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The Serotonergic Phenotype Is Acquired by Converging Genetic Mechanisms Within the Zebrafish Central Nervous System

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To gain knowledge about the developmental origin of serotonergic precursors and the regulatory cascades of serotonergic differentiation in vertebrates, we determined the spatiotemporal expression profile of the Ets-domain transcription factor-encoding gene *pet1* in developing and adult zebrafish. We show that it is an early, specific marker of raphe serotonergic neurons, but not of other serotonergic populations. We then use *pet1* expression together with tracing techniques to demonstrate that serotonergic neurons of rhombomeres (r) 1–2 largely originate from a progenitor pool at the midbrain–hindbrain boundary. Furthermore, by combining expression analyses of *pet1* and the raphe tryptophan hydroxylase (Tph2) with rhombomere identity markers, we show that anterior and posterior hindbrain clusters of serotonergic precursors are separated by r3, rather than r4 as in other vertebrates. Our findings establish the origin of r1–2 serotonergic precursors, and strengthen the evidence for molecular, ontogenic and phylogenic heterogeneities among the vertebrate brain serotonergic cell populations. *Developmental Dynamics 236: 1072–1084, 2007.* © 2007 Wiley-Liss, Inc.

Key words: *pet1*; *tph2*; serotonin; zebrafish; raphe

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INTRODUCTION

Serotonin (5HT) is a monoamine neurotransmitter involved in a wide range of behaviors and physiological processes. Accordingly, dysfunction of the serotonergic neurons in the anterior raphe, the main source of 5HT in the mammalian brain, has been implicated in several psychiatric diseases, including affective disorders, schizophrenia, abnormal anxiety, and addiction to psychostimulant drugs (reviewed in Lieberman et al., 1998; Lucki, 1998). Several observations suggest that 5HT might also have developmental functions by controlling embryonic growth, neuronal differentiation, neurite growth, synaptogenesis, and the migration of cells (Bailey et al., 1992; reviewed in Vitalis and Parnavelas, 2003; Fricker et al., 2005; Côté et al., 2007) as well as growth cone navigation (Zhou and Cohan, 2001; reviewed in Gaspar et al., 2003). In addition, recent studies in mammals have highlighted a role of the serotonergic system in neurogenesis (Brezun and Daszuta, 1999; Santarelli et al., 2003; reviewed in Djavadian, 2004).

In mammals, serotonergic innervation of the central nervous system (CNS) originates from two main clusters: the anterior and the posterior raphe. These clusters can be further subdivided into nine nuclei, B1 to B9,

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where B4–B9 project to more anterior brain areas and correspond to the anterior raphe complex (dorsal, median, and pontis raphe), while B1–B3 form the posterior raphe and project to the spinal cord (reviewed in Cordes, 2005). Ontogenetically, based on the expression of either 5HT, the 5HT-synthesizing enzyme tryptophan hydroxylase (Tph) or the 5HT transporter (Sert), it has been observed that the earliest serotonergic neurons are born in anterior rhombomeres, and it was suggested that they give rise to the anterior raphe (Lidov and Molliver, 1982; Aitken and Törk, 1988; Hansson et al., 1998). Among these, neurons forming the most dorsal cluster (B9, dorsal raphe) are interpreted to originate from rhombomere 1 (r1; Cordes, 2005). In contrast, the posterior raphe is believed to derive from posterior rhombomeres at a slightly later stage. A similar organization of the mature and developing raphe serotonergic system has been described in lower vertebrates such as teleosts, although fewer subnuclei are recognized at the adult stage (B6–B9 for the anterior raphe, B1–B2 for the posterior raphe; Kah and Chambolle, 1983; Ekström and Van Veen, 1984; Ekström et al., 1985; Kaslin and Panula, 2001). At early postembryonic stages in zebrafish, all caudal projections can be traced back to the caudal hindbrain cluster, suggesting that it is the origin of the posterior raphe (McLean and Fetcho, 2004a,b). The exact hindbrain localization and developmental origin of the presumptive anterior raphe neurons in zebrafish, as well as the regulatory steps leading to the development of the raphe serotonergic phenotype, have not been determined and are the subjects of the present study.

To address these issues, we first aimed to characterize an early and specific marker of presumptive raphe neurons in zebrafish. Despite the fundamental role of central serotonergic neurons, our knowledge about their developmental specification remains fragmentary, and few selective markers or processes have been found. In mammals and chicken, the generation of rostral hindbrain 5HT neurons depends on both the midline signal Sonic hedgehog (Shh), the isthmic organizer signal fibroblast growth factor 8 (Fgf8), and possibly also Fgf2 and/or Fgf4 from the primitive streak at early stages (Ye et al., 1998; Cordes, 2005). Recent results indicate that similar mechanisms control the induction of serotonergic raphe neurons in the zebrafish embryo (Teraoka et al., 2004). Studies in mammals and chicken identified several distinct transcription factors involved in induction or maturation of 5HT raphe neurons. These factors include intracellular targets for Shh signaling (Nkx2.2, 2.9, 6.1, and Gli2), GATA factors 2 and 3, the LIM domain factor Lmx1b, and the Ets-domain transcription factor Pet-1 (van Doorninck et al., 1999; Cheng et al., 2003; Ding et al., 2003; Craven et al., 2004). Pet-1 is exclusively expressed in postmitotic 5HT neurons and controls their final differentiation, i.e., the expression of the 5HT transmitter phenotype encoded by Tph, aromatic L-amino acid decarboxylase and Sert (Hendricks et al., 1999). Thus, in chicken misexpression of *pet-1*, *lmx1b*, and *nkx2.2* is necessary and sufficient to generate ectopic 5HT neurons in the dorsal spinal cord (Craven et al., 2004). Furthermore, in *Pet-1*-null mice the majority of the 5HT expression is lost (Hendricks et al., 2003). A small population of raphe neurons still produces 5HT, but shows reduced levels of Tph, Sert, and vesicular monoamine transporter 2. These observations suggest that Pet-1 is necessary for the final steps in the specification of the serotonergic phenotype, and can be used as a selective marker for raphe serotonergic neurons detectable earlier than Tph.

To identify the origin of anterior raphe 5HT neurons in zebrafish, and to start analyzing the 5HT regulatory cascade in this species, we cloned the zebrafish homologue of mouse *Pet-1* (Pfaar et al., 2002). We analyzed its temporal and spatial expression pattern during development and adulthood, thereby also verifying *pet1* as a specific marker for anterior raphe serotonergic neurons in zebrafish. Three Tph enzymes with largely nonoverlapping expression domains share 5HT synthesis in this species (Bellipanni et al., 2002; Teraoka et al., 2004), and *pet1* transcription precedes expression of the *tph* ortholog specific for the raphe, *tph2*. However, other *tph2*-positive 5HT clusters, for example, in the pretectum, appear not to rely on Pet1 expression for their development. Surprisingly, we found that anterior and posterior clusters of 5HT neuronal precursors in the zebrafish hindbrain are separate at the level of r3, unlike the situation in mouse or chicken where the gap is in r4. We then used *pet1* in combination with the *her5PAC:egfp* transgene (Tallafuss and Bally-Cuif, 2003) to trace the origin of anterior raphe serotonergic precursors. Our results demonstrate that the majority of the serotonergic precursors located in r1–2, but not those located further posteriorly (r4 and beyond), originate from a progenitor cell pool located at the midbrain–hindbrain boundary (MHB). Our findings highlight heterogeneity in the developmental origin of raphe 5HT neurons and in the regulatory cascades leading to *tph* (and 5HT) expression.

RESULTS

Cloning of Zebrafish *pet1*

To identify and clone zebrafish orthologs of mouse *Pet-1*, the mouse protein sequence (AAL 13055) was blasted against the zebrafish peptide database using Ensembl (www.ensembl.org, zv6). One single candidate was identified located on chromosome 9 at position 10.196.186-10.197.882 (ENSDARG00000009242). Genscan predicted in that location a 235 amino acid (aa) protein (GENSCAN0000-0022621) showing 60% overall identity to mouse Pet-1 and 89% identity within the Ets-domain (Fig. 1C), and clustering with Pet-1/FEV from other species, but not with Fli-1 or ERG (Fig. 1D). Hence, we refer to this protein as zebrafish Pet1 (GenBank Accession EF370169). We verified the sequence for this predicted protein and the intron/exon boundaries by combining direct reverse transcriptase-polymerase chain reaction (RT-PCR) -mediated cloning, 5′-rapid amplification of cDNA ends (RACE) and sequencing of a partial RZPD expressed sequence tag (EST) clone (IMAGp998C2214692Q). We found that zebrafish *pet1* consists of three exons as in other species (Fig. 1A,B). However, in contrast to mouse *Pet-1* and human *FEV*, our PCR analysis demonstrates that zebrafish *pet1* mRNA encompasses an upstream ATG (numbered 1 in Fig. 1A,B) likely at the

origin of 13 additional N-terminal aa not found in any other species by in silico search.

Sequencing of the RZPD clone showed that it spans exons 2 and 3, and links exon 2 with an in frame 27 base pair 5′ fragment distinct from exon 1 (GenBank Accession EF370170). This sequence matches to a genomic stretch located 870 bp upstream of ATG 1, and might, therefore, belong to an alternative 5′-exon (exon $E1_{up}$, Fig. 1A–C). We could verify the coexistence of transcripts $E1_{up}$ and E1 by RT-PCR at 1, 2, and 4 days postfertilization (dpf) using forward primers in exon $E1_{up}$ or exon E1 and reverse primers in exon E3 (not shown), but could not recover the $E1_{up}$ transcript in 5′-RACE experiments, suggesting that it might correspond to a minor proportion of *pet1* transcripts. Our expression studies below use a probe spanning exons 2–3, which will not distinguish between these two alternative splice variants of the *pet1* transcript.

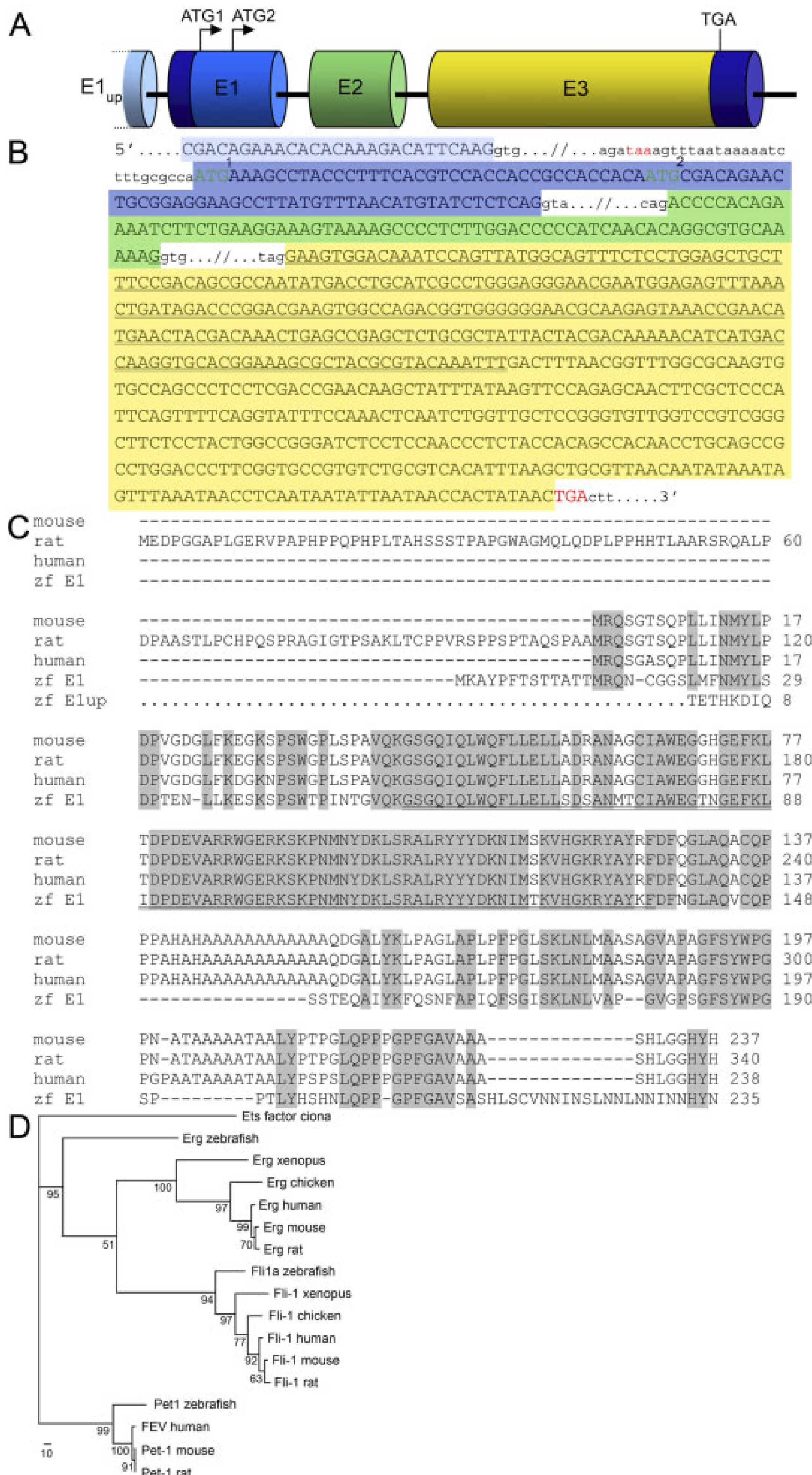

Fig. 1.

pet1 Expression Highlights Raphe Neurons and Precedes *tph2* Transcription in the Developing and Adult Zebrafish Brain

To determine whether *pet1* could be used as a specific marker for raphe serotonergic neurons, and to establish its relationship with *tph2*, we per-

Fig. 1. A: Organization of the *pet1* gene showing the three exons (E1–E3) giving rise to the major *pet1* transcript in embryos (transcript E1) with 5′- and 3′-untranslated regions (UTRs, dark blue boxes) and a partial exon ($E1_{up}$) producing an alternative transcript (transcript $E1_{up}$). The two consecutive ATGs (1 and 2) for transcript E1 are indicated. **B:** Genomic DNA sequence showing exonic sequences for transcripts E1 and $E1_{up}$ in capitals (color-coded as in A) and intronic sequences in lower case. ATG1 and ATG2 are colored in green and numbered, the closest in-frame 5′ stop codon for transcript E1 and the 3′ stop codon are in red, and the sequence encoding the Ets-domain is underlined. **C:** Alignment of Pet-1/FEV protein sequences from mouse (AAL13055), rat (NP_653354), human (NP_059991), and zebrafish (from transcript E1) using Clustal W. In addition, the partial amino acid sequence derived from zebrafish transcript $E1_{up}$ is included. Gray background indicates identical residues between the species. The conserved Ets-domain is underlined. **D:** Parsimony tree based on full-length sequences for vertebrate Ets-domain transcription factors of three closely-related members of the Erg subfamily: Pet-1/FEV, Erg and Fli-1. An Ets-domain factor from *Ciona* was used as an outgroup. Branch lengths are proportional to divergence between sequences. Numbers indicate bootstrap support for the nodes in percentages after 1,000 replicates.

formed a temporal analysis of its expression spanning development from 20 somites to 6 dpf (Fig. 2). In addition, we analyzed the presence and location of *pet1* transcripts in the adult brain (Figs. 3, 4).

At 20 somites, *pet1* expression was detected in cells located bilaterally along the midline and more posteriorly in the tail region (Fig. 2A). These cells might correspond to adrenal gland cells (Fig. 2Aa′ and see Bb″ for 25 hpf; Zhao et al., 2006) and blood precursors (Fig. 2Aa″; Detrich et al., 1995), respectively. Indeed, *Pet-1* has been detected in the mammalian adrenal medulla during embryonic development (Fyodorov et al., 1998). Expression of zebrafish *pet1* in presumptive adrenal gland cells and blood precursors was transient. In the anterior rhombencephalon, the first *pet1*-positive cells were detected at approximately 25 hours postfertilization (hpf; Fig. 2Bb′, and C,D, arrowheads). This time precedes the expression of *tph2* by approximately 5 hr (Fig. 2M–P, arrowheads). At 36 hpf, cells located in the posterior rhombencephalon also express *pet1* (Fig. 2E,F arrows), and at 48 hpf, both *pet1* and *tph2* expression domains are clearly organized in an anterior and a posterior cluster, separated by a gap, which is negative for expression. Both clusters consist of two bilaterally symmetrical adaxial columns lining the floor plate (Fig. 2G,H,S,T). In addition, *pet1* is transiently expressed in a cell cluster located just dorsal to the hindbrain population strongly positive for both *pet1* and *tph2* (Fig. 2H, stars in h′, compare with 2Tt′). The identity of these cells remains to be elucidated. Conversely, *tph2* (but not *pet1*) is also detectable in the epiphysis and at later stages in a pretectal area in the diencephalon (Fig. 2O–Y). At later stages (60 hpf and 6 dpf), expression of *pet1* and *tph2* in the hindbrain strongly resembles each other and have adopted an adult-like pattern (see below).

In the adult zebrafish brain, *pet1* transcripts are present in the anterior raphe (Fig. 3Aa′, 4A,B) as well as in scattered cells likely belonging to the posterior raphe (Fig. 3Aa″; Kaslin and Panula, 2001). *tph2* has a similar expression pattern (Figs. 3Bb″, b‴, 4D,E), but transcripts are also detected in the pretectal area (Figs. 3Bb′, 4C) and in the epiphysis (not shown). This finding is in contrast to *tph1*, expressed exclusively in the hypothalamus (Fig. 3C). To verify that *pet1* and *tph2* are coexpressed in the raphe, we raised an antibody against Tph2. The specificity of this antibody was demonstrated by double immunocytochemistry and in situ hybridization stainings, where it selectively labels neurons expressing *tph2* transcripts (Figs. 3B, all insets, and 4C–E). We observed that all cells expressing *pet1* were also positive for Tph2 (Figs. 3A, all insets, and 4A,B), proving the specificity of *pet1* expression for raphe serotonergic neurons. However, we could find in the raphe some cells with strong Tph2 immunoreactivity that displayed no or only a weak *pet1* in situ staining (not shown).

Mapping of the Border Between Anterior and Posterior Raphe Precursors Within the Developing Hindbrain

According to previous studies in the chicken and mouse, the precursor neurons for the anterior and the posterior raphe are segregated at early stages by a gap in r4, where no 5HT neurons are generated, but, instead visceral motor neurons of the facial nerve innervating the branchial arch derivates (Lumsden and Keynes, 1989; Marshall et al., 1992; Carpenter et al., 1993; Pattyn et al., 2003a). To map the gap separating the anterior and the posterior hindbrain *pet1*-positive clusters in zebrafish, we performed Tph2 immunohistochemistry on brains from *isl1:gfp* transgenic larvae, expressing green fluorescent protein (GFP) in cranial motor neurons of the hindbrain (Higashijima et al., 2000). Using this line at 6 dpf, we could readily identify the trigeminal central neurons (Va, Vp) located in r2 and r3, respectively (Fig. 5B; Chandrasekhar et al., 1997; Higashijima et al., 2000), as well as a third cluster immediately posterior (Fig. 5B, star). We mapped this third cluster to r4 by comparison with the position of retrogradely traced Mauthner neurons (Fig. 5A,C; Kimmel et al., 1981; O'Malley et al., 1996). Thus, *isl1:gfp* transgenic larvae allow precise positioning of r2, 3, and 4, and we subsequently labeled Tph2-positive neurons in this line (Fig. 5D–F). We found that the gap separating anterior and posterior serotonergic clusters (Fig. 5D, inset) overlaps with the position of Vp, in r3, and not with the third *isl1:gfp*-positive cluster in r4 (Fig. 5F). Hence, we conclude that, in contrast to earlier findings in other species, the gap separating the anterior from the posterior clusters of raphe precursors in zebrafish is located in r3.

pet1-Positive Cells Located in r1–2 Originate From the Midbrain–Hindbrain Boundary Progenitor Pool

Previous observations suggest that the different clusters of serotonergic neurons in the raphe complex might have different embryological origins (Craven et al., 2004; Cordes, 2005), but these neurons have not been directly traced. We, therefore, addressed whether the serotonergic precursors of the r1–2 group originate from common or distinct locations.

By combining *pet1* in situ hybridization with the detection of *rfng* transcripts to identify rhombomeric borders (Qiu et al., 2004), we observed that the first *pet1*-positive cells were located in r1, close to the MHB (data not shown). Because previous results from our laboratory also suggested that the MHB progenitor pool contributed neurons to the ventral anterior hindbrain (Tallafuss and Bally-Cuif, 2003), we specifically tested for the contribution of this pool to r1–2 serotonergic precursors. We used *her5PAC:egfp* transgenic embryos, which allow us to locate the early MHB pool by GFP expression at late gastrulation. The stability of GFP in these embryos is too short to permit direct tracing of MHB progeny cells until the appearance of the first *pet1*-positive cells. Thus, we injected caged-fluorescein at the one-cell stage and uncaged this compound between the 90% epiboly to tail bud stage in a few cells located either within the GFP-expressing domain, or immediately posterior to it (Fig. 6A). Cross-sections of 34–36 hpf embryos uncaged within the GFP-positive area (Fig. 6Ai) showed cells double labeled for un-

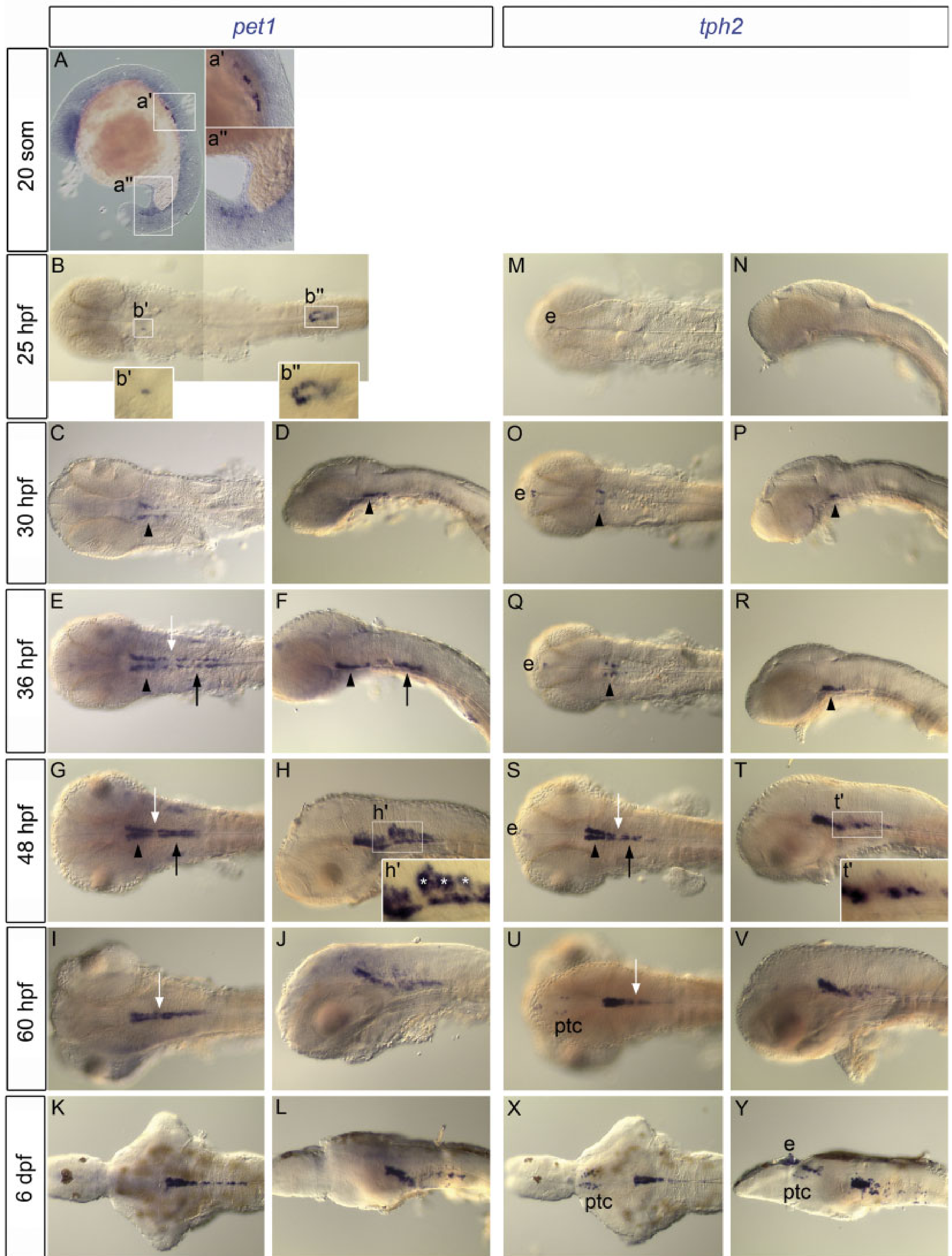

Fig. 2.

caged fluorescein and *pet1* in the anterior rhombencephalon (r1–2; Fig. 6B), but not more posteriorly (r4–7; not shown). This finding is in contrast to embryos where the uncaging was done posterior to the GFP-positive area (Fig. 6Aii). There, fluorescein-positive cells populated the floor plate in the anterior hindbrain region as well as a large domain roughly spanning the whole dorsoventral extent of the neural tube at the level of r3, where *pet1* is not expressed (not shown), while *pet1*-positive cells of r1 and r2 were unlabeled (Fig. 6C). Thus, *pet1*-positive serotonergic precursors of r1–2, but not those located more posteriorly, originate from the MHB progenitor pool.

DISCUSSION

In the present study, we cloned and analyzed the spatial and temporal expression pattern of the zebrafish Ets-domain transcription factor-encoding gene *pet1*. We showed that *pet1* is expressed in the subpopulation of *tph2*-positive cells located in the raphe, but not in other *tph*-positive clusters. We next used this marker in combination with tracing techniques to locate the origin of anterior hindbrain serotonergic precursors. Our results demonstrate that the serotonergic neurons of r1 and r2 largely originate from a progenitor cell pool located at the MHB.

Fig. 2. A–Y: Expression of *pet1* (A–L) and *tph2* (M–Y) revealed by in situ hybridization on whole-mount embryos/larvae (A–J and M–V) or brains (K–L and X–Y). Insets show higher magnifications of the boxed areas. The earliest *pet1*-positive cells are likely adrenal gland (Aa′ and Bb″) and blood (Aa″) precursors. B: The first *pet1*-expressing cells in the rhombencephalon are detected at 25 hours postfertilization (hpf). O: Expression of *tph2* in this location is not detected until a few hours later. Afterward, *pet1* and *tph2* expression highlights two parallel stripes of precursors lining the hindbrain floor plate. E,G,I,S,U: These are organized in an anterior (arrowhead) and posterior (arrow) cluster separated by a gap (white arrow). H: An additional column of *pet1*-positive cells runs along this domain in a slightly more dorsal location (stars in h′). Starting from 60 hpf *tph2* expression can also be seen in an additional cluster of cells corresponding to the pretectal complex (Kaslin and Panula, 2001). In addition, *tph2* was detected in the epiphysis at all stages examined. e, epiphysis; ptc, pretectal and thalamic complex.

This finding is in contrast to serotonergic neurons located more posterior in the hindbrain, which, according to previous findings, might originate from ventral neuroepithelial progenitors close to the floor plate at their respective anteroposterior level (reviewed in Goridis and Rohrer, 2002). Furthermore, by combining identification of the raphe serotonergic neurons using their specific expression of *pet1* and Tph2 with rhombomere identity markers we show that, in contrast to many other vertebrates, the gap separating the anterior from the posterior hindbrain clusters of serotonergic precursors is located in r3 in zebrafish and not in r4. Our findings strengthen the evidence for heterogeneity among the serotonergic cell populations of the vertebrate brain, both in the transcription factors presiding to their differentiation and in their developmental origin, with possible further differences between species.

Organization of the Zebrafish *pet1* Gene

Pet-1 belongs to the family of Ets transcription factors, which are characterized by a highly conserved DNA-binding Ets-domain (Peter et al., 1997; reviewed in Laudet et al., 1999; Oikawa and Yamada, 2003). The Ets family contains some 30 different members, of which Erg and Fli-1 are most closely related to Pet-1. In the present study, we have identified one zebrafish homologue of Pet-1 showing 89% identity to mouse Pet-1 within the Ets domain (Pfaar et al., 2002). As reported for mammals (Peter et al., 1997; Fyodorov et al., 1998; Pfaar et al., 2002), the main form of zebrafish Pet1 is encoded by three exons. However, we identified for this transcript 39 additional base pairs in the fish genome, giving rise to 13 additional N-terminal aa, which we could not find in other species by in silico search. The existence of this extra sequence was confirmed by RT-PCR. Furthermore, we demonstrated the existence of an alternative 5′-exon that is at least partially coding. The two alternative transcripts co-exist in vivo. Whether they are differentially expressed, however, remains to be determined, because our in situ probe does not distinguish between the two. A differential promoter usage in *Pet-1* has not been reported in other species to date.

pet1 Is a Selective Marker for Raphe Serotonergic Neurons in Zebrafish

Previous studies of chicken, mouse, rat, and human have identified *Pet-1*/*FEV* as a specific marker for postmitotic raphe serotonergic neurons preceding the expression of serotonergic neuron-specific proteins such as Tph and Sert (Hendricks et al., 1999; Pfaar et al., 2002; Craven et al., 2004; Maurer et al., 2004). Our findings that *pet1* has a similar expression pattern as *tph2* in the hindbrain during embryonic, larval, and adult stages, suggests that *pet1* is also a valid marker for this specific cell population in zebrafish. This assumption was verified by showing that *pet1* transcripts are colocalized with Tph2 protein in the adult brain. We further demonstrate that *pet1* transcripts can be detected approximately 5 hr earlier than *tph2* transcripts in the anterior rhombencephalon (r1), demonstrating that it is also a comparatively early marker in zebrafish. Finally, like in other vertebrates, zebrafish *pet1* is expressed in serotonergic precursors only at the postmitotic stage (C. Stigloher, unpublished observations).

Location of the Border Between Anterior and Posterior Raphe Precursors

Our finding that the gap separating the anterior from the posterior clusters of serotonergic precursors is located in r3 in zebrafish was unexpected considering observations from other species, where it has been found in r4 (Lumsden and Keynes, 1989; Marshall et al., 1992; Carpenter et al., 1993; Pattyn et al., 2003a). In agreement with our finding, Teraoka et al. (2004) described expression of *tph2* in the anterior cluster located just posterior to the trochlear nucleus (nIV) and anterior to the trigeminal motor nucleus (nV). We do not as yet have a mechanistic interpretation for this surprising interspecies difference. It is possible that the location of the border between anterior and posterior raphe precursors depends on the extent

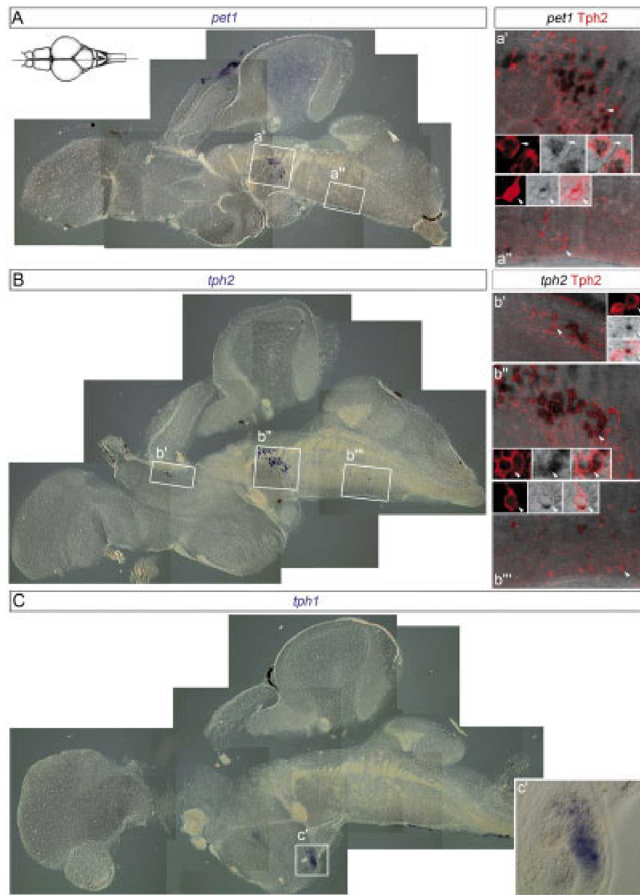

Fig. 3.

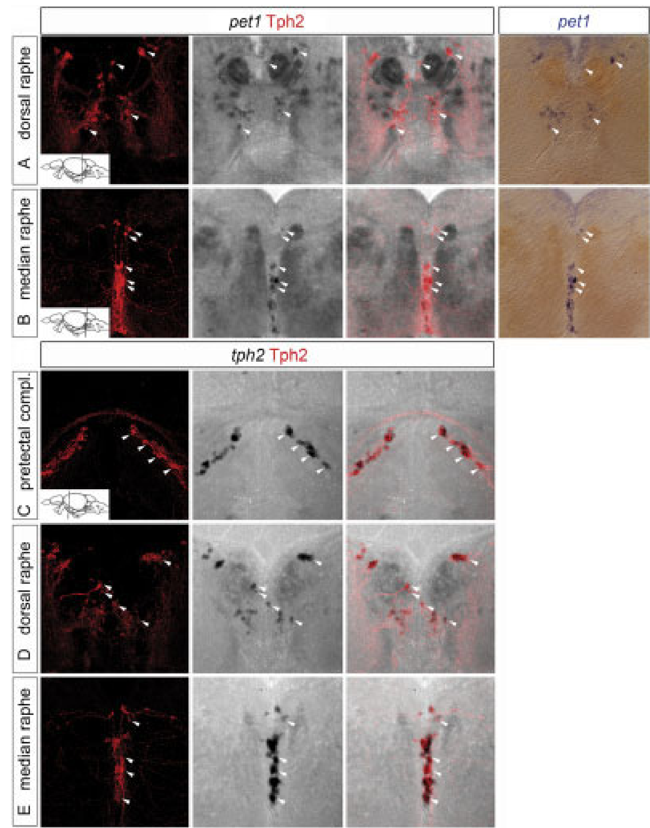

Fig. 4.

of the posteriorward migration of MHB-derived cells, on local specification cues, or both. In the mouse, a specific mechanism relying on r4-expressed genes (e.g., *Hoxb1* and *Phox2b*) accounts for the lack of serotonergic specification in this location (Pattyn et al., 2003a), and orthologous genes are also expressed in r4 in zebrafish (Guo et al., 1999; McClintock et al., 2002). However, we note that zebrafish-specific features have been observed in several specification or migration mechanisms in the hindbrain. For instance, a migration of the facial cranial nerve from r4 to r6 is observed in zebrafish but not in chicken (Chandrasekhar et al., 1997), and zebrafish r4 (but not mouse r4) expresses *fgf8* (Maves et al., 2002; Walshe et al., 2002). Thus, it remains possible, in particular, that the regulatory events involving Hoxb1 function differ between zebrafish and other vertebrates. Another important question will be to determine whether, in all species, the gap between ante-

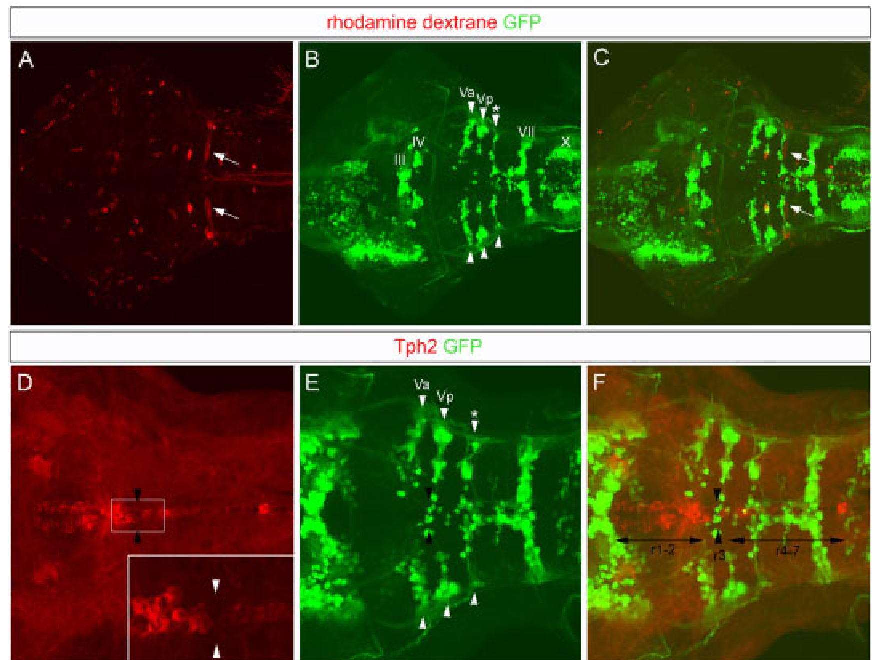

Fig. 5.

rior and posterior hindbrain clusters corresponds to the distinction between the precursors fated to form the anterior versus the posterior raphe, a conclusion currently mostly inferred from analyzing the spatiotemporal evolution of serotonergic markers during development (Lidov and Molliver, 1982; Aitken and Törk, 1988) and from back-filling caudal serotonergic projections in zebrafish (McLean and Fetcho, 2004a,b).

pet1-Positive Cells Located in r1–2 Originate From the MHB Progenitor Pool

According to the present literature, 5HT neurons arise from the ventral neuroepithelial progenitors located close to the floor plate (pMNv area) that, in r2 and beyond, also produce branchiomotor and visceromotor neuronal precursors (Goridis and Rohrer, 2002; Pattyn et al., 2003a; but see Craven et al., 2004; Cordes, 2005). The origin of r1 progenitors was unresolved, as no branchio- and visceromotor neurons arise from r1. Making use of the amenability of zebrafish embryos to direct lineage tracing, we demonstrate here that most, if not all, *pet1*-positive cells of r1 and r2 originate from the pool of progenitor cells located at the MHB at the end of gastrulation. Whether a single MHB progenitor gives rise to both r1 and r2 neurons cannot be concluded at this point. These results add to our previous observation that the early MHB progenitor pool generates cells populating r1 and r2 (and possibly also, to a low extent, r3 and r4; Tallafuss and Bally-Cuif, 2003). They also extend fate mapping data for *Wnt1*-positive cells in the embryonic day (E) 7.5 mouse embryo that gave rise to a few 5HT neurons of the anterior raphe (Zervas et al., 2004). In fish, the MHB pool partially overlaps with *wnt1* expression (Tallafuss et al., 2001).

Interestingly, the contribution of the MHB pool to serotonergic precursors appears to be precisely limited to the anterior hindbrain cluster. Thus, the anterior and posterior clusters are distinct not only in position, molecular specification, and fate (van Doorninck et al., 1999), but also in the developmental process that originally sets them aside within the neural tube.

Differential Regulation of the Serotonergic Phenotype in Zebrafish

In the zebrafish CNS, serotonergic neurons are found in several distinct clusters: in the epiphysis, the hypothalamus, a pretectal area of diencephalon, and the anterior and posterior raphe nuclei (Kaslin and Panula, 2001). The synthesis of 5HT in these different cell populations is controlled by at least three different Tph enzymes, all with a unique temporal and spatial expression pattern (Fig. 7). *tph1* is present in 5HT-containing cells of the embryonic and adult hypothalamus and transiently in cells along the floor plate of the spinal cord (Bellipanni et al., 2002). The expression of *tph1-like* is restricted to a preoptic cell cluster during late embryonic stages (Bellipanni et al., 2002). *tph2* is expressed by cells in the anterior and posterior raphe in embryonic until adult stages (Teraoka et al., 2004; present data). In addition, we detected *tph2* transcripts in a cluster of 5HT-positive cells in the pretectal area of the diencephalon from 3 dpf and onward (this study).

As opposed to *tph*, we could only identify one *pet1* gene in zebrafish, with expression limited to the anterior and posterior raphe. Furthermore, although *tph2* is expressed both in the diencephalon and in the hindbrain serotonergic neurons, *pet1* was only expressed at detectable levels in the hindbrain population. These findings demonstrate heterogeneity not only among 5HT nuclei but also within the Tph2-expressing population itself, with respect to the transcription factors required for activating serotonergic identity. In the mouse, the specification of 5HT progenitors further differs in r1 and r2, as r1 is not under control of Nkx2.2 and Phox2b functions (Briscoe et al., 1999; Ding et al., 2003;

Fig. 3. Comparison of the localization of *pet1*, *tph2*, and *tph1* transcripts (in situ hybridization, blue/black) and of Tph2 protein (immunohistochemistry, red) on adult brain sagittal sections. **A:** In situ staining for *pet1* in anterior (a′) and posterior (a″) raphe nuclei. a′ and a″ (optical projections) show higher magnification of boxed areas in A together with Tph2 immunostaining in red. Note the double-labeled cells, some of which (arrowheads) are further magnified in the small insets (optical sections). **B:** In situ staining for *tph2* in anterior (b″) and posterior (b‴) raphe nuclei and in the pretectal complex (b′). b′, b″, and b‴ (optical projections) show higher magnification of boxed areas in B together with Tph2 immunostaining. Double labeling was observed in all three regions for all cells, some of which (arrowheads) are further magnified in the small insets (optical sections). **C:** In situ staining for *tph1* in the hypothalamus. c′ shows higher magnification of boxed area in C. Schematic picture was modified from Wullimann et al. (1996).

Fig. 4. Compared localization of *pet1* and *tph2* transcripts (in situ hybridization, black/blue) and of Tph2 protein (immunohistochemistry, red) on adult brain coronal sections. Arrowheads indicate examples of double-labeled cells. **A,B:** *pet1* transcripts and Tph2 protein shown in optical projections of sections through the dorsal and the medial raphe, respectively, at the level indicated in the schematic pictures. Color brightfield pictures were included (right panels) to clarify the distinction between cross-cut fiber bundles (that appear dark on black–white images, but are negative for *pet1* transcripts) and blue in situ staining. The dorsal raphe (corresponding to cluster B6–B7) is located more dorsally and laterally than the medial raphe (B8–B9; Kaslin and Panula, 2001). **C–E:** The location of *tph2* transcripts and Tph2 protein in the pretectal complex (level of section indicated in schematic picture) is illustrated (C) in addition to the dorsal (D) and median (E) raphe in optical projections. Schematic picture was modified from Wulliman et al. (1996).

Fig. 5. Location of the gap separating the anterior and posterior *pet1*-positive raphe precursors in relation to green fluorescent protein (GFP) -positive cell clusters in the *isl1:gfp* transgenic line. Photomicrographs are confocal optical projections of dorsal views at hindbrain levels, anterior left. **A:** Mauthner neurons (arrows), located in rhombomere (r) 4, were labeled by retrograde tracing in 6 days postfertilization (dpf) *isl1:gfp* transgenic larvae using rhodamine dextran. **B,C:** Thereby, the spatial distribution of GFP-expressing cells (B, arrowheads) in relation to rhombomeres was determined (C, overlay of A and B). C: Note that the Mauthner neurons overlap with a cluster of GFP-positive cells (*) just posterior to the trigeminal nuclei (Va and Vp, arrows). **D:** The anterior and posterior clusters of serotonergic precursors were identified using an antibody against tryptophan hydroxylase (Tph) 2. The inset shows a higher magnification of the boxed area, and white arrowheads in the inset indicate the gap between the anterior and posterior Tph-positive clusters (optical section). **E,F:** Note, in F (overlay of D and E) that this gap overlaps with Vp, in r3 (black arrowheads), rather than with Mauthner neurons, in r4. Thus, the anterior Tph cluster spans r1–2, and the posterior cluster spans r4 and beyond.

Fig. 6.

Pattyn et al., 2003b). If a similar phenomenon occurs in zebrafish, the common origin of r1 and r2 precursors suggests that their acquisition of distinct genetic cascades to realize the serotonergic phenotype is an event that likely follows their exit from the MHB.

Together, these observations strongly support a model where the serotonergic phenotype is differentially regulated at multiple steps among the serotonergic populations within the zebrafish CNS, and would be mostly acquired by the progressive convergence of different

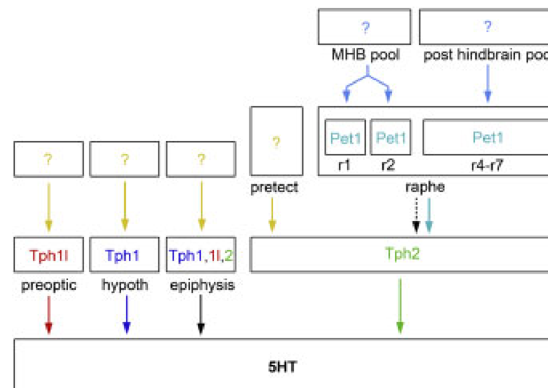

Fig. 7.

combinations of developmental factors toward expression of the 5HT neurotransmitter. The mechanisms that sustain the setting-up of these distinct genetic pathways and their convergence toward a common outcome remain an interesting future issue.

EXPERIMENTAL PROCEDURES

Cloning of Zebrafish *pet1*

A predicted zebrafish *pet1* ortholog (ENSDARG00000009242) was cloned and verified using a 5′-RACE protocol modified for GC-rich domains, according to the manufacturers' recommendations (Invitrogen). Briefly, total RNA was extracted from 56 hpf embryos using Trizol (Invitrogen). Total RNA was then treated with DNase I and reverse transcribed into cDNA using gene specific primer (GSP) 1 (5′caggttgtggctgtggtaga3′), purified on SNAP-columns and dA-tailed. Subsequently, the dA-tailed first-strand cDNA was reverse transcribed in a second-strand cDNA synthesis using a 3′-RACE adapter primer (5′ggccacgcgtcgactagtac(t)$_{17}$3′), and SNAP-column purified a second time. The 5′-RACE was followed by a nested PCR using an abridged universal amplification primer (AUAP; 5′ggccacgcgtcgactagtac3′) and GSP 2 (5′gctgtggtagagggttgga3′) and 3 (5′ctgaatgggagcgaagttg3′), generating a major amplification product corresponding to transcript E1 starting at ATG2 (Fig. 1B). To determine whether ATG1 was generally included in transcript E1, additional nested RT-PCR reactions were performed on cDNA reverse-transcribed from 56 hpf embryos with random hexamers. The forward PCR primers were located upstream, downstream, or spanning either of the two ATGs as follows: upstream of ATG1: primer 1, 5′atttattccagatcacagttttgag3′; primer 2, 5′-aaagtttaataaaaatctttgcgc3′; spanning ATG1: primer 3, 5′aaaatctttgcgccaatg3′; primer 4, 5′gtttaataaaaatctttgcgccaatga3′; downstream of ATG1: primer 5, 5′aaagcctaccctttcacgtc3′; primer 6, 5′tttcacgtccaccaccg3′; spanning ATG2; primer 7, 5′atgcgacagaactgcgg3′; downstream of ATG2: primer 8, 5′agaactgcggaggaagcc3′. GSP 2 and 3 were used as reverse primers. Specific amplification products were obtained using primers 4–8, demonstrating that ATG1 is contained within transcript E1.

Sequencing of an EST clone (IMAGp998C2214692Q, RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH, www.rzpd.de; used for subsequent in situ hybridizations) showed that it spans exons 2 and 3, and links exon 2 with a 27-base pair 5′-fragment from the same genomic locus but distinct from exon 1 (see below; Fig. 1A,B). The existence of such a transcript ("$E1_{up}$") was verified by PCR reactions performed on cDNA reverse transcribed from 1, 2, and 4 dpf embryos with random hexamers using a forward primer in exon $E1_{up}$ (5′cgacagaaacacacaaagacattca3′) and a reverse primer in exon 3 (5′cttcgtccgggtctatcagtttaa3′).

Fig. 6. Origin of rhombomere (r) 1–2 serotonergic precursors. **A:** Strategy for tracing the origin of r1–2 serotonergic precursors in *her5PAC:egfp* transgenic embryos injected with caged-fluorescein at one-cell stage (dorsal views, anterior left). An ultraviolet-light beam was focused along the midline (i) within or (ii) posterior to the green fluorescent protein (GFP) -expressing area at 90% epiboly/tail bud (left drawing; green GFP-positive midbrain–hindbrain boundary [MHB] progenitor pool). Photomicrographs: control embryos fixed directly after uncaging and processed for GFP (green) and fluorescein (red) immunostaining. Schematic pictures: two possible outcomes at 36 hours postfertilization (hpf). Top: uncaging within the GFP-positive area and *pet1*-positive cells of r1–2 fluorescein-labeled (red); origin within the MHB pool; Bottom: uncaging posterior to the GFP-positive area and *pet1*-positive cells of r1–2 fluorescein-labeled; origin posterior to the MHB pool. **B:** A 16-μm optical projection of a coronal cryosection through r1–2 of embryos uncaged within the GFP-expressing domain. Insets: High magnifications of 1-μm optical section of double-labeled cell indicated with an arrow. Arrowheads: *pet1*-positive cells on each side of the floor plate within r1–2. Note anti-fluorescein labeling of these *pet1*-positive cells. **C:** Same analysis in an embryo uncaged posterior to the GFP-expressing domain. mes, mesencephalon. Schematic picture modified from Mueller and Wullimann (2005).

Fig. 7. The serotonergic phenotype in the zebrafish central nervous system (CNS) is acquired by converging mechanisms. Schematic model of the genetic cascades encoding serotonin (5HT) neurotransmitter identity in the zebrafish CNS; the genes involved are color-coded, and their territories of expression are represented by boxes. The factors accounting for Tph1, 1l, and 2 expression in the pretectal, preoptic, hypothalamic, and epiphyseal clusters are unknown and, although depicted with a single color, might differ between these domains and/or the tryptophan hydroxylase (Tph) target gene. In addition to *tph1l* expression in the preoptic cluster a temporally nonoverlapping expression of *tph1* has been found in cells located in the preoptic area. Whether these cells are different from the *tph1l*-expressing cells or if there is a shift from *tph1l* to *tph1* is not known (Bellipanni et al., 2002). The differential regulation of *pet1* expression in r1 and r2 is inferred from studies in the mouse (Briscoe et al., 1999; Ding et al., 2003; Pattyn et al., 2003a). The dotted arrow leading to *tph2* expression in the raphe refers to the persistence of 5HT neurons in $Pet1^{-/-}$ mice (Hendricks et al., 2003), suggesting a partially redundant mechanism.

Molecular Phylogenetic Analysis

Multiple alignment of amino acid sequences for Pet-1/FEV, Erg, and Fli-1 were obtained by using Clustal Method in MegAlign software (version Power Macintosh 3.01, DNA Star, Inc.). A parsimony tree based on full-length sequences for the three different vertebrate Ets-domain transcription factors belonging to the Erg subfamily and a *Ciona* Ets-domain factor (BAE06415) as outgroup was constructed using PAUP* software (version 4.0b10, David L. Swofford, Florida State University, Sinauer Associates, Inc., Publishers). Probabilities were calculated using bootstrap with 1,000 replicates. The following protein sequences were used: Erg chicken (CAA54404), human (NP_004440), mouse (BAB69948), rat (AAH72519), *Xenopus* (CAB46567), zebrafish (AAH86811), Fli-1 human (AAH01670), mouse (NP_032052), rat (AAX83256), chicken (NP_001026079), *Xenopus* (CAA47389), Fli1a zebrafish (AAH66571), FEV human (NP_059991), Pet-1 rat (NP_653354), and mouse (AAL13055). Trees were printed using the program TreeviewPPC (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Generation of Monoclonal Antibodies (mAbs) Against Tph2

An internal peptide of Tph2 ($_{111}$CTKKEFNELVQHLKDHVNIV$_{130}$) was synthesized and coupled to KLH or ovalbumin (PSL, Heidelberg). Rats were immunized with 50 μg of pep-

tide-KLH using CPG 2006 (Tib Molbiol, Berlin) and IFA as adjuvant. After a 6-week interval, a final boost without adjuvant was given 3 days before fusion of the rat spleen cells with the murine myeloma cell line P3X63-Ag8.653. Hybridoma supernatants were tested in a differential enzyme-linked immunosorbent assay with the specific peptide or an irrelevant peptide coupled to ovalbumin. Positive-reacting hybridomas (IgG2a) were further analyzed by immunohistochemistry, and mAb 2E5 was found to specifically recognize Tph2.

In Situ Hybridization and Immunohistochemistry

Whole-mount in situ hybridization was performed on AB/AB embryos staged according to Kimmel et al. (1995) or on AB/AB adult brains as described elsewhere (Thisse et al., 1993; Adolf et al., 2006). The following probes were used: *tph2* (previously *tphR*; Teraoka et al., 2004), *tph1* (previously *tphD1*; Bellipanni et al., 2002), and *radical fringe* (*rfng*; Qiu et al., 2004). The RZPD EST clone IMAGp998C2214692Q was used to detect *pet1* transcripts. Immunocytochemical stainings were performed with the following antibodies: monoclonal rat α-Tph2 (1:8; see above), rabbit α-GFP (1:1,000; Torrey Pines Biolabs), or mouse α-fluorescein (1:200; Roche) and secondary antibodies coupled to Cy2 or Cy3 (Jackson ImmunoResearch). Flat-mounted preparations or cryostat sections of embryos, as well as Vibratome sections of adult brains, were photographed using a Zeiss Axioplan microscope equipped with a 3CCD color video camera (Sony) and processed with AxioVision 4.5 software (Zeiss), or a laser scanning confocal microscope (LSM510Meta, Zeiss). Subsequent image processing was done using LSM software (version 3.2 SP1.1, Zeiss) and Photoshop (version 9.0, Adobe Systems).

Mapping of the Gap Separating the Presumptive Anterior and Posterior Serotonergic Precursors in the Hindbrain

To locate the border between anterior and posterior raphe, larvae at 6 dpf from an *isl1:gfp* transgenic line (Higashijima et al., 2000) were processed for Tph2 immunohistochemistry (see above). For comparison and identification of r4, Mauthner neurons were retrogradely traced with fixable rhodamine dextran (10 kDa; Molecular Probes) by cutting the tail of *isl1:gfp* transgenic larvae with a scalpel soaked in dye. The larvae were fixed in 4% paraformaldehyde after 6 hr, and the brains were subsequently dissected, mounted, and imaged using a Zeiss Confocal microscope.

Tracing of *pet1*-Expressing Cells

The origin of the *pet1*-expressing cells located in r1–2 after 24 hpf was traced using uncaging of DMNB-caged fluorescein (10 kDa; 5 mg/ml; Molecular Probes) injected into one-cell stage embryos from a *her5PAC:egfp* transgenic line (Tallafuss and Bally-Cuif, 2003). Injected GFP-positive embryos were uncaged at 90% epiboly to tail bud stage, when a distinct GFP expression could be seen identifying the MHB progenitor pool (Fig. 6A). The uncaging was done using the ultraviolet (UV) excitation beam of a Zeiss Axioplan microscope, focused through a 0.1-mm pinhole and with a ×63 water immersion objective, leading to labeling of an area approximately five cell bodies in diameter. The UV beam was aimed at medial cells located either within the GFP-expressing domain or immediately posterior to it. To verify the location of the labeling, a set of embryos (n = 5 within the GFP-expressing domain, n = 6 posterior to the GFP-expressing domain) was fixed immediately after uncaging and processed for double immunocytochemistry against GFP and fluorescein (Fig. 6A i and ii). On average the posterior uncaging was done 2–3 cell rows behind the GFP-expressing area. The rest of the embryos were fixed at 33–35 hpf, a stage when *pet1* expression is clearly detectable in both anterior and posterior rhombomeric clusters, and processed for *pet1* in situ hybridization followed by immunodetection of uncaged fluorescein (see above). To visualize double-labeled cells, stained embryos were cryosectioned coronally at 25 μm.

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