Zebrafish bandoneon mutants display behavioral defects due to a mutation in the glycine receptor \( \beta \) subunit

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Zebrafish bandoneon mutants display behavioral defects due to a mutation in the glycine receptor β-subunit


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Bilateral alternation of muscle contractions requires reciprocal inhibition between the two sides of the hindbrain and spinal cord, and disruption of this inhibition should lead to simultaneous activation of bilateral muscles. At 1 day after fertilization, wild-type zebrafish respond to mechanosensory stimulation with multiple fast alternating trunk contractions, whereas bandoneon (beo) mutants contract trunk muscles on both sides simultaneously. Similar simultaneous contractions are observed in wild-type embryos treated with strychnine, a blocker of the inhibitory glycine receptor (GlyR). This result suggests that glycinergic synaptic transmission is defective in beo mutants. Muscle voltage recordings confirmed that muscles on both sides of the trunk in beo are likely to receive simultaneous synaptic input from the CNS. Recordings from motor neurons revealed that glycinergic synaptic transmission was missing in beo mutants. Furthermore, immunostaining with an antibody against GlyR showed clusters in wild-type neurons but not in beo neurons. These data suggest that the failure of GlyRs to aggregate at synaptic sites causes impairment of glycinergic transmission and abnormal behavior in beo mutants. Indeed, mutations in the GlyR β-subunit, which are thought to be required for proper localization of GlyRs, were identified as the basis for the beo mutation. These data demonstrate that GlyRβ is essential for physiologically relevant clustering of GlyRs in vivo. Because GlyR mutations in humans lead to hyperkplexia, a motor disorder characterized by startle responses, the zebrafish beo mutant should be a useful animal model for this condition.

channel | synapse | hyperkplexia | strychnine

Zebrafish embryos display three stereotyped behaviors by 36 h postfertilization (hpf) (1, 2). The earliest behavior consists of spontaneous, alternating coiling of the tail. This slow coiling behavior is independent of sensory stimulation and starts at 17 hpf and declines by 26 hpf. After 21 hpf embryos start to respond to mechanosensory stimulation with the two or three rapid trunk contractions that constitute the escape response. After 26 hpf, mechanosensory stimulation starts to initiate swimming episodes. The frequency of muscle contractions during swimming increases from 7 Hz at 26 hpf to 30 Hz at 36 hpf, the latter being comparable to the frequency of swimming in adult zebrafish (3).

The large-scale Tübingen mutagenesis screen isolated 63 zebrafish mutants with abnormal touch responses (4). Amongst them, mutations in seven genes, including accordion, zieharmonika/ache, and bandoneon (beo) were classified as accordion-type mutants. All mutations in this class displayed apparent simultaneous muscle contractions in both sides of the trunk, resulting in the shortening of the trunk in response to touch. Because this class of mutants was phenocopied by exposing wild-type animals to strychnine, a glycine receptor (GlyR) blocker, accordion-type mutants were predicted to have defects in inhibitory synaptic transmission within the CNS (4). However, the first accordion-type mutations that have been molecularly identified, accordion and zieharmonika/ache, were genes encoding an ATPase Ca2+ pump (5, 6) and acetylcholine esterase (7, 8), respectively, which were required by muscles. A dominant mutation, nic1b12, which carried a gain-of-function mutation in the α-subunit of the muscle nicotinic acetylcholine receptor, also displayed accordion-like phenotype due to the hypercontraction of trunk muscles (9).

The neural circuits in the hindbrain and spinal cord that mediate the earliest behaviors exhibited by vertebrate embryos have been extensively studied in lamprey, frog, and fish (10–12). The generation of reliable alternating activity requires a mechanism for reciprocal inhibition, which is mediated by glycinergic transmission (10–13). In mammals, the GlyRs in the adult spinal cord consist of a pentameric complex composed of three ligand-binding α1-subunits and two β-subunits, whereas fetal GlyRs are homomers composed of five α2-subunits (refs. 14 and 15; reviewed in ref. 16). In zebrafish, GlyR cDNAs encoding the α1-subunit and β-subunit have been cloned, and their expression patterns were described mainly in the adult CNS (17, 18). The β-subunit interacts with gephyrin, a cytoplasmic tubulin-binding protein found in postsynaptic densities, to localize GlyRs to the synapse (19–23).

In this paper, we use in vivo electrophysiology to show that the accordion-type phenotype of beo embryos results from mechano- sensitiv stimulation-induced simultaneous, bilateral activation of the trunk muscles. Furthermore, the coactivation of the muscles stems from a lack of glycinergic synaptic transmission due to an absence of GlyR clustering. The underlying basis for the beo phenotype are a putative null mutation and two missense mutations in the glrb2 gene that encodes the β-subunit of the GlyR. Mutations in the α1- or β-subunit of the GlyR cause an inherited human disorder known as hyperkplexia, which is characterized by exaggerated startle responses, neonatal hypertonia, and excessive falling in response to sudden acoustic or tactile stimuli (24–26). Because the beo embryos are accessible at stages that exhibit mutant phenotype, these zebrafish mutations may be useful as an animal model for hyperkplexia.

Materials and Methods

Recording from Muscle and Motoneurons. The dissection protocols for in vivo patch recordings have been described in refs. 3 and 27. Strychnine was applied at 5 μM. For the miniature currents, 1 μM tetrodotoxin (TTX; Sigma), 10 μM 6-cyano-7-nitroquinolin oxide-2,3-dione (CNQX), 50 μM d-2-amino-5-phosphonovaleric acid (APV), and 5 μM strychnine were applied in the bath to block the voltage-gated sodium channel, α-amino-3-hydroxy-5-methyl-4-

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Abbreviations: beo, bandoneon; GlyR, glycine receptor; CNQX, 6-cyano-7-nitroquinolinoxaline-2,3-dione; APV, d-2-amino-5-phosphonovaleric acid; hpf, hours postfertilization; MO, morpholino oligonucleotide; dpf, days postfertilization; TTX, tetrodotoxin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB195560).

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isoxazolepropionic acid receptor, NMDA receptor, and glycine receptor, respectively.

**Immunostaining, In Situ Hybridization, and Acridine Orange Labeling.** Immunostaining and *in situ* hybridization were done as described in refs. 5 and 28. Anti-slow muscle fiber (F59, Developmental Studies Hybridoma Bank, Iowa City, IA) at a concentration of 1:50 and anti-GlyRα (mAb4a, Synaptic Systems, Goettingen, Germany) at a concentration of 1:50 (29) were used for primary antibodies. Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) at a concentration of 1:50 (29) were used for primary antibodies. Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) at a concentration of 1:2,000, biotinylated anti-mouse IgG (Vector Laboratories) at a concentration of 1:250, and Qdot 605 streptavidin (Quantum Dot, Hayward, CA) at a concentration of 1:1,000 were used for visualization. The color of anti-GlyRα staining was false-colored green to make the labeling more apparent in the micrographs. For *in situ* hybridization, gfra1 (GenBank accession no. NM_131402) and grh2 (GenBank accession no. AB195560) probes covering all coding region was used. Acridine orange (Sigma) staining was performed as described in ref. 30.

**Additional Details.** Details for animals, behavioral assay, recording from muscle and motor neurons, electrophysiological recording, knockdown, mRNA rescue, mutagenesis of grh2, RT-PCR, and Western blotting are provided in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

**Results**

**beo Mutants Display Dorsal Bend in Response to Touch.** As part of an ongoing N-ethyl-N-nitrosourea mutagenesis screen, we isolated a new allele (mi106a) of *beo* that showed indistinguishable phenotype from a Tübingen mutant *beo*p221 (4). We performed phenotypic analysis on *beo*p221. The three early behaviors of *beo* embryos were analyzed to better clarify the nature of the mutation. *beo* mutants displayed normal spontaneous coiling at a frequency (0.27 ± 0.11 Hz, n = 10) at 22 hpf that was comparable with that of wild-type siblings (0.23 ± 0.08 Hz, n = 10). This finding indicates that the mutation does not perturb spontaneous coiling. However, when *beo* embryos are touched with forceps at 24 hpf, they appear to simultaneously contract the trunk muscles on both sides, resulting in the shortening of the body and a dorsal flexure of the trunk (Fig. 1B and Movie 1, which is published as supporting information on the PNAS web site) much like *accordion* embryos (5). In comparison, 24 hpf wild-type siblings respond to touch with two or three fast, alternating contractions of the trunk (Fig. 1A and Movie 2, which is published as supporting information on the PNAS web site). Unlike the relatively long lasting response of *accordion* mutants (~5 s), the bilateral response in *beo* mutants is fast and is over within 1 s of the touch, much like the escape response of wild-type siblings (wild-type: 0.91 ± 0.12 s, n = 10; *beo*: 0.86 ± 0.10 s, n = 10). Interestingly, wild-type embryos treated with 70 μM strychnine exhibited apparent, fast, bilateral contractions that were indistinguishable from *beo* mutants (data not shown). At 48 hpf, mutants failed to initiate swimming in response to touch and exhibited a response similar to the bilateral contractions observed at 24 hpf (data not shown). Thus, *beo* mutants exhibit normal spontaneous coiling, but they have defective touch responses and lack the ability to swim.

In addition to abnormal behavior, *beo* embryos exhibited morphological defects at 48 hpf. The head-to-tail length of *beo* embryos at rest was 15% shorter (2.58 ± 0.14 mm, n = 10) than that of wild-type siblings (3.03 ± 0.08 mm, n = 10; Student’s t test, P < 0.01) (Fig. 1C). Slow-twitch muscle fibers in *beo* were disturbed (Fig. 1D and E), but acridine orange labeling showed no significant increase in cell death (data not shown). Disruption of the notochord was also observed in *beo* (Fig. 1 F and G). These morphological defects were presumably secondary effects due to the mechanical stress caused by simultaneous, bilateral muscle contractions. Supporting this idea, suppression of motor behavior by tricaine (ethyl 3-aminobenzoate methanesulfonate), a weak Na<sup>+</sup> channel inhibitor, or N-benzyl-p-toluene sulfonamide, a specific inhibitor for muscle myosin, abolishes these morphological defects in *beo* (data not shown). As with the behavior, wild-type embryos treated with 70 μM strychnine showed these morphological defects that were indistinguishable from *beo* mutants (data not shown). The *beo* larvae died at ~10 days postfertilization (dpf), possibly from an inability to swim and feed effectively.

**Muscle in beo Receive Abnormal Input in Response to Touch.** One way to explain the abnormal behavior in *beo* is that trunk muscles on both sides seem to contract at the same time despite normal alternating output from motor neurons because muscles contract for longer than normal, as was the case with *accordion*, *zieharmonika/ache*, and *nic`. Refs. 10, 12] mutants, in which all of the defects were

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**Fig. 1.** *beo* embryos exhibit aberrant touch responses and morphological defects. (A) Frames from a movie showing a wild-type sibling (24 hpf) respond to mechanosensory stimulation with two alternating contractions of the trunk. The time of each frame is shown on the upper right corner of each frame. (B) A *beo* embryo (24 hpf) responds to touch with a strong bilateral trunk contraction that causes the trunk to bend dorsally. (C) At 48 hpf, *beo* mutants are shorter in length compared with wild-type siblings. (D) The normal pattern of trunk slow-twitch muscle fibers labeled with monoclonal antibody F59 seen in a side view of a wild-type sibling (48 hpf). (E) The trunk slow-twitch fibers are disarrayed in a *beo* mutant (48 hpf). (F) Micrograph showing the notochord in a wild-type sibling (48 hpf). (G) The notochord of a *beo* mutant (48 hpf) exhibits defects.
These results indicate that the trunk muscles were simultaneously activated by motor neurons on the two sides in beo mutants and in strychnine-treated wild-type embryos. Interestingly, the latency of beo was between that of ipsilateral stimulation and contralateral stimulation in wild-type embryos, suggesting that the absence of beo gene product slows down the response in addition to causing bilateral contractions.

To further characterize the output from the CNS, we examined the pattern of activity after mechanosensory stimulation. At 48 hpf, muscle recordings from wild-type embryos showed sustained episodes of rhythmic depolarizations after mechanosensory stimulation (Fig. 2D). The frequency of this rhythmic activity (28.9 ± 4.1 Hz, n = 10) was within the normal range of swimming (3). The duration of the muscle response in wild-type siblings was 2.53 ± 1.2 s with a peak depolarization of 7.0 ± 1.8 mV (n = 10). When strychnine was added to the bath solution, the pattern of activity was dramatically altered so that the responses were shorter in duration (0.51 ± 0.06 s, n = 6; Student’s t test, P < 0.01), greater in peak depolarization (12.9 ± 2.5 mV; Student’s t test, P < 0.01), and completely devoid of rhythmic activity (Fig. 2E). Similarly, muscle recordings from beo embryos showed a single peak of depolarization without rhythmicity that was shorter in duration (0.50 ± 0.05 s, n = 6; Student’s t test, P < 0.01) and larger in amplitude (13.2 ± 2.4 mV, n = 6; Student’s t test, P < 0.01) than in wild-type embryos without strychnine (Fig. 2F). Thus, the similarity of defective behavior and muscle physiology in beo mutants and strychnine-treated wild-type embryos is consistent with the hypothesis that glycinenergic transmission is aberrant in beo embryos.

**Glycinerinic Synaptic Transmission Is Aberrant in beo Mutants.** To see whether glycineergic transmission is defective in mutants, we measured spontaneous glycinerinic synaptic currents in motor neurons at 48 hpf. Spontaneous synaptic currents in the presence of TTX were less frequent in beo (0.30 ± 0.05 Hz, n = 4) (Fig. 3D) compared with wild-type embryos (0.64 ± 0.06 Hz, n = 4) (Fig. 3A). Application of blockers for the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (CNQX) and NMDA receptor (APV) removed a subset of the spontaneous currents in wild-type (0.33 ± 0.07 Hz, n = 10) (Fig. 3B). The remaining events were abolished by the addition of 5 μM strychnine (0 Hz, n = 6) (Fig. 3C), suggesting that they were glycineergic currents. In beo, spontaneous currents were completely eliminated by blocking glutamate receptors with CNQX and APV (0 Hz, n = 11) (Fig. 3E), and further application of strychnine had no effect (0 Hz, n = 11) (Fig. 3F). Thus, the frequency of glutamatergic currents in wild-type motor neurons (0.31 Hz) was comparable to that in mutant motor neurons (0.30 Hz). These results indicate that spontaneous glutamatergic synaptic currents were unperturbed and that spontaneous glycinenergic synaptic currents were missing in beo mutants.

The conclusion that beo mutations lack spontaneous glycineergic synaptic currents was corroborated by examination of the durations of the currents, which varied depending on whether they were glycineergic or glutamatergic. The duration when the currents were >50% of the peak currents was measured as the duration of spontaneous synaptic currents. The frequency histogram of the duration appeared to exhibit two populations of synaptic currents in wild-type embryos: a fast current with a peak at 0.8 ms and a slower current with a peak at 2.8 ms (Fig. 5, which is published as supporting information on the PNAS web site). Pharmacological removal of glutamatergic currents with CNQX and APV in wild-type siblings left the slow population, suggesting that the glycineergic synaptic currents are longer in duration than the glutamatergic ones. In beo, the frequency histogram of the duration showed only a fast population of glutamatergic synaptic currents. The removal of the glutamatergic synaptic currents with CNQX and APV abolished all spontaneous synaptic events in beo, demonstrating the lack of spontaneous glycineergic synaptic currents in beo mutants.

The absence of spontaneous glycineergic synaptic currents in beo
strychnine-sensitive conductance in whole-cell currents during voltage steps in the presence of bath-currents were also measured by comparing strychnine-sensitive
is common to all GlyR immunolabeling with an antibody (mAb4a) against an epitope that within the CNS beo
the defect is a lack of GlyR clusters at synapses.
(38.3 nA (Fig. 3) was about half of that in wild-type motor neurons (2.8 nA n/H11005, Fig. 3; Student’s t test, 0.01). Glycine-induced currents were also measured by comparing strychnine-sensitive whole-cell currents during voltage steps in the presence of bath-applied glycine (Fig. 6, which is published as supporting information on the PNAS web site). These experiments showed that the strychnine-sensitive conductance in beo motor neurons (1.5 nA/V, n = 3) was almost twice as large as that in wild-type motor neurons (2.8 nA/V, n = 3). These results demonstrate that functional GlyRs are expressed on the cell surface of motor neurons in beo and suggest that the defect is a lack of GlyRs clustering at synapses.

To test whether there is a lack of GlyR aggregation at synapses within the CNS in beo, localization of GlyRs was assayed by immunolabeling with an antibody (mAb4a) against an epitope that is common to all GlyR α-subunits (29). In wild-type embryos, clusters of GlyRs were found associated with the plasma membranes of cell bodies in the spinal cord, including the ventrolateral portion that contains the cell bodies of motor neurons (Fig. 3 I and J). In contrast, no clusters of GlyRs were visible in beo spinal cord (Fig. 3K). In these sections, it was not possible to determine whether there was increased labeling throughout the plasma membrane in the mutants. Furthermore, Western blotting with mAb4a showed that GlyRα was expressed comparably in wild-type and beo embryos (Fig. 5L). These results along with the electrophysiological evidence suggest that GlyRs are expressed on the surface of neurons but are not clustered at presumptive synaptic sites in beo mutants.

**beo Encodes for a β-Subunit of the GlyR and Is Expressed Early in the Hindbrain and Spinal Cord.** Because the β-subunit of the GlyR encoded by the glrb gene is essential for the clustering of GlyRs in mammals (16, 21), we focused on glrb as a candidate for the beo gene. Genomic sequences encoding glrb were identified from the ongoing *Danio rerio* sequencing project (available at www.sanger.ac.uk/Projects/D.rerio). Two positive genomic fragments that represented two β-subunit genes were found. One β-subunit gene in linkage group (LG1) was reported in ref. 18. This gene was termed glrb1, and the second β-subunit found in genomic fragment Scaffold1268 was termed glrb2. The glrb2 gene was physically mapped by using the LNS4 radiation hybrid panel and found to be located between two microsatellites, Z12080 and Z8801, in LG14 (Fig. 7A, which is published as supporting information on the PNAS web site). Furthermore, bulk segregant analysis by meiotic mapping showed that these two markers in LG14 were linked to the beo mutation. To see whether the glrb2 gene was the beo gene, glrb2 CDNA was cloned and sequenced from wild type and three alleles of the beo mutation. Wild-type glrb2 encodes 494 amino acid residues (GenBank accession no. AB195560) (Fig. 7B and C) that contain a signal peptide, four transmembrane domains (M1–M4) and a gephyrin-binding domain. In beo (H9221), Tyr-101 in the N terminus extracellular domain was changed to a stop codon to generate a severely truncated protein. Thus, there appears to be a null mutation of the glrb2 gene in beo (H9221). Missense mutations were found in the other alleles of beo; Leu-277 in M1 was changed to Arg in beo (W384), and Arg-297 in the cytoplasmic loop between M1 and M2 was changed to His in beo (W384). The molecular identification of beo as the glrb2 gene was confirmed by mutant rescue and antisense phenocopy. We injected wild-type glrb2 mRNA or glrb2 mRNA carrying the beo m106a point mutation into recently fertilized embryos of beo heterozygous carriers. Nearly all of the progeny injected with wild-type mRNA displayed normal escape responses after touch at 24 hpf (113 of 123 embryos, 92%) (Fig. 8B, which is published as supporting information on the PNAS web site), whereas ~25% of un.injected progeny (40 of 153 embryos, 26.1%) (Fig. 8A) and beo mRNA-injected progeny (41 of 147 embryos, 27.9%) (Fig. 8C) showed the mutant response. To knockdown synthesis of GlyRβ2, antisense morpholino oligonucleotides (MO) against glrb2 mRNA was injected into recently fertilized wild-type embryos. An overwhelming number of the antisense MO-injected wild-type embryos displayed the beo behavior after touch (84 of 95 embryos, 88.4%) (Fig. 8E), whereas none of the control MO-injected wild-type embryos exhibited aberrant responses (0 of 65 embryos, 0%) (Fig. 8D). A wild-type glrb2 mRNA was engineered with differences in the 5′-untranslated region, which should make it immune to the antisense glrb2 MO. When the antisense MO was co-injected with the engineered wild-type glrb2 mRNA into wild-type embryos, they responded normally to touch (73 of 81 embryos, 90.1%) (Fig. 8F). In contrast, embryos co-injected with antisense MO and the m106a MO exhibited the beo response (73 of 84 embryos, 86.5%) (Fig. 8G). Thus, mRNA rescue and antisense knockdown confirm that glrb2, encoding for the β2-subunit of GlyR, is the gene responsible for the beo phenotype.

Because beo mutants showed abnormal escape responses at 24 hpf, expression of glrb2 along with glra1 and glrb1 was assayed by RT-PCR at different stages. ghr1 and glrb1 were expressed starting at 1 day of development, whereas glrb1 begins expression at 3 days (Fig. 4A). This finding suggests that the β2- but not the β1-subunit
hindbrain are likely reticulospinal neurons, such as the Mauthner neurons in Xenopus and should be expressed in putative neurons in the hindbrain (Fig. 4 B–E), which was similar to glra1 expression in Xenopus (34, 35) and glra4α in zebrafish (36). The neurons in the hindbrain are likely reticulospinal neurons, such as the Mauthner cells, that are essential for the escape response in frog and fish (11, 32, 33). glra1 and glrb2 were also extensively expressed by neurons in the spinal cord (Fig. 4 F and G). By 48 hpf, expression of glra1 and glrb2 had extended to many other presumptive neurons in the hindbrain and spinal cord (data not shown). These data suggest that the GlyRβ2 normally serves to localize GlyRs to synapses within the hindbrain and spinal cord.

Discussion

beo Mutants Simultaneously and Bilaterally Contract Trunk Muscles.

The Tübingen screen found 7 accordion-type mutations that caused bilateral contractions of the trunk muscle (4). Two of these mutations, accordion and zieharmonika/ache, exhibited muscle defects in muscle ATPase Ca\(^{2+}\) pump and acetylcholine esterase, respectively (5–8). The present study demonstrates that one of the remaining five mutations, beo, is due to a defect in the CNS that causes the simultaneous activation of axial muscles on both sides of the trunk. In wild-type embryos, muscles contralateral to the side receiving mechanosensory stimulation received motor input 25 ms before ipsilateral muscles. This was similar to the latency difference between the two sides reported in ref. 31. In contrast, contralateral and ipsilateral muscles were activated simultaneously in beo embryos. Similar simultaneous activation of muscles on the two sides of the trunk examined in strychnine-treated wild-type embryos suggested that glycinergic synaptic transmission was defective in mutants. Indeed, glycinergic synaptic transmission mediates reciprocal inhibition between the two sides of the spinal cord to ensure antiphasic activation of motor neurons on the two sides in lamprey, Xenopus, and fish (10–12). Because the beo gene encodes for the GlyR β2-subunit of the GlyR (see below), beo provides genetic evidence for the importance of glycinergic transmission during alternating contractions of antagonistic muscle groups.

A recent study by Masino and Fetcho (37) used extracellular motor root recordings and found that fictive swimming activity in beo mutants was disorganized but still exhibited side-to-side alternation. There are two potential reasons for the disparity with our results, which show complete absence of alternating activity. First, the allele of beo analyzed in their experiment (beo\(^{p221}\)) may have been a weak allele. There are seven different alleles of beo (4). We examined beo\(^{p221}\), beo\(^{tw38f}\), and beo\(^{m106a}\) with the phenotypic analysis done with beo\(^{p221}\), which contained a stop codon at the position of amino acid 101 of the predicted 494-aa protein. This fact suggested that the beo\(^{p221}\) product was severely truncated and likely to be nonfunctional. Second, there might be some behavioral recovery from the early severe beo phenotype. Masino and Fetcho (37) assayed motor roots at 4–6 dpf, whereas we examined output from motor neurons at 2 dpf.

In beo embryos, the ipsilateral and contralateral muscles not only respond simultaneously but also show shorter and larger depolarizations lacking rhythmicity. This result suggests that glycinergic transmission is necessary for generation of the sustained rhythmic drive for swimming. Interestingly, the response to contralateral stimulation of beo mutants is delayed by 10 ms compared with that of wild-type siblings. The increased latency in mutants further suggests that glycinergic transmission can also accelerate the responses to stimuli in wild-type embryos. Although it is unclear how this acceleration occurs, one possibility is that glycinergic transmission is excitatory at early stages and that removal of the excitation can slow down the response rate of early neural circuits in beo mutants. In fact, glycinergic transmission is known to be excitatory at early stages in other organisms because of chloride ion levels that make the equilibrium potential for chloride ion with respect to the resting potential (38), which may also be the case in early zebrafish neurons.

beo Encodes the β-Subunit of the GlyR That Is Required for Synaptic Clustering of GlyRs.

We identified mutations in the glrb2 gene of beo mutants and confirmed that glrb2 mutation gave rise to the mutant phenotype by mRNA rescue and MO knockdown. In beo\(^{p221}\), a stop codon was located in the N-terminal extracellular domain, which was 5′ to the transmembrane domains, suggesting that this allele carried a null mutation. In beo\(^{m106a}\), an Arg-297-His mutation was found in the loop between M1 and M2. GlyRβ has a sequence similarity of 47% with GlyRα1, with transmembrane domains and loops between them being well conserved. A human glra1 mutation, which carries a homologous mutation in the residue corresponding to Arg-297 in GlyRβ2 (Arg-252-His in human GlyRα1), was reported to cause hyperekplexia (39). Functional analysis of GlyRα1 carrying the Arg-252-His mutation revealed that this mutation likely accelerated degradation of GlyRα1 (40). Thus, it is possible that beo\(^{m106a}\) is a hypomorph of GlyRβ2. In beo\(^{tw38f}\), Leu-277 in M1 was changed to Arg, M1 is highly conserved, and a
missense mutation in M1 of *glra1* was reported to be responsible for hyperekplexia because of the reduced expression of GlyRα1 at the cell surface (41). Thus, *beo* may also be a hydroporph of GlyRα1, GlyRs normally associate with gephyrin via direct binding to GlyRβ to aggregate GlyRs at synaptic sites (19–23). Indeed, the mouse mutation *spastic* is a GlyRβ hydroporph in which GlyRs and glycnergic inhibitory postsynaptic currents are dramatically decreased (42–45). Whether GlyRs are clustered in *spastic* mice, however, has not been reported.

In this study, we showed that *beo* embryos exhibited defects in glycnergic synaptic transmission. First, electrophysiological recordings from strychnine-treated wild-type muscles showed that blocking glycnergic transmission phenocopied the results from strychnine-treated wild-type muscles showed that block-gage of glycnergic synaptic transmission. First, electrophysiological record-
ing to glycine. In fact, coexpression of GlyRα1 subunits versus those made up of homomer GlyRα1 subunits (46, 47).

GlyRs made up of α1- and β-subunits have a lower single channel conductance but a lower Hill coefficient and EC50 compared with GlyRs made up of homomer α1-subunits. Another possibility is that surface expression of GlyRs is reduced in the absence of GlyRβ, much like the β-, γ-, and δ-subunits of the acetycholine receptor, which are retained in intracellular compartments in the absence of the α-subunit (48).

**beo Mutants Are an Animal Model for Hyperekplexia.** Hyperekplexia is a rare neurological disorder in humans that is characterized by exaggerated startle responses to unexpected acoustic or tactile stimuli (24). Various autosomal dominant and recessive mutations in the *glra1* gene have been identified as responsible for hyperekplexia (16, 25). Furthermore, a human patient carrying missense and nonsense mutations in *glrb* also exhibits hyperekplexia (26). Mice harboring mutations in *glra1* also exhibit a similar startle disease (49), with *spastic* mice carrying an insertion of the long interspersed nucleic element retrotransposon in an intron of the *glrb* gene (43, 44). Because *spastic* mice are hypomorphs for *glrb* expression (50), the *beo* mutation, which is likely a null allele, might be useful as a system to study synaptic clustering of GlyRs due to the putative absence of GlyRβ.

Zebrafish *beo* mutants and patients with hyperekplexia share the most critical feature of an exaggerated startle response due to impaired glycnergic synaptic transmission caused by mutations in GlyR genes. Because zebrafish embryos are readily accessible to molecular, genetic, pharmacological, and physiological interventions as well as in vivo visualization of fluorescent-tagged molecules, *beo* mutants could serve as an attractive model for hyperekplexia. Additionally, there are four other mutations of the *accordion*-type that remain to be characterized (4). It will be interesting to see whether any of these genes may be associated with synaptic clustering of GlyRs and could potentially be candidates for additional hyperekplexia genes.

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