4. Discussion

4.1. 5HT in early stages of zebrafish

The presence of 5HT in early stages of zebrafish embryos (8-cell-, blastula-, gastrula, 1dpf stages) (Fig. 3.1.) indicate that early fish embryos need prenervous 5HT to regulate some biological processes during the embryogenesis. Cell proliferation, cell movement during gastrulation, ciliary activity, secretion of hatching enzyme, neurulation and migration of neural crest cells have been shown to depend on 5HT like in other species such as Drosophila, molluscs, sea urchin, chicken, frogs, and mice (Colas et al. 1999 a, b, Buznikov et al. 2003, Buznikov et al. 2005, Fukumoto et al. 2005, Moiseiwitsch and Lauder1995). Although there is no report in the literature about the function of 5HT in those early stages of zebrafish.

The asymmetric distribution of 5HT in some cells at blastula stages of zebrafish (Fig. 3.2A) may have the function among others to pattern the left-right asymmetry of the internal organs during embryogenesis. Fukumoto et al. (2005) observed that 5HT at the 64-cell stage of frog embryos is progressively located at specific subset of blastomers. After inhibition of the 5HT synthesis at early stages of frog, around 15% of the embryos exhibit situs inversus in heart, bladder, and intestine. We could not detect this phenotype in the heart of TPH2 morphants by in situ hybridization using riboprobe of dHand as heart marker in early stages of development. This negative result may due to the low percentage of embryos with situs inversus, and to the low number of embryos we have tested.

We observed the localization of 5HT in the cells of the hatching gland located on the anterior part of the yolk at 30dpf zebrafish embryos (Fig.3.3C). Buznikov et al. (1996) detected that 5HT regulate the secretion of the hatching enzyme in blastula stage of sea urchin. We could not detect in details the function of 5HT in this tissue of zebrafish. But we observed the most embryos of TPH2 morphants even at 3 dpf could not hatch from its chorions in contrast to the wild type fish that could hatch easily from its chorions already maximum at 2dpf. We need always to help TPH2 morphants during the hatching from its chorions by using mechanical method (forceps). This problem in TPH2 morphants may due some defects in the secretion of hatching enzymes.

Interesting was the involvement of 5HT in primary embryonic induction in the echinoderm (Gustafson and Toneby 1970). The authors observed that 5HT was secreted by cells of the primary gut at the final stage of gastrulation and induce the transformation of some cells of ciliary bands into neuron cells. We could detect 5HT in the gastrulation stage and in 1dpf of zebrafish. 5HT at these stages may stimulate the same primary embryonic induction, special

at 1dpf stage when neural crest cells already were specified on the dorsal neural tube. Probably, 5HT here from unknown cells induce the transformation of some neural crest cells into e.g. neurons. TPH2 morphants are exhibiting defect in neural crest derived Rohon-Beard sensory neurons (Fig. 3.31.) that located close to some 5HT cells (McLean and Fetcho 2004). The transformation of some cells of the ciliary bands of echinoderms by external 5HT into neurons is similar from the mechanisms to the hypothesis of the evolution of neural crest cells, i.e. that neural crest cells during the evolution originated from HNK1 positive cells of ciliary band of echinodermata ancestors (Morikawa et al. 2001). Probably, 5HT-among other factors- played some roles in evolution of the vertebrate specific neural crest cells, and the gene duplication of the ancestral TPH in the invertebrate and the birth of two isoforms of TPH helped the establishment of the vertebrate animals. Two isoforms of TPH are restricted only to vertebrates, and TPH2 plays a developmental role in the formation of vertebrate specific skeletal elements of the pharyngeal arches (as shown in this work).

Taken together, 5HT appear to be a multifunctional regulator at blastula and gastrula and other early stages of zebrafish development. Its function in early stages of development become increasingly complex in connection with the spatio-temporal expression of its many different receptors.

4.2. Defects of the pharyngeal arches

In spite of significant anatomical differences between mammalians and fish, comparison of the organogenesis of their pharyngeal arches reveals several striking similarities. The mammalian pharyngeal arches give rise to the thymus, thyroid, and parathyroid glands. Fish also develop the thyroid and the thymus from their pharyngeal regions, but lack parathyroid glands and form gills.

In the TPH2 zebrafish morphants, the defects in the pharyngeal arches (PA) were due to multiple roles of TPH2. According to our results, the Knock down of TPH2 affected the normal development of at least three distinct tissues that participate in the PA morphgenesis, during three different time points of PA development.

First, we observed a reduction of numbers of the migrating cranial neural crest cells starting at 24 hpf in morphants.

Second, we observed the absence of endodermal pouches at the 36 hpf stage. This led to the absence of pouches that the migrating crest cells have to fill, and thus in consequence to the absence of many signaling factors necessary for PA morphogenesis,

Third, we observed the absence of the 5HT single cells in the PA of 5 days old embryos. We think that those cells demonstrate that the 5HT interaction with at least one 5HT receptor that is expressed in the epithelial-mesenchyme interaction site is of great importance, and the absence of the ligand serotonin, thus affects the normal development of the PA.

These three factors led the TPH2 morphants exhibit strong defects in the PA. In fact, these defects were more drastic than the defects observed in 5HT2B receptor morphants or the effect of the exposure of the fish to ritanserin, an inhibitor of the 5HT2B receptor.

Moiseiwitsch and Lauder (1995) showed by in vitro explantation experiments using mouse cranial neural crest cells from E9 embryos or mandibular mesenchyme cells from E12 embryos that 5HT regulates the migration of the mouse cranial neural crest cells or their mesenchymal derivatives, and demonstrated that the motility of the cells was dependent on the 5HT concentration. Low 5HT dose stimulates the motility of the cells, whereas this effect was lost as the dose of 5HT was increased. They could also inhibit the effect of the low dose of 5HT, when they exposed the explants to a 5HT1A receptor antagonist (NAN-190). In vivo, we could not detect 5HT in the dlx2a positive migrating cranial crest cells of the zebrafish embryos. This can be due to fast degradation of 5HT by enzymes like MAO. Experiments exposing the fish in the early stages to MAO inhibitors and then do 5HT immunostaining at different stages of the crest cell development to see if 5HT exists in those cells, may clarify this issue.

We think that the observed reduction of the migrating mouse cranial neural crest cells, when we follow dlx2a expression, was due to the defects in the hindbrain rhombomers, - the tissues where TPH2 expression correlates with the time of the crest cell migration into the pharyngeal arches. Wang et al. (2006) reported that TPH2 is expressed in the hindbrain at the 24 hpf stage of zebrafish embryos. Many publications (McNulty et al. 2005, Byrd and Meyers 2005) showed that there are relationships between hindbrain defects and abnormalities in crest cell migration (like changes in migration pattern and migration density).

We show here that Foxd3 morphants display a similar phenotype to TPH2 morphants with respect to the pharyngeal arches, suggesting that TPH2 and Foxd3 act in a common pathway in the formation of the pharyngeal arches.

The endodermal pouches play a much more important role in the pharyngeal arch development than previously thought (Veitch et al. 1999; Piotrowski and Nüsslein-Vorhard, 2000). Patterns of cranial NC cells along the anterior-posterior axis (Couly et al. 2002), and moreover, the signals produced in the ventral foregut endoderm are necessary for the normal patterning of the hyoid cartilage (Ruhin et al. 2003).

In the zebrafish mutant van gogh (vgo), the pharyngeal endoderm does not form, even though the hindbrain segmentation is unaffected, and the migrating neural crest cells initially form distinct streams but fuse when they reach the arches, due to the lack of pharyngeal pouches segmentations. The results from vgo suggest that the segmentation of the endoderm occurs without signaling from neural crest cells but that tissue interactions between the mesendoderm and the neural crest cells are required for the segmental appearance of the neural crest-derived cartilages in the pharyngeal arches (Piotrowski and Nüsslein-Vorhard, 2000).

We could not detect 5HT, 5HT2B receptor or any TPH isoform in the endodermal pouches at the 30 hpf stage. However, interestingly, nearly all essential signal molecules we identified in the endodermal pouches that are nessecary for the induction of serotonergic neurons in the hindbrain like: SHH, FGF8, and BMPs (Goridis and Roher 2002) (Fig. 4.1). May be these factors have the function to induce TPH2 expression in the neural crest derived 5HT single cells, that exist in the PA. Otherwise, they could promote the expression of TPH2 in the endodermal pouches. We need to improve our TPH2 riboprobe to perform more sensitive in situ hybridization experiments to clarify this issue.

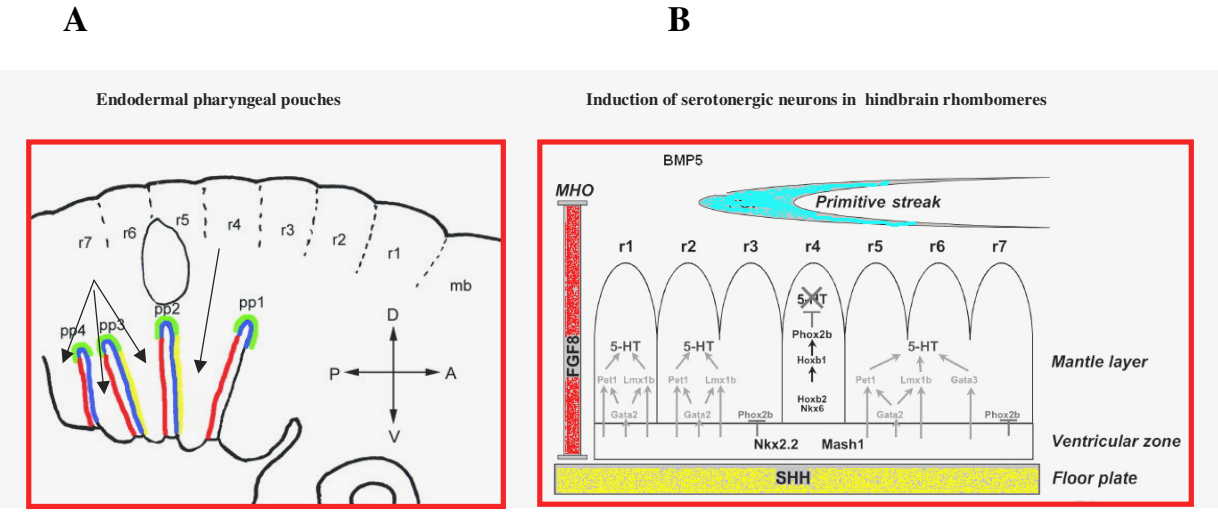

Fig. 4.1. Migrating cranial neural crest cells find the same signaling factor in the endermal pharyngeal pouches (A), that are essential for the induction 5HT cells in the hindbrain rhombomers (B). SHH (yellow), FGF8 (red), and BMPs (blue) (see text)

4.3. 5HT in the hindbrain

From about 36 hpf and onwards, 5HT was detectable in the anterior and posterior domain of the hindbrain by immunostaining. This correlated with TPH2 expression in the same time and tissue, as shown by in situ hybridization. Possibly, the real expression of TPH2 occured earlier, e.g. at least 6 h before this time, so that there was time for TPH2 protein synthesis and 5HT production. Wang et al. (2006) observed 5HT in the hindbrain already at 24 hpf. That is close to the time of migration of the cranial neural crest cells in the same tissue. Possibly, 5HT regulates their migration (Moiseiwitsch and Lauder; 1995) into the pharyngeal arches. This also explains the reduction of the cranial neural crest cells streams as shown in TPH2 morphants using the specific marker dlx2a at 24 hpf and 30hpf.

The 5HT signal did not disappear completely in the hindbrain of the TPH2 morphants. We can explain this observation due to one or more of the following reasons:

1) The assay that we used here – knock down by injection of morpholino antisense oligonucleotides has a limited efficiency in decreasing the target gene expression, and produces only reduction of gene expression for a maximum of 5 days after which the gene will be active again.
2) The 5HT antibody that we used here has a high sensitivity even in high dilutions. For example, we could hardly detect any 5HT signal difference in the intestine of wild type and TPH1 knock out mice, even when the knock out mice exhibited less than 5% of 5HT in comparison to the wild type (data not shown).
3) The 5HT transporter is still expressed in the hindbrain of the TPH2 morphants. Its function is to pump 5HT into the cells from the tissues around or from the blood circulation, when the cells need 5HT. This 5HT may be generated by other TPH isoforms in zebrafish.
4) We do not know about the expression pattern of the third TPH isoform-TPHD2-, which may be expressed close to the raphe nuclei.
5) Theoretically, due the gene duplication phenomena in the zebrafish, in contrast to the TPH genes that exist in mammals, there could be a fourth gene of TPH in the zebrafish. According to results of the Blast analysis of Genbank, the TPHD1 protein sequence is more similar to TPHD2 than to TPH2. By this means, the fourth principal gene could be duplicated from an TPH2 ancestral gene. The hypothetical fourth TPH isoform would then be similar to TPH2. In the zebrafish gene bank there are two predicted TPH2 isoforms, the first more similar to what we cloned (accession Nr.XM 67841) and our sequence (accession Nr. AY616134) and the other isoform (accession

Nr. XM 702088) and similar to what Teraoka et al. (2003) already cloned (accession Nr. AB12529). There are some differences between both sequences especially in the C-terminal domain, and moreover they have different start codons, and are both expressed -among other tissues - in the hindbrain. However since the major part of the sequence of the two genes is absolutely identical, we think both variants are generated by one single gene.

The role of Sox10 in the development of serotonergic neurons in the raphe nuclei

The defects in serotonergic neurons of Sox10 morphants raises many questions about the origin of this neurons in the CNS, and the role of Sox10 in their early development. We know that the expression of Sox10 in the first two days of zebrafish development is very dynamic. Sox10 is expressed in the neural crest cells and down regulated before their migration at 18 hpf. At 36 hpf, Sox10 expression is reinitiated in the region of the terminal nerve. At 48 hpf there a Sox10 expressing cells in the midbrain (Whitlock et al. 2005) and they most likely represent oligodendrocyte precursors (Woodruff et al., 2001). The oligodendrocytes in the central nervous system have the same function as the Schwann cells in the peripheral nervous system.

Both cells are characterized by Sox10 expression, and they produce myelin sheath surrounding the axons to stabilize the axons and to promote neurotransmission. In Sox10 knockout mice there is a defect in the both glial cells (oligodendrocytes and Schwann cells) and as consequence there are defects in the myelin sheath followed by defects in the development not only of the axons but also of the cell bodies of the neurons, especial of the motor neurons (Britsch et al. 2001). We know that the motor neurons are the precursor cells for central serotonergic neurons (Pattyn et al. 2003). In Sox10 zebrafish morphants there are also defects in neural crest derived central gonadotropin releasing hormone cells (White et al. 2005). Those cells are expressed in the same area as the central serotonergic neurons. We think, that the same mechanism is going on in the zebrafish. The Sox10 morphants have defects in the development of motor neurons, followed by defects in the serotonergic neurons.

4.4. 5HT in the hypothalamus

Serotonergic neurons in the hypothalamus have been reported in mammals (Lebrand et al. 1996) and also in the zebrafish (Bellipanni et al. 2002, McLean and Fetcho 2004). In mammals those neurons do not have serotonin synthesis activity and rather take up serotonin from serotonergic neurons, and, thus, these neurons are TPH negative but SERT-positive. The

pCPA treatment significantly reduced the hypothalamic levels of serotonin and its metabolite (5-hydroxyindole-3-acetic acid) in the rats (Aragŏn et al. 2005).

In zebrafish, serotonergic neurons of the hypothalamus express contrary to the situation in mammalians a tryptophan hydroxylase protein. It is TPHD1 according to the results of Bellipanni et al. (2002). However other authors (Teraoka et al. 2003, Wang et al. 2006) could not show expression of any TPH isoform in the hypothalamus, and those neurons do not express the SERT gene. In our experiments, we could detect 5HT in the hypothalamus of 2dpf wild type fish, but this signal was not affected by TPHD1 morpholino antisense. When the fish were injected with TPH2 morpholino antisense, the 5HT signal in the hypothalamus completely disappeared. This finding indicated first, that perhaps TPH2 was responsible for the 5HT existing in the hypothalamus, although TPH2 in hypothalamus was not detectable by in situ hybridisation probably due to its weak expression in this tissue in comparison to the raphe nucleus. Second, the development of the hypothalamus tissue could have been affected in TPH2 knock down fish. More experiments are needed to clarify these issues.

Interestingly the flexibility in the size and location of the mammalian serotonergic and dopaminergic neurons in the hindbrain, that express TPH2 and the midbrain, that express TH (Brodski et al. 2003) shifted to the hypothalamus, (Gou et al. 2003), as shown in the hypothalamus of zebrafish foggy mutant, that displays a reduction of dopaminergic neurons and a corresponding increase of the serotonergic neurons.

4.4. 5HT single cells

The origin of 5HT single cells and the source of their 5HT

5HT single cells are located in some neural crest derived tissues such as pharyngeal arches, dorsal root ganglia and enteric nervous system which lead us to hypothesise that those cells are also originating from neural crest cells. In particular, the TPH2 morphants exhibited defects in neural crest derived pharyngeal arches, which were similar to knockout in fish with Foxd3, an early gene marker of neural crest cells (Lister et al. 2006). A new observation is that the 5HT single cells in pharyngeal arches, dorsal root ganglia and intestine disappeared in 5dpf embryos by deletion of Foxd3, while the cells in the skin at 2 dpf of those fish were not affected. Also the Foxd3 morphants of Lister et al. (2006) exhibited defects in dorsal root ganglia and the enteric nervous system. It is known that the neural crest cells are migrating in two pathways, first laterally from dorsal neural tube to skin which gives rise to pigment cells and sensory neurons. The second pathway is ventral migration from which the pharyngeal arches, dorsal root ganglia and the enteric nervous system are derived. Therefore, we

speculate that the transcription factor Foxd3 regulate 5HT single cells that migrate ventrally, and not those cells in the skin that migrated laterally. Our search for a candidate gene that regulates laterally migrating neural crest cells led us to test Sox10 that is expressed early in neural crest cells. Sox10 mutant fish lacked melanocytes in the skin, and we observed that 5HT single cells in our Sox10 morphants disappeared from the skin. By tunel test that detect apoptotic cells, the skin of those Sox10 morphants was full of positive cells with different shapes (data not shown). Probably 5HT single cells were among those dead cells. Taken together, 5HT single cells are neural crest derived cells and during development of the fish are divided into two subgroups depending on the pathway of crest cell migration. The first group, regulated by Sox10 migrate laterally to the skin and show 5HT at an early stage as seen in the 36 dpf embryos. The cells of the second group migrate ventrally to three target tissues: pharyngeal arches, dorsal root ganglia, and intestine. We think then they differentiate depend signalling factors producing from the target tissues or adjacent to them. Probably, the cells in the pharyngeal arches will express Gcm2 (Fig. 3.10.A), and the cells in dorsal root ganglia and intestine become Hu positive (Fig. 3.14.A). 5HT single cells of this group show 5HT at later stages in contrast to the laterally migrated group. For instance, at 3 dpf in pharyngeal arches, and at 5 dpf in dorsal root ganglia and intestine.

In the early stages of mouse development (E15), TPH2 mRNA is expressed in the dorsal root ganglia (Gensat project). It is also interesting that the mouse bowel contained 5HT synthesized from both TPH isoforms. 5HT is produced by TPH1 in enterochromaffin cells and by TPH2 in the neurons of the enteric nervous system (Gershon, 2005). Both enterochromaffin cells and the neurons of the enteric nervous system are derived from the neural crest. Probably because that Foxd3 morphants lack 5HT single cells in the intestine of 5dpf embryos, while the TPHD1 and TPH2 morphants separately still show 5HT cells there, or because the efficiency of the morpholino antisens was already lost in those 5 dpf morphants. The double knock down embryos of both the TPHD1/TPH2 genes died before they reach the gastrulation stage.

We think 5HT single cells did not exhibit 5HT as they were located in the dorsal neural tube, and in the next stages of their development they delaminate from the epithelium and transform into mesenchymal form and migrate separately as single cells. At 24 hpf stage the laterally migrated cells reach their targeted tissue in the skin and after few hours, exactly at 36 hpf they become 5HT positive cells. This is the same time that we can detect 5HT in the raphe nucleus. This correlation in the time of 5HT signal let us speculate that TPH2 could be the enzyme synthesizing 5HT in single cells. Moreover, we could detect strong reduction in 5HT

single cells at 2dpf of TPH2 morphants in contrast to TPHD1 morphants or wild type. The results of in situ hybridisation of 24 hpf by TPH2 riboprobe gave weak positive reactivity in some cells that have the same distribution in the skin (data not shown). But we are still not sure if they are the same 5HT single cells. A specific antibody against zebrafish TPH2 which is not get available may identify the source of 5HT in those single cells.

We next tested if 5HT single cells receive 5HT by the plasma membrane 5HT transporter. In situ hybridisation experiments using 5HT transporter riboprobe gave no signal in those cells in contrast to a clear signal in the raphe nucleus. Moreover, when we exposed fish embryos to the 5HT transporter blocker, fluoxetine, we could not observe any differences in the amount of 5HT single cells in the skin of 2dpf fish or in pharyngeal arches of 5dpf fish (data not shown). However the effectiveness of fluoxetine against 5HT transporter of zebrafish is not clear.

Recently Jonz and Nurse (2006) also described 5HT single cells in the skin of 2dpf zebrafish and showed the cells are innervated, and postulated these cells are extrabranchial O_2 chemoreceptors as possible to maintain sufficient gas exchange across the skin in the earlier stages of zebrafish before appearance of the gill. However the authors could not detect any other cell marker for those cells other than 5HT. This postulated chemoreception is either oriented externally to sense oxygen changes in the water or internally to sense changes oxygen in blood. This chemoreception function is similar to the function of 5HT neurons in the rat medullary raphe that is reported by Risherson et al. (2004). These authors showed that 5HT neurons are highly sensitive to CO_2 and pH change in blood circulation and expressed thyrotropin releasing hormone (TRH) and substance P. We tested if both proteins also co-localized with 5HT in raphe or in 5HT single cells in the zebrafish, but there was no co-localization (data not shown). We should mentioned here, that Risherson et al. worked with brain of adult rat, and our experiments involved zebrafish embryos. More experiments are still needed to identify the function of 5HT single cells in skin of zebrafish embryos.

Jonz and Nurse also observed 5HT single cells in the gill arches of zebrafish embryos at 5 dpf and could not detect their function, but showed the respiratory function of those cells in the gill filaments at 9 dpf embryos. When the fish is exposed to hypoxia there is an increase of blood ventilation in the vessels of the gills in comparison to the control fish to maintain sufficient gas exchange under hypoxia conditions. Jonz and Nurse discussed a similarity in the function of those cells and glomus cells in the carotid bodies of mammals. Our immunostaining for tyrosine hydroxylase (TH), a marker for glomus cells gave no co-localization with 5HT in those cells. Jonz and Nurse could not detect any other cell marker for

all 5HT single cells, and also could not identify the origin of those cells and the source of its 5HT.

Our experiments could detect 5HT single cells in the pharyngeal arches of 3dpf embryos during neural crest differentiation to cartilage cells and identified their location in a specific pattern (pair formation) probably surrounding by Sox9a positive neural crest cells (Fig.3.10.D and E). This typical location becomes more obvious in later stages (5dpf) and is restricted to the posterior five branchial arches, while in the mandibular and the hyoid arches they form a pointed single line (Fig.3.10.B and C). We have also previously shown that 5HT2B receptors are localized in the pharyngeal arches of 3dpf embryos (Fig.3.34). We postulate that there is some kind of interaction during the pharyngeal arches morphogenesis between 5HT single cells and the cells surrounding them, which probably express 5HT2B receptors as shown in Fig.4.2B. This interaction may stimulate the signaling pathway of chondrogenesis in these Sox9a positive neural crest derived cartilage cells through 5HT2B receptors. 5HT2B receptors couple to phospholipase C to generate diacylglycerol that activate protein kinase C (Nebigil et al. 2001, Walker et al. 2007) thereby upregulating the biosynthesis of the cartilage proteoglycan core protein, a marker for cartilage matrix (Bhasin et al. 2004), which can be detected by alcian blue staining. We think when the precursors of 5HT single cells entered the pharyngeal pouches during neural crest migration they might have interacted with signaling molecules like SHH and Fgf8 produced locally there and essential for TPH2 expression and 5HT synthesis.

During our search for other markers for 5HT single cells, we found that Gcm2 was expressed in the same pattern as 5HT single cells in the branchial arches Fig.3.10A. Gcm2 is expressed in the ectodermal epithelium of the posterior pharyngeal arches 3-7, and in early gill filaments as they bud from this region (Hogan et al. 2004). Interestingly, the Gcm2 knock down zebrafish (Hogan et al. 2004, Hanaoka et al. 2004) exhibited exactly the same defects in the elements of the pharyngeal arches like TPH2 knock down fish. Probably Gcm2 and 5HT are sharing the same signaling pathway in the morphogenesis of pharyngeal arches and both cell types (Gcm2 expressing cells and 5HT single cells) are playing an additional role in gill filaments budding beside the role in pharyngeal arches development, so long the gill appears in the same tissues of the pharyngeal arches in early stages of zebrafish development.

The expression of Gcm2 is dependent on specific signaling molecules like Fgf3 and Fgf1 from the adjacent pharyngeal endoderm (Hanaoka et al. 2004, Hall et al. 2006), similarly as the 5HT single cells may be dependent on signaling proteins like SHH and Fgf8 from pharyngeal endoderm (Graham 2004).

Endothelin also co-localizes with 5HT in some gill cells (Zaccone et al. 1996) and play an important role in the development of the pharyngeal arches of the zebrafish throughout neural crest cells (Thomas et. al 1998, Miller et al. 2000) and required for this function phospholipase C as 5HT2B receptor (Walker et al. 2007, Nebigil et al. 2001). Probably there is an interaction between 5HT system and endothelin system during pharyngeal arches development.

Taken together, 5HT single cells originated from neural crest cells, but their function is still unclear.

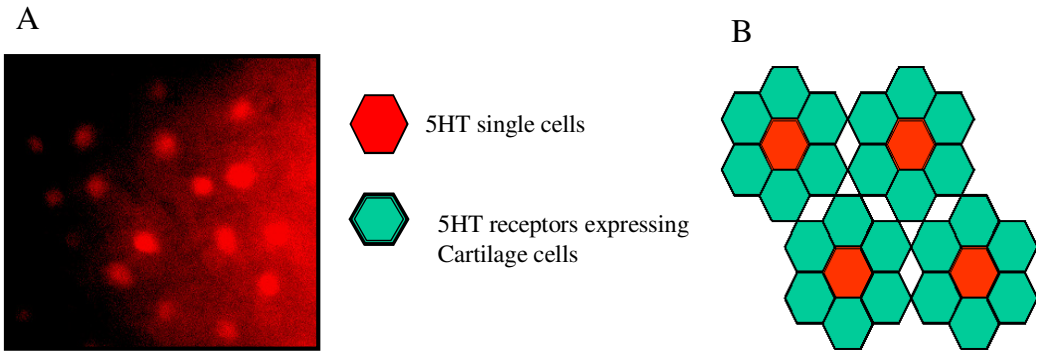

Fig. 4.2. 5HT immunostainig detects 5HT single cells (red) in 5th and 6th pharyngeal arches of 5dpf zebrafish embryos (A). A postulated scheme of one pharyngeal arch shows 5HT receptor expressing cartilage cells (green) which surround5HT single cells (red) during morphogenesis (B).

4.6. Independent gene encode TPH in Drosophila melanogaster

Neckameyer and White (1992) proposed, that there are only two aromatic amino acid hydroxylases (AAAH) in Drosophila, the first tyrosine hydroxylase and the second an enzyme (Henna) with both activities, phenylalanine hydroxylase (PAH) and tryptophan hydroxylase (TPH). But we could clone CG2199 a third AAAH in the Drosophila and termed it DmTPH, We showed that its amino acid sequence is more homologous to TPH of other species more than Henna. Also the phylogenetic tree is showing that DmTPH and TPH of other invertebrates (TPH of C.elegans and schistosoma manasoni) share one common ancestor enzyme (data not shown). Moreover, the ability of DmTPH to hydroxylate Trp to 5OHTrp in vitro is higher than DmPAH (Henna) as detected by the TPH enzyme activity assay (Fig.3.39), and Km values show that Trp is more a suitable substrate for DmTPH than for Henna (Table 3). Accordingly when we used 7OHTrp as substrate, we observed more cell death in DmTPH transfected cells than in Henna transfected cells, probably because more of the toxic product 5,7DHT was synthesized by hydroxylation of 7OHTrp in DmTPH cells than

in Henna cells. These results led us postulate that the protein product of the CG9122 gene (DmTPH) is the specific tryptophan hydroxylase enzyme in Drosophila melanogaster.

Alcaniz et al. (1997) have shown that PAH defect mutant of Drosophila have very low levels of 5HT. Moreover patients with PAH deficiency suffering from phenylketonuria (PKU) and accumulate phenylalanine which inhibit TPH and as a result PKU patients exhibit low 5HT level in the blood. Probably this regulation mechanism of led Alcaniz et al. (1997) think that PAH is necessary for 5HT synthesis in Drosophila.

In mammals in vivo, PAH that expressed massive in the liver can not synthesize 5HT as exhibit TPH1 knock out mice, they lack from 5HT in periphery blood circulation (Walther and Bader 2003), even their PAH in liver should be normal work, but (Alcaniz et al. 1997) reported that PAH of Drosophila can make in vivo 5HT, the authors means PAH work also as TPH because TPH did not exist in Drosophila as independing gene.

Parallel and independant from our work Coleman and Nekameyer reported 2005 that the gene product of CG9122 exhibit specific tryptophan hydroxylase activity in Drosophila, but they still believe that Henna can synthesize 5HT in vivo. The question if Henna can really synthesizes 5HT in Drosophila, can only be answered by generating a DmTPH deficient Drosophila mutant. In vitro, we observed that Henna transfected cells can hydroxylate Trp and exhibit around 50% of DmTPH activity (Fig. 3.39). However Trp is a bad substrate for mammalian PAH (Fitzpatrick 1991). This difference in the ability to hydroxylate Trp between mammalian PAH and dmPAH (Henna) may due some differences in their amino acid sequences. Two amino acid residues are important of Trp binding site in mammalian TPH. All TPHs from human and Drosophila have phenylalanine residues at both positions, and human PAH has a tryptophan residue at the first position (W326) and a phenylalanine residue at the second position (F331), but in Henna there is a tryptophan residue at the first position (W320) and a tyrosine residue at the second position (Y325) as shown in Table 4. Probably the replacement of F331 in human PAH with Y325 in Henna produces a suitable configuration to allow Trp to enter easily into the active center of Henna increasing its ability to hydroxylate Trp in vitro better than human PAH.

	hTPH1	hTPH2	DmTPH	hPAH	DmPAH (Henna)
1.	F313	F341	F382	W326	W320
2.	F318	F346	F387	F331	Y325

Table 4. Two amino acid residues involved in the binding of Trp in TPH from human and Drosophila, and the corresponding amino acid residues in PAH.

F: phenylalanine, W: tryptophan, Y:tyrosine. The number is indicating the position of the amino acid in the protein sequence.

4.7. Generation of TPH2 Knock out mouse

To investigate the role of 5HT system during mammalian embryogenesis, it was very important to generate TPH2 knockout. Especially, as the previous knockouts of 5HT system TPH1, SERT and 5HT receptors, except 5HT2B, did not show an important role in development. Pharmacological loss of function studies showed the role of 5HT in first cell divisions, and in many other developmental processes like cell proliferation, differentiation, and apoptosis (Buznikov et al. 1996, Azmitia 2001), and morphogenesis of different tissues and organs like central and enteric nervous system, craniofacial, bones, blood circulation system, and in embryonic left-right asymmetry (Sodhi and Sanders-Bush 2004, Gershon et al. 1990, Moiseiwitsch JR and Lauder JM. 1995, Nebigil et al. 2000, Azmitia 2007, Fukumoto et al. 2005). Nevertheless the role of 5HT in mammalian development remained unresolved until the generation of TPH2 knockout mice. We have generated 12 chimeric mice using ES cells after TPH2 gene targeting. PCR results showed that these manipulated ES cells were differentiated and distributed into different organs and tissues like skin, heart, brain and liver etc. of the generated chimeric mice (data not shown). But due to unknown reasons, this flexible ability of the manipulated ES cells in the generation of different kinds of differentiated cells was not involving the germ line (sperms or oocytes). This deficiency in germ line transmission of TPH2 manipulated ES cells may have been due a loss of pluripotency during cell culture. However the same ES cell line was already successfully used in our group in the generation of TPH1 knock out mice. This deficiency in germ line transmission led us to shift our orientation on a role of 5HT in the reproduction process since we knew about the massive expression of TPH2 in the testis. Then we started to follow the presence of TPH2 mRNA and protein in the sperms, and also followed the literature refering to this issue. We could show the expression of TPH2 and not TPH1 in the testis of the mice, and this expression start to appear only in puberty of the mice and not before, correlated with sperm production. Moreover immunostaning of rat testis sections showed positive signals for TPH in the spermatocytes, the precursors of the mature sperms, and this was supported by an other group using specific TPH2 riboprobe for in situ hybridisation of mice testis (Walther DJ. personal communication). Also the presence of TPH2 mRNA in human sperms combined with the above mentioned data, gave us enough clues to believe that TPH2 has an important role in the production of sperms in mammals. Others studies also observed many developmental roles of 5HT in the fertilization and reproduction processes, and also during oocytes and sperms maturation. Before fertilization, 5HT7 receptors participate in the egg

maturation. Eggs are arrested at late prophase (G2 arrest) of meiosis for oocytes maturation and 5HT7 receptors maintain this arrest (Sheng et al. 2004).

Rats injected with TPH inhibitor pCPA, have only 25 % of sperms as compared to control mice and only 4% of them are motile (Aragõn et al. 2005). The same authors have also studied the role of 5HT in the onset of puberty and spermatogenesis. They observed that the depletion of 5HT was accompanied by loss of germ cells in the testis and then by a decrease in sperm production, and also 5HT stimulates sperm motility (Parisi et al. 1984). During the fertilization process *in vitro*, 5HT stimulates the acrosome reactions of hamster sperm and this stimulation can be inhibited by 5HT receptors antagonists like quipazine or cyproheptadine (Meizel and Turner 1983).

5HT can also increase the intracellular calcium in rat 5HT1C receptors expressing Xenopus and starfish eggs. This intracellular calcium opens ion channels and causes cortical granule exocytosis leading to egg activation (Kline et al. 1988, Schilling et al. 1990). These fertilization events are normally initiated by sperms, maybe mediated by endogenous 5HT receptors. 5HT1D receptors are present in unfertilized oocytes, 2 cell embryos, morula, and blastocysts of mice (Vasela et al. 2003, Il'kova et al. 2004).

The results of Aragõn et al. (2005) had described the importance of 5HT for production of sperms. Together with our results that identified the expression of TPH2 in the spermatocytes we think TPH2 is the source of 5HT that plays a role in the spermatogenesis. Because we think that ES cells with a defect of TPH2 expression are not able to produce healthy sperms. To support the hypothesis as shown for germ line transmission we need to use more different ES lines in the targeting of TPH2.

Taken together, from the data above, we believe the developmental role of 5HT system in egg fertilization and early developmental stages of embryos due to interaction between the ligand 5HT synthesized by TPH2 in sperms and 5HT1D receptors expressed on unfertilised eggs. This interaction may lead to stimulate the process of fertilization and activate fertilized eggs and other fertilization events.