Hedgehog and Fgf Signaling Pathways Regulate the Development of *tphR*-Expressing Serotonergic Raphe Neurons in Zebrafish Embryos

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ABSTRACT: **Serotonin (5HT) plays major roles in the physiological regulation of many behavioral processes, including sleep, feeding, and mood, but the genetic mechanisms by which serotonergic neurons arise during development are poorly understood. In the present study, we have investigated the development of serotonergic neurons in the zebrafish. Neurons exhibiting 5HT-immunoreactivity (5HT-IR) are detected from 45 h postfertilization (hpf) in the ventral hindbrain raphe, the hypothalamus, pineal organ, and pretectal area. Tryptophan hydroxylases encode rate-limiting enzymes that function in the synthesis of 5HT. As part of this study, we cloned and analyzed a novel zebrafish *tph* gene named *tphR*. Unlike two other zebrafish *tph* genes (*tphD1* and *tphD2*), *tphR* is expressed in serotonergic raphe neurons, similar to *tph* genes in mammalian species. *tphR* is also expressed in the pineal organ where it is likely to be involved in the pathway leading to synthesis**

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of melatonin. To better understand the signaling pathways involved in the induction of the serotonergic phenotype, we analyzed *tphR* expression and 5HT-IR in embryos in which either Hh or Fgf signals are abrogated. Hindbrain 5HT neurons are severely reduced in mutants lacking activity of either Ace/Fgf8 or the transcription factor Noi/Pax2.1, which regulates expression of *ace/fgf8*, and probably other genes encoding signaling proteins. Similarly, serotonergic raphe neurons are absent in embryos lacking Hh activity confirming a conserved role for Hh signals in the induction of these cells. Conversely, over-activation of the Hh pathway increases the number of serotonergic neurons. As in mammals, our results are consistent with the transcription factors Nk2.2 and Gata3 acting downstream of Hh activity in the development of serotonergic raphe neurons. Our results show that the pathways involved in induction of hindbrain serotonergic neurons are likely to be conserved in all vertebrates and help establish the zebrafish as a model system to study this important neuronal class.
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INTRODUCTION

Serotonin (5HT) is an evolutionarily conserved neurotransmitter that has essential roles in numerous physiological functions, and serotonergic defects are implicated in many disorders in both invertebrates and vertebrates (Baumgarten and Grozdanovic, 1997). For instance, serotonergic neurons are implicated in cardiovascular and respiratory control, regulation of food intake, circadian rhythms, and the sleep cycle. Impaired function of 5HT neurons has been related to a number of neurological disorders including depression, aggression, schizophrenia, and anxiety (see for example, Hendricks et al., 2003). Furthermore, extensive degeneration of 5HT neurons and axonal terminals may occur following intake of methamphetamine and in Alzheimer's and Parkinson's disease (Gibb et al., 1994; Onodera et al., 1994). Together with various possible developmental roles for 5HT (Lauder, 1993; Whitaker-Azmitia et al., 1996), these observations highlight the importance of this neurotransmitter for nervous system development and function.

Despite the clinical and developmental significance of 5HT, the mechanisms by which 5HT neurons are produced and maintained during development are poorly understood, particularly compared to dopaminergic or adrenergic neurons (Goridis and Rohrer, 2002). The effects of several trophic factors on neurogenesis of 5HT neurons have been investigated using organ cultures of rodent brainstem. For instance, BDNF can increase numbers of tryptophan hydroxylase (Tph)-immunoreactive neurons as well as 5HT uptake ability (White et al., 1994; Mamounas et al., 1995). However, these and other related studies have not identified the factors required for the specification of 5HT neurons.

Based upon the location of 5HT neuronal clusters in the central nervous system (CNS), Rosenthal and colleagues have proposed that diffusible factors emanating from the midline and midbrain-hindbrain boundary (MHB) mediate serotonergic neuron development (Hynes et al., 1995; Ye et al., 1998). Candidates for such signals include Sonic hedgehog (Shh) emanating from the midline, Fgf8 emanating from the MHB (Irving and Mason, 2000), and Fgf4 from underlying tissues (Ye et al., 1998). In support of a role for Shh, 5HT neurons are reduced in numbers in mice lacking function of one of the Gli family transcriptional effectors of Hh signaling (Matise et al., 1998).

Several transcription factors are known to mediate the specification of 5HT neurons downstream of the signals that induce these cells. The homeodomain protein Nk2.2 is regulated by Hh activity (e.g., Barth and Wilson, 1995) and is probably expressed in precursors of the 5HT cells and is required for development of caudal 5HT neurons (Briscoe et al., 1999; Pattyn et al., 2003). The GATA family transcription factor Gata3 is likewise expressed in most nascent 5HT neurons and is required for development of at least some 5HT neurons (van Doorninck et al., 1999). Whereas Gli proteins Nk2.2 and Gata3 are widely expressed in the CNS, the Ets family transcription factor Pet1 shows expression tightly localized to serotonergic neurons (Fyodorov et al., 1998; Hendricks et al., 1999). Indeed, the majority of 5HT neurons fail to differentiate in mice lacking Pet1 function, confirming a key role for this regulatory protein (Hendricks et al., 2003).

Tphs are rate-limiting enzymes that catalyze the hydroxylation of tryptophan to 5HT. They also function in the biosynthesis of melatonin from serotonin in the pineal gland. In mammals, *tph* is expressed at high levels within the pineal gland and in 5HT-containing neurons of the raphe nuclei in the brainstem (Darmon et al., 1988; Stoll et al., 1990; Kim et al., 1991). In humans, alternative splicing of the *tph* gene in both 3′ and 5′ regions produces a variety of *tph* mRNA species (Boularand et al., 1995; Wang et al., 1998). The absence of expression of known *tph* genes in serotonergic neurons of the mammalian hypothalamus sug-

gests that these neurons may accumulate serotonin by active uptake mechanisms rather than through synthesis of the neurotransmitter themselves (Lebrand et al., 1996; Vanhatalo and Soinila, 1998). However, the recent identification of two *tph* genes with hypothalamic expression in fish (Bellipanni et al., 2002; this study) indicates that at least in this species, some diencephalic serotonergic neurons can synthesize 5HT.

The zebrafish (*Danio rerio*) is an excellent model system in which to study the genetic basis of neuronal development *in vivo*. Thousands of mutations with phenotypes affecting embryogenesis have been isolated, many of which affect nervous system development (Brand et al., 1996). Indeed, several forward genetic screens for mutations affecting specific classes of neuron have been undertaken and have identified novel gene functions (e.g., Guo et al., 1999). Furthermore, recent advances in techniques to abrogate function of known genes (Nasevicius and Ekker, 2000; Wienholds et al., 2002) further enhance the tractability of this species for studying gene function during CNS development. As many neuronal populations are conserved both in terms of their development and their function, then it is likely that the zebrafish will continue to grow in popularity as a model system to investigate issues related to human nervous system development, function, and disease.

Here, we study the early development of serotonergic neurons in wild-type and mutant zebrafish embryos, particularly focusing upon the raphe region, which is the major nucleus of 5HT neurons in mammals. To facilitate these studies, we cloned a third zebrafish *tph* gene named *tphR* (for raphe *tph*). In contrast to the two diencephalic *tph* genes, *tphD1* and *tphD2* (Bellipanni et al., 2002), *tphR* is expressed in raphe serotonergic neurons. We show that these neurons are severely depleted in the absence of Fgf8/Ace activity and are absent altogether in embryos lacking Hh activity. Our studies confirm an *in vivo* requirement of Fgf and Hh signals for the development of the raphe serotonergic neurons.

METHODS

Zebrafish

Fertilized eggs were obtained from natural mating of adult zebrafish bred according to the procedures described in Westerfield (1993). Adult and juvenile fish were maintained at 28.5°C under 14 h light and 10 h dark cycle. Adult carriers of *sonic you* (*syu*^{bx392}), *slow-muscle-omitted* (*smu*^{b641}), *you* (*you*^{ty97}), *acerebellar* (*ace*^{ti282}), and *no isthmus* (*noi*^{tu29a}) as well as other mutants were maintained and crossed to obtain homozygous mutant embryos. Transgenic *islet1*:GFP fish were used to localize the positions of cranial motor nuclei (Higashijima et al., 2000). 1-Phenyl-2-thiourea (PTU) was sometimes included in the water to prevent pigmentation. Stages of embryos/fry were determined as described by Schilling and Kimmel (1997).

Antibody Staining

Whole-mount antibody staining was carried out according to Wilson et al. (1990). Four percent paraformaldehyde (PFA)-fixed embryos were treated with 0.25% trypsin (Gibco) on ice in PBS for 15 min, and rinsed with PBS containing 0.5% Triton X-100 and 1% dimethylsulfoxide (PBTR). After blocking with 1% normal horse serum in PBTR, embryos were incubated overnight with rat anti-5HT monoclonal antibody (1:200; Chemicon). After washing in PBTR for several hours, embryos were treated with biotin-conjugated anti-rat IgG antibody (1:1000; Sigma), followed by overnight incubation with A-B solution provided by the Vectastatin Elite kit (Vector Laboratories, Ltd.). Color reaction was performed with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. After PFA fixation, the stained embryos were cleared in 70% glycerol and mounted for observation. Nomenclature of 5HT-immunoreactive neurons is according to adult zebrafish (Kaslin and Panula, 2001). For antibody staining after *tphR in situ* hybridization, mouse anti-opsin primary (1:1000; kindly provided by Paul Hargrave) and goat anti-mouse IgG secondary (1:200; Life-Tech) antibodies were used, followed by DAB color reaction. Anti-GFP monoclonal antibody (1:1000; AMS Biotech) and 39.4D5 islet monoclonal antibody (1:500; Developmental Studies Hybridoma Bank) were also used as primary antibodies.

cDNA Cloning and Sequencing

Zebrafish *tph* gene fragments were amplified by RT-PCR from brain RNA of adult fish using degenerate primers (forward: GAYCAYCCNGGNTTYAARGA; reverse: YTCRTGRCANGTRTCIGGYT). Two fragments were obtained and subcloned into T-vector (Promega). Three-prime and 5′ end sequences were obtained using RACE kits (Wako, Japan). cDNA sequences were determined using an automatic sequencer (310; ABI). Phylogenetic analysis was performed with Clustal from the DDBJ website (DNA Data Bank of Japan: http://www.ddbj.nig.ac.jp/E-mail/homology.html). One further sequence with homology to *tph* was obtained by RT-PCR with primers (forward: AGATCCCATACCACACGTAGAG; reverse: CGGTTCAGGAGTGTAAAGAGG) based upon genome sequence data in the Zebrafish Genome Database at the Sanger Institute (NA34579.1-1572).

In Situ Hybridization

Whole-mount *in situ* hybridization was carried out according to Barth and Wilson (1995). PFA-fixed embryos were

treated with proteinase K (Sigma) in PBS with 0.1% Tween.[20] After incubation with hybridization buffer containing 50% formamide, 5 X SSC, 2 mg/mL Torula RNA, and 200 μg/mL heparin (prehybridization), embryos were hybridized overnight with antisense probes diluted in hybridization buffer at 65°C. Following hybridization, embryos were washed with 2 X SSC and 0.2 X SSC for 30 min twice at 65°C, respectively. After blocking with a buffer containing 2% blocking reagent (Roche) in 100 m*M* maleic acid (pH 7.5) and 150 m*M* NaCl, embryos were incubated overnight in alkaline phosphatase conjugated anti-DIG antibody (1:4000; Roche) at 4°C. The color reaction was carried out by incubation in BM-purple substrate (Roche) after equilibrating with NTMT buffer (100 m*M* NaCl, 100 m*M* Tris-HCl pH 9.5, 50 m*M* $MgCl_2$) at room temperature for 15 min. Some stained embryos were embedded in paraffin and sectioned. For double *in situ* hybridization, the fluorescein labeled riboprobe was revealed with alkaline phosphatase conjugated antifluorescein antibody (1:2000) using a fast red color reaction substrate (Roche). Alkaline phosphatase was subsequently inactivated with 100 m*M* glycine buffer (pH 2.2) and embryos were then incubated with anti-DIG antibody as described above.

SU5402 and Cyclopamine Treatment

Dechorionated 24 h postfertilization (hpf) embryos were incubated in the dark in 9 μM SU5402 (Calbiochem) or 100 μM cyclopamine (Toronto Research Chemicals) until 48 hpf for *in situ* hybridization and 60 hpf for 5HT antibody staining. The embryos were rinsed three times in fish water and fixed immediately for *in situ* hybridization or antibody staining.

Misexpression Studies

For over-expression of Shh, capped mRNA (≈50 ng/embryo) was injected at the one or two cell stage using a fine glass needle connected to an automatic injector (IM 300 Microinjector; Narishige), as described previously (Barth and Wilson, 1995). Injected embryos were allowed to develop at 28.5°C and were used for whole-mount *in situ* hybridization or antibody staining.

RESULTS

Cloning of the Zebrafish *tphR* Gene

To identify cells that produce 5HT in the zebrafish CNS, we cloned and analyzed the expression of genes encoding Tph, the rate-limiting enzyme for 5HT production. Two different 426 bp fragments were obtained by degenerate RT-PCR, one of which was identical with *tphD1* isolated independently by Laure Bally-Cuif's group (Bellipanni et al., 2002). The second encoded a fragment of a gene we named *tphR* (raphe Tph) due to its expression (see below). The remaining coding sequence of *tphR* was isolated by 3′- and 5′-RACE procedures. Analysis of the Zebrafish Genome Database revealed one further *tph*-like sequence (NA34579.1-1572) different from *tphD1*, *tphD2*, and *tphR*. A 331 bp clone was obtained using RT-PCR with cDNA from 48 hpf embryos. However, with a probe made from this sequence, we were unable to detect expression by *in situ* hybridization in 48 hpf embryos (data not shown).

The complete cDNA sequence of *tphR* is 2216 bp encoding a protein of 473 amino acids. Alignment analysis showed high homology of TphR with Tphs from various species, as well as the other zebrafish Tphs (Fig. 1). The most conserved regions of *tphR* show 72 and 82% identity at the nucleotide and deduced amino acid sequence level, respectively, when compared to human *tph* [Fig. 1(A)]. Phylogenetic tree analysis of deduced amino acid sequences of Tph proteins suggests TphR occupies a position separate from other vertebrate and invertebrate Tph proteins [Fig. 1(B)]. In contrast, both TphD1 and TphD2 (for which sequence is still missing) fall within the same cluster as other vertebrate Tphs [Fig. 1(B) and data not shown].

tphR Is Expressed in Serotonergic Raphe Neurons and in the Pineal Organ

From about 35 hpf, *tphR* is expressed in bilateral longitudinal columns in the anterior hindbrain [Fig. 2(A,B)], which correspond to clusters of raphe neurons showing 5HT-immunoreactivity [5HT-IR; Fig. 2(C,D)]. Whereas serotonergic raphe cells in the adult zebrafish are arranged into dorsal (B6–B7) and ventral clusters (B1–B2; Kaslin and Panula, 2001), during larval stages these neurons instead form rostral and caudal clusters [Fig. 2(C,D)]. Adult zebrafish show an additional third small cluster of 5HT-IR neurons in the vagal lobe of the rhombencephalon. These cells are not detected by 5HT-IR or by *tphR* expression up to at least 4 dpf.

Hindbrain *tphR* expression is located just lateral to the *shh*-expressing floor plate and is within or adjacent to the *nk2.2*-expressing neuroepithelial domains of the ventral neural tube [Fig. 2(E,F) and data not shown; Barth and Wilson, 1995]. The anterior group of *tphR*-expressing cells is located in rhombomere 0/1, directly caudal to the trochlear nucleus and rostral to the trigeminal motor nucleus [Fig. 2(G,H); Chandrasekhar et al., 1997; Higashijima et al., 2000]. Caudal *tphR* expression occurred throughout more caudal rhombomeres. Overall, the pattern of *tphR* expression is highly reminiscent of that seen for *tph* genes in

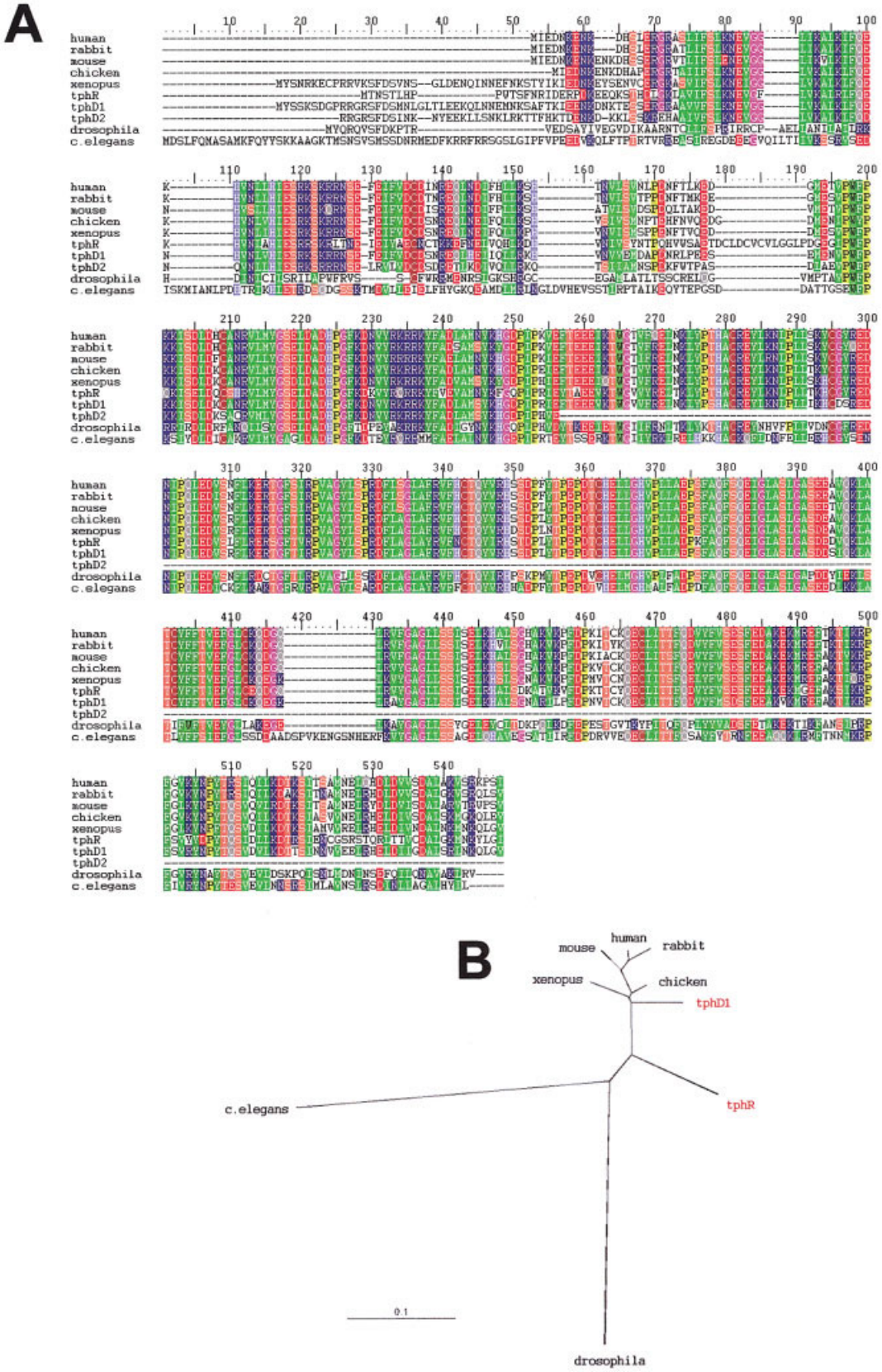

Figure 1 *tphR* is a novel tryptophan hydroxylase encoding gene. Amino acid sequence alignment (A) and phylogenetic tree (B) of TphR with tryptophan hydroxylase sequences of other species recorded in Genbank. Human (X52836), mouse (J04758), rabbit (L29305), chicken (U26428), *Xenopus* (L20679), zebrafish tphR (AB125219), zebrafish tphD1 (AF548566), zebrafish tphD2 (EST fl56d09.y1), *Drosophila* (PAH/TPH: M81833), and *C. elegans* (AF135186). The different colors in (A) highlight conserved amino acids.

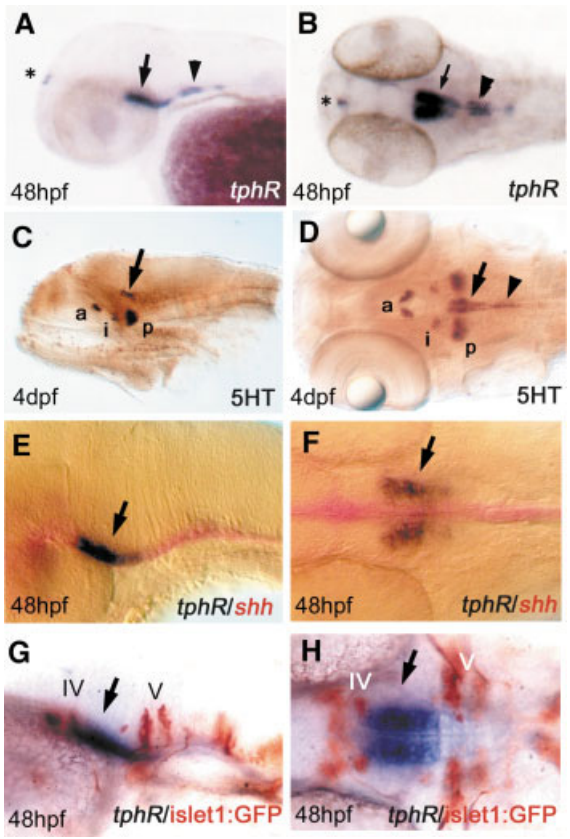

Figure 2 *tphR* is expressed in serotonergic raphe neurons. Lateral (A,C,E,G) and dorsal (B,D,F,H) views of brains of embryos labeled to detect the expression of genes or proteins (indicated bottom right). Typeface color matches reaction product color in double-labeled preparations. GFP-IR reveals cranial motor neurons expressing an Islet-1:GFP transgene (G,H). Age is indicated bottom left. Black arrows and arrowheads point to the anterior and posterior clusters of serotonergic raphe neurons. Asterisks indicate the pineal organ. Abbreviations: a, i, p, anterior, intermediate, and posterior regions of the paraventricular organ; IV, trochlear motor nucleus; V, trigeminal motor nucleus.

higher vertebrates (Briscoe et al., 1999; van Doorninck et al., 1999).

tphR expression is also detected in photoreceptor cells at the midline of the pineal organ [Fig. 3(A,B)]. The pineal organ shows relatively weak 5HT-IR in photoreceptor cells and strong expression in the pineal stalk, a feature that is maintained in the adult zebrafish brain [Fig. 3(G,H); Kaslin and Panula, 2001]. In contrast to 5HT-IR, *tphR* expression is restricted to opsin-immunoreactive photoreceptors and is absent from the pineal stalk [Fig. 3(A–D)]. The expression pattern of *tphR* in this region is very similar to that of *tphD1* [Fig. 3(E,F); Bellipanni et al., 2002] and *serotonin-N-acetyltransferase* (AANAT), which encodes one of the enzymes involved in synthesis of melatonin from 5HT (Gothilf et al., 1999).

In the hypothalamus, extensive and robust 5HT-IR is observed in three cell groups in the inferior lobe of the hypothalamus [Fig. 2(C,D); Bellipanni et al., 2002]. The most rostral of these corresponds to a cell group in the anterior part of the paraventricular organ. The other hypothalamic cell groups correspond to the intermediate and posterior serotonergic cell groups described in the adult paraventricular organ (Kaslin and Panula, 2001). Cells showing 5HT-IR in the diencephalon are in contact with the hypothalamic extension of the diencephalic third ventricle (data not shown). Besides these, a small cluster of serotonergic cells is also observed in the pretectal area (not shown). This cell group has also previously been described in the adult brain of zebrafish and other fish species (Ekstrom et al., 1985; Bolliet and Ali, 1992; Kaslin and Panula, 2001). However, *tphR* is not expressed in any of the three serotonergic cell clusters in

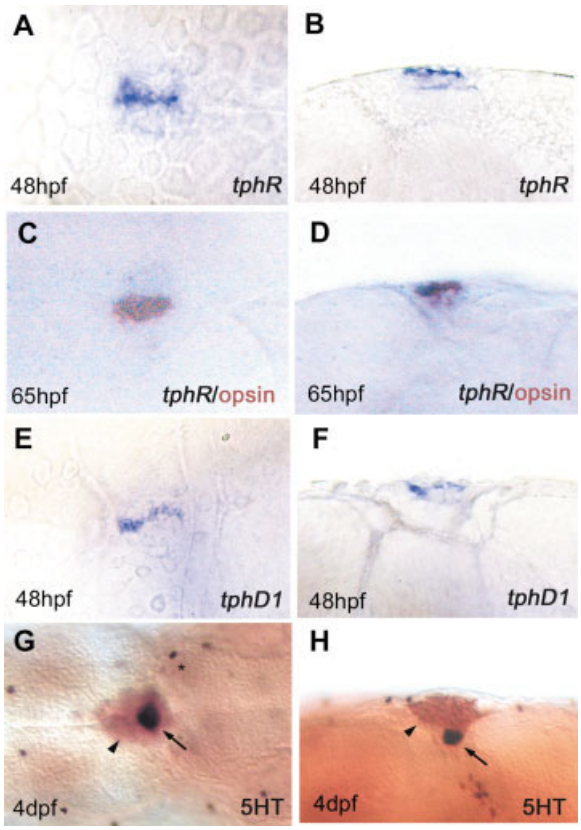

Figure 3 *tphR* is expressed in the pineal organ. Dorsal (A,C,E,G) and lateral (B,D,F,H) views of the epithalamus showing expression of *tphR*, Opsin-IR, *tphD1*, and 5HT-IR in the pineal organ. Age is indicated bottom left. The arrowhead indicates pineal organ cells and the arrow pineal stalk cells in (G) and (H). The asterisk indicates a 5HT-IR cell in the skin.

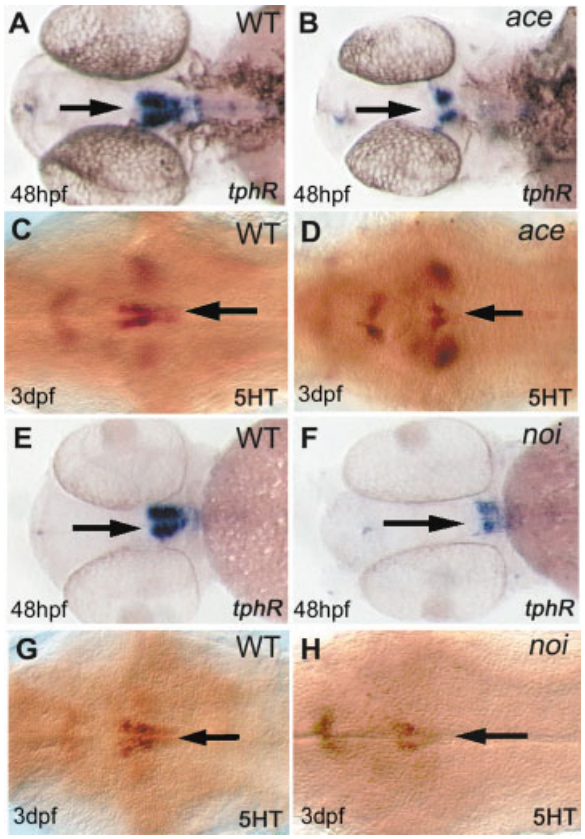

Figure 4 Ace (Fgf8) and Noi (Pax2.1) both promote development of serotonergic raphe neurons. Dorsal views of wild-type (wt), $ace^{-/-}$ (*ace*), and $noi^{-/-}$ (*noi*) embryos labeled to show *tphR* expression and 5HT immunoreactivity. Arrows point to serotonergic raphe neurons.

the hypothalamus or in the pretectal area up to at least 4 dpf. This is in contrast to *tphD1* and *tphD2*, which are both expressed in hypothalamus (Bellipanni et al., 2002).

The anti-5HT monoclonal antibody also labels unidentified cells in the skin [Fig. 3(G)]. These large round cells become immunoreactive at around the same stage as the cell groups showing 5HT-IR in the CNS.

Serotonergic Raphe Neurons Are Reduced in the Absence of Either Ace (Fgf8) or Noi (Pax2.1) Function

Zebrafish *acerebellar*$^{-/-}$ (*ace*) embryos lack functional Fgf8 due to a point mutation in the *ace* (*fgf8*) gene (Reifers et al., 1998). *ace* mutants show a significant reduction of hindbrain 5HT-IR neurons particularly in caudal regions [Fig. 4(C,D)]. Complementing this reduction, *tphR* expression in the hindbrain is also greatly reduced, again most notably in more caudal regions [Fig. 4(A,B)]. In contrast, 5HT-IR neurons in the diencephalon appear relatively unaffected in 48 to 65 hpf $ace^{-/-}$ embryos [Fig. 4(C,D) and data not shown].

The requirement of Fgf8 signaling could either be due to Fgf being required for the development of the territory within which serotonergic neurons are generated or due to Fgf mediating downstream events that lead to production of serotonergic neurons. To begin to address this issue, we incubated wild-type embryos from 24 hpf until they were fixed (after initial neuroepithelial patterning has occurred) in 9 or 21 μM SU5402 (Mohammadi et al., 1997) to inhibit Fgf receptor activity. At both concentrations of SU5402, embryos showed a reduction of both *tphR* expression and 5HT immunoreactivity in the raphe (Fig. 5). The phenotype was slightly weaker than in $ace^{-/-}$ embryos, suggesting that Fgfs are required both before and after 24 hpf for raphe development. In contrast to $ace^{-/-}$ embryos, 5HT immunoreactivity was also reduced in the intermediate and posterior serotonergic cell groups of the hypothalamus of SU5402-treated embryos (not shown), suggesting that Fgfs other than Fgf8 are required for the development of these cells.

In addition to modulating the timing of blocking Fgf activity, we examined the regional patterning of the rostral hindbrain of $ace^{-/-}$ mutants to determine if extensive deletion of tissue could account for the loss of serotonergic neurons. *ephA4* (Xu et al., 1995) is normally expressed in r1, r3, and r5, and three similar domains of expression are detected in $ace^{-/-}$ embryos [Fig. 6(A,B)]. This suggests that at least some rhombomeric regional character is retained in $ace^{-/-}$ mutants. Detailed fate mapping experiments have also

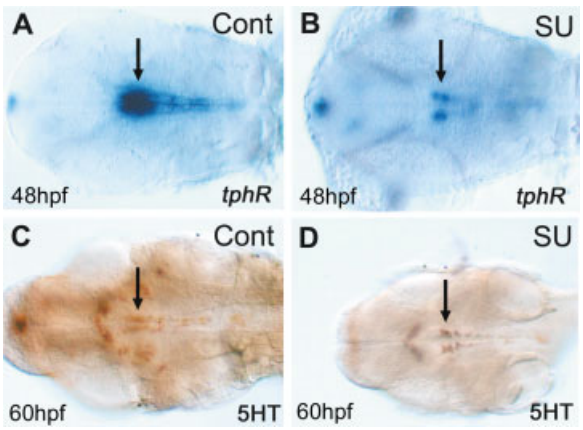

Figure 5 Inhibition of Fgf receptor activity leads to loss of serotonergic raphe neurons. Dorsal views of *tphR* expression and 5HT-IR in the brains of wild-type embryos treated with 9 μM SU5402 from 24 hpf to the stage indicated bottom left. Arrows point to serotonergic raphe neurons.

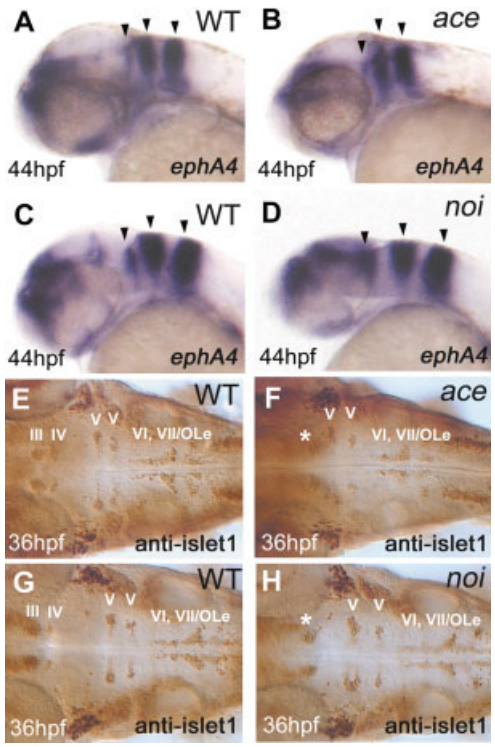

Figure 6 Rhombomeric patterning is still evident in embryo lacking Ace (Fgf8) and Noi (Pax2.1) function. Lateral (A–D) and dorsal (E–H) views of $ace^{-/-}$ (*ace*), $noi^{-/-}$ (*noi*), and sibling embryos (WT) labeled to show *ephA4* expression (A–D) and cranial motorneurons (E–H) revealed by expression of Islet1. Age is indicated bottom left. Arrowheads indicate *ephA4* expression in r1, r3, and r5 in (A–D). The asterisks indicate a fused nucleus that replaces nuclei III and IV in $ace^{-/-}$ and $noi^{-/-}$ embryos. Roman numerals refer to the identity of the cranial motor nuclei and OLe are otic and lateral line efferent neurons.

confirmed that CNS tissue is not deleted in $ace^{-/-}$ mutants, rather it is mis-patterned and fails to differentiate appropriately (Tallafuß and Bally-Cuif, 2003). This study also observed progressive alterations in regional patterning in $ace^{-/-}$ mutants that, together with our analysis and previous observations (Wilson et al., 2002), suggests that anterior hindbrain cells express combinations of both appropriate and inappropriate genes in the absence of Fgf8 activity. At later stages, the distance between midbrain somatic motor nuclei and the trigeminal motor nucleus is very compressed in $ace^{-/-}$ mutants, consistent with the severe neuronal defects in the region [Fig. 6(E,F); in our analysis, $ace^{-/-}$ mutants retained a reduced nucleus of oculomotor/trochlear neurons, whereas these cells are reported absent in the Tallafuß and Bally-Cuif study]. Although serotonergic neurons are absent from more caudal regions of the hindbrain of $ace^{-/-}$ mutants, branchiomotor neurons appear to form normally [Fig. 6(F)].

no isthmus (*noi*) is a mutation in the *pax2.1* gene that leads to reduced *fgf8* expression in the MHB area due to a requirement for Pax2 in the maintenance of *fgf8* expression (Reifers et al., 1998; Rhinn and Brand, 2001). Both 5HT-IR and *tphR* expression in the hindbrain are reduced in $noi^{-/-}$ embryos [Fig. 4(E–H)]. Similar to $ace^{-/-}$ mutants, the rhombomeric

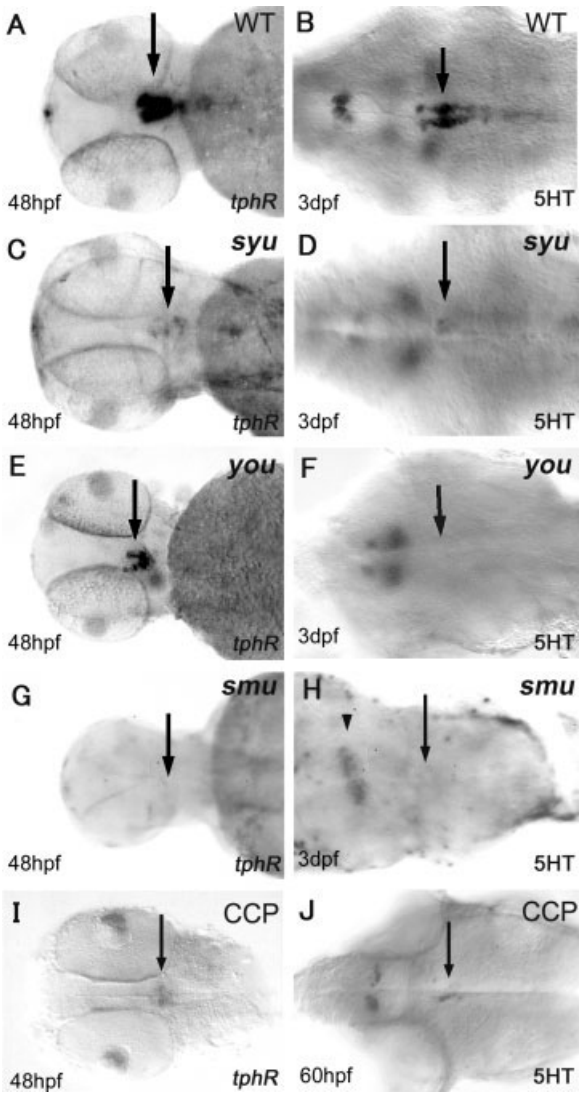

Figure 7 Hh signaling is required for the development of serotonergic raphe neurons. Dorsal views of brains of wild-type and mutant (indicated top right) embryos or wild-type embryos treated with 100 μM cyclopamine (CCP) from 24 hpf to the stage indicated bottom left labeled to detect the expression of genes or proteins (indicated bottom right). Black arrows point to serotonergic raphe neurons (or the position where they should be present) in (A–J), and the arrowhead points to anterior clusters of serotonergic neurons in the hypothalamus in (H).

stripes of *ephA4* expression are retained in *noi*$^{-/-}$ mutants and the oculomotor/trochlear nucleus is positioned closer to the trigeminal motor nucleus [Fig. 6(C,D,G,H)]. The presence of oculomotor/trochlear neurons again suggests that there is no major early deletion of tissue in *noi*$^{-/-}$ embryos.

Altogether, these results suggest that Fgf8 and Pax2.1 have complex roles in regulating both patterning of the anterior hindbrain and production of the serotonergic raphe neurons.

Hh Activity Is Required for Specification of Hindbrain 5HT Neurons

In vitro studies have shown that Shh can promote the development of serotonergic neurons in tissue explants (Ye et al., 1998) and so we assayed the development of these neurons in zebrafish embryos in which Hh activity is compromised.

sonic you$^{-/-}$ (*syu*) embryos lack Shh activity due to a mutation in the zebrafish *shh* gene (Schauerte et al., 1998), and although the mutation responsible for the *you*$^{-/-}$ phenotype has yet to be cloned, *you*$^{-/-}$ embryos show the U-shaped somites characteristic of embryos with reduced Hh activity (van Eeden et al., 1996). *syu*$^{-/-}$ embryos show a severe reduction and *you*$^{-/-}$ embryos show a milder and more variable reduction in *tphR* expression and 5HT-IR in hindbrain raphe neurons [Fig. 7(A–F)]. Both *syu*$^{-/-}$ and *you*$^{-/-}$ embryos maintain relatively normal 5HT-IR in the hypothalamus despite the reductions in hindbrain neurons.

The persistence of some hindbrain serotonergic cells in *syu*$^{-/-}$ embryos suggests either that Hh activity is not absolutely required for production of these neurons or that residual Hh activity is sufficient to specify the cells. *shh* is not the only Hh gene expressed in axial tissues in zebrafish and so some Hh activity mediated by other Hh proteins (Ekker et al., 1995; Currie and Ingham, 1996) is indeed likely to be present in the vicinity of the prospective raphe neurons in *syu*$^{-/-}$ embryos. We therefore analyzed the development of serotonergic neurons in embryos in which nearly all Hh activity is absent. *Slow-muscle-omitted* (*smu*) is a mutation in the zebrafish *smoothened* gene, which encodes a transmembrane protein essential for the transduction of all Hh signals (Chen et al., 2001; Varga et al., 2001; McMahon et al., 2003). At early stages, some residual Hh activity is present in *smu*$^{-/-}$ embryos due to the presence of maternally supplied Smu but all later Hh signaling in the brain and somites is likely to be completely abrogated in mutant embryos (Varga et al., 2001; Barressi et al., 2000). In support of this notion, *smu*$^{-/-}$ embryos exhibit a more severe phenotype than *syu*$^{-/-}$ embryos, lacking *tphR* expression and hindbrain serotonergic neurons [Fig. 7(G,H)]. Notably, unlike both *syu*$^{-/-}$ and *you*$^{-/-}$ embryos, *smu*$^{-/-}$ embryos also lacked diencephalic *tphD1* expression and most 5HT-IR intermediate and posterior paraventricular hypothalamic cell groups at least until 4 dpf [Fig. 7(H) and data not shown]. This is not due to an absence of the hypothalamic tissue, as dopaminergic neurons that colocalize with the diencephalic serotonergic neurons are still present in *smu*$^{-/-}$ embryos (Chen et al., 2001; Holzschuh et al., 2003; and data not shown).

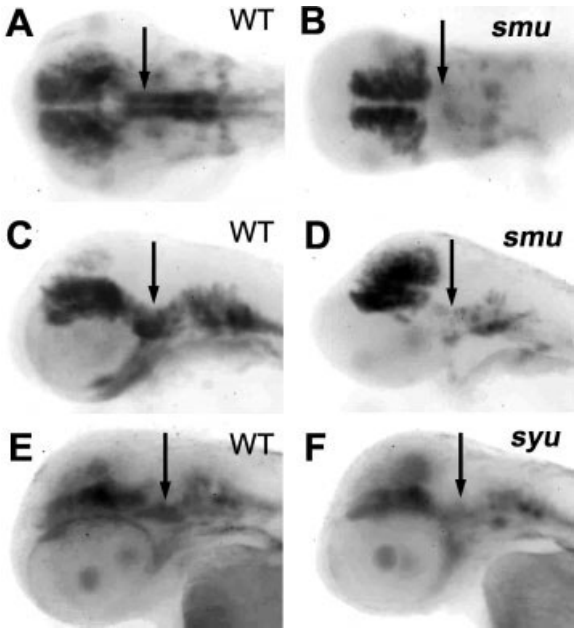

Figure 8 Requirement of Hh signals for *gata3* expression in the raphe region. Dorsal (A,B) and lateral (C–F) views of *gata3* expression in the brains of *smu*$^{-/-}$ (*smu*), *syu*$^{-/-}$ (*syu*), and sibling (wt) embryos at 48 hpf. Arrows indicate *gata3* expression in the raphe region.

The loss of serotonergic neurons in *smu*$^{-/-}$ mutants could be due to a requirement for Hh in the initial dorsoventral patterning of the neuroepithelium or due to a continuing role for Hh signaling in specifying neurons at later stages. To begin to address this issue, we incubated embryos from 24 hpf until they were fixed in 100 μM cyclopamine, which inhibits activity of the Smoothened protein (Incardona et al., 1998). These embryos showed a reduction of both *tphR* expression and 5HT immunoreactivity in the raphe [Fig. 7(I,J)], but not a complete absence as seen in *smu*$^{-/-}$ embryos. This suggests that Hh signaling promotes serotonergic neuron production both before and after 24 hpf.

Both 5HT-IR and *tphR* expression are markers of differentiated serotonergic neurons and so absence of

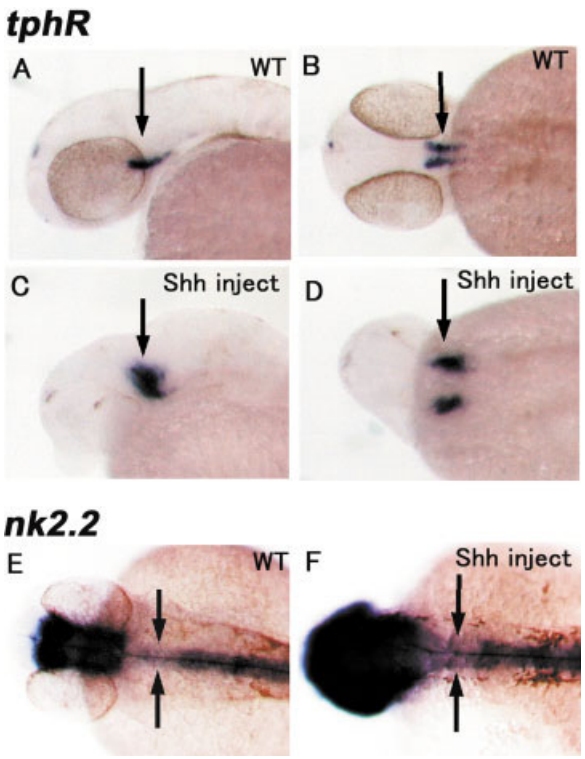

Figure 9 Exogenous Shh increases *tphR* expression. Lateral (A,C) and dorsal (B,D,E,F) views of *tphR* and *nk2.2* expression in the brains of 48 h wild-type and *shh*-injected embryos. Arrows in (A–D) indicate serotonergic raphe neurons and indicate the width of the medial expression domain of *nk2.2* in (E) and (F).

expression may indicate either an absence of neurons or a failure of the neurons to differentiate. We therefore analyzed expression of *gata3*, a transcription factor that is required for survival of at least some 5HT-IR neurons (van Doorninck et al., 1999). *gata3* transcripts are almost completely absent from the raphe region of $smu^{-/-}$ embryos [Fig. 8(A–D)] and are reduced in $syu^{-/-}$ embryos [Fig. 8(E,F)]. 5HT neurons arise from *nk2.2*-expressing precursors (Briscoe et al., 1999; Pattyn et al., 2003) and *nk2.2* transcripts are absent in the raphe region in $smu^{-/-}$ embryos and severely reduced in this region in $syu^{-/-}$ embryos (Varga et al., 2001; and data not shown). Altogether, these observations make it more likely that serotonergic neurons are absent in embryos lacking Hh activity than that they are present but fail to differentiate.

Exogenous Hh Activity Promotes Production of Serotonergic Neurons

To complement our analyses of serotonergic neuron production in situations of reduced Hh activity, we also assayed production of these cells in embryos with increased Hh activity. To do this, *shh* mRNA was injected into early blastomeres and its effect on production of serotonergic neurons was determined within the hindbrain at 48 hpf. *shh* overexpression led to a robust expansion of *tphR* and *nk2.2* expression within the hindbrain. Expression was absent from a wider midline domain but extended much further dorsally within the hindbrain neuroepithelium [Fig. 9(A–F)].

DISCUSSION

tphR Encodes a Novel Zebrafish Tph Protein

Here we report the cloning and expression pattern of a *tph* gene expressed in the raphe region of developing zebrafish. Together with *tphD1* and *tphD2* (Bellipanni et al., 2002), there are now three Tph-encoding genes in zebrafish (and a fourth *tph*-related sequence present in the genome sequence). It is interesting that the expression patterns of *tphR* and the two *tphD* genes are very different with *tphR* lacking hypothalamic expression and the *tphD* genes lacking expression in the raphe. This divergence in expression domains of paralogous genes is a common feature of likely duplicated genes in zebrafish (e.g., Rohr et al., 2001; Prince, 2002). It is thought that this divergence of expression contributes to the selective pressure to maintain both copies of duplicated genes within the genome (Force et al., 1999).

Phylogenetic tree analysis reveals that TphR occupies a position distant from the main clusters of vertebrate (and invertebrate) Tph proteins. Although *tphR* is expressed in the raphe region, which corresponds to the major site of *tph* expression in amniotes (Darmon et al., 1988; Stoll et al., 1990; Kim et al., 1991), it is *tphD1* and *tphD2* that are more closely related by sequence to other vertebrate *tph*s despite their divergent expression patterns (Bellipanni et al., 2003).

Expression of *tphR* mRNA precedes the development of 5HT–IR by about 10 h, and, as for *tph* genes in other species (e.g., Kim et al., 1991), they show a spatial distribution coincident with 5HT-IR. Within the pineal, the expression domains of *tphR* and *tphD1* overlap with AANAT-2, a gene that encodes the enzyme that converts 5HT into melatonin (Gothilf et al., 1999). Together, these data strongly suggest that *tphR* encodes a tryptophan hydroxylase involved in 5HT synthesis in the zebrafish raphe and pineal organ.

Some 5HT-IR Neurons May Not Synthesize 5HT

Expression of all known zebrafish *tph* genes is absent from the pretectal area and the pineal stalk, both sites of 5HT-IR. This suggests that there is another Tph enzyme in zebrafish and/or that serotonergic cells that do not express *tph* are able to take up 5HT from their environment. A 5HT uptake mechanism is suggested to occur in the dorsomedial nucleus of human hypothalamus where a small group of neurons contain 5HT but lack Tph immunoreactivity. The 5HT content of these neurons decreases after treatment with 5HT uptake inhibitors (Beaudet and Descarries, 1979; Vanhatalo and Soinila, 1998), which suggests that these neurons could take up 5HT from the cerebrospinal fluid. As 5HT cells within the pineal stalk and also the hypothalamus are near or in contact with the diencephalic ventricle, a similar uptake mechanism may be operating in zebrafish. It is also possible that 5HT produced in the pineal photoreceptors is directly taken up by the adjacent pineal stalk cells. Indeed, it appears that although *tphD* genes are expressed in the hypothalamus, the number of 5HT-IR cells may exceed the number of *tphD*- and *tphD2*-expressing cells in this region (Bellipanni et al., 2002).

Fgf Signals Are Required for Induction of Serotonergic Raphe Neurons

Analysis of experiments in which levels of Fgf activity were manipulated in mammalian brainstem cultures led Rosenthal and colleagues to suggest that Fgf activity is required for development of serotonergic neurons (Hynes et al., 1995; Ye et al., 1998). Our study provides *in vivo* confirmation of this hypothesis. The Rosenthal model suggests that Fgf8 from the MHB induces rostral but not caudal 5HT neurons (in combination with Hh signals and Fgf4). However, in our experiments, the absence of Fgf8 leads to complete absence of caudal 5HT neurons whereas a small number of serotonergic neurons still differentiate adjacent to the MHB. The retention of 5HT cells adjacent to the MHB is likely to be due to the presence of other secreted factors, including other Fgfs, from the MHB. For instance, Fgf3, Fgf17, and Fgf18 are all candidates for providing 5HT neuron-inducing signals from the MHB (Reifers et al., 2000; Meyers et al., 1998; Xu et al., 2000).

The complete loss of caudal serotonergic neurons in $ace^{-/-}$ embryos is not predicted by the Rosenthal model, which suggests a limited range of action of Fgf8 emanating from the MHB. One possibility is that neurons equivalent to the caudal serotonergic group of wild-type embryos are formed in $ace^{-/-}$ mutants but are displaced further rostrally than normal. However, detailed expression analyses (e.g., Reifers et al., 1998; Rhinn et al., 2003) have shown that *fgf8/ace* is transiently expressed throughout the anterior hindbrain and so the loss of caudal 5HT neurons could be explained by Fgf8 acting locally to promote the specification of all 5HT neuron precursors. If this were true, it would imply that Fgf signaling acts upon neuroepithelial cells long before they generate serotonergic neurons, perhaps in the regional patterning of the neuroepithelium. It is certainly true that some aspects of regional patterning are disrupted in $ace^{-/-}$ mutants (Wilson et al., 2002; Tallafuß and Bally-Cuif, 2003; Rhinn et al., 2003), but neuroepithelial tissue does not appear to be deleted (Tallafuß and Bally-Cuif, 2003) and at least some markers of appropriate rhombomeric identities are retained. Thus it is very unlikely that the serotonergic phenotype of $ace^{-/-}$ mutants is simply a result of absence of tissue within which the neurons are normally generated. Indeed, our experiments using a pharmacological inhibitor of Fgf receptor activity suggest a continued requirement for Fgf activity long after neural plate stage. It appears, then, that Fgf signals are likely to be required over a protracted period of time for the production of all serotonergic raphe neurons.

Hh Signals Are Required for Induction of Serotonergic Neurons

Our data show that abrogation of Hh activity through mutations in genes acting in the Hh pathway or through pharmacological blockade leads to loss of serotonergic raphe neurons. Loss of 5HT neurons in the raphe has been reported in mice lacking activity of Gli2, a direct downstream transcriptional mediator of Hh activity (Matise et al., 1998). Similarly, antibody blockade of Hh activity in rat tissue explants prevents formation of serotonergic neurons (Ye et al., 1998). Altogether, these data reveal a conserved essential requirement for Hh activity in the formation of serotonergic raphe neurons.

Perhaps surprisingly, one of the major roles for Hh activity in the promotion of ventral neural fates is to antagonize Gli3-mediated repression of these fates (Litingtung and Chiang, 2000; Persson et al., 2002; Rallu et al., 2002). Thus ventral cell fates are restored in the absence of Shh activity when Gli3 function is also removed. These results imply that the role of Hh activity is not to actively specify ventral cell identity but rather to relieve the suppression of ventral cell identity by dorsal repressors. Indeed, recent studies have identified several putative transcriptional repres-

sors of serotonergic neuronal fate (Pattyn et al., 2003). To our knowledge, it has not yet been addressed if serotonergic neurons are recovered in *shh;gli3* double mutant mice. However, our preliminary observations of fish embryos completely lacking Nodal activity (unpublished observations) suggest that at least some 5HT neurons can form in the likely complete absence of axially derived Hh signals. This raises the possibility that in certain conditions where the activity of other patterning genes is affected, 5HT raphe neurons may not require Hh signals for their formation.

Complementing the loss of function studies, overexpression of *shh* mRNA dorsally expands *tphR* expression. This expansion is restricted to the anteroposterior level of the CNS at which 5HT neurons normally form despite the ubiquitous Hh activity. This observation adds further weight to the notion that Hh signals must act in combination with regionally localized signals, such as Fgf8, to induce the serotonergic phenotype.

In mammals, Nk2.2 (Briscoe et al., 1999; Pattyn et al., 2003) and Gata3 (van Doorninck et al., 1999) are thought to function in serotonergic neurons or their precursors downstream of the signals that induce these cells. Our data are consistent with equivalent roles for these proteins in fish. *tphR*-expressing cells appear to arise adjacent to *nk2.2*-expressing precursor cells, and loss of *tphR* expression and 5HT neurons in Hh pathway mutants correlate with loss of *nk2.2* expression. *gata3* is expressed in the raphe region prior to differentiation of 5HT neurons (Neave et al., 1995) and colocalizes with *tphR* expression. Similar to *nk2.2*, reduced Hh activity leads to reduced *gata3* expression. Together, these data are consistent with the notion that equivalent transcriptional cascades mediate induction and differentiation of serotonergic raphe neurons in all vertebrates.

CONCLUSIONS

In conclusion, our data provide evidence of an *in vivo* requirement for Hh and Fgf signals in the specification of serotonergic raphe neurons. We suggest that the signals and transcription factors that regulate the development of these neurons are highly likely to be conserved across all vertebrates. We believe that the zebrafish is an attractive model system in which to further analyze the regulatory pathways that induce and maintain these clinically important neurons.

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