Identification and Expression of Voltage-Gated Calcium Channel β Subunits in Zebrafish

Weibin Zhou,† Eric James Horstick, Hiromi Hirata,‡ and John Y. Kuwada*

Voltage-gated calcium channels (VGCC) play important roles in electrically excitable cells and embryonic development. The VGCC β subunits are essential for membrane localization of the channel and exert modulatory effects on channel functions. In mammals, the VGCC β subunit gene family contains four members. In zebrafish, there appear to be seven VGCC β subunits including the previously identified β1 subunit. cDNAs for six additional VGCC β subunit homologs were identified in zebrafish, their chromosomal locations determined and their expression patterns characterized during embryonic development. These six genes are primarily expressed in the nervous system with cacnb4a also expressed in the developing heart. Sequence homology, genomic synteny and expression patterns suggest that there are three pairs of duplicate genes for β2, β3, and β4 in zebrafish with distinct expression patterns during embryonic development. Developmental Dynamics 237:3842–3852, 2008. © 2008 Wiley-Liss, Inc.

Key words: zebrafish; voltage-gated calcium channel; CACNB; spinal cord; nervous system; Rohon-Beard neuron; trigeminal ganglion; lateral line ganglion; heart; retina; VGCC

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INTRODUCTION
Voltage-gated calcium channels (VGCC) mediate Ca\(^{2+}\) influx into cells upon activation by membrane depolarization (Catterall, 2000). In excitable cells, such as muscles, neurons and endocrine cells, voltage-gated calcium channels play important roles in a variety of processes, including excitation–contraction coupling, synaptic transmission, and hormone secretion (Sheng et al., 1994; Rettig et al., 1997; Schredelseker et al., 2005). In addition, Ca\(^{2+}\) entering through voltage-gated calcium channels can serve as a second messenger in signaling pathways to regulate gene expression in developmental processes, including mesoderm patterning, neural induction, process outgrowth, neuronal migration, and cardiac cell differentiation (Komuro and Rakic, 1992; Moorman and Hume, 1993; Moreau et al., 1994; Leclerc et al., 1995, 1997, 2000; Brosenitsch et al., 1998; Haase et al., 2000; Palma et al., 2001; Rottbauer et al., 2001).

Each VGCC usually consists of a pore-forming α1 subunit, a cytoplasmic β subunit, an extracellular α2 subunit associated with a transmembrane δ subunit, and a transmembrane γ subunit (Dolphin, 2003). The α1 subunit contains four repeated domains, each having six transmembrane segments, which is sufficient to form the voltage-dependent Ca\(^{2+}\) channel. Among the auxiliary subunits, the β subunit modulates membrane targeting and the electrophysiological properties of the channel (Castellano et al., 1993; Herlitze et al., 2003). Coexpression of the β subunit with the α1 subunit is able to increase membrane expression and modify the pharmacological and biophysical properties of the channel (Bichet et al., 2000). How β subunits modulate these properties depends on the combination of α1 and β subunit types. For example, β subunits facilitate the voltage-dependence of the activation of L-type VGCCs but not non-L-type VGCCs. On the other hand, β subunits cause a hyperpolarizing shift of the inactivation of non-L-type VGCCs but not L-type VGCCs (Walker and De Waard, 1998).
In mammals, there are four VGCC β subunit genes, CACNB1-CACNB4 (Birnbaumer et al., 1998). Each VGCC β subunit protein (CAB) has five distinctive domains (D1–D5). D2 is a SH3 (Src Homology 3)-like domain and D4 is a MAGUK-like domain, which are found in the members of the membrane associated guanylate kinase (MAGUK) protein family (Dolphin, 2003). These two domains are highly conserved among CABs in different species. A motif at the beginning of D4 domain, known as β interaction domain (BID), is also highly conserved in all known VGCC β subunits and critical for the interaction between the VGCC α1 and β subunits. In contrast, D1, D3, and D5 are more variable.

Only a few nonmammalian CACNB genes have been identified (Dolphin, 2003). Recently the cacnb1 gene was identified in zebrafish (Schredelseker et al., 2005; Zhou et al., 2006). The loss of function of zebrafish cacnb1 causes the immotile phenotype of the zebrafish relaxed mutant, demonstrating the essential role of this gene for normal muscle function. Zebrafish cacnb1 is expressed in skeletal muscle and the nervous system, in agreement with its mammalian orthologs, but no obvious defect in motor output from the central nervous system (CNS) was detected by electrophysiological recordings of skeletal muscle after sensory stimulation of relaxed embryos (Zhou et al., 2006). This raises the possibility that other members of the VGCC β subunit family may compensate for the loss of cacnb1 in the nervous system of relaxed mutants.

RESULTS AND DISCUSSION

We queried the EST (http://www.ncbi.nlm.nih.gov) and zebrafish genomic (http://www.ensembl.org/Danio_rerio/) databases with the sequence of zebrafish cacnb1c (GenBank accession no. DQ198172) and identified six additional homologous sequences from zebrafish. The predicted protein sequences along with the mammalian orthologs, but no obvious defect in motor output from the central nervous system (CNS) was detected by electrophysiological recordings of skeletal muscle after sensory stimulation of relaxed embryos (Zhou et al., 2006). This raises the possibility that other members of the VGCC β subunit family may compensate for the loss of cacnb1 in the nervous system of relaxed mutants.

Zebrafish cacnb2a and cacnb2b

We identified an EST clone (GenBank accession no. CN324195) for cacnb2a and complete sequencing of this EST (GenBank accession no. DQ372944) revealed that this cDNA encoded a protein of 377 amino acids that lacked most of the D5 domain. Mammalian CACNB2 is alternatively spliced into many variants (Birnbaumer et al., 1998; Colecraft et al., 2002; Takahashi et al., 2003) including ones varying in the D1 domain that gave rise to distinct subcellular localizations and modulatory effects on L-type VGCC gating (Takahashi et al., 2003). However, no splice variant of β2 lacking the D5 domain was reported. Because the D5 domain is not required for interactions with the α1 subunit ( Qin et al., 1997), the protein encoded by zebrafish cacnb2a could still bind the α1 subunit, but the physiological function of this β2 isoform is unknown.

The cacnb2b gene was identified from the zebrafish genome database in Zv6_scaffold1007 and Zv6_scaffold3352. Two cDNA sequences (GenBank accession no. DQ372945) for cacnb2b were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR). cacnb2b.1 encodes a protein (CAβ2b.1) of 598 amino acids and cacnb2b.2 encodes for an identical protein except that it has an in-frame deletion of 27 amino acids, suggesting that the two forms are derived from the same gene (Fig. 2A). Because the deletion disrupts the critical BID domain, the isoform encoded by cacnb2b.2 is predicted not to interact
Zebrafish cacnb2a and cacnb2b genes. A: Alignment of proteins encoded by zebrafish and mammalian CACNB2 genes shows that they are highly homologous to each other. The red bar underlines the β interaction domain (BID). The blue bar underlines the deletion in the cacnb2b.2 variant. B: Ventral view of the head region showing that cacnb2a is expressed in the trigeminal ganglion (arrows) at 24 hours post fertilization (hpf). C: View of the dorsal surface of the brain showing expression of cacnb2a in the epiphysis at 24 hpf. D: View of ventral surface of the brain showing strong expression of cacnb2a along the optic stalks (white arrows) and weaker expression in the brain at 48 hpf. E: Dorsal view of head region of a 72 hpf embryo showing strong expression of cacnb2b in the retina. Anterior is left in all the panels except (H–I).
with VGCC α1 subunits. cacnb2a and cacnb2b are most closely related to mammalian CACNB2 genes. The proteins encoded by these two genes (zebrafish CAB2a and CAB2b.1) have 50% and 67% similarity with human CAB2.

We determined the chromosomal location of the two genes by radiation-hybrid mapping. cacnb2a was mapped to Chromosome 2, 16.37cR from EST marker fe08d03 (LOD = 10.8) and cacnb2b to Chromosome 7 at the location of EST marker fu93es09 (LOD = 16.5). In the mouse and human genomes, the CACNB2 gene is located close to the signal transducing adapter molecule (STAM) gene on Chromosome 2 (mouse) and Chromosome 10 (human), respectively (http://www.ncbi.nlm.nih.gov/Genomes/). In the zebrafish genomic sequence assembly (http://www.ensembl.org/Danio_rerio/), we found a zebrafish homolog of STAM (ENSDARG00000021945). This genomic synteny suggests that cacnb2a is an ortholog of mammalian CACNB2. We did not obtain sufficient syntenic information for cacnb2b because the genomic region containing cacnb2b is poorly assembled in the genomic database but the high level of sequence homology (63.9% identity) between CAB2b.1 and CAB2a suggests that cacnb2b is also an ortholog of mammalian CACNB2.

cacnb2a was expressed prominently by the trigeminal ganglia and the epiphysis at 24 hours post fertilization (hpf; Fig. 2B,C). At 48 hpf, cacnb2a expression in the brain increased with notable expression in the optic stalk (Fig. 2D). At 72 hpf, cacnb2b was still detectable in the epiphysis and was strongly expressed in the retina (Fig. 2E). No expression of cacnb2a was detected in the spinal cord from 24 hpf to 72 hpf (not shown).

cacnb2b was expressed extensively throughout the brain at higher levels compared with cacnb2a as well as in the spinal cord at 24 hpf (Fig. 2F,G). The expression of cacnb2b increased in the brain by 48 hpf (Fig. 2H,I) and remained high at 72 hpf (Fig. 2J). In addition to the CNS, there was weaker expression of cacnb2b in the olfactory placodes (Fig. 2F,H).

In mammals the VGCC β2 subunit is expressed in many tissues including heart, brain, lung, kidney, and pancreas (Hullin et al., 1992; Perez-Reyes et al., 1992). β2 is the predominant β subunit in the heart and targeted knockout of β2 results in prenatal death due to cardiac failure in mice (Ball et al., 2002). During embryonic development of the rat, however, β2 subunit in not detected in the heart until fetal day 15 and its abundance increases steadily with the maturation of the heart until birth (Haase et al., 2000). Similarly, as assayed by in situ hybridization expression of neither cacnb2a nor cacnb2b was detected in the developing cardiac tissue during the earliest stages of heart development in zebrafish.

Zebrafish cacnb3a and cacnb3b

The zebrafish cacnb3a gene was identified from genomic contig CR935846.7 in the Zv7 genome assembly. The cDNA (Genbank accession no. DQ372946) cloned by RT-PCR encodes a protein of 439 amino acids (CAB3a) that is most closely related to mammalian CAB3 (60% similarity; Fig. 3A). However, CAB3a has a shorter C-terminal domain than human and mouse CAB3 and could represent a splice variant of cacnb3a. cacnb3a was mapped by radiation-hybrid mapping to Chromosome 23, 5.23 cR from EST marker fd02b09 (LOD = 18.0).

cacnb3b was identified from genomic contig CT57349.6. The partial cDNA (GenBank accession no. DQ372947) cloned by RT-PCR encoded for 332 amino acid protein (CAB3b). The predicted protein sequence shared 55% similarity with human CAB3 and 72% similarity with zebrafish CAB3a. The nucleotide sequence identity between cacnb3a and cacnb3b was 79.8%, suggesting they were duplicated genes. cacnb3b was mapped to chromosome 23, 10.09 cR from SSLP marker z133368 (LOD = 10.9), approximately 85.16 cR from cacnb3a.

Although the D3 region is variable among the β subunits, a highly conserved motif (AKQKQKQ/5V) within D3 is found in neuronal β1, β3, and β4 but not in β2 (Dolphin, 2003). We found this conserved motif is also present in both zebrafish CAB3a and CAB3b (Fig. 3A).

Synteny between the region of mammalian and zebrafish genomes containing VGCCβ3 suggests that zebrafish cacnb3a and cacnb3b are orthologs of mammalian CACNB3. Mouse and human CACNB3 are closely linked to adenylate cyclase 6 (ADCY6), DEAD polypeptide 23 (DDX23) and Rho family GTPase 1 (RND1). Zebrafish cacnb3a is found in the contig located in chromosome 23 containing zebrafish homolog of ADCY6 (ENSDARG00000010558) and DDX23 (ENSDARG00000021945). The cacnb3b is also found closely linked to another zebrafish homolog of ADCY6 (ENSDARG00000027797) and a RND1 homolog (ENSDARG0000004218) in chromosome 23. The synteny thus suggests that cacnb3a and cacnb3b may be duplicated orthologs of mammalian CACNB3.

At 24 hpf, cacnb3a was expressed in two groups of cells located in the forebrain immediately adjacent to the olfactory placodes (Fig. 3B). In the hindbrain, it was expressed by rhombomERICALLY distributed pairs of cells that were likely to be reticulospinal neurons (Fig. 3C). In the spinal cord, cacnb3a was expressed by large, dorsally located cells likely to be mechanoSensory Rohon-Beard neurons. In addition, cacnb3a expression was also detected in segmentally distributed neurons with ventrally projecting axons. These neurons are ventral to the Rohon-Beard neurons and likely to represent commissural neurons in the dorsal spinal cord (Kuwada et al., 1990; Fig. 3D). By 48 hpf, cacnb3a was expressed in the brain (Fig. 3E) with high levels of expression in groups of cells in the hindbrain (Fig. 3F), the trigeminal ganglion and the posterior lateral line ganglion (Fig. 3G). At 72 hpf, cacnb3a was expressed throughout the brain and in the trigeminal and other cranial sensory neurons (Fig. 3H).

At 24 hpf, expression of cacnb3b could be detected in the trigeminal ganglia, two groups of sensory neurons in the otic vesicles (Fig. 3I) and Rohon-Beard neurons in the dorsal spinal cord (Fig. 3J). By 48 hpf cacnb3b was detected in the retina, the trigeminal ganglia, the otocysts and the posterior lateral line ganglia (Fig. 3K). Sections showed that cacnb3b was expressed by cells in the otocysts at 72 hpf (Fig. 3L). Thus,
Fig. 3. Zebrafish cacnb3a and cacnb3b genes. A: Alignment of proteins encoded by zebrafish and mammalian CACNB3 genes shows that they are highly homologous to each other. The red bar underlines the β interaction domain (BID). The blue bar underlines the AKQKQKQ/S/V motif that is conserved in β1, β3, and β4. B: Dorsal view of the head showing that cacnb3a is expressed in two groups of cells (arrows) in the forebrain adjacent to the olfactory placodes at 24 hpf. C: Dorsal view of the hindbrain showing the expression of cacnb3a in discrete bilateral groups of cells (arrows) that may represent reticulospinal neurons at 24 hpf. D: Lateral view of the spinal cord showing cacnb3a is expressed in cells in the dorsal cord likely to be Rohon-Beard neurons (arrows) and commissural neurons (arrowheads) at 24 hpf. E: Dorsal perspective of the head showing cacnb3a is expressed diffusely in the brain at 48 hpf. F: Dorsal view of the hindbrain showing that at 48 hpf cacnb3a is strongly expressed by discrete groups of cells in rhombomeres 4-6 based upon the location of the otocyst. G: Lateral view of the hindbrain showing expression of cacnb3a in the trigeminal ganglion (arrow) and posterior lateral line ganglion (arrowhead) as well as the cells in rhombomeres at 48 hpf. H: Lateral view of 72 hpf embryo showing strong expression of cacnb3a in the brain, the retina and the cells ventral to the otocyst (ot). I: Dorsal view of the hindbrain at 24 hpf showing cacnb3b expression in the trigeminal ganglion (arrow) and posterior lateral line ganglion (arrowhead) as well as the cells in rhombomeres at 48 hpf. J: Lateral view of trunk at 24 hpf showing cacnb3b expression in dorsal cells likely to be Rohon-Beard neurons in the spinal cord. K: Transverse section of 96 hpf embryo showing cacnb3b expression within the otocysts.
**Zebrafish cacnb3a and cacnb3b**

Our database search yielded two CACNB4 homologs in zebrafish. cacnb3a was initially identified from genomic contig BX548038.9 and the full-length cDNA (GenBank accession no. DQ372948) was cloned by RT-PCR. The predicted cacnb3a gene product (CAB4a) consists of 485 amino acids that shares 94% similarity with human CAB4 (Fig. 4A). Two cDNA clones (GenBank accession no. BQ260456 and CK362445) were first identified for cacnb4b and the genomic sequence of this gene was located in genomic contig BX072556.9. The cDNA cloned by RT-PCR (GenBank accession no. DQ372949) encoded for a protein of 489 amino acids (CAB4b) that was most homologous with human CAB4 (88% similarity; Fig. 4A). Previous structural analyses have shown that the D1 domain of the β4 subunit was unique compared with the D1 domains of the other VGCC β subunits (Vendel et al., 2006). This β4-specific D1 domain and the AKQRKQK/SV motif were found in both CAB4a and CAB4b (Fig. 4A).

We mapped cacnb4a to Chromosome 9, 30.11 cR from SSLP marker Z1273 (LOD = 12.0) and cacnb4b to Chromosome 6, 4.81 cR from EST marker f833h05 (LOD = 15.9). The genomic regions containing CACNB4 in mouse and human were highly syntenic. In these regions at least 7 genes (NMI, TNFAIP6, RIF1, NEB, ARL5A, CACNB4, and STAM2) are clustered in identical order on mouse Chromosome 2 and human Chromosome 2. We found a predicted zebrafish STAM2 homolog (ENSDARG00000005318) located immediately adjacent to cacnb4a in the Zv6 genome assembly. Thus the synteny corroborated the assignment of cacnb4a as a CACNB4 ortholog.

CACNB4 is essential for the function of neural circuits controlling motor behaviors because it is mutated in mouse lethargic mutants that exhibit an epilepsy-like phenotype (Burgess et al., 1997; Haase et al., 2000). Both zebrafish CACNB4 homologs are expressed widely in brain, consistent with the neural function of CACNB4. We detected a high level of cacnb4b transcript in the cerebellum in accordance with a role for CACNB4 in regulating motor behaviors. The β4 subunit is also expressed by the fetal heart in rats and precedes expression of the β2 subunit (Haase et al., 2000). Similarly in zebrafish cacnb4a was expressed by the embryonic heart during 24–72 hpf when neither cacnb2a nor cacnb2b expression was detected. Together, these data suggest that both zebrafish cacnb4a and cacnb4b are orthologs of mammalian CACNB4.

**Expression of VGCC β Subunits in Zebrafish Retina**

The zebrafish retina as with the other vertebrate retinas consists of three main layers: the ganglion cell layer (GCL) adjacent to the lens, the inner nuclear layer (INL) and the photoreceptors (PR; Pujic and Malicki, 2004). In the zebrafish retina, the CACNB family genes are expressed in distinct patterns during embryonic development. cacnb2a expression is barely detectable at 48hpf (Fig. 2D), but by 96 hpf, it is specifically expressed in photoreceptors and the outermost half of INL while absent in the GCL and the innermost tier of INL (Figs. 2E, 5A). In contrast, cacnb2b expression in the retina was detectable as early as 24 hpf (Fig. 2F). After 48 hpf, cacnb2b is strongly expressed in the ganglion cell layer in addition to its weak and diffused expression in the INL (Figs. 2H, 5B). The two CACNB3 homologs exhibited partially overlapping expression patterns. At 72 and 96 hpf, both cacnb3a and cacnb3b were expressed in the GCL with additional expression of cacnb3a in the innermost tier of the INL that is immediately adjacent to the inner plexiform layer (Figs. 3G, M, 5C,D). The expression of the two CACNB4 homologs also appeared to be partially overlapping. cacnb4a expression in retina...
**A:** Alignment of protein sequences encoded by zebrafish and mammalian CACNB4 genes shows that they are highly homologous to each other. The red bar underlines the β interaction domain (BID). The blue bar underlines the AKQKQKQ/S/V motif that is conserved in β1, β3 and β4. The green bar underlines the β4-specific D1 domain.

**B:** Lateral view showing cacnb4a expression in the brain and the cardiac tube (arrow) at 24 hpf.

**C:** Dorsal view showing expression of cacnb4a in the forebrain, retina, and midbrain at 24 hpf.

**D:** Dorsal view showing cacnb4a is expressed in the hindbrain and spinal cord at 24 hpf.

**E:** Dorsal view showing cacnb4b is strongly expressed in the forebrain, midbrain, and trigeminal ganglia (arrow) at 24 hpf.

**F:** Dorsal view showing strong expression of cacnb4b in the brain at 48 hpf.

**G:** Dorsal view of 72 hpf embryo showing expression of cacnb4b persists in the brain. The white arrows indicate stronger expression of cacnb4b in the cerebellum compared with the rest of the brain.

**H:** Lateral view at 72 hpf showing expression of cacnb4b by periodically located cells in the spinal cord. Anterior is left in all the panels.
was restricted to the innermost tier of the INL (Fig. 5E) while cacnb4b was expressed in this region as well as the GCL (Fig. 5F). Interestingly, cacnb3a and cacnb4b were expressed in a similar pattern that was complementary to that of cacnb2a.

In mice, the β2 subunit is essential for the retinal formation. Knocking out β2 in the CNS leads to impaired vision, abnormal ERGs, and morphological defects in the outer plexiform layer (Ball et al., 2002). Moreover, the expression of VGCC α1F in the outer segment (photoreceptor) of retina is abolished in these mutant mice. On the other hand, CNS knock-out of β1, β3, and β4 does not produce any obvious abnormality in the eye. Our results indicate that in the zebrafish retina the photoreceptors and the outermost layer of the INL express only the two β2 subunits while the other regions express multiple β subunits, suggesting that in the GCL and the inner layer of the INL these β subunits could function redundantly. This expression pattern is consistent with the loss of α1F expression in mice deficient for β2 in photoreceptors while there was little abnormality in mice deficient for the other β subunits.

Of the VGCC β subunits, cacnb1 appears to be the only one expressed in skeletal muscle in zebrafish (Table 1). This is consistent with the muscle phenotype found in relaxed mutants that is due to loss-of-function mutations in CACNB1 (Schredelseker et al., 2005; Zhou et al., 2006). Of interest, CACNB1 is also expressed throughout the CNS, including the hindbrain and spinal cord, yet spinal motor output following tactile stimulation of mutants is normal. Because the hindbrain and spinal cord are sufficient for motor responses to tactile stimuli, normal neural responsiveness of relaxed mutants suggests that these regions of the CNS function normally despite the loss of cacnb1. The normal response of the CNS to tactile stimulation in relaxed mutants could be due to the redundant actions of the other six CACNB genes, which are also extensively expressed in the nervous system including the hindbrain and spinal cord. Additionally, a duplicate cacnb1 might also act redundantly. At present, neither genome database searches nor degenerate PCR targeting the β1-specific domain produced any sequence other than the known cacnb1 gene (data not shown) suggesting a lack of duplication of cacnb1 in zebrafish. It is notable that we were able to identify only one cacnb1 gene in Takifugu rubripes genome as well, which suggests that there may not be a duplication of CACNB1 in teleosts. However, because the zebrafish genome has not been completely assembled, we cannot rule out that a duplicate cacnb1 might exist.

The zebrafish VGCC β subunit homologs are similar with their mammalian counterparts in sequence, genomic synteny, and expression pattern. Yet significant divergence of this gene family exists in zebrafish. With the possible exception of β1, the other β subunits appear to have undergone gene duplication and specification during evolution, as each pair of putative duplicates share significant sequence similarity but only partially overlapping expression patterns. The expression of the duplicates in different cells may be useful for revealing the specific function of CACNB genes.
in the cells that selectively express each of the duplicates.

**EXPERIMENTAL PROCEDURES**

**Fish Breeding and Maintenance**

Zebrafish (*Danio rerio*) were bred and maintained in a breeding facility following established procedures that meet the guidelines set forth by the University of Michigan Animal Care and Use protocols. Embryos were collected after natural spawns, kept at 28.5°C, and staged according to hours post fertilization (hpf; Westerfield, 1995).

**Database Search**

A BLAST search for zebrafish CACNB gene homologs was done using the sequence of zebrafish CACNB1c (GenBank accession no. DQ198172). The GenBank database was searched for EST clones and the zebrafish genome database (http://www.ensembl.org/Danio_rerio/) for genomic sequences. Each EST clone was completely sequenced to determine whether it contained a complete open reading frame. The acquired genomic sequences were analyzed with Genscan software (http://genes.mit.edu/GENSCAN.html) to determine potential exons, which were used to design primers for RT-PCR.

**RT-PCR and Cloning**

Total RNA was isolated from 24 to 30 hpf embryos by using Tri-reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed with oligo dT primers and Superscript II reverse transcriptase (Invitrogen) following the manufacturer’s instructions (Superscript II manual, version 11-11-203). The PCR products were gel-purified, cloned into the pGEM T-easy vector (Promega, Madison, WI) and sequenced at the University of Michigan Sequencing Core.

**TABLE 1. Expression Patterns of Mammalian CACNB Genes and Zebrafish Homologs**

<table>
<thead>
<tr>
<th>Mammalian Gene</th>
<th>Mammalian expression patterns</th>
<th>Zebrafish homolog</th>
<th>Chromosomal locus</th>
<th>Zebrafish protein</th>
<th>Embryonic expression in zebrafish</th>
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<tbody>
<tr>
<td><strong>CACNB1</strong></td>
<td>Skeletal muscle (β1a)1,2,3, neurons (β1b)3, brain, spinal cord, trigeminal ganglia, olfactory placodes4</td>
<td><strong>cacnb1</strong></td>
<td>Chr3 LOD=9.3</td>
<td>CAB1a (517aa)</td>
<td>CAB1c (603aa)</td>
</tr>
<tr>
<td><strong>CACNB2</strong></td>
<td>Heart, brain, aorta, lung, kidney, pancreas5-7</td>
<td><strong>cacnb2a</strong></td>
<td>Chr2 LOD=10.8</td>
<td>CAB2a (377aa)</td>
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<tr>
<td></td>
<td></td>
<td><strong>cacnb2b</strong></td>
<td>Chr7 LOD=16.5</td>
<td>CAB2a.1 (598aa)</td>
<td>CAB2b.2 (37aa)</td>
</tr>
<tr>
<td><strong>CACNB3</strong></td>
<td>Brain, aorta, trachea, lung, heart, pancreas, adrenal gland8-11</td>
<td><strong>cacnb3a</strong></td>
<td>Chr23 LOD=18.0</td>
<td>CAB3a (439aa)</td>
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<td></td>
<td></td>
<td><strong>cacnb3b</strong></td>
<td>Chr23 LOD=12.4</td>
<td>CAB3b (322aa, partial)</td>
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<tr>
<td><strong>CACNB4</strong></td>
<td>Brain (predominantly in cerebellum), kidney12,13</td>
<td><strong>cacnb4a</strong></td>
<td>Chr9 LOD=12.0</td>
<td>CAB4a (485aa)</td>
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<tr>
<td></td>
<td></td>
<td><strong>cacnb4b</strong></td>
<td>Chr6 LOD=15.9</td>
<td>CAB4b (489aa)</td>
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For degenerate RT-PCR of CACNB1 gene, the following primers were used for the amplification of β1-specific D2-D4 domain: forward primer, 5'-CCACCTCCAACTCCTGTMNGM-CARGG, reverse primer, 5'-CATG-GTCCGTCGACGARRGTTNGG. Other RT-PCR Primer sequences are available upon request to the authors.

In situ Hybridization and Sectioning
In situ hybridization was carried out following standard protocols (Li et al., 2004). To prevent pigmentation after 24 hpf, embryos were transferred to water containing 0.2 mM of 1-phenyl-2-thiourea at 20 hpf and fixed at appropriate stages. The antisense digoxigenin (DIG) -labeled probes for zebrafish CACNB genes were synthesized in vitro from the cloned cDNA sequences. The sense probes were used as negative controls and produced no significant signals (not shown). In some cases, after in situ hybridization, embryos were washed in phosphate buffered saline, dehydrated with 25%, 50%, 75%, 85%, and 100% ethanol and embedded in JB-4 plastics (Polysciences). Sectioning was performed with a Leica RM2265 automatic microtome. For double labeling, in situ hybridization was carried out using Fast Red (Roche Applied Science) as a coloration substrate, followed by immunohistochemical labeling with anti-acetylated tubulin antibody (1:500, Sigma) and secondary anti-mouse IgG (1:2,000; Molecular Probes). Fluorescence imaging was acquired with a Leica SP5 confocal microscope.

Radiation Hybrid Mapping
The LN54 radiation hybrid panel was used for physical mapping of zebrafish sequences (Hukriede et al., 1999). Mapping primers were designed according to the genomic sequence information acquired by searching the zebrafish genome database. Primer sequences are available upon request to the authors.

REFERENCES