

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 GlyR β is essential for physiologically relevant clustering of GlyRs *in vivo*. Because GlyR mutations in humans lead to hyperekplexia, a motor disorder characterized by startle responses, the zebrafish *beo* mutant should be a useful animal model for this condition.

channel | synapse | hyperekplexia | 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 with 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, *nic1^{dbn12}*, 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 α 1-subunits and two β -subunits, whereas fetal GlyRs are homomers composed of five α 2-subunits (refs. 14 and 15; reviewed in ref. 16). In zebrafish, GlyR cDNAs encoding the α 1-subunit and a 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 mechanosensory 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 is a putative null mutation and two missense mutations in the *grb2* gene that encodes the β -subunit of the GlyR. Mutations in the α 1- or β -subunit of the GlyR cause an inherited human disorder known as hyperekplexia, which is characterized by exaggerated startle responses, neonatal hyper-tonia, 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 hyperekplexia.

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 μ M. For the miniature currents, 1 μ M tetrodotoxin (TTX; Sigma), 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 μ M D-2-amino-5-phosphonovaleric acid (APV), and 5 μ M 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-nitroquinoxaline-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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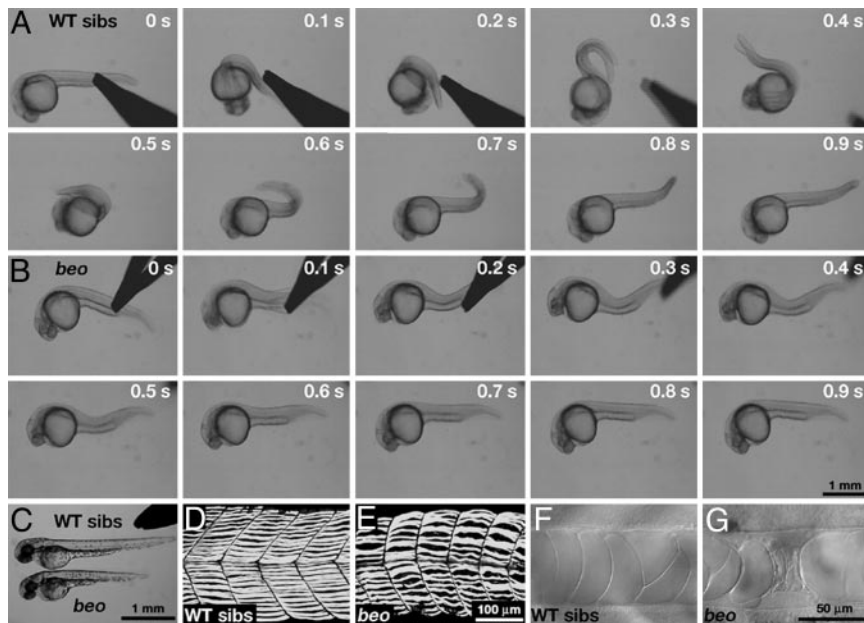


Fig. 1. *beo* embryos exhibit aberrant touch responses and morphological defects. (A) Frames from a movie showing a wild-type sibling (24 hpf) respond to mechanosensory stimulation with two alternating contractions of the trunk. The time of each frame is shown on the upper right corner of each frame. (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.

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-GlyR α (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 1:2,000, 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-GlyR α staining was false-colored green to make the labeling more apparent in the micrographs. For *in situ* hybridization, *glr1* (GenBank accession no. NM.131402) and *glrb2* (GenBank accession no. AB.195560) 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, meiotic physical mapping, cloning, knock down, mRNA rescue, mutagenesis of *glrb2*, 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*^{tp221} (4). We performed phenotypic analysis on *beo*^{tp221}. 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 $70 \mu\text{M}$ 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. 1F 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⁺ 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 $70 \mu\text{M}$ strychnine showed these morphological defects that were indistinguishable from *beo* mutants (data not shown). The *beo* larvae died at 7–10 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 *nic1*^{dbn12} mutants, in which all of the defects were

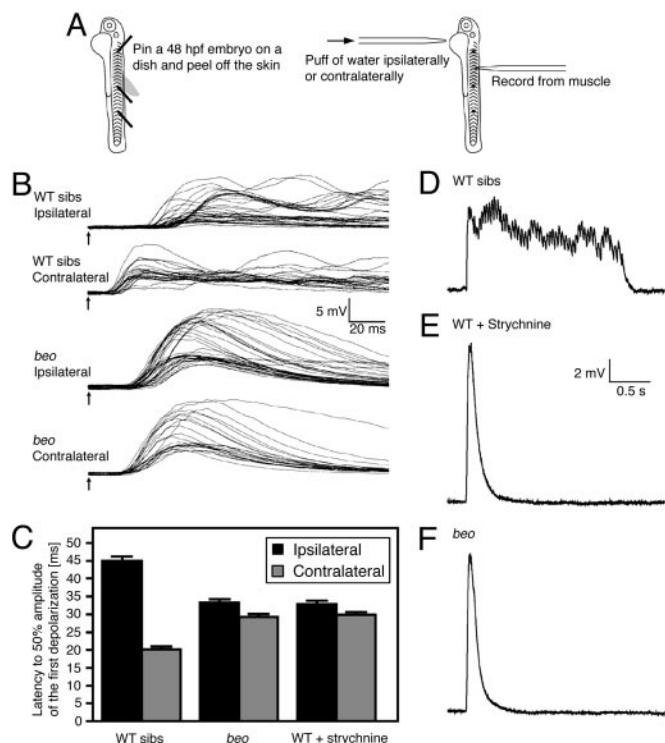


Fig. 2. Voltage response of *beo* muscles after mechanosensory stimulation is abnormal. (A) Schematic diagram of the experimental method. Embryos (48 hpf) were pinned on a dish, and their skin was peeled off to allow access to the muscle cells for electrophysiological recordings. Responses were evoked by mechanosensory stimulation delivered by a puff of bath solution on either side of the yolk ipsilateral or contralateral to the recorded muscle. (B) Superimposed traces of voltage responses of muscles evoked by mechanosensory stimulation. Arrows indicate the time of stimulation. The latency of the muscle response to contralateral stimulation was shorter than that to ipsilateral stimulation in wild-type siblings, whereas the latency to ipsilateral and contralateral stimulation was the same in *beo* embryos. (C) Histograms showing that the latency to half-maximal amplitude of the first depolarization was shorter to contralateral stimulation compared with ipsilateral stimulation in wild-type siblings and equal in duration to contralateral and ipsilateral stimulation in *beo* embryos and strychnine-treated wild-type embryos. Note that the latency in *beo* and strychnine-treated wild-type embryos is in between that of contralateral and ipsilateral stimulation in untreated wild-type embryos. (D) The long-lasting rhythmic depolarization recorded in a muscle after ipsilateral mechanosensory stimulation of a wild-type sibling. (E) The short and large response devoid of rhythmicity after ipsilateral stimulation in a strychnine-treated wild-type embryo. (F) The short and large response devoid of rhythmicity after ipsilateral stimulation in a *beo* embryo.

due to muscle defects (5–9). Alternatively, the output from motor neurons on the two sides could coincide, thereby causing simultaneous contraction on both sides of the trunk. To distinguish these two possibilities, we recorded from muscle cells and applied mechanosensory stimulation to the side of the yolk that was either ipsilateral or contralateral to the muscle (Fig. 2A). In wild-type siblings, the response to contralateral stimulation (latency to 50% of peak depolarization: 21.1 ± 1.7 ms, $n = 5$) (Fig. 2B and C) preceded that to ipsilateral stimulation (latency to 50% of peak depolarization: 44.9 ± 2.4 ms, $n = 5$; Student's *t* test, $P < 0.01$) by ≈ 25 ms, as shown in ref. 31. In contrast, the latencies of response to ipsilateral (31.9 ± 1.9 ms, $n = 4$) and contralateral (30.0 ± 1.5 ms, $n = 4$) stimulation in *beo* embryos were comparable. Furthermore, strychnine-treated wild-type embryos exhibited touch response latencies that were similar to that of *beo* mutants (ipsilateral: 32.9 ± 0.8 ms, $n = 3$; contralateral: 29.9 ± 0.6 ms, $n = 3$) (Fig. 2C). 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 glycinergic transmission is aberrant in *beo* embryos.

Glycinergic Synaptic Transmission Is Aberrant in *beo* Mutants. To see whether glycinergic transmission is defective in mutants, we measured spontaneous glycinergic 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 α -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 $5 \mu\text{M}$ strychnine (0 Hz, $n = 6$) (Fig. 3C), suggesting that they were glycinergic 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 glycinergic synaptic currents were missing in *beo* mutants.

The conclusion that *beo* mutations lack spontaneous glycinergic synaptic currents was corroborated by examination of the durations of the currents, which varied depending on whether they were glycinergic 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 glycinergic 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 glycinergic synaptic currents in *beo* mutants.

The absence of spontaneous glycinergic synaptic currents in *beo*

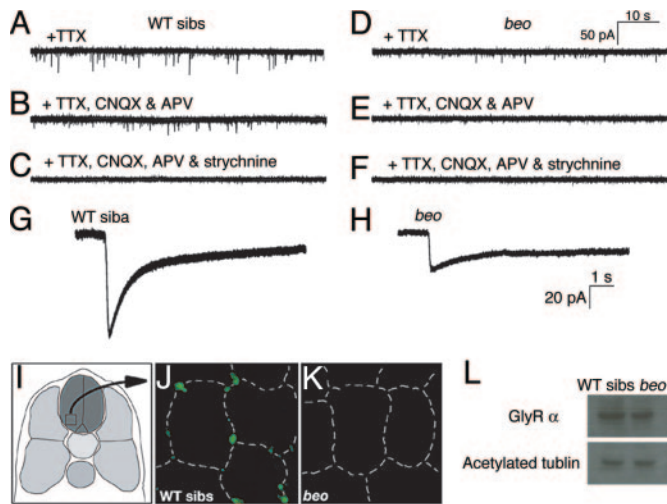


Fig. 3. Glycinergic synaptic transmission is missing in *beo* embryos because of a defect in GlyR clustering. (A) Spontaneous synaptic currents recorded in the presence of TTX in a wild-type motor neuron. (B) Nonglutamatergic spontaneous currents in the wild-type motor neuron after block of NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors with CNQX and APV, respectively. (C) The nonglutamatergic spontaneous currents are eliminated by strychnine in the wild-type motor neuron, showing that they are glycinergic currents. (D) Spontaneous synaptic currents recorded in the presence of TTX in a *beo* motor neuron. (E) Nonglutamatergic spontaneous currents in the presence of CNQX and APV are absent in the *beo* motor neuron, demonstrating that spontaneous glycinergic synaptic currents are missing in *beo*. (F) No further effect of strychnine on the *beo* motor neuron. (G and H) A puff of exogenous glycine induces a current in a wild-type motor neuron (G) and a smaller current in a *beo* motor neuron (H). (I) Cross sections of the spinal cord outlined by the square in the schematic illustration. (J) Labeling of GlyR α with mAb4a in wild-type marks clusters of GlyRs that are associated with the surface of presumptive neurons, which is outlined with dashed lines. (K) No clusters of GlyRs were labeled by mAb4a in the *beo* spinal cord. (L) Western blots labeled with mAb4a showing that GlyR α is expressed at levels comparable in wild-type siblings and *beo*. Blotting with antiacetylated tubulin is control.

mutants could result from the absence of functional GlyRs or the failure to aggregate GlyRs at synapses. To distinguish between these two possibilities, the sensitivity of motor neurons to exogenous glycine was assayed. Puffing glycine (100 mM, 20 ms) onto motor neurons *in vivo* revealed that wild-type (Fig. 3G) and *beo* (Fig. 3H) motor neurons responded to exogenous glycine. However, the amplitude of the glycine response in wild-type siblings (96.1 ± 6.8 pA, $n = 3$) was consistently larger than that in *beo* mutants (38.3 ± 18.3 pA, $n = 3$; Student's *t* test, $P < 0.01$). 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 about half of 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. 3I 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 GlyR α was expressed comparably in wild-type and *beo* embryos (Fig. 3L). 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 (LG)1 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 LN54 radiation hybrid panel and found to be located between two microsatellites, Z21080 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*^{tp221}, 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*^{tp221}. Missense mutations were found in the other alleles of *beo*; Leu-277 in M1 was changed to Arg in *beo*^{tw38f}, and Arg-297 in the cytoplasmic loop between M1 and M2 was changed to His in *beo*^{mi106a}.

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*^{mi106a} 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 $\approx 25\%$ of uninjected 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 GlyR β 2, 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 coinjected 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 coinjected with antisense MO and *beo*^{mi106a} mRNA exhibited the *beo* response (73 of 84 embryos, 86.9%) (Fig. 8G). Thus, mRNA rescue and antisense knockdown confirm that *glrb2*, encoding for the β 2-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 with RT-PCR at different stages. *glra1* and *glrb2* were expressed starting at 1 day of development, whereas *glrb1* begins expression at 3 days (Fig. 4A). This finding suggests that the β 2- but not the β 1-subunit

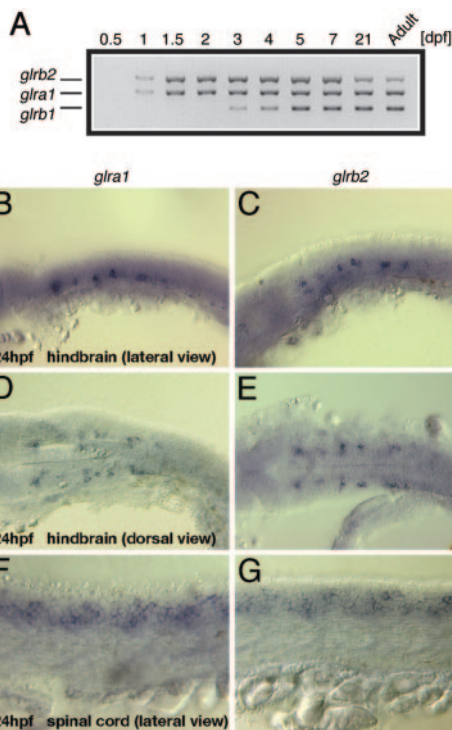


Fig. 4. Temporal and spatial expression of GlyR genes. (A) RT-PCR shows that *glra1* and *glrb2* are expressed starting on 1 dpf, whereas *glrb1* expression starts at 3 dpf. (B–G) Whole-mount *in situ* hybridization with a *glra1* (B, D, and F) or *glrb2* (C, E, and G) probe in 24-hpf embryos. Lateral views show expression of *glra1* and *glrb2* in putative neurons in the hindbrain (B and C, respectively) and spinal cord (F and G, respectively). Dorsal views show the bilaterally symmetric, presumptive hindbrain neurons expressing *glra1* (D) and *glrb2* (E).

is active in GlyRs before day 3. RT-PCR also found that *glrb1* expression was unchanged in *beo* mutants; i.e., it was not observed before day 3 and not increased at later stages (data not shown). Thus, *glrb1* does not compensate for the lack of *glrb2* in *beo* mutants. These data suggest that the lack of the GlyR β 2 during the first 3 days of development is responsible for the failure of GlyRs to aggregate at synapses (see above).

Because the hindbrain and spinal cord are necessary and sufficient for the escape response and swimming (2, 32, 33) and *beo* mutants are defective in these behaviors, *glrb2* should be expressed in these regions of the CNS. The patterns of expression of the *glra1* and *glrb2* genes were indistinguishable at 24 and 48 hpf. At 24 hpf, both genes were expressed in repeating bilateral clusters of cells in the hindbrain (Fig. 4 B–E), which was similar to *glra1* expression in *Xenopus* (34, 35) and *glra4a* 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, expressions 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 GlyR β 2 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 *ziehharmonika/ache*, exhibited muscle defects with mutations in muscle ATPase Ca²⁺ 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 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 β 2-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*^{ta86d}) may have been a weak allele. There are seven different alleles of *beo* (4). We examined *beo*^{tp221}, *beo*^{tw38f}, and *beo*^{mi106a} with the phenotypic analysis done with *beo*^{tp221}, which contained a stop codon at the position of amino acid 101 of the predicted 494-aa protein. This fact suggested that the *beo*^{tp221} 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*^{tp221}, 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*^{mi106a}, an Arg-297-His mutation was found in the loop between M1 and M2. GlyR β has a sequence similarity of \approx 47% with GlyR α 1, 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 GlyR β 2 (Arg-252-His in human GlyR α 1), was reported to cause hyperekplexia (39). Functional analysis of GlyR α 1 carrying the Arg-252-His mutation revealed that this mutation likely accelerated degradation of GlyR α 1 (40). Thus, it is possible that *beo*^{mi106a} is a hypomorph of GlyR β 2. 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 GlyR α 1 at the cell surface (41). Thus, *beo*^{tw38f} may also be a hypomorph of GlyR β 2. GlyRs normally associate with gephyrin via direct binding to GlyR β to aggregate GlyRs at synaptic sites (19–23). Indeed, the mouse mutation *spastic* is a GlyR β hypomorph in which GlyRs and glycinergic 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 glycinergic synaptic transmission. First, electrophysiological recordings from strychnine-treated wild-type muscles showed that blocking glycinergic transmission phenocopied the *beo* mutation, suggesting that the output from the CNS was aberrant in *beo* muscles because of a defect in glycinergic transmission. Second, motor neurons in *beo* embryos can respond to exogenous glycine, showing that they express functional GlyRs at the cell surface. Third, spontaneous glycinergic currents were completely absent in *beo* motor neurons, indicating that glycinergic transmission was impaired in *beo*. Fourth, immunostaining with an antibody against GlyR α revealed that GlyRs were not clustered in the *beo* spinal cord. Taken together, these results indicate that a mutation of the *glrb2* gene leads to impairment of clustering by GlyRs and defective function of neural circuits, leading to aberrant behavior starting with one of the earliest behaviors emitted by embryos. This study directly demonstrates that GlyR β is required for physiologically relevant clustering of GlyRs in the CNS.

Western blotting analysis suggested that the amount of GlyR α was not diminished in *beo* embryos despite the fact that they were not clustered in mutants. However, the response of *beo* motor neurons to exogenous glycine suggested a decrease in responsiveness to glycine. In fact, coexpression of GlyR α 1 with GlyR β significantly increased glycine-gated whole-cell currents compared with expression of GlyR α 1 alone in HEK-293 cells (46). The decreased response of *beo* motor neurons to exogenous glycine might be due to differences in the properties of GlyRs made up of α 1- and β -subunits versus those made up of homomeric α 1-subunits (46, 47). GlyRs made up of α 1- and β -subunits have a lower single channel conductance but a lower Hill coefficient and EC₅₀ compared with GlyRs made up of homomeric α 1-subunits. Another

possibility is that surface expression of GlyRs is reduced in the absence of GlyR β , much like the β -, γ -, and δ -subunits of the acetylcholine 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 nuclear element retrotransposon in an intron of the *glrb* gene (43, 44). Because *spastic* mice are hypomorphs for *glrb* expression (50), the *beo*^{tw221} 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 GlyR β .

Zebrafish *beo* mutants and patients with hyperekplexia share the most critical feature of an exaggerated startle response due to impaired glycinergic 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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