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Characterization and Expression of Serotonin Transporter Genes in Zebrafish

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WANG, Y., TAKAI, R., YOSHIOKA, H. and SHIRABE, K. *Characterization and Expression of Serotonin Transporter Genes in Zebrafish*. Tohoku J. Exp. Med., 2006, **208** (3), 267-274 —— To understand the development of serotonergic neurons in vertebrates, we used zebrafish as a model system. In this study we cloned two cDNAs (complementary DNAs) coding for serotonin transporter (SERT) from the zebrafish, named *serta* and *sertb*. The *serta* cDNA encodes a protein of 693 amino acids and showed high level of sequence identity with rat and human SERTs. In situ hybridization showed *serta* to be expressed in raphe nuclei, ventral posterior tuberculum and pineal organ. The expression of *serta* in raphe and ventral posterior tuberculum overlapped with the location of serotonin and expression of tryptophan hydroxylase, which is a key enzyme for serotonin synthesis. In the pineal organ *serta* is expressed in the cells in the vicinity of tryptophan hydroxylase-positive cells. We also cloned another zebrafish serotonin transporter, *sertb*, and found to be expressed in the medulla oblongata and in the inner nuclear layer of retina. The existence of two *sert* genes in the zebrafish genome indicates the gene was duplicated in the process of evolution as can be seen in other genes in the teleosts including zebrafish. The expression of the *serta* cDNA in cultured cells conferred a serotonin transport activity, thus indicating the validity of the cloned cDNA. We have established the expression system of zebrafish serotonin transporter in the cell culture in the present study, which is useful for the pharmacological analysis to determine the important residues for the interaction with serotonin and inhibitors. The expression system in the cell culture can be used to determine the effective concentration of inhibitors and addictive drugs. These information might be useful to evaluate the effect of those chemicals on serotonin neuron development and behavior of the animal. ———— zebrafish; serotonin transporter; pineal organ; raphe nuclei; ventral posterior tuberculum

The zebrafish *Danio rerio* is an excellent system for studying the development of the nervous system because of its visual access to neurons in living embryos. Serotonin (5-hydroxytriptamine, 5-HT) is a neurotransmitter implicated in the maturation of the locomotive network during development (Cazalets et al. 2000), as well as a variety of behaviors, including mood, sleep, pain, appetite and aggression in higher vertebrate (Gaspar et al. 2003; Luo et al. 2003). Serotonin is released into the synaptic cleft and exerts its effects by activating the pre- and post-synaptic

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receptors.

Serotonin transporter removes serotonin from the synaptic cleft into cells and terminates serotonin action, thereby modulating serotonergic signaling and neurotransmission. Accumulating evidence indicates that serotonin also acts as a morphogen in craniofacial and heart development (Moiseiwitsch and Lauder 1995; Vitalis et al. 2003). Serotonin exerts key neuromodulatory activities, but the development and function of this serotonergic system is still not completely understood. Tryptophan hydroxylase catalyzes the first step of serotonin and melatonin biosynthesis. The molecular cloning and expression of three tryptophan hydroxylases, tphD1, tphD2 and tphR in zebrafish have been reported (Bellipanni et al. 2002; Teraoka et al. 2004). An immunohistochemical approach was applied to study the development of a zebrafish serotonergic system (McLean and Fetcho 2004a, 2004b). Serotonin transporter is a target of antidepressant, selective serotonin re-uptake inhibitor (SSRI) and addictive drugs such as cocaine (Bauman et al. 2000). Zebrafish behavioral mutants were screened to explore cocaine sensitivity (Darland and Dowling 2001). As a result, zebrafish is an excellent model for the investigation of effect of antidepressant and addictive drugs on behavior and serotonergic system development.

In the present study we cloned two zebrafish serotonin transporter complementary DNAs (cDNAs), *serta* and *sertb*. We examined the expression by whole mount in situ hybridization of those genes during zebrafish development and showed that they are differentially expressed in raphe nuclei, pineal organ, ventral posterior tuberculum, retina and hindbrain. We further demonstrated the expression of full-length *serta* cDNA in human embryonic kidney cells (HEK) 293 cells to confer serotonin uptake activity.

Materials and Methods

Animals

Embryos of TL strain zebrafish (*Danio rerio*) were staged as described previously (Kimmel et al. 1995). The embryos were raised at 28.5˚C with 0.2 mM phenylthiourea (Sigma, St. Lous, MO, USA) in embryo media E3 (Nüsslein-Volhard and Dahm 2002).

Cloning and Sequencing of sert cDNAs

Poly A plus ribonucleoic acids (RNA) was isolated from 200 embryos of 72 hour post fertilization (hpf) using the QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Little Chalfont, UK). cDNA was synthesized by SuperScript III First-Strand Synthesis System (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. BLAST Basic Local Alignment Search Tool search of the zebrafish expressed sequence Tag (EST) database with human serotonin transporter (SERT) amino acid sequence identified two ESTs, AL913869 and AL914622. These two ESTs overlapped and cover a partial cDNA of 1074 bp. 5´-and 3´-RACE (rapid amplification of cDNA ends) were carried out to clone full-length SERT cDNA by using SMART RACE PCR amplification kit (BD Biosciences Franklin Lakes, NJ, USA). The polymerase chain reaction (PCR) primers for 5´-RACE and 3´-RACE of *serta* were 5´- ACCACCAGAGTCCTAAATGTTCCAG-3´ and 5´- CTCTTCCTTCATCTGTGTGCCTTCC-3´, respectively. The PCR primers for the cloning of full-length *serta* were 5´-AACCCTAACAGCAGTCCTCA-3´ and 5´-GGCCTCACCGTCACACAATA-3´. We blasted the zebrafish genome with the human SERT sequence and then found another sequence, BX547941, that codes for SERT. We named this sequence *sertb*. The PCR primers for the cloning of partial *sertb* were 5´-TCATTGGATTTGCGGTAGACCTCGG-3´ and 5´- TACTCGTCCATTACAGCTGTGATTA-3´. The PCR-amplified fragments were cloned onto pGEM-T easy vector (Promega Madison, WI, USA). The nucleotide sequences of four independent clones were also determined.

In situ hybridization and immunohistochemistry

Sense and anti-sense RNA probes labeled with Digoxigenin-labeled UTP (Roche diagnostics, Basel Switzerland) for *serta*, *sertb* and tphR (Teraoka et al. 2004) were generated by in vitro transcription from respective cDNAs. In situ hybridization and immunostaining to whole-mount embryos were carried out as we previously described (Li et al. 2004, 2005; Nüsslein-Volhard and Dahm 2002). Rabbit anti-5-HT antibody was purchased from Sigma and was used at 1 : 5,000 dilution. Secondary antibody was Alexa Fluor 568 goat anti-rabbit IgG (H + L) from Molecular Probe. For the observation of fluorescence of Alexa Fluor 568 con-focal laser microscope ZEISS LSM5 PASCAL was used.

Cell culture and expression of clones

HEK 293 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in humidified atmosphere comprising 5% CO_2 and 95% air at 37°C. *serta* cDNA was cloned into vector pBK-CMV (Stratagene La Jolla, CA, USA) at EcoRI and SmaI site to generate pBK-*serta* expression plasmid. The transfection of the expression vectors to HEK293 cells was performed using Lipofectamine Reagent (Invitrogen) by following the manufacturer's instructions. The cells were co-transfected with an enhanced green fluorescent protein EGFP expression vector, pEGFP-N1 (Clontech Mountain View, CA, USA), to facilitate the identification of transfectant by fluorescence of EGFP. The transfectants were selected in the a medium containing 600 μg/ml of G418 (Sigma St. Louis, MO, USA) for 10 to 15 days. The colonies with fluorescence were selected and subjected to serotonin uptake measurements.

Transport Assay

The cells (1.25×10^6) were plated in each well of 24-well tissue culture plates coated with poly-L-lysine (Roche diagnostics). The transport assay was carried out essentially as described before (Bernstein and Quick 1999). In brief the vector-transfected HEK 293 cells and pBK-*serta* transfectants were grown until they were confluent. After washing two times with 1 ml of assay buffer (phosphate buffered saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4 with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$), cells were incubated in an assay buffer containing six different concentration of [^{3}H]-labeled serotonin (5-Hydroxy[G-^{3}H]tryptamine creatine sulfate from Amersham Bioscience) diluted with unlabeled serotonin for 10 min for Km and Vmax determinations. At the end of the incubation, the cells were washed three times with 0.5 ml of ice-cold PBS and then dissolved in 0.5 ml of 0.1% sodium dodecyl sulfate (SDS). After 30 min at room temperature, then the solution was removed from each well and the radioactivity was measured in vials containing 4 ml of scintillation cocktail, Clear-SolII (nacalai tesque). Assuming the Michaelis-Menten kinetics, the data were then plotted and analyzed by a non-linear least squares curve fit (GraphPad Prism, San Diego, CA, USA). In inhibition studies, 25 nM [^{3}H]-serotonin was incubated with increasing concentrations of inhibitor and then was incubated for 10 min at room temperature. The assays were carried out in triplicate and IC_{50} values are given as mean values with 95% confidence intervals from six independent measurements. The protein concentration was determined using Protein Assay Reagent (BIO-RAD). The fluvoxamine maleate was provided by Solvay pharmaceuticals.

Results and Discussion

Cloning of serotonin transporter from zebrafish

We isolated two zebrafish SERT genes. First, we used reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify a partial cDNA fragment of *serta* (1310 bp). We extended this fragment to a 2168 bp full-length clone by 5´- and 3´-rapid amplification of cDNA ends (RACE) (GenBank accession number DQ285098). *serta* encodes 693 amino acids, which contains twelve potential transmembrane domains, N-linked glycosylation and kinase-mediated phosphorylation sites. We then blasted zebrafish genome with the human SERT sequence and found another sequence BX547941 that potentially codes for SERT. We cloned a partial sequence of cDNA of 1083 bp, encoding 361 amino acids and the sequence thus showed a significant homology to vertebrate SERT. We named this sequence *sertb* (GenBank accession number DQ285099). The sequence identity between *serta* and *sertb* was 76.5%. Alignment of *serta* amino acid sequence to human, mouse, rat, chick and cow SERT (Fig. 1A) showed a sequence similarity ranging from 66 to 69%, while the similarity of *serta* with other transporter genes (dopamine transporter and noradrenalin transporter) is less than 50%. *sertb* amino acids sequence has about 75% similarity to other vertebrate SERTs. A phylogenetic analysis indicates that both *serta* and *sertb* are vertebrate serotonin transporters, but neither dopamine nor noradrenalin transporters (Fig. 1B). In zebrafish, many genes are duplicated due to genome duplication in the course of evolution and two genes are both functional in most cases. A high level of identity and the functional conservation of the amino acid sequence in *serta* and *sertb* together suggest that both genes are functional.

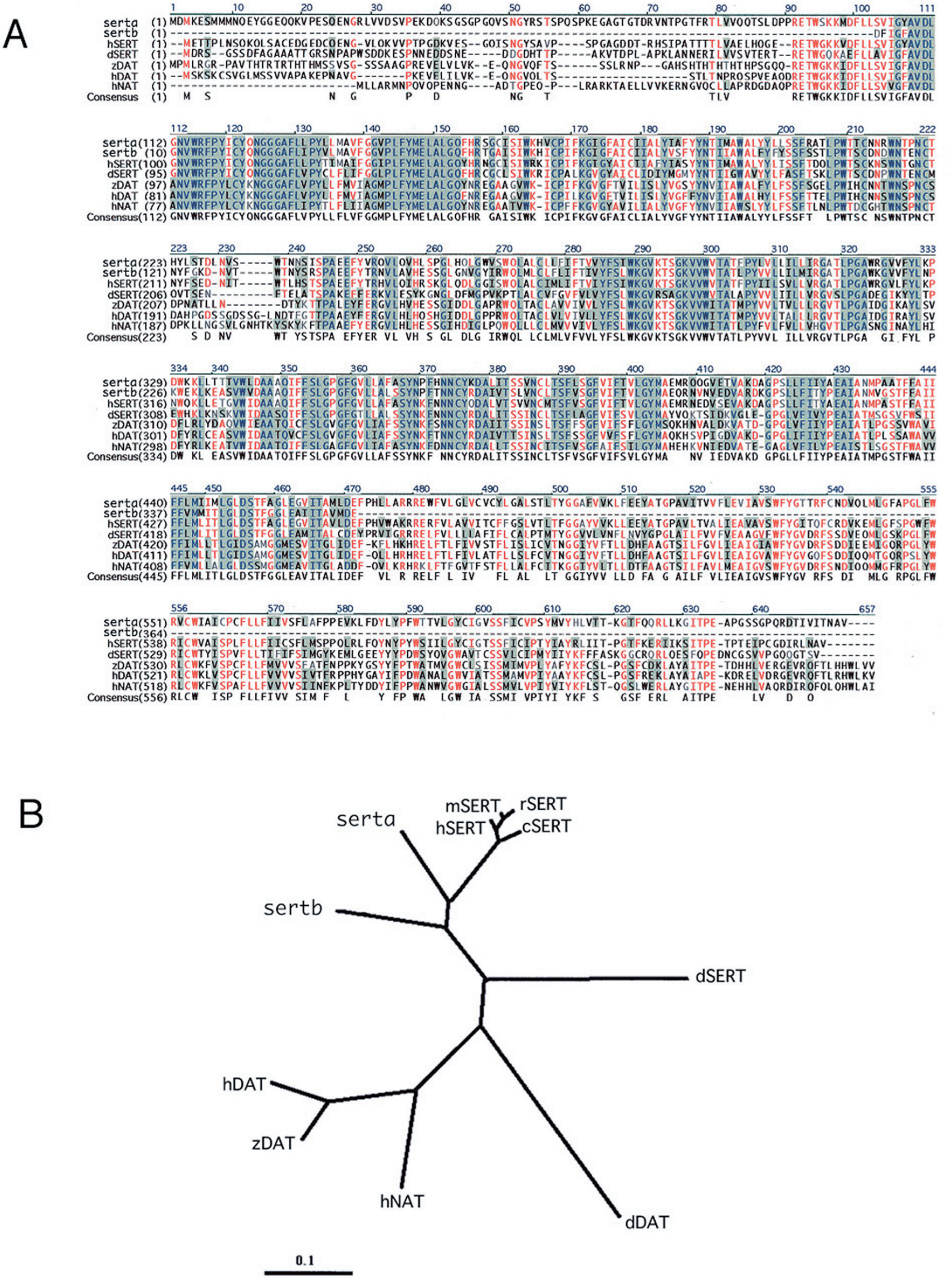

Fig. 1. (A) The aligned protein sequences of SERT, dopamine transporter (DAT) and noradrenaline transporter (NAT) using the ClustalW program. Sequences of human SERT (hSERT, X70697), dSERT (drosophila SERT, U04809), zebrafish dopamine transporter (zDAT, AF318177), human dopamine transporter (hDAT, L24178), human noradrenaline transporter (hNAT, M65105) are from the GenBank database. The shared colors indicate conserved (identical or similar) amino acid residues. (B) Phylogenetic analysis of the SERT, DAT, NAT sequences. The partial sequences corresponding to the sequence available in *sertb* were used for the analysis.

Expression of SERT during zebrafish development in comparison to serotonin and tryptophan hydroxylase

The expression pattern of *serta* by in situ hybridization was compared with the expression of tphR which is a key enzyme catalyzing the first step of the serotonin biosynthesis and with distribution of serotonin detected by immunostaining using anti-serotonin antibody (Fig. 2). We chose 5 different time points 24, 36, 48, 72 and 96 *hpf* to do an expression analysis. The *serta* expression (Figs. 2B, 2C, and 2E) overlapped with the expression of tphR (Fig. 2D) and the distribution of serotonin in the neurons was located close to

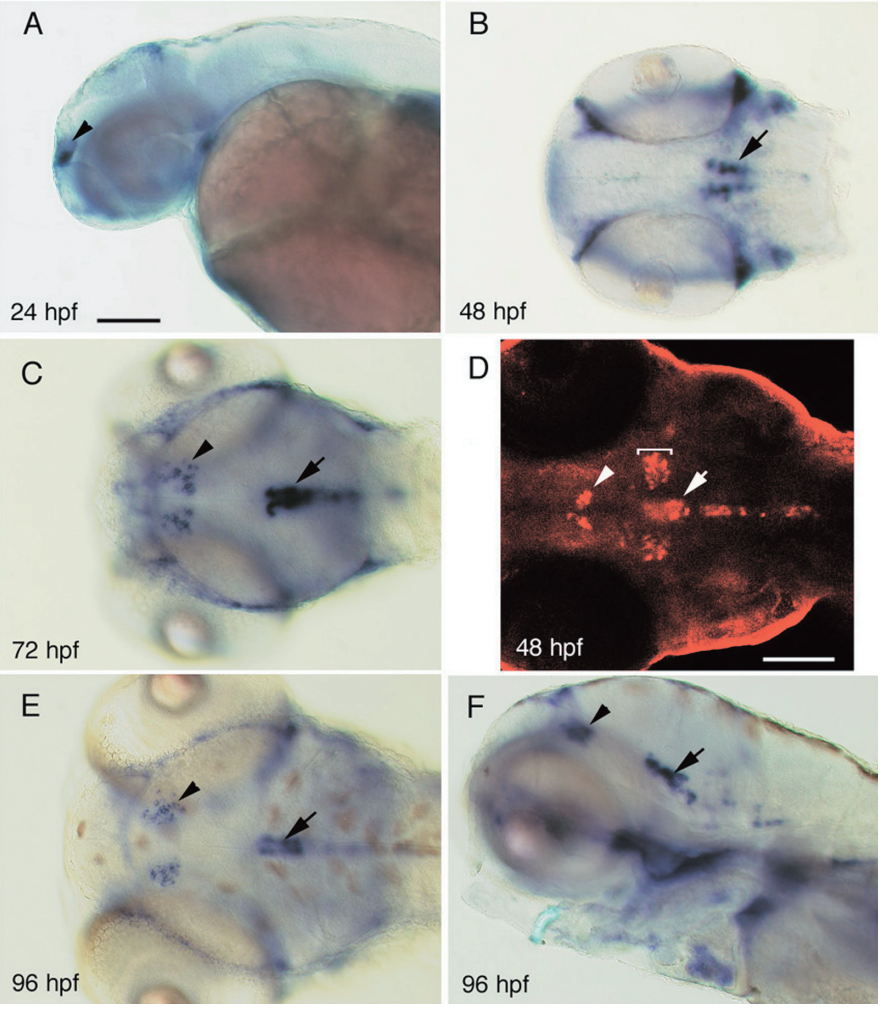

Fig. 2. *serta* expression during zebrafish embryogenesis. Side views (A and F) and dorsal views (B, C and E) of whole mount in situ hybridization using the *serta* probe and whole mount immunostaining using anti-serotonin antibody (D). (A) Twenty-four hpf embryo. The arrowhead indicates *serta* expression in pineal organ. The scale bar designates 100 μm. (B, D) Forty-eight hpf embryo. The arrows designate the raphe nuclei and the arrowhead indicates the ventral posterior tuberculum. (C) Seventy-two hpf embryo. The arrowhead and arrow designate the ventral posterior tuberculum and raphe nuclei, respectively. (D) Whole mount immunostaining of serotonin for 48 hpf embryo. The arrowhead and arrow designate ventral posterior tuberculum and raphe nuclei, respectively. The brackets indicate the hypothalamus. Serotonergic neurons in hypothalamus had no expression of SERT at least until 96 hph, although serotonin was detected from 48 hpf. The scale bar designates 100 μm. (E, F) Dorsal (E) and side view (F) of 96 hpf embryo. The arrowhead and arrow designate ventral posterior tuberculum and raphe nuclei, respectively.

the midline of the ventral hindbrain from 24 hpf (Fig. 2D), where it persists at least until 96 hpf. These *serta*-positive neurons were thus classified as serotonin neurons in the raphe nuclei based on the expression pattern and localization of serotonin and tphR. The expression of *serta* was also observed in the cells of pineal organ in the vicinity of tphR-positive cells. TphR is expressed at the center of the pineal organ (Fig. 3A), probably consisting of the photoreceptor cells, while *serta* is expressed around tphR-positive cells in the pineal organ (Fig. 3B). In the pineal organ melatonin is synthesized via serotonin from tryptophan. In some of the cells in the pineal organ which express *serta*, serotonin might be used as a neurotransmitter. At 48 hpf expression of *serta* in the ventral posterior tuberculum (Fig. 2C) starts and the expression overlaps with the serotonin distribution (Fig. 2D).

Sertb was expressed in a inner nuclear layer *in retina* (Fig. 4A) and in the dorsal medulla oblongata (Fig. 4B). The expression of tphD1, one of the three tryptophan hydroxylases in zebrafish, was previously reported in the subset of the inner nuclear layer neurons (Bellipanni et al. 2002). Serotonin was detected in the medulla oblongata of zebrafish embryo (McLean and Fetcho 2004a). Given that both *serta* and *sertb* are functional, the sum of the expression pattern of two genes should reflect the cells harboring the

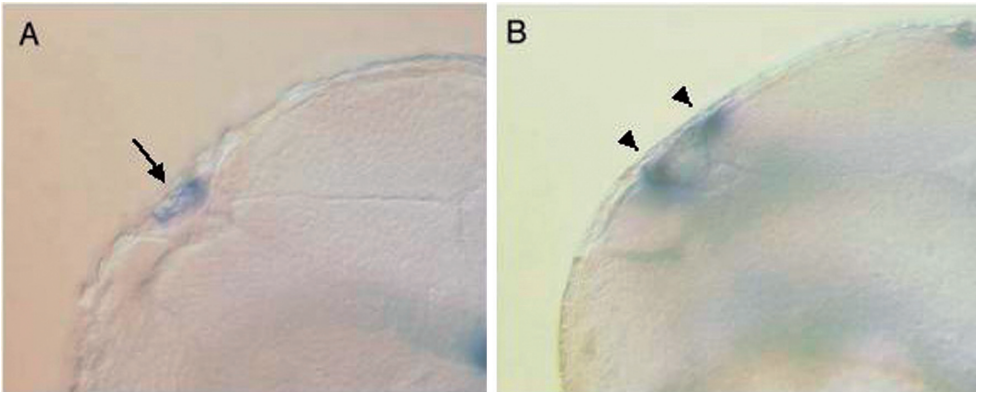

Fig. 3. *serta* expression in comparison to tphR. Side views of the pineal organ of whole mount in situ hybridization of 48 hpf embryo. (A) tphR was used as a probe. The neurons in the pineal organ were stained (arrow). (B) *serta* was used as a probe. The cells in the vicinity of tphR-positive cells were stained (arrow heads).

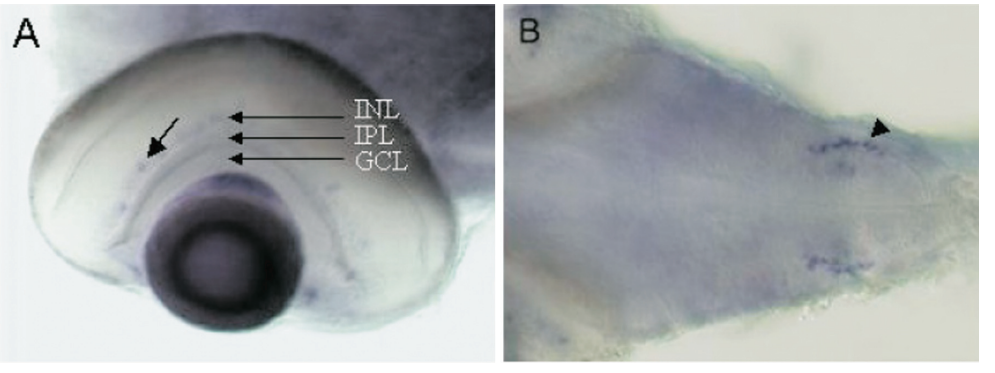

Fig. 4. *sertb* expression at 72 hpf zebrafish embryo. (A) A dorsal view of embryo of whole mount in situ hybridization. The cells in the inner nuclear layers were stained (arrow). INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (B) Dorsal view of hindbrain. The cluster of cells in the area postrema was stained.

serotonin transporter activity in zebrafish.

Serotonergic neurons in the hypothalamus have been reported in mammals (Lebrand et al. 1996) and also in zebrafish (Bellipanni et al. 2002; McLean and Fetcho 2004a). In mammals those neurons do not have serotonin synthesis activity rather they borrow serotonin through serotonin uptake from other serotonergic neurons, and these neurons are tryptophan hydroxylase-negative but SERT-positive. In zebrafish, serotonergic neurons in hypothalamus express tryptophan hydroxylase in contrast to the mammalian hypothalamus (Bellipanni et al. 2002). We found that these neurons do not express the SERT gene.

Transport assay

To verify the authenticity of the putative *serta* cDNA, full-length cDNA was transfected into HEK 293 cells and then were assessed for serotonin transport activity. The cells transfected with the transporter cDNA reveal a specific [^{3}H]-5HT uptake activity while the mock-transfected cells did not show any activity (data not shown). These observations are consistent with a lack of endogenous 5-HT transport activity in HEK 293 cells and further strengthen the notion that the observed activity is rendered by the heterologous expression of the serotonin transporter cDNA. A kinetic analysis revealed the initial velocity of the [^{3}H]-5HT uptake to be a saturating function of the ligand concentration (Fig. 5A) which is characterized by the Michaelis–Menten parameter: K_m= 4.2 ± 0.74 μM. These findings clearly indicate that the *serta* cDNA encodes the 5-HT transporter.

We next tested whether the 5-HT uptake activity observed in the transfected HEK 293 cells is sensitive to fluvoxamine, an inhibitor of serotonin transport, which is used as an antidepressant. An increasing amount of fluvoxamine inhibited serotonin transport activity with about IC_{50} = 183.4 nM (164.4 to 204.4 nM in 95% confidence intervals)(Fig. 5B) which is comparable to those of human and bovine SERT (Mortensen et al. 2001). These stable transfected cell lines are an excellent tool for comparing the species-difference of the kinetics, inhibitor sensitivities, ligand binding affinities and to identify the critical regions for inhibitor binding. The application of fluvoxamine to the zebrafish embryo at equal or higher concentrations than IC_{50} may thus provide useful information for evaluating the effect of the serotonin reuptake inhibitor on the development of the nervous system.

The spatio-temporal distribution of serotonin-positive clusters has been reported in the brain of zebrafish embryo (McLean and Fetcho 2004a). In the present study zSERT-positive clusters were detected in epiphysis, ventral posterior tuberculum, raphe nuclei and area postrema in medulla oblongata in agreement with tphR

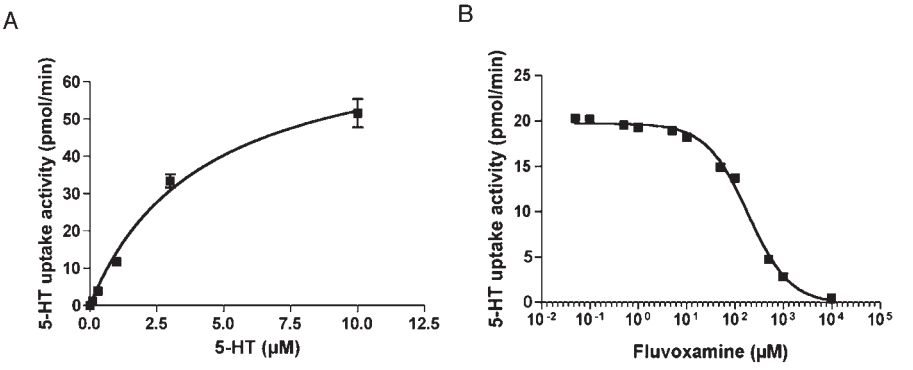

Fig. 5. Serotonin transporter activity measurement. (A) The stably transfected HEK293 cells were incubated with increasing concentrations of [^{3}-H]Serotonin for 10 min at room temperature. The non-specific serotonin uptake was determined by a parallel transfection with a pBK-CMV vector. (B) [^{3}H]-serotonin (25 nM) was incubated with increasing concentrations of inhibitor and incubated for 10 min at room temperature. The assays were carried out in six wells.

expression and 5-HT distribution in zebrafish embryos and larvae except for serotonergic neurons in the hypothalamus where the expression of SERT was not detected by in situ hybridization. As a result, *serta* and *sertb* cDNA bear significant homology with the rat and human serotonin transporters previously reported. The stable expression of the *serta* cDNA reveals serotonin transport activity from serotonin uptake studies. Since the blockade of serotonin transport is intimately linked to the alleviation of depression and associated disorders, and it may mediate some of the neurophysiological and behavioral effects of substance addiction, such as those associated with cocaine, the zSERT cDNA should thus facilitate the performance of analyses of the physiological significance of this transport system in the etiology of disorders using the zebrafish as a model.

Acknowledgments

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