Distinct phenotypes in zebrafish models of human startle disease ☆

Lisa R. Ganser [d,1], Qing Yan [a,1], Victoria M. James [b,c], Robert Kozol [a], Maya Topf [c], Robert J. Harvey [b], 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, Birkbeck College, London WC1E 7HX, UK
[d] Department of Biology and Physics, 1000 Chastain Rd, #1202, Kennesaw State University, Kennesaw, GA 30144 USA

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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 glycinergic 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 glrbb (one of two zebrafish orthologs of human GLRB) that cause compromised glycinergic 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 zebrafish 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 glrbb morphants exhibited embryonic spasticity, only glrbb morphants exhibited bilateral contractions characteristic of beo mutants. Likewise, zebrafish over-expressing a dominant startle disease mutation (GlyR $\alpha1^{R271Q}$) exhibited spasticity but not bilateral contractions. Since GlyR βb can interact with GlyR α subunits 2–4 in addition to GlyR α1, loss of the GlyR βb 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 glycinergic 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.

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 glycinergic 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 glycinergic 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

* Corresponding author.
E-mail address: jdallman@bio.miami.edu (J.E. Dallman).
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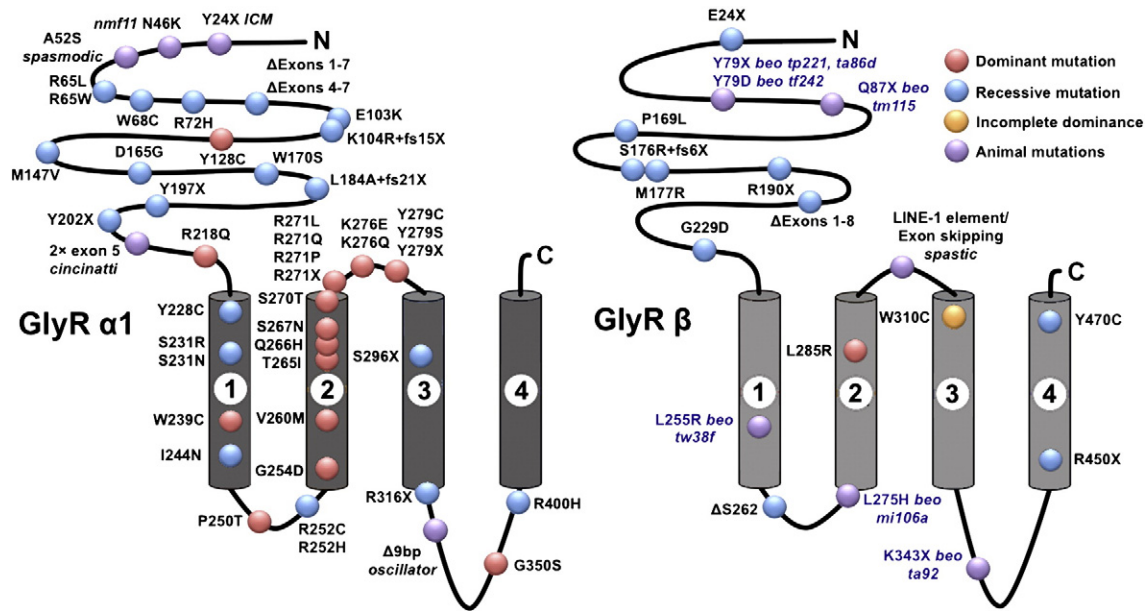[1] These authors contributed equally to this work.

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 known to cause human startle disease. Purple circles indicate the relative positions of amino acid alterations found in mouse, cattle and zebrafish glycinergic 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; Mülhardt 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*, *glrba*, and *glrbb*; Hirata et al., 2010). The two duplicate genes, *glra4b* and *glrbb* 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 (*glrbb*), resulting in touch-induced simultaneous bilateral contractions of the axial muscles due to the loss of reciprocal glycinergic inhibition of motor circuits (Hirata et al., 2005, 2010). Despite the duplication of the GlyR β subunit genes in zebrafish, *glrba* is unable to compensate for the loss of *glrbb* 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 *glrbb*.

To directly compare zebrafish *glra1* and *glrbb* startle disease models, we designed splice-site morpholinos to knockdown the expression of these genes (Draper et al., 2001). As expected, injecting *glrbb* morpholinos produced a phenotype similar to *beo* mutants, characterized by simultaneous bilateral contractions strong enough to shorten the body. In addition, both *glrbb* and *glra1* morphants produced spastic and erratic behaviors at early stages, but in contrast to *glrbb* morphants, *glra1* morphants only rarely produced bilateral contractions and by 48 hpf *glra1* morphants produced normal behaviors. Immunohistochemistry of *glrbb* and *glra1* morphants also demonstrated distinct GlyR immunostaining patterns on early-differentiating spinal neurons. While *glra1* morphants exhibited reduced but still synaptic GlyR staining, *glrbb* 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 glycinergic 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