1. Introduction

1.1. Serotonin system

1.1.1. Discovery of serotonin

In the 1930s Dr. Erspamer extracted from the rabbit gastric mucosa a substance that can induce smooth muscle contraction. He named this substance enteramine (Erspamer et al. 1952). In the late 1940s Dr. Page at the Cleveland Clinic isolated a vasoconstricting substance in serum and named it serotonin. The structure of serotonin was reported in 1949. Around 1952 it was realized that enteramine and serotonin were the same substance. In 1952 Twarog tested the idea that serotonin might also be used as neurotransmitter in vertebrates. Her research resulted in the identification of serotonin in the brain (Twarog and Page 1953).

1.1.2. Biosynthesis and metabolism of serotonin

1.1.2.1. Biosynthesis

Serotonin is made from the essential amino acid tryptophan. This amino acid contains an indole ring and is obtained from dietary sources (humans can not make tryptophan). It is the least common amino acid in natural proteins. The rate-limiting step for the synthesis of serotonin from tryptophan catalysed by tryptophan hydroxylase (TPH; EC 1.14.16.4) (Grahame-Smith 1964; Lovenberg et al. 1967). TPH uses Fe^{2+} as cofactor and O_2 and tetrahydrobiopterin (BH4) as co-substrate to hydroxylate tryptophan generating 5-hydroxytryptophan (Kaufmann 1967). This metabolite is decarboxylated by aromatic amino acid decarboxylase (AAAD) to 5-hydroxytryptamine (5HT) or serotonin (Fig.1.1).

The enzyme TPH belongs to a superfamily of aromatic amino acid hydroxylases (AAAH), together with phenylalanine (PAH) and tyrosine hydroxylase (TH) (Fitzpatrick 1999).

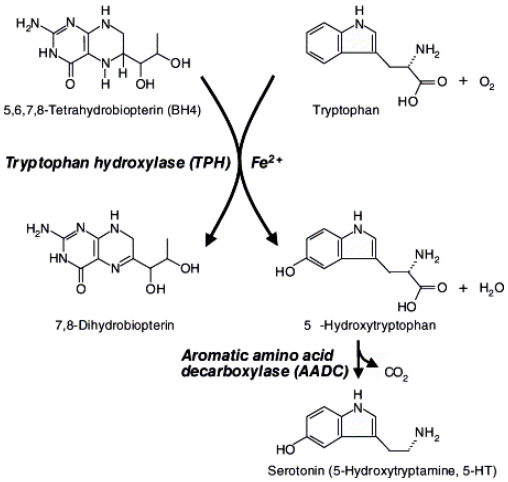

Fig. 1.1. Catalytic mechanisms in serotonin synthesis. Tryptophan hydroxylase (TPH) is the rate limiting enzyme in serotonin synthesis and catalyzes the hydroxylation of tryptophan using the cofactor Fe^{2+} and the co-substrates O_2 and tetrahydrobiopterin (BH4). Aromatic amino acid decarboxylase (AADC) decarboxylates the resulting 5-hydroxytryptophan to yield 5-hydroxytryptamine (5-HT) or serotonin (Walther and Bader 2003).

1.1.2.1.1. Tryptophan hydroxylases

To study the developmental and physiological effect of the loss of 5HT synthesis our group generated TPH Knock out mice (Walther et al. 2003). We expected a lethal phenotype as had been found for TH Knock out mice (Zhou et al. 1995), or at least strong developmental defects as pharmacological studies initially showed that 5HT can modulate a number of developmental events, including cell division, neuronal migration, cell differentiation and synaptogenesis (Gaspar et al. 2003). It therefore came as a surprise that TPH1 mutant mice had no abnormalities of brain development. The reason was that they lack only 5HT in the periphery, in particular in the gut, in the blood and in the pineal gland, but there was only a minor reduction in the 5HT level in the serotonergic brain regions, suggesting the existence of a second TPH gene not affected by the gene manipulation. Therefore our group screened the Human and Zebrafish Genome Database and detected a homolog to the human TPH in both species and named it TPH2, and the original TPH was renamed TPH1.

TPH2 full length cDNA was isolated from zebrafish, mouse, rat, and human, and the expression of TPH2 cDNA in cell culture enabled the expressing cells to synthesize the direct product of tryptophan hydroxylation 5-hydroxytryptophan, proving the identity of TPH2 as a real tryptophan hydroxylase (Walther et al. 2003).

1.1.2.1.2. Characteristics of TPH1 and TPH2

TPH isoforms are organized into N-terminal regulatory and C-terminal catalytic domains, and show highly homologous protein sequence with 71% identity in humans. In TPH2 all amino acids which have been detected to be important for the structural and functional properties of TPH1 are conserved. Already in previous reports using purified TPH samples prepared from brain stem have shown that this TPH (TPH2) can be phosphorylated by Ca^{2+} /calmodulin-dependent kinase II (CaMKII) and protein kinase A (PKA) like TPH samples prepared from the mouse mast cell line P815 (TPH1) (Ehret et al. 1989). The phosphorylation sites for CaMKII have been identified to serine 58 and 260 and for PKA to serine 58 in recombinant TPH1 (Johansen et al. 1996; Kuhn et al. 1997). Both are conserved in TPH2 suggesting that these are also the phosphorylation sites in this new neural isoform. After phosphorylation, a 14-3-3 protein binds to the phosophoserine residue 58 in TPH1, increasing the activity and inhibiting its dephosphorylation (Meek and Neff 1972).

Moreover, a C-terminal leucine zipper involved in formation of the tetrameric holoenzyme is also conserved in both isoforms (Fig. 1.2).

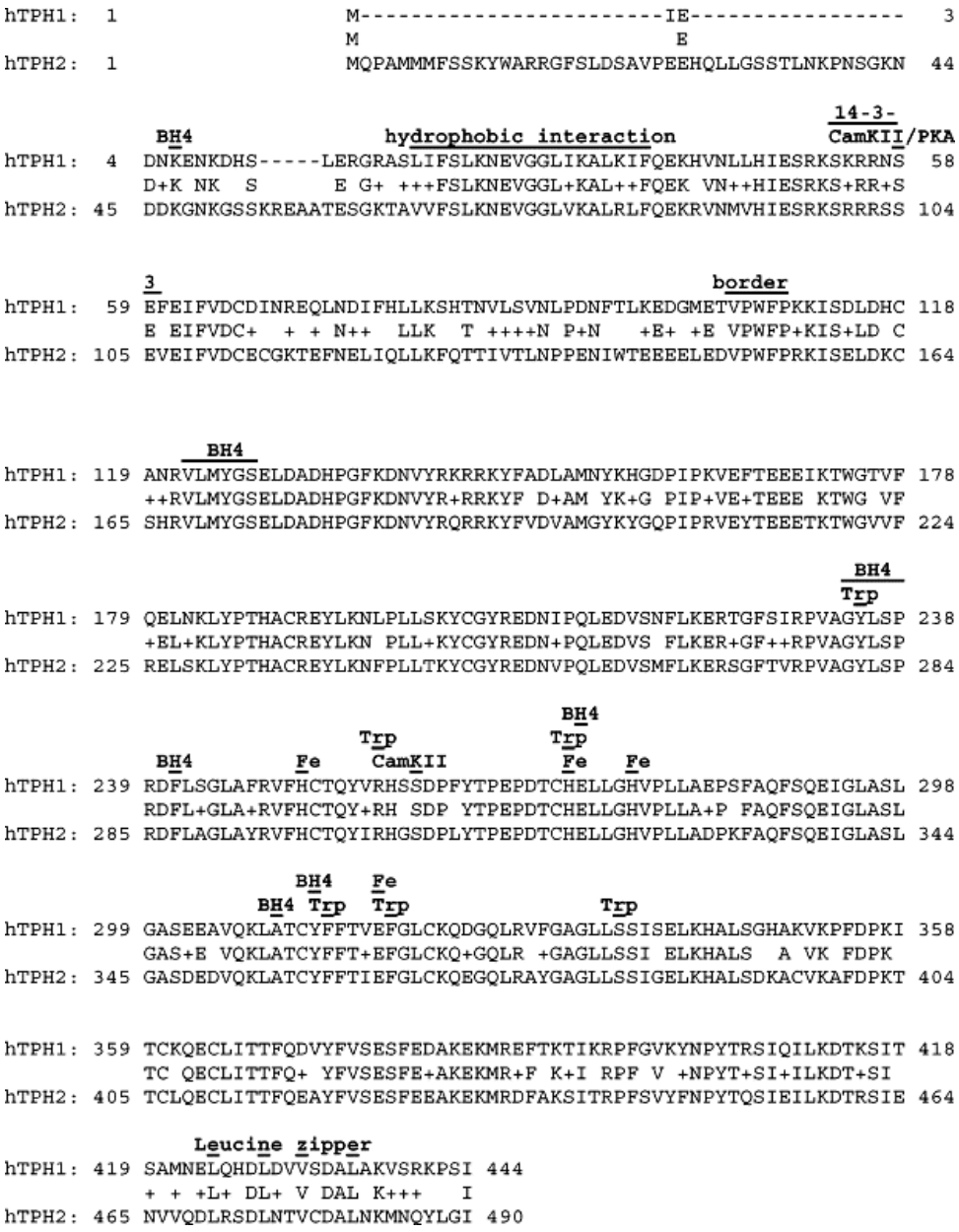

Fig. 1.2. Comparison of human TPH1 and TPH2. The central line indicates identical and similar (+) amino acid residues. Functionally important residues of TPH1 are marked. Fe: iron (Fe^{2+}) binding site, Trp, tryptophan binding site, BH4, co-substrate binding site, 14-3-3, binding site for 14-3-3 proteins, PKA: protein kinase A phosphorylation site; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation site; also, the hydrophobic interaction domain and the leucine zipper involved in multimerization and the border between the regulatory and the catalytic domains are shown (Walther and Bader 2003).

1.1.2.2. Metabolism of serotonin

1.1.2.2.1. Monoamine oxidase (MAO)

The main pathway of 5HT catabolism is oxidative deamination by the monoamine oxidase (MAO). MAO converts 5HT to 5-hydroxyindoleacetaldehyde, and this product is oxidized to the form 5-hydroxyindoleacetic acid (5HIAA) (Fig.1.3).

MAO is a flavoprotein enzyme, located in the outer membrane of the mitochondria, and participates in the degradation of several monoamines like serotonin, dopamine and noradrenalin. In the central nervous system, MAO plays an important physiological role by regulating the concentration of the biogenic amines and keeping them in the normal range. This avoids accumulation of the neurotransmitter leading to neurotransmission overstimulation.

In mammals there are two MAO isoforms, A and B. In the brain, MAO A expressing cells are located in cells containing catecholamines, whereas MAO B positive cells are observed in serotonergic neurons, like the nucleus raphe dorsalis and nucleus centralis superior (Westlund et al. 1985).

In the hypothalamus, MAO A and B, are also expressed in different cell groups, in the lateral and tuberal regions, respectively (Westlund et al. 1988).

In the zebrafish, surprisingly, there is only one single MAO gene, expressed mainly in the brain and liver. Its FAD cofactor and substrate binding site sequences are similar to mammals, but with different sequences of the transmembrane domains (Setini et al. 2005; Anichtchik et al. 2006).

1.1.2.2.2. Arylalkylamin-N-Acetyltransferase (AANAT)

AANAT also called serotonin -N-acetyltransferase, belongs to the superfamily of acetyltransferases. AANAT is distributed in different species and is an important enzyme in melatonin synthesis (Fig.1.3), controls the night/day rhythm in the vertebrate pineal gland, and regulates reproduction in different seasons during the whole year (Smith 1990). Melatonin modulates the function of the circadian clock in the suprachiasmatic nucleus (McArthur et al. 1997). AANAT influences activity and sleep, its enzyme activity is blocked when animals are exposed to light at night (Wang et al. 2004).

The zebrafish have two aanat genes, aanat1 and aanat2; the former is expressed only in the retina and the second is expressed in both the retina and the pineal gland (Begay et al. 1998; Gothilf et al. 1999).

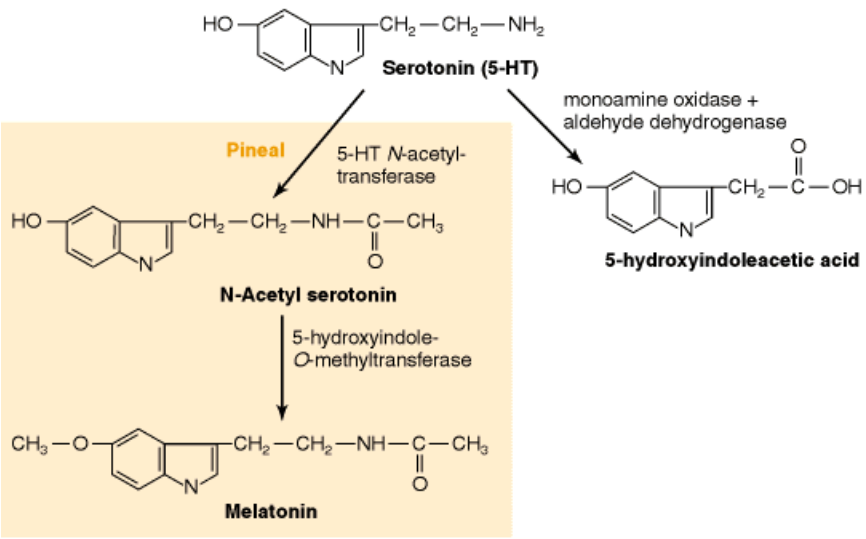

Fig. 1.3. The two main catabolism pathways of serotonin. The first is mediated by MAO and the end product is 5HIAA, and the second pathway converts 5HT in the pineal gland by AANAT to melatonin (Basic Neurochemistry 1996).

1.1.3. 5HT receptors

Serotonin mediates a wide range of physiological functions by interacting with many receptors that exist almost in all species, invertebrates and vertebrates, and these receptors have been implicated in playing important roles in certain pathological and psychopathological conditions. These receptors are located on the cell membrane of nerve cells and other cell types.

1.1.3.1. Classification

Till now 16 mammalian 5HT receptors are detected, and divided in seven distinct groups according to their amino acid sequence homology and the signalling pathways, 5HT1, 5HT2, 5HT3, 5HT4, 5HT5, 5HT6, and 5HT7 (Hoyer and Martin 1997). Group 5HT1 comprises seven members identified as 5HT1A, 5HT1B, 5HT1D, 5HT1E, 5HT1F, 5HT1P, and 5HT1S. The 5HT2 receptor group contains 3 subgroup, 5HT2A, 5HT2B, and 5HT2C, and there are two receptors in the 5HT5 receptor group, 5HT5A and 5HT5B, which is missing in mammals (Grailhe et al. 2001). For the rest of the 5HT receptor groups, 5HT3, 5HT4, 5HT6, and 5HT7 every group contains only one receptor.

1.1.3.2. Mechanism of action

With the exception of the 5-HT3 receptor, a ligand gated ion channel, all other 5-HT receptors belong to the group of G protein coupled seven transmembrane receptors (Fig.1.4), and

mediate cellular signalling effects by activating an intracellular second messenger cascade like adenylate cyclase or phospholipase C as following:

- 5-HT1 receptors are Gi/Go coupled and reduce cellular cAMP (Lanfumey and Hamon 2004).
- 5-HT2 receptors are Gq/G11 coupled, and increase cellular level of inositol trisphosphate (IP3) and diacylglycerol (DAG) (Nebigil et al. 2001).
- 5-HT3 receptors are ligand-gated Na+ and K+ cation channels and induce direct plasma membrane depolarization (Barnes and Sharp 1999).
- The 5-HT4 receptor is Gs coupled and increases cellular cAMP.
- The 5-HT5 receptor is Gi/o coupled and inhibits adenylate cyclase reaction (Nelson 2004).
- The 5-HT6 receptor is also G protein coupled and increases adenylate cyclase activity (Woolley et al. 2004).
- The 5-HT7 receptor is Gs coupled and increases cAMP (Thomas and Hagen 2004).

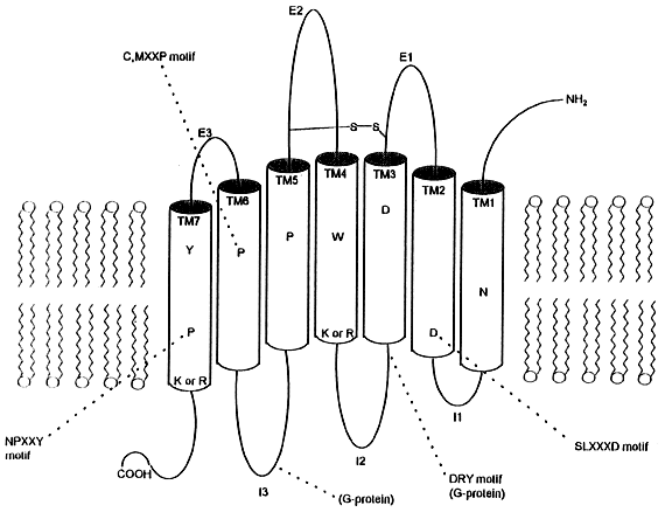

Fig. 1.4. Structure of G protein coupled seven transmembrane receptors. The indicated amino acids are highly conserved in serotonin receptors.

1.1.3.3. Localization

5HT receptors in neuronal synapses can be located presynaptically or postsynaptically. 5HT1A receptors are expressed in different regions of the mammalian brain, mainly in the hippocampus, septum, amygdala, and cortical limbic areas. 5HT1A receptors located in the raphe nuclei correspond to somatodendritic autoreceptors. 5HT1A is also found during embryonal development in craniofacial tissues (Moiseiwitsch and Lauder 1995).

5-HT1B receptors are expressed in the brain and located presynaptically, to control the release of 5HT, and postsynaptically in the substantia nigra, globus pallidus, and dorsal subiculum. 5HT1D receptors are expressed also in central nervous system (CNS). The neocortex is the highest expression region of 5HT2A receptors in the brain. 5HT2B receptors are expressed during mouse embryogenesis in heart and craniofacial tissues (Choi et al. 1997), and in embryonal chicken bone cells (Westbroek et al. 2001). 5HT3 receptors are found in the periphery and central nervous system. 5HT4 receptors are found in the hippocampus, and peripherally in the ileum. 5HT5A receptors are detected in the CNS mainly in the astrocytes. The expression of 5HT6 receptors is restricted to the CNS. 5HT7 receptors are found mainly in the CNS, but also at low level peripherally (Glennon et al. www.acnp.org).

1.1.4. Serotonin transporter

Serotonin transporter (SERT) is expressed in vertebrates and invertebrates. In mammals, SERT is expressed in the raphe nucleus and also in other brains tissues like amygdala, thalamus and hypothalamus. SERT was found also in nonneuronal tisses for instance in the placenta, lungs, blood platelets, mast cells and neural crest cell derived tissues like autonomic ganglia, tooth primordia, adrenal medulla, chondrocytes and neuroepithelial cells in the skin, heart, intestine and lungs, and also in some peripheral sensory cells like cochlear and retinal ganglionic cells, taste buds and hair follicles. In the zebrafish, due the whole genome duplication phenomena (Amores et al. 1998), Wang et al. (2006) could clone two cDNAs for SERT named SERTa and SERTb. SERTa amino acid sequence showed high identity with the SERT of mammals and is expressed in the raphe nucleus, tuberculum and in the pineal gland and moreover the 5HT reuptake inhibitor fluoxetine can inhibit the activity of SERTa. SERTb is expressed in the medulla oblongata and in the retina.

SERT is a transmembrane protein and human SERT has 12 transmembrane domains (TM). Both N- and C- termini are located in the cytoplasma. The 5HT binding site is localized within TMs 1-3 and TMs 8-12. SERT belongs to the family of sodium and chloride dependent transporters like dopamine and norepinephrine transporters and is regulated by protein kinases, there are six sites for the phosphorylation by protein kinase A and protein kinase C. According to a simple model for SERT function in the neurons. In the synaptic transmission process 5HT released into the synaptic cleft induces effects by selective 5HT receptors on the postsynaptic neurons. SERT localized in the presynaptic membrane, recycles 5HT to avoid constant stimulation of postsynaptic cells by accumulation of 5HT in the synaptic cleft. First a sodium ion binds to SERT followed by the binding of 5HT and a chloride anion, this complex

induces a conformational change in the SERT protein. The transporter then flips inside the cell, releasing serotonin. In the cytoplasm a potassium ion binds to SERT, and the transporter flips back out into the synaptic cleft, ready to receive another serotonin molecule (Basic Neurochemistry; 1998).

After 5HT biosynthesis in the presynaptic neuron 5HT is removed from the cytoplasm and transported into vesicles by the neuronal vesicular monoamine transporter (VMAT2) (Fig.1.5). Two vesicular monoamine transporters (VMAT) have been cloned, containing 12 transmembrane domains. The first transporter, cloned from chromaffin granules, has been termed VMAT1. It contains 521 amino acids and a large loop between transmembrane domains 1 and 2, which faces the lumen of the vesicle, contains sites for glycosylation. Both the NH2 and COOH termini are oriented to the cytoplasm. A second vesicular transporter (VMAT2), cloned from rat brain, is 62% identical to VMAT1. Human chromosome 8 contains the gene for VMAT1 and chromosome 10 contains the gene for VMAT2. Storage of 5HT in vesicles requires its active transport from the cytoplasm. The vesicular transporter uses the electrochemical gradient generated by a vesicular H^+-ATPase to drive transport, such that a cytoplasmic amine is exchanged for a luminal proton; that is, uptake of 5HT is coupled to efflux of H^+.

Fluoxetine inhibits the membrane transport of 5HT by SERT, and fenfluramine blocks the vesicular transport by VMAT (Fig.1.6).

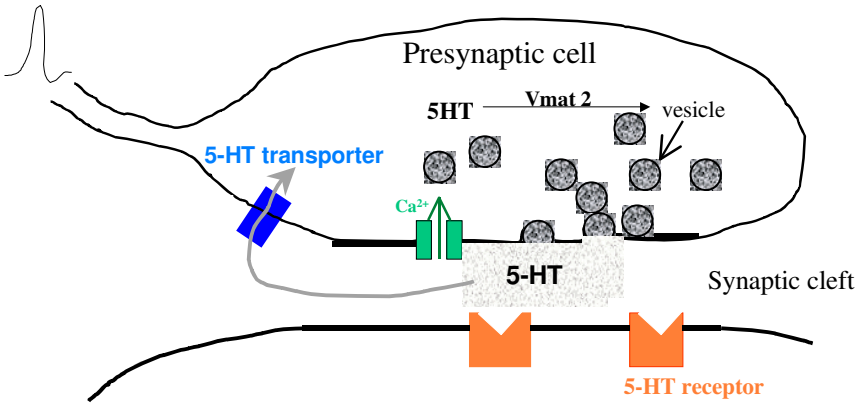

Fig. 1.5. 5HT recycling in the synapse. 5HT is synthesized in the presynaptic cell then transported into vesicles by the neuronal vesicular monoamine transporter (VMAT 2) to eventually undergo Ca^{2+} -mediated release. Extracellular 5HT acts on 5HT receptors located on the membrane of the postsynaptic cell. 5HT activity is terminated by re-uptake through the serotonin transporter (SERT), which under certain conditions can also reverse its orientation and mediate vesicle independent neurotransmitter release.

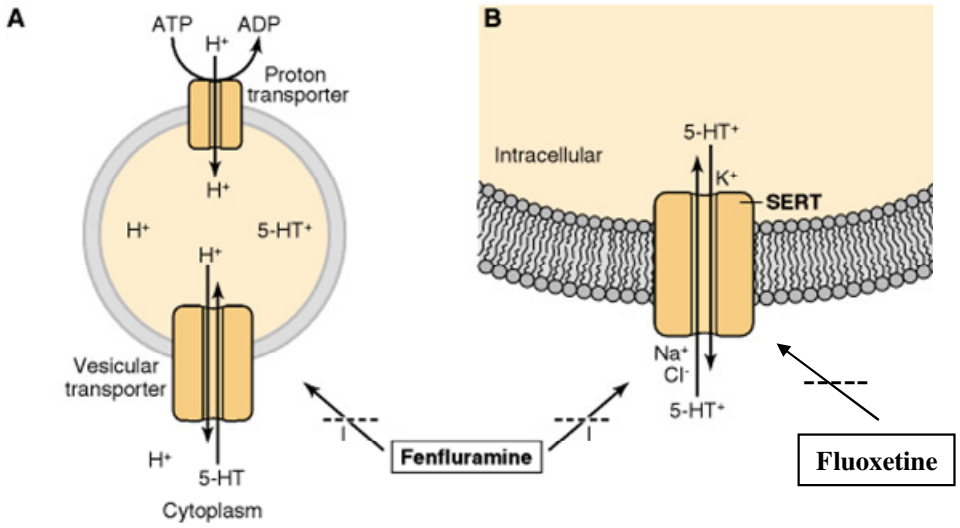

Fig. 1.6. Fluoxetine binds to SERT with a higher affinity than 5HT and blocks 5HT from binding and the transporter from flipping inside the presynaptic cell. The amphetamine fenfluramine inhibits the transport of 5HT by both (A) the vesicular transporter (VMAT) and (B) the serotonin transporter (SERT). Fenfluramine stimulates the release of 5HT from serotonergic terminals, by blocking the vesicular transporter and disrupts the proton gradient across the vesicle membrane. The increase in intracellular 5-HT favors the release of 5HT by the reverse action of the SERT (Basic Neurochemistry, 1998).

1.2. Serotonin system in the brain

1.2.1. Localization of serotonin in the brain

1.2.1.1. Raphe nuclei

Serotonergic neurons in the raphe nuclei were first studied by Dahlstrom and Fuxe. In 1964 they reported the distribution of serotonin neuron cell bodies using the B nomenclature (Dahlstrom and Fuxe 1964). The system of Dahlstrom-Fuxe describes a small population of neurons whose cell bodies are present within the raphe nuclei (Fig.1.7). The midline raphe nuclei consist of the caudal nucleus (B8), the dorsal raphe nucleus (B6, B7), the median raphe nucleus (B5, B8), raphe magnus nucleus (B3), raphe pallidus nucleus (B1), and the raphe obscurus nucleus (B2). Outside the raphe nuclei there are collections of 5-HT containing cell bodies in a region adjacent to the medial lemniscus (B9), in the ventrolateral medulla (B3), and in the central gray of the medulla oblongata (B4) (Jacobs and Azmitia 1992).

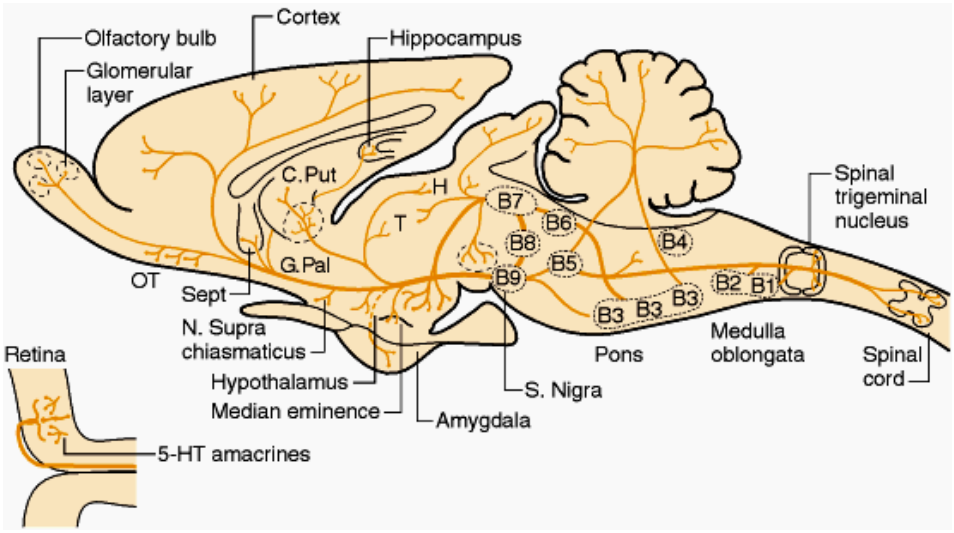

Fig. 1.7. Schematic drawing indicating the location of the serotonergic neorons groups in the raphe nuclei of the rat central nervous system and their major projections. (Consolazione and Cuello 1982)

1.2.1.2. Hypothalamus

The main function of the hypothalamus is the regulation of homeostasis including food intake, body temperature, and blood pressure linking the central nervous system to the hormone system. The hypothalamus regulates the secretion of hormones of the pituitary gland by so called releasing hormones.

5HT in the hypothalamus of mammals has been reported (Lebrand et al. 1996), but there is no 5HT synthesis, only uptake by SERT. Moreover the hypothalamus receives serotonergic projections from the raphe nuclei. In the zebrafish in contrast to mammals, the serotonergic neurons of the hypothalamus express tryptophan hydroxylase and synthesize 5HT (Bellipanni et al. 2002), but they are negative for SERT (Wang et al. 2006).

Several 5HT receptor agonists stimulate the hypothalamus-pituitary-adrenal axis in the rat (Calogero et al. 1990) and 5HT1A, 5HT2A and 5HT2C mediated oxytocin, prolactin and ACTH/corticosterone responses are observed in the hypothalamic paraventricular nucleus (Bagdy 1996). Moreover 5HT in the hypothalamus controls the food metabolism and body weight. Factors increasing 5HT signaling in hypothalamus lead to reduced food intake and weight gain and increase energy output in the both animals and human. This stimulation is mediated by 5HT receptors located in distinct hypothalamic tissues (Leibowitz and Alexander 1998).

1.2.1.3. Thalamus

During the prenatal stage of mammals, the thalamocortical neurons expressed transiently SERT and VMAT2, despite their later glutaminergic phenotype. In this stage, these neurons do not synthesize 5HT but they take up exogenous 5HT and store it in vesicles, and the 5HT innervation of somatosensory, auditory, visual cortices originates in the thalamus and not in the raphe nuclei (Lebrand et al. 1996).

1.2.2. Development of the brain serotonin system

The embryonic precursor of the brain is the neural plate, that during neurulation converts into neural tube, which is subdivided into the major brain regions: the forebrain, midbrain, and hindbrain. The serotonergic neurons arise in the hindbrain. The midbrain-hindbrain organizer (MHO) defined by the expression of the transcription factors Otx2 and Gbx2 is a major determinant for the development of serotonergic neurons. They appear caudal of it and dopaminergic neurons are generated rostrally (Fig.1.8). When the organizer is moved the area of one transmitter is increased to the expense of the other (Brodski et al. 2003). One important diffusible factor generated at the MHO is fibroblast growth factor 8 (FGF8) (Ye et al. 2001) which together with FGF4 produced by the primitive streak and sonic hedgehog (SHH) synthesized by the floor plate define the inductive center for serotonergic differentiation (Ye et al. 1998). In this zone in rhombomeres 1 to 7 (r1-r7), neural progenitors, which have produced branchiomotor and visceromotor neuronal precursors before, start to differentiate to serotonergic neurons at embryonic day 10.5 (E10.5) in the mouse. Exceptions are r1, which never generates motoneurons, and r4, which carries on producing motoneurons and never gets serotonergic. The lack of serotonergic differentiation in r4 is due to the persistence of the expression of Phox2b, an inhibitor of serotonergic differentiation and separates two zones of serotonergic fate with partly distinct mechanisms of differentiation forming the basis for the two clusters of adult serotonergic neurons (Pattyn et al. 2003). In all other rhombomeres (except r1, where it is never expressed), Phox2b is switched off at E10.5 probably by Nkx2.2. In conjunction with Mash1, Nkx2.2 also postmitotically activates the transcription factors Gata3, Gata2, Lmx1b, and Pet1 (Pattyn et al. 2004), which together define the serotonergic cell type by activating marker genes such as for TPH2, aromatic amino acid decarboxylase (AAAD), SERT and VMAT2, and some serotonin receptors (Hendricks et al. 1999). Thereby, Gata3 is only essential for the development of the caudal cluster but not the rostral one, and the function of Pet1, which is nevertheless expressed very specifically in all serotonergic neurons and until adulthood, is partially redundant since Pet1 deficient mice still retain about 30% of such cells (Hendricks et al. 2003). Thus, only Mash1, Lmx1b, and probably Gata2 are

indispensable for all serotonergic neurons to develop properly.

1.2.2.1. Transcription factors involved in serotonergic neurons differentiation

1.2.2.1.1. Phox2b

Phox2b (paired-like homeodomain protein 2b) is a transcription factor belonging to the Q50 paired-like class (Brunet and Pattyn 2002) which represses serotonergic differentiation in r2-r7. Phox2b deficient mice lack all visceromotor neuron precursors and serotonergic neurons are extensively produced in r2-r7, including r4 (Pattyn et al. 2003), confirming that Phox2b is a central repressor of serotonergic fate (Fig.8). The formation of serotonin neurons is enabled in r2-r3 and r5-r7 through inhibition of Phox2b by Nkx2.2, whereas in r4, Hoxb1, Nkx6.1, and Nkx6.2 sustain its expression and thereby block serotonergic differentiation (Pattyn et al. 2003).

1.2.2.1.2. Mash1

Mash1 (mouse achaete-scute homolog 1) is a basic-helix-loop-helix (bHLH) transcription factor, which is already detected in r1-r7 during motor neuron generation but becomes only essential when serotonergic neurons are developed in this zone. Thus, in Mash1-deficient mice no cells expressing the downstream factors Pet1, Lmx1b, Gata2, Gata3, and also no serotonergic neurons appear (Pattyn et al. 2004). However, Nkx2.2, Phox2b, and SHH retain their normal pattern of expression in these mice.

Furthermore, Mash1 specifies the serotonergic phenotype in neural crest derivatives like enteric and other peripheral neurons (Blaugrund et al. 1996).

1.2.2.1.3. Nkx2.2

Nkx2.2 (NK transcription factor related, locus 2) is expressed transiently starting at E10.5 in all serotonergic precursors. Mice lacking this factor do not express Gata3, Lmx1b, and Pet1 in caudal raphe nuclei and no serotonergic neurons develop in this area in contrast to the dorsal raphe nuclei where all these factors and such neurons persist (Pattyn et al. 2003, Briscoe et al. 1999, Ding et al. 2003). Together with Lmx1b and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube (Cheng et al. 2003).

1.2.2.1.4. Lmx1b

Lmx1b (LIM homeobox transcription factor 1β) is required for the formation of the entire serotonin system in the hindbrain, since its deletion in mice leads to the absence of such neurons in the brain (Ding et al. 2003, Cheng et al. 2003). It is expressed in developing serotonergic neurons together with Pet1 starting around E11 in the rostral cluster of

serotonergic differentiation and one day after in the caudal one consistent with the delayed appearance of serotonergic cells in the latter region (Cheng et al. 2003).

Its ablation does not affect the expression of Nkx2.2, Gata3, and SHH and only partly the one of Pet1, putting these factors upstream or in parallel to Lmx1b (Ding et al. 2003, Cheng et al. 2003). Together with Nkx2.2 and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube (Cheng et al. 2003).

In addition, Lmx1b is important for the development of dopaminergic neurons (Smidt et al. 2000).

1.2.2.1.5. Pet1

The ETS domain transcription factor Pet1 (Pheochromocytoma 12 ETS (E26 transformation-specific)) is a specific marker for all serotonergic neurons from E11 until adulthood (Samad et al. 2004). Recently, this unique specificity was confirmed by the use of the Pet1 promoter to target marker genes exclusively to 5-HT neurons in transgenic mice (Scott et al. 2005). Pet1 binding sites are found in the promotor regions of several genes expressed in serotonergic neurons such as AAAD and SERT (Hendricks et al. 1999).

In mice lacking Pet1, 70% of serotonergic neurons fail to differentiate, whereas in the remaining Pet1-deficient neurons diminished expression of VMAT2, TPH and SERT was observed (Hendricks et al. 2003). These animals survive but show anxiety-like and aggressive behavior.

1.2.2.1.6. Gata2 and Gata3

Six Gata (GATA-motif binding) transcription factors exist in vertebrates characterized by C4-type zinc-finger motifs and two of them, Gata2 and Gata3, are expressed in the developing brain (Patient and McGhee 2002). Experiments in chicks show, that Gata2 is necessary and sufficient for the induction of Lmx1b and Pet1 and serotonergic neurons in r1, but not more caudally (Craven et al. 2004). In hindbrain explant cultures of Gata2 deficient mice, no 5-HT neurons are developed indicating that Gata3 maybe also pivotal for serotonergic differentiation in general. In contrast, Gata3 is not required for the differentiation of the rostral 5-HT neurons (van Doorninck et al. 1999) and appears unable to substitute for the loss of Gata2 in r1. However, in Gata3-deficient mice, around 80% of serotonergic neurons in the caudal clusters and 30% in the rostral clusters are missing (Pattyn et al. 2004). Nevertheless, the expression of Pet1 and Lmx1b was unchanged in Gata3 knockout mice showing that these factors act in parallel.

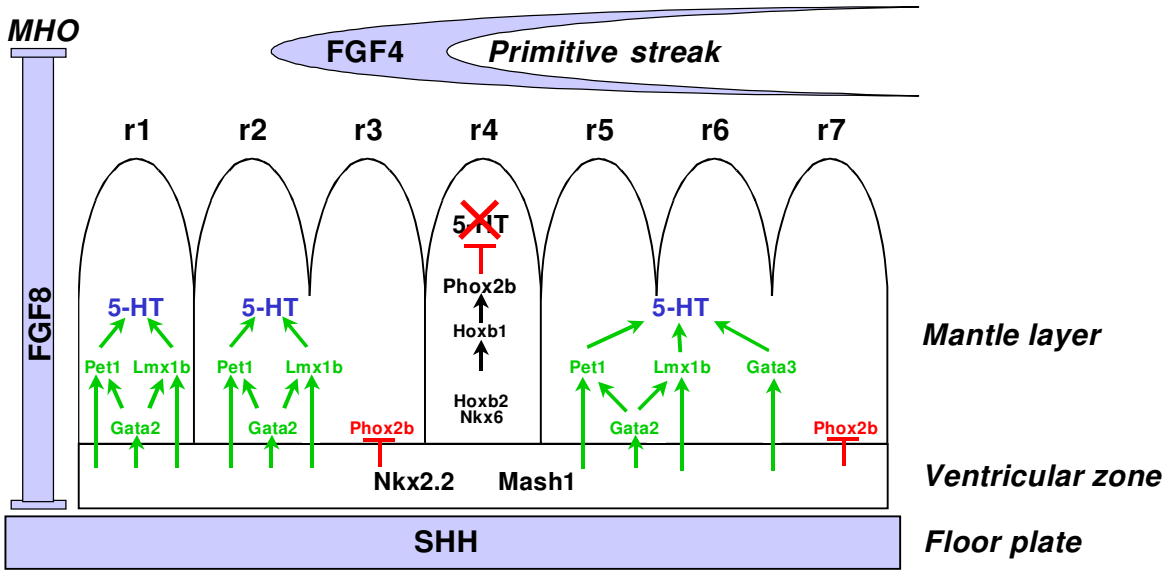

Fig. 1.8. Induction and differentiation of serotonergic neurons in vivo (see text for explanation). MHO, midbrain-hindbrain organizer; r1 – r7, rhombomeres 1 – 7; SHH, sonic hedgehog, FGF, fibroblast growth factor (Alenina et al. 2006).

1.3. Developmental role of 5HT during embryogenesis

5HT has been detected in embryos before the appearance of neurons. This pre-nervous 5HT in early stages of embryogenesis modulates cell proliferation, migration, cell shape and cell-cell coupling. Moreover 5HT is involved in cytoskeletal function, in the cell cycle and at the same time in apoptosis (Buznikov et al. 1996). This multifunctional role is dependent on many factors. The serotonin system includes two enzyme isoforms which synthesize 5HT (Walther et al. 2003), and more than 15 membrane receptors which transduce the 5HT signal into different downstream cellular partners (Hoyer et al. 2002), as well as an enzymatic degradation system with different enzymes like MAO (Gershon et al. 1990), a storage and release system by vesicles, and a membrane 5HT transporter that pumps extracellular 5HT into the cells again and at the same time serve as a negative regulator of 5HT signals for surrounding cells. Initially this system let 5HT modulate a wide range of developmental events by different effect gradient (dose dependent effect via different receptors) (Lauder 1988). 5HT play this developmental role in both vertebrate and invertebrate animals.

1.3.1. Invertebrates

Many studies showed that 5HT exist in invertebrates before the development of neurons, in oocytes, in early embryos, and in larvae of ascidians, echinoderms, insects, molluscs and nemertean worms (Buznikov et al. 2005). Immunostaining experiments with 5HT antibody have demonestrated the presence of 5HT in mature eggs, one-cell and cleaving division embryos of nudibranch molluscs and also during different early stages, eight-cell- till blastula stages, whereas at the gastrula stage, 5HT could not be detected, but 5HT positive cells appeared again at the early veliger stage. Interestingly, during the different stages of embryogenesis, 5HT changes its location, for instance in the 8-16 cell embryos 5HT localized in the micromeres but, in the blastula stage 5HT can be observed in all blastomeres. The 5HT2 receptor antagonist, ritanserin, blocked the cleavage divisions in the mollusc embryos, and arachidonnoylserotonin (serotoninamide AA-5HT) prevent this cytostatical action of ritanserin. This 5HT amide alone has no effect on the cleavage divisions of the mollusc embryos (Buznikov et al. 2003).

In the late gastrula and prism stages of the sea urchin, 5HT cells could be observed by immunostaining, and were localized to the archenteron (primary gut), and mesenchyme-like cells and the apical ectoderm. Immunostaining with some 5HT receptor antibodies (5HT1A, 5HT2A, 5HT2B, and 5HT7) and also with SERT antibodies showed immunoreactivity in the gastrula stage of the sea urchin, moreover antagonists against those receptors and SERT caused many malformations when they are applied at the mid blastula stages of sea urchin embryos. Those embryos show no sign of gastrulation, where the central region of the wild type gastrula was filled with uniformaly distributed mesenchyme like cells (Buznikov et al. 2005).

In the earthworm embryos (Eisenia fetida), the first 5HT expressing neurons appear at the embryonic stage 2 (E2; day7) in the subesophageal ganglion, and the next serotonergic neurons in this stage appear in the rostral part of the ventral ganglion. The innervation of their body wall starts at E3 (day 8-9) to E4 (day 10-11). 5HT positive cells in the cerebral ganglion, can be detected only from E5 (day 12-13), and their number increases in each ganglia until the hatching stage, and also at E5, 5HT can be detected in the stomatogastric nervous system. At E9 (day 20-21) serotonergic neurons appear in both the stomatoganglia and the enteric plexus (Koza et al. 2006).

In nematodes (Caenorhabditis eleganes; C. elegans), 5HT regulates many biological processes like male mating behaviour, pharyngeal pumping, the feeding process and egg laying (Sze et al. 2000, Xiao et al. 2006, Hobson et al. 2006). There are till now 4 5HT receptors identified

in C. elegans: 5HT1-, 5HT2-, 5HT3- (5HT gated Cl^- channel), and 5HT7 like receptors. 5HT2 like receptors are expressed in some muscles that are postsynaptic to serotonergic neurons, and regulate the egg laying and male mating behaviour. Mutants for 5HT2 like receptor exhibit defects in those both functions (Xiao et al. 2006), whereas 5HT7 like receptor mutants are showing defects in the pharyngeal pumping and egg laying behaviour (Hobson et al. 2006). The nematode *mod-1* mutant (for modulation of locomotion defective) shows mutations in the 5-HT3 like receptor with defects in the ability to move (Ranganathan al et. 2000).

1.3.2. Vertebrates

5HT controls cell proliferation and differentiation and morphogenesis during early stages of embryogenesis before the existence of neurons. Starting from the reproductive processes, 5HT regulates these processes not only through the hypothalamo-hypophysial system, but also by direct effects on reproductive organs. Local 5HT synthesis has been reported in rat ovary, uterus, testis, and mouse placenta (Vesela et al. 2003). Inhibition of 5HT synthesis in rat reduces the amount of sperms by 60% (Aragon et al. 2005), 5HT regulates oocyte maturation and early cleavage division (Byrden and Lawrence 1973). 5HT has been detected in mouse embryonic stem cells (Walther and Bader 1999). It plays roles in blastomere adhesion and gastrulation movement in amphibia (Hamalianen and Kohenen 1989), also regulates the migration of mouse cranial neural crest cells (Moiseiwitsch and Lauder 1995), as well as the development of the mouse craniofacial region, limb, and heart (Moiseiwitsch 2000, Bhasin et al. 2004a, Nebigil et al. 2000). Recently it has been shown that 5HT plays a role in the left-right patterning of asymmetrical organs in chick and frog (Fukumoto et al. 2005). Finally 5HT is a very important element in mammalian brain development being involved in dendrites maturation and axon connections (Gaspar et al. 2003)

1.4. The pharyngeal arches

The pharyngeal arches comprise a number of different embryonic cell types. Each arch has an outer covering of ectoderm, an inner covering of endoderm and mesenchymal core derived from both neural crest and mesoderm (Fig.1.9). The different embryonic populations of the arches generate distinct components of the pharynx. The ectoderm produces the epidermis and the sensory neurons of the arch associated ganglia, while the endoderm gives rise to the epithelial cells lining the pharynx and endocrine glands that form in the pharyngeal region: the thyroid, parathyroid and thymus. The neural crest forms the connective and skeletal tissues, whereas the mesoderm forms the musculature and endothelial cells of the arch

arteries. In all vertebrates, the first most anterior arch forms the jaw, while the second forms the hyoid apparatus. The more posterior arches, of which there are up to five in anamniotes, either develop gills in fish or contribute to the throat in birds and mammals (Graham 2003).

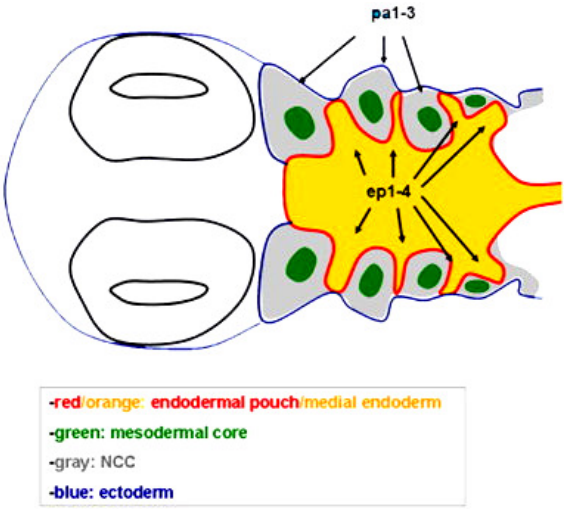

Fig. 1.9. Schematic drawing of the pharyngeal region of a 24-hpf zebrafish embryo. The pharyngeal arches are comprised of mesoderm (green), neural crest cells (gray), endodermal pouches (red), medial pharyngeal endoderm (orange), and lateral ectoderm (blue). Zebrafish possess 6 endodermal pouches and 7 pharyngeal arches. Ventral view. ep, endodermal pouches; pa, pharyngeal arches (Kopinke et al. 2006).

1.4.1. Neural crest cells

During the neurulation stage of the vertebrates, the neural crest cells arise from the border between the neural ectoderm, that form the neural tube and the non-neural ectoderm, that form the epidermis (LeDouarin and Kalcheim 1999) (Fig.1.10). Many members of different signaling factor families like Wnt, Fgf and Bmp from the neural plate and the underlying mesoderm participate in their induction. These cells are located adjacent to the dorsal neural tube, and can be identified by several protein markers as: Foxd3, Sox9, Sox10, Snail, crestin and others. During or after the neural tube closure, the neural crest cells migrate throughout the body to yield a wide range of different tissues, like the dorsal root ganglia, peripheral nervous system, sensory cells, chromaffin cells of the adrenal medulla, pharyngeal arches, melanocytes and other different cells (Bronner-Fraser and Fraser 1988). Neural crest cell differentiation is dependent on signaling factors in the environment of the cell migration pathway or in the target tissues of their migration. Those signaling proteins include Bmp, Wnt, endothelin and glucocortocoid hormones (Fig.1.11). Neural crest cells are responsible for many congenital heath problems like craniofacial and cardiac outflow tract abnormalties.

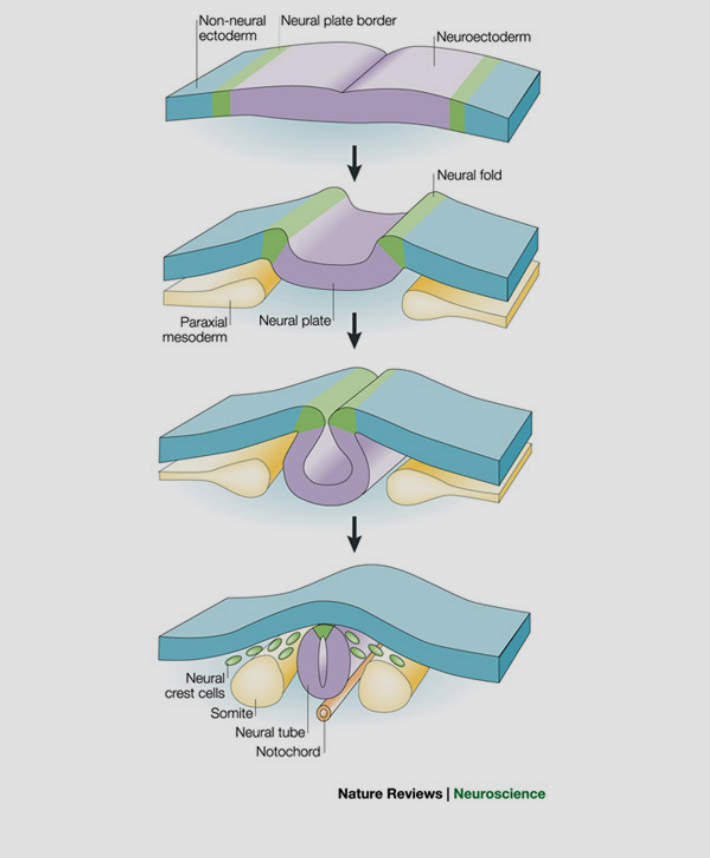

Fig. 1.10. The neural crest cells arise from the neural folds (green) between the neuroectoderm (blue) and the non-neural ectoderm (purple) and are induced by signaling factors from the ectoderm and the underlying mesoderm (yellow). During neurulation the neural crest cells start to migrate through the whole body (Gammill and Bronner-Fraser 2003)

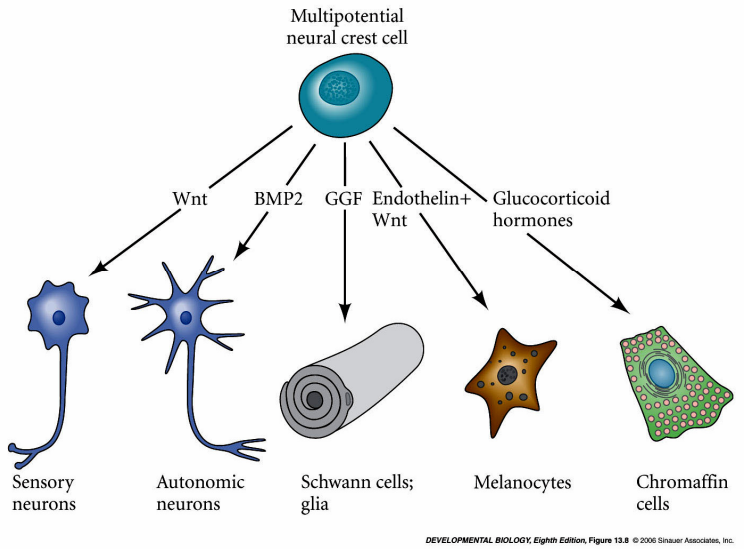

Fig.1.11. Paracrine factors in the environment help to specify the different neural crest derived lineages (see text).

1.4.1.1. Cranial neural crest cells

The migration of the cranial neural crest cells is distinct from that of the trunk neural crest cells. The cranial neural crest cells do not migrate as a single mass but are organised into streams, three of which can be identified in the developing head of all vertebrate embryos: trigeminal, hyoid, and post-otic (Graham et al. 2005) (Fig.1.12).

1) The first stream, the trigeminal crest stream arises from the midbrain and rhombomeres 1 and 2 of the hindbrain and forms neurons with the trigeminal ganglion and the components of the orofacial prominences and mandibular arch, the skeleton of the lower jaw and upper jaw.
2) The second crest stream is the hyoid stream, arises from the rhombomere 4 of the hindbrain and forms neurons of the proximal facial ganglion as well as the constituents of the pharyngeal arch, the hyoid skeleton.
3) The post-otic crest stream is generated by rhombomeres 6 and 7 of the hindbrain and forms the neurons of the proximal and jugular ganglia, and skeletal components of the posterior pharyngeal arches, also known as branchial (gill) arches in fish and amphibians, where they form the cartilage and bone of the jaw, middle ear and neck in mammals (Trainor and Krumlauf 2000).

In chick embryos neural crest cells from rhombomeres 3 and 5 undergo large–scale apoptosis in response to BMP4 through regulation of msx2 expression. Expression of noggin in rhombomere 4 antagonizes the BMP signal and prevents neural crest death in this region (Graham et al. 1994).

In the head there is also a correlation between the timing of the crest cell migration and the fates the crest cells follow. Early migratory cells populate the pharyngeal arches and facial prominences, and these groups will generate ectomesenchymal derivatives: bone cartilage, and connective tissue. Later migratory crest cells stay closer to the developing central nervous system and generate neurons and glia of the cranial ganglia (Begbie and Graham 2001).

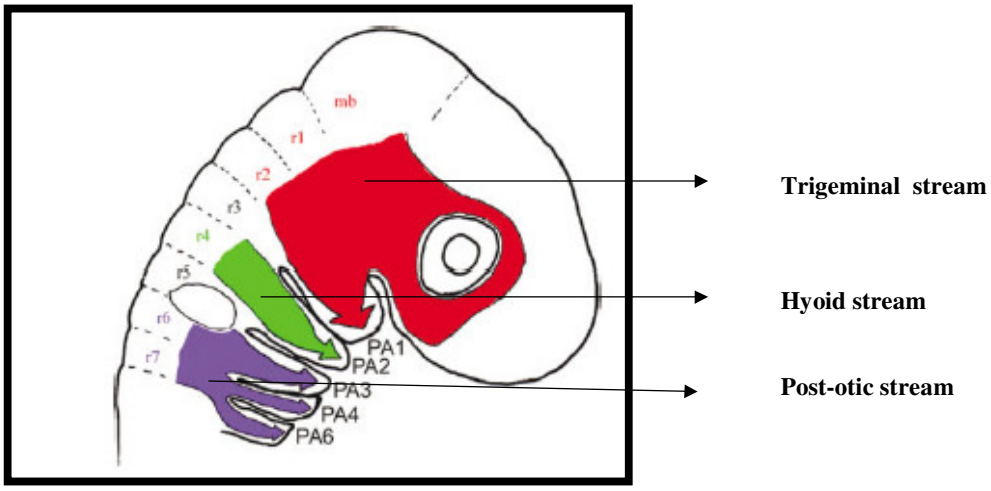

Fig. 1.12. The cranial neural crest cells migrate from the hindbrain rhombomers in 3 streams into the pharyngeal arches (see text) (Graham et al. 2004).

1.4.1.2. Trunk neural crest cells

The trunk neural crest cells migrate from the neural tube ventrally through the anterior half sclerotome. These cells have a neuronal fate. Those that move furthest ventrally form the sympathic ganglia, while the remainder cease migration in the anterior sclerotome and form the dorsal root ganglia. The later migratory neural crest cells, however move dorsolaterally between the dermamyotome and the overlying ectoderm and these cells will form the melanocytes (Lallier et al. 1992) (Fig.1.13).

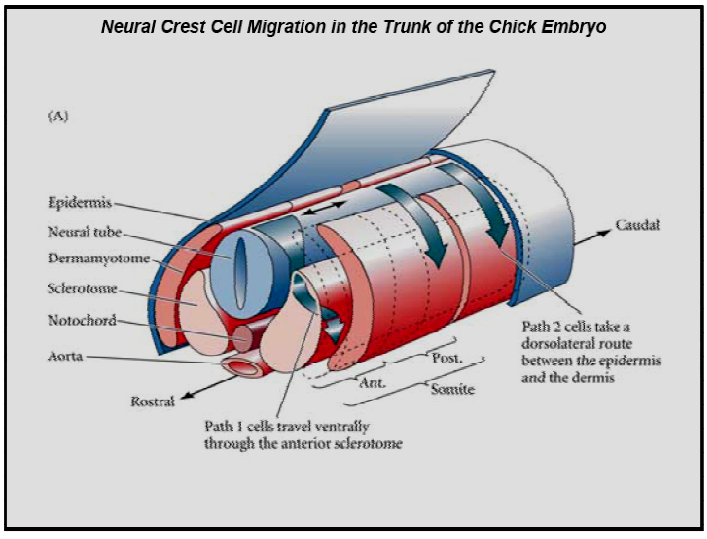

Fig. 1.13. The trunk neural crest cells migrate in 2 pathways: dorsal-ventral and dorsal-lateral (see text).

1.4.2. Endodermal pharyngeal pouches

Recent studies suggest an important role of the pharyngeal endoderm in organizing the development of the pharyngeal arches (Piotrowski and Nusslein-Volhard 2000, Graham et al. 2004). The formation of the endodermal pharyngeal pouches is the first indication of the pharyngeal arches development. In normal pharyngeal arches Bmp-7 is expressed at the posterior endodermal margin, FGF8 in the anterior endoderm of the pharyngeal endoderm, Pax-1 in the most dorsal endoderm of the pharyngeal pouches and sonic hedgehog is expressed in the posterior endoderm of the second arches and later in the posterior endoderm of the third arches (Fig.1.14).

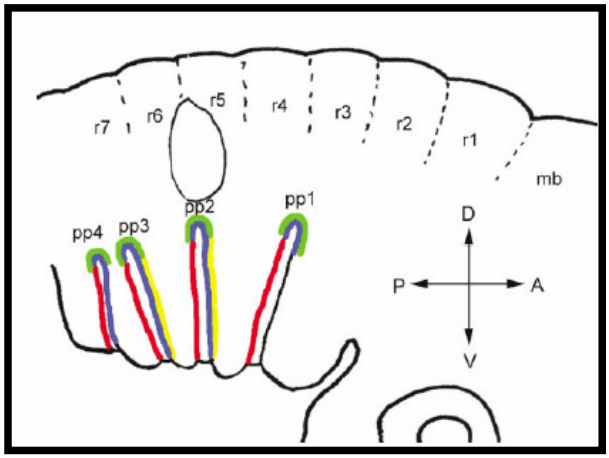

Fig. 1.14. The signaling proteins in the endodermal pouches:
Green : Pax-1 is expressed at the dorsal tip of each pouch.
Blue : BMP-7 is expressed in the posterior endodermal margin.
Red : Fgf8 is expressed in anterior endodermal margin.
Yellow: SHH is expressed at the posterior margin of pouches 2 and 3 (Graham et al. 2004).

1.5. Relationship between serotonin system and neural crest cells

The cranial neural crest cells and the serotonergic neurons arise almost from the same rhombomers of the hindbrain. Moreover neural crest cells and 5HT neurons share many transcription factors, signaling factors and enzymes for their development or function, as listed in the following:

1.5.1. MAO

Some neural crest derived tissues exhibit 5HT during development, but others do not, due the localization and the expression of the 5HT degradation enzymes monoamine oxidase (MAO)

A and B.

MAOB is expressed early in a variety of neural crest derivatives, such as the facial sensory organs, the heart, the aorta, cranial mesenchyme (developing bones, sensory neurons of the cranial ganglia, cartilages, thyroid), and dental mesenchyme. Finally MAOB was found massively expressed in the pharyngeal organ, heart, liver, and mast cells. In contrast, MAOA expression was restricted to the sympathic ganglia (Vitalis et al. 2003).

1.5.2. Gbx2

Gbx2 is expressed caudal to the midbrain hindbrain organizer (MHO), in the anterior hindbrain, where the rostral cluster of the serotonergic neurons is located. The Gbx2 homozygous null mice are missing the hindbrain rhombomeres 1-3, and as consequence are also missing the rostral cluster of serotonergic neurons. This is associated with a reduction and fusion of the cranial neural crest cell streams during their migration and those mice are displaying defects in the neural crest cell patterning and pharyngeal arch artery (Byrd and Meyers 2005).

1.5.3. SHH

Inhibition of sonic hedgehog signalling in vivo results in craniofacial neural crest cell death (Ahlgren and Bronner-Fraser 1999). Targeted disruption of SHH in mouse leads to near complete absence of the neural crest derived craniofacial skeletal elements at birth (Yamagishi et al. 2006). At the same time SHH is an essential factor upstream in the cascade of serotonergic neurons induction.

But on the other hand, an early role for sonic hedgehog from the ventral foregut endoderm is important in the lower jaw development. The cranial neural crest cells migrated normally in SHH KO mice into the pharyngeal arches but they were subjected to massive apoptosis (Brito et al. 2006).

1.5.4. FGF8

Fibroblast growth factor 8 (FGF8) is expressed specifically at the midbrain-hindbrain boundary, or isthmus, and plays an important role in the induction cascade of the serotonergic neurons in the hindbrain, and it is also expressed within the developing pharyngeal arch ectoderm and endoderm during neural crest cells migration through the arches. Analysis of neural crest cells development in FGF8 mutant embryos demonstrated that neural crest cells

are specified and migrate, but undergo cell death in areas both adjacent and distal to where FGF8 is normally expressed (Abu-Issa et al. 2002).

1.5.5. BMPs

BMP 2, 6 and 7 are expressed in the embryonic rat hindbrain raphe and define roles in the regulation of serotonergic neuron development (Galter et al. 1999). At the same time BMP signalling is necessary for neural crest cell migration and ganglion formation in the enteric nervous system (Goldstein et al. 2005). The segregation of the cranial neural crest into three streams, is a prominent feature of the developing vertebrate pharyngeal arches, and involves the focal depletion of neural crest from rhombomeres 3 and 5 through apoptosis mediated by the induction of BMP 4 expression in these two rhombomeres. However rhombomere 4 is not susceptible to BMP 4- mediated neural crest cell death due an elevated expression of noggin, a BMP 4 antagonist (Smith and Graham 2001). Interestingly this rhombomer, in contrast to all other rhombomers, produce no serotonergic neurons due the expression of the repressor Phox2b.

1.5.6. Mash1

All the central serotonergic neurons were not developed in Mash1 null mice (Pattyn et al. 2004) and, at the same time, those mice lacked the neural crest derived noradrenergic neurons and adrenochromaffin cells (Unsicker et al. 2005).

Mash1 is expressed in the sympathetic, parasympathetic and enteric ganglia which are the main components of the peripheral autonomic nervous system, all derived from the neural crest (Pattyn et al. 1999). Furthermore, Mash1 gene is required for the genesis of the neural crest derived, 5HT immunoreactive glomus cells in the carotid body (Kameda 2005). Mash1 is expressed in the neural crest derived rat thyroid C-cells, and those cells acquire a phenotype similar to serotonergic neurons, they express neurofilaments, and show 5-HT immunostaining, expression of tryptophan hydroxylase (TPH), 5-HT1B receptor, and SERT (Clark et al. 1995)

1.5.7. Phox2b

Phox2b plays different roles in the developmental regulation of serotonergic neurons and neural crest derived tissues. While Phox2b is the master repressor of specification of the central serotonergic neurons (Pattyn et al. 2004), it is required for the development of some neural crest derived tissues.

In the Phox2b knock out mice, rhombomere 4 can produce 5HT again, but the neural crest derived carotid body and the peripheral chemoreceptor neurons degenerate. In this case the effects of Phox2b on serotonergic neurons and neural crest cells are discrepant. But on the other hand, Phox2b and serotonergic neurons are necessary in the central respiratory chemoreception. Phox2b heterozygous mutants have an altered response to hypoxia and hypercapnia at birth (Dauger et al, 2003). Congenital central hypoventilation syndrome, a unique disorder of respiratory control associated with tumors of neural crest origin, results from polyalanine repeat expansion mutations in the Phox2b gene in over 90% of cases (Berry-Kravis et al. 2006). A role in the central chemoreception is also postulated for the central serotonergic neurons. They are located in association with large medullary arteries and almost devoid of veins. When they are stimulated by an increase in CO_2 in the blood, they induce hyperventilation (Bradley et al. 2002). However, there are other reports which do not accept this central chemoreception function for serotonergic neurons (Stornetta et al. 2006) showing that the central Phox2b positive chemoreceptors are not serotonergic but glutamatergic neurons.

1.6. Aims

1) To characterize the 5HT synthesizing genes TPH and Henna (PAH) in Drosophila melanogaster by sequence analysis and comparison of biochemical studies on their enzymatic activities to hydroxylate tryptophan, and phenylalanine.

2) To characterize all TPH genes in zebrafish, compare their sequences with mammalian TPH genes and follow their expression pattern in zebrafish embryos in correlation with the localization of 5HT and 5HT receptors and 5HT transporter.
   To study the functional roles of TPH2 and 5HT2B receptors during embryogenesis of zebrafish by genetic approach using morpholino antisense oligonucleotides to knock down these genes.
   To dissect the developmental roles of 5HT, 5HT2B receptors, and the 5HT transporter in the zebrafish embryos by pharmacological methods using pCPA the specific inhibitor of TPH, and the 5HT2B blocker ritanserin, and the 5HT transporter blocker fluoxetine.

3) To study the functional roles of TPH2 in testis and brain of mice by deleting this gene using the homologous recombination technique.