Characterization of zebrafish GABA<sub>A</sub> receptor subunits

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γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system, exerts its effect through the activation of GABA receptors. GABA<sub>A</sub> receptors are ligand-gated chloride channels composed of five subunit proteins. Mammals have 19 different GABA<sub>A</sub> receptor subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ, and ρ1–3), the physiological properties of which have been assayed by electrophysiology. However, the evolutionary conservation of the physiological characteristics of diverged GABA<sub>A</sub> receptor subunits remains unclear. Zebrafish have 23 subunits (α1, α2a, α2b, α3–5, α6a, α6b, β1–4, γ1–3, δ, π, ζ, ρ1, ρ2a, ρ2b, ρ3a, and ρ3b), but the electrophysiological properties of these subunits have not been explored. In this study, we cloned the coding sequences for zebrafish GABA<sub>A</sub> receptor subunits and investigated their expression patterns in larval zebrafish by whole-mount in situ hybridization. We also performed electrophysiological recordings of GABA-evoked currents from Xenopus oocytes injected with one or multiple zebrafish GABA<sub>A</sub> receptor subunit cRNAs and calculated the half-maximal effective concentrations (EC50s) for each. Our results revealed the spatial expressions and electrophysiological GABA sensitivities of zebrafish GABA<sub>A</sub> receptors, suggesting that the properties of GABA<sub>A</sub> receptor subunits are conserved among vertebrates.

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system of vertebrates, controls the excitability of neural networks mainly through GABA<sub>A</sub> receptors<sup>1</sup>. The GABA<sub>A</sub> receptor mediates two types of inhibition, known as phasic and tonic inhibition<sup>2</sup>. Phasic inhibition occurs at postsynaptic sites, where the GABA concentration transiently rises to more than 1 mM during synaptic transmission<sup>3</sup>, while tonic inhibition occurs at extrasynaptic sites, where the concentration of spillover GABA increases to ~0.5 µM<sup>4,5</sup>. Regardless of synaptic or extrasynaptic sites, GABA<sub>A</sub> receptors comprise five subunits forming Cl<sup>-</sup>-conducting channels. Each subunit has a large extracellular N-terminal domain that contributes to GABA binding, followed by four transmembrane domains, with the second one forming the channel pore. Mammals have 19 different GABA<sub>A</sub> receptor subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ, and ρ1–3). Although this diversity may allow for numerous possible combinations of subunits, most GABA<sub>A</sub> receptors are composed of two α, two β, and one γ subunit<sup>6</sup>. In fact, experimental evidence of native GABA<sub>A</sub> receptors suggests that there are fewer than 20 receptor subtypes, with the major synaptic GABA<sub>A</sub> receptor combinations being α1β2γ2, α1β3γ2, α2β3γ2, and α3β3γ2<sup>7,8</sup>. The extrasynaptic GABA<sub>A</sub> receptors appear to contain specific subunits such as α4, α5, α6, and δ, forming α4β3δ, α5β3γ2, and α6β3γ2<sup>7</sup>. The other subunits—ε, π, and θ—also assemble with α and β subunits and are located at extrasynaptic sites<sup>2</sup>. The ρ subunits form homopentameric GABA<sub>A</sub> receptors that are predominantly expressed in the retina<sup>8</sup>. The β3 subunit can also form homopentameric channels that are activated by the anesthetic agent etomidate and a high concentration (~10 mM) of GABA in Xenopus oocytes<sup>10</sup>. While the physiological properties of GABA<sub>A</sub> receptor isoforms in mammals have been addressed, the evolutionary conservation and physiological significance of diverged subunits in vertebrates remain largely unclear.

GABA<sub>A</sub> receptors have also been studied in zebrafish, a vertebrate model that offers advantages such as the production of many offspring, fast embryonic development, optical transparency during embryogenesis, rapid acquisition of locomotor behaviors, and the ease of pharmacological treatment<sup>11</sup>. Cocco and colleagues identified 23 putative GABA<sub>A</sub> receptor subunits (eight α, four β, three γ, one δ, one ε, one θ, and five ρ) in the zebrafish genome<sup>12</sup>. They also investigated the transcript levels of GABA<sub>A</sub> receptor subunits in the adult zebrafish brain using reverse transcription-polymerase chain reaction (RT-qPCR). Another recent study assayed the spatial expression patterns of eight GABA<sub>A</sub> receptor α subunits in zebrafish embryos by in situ hybridization and showed that most α subunits are expressed during embryogenesis<sup>13</sup>. Several loss-of-function studies have revealed the physiological function of GABA<sub>A</sub> receptors in zebrafish. Antisense morpholino-mediated knockdown of the α1 subunit caused reduced spontaneous locomotor activity in larvae at 5 days post-fertilization (dpf)<sup>14</sup>, while CRISPR/Cas9-mediated knockout of α1 caused seizure phenotypes in juveniles at 35 dpf<sup>15</sup>. Knockdown of the...
α2 subunit perturbed the expression of the proneural gene *neurod* and a GABA-synthesizing enzyme gene *gad1b* within 1 day of development. Zebrafish larvae lacking the β3 subunit showed reduced sensitivity to anesthetic drugs such as etomidate and propofol. Patch-clamp recordings of GABA$_A$ receptor-mediated miniature inhibitory postsynaptic currents from zebrafish Mauthner cells revealed three different types of gating kinetics, suggesting that zebrafish also have multiple GABA$_A$ receptor subtypes comprising different subunit combinations. However, the electrophysiological characteristics of the zebrafish GABA$_A$ receptor subunit have not yet been explored.

In this study, we performed phylogenetic analysis and cloned cDNAs for zebrafish GABA$_A$ receptor subunits. Our whole-mount in situ hybridization revealed the spatial expression patterns of GABA$_A$ receptor subunit genes in 5 dpf larvae. We also assayed GABA-mediated gating of zebrafish GABA$_A$ receptors composed of various combinations of receptor subunits in *Xenopus* oocytes. These attempts provide useful information on the spatial expressions and electrophysiological GABA sensitivities of zebrafish GABA$_A$ receptors and suggest that the properties of GABA$_A$ receptor subunits are conserved among vertebrates.

**Results**

**Phylogenetic analysis and cloning of zebrafish GABA$_A$ receptor subunits.** Nineteen GABA$_A$ receptor subunits have been identified in mammals (α1–6/gabra1–6, β1–3/gabrb1–3, γ1–3/gabrg1–3, δ/gadrd, ε/gadre, π/gabrp, θ/gabrq, and ρ1–3/gabrr1–3)\(^{19}\). Previous searches for GABA$_A$ receptor subunits in the zebrafish genome database have suggested 23 GABA$_A$ receptor subunit genes comprising eight α (α1/gabra1, α2a/gabra2a, α2b/gabra2b, α3/gabra3a, α4/gabra4a, α6a/gabra6a, and α6b/gabra6b), four β (β1–4/gabrb1–4), three γ (γ1–3/gabrg1–3), one δ/gadrd, one π/gabrp, and five ρ (ρ1–5/gabrr1–5) as well as additional ζ/gabrz subunits, but neither ε nor θ subunits\(^{12,13}\). Some subunits that have a or b at the end of the subunit/gene name are paralogs generated by a suspected duplication of the whole genome during fish evolution.\(^{18}\) We recapitulated in silico analysis using human and mouse GABA$_A$ receptor protein sequences as queries to obtain zebrafish GABA$_A$ receptor protein sequences. We successfully cloned cDNAs for all zebrafish GABA$_A$ receptor subunits except for α2b from an RNA mixture extracted from a pool of 1-5 dpf zebrafish embryos/larvae. The previously annotated zebrafish α2b subunit (XP_017214538.1) showed 86% amino acid identity to the zebrafish α2a subunit in the N-terminus (exons 1–8) and only 10% identity in the C-terminus (exon 9). Therefore, the α2b subunit has been removed from the National Center for Biotechnology Information (NCBI) annotation as it was presumably caused by an incorrect annotation of the last exon. We then searched for another exon encoding the C-terminus of α2b in the genome database using the C-terminus protein sequence of zebrafish α2a as a query and identified the other last exon encoding a possible α2b C-terminus that showed 76% amino acid identity to zebrafish α2a. We successfully cloned the intact coding sequence of this newly annotated subunit and named zebrafish GABA$_A$ receptor α2b subunit (LC596832), which differed from the previous annotation only in the last exon. We then updated the phylogenetic tree of human, mouse, and zebrafish GABA$_A$ receptor subunits (Fig. 1). Our amino acid alignments of the GABA$_A$ receptor subunit genes showed that each subunit is conserved among vertebrates, especially in four transmembrane domains (Supplementary Figs. 1–17; Supplementary Table 1). We also confirmed that the ζ subunit, which is found in zebrafish but not in mammals, belongs to the π subfamily, with the highest similarity to the zebrafish π subunit, indicating that the ζ subunit is a paralog of the π subunit. Thus, we suggest renaming the π and ζ subunits to πa and πb subunits, respectively. We hereafter refer to π and ζ as π/πa and ζ/πb, respectively.

**Spatial expression of zebrafish GABA$_A$ receptor genes.** A previous RT-PCR analysis described the expression of some, but not all, GABA$_A$ receptor genes in the brain and eye of adult zebrafish.\(^{12}\) Another whole-mount in situ hybridization study reported the spatial expression patterns of eight subunit genes in zebrafish embryos at 1, 2, and 4 dpf.\(^{13}\) Since zebrafish larvae with a defect in the α1 gene showed seizure-like motor activity as early as 4 dpf\(^{15}\) and GABA$_A$ receptor antagonist-induced zebrafish seizure can be assayed at 7 dpf\(^{21}\), we investigated the spatial expression of GABA$_A$ receptor subunit genes by whole-mount in situ hybridization in zebrafish larvae at 5 dpf, when the deficiency of GABA$_A$ receptor is likely correlated with seizure. The α1 gene expression of some, but not all, GABA$_A$ receptor genes in the brain and eye of adult zebrafish.\(^{12}\) Another whole-mount in situ hybridization study reported the spatial expression patterns of eight subunit genes in zebrafish embryos at 1, 2, and 4 dpf.\(^{13}\) Since zebrafish larvae with a defect in the α1 gene showed seizure-like motor activity as early as 4 dpf\(^{15}\) and GABA$_A$ receptor antagonist-induced zebrafish seizure can be assayed at 7 dpf\(^{21}\), we investigated the spatial expression of GABA$_A$ receptor subunit genes by whole-mount in situ hybridization in zebrafish larvae at 5 dpf, when the deficiency of GABA$_A$ receptor is likely correlated with seizure. The α1 gene was predominantly expressed in the forebrain, midbrain, hindbrain, and eye, while the other subunit genes were expressed by different but restricted patterns in the olfactory bulb, forebrain, midbrain, and eye at low levels (Fig. 2a–w). Probes for β subunits showed broad labeling in the whole brain (Fig. 2h–k). Expression of all three γ subunit genes was observed in broad brain regions including the olfactory bulb, forebrain, midbrain, hindbrain, and eye (Fig. 2l–n). The δ and ζ/πb genes were also expressed in the broad brain regions, while the π/πa gene was expressed in the restricted pattern in the midbrain and eye (Fig. 2o–q). Among the five ρ subunits, the ρ2a gene was predominantly expressed in the olfactory bulb, forebrain, midbrain, hindbrain, and eye (Fig. 2s). Expression of the other ρ subunit genes was also observed at low levels in the broad brain regions (Fig. 2r–v). We have summarized the spatial expressions with staining intensities indicated by ++, +, +, or + in Table 1. These different but overlapping expressions of GABA$_A$ receptor subunit genes suggest the formation of various GABA$_A$ receptor subtypes comprising different subunit combinations.

**GABA concentration–response of zebrafish GABA$_A$ receptor subtypes.** To assess the electrophysiological properties of zebrafish GABA$_A$ receptor subunits, we employed two-electrode voltage-clamp recordings and recorded GABA-evoked currents from *Xenopus* oocytes expressing single or multiple GABA$_A$ receptor subunits. We first recorded GABA currents from oocytes injected with one type of subunit cRNAs. The expression of single α, γ, δ, π/πa, or ζ/πb subunit did not generate GABA-evoked currents at any GABA concentration, while that of either single β subunit yielded small currents (β1: 27.5 ± 10.7 nA, n = 4; β2: 29.4 ± 6.3 nA, n = 5; β3: 81.7 ± 7.5 nA, n = 5; β4: 28.3 ± 3.6 nA, n = 5) only in the presence of GABA at 10 mM, which is a...
non-physiological concentration at both synaptic and extrasynaptic sites. Expression of the ρ2a subunit alone generated sufficient GABA-mediated currents (> 200 nA) with an EC50 of 0.6 ± 0.1 μM (Fig. 3a; Table 2). However, for unknown reasons, we failed to obtain GABA-evoked currents from oocytes injected with ρ1, ρ2b, ρ3a, or ρ3b subunit cRNAs.

In mammals, heteropentameric GABAA receptors are composed of two α and two β subunits, along with another one chosen from the γ, δ, ε, π or θ subunit2,7, with α1β2γ2 and α1β3γ2 being the two most prevalent subtypes in rat brain neurons22. Mammalian GABAA receptors composed of only α and β subunits can also function as GABA-dependent Cl− channels with lower EC50 values compared to those of αβγ GABAA receptors in Xenopus oocytes23. To explore the electrophysiological properties of GABAA receptor subtypes in zebrafish, we next recorded the GABA-mediated currents from oocytes injected with ρ1, ρ2b, ρ3a, or ρ3b subunit cRNAs.

Figure 1. A phylogenetic tree of GABAA receptor subunits. Amino acid sequences of GABAA receptor subunits from humans, mice, and zebrafish were used to create a phylogenetic tree. This phylogenetic tree detected the subfamilies of α, γ/ε, β/θ, δ/π, and ρ. H: human; M: mouse; Z: zebrafish.
Figure 2. Spatial expression of zebrafish GABA<sub>A</sub> receptor subunits. Whole-mount in situ hybridization of 5 dpf zebrafish larvae using antisense probes for gabra1 (a), gabra2a (b), gabra3 (c), gabri4 (d), gabra5 (e), gabra6a (f), gabra6b (g), gabri7 (h), gabri2 (i), gabri3 (j), gabri4 (k), gabri1 (l), gabri2 (m), gabri3 (n), gabri (o), gabrp (p), gabrz (q), gabrr1 (r), gabrr2a (s), gabrr2b (t), gabrr3a (u), and gabrr3b (v). Negative control without probe showing no signals (w). Regions of the olfactory bulb, forebrain, midbrain, hindbrain, and eye are indicated in the image (x). Each labeling image is composed of a whole lateral view (left top), a whole dorsal view (right top), a magnified lateral view of the head region (left bottom), and a magnified dorsal view of the head region (right bottom). The scale bars in the whole and magnified views are 1 mm and 200 μm, respectively. OB: olfactory bulb; FB: forebrain; MB: midbrain; HB: hindbrain.
either β1 or β2 with any a subunit in the absence or presence of the γ2 subunit failed to elicit currents following exposure to GABA. We also tested all γ, δ, π/πa, and ζ/πb subunits to determine whether their co-expression changed the EC50 of α1β3 GABAA receptors, implying the incorporation of these subunits into the functional heteropentameric channels. Co-expression of the α1 and β3 subunits with the γ1, γ2, or γ3 subunit generated GABA-evoked currents with higher EC50 values compared to those in α1β3 GABAA receptors, while those with the π/πa subunit yielded currents with lower EC50 values (Fig. 3f). Interestingly, co-expression of α1 and β3 with either the δ or ζ/πb subunit eliminated GABA-dependent currents. These results showed that the electrophysiological sensitivity of zebrafish GABAA receptors to GABA differed according to the subunit combination, providing the functional diversity of GABAA receptor subtypes in zebrafish, as observed in mammals.

**Discussion**

In this study, we investigated the phylogeny, expression, and electrophysiology of zebrafish GABAA receptor subunits using in silico analysis, in situ hybridization, and in vitro current recording, respectively. These analyses revealed differences in the spatial expression and electrophysiological properties of GABAA receptors in zebrafish and suggested the conservation of receptor characteristics with minor differences in vertebrates.

**Conservation of GABA receptor genes in vertebrates.** Previous database searches have suggested the presence of 23 GABAA receptor subunits genes in zebrafish. However, one of the annotated α2b/gabra2b exons was removed from the database as a result of standard genome annotation processing in NCBI (https://www.ncbi.nlm.nih.gov/protein/1040662547). In this study, we identified a new exon and corrected the α2b/gabra2b annotation. Our cloning of 23 cDNAs of zebrafish GABAA receptor subunits confirmed that all of the exon–intron junctions were correct for the 22 previously suggested and 1 newly identified GABAA receptor subunit. We noticed that the β4 subunit is found in zebrafish, amphibians, reptiles, and birds but not in mammals. Interestingly, the spatial expression pattern and electrophysiological properties of the β4 subunit were similar to those of the β3 subunit in zebrafish. Thus, β4 may serve as a reserve of β3 to form functionally indistinguishable GABAA receptor subtypes. Our phylogenetic analyses also suggested that the zebrafish-specific ζ subunit is a paralog of the π subunit, presumably generated by gene duplication in teleosts. Thus, our nomenclature of changing the π and ζ to πa and πb, respectively, is reasonable. We also noted that neither ε nor θ subunit is found in zebrafish, while the ε subunit is found only in mammals and birds and the θ subunit is found in mammals, birds, and reptiles.

A recent study proposed that a subfamily of ρ subunits is phylogenetically close to a subfamily comprising α, γ, and ε subunits. However, our phylogenetic tree suggested that the ρ subfamily is instead close to a subfamily comprising the β, δ, ε, and π subunits, consistent with an old phylogenetic study. This discrepancy could be caused by a difference in phylogenetic methods and, thus, will be solved in future development of phylogenetic methods.

|         | Olfactory bulb | Forebrain | Midbrain | Hindbrain | Eye |
|---------|---------------|-----------|----------|-----------|-----|
| α1      | +++           | +++       | +++      | +++       | +++ |
| α2a     | +             | +         | +        | +         | +   |
| α3      | +             | +         | +        | +         | +   |
| α4      | +             | +         | +        | +         | +   |
| α5      | +             | +         | +        | +         | +   |
| α6a     | +             | +         | +        | +         | +   |
| α6b     | +             | +         | +        | +         | +   |
| β1      | +             | +++       | +++      | +++       | +++ |
| β2      | +             | +++       | +++      | +++       | +++ |
| β3      | +             | +         | +        | +         | +   |
| β4      | ++            | ++        | +        | +         | +   |
| γ1      | ++            | ++        | +        | +         | +   |
| γ2      | +             | ++        | +        | +         | +   |
| γ3      | +             | ++        | +        | +         | +   |
| δ       | ++            | +         | +        | +         | +   |
| π/πa    | +             | +         | +        | +         | +   |
| ζ/πb    | +             | ++        | +        | +         | +   |
| ρ1      | ++            | +         | ++       | +         | +   |
| ρ2a     | ++            | +         | ++       | +         | +   |
| ρ2b     | +             | +         | +        | +         | +   |
| ρ3a     | +             | +         | +        | +         | +   |
| ρ3b     | +             | +         | +        | +         | +   |

Table 1. Expression patterns of GABA<sub>α</sub> receptor subunit genes in zebrafish larvae at 5 dpf. Note that ++++, ++,+ indicate the intensity of staining.
Spatial distributions of $\text{GABA}_A$ receptor genes. A previous in situ hybridization study reported the expression patterns of $\text{GABA}_A$ receptor subunit genes in zebrafish at 1, 2, and 4 dpf. Here, we assayed the spatial expressions of $\alpha$ and the other subunit genes in zebrafish at 5 dpf. The nucleotide sequence identity of the $\text{GABA}_A$ receptor coding regions was 41.6–75.0%, with the highest between $\rho_3a$ and $\rho_3b$ paralogs and the second highest (72.1%) between $\rho_2a$ and $\rho_2b$ paralogs (Supplementary Fig. 18). Although the expression patterns of $\rho_3a$ and $\rho_3b$ were almost identical, those of $\rho_2a$ and $\rho_2b$ were different at least in the olfactory bulb. Thus, we assume that each antisense probe presumably recognizes its specific target. The results of in situ hybridization showed that
| GABA4 receptor | EC50 (μM) | Hill coefficient |
|----------------|-----------|------------------|
| a1 β1           | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | 1.2 ± 0.2 | 1.6 ± 0.1        |
| β3γ2            | 126.9 ± 10.0 | 1.3 ± 0.1    |
| β4              | 3.6 ± 0.6 | 1.0 ± 0.1        |
| β4γ2            | 143.4 ± 12.5 | 1.2 ± 0.1    |
| β1              | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | 4.6 ± 0.4 | 1.2 ± 0.1        |
| β3γ2            | 99.9 ± 14.1 | 1.3 ± 0.1    |
| a3 β1           | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | 11.5 ± 2.1 | 1.1 ± 0.1        |
| β3γ2            | 239.6 ± 24.2 | 0.9 ± 0.0    |
| β1              | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | 1.9 ± 0.2 | 1.4 ± 0.1        |
| β3γ2            | 66.6 ± 7.5 | 1.0 ± 0.1        |
| a5 β1           | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | ND        | ND               |
| β3γ2            | ND        | ND               |
| a6a β1          | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | ND        | ND               |
| β3γ2            | ND        | ND               |
| a6b β1          | ND        | ND               |
| β1γ2            | ND        | ND               |
| β2              | ND        | ND               |
| β2γ2            | ND        | ND               |
| β3              | 0.6 ± 0.2 | 1.6 ± 0.2        |
| β3γ2            | 7.0 ± 1.3 | 0.6 ± 0.0        |
| γ1 a1β3         | 58.0 ± 4.0 | 1.5 ± 0.1        |
| γ3 a1β3         | 9.0 ± 0.8  | 2.1 ± 0.3        |
| δ a1β3          | ND        | ND               |
| π/πa a1β3       | 2.8 ± 0.6  | 1.2 ± 0.2        |
| ρ/πb a1β3       | ND        | ND               |
| ρ1              | ND        | ND               |
| ρ2a             | ND        | ND               |
| ρ2b             | 0.6 ± 0.1  | 0.9 ± 0.0        |
| ρ3a             | ND        | ND               |
| ρ3b             | ND        | ND               |

**Table 2.** Summary of the gating properties of zebrafish GABA4 receptors. Values represent the mean ± SEM. ND: the maximum current smaller than 200 nA and, thus, not determined.
α1 was predominantly expressed in the larval brain at 5 dpf. Depletion of the α1 subunit in zebrafish affected spontaneous behavior at 5 dpf. Thus, the α1 appears to be the major isoform in zebrafish, similar to that in mammals. We also confirmed the overlapping expression patterns of the β3 and the γ2 with the α1 subunit in the larval forebrain, midbrain, and hindbrain, suggesting that the α1β3γ2 GABA<sub>A</sub> receptor, which is the prevalent subtype in mammals, may be formed in zebrafish. However, our RNA labeling does not provide insights at the cellular level and thus, we cannot determine the actual co-expression.

**Electrophysiological properties of zebrafish GABA<sub>A</sub> receptor subunits.** Previous electrophysiological recordings of GABA-evoked currents from *Xenopus* oocytes expressing the human β3 subunit with the human α (α1–6) subunit enabled EC50 comparisons of different GABA<sub>A</sub> receptor subtypes. The order of the EC50 values for the human GABA<sub>A</sub> receptor was α4β3 < α6β3 < a5β3< α1β3, α2β3, α3β3. Co-expression of the human γ2 subunit increased the EC50 values and mostly maintained the order: α6β3γ2, a5β3γ2 < α4β3γ2, α1β3γ2, α2β3γ2, α3β3γ2. This finding is consistent with the fact that α1/2/3- and α5/6b-containing GABA<sub>A</sub> receptors localize at synaptic sites where the GABA concentration increases to more than 1 mM during inhibitory transmission, while α4/5/6 subunit-containing tonic GABA<sub>A</sub> receptors function at extrasynaptic sites where the GABA concentration is low (~0.5 μM). Similar oocyte recordings using zebrafish GABA<sub>A</sub> receptor subunits in this study revealed an order of EC50 values of α6β3 < α5β3, α1β3, α2αβ3 < α3β3 in the absence of the γ2 subunit and α6β3γ2 < α5β3γ2, α2αβ3γ2, α1β3γ2 < α3β3γ2 in the presence of the zebrafish γ2 subunit. Thus, the electrophysiological properties of each subunit appeared to be conserved among vertebrates, suggesting that α1/2/3- and α5/6b-containing GABA<sub>A</sub> receptors are synaptic and extrasynaptic, respectively, in zebrafish. Co-expression of the γ2 subunit increased the EC50 values in both human and zebrafish, further supporting the notion that the electrophysiological properties of GABA<sub>A</sub> receptor subunits are conserved in vertebrates.

Previous studies of oocyte electrophysiology have shown that β3 homopentameric GABA<sub>A</sub> receptors produce small currents when exposed to 10 mM GABA and much larger currents when exposed to 100 μM etomidate. We recapitulated that zebrafish β3 homopentameric GABA<sub>A</sub> receptors elicited small currents upon exposure to 10 mM GABA and large currents upon exposure to 100 μM etomidate, with the latter showing an EC50 of 30.3 ± 5.7 μM (data not shown). Zebrafish homopentameric GABA<sub>A</sub> receptors comprising the β1, β2 or β4 also produced small currents following exposure to 10 mM GABA, suggesting that either these β subunits can be expressed in *Xenopus* oocytes. However, heteropentameric zebrafish GABA<sub>A</sub> receptors containing β3 or β4 yielded GABA-evoked currents, whereas those containing β1 or β2 did not. We were unable to determine why zebrafish β1 and β2 subunits failed to function in heteropentameric GABA<sub>A</sub> receptors. A triple-knockout study of the GABA<sub>A</sub> receptor β subunit in mice suggested that β3 plays an indispensable role in inhibitory synaptic transmission in mammals. CRISPR-mediated disruption of the β3 gene in zebrafish increased spontaneous larval movements, also implying an essential physiological function of the β3 subunit in zebrafish. Thus, β3 is presumably the primary β isoform not only in mammals but also in zebrafish.

The mammalian δ subunit can form homopentameric GABA<sub>A</sub> receptors, which were initially referred to as GABA<sub>B</sub> receptors and eventually recategorized as GABA<sub>A</sub> receptors. Our electrophysiology also showed that zebrafish p2α subunit forms functional homopentameric GABA<sub>A</sub> receptors. The expression of the p2α subunit was observed in zebrafish eyes, similar to the expression of the p2 subunit in mice.

Although heteropentameric zebrafish α1β3 GABA<sub>A</sub> receptors elicited GABA-evoked currents in *Xenopus* oocytes, the additional expression of either the δ or ζ/πb subunit eliminated the currents inconsistent with the findings in mammalian GABA<sub>A</sub> receptor cases. The zebrafish δ and ζ/πb subunits may suppress the formation of α1β3 heteropentameric channels. We also failed to record GABA-mediated currents from oocytes injected with α4, α6α, β1, β2, p1, p2b, p3a, or ρ3b cRNA consistent with the previous electrophysiology using mammalian orthologs of these subunits. The efficiency of zebrafish protein synthesis may differ among receptor subunits in zebrafish oocytes.

Taken together, our current study provides basic information on the expression and gating properties of zebrafish GABA<sub>A</sub> receptors. Since recent development in CRISPR/Cas9 technology have enabled easy and multiple targeted gene disruption, future studies of GABA<sub>A</sub> receptor knockout in zebrafish will clarify the physiologically relevant function of each GABA<sub>A</sub> receptor subunit and unveil the significance of GABA<sub>A</sub> receptor diversity.

**Materials and methods.**

**Animals.** Zebrafish (Danio rerio) were reared and maintained in 1.7 L tanks in a recirculating Meito System (Meito System) at 28.5 °C under a 14 h light and 10 h dark photoperiod according to the standard protocol. Larvae were fed brame and Gemma Micro ZF 75 (Funakoshi) twice daily from 5 to 30 dpf. Juvenile fish were fed brine shrimp (Toki Guppy) and Gemma Micro ZF 75 twice daily from 30 to 90 dpf. Adult fish were fed brine shrimp and Otohime B2 (Marubeni Nissin Feed) twice daily after 90 dpf. Zebrafish AB line was purchased from the Zebrafish International Resource Center (https://zebrafish.org/home/guide.php) and used for line maintenance.

**Phylogenetic analysis.** Amino acid sequences of human and mouse GABA<sub>A</sub> receptor subunit proteins were obtained from the NCBI database. The accession numbers are as follows. Human GABA<sub>A</sub> receptor subunit: α1: NP_000797; α2: NP_000798; α3: NP_000799; α4: NP_000800; α5: NP_000801; α6: NP_000802; β1: NP_000803; β2: NP_000804; β3: NP_000805; γ1: NP_775807; γ2: NP_944494; γ3: NP_150092; ε: NP_004952; δ: NP_000806; π: NP_055026; θ: NP_061028; ρ1: NP_002033; ρ2: NP_002034; and ρ3: NP_001099050. Mouse GABA<sub>A</sub> receptor subunit: α1: NP_001345964; α2: NP_002092; α3: NP_001344743; α4: NP_000804; α5: NP_000803; α6: NP_001093111; β1: NP_002095; β2: NP_001349575; β3: NP_002097; γ1: NP_003482; γ2: NP_002099; γ3: NP_032100; ε: NP_059065; δ: NP_032098; π: NP_666129; θ: NP_065234; ρ1: NP_032101; ρ2: NP_032102;
and ρ3: NP_001074659. Zebrafish GABA<sub>A</sub> receptor subunit: α1: NP_001070794; α2a: XP_009305482; α2b: LC596832; α3: XP_021324930; α4: NP_001017822; α5: XP_005166139; α6a: NP_957025; α6b: XP_002667403; β1: XP_002664179; β2: XP_016092780; β3: XP_005166138; β4: XP_017208500; γ1: XP_009305483; γ2: NP_0010143179; γ3: XP_009300843; δ: XP_700099; π/πa: XP_002664479; ζ/πb: NP_001108214; p1: NP_001020724; p2a: XP_017207163; p2b: XP_697486; p3a: XP_009295726; and p3b: NP_001122232. To create a phylogenetic tree, we used the Interactive Tree of Life (iTOL) online tool v5 (https://itol.embl.de).

Cloning of GABA<sub>A</sub> receptor subunits. Total RNA was extracted from mixtures of 1, 2, and 3 dpf zebrafish embryos and 4 and 5 dpf larvae using Sepasol RNA II Super (Nacalai Tesque) as described previously. Digoxigenin-labeled probes covering the complete coding sequences were used (α1: 1377 bp; α2a: 1353 bp; α2b: 1353 bp; α3: 1425 bp; α4: 1671 bp; α5: 1359 bp; α6a: 1332 bp; α6b: 1305 bp; β1: 1452 bp; β2: 1416 bp; β3: 1494 bp; β4: 1446 bp; γ1: 1362 bp; γ2: 1392 bp; δ: 1377 bp; π/πa: 1335 bp; ζ/πb: 1341 bp; ρ1: 1398 bp; ρ2a: 1422 bp; ρ2b: 1392 bp; ρ3a: 1416 bp; and ρ3b: 1413 bp).

Whole-mount in situ hybridization. In situ hybridization of whole-mount zebrafish embryos with a digoxigenin-labeled antisense RNA probe was performed as described previously. Digoxigenin-labeled probes covering the complete coding sequences were used (α1: 1377 bp; α2a: 1353 bp; α2b: 1353 bp; α3: 1425 bp; α4: 1671 bp; α5: 1359 bp; α6a: 1332 bp; α6b: 1305 bp; β1: 1452 bp; β2: 1416 bp; β3: 1494 bp; β4: 1446 bp; γ1: 1362 bp; γ2: 1392 bp; δ: 1377 bp; π/πa: 1335 bp; ζ/πb: 1341 bp; ρ1: 1398 bp; ρ2a: 1422 bp; ρ2b: 1392 bp; ρ3a: 1416 bp; and ρ3b: 1413 bp).

In vitro synthesis of capped cRNAs. Capped zebrafish GABA receptor mRNAs were synthesized from pCS2 + based plasmids using the mMessage mMachine SP6 Transcription Kit (Thermo Fisher Scientific) as described previously.

Electrophysiology. Electrophysiology was performed as described previously. In brief, oocytes were injected with five femtomoles of cRNAs using a Nanoject II (Drummond Scientific) and incubated in Barth’s solution (88 mM NaCl, 1 KCl, 2.4 mM NaHCO<sub>3</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, and 10 mM HEPES at pH 7.5 with NaOH) and GABA solutions of different concentrations (EC50). y: normalized current.

\[ y = \text{min} + \frac{\text{max} - \text{min}}{1 + \left( \frac{x}{\text{EC}_{50}} \right)^n} \]

Statistics. Quantitative data are presented as means ± SEM. All error bars in the graphs represent the SEM values. Statistical significance was determined by pairwise analysis of variance.

Ethics statement. This study was approved by the Animal Care and Use Committee of Aoyama Gakuin University (A9/2020) and carried out according to the Aoyama Gakuin University Animal Care and Use Guidelines and the Animal Research of in vivo Experiments (ARRIVE) guidelines.

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Author contributions
H.H. designed the research; K.S., I.S., M.S., D.I., M.K., and H.H. performed the research and analyzed the data; K.S. and H.H. wrote the manuscript.

Competing interests
The authors declare no competing interests.
Additional information

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