DETERMINING THE FUNCTIONS OF *GAD1* AND *GAD2* IN EMBRYONIC ZEBRAFISH USING SPLICE BLOCKING MORPHOLINOS

by

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(Under the Direction of James D. Lauderdale)

ABSTRACT

GABA is a major inhibitory neurotransmitter in the central nervous system (CNS), which is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD). There are two isoforms of GAD encoded by two genes *gad1* and *gad2*. In mammals, *gad1* encodes GAD67 and *gad2* encodes GAD65. A decrease in GAD67 in the human brain is associated with schizophrenia and related neurological disorders. In mice, a loss of GAD65 causees spontaneous seizures. We studied the role of GAD in modulating neural activity in zebrafish. Splice blocking morpholinos were used to manipulate expression of *gad* in embryonic zebrafish. *gad1-gad2* double morphants exhibited substantial reduction in GABA, leading to spontaneous seizures-like neural activity. Furthermore, *gad1* and *gad2* single morphants also exhibited seizure-like activity, suggesting that both GADs are important for the normal functioning of the brain.

INDEX WORDS: GABA, *gad1*, *gad2*, GAD67, GAD65, zebrafish, knockdown, splice blocking morpholinos, neural activity, seizures, electrophysiology

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

GABA and its functions in the organism

GABA (γ-aminobutyric acid) is a major inhibitory neurotransmitter in the central nervous system (CNS). As an inhibitory neurotransmitter, GABA binds to the inotropic GABA A and C receptors in the active post-synaptic neuron and activates a Cl^- ion channel. This causes an influx of Cl^- ions in the active neuron, which results in hyperpolarization of the neuron and inhibition of action potential [3, 4]. When bound to the metabotropic GABA B receptor of active post-synaptic neuron, GABA activates K^+ ion channel, resulting in the hyperpolarization of the neuron and inhibition of action potential [53].

In the CNS, GABA has several functions, apart from its function as the major inhibitory neurotransmitter. In the developing embryo, GABA acts as an excitatory neurotransmitter [2, 5], and is thought to be involved in the development of the nervous system. GABA is also thought to play a role in migration and maturation of neuronal precursors [2, 6, 7].

GABA is not only found in the CNS, but also in the peripheral nervous system and other parts of the body, including in the pancreas, testis and ovaries. GABA also has endocrine functions in the insulin-producing pancreatic β cells, and in the adrenal medulla and gastrointestinal tract [1, 2].

In mature neurons, GABA is present in two distinct compartments in the neuron: the synaptic terminal and the cell body. The pool of GABA in each compartment is thought to perform different functions. The GABA present in the synaptic terminals functions as a neurotransmitter. The GABA in the cell body has a metabolic function where it participates in the Tricarboxylic Acid (TCA) cycle [Figure 1] [1, 8]. Three enzymes, glutamic acid decarboxylase (GAD), GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) are involved in the GABA shunt pathway of the TCA cycle. GAD synthesizes GABA, and the other two enzymes are responsible for the catabolism of GABA. [1, 8]. The GABA shunt pathway is an important disposal pathway for GABA and also provides energy upon the catabolism of GABA [8, 9]. Minor sources for GABA (other than glutamate) include ornithine and putrescine [10, 11].

Glutamic acid decarboxylase (GAD)

The major pathway through which GABA is synthesized is through the conversion of glutamate to GABA by the action of GAD in a single step reaction. GAD has two protein isoforms in the vertebrate system, encoded by two separate genes [1, 8, 12]. In mammals, the *gad1* gene encodes the protein isoform called GAD67, which has a molecular weight of ~67 kilodalton (kDa), and the *gad2* gene encodes the protein isoform called GAD65, which has a molecular weight of ~65 kDa [1, 8, 12]. There is evidence of a third GAD gene called *gad3*, in the armored grenadier (*Coryphaenoides armatus*), a scavenging deep sea fish [13], but the zebrafish genome has only two genes *gad1* and *gad2* [14].

Expression of GAD

In the mammalian brain, though *gad1* and *gad2* are co-expressed in most of the GAD expressing neurons, there are differences in expression levels and subcellular localization [15, 16]. In the rat brain the overall expression level of *gad1* is higher, but many regions have higher levels of *gad2*, especially regions corresponding to the visual and neuroendocrine systems [15, 16]. The cellular expression of the GAD proteins are similar to the expression of their respective mRNAs [16]. However, the two proteins have different subcellular localization within the neuron [15, 16].

GAD65 expression is localized in the terminals of the neurons, and appears to associate with synaptic vesicles [17-20], and GAD67 is more distributed in the cell body of the neurons [15-17]. The N-terminal domain of the two proteins appears to be responsible for this differential subcellular distribution. The N-terminal domain of GAD65 contains signals that target it to the Golgi complex [20, 21]. Apart from the expression in the CNS, GAD is expressed in non-neuronal tissues. In the developing mouse embryo, *gad1* mRNA is expressed in the ventral neural tube and notochord, tail bud, developing limb buds, pharyngeal arches and in the vibrissae placodes [Figure 2] [22]. Non-neuronal GAD65 expression is detected in the Islets of Langerhans of the rat pancreas [43].

In zebrafish, GABA immunoreactive cells have been reported in the embryonic zebrafish brain as early as 16 hours post fertilization (hpf) [23]. At 16 hpf GABA immunoreactive cells are found in the telencephalic nucleus, nucleus of the tract of post optic commissure (nTPOC) of the ventral diencephalon, in the nucleus of medial longitudinal fascicle (nMLF) of the midbrain, as well as clusters of cells in the hindbrain

and spinal cord [23]. At 24 hpf, these clusters of GABA positive cells increase, and new cells begin expressing GABA [14, 23-25]. In the spinal cord, GABA labeling is detected at 18-19 hpf, in four types of neurons: commissural secondary ascending (CoSA), dorsal longitudinal ascending (DoLA), ventral longitudinal descending (VeLD) and Kohmer-Agduhr (KA) [Figure 3] [14, 26].

From in situ hybridization data on 24 hpf embryos, *gad1* and *gad2* are shown to be mostly co-expressed in the same regions in the brain, except in the caudal hindbrain posterior to the otic vesicle where expression of only *gad1* mRNA is detected. The level of *gad2* is higher in the midbrain than *gad1* [14]. However, in the spinal cord, the level of *gad1* expression is higher than *gad2* expression [14]. The neurons in the spinal cord expressing *gad1* and *gad2* are similar to the ones expressing GABA [14, 24].

In adult zebrafish, similar labeling for GABA and GAD is observed in the retina [27].

Isoforms of *gad1*

In mouse and rat, in addition to the full length isoform, two splice variants of GAD67 have been observed during developmental stages [6, 30, 31]. In the developing rodent embryo, there is an insertion of two alternatively spliced exons (exons 7a and 7b) in the *gad1* cDNA. Both inserts are almost identical and contain overlapping start and stop codons. One variant gives rise to a 25 kDa isoform that contains the N-terminal region of the protein, and a 44 kDa isoform that contains the rest of the protein, which includes the C-terminal domain. The other variant gives rise to only the 25 kDa isoform [Figure 4] [2, 6]. These variants have been detected on Western blot, in RNA *in situ* hybridization and immunofluorescence in the rat spinal cord, in the early embryonic

stages [6, 31, 32]. The 25 kDa form is enzymatically inactive and the 44 kDa form is thought to be functional since it still contains the catalytic domain. Apart from the full length isoform of *gad1*, the 25 kDa variant has also been detected in adult zebrafish using the antibody that recognizes the 25 kDa variant in the mouse [32]. The 25 kDa is found mainly in the retina, where it is thought to regulate GABA levels in the light/dark cycle. The full length GAD67 is abundant during the light period, while the 25 kDa form is more abundant during the dark period [32]. In contrast to GAD67, no other variants of GAD65 have been detected.

Structure of GAD

GAD has two main functional domains: the N-terminal domain and the C-terminal domain. The C-terminal domain is the catalytic domain of the proteins [2, 18, 19, 28]. The N-terminal domain for human GAD65 is composed of residues 1-95, and the N-terminal domain for human GAD67 is composed of residues 1-101. The N-terminal domains of the two proteins have only a 23% identity, whereas the C-terminal domains have a 73% identity [8, 18]. GAD requires a cofactor pyridoxal phosphate (PLP) for its activity [8, 18, 29]. GAD is a part of a large family of proteins that bind to PLP as a cofactor, and the members of this superfamily have highly conserved amino acids that form the binding site and interact with the cofactor. The motif containing the residues NPHK interacts directly with PLP [18]. Using a sequence alignment algorithm called PROBE, Qu and co-workers [29] identified nine highly conserved residues that interact with the cofactor, and six statistically significant conserved motifs in the two human GADs, including the NPHK motif. These motifs appear to play an important role in the stabilization of the protein structure and its interaction PLP [29]. The functional form of

GAD is a dimer connected by non-covalent linkages [18]. The enzymes form homodimers as well as heterodimers [18, 28, 42]. The two enzymes interact differently with the cofactor. About 50% of the GAD in the rat brain is present as apoenzyme (without cofactor), and it has been shown that majority of apoGAD is the GAD65 isoform, and GAD65 more highly regulated by PLP than GAD67 [8, 17]. The differential interaction with the cofactor is thought to be one of the reasons for functional differences between GAD65 and GAD67. The interchange between apoGAD and holoGAD plays an important role in the regulation of GAD activity and in the synthesis of GABA as a neurotransmitter.

From studies in a culture system, the N-terminal domains of the two proteins appear to be responsible for the differential subcellular distribution. The N-terminal domain of GAD65 undergoes posttranslational modifications that are required for targeting of the enzyme. GAD65 is targeted to the Golgi apparatus and to synaptic vesicles in the presynaptic terminal [33, 34]. Cysteines 30 and 45 of GAD65 undergo posttranslational palmitoylation [35, 36], which is required for targeting GAD65 from the Golgi to presynaptic clusters [37, 38]. The first four serine residues of GAD65 undergo phosphorylation, and are associated with the insoluble membrane-bound form of GAD65 [38]. Studies show that the first 69 amino acid residues in GAD65 and GAD67 are not necessary for homodimerization of both the enzymes [28, 42]. However, the N-terminal region of GAD65 is involved in heterodimerization with GAD67 [41]. The N-terminal region of GAD67 also appears to be important for GAD65-independent trafficking and subcellular localization of GAD67 [39, 40].

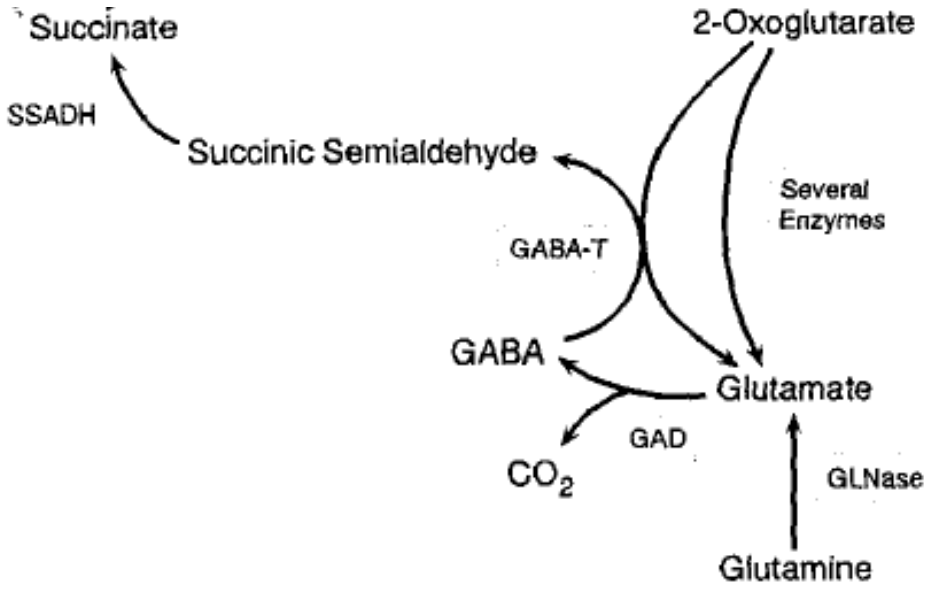


Figure 1: *GABA metabolism*. Adapted from [8]. Production of GABA by the GABA shunt pathway of the TCA cycle involves conversion of oxyketoglutarate (2-oxoglutarate) to glutamate, which is then converted to GABA by GAD. For GABA disposal, GABA-T and SSADH catabolize GABA into succinate. This pathway occurs in the cellular compartment of the neuron.

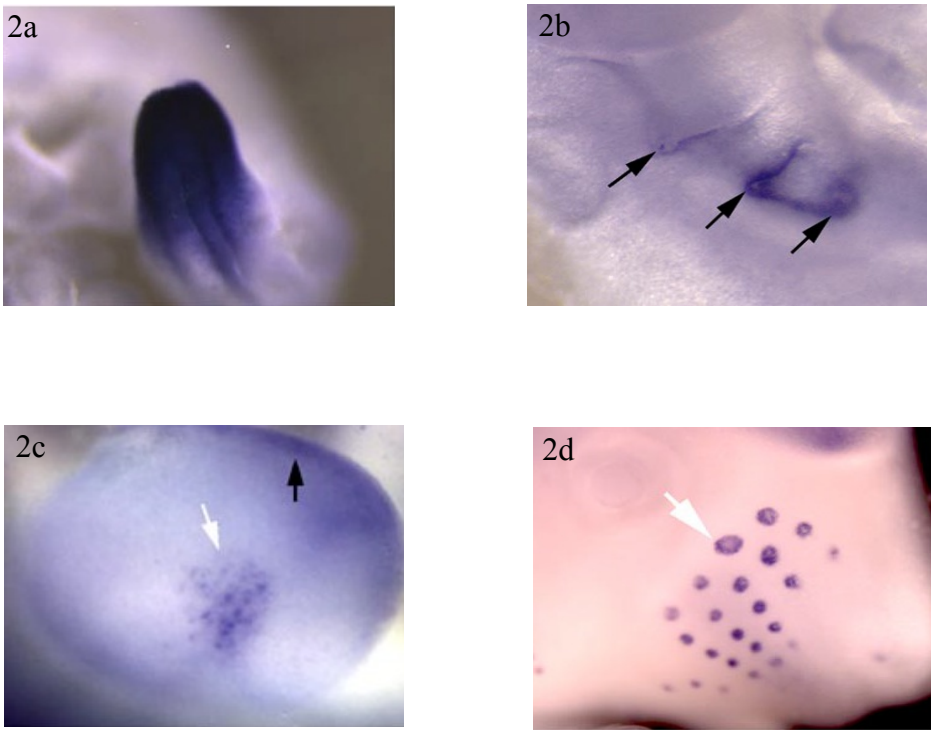


Figure 2. *Non-neuronal expression of* gad1 *mRNA in mouse embryo.* Adapted from [22]. 2a: *gad1* expression in the tail bud at E.9.0. 2b: *gad1* expression in the pharyngeal pouches at E.9.5. 2c: *gad1* expression in the forelimb (white arrow) and in the apical ectodermal ridge (black arrow) at E.10.5. 2d: *gad1* expression in the vibrissae placodes at E.12.5.

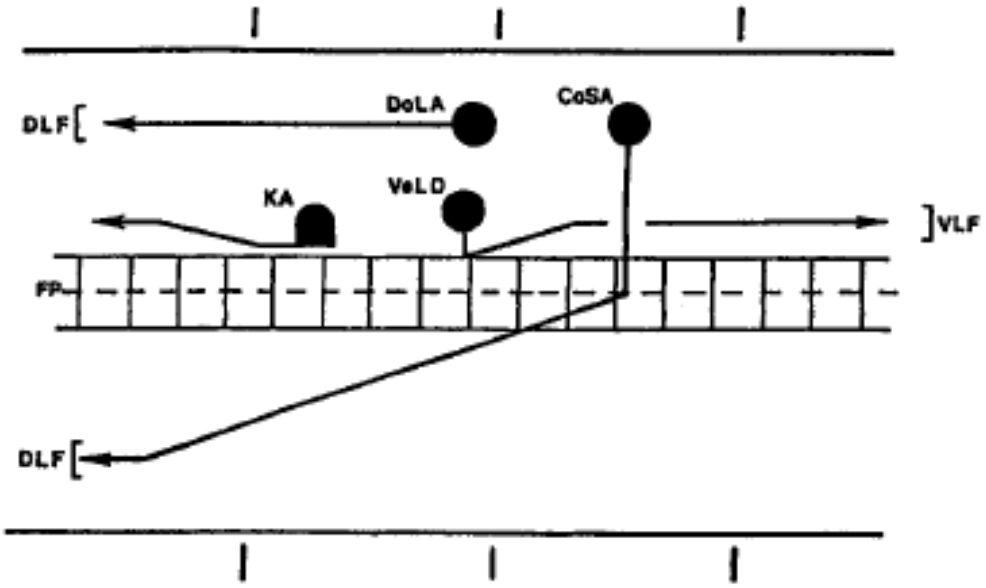


Figure 3. *Schematic of GABA expressing neurons in spinal cord of 22 hpf zebrafish embryo*. Adapted from [26]. Spinal cord represented in an 'open book' configuration as if it is cut longitudinally along the dorsal midline and flattened. GABA immunoreactivity seen in CoSA, DoLA, VeLD and KA neurons, as well as dorsal longitudinal fasciculus (DLF) and ventral longitudinal fasciculus (VLF) axons. FP indicates midline floor plate cells and the dashed line indicates ventral midline.

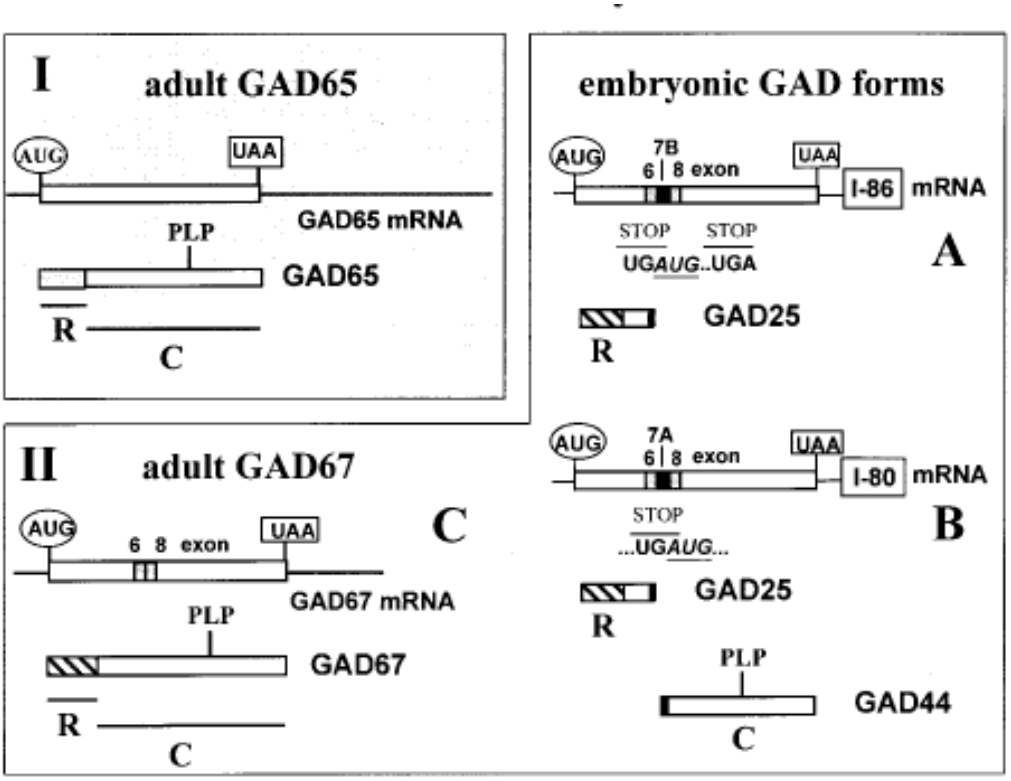


Figure 4. *Isoforms of GAD.* Adapted from [2]. (I) Adult GAD65 (*gad2*) has only one known isoform. (II) GAD67 (*gad1*) has three known isoforms: two embryonic isoforms that are 25kDa and 44kDa in size arising due to the insertion of exons 7a and 7b, and the full length isoform.

CHAPTER 2

CHARACTERIZATION OF *GAD1* AND *GAD2* EXPRESSION IN ZEBRAFISH[1]

[1] Hariharan, Vani and Lauderdale, James D. To be submitted to Developmental Neurobiology.

Abstract

In the developing rodent embryo, in addition to the full length form, GAD67 has two other splice variants arising due to the insertion of an embryonic exon. The existence of these variants has not conclusively been confirmed in the zebrafish. We tested to see if there was an embryonic exon insertion the same region in the zebrafish *gad1* mRNA through RT-PCR and showed that the embryonic exon is not inserted into the same region as in the rodent. Additionally, we show the possible existence of a previously undescribed splice variant of *gad1*.

In the rodent brain, GAD65 and GAD67 have different expression patterns as well as subcellular localization in the neurons. We show differences in the expression pattern of the GAD65 and GAD67 orthologs in the zebrafish embryo.

Introduction

GAD has two main functional domains: the N-terminal domain and the C-terminal domain. The C-terminal domain is the catalytic domain of the proteins [2, 18, 19, 28]. The N-terminal domain for human GAD65 is composed of residues 1-95, and the N-terminal domain for human GAD67 is composed of residues 1-101. GAD requires a cofactor pyridoxal phosphate (PLP) for its activity [8, 18, 29]. GAD interacts with PLP at the C-terminal domain through the motif NPHK, which is highly conserved throughout the PLP binding protein superfamily [18, 29]. From studies in a culture system, the N-terminal domains of the two proteins appear to be responsible for the differential subcellular distribution. The N-terminal domain of human GAD65 undergoes posttranslational modifications that are required for targeting of the enzyme. GAD65 is targeted to the Golgi apparatus and to synaptic vesicles in the presynaptic terminal [33,

34]. Cysteines 30 and 45 of GAD65 undergo posttranslational palmitoylation [35, 36], which is required for targeting GAD65 from the Golgi to presynaptic clusters [37, 38]. The first four serine residues of GAD65 undergo phosphorylation, and are associated with the insoluble membrane-bound form of GAD65 [38]. The N-terminal region of GAD67 also appears to be important for GAD65-independent trafficking and subcellular localization of GAD67 [39, 40].

In this study we have characterized the expression patterns of *gad1* and *gad2* in embryonic zebrafish by in situ hybridization, as well as antibody labeling. We used antibodies that specifically recognized zebrafish GAD65 and GAD67. The antibody labeling shows that although GAD65 and GAD67 are mostly co-expressed in the embryonic zebrafish brain, there are subtle differences in expression of the two proteins at the cellular level. We have also tried to identify the different isoforms of GAD67 that have been described in the mouse and rat in the zebrafish by RT-PCR. We did not find evidence for the presence of the embryonic exon in the developing zebrafish. However, we think that we have discovered another splice variant of GAD67 (*gad1*) that has not been described before in the literature.

Methods and Materials

Bioinformatics analysis of GAD sequences in zebrafish, mouse and human

Using publically available gene, transcript and protein data, bioinformatics analysis was carried out on *gad1* and *gad2* of zebrafish, mouse and human. Pairwise sequence alignment was carried out using the EMBOSS Needle global alignment algorithm and LALIGN local alignment algorithm of the EMBL-EBI. The gene and protein structures of zebrafish *gad1* and *gad2* were determined by comparing the

transcript sequences with the respective genomic sequences and marking the exon-intron boundaries using the MacVector software (MacVector, Inc., Cary, NC).

RNA extraction and cDNA synthesis

Total RNA from 3dpf embryonic zebrafish was extracted using the TRIzol Reagent (Ambion, Life Technologies, Grand Island NY) following the standard protocol from Ambion. First strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA).

In Situ hybridization

Probe Synthesis. PCR was used to amplify the first 600-800 bp of *gad1* and *gad2* cDNA in order to make digoxigenin (DIG) labeled probes for hybridization. The primers were designed with SP6 and T7 promoter sequences as 5' overhangs in the forward and reverse primers, respectively (Invitrogen, Life Technologies, Grand Island NY). The T7 minimum promoter sequence is 5' TAATACGACTCACTATAGGGAGA and the SP6 minimum promoter sequence is 5' ATTTAGGTGACACTATAGAAGNG.

The primer sequences for *gad1* were:

Gad1 IS LP2: 5' GAGCGTCTTCTGCACCTTCTTCC

Gad1 IS RP2: 5' AGATTCTGACCCCGTATTTCAGC, amplifying the first 565 bp of *gad1* cDNA.

The primer sequences for *gad2* were:

Gad2 IS LP2: 5' GAGGTGTCTCTGTCGCTGCTCTG

Gad2 IS RP1: 5' AGACTGGGATGCGCAGTTTTTAT, amplifying the first 600 bp of *gad2* cDNA.

PCR amplification was carried out on total cDNA synthesized from 3dpf zebrafish using the primers containing the SP6 and T7 promoter regions (see above for sequences). The PCR product obtained was purified using the QIAquick PCR Purification Kit (QIAGEN). Antisense and sense probes were synthesized from the purified PCR products. In vitro transcription was carried out using DIG RNA Labeling Kit (SP6/T7) from Roche Applied Science (Roche Applied Science, Indianapolis, IN) that labeled UTP with digoxigenin. To obtain antisense probes, the T7 polymerase was used and to obtain sense probes, the SP6 polymerase was used. The probes were purified by mixing 3.1 μl 1M Lithium Chloride solution and 90 μl of 100% ethanol (for a 20 μl transcription reaction volume) and incubating at -80 ^{o}C overnight and centrifuging. 2 μl of 1 mg/mL glycogen was used as a carrier for the labeled RNA probes. To test the labeling efficiency of the sense and antisense probes, serial dilutions of the probes were made up to 1:10,000 dilution of the probes and detected by dot blot on nitrocellulose membrane by exposing the membrane to nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt (BCIP) (Roche Applied Science, Indianapolis, IN) substrates and incubating at 37 ^{o}C till the color developed.

Hybridization and detection. In situ hybridization was carried out in 15-20 3dpf wild type WIK zebrafish embryos. The chorions around the embryos were removed and the embryos were fixed in 4% Paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS) for 1 hour at room temperature (RT). The fixed embryos were dehydrated in methanol-PBS series and stored in 100% methanol at -20 ^{o}C till hybridization. The dehydrated embryos were rehydrated in a PBS methanol series and washed 5x 5 min in 1X PBSTw (with 0.01% Tween-20 detergent) made in RNAse-free diethylpyrocarbonate

(DEPC) treated water. The embryos were then incubated in pre-hybridization solution for 2 hours at 70 ^{o}C. The sense and antisense probes were used at a concentration of 1:200 μl in pre-hybridization solution. The embryos were incubated with the diluted probes overnight at 70 ^{o}C. Following hybridization, the embryos were washed with 2X sodium citrate solution (SSC) 2x 10 min at 70 ^{o}C and 0.2X SSC 2x 30 min. After these, the embryos were washed in 1X PBSTw for 5x 10 min at RT. Following the washes, the embryos were incubated in blocking solution containing 1% bovine serum albumin (BSA) and 5% heat inactivated sheep serum in 1X PBSTw for 2 hours at RT. The Anti-Digoxigenin-AP Fab Fragments (Roche Applied Science, Indianapolis, IN) was pre-absorbed on 1dpf WIK zebrafish embryos (treated the same was as embryos for in situ hybridization) for 1 hour at RT. The pre-absorbed anti-DIG antibody was used at a concentration of 1:3000 μl in the blocking solution. After blocking, the embryos were exposed to the diluted anti-DIG overnight at 4 ^{o}C in the dark. After the overnight incubation, the embryos were washed with 1X PBSTw for 6x 15 min at RT. The embryos were then washed with alkaline phosphatase buffer for 2x 5 min at RT in the dark. For the color reaction, the substrates NBT/BCIP were diluted at a concentration of 4.5 μl/ 3.5 μl in the alkaline phosphatase buffer. The embryos were exposed to the NBT/BCIP substrate solution in the dark for up to 3.5 hours (with constant checking for the color development). To stop the color reaction, the embryos were washed with 1X PBSTw and dehydrated with methanol. The reaction was stopped at 1 hour intervals by removing about 5 embryos at each time point and washing with 1X PBSTw. The yolks were carefully removed from the embryos using dissection needles. The embryos were then cleared in glycerol-PBSTw series and stored in 100% glycerol. The embryos were

mounted on bridged glass slides (made by fixing 2 coverslips on either side of the glass slide, leaving some space for mounting the embryo) and imaged under a Zeiss Axio Imager. D2 microscope with a Zeiss Axio HRc camera. The software used was Zeiss AxioVision Rel. 4.8.

Immunofluorescence

Craniotomy of 30 dpf and adult zebrafish. The 30 dpf and adult zebrafish were anaesthetized using MS-222 (commonly known as Tricaine) (A5040, Sigma-Aldrich, St. Louis MO) and euthanized by decapitation. The heads were placed in ice cold 1X PBS for dissection. The craniotomy was performed using two pairs of fine forceps under the dissecting microscope by carefully removing the cranium and the surrounding muscular tissue. The brains were gently removed and placed in 1.5 mL Eppendorf tubes.

Cryosections. 3dpf wild type WIK zebrafish embryos, 30 dpf and adult zebrafish brains were fixed in 4% PFA in 1X PBS for 30- 45 min at RT. After fixation, the embryos were washed 3x 10 min in 1X PBS at RT. The embryos were dehydrated in 5% sucrose solution in 1X PBS for at least 1 hour at RT and then in 15% sucrose solution in 1X PBS for at least 1 hour. The embryos were stored in 15% sucrose solution at 4 ^{o}C till sectioning. The embryos were embedded in OCT (Tissue-Tek) 20% sucrose mixture (1:2 ratio of 20% sucrose: OCT) and flash-frozen in a slurry of dry ice and 2-methylbutane or placed in -80 ^{o}C till the blocks were hardened. Transverse cryosections 10 μm thick of 3dpf zebrafish brains were taken on a cryostat. Serial sections were then used for antibody labeling.

Antibody labeling of cryosections. To dissolve the embedding OCT sucrose from the sections, the slides were incubated in 1X PBS at 37 ^{o}C for 10 min or till the medium

was dissolved. A blocking solution of 1% BSA was made in 1X PBS and the sections were blocked for 1 hour at RT. The primary antibodies were diluted in the blocking solution. The antibodies used were rabbit polyclonal anti-GABA antibody (A2052, Sigma-Aldrich, St. Louis MO) used in a dilution of 1:1500 μl, rabbit polyclonal anti-GAD65+GAD67 (ab11070, Abcam, Cambridge MA) used in a dilution of 1:1000 μl, rabbit polyclonal anti-GAD-1 (IN) (55771, AnaSpec Inc., Freemont CA) used in a dilution of 1:50 μl and rabbit polyclonal anti-GAD-2 (IN) (55772, AnaSpec Inc., Freemont CA) used in a dilution of 1:50 μl. The antibody anti-GAD-1 (IN) is specific for GAD67 and the antibody anti-GAD-2 (IN) is specific for GAD65. The sections were exposed to the anti-GABA antibody for 1 hour at RT and to the other antibodies overnight at 4 ^{o}C. After exposing to the antibodies, the sections were washed with 1X PBS for 3x10 min at RT. The anti-rabbit secondary antibody conjugated with a fluorophore (Alexa Fluor 488 or 594) (Invitrogen, Life Technologies, Grand Island NY) was diluted in the blocking solution and used at a dilution of 1:1000 μl. Following the washes after exposure to the primary antibodies, the sections were incubated with the secondary antibodies for 30 min as RT in the dark. After incubation with the secondary antibody, the sections were washed with 1X PBS for 3-5x 10 min. The nuclei were counterstained with diamidino-2-phenylindole (DAPI) and coverslips were fixed on the slide using the VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame CA). The slides were imaged on a Zeiss Axio Imager. D2.

RT-PCR to determine splice variants of gad1

To identify the embryonic insertion between exons 6 and 8, two primer sets were designed to amplify the sequences between exons 6 and 8 for the zebrafish *gad1* cDNA. The primer sequences were:

Emb spl. LP: 5' TCGACAGATCCACCAAAGTG

Emb spl RP1: 5' GAAGAGCGCATCTCCATCTC

Emb spl RP2: 5' GCAGACATGCCTTTGGTTTT

The LP was designed to bind in exon 5, RP1 in exon 7 and RP2 in exon 8 of *gad1* cDNA. LP used with RP1 gave an amplicon of 350 bp and LP used with RP2 gave an amplicon of 440 bp.

Total RNA was extracted from 1dpf, 2dpf and 3dpf wild type WIK zebrafish embryos and converted to cDNA. PCR was then carried out on the 1dpf, 2dpf and 3dpf zebrafish cDNA using these two primers. The result of the PCR was determining by carrying out gel electrophoresis on a 1% agarose gel dissolved in 1X Tris-acetate-EDTA (TAE) buffer. The gel was run at 100-110V and the running buffer used was 1X TAE. 1 μl of Ethidium Bromide was added to the agarose gel, to detect the bands. A 100 bp DNA marker was added to determine the size of the PCR products.

Primers were designed to amplify the region between exons 1 and 5 of the zebrafish *gad1* cDNA. The primer sequences were:

GAD67 LP: 5' TATTTAGGTGACACTATAGAGAGTCTCGCAGCAACATCA

GAD67 FP3: 5' TAATACGACTCACTATAGGGGGATCTGTCGAAGGTCTTGC

The amplicon was ~560 bp in length.

To confirm if the *gad1* variant in which exon 3 was skipped, primers were designed to span the exon 2-exon 4 splice junction. Three left primers were designed to bind to exon 2- exon 4 junction within a few nucleotides of each other and the right primer was designed to bind to exon 5. The primer sequences were:

67 alt spl LP1: 5' TATTTAGGTGACACTATAGCAACCTGCCACAAGTTTCCT

67 alt spl LP2: 5' TATTTAGGTGACACTATAGCCTGCCACAAGTTTCCTGCA

67 alt spl LP3: 5' TATTTAGGTGACACTATAGGACAACCTGCCACAAGTTTC

FP3: 5' TAATACGACTCACTATAGGGGGATCTGTCGAAGGTCTTGC

The amplicon sizes were 298 bp, 295 bp and 300 bp for 67 alt spl left primers 1, 2 and 3 with the right primer FP3, respectively.

3dpf wild type WIK zebrafish cDNA was used for the PCR reaction.

Results

Bioinformatics comparison of zebrafish, mouse and human gad sequences

Bioinformatics analysis of gene and mRNA sequences of *gad1* and *gad2* confirmed that the zebrafish genome has two *gad* genes. *Gad1* is located on chromosome 6 and *gad2* is located on chromosome 24 in zebrafish [14]. During our search for *gad* sequences, we encountered a zebrafish GAD1-like gene. Comparison of the GAD1-like gene with the established zebrafish *gad1* and *gad2* sequences showed that the GAD1-like gene is the same as *gad1*. Using publically available genome and transcript data and the MacVector software, we determined the gene structures of the *gad1* and *gad2* by comparing the transcript sequences with the genomic sequences. The gene structures of *gad1* and *gad2* are similar. *Gad1* has 17 exons, and *gad2* has 16 exons. Two translation start sites were identified for *gad1*. In addition to *gad1*'s start site on exon 2,

bioinformatics analysis uncovered a putative start site towards the end of exon 3. In contrast, *gad2* had only one translation start site. Pairwise sequence comparison of zebrafish, mouse and human *gad* sequences showed that *gad1* mRNA and protein sequences of zebrafish had higher homology with *gad1* sequences of mouse and human than with *gad2* sequences of zebrafish. The N-terminal domains is less conserved in all the three species, and has low percentage identity, compared to the highly conserved C-terminal domains. Also, the C-terminal domains of the zebrafish *gad1* and *gad2* show higher homology with the C-terminal domains of *gad1* and *gad2* of human and mouse respectively, than with each other. The results of these comparisons is tabulated in Table 1, and also represented by Figure 5.

The proteins GAD65 and GAD67 have two functional domains: the N-terminal domain, and the C-terminal domain. The C-terminal domain contains the cofactor binding site and is the catalytic domain. Zebrafish GAD65 is composed of 583 amino acids and GAD67 is composed of 587 amino acids. The predicted molecular weight of zebrafish GAD67 is 66.155 kDa and that of zebrafish GAD65 is 65.458 kDa. From bioinformatics analysis, we determined that the PLP binding site is present in exon 13 of GAD67 and in exon 12 in GAD65. The N-terminal domain of GAD67 is composed of 94 residues, ending at exon 4, and the N-terminal domain of GAD65 is composed of 93 residues, ending at exon 3 [Figure 6]. When compared with the known posttranslational modifications on the N-terminal domain of the human GAD65, the posttranslational modifications of zebrafish GAD65 occupied very similar positions. The phosphorylation of the first 4 serines in the human GAD65 occupy positions 3, 6, 10 and 13, and these serines are all found on exon 1. Cysteines 30 and 45 of human GAD65 undergo

palmitoylation, and both are found on exon 2. In the zebrafish GAD65, the cysteines that are palmitoylated occur at positions 29 and 45, both found on exon 2. However, only the first three serines of zebrafish GAD65 are present on the first exon. The fourth serine is present in exon 2 [Figure 7]. The protein structure of human and zebrafish GAD65 are highly similar in terms of the exon boundaries.

Expression of gad1 and gad2

Antibody labeling of 3dpf zebrafish brain for GABA and GAD (using the antibody that recognizes both GAD67 and GAD65) confirmed that GABA and GAD have similar expression patterns, and are mostly co-expressed in all the regions of the brain. According to previous studies, at 16-24 hpf, GABA labeled neurons found in the telencephalic nucleus, nucleus of the tract of post optic commissure (nTPOC) of the ventral diencephalon, in the nucleus of medial longitudinal fascicle (nMLF) of the midbrain, as well as clusters of cells in the hindbrain and spinal cord [14, 23-25]. Our results from antibody labeling of the 3 dpf zebrafish brain confirmed that the regions expressing GABA and GAD were similar to the 1dpf and 3 dpf zebrafish brain [Figure 8] [14, 24, 25].

Use of the antibodies specific to GAD67 and GAD65 revealed that though the labeling of GAD67 and GAD65 showed significant similarities, there were also differences in the expression patterns. In serial sections that were labeled with anti-GAD-1 (IN) and anti-GAD-2 (IN), GAD65 (*gad2*) appeared to be expressed in a few cells that were not labeled with anti-GAD-1 (IN) (specific for GAD67 or *gad1*) in the 3 dpf brain. Labeling of 30 dpf and adult zebrafish brains with anti-GAD-1 (IN) and anti-GAD-2 (IN) also showed significant differences in expression in serial sections. The anti-GAD-1 (IN)

labeled cell bodies and axons, whereas the anti-GAD-2 (IN) antibody showed specific punctate staining around the cell bodies and strong labeling in the axons [Figure 9]. In situ hybridization showed that *gad1* and *gad2* expression patterns in the 1 dpf embryonic zebrafish brain were similar, except in the caudal hindbrain. *Gad1* expression was detected in the neurons in the rhombomeric region behind the otic vesicle, whereas *gad2* expression was not detected in those neurons. *Gad1* was expressed in the neurons in the rhombomeres 2 and 3, as well as the neurons above the first two somites, and *gad2* was not [Figures 10A & 10A']. This confirmed the results from previous studies [14].

In the spinal cord, GABA is expressed in four types of neurons: commissural secondary ascending (CoSA), dorsal longitudinal ascending (DoLA), ventral longitudinal descending (VeLD) and Kohmer-Agduhr (KA) [14, 26]. RNA in situ hybridization of *gad1* in the 1 dpf zebrafish spinal cord showed strong labeling in these neurons, whereas in situ hybridization for *gad2* showed very low labeling in these neurons [Figures 10B & 10B']. This confirmed the mRNA expression of *gad1* and *gad2* in 24 hpf zebrafish embryos from previous studies [14].

Isoforms of gad1 in zebrafish

In the rodent, GAD67 has two isoforms that are present during development. The two isoforms are 25 kDa and 44 kDa in size and arise due to the insertion of an 80 bp and 86 bp embryonic exon. This insertion was shown to be between exons 6 and 8 in the rodent embryo [2, 6, 30-32]. In the zebrafish, according to previous studies, the 25 kDa isoform is expressed in the retina of adult zebrafish [32]. In this study, in order to identify the embryonic splice variants of *gad1*, we designed primers spanning exons 6 and 8 [Figure 11A]. We performed PCR on total cDNA extracted from 1, 2 and 3 dpf zebrafish

embryos. If the zebrafish also have an embryonic insertion between exons 6 and 8, the PCR results would show two bands in the agarose gel: one band that is from the full-length splice variant, and another band that is ~80 bp higher than the first band, corresponding to the embryonic exon. Our results do not show the band that is higher by ~80 bp [Figure 11A'].

However, we believe that we have found a new splice variant of *gad1* in which exon 3 is skipped. PCR results of primers GAD67 LP and GAD67 FP3 showed the presence of an additional band that was ~100 bp smaller than the band of the expected size. Sequencing of this band showed that exon 3 was completely skipped [Figures 11B & 11B']. PCR performed on 3 dpf zebrafish cDNA with the primers designed to bind to exon 2- exon 4 junction of *gad1* resulted in bands of expected size on the agarose gel shown in figure 11C'.

Conclusion

From the bioinformatics analysis of the structures of the *gad* mRNA and proteins in zebrafish, mice and humans, we can conclude that across the vertebrate system, *gad1* is strongly conserved, as well as *gad2*. However, *gad1* and *gad2* show lesser homology in the same system, than *gad1* or *gad2* across the species. Even the mRNA and protein structures, including the exon structure, posttranslational modifications, and the catalytic domains are well conserved across the vertebrates.

From previous studies as well as the results from the in situ hybridization and antibody labeling, it is clear that the *gad1* and *gad2* expression patterns have subtle differences. From previous studies of subcellular expression of *gad1* and *gad2*, it is known that GAD67 is mainly present in cell bodies, and GAD65 is mainly present in the

axon terminals in synaptic vesicles [8, 15, 16]. Our results from the antibody labeling of 30 dpf and adult zebrafish brain seem to correlate with the previously described subcellular localization patterns of GAD65 and GAD67. It is possible that the punctate staining seen with the anti-GAD-2 (IN) antibody is in the synaptic vesicles. Also, from previous studies, GAD65 is also targeted to the vesicles in the Golgi apparatus in the cell body of the neurons [21, 28, 33-38]. It is equally possible that the punctate staining seen with the anti-GAD-2 (IN) antibody is the GAD65 that is bound in vesicles in the Golgi. However, without closer analysis, it is not possible to conclude that the punctate staining is associated with the vesicles in the Golgi and the synapses of the neurons. An important experiment to determine that would be to co-label GAD65 with a Golgi marker and a synapse marker such as Synapsin. Coexpression of GAD65 and either the Golgi marker or the synaptic marker will confirm the presence GAD65 in vesicles in the zebrafish brain. This will also provide strong correlative evidence for the role of GAD65 in the production of GABA as a neurotransmitter, and for GAD67 in the production of GABA for maintaining basal levels in the CNS.

The RT- PCR experiments to determine the different isoforms of *gad1* show that with the experimental design that we followed, there is no direct evidence as yet for the embryonic splice variants of *gad1*. The absence of a second, higher weight band in the PCR amplifying the region between exons 6 and 8 of zebrafish *gad1* shows that the particular splice variants described in the mouse [6] are probably not present in the embryonic zebrafish. However, this does not prove that these splice variants do not exist in the embryonic zebrafish. It is very possible that the insertion is on another exon, or that there is a cryptic stop site that is activated in other developmental stages that give rise to

the 2kDa and the 44 kDa isoforms. Looking at earlier, and later developmental stages in the zebrafish might be useful. Also, a closer analysis of the sequence and exon-intron structure of zebrafish and mouse *gad1* in all the transcript variants will be necessary in order to rule out the absence or presence of the embryonic exon in the zebrafish.

Table 1. Comparison of *gad* sequences from human, mouse and zebrafish

	Zebrafish	Mouse	Human	% Identity
cDNA	Gad1			**34.6**
	Gad2			
	Gad1	Gad1		57.5
	Gad2	Gad2		49.5
	Gad1		Gad1	53.8
	Gad2		Gad2	48.9
Protein	Gad1			**63**
	Gad2			
	Gad1	Gad1		83.5
	Gad2	Gad2		77.9
	Gad1		Gad1	82.7
	Gad2		Gad2	78.3
	Gad1 N-terminal			**21.8**
	Gad2 N-terminal			
	Gad1 C-terminal			**71.8**
	Gad2 C-terminal			

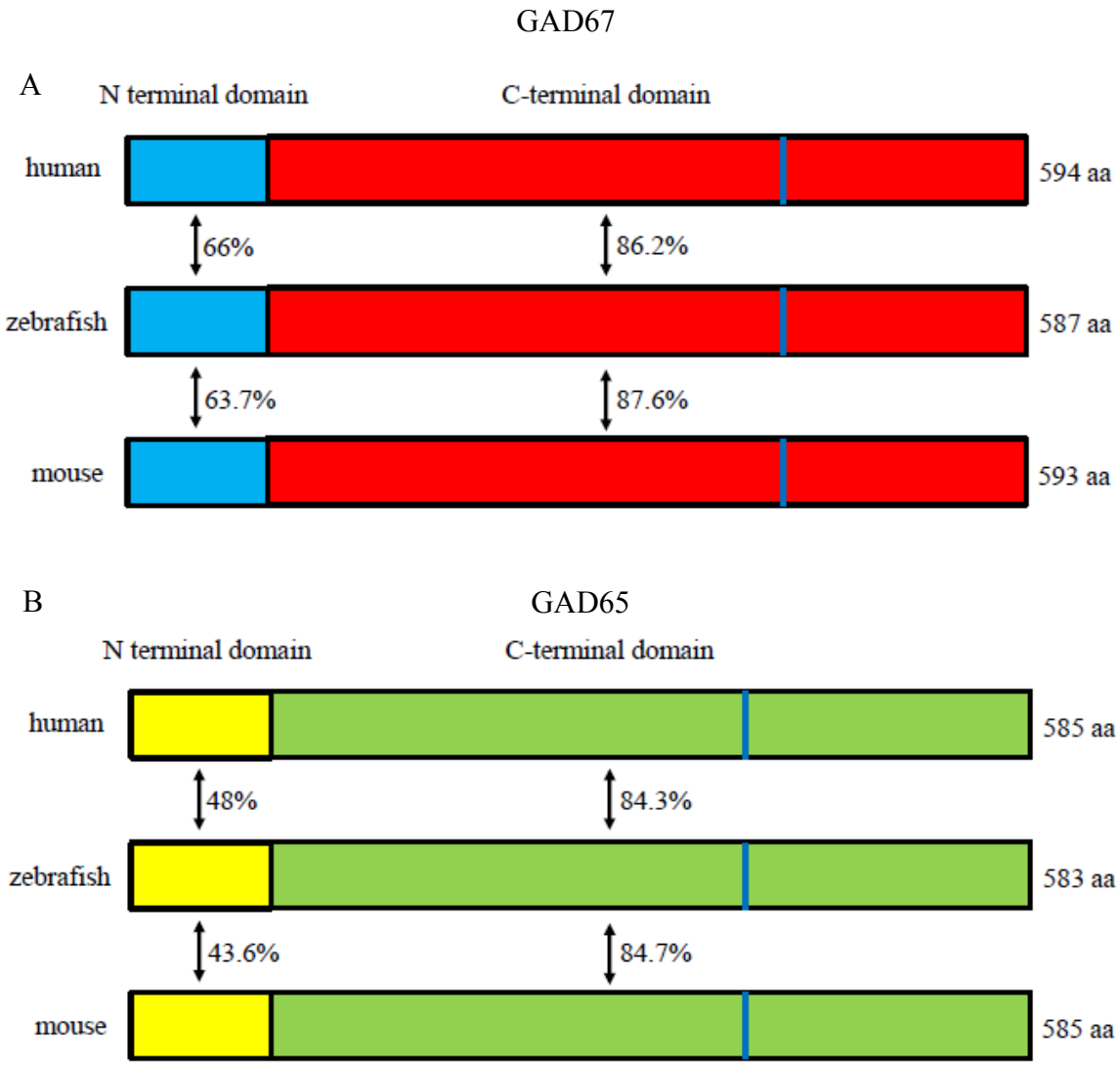


Figure 5. *Comparison of GAD protein structure in human, mouse and zebrafish.* Figure 5A shows the comparison of the N-terminal and C-terminal domains of GAD67 in zebrafish, human and mouse. Figure 5B shows the comparison of the N-terminal and C-terminal domains of GAD67 in zebrafish, human and mouse.

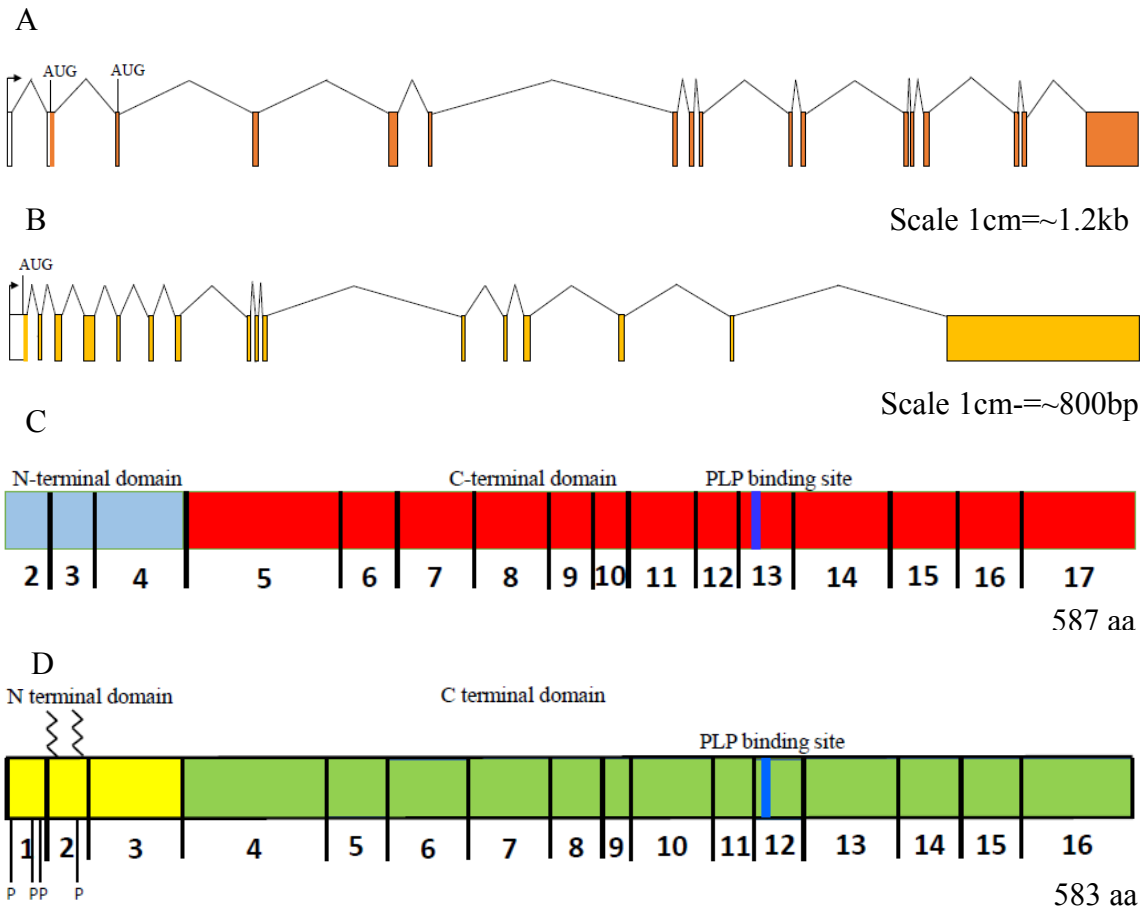


Figure 6. *Gene and protein structures of zebrafish gad1 and gad2.* Figures 6A and 6B are representations of the gene structure and splicing of the full length *gad1* and *gad2* genes, respectively. *Gad1* has a second putative start site on exon 3. Figures 6C and 6D are representations of the protein structures of GAD67 and GAD65, respectively. The cofactor binding site for GAD67 is in exon 13 and the cofactor binding site for GAD65 is on exon 12. The posttranslational modifications of the N-terminal domain of GAD65 have been mapped out in the diagram. The palmitoylation is indicated by the zigzag lines, and the phosphorylation is indicated by P.

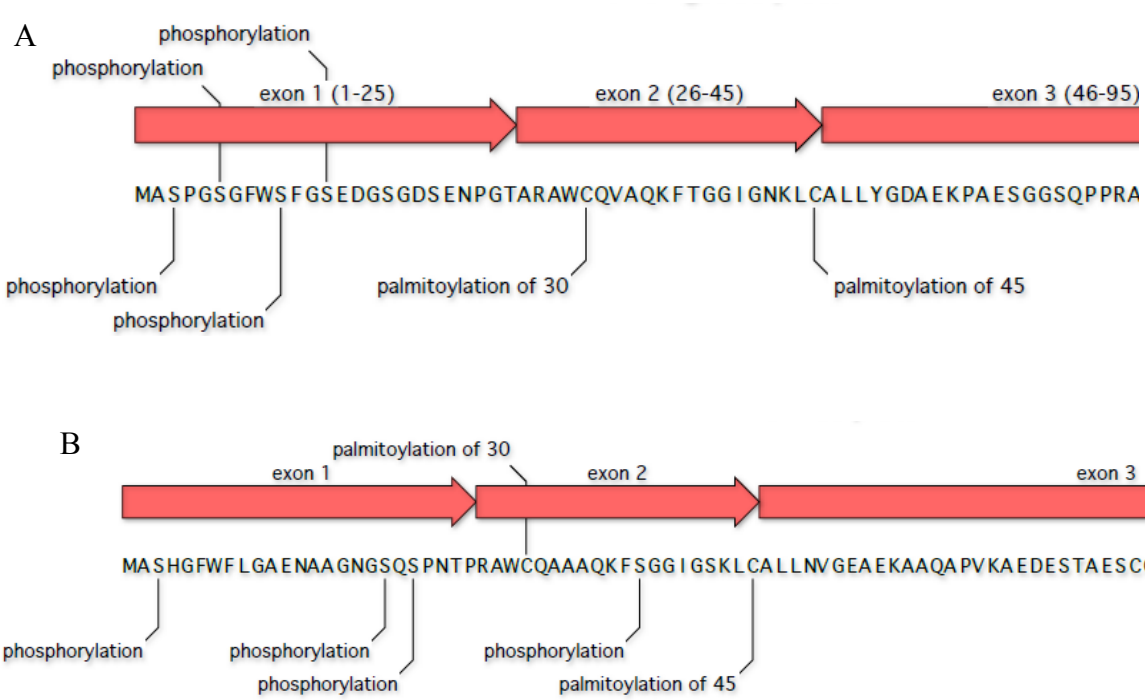


Figure 7. *Comparison of human and zebrafish GAD65 N-terminal domains.* Figure 7A shows the posttranslational modifications on the N-terminal domain of human GAD65 protein. Figure 7B shows corresponding posttranslational modifications on the N-terminal domain of zebrafish GAD65.

Figure 8. *Antibody labeling of GAD and GABA in 3dpf zebrafish brain.* Figures A, B and C are labeled for GABA and figures A', B' and C' are labeled for GAD65+67. The sections labeled for GABA and GAD are corresponding or serial sections. 8A and 8A' show labeling in the forebrain. GABA and GAD are expressed in the lateral forebrain bundle and the dorsal thalamus. 8B and 8B' show labeling in the midbrain where GABA and GAD are expressed in the nucleus of longitudinal medial fasciculus. 8C and 8C' are sections through the hindbrain, where GABA and GAD label the medulla oblongata, and show expression in alternating columns throughout the hindbrain. Scale bar for all images is 10 μm.

GABA GAD 65+67

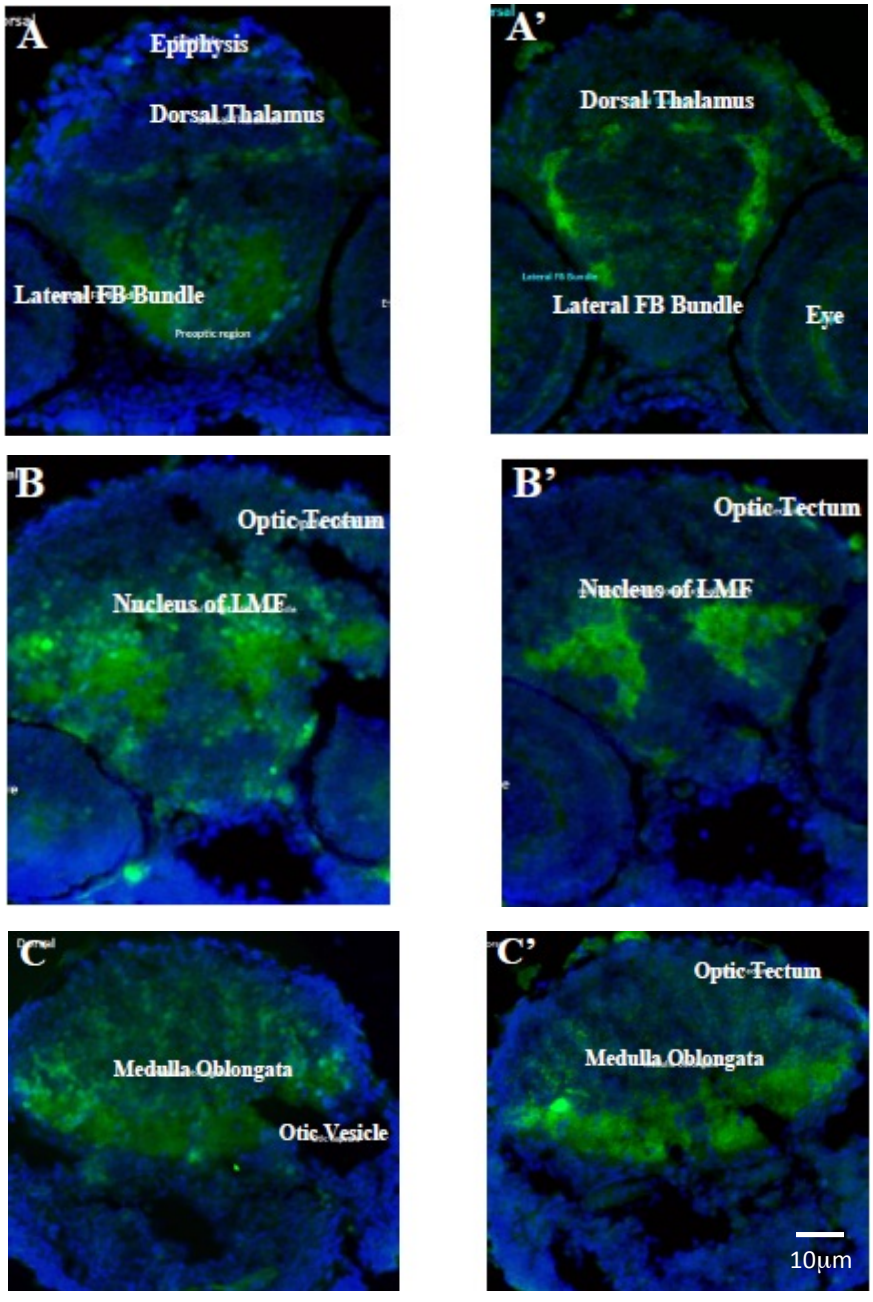
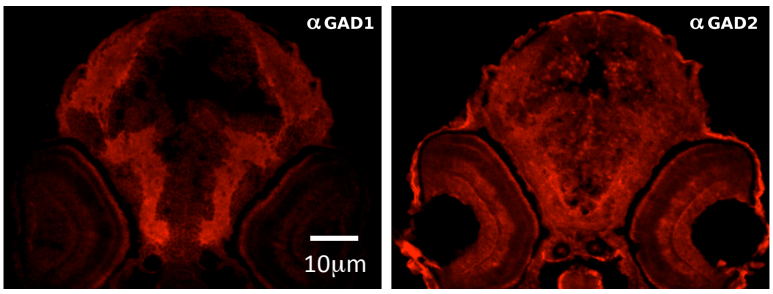


Figure 9. *Comparison of GAD65 and GAD67 expression using specific antibodies.* Figure 9A shows labeling of GAD in serial sections of 3dpdf zebrafish caudal forebrain using the anti-GAD-1 (IN) and the anti-GAD-2 (IN) antibodies. Though the post optic commissure is strongly labeled in both sections, the cells in the midline region express GAD65 but not GAD67. Figure 9B shows GAD labeling in 3 dpf, 30 dpf and adult zebrafish brain sections. Anti-GAD-1 (IN) and anti-GAD-2 (IN) antibodies label GAD67 and GAD65 in serial sections. In the 30 dpf and adult brain sections, GAD65 shows punctate staining, and strong staining in the axons, whereas GAD67 staining is seen more in the cell bodies. Scale bar for all images is 10 μm. [Staining and images done by Tyler Page].

GAD1 GAD2

A



B

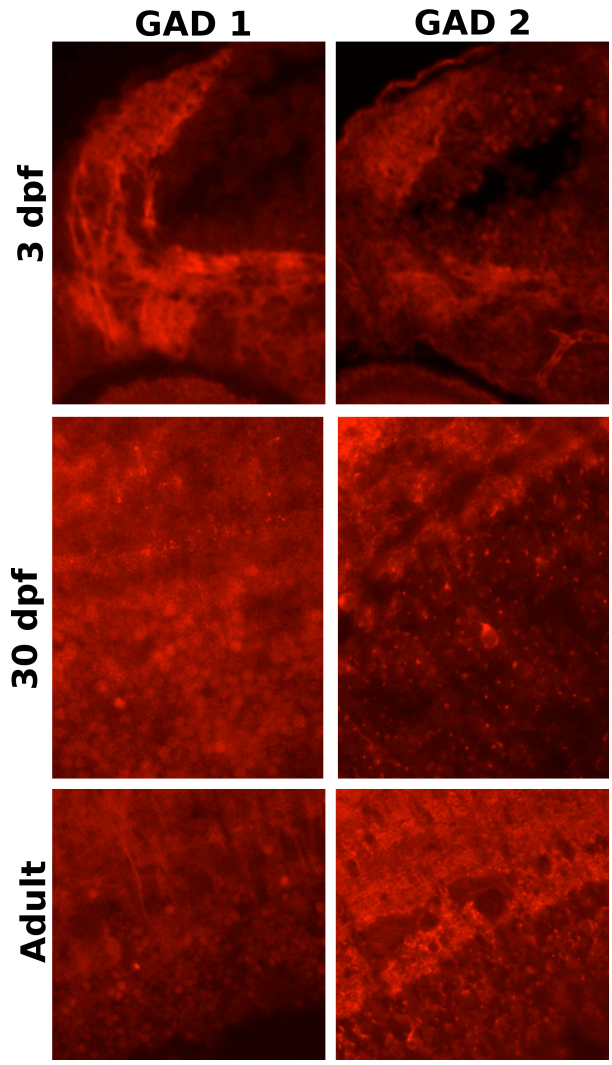


Figure 10. *RNA In situ hybridization of gad1 and gad2 in 1 dpf zebrafish embryos.* Figures 10A and 10B show *gad1* expression and 9A' and 9B' show *gad2* expression. 10A and 10A' show the expression of *gad* in the caudal hindbrain. *gad1* is expressed in the neurons in rhombomeres 2 and 3, and in the neurons above somites 1 and 2, whereas *gad2* is not. 10B and 20B' show *gad* expression in the spinal cord. *gad1* is strongly expressed in the dorsal longitudinal ascending (DoLA), commissural secondary ascending (CoSA), ventral longitudinal descending, and the Kohmer-Agduhr neurons. *gad2* is also expressed in the neurons in the spinal cord, but the expression level at 1 dpf is very low.

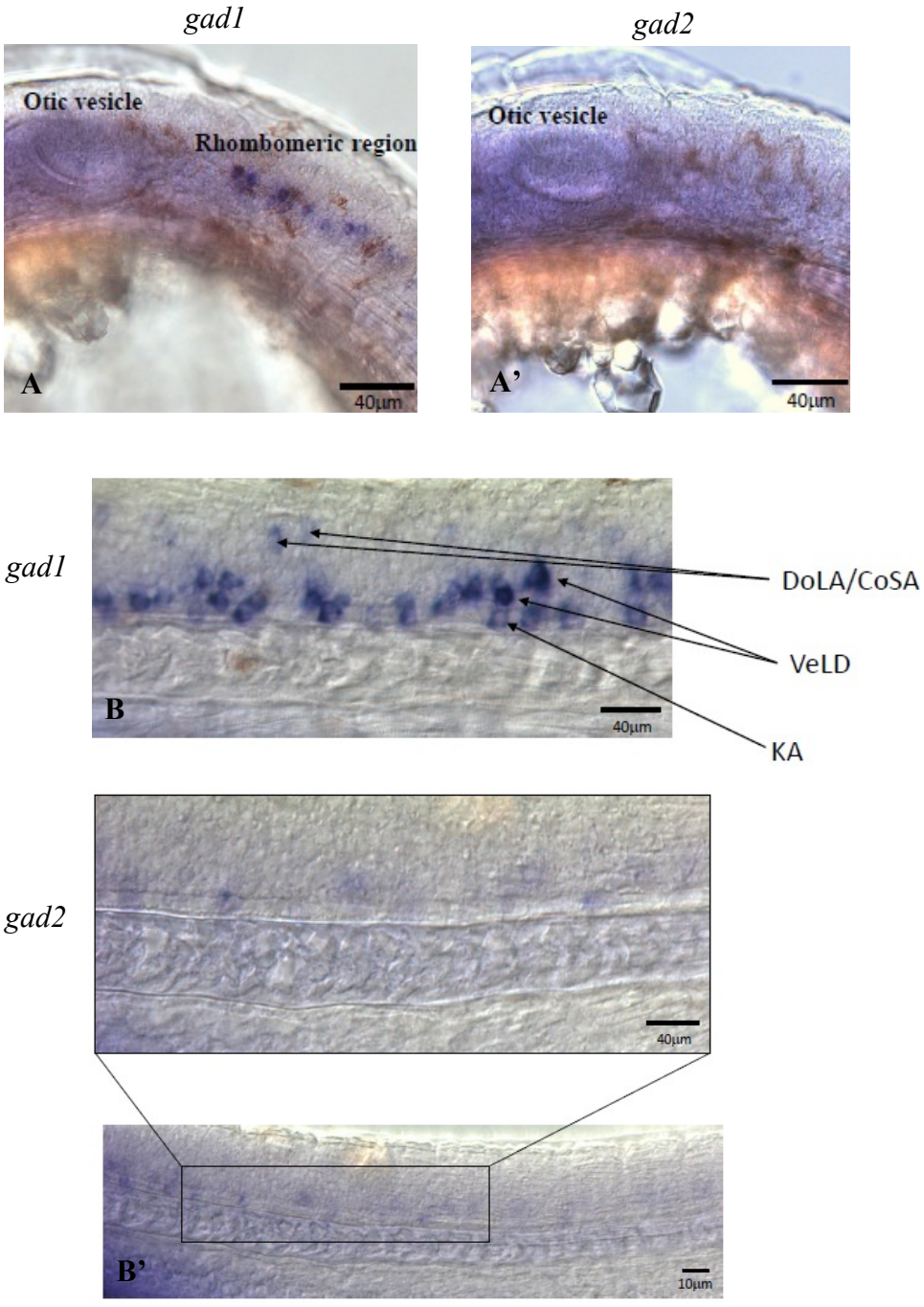
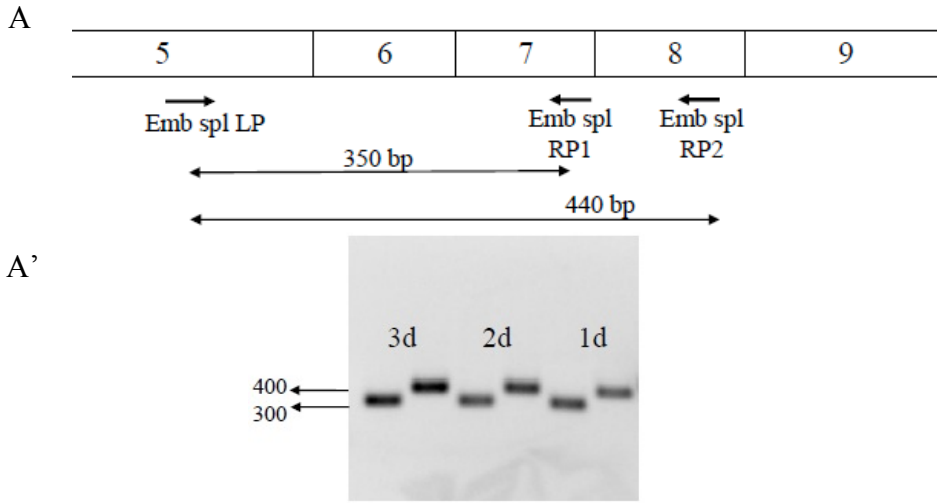
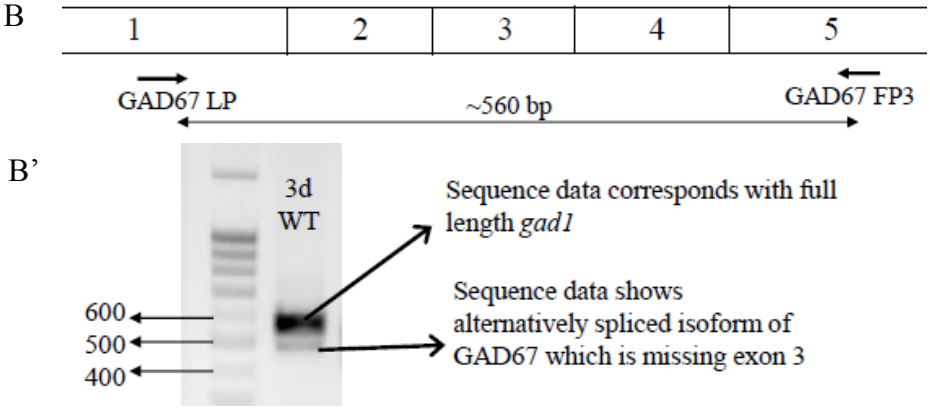
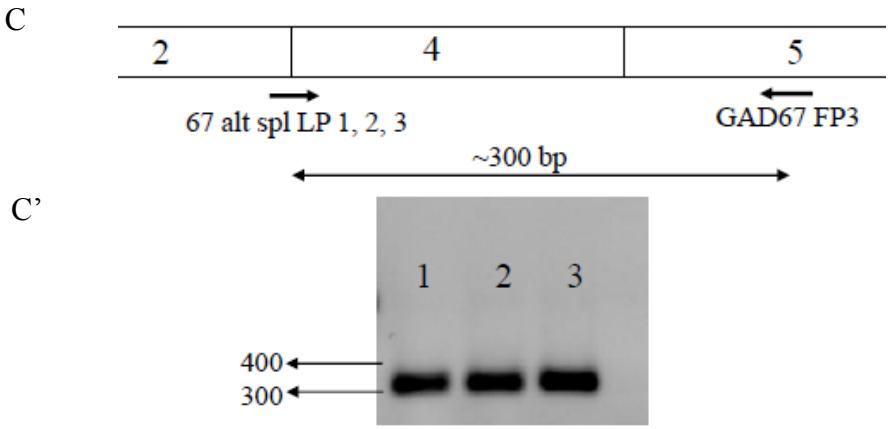

gad1
gad2
Otic vesicle
Rhombomeric region
A
40µm
Otic vesicle
A'
40µm
gad1
B
40µm
DoLA/CoSA
VeLD
KA
gad2
40µm
B'
10µm

Figure 11. *Isoforms of gad1*. Figure 11A is a schematic of the PCR to determine the embryonic splice variant of *gad1*. Figure 11A' shows that in the 1, 2 and 3 dpf embryonic cDNA, the band corresponding to the embryonic exon is absent. The first band in each developmental stage is the amplicon from the primers Emb spl LP and Emb spl RP1, and the second band corresponds is the amplicon from the primers Emb spl LP and Emb spl RP2. Figure 11B is a schematic for the PCR with the primer pair GAD67 LP and GAD67 FP3. Figure 11B' shows an additional band that is smaller than the expected 560 bp band. Sequencing of this band showed that exon 3 was missing, leading to the possibility of an additional splice variant. Figure 10C is a schematic for PCR that confirmed the splice variant of *gad1* in which exon 3 is skipped. The left primers were designed to bind to the exon 2- exon 4 junction. Figure 10C' shows that the PCR with primer pairs the 67 alt spl LP1, 2, and 3 with GAD67 FP3 all gave bands of the expected size, which is around 300 bp for all the three primer pairs.


A
5
6
7
8
9
Emb spl LP
Emb spl RP1
Emb spl RP2
350 bp
440 bp
A'
3d
2d
1d
400
300


B
1
2
3
4
5
GAD67 LP
~560 bp
GAD67 FP3
B'
3d
WT
600
500
400
Sequence data corresponds with full length gad1
Sequence data shows alternatively spliced isoform of GAD67 which is missing exon 3


C
2
4
5
67 alt spl LP 1, 2, 3
GAD67 FP3
~300 bp
C'
1
2
3
400
300

CHAPTER 3

DETERMINING THE FUNCTIONS OF GAD1 AND GAD2 IN EMBRYONIC ZEBRAFISH USING SPLICE BLOCKING MORPHOLINOS[1]

[1] Hariharan, Vani and Lauderdale, James D. To be submitted to Developmental Neurobiology.

Abstract

A decrease in GAD67 in the human brain is associated with schizophrenia and related neurological disorders. In mice, a loss of GAD65 causes spontaneous seizures. We studied the role of GAD in modulating neural activity in zebrafish. Splice blocking morpholinos were used to manipulate *gad* in embryonic zebrafish. The *gad1-gad2* double morphants showed a substantial reduction in GABA, leading to spontaneous seizure-like neural activity, which was confirmed by electrophysiology. Furthermore, knockdown of either one of the *gads* also resulted in seizure-like activity, suggesting that both isoforms are important for the normal functioning of the brain.

Introduction

The two GADs are thought to have different functions, due to the fact that they have different subcellular localization, and bind differently with the cofactor pyridoxal phosphate [1, 2, 8]. GAD65 expression is more concentrated in the terminals of the neurons, and appears to associate with synaptic vesicles [17], and is thought to produce the GABA that functions as a neurotransmitter. GAD65 is more highly regulated by the cofactor PLP than GAD67 [8, 17]. GAD67 is more evenly distributed in the neuron, perhaps a little more in the cell body, and is thought be the enzyme that is involved in the GABA shunt pathway of the TCA cycle. GAD67 is thought to produce basal levels of GABA in the brain [8, 15-17].

The loss of even one of the GAD enzymes has adverse effect on the nervous system. Dysregulation of GAD67 is implicated in the autism spectrum disorders, including schizophrenia, autism and bipolar disorders in humans [44]. A decrease in the mRNA levels of GAD67 in several brain regions including the prefrontal cortex and the

temporal cortex was seen in postmortem brain of patients diagnosed with these disorders [44]. Knockout studies for *gad1* and *gad2* in mice revealed that the two enzymes were functionally different and the presence of both alleles of one of the genes did not affect the phenotypic effects seen with the knockout of the other gene. Mice that had a homozygous mutation for *gad1* died shortly after birth due to severe cleft palate [45, 46]. In GAD67-/- mice pups at P0.5, GABA levels were reduced to 7% of the levels in the wild type pups. In GAD67+/- mice, the GABA levels were about 65% of the wild type levels. In contrast, GAD65-/- mice at P0.5 had normal levels of GABA [45]. However, two months after birth, the GAD65-/- mice developed a deficit in GABA, particularly in the amygdala and the hypothalamus. The GAD65-/- mice showed susceptibility to seizures and occasional spontaneous seizures were observed. In EEG recordings, the GAD65-/- mice showed long lasting, spontaneous seizures that was not observed in GAD65+/- mice or wild type mice [47]. The GAD65-/- mice also showed anxiety-like behavior [2, 45, 47].

In this study, we decided to test the functions of *gad* in the zebrafish embryo. We took a loss of function approach to study the individual functions of *gad1* and *gad2* by knocking out the proteins using Morpholino (MO) oligos. Morpholinos are modified antisense oligomers that have the four nucleotide bases and can be designed to complementarily bind to specific mRNA. Instead of ribose sugar, they have a morpholine moiety, and a phosphorodiamidate linkage, giving a neutral charge backbone [48, 49, 50]. Morpholinos are typically 25 bases long, and contain bases that are complementary to the mRNA that is to be knocked down. There are two kinds of Morpholinos: Morpholinos that bind to the translation start site of the mRNA, and Morpholinos that bind to exon-

intron junctions of prespliced mRNA. The MOs that bind to the translation start site prevent the RNA polymerase from binding to the AUG by steric hindrance, and prevent the protein from being translated. The MOs that bind to exon-intron junctions, prevent the splicing of that particular intron in the mRNA, resulting, in most cases, the retention of the intronic sequence in the mRNA. More than one splice blocking MO can be used, targeting different internal introns. In both cases, using the splice blocking MOs and the translation blocking MOs will result in the knockdown of protein function, giving a loss of function phenotype. The advantage of using splice blocking MOs is that the knockdown can be quantified by RT-PCR [51, 52].

In this study, we have used the splice blocking MOs against *gad1* and *gad2*. We used the Morpholinos individually, and together, to effect complete prevention of GABA synthesis. We quantified the knockdown by RT-PCR and Western blot. We saw that the Morpholinos, when used individually, as well as in conjunction, affected the behavior of the embryonic zebrafish. We confirmed abnormal and increased neural activity in the morphant zebrafish embryos by electrophysiology for the individual Morpholinos as well as the co-injections, suggesting that both proteins are essential for normal neural activity, and are non-redundant in the zebrafish.

Methods and Materials

Design of Translation Blocking and Splice Blocking Morpholinos

25bp splice blocking and translation blocking Morpholinos (MO) were designed to target *gad1* and *gad2* mRNA with the help of Gene Tools (Gene Tools, LLC, Philomath OR). The translation blocking morpholinos were designed to bind complementarily to the translation start site of the mRNA. The splice blockers were

designed to target the N-terminal domain of the two proteins. For *gad1*, the splice blocker was designed to bind to the exon 2-intron 2 junction of the mRNA. For *gad2* the splice blocker was designed to bind to the exon 3- intron 3 junction of the mRNA. The sequences of the MOs are:

Gad1 Translation Blocker: 5' GAAACCAAAACCCGTGTGATGCCAT

Gad1 Splice Blocker: 5' TGTGATTTGTGGTGATTTACCTGTT

Gad2 Translation Blocker: 5' AAGGTGCAGAAGACGCCATCAGTCC

Gad2 Splice Blocker: 5' GCGTTATCCAGAGAGACCTACTTGT

Controls for the MOs were 5 base mispair control MOs, in which 5 bases of the antisense MO sequences were changed. These MOs were designed specificity controls for the antisense MOs. The sequences of the 5-mispair specificity controls are:

Gad1 Translation Control: 5' GAtAgCAAAAgCCGTcTcATGCCAT

Gad1 Splice Control: 5' TGTcATTTcTGGTcATTTACgTcTT

Gad2 Translation Control: 5' AAGcTcCAGAAcACcCCATCAcTCC

Gad2 Splice Control: 5' GCcTTATgCAcAGAcACCTACTTcT

The mispair bases are represented in small letters and the complementary bases are represented in capital letters.

Dilution and Determination of concentration of Morpholinos

The MOs were delivered in powder form. 300 nanomol of MOs were dissolved in 300 μl of distilled deionized water to give a 1 milli Molar (1mM) stock solution. The final concentration of the MOs in milli gram per milli liters was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The MO stock solutions were diluted to a concentration of 20X in 1M HCl. The absorbance

wavelength used was 265 nm. The concentration of the stock MOs were calculated in mg/mL from the readings obtained from the spectrophotometer.

Microinjection in zebrafish embryos

The injections were done in single cell to 4-cell stage zebrafish embryos using a pressure injection system. The injection needles were made from glass capillary tubes containing filament, and the tips of the needles were broken to give approximately 1 μm diameter opening. The injection mold was made with 3% agarose. The embryos were transferred to the rows in mold using a plastic pipette. Embryos that had gone past the 4-cell stage were not used for injection. The injection solution contained the morpholino diluted in 2M KCl (to maintain an isotonic state within the embryo), Phenol Red (to visualize the injections within the embryo), and Texas Red-Dextran (to sort for injected/uninjected embryos as well as to confirm that the morpholinos have been taken up by all cells of the embryo). The MO solution was loaded in the microinjection needles by a 20 μL micropipette. The droplet size of the MO solution was adjusted to approximately 1.25 μm in order to deliver a volume of 1 nL of the MO into each embryo. The solution was injected into mineral oil and the size was measured by a micrometer under seen under a dissecting microscope. The size was increased or decreased by increasing or decreasing the injection pressure and the injection time in milli seconds. After adjusting the droplet volume to 1 nL, the zebrafish embryos on the mold were injected individually in their yolks. After injection, the embryos were gently transferred into a Petris dish by washing the mold with egg water (Salt stock solution containing 40g of "Instant Ocean" Sea Salt dissolved in 1 liter of distilled water, and diluted to a working

concentration of 60mg/mL in distilled water). The injected embryos were incubated at 28 ^{o}C until other experiments were performed.

RT-PCR

RNA extraction and cDNA synthesis. Total RNA from 3, 5 and 7 dpf wild type and morphant zebrafish embryos was extracted using the TRIzol Reagent (Ambion, Life Technologies, Grand Island NY) following the standard protocol from Ambion. The RNA was treated with TURBO DNA-*free* Kit (Ambion, Life Technologies, Grand Island NY) for genomic DNA contamination. First strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA). The cDNA was diluted 5 times before use for PCR.

Genomic DNA extraction. 7 dpf wild type zebrafish larvae were used for genomic DNA (gDNA) extraction. 20-30 larvae were transferred to a 1.5 mL Eppendorf tube. 100-150 μL of STE extraction buffer was added to the tube (STE: 0.1M NaCl, 10mM Tris-HCl pH 8, 1mM EDTA, 5% Triton X-100, final pH 7.5). 20% SDS detergent was added to give a final concentration of 1% SDS, and the contents of the tube were vortexed for about 10 s. 20 mg/mL Proteinase K was added to give a final concentration of 0.5 mg/mL of Proteinase K, and vortexed briefly. The tube was then incubated at 55 ^{o}C for 2 hours with frequent vortexing, or overnight at 55 ^{o}C. After the 2 hour or overnight incubation, the tube was centrifuged at maximum speed for 5 min at RT, and the supernatant was carefully collected into a new tube. The supernatant was centrifuged again to remove all particulate matter. 100- 150 μL of 100% isopropanol was added to the supernatant, and the tube was inverted 10-15 times, and centrifuged for 10 min at 13000- 15000 rpm at 4^{o}C. The supernatant after centrifugation was discarded very carefully so as to not lose

the pellet containing the gDNA. 200-300 μL of 70% ethanol was added to wash the DNA pellet, and the tube was centrifuged for 2 min at 5000- 8000 rpm and the ethanol was discarded. The pellet was air dried for 15-20 min, and the DNA was suspended in 50-100 μL of Tris EDTA buffer (10mM Tris-HCl pH 7.5, 1mM EDTA).

PCR. PCR was performed on 2 μL of the diluted cDNA extracted from the wild type and morphant embryos. The primers used for quantifying the knockdown of the *gads* were designed to amplify exons 1-5 of *gad1* and *gad2* cDNA. The sequences were:

For *gad1*:

GAD67 LP: 5' TATTTAGGTGACACTATAGAGAGTCTCGCAGCAACATCA

GAD67 FP3: 5' TAATACGACTCACTATAGGGGGATCTGTCGAAGGTCTTGC

The amplicon was ~560 bp in length.

For *gad2*:

GAD65 LP: 5' TATTTAGGTGACACTATAGCGTTTGGACAGGTGTGATTG

GAD65 FP1: 5' TAATACGACTCACTATAGGGCTGGGATGCGCAGTTTTTAT

The amplicon size was ~680 bp.

To confirm exon skipping in the morphants, and also that the intronic sequence was not included, additional primers were designed to amplify exon-intron sequences. For *gad1*, primers were designed to amplify exon 2-intron 2 region, and for *gad2*, primers were designed to amplify exon 3- intron 3 region.

The primer sequences for *gad1* MO splicing were:

Gad1 E2 LP: 5' CGTCTTCTGCACCTTCTTCC

Gad1 E2I2 RP: 5' TTGAAAGCAGATGATGGCTG, giving an amplicon of 716 bp with *Gad1* E2 LP.

Gad1 I2E3 LP: 5’ CTGTCTGTGCCTCATCCTGA

Gad1 E3 RP: 5’ TCTTCATGCCCAGTTTCCTC, giving an amplicon of 748 bp with *Gad1* I2E3 LP.

The primer sequences for *gad2* MO splicing were:

Gad2 E3 LP: 5’ TCTGTTAAATGTCGGGGAGG

Gad2 E3I3 RP: 5’ TGGCCCTAAAAAGAATACGA, giving an amplicon of 393 bp with *Gad2* E3 LP.

Gad2 E3I4 LP: 5’ CAATCTGCCTTTTCCCGTTA

Gad2 E4 RP: 5’ CGCGACAGCTGATCAGAATA, giving an amplicon of 574 bp with *Gad2* E3I4 LP.

The result of the PCR was determining by carrying out gel electrophoresis on a 1% agarose gel dissolved in 1X Tris-acetate-EDTA (TAE) buffer. The gel was run at 100-110V and the running buffer used was 1X TAE. 1 μl of Ethidium Bromide was added to the agarose gel, to detect the bands. A 100 bp DNA marker was added to determine the size of the PCR products.

Sequencing. The PCR products of primer pairs GAD67 LP + GAD67 FP3 and GAD65 LP + GAD65 FP1 with the morphant cDNA was purified using the QIAquick PCR Purification Kit (QIAGEN) or by excising the band from the agarose gel and performing gel extraction using the GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis MO). The purified PCR products were then sent for sequencing at Georgia Genomics Facility that is part of the University of Georgia. Single tube Sanger sequencing was performed.

Western Blot

Extracting protein. For western blot, 3, 5 and 7 dpf wild type and morphant zebrafish were used. 15-20 embryos were taken in an Eppendorf tube. 60-100 μL of RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA) with 1 mM EDTA solution and 1 mM protease inhibitor cocktail (Sigma-Aldrich, St. Louis MO) was added to the tube, and the embryos were homogenized using a small pestle. The tubes were centrifuged for 10 min at high speed and the supernatant was collected and stored at -80 ^{o}C until further use. The protein concentration was measured by a spectrophotometer and determined using the Bradford assay. 1 μL of protein was added to 1 mL of 1:5 dilution of Bradford Reagent (Bio Rad, Hercules, CA), and the absorbance was measured at 280 nm by a spectrophotometer. The protein concentration was calculated by using a standard curve in which the absorbance wavelengths of bovine serum albumin (BSA) dilutions of known concentration were used.

SDS-PAGE. SDS-polyacrylamide gels were made using an upright gel casting apparatus. The stacking gel was made at a concentration of 4% and the resolving gel was made at a concentration of 10%. 10-15 μg of each protein sample was loaded in the gel. Prior to loading the gel, the protein samples were denatured by adding equal volume of Laemmli sample buffer (Bio Rad, Hercules, CA) with 10% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and boiling in a water bath for 10 min. The denatured protein samples were loaded in the gel and the gel was run at 75 volts for 3.5-4 hours. A 1X Tris-glycine running buffer was used (10X Tris-glycine buffer: 250 mM Tris, 2 M glycine, 10% SDS) as running buffer for the SDS-PAGE. The protein bands were transferred to a 0.45 μm nitrocellulose membrane (Bio Rad, Hercules, CA) using a transfer rig that was

cooled with ice. Ice cold Towbin transfer buffer (28.8 g glycine, 6.06 g Tris base, 200 mL methanol made up to 2000 L with deionized distilled water) was used for the transfer. The transfer was carried out at 130 volts for 1-1.5 hours.

Immunodetection. After transfer, the nitrocellulose membrane was blocked with 5-10% milk block (Bio Rad, Hercules, CA) overnight at 4 ^{o}C with gently rocking. Proceeding blocking, the membrane was exposed to primary antibodies for 1 hour at RT with gently rocking. The antibodies used for immunodetection were polyclonal anti-GAD65+GAD67 (ab11070, Abcam, Cambridge MA) used at a dilution of 1:2000 μl in 5% milk block, rabbit polyclonal anti-GAD-1 (IN) (55771, AnaSpec Inc., Freemont CA) used in a dilution of 1:50 μl in 5% milk block, and rabbit polyclonal anti-GAD-2 (IN) (55772, AnaSpec Inc., Freemont CA) used at a dilution of 1:1000 μl in 5% milk block. Anti- GAPDH (ab9484, Abcam, Cambridge, MA) was used as loading control. Following exposure to the primary antibody, the membrane was washed 4x 10 min in 1X TBSTw (1 L of 10X TBS: 24 g Tris base, 88g NaCl, pH 7.6. To 1X TBS, 0.1% Tween-20 was added). HRP conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific Inc., Waltham, MA) was used at a dilution of 1:10,000 in 5% milk block. The membrane was exposed to the HRP secondary antibody for 1 hour at RT with gently rocking. After exposure to the secondary antibody, the membrane was washed 4x 10 min in 1X TBSTw. Following the secondary antibody, the membrane was exposed to the chemiluminescence substrate Immune-Star WesternC Kit (Bio Rad, Hercules, CA) for 1-5 min, and the chemiluminescence was developed on an X-ray film in a dark room.

GABA dot blot

Protein extracts from 2, 3, 4, 5 and 7 dpf wild type and double morphant zebrafish were used to detect amount of GABA by immunodetection on a nitrocellulose membrane. 1-2 μL of 2 mg/mL protein sample was blotted on a 0.45 μm nitrocellulose membrane. The membrane was blocked in 10% milk block in 1X TBSTw for 1 hour at RT with gentle rocking. After blocking, the membrane was exposed to the primary antibody anti-GABA antibody (A2052, Sigma-Aldrich, St. Louis MO) used in a dilution of 1:2000 μl for 1 hour at RT with gentle rocking. After incubation with the primary, the membrane was washed 3x 10 min with 1X TBSTw at RT with gentle rocking. Following the washes, the membrane was incubated with HRP conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific Inc., Waltham, MA) at a dilution of 1:10,000 in 5% milk block for 1 hour at RT with gentle rocking. The membrane was washed 4x 10 min with 1X TBSTw, and was exposed to the chemiluminescence substrate Immune-Star WesternC Kit (Bio Rad, Hercules, CA) for 1-5 min, and the chemiluminescence was developed on an X-ray film in a dark room.

Immunofluorescence

Cryosections. 3dpf wild type and double morphant zebrafish embryos were fixed in 4% Paraformaldehyde (PFA) in 1X PBS for 30- 45 min at RT. After fixation, the embryos were washed 3x 10 min in 1X PBS at RT. The embryos were dehydrated in 5% sucrose solution in 1X PBS for at least 1 hour at RT and then in 15% sucrose solution in 1X PBS for at least 1 hour. The embryos were stored in 15% sucrose solution at 4 ^{o}C till sectioning. The embryos were embedded in OCT (Tissue-Tek) 20% sucrose mixture (1:2 ratio of 20% sucrose: OCT) and flash-frozen in a slurry of dry ice and 2-methylbutane or

placed in -80 ^{o}C till the blocks were hardened. Transverse cryosections 10 μm thick of 3dpf zebrafish brains were taken on a cryostat. Serial sections were then used for antibody labeling.

Antibody labeling of cryosections. To dissolve the embedding OCT sucrose from the sections, the slides were incubated in 1X PBS at 37 ^{o}C for 10 min or till the medium was dissolved. A blocking solution of 1% BSA was made in 1X PBS and the sections were blocked for 1 hour at RT. The primary antibodies were diluted in the blocking solution. The antibody used was rabbit polyclonal anti-GABA antibody (A2052, Sigma-Aldrich, St. Louis MO) used in a dilution of 1:1500 μl. The sections were exposed to the anti-GABA antibody for 1 hour at RT. After exposing to the antibody, the sections were washed with 1X PBS for 3x10 min at RT. The anti-rabbit secondary antibody conjugated with a fluorophore (Alexa Fluor 488 or 594) (Invitrogen, Life Technologies, Grand Island NY) was diluted in the blocking solution and used at a dilution of 1:1000 μl. Following the washes after exposure to the primary antibodies, the sections were incubated with the secondary antibodies for 30 min as RT in the dark. After incubation with the secondary antibody, the sections were washed with 1X PBS for 3-5x 10 min. The nuclei were counterstained with diamidino-2-phenylindole (DAPI) and coverslips were fixed on the slide using the VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame CA). The slides were imaged on a Zeiss Axio Imager. D2 with a Zeiss Axio HRc camera. The software used was Zeiss AxioVision Rel. 4.8.

Electrophysiology

3 dpf wild type and morphant zebrafish embryos were anaesthetized using MS-222 (commonly known as Tricaine) (A5040, Sigma-Aldrich, St. Louis MO) before

paralyzing. Paralysis was carried out using α-bungarotoxin (B-1601, Invitrogen, Life Technologies, Grand Island NY). Each embryo was placed on a sloping agarose mold and the embryos were paralyzed with 250 μL α- microinjected into the yolk or with bath application of 0.05 mM Pancuronium Bromide (P1918, Sigma-Aldrich, St. Louis MO). The embryos injected with the α-bungarotoxin were then transferred back into a dish containing egg water. With α-bungarotoxin, paralysis occurred within about 1-2 hours after injection. With Pancuronium Bromide, paralysis occurred withing 10-15 min after bath application.

After paralysis, the fish were mounted in 1.2% agarose made with normal Ringer's (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM HEPES, pH 7.2.) on a 22x22mm cover slip mounted in a PC-H perfusion chamber (Siskiyou, Grants Pass, OR) with silicone grease. The perfusion chamber was filled with 2 mL normal Ringer's solution. A sharp glass pipet microelectrode (10–20 MΩ impedance), loaded with normal Ringer's solution, was inserted into the optic tectum and the chloride-coated silver reference wire was placed in the Ringers solution.

Electrical activity was recorded using an Axoclamp 900a amplifier (Axon Instruments, Union City, CA). The amplified voltage was passed through a Hum Bug Noise Eliminator (AutoMate Scientific, Berkley, CA), band-pass filtered from 1 Hz–0.1 kHz, and digitized at 10 kHz using a Digidata 1440 interface and stored on a PC using pClamp software (version 10.3 and 10.4, Molecular Devices).

Results

Titration of splice blocking Morpholinos

The stock concentrations of the MOs were determined using the NanoDrop 2000. Titration of the splice blocking morpholinos was carried out to determine the optimum concentration of MO to inject into each embryo. The criteria for determining the optimal concentration was to observe the death rate and rate of deformity one day after injection. The control for the injection was injections of 2M KCl with Phenol Red and Texas Red Dextran, without the MO. Increase in the MO concentration would result in increase in the death and deformity rates. Titration was carried out for each splice blocking MO. Initial titrations were done using concentrations of 1 ng/nL, 3 ng/nL, and 6 ng/nL for each MO. Depending on the death rate, the concentrations were increased or decreased. The deformity rate for each concentration was observed. Mild deformity was defined as small deformities to the tail, body axis, and head, including the brain structure and eye of the embryos. Severe deformity was defined as highly abnormal embryos which were either missing essential structures, or which had severe development delay. While observing the deformity rates, it was noticed that the embryos injected with the morpholinos had developmental delay. This was not seen in the KCl dye injection controls or in the mispair specificity controls. For *gad1* splice blocker, the injecting concentration was determined to be 0.5 ng/nL and for *gad2* splice blocker, the concentration was determined to be 1 ng/nL after the titrations [Figure 12].

RT-PCR of gad1 and gad2 morphants

In order to assess knockdown of *gad1* and *gad2*, PCR was carried out on cDNA from 3 dpf wild type and morphant embryos. Our assumption was that the MOs would

cause the addition of intronic sequences in the cDNA of the morphants, which would appear as bands of higher molecular weight on the agarose gel. However, from the PCR, we saw that the bands of the morphants was smaller by about 100 bp compared to the wild type cDNA bands for the GAD67 LP+FP3 and GAD65 LP+FP1 primer pairs. Sequencing of the band from the *gad2* morphant showed that exon 3 was completely skipped. Exon 3 in *gad2* is about 145 bp long. Bioinformatics analysis showed that the deletion of exon 3 was in frame with the ORF of *gad2*, giving a potential translated sequence that is smaller by 48 amino acid residues. For the *gad1* morphants, RT-PCR showed two bands, both of different sizes from the wild type bands. Sequencing of the upper band in the *gad1* morphant showed a partial loss of exon 2, leading to a deletion of about 45 bp of exon 2. Bioinformatics analysis showed that this deletion was in frame, giving a potential translated sequence that would be smaller by 15 amino acid residues. Sequencing of the lower band in the *gad1* morphant showed that the entire exon 2 was skipped. Exon 2 of *gad1* is about 126 bp long. Bioinformatics analysis showed that this deletion was also in frame, and in addition, would possibly uncover the putative start site on exon 3 [Figure 13].

To test if the intronic sequences was included in the morphants, we designed primers that spanned the exon 2- intron 2 junction of *gad1* and exon 3- intron 3 junction of *gad2*. The exon-intron primers were first tested on gDNA, and all the primers except for Gad2 I3E4 LP + Gad2 E4 RP worked on zebrafish gDNA. We performed PCR on the morphant cDNA using these primers. For *gad2* morphants, we did not see any bands, with the Gad2 E3 LP + Gad2 E3I3 RP, confirming that the intron 3 was not included in the morphant cDNA. For the *gad1* morphants, however, the results were a little

surprising. From the previous PCR, on the assumption that the intron was not included in the morphants, we did not expect to see any bands for Gad1 E2 LP + Gad1 E2I2 RP and Gad1 I2E3 LP + Gad1 E3 RP. However, the PCR showed bands of the appropriate size for the *gad1* morphants for both the exon- intron primers. This led us to the conclusion that apart from inducing exon skipping, the *gad1* MO is also preventing the splicing of intron 2, leading to the inclusion of intron 2 between exons 2 and 3 [Figure 14].

Western Blot of gad1, gad2 and double morphants

To determine if there was a reduction or loss of protein in the *gad1* and the *gad2* morphants, Western blot was carried out using protein extract of morphants at 1, 2, 3, 4, 5 and 7 days post injection. A decrease in protein was seen for the co-injected double morphants, as well as for *gad1* and *gad2* morphants. Furthermore, as the concentration of the MOs were increased, there was greater loss of protein. A time series of the morphants showed that the MOs were effective until about 3 days, after which there was recovery of the protein. By 7 days, the protein levels were almost to that of the wild type [Figures 15A & 15B].

The anti-GAD65+GAD67 antibody detected two bands of protein around the range of 65 kDa. Initially, we thought that the two bands were GAD65 and GAD67, but according to the predicted molecular weights of GAD65 and GAD67 in the zebrafish (GAD65 = 65.458 kDa, GAD67 = 66.155 k Da), the molecular weight difference might be too little to be resolved into two bands on a 10% gel. Hence it is possible that these one two bands might be an isoform of GAD67, or even possibly, of GAD65, and the main band might be a combination of the two proteins. The anti-GAD-2 (IN) antibody showed additional bands, apart from the 65 kDa band. Since the embryos were not de-

yolked at the time of extracting protein, it is possible that these additional bands are yolk proteins. The anti-GAD-1 (IN) antibody however, did not detect the 67 kDa band well. Instead, additional bands, of different sizes were detected in the wild type protein extract by the antibody. These additional bands disappear in the morphants protein extracts [Figures 15C & 15D]. Since *gad1* has have at least 3 different isoforms, it is possible that these isoforms are being detected in the zebrafish wild type extracts. Alteration of the mRNA by the *gad1* splice blocking MOs may lead to alteration of the protein, and hence the epitope detected by the antibody, leading to a loss of these bands.

GABA dot blot

A dot blot for GABA was carried out to see if there was reduction or loss of GABA in the morphants compared to the wild type zebrafish embryos. The double morphants were used for this experiment, and a time series of 2, 3, 4, 5 and 7 dpf zebrafish was carried out. The results of the dot blot showed that at 2 and 3 days, the GABA levels were almost not detectable, but by day 4, the GABA levels started to increase and by day 7, the levels were almost to that of wild type [Figure 15E]. This confirmed that the MOs were effective until about 3 days.

Immunofluorescence

Immunofluorescence for GABA was performed on 3 dpf wild type and double morphant zebrafish embryos to see if there was a reduction of GABA levels or a loss of GABA expressing cells in the brain of the morphants. From the results, there seems to be a reduction in the levels of GABA in the brain of the morphant zebrafish compared to the wild type embryos. There did not seem to be a loss in the GABA cell types, but the intensity of the staining in the cells expressing GABA were reduced in the morphant

brains. This reduction was seen throughout all regions in the brain. The reduction in GABA levels was especially noticeable in the retina, and the hindbrain of the morphant zebrafish [Figure 16].

Electrophysiology

The electrophysiology recordings were carried out in the optic tectum of the zebrafish embryos. From the 5-10 min recordings on wild type, *gad1, gad2,* and the double morphant zebrafish, neural activity in the morphant zebrafish appeared abnormal and increased. The abnormal electrophysiological activity was not observed in the 5-mispair control injected zebrafish. The individual morphants showed abnormal activity as well, and interestingly, the neural activity of the individual morphants was different from each other. The *gad1* morphants appeared to have increased neural activity compared to the *gad2* morphants [Figure 17]. We defined this abnormal electrical activity as spontaneous seizures in the morphant zebrafish embryos.

Conclusion

In this study, we used splice blocking Morpholinos against *gad1* and *gad2* to knock down the proteins and determine their functions in the zebrafish embryo. We designed the splice blockers to bind to the exon 2-intron 2 junction of *gad1* and to exon 3-intron 3 junction of *gad2.* Both these exons are in the N-terminal domain of the proteins. We expected that the splice blockers would sterically hinder the splicing machinery of the cell, leading to inclusion of intron 2 in the *gad1* mRNA and the inclusion of intron 3 in the *gad2* mRNA. Since introns have several stop codons, this would have resulted in the premature stopping of translation of *gad1* and *gad2*, resulting in non-functional truncated proteins. This would be considered as a knockdown of the

gene, giving essentially a loss of function phenotype. However, from the RT-PCR results of *gad2* morphants, it was seen that instead of inclusion of intronic sequences, the entire exon 3 was being skipped. This would have still resulted in truncation GAD65, but bioinformatics analysis suggested that the deletion of exon 3 was in frame, and that the protein would still potentially be made. From previous studies, it was shown that the N-terminal domain of *gad2* undergoes several post translational modifications, and is responsible for targeting the protein to vesicles in the Golgi apparatus and the synapse [20, 21, 35-38, 40, 42]. But all of these modifications are on the first two exons of the protein. Exon 3 does not seem to play a role in either targeting the enzyme to vesicles or to specific subcellular localizations. Also, it was shown that the loss of some residues of the N-terminal domain did not affect the activity of the enzyme. However, from the western blots of *gad2* morphants, it was seen that there is a definite reduction in the protein.

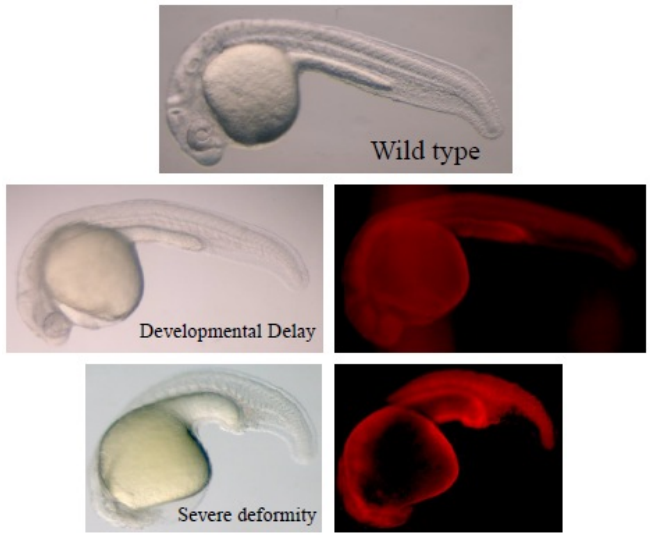
The knockdown of *gad1* painted a much more complicated picture. From the RT-PCR, it was seen that not only exon 2 was being skipped, but in some of the mRNA, exon 2 was partially deleted, and also, in some of the mRNA, intron 2 was being included. From the bioinformatics analysis, the partial and complete skipping of exon 2 both resulted in in-frame deletions, and the protein could potentially be made. However, the western blots of *gad1* morphants also show reduction in the protein. What was also seen in the western blots of *gad1* using the antibody specific to *gad1* was that there were several bands in the wild type protein extract, which disappeared in the *gad1* morphants. Since *gad1* has at least 3 different splice variants in the mammalian system, it is possible

that it has variants in the zebrafish as well. The MO against *gad1* might be affecting the expression of the splice variants as well.

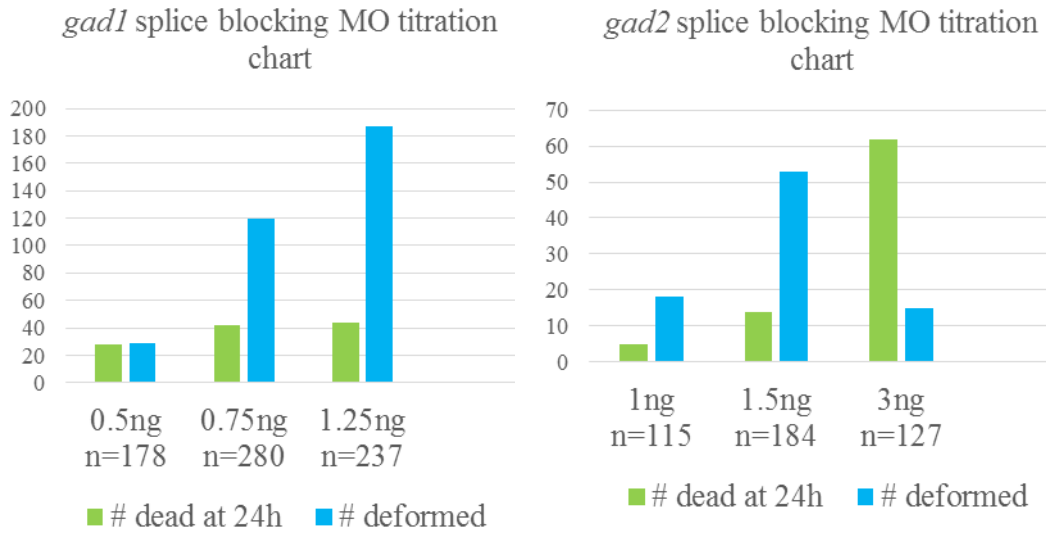
In the zebrafish, use of the splice blockers against *gad1* and *gad2* shows a definite reduction in the protein as well as GABA levels, as seen from the western blots, the dot blot and the immunolabeling. Also, the MOs have an adverse effect on the neural activity of the embryo, as seen from the electrophysiology. From this it is clear that the alteration of the GAD proteins is affecting the functioning of the two enzymes. Partial or complete loss of an exon might change the conformation of the enzyme structure, leading to reduced catalytic activity, and hence a reduction in GABA. Another distinct possibility is that loss of the exons 2 and 3 in *gad1* and *gad3* respectively may result in changes in the subcellular localizations of the two enzymes. This might also result in abnormal neural activity. By taking this study further, it is possible to characterize the functions of exons 2 and 3 in *gad1* and *gad2* of the zebrafish. Regarding the subcellular localization, further experiments can be done in the *gad* morphants to see if there are any changes in the subcellular localization of the proteins. Co-localization studies with a Golgi marker and synapse marker can determine if the subcellular localization of the two GADs in the morphants is affected.

Figure 12. *Titration of gad1 and gad2 splice blocking Morpholinos.* Figure 12A shows embryos deformed by the MO injections compared with the wild type embryos. The fluorescent images show the distribution of the Texas Red Dextran in the embryo. It is assumed that the dye is an indication of the distribution of the MO. Figure 12B shows the rate of deformity and death for each concentration of the MO observed during the titration. Figure 12C shows PCR for *gad1* MO titration using GAD67 LP and GAD67 FP3 primer pair. The concentrations of MOs were 0.25, 0.5 and 0.75 ng/nL of *gad1* MO. The results show that while there is little alteration of morphant cDNA for 0.25 ng/nL injection, there is alteration of almost 100% of morphant cDNA for the 0.5 and 0.75 ng/nL injections. Figure 12D is the PCR for *gad2* MO titration using the GAD65 LP and GAD65 FP1 primer pair. The concentrations used were 1, 1.5 and 3 ng/nL of MO. For the 1 ng/nL concentration, most of the cDNA is altered, and for the higher concentrations, almost 100% of the cDNA is altered. Taking into account the death and deformity rates of the two MOs, the working concentrations of the *gad1* and *gad2* MOs were set at 0.5 ng/nL for *gad1* MO and 1 ng/nL for *gad2* MO.

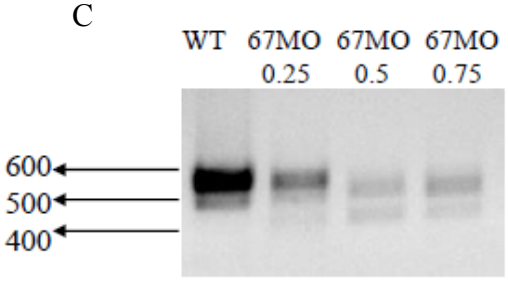
A


Wild type
Developmental Delay
Severe deformity

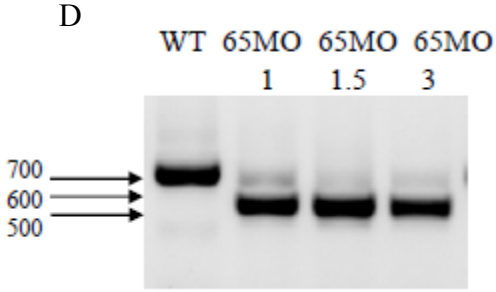
B


gad1 splice blocking MO titration chart
200
180
160
140
120
100
80
60
40
20
0
0.5ng n=178
0.75ng n=280
1.25ng n=237
dead at 24h
deformed
gad2 splice blocking MO titration chart
70
60
50
40
30
20
10
0
1ng n=115
1.5ng n=184
3ng n=127
dead at 24h
deformed

C


WT
67MO 0.25
67MO 0.5
67MO 0.75
600
500
400

D


WT
65MO 1
65MO 1.5
65MO 3
700
600
500

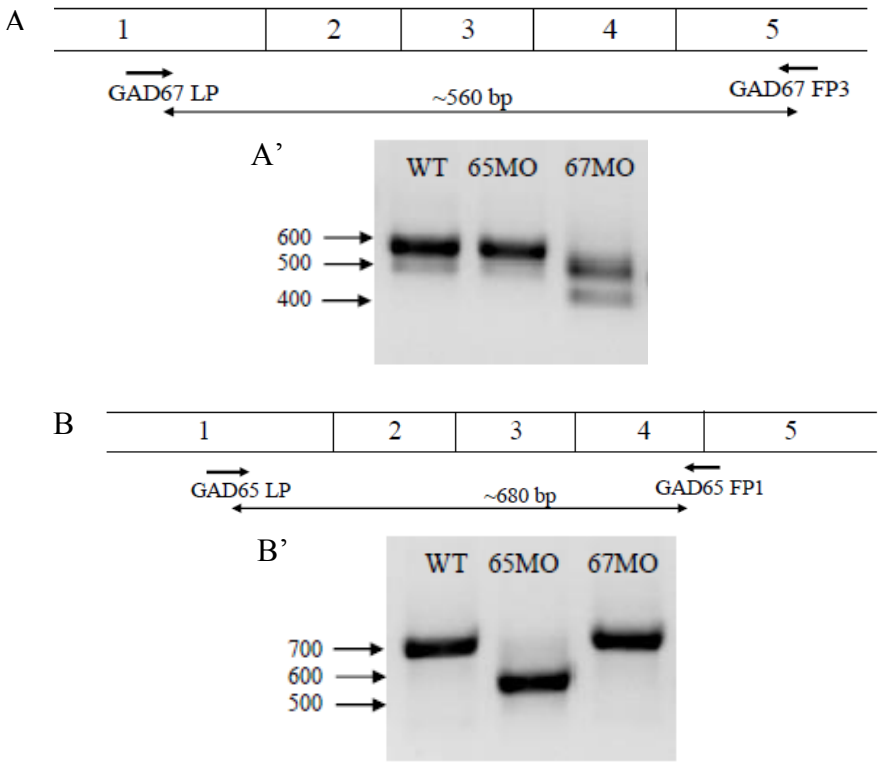


Figure 13. *gad1 and gad2 morphant PCR*. Figure 13A is a schematic of the PCR using the primers GAD67 LP and GAD67 FP3. Figure 13A' is the agarose gel showing that the two bands in the *gad1* morphant (denoted as 67MO) are of different sizes than the two wild type bands. The *gad2* morphant (denoted as 65MO) is included to show that the *gad2* band is the same size as wild type, and confirms that the *gad1* MO is specific for *gad1* and does not alter *gad2*. Figure 13B is a schematic of the PCR using the primers GAD65 LP and GAD65 FP1. Figure 13B' is the agarose gel showing the decrease in size of the *gad2* morphant band compared to the wild type. The *gad1* morphant is included to show specificity of the *gad2* MO, which alters only *gad2* and not *gad1*.

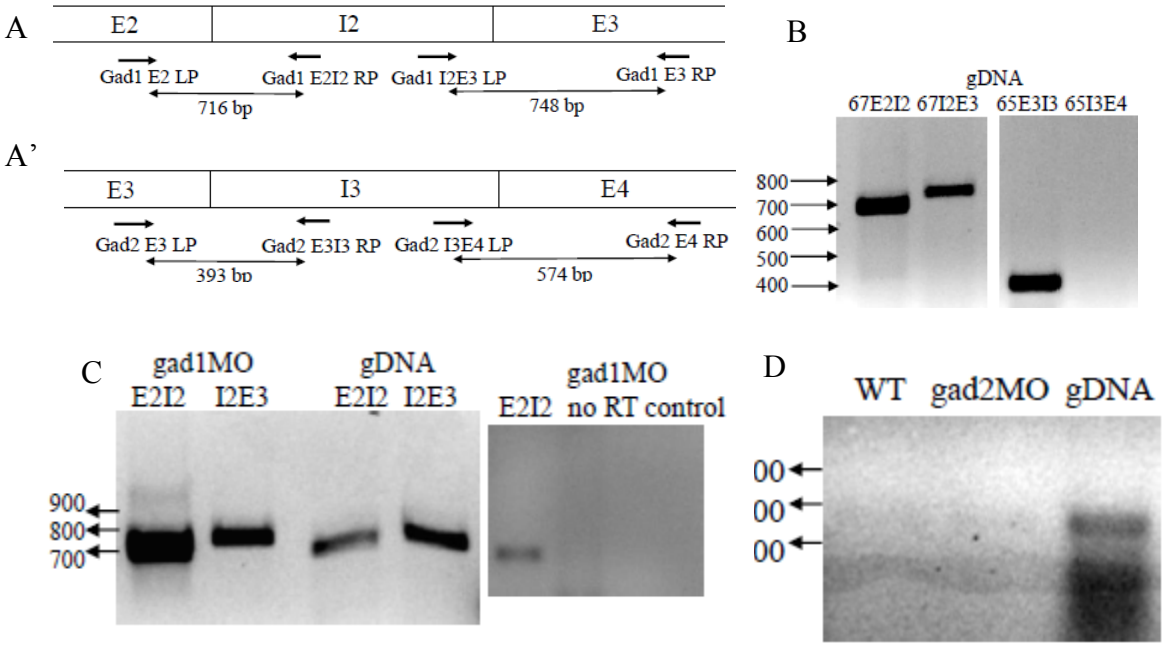
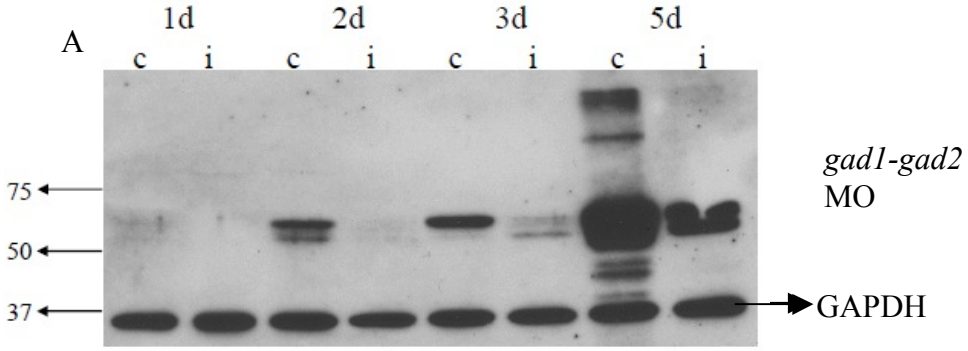
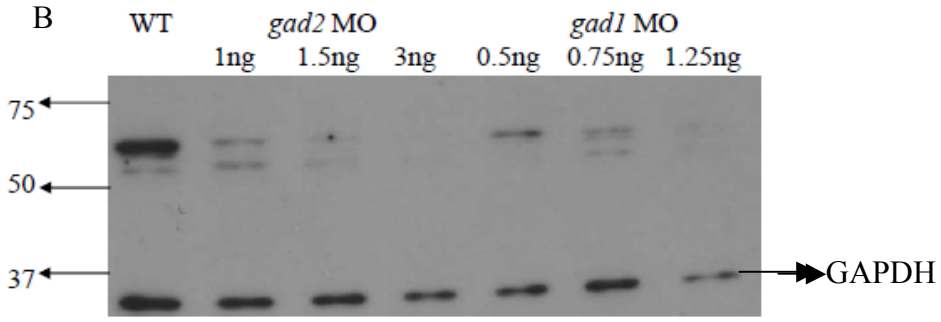
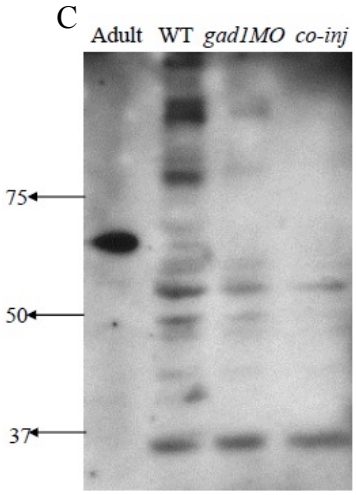


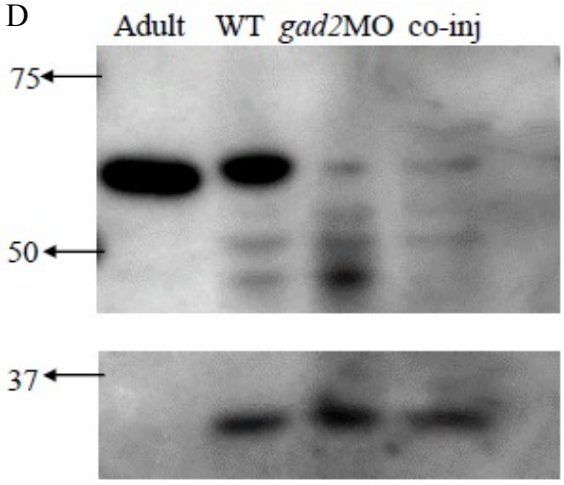
Figure 14. *Exon-intron PCR for morphants*. Figures 14A and 14A' are schematics for the PCR with the exon-intron spanning primers for testing the splicing of cDNA in the *gad* morphants. Figure 14A shows the primers for *gad1* morphants spanning exon 2-intron 2 junction and figure 14A' shows the primers for *gad2* morphants spanning exon 3-intron 3 junction. Figure 14B shows the PCR using the exon-intron spanning primers on genomic DNA. The Gad2 I3E4 primer pair did not work. Figure 14C shows the PCR for *gad1* morphants with the exon-intron primers. The *gad1* morphant cDNA gives a positive result for intron 2 sequence. Figure 14D shows the PCR for *gad2* morphants with primer pair E3I3. The wild type and the *gad2* morphant cDNA are not amplified but the genomic DNA (gDNA) is.

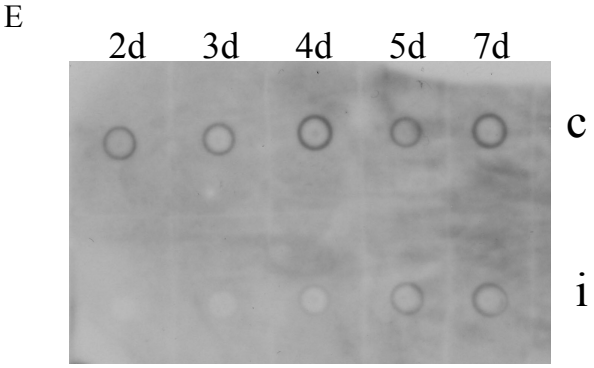
Figure 15. *Western blot showing reduction of GAD in morphants.* Figure 15A shows a time series for double morphants. The antibody anti GAD65+GAD67 detects both proteins. It shows that there is significant loss of protein until 4 days, after which the protein levels begin to recover. Figure 15B is a titration for *gad1* and *gad2* MO. As the MO concentration was increased, the levels of GAD decreased. The anti GAD65+GAD67 antibody was used to detect both the GADs. Figure 15C shows reduction of GAD67 in the *gad1* morphants and the double morphants. The antibody anti the GAD-1 (IN) detects GAD67 specifically. The additional bands are possibly splice variants of GAD67. Figure 15D shows the reduction of GAD65 in *gad2* morphants and double morphants. The antibody anti-GAD-2 (IN) detects GAD65 specifically. The additional bands are possibly yolk proteins that are being detected by the antibody. Figure 15E is a dot blot for GABA, showing that there is significant reduction of GABA in the 2 and 3 day double morphants compared to the wild type levels, and the GABA levels recover from 4 days post injection. Anti-GAPDH was used as a loading control in all the blots. The band size is 35 kDa.


A
1d
2d
3d
5d
c
i
c
i
c
i
c
i
75
50
37
gad1-gad2
MO
GAPDH


B
WT
gad2 MO
1ng
1.5ng
3ng
gad1 MO
0.5ng
0.75ng
1.25ng
75
50
37
GAPDH


C
Adult
WT
gad1MO
co-inj
75
50
37


D
Adult
WT
gad2MO
co-inj
75
50
37


E
2d
3d
4d
5d
7d
c
i
GABA dot
blot

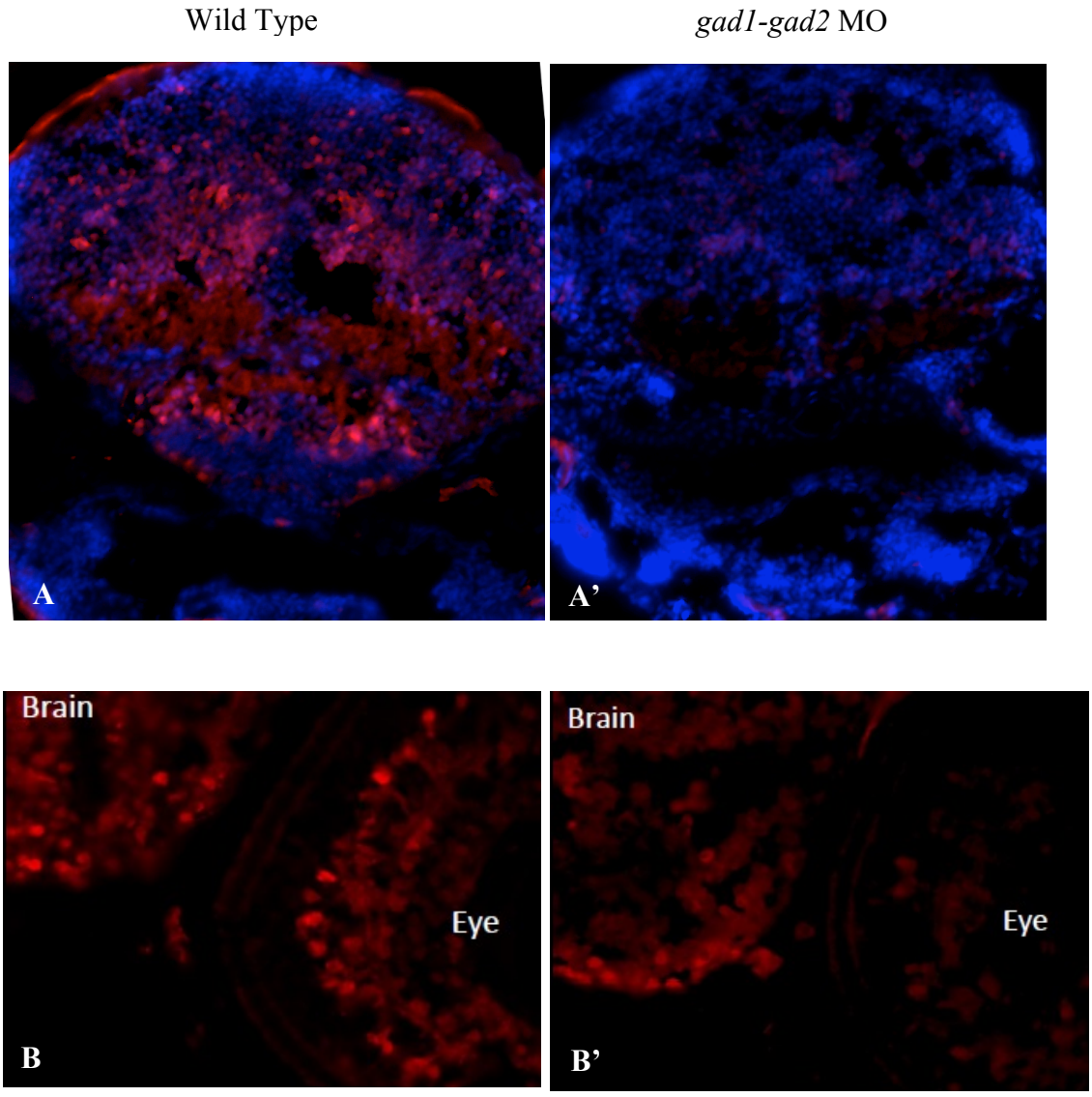
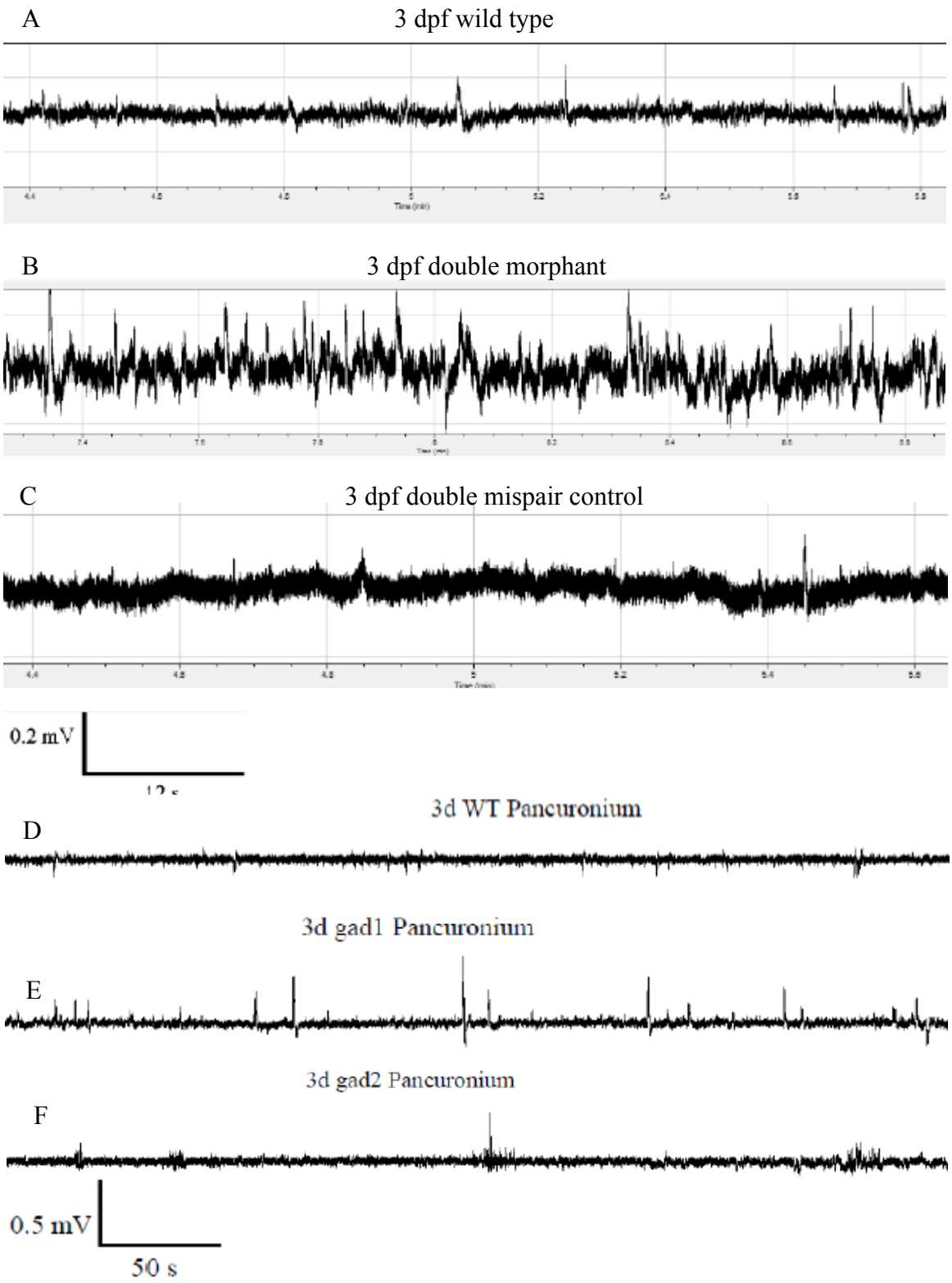


Figure 16. *Immunofluorescence shows reduction in GABA in brain of morphants*. Figures 16A and 16A' shows reduction in GABA in a transverse section through the hindbrain of the 3 dpf wild type and double morphant zebrafish. Figures 16B and 16B' shows reduced GABA in retina of 3 dpf double morphants. Scale bar for all images is 10 μm. [Staining was done by Cindy Bledsoe].

Figure 17. *Electrophysiology of morphants.* Figures 17A, 17B and 17C shows the electrophysiological traces of 3 dpf wild type, double morphants, and the mispair control double morphants, respectively. The paralytic used was α-bungarotoxin. The traces show increased and abnormal neural activity in the morphants, compared to the neural activity of the wild type and the mispair control. The scale bar for all the images is 0.2 mV on the Y-axis and 12 s on the X-axis. Figures 17C, 17D and 17E shows the electrophysiology for 3 dpf wild type, *gad1* and *gad2* morphants. The paralytic used was Pancuronium Bromide. The traces show increased neural activity even for the single morphants. Neural activity of *gad1* morphants was different from that of the *gad2* morphants, indicating the possibility of differences in function. [Electrophysiology done by Rebecca Ball].


A
3 dpf wild type
B
3 dpf double morphant
C
3 dpf double mispair control
0.2 mV
3d WT Pancuronium
D
3d gad1 Pancuronium
E
3d gad2 Pancuronium
F
0.5 mV
50 s

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