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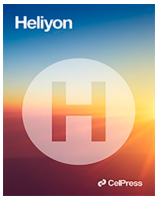

Research article

Conserved expression of the zebrafish *syt4* gene in GABAergic neurons in the cerebellum of adult fishes revealed by mammalian SYT4 immunoreactive-like signals

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ABSTRACT

Synaptotagmin 4 (*syt4*) belongs to the synaptotagmin protein family, which has 17 and 28 family members in human and zebrafish, respectively. In zebrafish and rodents, *syt4* is known to express abundantly in the entire central nervous system in the early developmental stages. In adult rodents, the gene expression shifts to be predominant in the cerebellum, mostly in Purkinje cells, a type of GABAergic neurons. However, there is no report of the expression pattern of *syt4* in the adult zebrafish brain. Therefore, we hypothesize that the expression of *syt4* is conserved in adult zebrafish and is specific to the GABAergic neurons, likely Purkinje cells, in the cerebellum. To examine the hypothesis, we first show that only one copy of *syt4* gene remains in the zebrafish genome, and it is orthologous to the gene in other vertebrates. We further observe mammalian SYT4 antibody immunoreactive-like (mSYT4-ir) signals in several structures in the hindbrain including the medial divisions of the valvula cerebelli and the corpus cerebelli. In addition, our observations indicate the presence of mSYT4-ir signals in GABAergic neurons, most notably in the Purkinje cell layer of the molecular layer in the aforementioned structures. Conversely, mSYT4-ir signals are not observed in glutamatergic or cholinergic neurons. Therefore, we deduce that the *syt4* gene in zebrafish exhibits a homologous expression pattern to those of previously studied vertebrate species, which is revealed by the positive immunoreactive-like signals of mammalian SYT4 antibodies.

1. Introduction

Conservation in a specific gene revealed between zebrafish, a model organism, and other vertebrate groups (including humans), not only signals important functions of the gene but also enables studies in zebrafish for human diseases caused by alterations in the gene.

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As an important model organism in studying human diseases, it is critical to elucidate whether a gene being studied is conserved between zebrafish and other mammalian species. Conservation can be examined at different levels: homology based on DNA and protein sequences, and expression patterns across different developmental stages. Notably, zebrafish often possess one or more duplicate genes homologous to a gene in other species due to the teleost-specific genome duplication [1]. Therefore, it is essential to analyze the phylogeny of the gene with vertebrate homologs before studying it in zebrafish as a model for human diseases. In this study, we determined the conservation of a synaptotagmin gene in zebrafish with its mammalian orthologous gene.

Synaptotagmin gene family (*SYT*) has 17 family members in mammalian species, i.e. humans and rodents. The large gene family with different isoforms ensures the normal function of brains by regulating various biological functions including presynaptic vesicle exocytosis and postsynaptic receptor endocytosis in different organisms [2–6]. Although the knowledge of the protein family was mostly contributed by studying *SYT1* and *SYT2*, more and more studies suggested that the homologous genes in this family play various and diverged roles in the central nervous system [7].

Synaptotagmin 4 (*SYT4*) is one of the most special members of the gene family. Instead of being responsible for vesicle exocytosis and sensing calcium signals as other *SYT* genes, *SYT4* was found to play a role in vesicle trafficking and synaptic plasticity in the neuromuscular junctions of fruit flies and neurons of rodents [3,8–11].

The expression of *Syt4* is dynamic through developmental stages in rodent brain. It was observed in most of the regions of the central nervous system in the early developmental stages whereas in adults, it is mainly restricted to the pituitary gland, hippocampus, and hypothalamus, and the highest expression was observed in the cerebellum in adults [12–15]. Furthermore, *SYT4* is expressed in a fine granular pattern throughout the soma and dendrites of Purkinje neurons, a group of GABAergic inhibitory neurons, in the cerebellum in rodents [12], and also in cerebellar granule and Purkinje cell layers in zebra finches [16]. A series of studies showed that the lack of *SYT4* resulted in impaired motor coordination, disrupted passive avoidance, and reduced anxiety and depression-like behavior [17–19]. The studies suggested that *Syt4* was critical for synaptic regulation within neuronal circuits and brain regions involved in movement, learning, and memory in rodents.

Interestingly, some fundamental molecular functions of the gene were found to be opposite between fruit flies and rodents. Despite both fruit flies and rodents exhibiting a nonsynonymous mutation that impairs the calcium-sensing capability of the C2A domain of SYT4, the capacity to bind calcium persists in fruit flies but not in rodents [20,21]. In addition, the gene plays opposite roles in fruit flies and rodents in postsynaptic retrograde signals, rate of vesicle exocytosis, and SNARE-catalyzed membrane fusion [3,8–11,21]. This raises an interesting question: there might be a functional divergence of the gene between invertebrates and vertebrates. Nonetheless, there is a lack of information on this gene in zebrafish, which undermines its potential as a pertinent vertebrate model for human diseases.

In zebrafish, as a relevant model organism to study human genes, the knowledge of *syt4* is limited. The expression of *syt4* was observed specifically in the central nervous system including the brain and spinal cord in embryos and larvae, which is conversed as it was in rodents [22]. However, knowledge on the distribution of *syt4* in the adult brain regions and cells remains unknown. To understand whether and how the divergence of the function of *syt4* occurred between vertebrates and invertebrates, basic knowledge of the gene in the adult zebrafish brains is essential. We hypothesized the expression of the zebrafish *syt4* may show a conserved expression pattern in the adult brains as seen in the rodents. In this study, we answered the question by the following experiments: (1) analyzing the phylogenetic relations of *syt4* in zebrafish with orthologous *Syt4* in rodents and *SYT4* in humans to confirm their homology, and (2) revealing the distribution of the expression of *syt4* in the adult brains and neural cell types. This study will serve as fundamental knowledge for studying the gene function in zebrafish in the future and for further understanding of functional divergence of the gene across invertebrate and vertebrate taxa.

2. Materials and Methods

2.1. SYT sequence retrieval from database and phylogenetic analyses

Amino acid and coding nucleotide sequences were obtained from Ensembl (https://asia.ensembl.org/index.html, data retrieved in July 2020). Sequences of homologs of human *SYT4* in several vertebrates species were retrieved from common carp (*Cyprinus carpio*), climbing perch (*Anabas testudineus*), goldfish (*Carassius auratus*), clown anemonefish (*Amphiprion ocellaris*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), degu (*Octodon degus*), golden hamster (*Mesocricetus auratus*), guinea pig (*Cavia porcellus*), chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), macaque (*Macaca mulatta*), olive baboon (*Papio anubis*), and orangutan (*Pongo abelii*). The ensemble numbers of *syt4* and homologs were listed in Supplementary Table 1. If more than one transcript was found in the database, the longest coding sequences and their corresponding amino acid sequences were used for the analyses.

The amino acid and nucleotide sequences were aligned separately based on their sequence retrieval gene group via MEGA-X software and using the MUSCLE algorithm. Gaps were removed after the alignment, and each sequence retrieval gene group of amino acid and nucleotide sequences was analyzed separately to determine the mean distance. MEGA-X's "Find Best DNA/Protein Models (ML)" option was used to select the best model, and two phylogenetic trees were constructed, one using maximum likelihood (ML) method and another using maximum parsimony (MP) method for the amino acid and nucleotide sequences of each gene group.

2.2. Animals

Animals (*Danio rerio*) were reared in tap water at 28 °C and kept under a 14 h light/10 h dark photoperiod cycle. The water quality was monitored daily. The transgenic fish lines used for GABAergic neurons and glutamatergic neurons were *Tg(Gad1b:GFP)* [23] and

Tg(Vglut2a:GFP) [24], respectively. Transgenic fish lines were reared in the same conditions as the wild-type fishes. All the procedures follow IACUC protocol NTU-110-EL-00141.

2.3. Brain tissue section preparation for immunohistochemistry

Fishes were anesthetized in 250 mg/L MS-222 (Sigma-Aldrich). Transcardiac perfusion was performed with Ringer's solution until the circulating blood was flushed out and the gills turned clear. After perfusion, fishes were decapitated and the whole heads including the skull and brain were fixed in 4% paraformaldehyde (PFA) over two days. The fish brain was dissected by carefully removing the skull and embedded in 1% agarose in phosphate-buffered saline (PBS) before being cut coronally into 50 μm slices using a vibratome. The sections were stored in PBS before further experiments.

2.4. Immunofluorescence (IF) experiments

The sections were washed with PBST (PBS with 0.1% Tween 20) for 10 min three times at room temperature. The sections were incubated in PBST containing 1% blocking reagent (Roche) at room temperature for 2 h. After blocking, the sections were incubated in primary antibodies in PBST containing 1% blocking reagent, respectively, at 4 °C for 8 h overnight. The sections were then washed with PBST for 10 min three times at room temperature. Sections were incubated in secondary antibodies at room temperature for 2 h. The sections were incubated in DAPI (Sigma-Aldrich) at 1:10,000 dilution in PBST at room temperature for 20 min. After DAPI staining, the sections were washed with PBS for 5 min three times at room temperature and stored in 75% glycerol.

The following primary antibodies were used: rabbit polyclonal antibody against SYT4 (Synaptic Systems 105,143, peptide sequence from rat SYT4) at 1:100 dilution (most of the results were done by using this antibody); rabbit polyclonal antibody against SYT4 (ABclonal A7737, peptide sequence from human SYT4) at 1:100 dilution (for confirmation in Supplementary Fig. 6); goat polyclonal antibody against ChAT (Sigma-Aldrich AB144P) at 1:250 dilution. Antibody against GFP (Cell Signaling mouse *anti*-GFP (4B10) cell signaling 2955 S) at 1:1000 dilution was used to amplify GFP signals in *Tg(Gad1b:GFP)* and *Tg(Vglut2a:GFP)* transgenic fish lines. Antibody against oxytocin (Peninsula Laboratories Internation, Inc. guinea pig anti-oxytocin T-5021) was used at 1:500 dilution. Secondary antibodies used in this study included: goat anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, A-11008) at 1:500 dilution; goat anti-rabbit Alexa Fluor 594 (Thermo Fisher Scientific, A-11012) at 1:500 dilution; goat anti-guinea pig Alexa Fluor 488 (Thermo Fisher Scientific, A-11073) at 1:500 dilution.

2.5. In situ hybridization

Please see Supplementary Materials and Methods for the details of *in situ* hybridization.

2.6. Imaging analysis

The brain sections were mounted onto a microscope slide using 75% glycerol as the mounting solution. Fluorescence microscopy

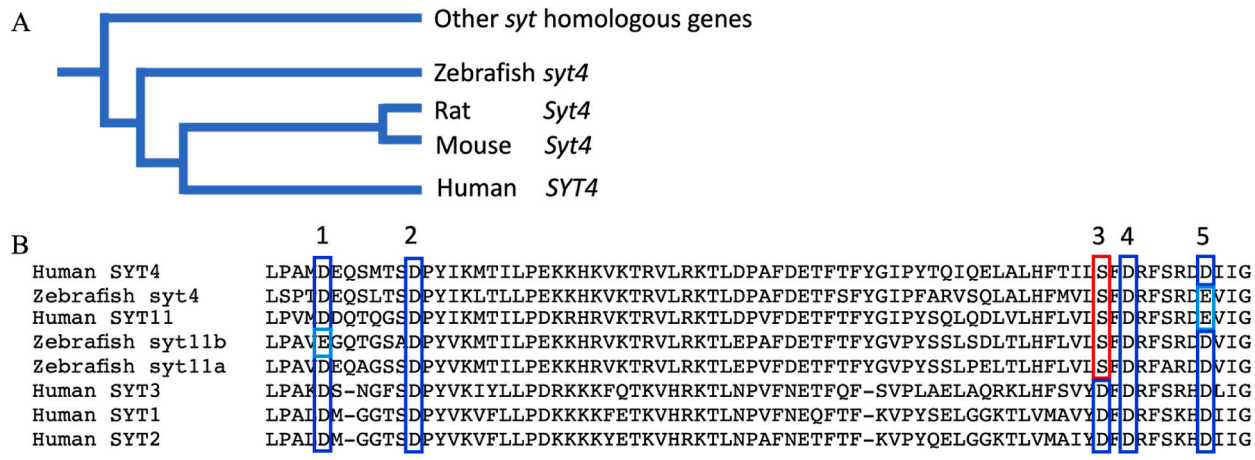

Fig. 1. Homology of *syt4* with homologs in other vertebrate species. (A) Simplified phylogenetic tree of *syt4* and *syt* genes in zebrafish and other vertebrate species including rodents and humans. The complete phylogenetic trees were shown in Supplementary Fig. 1. **(B)** Alignment of partial peptide sequences of domain C2A, which has 5 calcium binding site labeling 1–5 in the figures. We included several homologs from human (SYT1-3) with no mutation at the third position of calcium biding site to compare with SYT4 and another homolog (SYT11) with substitution. The substitution of calcium biding (aspartic acid (D) to serine (S)) is conserved in zebrafish gene *syt4*, *syt11a* and *syt11b* (third amino acid, red box). We observed an extra substitution at the fifth binding site in zebrafish *syt4* (aspartic acid (D) to glutamic acid (E), light blue box) and human *SYT11*. Conserved amino acids between different species were labeled in different color for an easy visualization.

images were obtained using a Zeiss Axioskop microscope and iNS-EDF software. Confocal microscopy images were obtained using a Leica SP8 or Nikon ECLIPSE Ti microscope. Fiji (ImageJ) software was used to process and layer the images. The topology and nomenclature of the zebrafish brain areas described follows Neuroanatomy of the Zebrafish Brain: A Topological Atlas [25].

3. Results

3.1. Synaptotagmin 4 (syt4) is the only homolog in zebrafish with SYT4 genes in other vertebrates

It is important to confirm the homology of *syt4* in zebrafish before using zebrafish as a model organism to study the function of *SYT4* in humans. The *SYT* gene family has 17 family members in humans: *SYT1* to *SYT17* (human genome draft GRCh38.p13, Ensembl database). In zebrafish, 28 family members were identified (ZFIN database. Supplementary Table 1). Several gene members have duplicated paralogs, e.g. *syt1a* and *syt1b*, preserved in the zebrafish genome due to the teleost-specific genome duplication [1]. Although only one *syt4* gene was identified in zebrafish, we set out the goal to confirm the homology of *syt4* with its homologs in vertebrates using phylogenetic analyses. Several zebrafish *syt* genes reported in the ZFIN database were not included in the analyses due to the reason they were not identified in the Ensembl database, i.e. *syt2b*, *syt 8*, *syt18a*, *syt18b,* and *syt 19,* as of the date we retrieved and analyzed the data.

Phylogenetic analyses summarized the following two conclusions: (1) zebrafish *syt4* is clustered together with other *SYT4* genes in humans and rodents, while all the other *SYT* genes are presented as outgroups (Fig. 1A, Supplementary Fig. 1). The results were consistent using two different algorithms, i.e. maximum likelihood (ML) and maximum parsimony (MP), and with DNA coding and peptide sequences (Supplementary Fig. 1). (2) *SYT11* and *syt11a/*b were the closest paralogous genes of *SYT4* and *syt4* in humans and zebrafish, respectively, as proposed in a previous study [26]. It should be noted that the DNA coding sequences of the human *SYT11* and the zebrafish *syt11a* and *syt11b* cluster together with other *SYT4* genes when analyzed using MP (Supplementary Fig. 1B). However, this finding did not alter the conclusions previously stated: *SYT4* shared the most recent common ancestor with *SYT11* compared to other *SYT* genes in the species analyzed.

In conclusion, *syt4* in zebrafish is the orthologous gene of *SYT4* in humans and selected vertebrate species. Therefore, the zebrafish *syt4* gene can represent a suitable model to study the functions of the orthologous human *SYT4* gene. This further implies the conserved and important function of *SYT4* in vertebrates.

3.2. Amino acid substitution at the calcium-binding site is conserved in zebrafish SYT4 C2A domain

It was proposed that the C2A domain of human SYT4 does not have calcium-binding ability due to the third calcium binding site mutated from aspartic acid (D) to serine (S). The mutation (D to S) was found to be conserved in rodents *SYT4* [26]. The substitution was also identified in the closest related gene *SYT11* in humans and rodents. By comparing the calcium-binding region of the C2A domain, we identified the conserved substitution at the third calcium-binding site from aspartic acid to serine in zebrafish SYT4 -(Fig. 1B). In addition, we identified a substitution from aspartic acid (D) to glutamic acid (E) at the fifth calcium binding site in zebrafish *syt4*, which was also seen in human *SYT11*. From the literature, the calcium-binding ability may vary between aspartic acid and glutamic acid [27]. However, it requires further studies to elucidate the ion binding ability of the novel substitution in the protein.

3.3. Antibody selection and immunoreaction tested in zebrafish brains

While *syt4* exhibited abundant expression throughout the central nervous system during embryonic stages in both zebrafish and rodents, its most substantial expression in rodents was confined to the cerebellum in adulthood [12,13,15,28]. Given the limited exploration of *syt4* in adult zebrafish, our study was designed to ascertain the expression pattern and antibody specificity of SYT4 in the zebrafish brain.

Initial attempts at identifying RNA expression through *in situ* hybridization in zebrafish adult brains proved inconclusive (Supplementary Fig. 2). Consequently, efforts were redirected towards elucidating the protein expression of the gene in adult brains. Therefore, we set out the experiments to identify the protein expression of the gene in adult brains. The experimental workflow is delineated in Supplementary Fig. 3.

In the context of immunofluorescence staining, comprehensive negative controls were implemented. Fish brains were stained with only secondary antibodies (Alexa Fluor 488 and 594 from Thermo Fisher Scientific) without primary antibodies, and a negative control devoid of both primary and secondary antibodies was included (Supplementary Fig. 4). These results aimed to ensure the reliability of subsequent positive signals observed in SYT4 staining.

Given the absence of antibodies specifically targeting zebrafish SYT4, multiple antibodies against mammalian SYT4 from different manufacturers, designed for rat and human SYT4, were evaluated and tested in this study. Most of the results were performed with *anti*-SYT4 produced by Synaptic Systems (#105143) designed from rat SYT4 peptides, and confirmation was done with *anti*-SYT4 produced by ABClonal (#A7737) designed from human SYT4 peptides (the results will be described in the later paragraph). It was crucial to note that although protein sequences of mammalian and fish SYT4 exhibit high similarity (approximately 70%), our designation as mammalian SYT4 immunoreactive-like (mSYT4-ir) signals in zebrafish was provisional, as other SYT antibodies in zebrafish were not tested.

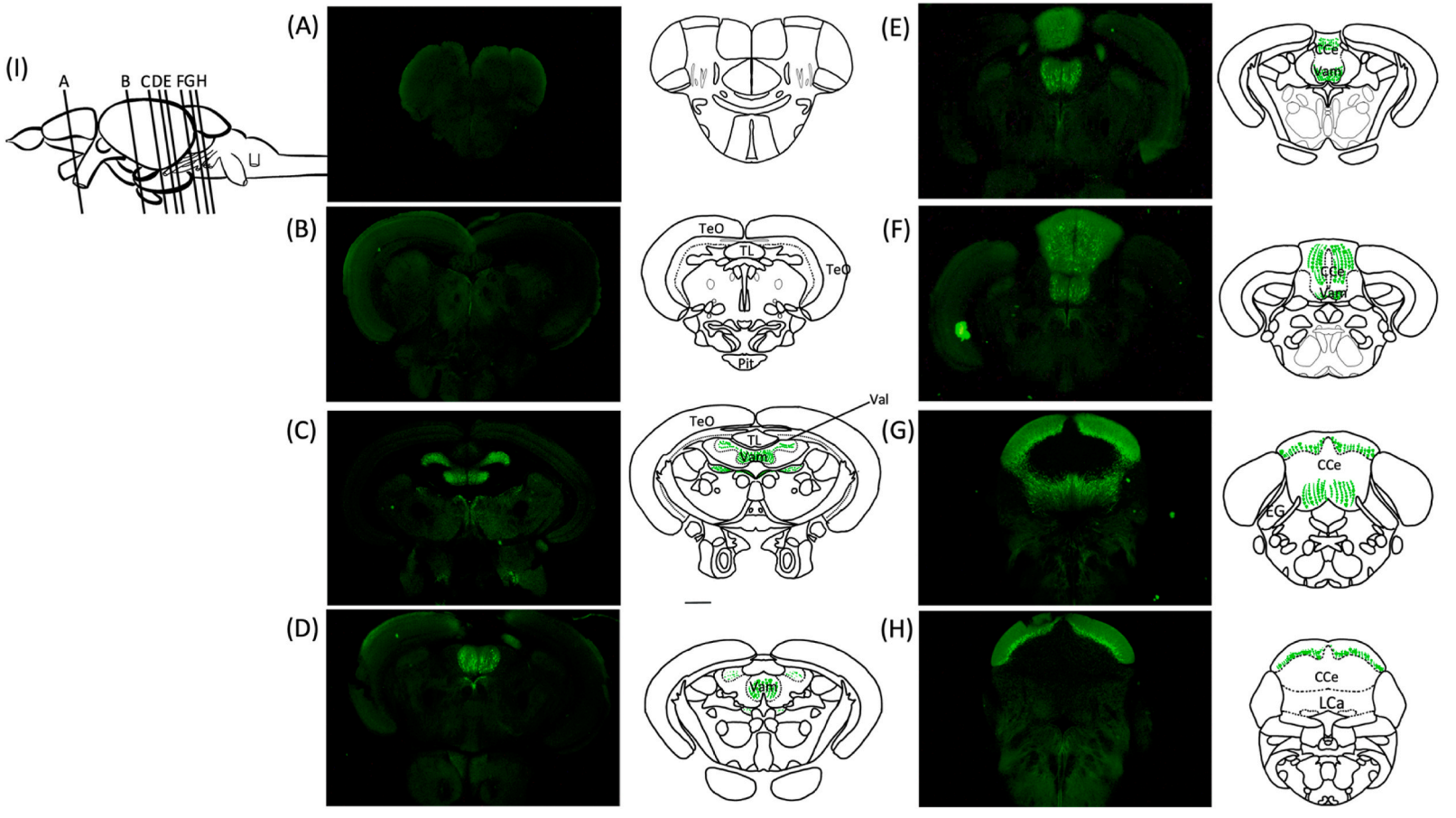

Fig. 2. SYT4 is predominantly expressed in cerebellum in adult zebrafish. The corresponding location of each section (A–H) were indicated in (I). TeO: tectum opticum; TL: torus longitudinalis; Pit: pituitary; Val: lateral division of valvula cerebelli; Vam: medial division of valvula cerebelli; CCe: corpus cerebelli, EG: eminentia granularis; Lca: lobus caudalis of cerebellum. Green color represents mSYT4-ir signals.

3.4. Positive mSYT4-ir signals are observed in the hindbrain, mostly in the cerebellum, of adult zebrafish

We first aimed to identify the brain regions that express the *syt4* gene by analyzing the distribution of mSYT4-ir signals throughout the brain of wild-type (WT) zebrafish with at least 3 replicates (one replicate is shown in Supplementary Fig. 5). In this article, we described only those immunopositive areas consistently labeled in all the replicated experiments in this study.

No mSTY4-ir signals were observed in the forebrain (including telencephalon and diencephalon), and tectum opticum (Fig. 2A and B, Supplementary Fig. 5). Positive signals were observed in the medial divisions of valvula cerebelli (Vam) with lower fluorescence levels of signals observed in lateral divisions of valvula cerebelli (Val) (Fig. 2C and D). Intense mSYT4-ir signals of Vam could be observed also in the corpus cerebelli (CCe) (Fig. 2C–H), particularly the molecular layer (ML) (Fig. 2E–H). This implied that the most abundant expression of the *syt4* gene in the adult zebrafish brains was likely to be in the cerebellum, which was conserved as it was seen in mammals. We conclude that the expression pattern of the *syt4* gene is conserved in teleost and mammals: it is abundant and can be observed in most of the regions of the central nervous system in larvae based on the literature [22] but changed to be mainly circumscribed to the cerebellum in adults.

3.5. Positive mSYT4-ir signals are mainly circumscribed in GABAergic neurons

In rodents and birds, the *Syt4* gene was proposed to be expressed in Purkinje cells, a type of GABAergic neuron, in the cerebellum [12,13,16]. We further asked whether (1) the zebrafish *syt4* gene is expressed in GABAergic neurons, and (2) it is mainly expressed in GABAergic neurons, in adult zebrafish brains. We designed three experiments using two transgenic fish lines with reporter genes specific to GABAergic and glutamatergic neurons, respectively, alongside the antibody of a marker of cholinergic neurons to answer the questions.

First, we aimed to confirm the localization of mSYT4-ir signals in the GABAergic neurons in the *gad1a* transgenic line, *Tg(gad1a:GFP)*. From the literature, *gad1a* was predominantly expressed in Purkinje and stellate cells in the molecular layer (ML) of the cerebellum [29]. The results showed that mSYT4-ir signals were enriched and colocalized in GABAergic neurons in Vam and CCe (Fig. 3A–C). In Vam, mSYT4-ir signals were observed in both cell bodies, mostly cytoplasm, and dendrites of GABAergic neurons (Fig. 4A and B). In CCe, we observed mSYT4-ir signals in GABAergic neurons in ML but not in GCL (Fig. 4C–E). Particularly, we observed mSYT4-ir signals in the cell bodies at the basal layer of ML, which were proposed to be the Purkinje cell layer (PCL) [29] (Fig. 4F). In the ventral part of ML, robust mSYT4-ir signals were seen in the dendrites (Fig. 4G). Similar to rodents, we also observed a fine granule pattern of mSYT4-ir signals in neurons. Based on the morphologies of the cells showing positive mSYT4-ir signals in most of the regions in the cerebellum including Vam and CCe, they were likely to be Purkinje cells. The positive mSYT4-ir signals in the GABAergic neurons were further confirmed by using an alternative human SYT4 antibody from a different manufacturer (Supplementary Fig. 6). To be noted, we did not observe positive mSYT4-ir signals in the eminentia granularis (EG in Fig. 2G) and associated Purkinje cells in the lobus caudalis (LCa of Fig. 2H) of cerebellum.

To answer the second question of whether the zebrafish *syt4* was mainly expressed in GABAergic neurons, we aimed to observe mSYT4-ir signals in the glutamatergic neurons and cholinergic neurons. Here, we used *vglut2a* reporter line, (*Tg(vglut2a:GFP)*), which

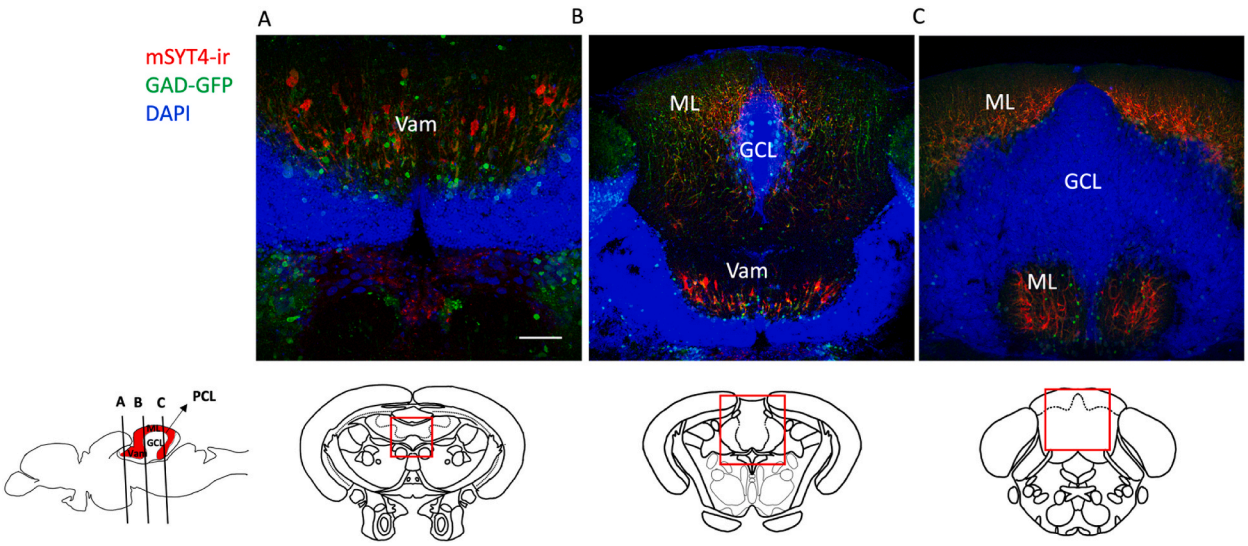

Fig. 3. SYT4 is specific to GABAergic neurons in adult zebrafish. (A) Co-localization of mSYT4-ir signals (red) and GAD (green, a marker of GABAergic neurons) in Vam of cerebellum. (B) Co-localization of SYT4 and GAD in ML and Vam. (C) Co-localization of mSYT4-ir signals and GAD in ML. Relative locations of sections were indicated in the sagittal view of the brain illustration on the left (red color indicates ML in cerebellum). Vam: medial division of valvula cerebelli; ML: molecular layer; GCL: granule cell layer.

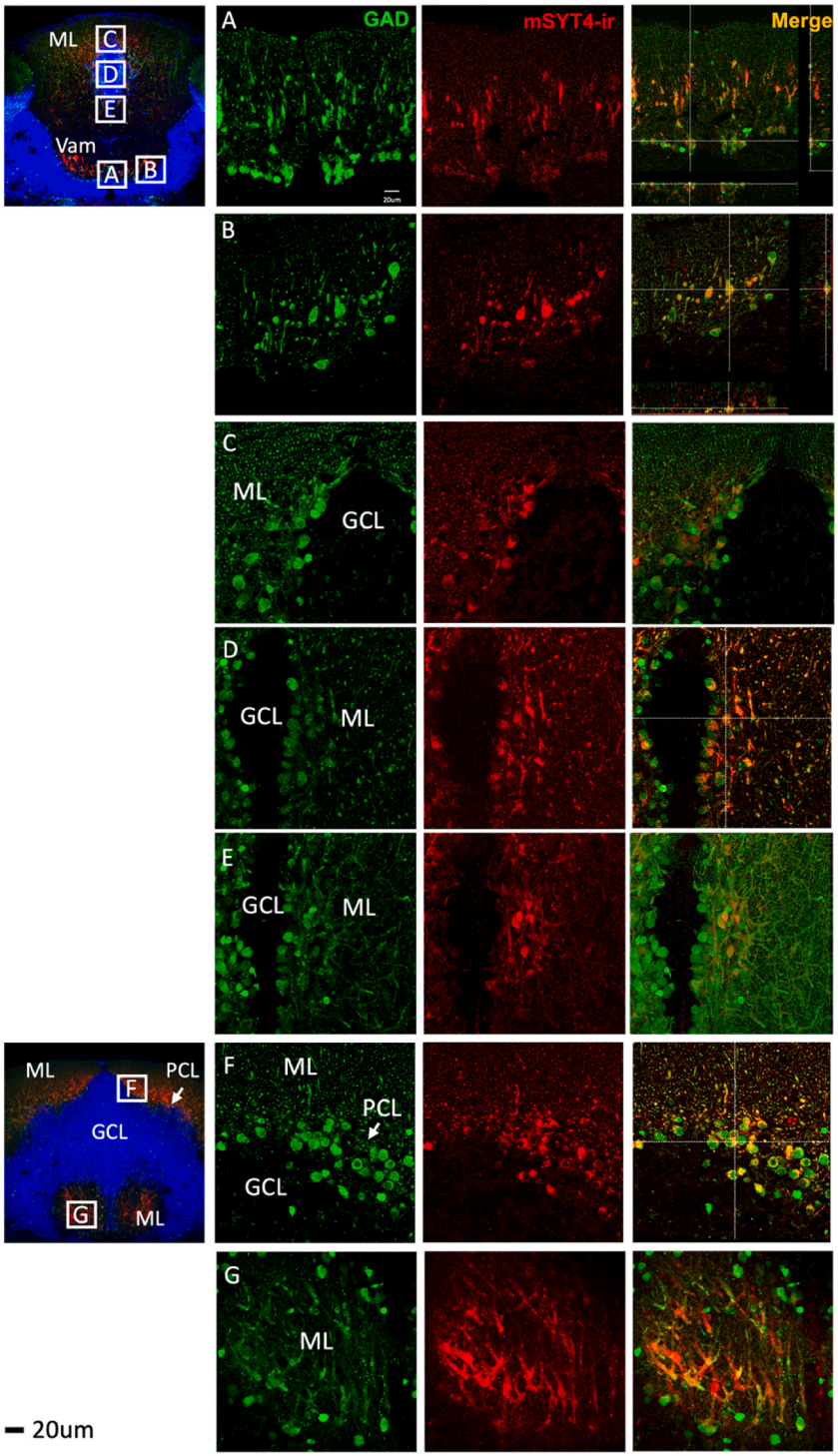

Fig. 4. Co-localization of GAD (green) and mSYT4-ir signals (red) in the cell bodies and dendrites in different regions of the adult cerebellum. **(A and B)** GAD and mSYT4-ir signals in Vam. **(C–E)** GAD and SYT4 in CCe. Signals were only observed in the cell bodies and dendrites of the molecular layer (ML) but not in the granule cell layer (GCL). **(F and G)** GAD and mSYT4-ir signals in ML. The basal layer of ML is the Purkinje cell layer (PCL) indicated by the arrows. Relative locations of sections were indicated in the sagittal view of the brain illustration on the left (red color indicates ML in the cerebellum).

expressed GFP proteins specifically in glutamatergic neurons in the granule cell layer (GCL) of CCe [29]. The results showed that mSYT4-ir signals were enriched in ML of Vam and CCe, while the positive GFP signals from the *vglut2a* reporter gene were found in GCL but absent in ML (Fig. 5A–C). We conclude that the zebrafish *syt4* gene is not expressed in glutamatergic neurons.

By double staining antibodies of Choline Acetyltransferase (ChAT) and mammalian SYT4, no colocalization of the two antibodies in the cholinergic neurons was observed (Fig. 6A–C). In particular, mSYT4-ir signals were not co-localized with cholinergic neurons in NIII (Fig. 6D).

We concluded that mSYT4-ir signals, implying the expression of the zebrafish *syt4* gene, were conserved in and specific to GABAergic neurons in the structures of the cerebellum in the hindbrain as seen in rodents. The expression was mainly circumscribed to ML of Vam and CCe, which was likely to be Purkinje cells. In addition, the zebrafish *syt4* gene was not expressed in glutamatergic or cholinergic neurons in the brain structures we verified.

3.6. *SYT4 in not expressed in oxytocin neurons in the adult brain*

It was shown that SYT4 is expressed abundantly in oxytocin neurons in the hypothalamus in mice [30]. The authors proposed that *Syt4* negatively regulated oxytocin exocytosis through an increased vesicle binding of SYT4 and thus enhanced the negative regulation of oxytocin release. However, we did not observe the expression of *SYT4* in the hypothalamus, nor the colocalization of mSYT4-ir signals in the oxytocin neurons in zebrafish (Supplementary Fig. 8).

4. Discussions

In this study, we hypothesized that *syt4* in zebrafish is conserved and homologous to mammalian *Syt4*, i.e. the sequences and the expression pattern of *syt4* in zebrafish brains should be homologous to other vertebrate species reported in literature. We first showed that there was only one copy of *syt4* in the zebrafish genome and it was orthologous to *SYT4* and *Syt4* in humans and rodents. We also showed that the mutation in the calcium-binding site was conserved in zebrafish *syt4*. As for the expression pattern, zebrafish *syt4* showed the same pattern as rodents: it is expressed mostly circumscribed in GABAergin neurons but not in glutamatergic or cholinergic neurons in the cerebellum in adults.

Based on the literature, certain fundamental molecular functions of SYT4 differ between fruit flies and rodents, including calcium binding ability and postsynaptic retrograding signals [3,8–11,20,21]. Although the molecular functions were not assessed in this current study, we aim to provide some insights by revealing the expression pattern of *syt4* in zebrafish. From our observation, we suggest that the expression patterns are conserved across the vertebrate species, indicating the divergence observed in *Drosophila* and

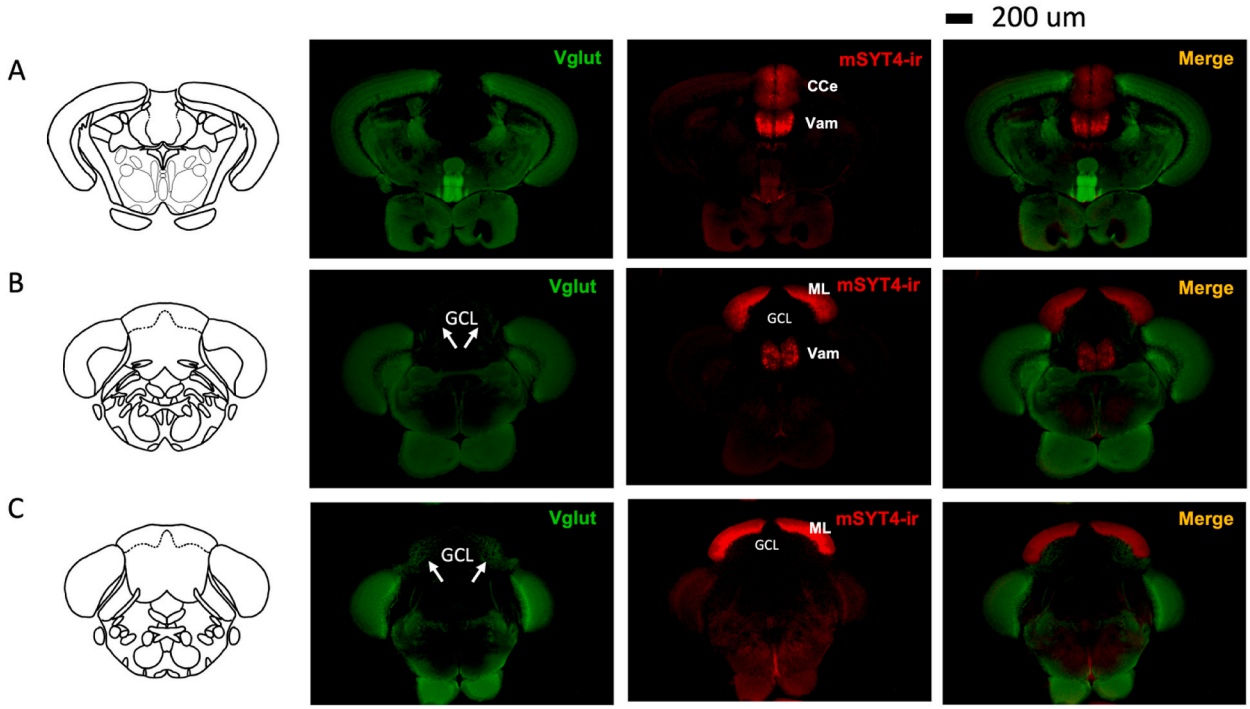

Fig. 5. No co-localization of Vglut (glutamatergic neurons) was observed with SYT4 in the cerebellum. (A–C) Overview of ChAT and mSYT4-ir signals in the cerebellum. The strong signals of Vglut were seen in the granule cell layer (GCL) indicated by arrows. Relative locations of sections were indicated in the sagittal view of the brain illustration on the left (red color indicates ML in the cerebellum). Vam: medial division of valvula cerebelli; CCe: corpus cerebelli; ML: molecular layer; GCL: granule cell layer.

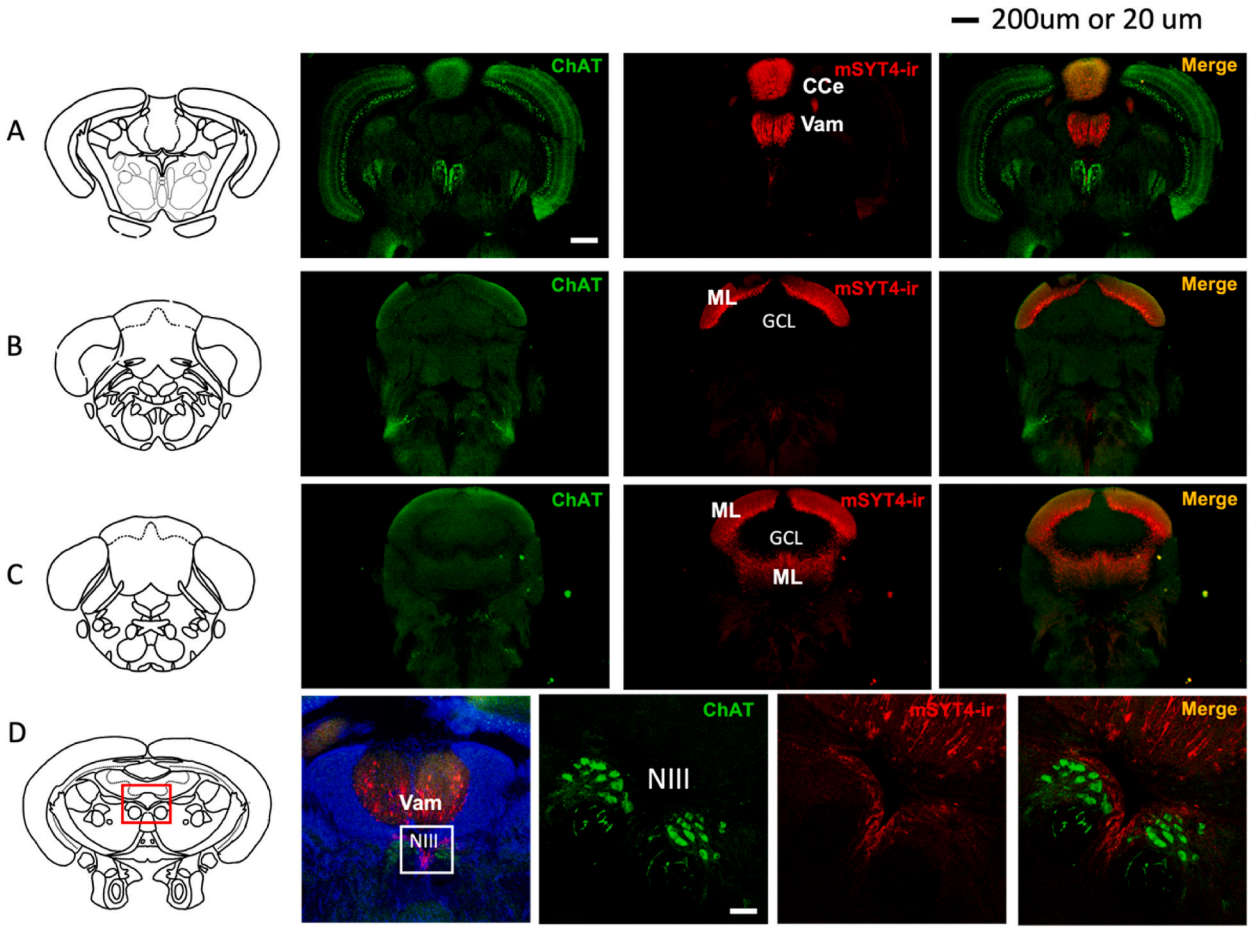

Fig. 6. No co-localization of ChAT (cholinergic neurons) was observed with SYT4 in the cerebellum. (A–C) Overview of ChAT and mSYT4-ir signals in the cerebellum. **(D)** Signals of ChAT and SYT4 in oculomotor neurons (NIII). Relative locations of sections were indicated in the sagittal view of the brain illustration on the left (red color indicates ML in the cerebellum). Vam: medial division of valvula cerebelli; CCe: corpus cerebelli; ML: molecular layer; GCL: granule cell layer.

potentially other invertebrate lineages may occurred in the common accestor of vertebrates. However, further studies and experiments are required to confirm the hypothesis and examine whether the molecular functions align with the conserved expression pattern in the future.

Unlike *Syt1*, which is expressed in most of the regions of rat brains throughout their entire lifespan, *Syt4* is predominantly expressed in several regions in adults [13]. It was expressed universally only in the early developmental stages but changed to be predominantly expressed in several structures in adulthood including the cerebellum, hypothalamus, hippocampus, and pituitary gland, and with the most significant expression observed in the cerebellum in rats [13,14]. In line with the literature, we observed the expression of *syt4* mainly in several structures of the cerebellum of adult zebrafish. However, we did not observe convincing signals in the neuroendocrine structures, i.e. oxytocin neurons, as it was seen in mammals. Although the expression of *Syt4* is observed in the neuroendocrine system in rodents, the expression levels in these structures are relatively low compared with early developmental stages and with the cerebellum in adults [13]. In addition, it was shown that the *Syt4* expression could be induced by kainic acid-induced seizures in the cells in the hippocampus in rodents [31]. We can't rule out the possibility that there is no expression of *syt4* in these structures, or it remains low expression level but will be activated by certain stimulations.

From the literature, *Syt4* was expressed abundantly in oxytocin neurons in the hypothalamus [30]. The authors postulated that *Syt4* mediates a downregulatory impact on oxytocin exocytosis by enhancing vesicle binding to SYT4, thereby reinforcing the inhibitory modulation of oxytocin release. However, we did not observe the co-localization of mSYT4-ir signals in the oxytocin neurons in the posterior part of the parvocellular preoptic nucleus (PPp) (Supplementary Fig. 8). This may imply a diverged function of *syt4* in oxytocin neurons in zebrafish. Further studies are required to elucidate the unsolved questions of the gene in vertebrate species.

The cerebellum regulates motor functions and learning in mammals and the function is conserved in teleost which includes zebrafish [32]. The absence of *Syt4* in the cerebellum led to deficits in motor control, disrupted passive avoidance, and decreased anxiety and depression-like behavior in mice. These findings underscore the crucial role of the gene in cerebellar function [17–19].

In the cerebellum, *Syt4* was found to be robustly expressed in Purkinje cells in rats, which is a population of GABAergic neurons

[12]. The same expression pattern was discovered in songbirds (zebra finches) as well [16]. In the two species, the expression of *Syt4* can be induced by kainic acid-induced seizures and singing, respectively [16,31]. We confirmed that *syt4* is expressed in GABAergic neurons in adult zebrafish, mostly in the cerebellum including ML of Vam and CCe. In zebrafish, Purkinje cells were found in Val, Vam, and CCe [33]. Recently, scientists found that Purkinje neurons may have different physiological functions and morphologies in the zebrafish cerebellum [34]. Three different types of Purkinje neurons were found to respond to different ions (sodium or calcium) with different spectra of stimulus and showed different morphologies [34]. Although we are not able to elucidate which subtype of Purkinje neurons express *syt4* in zebrafish, we may hypothesize that it is expressed in the subtypes that respond to calcium stimulus as the studies found in fruit flies and rodents [3,8–11,20,21]. However, further studies to confirm the expression of zebrafish *syt4* in Purkinje cells are required to elucidate the question.

Other than colocalizing with GABAergic neurons, we also observed mSYT4-ir signals in a population of cells in Vam, which were not glutamatergic or cholinergic neurons (Supplementary Fig. 7, indicated by arrows). It was found that *Syt4* was expressed in astrocytes, a subtype of glial cells, in rodents [35]. The reduction of *Syt4* in astrocytes decreased calcium-dependent glutamate release, which was the gliotransmission pathway regulating synaptic transmission. It provides a future direction in that we may examine whether SYT4 is expressed in glial cells in zebrafish brains.

As we have clarified, only regions with signals consistently observed in different staining replicates were included in the Results. However, certain regions may be misinterpreted as positive signals in our figures, such as the ventricular zone of the telencephalon and superficial layers of the optic tectum (TeO) in Fig. 2B, and the dorsal thalamic nucleus (DTN) in Fig. 2C. We scrutinized the signals at higher magnifications in additional staining results to confirm their validity. It was discovered that the signals observed in TeO and DTN in Fig. 2B and C might be background signals, as they did not appear to be genuine signals under higher magnification (Supplementary Figs. 9A and 9B).

Finally, we would like to share our experience with another *anti*-SYT4 antibody. We also tested an antibody from Sigma-Aldrich (HP010574). However, no validated signals were observed in several replicates of the staining. This lack of signals might be attributed to a greater divergence in the region of the clone compared to the region in zebrafish.

5. Conclusion

We identified that *syt4* in the zebrafish genome is the orthologous gene with *Syt4* in rodents and *SYT4* in humans. Furthermore, the expression pattern of SYT4 is conserved in vertebrate adult brains: it is expressed the most abundantly in GABAergic neurons in the cerebellum. We concluded that *syt4* in zebrafish can serve as a model to study the *SYT4* gene in humans.

Data availability statement

DNA and amino acid sequences of the genes were retrived from Ensembl database as stated in the article. No other data was retrieved from an online database and used for the research described in the article.

CRediT authorship contribution statement

Meng-Shin Shiao: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Sian-Tai Liu:** Resources, Methodology. **Ganchai Siriwatcharapibool:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Supranee Thongpradit:** Methodology, Data curation. **Punnakorn Khunpanich:** Writing – original draft, Formal analysis, Data curation. **Sok-Keng Tong:** Writing – review & editing, Formal analysis, Data curation. **Chih-Hsuan Huang:** Formal analysis, Data curation. **Natini Jinawath:** Project administration, Investigation, Conceptualization. **Ming-Yi Chou:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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M.S.S.: project management and manuscript writing. S.T.L., G.S., S.T., S.K.T. and C.H.H.: performed experiments and prepared figures. G.S. and P.K. performed data analyses and manuscript writing. N.J.: manuscript writing. M.Y.C.: project management, manuscript writing, and provided funding resources. This work was supported by grants from the National Science and Technology Council of Taiwan (111-2313-B-002-001-MY3 and 111-2313- B-002-060-MY3) which were awarded to Dr. Ming-Yi Chou.

Appendix A. Supplementary data

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