Distinct Phenotypes in Zebrafish Models of Human Startle Disease

Lisa R. Ganser
Kennesaw State University, lganser@kennesaw.edu

Qing Yan
University of Miami

Victoria M. James
Birkbeck College, University of London

Robert Kozol
University of Miami

Maya Topf
Birkbeck College, University of London

See next page for additional authors

Follow this and additional works at: http://digitalcommons.kennesaw.edu/facpubs

Part of the Medical Genetics Commons, Neurology Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation

This Article is brought to you for free and open access by DigitalCommons@Kennesaw State University. It has been accepted for inclusion in Faculty Publications by an authorized administrator of DigitalCommons@Kennesaw State University. For more information, please contact digitalcommons@kennesaw.edu.
Distinct phenotypes in zebrafish models of human startle disease

Lisa R. Ganser d,1, Qing Yan a,1, Victoria M. James bc, Robert Kozol a, Maya Topf c, Robert J. Harvey b J, Julia E. Dallman a,⁎

a Department of Biology, Cox Science Center, 1301 Memorial Drive, University of Miami, Coral Gables, FL 33124-0421, USA
b Department of Pharmacology, UCL School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, UK
c Institute for Structural and Molecular Biology, Department of Biological Sciences, Birckbeck College, London WC1E 7HX, UK
d Department of Biology and Physics, 1000 Chastain Rd, #1202, Kennesaw State University, Kennesaw, GA 30144 USA

ARTICLE INFO

Article history:
Received 29 May 2013
Accepted 1 September 2013
Available online 9 September 2013

Keywords:
glra1
ghrb
Glycine receptor
Startle disease
Hyperekplexia
Zebrafish

ABSTRACT

Startle disease is an inherited neurological disorder that causes affected individuals to suffer noise- or touch-induced non-epileptic seizures, excessive muscle stiffness and neonatal apnea episodes. Mutations known to cause startle disease have been identified in glycine receptor subunit (GLRA1 and GLRB) and glycine transporter (SLC6A5) genes, which serve essential functions at glycinerergic synapses. Despite the significant successes in identifying startle disease mutations, many idiopathic cases remain unresolved. Exome sequencing in these individuals will identify new candidate genes. To validate these candidate disease genes, zebrafish is an ideal choice due to rapid knockdown strategies, accessible embryonic stages, and stereotyped behaviors. The only existing zebrafish model of startle disease, bandoneon (beo), harbors point mutations in ghrb (one of two zebrafish orthologs of human GLRB) that cause compromised glycinerergic transmission and touch-induced bilateral muscle contractions. In order to further develop zebrafish as a model for startle disease, we sought to identify common phenotypic outcomes of knocking down glycinerergic orthologs of two known startle disease genes, GLRA1 and GLRB, using splice site-targeted morpholinos. Although both morphants were expected to result in phenotypes similar to the zebrafish beo mutant, our direct comparison demonstrated that while both glra1 and ghrb morphants exhibited embryonic spasticity, only ghrb morphants exhibited bilateral contractions characteristic of beo mutants. Likewise, zebrafish over-expressing a dominant startle disease mutation (GlyR α1R271Q) exhibited spasticity but not bilateral contractions. Since GlyR β2 can interact with GlyR α subunits 2–4 in addition to GlyR α1, loss of the GlyR β2 subunit may produce more severe phenotypes by affecting multiple GlyR subtypes. Indeed, immunohistochemistry of glra1 morphants suggests that in zebrafish, alternate GlyR α subunits can compensate for the loss of the GlyR α1 subunit. To address the potential for interplay among GlyR subunits during development, we quantified the expression time-course for genes known to be critical to glycinerergic synapse function. We found that GlyR α2, α3 and α4a are expressed in the correct temporal pattern and could compensate for the loss of the GlyR α1 subunit. Based on our findings, future studies that aim to model candidate startle disease genes in zebrafish should include measures of spasticity and synaptic development.

© 2013 The Authors. Published by Elsevier Inc. Open access under CC BY-NC-ND license.

Introduction

Rhythmic motor behaviors require a balance between nervous system excitation and inhibition (E/I balance). The importance of E/I balance is illustrated by genetic mutations that selectively disrupt either excitation or inhibition and result in nervous system dysfunction (Ganser and Dallman, 2009; Gatto and Broadie, 2010). For example, in humans, startle disease/hyperekplexia results from excessive excitation due to damaging mutations in genes encoding key components of the inhibitory glycinerergic synapse (Harvey et al., 2008). As the major inhibitory neurotransmitter in vertebrate hindbrain and spinal cord, glycine plays a critical role in the control of motor behaviors and reflexes. When glycinerergic signaling is disrupted in newborn children, the result is exaggerated startle reflexes and hypertonia in response to unexpected auditory, tactile or visual stimuli. This abnormal startle response may also be accompanied by apnea episodes, i.e. the suspension of breathing (Thomas et al., 2010).

The majority of human startle disease cases are caused by dominant and recessive mutations in GLRA1, encoding the α1 subunit of the glycine receptor, GlyR α1 (Chung et al., 2010; Shiang et al., 1993; Fig. 1A). Mutations in this gene cause similar disorders in mice (Buckwalter et al., 1994; Holland et al., 2006; Ryan et al., 1994; Traka et al., 2006) and Poll Hereford cattle (Pierce et al., 2001). Dominant and recessive mutations in SLC6A5, encoding the presynaptic glycine transporter GlyT2, are now emerging as a second major cause of startle...
Fig. 1. Pathogenic mutations in the postsynaptic GlyR α1 and β subunits in startle disease. The predicted four-membrane spanning domain (M1–M4) topology of GlyR α1 and GlyR β subunits is depicted. Numbered columns indicate four predicted membrane-spanning domains 1–4 in each subunit. Red (dominantly inherited) and blue circles (recessively inherited) indicate the relative positions of amino acid alterations observed in human startle disease. Purple circles indicate the relative positions of amino acid alterations found in mouse, cattle and zebrafish glycergic disorders. For primary references to previously reported specific mutations, see Harvey et al. (2008), Chung et al. (2010, 2013) and James et al. (2013).

disease (Carta et al., 2012; Giménez et al., 2012; Rees et al., 2006), and also occur in Belgian Blue cattle (Charlier et al., 2008) and Irish wolfhounds (Gill et al., 2011). Mutations in GLRB, encoding the GlyR β subunit were thought to be a rare cause of human startle disease (Rees et al., 2002), although mutations in this gene were also reported in the mouse mutant spastic (Kingsmore et al., 1994; Müller et al., 1994) and the zebrafish mutant bandoneon (beo; Granato et al., 1996; Hirata et al., 2005). However, several recent reports have identified novel dominant and recessive mutations in GLRB (Al-Owain et al., 2012; Chung et al., 2013; James et al., 2013; Lee et al., 2013; Fig. 1B), often associated with additional phenotypic consequences, including gaze disorders, apnea episodes, learning difficulties and developmental delay. Since GlyR α1 and β subunits occur in the same pentameric GlyR complex, it is currently unclear why mutations in GLRB can give rise to a more severe clinical phenotype than mutations in GLRA1.

To model human startle disease, zebrafish are an attractive model because genes can be ‘inactivated’ using antisense morpholino knockdown (Eisen and Smith, 2008). Moreover, the resulting swimming phenotypes are readily quantified (Burgess and Granato, 2007). In contrast to mammalian genomes with five known GlyR subunit genes (GLRA1, GLRA2, GLRA3, GLRA4 and GLRB), the zebrafish genome encodes seven GlyR subunit genes (glra1, glra2, glra3, glra4a, glra4b, glrb, and glrb); Hirata et al., 2010). The two duplicate genes, glra4b and glrb resulted from whole genome duplication early in the evolution of teleosts (Hurley et al., 2007a). This phenomenon can be advantageous, since individual paired genes often differ in terms of expression patterns and functional roles (Hurley et al., 2007b; Ogino et al., 2011). For example, the zebrafish mutant bandoneon (beo) harbors mutations in one of the paired GlyR β subunit genes (glrb), resulting in touch-induced simultaneous bilateral contractions of the axial muscles due to the loss of reciprocal glycnergic inhibition of motor circuits (Hirata et al., 2005, 2010). Despite the duplication of the GlyR β subunit genes in zebrafish, glrb is unable to compensate for the loss of glrb function due to a different expression pattern, suggesting that it forms part of a distinct GlyR with a unique function (Hirata et al., 2005). Since mutations in human GLRA1, GLRB and SLC6A5 all result in startle disease (Harvey et al., 2008), it is unusual that to date no mutations in glra1 or slc6a5 have been discovered that produce bilateral contractions in larval zebrafish. We therefore sequenced the remaining beo alleles and have confirmed that they all harbor damaging mutations affecting glrb.

To directly compare zebrafish glra1 and glrb startle disease models, we designed splice-site morpholinos to knockdown the expression of these genes (Draper et al., 2001). As expected, injecting glrb morpholinos produced a phenotype similar to beo mutants, characterized by simultaneous bilateral contractions strong enough to shorten the body. In addition, both glrb and glra1 morphants produced spastic and erratic behaviors at early stages, although in contrast to glrb morphants, glra1 morphants only rarely produced bilateral contractions and by 48 hpf glra1 morphants produced normal behaviors. Immunohistochemistry of glrb and glra1 morphants also demonstrated distinct GlyR immunostaining patterns on early-differentiating spinal neurons. While glra1 morphants exhibited reduced but still synaptic GlyR staining, glrb morphants exhibited GlyR α subunit trapping in intracellular, non-synaptic compartments, suggesting that the more severe beo phenotype results from a loss of multiple GlyR subtypes. Our quantitative analysis of mRNA expression for glycnergic genes in early development identified glra2 and glra3 as having high expression levels early in development that could ameliorate the glra1 morphant phenotype. In summary, we identify a phenotypic range in zebrafish startle disease models that should be taken into account when using zebrafish to study novel candidate startle disease genes.

Materials and methods

Fish care and embryo rearing

Experiments were carried out using Danio rerio wild type strains AB, Tubingen, and BWT (a fish store strain from Long Island). Adults were kept on a 14 hour light/10 hour dark circadian cycle at 28.5 °C. Embryos were collected from natural crossings shortly after removing a divider at first light. Embryos were then reared in glass Petri dishes containing system water (water that houses the adult fish) in a 28.5 °C incubator with the same 14 hour light/10 hour dark cycle. All experiments were
conducted in accordance with the University of Miami Institutional Animal Care and Use Committee guidelines.

**Detection of beo mutations**

DNA was extracted from fin clips of beo mutation carriers using a QIaAmp DNA mini kit (Qiagen, Manchester, UK). The nine coding exons and flanking sequences of *glrb* were amplified from purified genomic DNA. Forward and reverse primers each of 20–30 bp in length were designed to cover the start and end of the regions to be amplified and ordered from Eurofins MWG Operon (Ebersberg, Germany). DNA polymerase was ordered from Clontech, Saint-Germain-en-Laye, France. PCR products were gel purified and cloned into pCR4-TOPO (Invitrogen, Paisley, UK). Sanger sequencing was performed by the DNA Sequencing Service (University of Dundee, UK). DNA sequences were analyzed using Sequencer 5.1 software (GeneCodes Corporation, Ann Arbor, USA) by alignment with reference sequences downloaded from NCBI or UCSC databases. For mutation identification, single nucleotide variants (SNVs) were noted and the effect of these changes on the encoded protein were examined using bioinformatics software including SIFT (Kumar et al., 2009) and Polyphen-2 (Adzhubei et al., 2010).

**Morpholino design and injection**

Splice site-targeted morpholinos (MOs) were designed against *glra1* and *glrb*. Intron/exon junctions were selected for MO design based both upon the appropriateness of the sequence for effective MO knockdown (i.e. 50% GC content and multiple mismatches to related genes). Two distinct MOs against *glra1* were tested to control for possible off-target effects: *glra1*MOex4 5′-GAATTGTCTCCACCTCTATGCT-3′ and *glra1*MOex7 5′-CTCTCCGTGAACAGAAGACTTG-3′. Off-target effects were not a concern in the case of the *glrb* splice morpholino *glrb* MOex5 5′-GAGAGCATTTAAGTCACCTCAATG-3′ because of the previously described *beo* mutant phenotype (Hirata et al., 2005). For control-injected embryos, we used the standard control MO provided by Gene Tools (Gene Tools, LLC; Philomath OR). Lyophilized MOs were resuspended in water as 1 mM stocks and stored at room temperature. Prior to use, the MO stock solution was heated for 5 min to 65 °C. For injection, stock solutions were diluted in a filtered solution of 1% (w/v) fast-green dye. MOs were injected using filament-lined Kwik-Fil borosilicate capillary glass (World Precision Instruments, Sarasota FL) pulled on a P-97 micropipette puller (Sutter Instruments, Novato CA) to a long taper with a 30 μm diameter bolus. MOs were injected into wild type embryos at the 1–2 cell stage. Embryos were sorted 6–8 h after injection so that only morphants in which the MO bolus had evenly dissipated were later analyzed for behavioral phenotypes.

**RT-PCR**

Unless otherwise stated, all reagents were from Life Technologies, Grand Island NY. Once behavioral analyses were completed, embryos were ground in TRIzol for RNA extraction. RNA preparations were then DNase-treated using DNA-free. The reverse transcriptase SuperScript III with either an anchored oligo(dT) primer or a gene specific primer was used to synthesize cDNA for subsequent PCR analysis. Diagnostic PCR primers were designed to detect mis-splicing events induced by MO masking of intron/exon junctions for *glra1* (Fex3 5′-TGGGATCC ATGCTGAAACA-3′; Rex8 5′-ATACTCCAGGGCCAGAGA-3′) and *glrb* (Fex5 5′-CTGAGCAACCTCTGGAAATG-3′; Rex8 5′-CTCCAGCAGCGTCTAGTACG-3′) (All primers were synthesized by Integrated DNA Technologies, Coralville, IA). Primers targeting *slc6a5* (F: 5′-AG GATCATGCTGCGCCAG-3′; R: 5′-CAAGGTTCAATCTCTGACCT-3′) were utilized as internal controls. Shifted RT-PCR products caused by MO-induced alterations in pre-mRNA splicing were gel purified using Wizard Gel and PCR Clean-up System (Promega, Madison, WI). Purified bands were then re-amplified and sequenced using Sanger DNA sequencing to determine the predicted impact on targeted proteins.

**Behavioral analyses**

A high-speed camera (1024 Photron FASTCAM, San Diego, CA) was used to record spontaneous and touch-evoked behaviors of 17 to 50 hpf control, *glra1*, and *glrb* morphants. Embryos were manually dechorionated. Spastic behaviors were characteristic of all groups just after dechorionation. Therefore, behavioral assays were conducted at least 1 h after dechorionation. Videos were scored by hand to generate ethograms that highlight rhythmic and spastic aspects of behaviors.

**Immunohistochemistry**

Cryosectioning and antibody staining were performed as previously described (Ogino et al., 2011). Briefly, anesthetized fish embryos were embedded in O.C.T. compound (Tissue-Tek, Torrance, CA) and gradually frozen in liquid nitrogen. Samples were then sectioned on a cryostat (CM-1850, Leica) and mounted on poly-l-lysine coated slides (Newcomer Supply, Middleton, WI) prior to a 10 min fixation in 4% (w/v) formaldehyde (diluted from 16%, Pierce Biotechnology, Rockford, IL). Anti-glycinergic (clone mAb7a, mouse IgG1, 1:500, Synaptic Systems, Göttingen, Germany), and a pan anti-GlyR α subunit (clone mAb4a, mouse IgG1, 1:100, Synaptic Systems) were used as primary antibodies. Alexa 488- and Alexa 568-conjugated donkey anti-mouse IgGs were used as secondary antibodies (1:2000, Life Technologies, Carlsbad, CA). Double staining with anti-glycinergic and anti-GlyR α subunit antibodies was performed sequentially. Stained sections were mounted in Vectashield/DAPI (Vector Laboratories, Burlingame, CA) and images were captured on a confocal microscope using a 1.4 NA 63 × oil objective (SP5, Leica Wetzlar Germany).

**Quantification of synaptic staining**

Images were processed using ImageJ (NIH). The brightness and contrast were adjusted to maximize dynamic range and reduce background noise before analyzing the density of GlyR α subunit and gephyrin puncta. For puncta density analysis, to avoid the impact of variation along the anterior–posterior axis, three images were picked in each stack: one each near top, middle, and the bottom of the stack. The area with puncta expression in each image was calculated in ImageJ. GlyR α subunit and gephyrin puncta in each image were identified and counted by custom MatLab (MathWorks, Natick, MA) programs (Morgan et al., 2008; Soto et al., 2011). For GlyR α subunit and gephyrin puncta co-localization analysis, puncta in the three-dimensional stack were first found by custom MatLab programs (Morgan et al., 2008; Soto et al., 2011). Then the three-dimensional location of each voxel in all GlyR α subunit puncta and gephyrin puncta were compared in MatLab to identify the colocallized puncta.

**Quantitative PCR**

Primers were designed against genes associated with the glycinergic synapse (Table 1). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) to span introns with the goal of excluding the possibility that residual genomic DNA contributed to estimates of transcript abundance. To generate a developmental time series, RNA from 24, 32, 48 and 72 hpf wild type embryos was harvested. RNA extraction method followed the manufacturer’s protocol (TRizol, Life Technologies, Carlsbad, CA). Extracted RNAs were then DNase-treated (DNA-Free, Life Technologies), quantified on a NanoDrop 2000 (ThermoScientific,
West Palm Beach, FL) and electrophoresed on a 1% (w/v) agarose gel to assess quality.

The SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad CA) was used for cDNA synthesis. However, gene specific primers (GSPs) were used in place of oligo(dT) to better detect rare transcripts encoding synaptic proteins. Each synthesis reaction contained 1 µg of RNA template. To generate standard curves, serial dilutions of gel-purified PCR products were used as template. Products were purified using the Wizard SV Gel and PCR purification kit (Promega, Madison WI). Stocks were made from a 1:100 dilution of purified PCR product in DNase/RNase free H2O. Serial dilutions were adjusted to encompass the range of expression levels for each gene. qPCR reactions contained 1 µL of cDNA template to a final volume of 10 µL. The PCR protocol was as follows: 1 step of 95 °C for 1 min; 40 cycles of 95 °C for 15 s, 55 °C for 20 s, 68 °C for 30 s; and a melting curve and a final step of 95 °C. Three samples of each time point were run in duplicate on the same plate with a standard curve consisting of four 1:10 serial dilutions of purified PCR product as template. Relative mRNA copy numbers were calculated based on the expression levels of the housekeeping gene encoding EF1 (Tang et al., 2007).

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotides</th>
<th>Tm (bp)</th>
<th>Size (bp)</th>
<th>Ensembl protein ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>glra1</td>
<td>F: CGAATGCAGGTCCTTCTC</td>
<td>57</td>
<td>67</td>
<td>ENSDARP00000136733</td>
</tr>
<tr>
<td></td>
<td>R: CACACGTGCAGTACAAATGTC</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glra2</td>
<td>F: CTTGTCACCAAAAATCACAGG</td>
<td>55</td>
<td>115</td>
<td>ENSDART00000066192</td>
</tr>
<tr>
<td></td>
<td>R: GGTTGATGCCTCAGTATCCC</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glra3</td>
<td>F: CGCTAACTTGGTGCCAGACAG</td>
<td>54</td>
<td>116</td>
<td>ENSDARPO0000009777</td>
</tr>
<tr>
<td></td>
<td>R: TGGTCTGAGTCTGCTGACG</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glra4a</td>
<td>F: CACAGCAAAAGCTGCTACATCA</td>
<td>50</td>
<td>90</td>
<td>ENSDARP0000146445</td>
</tr>
<tr>
<td></td>
<td>R: TGGTCTCCTGGCAGAGATAA</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glrb</td>
<td>F: CCGGAAACACAGACACAGAGC</td>
<td>54</td>
<td>112</td>
<td>ENSDARP00000074685</td>
</tr>
<tr>
<td></td>
<td>R: AAGTGGAAAAGGACAGCACAGC</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glrbb</td>
<td>F: TGTCATCATCTGCTGTTGG</td>
<td>55</td>
<td>73</td>
<td>ENSDARP0000127692</td>
</tr>
<tr>
<td></td>
<td>R: CTTCTTCTTCTCCTTTTTAC</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gphna</td>
<td>F: CCACCCCATCATAAAGACCC</td>
<td>54</td>
<td>65</td>
<td>ENSDARP0000087952</td>
</tr>
<tr>
<td></td>
<td>R: CATGGTACCTGCGAGCTGGA</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gphnb</td>
<td>F: TACAGACACAGTACAGTGAC</td>
<td>57</td>
<td>85</td>
<td>ENSDARP000005053</td>
</tr>
<tr>
<td></td>
<td>R: TTTCGAGCCCTCCACTATGC</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>slc6a5</td>
<td>F: GGATCCATCCATGTTGCTC</td>
<td>53</td>
<td>87</td>
<td>ENSDARP0000071929</td>
</tr>
<tr>
<td></td>
<td>R: GGGTTATCAGGTAGGGAGCA</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>slc6a6</td>
<td>F: AAGATGGCCATGCCTGAAAGA</td>
<td>54</td>
<td>65</td>
<td>ENSDARP0000087855</td>
</tr>
<tr>
<td></td>
<td>R: GGTTGGCGTTCAGACAGCTC</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>slc3a21</td>
<td>F: ACAAGCCCAAGAATCAGCTG</td>
<td>57</td>
<td>95</td>
<td>ENSDARP0000083453</td>
</tr>
<tr>
<td></td>
<td>R: CTGAGCAAAATGGAGTGGGTA</td>
<td>57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eef1a1l1</td>
<td>F: GTCGCCGCCAGCCCTAACAAT</td>
<td>58</td>
<td>82</td>
<td>ENSDARG00000020850</td>
</tr>
<tr>
<td></td>
<td>R: ATCCAGTGGAGTCAGCATCAGCA</td>
<td>58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>beo allele</th>
<th>cDNA</th>
<th>Substitution</th>
<th>Consequence</th>
<th>SIFT</th>
<th>Polyphen-2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tu230°</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Hirata et al. (2010)</td>
</tr>
<tr>
<td>tm115</td>
<td>c.C232T</td>
<td>p.Q87X</td>
<td>Truncation</td>
<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>tf242</td>
<td>c.T301G</td>
<td>p.Y79D</td>
<td>Missense</td>
<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>ts242</td>
<td>c.A1093T</td>
<td>p.K343X</td>
<td>Truncation</td>
<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
</tbody>
</table>

° Mutant lost.

References

Mutations in glrbb were previously identified as the basis for the beo mutant phenotype (Hirata et al., 2005). In the original study, the underlying mutations for some, but not all beo alleles were identified (Hirata et al., 2005; Table 2). These resulted in either missense (tw38°, p.L255R in TM1, mi106a, p.L275H in TM2) or nonsense (tp221, p.Y79X) mutations. Since glra1 and glrbb genes are both located on zebrafish chromosome 14 and participate in the same GlyR complex, we speculated that some of the remaining beo alleles might actually be mutations in glra1 that could fail to complement mutations in glrbb due to linkage. To determine the types of mutations in remaining beo alleles ta86d, td29, tm115 and tf242, we obtained these lines from the zebrafish Stock Center in Tübingen, Germany. We first amplified the nine coding exons of glrbb, which revealed unique missense mutations (tf242, c.T301G, p.Y79D) or nonsense mutations resulting in premature termination of the GlyRβ polypeptide (tw86d, c.T303A, p.Y79D; tm115, c.C325T, p.Q87X and ta92, c.A1093T, p.K343X respectively; Fig. 1, Table 2). It is also noteworthy that both tp221 and ta86d alleles harbor the same mutation — p.Y79X. Since the remaining beo allele, tu230, was lost (Granato et al., 1996), we conclude that all available beo alleles harbor mutations in glrbb.

Splice site-targeted morpholino knockdown of glr1 and glrbb genes

Given that all available beo alleles can be explained by mutations in glrbb, we investigated the phenotype resulting from glr1 inactivation in zebrafish. Two independent, splice-site-targeted morpholinos (MOs), glr1MOex4 and glr1MOex7, were designed...
against intron/exon boundaries shared among all glra1 transcripts (Fig. 2A). The glra1MOex4 targets the 3′ acceptor site of exon 4, an exon that encodes determinants of glycine binding (Lynch, 2004; as well as the epitope recognized by the anti-GlyR antibody mAb4a) while glra1MOex7 targets the 5′ donor site of exon 7 that encodes the first two membrane-spanning domains (M1 and M2) of the GlyR α1 subunit. Both MOs would be expected to disrupt GlyR function by causing exon-skipping or intron- retention, introducing a frameshift, and truncating translated proteins. RT-PCR analysis of RNA harvested from 28 hpf glra1 morphants revealed the nature of mis- splicing events induced by MO injections. Diagnostic primers were designed to detect both exon-skipping and intron- retention events and morphant-specific bands would be subsequently gel purified and sequenced to determine the exact nature of the MO-induced disruption (Eisen and Smith, 2008). For glra1MOex4, we identified a single morphant band caused by a 61 bp deletion due to a cryptic-splice-donor site towards the 3′ end of exon 4 (Fig. 2B; middle lane). For glra1MOex7, we identified two morphant-specific PCR products, both smaller than the wild type product (Fig. 2B; right-most lane). Sequencing of glra1MOex7 morphant-specific bands demonstrated that the lower of the PCR products is a result of skipping exon 7 entirely, while the middle band is the result of a cryptic splice-site in the middle of exon 7. The cryptic splice-site results in an in-frame transcript. Nonetheless, the resulting protein would still be expected to lack function because of deletion of the transmembrane domain M1.

Although a translation-blocking MO had previously been designed to knock down glrbb (Hirata et al., 2005), this MO did not work in our hands, possibly due to sequence variability surrounding the start methionine in different wild-type strains. In addition, we cannot monitor the efficacy of this translation-blocking MO, due to the lack of a specific GlyR β antibody. Therefore, we designed a new splice-site morpholino against 3′ acceptor site of glrbb exon 5, one of the largest exons upstream of the transmembrane domains (Fig. 3A). Using glrbbMOex5, we were able to faithfully recapitulate the beo mutant accordin phenotype resulting from tonic bilateral muscle contractions (Fig. 3D). RT-PCR analysis of RNA harvested from glrbbMOex5-injected embryos at 28 hpf demonstrates that glrbbMOex5 caused a 156 bp deletion and sequencing this PCR product demonstrated that a cryptic splice-donor site in exon 5 accounted for morpholino-induced mis-splicing (Fig. 3B; right lane).

Quantification of the onset of swimming behavior

From 24 to 36 hpf, zebrafish larvae transition from coiling behaviors (not propulsive) to rhythmic alternating motor behaviors that can propel the larva away from a threat (Saint-Amant and Drapeau, 1998). Therefore analysis of morphant behaviors at these stages occurs against a rapidly changing but highly stereotyped control baseline. To quantify behaviors, we determined the duration of discrete components of two, sequential touch-induced behaviors in each larva (Fig. 4). For example, in response to touch, a control morphant coiles in one direction (light blue bar) until tail touches head (black column), coiles in the opposite direction (dark blue bar) until tail touches head (black column) and slowly returns to rest (tan bar). While the exact timing of the two independent behaviors varies, the sequence of components is the same. To capture the developmental progression of control morphant behaviors from 24 to 36 hpf, we plotted single behaviors for 50 individual larva that span the behavioral transition from coiling to propulsive swimming (Fig. 5; left column). We split the 24–36 hpf time period into two qualitatively distinct early, 24–28 hpf, and late, 29–36 hpf bins. Staging is
Fig. 3. Efficacy and target specificity of splice-site-blocking glrbb exon 5 morpholinos. A. Schematic representation of the zebrafish GlyRβb subunit gene (glrbb). Exons, shown as black boxes, are connected by introns, shown as lines. Exons that encode membrane-spanning domains M1–M4 are indicated. The glrbbMOex5 splice-site-blocking MO (thick black bar) was designed to knockdown glrbb expression by masking exon/intron junction intron4/exon5. Diagnostic primers designed to amplify exons 5 to 8 were used to detect MO-induced mis-splicing events. B. RT-PCR results demonstrate specificity of altered glrbb pre-mRNA splicing caused by MO injection. The leftmost lane contains a size standard ladder followed by two lanes of RT-PCR from RNA samples of 28 hpf embryos injected with 2 nL 0.5 mM control MO and 2 nL 0.5 mM glrbbMOex5 respectively. In the upper gel, glrbb diagnostic primers are used for PCR from cDNA synthesized using a gene-specific primer for glrbb. In contrast to control morphants, where a single strong PCR product of 430 bp is detected, glrbbMOex5 morphants exhibit reduced levels of the wild type PCR product and a new product of 274 bp, indicating exon skipping. In the lower gel, PCR for a GlyT2 cDNA (slc6a5), amplified from cDNA synthesized using anchored oligo-dT primers, is used as a loading control. C. Percentages of glrbb MOex5 morphants exhibiting wild type (wt), spastic or accordion phenotypes are plotted at 24–28 hpf and 29–36 hpf. The number of embryos analyzed is indicated at the center of each plot. D. Pictures of 5 representative 48 hpf larvae demonstrate shortening of the body axis produced by tonic bilateral contraction in glrbb but not glra1 morphants. Length and width (yolk to back) measurements (average n = 5, error bars indicate standard deviation). A Student’s t-test indicates that glrbb morphants are significantly shorter ($p < 0.05$) and wider ($p < 0.005$) than their control and glra1 morphant counterparts.

Fig. 4. Repeated stimulation evokes erratic, sometimes spastic behaviors in glra1 and glrbb morphants. Two sequential, touch-evoked behaviors are plotted for four, representative morphant larvae: control, A; glra1MOex4, B; glra1MOex7, C; and glrbbMOex5, D. Behaviors were recorded using a high-speed camera and plotted as lines with the different behavioral components indicated by line color and each component’s duration indicated by line length (scale bar = 60 ms; light blue = initial bend; dark blue = counter-bend; black column = top of coil, when the tail comes closest to touching the head; tan = return to rest; red = stuck or bend to same side; boxed diagonal lines = bilateral contraction). Still frames are included above the first of two plots with the number of pictures/length reflecting relative rates of change in the movement. A. The control morphant exhibited behavioral stereotypy with repeat stimulation: both behaviors consist of two coils in alternating directions followed by relaxation. B. The glra1MOex4 morphant exhibited erratic, spastic behaviors. In the first behavior, the glra1MOex4 morphant produced spastic behavior, getting stuck in the first coil and producing a second coil to the same side as the first. The second behavior was different from the first—the glra1MOex4 larva produced only one coil, and still spastic—the larva got stuck in the coil. C. The glra1MOex7 morphant also exhibited erratic, spastic behaviors. In the first behavior, the glra1MOex7 larva produced three coils, all to the same side. The second behavior was different from the first—this behavior was not spastic, consisting of two smooth alternating coils that return to rest. D. The glrbbMOex5 morphant larvae exhibited erratic, bilateral contraction and spastic behaviors. In the first behavior, the glrbbMOex5 morphant produced bilateral contraction that actually shortened the body axis—the classic accordion phenotype. The second behavior was different from the first—this time the glrbbMOex5 morphant produced two coils, getting stuck in each of these coils.
Spasticity is characteristic of both glra1 and glrbb morphants but bilateral contraction mainly occurs in glrbb morphants. A. One behavior per larva is plotted for representative populations of 50 control (left column), 48 glra1 (middle column), and 47 glrbb (right column) morphants that span the 24–36 hpf time period. All larvae were stimulated twice but only one of each pair of behaviors was plotted here. (Scale bar = 200 ms; light blue = initial bend direction; dark blue = counterbend; black column = top of coil, when the tail comes closest to touching the head; tan = return to rest; red = stuck or bend to same side; boxed diagonal lines = bilateral contraction). Larval staging was based on using the angle of the head to the long tail axis as diagnostic of stage (Kimmel et al., 1995). B. Time from rest to first coil is plotted for all recorded control (n = 83), glra1ex4 (n = 92), glra1ex7 (n = 92), and glrbbex5 (n = 68) morpholino-injected larvae. ANOVA indicated a significant effect of morpholino injected on time to first coil [F(3,334) = 15.8225, p = 0.0001]. Post hoc Bonferroni-corrected Student’s t-tests compared all means and showed significant differences at p < 0.05 (**) that both glra1 morphants and glrbb morphants are significantly different from control morphants. In addition, glra1ex7 morphant times to first coil were significantly longer (p = 0.05, black triangle) than glra1ex4 and glrbbex5 morphants. C. Hitch durations in milliseconds are plotted for any of the control, glra1, or glrbb larva that exhibited this behavior. ANOVA indicated no significant effect of morpholino injected on hitch time [F(3,135) = 2.41, p = 0.07], thus there is no subsequent post hoc analysis. D. ‘Accordion’ durations in milliseconds are plotted for any of the control, glra1, or glrbb larva that exhibited this behavior. The ‘accordion’ behavior is clearly more frequent in glrbbex5 morphants, occurring 27 times compared to never in controls and 5 times in all glra1 morphant larvae.

Based on a two criteria: time in hpf and morphology (Kimmel et al., 1995). Single coils first transitioned to alternating coils, 24–28 hpf, and then, 29–36 hpf, to swimming characterized by shallower and more rapid alternating bends (Fig. 5; left column).

**glra1 and glrbb morphants exhibit spasticity**

Both glra1 and glrbb morphants exhibit spastic and erratic behaviors during 24–36 hpf (Figs. 2C & 3C). Spastic behaviors (indicated by red bars) were characterized by hitches. We defined hitches as pauses that disrupted the smooth progression of a movement and/or repeated bends to one side (Figs. 4B, C & D; Fig. 5, middle & right columns). Behaviors were also erratic, lacking stereotypy when elicited multiple times (Supplemental movies; Figs. 4B, C & D). In addition, the duration of the time to first coil was significantly longer in glra1 morphants (glra1MOex4: 296 ± 18 ms, n = 93; glra1MOex7: 444 ± 56 ms, n = 93) and glrbb morphants (glrbbMOex5: 241 ± 33 ms, n = 69) compared to control morphants (Control MO: 109 ± 11 ms, n = 83, means ± standard deviation for all treatments; Fig. 5B). Spasticity in both glra1 and glrbb morphants (Fig. 5C) was associated with a delay in the onset of rhythmic behaviors seen in control morphants at 30–36 hpf. In addition to spasticity, glrbb morphants exhibited simultaneous bilateral contractions strong enough to shorten the body axis—the diagnostic accordion phenotype (Granato et al., 1996; Figs. 3D, 4D & 5D; Supplemental movies). Moreover, in contrast to glra1 morphants in which behavioral disruptions were transient, glrbb morphant phenotypes persisted, resulting in larvae with a shortened axis by 48 hpf (Fig. 3D). Spastic phenotypes were recapitulated by injecting mRNA encoding a dominant-negative GlyR α1 subunit mutant R270Q (Figs. 6B & C). Curiously, by contrast to the dominant-negative glra1 R270Q RNA that lacked a morphological phenotype, over-expressing the wild type GlyR α1 subunit mRNA produced shortened-axis and Cyclops phenotypes (Figs. 6A & C). Cyclopic phenotypes are commonly associated with disrupted sonic hedgehog (shh) signaling in early embryos and likely reflect the disruptive effect of mis-expressing functional glycine receptors.

**grrb is epistatic to glra1 at the level of behavior**

To formally test for epistatic relationships between glra1 and glrbb, we co-injected glrbbMOex5 with wither glra1MOex4, glra1MOex7, or control morpholinos and analyzed the resulting phenotypes (Table 3).
In summary, punctate GlyR immuno-staining was reduced in both *glra1* and *glrbb* morphants at both 24 and 48 hpf. At 24 hpf, GlyR puncta in *glra1* morphants were dramatically reduced in the medial spinal cord, although some remaining puncta – presumably containing other GlyR α subunits – co-localized with gephyrin and could be detected in the lateral spinal cord. This staining pattern contrasts with *glrbb* morphants, in which loss of both GlyR clusters and co-localization with gephyrin was evident.

Quantification of gene expression for components of the glycnergic synapse in developing zebrafish

To quantify the relative mRNA expression levels of genes known to be critical to glycnergic synapse function, we generated a developmental time-course for eleven zebrafish genes implicated in the function of glycnergic synapses using quantitative PCR (Fig. 9). While quantitative PCR yields no spatial information, it does reveal two distinct groupings of genes that share developmental expression trajectories. Expression of the majority of genes including *glra1*, *glrba*, *glrbb*, *gphnb*, *gphna*, and *slc6a9* exhibited a steady increase with development. By contrast, expression of *gphna3*, *slc6a9* (*GlyT1*) and *slc32a1* (*VIAAT*) increased until 48 hpf and then either leveled off (*GlyT1/VIAAT*) or decreased (*gphna3, gphna*). These shared developmental expression trajectories could reflect shared transcriptional regulation. In the context of multiple duplicated subunits, qPCR also provides information about the dominant subunits expressed at different developmental time periods at the mRNA level. For example, in the case of the GlyR β subunits, the β1b subunit mRNA is ~5 fold more abundant.
at both 48 and 72 hpf than βa. Likewise, the expression of gphna mRNA dominates at 48 hpf being 35 times more abundant than gphnb while at 72 hpf, it is only four times more abundant. The levels of GlyRα subunit transcripts differ with the most abundant being glra2 and glra3. Expression of glra1, glra4a and glrbb, is barely detectable at 24 and 32 hpf, but increases onwards from 48 hpf.

Discussion

Our study demonstrated that all available alleles of the GlyR mutant bandoneon correspond to defects in glrbb, encoding the GlyRβb subunit. Consistent with recent studies on human startle disease mutations in the GlyRβ subunit gene (Al-Owain et al., 2012; Chung et al., 2013; James et al., 2013; Lee et al., 2013), mutations resulting in protein truncation are the predominant mechanism disrupting GlyRβb subunit function in the beo allele series (4/7 alleles), followed by missense mutations affecting residues in the membrane spanning domains M1/M2 (2/7 alleles) or large extracellular domain (1/7 alleles). Y79D introduces a negative charge and a hydrophilic residue into a packed area of hydrophobic side-chains, which is likely to disrupt the local β-sheet fold on which it resides in the extracellular domain, possibly indirectly affecting glycine binding. The substitution L255R may disrupt packing of hydrophobic and aromatic residues between TM helices due to the large, positively charged side-chain of arginine, thus affecting the correct fold of the subunit or insertion into the membrane. R275 is equivalent to R252 in the human GlyRα1 subunit (James et al., 2013). A spontaneous mutation causing the same substitution in this residue (R252H) is known to cause startle disease when inherited in compound heterozygote manner (Vergouwe et al., 1999). Substitutions at R252 in the GlyRα1 subunit have been shown to severely disrupt the correct function of

Fig. 7. GlyRα puncta are reduced in glra1 morphants and absent in glrbb morphants. Transverse sections through fast-frozen 24 hpf (left; A–D) and 48 hpf (right; A′–D′) larvae were stained for GlyRα subunits (red), gephyrin (green), and nuclei (blue). Diagrams above the micrographs show the tight packing of spinal cord cells. In the diagrams, cells with purple cytoplasm represent the differentiated neurons: primary motor neurons (PMNs) that exit the spinal cord to innervate muscle, early differentiating interneurons, and dorsal sensory Rohon Beard neurons. These differentiated neurons form in a ring that surrounds the medial stem cells, outlined in gray. By 48 hpf more neurons have differentiated and become incorporated into the circuit as reflected by an increased synaptic staining on lateral neurons. Representative images for each stage and morpholino treatment are displayed as groupings of four images with the leftmost image giving a low magnification view of the entire spinal cord with the motor/inter-neuron region boxed in white magnified in the subsequent three images. A. In 24 hpf control morphants, GlyRα and gephyrin staining are non-overlapping. GlyR puncta decorate precursor cells near the spinal cord midline (red arrowheads) as well as lateral neurons (red arrows). Gephyrin puncta (green arrows) are solely associated with lateral neurons, but do not co-localize with GlyRs at this developmental time point. B, C. In both glra1 morphants, GlyRα subunit staining is absent in precursors near the midline. Curiously, in glra1 morphants, residual GlyRα subunits did co-localize with gephyrin (yellow arrows) in lateral neurons. D. In 24 hpf glrbbMOex5 morphants, gephyrin puncta (green arrows) occur in the absence of GlyRα subunit puncta. A. In 48 hpf control morphants, GlyRα subunit and gephyrin puncta commonly co-localized. B′, C′. In 48 hpf glra1 morphants, a subset of GlyRα subunit and gephyrin puncta commonly co-localized but these co-localized puncta are positioned more laterally than in control morphants. D′. 48 hpf glrbbMOex5 morphants lacked clear GlyRα subunit puncta, but exhibited smaller gephyrin puncta that tended towards the lateral neuropil rather than circum-nuclear as in control morphants. Scale bars: 10 μm.
The protein, specifically affecting GlyR membrane trafficking (Rea et al., 2002). Thus, R275H in the zebrafish GlyR β subunit is predicted to operate by a similar pathogenic mechanism.

Since mutations in GlyR α1, β3 subunit or GlyT2 genes all cause startle disease in dogs, cattle, mice and humans, one mystery is why no mutations in glrα1 or slc6a5 have been discovered to date in phenotypic screens of zebrafish mutants generated by ENU mutagenesis (Granato et al., 1996). We now have a potential explanation for this finding, since knocking down glrα1 (encoding the GlyR α1 subunit) produced a relatively mild phenotye compared to beo, characterized by spasticity that delayed the onset of rhythmic motor behaviors. This phenotype is likely to be genuine, since it was reproduced using two different MOs, and using a dominant-negative zebrafish GlyR α1 subunit mutant, R270Q — equivalent to the most common dominant startle disease mutation found in humans (R271Q; Chung et al., 2010; Shiang et al., 1993).

Notably, this phenotype is distinct from the ‘accordion’ behavior produced by inactivation of glrbb, encoding the GlyR β3 subunit (Hirata et al., 2005). While our study clearly implicates GlyRs containing the α1 subunit in the smooth transition from spontaneous coiling behaviors to propulsive locomotion, the finding that glrα1 loss-of-function causes this transient phenotype in zebrafish was unexpected. Several potential compensatory mechanisms have been observed in animals with loss-of-function mutations in the GlyR α1 subunit gene, including increased presynaptic glycine uptake, increased inhibitory GABAergic transmission, or up-regulation of additional GlyR α subunit genes (Graham et al., 2003; Gundlach et al., 1988; Kling et al., 1997; Lunnis et al., 1990; Wässle et al., 1998). However, a strong visible phenotype is always observed in these animal models and in recessive human startle disease caused by GLRA1 mutations (Chung et al., 2010). So what could be different in the zebrafish? Since the beo phenotype can also be reproduced by applying the GlyR antagonist strychnine (Granato et al., 1996; Hirata et al., 2005), our interpretation is that the beo phenotype results from the loss-of-function of multiple GlyR subtypes. This supported by epistasis experiments that demonstrate glrbb is epistatic to glrα1 at the level of behavior. Consistent with epistasis, immunohistochemistry showed that glrα1 morphants exhibited residual GlyR α subunit puncta, while in glrβ3 morphants, GlyR α subunits were trapped in intracellular aggregates. Moreover, qPCR results also identified glrα2, glrα3, and glrα4a in developing embryos, which could participate in α2/β3 or α3/β3 GlyRs. Certainly, compared with mammals, the zebrafish has additional opportunities for compensating for the loss of glrα1, due to the duplication of the α4 subunit genes (glrα4a, glrα4b; Hirata et al., 2010). In our view, the zebrafish GlyR gene expansion (Hirata et al., 2010) reflects strong selective pressure...
for effective escape responses in this organism that develops externally with minimal parental care. Taken together, our data suggest that knocking down zebrafish orthologs of human hyperekplexia genes will likely produce a phenotypic range from more mild spasticity (glra1 and glrbb) to bilateral contractions (glrrb).

The behavioral transition from slow coiling to fast rhythmic flexions (Saint-Amant and Drapeau, 1998; Warp et al., 2012), which is delayed in glra1 morphants, reflects changes taking place in the connectivity of neural circuits. While embryonic slow coiling behaviors rely upon gap-junctional coupling among neurons (Brustein et al., 2003; Ganser and Dallman, 2009; Saint-Amant and Drapeau, 2001), larval rhythmic flexions rely upon synaptic inputs (Brustein et al., 2003; Kinkhabwala et al., 2011; Koyama et al., 2011; McLean and Fetcho, 2009; McLean et al., 2008). Our data show that zebrafish express α1ββ GlyRs throughout this period of behavioral transition. These findings contrast with rodent spinal cord (Agaki et al., 1991; Becker et al., 1988; Harvey et al., 2004; Singer et al., 1998) and embryonic spinal cultures (Hoch et al., 1989) in which the GlyR α2 subunit predominates at early stages with the GlyR α1 and α3 subunits appearing postnatally. By contrast, in zebrafish, we show that GlyR α subunit immunostaining on neuronal precursors and differentiating neurons was disrupted by knocking down either glra1 or glrrb, suggesting that the majority of early GlyR puncta in 24 hpf zebrafish spinal cord are composed of α1 and ββ subunits.

In addition to glycine (Flint et al., 1998), non-synaptic release of both glutamate and GABA are known to cause Ca2+ transients in neural precursors that promote growth and maturation (Akerman and Cline, 2006; Ben-Ari and Spitzer, 2010; Haydar et al., 2000; Komuro and Rakic, 1996; Owens and Kriegstein, 2002). Both immature neurons and muscle express a variety of neurotransmitter receptors prior to innervation (Borodinsky et al., 2004; Moody and Bosma, 2005) at which glutamate and GABA are known to cause Ca2+ transients in the neuronal cytoplasm. These GlyRs are shown assembled into pentameric, ligand-gated Cl− channels with a 2α:3β subunit stoichiometry. These GlyRs are shown clustered at the synapse via interactions between GlyR β subunit and the scaffolding protein gephyrin. B-D. Average expression levels for all genes and time points (24, 32, 48, 72 hpf) are shown for an average of three independent mRNA preparations ± standard deviation. Genes are grouped into graphs according to their levels of expression and all gene expression is normalized to the housekeeping gene EF1. B. GlyT1 (slc6a9) and VIAAT (slc32a1) expression levels. C. GlyR α1, α4a, βα and ββ subunit (glra1, glra4a, glrba, glrbb) and GlyT2 (slc6a9) expression levels. D. GlyR α2 subunit, α3 subunit, gephyrin-a and gephyrin-b (glra2, glra3, gphna, gphnb) expression levels.

Conclusions

Recent proteomic analyses of glutamatergic synapses suggest that up to a thousand proteins participate in this neuronal specialization (Grant, 2012). Even if glycineergic synapse function requires a fraction of this number of specialized proteins, the door is certainly open to complex forms of inheritance for startle disease. Despite the significant successes in identifying genetic defects that cause startle disease, many idiopathic cases remain in which the genetic cause is unresolved (Carta et al., 1998; Moody and Bosma, 2005) creating sequential checkpoints so that development only progresses when coordinate regulation of channels, receptors, and transporters successfully produce signals that feedback to promote maturation and qualitatively change excitability of the circuit (Ben-Ari and Spitzer, 2010). One possibility is that by knocking down glra1 we delay the onset of rhythmic behaviors by disrupting signaling associated with depolarizing glycine (Ben-Ari and Spitzer, 2010).
in overexpression models using proteins harboring potential disease-causing mutations.

Acknowledgments

We would like to thank UM Biology Molecular Core manager Dr. Hurt and Imaging Core manager Dr. Baker for their help with sequencing and confocal imaging respectively. We thank Drs. Baker and Wikramanayake for their feedback on writing. Anthony Sanchez for his assistance in analyzing embryonic movements, and Ricardo Cepeda (UM), Prof. Steve Wilson and Carole Wilson (UCL) for fish care. We would like to acknowledge Dr. Hiromi Hirata for his assistance in generating fast-frozen tissue sections and members of Dr. R. O. Wong’s lab, a Travel Grant from the Company of Biologists, and Dr. Jiang for teaching Qing Yan to program MatLab to quantify immunohistochemistry. We also thank Lingyu Wang for assisting in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station. Grant/Other Support: Medical Research Council (G0601585 to RJH), University in the development of an image analysis work station.


