UNDERSTANDING THE ROLE OF THE *GAD* GENES IN NEURAL DEVELOPMENT AND NERVOUS SYSTEM FUNCTION IN ZEBRAFISH

by

ARIEL J. VANLEUVEN

(Under the Direction of James D. Lauderdale)

ABSTRACT

The nervous system requires a fine balance of excitatory and inhibitory signals for proper development and functioning. Γ-Aminobutyric Acid (GABA) is the major inhibitory neurotransmitter in the central nervous system of all vertebrates. GABA is made by the glutamic acid decarboxylase (GAD) enzyme, which exists in two isoforms, GAD67 and GAD65, each of which is encoded by an independent gene, *GAD1* and *GAD2*, respectively. Disruptions in the *GAD* genes, namely *GAD1*, have been implicated in several neurological disorders, however little is known about the mechanism(s) that cause these disorders. Like mammals, zebrafish have both known *gad* genes; however, we recently found molecular evidence for a *gad1* paralog in zebrafish. This discovery could allow for studies addressing the mechanistic and functional questions of *gad1* gene function in a way that has not been previously possible in mammalian models. We used standard reverse transcription polymerase chain reaction (RT-PCR) and mRNA *in situ* hybridization to sequence and localize expression of this *gad1a* gene during zebrafish neural development. We used double fluorescent *in situ* hybridization (F.I.S.H.) to assess the *gad* expression pattern both between zebrafish *gad* genes and to known markers of spinal cord interneurons during development. We also used CRISPR-Cas9 to create different *gad1* mutant zebrafish to address questions of *gad* gene function. We have found that there is differential temporal and spatial expression between the *gad1* paralogs during development, while *gad1b* and *gad2* seem to be co-expressed. The presumably distinct expression pattern

among this family of genes suggests a complex regulation of the zebrafish *gad* genes. Analyses of *gad1a* -/- and *gad1b* -/- mutant zebrafish show that *gad1b* -/- zebrafish have significantly reduced levels of GABA as measured by high-performance liquid chromatography (HPLC). The *gad1b* -/- larvae are hypersensitive to seizure-like behavior and exhibit increased and abnormal brain activity as measured by electrophysiology. Comparatively, the *gad1a* -/- larvae are somewhat hypersensitive and show less abnormal neurological activity. Taken together, these findings have uncovered a previously unknown genome duplication that has separated *gad1* into two genes in zebrafish that both contribute, though in different ways, to proper nervous system development and function.

INDEX WORDS: GABA, *gad1a*, *gad1b*, *gad2*, Gad67, Gad65, zebrafish, CRISPR-Cas9, neural activity, seizures, neural development

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DEDICATION

I dedicate this work to my family: David W. VanLeuven, Joanne VanLeuven, and David A. VanLeuven and to Erick Melendez. My family and Erick have been my greatest supporters throughout my graduate school career. They have always been there to provide me with the support, encouragement, and motivation that I needed to persevere on this journey; I would not have made it without them.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

GABA in the nervous system

Γ-aminobutyric acid (GABA) is the most abundant and therefore the primary inhibitory neurotransmitter in the central nervous system (CNS) of all vertebrates. It is prevalent in the CNS, being made by 20-30% of neurons in the adult brain (Varju, Katarova et al. 2001). The major biochemical pathway through which GABA is made *in vivo* is by the activity of the glutamate decarboxylase or glutamic acid decarboxylase (GAD) enzyme (IUBMB Enzyme Nomenclature EC 4.1.1.15) (Martin and Rimvall 1993, Tillakaratne, Medina-Kauwe et al. 1995). The GAD enzyme catalyzes the α-decarboxylation of L-glutamate, or glutamic acid, into GABA in a single step (Awapara 1950, Roberts and Frankel 1950, Roberts and Frankel 1951, Roberts and Frankel 1951, Roberts 1974, Roberts 1988, Erlander and Tobin 1991).

Once GABA is synthesized, it functions as an inhibitory neurotransmitter by binding to GABA receptors. Synaptic activity occurs when GABA binds either to its ionotropic receptor, namely $GABA_A$ and $GABA_C$, or its metabotropic receptor, $GABA_B$. The ionotropic GABA receptors are ligand-gated ion channels, so when GABA binds, the channel opens leading to an influx of chloride (Cl^-) ions down their concentration gradient into the neuron thereby hyperpolarizing the membrane and preventing initiation of an action potential (Bowery 1992, Macdonald and Olsen 1994, Johnston 1996, McKernan and Whiting 1996, Enz and Cutting 1999, Bormann 2000, Enz 2001, Zhang, Pan et al. 2001). The $GABA_A$ receptor is found throughout the CNS and is responsible for most of the fast-acting inhibitory neurotransmission while $GABA_C$ appears to be namely in the retina or non-neural locations (Enz, Brandstatter et al. 1996, Lukasiewicz 1996, Fletcher, Clark et al. 2001, Connaughton, Nelson et al. 2008).

When GABA binds the metabotropic $GABA_B$ receptor, there is either down-regulation of pre-synaptic calcium (Ca^{2+}) channels or activation of inward rectifying (K^+) currents on the post-synaptic neuron. This activity is responsible for the late inhibitory potentials and is mostly associated with neuromodulation and the activity of mature neural circuits (Bowery 1992, Mott and Lewis 1994, Kaupmann, Malitschek et al. 1998, Kaupmann, Schuler et al. 1998).

The neuron's supply of GABA for neurotransmission largely resides at the synaptic terminal, such that it can be packaged into vesicles and released into the synaptic cleft. However, there is another major subcellular pool of GABA in the soma or cell body (Martin and Barke 1998). The fact that there is a second distinct location for GABA in the neuron indicates that GABA likely serves a function not just as a neurotransmitter, but also in cellular metabolism, likely as part of the Tricarboxylic Acid (TCA) cycle (Martin and Rimvall 1993, Tillakaratne, Medina-Kauwe et al. 1995, Watanabe, Maemura et al. 2002). Several enzymes, including GAD, are involved in the TCA cycle's GABA shunt pathway, which assists in GABA breakdown to provide energy for the neuron (Hassel, Johannessen et al. 1998). While the metabolic component of GABA is important, it is secondary to its role as an inhibitory neurotransmitter.

It is well established that the CNS must maintain a delicate balance between excitatory and inhibitory neural signaling events both during development and in adulthood to function normally. Not surprisingly, the regulation of GABA synthesis by the *GAD* genes plays a critical role in this process.

GAD genes and enzymes in vertebrates

In all vertebrates, there are two *GAD* genes, *GAD1* and *GAD2* each of which encode a distinct GAD enzyme, known as Gad67 and Gad65, respectively (Legay, Pelhate et al. 1986, Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Feldblum, Erlander et al. 1993, Soghomonian and Martin 1998). These *GAD1* and *GAD2* genes likely arose from an ancestral *GAD* gene that duplicated very early in the vertebrate lineage, before the divergence of teleost and cartilaginous fish (Erlander, Tillakaratne et al. 1991, Bosma, Blázquez et al. 1999, Lariviere,

MacEachern et al. 2002, Grone and Maruska 2016). The *GAD1* gene in humans has been mapped to chromosome 2 while the human *GAD2* gene has been mapped to chromosome 10. In mice, both the *Gad1* and *Gad2* genes have been mapped to chromosome 2 (Edelhoff, Grubin et al. 1993). There is also evidence for a third *GAD* gene in most vertebrates; it was lost in the hominids, but might be present in some teleost fish species (Bosma, Blázquez et al. 1999, Grone and Maruska 2016). There has also been some recent molecular evidence for a *gad1a* gene in adult zebrafish (Grone and Maruska 2016, Cocco, Ronnberg et al. 2017). Despite this, the most discussed and characterized *GAD* genes in the literature are *GAD1* and *GAD2.*

In humans and mice, the exon/intron boundaries and the promoter regions of the *GAD1* and *GAD2* genes have been described (Bu and Tobin 1994, Szabo, Katarova et al. 1996, Yanagawa, Kobayashi et al. 1997, Makinae, Kobayashi et al. 2000, Matsukawa and Ueno 2007). The human *GAD1* gene spans over 45 kilobases (kb) of genome space and has 17 exons, 16 of which are the coding regions (Bu and Tobin 1994). The mouse *Gad1* gene also spans ~45 kb of genome space and has 19 exons, all but two (exon 0B and exon F) which are conserved in humans (Szabo, Katarova et al. 1996, Yanagawa, Kobayashi et al. 1997). There were also efforts to map the promoter and the 5'-untranslated region (5'-UTR) of the mammalian *GAD1* gene and identified some small species-specific differences between human and mouse sequences in this region (Szabo, Katarova et al. 1996, Yanagawa, Kobayashi et al. 1997, Chen, Dong et al. 2011).

The *GAD2* gene in both humans and mice spans over 75 kb and contains 16 exons all of which contribute to the coding sequence (Bu and Tobin 1994, Makinae, Kobayashi et al. 2000). Based upon the gene and exon structure between humans and mice, the *GAD2* gene appears to be highly conserved between species. The promoter region of the mouse *Gad2* gene has also been studied and is unique from that of *Gad1* and appears to have complex transcriptional regulation (Pinal, Cortessis et al. 1997, Makinae, Kobayashi et al. 2000).

As mentioned, the *GAD1* gene encodes for the Gad67 enzyme while the *GAD2* gene encodes for the Gad65 enzyme. These enzymes are named based upon the protein's molecular weight in kilodaltons (kDa): the Gad67 enzyme is ~67 kDa while the Gad65 enzyme is ~65 kDa (Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Bosma, Blázquez et al. 1999). These GAD enzymes are highly conserved and have been described in *Drosophila*, avian, teleost fish, and mammals (Jackson, Newby et al. 1990, Bu, Erlander et al. 1992, Åhman, Wagberg et al. 1996, Martin, Heinrich et al. 1998, Trabucchi, Trudeau et al. 2008). The Gad67 and Gad65 enzymes have two domains, an N-terminal domain and a catalytic domain. The GAD enzymes' N-terminal domain is relatively small and have low sequence identity both within and between species, while the C-terminal domain is the catalytic domain which is large, well-conserved, and contains a nine amino acid residue pyridoxal 5'-phosphate (pyridoxal-P) co-factor binding site (Martin, Liu et al. 2000).

Both GAD enzymes require the co-factor pyridoxal-P for full functionality of the enzyme (Kaufman, Houser et al. 1991, Qu, Martin et al. 1998). While both GAD enzymes require pyridoxal-P, the Gad67 enzyme is almost always bound to pyridoxal-P while Gad65 only binds its co-factor when neurotransmission is needed (Kaufman, Houser et al. 1991, Martin, Liu et al. 2000, Battaglioli, Liu et al. 2003). This indicates that the interaction of GAD enzyme with its co-factor is a critical step in the regulation of GABA synthesis.

While both Gad67 and Gad65 are known to be involved in GABA synthesis, they have different subcellular localizations in the neuron. It is thought that the N-terminal sequence of the Gad67 and Gad65 enzyme is responsible for this subcellular targeting (Martin, Liu et al. 2000). The Gad67 enzyme is found throughout the neuron, both in the soma and the synapse, while Gad65 is almost exclusively found in the synaptic terminal (Kaufman, Houser et al. 1991, Pinal and Tobin 1998). This distribution of the different Gad enzymes throughout the neuron suggests that they play slightly different roles in the context of cellular metabolism and neurotransmission

as well as other cellular processes like neurotrophic factors and autocrine/paracrine signaling during development (Feldblum, Erlander et al. 1993, Pinal and Tobin 1998).

GABA and GAD during development

GABA is well-characterized as an inhibitory neurotransmitter, but there is also an apparent role for GABA, and by extension the *GAD* genes, during development. One of the most interesting aspects of this developmental role is the fact that GABA provides excitatory activity and a neurotrophic role in early neural development (Cherubini, Gaiarsa et al. 1991, Ben-Ari 2002, Ben-Ari, Gaiarsa et al. 2007, Khazipov, Valeeva et al. 2014). The fact that GABA is excitatory during development but inhibitory in adulthood is due to the intracellular and extracellular concentrations of Cl^-. There are elevated intracellular levels of Cl^- in immature neurons as well as expression of proteins called NKCC1 (a Cl^- transporter) and KCC2 (a K^+/Cl^- co-transporter) (Rivera, Voipio et al. 1999, Yamada, Okabe et al. 2004, Dzhala, Talos et al. 2005). In this situation, GABA release and binding at the post-synaptic neuron causes Cl^- to flow out of the neuron which ultimately depolarizes the membrane and is associated with excitatory activity. The system can be altered through removing GABAergic or glutamatergic signaling during development which leads to up-regulation of KCC2 (Ludwig, Li et al. 2003). These findings again hint at the fine balance of excitation and inhibition that is required of the developing nervous system.

This balance is ever-changing though as there is evidence that the ratio of neurons that respond to excitatory signals decreases quickly as the organism develops (Khazipov, Khalilov et al. 2004, Tyzio, Cossart et al. 2006, Tyzio, Holmes et al. 2007). In mice, GABA depolarizes membranes and is excitatory during the first post-natal week of development (Mueller, Taube et al. 1984, Ben-Ari, Cherubini et al. 1989, Cherubini, Gaiarsa et al. 1991). This is likely due to the reversed concentrations of intracellular and extracellular Cl^- which cause the Cl^- transporters to move Cl^- ions in the opposite direction (Misgeld, Deisz et al. 1986). As development progresses, the expression of the Cl^- transporters changes as does the Cl^- ion gradient and by the second

post-natal week of mouse development, GABA responses become hyperpolarizing and inhibitory potentials are observed (Cherubini, Gaiarsa et al. 1991).

GABA signaling during development appears to be mediated by the $GABA_A$ receptor and activity of this $GABA_A$ receptor has been implicated in other systems besides neurotransmission (Khazipov, Valeeva et al. 2014). In stem cell culture, if the $GABA_A$ receptor is activated by GABA, there are hyperpolarizing currents seen, but there is also evidence of larger cell volumes and an increased number of cells in the DNA replication phase (S-phase) of the cell cycle (Andäng, Hjerling-Leffler et al. 2008). These data suggest that there is autocrine/paracrine GABA signaling that negatively controls cell proliferation and growth (Andäng, Hjerling-Leffler et al. 2008).

Similarly, there is evidence for GABA's contribution as a neurotrophic factor during neural development, particularly in synapse formation and differentiation (Lauder 1993, Martin and Rimvall 1993, Kim, Nam et al. 2004). Addition of GABA to developing ganglion cells lead to changes such as aberrant dendritic protrusions and membrane thickening (Wolff, Joó et al. 1978). Incubation of neuroblastoma cells with GABA was also found to increase synaptogenesis and synaptic terminal swelling as well as promote vesicle proliferation and abnormal intermediate junctions (Wolff 1982, Madtes Jr and Redburn 1983). These data indicate that GABA is playing a role during development that is different than its role in neurotransmission later in life.

While GABA is important in a distinct, developmental context, there is also evidence for developmentally-specific roles of the *GAD* genes and GAD enzymes. For example, there is evidence in the literature from both rat and mouse for alternative splicing of the *Gad1* gene during development (Bond, Wyborski et al. 1990, Szabo, Katarova et al. 1994, Trifonov, Yamashita et al. 2014). Experiments in these studies showed that there is a bicistronic start/stop site in an embryonic exon (exon 7A/B) of the *Gad1* gene which is only present during embryonic stages; it is alternatively spliced out in adults (Bond, Wyborski et al. 1990, Szabo, Katarova et

al. 1994). It is hypothesized that during development, the leaky bicistronic site will create two different transcripts that will translate into a leader 25 kDa form of Gad67 and a truncated 44 kDa form of Gad67. The 25 kDa form of Gad67 is enzymatically inactive because it lacks the pyridoxal-P co-factor binding site, but it is thought to be important for proper GABAergic neural development (Bond, Wyborski et al. 1990, Szabo, Katarova et al. 1994). This alternative splicing is also seen in other non-neural tissues like the retina and islet cells (Chessler and Lernmark 2000, Connaughton, Dyer et al. 2001). Though unpublished, there is also evidence in EST databases for alternative splicing of the human *GAD1* gene that likely also leads to 25 kDa and 44 kDa forms of Gad67. However, occurrences of alternative splicing for the *Gad2* gene and truncated forms of the Gad65 enzyme have not been reported for any organism.

In addition to the alternative splicing of *Gad1* during development, there is also evidence for differential expression of the *Gad* genes to specific domains during mouse gestation (Katarova, Sekerková et al. 2000). These domains of *Gad* expression are largely overlapping, and all regions were shown to produce GABA indicating that both GAD and GABA play a role as signaling factors during development (Katarova, Sekerková et al. 2000).

There are also differential levels of expression between *Gad1* and *Gad2* in various cell-specific manners (Esclapez, Tillakaratne et al. 1993, Feldblum, Erlander et al. 1993, Sheikh, Martin et al. 1999). For instance, both *Gad* genes are expressed in several subregions of the brain as well as interneurons and projection neurons, but *Gad2* appears to be more highly expressed in the visual and neuroendocrine-related brain regions, while *Gad1* appears to be more highly expressed in cortical, neocortical, basal ganglia and cerebellum (Esclapez, Tillakaratne et al. 1993, Feldblum, Erlander et al. 1993). Differential levels of expression of the *Gad* genes, particularly *Gad1*, have also been reported in non-neural tissues like the lens, pancreatic islets, kidneys, pharyngeal arches/pouches, tail bud mesenchyme, apical epidermal ridge (AER), limb bud regions, testis and oviducts (Erdö, Joo et al. 1989, Párducz, Dobo et al. 1992, Tillakaratne, Erlander et al. 1992, Kim, Richter et al. 1993, Maddox and Condie 2001,

Kwakowsky, Schwirtlich et al. 2007). Due to the importance of *GAD* and GABA both in development and in adulthood, it is to be expected that any irregularities in the *GAD* genes, GAD enzymes, or GABA would likely have detrimental effects.

GAD and GABA dysregulation in both neural and non-neural disorders

In humans there is evidence that decreased expression of genes involved in GABAergic signaling are correlated with epilepsy (Brooks-Kayal, Shumate et al. 1999). Epilepsy, or periodic seizures, is one of the most common neurological disorders both in the United States and worldwide and is usually caused either by an increase in excitatory neurotransmission or a decrease in inhibitory neurotransmission (Bromfield, Cavazos et al. 2006, Theodore, Spencer et al. 2006, Mirza, Appleton et al. 2015). Considering that GABA is so prevalent in the brain, any dysregulation in the GABAergic pathway could cause a seizure.

There has been evidence that has linked mutations in the human *GAD* genes with several neurological disorders. Gene polymorphisms and mutations in *GAD1* have been associated with spastic cerebral palsy, Stiff-person syndrome, and attention deficit and hyperactivity disorder (ADHD), among other types of neurological disorders (McHale, Mitchell et al. 1999, Lynex, Carr et al. 2004, Bruxel, Akutagava-Martins et al. 2016). There is also a report of polymorphisms in the *GAD* genes that lead to increased susceptibility to seizures after traumatic brain injury (Darrah, Miller et al. 2013). Mutations in *GAD2* have also been linked to cerebellar ataxia, Stiff-person syndrome and other movement disorders (Lynex, Carr et al. 2004). Disruptions in the *GAD1* gene have also been widely investigated as being linked to schizophrenia and bipolar disorder (Akbarian, Kim et al. 1995, Guidotti, Auta et al. 2000, Addington, Gornick et al. 2005, Hossein Fatemi, Stary et al. 2005, Lundorf, Buttenschøn et al. 2005, Akbarian and Huang 2006, Benes 2010). There is a recent report showing evidence for alternative transcripts of human *GAD2* being associated with schizophrenia (Davis, Tao et al. 2016). Furthermore, reductions in both Gad65 and Gad67 protein have also been associated with autism (Fatemi, Halt et al. 2002).

There have also been reports of mutations or polymorphisms in the *GAD* genes which lead to non-neural disorders in humans. Mutations in *GAD1* have been linked to abnormal craniofacial development and cleft palates (Kanno, Suzuki et al. 2004, Vieira, Howe et al. 2008). It is also interesting to note that other perturbations to GABA signaling during development, namely through the maternal use of GABA receptor modulator compounds as anti-epileptic drugs (AEDs like diazepam), are associated with craniofacial deformities and cleft palates (Aarskog 1975, Safra and Oakley JR 1975, Scapoli, Martinelli et al. 2002, Vieira, Howe et al. 2008, Marinucci, Balloni et al. 2011). Polymorphisms in *Gad2* have also been linked to increased susceptibility to type 2 diabetes mellitus and obesity (Baekkeskov, Aanstoot et al. 1990, Choquette, Lemieux et al. 2009, Chen, Lin et al. 2012, Li, Qiao et al. 2015). Due to the presence of many disorders associated with dysregulations in the *GAD* genes, researchers sought to use the mouse model to address more mechanistic questions about these disorders.

In the mouse model, *Gad1* -/- had a severe cleft palate and died as neonates at P0 (Asada, Kawamura et al. 1997, Condie, Bain et al. 1997, Oh, Westmoreland et al. 2010). Interestingly, *Gad2* -/- mice had no abnormal craniofacial features, but were hypersensitive to drug induced seizures, exhibited some hyperactive behavior, had temporal lobe epilepsy, and were prone to sudden death (Asada, Kawamura et al. 1996, Kash, Johnson et al. 1997). In the *Gad1/Gad2* double knockout mouse, these mice showed cleft palates at 100% penetrance which were more severe in nature as compared to the *Gad1*-/- single knockout (Asada, Kawamura et al. 1997, Kakizaki, Oriuchi et al. 2015). There is evidence that these abnormal craniofacial phenotypes may be correlated to GABA signaling through the $GABA_A$ receptor. Mutations in the *Gabrb3* gene in mice, which encodes the β3 subunit of the $GABA_A$ receptor, also lead to cleft palates as well as seizures (Culiat, Stubbs et al. 1993, Culiat, Stubbs et al. 1995, Homanics, DeLorey et al. 1997, Hagiwara, Katarova et al. 2003). Considering the limitations of neonatal or premature death in *Gad1* and *Gad2* mutant mice, respectively, as well as some technical limitations for studying GABAergic neural development in a mammalian

model, there is still an area of research need in the field to understand the role of GABA and the *Gad* genes in development and nervous system function.

GABAergic neural development in zebrafish

Zebrafish provide a great vertebrate model system for studying and better understanding many aspects of the nervous systems and the mechanisms which govern its development (Schier, Neuhauss et al. 1996). Zebrafish have been used to study neural development and axonogenesis for years (Kimmel, Powell et al. 1982, Bernhardt, Chitnis et al. 1990, Chitnis and Kuwada 1990, Kuwada, Bernhardt et al. 1990, Wilson, Ross et al. 1990, Bernhardt, Patel et al. 1992, Ross, Parrett et al. 1992). A large component of this work was in identifying subclasses of GABAergic neurons and in the brain and spinal cord of developing zebrafish and following their development (Bernhardt, Chitnis et al. 1990, Chitnis and Kuwada 1990, Kuwada, Bernhardt et al. 1990, Wilson, Ross et al. 1990, Ross, Parrett et al. 1992, MacDonald, Xu et al. 1994, Mueller, Vernier et al. 2006). There are also reports documenting the distribution of GABAergic neurons in the spinal cord and hindbrain of embryonic and larval zebrafish (Higashijima, Mandel et al. 2004, Higashijima, Schaefer et al. 2004).

In recent years, it has also been determined that the *dlx* genes are involved in the specification of GABAergic neurons, particularly in the zebrafish forebrain (MacDonald, Debiais-Thibaud et al. 2010, Yu, Xi et al. 2011, MacDonald, Pollack et al. 2013). This mechanism appears to be conserved in mammals and animals that are lacking *Dlx1* in particular have poor interneuron development and are prone to epilepsy (Cobos, Calcagnotto et al. 2005).

The studies of GABAergic neurons in the zebrafish brain have largely focused on identifying the distribution of GABAergic neurons in 1-3 days post fertilization (dpf) zebrafish brains (Doldan, Prego et al. 1999, Mueller, Vernier et al. 2006). It was found that GABA is present in many regions of the brain, including the olfactory bulb (OB) and pre-optic regions of the telencephalon; the epiphysis (E), habenula (H), ventral thalamus (VT), pretectum (Pr), posterior commissure (ptc), postoptic commissure (poc), nucleus of the medial longitudinal

fasciculus (N) and the hypothalamus (Hi) of the diencephalon; and the optic tectum (TeO) and tegmentum (T) of the mesencephalon (MacDonald, Xu et al. 1994, Mueller, Vernier et al. 2006). This information can also be found in Figure 1.1. GABA was also found in the pituitary, the medulla oblongata, portions of the cerebellum, and the retina (Mueller, Vernier et al. 2006).

It was also shown that in the early zebrafish spinal cord (~18-24 hours post fertilization (hpf)), there are six classes of neurons, four of which are interneurons. These interneurons were classified by the location of their soma both along the dorsal-ventral axis and in relation to other cell types as well as by the directionality of their axons (Bernhardt, Chitnis et al. 1990, Bernhardt, Patel et al. 1992). These four types of interneurons were then shown to be GABAergic (Bernhardt, Patel et al. 1992) and are named as follows: the Dorsal Longitudinal Ascending (DoLA), Commissural Secondary Ascending (CoSA), Ventral Longitudinal Descending (VeLD) and Kolmer-Agduhr (KA) neurons. A schematic of these GABAergic neurons in the early zebrafish spinal cord is shown in Figure 1.2 and Figure 1.3.

These studies have focused primarily on all GABAergic neurons using GABA antibody labeling. In 1998, a study was published describing the sequence and expression characteristics of the *gad* genes in zebrafish (Martin, Heinrich et al. 1998). This study showed that like mammals, zebrafish have *gad1* and *gad2* genes, these genes are largely co-expressed during early neural development and axonogenesis when looked at by *in situ* hybridization, and all *gad* expressing cells make GABA (Martin, Heinrich et al. 1998). A representative schematic of *gad* expression in the early zebrafish spinal cord neurons is shown in Figure 1.3. This work represented a thorough assessment of the *gad1* and *gad2* genes in early zebrafish neural development. There has recently been hints for a paralogous *gad1* gene, called *gad1a,* but has yet to be characterized (Grone and Maruska 2016, Cocco, Ronnberg et al. 2017)

Understanding the role of the *gad* genes in zebrafish using CRISPR/Cas9

The possibility of a *gad1a* gene is not implausible, it is well known that zebrafish and other teleosts had a whole genome duplication event which has given rise to many paralogous

genes (Sidow 1996, Postlethwait, Yan et al. 1998, Wittbrodt, Meyer et al. 1998, Holland 1999, Meyer and Schartl 1999, Christoffels, Koh et al. 2004, Hoegg, Brinkmann et al. 2004, Jaillon, Aury et al. 2004). Some of these duplicated genes remain as functional genes while others were lost entirely or are pseudogenes. The question now is "does *gad1a* seem to be present and important in the zebrafish and if so, how does this play a role in zebrafish neural development and function?"

The only published evidence for *gad1a* shows low levels of expression in adult zebrafish brains by qRT-PCR (Cocco, Ronnberg et al. 2017). While this is the only expression data for *gad1a* in the zebrafish, it does not provide any evidence about the presence or the role of this gene during development. We know from the published literature that the *gad* genes are important during various developmental processes and likely serve several functions aside from their characteristic role in GABA production for inhibitory neurotransmission. In order to address our major questions, we must first characterize the expression of *gad1a* during development using standard molecular approaches.

Additionally, there are still questions about *gad1* gene function and the mechanisms of how the *gad* genes can lead to diseases/disorders that also affect humans. Previous work in our lab used morpholino knockdown approaches to study the *gad* genes during zebrafish development, but with the introduction of improved methods for targeted mutagenesis and the requirement to distinguish *gad1a*, *gad1b* and *gad2*, we must evaluate these results in a more rigorous way (Hariharan 2013, Beebe 2015). To better address these questions of *gad* gene function, we must knock-out all three zebrafish *gad* genes (*gad1a, gad1b*, and *gad2*) and carefully assess the development and function of these mutant zebrafish which will provide helpful insight into the role of the *gad* genes during development. Fortunately, the CRISPR-Cas9 technology for targeted mutagenesis has revolutionized the genome editing abilities for the research community since 2013 and has been widely used ever since, particularly in the zebrafish (Chang, Sun et al. 2013, Hwang, Fu et al. 2013, Hwang, Fu et al. 2013, Jao, Wente et

al. 2013, Varshney, Lu et al. 2013, Irion, Krauss et al. 2014, Sung, Kim et al. 2014, Varshney, Pei et al. 2015, Burger, Lindsay et al. 2016).

Our major hypothesis is that there are paralogous *gad1a* and *gad1b* genes as well as *gad2* in zebrafish which are all expressed and important in neural development and nervous system function, though in distinct ways. The use of both classic and modern tools for molecular and developmental biology in this study can considerably expand our understanding of the *gad* genes both in the context of neural development and nervous system function and can hopefully lead to a more mechanistic understanding of how the *gad* genes are dysregulated in disease.

Figures

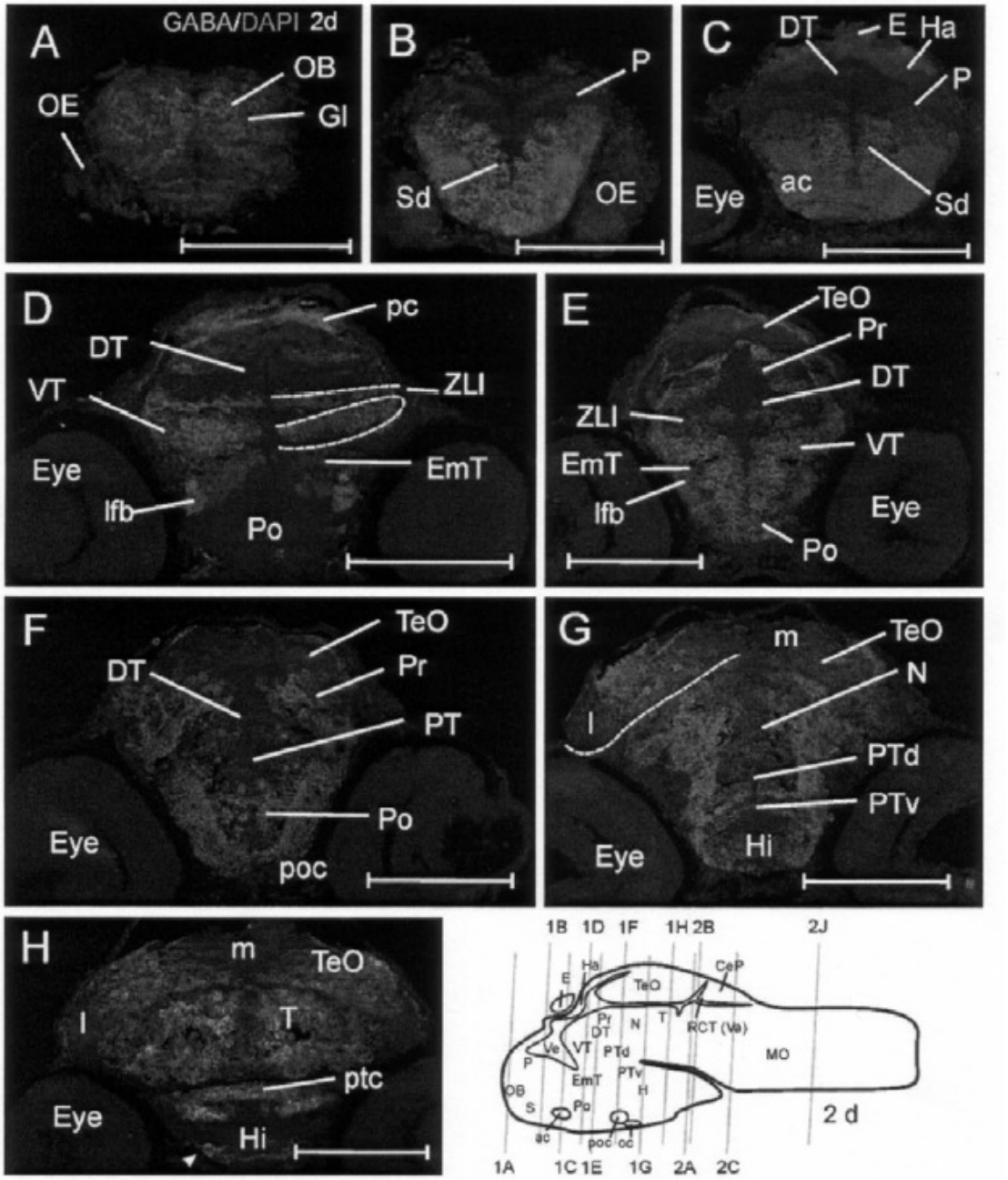

Figure 1.1: GABAergic neurons in the 2 dpf zebrafish brain. Adapted from (Mueller, Vernier et al. 2006). Transverse sections through a 2 dpf zebrafish brain from the olfactory bulb (Panel A) through the mesencephalon (Panel H), with the detailed plane of section in the lower right panel. Abbreviations: olfactory bulb (OB); epiphysis (E); habenula (H); ventral thalamus (VT); pretectum (Pr); posterior commissure (ptc); postoptic commissure (poc); nucleus of the medial longitudinal fascicle (N); the hypothalamus (Hi); optic tectum (TeO); tegmentum (T).

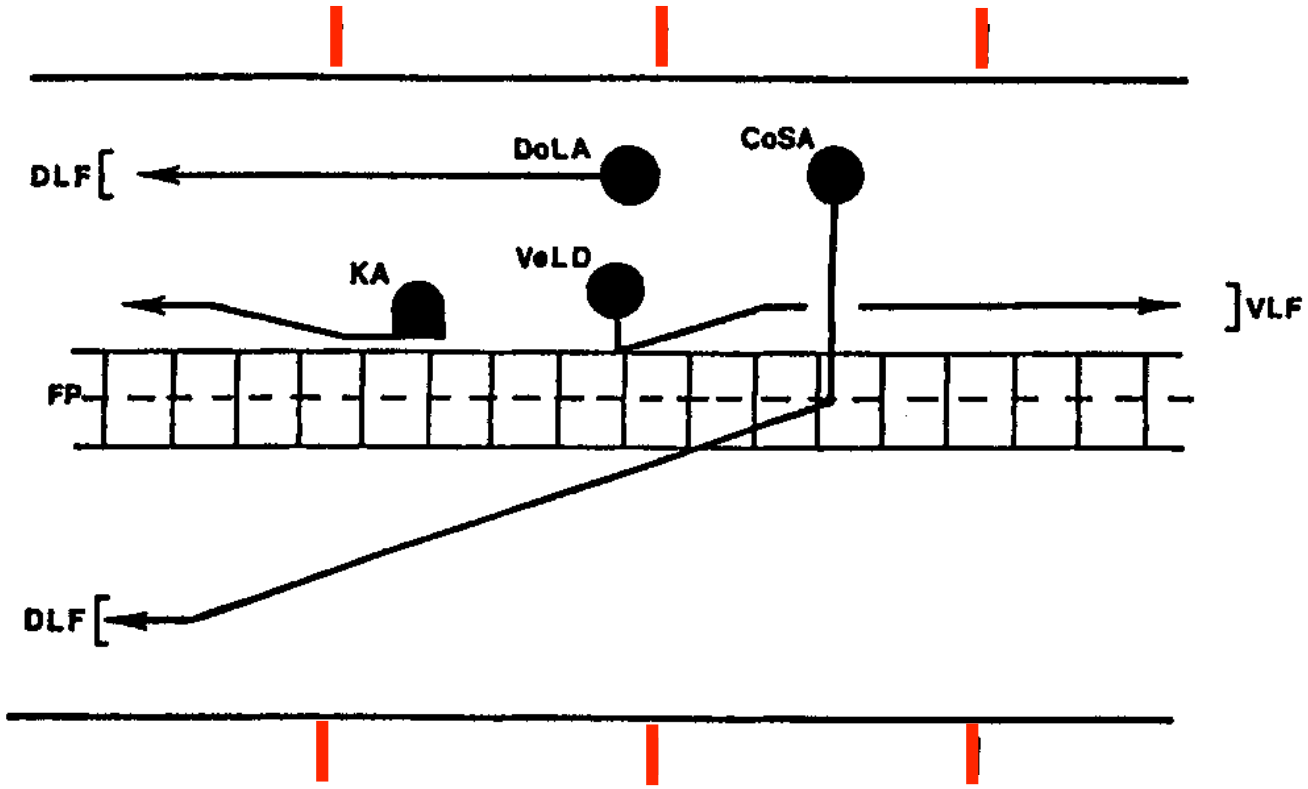

Figure 1.2: GABAergic neurons in the developing zebrafish spinal cord. Adapted from (Bernhardt, Patel et al. 1992). The spinal cord of an ~22 hpf zebrafish embryo is shown here in an open-book format where the dashed line represents the ventral midline of the floor plate (fp) and the segment borders are marked with red bars. There are four subclasses of GABAergic neurons: the Dorsal Longitudinal Ascending (DoLA) and Commissural Secondary Ascending (CoSA) whose axons are part of the Dorsal Longitudinal Fasciculus (DLF); the Ventral Longitudinal Descending (VeLD) whose axons are part of the Ventral Longitudinal Fasciculus (VLF); and the Kolmer-Agduhr (KA) neurons.

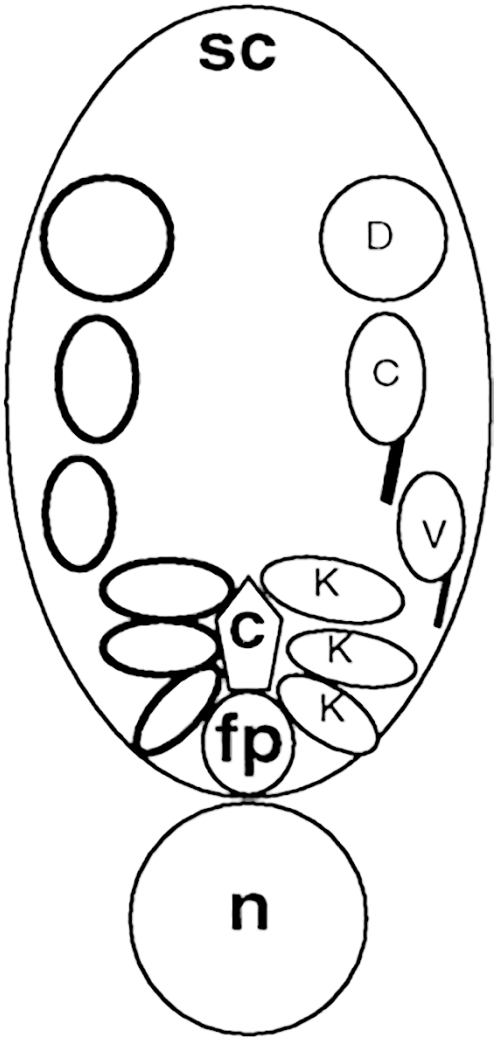

Figure 1.3: *gad* and GABAergic neurons in a transverse section of a 1 dpf zebrafish spinal cord. Adapted from (Martin, Heinrich et al. 1998). This illustrates the locations along the dorsal-ventral axis of the four types of both *gad* positive and GABA positive interneurons in the 1 dpf zebrafish spinal cord. They are the Dorsal Longitudinal Ascending (DoLA) and Commissural Secondary Ascending (CoSA), the Ventral Longitudinal Descending (VeLD), and the Kolmer-Agduhr (KA) neurons, the latter which directly contact the central canal (c) and floorplate (fp). The notochord (n) is ventral to the spinal cord.

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CHAPTER 2

A NOVEL *gad1* PARALOG IN THE ZEBRAFISH IS DIFFERENTIALLY EXPRESSED IN THE DEVELOPING NERVOUS SYSTEM[1]

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[1]VanLeuven, A.J., Ball, R.E., Gunderson, C.E. and J.D. Lauderdale. To be submitted to *Neural Development.*

Abstract

Background: Γ-Aminobutyric Acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system of all vertebrates. GABA is made by the glutamic acid decarboxylase (GAD) enzyme, which exists in two isoforms, GAD67 and GAD65. The genes *Gad1* and *Gad2* encode these isoforms, respectively, and both function in GABA synthesis. Like mammals, zebrafish have both known *gad* genes; however, we recently found evidence molecular evidence for a *gad1* paralog in zebrafish.

Methods: We used standard RT-PCR, mRNA *in situ* hybridization and antibody labeling to sequence, localize and validate gene activity of this *gad1a* gene during zebrafish neural development. We used double fluorescent *in situ* hybridization to assess the expression pattern between the zebrafish *gad* genes as well as to compare the expression pattern of the *gad* genes to well-known markers of zebrafish spinal cord development.

Results: This *gad1a* paralog is first detected by *in situ* hybridization at the 14-16 somite stage, while *gad1b* and *gad2* are not detected at this timepoint. At 1-day post fertilization (dpf), the *gad* genes are all expressed in the developing zebrafish brain and spinal cord, though this newly characterized *gad1a* gene is expressed in a distinct, pattern as compared to *gad1b* and *gad2.* Through double fluorescence *in situ* hybridization (F.I.S.H.) it is evident that *gad1b* and *gad2* are largely co-expressed, while *gad1a* is has its own specific expression pattern in the spinal cord, with very little if any overlapping expression with *gad1b/gad2*. This differential spatial expression of the *gad* genes persists to 3 dpf.

Conclusions: Through standard approaches, we have characterized the *gad1a* gene and discovered that there is differential but partially overlapping temporal and spatial expression both between the *gad1* paralogs and when compared to *gad2* during development. This description of a *gad1* paralog in zebrafish is another example of a genome duplication event that has apparent significance. The expression pattern of this family of genes indicates a complex regulation of the *gad* genes. This has interesting implications in terms of neural

development and gene regulation and requires that we consider this when we think about how the nervous system develops and functions.

Background

It is well-established that Γ-Aminobutyric Acid (GABA) is the major inhibitory neurotransmitter in the central nervous system of all vertebrates. GABA is made *in vivo* through the α-decarboxylation of glutamic acid, a process catalyzed by the enzyme glutamate decarboxylase (GAD, IUBMB Enzyme Nomenclature EC 4.1.1.15) (Awapara 1950, Roberts and Frankel 1950, Roberts, Frankel et al. 1950, Roberts and Frankel 1951, Roberts and Frankel 1951, Martin and Rimvall 1993). This GAD enzyme exists in two isoforms, Gad67 and Gad65, each of which are encoded by their own gene (Legay, Pelhate et al. 1986, Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Soghomonian and Martin 1998). Both of these GAD enzymes are found in both neural and non-neural tissues and are highly conserved from flies to mammals (Jackson, Newby et al. 1990, Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Feldblum, Erlander et al. 1993, Åhman, Wagberg et al. 1996, Martin, Heinrich et al. 1998, Maddox and Condie 2001, Trabucchi, Trudeau et al. 2008).

There is evidence for *GAD* expression in embryonic development that indicates a possible role for *GAD* and GABA in development that is separate from its role as a neurotransmitter (Martin, Heinrich et al. 1998, Maddox and Condie 2001). Additionally, in rodent literature there are reports that *Gad1* is alternatively spliced during development to produce a 25 kilodalton (kDa) and a 44 kDa form of Gad67, the latter of which is enzymatically active (Bond, Wyborski et al. 1990, Szabo, Katarova et al. 1994). This alternative splicing has been published for rodent *Gad1* and there is evidence in EST databases for similar alternative splicing of human *GAD1;* however, alternative splicing of *GAD2* has not been identified or reported in any organism. It is unclear what if any developmentally distinct isoforms of GAD are present in non-mammals. All that is known about *gad1* and *gad2* in non-mammalian vertebrates is that they encode GAD proteins that are clearly orthologous to mammalian GAD67 and GAD65 based

upon amino acid sequence, antigenicity and other criteria, but the molecular size of the encoded isoforms can vary between species.

At the genetic level, there is strong evidence for an ancestral gene duplication that gave rise to the two *GAD* genes in vertebrates that are known as *GAD1* and *GAD2* (Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Bosma, Blazquez et al. 1999). This hypothesis was first based upon similarity of the intron-exon organization in humans and mice (Bu and Tobin 1994, Yanagawa, Kobayashi et al. 1997, Makinae, Kobayashi et al. 2000, Matsukawa and Ueno 2007). Subsequent molecular phylogenetic analyses indicated that the duplication event occurred early in vertebrate evolution after the branching of the agnathans, but before the divergence of the cartilaginous fish, which was approximately 450 million years ago (Bosma, Blazquez et al. 1999, Lariviere, MacEachern et al. 2002). In humans, mice, zebrafish, and most other organisms who have suitable genomic sequence data available, the *GAD* genes are distinct genes and usually are located on different chromosomes and have slight differences in their promoter regions and 5'- untranslated regions (UTR's) (Edelhoff, Grubin et al. 1993, Bu and Tobin 1994, Szabo, Katarova et al. 1996, Yanagawa, Kobayashi et al. 1997, Martin, Heinrich et al. 1998, Makinae, Kobayashi et al. 2000).

In addition to the duplication that gave rise to paralogous *Gad1* and *Gad2* genes, there is evidence that suggests that fish species such as zebrafish, medaka, three-spined sticklebacks, fugu, and African cichlids also have a duplicated *gad1* gene (Grone and Maruska 2016, Lai, Fagernes et al. 2016). These *gad1* paralogs, designated as *gad1a* and *gad1b* most likely arose as a result of a genome duplication that occurred in the teleost lineage (Sidow 1996, Postlethwait, Yan et al. 1998, Wittbrodt, Meyer et al. 1998, Holland 1999, Meyer and Schartl 1999, Christoffels, Koh et al. 2004, Hoegg, Brinkmann et al. 2004, Jaillon, Aury et al. 2004, Amores, Catchen et al. 2011). Although the existence of *gad1a* and *gad1b* paralogs is recognized by researchers who use these fish as model organisms, available evidence in the published literature has been somewhat inconsistent.

The use of *gad1a* and *gad1b, gad1,* or simply *gad* has often been used either interchangeably or ambiguously in zebrafish literature (Higashijima, Mandel et al. 2004, Higashijima, Schaefer et al. 2004, MacDonald, Debiais-Thibaud et al. 2010, Yu, Xi et al. 2011, Zhang, Vanmeert et al. 2017). Furthermore, the sequences used for molecular biological experiments in much of the literature are either not provided or reference the first publication describing the zebrafish *gad* genes which was published nearly twenty years ago. In this study, it was shown that all *gad* expressing cells in the zebrafish spinal cord were GABAergic (Martin, Heinrich et al. 1998). Previous studies of GABAergic neurons in the zebrafish show that during early development, ~18-25 hours post fertilization (hpf), there are four subclasses of GABAergic neurons. These are interneurons and are known as Dorsal Longitudinal Ascending (DoLA), Commissural Secondary Ascending (CoSA), Ventral Longitudinal Descending (VeLD), and Kolmer-Agduhr (KA) neurons (Bernhardt, Patel et al. 1992). In the study by Martin *et al.* 1998, they suggest that Gad67 and Gad65 are both expressed in KA neurons. They also suggest that Gad67 is found in VeLD and CoSA neurons, though the data for Gad67 in CoSA neurons is not entirely convincing.

Taking this and what is written in the literature into account, a survey of these papers indicates that '*gad1*' is *gad1b,* but it is not known what, if any, role *gad1a* plays in the zebrafish. Recently, there was a paper which provided the first published account of a sequence for *gad1a* (Grone and Maruska 2016) and there has since been one other paper that clearly differentiates *gad1a* and *gad1b* (Cocco, Ronnberg et al. 2017), but does so in the adult zebrafish by qRT-PCR. As of now, there is no molecular characterization of *gad1a* in the developing zebrafish.

We do not think that *gad1a* and *gad1b* can or should be used interchangeably and one of the goals of this study is to determine whether there are distinct *gad1* paralogs in addition to *gad2* in the zebrafish in an effort to ensure that zebrafish researchers around the world refer to the proper gene, particularly in the context of the nervous system. Another goal of this study is to provide a careful and thorough study of the zebrafish *gad* genes during neural development

to determine if there are relevant differences in *gad* gene expression. It is known that at 1 dpf, the four types of GABAergic neurons in the developing zebrafish spinal cord have a stereotypical organization along the dorsal-ventral and medial-lateral axes (Bernhardt, Patel et al. 1992). This study will add *gad1a* into these types of analyses performed by Martin *et al*. 1998 and help us better understand the expression of the zebrafish *gad* genes. We hypothesize that there are paralogous *gad1* genes in zebrafish, as well as *gad2*, which exhibit distinct expression patterns in the developing nervous system.

Methods

Zebrafish care and maintenance

Adult zebrafish (*Danio rerio*) of the WIK strain were obtained from the Zebrafish International Research Center (ZIRC) and maintained in an Aquatic Habitats (Apopka, FL) multi-rack system according to standard procedures (Westerfield 1993). Habitat water consisted of reverse osmosis filtered/sterilized water to which sodium bicarbonate and other salts (Instant Ocean, Aquarium Systems, Inc., Mentor, OH, USA) were added to maintain pH from 7.0 -7.4 and conductivity between 400 and 430 µS. All experimental procedures were conducted in accordance with 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 (A2016 09-004-Y1-A1).

Staging and animal fixation

Zebrafish for all experiments were collected and staged accordingly (Kimmel, Ballard et al. 1995). Animals used for *in situ* hybridization and immunofluorescence were anesthetized in 0.4% Tricaine-S pH 7.4 (Pentair TRS4) (Westerfield 1993) and then fixed in 4% PFA/1X PBS, pH 7.4 at room temperature (RT) for 1 hour with gentle rocking. Fixed samples were washed with 1X phosphate buffered saline, pH 7.4 (1X PBS) and dehydrated into 100% Methanol and stored at -20°C at least overnight prior to use for *in situ* hybridization or immunofluorescence experiments.

Gene assemblies

Genomic and RNA nucleic acid sequences of the *gad1a, gad1b* and *gad2* genes were obtained from the Zebrafish Model Organism Database (ZFIN). Gene and protein assemblies were made in MacVector and Adobe Illustrator. The accession number for genomic DNA and mRNA sequences for *gad1a* are CR394567 and XM_005167412, respectively. We have also generated and will make available a 2012 base pair (bp) sequence that we collected from 1 dpf zebrafish RNA; it encompasses the entire *gad1a* CDS for comparison with the predicted sequence. The accession number for genomic DNA and mRNA sequences for *gad1b* are NW_018394195 and NM_194419.1, respectively. The accession number for genomic DNA and mRNA sequences for *gad2* are NW_001878476.5 and NM_001017708, respectively.

Revere Transcription PCR (RT-PCR)

Total RNA was extracted from pool of 50-100 embryonic and larval zebrafish using TRIzol® Reagent (Thermo Fischer Scientific, Inc. 15596-026) according to the manufacturer's protocol. Reverse transcription was performed using the ThermoScientific RevertAid First Strand cDNA Synthesis Kit (K1621) according to the manufacturer's protocol using the supplied Oligo$(dT)_{18}$ primers. The primer sequences for amplifying *gad1a* message are Fwd: 5'-GACGACAAGGGTCGAATTGT-3' and Rev: 5'-ATGTCTACATGACGGCCACA-3' with a T_m of 55°C. The primer sequences for amplifying *gad1b* message are Fwd: 5'-TTCACATATGAGATTGCGCC-3' and Rev: 5'-GAGACCACCATTCGGAAGAA-3' with a T_m of 55°C. The primer sequences for amplifying *gad2* message are Fwd: 5'-CAGTCTTCGTGCTGTTGGAA-3' and Rev: 5'-ACAAAGAACGGCACGTATCC-3' with a T_m of 55°C. The primer sequences for amplifying *β-actin* message are Fwd: 5'-TGTTTTCCCCTCCATTGTTG-3' and Rev: 5'-CTTCTCCTTGATGTCACGGAC-3' with a T_m of 58°C. All of the RT-PCR reactions were performed with 100 ng of starting cDNA template and were amplified with the following conditions: 95°C for 2 minutes followed by 35 cycles of 95°C for 30 seconds, T_m°C for 30 seconds, 72°C for 1 minute then a final extension at 72°C for 5

minutes and then held at 4°C. samples were run on a 1.5% agarose (50002, SeaKem® LE Agarose) gel in 1X TAE (40 mM Tris-Base, 20 mM Acetic Acid, 1 mM EDTA) at 105 Volts for 30 minutes.

Sequencing of gad1a message

Total RNA was extracted from a pool of 50 1 dpf wild-type zebrafish using TRIzol® Reagent (Thermo Fischer Scientific, Inc. 15596-026) according to the manufacturer's protocol. Reverse transcription was performed using the ThermoScientific RevertAid First Strand cDNA Synthesis Kit (K1621) according to the manufacturer's protocol using the supplied Oligo$(dT)_{18}$ primers. The primer sequences for amplifying the intermediate portion of *gad1a* message are Fwd: 5'-GACGACAAGGGTCGAATTGT-3' and Rev: 5'-ATGTCTACATGACGGCCACA-3' with a T_m of 55°C. The primer sequences for amplifying the 3' portion of *gad1a* message are Fwd: 5'-GCTTTGTGCCGATGTTTG-3' and Rev: 5'-TAATACGACTCACTATAGGGGTTTCTGTGGACAGGGCCTA-3' with a T_m of 60°C. The primer sequences for amplifying the 5' portion of *gad1a* message are Fwd: 5'-CTGGTGCTGAAGCTTCTGGA-3' and Rev: 5'-CCTCACTAACTACGTGCGCA-3' with a T_m of 54.5°C. The RT-PCR reaction was performed with 100 ng of cDNA template and were amplified with the following conditions: 95°C for 2 minutes followed by 35 cycles of 95°C for 30 seconds, T_m°C for 30 seconds, 72°C for 1 minute then a final extension at 72°C for 5 minutes and then held at 4°C. After the PCR, samples were purified using the GeneClean II Glassmilk Purification Kit (MP Biomedicals 111001400). Samples were sent for Sanger Sequencing to GENEWIZ (South Plainfield, NJ). Sequencing data from GENEWIZ was analyzed in MacVector.

Colorimetric in situ hybridizations

Antisense probes were constructed from whole-embryo cDNA for the *gad1b* gene (primers for amplification of *gad1b* are Fwd: 5'-ATTTAGGTGACACTATAGTTCTTCCGAATGGTGGTCTC-3' and Rev: 5'-TAATACGACTCACTATAGGGTTCACCTCACAAAGGTGCTG-3'. The T7 promoter (Rev) and

Sp6 promoter (Fwd) were added to these primers to allow for the construction of antisense and sense *in situ* probes, respectively. The antisense *in situ* probes for *gad1a* and *gad2* were constructed from a gBlock Gene Fragment (see Supplemental Table 2.1 for sequences). The amplicons from all PCR reactions were cleaned with GeneClean II Glassmilk Purification Kit (MP Biomedicals 111001400) following the manufacturer protocol prior to use as a template in transcription reactions. DIG-labeled riboprobes for all genes were synthesized with a DIG-Labeling Kit (Roche 11175025910) per the manufacturer's protocol. Probes were precipitated using LiCl/ethanol according to the manufacturer protocol and resuspended in 22 µL nuclease free water. Probe concentration was assessed on a NanoDrop ND-2000 spectrophotometer and subjected to dot blot analysis to ensure proper labeling. Probes for *gad1b* and *gad2* were made into a 1:10 stock by adding 130 µL of Hybridization Buffer (Thisse and Thisse 2008). The *gad1a* probe was made into a 1:100 stock by adding 1,280 µL of Hybridization Buffer (Thisse and Thisse 2008). Probe stocks were stored at -80°C until use. The *gad* riboprobes were diluted from their appropriate stocks at 1:250, 1:200 and 1:100 dilutions for *gad1a, gad1b* and *gad2*, respectively for working aliquots. Whole-mount RNA *in situ* hybridization (ISH) was performed as previously described (Thisse and Thisse 2008). Images were collected using an AxioCamHRC fluorescent microscope with color camera (Carl Zeiss Microscopy, LLC, Thornwood, NY) connected to a computer running AxioVision image capture software (Carl Zeiss Microscopy, LLC, Thornwood, NY).

Double fluorescent in situ hybridizations (F.I.S.H.)

Antisense probes for the *gad1a, gad1b*, and *gad2* genes were the same as those designed for colorimetric *in situ* hybridizations (see previous section). The zebrafish spinal cord interneuron markers *tbx16, pax2a, mnx1,* and *pkd2l1* which label DoLA, CoSA, VeLD and KA neurons, respectively were identified from published literature (Mikkola, Fjose et al. 1992, Tamme, Wells et al. 2002, Wendik, Maier et al. 2004, Djenoune, Khabou et al. 2014). Antisense probes for these interneuron markers were constructed from gBlock Gene Fragments (see

Supplemental Table 2.1 for sequences). The amplicons from all PCR reactions were cleaned with GeneClean II Glassmilk Purification Kit (MP Biomedicals 111001400) following the manufacturer protocol prior to use as a template in transcription reactions. The *gad1a*, *gad2, tbx16, pax2a, mnx1,* and *pkd2l1* gBlock fragments were used to create FITC-labeled riboprobes (Roche 11685619910) for use in double F.I.S.H in combination with the previously constructed DIG-labeled riboprobes. Probes were precipitated using LiCl/ethanol according to the manufacturer protocol and resuspended in 21 µL nuclease free water. Probe concentration was assessed on a NanoDrop ND-2000 spectrophotometer. Probes for *tbx16, pax2a, mnx1,* and *pkd2l1* were made into 1:10 stocks by adding 130 µL of Hybridization Buffer (Thisse and Thisse 2008) and stored at -80°C until use. The *gad* probes are still diluted from their stocks at 1:250, 1:200 and 1:100 dilutions for *gad1a, gad1b* and *gad2*, respectively for working aliquots. Probes for *tbx16, pax2a, mnx1,* and *pkd2l1* were all diluted from their stocks at 1:100 dilutions for working aliquots. These experiments were performed essentially as described in (Thisse and Thisse 2008) with the following significant modifications adapted from (King and Newmark 2013): after probe incubation and washing, samples were equilibrated in TNTx (0.1 M Tris pH 7.5, 0.15 M NaCl, 0.3% Triton X-100) followed by blocking and antibody incubation for peroxidase-conjugated antibodies (1:2000 Anti-Digoxigenin-POD [Roche 11207733910] and 1:2000 Anti-Fluorescein-POD [Roche 11426346910]) were performed with 5% horse serum (Sigma-Aldrich H1270) and 0.5% blotting grade block powder (BioRad 170-6404) in TNTx. Post-antibody washes were with TNTx.

TSA reaction

The TSA Plus Cyanine 3 and Fluorescein System [Perkin Elmer NEL753001KT]) were used for fluorescence detection of labeled riboprobes. Tyramide signal amplification was performed by incubating zebrafish for 10 minutes in fluorophore-conjugated tyramides diluted 1:50 in 1x Amplification Diluent (Perkin Elmer NEL753001KT). For double F.I.S.H., residual

peroxidase was quenched by incubating for 45 minutes in 100 mM sodium azide in 1X PBS + 0.1% Triton X-100 followed by re-equilibration, blocking and antibody incubation as described.

Immunofluorescence

Fixed 1 dpf zebrafish were mounted in TissueTek O.C.T. Compound (25608-930), frozen at -80°C and cryosectioned using a Leica CM1850 (Leica Microsystems, Buffalo Grove, IL) at 8 µm. Sections were collected on charged slides and then washed with 37°C 1X PBS until all gelatin had dissolved. Slides were blocked with 3% Bovine Serum Albumin (BSA; Thermo Fischer Scientific, Inc. 9048-46-8) plus 10% donkey serum (Sigma-Aldrich D9663) in 1X PBS for at least 1 hour at RT. For Gad67b, we used mouse monoclonal anti-GAD-67 (Santa Cruz Biotechnology Inc. 58531) that detects zebrafish Gad67b at a dilution of 1:50-1:100 in block. Slides were incubated in the primary antibody overnight at 4°C. After incubation with the primary antibody, slides were washed with 1X PBS and incubated with Alexa Fluor® 594-conjugated donkey anti-mouse immunoglobulin G secondary antibody (Thermo Fischer Scientific, Inc. R37115) diluted 1:1000 in block and incubated for 1 hour at RT. Slides were washed with 1X PBS and incubated with 1:10,000 DAPI (4',6-Diamidino-2-phenylindole; Sigma-Aldrich D9542) for 1 minute then washed with 1X PBS and coverslipped with EMS-Fluorogel (Thermo Fischer Scientific, Inc. 50-247-04). Images were collected using an AxioCamHRC fluorescent microscope with color camera (Carl Zeiss Microscopy, LLC, Thornwood, NY) connected to a computer running AxioVision image capture software (Carl Zeiss Microscopy, LLC, Thornwood, NY).

Results

There are 3 gad genes in zebrafish

In our investigations on the zebrafish genome database (ZFIN) and through nucleotide BLAST analyses performed on NCBI, there is EST and mRNA evidence for *gad1a, gad1b* and *gad2* genes in zebrafish. These annotations show genomic information and molecular characterization for *gad1b* and *gad2*, but only genomic information and predicted mRNA

sequences for *gad1a*. To confirm that *gad1a* is expressed in the developing zebrafish, we extracted total RNA from a pool of 1 dpf zebrafish and performed RT-PCR to sequence the entire predicted coding region of *gad1a*. Our results confirm that *gad1a* is indeed expressed in the 1 dpf zebrafish, as is *gad1b* and *gad2*. This nearly full-length sequence we obtained is the first hard evidence for *gad1a* expression in the developing zebrafish.

The genome databases show that *gad1a* is located on chromosome 9 while *gad1b* is located on chromosome 6 and *gad2* is located on chromosome 24 (Figure 2.1, Panel A). To help classify the previously uncharacterized *gad1a* gene as a *gad1* paralog, we looked at synteny, gene structure, and molecular similarity between the zebrafish *gad* genes. The syntenic organization of genes surrounding zebrafish *gad1a, gad1b* and *gad2* compared to human synteny, shows that the presence and organization of many genes are conserved between zebrafish and mammals (Figure 2.1, Panel B). Furthermore, the genes surrounding *gad1a* and *gad1b* have essentially been parsed out equally for *gad1a* and *gad1b* (Figure 2.1, Panel B). We also find that the syntenic organization of genes surrounding *gad1a* is well-conserved in teleosts; however, there is less conservation in the genes surrounding *gad1b* in teleosts (Supplemental Figure 2.1).

When we look at the gene structure of the zebrafish *gad* genes, we see that *gad1a* and *gad1b* each have 17 exons and take up approximately the same amount of genomic space while *gad2* has 16 exons and is considerably more compact in terms of gene size. The translation start site resides in exon2 for both *gad1a* and *gad1b*, while this site is in exon1 of *gad2* (Figure 2.1, Panel A). The intron sizes between *gad1a* and *gad1b* are different, but the overall exon organization and the exon/intron junction sites are conserved between zebrafish and mammals (Figure 2.1, Panel A and Supplemental Table 2.2). In terms of molecular similarity, the nucleic acid sequence identity between the zebrafish *gad* genes is not significantly similar (data not shown). We also found evidence for a putative bicistronic start/stop site in the zebrafish *gad* genes (Supplemental Table 2.3).

Bioinformatic analyses of the amino acid sequence identities between the zebrafish Gad67a, Gad67b and Gad65 proteins indicate that they are distinct proteins yet are of similar size and have similar structures (Table 2.1 and Supplemental Figure 2.2). The amino acid sequence identity between Gad proteins shows that Gad67a and Gad67b are 87% identical while each are only 65% and 71% identical to Gad65, respectively (Table 2.2). Furthermore, the catalytic domains of zebrafish Gad67a and Gad67b are significantly conserved and the cofactor binding site is the same, suggesting that these proteins likely have similar functions.

The 3 gad genes are all present during early embryonic development

There is evidence that the *GAD* genes in other organisms are likely important for processes in development as well as during neurotransmission. To address whether the zebrafish *gad* genes are expressed during development, we first took an RT-PCR approach. These RT-PCR experiments suggest that *gad1b* and *gad2* are expressed at the 1-4 cell stage, indicating that they are maternally expressed, whereas *gad1a* is not detected at this timepoint. Both *gad1a* and *gad1b* are expressed, but at different relative levels, during gastrulation and early somitogenesis, while *gad2* exhibits very low expression at these timepoints. By the 14-16 somite stage (~16 hpf), all three of the *gad* genes are expressed at significant levels. By 1 dpf and 3 dpf, the expression of all three *gad* genes continually increases (Figure 2.2). These data show that *gad1a, gad1b* and *gad2* are all expressed though at different times and at different relative levels throughout embryonic zebrafish development.

The 3 gad genes have distinct expression during neural development

To ask where the *gad1a* gene is expressed in the developing zebrafish, we took an *in situ* hybridization approach. Because of its relation to the other *gad* genes and based upon experiments done in previous studies, we chose to focus on important timepoints in neural development. At the 14-16 somite stage (~16 hpf), which is when the first GABAergic neurons can be detected, we see robust and bilateral expression of *gad1a* in the developing spinal cord

(Figure 2.3) We also see bilateral expression in the tail bud (Figure 2.3 and Supplemental Figure 2.3). These patterns are not seen in *gad1b* or *gad2* at this timepoint.

At 1 dpf, which is in the middle of axonogenesis, we see that the expression of *gad1a* in the brain appears to be localized to two small regions in the diencephalon, likely the tract of the postoptic commissure (tpoc) and the medial longitudinal fasciculus (mlf) (Figure 2.4). There is expression of *gad1b* in the telencephalon and in what is likely the tpoc in the diencephalon. The expression of *gad2* is in the telencephalon and likely the tpoc, like what is seen for *gad1b*, but there is also expression of *gad2* in the mlf (Figure 2.4). In the spinal cord at 1 dpf, *gad1a* is expressed in a subset of cells in more dorsal portions of the developing spinal cord, while *gad1b* and *gad2* appear to be localized to more ventrally located cells (Figure 2.4). High magnification views of *gad1a, gad1b* and *gad2* in the spinal cord show that there are clearly *gad1b* and *gad2*, and possibly *gad1a*, expressing cells which contact the central canal, presumably KA neurons. These findings for *gad1b* and *gad2* are consistent with previous reports (MacDonald, Xu et al. 1994, Martin, Heinrich et al. 1998). Taking these *in situ* data at 1 dpf together, there appears to be distinct expression of *gad1a*, *gad1b*, and *gad2* in the brain and spinal cord. We note that the expression of the three zebrafish *gad* genes is likely in overlapping patterns in discrete regions of the brain and spinal cord, and there may be slightly different levels of *gad* expression in these regions.

When we look at 3 dpf, which is when the zebrafish nervous system is essentially fully developed, we see that *gad1a* is expressed in deep regions of the hindbrain at the otic level. At this timepoint, we see similar patterns for *gad1b* and *gad2* which show high expression in the telencephalon, midbrain and through the tectum and tegmentum (Figure 2.5). The expression patterns at 3 dpf suggest that *gad1a, gad1b* and *gad2* have distinct expression, specifically that *gad1a* is expressed in a different population of cells in the brain as compared to *gad1b* and *gad2* which appear to be expressed in the same regions of the developing brain.

To better address this idea about *gad* gene expression, we performed double fluorescent *in situ* hybridization (F.I.S.H.) experiments to visualize any co-expression of the *gad* genes during nervous system development. In whole-mount double F.I.S.H. experiments, we see that *gad1b* and *gad2* are essentially co-expressed in more ventral cells in the developing spinal cord, presumably KA and VeLD neurons (Figure 2.6, Panel B). We see that *gad1a* is expressed in more dorsal cells of the developing spinal cord, presumably DoLA and CoSA neurons (Figure 2.6, Panel A). There may be some low levels of co-expression between *gad1a* and a subset of these ventrally located, *gad1b/gad2* expressing cells; however, the overall expression pattern between *gad1a* and *gad1b/gad2* appear to be in different subsets cells in the spinal cord, which is consistent with the colorimetric *in situ* hybridization results.

gad expression in the developing zebrafish spinal cord

To assess the GABAergic cell types of the early spinal cord with the inclusion of *gad1a*, we looked at the pattern of *gad* expression by *in situ* hybridization in transverse cryosections through the spinal cord of 1 dpf zebrafish. Analyses of these *in situ* hybridizations in sections, allow us to look at the location of staining in both the dorsal-ventral and medial-lateral axes of the spinal cord to infer about the subclasses of GABAergic neurons. We find that *gad1a* appears to be expressed in more dorsal GABAergic cells in the spinal cord, likely DoLA or possibly CoSA neurons (Figure 2.7, Panel A). At this timepoint, *gad1b* and *gad2* appear to be expressed in more ventral GABAergic cells in the spinal cord, likely KA and VeLD neurons. Antibody analysis shows that Gad67b is expressed in more ventral cell types, likely the KA and VeLD neurons of the developing spinal cord (Figure 2.7, Panel B).

To validate expression of the *gad* genes with the four subclasses of GABAergic interneurons, we performed double F.I.S.H. experiments with known markers for these four types of interneurons. Preliminary results so far suggest that *gad1a* is co-expressed in most *tbx16* and *pax2a* expressing cells which label DoLA and CoSA neurons, respectively (Figure 2.8). It does not appear that *gad1a* is co-expressed with either *mnx1* or *pkd2l1,* which label

VeLD and KA neurons, respectively (Figure 2.8). Additional experiments of *gad1b* with the interneuron markers are in progress, but preliminary assessments indicate that *gad1b* is co-expressed with *pkd2l1* marking KA neurons, but not with *mnx1*, *pax2a* or *tbx16*, marking VeLD, CoSA and DoLA neurons, respectively (data not shown).

Discussion

The gene, molecular, and synteny data provide evidence for an ancestral whole-genome duplication event that took place in teleost fish to give rise to *gad1a* and *gad1b* as paralogous genes. These *gad1a* and *gad1b* are paralogous genes whose nucleic acids have changed throughout zebrafish evolution. The finding of a putative bicistronic start/stop site suggests that *gad1a* and *gad1b* may be differentially regulated during development similar to what was found for *Gad1* in rodents (Bond, Wyborski et al. 1990, Szabo, Katarova et al. 1994). Taken together, this information shows that there are paralogous *gad1a* and *gad1b* genes as well as *gad2* in the zebrafish, all of which are on different chromosomes and have different genomic structures.

The amino acid sequence analyses show that Gad67a and Gad67b are distinct proteins and not a result of an incorrect annotation. If these proteins were indeed resulting from the same gene or a splice variant of a single gene, we would expect to see either the same amino acid sequence identity or a largely identical sequence but with several amino acids missing reflecting the alternative splice event, respectively. Instead we see a relatively high sequence identity and the proteins are nearly the same size and molecular weight (Table 2.1, Table 2.2 and Supplemental Figure 2.2). It is also noteworthy that zebrafish Gad67a and Gad67b are 82-85% identical to mouse and human Gad67, while zebrafish Gad65 is ~78% identical to mouse and human Gad65 (Table 2.3). These data suggest that the Gad67 proteins are slightly more evolutionarily conserved than the Gad65 protein. The only remaining limitation with studying the Gad proteins in zebrafish is that we have not been able to find an antibody that can preferentially recognize Gad67a.

It is interesting that *gad1b* is the more characterized gene and is often recognized as the zebrafish *gad1* gene, because these data suggest that the zebrafish *gad1a* gene and Gad67a protein are more closely related to the mammalian *GAD1* gene and Gad67 protein than *gad1b* and Gad67b. Considering that the zebrafish *gad1* gene seems to have been parsed out into two genes allows for investigation of questions regarding how the *gad* genes are involved in GABAergic neural development.

In the context of development, our RT-PCR results suggest that the *gad* genes are detected at early developmental timepoints, namely at timepoints before the 14-16 somite stage (~16 hpf) when there is not yet a nervous system in the zebrafish. This suggests that all the *gad* genes, particularly *gad1a* and *gad1b*, may play a role in non-neural processes during development. All *gad* genes are detected from the 14-16 somite stage on, suggesting all three zebrafish *gad* genes are involved in neural development, which is to be expected. The fact that we detect *gad1b* and *gad2* via RT-PCR but not by *in situ* hybridization at the 14-16 somite stage suggests that these genes are too low to detect using this approach. The *gad1b* and *gad2* genes likely still play a role in development, but perhaps *gad1a* is the critical *gad* gene in terms of GABAergic neuron development.

Our *in situ* hybridization results suggest that *gad1a* appears to be expressed both in the early spinal cord and in non-neural cells in the tail bud (Figure 2.3 and Supplemental Figure 2.3). The expression of *gad1a* in the tail bud is consistent with a similar report of non-neural *Gad1* expression in the mouse tail bud (Maddox and Condie 2001). This is particularly interesting because it was not previously known what, if any, presence *gad1a* had during development. It has been shown in the literature that the more dorsal GABAergic interneuron types in the spinal cord develop first (between 16-18 hpf) while the most ventral KA neurons do not appear until several hours later (Bernhardt, Chitnis et al. 1990, Kuwada, Bernhardt et al. 1990, Bernhardt, Patel et al. 1992). It is interesting to note that *gad1a*, but not *gad1b* or *gad2,* is highly expressed in the spinal cord at the time when these more dorsal cell types are emerging.

This suggests that it is likely *gad1a* that is important for these early GABAergic neurons during their development. The report that expression levels of *gad1a* transcripts in the adult are quite low suggests that *gad1a* may be more important developmentally, which is consistent with our data (Cocco, Ronnberg et al. 2017).

The expression pattern of the *gad* genes in the 1 dpf zebrafish brain and spinal cord suggest a complex and overlapping distribution of the *gad* genes (Figure 2.4). There is a high degree of overlapping expression in distinct regions of the telencephalon and diencephalon between zebrafish *gad1a* and *gad1b* when compared to *gad2*. It is known in both zebrafish and mammalian literature that the *GAD1* and *GAD2* genes are co-expressed in the nervous system (Esclapez, Tillakaratne et al. 1993, Martin, Heinrich et al. 1998). The pattern that we see in the brain where *gad1a* and *gad1b* expression is essentially parsed out in comparison to *gad2* supports that these genes as paralogs. However, there is less overlap of *gad1a* with *gad1b* or *gad2* in the spinal cord as the *gad1a* gene appears to be expressed in a different subset of cells as compared to *gad1b* and *gad2*. It is possible that *gad1a* is co-expressed at a low level in some *gad1b/gad2* expressing cells as well as in more dorsal cells of the spinal cord. The differences in expression levels between the *gad* genes, particularly at 1 dpf, were present to an extent in both our colorimetric and fluorescent *in situ* experiments and are consistent with other reports of expression level differences between *Gad1* and *Gad2* in the same cells in the mammalian brain (Feldblum, Erlander et al. 1993, Sheikh, Martin et al. 1999).

The conclusions we drew from *gad* gene expression in the 1 dpf spinal cord are further supported by the analyses of *gad in situ* hybridization sections and double F.I.S.H. experiments. These data suggest that *gad1a* is expressed more in dorsal cell types, likely DoLA and CoSA neurons, while *gad1b/gad2* are expressed in more ventral cell types, likely VeLD and KA neurons. These sections analyses are consistent with double F.I.S.H. experiments for markers of these DoLA, CoSA, VeLD and KA neurons that we have performed so far. Work in other labs supports that *gad1b* is expressed in KA neurons so our results are consistent with these

findings (Djenoune, Khabou et al. 2014). When comparing our results to those from other reports of *gad* genes during early zebrafish development, we conclude that what is referred to as *gad1* is actually *gad1b* and our findings are consistent with these reports (Martin, Heinrich et al. 1998). We are adding to the published literature by introducing *gad1a* in this study and suggesting that it is differentially expressed from *gad1b* and *gad2* in early zebrafish neural development and has an overlapping expression pattern in the brain but distinct expression in the spinal cord at subsequent timepoints during neural development. These data suggest that *gad1a* and *gad1b* have evolved slightly different gene regulatory networks and there is a more complex involvement of the *gad* genes in the nervous system than was previously assumed.

While these findings may complicate the story for neural development, they also open opportunities to study *gad1* gene function that are not possible in mammalian models due to technical limitations as well as neonatal lethality in *Gad1* -/- mice (Asada, Kawamura et al. 1997, Condie, Bain et al. 1997, Oh, Westmoreland et al. 2010). In future studies, we will create genetic mutants for *gad1a, gad1b* and *gad2* to observe what, if any, differences are seen during nervous system development and function in zebrafish.

Conclusions

In conclusion, our study has shown that there is indeed a novel *gad1* paralog in zebrafish that is expressed during development. This *gad1a* gene is highly expressed when the first GABAergic neurons in the spinal cord are detectable, meaning it is likely critical for early GABAergic neural development. The differential expression pattern of *gad1a* indicates a distinct regulatory mechanism as compared to *gad1b* and *gad2*.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 2.1: Zebrafish Gad protein's amino acid length and weights in kilodaltons (kDa).

Protein	Bioinformatics Data
GAD67a	591aa = 66.8 kDa
GAD67b	587aa = 66.2 kDa
GAD65	583aa = 65.5 kDa

Table 2.2: Zebrafish Gad amino acid identity comparisons.

Protein	Bioinformatics Data
GAD67a v. GAD67b	87% identity
GAD67a v. GAD65	71% identity
GAD67b v. GAD65	65% identity

Table 2.3: Gad amino acid identity comparisons of and between humans (as a mammalian representative) and zebrafish.

	Human GAD67	Human GAD65	Zebrafish GAD67A	Zebrafish GAD67B	Zebrafish GAD65
Human GAD67	100%	66%	81%	82.7%	65%
Human GAD65	66%	100%	65%	66%	78.30%
Zebrafish GAD67A	81%	65%	100%	85%	70%
Zebrafish GAD67B	82.7%	66%	85%	100%	65%
Zebrafish GAD65	65%	78.30%	70%	65%	100%

Figures

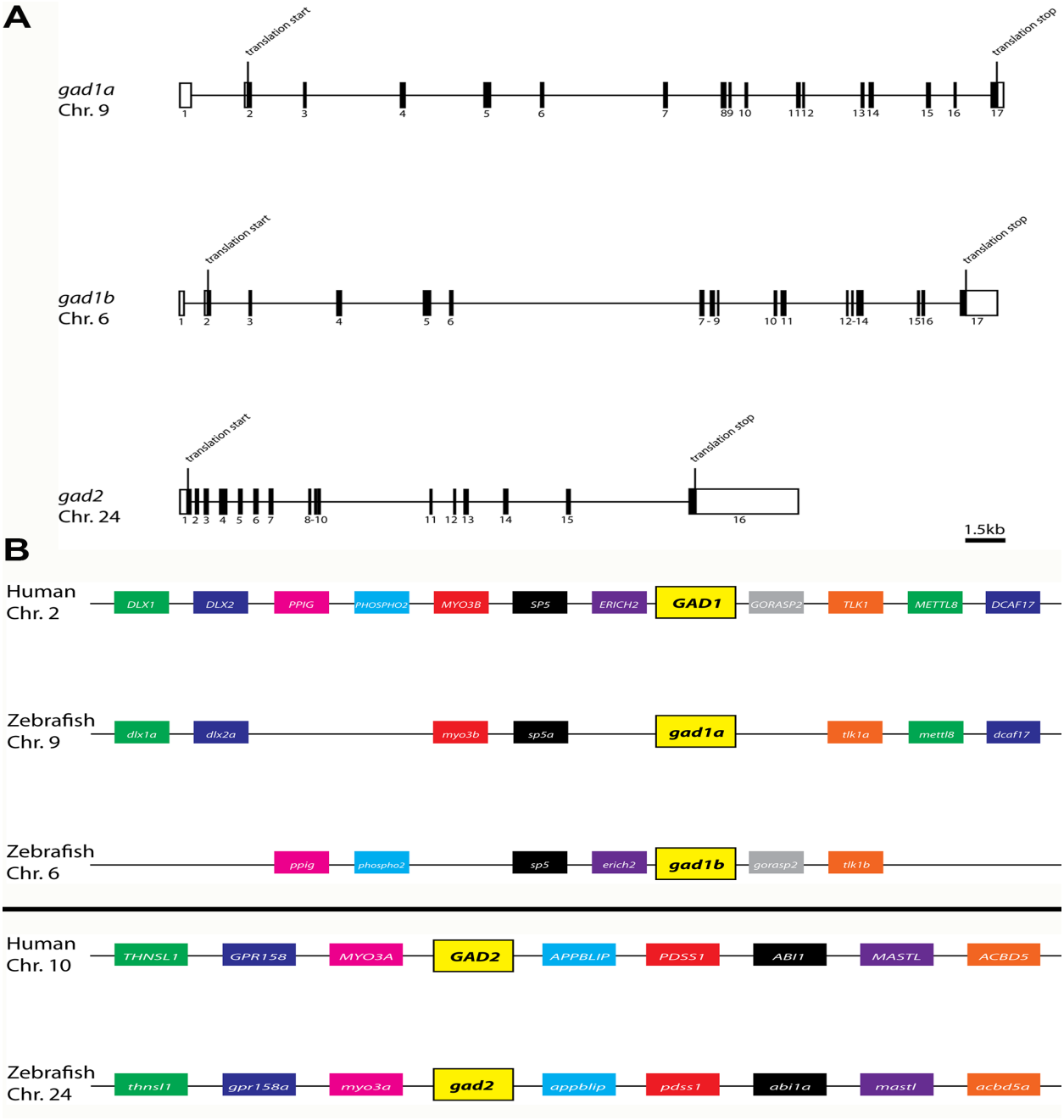

Figure 2.1: Zebrafish *gad* gene maps and synteny. A) The exon/intron organization of the zebrafish *gad* genes in genome space, to scale. *gad1a* and *gad1b* take up approximately the same amount of space and have the same distribution of the coding region between exons 2-17. *gad2* is considerably more compact and its coding region is between exons 1-16. B) The syntenic organization of zebrafish chromosomes 9, 6, and 24 compared to human chromosomes 2 and 10. The genes around *gad1a* and *gad1b* have essentially been equally parsed out, while the genes around *gad2* are conserved between mammals and zebrafish.

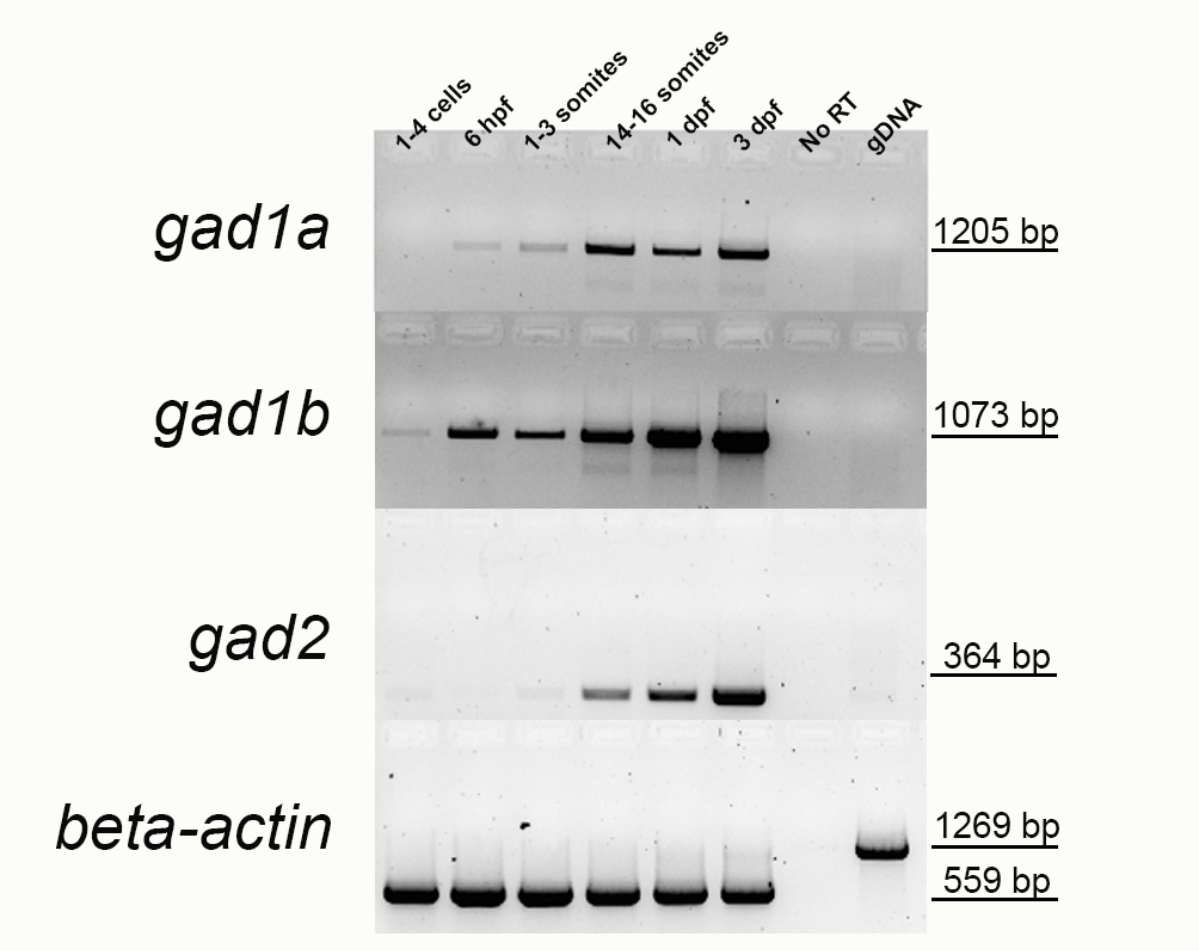

Figure 2.2: RT-PCR of the zebrafish *gad* genes during early development. Samples were taken at the listed timepoint for RNA extraction and RT-PCR for *gad1a, gad1b* and *gad2* to assess the presence and relative levels of expression during early embryonic and larval development. Beta-actin was used as a loading control; a genomic DNA control is included for all genes to show that amplification is from reverse transcribed RNA. The expected amplicon size is listed on the right of the image. *gad1a* does not appear to be maternally expressed but is detected at all other timepoints we looked at. *gad1b* is detected at all timepoints we looked at while *gad2* is detected at very low levels until the 14-16 somite stage, 1 dpf and 3 dpf. There are differences in relative levels of the *gad* genes during early development in zebrafish.

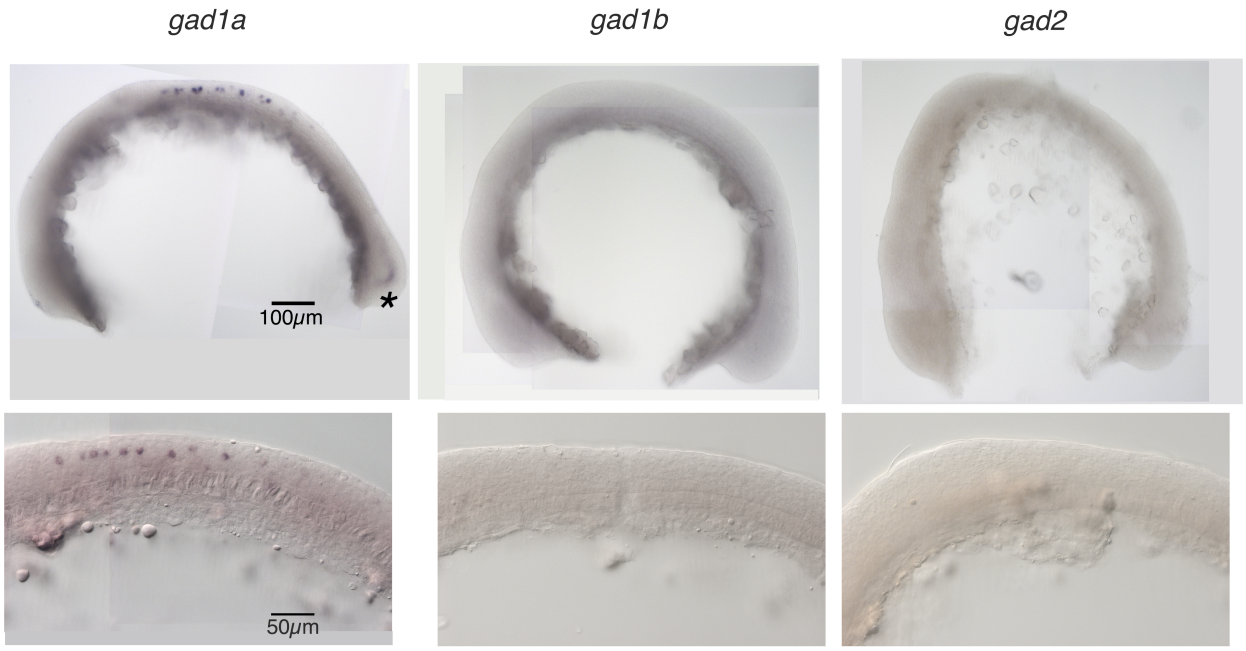

Figure 2.3: Zebrafish *gad* expression at the 14-16 somite stage by colorimetric *in situ* hybridization. Lateral views (anterior to the left) of ISH for *gad1a, gad1b* and *gad2* on wild-type embryos at 14-16 somites (~16 hpf) show that *gad1a* is present in the early spinal cord while *gad1b* and *gad2* are not detectable by ISH in this pattern. The asterisk is noting *gad1a* expression bilaterally in the tail bud that is not detectable for either *gad1b* or *gad2*.

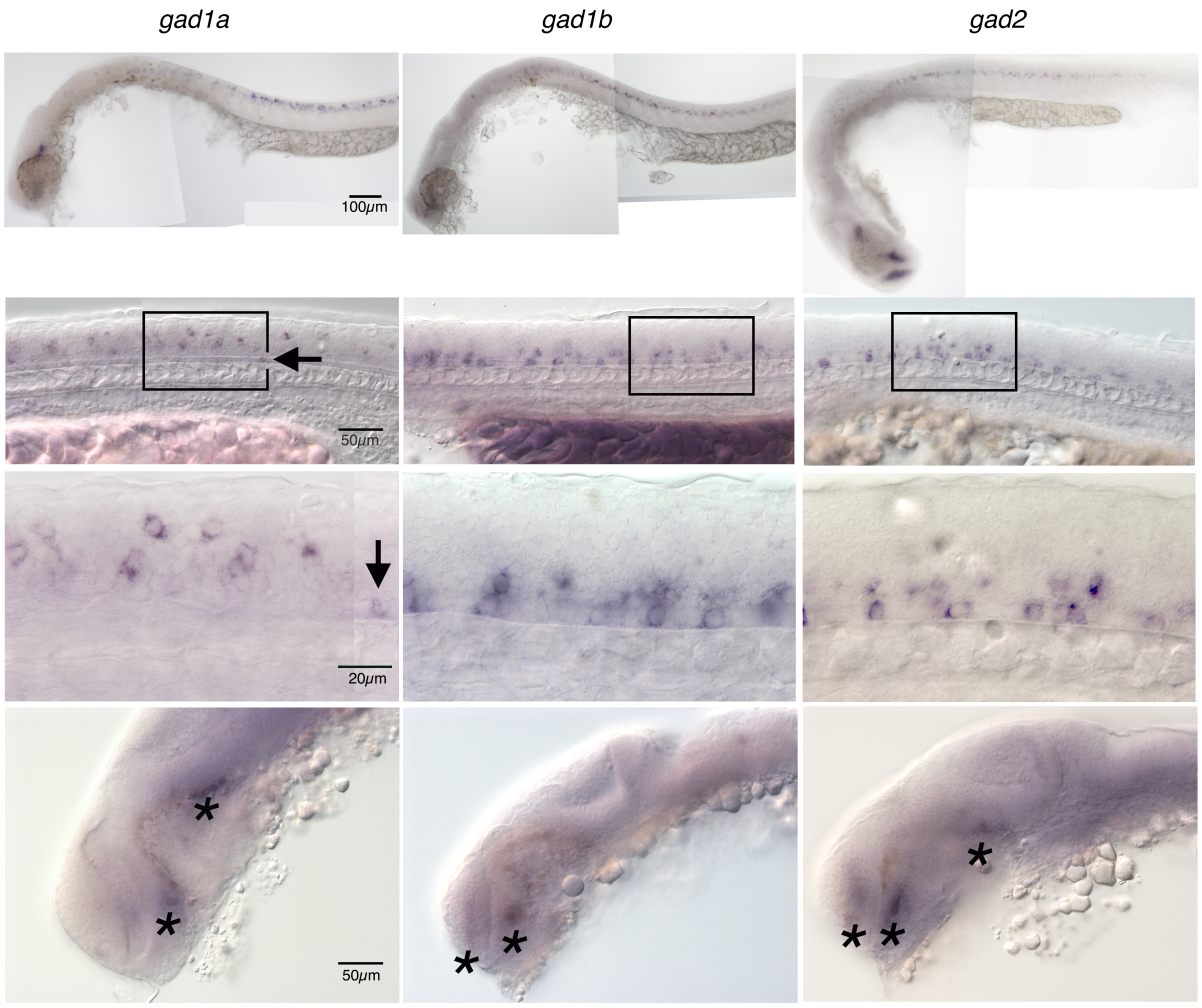

Figure 2.4: Zebrafish *gad* expression at 1 dpf by colorimetric *in situ* hybridization. Lateral views (anterior to the left) of ISH for *gad1a, gad1b* and *gad2* on wild-type embryos at 1 dpf show that all three genes are expressed in the brain and spinal cord. In the spinal cord, *gad1a* is expressed in more dorsal and medial cell types, while *gad1b* and *gad2* appear to be expressed in the same cells that are more ventrally located and appear to contact the central canal. The arrows are noting a *gad1a* expressing cell that looks like it may be contacting the central canal. In the brain, *gad1a* appears to be expressed in discrete populations of the diencephalon, likely the tpoc and the mlf. *gad1b* appears to be expressed in the ventral telencephalon and the tpoc. *gad2* appears to be expressed in all three of these regions: the telencephalon, the tpoc and the mlf. There also may be slightly different expression levels of the gad genes in the brain. Abbreviations: tpoc = tract of the postoptic commissure; mlf = media longitudinal fasciculus.

Figure 2.5: Zebrafish *gad* expression at 3 dpf by colorimetric *in situ* hybridization. Dorsal views (anterior to the left) of ISH for *gad1a, gad1b* and *gad2* on wild-type embryos at 3 dpf highlights spatial expression differences between *gad1a* in the hindbrain and the largely co-expressed *gad1b* and *gad2* genes across the telencephalon, midbrain, tectum and hindbrain. There appears to be distinct *gad1a* expressing cells in the hindbrain at the level of the otocyst that either do not express *gad1b* or *gad2* or do so at a level below the threshold of detection by ISH.

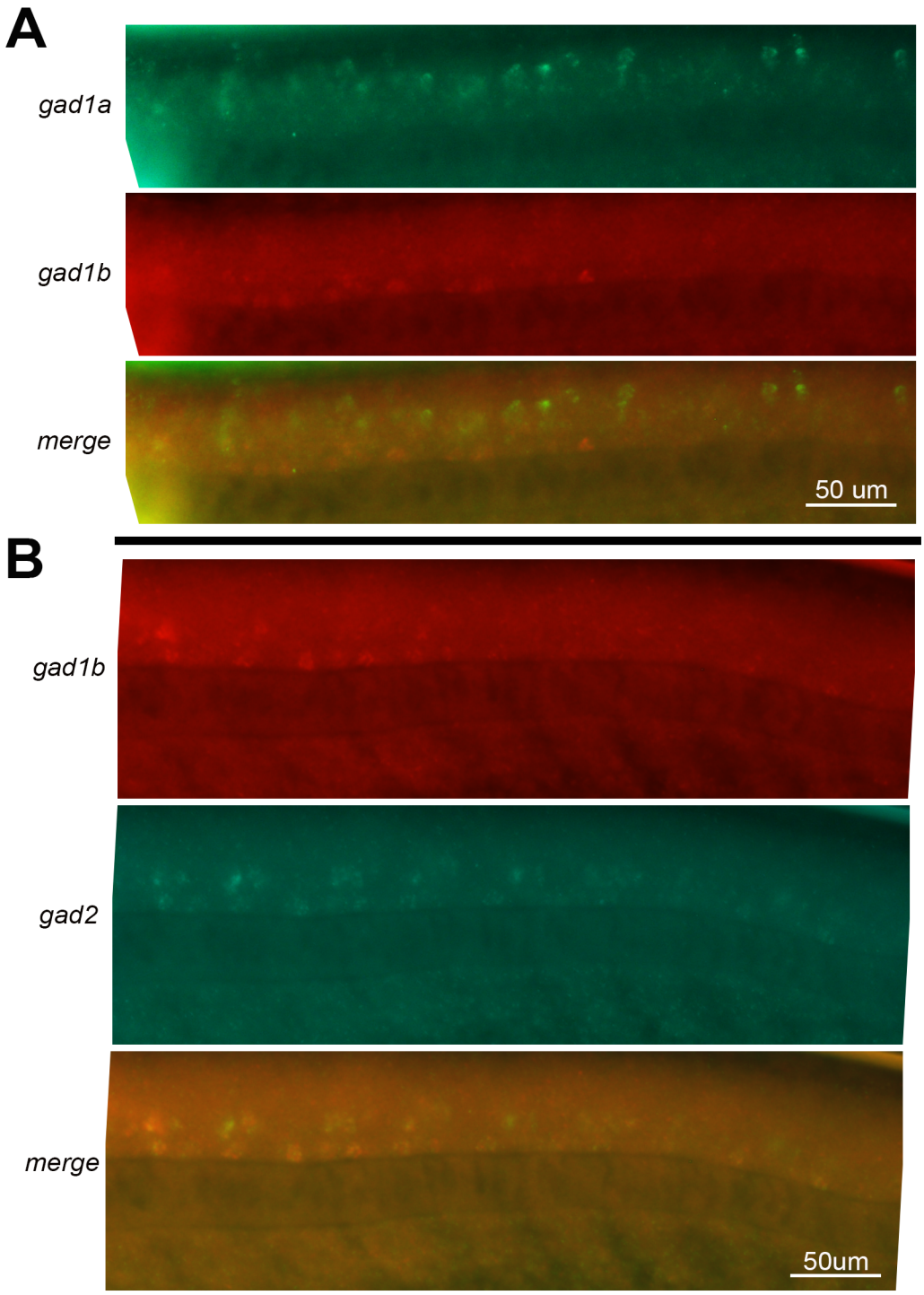

Figure 2.6: Expression of the zebrafish *gad* genes by double fluorescent *in situ* hybridization at 1 dpf. Lateral views (anterior to the left) of whole-mount F.I.S.H. experiments on wild-type embryos. A) *gad1a-FITC+gad1b-DIG* in the spinal cord. *gad1a* is expressed in a distinct pattern in the more dorsal portions of the developing spinal cord while *gad1b* is in more ventral portions of the spinal cord. B) *gad1b-DIG+gad2-FITC* in the spinal cord. *gad1b/gad2* are essentially co-expressed in more ventral spinal cord neurons.

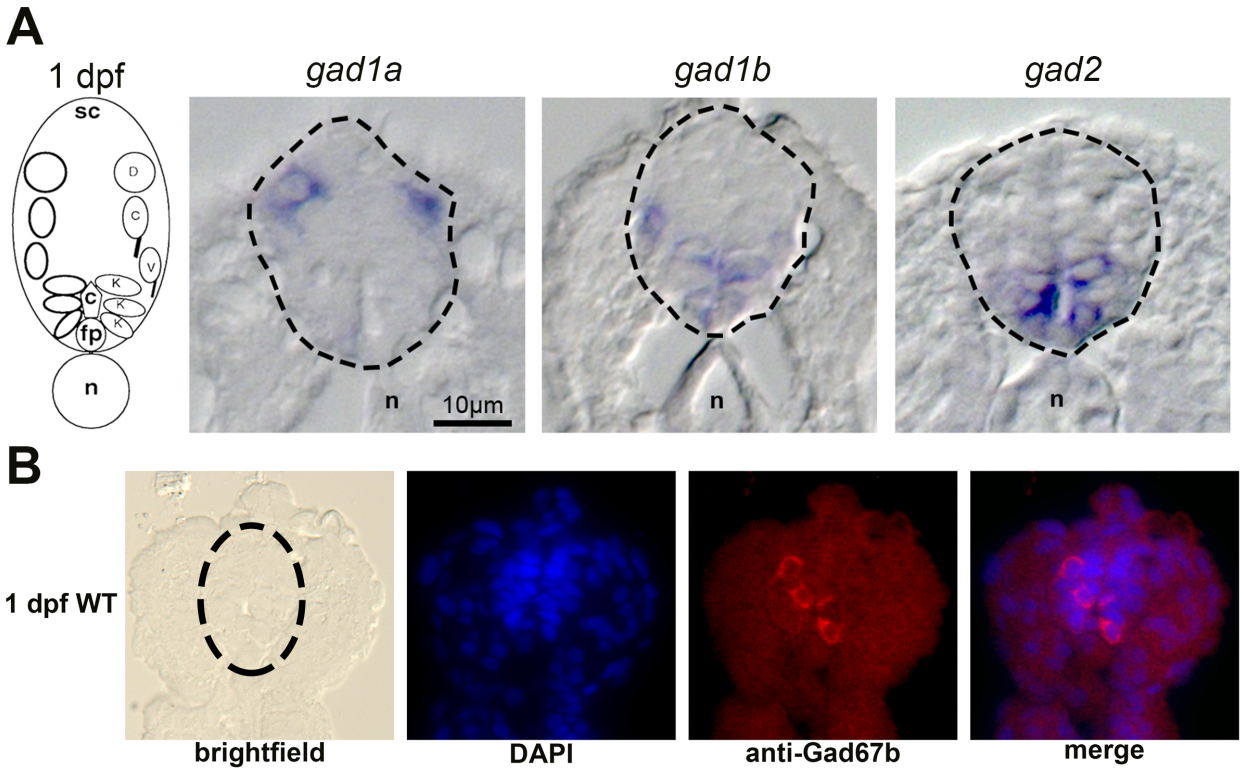

Figure 2.7: Section analyses of the zebrafish *gad* genes in the 1 dpf spinal cord. A) A schematic showing the orientation of the four subclasses of GABAergic neurons in the developing spinal cord is shown for reference. Colorimetric ISH for *gad1a, gad1b* and *gad2* were performed and then were cut in 8 µm transverse sections. *gad1b* and *gad2* are expressed in more ventral cells, likely KA and VeLD neurons. *gad1a* is expressed in more dorsal cell types, likely DoLA or CoSA neurons. B) Antibody staining for Gad67b (*gad1b*) on 8 µm transverse sections of a WT zebrafish spinal cord. Gad67b appears to be expressed in ventral spinal cord neurons, likely KA and VeLD. n = notochord, fp = floor plate, c = central canal, K = KA neuron, V = VeLD neuron, C = CoSA neuron, D = DoLA neuron.

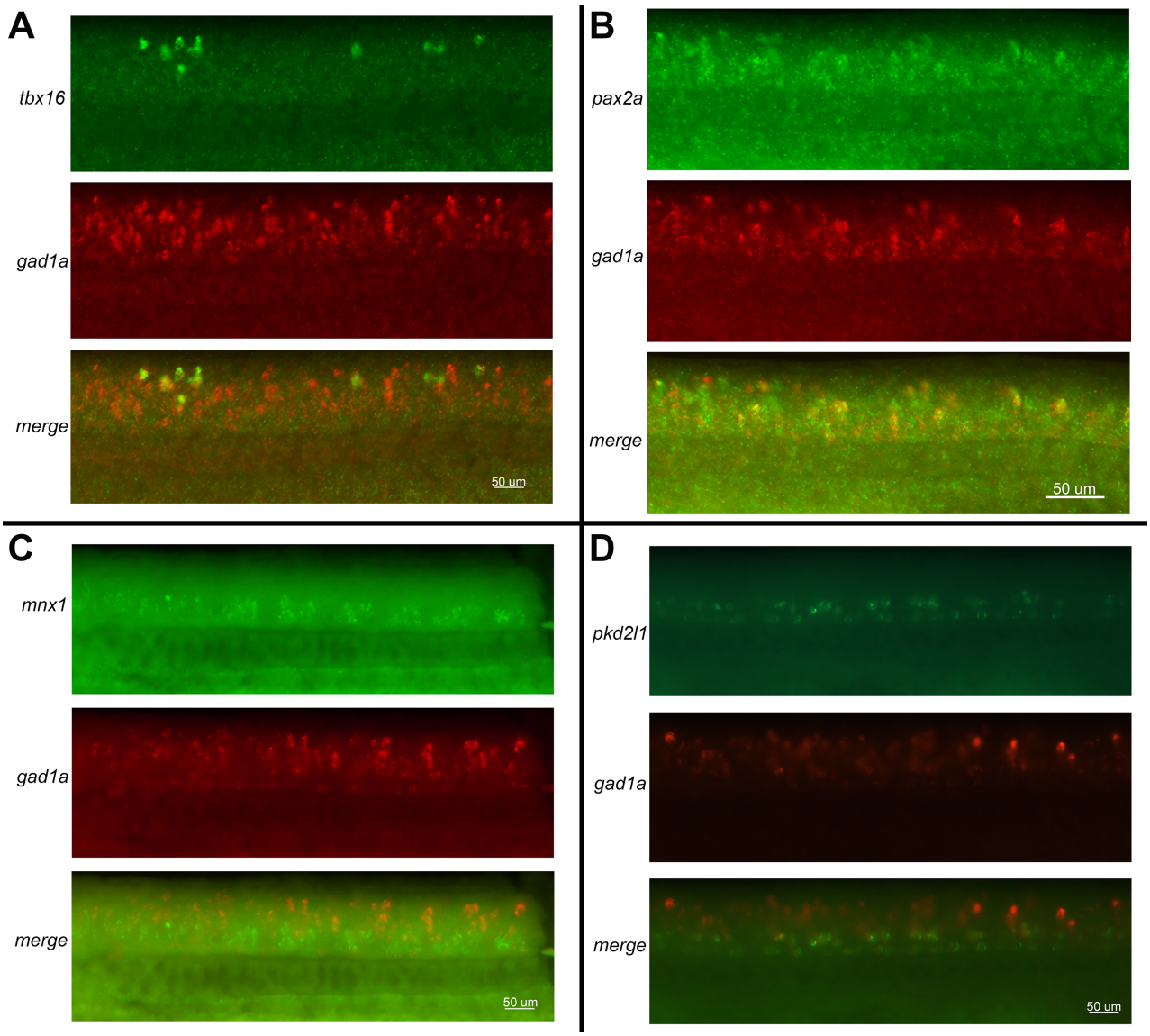

Figure 2.8: *gad1a* with interneuron markers via double fluorescent *in situ* hybridization in 1 dpf spinal cord. Lateral views (anterior to the left) of whole-mount F.I.S.H. experiments on wild-type embryos. A) *gad1a-DIG+tbx16-FITC* indicate co-expression in most *tbx16* cells suggesting *gad1a* is in DoLA neurons. B) *gad1a-DIG+pax2a-FITC* indicate co-expression in most *pax2a* cells suggesting *gad1a* is in CoSA neurons. C) *gad1a-DIG+mnx1-FITC* do not indicate co-expression suggesting *gad1a* is not in VeLD neurons. D) *gad1a-DIG+pkd2l1-FITC* do not indicate co-expression suggesting *gad1a* is not in KA neurons.

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Supplemental Tables and Figures

Supplemental Tables

Supplemental Table 2.1: Sequences used to make gBLOCK gene fragments for *in situ* hybridization experiments.

gene	**gBLOCK fragment/sequence, does not include Sp6 or T7 promoters for *in vitro* transcription**
gad1a	TGTCTGGAGCTGTCGGAGTATCTCTACCACAAGATCAAGAACAGAGAAGGATATGA GATGGTGTTTCAAGGGGAGCCACAGCACACAAATGTATGTTTCTGGTACATTCCTCC AAGCCTGCGGCTTCTGCCAGATGGAGAGGAGAAACGACATCGGCTTCATAAGGTC GCCCCAAAGATCAAGGCACTGATGATGGAGTGCGGGACAACAATGGTGGGCTACC AGCCTCAGGGTGAGAAGGTTAACTTCTTCAGGATGGTGGTCTCCAATCCGGCGGTT ACCAGGTCTGACATTGACTTCCTGATCGATGAGATAGAAAGACTGGGACAGGATTT ATAGAGAACGCAGAACAAGTTCAGTAGATGTAAATCATCTGGAAATGGAGGAGGCA ATCAGACGTGTTGTATCAGCTCGATGTTCCAGAGAATTAGGCCCTGTCCACAGAAA CACAGCTA
gad2	TCTACAACAAGATTAAGGACAGGGAAGGATATCAGATGGTGTTTGATGGAAAGCCG CAGCATACCAATGTGTGTTTCTGGTACCTTCCACCGGGCGTGCGCTACCTGGAGGA CAAAGTGGAGAGGATGAAGCGTCTGCACAAGGTTGCCCCTGTAATCAAAGCCAGAA TGATGGAGTACGGCACGACCATGGTGAGCTACCAGCCACAGGGAGACAAGGTCAA CTTCTTCCGCATGGTCATCTCCAATCCAGCCGCTACCTTTGAAGACATTGACTTCCT CATTGAAGAGATCGAGCGACTGGGGCAGGATCTTTAAAACTTACCGCACCAAAACC TGTTACTCCCGTGTCCCTGGATGGATTGCATATTTGTTGTGAATGTAACGGTAAATC TCTGATTCCTCTTCTCCAAAGTCACATTTAAAC
tbx16	CACATACCAGCCGTACAGATTTCACGAATACGGCAAATCCCCGTCTCCATCTTCATC TTCCAGCGTTGGCGGCAGCAGTGCATGTGGCAGCGCGGGACGTCCCAGCTTTGAG TCCCGAGTCTTGGACGTGGCCACCGTGCCCGACACAGACAGCTCCAGCAAGCCCT CCTCCGCTCCAGAGTTCAGCCTCCCTCCGCATCCCTCTGCAGGACACCAGGAGTAC GCTGGAGTGCTAAACATGGCCATCACCCAGGCCAAACCAGGCATGCTGGGAACTC ATCCGCTTTACAGCCACTACAGCACAGAACAGTCTCTGGGGCAGTGGAGCGGGGC AGCAGCATCCCAATACCCGCCTCCACCTCCGCCCCATCATCACCTTCCCACCGAAT ACAGCAGCCAAGCTGTCCATCATGGCTATCACCATGGAAACGTTGGCGATTGGAGC CAGTATCCGCTTTTCTCATACTCGTGCTGGTGAGCCGGACACCGATTGAGAGACCA TTTTGAGGATGCTTATTTTTAATTTTCTGCACGATGAGGTCTGGATGGTTTTTCATGA CCTGTAAAATGGCACACGAAGTCGCCCGGTACTGAGTGTAC
pax2a	ACTCAACAGCAGCTGGAGGCTCTGGATCGGGTGTTTGAGCGGCCGTCATACCCCG ACGTCTTCCCCACGTCAGAACACATCAAGCCAGAGCAGGCTAATGAGTACTCGCTA CCAGCACTGAACCCTGGACTGGACGAAGTCAAGCCCAGTCTGTCAACCAGCGTCA GCTCAGATTTGGGCTCCAGCGTGTCACAGAGCTACCCAGTAGTGACAGGTCGAGA GATGGCGAGCACGACCCTACCAGGATATCCACCTCACGTTCCCCCTACTGGGCAG GGCAGCTACCCCACCTCTACACTTGCTGGAATGGTCCCTGGAAGCGACTTTTCAGG AAATCCCTACTCTCACCCGCAGTACACAACTTACAATGAAGCTTGGCGGTTCAGCAA CCCCGCGTTATTAAGTTCCCCTTATTATTATAGTGCCGCATCCCGGGGCTCCGGGC CTCCCACTGCTGCCACTGCCTATGACCGCCACTAGTTACCATCGCAGCCCAGTCAA ACTGCAGGACCACGGCCGCGGCCTCCATATCGTACCCGTCTGAATGGTCAGAGGG ATTGAAGATGGATACGCCATCTTCACTTTCATGGAGCCGAAAT
mnx1	TCCACCGCCGGAATGATGCTGCCTAAAATGGCAGATTTCAATGGCCAGGCGCAGTC GAACTTACTCGGCAAGTGCAGAAGACCAAGAACTGCATTCACAAGCCAGCAGCTCC TTGAACTTGAGCATCAGTTTAAGCTGAATAAATATCTATCCAGACCAAAACGCTTTGA AGTGGCCACGTCATTGATGCTAACAGAGACGCAGGTGAAAATCTGGTTTCAGAACA GGCGCATGAAATGGAAGCGCAGTAAAAAGGCCAAAGAACAAGCCGCTCAAGATGC CGAGAAGCAAAAGGGAAAAGGAAACCACGACAAAATGGACGGACTGGAAAAGGAC TACCAGAAGGTAGATTCAGGGAAAAGTAACAGAATACGGGACTTTAGGGACAGTGA

	CGACGAAGAAGGAGATAACTATATGCTGAATTCATCTGATTGTTCCTCTGAGGATGA ACGAACCAATGACATAAGTCCACAACCATGAGACCTTATAAAAACGAAAGACTATGA GTGAATTATGTGATTTGTATGTATCATCATCTTGCAGTCAATATGGAAATGCAGAGGT GACATCCACATGAGCTGTTTGTACACATACAAACATC
pkd2l1	TAGTGGTGATACTGCTTGCTGTGGTGGCAATTGTTTTCAGTGCATTTCGGACCATCA AAGTGGATGGATTACTCGGAAACCTTCTGAAACAACCAGACATCTATGCTGATTTTG AATTTTTGGCATTTTGGCAAACCCAGTACAACAACATGAATGCAGTCAATTTGTTTTT TGCTTGGATTAAGATCTTCAAGTACATCAGCTTCAATAAGACGATGACTCAGCTGAC GTCCACACTGGCTCGCTGTGCTCTGGACATTTTTGGATTTGCCATCATGTTCTTCAT TGTGTTTTTTGCGTATGCTCAGCTTGGGTACCTGCTCTTTGGGACAGAGGTGGAAA CTTTCAGCACGTTTAACAAATGCATTTTCACACAGTTTCGAATCATCCTTGGAGATTT CGATTATGATGCCATTGACAGAGCAAATCGAGTGCTTGGACCCATTTACTTCTTCTC CTATGTATTCTTTGTCTTCTTTGTGTTACTGAACATGTTTCTGGCCATCATCAATGAC ACATACTCTGAAGTGAAGTCAGAGCTCGCATCTCAGAAAGACGAGTTCCAGATTGC AGATCTCATCAAGCAGAGTTATGCTAAGACTTTCATGAAGCTGAAGCTTAAAAAGGA AAAGATCTCTGATGTTCAGAAAGCTCTGGATTCAGGCGCAAGTGAGCTGGAGTTTA AAGATTTCAGGAACGCGTTGAAAGAGATGGGTCACAGTGACCGAGAGATCTCAGCC GCTTTCTCCAAATTCGACCAGGATGGAAACCAGACTTTAGACAAGCAGGAGCAGGA GAACATGAAGCAGGAGCTGGAGGAGAAAAGGGATGCTCTCAGTGCAGAGCTGCGT GACCTCGAGAACAGTGTGGAACCA

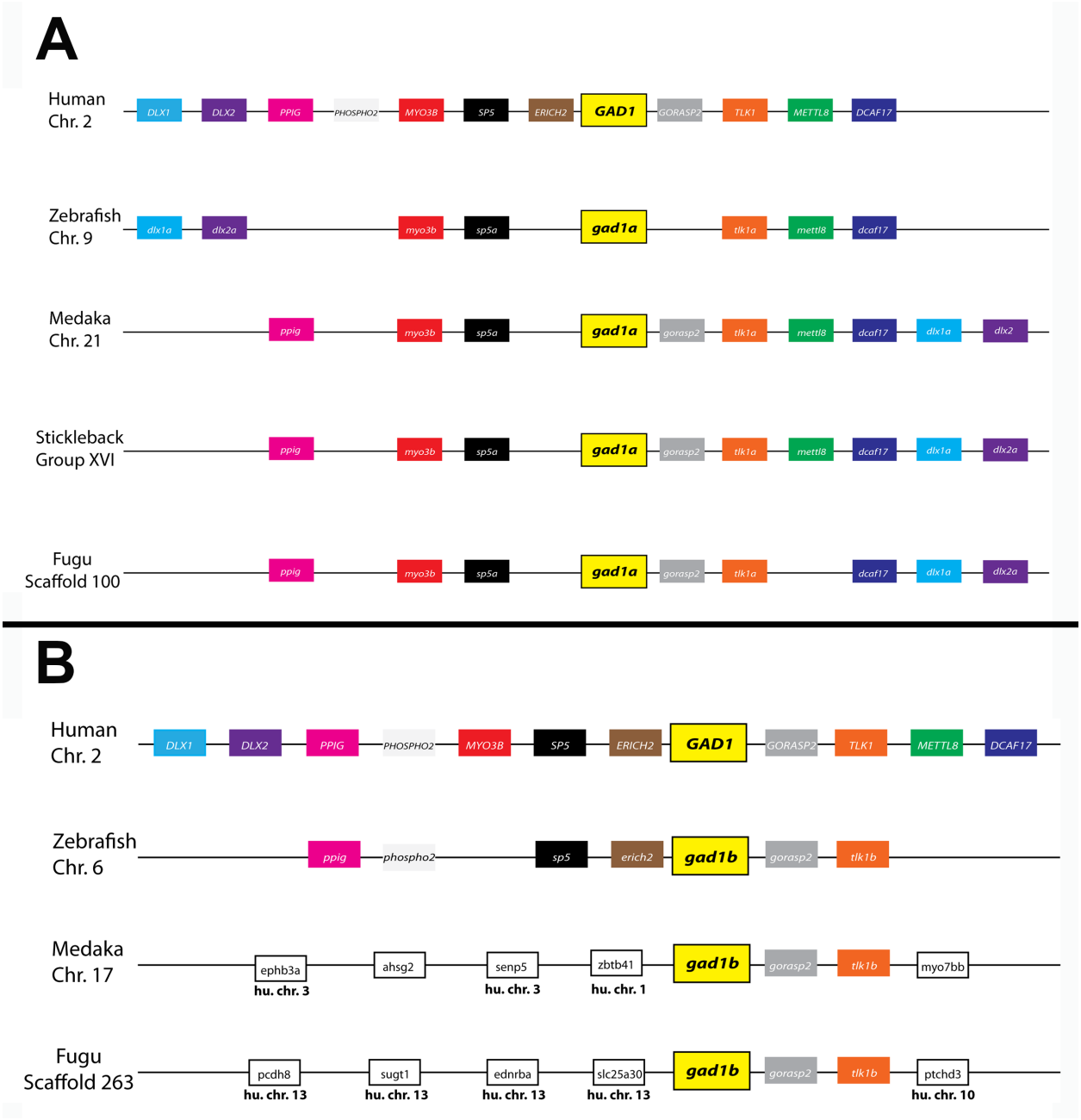
Supplemental Table 2.2: Exon/intron junctions between mammalian and zebrafish *GAD1* and *GAD2* genes. Exons are noted in red.

exon/intron junctions	human *GAD1*	mouse *Gad1*	zebrafish *gad1a*	zebrafish *gad1b*
exon 1	…GCCGAGGTGGGTAA	…ACCGAGGTGGGTAA	…CACAAGGTAAGCCG	…CGTAAGGTAAGCGC
exon 2	CTTTGCAGCCTGTT… CCACAAGTAGGTCC	GTTTGCAGCCTGTT… CTACAAGTAGGTCC	CCCCTCAGCCAGCC… CCTCAAGTAGGTCC	CCGTGTAGTGTCAG… CCACAAGTAGGTCT
exon 3	CGTCCTAGCGTACG… TCTGCGGTAAGTGA	GCTCCTAGCGTATG… TCTGTGGTAAGTAA	CCCTTTAGACTACG… TATGTGGTGAGTTG	CGTTTTAGCCTCGG… TCTGTGGTAAGTAT
exon 4	TGCCATAGGCTTCT… CTAGAGGTAGCCCC	CACTTTAGGCTTCT… CTCAAGGTGGTTTC	TGATATAGGGTTCT… CAAGAGGTGAGCGA	TGATGCAGGTTTCC… CGCGAGGTAAACTC
exon 5	CAATTCAGATCTG… GCACAGGTAAGGAG	CTGCTTAGATCTGC… GCACAGGTAAGAGT	CCTCACAGATCTGC… GGACAGGTGATGAA	CATTCCAGATCTAC… GAACAGGTACAGTG
exon 6	CTATCTAGGTCATC… CAACATGTAAGTCT	CTCTCTAGGTCACC… CAATATGTAAGTCT	ACATTCAGGTCACC… CAACATGTGAGTTA	CTTCTCAGGTCATC… CAACATGTGAGTTT
exon 7	CTTTCCAGGTTTAC… CTCCTGGTAGGGTT	CACTCTAGGTTCAC… CTCCTGGTAGGTTT	GTTTTCAGGTTCAC… CACCAGGTGTGGCC	GTGTTTAGGTTCAC… CACCTGGTAAACTG
exon 8	GCTGACAGGGGGCG …GAACAGGTGAGTCG	GTTGATAGGGGGAG… GAACACGTGAGTTG	TTGTGCAGGAGGAG… GAACACGTAGGTCA	TTTCACAGGTGGTG… GAACATGTAAGTGC
exon 9	TGTTGCAGAGTCAC… TGAAAGGTAGGCAG	TGTTGCAGAGTCAC… TGAAAGGTAGGCAG	TTTCCCAGAGTCAC… TGAAAGGTGAGTTT	TTTCGCAGAGTCAT… TGAGAGGTATGGCG
exon 10	AATTATAGGGGGAA… CAGAAGGTATGTAC	AATTATAGGGGGAA… CAAAAGGTAGGTAT	TCTGTTAGAGGTCG… CAGAAGGTGAATAA	TGTTTCAGGGGGCG… CAGAAGGTATGTA
exon 11	CCTTGTAGGGATAT… GTCGATGTAAGTGC	CCTTGTAGGGCTAT… GTGGATGTAAGTGT	CTGTTCAGGGCTTT… GTAGATGTACGTGT	TGTTTTAGGGATAT… GTGGATGTAAGTTT
exon 12	CCCCACAGGCTGCC… AGAAAGGTAACGGC	CTTTGCAGGCTGCC… AGAAAGGTACAGTA	TTTTTAAGGGAGCG… TGAGAGGTATTCCA	TTATTCAGGGTGCG… TGAGAGGTCTGTAT
exon 13	TCTTTCAGGGCCAA… GAAAAGGTCTGTAC	TCTTCCAGGGCCAA… GAAAAGGTTTGTAC	TTCTACAGAGCAAA… GAGAAGGTGAGAAA	TGTTTCAGGGCAAA… GAGAAGGTGAGACC
exon 14	CATGATAGGGTATA… GCAAAGGTATGAAG	TATGACAGGGTATA… GCAAAGGTATGGAG	TTTACCAGGGTCTT… TCAAAGGTGTGTGA	TGTTCCAGGGCATT… GCCAAGGTTGGTCA
exon 15	TAAAACAGGGCACA… GGCGAGGTAGGTAA	TGAAACAGGGCACC… GGTGAGGTAGGTTG	TCCAACAGGGCACT… GGGGAGGTGAGTGA	TTGTGCAGGGCACG… GGCCAGGTTTGTAT
exon 16	CTCTGCAGCCTGAG… CACAAGGTATGGAC	TTCTGCAGCCTGAG… CACAGGGTAAGGAT	TGTTTCAGCCACAG… CATAAGGTGAGACG	CTCCATAGCCCCAG… CACAGGGTACGTCG
exon 17	ATCACAAGGTGGCT…	ATCTCAAGGTGGCT…	TTTTGCAGGTCGCC…	TCCTGCAGGTGGCG…
exon/intron junctions	human *GAD2*	mouse *Gad2*	zebrafish *gad2*	
exon 1	…GCACAGGTAGGAAA	…GCACAGGTAGGAAA	…ACACGCGTAAGTGA	
exon 2	CCTTGCAGCGCGAG…	GATTGCAGCGAGAG…	CGTTTCAGCTAGAG…TATGTGGTACGTTT	

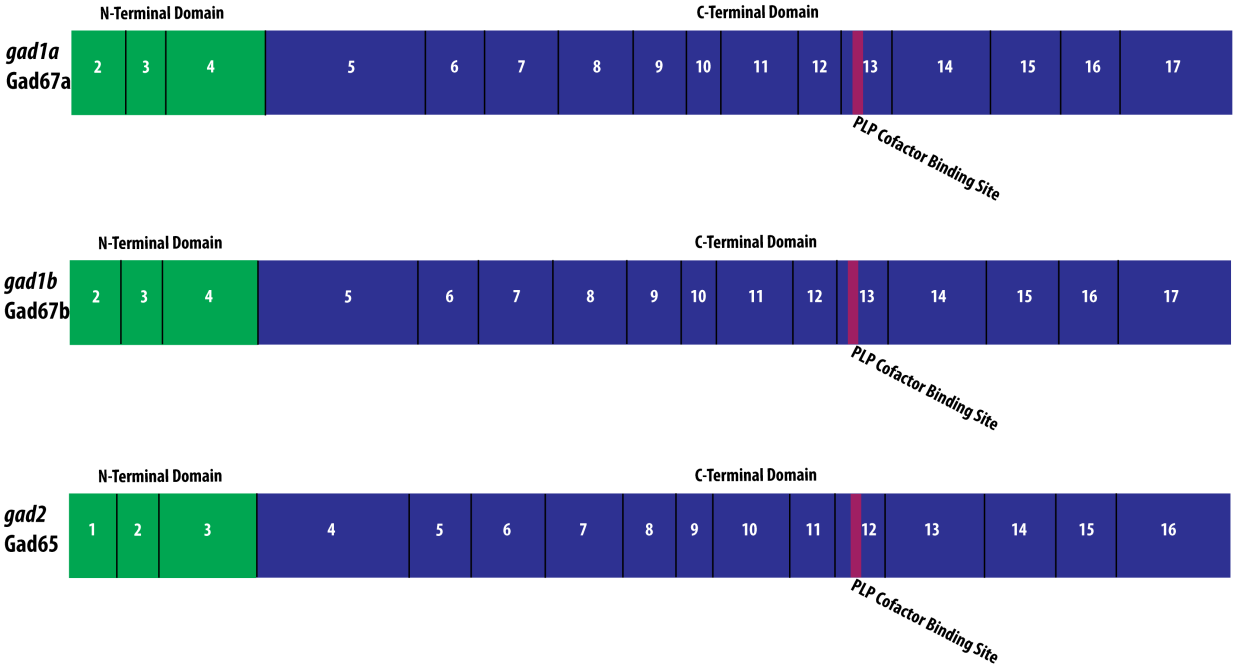
	TGTGCGGTGAGTGC	TGTGCGGTGAGTGG	
exon 3	TTACCCAGCCCTGC… CAACAGGTAAAGAC	TTGTCCAGCTCTGC… CAACAGGTAAATGG	TTTTGTAGCTCTGT…CAACAGGTAAATCA
exon 4	TTCTTTAGACCTGC… AAACAGGTATTGTC	TTCTTTAGACCTGC… AAACAGGTACTGTG	TTTTCCAGATCTGT…AACTGGGTGAGTGT
exon 5	TTATTCAGGGCATC… TAACATGTAAGTAG	TTCTTCAGGGCATC… TAACATGTAAGTAA	ATCTTCAGCGCATC…CAATATGTGAGTCT
exon 6	CATTTCAGGTTCAC… CTCCCGGTACATGA	TATTTTAGGTTCAC… CTCCTGGTATATAT	GCAAACAGGTTCAC…CTCCGGGTAAGCAG
exon 7	CACTTTAGGTGGCG… GAACATGTATGTGT	CCCTCTAGGTGGCG… GAGCATGTATGTGT	TCCTCCTAGGTGGCG…GAACATGTATGTCC
exon 8	TTTTACAGAGTCAT… TGAGAGGTGAGCAC	CTTCACAGAGTCAC… CGAGAGGTGAGCAC	TCTTTCAGAGCCAT…TGAGAGGTCAGTCT
exon 9	CCTTCCAGAGGGAA… CAGAAAGTAAGTTT	TCTTACAGAGGGAA… CAGAAAGTAAGTGA	TCTGCCAGGGGTAA…CAGAAGGTAATTTC
exon 10	TCGCACAGGGGTTT… GTGGATGTAAGTAT	TTTTATAGGGATTT… GTGGATGTAAGTAA	TCTCACAGGGATAC…GTGGATGTAAGTAG
exon 11	TTTTTCAGGCAGCT… GGAGAGGTATGTTG	TTTTTCAGGCTGCT… AGAGAGGTATGTGT	TCATACAGGGAGCA…TGAGAGGTTAGAAA
exon 12	TATTTTAGGGCCAA… GAAGAGGTATGTCT	TCTTTTAGGGCCAA… GAGGAGGTAAGCTT	CATTTTAGGGCTAA…GAGGAGGTGAGACC
exon 13	TCTTGTAGGGATTG… GCAAAGGTGAGTAT	TCCTGTAGGGACTG… GCAAAGGTAAGCCC	TGATCCAGGGACTG…GCTAAGGTATTATA
exon 14	TTACCTAGGGGACT… GGGAAGGTATGTAT	TTTCATAGGGGACT… GGGAAGGTACGTGT	CTCTGAAGGGCACG…GGAAAGGTCAGTCA
exon 15	CTTTGTAGCCTCAG… TCGAAGGTCAGTGC	CCTTTTAGCCTCAA… TCAAAGGTAGGCAC	CCTTGCAGCCGCAG…CACAAGGTGAGAGA
exon 16	TCCCACAGGTGGCT…	TCTCAAAGGTGGCG…	CCTCCCAGGTTGCC…

Supplemental Table 2.3: *GAD* exon 6 and 7 in mammals and zebrafish. Putative bicistronic start/stop sites in red.

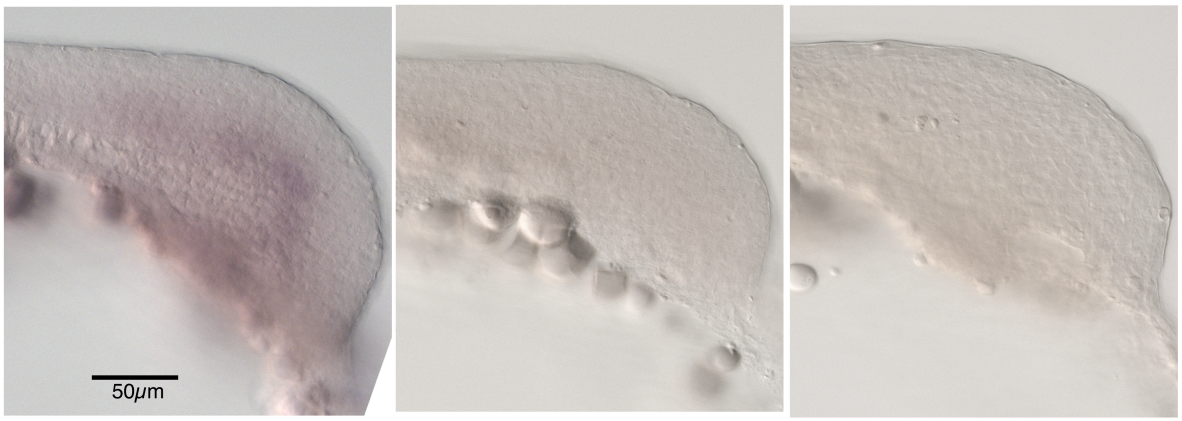
	Exon 7
Zebrafish *gad1a*	GTTCACGTATGAGATTGCGCCAGTGTTTGTCCTGATGGAGCAGCTCACACTG AAGAAGATGCGAG…
Zebrafish *gad1b*	GTTCACATATGAGATTGCGCCTGTGTTTGTCCTGATGGAGCAGCTCACACTCA AGAAGATGCGGG…
Mouse *Gad1*	…TCTTAAGAAGATGAGAGAGATCGTTGGATGGTCAAATAAAGATGGTGATGG GATATTTTCTCCTG
Human *GAD1*	…ACTTAAGAAGATGAGAGAGATAGTTGGATGGTCAAGTAAAGATGGTGATGG GATATTTTCTCCTG
	Exon 6
Zebrafish *gad2*	…GCTGAAGAAGATGAGGGAGATCATTGGCTGGCAGGACGGCCACGGTGATG GAATATTCTCTCCGG
Mouse *Gad2*	…CACTAAAGAAAATGAGAGAAATCATTGGCTGGCCCGGGGGCTCTGGCGAT GGAATCTTTTCTCCTG
Human *GAD2*	…CACTAAAGAAAATGAGAGAAATCATTGGCTGGCCAGGGGGCTCTGGCGAT GGGATATTTTCTCCCG



Supplemental Figure 2.1: Teleost *gad1a* and *gad1b* synteny. A) The syntenic organization of *gad1a* in several teleost species (zebrafish, medaka, stickleback and fugu) as compared to human *GAD1*. The genes around *gad1a* are well conserved among teleosts. B) The syntenic organization of *gad1b* in several teleost species (zebrafish, medaka and fugu) as compared to human *GAD1*. Two genes immediately around *gad1b* are conserved, but it looks like a chromosomal translocation occurred in medaka and fugu. Stickleback *gad1b* has either not been identified or has not been annotated.



Supplemental Figure 2.2: Zebrafish Gad protein structures. The N-terminal domain is shown in green and the C-terminal or catalytic domain is shown in blue. The numbers represent the exons which give rise to these regions/domains of the protein. The co-factor binding site is conserved in all zebrafish Gad proteins.



Supplemental Figure 2.3: Zebrafish *gad* expression in the tail bud at the 14-16 somite stage. High magnification, lateral views (anterior to the left) of ISH for *gad1a, gad1b* and *gad2* on wild-type embryos at 14-16 somites (~16 hpf) show that *gad1a* is detected in the tail bud, while *gad1b* and *gad2* are not.

CHAPTER 3

A POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) SCREENING APPROACH FOR IDENTIFYING CRISPR-CAS9 INDUCED MUTATIONS IN ZEBRAFISH[1]

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[1]VanLeuven, A.J. Park, S., Menke, D.B. and J.D. Lauderdale. Submitted to *BioTechniques,* 03/07/18.

Abstract

The introduction of CRISPR-Cas9 technology for targeted mutagenesis has revolutionized reverse genetics and made genome editing a realistic option in many model organisms. One of the difficulties with this technique is screening for mutations in large numbers of samples. Many screening approaches for identifying CRISPR-Cas9 mutants have been published; however, in practice these methods are time consuming, expensive, or often yield false positives. This report describes a PCR-based screening approach using non-denaturing polyacrylamide gel electrophoresis (PAGE). This approach does not depend on the formation of heteroduplexes and reliably detects changes as small as 1 base pair (bp) in nucleic acid length at the target site. This approach can identify novel mutations and is also useful as a routine genotyping method.

Method Summary

Our approach implements a polyacrylamide gel electrophoresis (PAGE) technique that is known to provide resolution of as small as 1 bp in 1000 bp (Raymond and Weinstraub 1959, Sambrook and Russell 2001) as an inexpensive and robust screening approach for identifying CRISPR-Cas9 induced mutations in zebrafish. In this approach, we PCR amplify a small region (<150 bp) encompassing the CRISPR-Cas9 target site and the PCR product is then run directly on a 10 x 8 centimeter (cm), 15% polyacrylamide gel at 200 Volts for 2-2.5 hours. Using this approach, we routinely detect 1-14 bp indels without relying on the formation of heteroduplexes prior to PAGE.

Report

The CRISPR-Cas9 genome editing technique is widely used in many labs, especially in the zebrafish community (Hwang, Fu et al. 2013, Jao, Wente et al. 2013, Burger, Lindsay et al. 2016). In our experience, the rate-limiting step when using this technology is the screening of zebrafish for CRISPR-Cas9 induced mutations. Several techniques describing the identification of CRISPR-Cas9 induced mutations have been reported, each with their own strengths and

limitations (Ota, Hisano et al. 2013, Kim, Kim et al. 2014, Yu, Zhang et al. 2014, Zhu, Xu et al. 2014, Varshney, Pei et al. 2015, Vouillot, Thelie et al. 2015, D'Agostino, Locascio et al. 2016, Samarut, Lissouba et al. 2016, Hua, Wang et al. 2017). T7 Endonuclease I (T7E1) and Surveyor Mismatch Cleavage Assays, both PCR- and molecular-based assays, are efficient in identifying mismatched DNA at a specific locus; however, these assays also detect single-nucleotide polymorphisms (SNPs). SNPs are prevalent in the zebrafish genome, and in our hands, use of the T7E1 assay leads to false positive results for our genes of interest. High Resolution Melting Analysis (HRMA) and derivative melting curves require a quantitative PCR machine which can be expensive to implement if the equipment and software are not already in a lab. Furthermore, the derivative melting curve assay is best used to detect mutations that have a change of greater than 15 bp in nucleic acid length at the target site (D'Agostino, Locascio et al. 2016); however, the median CRISPR-Cas9 induced indel size ranges from 4-9 bp depending on the length of the single-stranded guide RNA (sgRNA) (Zhang, Li et al. 2016). Sequencing is definitive in identifying indels of any size but can be expensive and slow for a primary screening approach.

In our laboratory, CRISPR-Cas9 is used as a tool to create and establish mutants for specific genes of interest in zebrafish. To facilitate screening, we tested neutral PAGE as a rapid and sensitive method for identifying CRISPR-Cas9 mutants. There are assays that use PAGE to identify CRISPR-Cas9 induced mutations in zebrafish, mice, and human cells; however, these assays require heteroduplex formation prior to PAGE (Ota, Hisano et al. 2013, Zhu, Xu et al. 2014). We reasoned that it would be possible to directly run PCR products via PAGE to identify mutations and genotype zebrafish with known mutations based solely upon a size difference in amplicon length rather than through formation and detection of heteroduplexes or enzymatic cleavage of DNA mismatches. The detection of small changes in nucleotide length, such as those of a typical CRISPR-Cas9 indel, requires a high-percent polyacrylamide gel. We use gels containing a 15% concentration of acrylamide monomer to obtain sufficient

resolving power in amplicons ranging from 25-150 bp (Sambrook and Russell 2001). Importantly, we find that an acrylamide monomer to N,N'-methylenebisacrylamide crosslinker ratio of 19:1 (or 5%) is essential to resolve 1-2 bp indels (supplemental protocol).

In a typical screening experiment, CRISPR-Cas9 injected embryos, referred to as F0 injected, are grown to sexual maturity and then outcrossed to a wild-type zebrafish to obtain putative F1 heterozygous progeny. Because a single founder could harbor many germline mutations, we screen zebrafish individually at 2 days post fertilization (dpf) via PCR and PAGE analyses. If an animal has a CRISPR-Cas9 induced mutation at the target site, there will be two bands on the gel: one band of known size that represents the wild-type allele and an additional band that represents a CRISPR-Cas9 induced indel (Figure 1).

To perform these experiments, we select 12 zebrafish per F0 outcross, and then perform a standard DNA extraction and ethanol precipitation on individual embryos. We use ~100 ng of genomic DNA for a standard PCR reaction with gene specific primers. We directly load 5 µL of the PCR product into a 10 x 8 cm, 15% polyacrylamide gel and run the gel at 200 V for 2-2.5 hours in freshly prepared 1X TBE buffer. The gel is stained with ethidium bromide and analyzed under UV light. A detailed supplemental protocol is also available for this assay.

We typically detect mutations in 8-25% of our F0 injected zebrafish with a germline transmission rate between 25-67%. Using this approach, we have identified 7 novel alleles at 3 different loci. Based upon the resolution power of the gels described above, we have tested amplicons ranging between 86-126 bp under these conditions and were consistently able to detect indels as small as 1 bp. Representative gels for an F0 outcross screening that lead to the identification of 4 novel alleles as well as how this approach is used to genotype the F2 generation are shown in Figure 2.

The major benefits of this protocol as compared to similar approaches are the improvements in sensitivity of the assay and the time and cost effectiveness. For instance, the genomic DNA extraction, PCR, and analysis of the polyacrylamide gel can be performed within

48 hours, while the use of sequencing as a primary screening approach takes up to three days and is more expensive. Similarly, there is no enzymatic cleavage step like in T7E1 nor is there a need to heat the samples to form heteroduplexes between the PCR and PAGE (Ota, Hisano et al. 2013, Zhu, Xu et al. 2014, Vouillot, Thelie et al. 2015). Therefore, our approach saves at least one step that is required in other protocols and does not rely on analysis of mismatches that may also report SNPs.

Our approach provides the ability to elucidate multiple pieces of mutagenesis information in a single experiment. Using PCR and PAGE as described here will show if a F0 injected animal is carrying a germline mutation at a frequency of at least 8% (if there is an indel in at least 1 of 12 putative F1 animals that are screened). This approach also shows the relative type of mutations present (insertions versus deletions) and how many types of mutations come from each F0 injected zebrafish (Figure 1 and Figure 2, Panel A).

This approach is also well-suited as a genotyping method once known alleles are identified since we can discriminate between wild-type, heterozygous, and homozygous mutations under the same conditions as those for which we screen for novel mutations (Figure 2, Panel B).

Finally, this protocol has been successfully used to detect CRISPR-Cas9 induced indels in other vertebrates in addition to zebrafish, including mice (Sumadra and Condie, personal communications), human iPSCs (Lauderdale, unpublished), and lizards (supplemental protocol).

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Figures

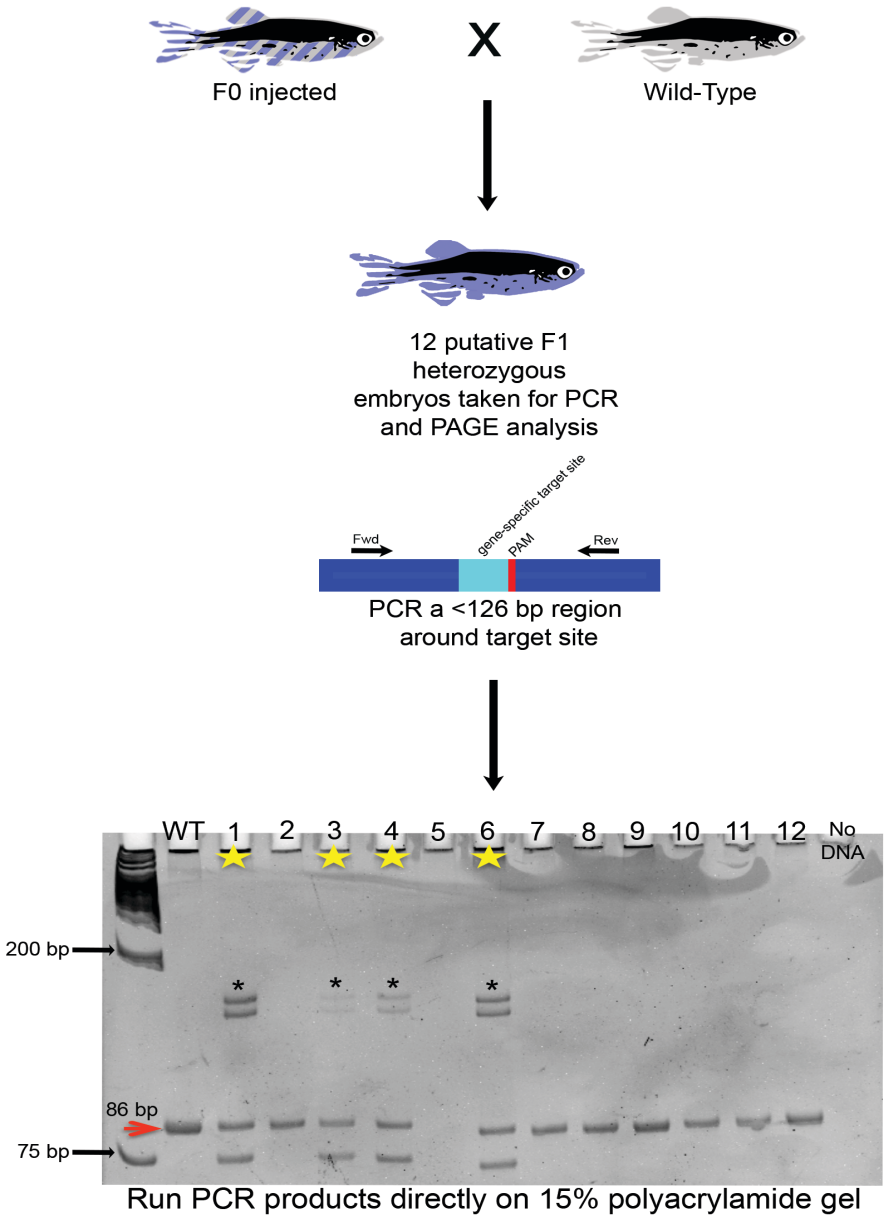

Figure 3.1: Workflow overview of PCR and PAGE for screening CRISPR-Cas9 induced mutations in zebrafish. F0 injected zebrafish are grown to adulthood and outcrossed to a wild-type zebrafish. Twelve embryos from this outcross are sacrificed for genomic DNA extraction and PCR analysis of the region encompassing the target site. PCR products are directly run on a 15% polyacrylamide gel. This gel represents an outcross in which the F0 injected founder is carrying a single 10 bp deletion at *gad2* exon1 that is transmittable at a frequency of ~33% to the F1 generation. The second pair of bands that are noted with an asterisk are heteroduplexes. These heteroduplexes are seen with all heterozygous samples for all alleles and are not a reflection of non-specific primer binding.

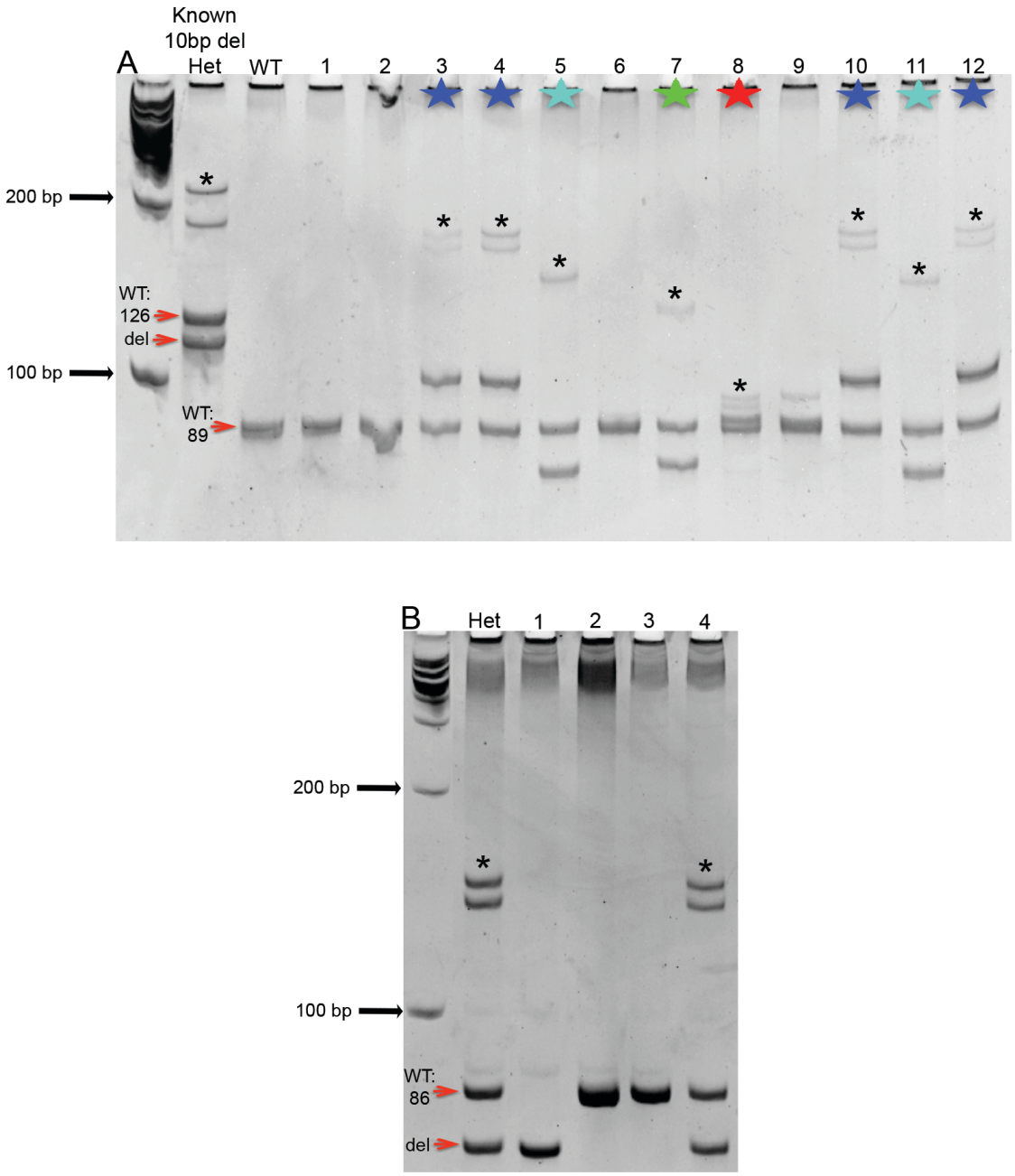

Figure 3.2: Representative ways in which neutral PAGE can be used to screen at the F1 and F2 generation. (A) PAGE results showing 12 individual embryos from an outcross in which the F0 injected founder transmits 4 different germline mutations in *gad1a* exon5 at a frequency of ~67%. The first lane is a known heterozygous zebrafish for a different allele which serves as a positive control. The starred samples were sequenced and determined to have the following types of mutations: embryo numbers 3, 4, 10, 12 have a 14 bp insertion; embryos 5 and 11 have a 10bp deletion; embryo number 7 has a 9 bp deletion; embryo number 8 has a 2 bp insertion. (B) PAGE results from an incross of a line of fish (*gav2501*) that are heterozygous for a CRISPR-Cas9 induced 10 bp deletion at *gad2* exon1. Fish number 1 is a homozygous mutant, fish numbers 2 and 3 are wild-type and fish number 4 is a heterozygous mutant. In both gels, the second pair of bands that are noted with an asterisk are heteroduplexes.

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Supplemental Methods

Protocol For:

A polyacrylamide gel electrophoresis (PAGE) screening approach for identifying CRISPR-Cas9 induced mutations in zebrafish

LEGEND

ATTENTION

* ***HINT***

REST

REAGENTS

10X TBE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) Cat. # 161-0733

Dream Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) Cat. # EP0702

10X Dream Taq Green Buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplied with Dream Taq DNA Polymerase

100 mM dNTPs (Thermo Fisher Scientific, Waltham, MA, USA) Cat. # 10297018

100 µM Oligonucleotide Primers (Integrated DNA Technologies, Inc., Redwood City, CA, USA)

100% Ethanol (Koptec, King of Prussia, PA, USA) CAS # 64-17-5

30% Acrylamide/Bis Solution 19:1, 5% crosslinker (Bio-Rad Laboratories, Inc., Hercules, CA, USA) Cat. # 161-0154

TEMED N,N,N',N'-Tetramethylethylenediamine (Research Organics, Inc., Cleveland, OH, USA) CAS # 110-18-9

Ammonium persulfate (Sigma-Aldrich, St. Louis, MO, USA) CAS # 7727-54-0

Quick-Load Purple 2-Log DNA Ladder (New England BioLabs, Inc., Ipswich, MA, USA) Cat. # N0550S

10 mg/mL Ethidium bromide (Bio-Rad Laboratories, Inc., Hercules, CA, USA) Cat. # 161-0433

PROCEDURE

Genomic DNA Extraction

1. Place a dechorinated zebrafish embryo (or a piece of zebrafish tail fin) into a 1.5 mL microfuge tube and remove the excess liquid.
2. Add 48 µL of DNA Extraction buffer & 2 µL of 10 mg/mL ProK in glycerol to each tube.
3. Incubate at 55°C in a water bath.

REST: Leave the solution overnight in 55°C.

4. After the overnight incubation, centrifuge at 13,000 rpm for 10 min at room temperature.
5. Remove the pellet of debris, add 100 µL of 100% Ethanol to precipitate genomic DNA.
6. Shake to mix and place tube(s) in -20°C for at least 30 minutes.

REST: Samples can be left/stored in -20°C as long as needed.

7. Centrifuge at 13,000 rpm for 10 minutes at room temperature.
8. Remove the supernatant and air-dry the pellet for ~1 minute.
9. Resuspend the genomic DNA in 21 µL of 1X TE Buffer.

*** HINT: At this point, you can check the concentration of the genomic DNA spectrophotometrically and/or run a small aliquot on a 1.5% agarose gel, but for more experienced users, this step is not necessary.**

REST: Either store the genomic DNA at 4°C or use immediately in PCR.

PCR

1. Assemble a PCR reaction to detect a CRISPR-induced mutation at a gene-specific target site as follows for a single reaction:

    2.0 µL genomic DNA (this is ~100 ng of DNA)

    0.2 µL 20 µM Forward primer

0.2 µL 20 µM Reverse primer

2.0 µL 10X Dream Taq Green Buffer

0.5 µL 10 mM dNTPs

0.1 µL Dream Taq (stock = 5 U/µL)

15.0 µL Milli-Q Water

20 uL

* **HINT: Make a master mix for however many samples you have with all these components except the genomic DNA, shake to mix, and aliquot 18 µL of the master mix to each tube.**

2. Run the PCR in a Thermal Cycler.

⇨ **ATTENTION: Make sure to use the appropriate denaturing, annealing and extension temperatures which are dependent upon the type of Taq and the gene-specific primers. After the initial denaturation, use 30 cycles of denaturing, annealing and extension followed by a 2-minute 72°C extension.**

REST: The PCR product can be kept at 4°C or used immediately in PAGE.

Polyacrylamide Gel Electrophoresis (PAGE)

⇨ **ATTENTION: Wear gloves at all times when handling the polyacrylamide gels as some of the reagents are neurotoxins.**

1. Use an upright electrophoresis setup (see equipment below for the model we use).

* **HINT: This is an apparatus commonly used for SDS-PAGE and western blotting; glass plates and notched Alumina plates are both 10 x 8 cm; spacers are 10 cm x 1mm width T-shaped spacers.**

2. Rinse the glass plates, notched Alumina plates, spacers, and gel caster with water to remove any residual gel debris.
3. Assemble the gel caster following the manufacturer protocol before preparing the gels.
4. Make two 15% polyacrylamide gels (see recipe below).

* **HINT: The acrylamide solution should be 19:1 or a 5% ratio of acrylamide monomer to crosslinker.**

* **HINT: We do <u>not</u> place these solutions under a vacuum to de-gas/de-aerate before proceeding. One can de-gas the gel to remove oxygen if desired; this will speed up the polymerization process and make polymerization more homogeneous, but it is not essential for this application.**

5. Once all ingredients are added, invert a couple times and quickly pour the mixture between the glass plates in the gel caster and fill it all the way to the top of the glass.
6. Place a 15-well comb (1 mm width) between the glass plates and into the liquid until the lips rest on the plates.
7. Allow the gels to polymerize/harden for ~45 minutes.

* **HINT: To ensure the gel is hardened, keep any remaining solution in the 15 mL conical tube to monitor the polymerization process.**

REST: Once made, the gel can be used immediately or stored in 1X TBE in 4°C.

8. Once the gel(s) have hardened, assemble the electrophoresis unit following the manufacturer protocol.
9. Pour fresh 1X TBE into the electrophoresis unit in both buffer chambers and the bottom reservoir.

* **HINT: Do not re-use 1X TBE in the buffer chambers.**

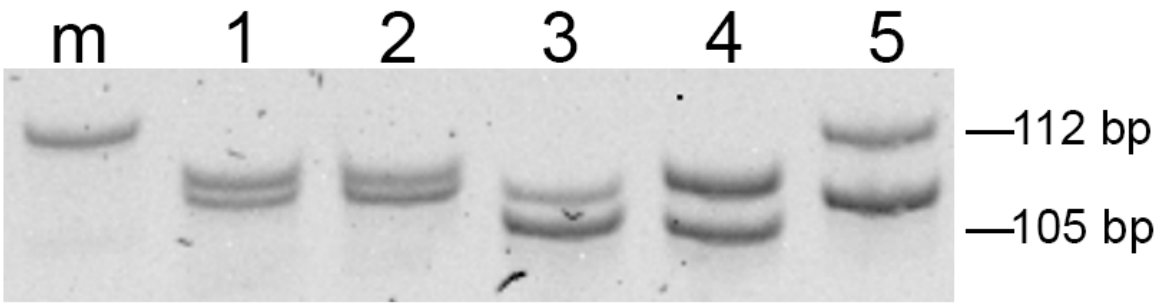
10. When the PCR is complete, directly load 5 µL of each sample into individual wells on the gel; load 3 µL of DNA ladder as well.
11. Set the voltage to 200 V and run the gel for 2-2.5 hours.

ATTENTION: Do not run the gel longer than 3 hours at 200 V or the buffer will overheat which results in poor gel resolution; image the gel immediately after the run.

Gel Analysis

1. Carefully remove the polyacrylamide gels from between the plates.
2. Stain the gels in ethidium bromide staining solution (see recipe below) for ~1 min.
3. De-stain the gels in cold tap water for ~1 min before imaging.
4. Place one gel at a time on the tray in the gel imager (see equipment below).
5. Adjust the camera & settings as prompted by the software to visualize the entire gel.
6. When positioned, press Capture to get a still image of the gel.
7. Under the Image Tools icon, invert the data/display.
8. Take a picture of the gel and save it both as a .scn and as a .tiff or .jpg file.
9. Repeat for the second gel.

A representative gel is shown below in the figure below:



A representative 15% polyacrylamide gel demonstrating resolution of 1-5 bp differences in DNA length. The marker (m) is at 112 bp. All samples are lizard DNA. Lane 1 contains amplicons of 107 and 108 bp. Lane 2 contains amplicons of 107 and 109 bp. Lane 3 contains amplicons of 105 and 108 bp. Lane 4 contains amplicons of 105 and 109 bp. Lane 5 contains amplicons of 107 and 112 bp.

RECIPES

DNA Extraction Buffer (50 mL)

1 M Tris pH 8	500 µL	10 mM
0.5 M EDTA	1 mL	10 mM
5 M NaCl	2 mL	200 mM
20% SDS	1.25 mL	0.5%
Milli-Q Water	45.25 mL	

* **HINT: If the SDS doesn't go into solution, heat it at 37°C until it dissolves.**

10 mg/mL ProK (200 µL)

20 mg/mL ProK	100 µL	10 mg/mL
100% Glycerol	100 µL	50%

1X TE Buffer (50 mL)

1 M Tris pH 8.2	500 µL	10 mM
0.5 M EDTA	100 µL	1 mM
Milli-Q Water	49.4 mL	

10% w/v Ammonium persulfate (APS) (10 mL)

APS	1 g	
Milli-Q Water	up to 10 mL	

5X TBE (10 mL)

10X TBE	5 mL	5X
Milli-Q Water	5 mL	

1X TBE (1 L)

10X TBE	100 mL	1X
Milli-Q Water	900 mL	

20 µM Oligonucleotide Primers (50 µL)

100 µM primer	10 µL	20 µM
10 mM Tris pH8	40 µL	

10 mM dNTPs (50 µL)

100 mM dATPs	5 µL	10 mM
100 mM dCTPs	5 µL	10 mM
100 mM dGTPs	5 µL	10 mM
100 mM dTTPs	5 µL	10 mM
10 mM Tris pH8	30 µL	

15% Polyacrylamide Gel (12 mL, for 2 gels)

30% Acrylamide/Bis Solution 19:1	6 mL	15%
5X TBE	2.4 mL	
Milli-Q Water	3.6 mL	
10% w/v APS	60 µL	

TEMED 12 µL

Add the water, TBE and Polyacrylamide first (no particular order is necessary) followed by the APS and finally the TEMED in a 15 mL conical tube. We do not de-gas the gel.

Ethidium Bromide Staining Solution

10 mg/mL Ethidium bromide 10 µL 0.5 µg/mL

Distilled Water up to 200 mL

TROUBLESHOOTING

Smeared or poorly resolved bands on gel

1. Ensure that the proper concentration of polyacrylamide monomer and ratio of crosslinker to acrylamide monomer are used (see the steps outlined above).
2. Poor electrophoresis conditions; use fresh 1X TBE for each gel run or cool the buffer before use. Avoid running the gel longer than 3 hours.

Samples do not travel down the gel

1. Improper apparatus setup; make sure the seals are tight so that buffer doesn't leak out during the run.

EQUIPMENT

Vapo.Protect Thermal Cycler (Eppendorf, Hamburg, Germany, 6325ZJ104747)

SE 250 Mighty Small II Upright Electrophoresis Setup (Hoefer, Holliston, MA, USA, SE250-10A-1.0)

Molecular Imager® Gel Doc™ XR+ with Image Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA, USA, 721BR05583)

CHAPTER 4

GENERATION OF *gad1* MUTANT ZEBRAFISH FOR UNDERSTANDING NERVOUS SYSTEM ACTIVITY[1]

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[1]VanLeuven, A.J., Ball, R.E., Carpenter, J.M., Filipov, N.M. and J.D. Lauderdale. To be submitted to *Journal of Neuroscience.*

Abstract

Normal nervous system development and function requires a fine balance of excitatory and inhibitory activity. It is known that the amino acid neurotransmitter GABA plays a critical role both in normal nervous system activity and during development. In all vertebrates, GABA is made *in vivo* largely through the enzymatic activity of glutamic acid decarboxylase (GAD). There are two GAD isoforms, GAD67 and GAD65, each encoded by an independent gene, *GAD1* and *GAD2*, respectively, and both function in GABA synthesis. There is evidence that disruptions in the *GAD* genes, namely *GAD1*, are implicated in several neurological diseases, but little is known about the mechanism(s) through which disruptions in the *GAD* genes cause developmental and/or neurological disorders. To begin to address this, we generated *gad1a* and *gad1b* mutant zebrafish using CRISPR-Cas9 targeted mutagenesis and analyzed these fish for any changes in GABA production and neural activity. So far, *gad1b* +/- and *gad1b* -/- adults have significantly reduced levels of GABA as measured by high-performance liquid chromatography (HPLC) when compared to age and gender matched wild-type zebrafish. Both 7 days post fertilization (dpf) *gad1a* and *gad1b* mutant zebrafish show increased and abnormal neural activity as measured by hypersensitivity to the chemiconvulsant drug PTZ and by extracellular electrophysiology when compared to 7 dpf wild-type zebrafish. Interestingly, these the two mutants show distinct profiles of the pattern and frequency of abnormal neurological activity. Overall, these two zebrafish *gad1* mutants have less GABA, are more sensitive to perturbations in GABA signaling, and exhibit higher neural activity.

Significance Statement

The goal of this study is to better understand the role of *gad1* both in GABA production and nervous system activity using the zebrafish as a model system. Zebrafish provide a unique and advantageous model in which to study the *gad1* gene due to a genome duplication event which has resulted in paralogous *gad1* genes: *gad1a* and *gad1b*. Since *gad1* is duplicated in zebrafish, we are now able to study *gad1* gene function, something that cannot be performed in

the mouse model due to neonatal lethality of Gad1 mutant mice. The *gad1a* and *gad1b* mutant fish generated and discussed in this study can be used as models to better understand aspects of neural development and seizure-like activity.

Introduction

GABA is the primary inhibitory neurotransmitter in all vertebrate animals. GABA is made by the conversion of glutamate (or glutamic acid) into GABA catalyzed by the glutamic acid decarboxylase (GAD) enzyme (Roberts and Frankel 1950, Roberts, Frankel et al. 1950, Roberts and Frankel 1951, Roberts and Frankel 1951, Roberts 1974, Roberts 1988, Martin and Rimvall 1993). In vertebrates, there are two *GAD* genes, known as *GAD1* and *GAD2,* which each encode a distinct isoform of the GAD enzyme, Gad67 and Gad65, respectively (Legay, Pelhate et al. 1986, Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Martin, Heinrich et al. 1998). The *GAD* genes are largely co-expressed in vertebrates though they are found in different subcellular localizations and sometimes even at different levels in the same tissue (Kaufman, Houser et al. 1991, Esclapez, Tillakaratne et al. 1993, Feldblum, Erlander et al. 1993, Martin, Heinrich et al. 1998, Pinal and Tobin 1998, Sheikh, Martin et al. 1999, Martin, Liu et al. 2000). Both of these observations suggest that while both *GAD* genes make GAD enzymes that function in GABA synthesis, there may be slight differences in how they contribute to cellular and neuronal function. Additionally, the *GAD* genes have been detected both during development and in adults, suggesting they are important for various developmental processes and neurotransmission (Martin, Heinrich et al. 1998, Katarova, Sekerkova et al. 2000, Varju, Katarova et al. 2001, Cocco, Ronnberg et al. 2017).

At the protein level, these GAD enzymes are highly conserved and have been described in many species, including zebrafish, rodents and humans (Erlander, Tillakaratne et al. 1991, Bu, Erlander et al. 1992, Martin, Heinrich et al. 1998). In their most characterized role, the GAD enzymes sit at a critical point in the regulation of nervous system activity, as they convert an excitatory neurotransmitter (glutamate) into an inhibitory neurotransmitter (GABA). Because the

GAD enzymes function at this significant junction, the implications of mutations or polymorphisms in these genes can be quite detrimental.

There are many reports in which humans have mutations in the *GAD1,* and also *GAD2,* genes that are linked to various neurological disorders. Many of the *GAD1* mutations are linked to schizophrenia and bipolar disorder (Guidotti, Auta et al. 2000, Addington, Gornick et al. 2005, Hossein Fatemi, Stary et al. 2005, Lundorf, Buttenschøn et al. 2005, Akbarian and Huang 2006, Benes 2010, Davis, Tao et al. 2016). There is also evidence for human *GAD1* and *GAD2* mutations that are correlated to spastic cerebral palsy, Stiff-Person Syndrome and other neurodevelopmental and movement disorders (McHale, Mitchell et al. 1999, Lynex, Carr et al. 2004). However, the mechanisms by which mutations in the *GAD* genes contribute to or cause neurological disorders are poorly understood.

To address these mechanistic questions, researchers can use model organisms to better study the role of the *GAD* genes in disease. However, *Gad1* -/- mutant mice die at birth due to a severe cleft palate (Asada, Kawamura et al. 1997, Condie, Bain et al. 1997, Oh, Westmoreland et al. 2010). *Gad2* -/- mutant mice do not have this craniofacial phenotype, thus they survive as neonates, but are either hypersensitive to drug-induced seizures or exhibit spontaneous seizures and are prone to sudden death (Asada, Kawamura et al. 1996, Kash, Johnson et al. 1997). *Gad1-/-;Gad2-/-* double mutant mice also showed severe cleft palates and did not survive past birth (Kakizaki, Oriuchi et al. 2015).

Since the *Gad1* -/- mice die at birth, further studies of the *Gad1* gene were not pursued and little remains known about how the *GAD* genes are involved in development, their regulation, nor any mechanistic details about their role in neurological disorders. In light of these limitations, our lab is using the zebrafish as a model organism for such studies. The GAD enzymes are conserved in zebrafish and it is well-established that zebrafish are good organisms for studying the mechanisms of neural development and neurological disorders (Schier, Neuhauss et al. 1996, Stewart, Desmond et al. 2012). Additionally, we hypothesize that there

are paralogous *gad1a* and *gad1b* genes in zebrafish which may permit studies of *GAD1* gene function in this vertebrate model system that are not possible in mammalian models (VanLeuven, Ball et al. In Preparation).

The introduction of CRISPR-Cas9 for targeted mutagenesis has also been a huge benefit to the pursuit of research questions regarding gene function. This technique has been widely used in the zebrafish community and is an efficient method for generating targeted mutations in a gene of interest (Chang, Sun et al. 2013, Hwang, Fu et al. 2013, Hwang, Fu et al. 2013, Varshney, Lu et al. 2013, Irion, Krauss et al. 2014, Sung, Kim et al. 2014, Varshney, Pei et al. 2015, Burger, Lindsay et al. 2016). Using CRISPR-Cas9, we can create mutations in the zebrafish *gad1a* and *gad1b* genes to better understand the role of the *gad1* genes in zebrafish which can hopefully be extended to higher vertebrates. One of the goals of this study is to show that zebrafish *gad1a* and *gad1b* are both functional genes, though we predict they will be unequal contributors to GABA production, and both have distinct roles in nervous system function. We also want to assess whether there are any evident non-neural developmental abnormalities like what was found in the mouse model.

Materials & Methods

Zebrafish care and maintenance

Adult zebrafish (*Danio rerio*) of the WIK strain were obtained from the Zebrafish International Research Center (ZIRC) and maintained in an Aquatic Habitats (Apopka, FL) multi-rack system according to standard procedures. Habitat water consisted of reverse osmosis filtered/sterilized water to which sodium bicarbonate and other salts (Instant Ocean, Aquarium Systems, Inc., Mentor, OH, USA) were added to maintain pH from 7.0 -7.4 and conductivity between 400 and 430 µS. All experimental procedures were conducted in accordance with 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 (A2017 10-018-Y1-A2).

sgRNA design

The sequences for CRISPR target sites in the *gad1a* and *gad1b* genes were designed using the ZiFiT Targeter program website (http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx) (Hwang, Fu et al. 2013, Hwang, Fu et al. 2013). The resultant target sequences for each gene used in this study can be found in Figure 4.1. These target sequences were evaluated for the probability of causing off-target effects using the ZiFiT program. These sequences were confirmed via nucleotide BLAST searches against the zebrafish genome to contain at least three or more mismatches at any other loci thereby limiting the possibility of off-target effects.

sgRNA, Cas9 and RNP construction

The sgRNA for targeting *gad1b* (*gav2303* allele) was made from a PCR-amplified template as described in (Nakayama, Fish et al. 2013) and transcribed using the MEGAshortscriptTM T7 Kit (AM1354, Thermo Fischer Scientific, Inc., Waltham, MA) following the manufacturer protocol. The Cas9 mRNA was transcribed from an optimized expression vector as described in (Jao, Wente et al. 2013) using the mMessage mMACHINE Sp6 Kit (AM1340, Thermo Fischer Scientific, Inc.) following the manufacturer protocol. Both the sgRNA and the Cas9 mRNA were purified by LiCl precipitation and re-dissolved in DEPC-treated water.

The *gad1a (gav2404)* allele was made using a ribonucleoprotein (RNP) complex (Burger, Lindsay et al. 2016). To synthesize this sgRNA, we ordered gene-specific crRNA targeting *gad1a* exon5 as well as trRNA which can be used with any crRNA. Both of these RNA oligonucleotides were purchased from Integrated DNA Technologies (IDT, Skokie, IL) following the online ordering protocol and upon arrival were resuspended to a concentration of 100 µM in the provided Nuclease Free Duplex Buffer. Purified Cas9 nuclease was also purchased from IDT (1074181). Immediately before performing embryonic microinjections, we made a working dilution of Cas9 nuclease in Cas9 working buffer (20 mM HEPES, 150 mM KCl, pH 7.5). The sgRNA duplex was made by combining equimolar amounts (3.4 µL each) of crRNA and trRNA plus 3.2 µL of Nuclease Free Duplex Buffer, incubating at 95°C for 5 minutes, and cooling to

room temperature on the benchtop. The RNP complex is assembled by combining 1 µL each of the sgRNA duplex and the Cas9 protein dilution, 0.5 µL DEPC-treated water and 2.5 µL 0.4M KCl with phenol red and incubating at 37°C for 10 minutes.

Microinjections

sgRNA for *gad1b* exon4 plus Cas9 mRNA were co-injected into 1-2 cell stage zebrafish embryos in an approximately 1 nL solution that contained 100 ng/µL of sgRNA and 600 ng/µL of Cas9 mRNA. The RNP complex for *gad1a* was injected into 1-2 cell stage zebrafish embryos in an approximately 1 nL solution such that the concentration of the sgRNA duplex is 234 pg (156 pg trRNA, 78 pg crRNA) and the concentration of the Cas9 enzyme is 736 pg. In all cases, only animals that exhibited normal development post-injection were grown to adulthood as potential F0 founders.

HPLC-ECD sample preparation

Adult and 7 dpf larval zebrafish were anesthetized in 0.4% Tricaine-S pH 7.4 (Pentair TRS4) (Westerfield 1993) and then placed on a pre-chilled metal block. For larval samples, single heads were removed, rinsed with 40 µL of Hank's Final solution (Westerfield 1993) and then placed in a pre-weighed 1.5 mL microcentrifuge tube to record the wet mass in milligrams (mg). For adult samples, the heads were removed and brains were dissected out with forceps and rinsed with ~40 µL of Hank's Final solution. Adult brains were briefly blotted on a piece of filter paper and then placed in a pre-weighed 1.5 mL microcentrifuge tube to record the wet mass in mg. For these preparations, we either added 200 µL of 0.2 N perchloric acid to detect catecholamine neurotransmitters or 200 µL of 18.2 Ω Milli-Q Water to detect amino acid neurotransmitters. Once the solution is added to the tube and samples are fully immersed into the solution, the tubes were immediately frozen on dry ice and stored at -80°C until they were run in HPLC with electrochemical detection (HPLC-ECD). Samples were normalized and run as described previously (Ross and Filipov 2006, Coban and Filipov 2007).

PTZ dose response assay

For assays with wild-type, *gad1b* +/- and *gad1b* -/-, we either performed a *gad1b* heterozygous incross and post-hoc genotyped each fish from each per treatment group or we crossed several zebrafish of known genotype (wild-type, *gad1b* -/- or *gad1b* -/- x wild-type). Each experimental replicate was performed on separate occasions. For assays with *gav2404* +/- and *gav2404* -/- we crossed several zebrafish of known genotype (*gad1a* -/- or *gad1a* x wild-type) and performed the experiment on three rounds of larvae from these crosses on one day. Embryos were grown in standard egg water (Westerfield 1993).

To perform the assay, we divided 7 dpf larvae of the desired genotype into six petri dishes each with 15-20 fish and labeled each dish corresponding to the dose that would be assayed. We remove as much egg water as possible and pour 15 mL of pre-measured pentylenetetrazole (PTZ; P6500, Sigma-Aldrich), a known $GABA_A$ receptor antagonist, diluted in standard egg water at the following concentrations into the appropriately labeled dish: 0 mM (egg water only control), 1 mM, 2.5 mM, 5 mM, 10 mM and 15 mM (positive control). Once the solution is bath applied to the dishes, we began a timer for 10 minutes and monitored all dishes for abnormal behavior as defined by stage II and stage III seizure-like behavior (Baraban, Taylor et al. 2005). To control for double counting of responding fish, when we saw a fish that exhibited abnormal behavior, we removed that fish and placed it in a separate dish. At the end of 10 minutes, we counted how many fish responded with stage II or stage III behavior and how many fish did not respond at each treatment group.

Extracellular Electrophysiology

Zebrafish of the desired genotype were grown to 7 dpf and immobilized with 250 μM of α-bungarotoxin (B1601, Life Technologies) in 1X E3 media with 1 mM HEPES (Westerfield 1993). Once paralyzed, we moved single larvae to the lid of a 35 mm non-TC-treated petri dish and oriented the fish laterally. Once properly positioned, we added warm, but not hot, 0.4% agarose in 1X E3 media onto the fish and let it cool for ~2 minutes. We added ~3.5 mL of 1X E3

media to the lid and then inserted a sharp glass pipet microelectrode (15-20 MΩ impedance), loaded with 2-3 µL of normal Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM HEPES, pH 7.2) into the optic tectum (TeO). The optic tectum was chosen to facilitate comparison with previously published data obtained from larval zebrafish (Baraban, Taylor et al. 2005). A chloride-coated silver wire (0.010" A-M Systems, Inc., Sequim, WA) reference electrode was placed touching the surrounding solution. Field recordings were collected using Molecular Devices' Axoclamp software and data were digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with CLAMPEX 10.4 software (Axon Instruments, Sunnyvale, CA). We performed field recordings from each fish for 20 minutes.

Experimental Design and Statistical Analysis

For the HPLC-ECD experiments with 7 dpf larval samples, we assayed 5 replicates, in this case 5 single larval heads. For the adult samples, we used 5 male and 5 female brains to account for potential gender differences across the genotypes tested. The selection of 5 biological replicates came from discussions with HPLC experts to capture sufficient amounts of data for proper statistics. Results of these experiments were analyzed and plotted using GraphPad Prism (La Jolla, CA). We plotted normalized values from all replicates for each genotype with the mean and standard deviation. We performed a one-way ANOVA to test statistical significance across the groups.

For the PTZ dose response assay, we used 15-20 7 dpf zebrafish per treatment group for each genotype tested. When we assayed a *gad1b* heterozygous incross, we randomly sorted zebrafish into pools of 20 with the assumption that ~5 fish per genotype would be in each treatment group. When we assayed crosses of known genotypes, we used 20 zebrafish per treatment group when assaying the *gad1b* allele and 15 zebrafish per treatment group when assaying the *gad1a* allele. The *gad1b* heterozygous incross resulted in unequal numbers of each genotype for that experiment, thus the resulting N value is not the same across each genotype. However, each of the 10-minute assays was performed 3 times for each genotype at

each dose. Taking all experiments into account, we assayed at least 42 larvae for each genotype at each PTZ treatment which provides more than sufficient biological and technical replicates for statistics. Gender is not determined in 7 dpf larvae, so we did not consider sex differences in this experiment. Results of these experiments were plotted using GraphPad Prism (La Jolla, CA). Due to unequal numbers across the genotypes, we plotted the percentages of responding fish of each genotype at each dose with standard deviation across the three trials. We performed no additional statistical analyses on these data.

For the electrophysiology experiments, we used at least ten fish per genotype or treatment group. This is the standard number of replicates used in the field to capture any natural variation that occurs across a population. The raw trace data is reported, so there were no statistical analyses performed on these data.

Results

Identification and description of gad1a -/- and gad1b -/- mutant zebrafish

Potential founders that were injected with CRISPR-Cas9 material were crossed with wild-type fish and the F1 progeny were genotyped via PCR and PAGE analyses (VanLeuven, Park et al. Under Review). Animals that showed evidence of a mutation based upon this initial screening were then sequenced via Sanger sequencing to determine the nature of the mutation (Figure 4.1). Animals with the same mutation were housed together and bred to identify fish with homozygous mutations for each allele. These F2 generations were sequence-verified before being established as secured homozygous mutant lines (Supplemental Figure 4.1). Their inbred progeny were grown to adulthood and were used for all behavioral and molecular aspects of this study. Screening and sequencing primers used in this study can be found in Supplemental Table 4.1.

Zebrafish with the confirmed mutations described in Figure 4.1 (*gad1a*^{gav2404} and *gad1b*^{gav2303}) are viable and fertile into adulthood; these animals live more than one year and regularly produce viable progeny. Since these are the first *gad* mutant zebrafish ever created,

we first observed these mutant zebrafish during development. Starting at 3 dpf, *gad1b* -/- zebrafish exhibited hyperactive swimming behavior as compared to *gad1b* +/- or wild-type fish (data not shown). This abnormal and hyperactive behavior in *gad1b* -/- persisted through 7 dpf. Even as adults, the *gad1b* -/- are more active and often die sooner than *gad1b* +/- or wild-type zebrafish. The *gad1a* -/- zebrafish are slightly more active at 2 dpf as compared to wild-type, *gad1a* +/-, and *gad1b* mutant zebrafish (data not shown). From 3 dpf through larval development and as adults, however, there are not any extreme behavioral or swimming phenotypes of *gad1a* mutant zebrafish when compared to *gad1b* mutant or wild-type zebrafish. Neither the *gad1a* nor *gad1b* mutants exhibit any other abnormal phenotypes or developmental defects that we could detect.

***gad1b* mutant zebrafish have decreased GABA**

To quantify the levels of GABA in *gad1b* mutant zebrafish, we performed standard HPLC-ECD analyses on larval zebrafish heads and adult zebrafish brains. We tested ten adult brains for each genotype, all of which were age and gender matched. The average normalized concentration of GABA in adult wild-type, *gad1b* +/- and *gad1b* -/- brains is 987 ng/mg, 743.3 ng/mg and 644.6 ng/mg of tissue, respectively (Table 4.1 and Figure 4.2). There is a statistically significant decrease in GABA between adult wild-type and *gad1b* +/- brains ($p = 0.0324$) as well as between adult wild-type and *gad1b* -/- brains ($p = 0.0023$). However, there is no statistically significant change in GABA between *gad1b* +/- and *gad1b* -/- zebrafish (Table 4.1). There does not appear to be any noticeable contribution of gender or age in these values. Additionally, there is not a statistically significant change in the levels of any other neurotransmitter tested across wild-type and *gad1b* mutant adult zebrafish brains, except for a decrease in glutamate in adult *gad1b* +/- brains as compared to wild-type. This decrease in glutamate is not present when comparing wild-type to *gad1b* -/- or when comparing *gad1b* +/- to *gad1b* -/- (Supplemental Figure 4.2 and Supplemental Table 4.2). There is also no statistically significant change in any

neurotransmitter, including GABA, between wild-type, *gad1b* +/-, *gad1b* -/- and *gad1a* -/- in 7 dpf larvae (Supplemental Figure 2.3 and Supplemental Table 4.3).

***gad1a* and *gad1b* mutant zebrafish are sensitive to perturbations in GABA signaling**

To address the functional implications of these *gad* mutants, we first designed a PTZ-dose response experiment to assess seizure-like behavior across *gad1* mutants when exposed to sub-clinical doses of the chemiconvulsant drug PTZ. These data are displayed in Figure 4.3. As expected, no fish of any genotype exhibit seizure-like behavior at 0 mM PTZ. Interestingly, at 1 mM PTZ, almost 50% of *gad1b* -/- exhibit seizure-like behavior as compared to only 7% of *gad1a* -/-. Neither the *gad1a* +/-, *gad1b* +/- nor wild-type fish showed any response at this dose. At 2.5 mM PTZ, ~85% of *gad1b* -/- and ~30% of *gad1b* +/- responded as compared to only ~4-18% of *gad1a* -/-, *gad1a* +/- and wild-type fish at this dose. At 5 mM, 10 mM PTZ, and 15 mM PTZ the *gad1b* -/- are essentially all exhibiting seizure-like behavior. Approximately 70% of both *gad1b* +/- and *gad1a* -/- show abnormal behavior at 5 mM PTZ as compared to ~40% of wild-type. At 5mM ~82% of *gad1a* +/- seem to be exhibiting this behavior. At 10 mM and 15 mM PTZ, essentially all *gad1a* and *gad1b* mutants and almost all wild-type fish show seizure-like behavior, which is to be expected based upon the reports in the literature.

***gad1a* -/- and *gad1b* -/- zebrafish have increased and abnormal neural activity**

We performed extracellular electrophysiology to measure local field potentials in the optic tectum as a metric of neural activity. If the electrode is placed very close to or in a neuron, these recordings provide information about the activity of this and the few surrounding neurons. In these experiments, we saw spontaneous, epileptiform-like discharges in both 7 dpf *gad1a* -/- and *gad1b* -/- zebrafish as compared to wild-type animals (Figure 4.4). More specifically, *gad1a* -/- and *gad1b* -/- both show an overall increase in both high and low amplitude discharges in a 20-minute recording period when compared to wild-type zebrafish. This means that both *gad1a* -/- and *gad1b* -/- have increased and abnormal neural activity though in slightly different ways.

In *gad1a* -/-, we see a more active baseline than wild-type, as indicated by a general increase in amplitude. There are infrequent larger amplitude electrical discharges that we have never seen in a wild-type fish, but overall we conclude that there is only a small increase in neural activity in *gad1a -/-.*

In *gad1b* -/-, we see frequent, low amplitude discharges as well as high amplitude discharges as compared to wild-type and *gad1a -/-*. We observed some type of increased neural activity in every *gad1a* -/- fish (n = 10) and *gad1b* -/- (n= 10) sampled.

Discussion

This is the first published examples of viable *gad1a* and *gad1b* mutant zebrafish in the research community. These fish can be used to study the role of *gad* and GABA both during development and in the regulation of nervous system activity. As stated previously, earlier work in *Gad1* mutant mice was limited due to the neonatal lethality in *Gad1* -/- mice (Asada, Kawamura et al. 1997, Condie, Bain et al. 1997, Oh, Westmoreland et al. 2010). It is interesting that in this study, we found that neither *gad1a* -/- nor *gad1b* -/- zebrafish have abnormal craniofacial structures nor do they have neonatal lethality. This suggests that in zebrafish, the paralogous *gad1* gene function has been divided out among two genes (VanLeuven, Ball et al. In Preparation). However, both *gad1a* -/- and *gad1b* -/- zebrafish have a neurological phenotype, though these phenotypes manifest in different ways. This has interesting implications both in the context of gene regulatory networks, neural development, and in nervous system activity. In future studies, we will make double *gad1a* -/-;*gad1b* -/- and assess both craniofacial and neurological phenotypes. These *gad* mutant zebrafish can serve as models for future studies addressing these topics.

The data from our HPLC experiments support part of our hypothesis that *gad1b* gene function is altered and therefore GABA levels in *gad1b* mutant zebrafish are lower than wild-type. The fact that we see a reduction in glutamate in adult *gad1b* +/- is surprising to us. One possible mechanism that could explain this result is that we are altering some sort of enzyme

feedback loop and a small perturbation to *gad1b* changes the Gad enzyme's role in a cellular process that impacts glutamate (GABA's precursor) in some way. The preliminary HPLC experiments using single larval heads showed large amounts of variation in levels of all neurotransmitters for all genotypes, and Dopamine (DA) could not be measured (Supplemental Figure 4.3 and Supplemental Table 4.2). This is likely due to insufficient amounts of sample, so we will repeat these experiments using 5 pools of 10 larvae. HPLC-ECD will also be performed on adult *gad1a* mutants to address what if any changes in GABA and other neurotransmitters are present. We predict, based upon previous reports of *gad1a* having low expression in adults, that there may not be a significant change in GABA in adult *gad1a* -/- brains (Cocco, Ronnberg et al. 2017). There may be a noticeable change in GABA in the 7 dpf *gad1a* -/- larval samples though. In the future, we will also create *gad2* -/- zebrafish and perform HPLC on these zebrafish to fully complete the story on *gad* gene contribution to GABA production.

Similar types of experiments were performed in mouse *Gad1* -/- and reported over 90% reduction of GABA levels in P0.5 *Gad1* -/- as compared to wild-type mice (Asada, Kawamura et al. 1997). This group also reported no statistically significant difference in GABA levels *Gad2* -/- as compared to wild-type mice (Asada, Kawamura et al. 1996, Asada, Kawamura et al. 1997). However, a different report shows statistically significant reduction of GABA in *Gad2* -/- (Kash, Johnson et al. 1997). They concluded that the genetic background of the mouse must be taken into account and can lead to differences in the observed GABA levels (Kash, Johnson et al. 1997). We do not see such a dramatic decrease in GABA in our *gad1b* mutant zebrafish. One explanation for such a difference between zebrafish and mice could be due to the gene duplication in zebrafish leading to *gad1a* and *gad1b*, or perhaps some other species-specific difference. The issue of genetic background in mice and potential modifier effects could be a factor as well. To fully address this issue, we will have to perform these experiments in *gad1a* -/- and *gad2* -/- zebrafish and consider how all three zebrafish *gad* genes are involved in GABA production. The data we have so far suggest that there is a defined relationship between

genotype and GABA production. Additionally, the mutations we are making are specific to the GABAergic pathway and no other neurotransmitter system.

For the PTZ dose response experiments, we wanted to ask whether changes in GABA production *in vivo* make a zebrafish more sensitive to further alterations in GABA signaling in an allele specific manner. We hypothesized that *gad1a* and *gad1b* mutant zebrafish will be more sensitive to further perturbations in GABA signaling. This means that *gad1* mutants, specifically homozygous mutants, would exhibit seizure-like behavior in the presence of low or sub-clinical doses of PTZ while wild-type fish will not. This is an important concept to understand when trying to discover whether there is an allelic contribution of the *gad* genes to behavior and by extension neural activity. These data suggest a more-or-less stepwise and relatively predictable contribution of the *gad1* genes in the context of behavioral responses to varying subclinical doses of PTZ. These behavioral data also pair well with the HPLC data suggesting that statistically significant reductions in GABA level cause almost half of a pool of *gad1b* -/- animals to respond to 1mM PTZ, a 15-fold smaller dose than is usually administered. Even *gad1b* +/- and *gad1a* mutants exhibit a clear increase in the likelihood for seizure-like behavior when exposed to subclinical doses of PTZ. This suggests that there is a delicate, fine-tuning of the GABAergic inhibitory networks in the central nervous system. There is relatively high variation in all genotypes among trials, especially for the 2.5 mM and 5mM doses. This suggests that perhaps these doses of PTZ are approaching a threshold of GABAergic neural activity in zebrafish that we are manipulating. A more likely explanation for this variation is simply due to normal genetic variation.

The *gad1b* -/- line is more inbred than the *gad1a* -/- line due to how we had to maintain the lines early in the production of these mutants. Additionally, when we generated the heterozygous and wild-type fish for these experiments, we used zebrafish raised from different clutches and therefore sampled across a larger population of our zebrafish colony. We chose to set up the experiment for most of the dose response assays in this way to capture the response

variability present in our normal population. It is known that even among wild-type fish, there is some variation in how zebrafish respond to PTZ so we wanted to ensure that even considering the mutations that we were accurately sampling the population (Ball, Page et al. In Preparation).

Since we had evidence that these *gad1b* mutant zebrafish had less GABA and *gad1a/gad1b* mutants in general showed increased sensitivity to alterations in GABA signaling, we next wanted to characterize their neural phenotypes via electrophysiology. This approach has been used in several laboratories and on both drug-induced and genetic zebrafish models of epilepsy (Baraban, Taylor et al. 2005, Baraban, Dinday et al. 2013). We found that both *gad1a* -/- and *gad1b* -/- had increased and abnormal neural activity as compared to wild-type. The 15mM PTZ treated zebrafish had very frequent, stereotyped high amplitude electrical discharges. This is not surprising considering that PTZ acts on the $GABA_A$ receptor, therefore all GABA signaling is blocked. This treatment of 15mM PTZ in our hands is consistent with published results from other labs (Baraban, Taylor et al. 2005). We did note that the electrical signatures of *gad1a* -/-, *gad1b* -/- and 15mM PTZ indicate that they are acting on the same, GABAergic signaling pathway. To further test this, we used *scn1b* -/-, a known genetic model of zebrafish epilepsy (Schoonheim, Arrenberg et al. 2010, Baraban, Dinday et al. 2013). We see a different electrical signature in *scn1b* -/- as compared to *gad1a* -/- or *gad1b* -/-, which is expected since this mutation is in a voltage-gated sodium channel and not related to GABA signaling.

We noted differences between electrical signatures recorded for *gad1a* -/- and *gad1b* -/-. The observed differences in the pattern of discharges between *gad1a* -/- or *gad1b* -/- are likely not attributed simply to the placement of the electrode because we tested several locations throughout the tectum of each fish we sampled. Additionally, the signatures are consistent within each genotype (*gad1a* -/- or *gad1b* -/-) and both are distinct from wild-type. The more likely conclusion is that *gad1a* -/- and *gad1b* -/- zebrafish exhibit differences in neural activity

due to the different mutations which presumably result in different amounts of GABA and/or differences in neural development.

It should also be noted that we did not observe the stereotyped, consistent pattern of electrical discharges like what is observed with 15mM PTZ for either the *gad1a* -/- or *gad1b* -/- zebrafish. We did not expect this, as we were manipulating GABA signaling in these instances at the level of the ligand rather than the receptor. Additionally, we knew that each of these mutants still have unaltered forms of two of the three *gad* alleles and enzymes with approximately 60% of the normal amount of GABA. The combination of these factors makes it difficult to predict a consistent pattern of discharges, but in our 20-minute recording periods, we always observed at least 1-2 low amplitude events for *gad1a* -/- and 3-4 high amplitude events for *gad1b* -/-.

We think, based upon these results, that these *gad1a* -/- and *gad1b* -/- zebrafish mutants provide a useful model for future studies of seizure disorders because these spontaneous electrical discharges are similar to what often occurs in human patients who suffer from seizure disorders. Therefore, using these *gad1a* -/- and *gad1b* -/- zebrafish, we can perform further studies to better elucidate a mechanism of seizure onset and propagation which can be extended to other vertebrates, including humans. These mutants can also be used to study the differences in *gad1a* and *gad1b* involvement in neural development and how they impact neural activity both as larvae and adults.

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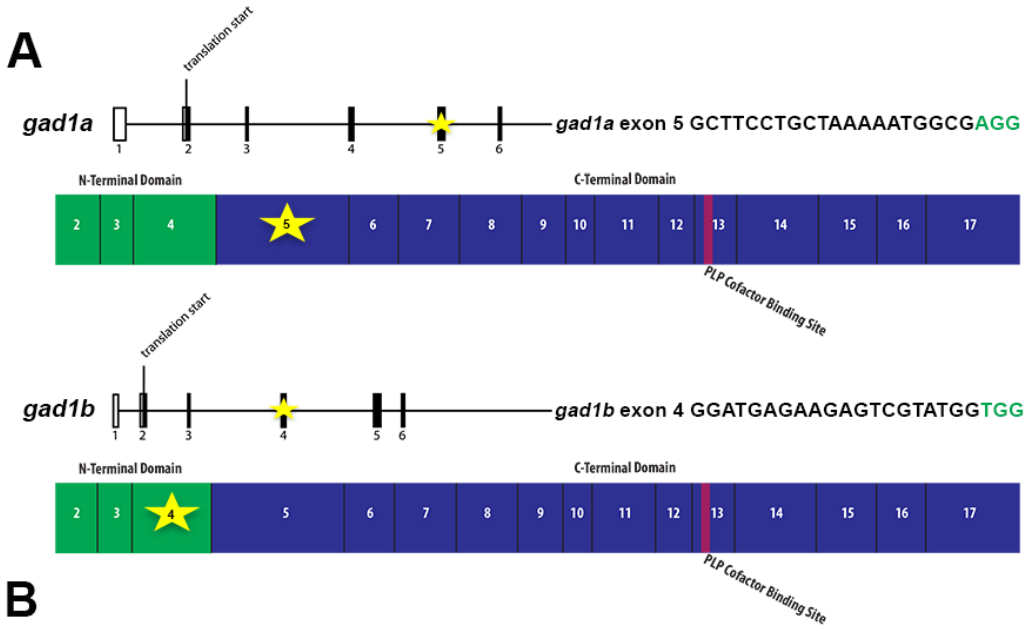
Tables

Table 4.1: Mean normalized concentration of GABA in adult brains by genotype and comparisons of the mean by one-way ANOVA.

	WT	*gad1b*$^{gav2303\ +/-}$	*gad1b*$^{gav2303\ -/-}$	WT and Het ($p < 0.05$)	WT and -/- ($p < 0.05$)	Het and -/- ($p < 0.05$)
GABA	987 ng/mg	743.3 ng/mg	644.6 ng/mg	Yes: $p = 0.0324$	Yes: $p = 0.0023$	No

Figures



B

gad1a	Exon 5 locus - Nucleic Acid Sequence	Indel
WT	TTCCTGCTAAAAATGGCGAGGAGTACACCATGCAGTTCCTG	
gav2404	TTCCTGCTAAAAAgtgctagtctgtgcTGGCGAGGAGTACACCATGCAGTTCCTG	14 bp ins

gad1b	Exon 4 locus - Nucleic Acid Sequence	Indel
WT	ACAGTTTGGATGAGAAGAGTCGTATGGTGGGCTCCTTCAAGGAGAGC	
gav2303	ACAGTTTGGATGAGAAGAGTCG----------TCCTTCAAGGAGAGC	10 bp del

Figure 4.1: *gad1a* and *gad1b* loci with CRISPR-Cas9 target sites and the resultant mutations. A) The stars represent the exon in each gene which was targeted for mutagenesis and the corresponding location in the protein. The 20-nucleotide target site is listed to the right of the gene; the PAM motif is in green. B) The nucleotide sequence for the wild-type locus and the resulting mutation that was generated by CRISPR-Cas9 mutagenesis. Deletions are noted as dashes and insertions are noted as lowercase, red letters. Both types of indels generated are frameshift mutations that are predicted to cause truncated, non-functional proteins.

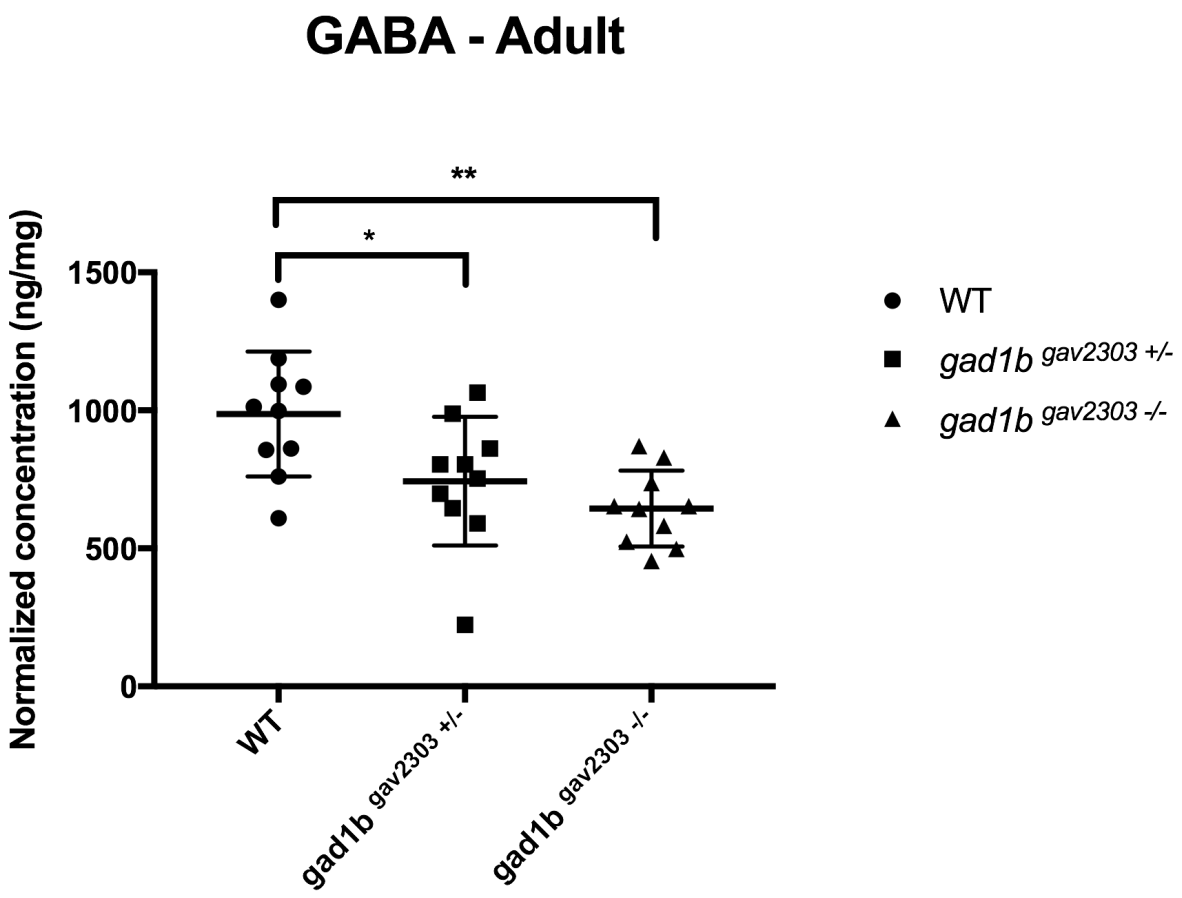


Figure 4.2: HPLC-ECD results of GABA levels in WT, *gad1b*$^{gav2303\ +/-}$ and *gad1b*$^{gav2303\ -/-}$ adult zebrafish brains. The normalized concentration of GABA is plotted for 10 biological replicates for each genotype. There is a statistically significant decrease in GABA in both heterozygous and homozygous mutants as compared to wild-type zebrafish. *p = 0.0324, **p = 0.0023. See Table 4.1 for the mean values.

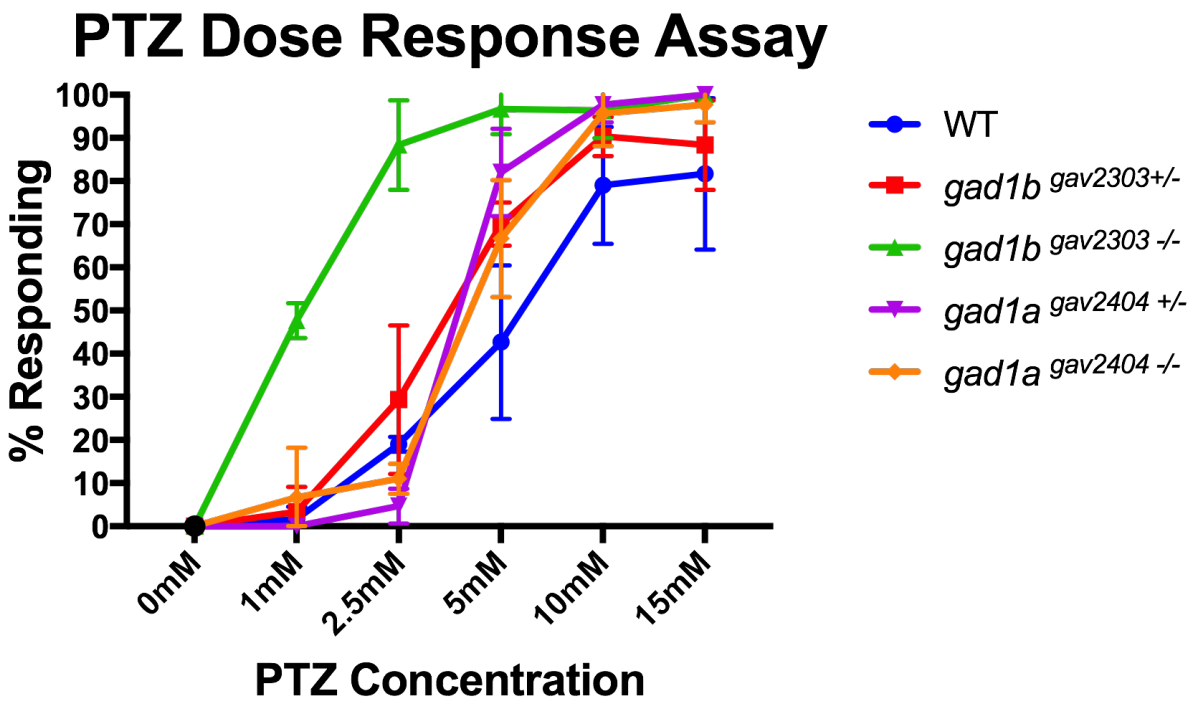


Figure 4.3: PTZ dose response assay on 7 dpf *gad1a* and *gad1b* mutants. Zebrafish were sorted into groups of 10-20 per genotype for each treatment group and assayed for stage II and stage III behavioral phenotypes for 10 minutes. Assays were performed three times for biological and technical replication. N>42 for each genotype at each dose. Both *gad1a* and *gad1b* mutants are hypersensitive to seizure-like behavior when exposed to subclinical doses of PTZ as compared to wild-type. The *gad1b* -/- are much more hypersensitive with almost a 50% response rate at 1 mM PTZ and more than 85% response rate at remaining doses of PTZ.

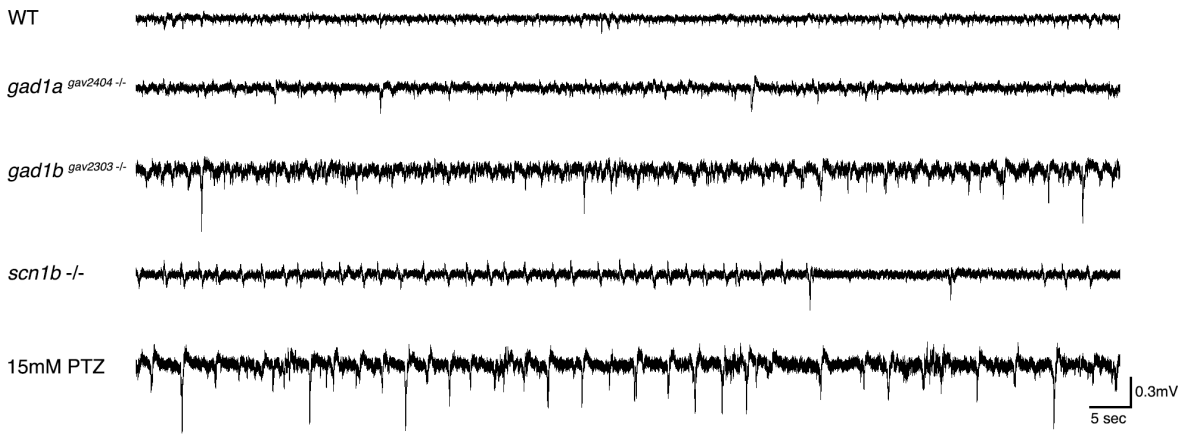


Figure 4.4: Extracellular electrophysiology to measure 7 dpf *gad1a* -/- and *gad1b* -/- neural activity. Representative traces of *gad1a* -/- and *gad1b* -/- showing neural activity compared to wild-type (WT) fish, *scn1b* -/- fish and WT fish treated with 15 mM PTZ. N > 10 for each genotype/treatment. *gad1a* -/- have low amplitude increases in neural activity but are more active than WT. *gad1b* -/- have higher amplitude and more frequent increases in neural activity as compared to wild-type. *scn1b* -/- are an established genetic seizure model in zebrafish but act through a different pathway than alterations in GABA signaling. 15mM PTZ blocks all GABA signaling and produces consistent, high amplitude electrical discharges. The latter two experiments show that these *gad1a* -/- and *gad1b* -/- zebrafish are abnormal, work through a different mechanism than *scn1b* -/- and are not as abnormal as treatment with 15mM PTZ.

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Supplemental Tables and Figures

Supplemental Tables

Supplemental Table 4.1: Screening and sequencing primers.

	Forward (5'-3')	Reverse (5'-3')
***gad1a* screening**	CATTAGCATTGACTTGACCGAG	AGCAGGAACTGCATGGTGTA
***gad1a* sequencing**	ACTCAGCGATGCAATGTCAG	TGTGCATGGTCTTCATCACC
***gad1b* screening**	CCCGTGTTGTAATGATGCAG	GTGAAGCGCTCATTGTTGTC
***gad1b* sequencing**	TCCCAGTAAAACTCCAACCG	GCGAACAGGTTGGAGAAATC

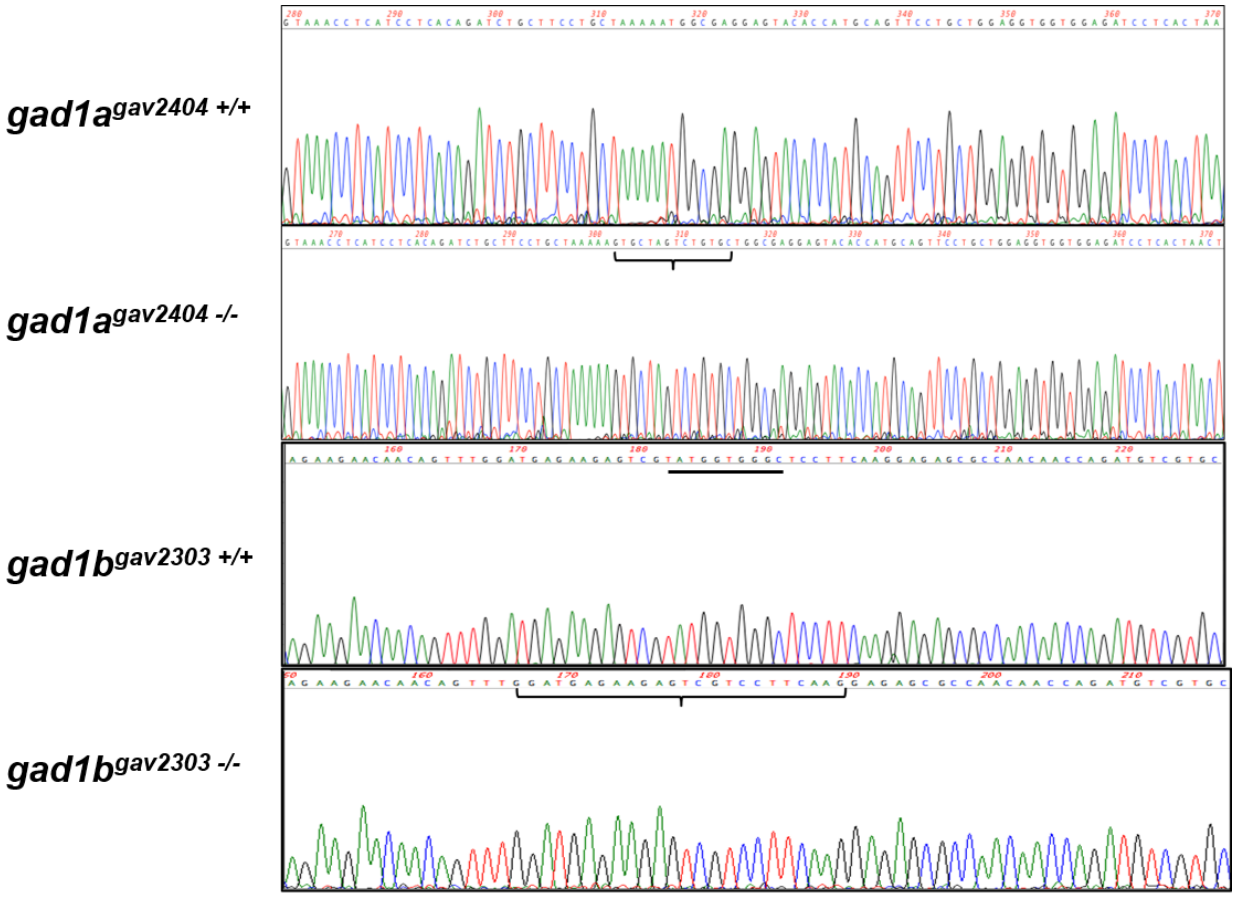
Supplemental Table 4.2: Mean normalized concentration of neurotransmitters in adult brains by genotype and comparisons of the mean by one-way ANOVA.

	WT	*gad1b*$^{gav2303\ +/-}$	*gad1b*$^{gav2303\ -/-}$	WT:Het ($p < 0.05$)	WT:-/- ($p < 0.05$)	Het:-/- ($p < 0.05$)
Serotonin (5-HT)	0.3025 ng/mg	0.261 ng/mg	0.3166 ng/mg	No	No	No
Serotonin Metabolite (5-HIAA)	0.2186 ng/mg	0.2254 ng/mg	0.1912 ng/mg	No	No	No
Dopamine (DA)	0.1536 ng/mg	0.205 ng/mg	0.1575 ng/mg	No	No	No
Glutamate	1,423 ng/mg	1,046 ng/mg	1,252 ng/mg	Yes $p = 0.0022$	No	No
Glutamine	1,342 ng/mg	1,138 ng/mg	1,155 ng/mg	No	No	No
Norepinephrine (NE)	1.051 ng/mg	1.007 ng/mg	1.113 ng/mg	No	No	No
Norepinephrine Metabolite (MHPG)	26.81 ng/mg	31.34 ng/mg	24.56 ng/mg	No	No	No

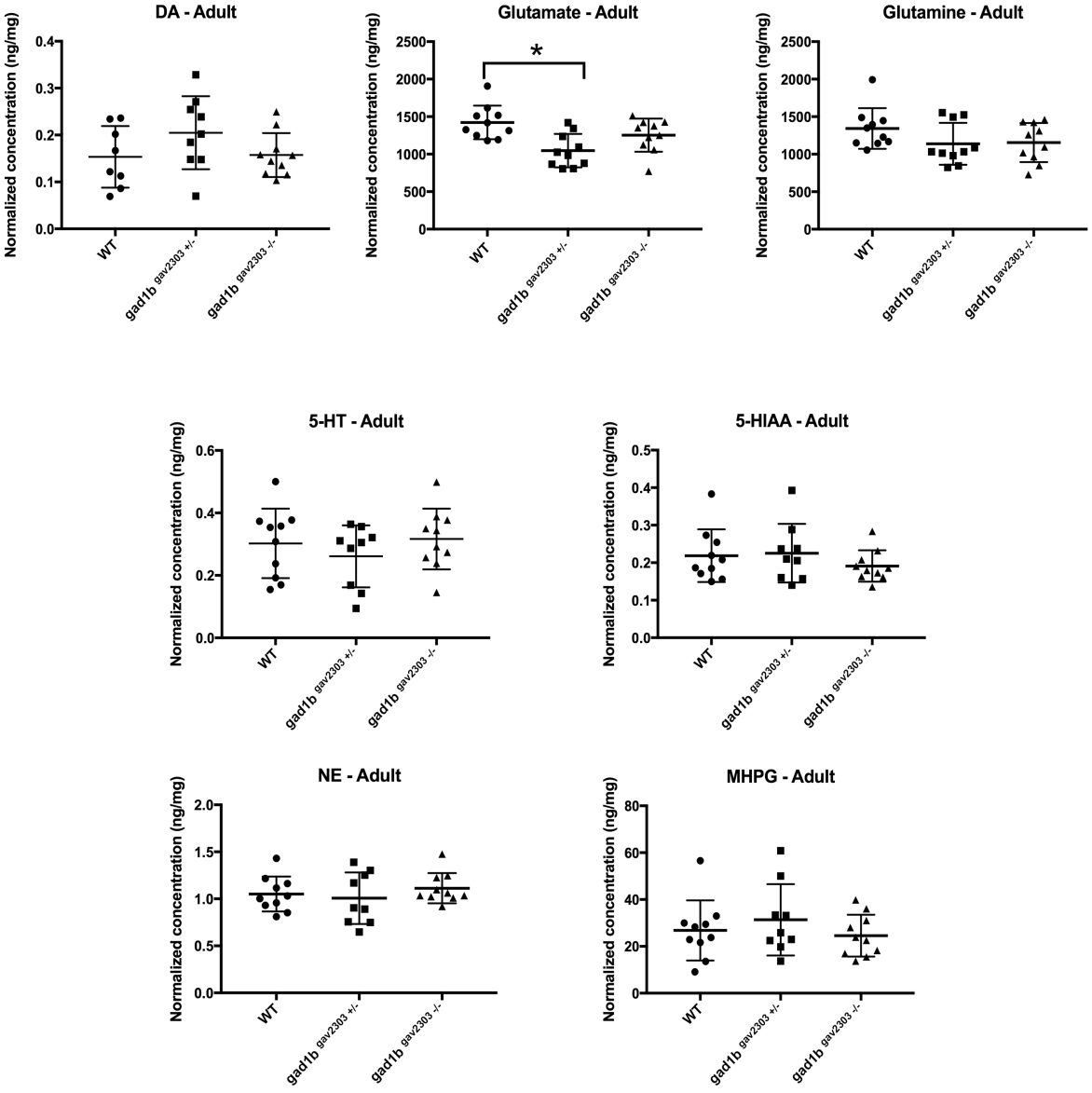
Supplemental Table 4.3: Mean normalized concentration of neurotransmitters in 7 dpf larvae by genotype and comparisons of the mean by one-way ANOVA.

	WT	*gad1b*$^{gav2303}_{+/-}$	*gad1b*$^{gav2303}_{-/-}$	*gad1a*$^{gav2404}_{-/-}$	WT:Het ($p < 0.05$)	WT:-/- ($p < 0.05$)	Het:-/- ($p < 0.05$)
Serotonin (5-HT)	0.073 ng/mg	0.035 ng/mg	0.015 ng/mg	n/a	No	No	No
Serotonin Metabolite (5-HIAA)	1.485 ng/mg	1.625 ng/mg	0.8693 ng/mg	n/a	No	No	No
GABA	60.59 ng/mg	25.17 ng/mg	56.61 ng/mg	0.6367 ng/mg	No	No	No
Glutamate	324.1 ng/mg	477.1 ng/mg	357.8 ng/mg	294.8 ng/mg	No	No	No

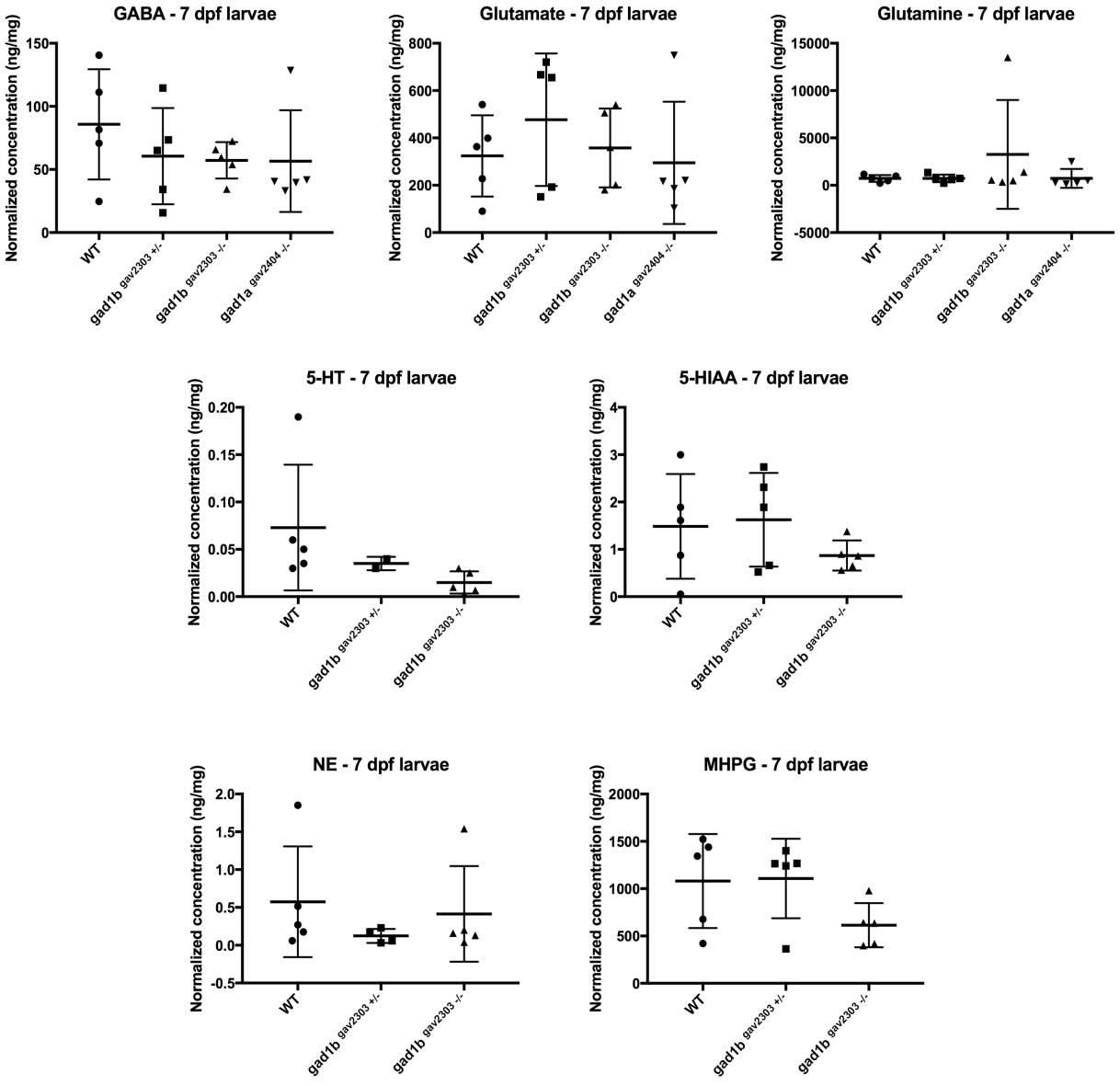
Glutamine	716.8 ng/mg	724.8 ng/mg	3,259 ng/mg	724.8 ng/mg	No	No	No
Norepinephrine (NE)	0.5743 ng/mg	0.1233 ng/mg	0.414 ng/mg	n/a	No	No	No
Norepinephrine Metabolite (MHPG)	1,081 ng/mg	1,107 ng/mg	614.5 ng/mg	n/a	No	No	No
Dopamine (DA)	n/a	n/a	n/a	n/a	n/a	n/a	n/a



Supplemental Figure 4.1: Chromatograms showing confirmation of homozygous mutations for *gad1a*^{gav2404} and *gad1b*^{gav2303} alleles. Sequence verification of homozygous mutations in *gad1a* and *gad1b* that we generated. The bracket in *gad1a* -/- represents the 14 bp insertion that is not present in wild-type. The black line in *gad1b* +/+ indicates the 10 bp that are deleted. The bracket in *gad1b* -/- shows the sequence with the 10 bp deletion.



Supplemental Figure 4.2: HPLC-ECD showing the levels other neurotransmitters tested in WT, *gad1b*$^{gav2303\ +/-}$ and *gad1b*$^{gav2303\ -/-}$ adult zebrafish brains. Normalized concentrations of 7 other neurotransmitters tested in the three genotypes. Abbreviations: 5-HT = serotonin; 5-HIAA = a serotonin metabolite; DA = dopamine; NE = norepinephrine; MHPG = a norepinephrine metabolite. There is no statistically significant change in any of these levels of neurotransmitter across these genotypes except for a decrease in glutamate between WT and *gad1b* +/- (noted with an asterisk).



Supplemental Figure 4.3: HPLC-ECD showing the levels other neurotransmitters tested in 7 dpf WT, *gad1b*$^{gav2303\ +/-}$, *gad1b*$^{gav2303\ -/-}$ and *gad1a*$^{gav2404\ -/-}$ larvae. Normalized concentrations of all neurotransmitters tested in the three genotypes. Abbreviations: 5-HT = serotonin; 5-HIAA = a serotonin metabolite; NE = norepinephrine; MHPG = a norepinephrine metabolite. There is no statistically significant change in any of these levels of neurotransmitter across these genotypes. There is frequently a large amount of variation among these samples.

CHAPTER 5

CONCLUSION

In this body of work, I have identified a *gad1* paralog (*gad1a*) in the zebrafish and shown that it is indeed present in a distinct expression pattern in the developing zebrafish. Additionally, I generated both *gad1a* and *gad1b* mutant zebrafish and uncovered that the function of these genes during development and in neurotransmission seems to be parsed out and they play slightly different roles. This notion of paralogous genes is not uncommon in zebrafish, but to see both genes with functionality that does not appear to be totally redundant is fascinating (Sidow 1996, Wittbrodt, Meyer et al. 1998, Meyer and Schartl 1999, Hoegg, Brinkmann et al. 2004). The mechanisms of both the regulation of these genes and how these genes are involved in development and in neurological disorders remain unknown, but we now have a model in which to address these questions in future studies.

Previous work both from our lab and others has shown that there is a role for the *GAD* genes and other aspects of GABA signaling in craniofacial development (Culiat, Stubbs et al. 1993, Culiat, Stubbs et al. 1995, Asada, Kawamura et al. 1997, Condie, Bain et al. 1997, Oh, Westmoreland et al. 2010, Hariharan 2013, Beebe 2015). These studies showed that *GAD1*, but not *GAD2*, is important for proper craniofacial development as *Gad1* -/- mice have a severe cleft palate and die at birth, while *Gad2* -/- mice do not have this phenotype (Asada, Kawamura et al. 1996, Asada, Kawamura et al. 1997, Condie, Bain et al. 1997, Oh, Westmoreland et al. 2010). Similar results were seen with the use of translation blocking morpholinos in zebrafish in our lab. Knockdown of *gad1*, but not *gad2*, resulted in craniofacial abnormalities in zebrafish which resulted in smaller craniofacial cartilages and abnormal chondrocyte stacking (Beebe 2015). In both *gad1* and *gad2* knockdown, zebrafish had abnormal neurological activity as measured by extracellular electrophysiology (Hariharan 2013, Beebe 2015).

In our zebrafish CRISPR-Cas9 mutants however, neither the *gad1a* -/- nor the *gad1b* -/- have any noticeable craniofacial abnormalities. For *gad1b* -/-, we looked at live images and Alcian blue staining of 7 dpf zebrafish larvae as well as patterns of *dlx2a* staining by *in situ* hybridization in early embryos and we could not detect any noticeable changes in staining pattern nor in craniofacial structural changes (data not shown). When we look closer at the *gad1* translation blocking morpholino that was used in previous studies in our lab, we hypothesize that the morpholino is acting on both *gad1a* and *gad1b.* This could explain why neither the *gad1a* -/- nor the *gad1b* -/- zebrafish have any abnormal craniofacial phenotypes: the CRISPR-generated mutants are only targeting one gene, while the morpholino was targeting both *gad1a* and *gad1b*. It is also important to note that while neither of the *gad1a* and *gad1b* CRISPR-Cas9 mutants have abnormal craniofacial phenotypes, both show increased and abnormal neural activity. These results further support the conclusion from previous work in our lab which indicates that at least the zebrafish *gad1a* and *gad1b* genes have different roles both during development and in neurotransmission.

Thus far, I have identified the paralogous *gad1a* and *gad1b* genes and shown that they are both developmentally and functionally distinct. It will be interesting to see if *gad1* double mutants (*gad1a -/-; gad1b -/-*) show similar craniofacial problems like what was seen in the morphant zebrafish and/or the mouse model. I hypothesize that these double *gad1* mutants will have abnormal craniofacial structures, and additional studies will be required to elucidate the mechanism(s) for *gad1a* and *gad1b* gene function in this developmental process. Such studies could look either at differences in the cell cycle or in the cranial neural crest cells which give rise to the craniofacial cartilage to provide more insight into a possible mechanism (Eisen and Weston 1993, Knight and Schilling 2006, Andang, Hjerling-Leffler et al. 2008).

It is interesting to note that I have also generated a *gad2* mutant zebrafish, however when I breed these zebrafish, the *gad2* -/- are not surviving to or through sexual maturity. The oldest *gad2* -/- animal I have found is 1.5 months old and it did not survive long enough to

breed. This is similar to what occurred in the mouse model which reported that *Gad2* -/- died much earlier than their heterozygous or wild-type littermates (Asada, Kawamura et al. 1996, Kash, Johnson et al. 1997). When looking at the exon/intron junctions and synteny for *gad2* as well as the surveys of the molecular databases, it appears that *gad2* is well conserved between mammals and zebrafish and there is no evidence of a paralogous *gad2* gene. Taking this and what I see with the *gad2* -/- zebrafish could indicate a conserved mechanism, at least of *gad2* gene function, across vertebrates. Further work would need to be performed to address this.

A large portion of this work involved generating the *gad* mutant zebrafish that were used in this study. In the process of creating these mutant zebrafish, I also developed a fast, sensitive, and inexpensive screening method for identifying CRISPR-Cas9 induced mutations in zebrafish (VanLeuven, Park et al. Under Review). This approach is based on neutral polyacrylamide gel electrophoresis (PAGE) which has been used since the 1950s to distinguish changes as small as 1/1000 nucleotides in a given sample of DNA (Raymond and Weinstraub 1959). We adapted this approach to identifying putative F1 heterozygous zebrafish which are the progeny of an injected F0 founder crossed to a wild-type. We deduced that if a CRISPR-Cas9 injected founder is carrying a germline mutation and is crossed to a wild-type fish, one allele will be of known size and the other may have an altered size. With a small enough amplicon (< 150 base pairs (bp)) and the appropriate gel conditions, we can separate these bands and identify heterozygous fish from wild-type fish. We have established this methodology as both a screening approach for identifying novel mutations as well as a genotyping method that we continue to use in the lab for detecting indels as small as 1 bp in length.

As mentioned, I have identified a gene, *gad1a*, in the zebrafish that is expressed in a distinct way from its *gad1b* paralog. The finding that *gad1a* is expressed in a distinct pattern in the early zebrafish spinal cord at ~16 hours post fertilization (hpf) while *gad1b* and *gad2* are not detected in this pattern indicates that early GABAergic neural development is likely mediated more by *gad1a* than the other zebrafish *gad* genes. This makes sense, for example, in the

context of how the GABAergic cells in the spinal cord develop (Bernhardt, Chitnis et al. 1990, Kuwada, Bernhardt et al. 1990, Bernhardt, Patel et al. 1992). The more dorsal interneuron subclasses (the DoLA, CoSA, and even VeLD neurons) appear first, and at least two of these subtypes have been shown to express *gad1a.* The more ventral interneuron subclass (the KA) appears to express *gad1b/gad2*, but likely not *gad1a.* The CoSA and VeLD neuron classes may have some low-level overlap in expression of *gad1a* v. *gad1b* v. *gad2*, but this would need to be assessed in greater detail. Additionally, there is expression of *gad1a*, but not *gad1b* or *gad2* in the tail bud at 16 hpf, suggesting an additional non-neural role for *gad1a*. A similar report in mice shows non-neural expression of *Gad1* in the tail bud, so our results are consistent with this data and suggest that *gad1a* rather than *gad1b* may be more related to mammalian *Gad1* (Maddox and Condie 2001). In any case, the co-expression studies we have performed so far indicate that there are complex regulatory mechanisms of the zebrafish *gad* genes during development. Future studies investigating the regions around the *gad1a* and *gad1b* gene could uncover enhancers that are responsible for the differential gene expression that we observe.

When we compare our results of *gad* expression, particularly in the spinal cord, to what was reported in (Martin, Heinrich et al. 1998), we conclude that what they reported as *gad1* is really *gad1b.* Our results are consistent with their results for *gad1* (*gad1b*), but we go a step further by including *gad1a* in such analyses. Taken together, even if the expression pattern between the zebrafish *gad* genes is partially overlapping, there is still a clear distinction between *gad1a* and *gad1b/gad2*. This has important implications in terms of development and how we think about the regulation and redundancy of the *gad* genes, specifically in the zebrafish.

In terms of identifying *gad1a* and its expression and role in the zebrafish, we used primarily established techniques like RT-PCR and *in situ* hybridization. The major limitation we have experienced is not being able to differentially detect Gad67a protein by antibody stain or western blot. This can be dealt with by using other methods, namely alternate antibodies to help characterize Gad67a expression or activity and by high-performance liquid chromatography with

electrochemical detection (HPLC-ECD) to detect changes in neurotransmitter levels across different genotypes (Ross and Filipov 2006, Coban and Filipov 2007). Using HPLC-ECD, we have been able to conclude that both *gad1b* +/- and *gad1b* -/- have significantly less GABA than wild-type zebrafish. This technique was also used in the *Gad1* -/- and *Gad2* -/- mouse mutants and it was shown that *Gad1* -/- have an ~90% reduction in GABA. The data for *Gad2* -/- was inconsistent across studies; one study reported no change at all in GABA levels and another showed there was a significant reduction in GABA levels, but not as robust as was seen in *Gad1* -/- mice (Asada, Kawamura et al. 1996, Kash, Johnson et al. 1997). The differences seen in *Gad2* -/- were concluded to be from the genetic background of the mice and likely indicate modifier effects in how *Gad2* (the Gad65 isoform of the enzyme) contributes to GABA production (Kash, Johnson et al. 1997).

We will be performing these assays on *gad1a* -/- and *gad2* -/- to complete the story on how the zebrafish gad genes contribute to GABA production. We hypothesize that *gad1a* will likely not have a huge reduction in GABA in adults, especially based upon a study which showed by qRT-PCR that *gad1a* levels in the adult brain are quite low (Cocco, Ronnberg et al. 2017). We do hypothesize that GABA levels in both *gad1a* -/- and *gad1b* -/- at 7 dpf will be lower than in wild-type. In terms of *gad2*, we hypothesize that there will be less GABA in the *gad2* -/-, but likely not as much of a reduction as we see in the *gad1b* -/-. Even though I have to speculate as to what the HPLC-ECD results will teach us about GABA levels across the *gad1a*, and *gad2* mutants, our ability to use HPLC-ECD can provide critical pieces of data to help us understand GABAergic neural activity in zebrafish.

Additionally, since the expression of *gad1a* and *gad1b* are distinct and the *gad1a* -/- and *gad1b* -/- mutants have differences in sensitivity to PTZ and in overall neural activity, this indicates that there are likely different mechanisms underlying the regulation, and/or activity of these genes. Future studies can address these questions from a neural developmental point of view or from a neural activity point of view. One way we can do this is by using calcium imaging

plus light sheet microscopy to compare wild-type, *gad1a* -/- and *gad1b* -/- neural activity in live zebrafish and begin to better understand the neural mechanisms at play. Our lab has experience with analyzing calcium imaging data and we are actively pursuing this approach (Tao, Lauderdale et al. 2011).

Based upon the combination of the results from these studies, I speculate that the reason why *gad1b* -/- zebrafish do not exhibit a craniofacial phenotype is because there are wild-type levels of *gad1a* expressed during development. It has been shown that there are relatively low levels of *gad1a* expression in adult zebrafish (Cocco, Ronnberg et al. 2017), but this is after the critical developmental window for craniofacial structures. It is clear from our electrophysiology data that both *gad1a* -/- and *gad1b* -/- exhibit increased and abnormal neural activity as 7 dpf larvae. Even though I have not specifically looked at neural activity of *gad1a* nor *gad1b* in adult zebrafish, I hypothesize that neither can fully compensate for one another neither during development nor in adulthood. In conclusion, this notion of differentially regulated paralogs offers a unique opportunity for studies of *gad1* gene function in both neural and non-neural processes in the zebrafish.

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