGAGCCAGTATCCGCTTTTCTCATACTCGTGCTGGTGAGCCGGACAC CGATTGAGAGACCATTTTGAGGATGCTTATTTTTAATTTTCTGCACGATGAGGTCTGGATGGTTTTTCATGACCTGTAAAATGGCACACGAAGTCGCC CGGTACTGAGTGTAC

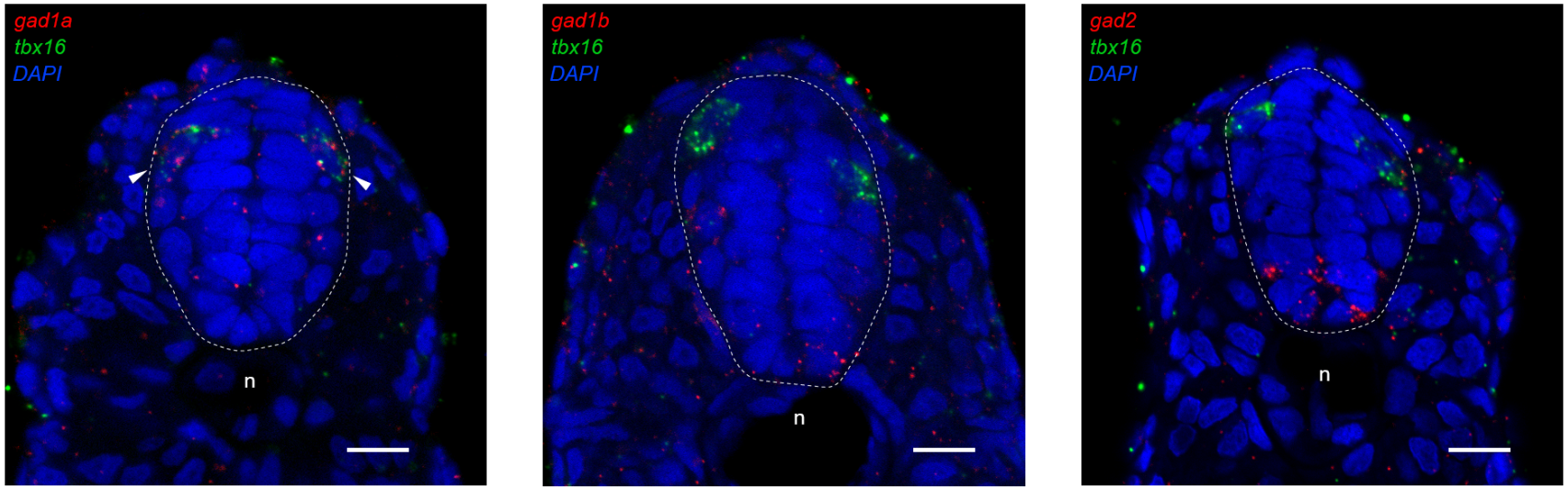

Figure 2.1 Double fluorescent *in situ* hybridization of DoLA interneurons and GAD. Transverse cross sections of 1dpf zebrafish spinal cords show co-expression of *gad1a*, but neither *gad1b* or *gad2* with the DoLA interneuron marker, *tbx16*. Dashed lines indicate spinal cord; arrowheads indicate co-expression. Scale bars = 10 µm.

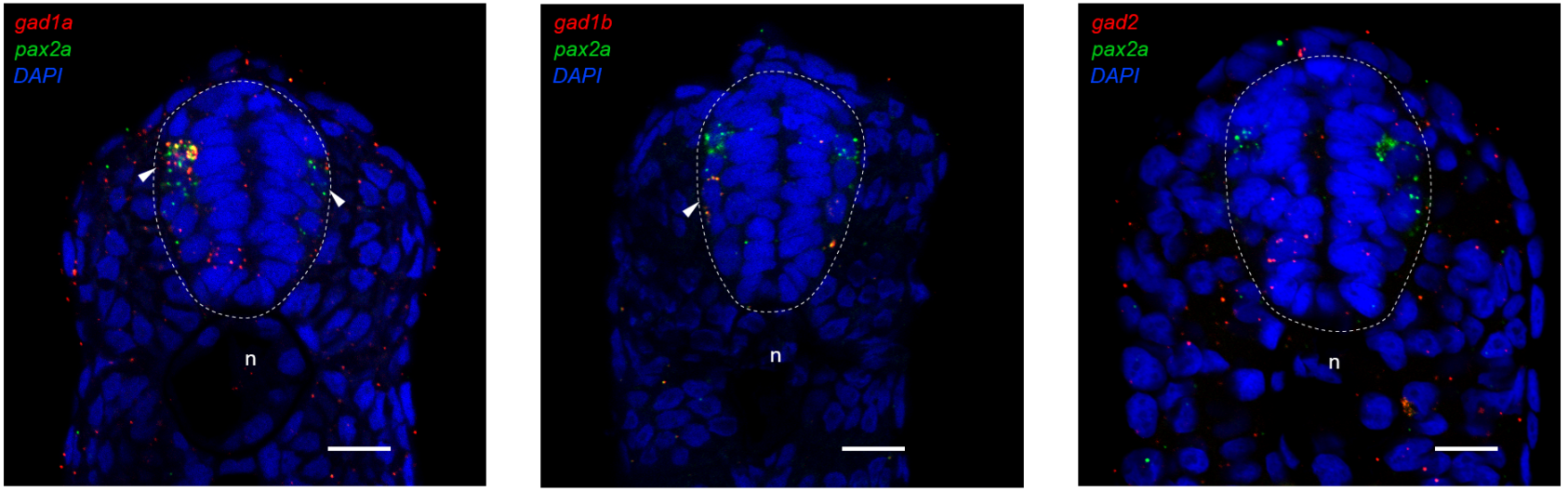

Figure 2.2 Double fluorescent *in situ* hybridization of CoSA interneurons and GAD. Transverse cross sections of 1dpf zebrafish spinal cords show co-expression of *gad1a* and *gad1b*, but not *gad2* with the CoSA interneuron marker, *pax2a*. Some *pax2a*-positive cells likely express both *gad1a* and *gad1b* at 1dpf, but it seems that the more dorsal CoSA interneurons only express *gad1a* at this stage (see middle panel). Dashed lines indicate spinal cord; arrowheads indicate co-expression. Scale bars = 10 µm.

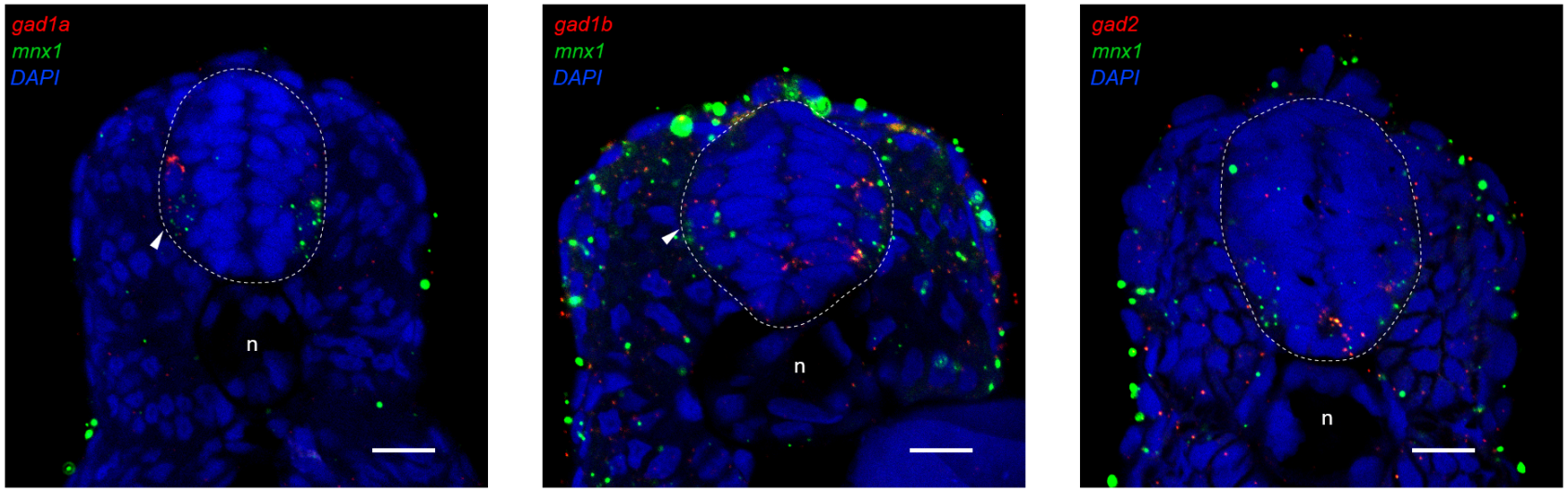

Figure 2.3 Double fluorescent *in situ* hybridization of VeLD interneurons and GAD. Transverse cross sections of 1dpf zebrafish embryos appear to show *mnx1* co-expression with some *gad1a and gad1b* cells at this stage. We cannot definitively conclude that *gad2* is not expressed in VeLD interneurons (see right panel), although we have never observed *mnx1* co-labeled with *gad2*. Dashed lines indicate spinal cord; arrowheads indicate co-expression. Scale bars = 10 µm.

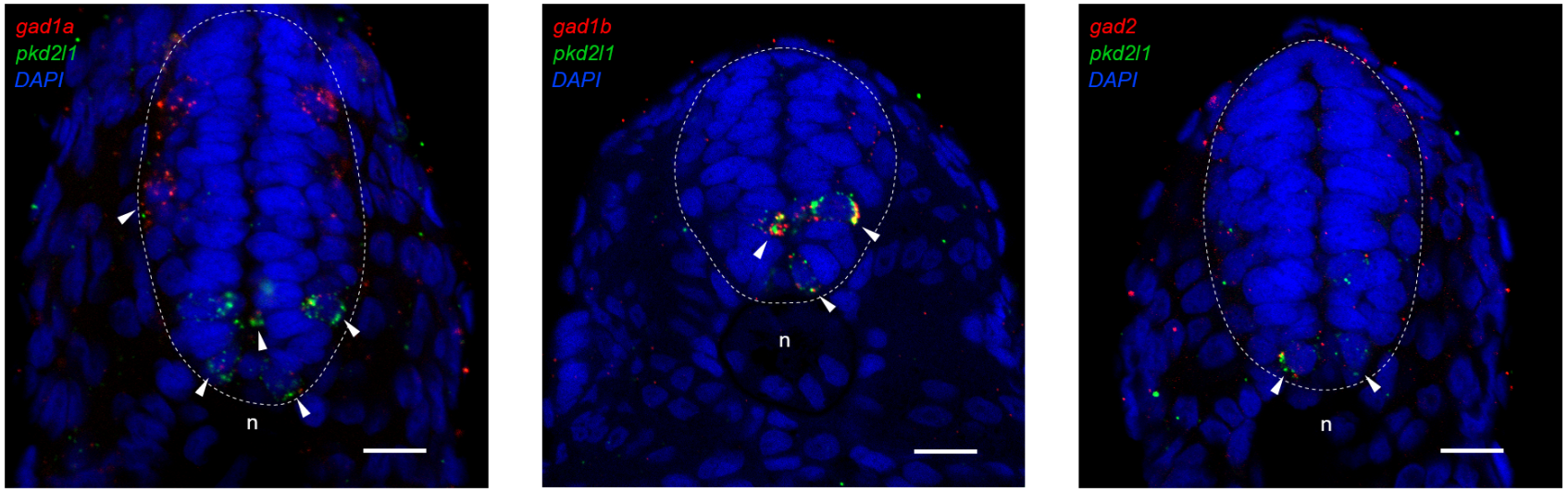

Figure 2.4 Double fluorescent *in situ* hybridization of KA interneurons and GAD. Transverse cross sections of 1dpf zebrafish embryos show co-expression of all zebrafish *gad* genes with the KA interneuron marker, *pkd2l1*. VeLD interneurons positive for *pkd2l1* also express *gad1a* (see left panel). Dashed lines indicate spinal cord; arrowheads indicate co-expression. Scale bars = 10 µm.

CHAPTER 3:

CHARACTERIZATION OF *GAD3* EXPRESSION IN THE DEVELOPING ZEBRAFISH EMBRYO

3.1 Introduction

While most vertebrates have been shown to have just two GAD genes, *GAD1* and *GAD2*, previous studies in other fish species have shown the existence of a third GAD gene. Through RT-PCR of adult brain cDNA from a type of deep sea teleost, the abyssal grenadier (*Coryphaenoides armatus*), researchers found *GAD1* and *GAD2* vertebrate orthologs, as well as another GAD gene termed *GAD3* [23]. This GAD3 was found to be co-expressed with GAD67 and GAD65 in the grenadier cerebellum, telencephalon, and hypothalamus [108]. Additionally, an orthologous GAD3 in goldfish (*Carassius auratus*) has been found, which is 86% identical to the grenadier GAD3, and also expressed in the telencephalon and hypothalamus [109, 110].

Phylogenetic analysis determined that the abyssal grenadier GAD3 was equally divergent from both GAD1 and GAD2 and has evolved at a similar rate. Thus, has been was hypothesized that GAD3 must have emerged prior to the divergence of teleosts from other vertebrates, possibly from an ancient gene duplication [23, 87, 108]. Furthermore, recent bioinformatic evidence has shown that highly conserved but uncharacterized GAD3 sequences exist in many vertebrate genomes, including one in zebrafish identified as *zgc:163121* [87], which we refer to as *gad3*. The zebrafish *gad3* is located on chromosome 2 and contains 15 exons (ENSDARG00000055370) [130]. Three possible transcripts have been reported for this gene, one of which is a retained intron

and is not translated. The principle isoform contains all 15 exons, and an alternative transcription start site located in exon 2 may yield the other isoform, which contains only 14 exons. The resulting transcript is truncated in comparison to *gad1a, gad1b,* or *gad2* transcripts (see Figure 3.1 for gene maps), but the PLP-binding domain is conserved and therefore likely yields a functional GAD enzyme [87, 111, 112]. Very little is known about *gad3* other than the studies referenced above, but one study found that *gad3* (*zgc:163121*) was one of a few of genes which was downregulated in response to treatment with the glucocorticoid antagonist, dexamethone, in zebrafish [131].

Because of our understanding of the importance of the other three GAD genes in zebrafish development, we wanted to determine the expression pattern of *gad3.* Therefore, we designed probes targeting *gad3* mRNA and performed *in situ* hybridization in order to visualize a possible *gad3* expression pattern in the developing zebrafish. We also performed RT-PCR with whole organism cDNA and adult brain cDNA generated from our wildtype zebrafish population to determine the presence of *gad3* expression at various timepoints important for neural development and to ascertain whether *gad3* is also expressed in the adult zebrafish brain. This work will serve as a foundation for further investigation of the role of *gad3* and its relation to the other zebrafish GAD genes.

3.2 Methods

3.2.1 Animal Care and Maintenance

Adult zebrafish (*Danio rerio*) of the WIK strain were cared for and maintained in accordance with standard procedures [124, 125]. All experimental procedures were conducted in accordance with the National Institutes of Health guidelines for use of zebrafish in research under protocols approved by the University of Georgia Institutional Animal Care and Use Committee.

3.2.2 Staging and Fixation of Zebrafish Embryos:

Zebrafish used in all experiments were staged appropriately according to standard protocols [125]. Animals were anesthetized with 0.4% tricaine in PBS, and then transferred to a 1.5 mL microfuge tube. Anesthetized animals were treated with 4% PFA in PBS for 2 hours at room temperature (RT) or overnight at 4 ºC, followed by washes in PBS and then dehydration into 100% methanol for storage at -20 ºC.

3.2.3 Developmental Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

Total RNA was collected from the pools of 50-60 embryonic or larval stage zebrafish using TRIzol Reagent (Life Technologies, Carlsbad, CA). Animals were anesthetized appropriately with 0.4% tricaine in PBS, and RNA extraction was performed according to the TRIzol RNA extraction protocol, followed by precipitation with glycogen and isopropanol. RNA was stored at -80ºC. Adult brain RNA was collected by pooling 4 adult zebrafish brains and performing the RNA extraction as above. RNA was subsequently treated with DNase I to eliminate genomic DNA contamination. cDNA was generated from each RNA sample with the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA) with the oligo$(dT)_{18}$ primer according to the manufacturer's instructions, and stored at -20 ºC. The following primer sequences were used to amplify *gad3* and *ß-actin*, respectively: *gad3* Fwd (5'-CAACAAACACCGCGTGAAAC-3') and *gad3* Rev (5'-GCAATAACAACTGGCATCAGC C-3'); *ß-actin* Fwd (5'-TGTTTTCCCCTC CATTGTTG-3') and *ß-actin* Rev (5'-CTTCTCCTTGATGTCACGGAC-3'). All RT-PCR reactions were performed with 40 – 100 ng of cDNA template under the following conditions: initial denaturation at 95 ºC for 2 minutes, followed by 32 cycles of denaturation at 95 ºC for 30 seconds, annealing at 58 ºC (T_m for *gad3* and *ß-actin* primer pairs) for 30 seconds, and extension at 72 ºC for 1 minute, and then final extension at 72 ºC for 2 minutes preceding termination. RT-

PCR products were stored at 4 ºC. Products were visualized by gel electrophoresis with SYBR Safe DNA gel stain (Life Technologies) on a 1.5% agarose gel made with 1X TAE buffer. Electrophoresis was performed for 30 minutes at 105V. *gad3* RT-PCR products amplified from 3dpf whole organism cDNA and adult brain cDNA were sequenced by Sanger sequencing (GENEWIZ, South Plainfield, NJ) to confirm sequence identity to *gad3* mRNA (see Table 3.S.2 for sequences).

3.2.4 Probe Synthesis:

Probes for *gad3* were synthesized from both cDNA and gBlock Gene Fragment templates. The RT-PCR primer sequences used for amplifying *gad3* from cDNA were modified by the addition of the Sp6 and T7 promoter sequences to the 5' ends of the Fwd and Rev primers, respectively. These modified primers were then combined with the *gad3* RT-PCR Fwd or Rev primers appropriately to amplify a *gad3* product containing either the Sp6 recognition sequence at the 5' end or the T7 recognition sequence at the 3'end. Thus, the Sp6-containing product serves as the template for the sense probe, while the T7-containing product serves as the template for the antisense probe. The primer sequences for generating the sense amplicon are *gad3* Sp6 Fwd (5'-ATTTAGGTGACACTATAGCAACAAACACCGCGTGAAAC-3') and *gad3* Rev (5'-GCA ATAACAACTGGCATCAGCC-3'). The primer sequences for generating the antisense amplicon are *gad3* Fwd (5'-CAACAAACACCGCGTGAAAC-3') and *gad3* T7 Rev (5'-TAATACGACT CACTATAGGGGCAATAACAACTGGCATCAGCC-3'). Products were amplified under the same conditions as described above. Amplicons were then purified by gel extraction using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

The *gad3* gBlock Gene Fragment (Integrated DNA Technologies, Coralville, IA) was designed from the 510 bp mRNA sequence located directly upstream of the *gad3* RT-PCR primer binding sites. The *gad3* gBlock was also designed to contain the T7 promoter sequence on the 5' end, and the T3 promoter sequence on the 3' end, with random stabilizing sequences on each end (see Table 3.S.1 for sequence). The *gad3* gBlock template was amplified using T7 Fwd primer (5'-CCTGGCACTGTAATACGACTCACTATAGGG-3') and T3 Rev primer (5'-CTACTCTGG ACAATTAACCCTCACTAAAGG-3'). PCR was performed with 1 ng of gBlock template under the following conditions: initial denaturation at 95 ºC for 5 minutes, followed by 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 72 ºC for 30 seconds, and extension at 72 ºC for 1 minute, and then final extension at 72 ºC for 5 minutes preceding termination. gBlock amplicons were stored at 4 ºC and then purified by gel extraction. *gad3* gBlock and cDNA-derived amplicons were sequenced by Sanger sequencing (GENEWIZ) to confirm sequence identity to *gad3* mRNA (see Table 3.S.2 for sequences).

Two EVL markers, *keratin 4 (krt4)* and *periplakin (ppl)* were chosen as controls for determining proper visualization of EVL cells by *in situ* hybridization. We designed gBlocks for each of these genes based on the cb59 (*krt4* probe) and cb180 (*ppl* probe) sequence data made available through ZFIN (see Table 3.S.1 for sequences) [132, 133]. gBlocks were designed and amplified in the same manner as described above.

Sense and antisense riboprobes were synthesized by *in vitro* transcription with the DIG RNA Labeling Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Sp6 and T7 RNA polymerases were used to transcribe the DIG-labeled sense probes from the cDNA-derived and gBlock amplicons, respectively. Likewise, T7 and T3 RNA polymerases were used to transcribe the DIG-labeled antisense probes from the cDNA-derived and gBlock amplicons.

Riboprobes were purified by LiCl precipitation with glycogen overnight, followed ethanol washes and resuspension into nuclease-free water. Concentration for each probe was determined by fluorescence-based quantitation with the Qubit RNA HS Assay kit and Qubit 3 Fluorometer (Life Technologies). RNA dot blot analysis was performed to determine the relative strength of each probe when stained with NBT/BCIP (NBT, Roche; BCIP, Bio-Rad Laboratories, Hercules, CA). Probes were then stored as full-strength stocks or diluted 1:10 in 10mM Tris (pH 8) and stored at -80 ºC.

3.2.5 Colorimetric in situ Hybridization

Whole mount *in situ* hybridization was performed according to standard protocols [126] with modifications. Prior to rehydration from storage, fixed embryos were incubated in 6% H_2O_2 in methanol for 30 minutes at RT to quench endogenous peroxidases. Following rehydration, permeabilization, and post-fixation, specimens were incubated in Hybridization (Hyb) buffer for ≥ 2 hours at 70 ºC. Probes were diluted into Hyb buffer and incubated with specimens overnight at 70 ºC. After probe removal and stringency washes, specimens were incubated for ≥ 2 hour at RT in blocking solution (2 mg/mL BSA + 2% heat-inactivated sheep serum in PBST), and then incubated in 1:3000 anti-DIG-AP (Roche) antibody overnight at 4 ºC. After antibody removal, target gene expression was visualized by treatment with NBT/BCIP in alkaline phosphatase buffer in the dark until desired color development was obtained (≤ 5 hours). Stained specimens were mounted in 100% glycerol and imaged with a Zeiss Compound Brightfield and Fluorescent/Colibri microscope and a Zeiss Dissecting Brightfield/Fluorescent microscope.

3.2.6 Fluorescent in situ *Hybridization (FISH)*

Whole mount fluorescent *in situ* hybridization was performed similarly to methods described above [126] for steps through hybridization, with the following modifications from other

FISH protocols [127–129]: after probe removal and stringency washes, specimens were washed in TNT (0.1M Tris-HCl pH 7.5; 0.15M NaCl; 0.5% Tween-20), incubated in FISH block (0.5% Roche Blocking Reagent + 5% horse serum in TNT) for ≥ 2 hours at RT, and then incubated in 1:5000 anti-DIG-POD (Roche) antibody overnight at 4 ºC. Following antibody removal and subsequent washes in TNT, target gene expression was detected by Tyramide Signal Amplification (TSA) with the TSA Plus Cyanine 3 and Cyanine 5 system (Perkin Elmer). Specimens were incubated in 1:50 Tyr-Cy3 conjugate in 1X Amplification Diluent for 30 minutes – 1 hour at RT. TSA reaction was stopped by washes in TNT, and then specimens were treated with 1 µg/mL DAPI in PBST for 10 min at RT. After subsequent washes in PBST, embryos were cleared in 70% antifade glycerol (4% n-propyl gallate in PBST) overnight at 4 ºC. Whole mount specimens were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and confocal microscopy was performed using a Zeiss LSM 880 Confocal Microscope, with image processing performed in Zeiss ZEN Black software (Carl Zeiss Microscopy LLC, White Plains, NY).

3.3 Results

3.3.1 gad3 *is present in early zebrafish development through adulthood*

Our RT-PCR experiments have shown that *gad3* is expressed as early as the 6hpf (Figure 3.2), which is in agreement with other studies that show *gad3* expression at 4hpf [134–136]. Here, we have shown *gad3* expression from 6hpf through larval day 7 from whole organism cDNA. We also observe a faint band present from RT-PCR with cDNA generated from 1-4 cell stage total RNA with multiple technical replicates (data not shown), but this is inconclusive. In addition to these findings, we have shown that *gad3* is in fact expressed in the adult zebrafish brain (Figure 3.2), which is consistent with studies shown in other teleost species [23, 108, 109].

3.3.2 gad3 *is present in EVL cells through 1dpf*

In order to determine the expression pattern of *gad3* in zebrafish tissues, we designed two *in situ* hybridization probes, one derived from whole organism cDNA and another from a synthesized gBlock gene fragment, each of which targeted separate regions of the *gad3* transcript (see Table 3.S.2 for probe sequences). Colorimetric *in situ* hybridization for *gad3* with both cDNA-derived and gBlock probes revealed an identical expression pattern in 1dpf zebrafish (see Figure 3.3 and Figure 3.S.2 for *gad3* staining). *gad3* signal was detected in the most superficial cells, which were determined to be enveloping layer (EVL) cells based on location and morphology. *gad3* also appears to be expressed in the midbrain at 1dpf, but not in the spinal cord. Possible signal was observed in the optic tectum, in the cells lining the third ventricle (see Figure 3.3), but further imaging analysis will need to be conducted in order to confirm this observation.

Since *gad3* appeared to be most highly expressed in the EVL at 1dpf, we repeated the colorimetric *in situ* hybridization experiments with the *gad3* cDNA-derived probe using embryos fixed at 6hpf, 18hpf, and 24hpf in development. We observed strong staining for *gad3* in the EVL at each of these stages (see Figure 3.4). In order to better evaluate the observed *gad3* expression pattern in the EVL and verify that we were able to properly detect genes expressed in the EVL, we performed another set of *in situ* hybridization experiments using probes for two EVL markers, *krt4* and *ppl*, with 18hpf and 24hpf embryos. The results of these experiments showed the expected expression patterns for both *krt4* and *ppl* (see Figure 3.S.3 for 24hpf embryos), matching available expression data [132, 133].

3.3.3 gad3 *is not present in the spinal cord at 1dpf*

We repeated these experiments by fluorescent *in situ* hybridization to visualize any low expression of *gad3* in 1dpf zebrafish. We observed strong expression of *gad3* in the EVL, but *gad3* was not detected in the spinal cord at this timepoint (Figure 3.5).

3.4 Discussion

We have determined that *gad3* is expressed as early as 6hpf, and possibly even in the 1-4 cell stage prior to zygotic transcription, with continuous expression through larval day 7. These findings are in agreement with data made available through various RNA sequencing (RNA-seq) studies, most of which focus on timepoints in early development [134–137]. Data from a recent study which provides a developmental time course for mRNA expression [134], and made available through the Expression Atlas [138], reveals documented expression of *gad3* (*zgc:163121)* during the blastula stages up to larval day 5 (see Figure 3.S.1 for this mRNA expression data). The expression levels depicted in this study show increased expression at 50% epiboly through 75% epiboly and at prim-15 relative to the other timepoints [134], which could explain the some of the fluctuation of the relative band intensities shown for different timepoints in our RT-PCR results (Figure 3.2). In addition to showing *gad3* expression through larval day 7, we have also presented the first evidence, to the best of our knowledge, that *gad3* is expressed in the adult zebrafish brain. Further experiments will need to be conducted in order to determine which regions of the brain express *gad3* and to begin to elucidate the role of *gad3* in adult zebrafish.

We have also shown the first reported expression pattern by *in situ* hybridization for *gad3* in early zebrafish development. While our data suggests that *gad3* may be expressed in the brain at 1dpf, *gad3* is primarily expressed by EVL cells from 6hpf to 1dpf. These findings are supported by recent single cell RNA-seq (scRNA-seq) studies, which have each provided transcriptome

atlases for the developing zebrafish [135, 136]. These data are invaluable and have allowed us to search for *gad3*-expressing cells that may not be detectable by *in situ* hybridization. According to this information, *gad3* (*zgc:163121*) appears to be most highly expressed in the EVL or cells in the periderm lineage up to the first day of development, as well as some expression in neural tissue [135, 136]. Likewise, data from another recent scRNA-seq study lists *gad3* (*zgc:163121*) as one of 16 upregulated genes in the periderm at larval day 2 [137]. Thus, our detection of *gad3* expression predominantly in the EVL of the developing zebrafish embryo is corroborated.

What we refer to as the zebrafish *gad3*, *zgc:163121*, has been annotated as encoding a putative GAD1-like protein (NP_001083039) [111, 112], rather than another canonical GAD enzyme. GAD1L is another PLP-dependent carboxylase, and is considered paralogous to cysteine sulfinic acid decarboxylase (CSAD). Mammalian GAD1L has not been shown to have decarboxylase activity for glutamate, and instead has been shown to catalyze the substrates needed for the synthesis of taurine, hypotaurine, and ß-alanine [139, 140]. A recent study has also reported that both mouse CSAD and GAD1L can catalyze the decarboxylation of both aspartate and cysteic acid, but display a preference for cysteine sulfinic acid as a substrate [141]. Furthermore, the same study had determined that there is no sequence for a GAD1L gene in the zebrafish genome [141], and phylogenetic analyses have shown that vertebrate GAD3 is more closely related to GAD1 and GAD2 than to GADL1 [87]. While much more information is needed in order to conclude that *zgc:163121* encodes another GAD enzyme, the literature does not currently suggest that this is unlikely or implausible.

Enveloping layer (EVL) cells are a specialized embryonic cell type found in vertebrates such as *Xenopus* and zebrafish. The developing zebrafish epidermis consists of two layers, the superficial EVL and the basal epidermal layer (EBL). The EVL cell lineage emerges during the

blastula period, and serves as a protective monolayer that totally covers the embryo by the end of this period [125, 142]. The EVL also has an important developmental role, as it helps to pull the deep cells along with the yolk syncytial layer during epiboly [125]. The EBL is established after the EVL and resides underneath to form a bilayered epidermis which lasts until the end of the larval stages [142]. The EVL is said to give rise to the zebrafish periderm in larval development, however these terms have been used interchangeably and the process of periderm development and replacement of the EVL has not been extensively studied. However, it has been shown that EVL cells can persist through larval stages [143] and to at least until 1 month of development but are then not found after metamorphosis begins [144]. Throughout this time, EVL cells that are lost are replaced by basal cells, which are derived from a different lineage but fully take over for this protective monolayer [144].

Why would a GAD enzyme be needed in the enveloping layer cells? We know that GAD is present in other tissues outside of the vertebrate CNS, but it is interesting that this gene is expressed so early in development without strong support for its expression in the developing nervous system. While scRNA-seq atlases do contain data showing that *gad3* is expressed in some neural cell types in embryonic zebrafish development [135, 136], the highest expression values and clusters of *gad3*-expressing cells are found in EVL populations, or periderm and epidermal lineages [135–137]. Some research has implicated a role for GABA in epidermal maintenance, as well as use as a potential treatment of inflammatory skin conditions. Two studies have provided evidence of GABAergic involvement in wound healing. Treatment with GABA on rat wounds was correlated with suppressed inflammation and increased cell proliferation and re-epithelization of the wound area [145], and treatment of GABA and $GABA_A$ receptor-agonists was associated with rapid recovery rates, as well as prevention of hyperplasia [146]. Additionally, immunoreactivity

for $GABA_A$ antibody and localization of the $GABA_A$ receptor were observed in mouse epidermis [146]. Another group reported expression of GAD67 in both murine and human dermal fibroblasts by RT-PCR, and has provided evidence for the importance of GAD67 expression in the production of hyaluronic acid and type I collagen in human dermal fibroblasts [147, 148].

Future directions for this work should include experiments designed to determine which, if any, components of vertebrate GABAergic inhibition might be co-expressed or localized near *gad3* expression in zebrafish development and adulthood. In addition to determining the expression pattern of *gad3* in the adult zebrafish brain, studies should be conducted similarly to those previously performed in other teleosts to better understand what role *gad3* plays that may be coordinated with the other zebrafish GAD genes. The evidence of *gad3* expression in the adult zebrafish brain from our research, as well as the complementary findings that *gad3* is expressed in EVL cells, makes this "new" GAD gene even more interesting and warrants further investigation in the functionality of *gad3* in zebrafish development, as well as how this may add to our understanding of GABAergic signaling in vertebrates.

Table 3.S.1 Chosen sequences for *gad3* cDNA-derived and gBlock *in situ* hybridization probes. Sequences below do not contain Sp6/T7/T3 promoter sequences or random stabilizing ends.

PROBE	*GAD3* CDNA FRAGMENT / GBLOCK SEQUENCE (5' – 3')
gad3* Sp6/T7 cDNA fragment	CAACAAACACCGCGTGAAACTGCACGGAATTGAAAGAGCCCATTCTGTGACCTGGAACCC ACACAAGATGATGGGCGTTCCACTGCAATGCTCCACCATTCTAGTCAAGAGGAAGGGCCT CCTGCAGCAGTGTAATCAGCTGTGTGCCGAATATCTCTTCCAACCTGACAAGCACTACGAA GTGTCCTACGACACTGGGGACAAAAGCATTCAGTGCGGACGACACGTGGATATCTTTAAA CTGTGGCTCATGTGGAAGGCGAAGGGCTCCGAGGGTTTTGAATCACAGGTCAACCACTGC CTGGAGAATGCAGAGTATCTTTACTACAAGCTGAAGAGAAGAACAGATTTTCAGCTCGTCT TCAAGGGAAAACCTGAACACAGTAATGTGTGCTTTTGGTATCTCCCCAAACGAGTGCAAA ACATTCCTCTAGGCCCAGAGCGAGAGAAAGAACTTCATATGGTGGCTCCGAAGATCAAGA CAAAGATGATGGAGGAAGGATTCACCATGATTGGCTATCAGCCTTTGGAAGACAAAGTGA ATTTCTTCCGTTGCGTTTTCTCCAACCCAGCGACACAGAGGGAAGACGTCGACTTCCTGCT GGATGAAATCGTCCGTCTGGGCTGTGAACTCTAGGAAGACACATTTCTTTTAAAAAATCAC TCTTTTTATGTGTGAAAGTTAATGTATGTTAGAAAAATATTTATTTATTTAAGGCTGATGCC AGTTGTTATTGC
***gad3* T7/T3 gBlock**	GTTTACTTATGAAATATCCCCAGTCTTCATCCTAATGGAGGAGGTAGTTCTCAGAAAAATG CACACAATTATTGGATGGCCAGAGGAAGATGGAGATGGCATCTTTTGTCCTGGAGGCTCCA TGTCCAATCTCTACAGTGTGTTGTTGGCTAGGTTTCATCTCTTTCCGGCAGTGAAGACCCAC GGGATGTGTGCGATCCCTCGCCTAGCGATGTTCACCTCCGCGCACAGTCATTATTCAATCA AGAAGTCTGCGGCTGTTCTCGGCATCGGCACTGAAAATGTGATAGTCGTCAGATGTGACGA AAGGGGGAAGATGATCTCATCAGAACTCAACTCCAGCATTGAGGAAGCAAAGTCGAAGGG CCTCGTCCCATTCTATGTCAACGCAACCGCCGGAACTACAGTCTATGGAGCCTTTGACCCT TTGCATAAAATAGCAGATATTTGTGAGCACCATGGCCTCTGGATGCACGTAGATGCTGCTT GGGGTGGAGGCTTGCTTCTATC
***krt4* T7/T3 gBlock**	GTCAACCAGAGCCTGTTGGCCCCCCTCAACCTGGAAATTGACCCCACAATTCAGGCTGTCC GCACTCAGGAGAAAGAGCAAATTAAGACCCTCAACAACCGCTTCGCTTCCTTCATCGACA AAGTGCGCTTCCTGGAACAGCAGAACAAGATGCTGGAGACCAAATGGAGTCTTCTCCAAG AACAGACAACCACACGTTCCAACATCGATGCCATGTTTGAGGCATACATCTCTAACCTGCG CAGACAGCTCGATGGACTGGGAAATGAGAAGATGAAGCTGGAGGGAGAGCTGAAGAACA TGCAAGGCCTGGTTGAGGACTTCAAGAACAAGTACGAGGATGAGATCAACAAGCGTGCTT CCGTAGAGAATGAGTTTGTCCTTCTCAAGAAGGATGTTGATGCAGCCTACATGAACAAGGT TGAGCTTGAAGCCAAGGTTGATGCTCTTCAGGATGAGATCAACTTCCTCAGGGCAGTCTAT GAGGCTGAACTCCGGGAGCTCCAGTCTCAGATCAAGGACACATCAGTTGTTGTAGAAATG GACAACAGCAGAAACCTGGATATGGACTCCATCGTGGCTGAAGTTCGTGCTCAGTATGAA GACATCGCCA
ppl T7/T3 gBlock	AAGAGTCTGGCTGCAATGCGAGTGAAGAGGGAACAGCGAGAAAGTCACCTCCGCCGTTCA ATCGTCGTCATTGATCCAGACACCGGAAAGGAGATGAAGCCCGAGGAGGCTTACAAGCTG GGCCTAATCGAATGGAAAATGTTTGTCAACCTTCAAAGTCAAGAGTGCGATTGGGAGGAG ATCACCGTCAAGGGTCCAAGTGGCGAGTCCTCCGTTCTTCACGACAGAAAATCAGGCAAG AAGTTTTCCATTGAAGATGCTCTGAAGGCAGGAAACATCACAAACCGTCAACTGCAACAG TACCAGAACAAAGAAATCAGCATCCAAGAGTTTGGCGTCATGCTGTCAGGCAGAGGCAAA TGAATCCATTAATTAGACAAATACAACCAATTTTATATTCTGTTTACTAGTATTAAGATCTA GAGTAAACATGCAGTTTGCAGTCCCAAATTGCATTTTTTTATTCCCTTTCTCAATACAGTAT GTATTTATTTTCAAATGCATTCTTATACCAATGCAAGAAGGTGTTTCTTTGGTTATGGTGCT GAATTTGGGTTAATG

* indicates target *gad3* sequence based on RefSeq data (NM_001089570.2).

Table 3.S.2 Deconvolved Sanger sequencing results for each gad3 *in situ* hybridization probe. Sequences below represent the consensus between sequencing results from both the forward and reverse primers. Bold bases indicate Sp6/T7/T3 promoter identity. Highlighted bases are indicative of SNPs present in cDNA-derived probe sequence.

PROBE	*GAD3* CDNA FRAGMENT / GBLOCK SEQUENCE (5' – 3')
***gad3* Sp6 cDNA-derived template**	**TAG**CAACAAACACCGCGTGAAACTGCACGGAATTGAAAGAGCCCATTCTGTGACCT GGAACCCACACAAGATGATGGGCGTTCCACTGCAATGCTCCACCATTCTAGTCAAGA GGAAGGGCCTCCTGCAGCAGTGTAATCAGCTGTGTGCCGAATATCTCTTCCAACCTG ACAAGCACTACGAAGTGTCCTACGACACTGGGGACAAAAGCATTCAGTGCGGACGA CACGTGGATATCTTTAAACTGTGGCTCATGTGGAAGGCGAAGGGCTCCGAGGGTTTT GAATCACAGGTCAACCACTGCCTGGAGAATGCAGAGTATCTTTACTACAAGCTGAAG AGAAGAACAGATTTTCAGCTCGTCTTCAAGGGAAAACCTGAACACAGTAATGTGTGC TTTTGGTATCTCCCAAAACGAGTGCAAAACATTCCTCTAGGCCCAGAGCGAGAGAAA GAACTTCATATGGTGGCTCCAAAGATCAAGACAAAGATGATGGAGGAAGGATTCAC CATGATTGGCTATCAGCCTTTGGAAGACAAAGTGAATTTCTTCCGTTGCGTTTTCTCC AACCCAGCGACGCAGAGGGAAGACGTCGACTTCCTGCTGGATGAAATCGTCCGTCTG GGCTGTGAACTCTAGGAAGACACATTTCTTTTAAAAAATCACTCTTTTTATGTGTGAA AGTTAATGTATGTTAGAAAAATATTTATTTATTTAAGGCTGATGC
***gad3* T7 cDNA-derived template**	GAAACTGCACGGAATTGAAAGAGCCCATTCTGTGACCTGGAACCCACACAAGATGA TGGGCGTTCCACTGCAATGCTCCACCATTCTAGTCAAGAGGAAGGGCCTCCTGCAGC AGTGTAATCAGCTGTGTGCCGAATATCTCTTCCAACCTGACAAGCACTACGAAGTGT CCTACGACACTGGGGACAAAAGCATTCAGTGCGGACGACACGTGGATATCTTTAAAC TGTGGCTCATGTGGAAGGCGAAGGGCTCCGAGGGTTTTGAATCACAGGTCAACCACT GCCTGGAGAATGCAGAGTATCTTTACTACAAGCTGAAGAGAAGAACAGATTTTCAGC TCGTCTTCAAGGGAAAACCTGAACACAGTAATGTGTGCTTTTGGTATCTCCCAAAAC GAGTGCAAAACATTCCTCTAGGCCCAGAGCGAGAGAAAGAACTTCATATGGTGGCT CCAAAGATCAAGACAAAGATGATGGAGGAAGGATTCACCATGATTGGCTATCAGCC TTTGGAAGACAAAGTGAATTTCTTCCGTTGCGTTTTCTCCAACCCAGCGACGCAGAG GGAAGACGTCGACTTCCTGCTGGATGAAATCGTCCGTCTGGGCTGTGAACTCTAGGA AGACACATTTCTTTTAAAAAATCACTCTTTTTATGTGTGAAAGTTAATGTATGTTAGA AAAATATTTATTTATTTGAAGGNTGATGCCAGTTGTTATTGC**CCCTAT**
***gad3* T7/T3 gBlock template**	**TAATACGACTCACTATAGGG**GTTTACTTATGAAATATCCCCAGTCTTCATCCTAATG GAGGAGGTAGTTCTCAGAAAAATGCACACAATTATTGGATGGCCAGAGGAAGATGG AGATGGCATCTTTTGTCCTGGAGGCTCCATGTCCAATCTCTACAGTGTGTTGTTGGCT AGGTTTCATCTCTTTCCGGCAGTGAAGACCCACGGGATGTGTGCGATCCCTCGCCTA GCGATGTTCACCTCCGCGCACAGTCATTATTCAATCAAGAAGTCTGCGGCTGTTCTCG GCATCGGCACTGAAAATGTGATAGTCGTCAGATGTGACGAAAGGGGGAAGATGATC TCATCAGAACTCAACTCCAGCATTGAGGAAGCAAAGTCGAAGGGCCTCGTCCCATTC TATGTCAACGCAACCGCCGGAACTACAGTCTATGGAGCCTTTGACCCTTTGCATAAA ATAGCAGATATTTGTGAGCACCATGGCCTCTGGATGCACGTAGATGCTGCTTGGGGT GGAGGCTTGCTTCTATC**CCTTTAGTGAGGGTTAATT**G

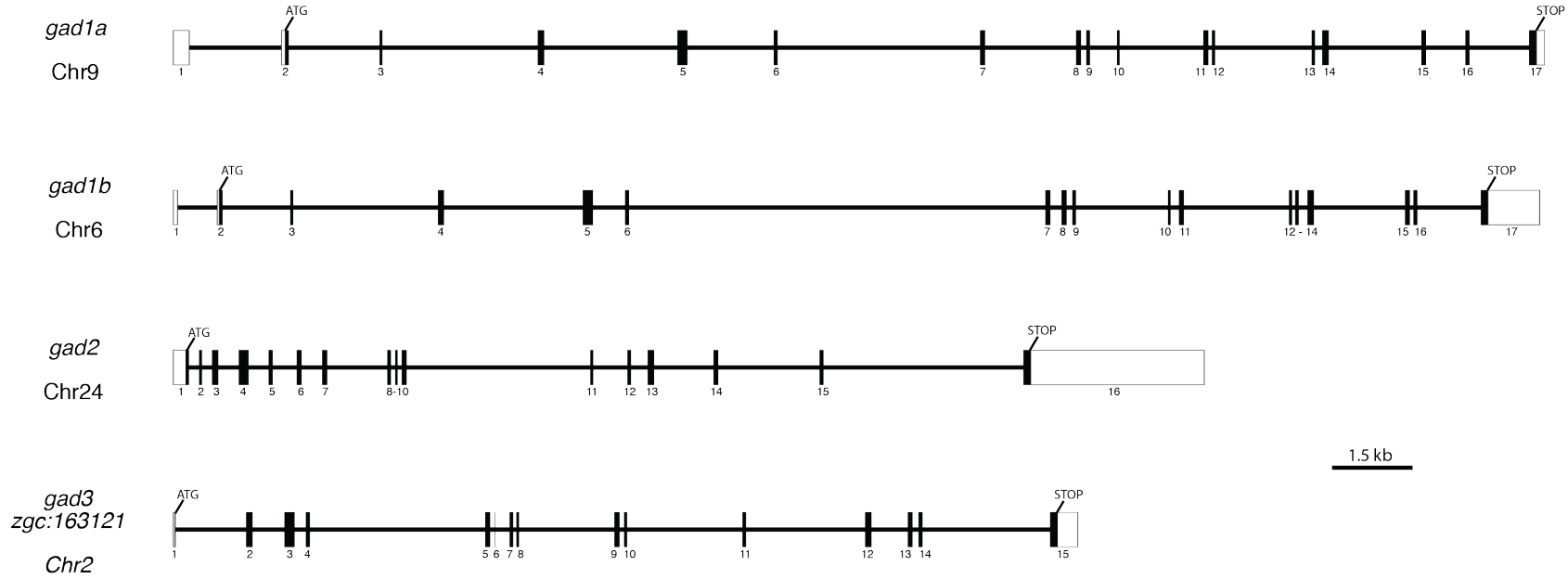

Figure 3.1 Gene maps of all zebrafish *GAD* genes. All genes are organized 5' to 3' with exons numbered appropriately. Exons are indicated by black rectangles, and untranslated regions (UTRs) are indicated by white rectangles.

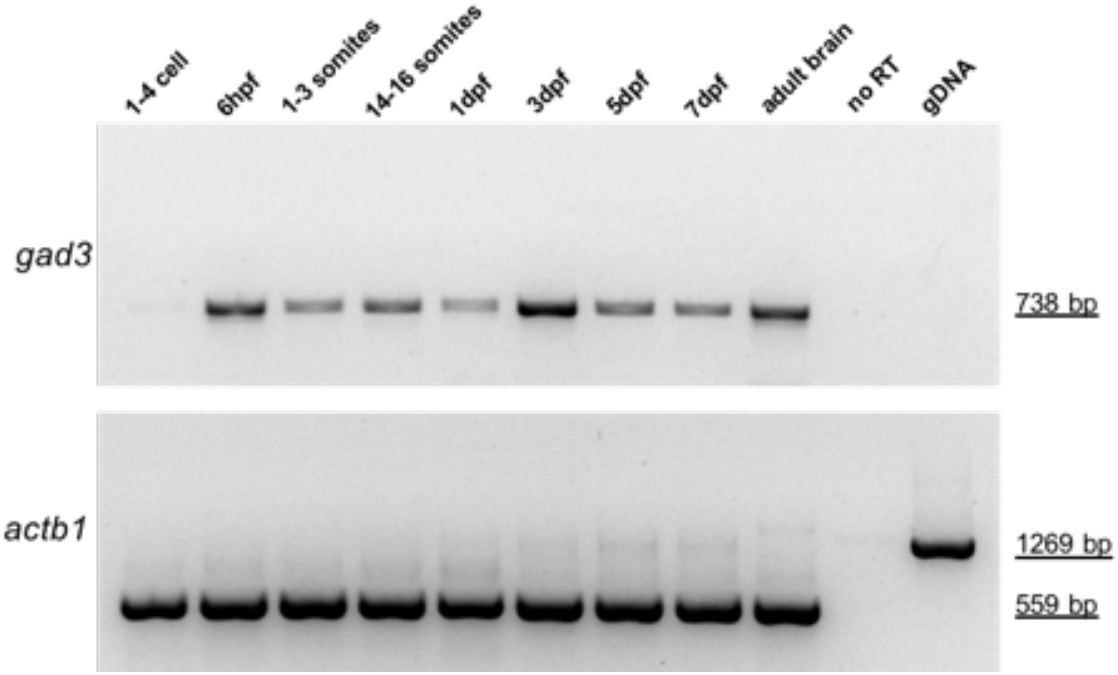

Figure 3.2 Developmental RT-PCR of *gad3* expression. Whole organism cDNA from the WT cleavage stages up to larval day 7 and adult brain cDNA was used to perform RT-PCR to visualize zebrafish *gad3* (*zgc:163121*) and *ß-actin* (*actb1*) expression over developmental time. *gad3* in expressed as early as 6hpf and continues into larval day 7. A faint band is present in the 1-4 cell stage, and this been reproduced in technical replicates (data not shown). *gad3* is also present in the adult zebrafish brain. Inclusion of the genomic DNA and no RT controls ensure that amplicons generated are from reverse-transcribed RNA. Expected sizes for *gad3* and *ß-actin* are 738 bps and 559 bps, respectively, when amplified from cDNA.

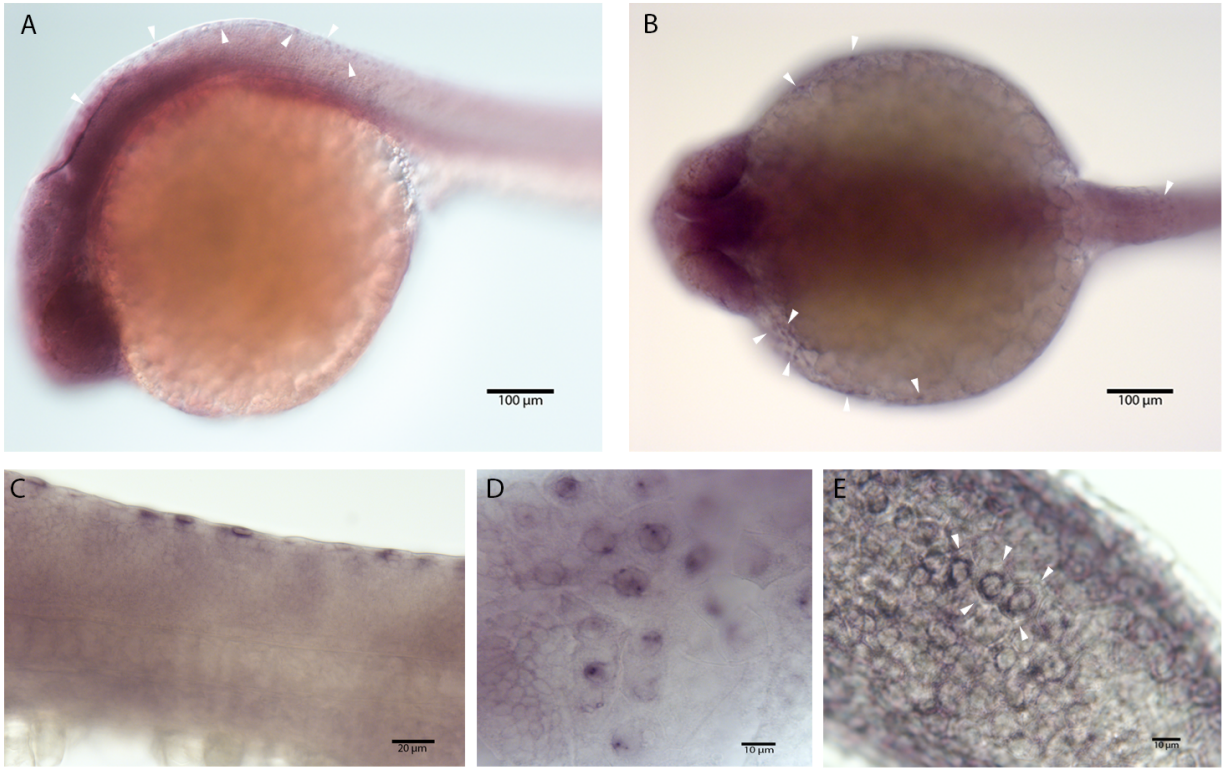

Figure 3.3 *gad3* expression pattern in 1dpf zebrafish. *gad3* expression was detected by colorimetric *in situ* hybridization in EVL cells at 1dpf with two different *in situ* hybridization probes targeting *gad3* mRNA. Panels A and B show the lateral and dorsal views of a 24hpf embryo which underwent hybridization with the *gad3* cDNA-derived probe. We observed apparent signal in neural tissues, as well as the superficial "dotted" patterning in the EVL around the zebrafish head and the yolk. The same expression pattern was found in embryos hybridized with the gBlock probe for *gad3*. Panels C and D show high magnification views of *gad3* staining in the EVLs from a lateral and dorsal view, respectively. Panel C shows *gad3* expression in the EVL, but *gad3* does not appear to be present in the spinal cord in this stage. D shows the geometric shapes of the EVL cells with *gad3* staining centered around the nucleus. Panel E shows a high magnification view of *gad3*-expressing EVL cells in the tail of a specimen hybridized with the cDNA-derived probe. White arrowheads indicate signal in EVL cells in panels A, B, and E.

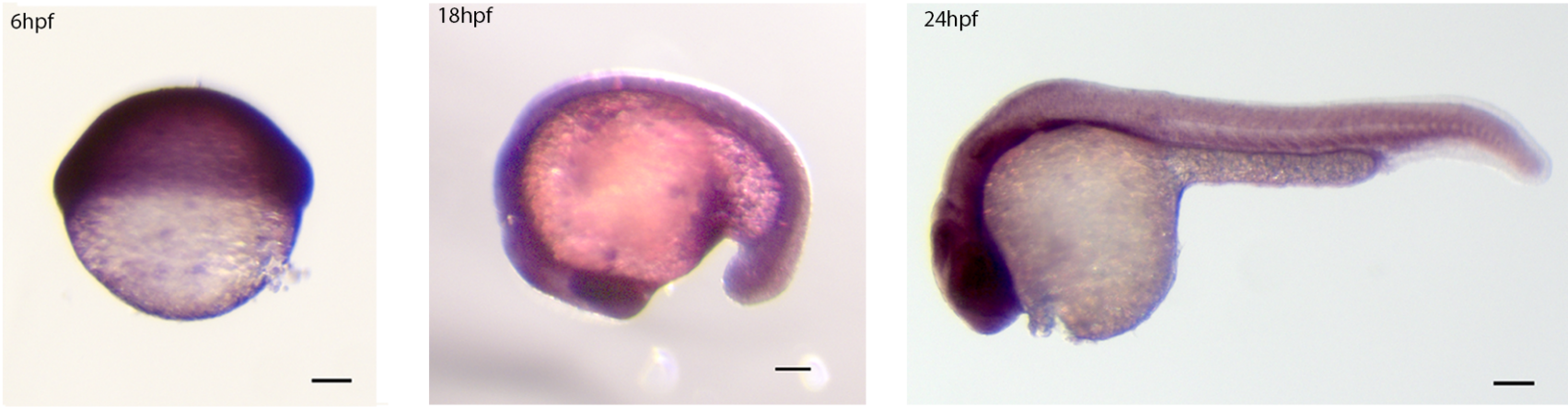

Figure 3.4 *gad3* expression in 6hpf, 18hpf, and 24hpf embryos. Developmental time course of *gad3* expression was observed by *in situ* hybridization. The probe used for *gad3* detection in this set of experiments was derived from WT zebrafish whole organism cDNA. *gad3* is highly expressed at shield stage, as the EVL spreads over the yolk in epiboly. The EVL completely covers the embryo and most of the yolk at 18hfp, and continues to cover the embryo through 1dpf. It appears that there is detection of *gad3* in the brain, but not the spinal cord at 24hpf. Scale bars = 100 μm.

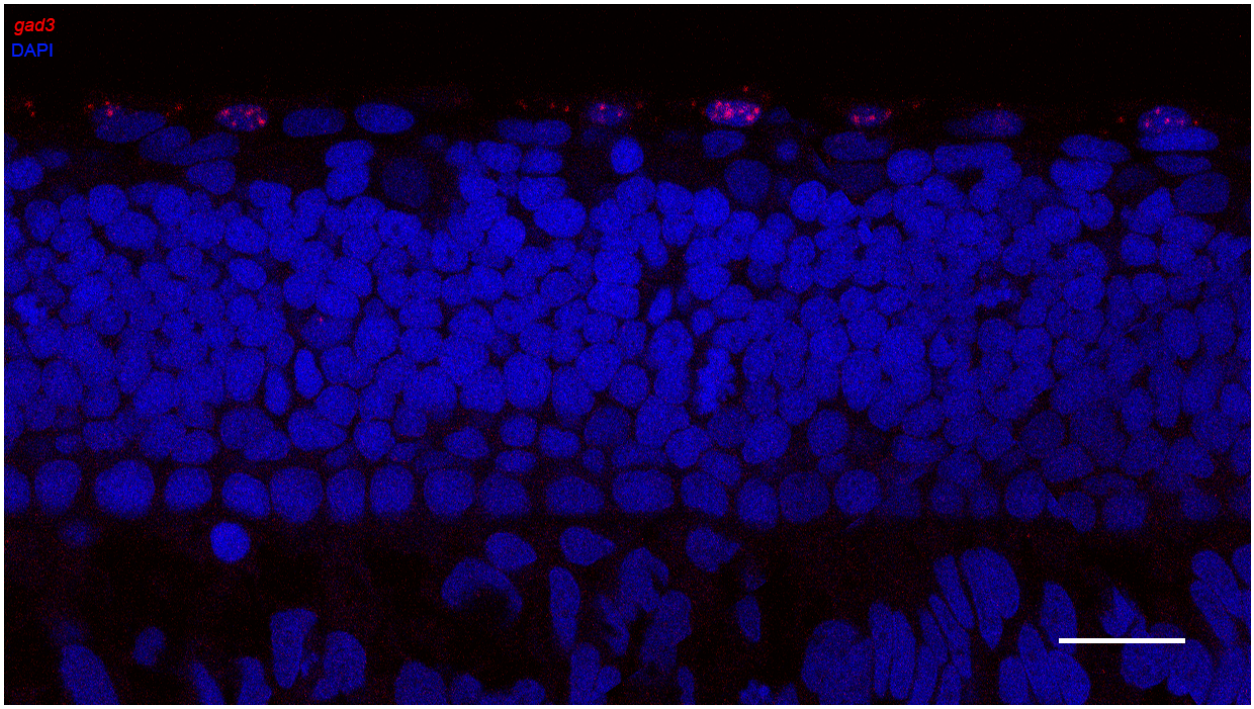

Figure 3.5 *gad3* is not expressed in the spinal cord at 1dpf. Lateral view of a 1dpf zebrafish spinal cord taken by confocal microscopy. *gad3* expression was detected by fluorescent *in situ* hybridization in EVL cells, but not in the spinal cord at 1dpf. Scale bar = 20 µm.

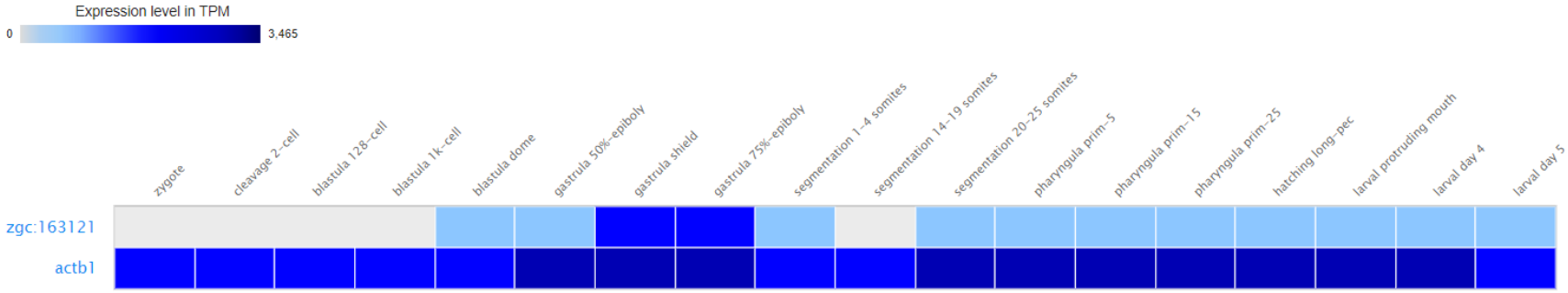

Figure 3.S.1 mRNA expression time course data for *gad3* and *ß-actin*. Expression levels of *gad3 (zgc:163121)* and *ß-actin* (*actb1*) support our findings that *gad3* is expressed early in development, and persists through the larval stages. Information was provided via the Expression Atlas [134, 138].

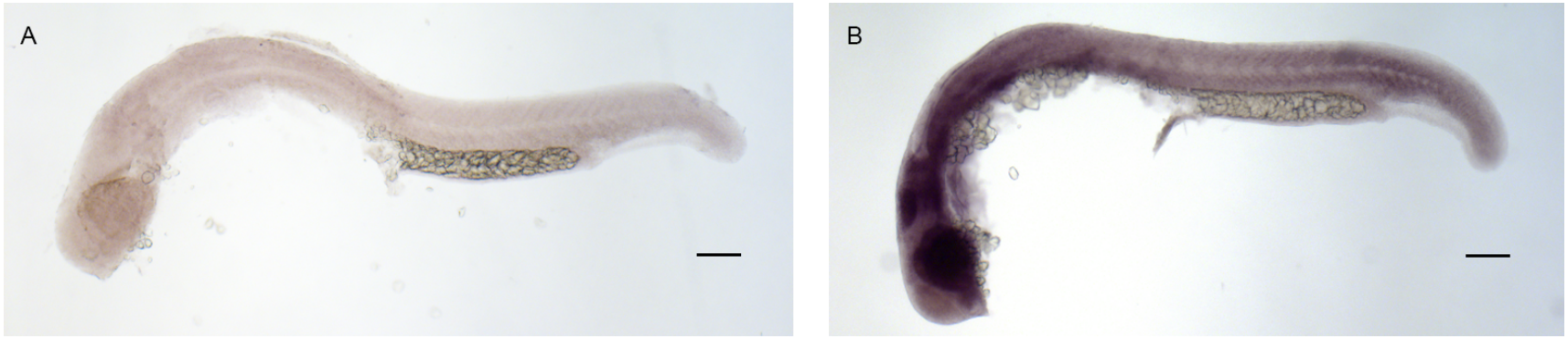

Figure 3.S.2 Comparison of *gad3 in situ* hybridization sense and antisense probes. *gad3* expression was detected by colorimetric *in situ* hybridization with the cDNA-derived antisense probe (B), but not the sense probe (A). Scale bars = 100 µm.

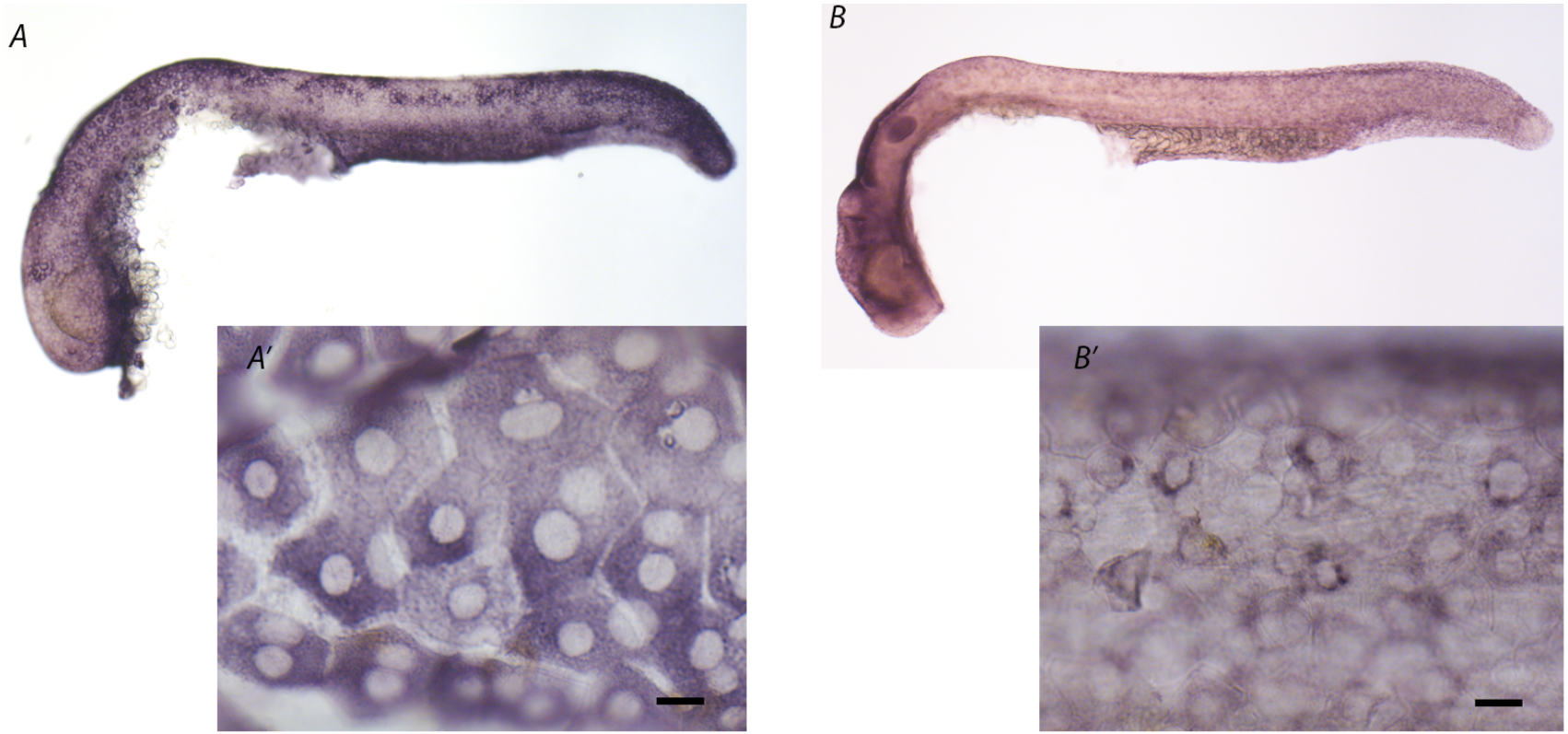

Figure 3.S.3 Colorimetric *in situ* hybridization for EVL markers *krt4* and *ppl.* *keratin-4 (krt4)* and *periplakin (ppl)* have been identified as strong EVL markers in zebrafish [132, 133]. We performed *in situ* hybridization to determine that the observed superficial *gad3* expression pattern was a probable EVL signal, and to control for our ability to perform the experiments without damaging the EVL cells. Both markers provide insight into labelling of EVLs, as detection of *gad3* expression did not give us the expected pattern, such as that shown by detection of *krt4*. *gad3* in EVL cells more closely resembles the nuclear-centric pattern given by *ppl* expression. Panels A/A' display *krt4* expression, and panels B/B' display *ppl* expression. Scale bars = 10 μm.

CHAPTER 4:

CONCLUSIONS

The work presents a characterization of the differential expression patterns of four distinct zebrafish GAD genes in embryonic development. Our results show that *gad1a, gad1b,* and *gad2* are differentially expressed in the spinal cord at 1dpf, and appear to be segregated by GABAergic neuronal positioning along the dorsoventral axis. We have also shown that *gad3* is expressed primarily in EVL cells at 1dpf, and may also be expressed in neural tissue. Much work remains in order to better understand *GAD* expression in the context of embryonic development in zebrafish and how the products of these genes may work to contribute to GABA signaling in development.

Work from our lab and from others has previously shown that the zebrafish GAD genes turn on early in development, and are expressed in GABAergic interneurons in the 1dpf spinal cord [85, 86]. Additionally, we have shown that *gad1a* expression can be detected in the spinal cord at ~16hpf in dorsally located interneurons, prior to the detection of either *gad1b* or *gad2* expression by *in situ* hybridization [85]. Herein we have shown that *gad1a* expression appears to be relatively restricted to more dorsally positioned interneurons, with *gad1b* being expressed in medially and ventrally located cells, and apparent confinement of *gad2* expression to ventral interneurons. However, we have not determined whether the expression of these genes continues to be spatially distributed in the spinal cord at later timepoints. Future experiments will focus on determining whether the observed *GAD* expression patterns change in the developing zebrafish spinal cord through embryonic and larval development. Our lab has also previously generated

GAD-deficient zebrafish [85], but the expression patterns of these genes have not yet been characterized in mutant fish. Investigations into *GAD* expression in the embryonic and early larval spinal cord of GAD mutants will be essential for understanding the role that *gad1a, gad1b,* and *gad2* play in proper spinal cord development and provide insight into the regulation of their differential expression.

Our investigation of *gad3* revealed high expression in early embryonic stages as detected by RT-PCR and *in situ* hybridization, and is consistent with available mRNA expression data and scRNA-seq data [134–137]. Interestingly, our data and scRNA-seq data point to *gad3* being mostly expressed in the EVL during development, but we also have evidence for some expression in developing neural tissues [135, 136]. We have observed possible *gad3* expression in the zebrafish brain at 1dpf by *in situ* hybridization, but our evidence is inconclusive at this point in time. Further imaging analysis with wholemount and sectioned tissues will be performed in order to determine whether any *gad3* expression is detected in the brain at 1dpf. Likewise, we intend to perform *in situ* hybridization with 3dpf zebrafish embryos to more fully characterize *gad3* expression during development. We have shown the first evidence that *gad3* is expressed in the adult zebrafish brain, and therefore future experiments must be conducted in order to determine which regions of the brain express *gad3*, and whether it is co-expressed with *gad1a, gad1b,* or *gad2.* This will be the first step in determining whether there is any cooperation of *gad3* with the other GAD genes and what role it may serve in GABAergic signaling.

In addition to determining whether *gad3* is expressed in zebrafish neural tissues, it is important to understand what function *gad3* serves in the developing epidermis. Studies have shown that EVL cells persist as the outermost epidermal cell type through larval development, and may even be found at 1 month of development [143, 144]. One study has shown that *gad3* is one

of 16 highly upregulated genes in the periderm at larval day 2 [137], and we have shown that *gad3* is expressed through larval day 7. Therefore, future efforts will work to determine whether *gad3* expression persists in the EVL, and if it is present in the developing or mature epidermis. Eventual generation of *gad3* mutants will be crucial in determining the importance of *gad3* in zebrafish.

The findings presented by this work again point to the importance of all four GAD genes in zebrafish development, as well as suggest the existence of complex regulatory mechanisms for *GAD* expression. The presence of *gad3* further complicates the story of GABAergic signaling in teleost and greater vertebrate development and introduces even greater potential for research in the field.

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