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Characterization of zebrafish $GABA_A$ 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. $GABA_A$ receptors are ligand-gated chloride channels composed of five subunit proteins. Mammals have 19 different $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors, suggesting that the properties of $GABA_A$ 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 $GABA_A$ receptors[1]. The $GABA_A$ receptor mediates two types of inhibition, known as phasic and tonic inhibition[2]. Phasic inhibition occurs at postsynaptic sites, where the GABA concentration transiently rises to more than 1 mM during synaptic transmission[3], while tonic inhibition occurs at extrasynaptic sites, where the concentration of spillover GABA increases to ~0.5 μM[4,5]. Regardless of synaptic or extrasynaptic sites, $GABA_A$ receptors comprise five subunits forming Cl^--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 $GABA_A$ 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 $GABA_A$ receptors are composed of two α, two β, and one γ subunit[6]. In fact, experimental evidence of native $GABA_A$ receptors suggests that there are fewer than 20 receptor subtypes, with the major synaptic $GABA_A$ receptor combinations being α1β2γ2, α1β3γ2, α2β3γ2, and α3β3γ2[7,8]. The extrasynaptic $GABA_A$ receptors appear to contain specific subunits such as α4, α5, α6, and δ, forming α4β3δ, α5β3γ2, and α6β3δ[2,7]. The other subunits—ε, π, and θ—also assemble with α and β subunits and are located at extrasynaptic sites[2]. The ρ subunits form homopentameric $GABA_A$ receptors that are predominantly expressed in the retina[9]. 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[10]. While the physiological properties of $GABA_A$ receptor isoforms in mammals have been addressed, the evolutionary conservation and physiological significance of diverged subunits in vertebrates remain largely unclear.

$GABA_A$ 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[11]. Cocco and colleagues identified 23 putative $GABA_A$ receptor subunits (eight α, four β, three γ, one δ, one π, one ζ, and five ρ) in the zebrafish genome[12]. They also investigated the transcript levels of $GABA_A$ 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 $GABA_A$ receptor α subunits in zebrafish embryos by in situ hybridization and showed that most α subunits are expressed during embryogenesis[13]. Several loss-of-function studies have revealed the physiological function of $GABA_A$ receptors in zebrafish. Antisense morpholino-mediated knockdown of the α1 subunit caused reduced spontaneous locomotor activity in larvae at 5 days post-fertilization (dpf)[14], while CRISPR/Cas9-mediated knockout of α1 caused seizure phenotypes in juveniles at 35 dpf[15]. Knockdown of the

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α2 subunit perturbed the expression of the proneural gene *neurod* and a GABA-synthesizing enzyme gene *gad1b* within 1 day of development[16]. Zebrafish larvae lacking the β3 subunit showed reduced sensitivity to anesthetic drugs such as etomidate and propofol[17]. 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[18]. 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/genes have been identified in mammals (α1–6/*gabra1–6*, β1–3/*gabrb1–3*, γ1–3/*gabrg1–3*, δ/*gabrd*, ε/*gabre*, 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 subunits/genes comprising eight α (α1/*gabra1*, α2a/*gabra2a*, α2b/*gabra2b*, α3–5/*gabra3–5*, α6a/*gabra6a*, and α6b/*gabra6b*), four β (β1–4/*gabrb1–4*), three γ (γ1–3/*gabrg1–3*), one δ/*gabrd*, one π /*gabrp*, and five ρ (ρ1/*gabrr1*, ρ2a/*gabrr2a*, ρ2b/*gabrr2b*, ρ3a/*gabrr3a*, ρ3b/*gabrr3b*) 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[20]. 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 information 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 subunits 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 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–g,w,x). 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 subunits 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

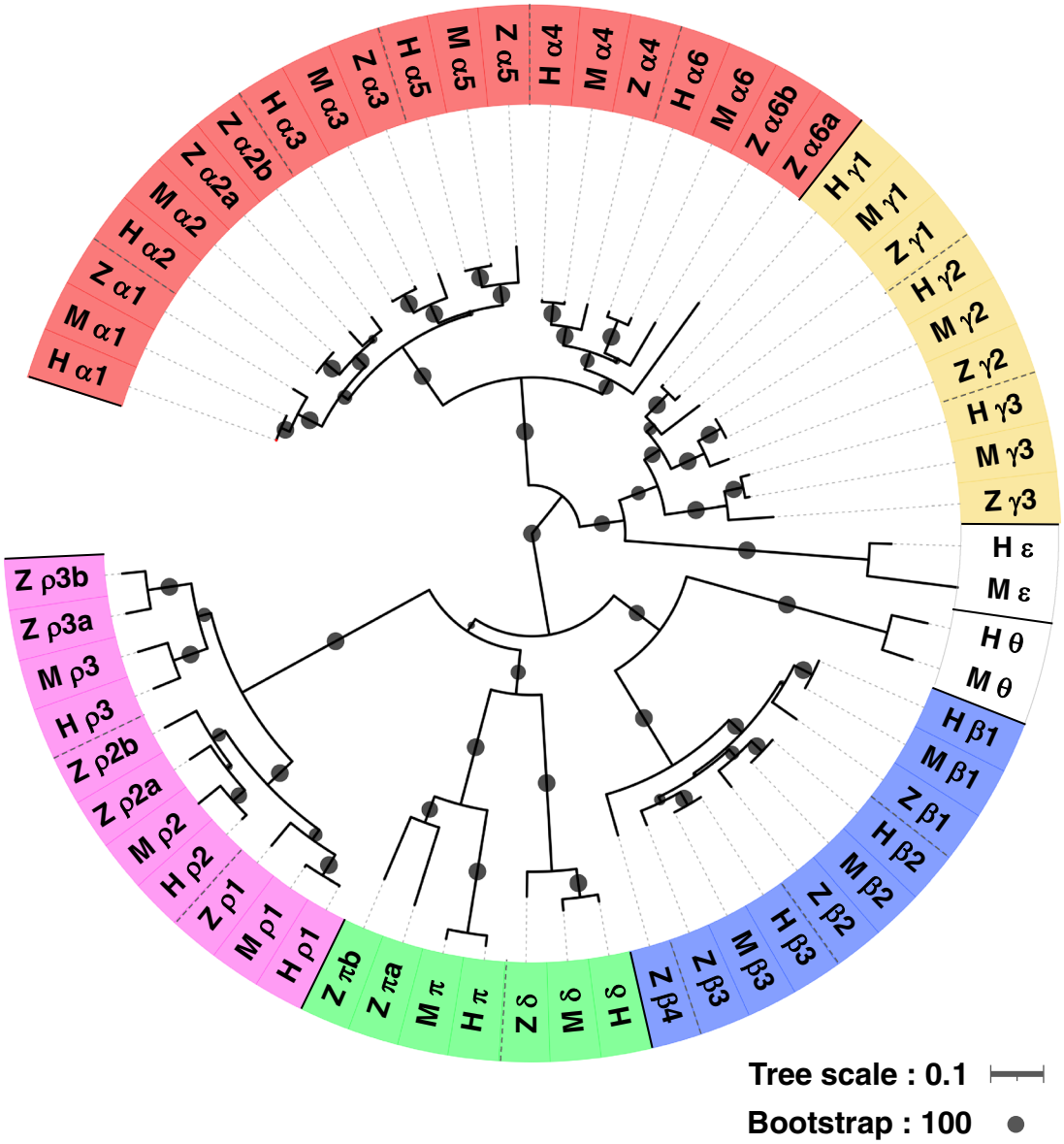

Figure 1. A phylogenetic tree of $GABA_A$ receptor subunits. Amino acid sequences of $GABA_A$ receptor subunits from humans, mice, and zebrafish were used to create a phylogenetic tree. This phylogenetic tree detected the subfamilies of α, γ/ ε, β/ θ, δ/π, and ρ. H: human; M: mouse; Z: zebrafish.

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 $GABA_A$ receptors are composed of two α and two β subunits, along with another one chosen from the γ, δ, ε, π or θ subunit[2,7], with α1β2γ2 and α1β3γ2 being the two most prevalent subtypes in rat brain neurons[22]. Mammalian $GABA_A$ 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 $GABA_A$ receptors in *Xenopus* oocytes[23]. To explore the electrophysiological properties of $GABA_A$ receptor subtypes in zebrafish, we next recorded the GABA-mediated currents from oocytes injected with one α and one β cRNAs with or without one γ cRNA. Co-expression of the β3 subunit with the α1, α2a, α3, α5, or α6b subunit yielded GABA-evoked currents, whereas that with the α2b, α4, or α6a subunit did not (Fig. 3b). Similarly, co-expression of β3 and γ2 subunits with the α1, α2a, α3, α5, or α6b subunit generated GABA-mediated currents, while that with the α2b, α4, or α6a subunit did not (Fig. 3c). In all cases, co-expression of the γ2 subunit increased the EC50 value. Co-expression of the β4 with the α1 subunit in the absence or presence of the γ2 subunit also yielded GABA-evoked currents, the latter with a higher EC50 value (Fig. 3d, e). By contrast, for unknown reasons, co-expression of

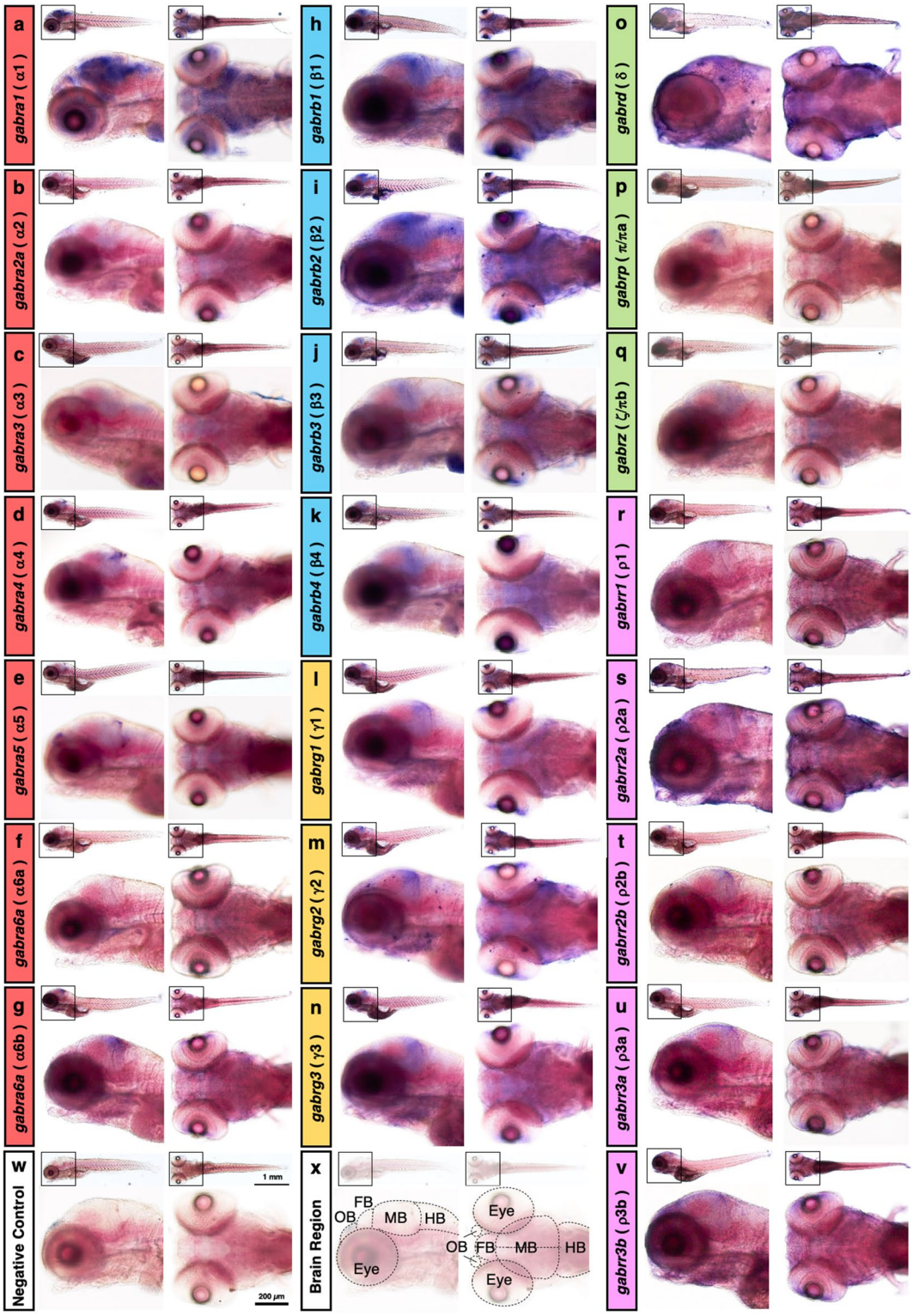

Figure 2. Spatial expression of zebrafish $GABA_A$ receptor subunits. Whole-mount in situ hybridization of 5 dpf zebrafish larvae using antisense probes for *gabra1* (**a**), *gabra2a* (**b**), *gabra3* (**c**), *gabra4* (**d**), *gabra5* (**e**), *gabra6a* (**f**), *gabra6b* (**g**), *gabrb1* (**h**), *gabrb2* (**i**), *gabrb3* (**j**), *gabrb4* (**k**), *gabrg1* (**l**), *gabrg2* (**m**), *gabrg3* (**n**), *gabrd* (**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.

	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 $GABA_A$ receptor subunit genes in zebrafish larvae at 5 dpf. Note that +++, ++, and + indicate the intensity of staining.

either β1 or β2 with any α 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors to GABA differed according to the subunit combination, providing the functional diversity of $GABA_A$ receptor subtypes in zebrafish, as observed in mammals.

Discussion

In this study, we investigated the phylogeny, expression, and electrophysiology of zebrafish $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptor subunits/genes in zebrafish[12]. 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 $GABA_A$ receptor subunits confirmed that all of the exon–intron junctions were correct for the 22 previously suggested and 1 newly identified $GABA_A$ 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 $GABA_A$ 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[20]. 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[13]. However, our phylogenetic tree suggested that the ρ subfamily is instead close to a subfamily comprising the β, θ, δ, and π subunits, consistent with an old phylogenetic study[24]. This discrepancy could be caused by a difference in phylogenetic methods and, thus, will be solved in future development of phylogenetic methods.

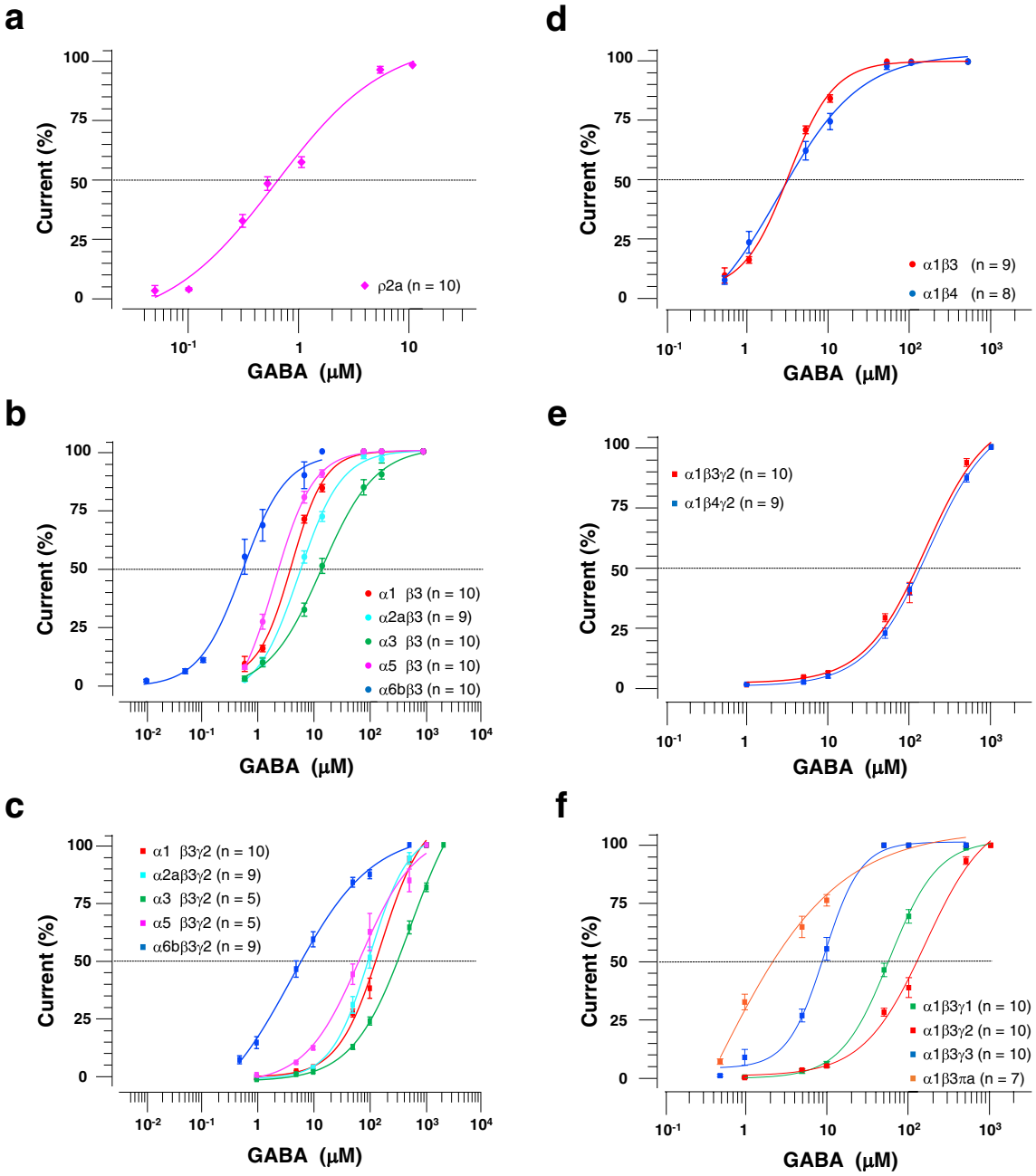

Figure 3. Electrophysiological sensitivities of zebrafish $GABA_A$ receptors to GABA. (**a**) Cumulative dose–response relationship of GABA-evoked currents recorded from *Xenopus* oocytes expressing the ρ2a subunit. (**b**) Cumulative dose–response relationship of GABA currents by the recombinant expression of the α subunit with the β3 subunit. (**c**) Cumulative dose–response relationship of GABA currents by the expression of the α subunit with the β3 and γ2 subunits. (**d**) Cumulative dose–response relationship of GABA currents by the expression of the β subunit with the α1 subunit. (**e**) Cumulative dose–response relationship of GABA currents by the expression of the β subunit with the α1 and γ2 subunits. (**f**) Cumulative dose–response relationship of GABA currents by the expression of γ or π subunits with α1 and β3 subunits. The amplitude of each GABA-evoked response was normalized to the maximally evoked current for each oocyte.

Spatial distributions of $GABA_A$ receptor genes. A previous in situ hybridization study reported the expression patterns of $GABA_A$ receptor subunit genes in zebrafish at 1, 2, and 4 dpf[13]. Here, we assayed the spatial expressions of α and the other subunit genes in zebrafish at 5 dpf. The nucleotide sequence identity of the $GABA_A$ receptor coding regions was 41.6–75.0%, with the highest between ρ3a and ρ3b paralogs and the second highest (72.1%) between ρ2a and ρ2b paralogs (Supplementary Fig. 18). Although the expression patterns of ρ3a and ρ3b were almost identical, those of ρ2a and ρ2b 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

	$GABA_A$ receptor	EC50 (μM)	Hill coefficient
α1	β1	ND	ND
	β1γ2	ND	ND
	β2	ND	ND
	β2γ2	ND	ND
	β3	3.2 ± 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
α2a	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
α3	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
α4	β1	ND	ND
	β1γ2	ND	ND
	β2	ND	ND
	β2γ2	ND	ND
	β3	ND	ND
	β3γ2	ND	ND
α5	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
α6a	β1	ND	ND
	β1γ2	ND	ND
	β2	ND	ND
	β2γ2	ND	ND
	β3	ND	ND
	β3γ2	ND	ND
α6b	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	α1β3	58.0 ± 4.0	1.5 ± 0.1
γ3	α1β3	9.0 ± 0.8	2.1 ± 0.3
δ	α1β3	ND	ND
π/πa	α1β3	2.8 ± 0.6	1.2 ± 0.2
ζ/πb	α1β3	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 $GABA_A$ 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[14,15]. Thus, the α1 appears to be the major isoform in zebrafish, similar to that in mammals[22]. 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptor subtypes[23]. The order of the EC50 values for the human $GABA_A$ receptor was α4β3 < α6β3 < α5β3<α1β3, α2β3, α3β3. Co-expression of the human γ2 subunit increased the EC50 values and mostly maintained the order: α6β3γ2, α5β3γ2 < α4β3γ2, α1β3γ2, α2β3γ2, α3β3γ2. This finding is consistent with the fact that α1/2/3 subunit-containing phasic $GABA_A$ 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 $GABA_A$ receptors function at extrasynaptic sites where the GABA concentration is low (~ 0.5 μM)[2,7]. Similar oocyte recordings using zebrafish $GABA_A$ receptor subunits in this study revealed an order of EC50 values of α6bβ3 < α5β3, α1β3, α2aβ3 < α3β3 in the absence of the γ2 subunit and α6bβ3γ2 < α5β3γ2, α2aβ3γ2, 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 $GABA_A$ 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 $GABA_A$ receptor subunits are conserved in vertebrates.

Previous studies of oocyte electrophysiology have shown that β3 homopentameric $GABA_A$ receptors produce small currents when exposed to 10 mM GABA and much larger currents when exposed to 100 μM etomidate[17,25]. We recapitulated that zebrafish β3 homopentameric $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors. A triple-knockout study of the $GABA_A$ receptor β subunit in mice suggested that β3 plays an indispensable role in inhibitory synaptic transmission in mammals[26]. 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[17]. Thus, β3 is presumably the primary β isoform not only in mammals but also in zebrafish.

The mammalian ρ subunit can form homopentameric GABA receptors, which were initially referred to as $GABA_C$ receptors[27] and eventually recategorized as $GABA_A$ receptors[19]. Our electrophysiology also showed that zebrafish ρ2a subunit forms functional homopentameric $GABA_A$ receptors. The expression of the ρ2a subunit was observed in zebrafish eyes, similar to the expression of the ρ2 subunit in mice[28].

Although heteropentameric zebrafish α1β3 $GABA_A$ 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 $GABA_A$ receptor cases[23]. 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, α6a, β1, β2, ρ1, ρ2b, ρ3a, or ρ3b cRNA inconsistent with the previous electrophysiology using mammalian orthologs of these subunits. The efficiency of zebrafish protein synthesis may differ among receptor subunits in *Xenopus* oocytes.

Taken together, our current study provides basic information on the expression and gating properties of zebrafish $GABA_A$ receptors. Since recent development in CRISPR/Cas9 technology have enabled easy and multiple targeted gene disruption, future studies of $GABA_A$ receptor knockout in zebrafish will clarify the physiologically relevant function of each $GABA_A$ receptor subunit and unveil the significance of $GABA_A$ 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[29]. Larvae were fed paramecia and Gemma Micro ZF 75 (Funakoshi) twice daily from 5 to 30 dpf. Juvenile fish were fed brine shrimp (Tokai 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 $GABA_A$ receptor subunit proteins were obtained from the NCBI database. The accession numbers are as follows. Human $GABA_A$ 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 $GABA_A$ receptor subunit: α1: NP_001345964; α2: NP_032092; α3: NP_001344743; α4: NP_034381; α5: NP_001349090; α6: NP_001093111; β1: NP_032095; β2: NP_001349575; β3: NP_032097; γ1: NP_034382; γ2: NP_032099; 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 $GABA_A$ 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_001243179; γ3: XP_009300843; δ: XP_700099; π/πa: XP_002664479; ζ/πb: NP_001108214; ρ1: NP_001020724; ρ2a: XP_017207163; ρ2b: XP_697486; ρ3a: XP_009295726; and ρ3b: NP_001122232. To create a phylogenetic tree, we used the Interactive Tree of Life (iTOL) online tool v5 (https://itol.embl.de).

Cloning of $GABA_A$ 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[30]. Oligo(dT)18 Primer (Thermo Fisher Scientific), SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific), and Phusion DNA polymerase (New England Biolabs) were used for RT-PCR as described previously[31]. The primer sequences are listed in Supplementary Table 2. The following program was used for amplification: 94 °C for 2 min; 94 °C for 10 s, 63 °C for 20 s, 72 °C for 30 s, 35 cycles of 72 °C for 1 min, and 4 °C forever. The PCR products were cloned into the pCS2 + expression vector as described previously[32].

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[33]. 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: 1428 bp; γ3: 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[34].

Electrophysiology. Electrophysiology was performed as described previously[35]. 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 $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 10 mM HEPES at pH 7.5 with NaOH supplemented with gentamicin at 50 µL/mL and penicillin/streptomycin at 100 units/mL) at 17 °C for 24–72 h before recording. Oocyte recording solution (90 mM NaCl, 1 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES at pH 7.5 with NaOH) and GABA solutions of different concentrations were applied to oocytes using a BPS-8 solution switcher (ALA Scientific). The borosilicate electrodes had resistances of ~ 0.5 MΩ when filled with 3 M KCl. Two-electrode voltage-clamp recordings were made from oocytes held at -50 mV using pClamp 10.2 to control GeneClamp 500B amplifier via Digidata 1440A digitizer (Molecular Devices). Signals were low-pass filtered at 10 Hz and sampled at 100 Hz. The recordings were analyzed using Clampfit 10.7 (Axon Instruments) and SigmaPlot 11.0 (Systat Software). The sample numbers are indicated in the figures. The EC50s and Hill coefficients were calculated using the sigmoid standard curve as below. *x*: GABA concentration (EC50). y: normalized current.

$$y = \min + \frac{(\max - \min)}{1 + (x/EC50)^{-Hillslope}}$$

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., L.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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