Identification and characterisation of novel zebrafish brain development mutants obtained by large-scale forward mutagenesis screening

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During the 4 1/2 years of my Ph.D., I did not only learn to perform the first steps as a scientist, but actually I achieved a lot more than that: I learned how to focus from a vague idea onto a specific problem, which is not impossible to solve. I learned how to keep myself motivated even in hard times of being unsuccessful and stressed. I learned to organise not only myself but also other people closely associated with my projects and assisting me. After all, I learned to be patient and to believe in my work and my personal abilities.

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Die Fragen sind es,
aus denen das, was bleibt,
entsteht.

Erich Kästner
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Summary

Developmental biology addresses how cells are organised into functional structures and eventually into a whole organism. It is crucial to understand the molecular basis for processes in development, by studying the expression and function of relevant genes and their relationship to each other. A gene function can be studied by creating loss-of-function situations, in which the change in developmental processes is examined in the absence of a functional gene product, or in gain-of-function studies, where a gene product is either intrinsically overproduced or ectopically upregulated. One approach for a loss-of-function situation is the creation of specific mutants in single genes, and the zebrafish (*Danio rerio*) has proven to be an excellent model organism for this purpose.

In this thesis, I report on two forward genetic screens performed to find new mutants affecting brain development, in particular mutants defective in development and function of the midbrain-hindbrain boundary (MHB), an organizer region that patterns the adjacent brain regions of the midbrain and the hindbrain.

In the first screen, I could identify 10 specific mutants based on morphology and the analysis of the expression patterns of *lim1* and *fgf8*, genes functioning as early neuronal markers and as a patterning gene, respectively. Three of these mutants lacked an MHB, and by complementation studies, I identified these mutants as being defective in the *spg* locus.

The second screen produced 35 new mutants by screening morphologically and with antibodies against acetylated Tubulin, which marks all axonal scaffolds, and anti-Opsin, which is a marker for photoreceptors in the pineal gland. According to their phenotype, I distributed the mutant lines into 4 phenotypic subgroups, of which the brain morphology group with 18 mutant lines was studied most intensively.

In the last part of my thesis, I characterise one of these brain morphology mutants, *broken heart*. This mutant is defective in axonal outgrowth and locomotion, and shows a striking reduction of serotonergic neurons in the epiphysis and in the raphe nuclei in the hindbrain, structures involved in serotonin and melatonin production. Studies in other model organisms suggested a role of factors from the floor plate and the MHB in induction of the serotonergic neurons in the hindbrain, and using *broken heart*, I show that Fgf
molecules such as Fgf4 and Fgf8 can restore partially the loss of serotonergic neurons in the mutant.

I conclude that forward genetic screens are an invaluable tool to generate a pool of mutations in specific genes, which can be used to dissect complex processes in development such as brain development.
Abbreviations

A-P anterior-posterior
bp basepair(s)
C celsius
CA catecholaminergic
DAB 3’-3’-diaminobenzidine
DNA desoxyribonucleic acid
dNTP desoxy-A/C/G/T-triphosphat
D-V dorso-ventral
EDTA ethylendiamintetraacetat
EST expressed sequence tag
Fig. Figure
Fgf fibroblast growth factor
hpf hours post fertilisation
hrs hours
5-HT 5-Hydroxytryptamine (= serotonin)
ISH in situ hybridisation
LG linkage group
MHB midbrain hindbrain boundary
min minutes
mRNA messenger ribonucleic acid
n number
o/n over night
pf post fertilisation
rh rhombomere
RT room temperature
sec seconds
som somites (stage)
TH tyrosine hydroxylase
Wt wild-type
1 Introduction

In the introduction of my thesis, I will first focus on general developmental concepts, before I introduce the zebrafish as an established vertebrate model organism. Then, I will give an overview on its advantages as a genetic system, and in particular the different methods one can use to perform genetic screens. My project aimed to understand neural development, therefore I will explain basic steps in neural development with the focus on the differentiation of particular neural structures.

1.1 Developmental biology and its elementary concepts

Developmental biology is a discipline that combines molecular biology, genetics and morphology, but also classical experimental and descriptive embryology. The aim is to understand how a multicellular organism emerges from a single cell, the zygote. The goal in molecular terms is to understand the genetic cascades, how genes influence each other in this highly dynamic process, modulated in space and time. In addition it has to be taken into account that it is not only the state of gene expression within a cell which should be considered, but also the protein composition plays an important role in the function of molecular networks.

One of the basic concepts in developmental biology is regional specification or pattern formation, which involves the induction of distinct patterns in a cell population which had been previously similar and therefore diversifies the cells. Different body parts such as head, trunk and tail are formed through embryonic induction: signaling between cells activates a different combination of regulatory genes in each particular region. Cells that program the fate of other cells can be called organisers. Then, cells undergo differentiation. Each cell type activates a specific set of genes to express and maintain positional information. To facilitate a crosstalk between the cells, they have to be competent, which means they have to be able to receive and interpret an inductive signal and respond to it accordingly. Later, morphogenesis takes place, comprising cellular movements and the movement of whole tissues to build up the three dimensional shape of
an organism. The movements are based on the dynamics of the cytoskeleton and other mechanical properties of the cells. During the growth phase, the organism increases in size.

1.2 Why is the zebrafish a preferred model organism?

The interest in the zebrafish (Danio rerio) as a vertebrate model organism started in the early 1980s with George Streisinger, who introduced it as an ideal vertebrate to study cellular, molecular and genetic mechanisms of development (Streisinger et al., 1981). There are numerous advantages that make the zebrafish a preferred model organism. Adult zebrafish are small (3-4 cm), produce a large number of offspring in one mating and have a short generation time of 3-4 months (Nüsslein-Volhard and Dahm, 2002). Zebrafish embryos are relatively large in size (a fertilised egg is about 0.7 mm in diameter), and develop rapidly. In addition, the ex utero fertilization and the fact that the chorion and the embryos are translucent during embryonic stages of development allow live morphology observations and easy manipulation. Micromanipulation experiments can be conducted, such as dye injections into single cells to track cell lineages (Kimmel and Warga, 1986; Eisen et al., 1986), DNA and RNA injections, cell ablations (Eisen et al., 1989) and cell transplantations (Eisen, 1991). Furthermore, mutagenesis and genetic mapping tools are available that allow identification of mutants in novel genes that control different aspects of embryonic development (Geisler et al., 1999; Stickney et al., 2002; Geisler, 2002).

1.3 Ontogenetic development of the zebrafish

Important stages of embryonic development of the zebrafish, which were previously described (Kimmel et al., 1995), are lined out in the following.

Ontogenesis of the zebrafish starts with the external fertilisation of the egg by a spermatozoon that enters the oocyte at the future animal pole. The freshly laid egg is surrounded by a transparent chorion. At the one-cell-stage, contractile forces cause yolk-
free cytoplasm to accumulate at the animal pole of the zygote and form the blastodisc, which is situated on top of a giant vegetal yolk cell, rich in granules. During early cleavage stages the zygote starts to divide, leaving newly formed blastomeres connected by cytoplasmatic bridges. Blastomeres are cleaved radial-symmetrically without cell growth, thereby diminishing in size in the course of early development. Already during early cleavage stages the embryo undergoes its first division into two cell lineages: (a) germ line cells, which inherit unique maternal transcripts demarcating them as primordial germ cells (PGCs), and (b) somatic cells, forming the large bulk of the embryo. During early blastula stages, when the embryo consists of 1000 cells, it undergoes various cellular changes, collectively termed as midblastula transition (MBT). This period is characterised by an increase in cell cycle length, a loss of cell synchrony and the beginning of cell motility. Until MBT, the embryo lives exclusively on maternal products and at MBT, zygotic transcription starts. Marginal most cells collapse and release their cellular content, including nuclei, into the underlying cytoplasm of the yolk cell, thereby constituting an teleost-specific extraembryonic structure, the yolk syncytial layer (YSL). At late blastula stages, distinct morphogenetic movements begin: epiboly starts with the doming of the yolk cell and causes a specific distribution of cells whereby cells move and spread over the yolk cell in an animal-to-vegetal direction. At 50% epiboly the gastrulation period starts, which gives rise to the re-organisation of the embryo into three germ layers and involves a concert of morphogenetic movements. Outer, prospective ectodermal cells that interact directly with the environment are separated from inner cells. Marginal most cells start to involute around the germ ring in an vegetal-to-animal direction, leading to a thickening of the blastodermal margin, the germ ring. At the same time, cells start convergence and move to the prospective dorsal side of the embryo, leading to the formation of a knob-like thickening at the dorsal margin, which is called the shield, the equivalent of Spemann’s organiser in the fish.

Involuting cells give rise to the hypoblast, comprising the mesoderm and the endoderm, which is continuously fed by cells from the overlying epiblast or ectoderm. In addition to convergence movements along the DV axis, medio-lateral intercalation - extension movements of cells near the dorsal side occur along the animal-vegetal, or future anterior-posterior axis, which act in concert with epiboly to extend the embryo along the A-
P axis. When epiboly is completed at the end of gastrulation, the yolk is completely covered by the blastoderm, and the concerted movements have established the D-V and A-P body axes. After gastrulation the embryo is further patterned and elongated in the course of segmentation along its D-V and A-P axes. In particular, the tailbud extends away from the yolk cell to produce the embryonic tail region. The gut tube forms in close proximity to the yolk surface and the notochord primordium separates from the adjacent somitic mesoderm, which is progressively subdivided into the segmentally arranged somites. Anteriorly, the forebrain, midbrain, hindbrain and, posteriorly, the spinal cord primordia become morphologically distinct, reflecting the A-P regionalisation along the central nervous system. After 1 dpf the pharyngula period starts and the basic body plan structures become visible and the embryo subsequently develops a beating heart, circulating blood cells and a partly functional neural circuitry. The embryonic axis has straightend and organogenesis continues for the next days. After 2 dpf the embryo hatches from the chorion and has completed most of its morphogenesis. At 5 dpf larvae develop a swim bladder and start to swim and to feed. Sexual maturity is reached within 3-4 months, and adults can live for 2.5 – 4 years.

1.4 Genetic screens

Genetic mutagenesis screens are an invaluable tool for the identification of genes required for specific cellular and developmental processes, encoding transcription factors, housekeeping genes or signaling molecules. Screens have been applied in a number of animals used as developmental model organisms, such as Drosophila melanogaster, Caenorhabditis elegans and mouse. The key studies pioneering the field of genetic screens had been carried out in Drosophila in a forward screen (Nüsslein-Volhard and Wieschaus, 1980; Jürgens et al., 1984; Nüsslein-Volhard and Kluding, 1984; Wieschaus and Kluding, 1984). The researchers made advantage of the highly developed genetics of the fruitfly to establish a saturating screen in which most of the early genes, which control patterning along the A-P and D-V axes, could be identified. Forward screening allows the
identification and characterisation of a gene by scoring the phenotype caused by the mutation of the gene (Haffter and Nusslein-Volhard, 1996). In contrast, reverse genetics used in mouse involves the replacement of genes via homologous recombination in murine ES-cells, the so called knock-outs.

Information about possible gene functions to test in knock-outs was based on knowledge obtained in studies with *Drosophila* and the fact that developmentally important genes are conserved throughout evolution and might have homologues in vertebrates. It is obvious that genes without homology to invertebrate genes, e.g. genes that appeared later in evolution with the creation of the vertebrate tree, will not be discovered. Therefore, it was feasible to introduce an organism in which forward genetics is possible and which is also a vertebrate, the zebrafish. In 1981, George Streisinger published his basic studies about methods of *in vitro* fertilisation, the creation of haploid embryos and the establishment of homozygous lines (Streisinger et al., 1981).

1.4.1 Mutagenic agents used in zebrafish

There are a variety of mutagenic agents that have been used to generate fish to be screened for novel phenotypes. The two main points that have to be taken into account when choosing a mutagen are the efficiency and the ability to make specific point mutations. X-rays, transposons and gamma radiation have all been used for this purpose (Kimmel, 1989), but display a number of disadvantages, such as they often produce aberrations or deletions that are difficult to discover and analyse (Mullins et al., 1994). In addition, these mutagens are biased in the chromosomal sites at which they introduce mutations. Therefore, the more favoured agents in zebrafish are the chemical mutagens, EMS (Ethylmethanesulphonate) and ENU (N-ethyl-N-nitrosourea). Both EMS and ENU frequently produce intragenic point mutations making them ideal for the setting up of a genetic screen. The frequency of mutagenesis depends on the concentration of the mutagen and can be estimated from the frequency of mutations in known pigment loci, e.g. the golden locus, or by measuring the number of embryos with an embryonic lethal phenotype (Grunwald and Streisinger, 1992). An average mutation frequency in a screen is usually
around 1 mutation in a defined pigmentation locus per every 700 – 800 genomes screened (Solnica Krezel et al., 1994). ENU has the higher mutation potency and is now the preferred mutagen in genetic screens (Mullins et al., 1994; Pelegri, 2002). One of the main problems to be addressed when setting up a screen is that of mosaicism in the spermatozoa of the male fish, although work had been reported which favoured mutation of postmeiotic gametes (Riley and Grunwald, 1995). To generally avoid the possibility of mosaicism, the germ cells in the male fish are mutagenised at a premeiotic stage during spermatogenesis. A point mutation induced in one DNA strand of a premeiotic germ cell will be fixed in both strands during DNA replication. In the three or four weeks following the ENU treatment the mutagenised male fish are mated to wild-type female fish and the progeny from these crosses is discarded. After one month of this breeding regime, the sperm that was present at the timepoint of mutagenesis should have been removed. The fish will then be able to produce new spermatozoa carrying non-mosaic copies of the mutation of interest (Solnica Krezel et al., 1994; Mullins et al., 1994).

ENU has an active O\textsuperscript{6} – ethylguanine group that causes alkylation of one strand of DNA (Mullins et al., 1994). The major change that occurs as a result of this is thought to be a shift from a G-C pair to an A-T pair in the DNA code. Comparing the sequence of genes of interest from mutant fish compared to wild-type has also confirmed this shift of base pairs.

An alternative approach to chemical-mediated mutagenesis is insertional mutagenesis which has been established for a long time in \textit{Drosophila} and, recently, also in the zebrafish, where retroviral elements are mutagenic by integration into the zebrafish germline genome after injection of the virus into the embryo, leading to the generation of hundreds of insertions (Gaiano et al., 1996). The advantage of insertional mutagenesis is that it greatly speeds the cloning of mutant genes: the integrated viral DNA serves as a tag to clone mutated genes. Limitations are that insertional mutagens provoke a less efficient mutagenic rate than chemical mutagens and do not integrate entirely randomly into the host DNA. Recently, a pilot screen discovered 75 new mutants (Golling et al., 2002).
1.4.2 Genetic screening strategies

In the next section, I will focus on the different screening strategies one can choose depending on the screening purpose and the availability of space, equipment and manpower.

1.4.2.1 Non-mosaic haploids

There are two types of mutagenesis screen that can be undertaken using the zebrafish: haploid or diploid screens. Haploid screens are less labour intensive, need less aquarium space and are less time consuming. Haploid embryos can be produced gynogenetically or androgenetically (Westerfield, 1994; Corley-Smith et al., 1996). In gynogenetic haploid screens, mutagenised females are squeezed to produce a clutch of unfertilised embryos. Sperm to be used in the fertilisation is treated with a pulse of UV irradiation, which destroys the DNA of the sperm (and therefore deactivates the nucleus) but leaves sperm that is still able to activate the egg. Fertilisation is performed in vitro by careful mixing of the sperm and eggs and the embryos develop with only a maternal contribution to the genome (Westerfield, 1994). Conversely, in androgenetic screens, the unfertilised eggs are UV irradiated and are subsequently fertilised with untreated sperm from mutagenised males. This in turn produces haploid embryos with only a paternal contribution to the genome (Corley-Smith et al., 1996).

Haploid screens produce embryos that contain a background phenotype which itself can make the scoring of novel phenotypes difficult. The embryos have a reduced body length, a kinked neural tube and smaller melanocytes than diploid wild type embryos (Streisinger et al., 1981). Although present, these differences are consistent enough to be discounted in the screening process making the finding of new mutations possible. An alternative of screening haploid progeny is to screen diploid mutants.
1.4.2.2 Diploid screens

To circumvent the disadvantages of haploid phenotypes, diploid screens are often favoured, although they require a lot more aquarium space and are very time consuming. There are different possibilities to perform diploid screens, one would be to do a homozygous diploid screen, the other one is the classical three-generation diploid screen.

In homozygous (gynogenetic) diploid screens, eggs from a mutagenised mother are fertilised with sperm that has been UV treated (Streisinger et al., 1981). The egg is then subjected to either heat shock or to early or late hydrostatic pressure to restore a full complement of chromosomes. By either suppressing meiosis II (early pressure) or the first cell cleavage (late pressure) the maternal chromosomes are duplicated and a homozygous diploid embryo is produced (Streisinger et al., 1981). In theory, as there is no sexual contribution to this process, all the embryos produced in a clutch will be clones of each other, although recombination in the germ line will produce some differences between embryos.

A schematic of a classical three-generation screen is shown in Figure 1. It implies the most time and space consuming screening method, but is on the other hand less subject to additional phenotypes introduced by treatment like heat shock, pressure or haploidy. The first large scale screens using this method were undertaken in the early 1990s in Tuebingen and Boston, which supplied the zebrafish community with over 2000 novel mutants which had been identified scoring morphological abnormalities (Development 123, 1996). These mutants were grouped into more than 20 phenotypic subgroups, among these the group of midbrain-hindbrain boundary (MHB) mutants, *ace*, *noi* and *spg* (*acerebellar, no isthmus* and *spiel-ohne grenzen*), which all lacked a functional MHB-organiser. In the following years, these mutants could be assigned to genes: *ace* is a mutation in the *fgf8*, *noi* in the *pax2.1*, and *spg* in the *pou2* locus (Brand et al., 1996b; Reifers et al., 1998; Lun and Brand, 1998; Burgess et al., 2002). Although invaluable insights in the function of the organiser could be obtained with these mutants, the need for additional mutants to refine already established pathways is obvious. A new screen was therefore designed in Tuebingen in the year 2000, which was aimed again for saturation, but far more specific than the first one, e.g. not only morphology, but also invasive screening methods such as antibody stainings.
and in situ hybridisation were used. 20 screening groups screened over the period of one year for mutants defective for example in the development of cartilage, angiogenesis, fins, bone, pigmentation, neural crest, germ cells and brain. The results of the brain group, which comprised our lab and people from the laboratory of Steve Wilson, will be discussed in this thesis.

**Figure 1:** Schematic drawing of a gynogenesis based (A) and a classical three-generation screen (B).

### 1.4.2.3 Alternative screening methods

More recently, the TILLING (Targeting Induced Local Lesions In Genomes) method was introduced as an alternative screening system in *Arabidopsis thaliana* (McCallum et al., 2000; Till et al., 2003). In zebrafish, TILLING was started large scale in 2000 (Wienholds et al., 2002), and is now going to be established in many other laboratories. Here, chemical mutagenesis is combined with PCR-based screening to identify mutations in a gene of interest, therefore being the first reverse screening method applied in zebrafish.
In addition, another forward genetic method that blocks gene function only transiently is favoured to test the function of a known gene of interest: Morpholinos, chemically modified oligonucleotides that are able to bind to mRNAs, are injected to block translation or splicing of the target mRNA (Genesis 3, July 2001).

1.4.2.4 Identification of the mutation

The first step to identify the disrupted gene in the mutant of interest derived from ENU treatment is to place its mutant locus onto a genomic map. Two types of genomic maps exist, the genetic map and the radiation hybrid map (Geisler, 2002). Radiation hybrid maps were created by fusing lethally irradiated cells of the zebrafish to a hamster cell line to obtain somatic cell hybrid lines. Random fragments of the zebrafish chromosomes are integrated into the hamster chromosomes or retained as separate microchromosomes and the breakpoints induced by the radiation are used for mapping (Geisler et al., 1999). The radiation hybrid map is used to map candidate genes and to increase the number of markers for positional cloning, mutants are placed onto genetic maps. Such maps are produced by scoring a large number of polymorphic markers on a panel of F2 fish form a reference cross. Polymorphic markers are for example SSLPs (simple sequence length polymorphisms), (CA)n repeats of variable length flanked by two primers. Alternatively, SNPs, single nucleotide polymorphisms, can be used (Stickney et al., 2002).

To map a mutant, a mapping cross between the line carrying the mutation in the Tuebingen background and the reference Wik line is performed, the first generation incrossed and F2 embryos sorted by phenotype into homozygous mutants and wildtypes (homo- and heterozygotes) (‘Tuebingen’ or ‘Tue’ and ‘Wik’ are the names of two established wildtype lines). The mutants and their wildtype siblings are then subjected to PCRs using SSPLs as PCR primers that span the whole genome at an equal distance. If one of the markers is linked to the mutation, a distinct PCR-pattern preferably segregates with the Tuebingen allele.
1.4.3 Methods of screening used in theTuebingen screen 2000

In the first Tuebingen mutant screen, morphological criteria had been almost exclusively used (Haffter et al., 1996). With a screen design like this, screening itself is very rapid and easy, but specific phenotypes, such as defects in the formation of axonal circuits, will be almost certainly missed. Since our screen group was particularly interested in screening for defects in early brain development, not only morphology was analysed, but also antibody stainings against phosphorylated Tubulin and Opsin had been performed, which could help to detect more subtle defects. Anti-acetylated Tubulin labels early developing cytoskeletal elements in axons (Piperno and Fuller, 1985; Chitnis and Kuwada, 1990) and Opsin the photoreceptors of the epiphysis, a highly specialised structure in the roof of the forebrain.

In the following section, I will shortly describe the main features in neuronal development before I describe distinct features that are important for our analysis, such as axon tract formation, motoneuron specification, the development of the epiphysis and the serotonergic system.

1.5 Development of the vertebrate nervous system

The development of the nervous system can be subdivided into four parts: (i) neural initiation, (ii) neurulation, (iii) differentiation and (iv) the establishment of ordered neuronal connectivity.

1.5.1 Neural initiation

The first step in the development of the nervous system in vertebrates is initiated during gastrulation and results in the specification of neuroectoderm. During induction, neural fate is induced by both vertical signals coming from the mesendoderm and planar signals travelling within the plane of the neuroectoderm (Ruiz i Altaba, 1994; Kelly and
Melton, 1995; Lumsden and Krumlauf, 1996; Wilson et al., 2002). In zebrafish, this model needs to be modified, since several zebrafish mutations affecting mesoderm development do not disrupt initiation of neuroectoderm (Barth et al., 1999; Fekany-Lee et al., 2000). The cells in the ectoderm have a tendency to become neuronal (Weinstein and Hemmati-Brivanlou, 1999). This default state can be changed in the transformation phase by neural suppressor genes encoding the bone morphogenetic protein (BMP) family, BMP2 and BMP4 (Sasai and De Robertis, 1997; Wilson et al., 1997; reviewed in Chitnis, 1999). Then, patterning and posteriorisation occurs, mediated by secreted factors of the Wnt and Fgf families (Streit et al., 2000; reviewed in Wilson and Edlund, 2001).

1.5.2 Neurulation

During the course of neurulation of higher vertebrates, the neural plate is folding up at its lateral edges, the neural ridges, and eventually fuses at the midline. This process gives rise to the neural tube and its lumen, the neurocoel, and is called primary neurulation. The dorsal most region of the neural tube, which is derived from the neural ridges, gives rise to the neural crest cells that migrate out from the neural tube and contribute to diverse organs and structures (see below).

In contrast, in zebrafish and other teleosts, the neural tube is formed by secondary neurulation: from late gastrulation stages onwards the neural plate cells move centripetally to form a solid neural keel. It hollows then secondarily to form the neural tube and its neurocoel. During formation of the neural keel, the topological arrangement of cells is very similar to that of other vertebrates (Papan and Campos Ortega, 1994).

1.5.3 Differentiation and morphogenesis

After the neural tube has been formed, it is continuously segmented during morphogenesis: along the anterior-posterior axis the three primary brain vesicles (forebrain, midbrain and hindbrain) appear, and along the dorso-ventral axis it is divided into the roof plate, alar plate, basal plate and floor plate. Subsequently, as proliferation and
morphogenesis proceeds, the primary brain vesicles get further subdivided and regionalised, and this subdivision is reflected by gene expression in distinct domains along the anterior-posterior axis (reviewed in Rhinn and Brand, 2001).

1.5.4 Patterning along the anterior-posterior axis

Originally, the early neural tissue is anterior in character (reviewed in Stern, 2001). Then, neuroepithelial organisers, consisting of special cells that produce molecules to control cell fate of the surrounding cells, pattern the neural epithelium. Ablation and transplantation experiments in chick and zebrafish revealed the existence of two of these organisers: one at the anterior end of the neural plate, the anterior neural ridge or row 1 (Shimamura and Rubenstein, 1997; Houart et al., 1998), which acts on the forebrain, and the midbrain-hindbrain boundary organiser (MHB, or isthmic organiser), which patterns the midbrain and the hindbrain primordium (Martinez and Alvarado-Mallart, 1990; Marin and Puelles, 1994; reviewed in Rhinn and Brand, 2001). Additional organising centers have been described more recently in the zebrafish: the rhombomere 4, the first rhombomere to form and an organiser in the hindbrain (Maves et al., 2002), and the tail organiser, which is derived from the ventral margin (Agathon et al., 2003).

1.5.5 Cellular diversification

An important aspect of development following patterning is the differentiation of cells to achieve distinct identities and specialised functions (Wolpert et al., 1998). Differentiated cells are distinct in their gene expression and protein content and often are also distinguishable by their structure. A good example in which differentiation has been studied extensively is the neural crest. It arises from the ectoderm and differentiates into a great variety of cells, including glia, cartilage, pigmented cells and neurons. The fate and development of neural crest cells could be studied with the help of chick-quail chimeras, which facilitated the tracking of individual cells (reviewed in Selleck and Bronner-Fraser, 1996). However, the factors influencing the fate of the individual cells are largely
unknown, but since neural crest cells have to migrate to reach their target area in the embryo, it is thought that the factors they encounter during migration affect their differentiation.

Among the neurons arising from the neural crest, there are adrenergic, cholinergic and serotonergic neurons. Adrenergic neurons for example are specified via a combination of FGF (fibroblast growth factor) and NGF (nerve growth factor) from a common precursor pool (Stemple and Anderson, 1993).

The process that produces neurons is called neurogenesis and has been most extensively studied in *Drosophila*. At the beginning, proneural genes are expressed in a proneural cluster of cells in the ectoderm before any sign of neuronal differentiation (reviewed in Bertrand et al., 2002). Within this proneural cluster, a second set of genes, the neurogenic genes, is expressed to allow only a subset of the cells to become committed to neural differentiation. Delta-Notch signaling is crucial for this process, and several basic helix-loop-helix- (bHLH) transcription factors, e.g. genes of the *achaete-scute* complex and *neurogenin*, are induced (reviewed in Korzh and Strahle, 2002; reviewed in Bertrand et al., 2002). In particular, the transmembrane protein Notch acts as a receptor, and the transmembrane protein Delta acts as a ligand in lateral inhibition within a proneural cluster (reviewed in Beatus and Lendahl, 1998). Delta in one cell activates Notch signaling in its neighbour, thereby inhibiting the neighbour from becoming committed to neural fate.

**1.5.6 Neural connectivity**

Once pattern formation and neuronal differentiation had occurred, a fully functioning nervous system depends on the guided outgrowth of axons from neurons towards their target cells and the establishment of correct synaptic connections. The leading edges of the axons, the growth cones, navigate over long distances along specific pathways to direct the axon to its correct target. The growth cone senses its environment and is guided by different mechanisms acting as guidance cues, which are mediated by different molecules that are encountered by the growth cone on these pathways (reviewed in Tessier-Lavigne and Goodman, 1996). The guiding signals are either repellent or attractive, and, in addition, can be diffusible or attached to the cell membrane or to the extracellular matrix.
(reviewed in Nieto, 1996; Goodman, 1996). In addition, it has been proposed that boundaries of embryonic gene expression in the CNS function as guides for early axons in chick and zebrafish (Lumsden and Keynes, 1989; Macdonald et al., 1994).

1.6 Formation of axonal tracts in the zebrafish

The formation of tracts in the fish brain appears to proceed in a similar way to invertebrate tract formation (Wilson and Easter, 1991). Brain tracts are pioneered by small numbers of neurons extending growth cones. The extended growth cone navigates through an environment of columnar epithelial cells to reach its target. Within two days of development thick axon tracts have formed (Wilson et al., 1990). It has been observed that many axons appear to grow along the boundary between domains of neuroepithelial cells in the CNS, which can be repulsive or attractive for the growth cones, according to the character of the induced guidance molecule. The character of the induced guidance molecule will then determine the direction of growth cone extension.

There are several families of proteins that may be involved in the promotion or inhibition of growth cone extension. These include the Eph family, netrins, semaphorins, cadherins and immunoglobulin superfamily (reviewed in Kolodkin, 1996; Tessier-Lavigne and Goodman, 1996; Brennan et al., 1997; Wilson et al., 1997). Some of these proteins can act as long-distance guidance cues, for example netrins, others are secreted or are found on the cell surface and act in the vicinity of the growth cone, e.g. Eph receptor tyrosine kinases, signaling molecules, semaphorins, cadherins and immunoglobulins (reviewed in Nieto, 1996). Furthermore, proteins expressed on glia, such as the glial fibrillary acidic protein (GFAP), also contribute to axonal guidance (Marcus and Easter, 1995) and specific glial subtypes serve in fasciculation of the posterior lateral line (Gilmour et al., 2002).

Additional gene families involved in axon guidance could be revealed by the study of mutants defective in the formation of one or more axon tracts. In order to form commissures, pioneering growth cones are first attracted to, and then repelled from, the midline cells. In *ace*, the anterior commissure (AC) and the post-optic commissure (POC)
are seen to be initially absent and then to be fused together at later stages and the optic chiasm is frequently not formed (Shanmugalingam et al., 2000). Cuboidal cells of the anterior midline are mis-specified in ace and fail to induce expression of midline genes that either influence axon extension, such as net1 and sema3D (Strahle et al., 1997, Halloran et al., 1999) or in genes that can indirectly influence guidance cues such as pax2.1 (Macdonald et al., 1997), twhh (Ekker et al., 1995) and six3.

Another zebrafish mutant, you-too (yot) (Brand et al., 1996a; van Eeden et al., 1996; Karlstrom et al., 1996; Karlstrom et al., 1999) has been found to have defects in commissure formation. yot mutants have no POC, reduced AC and optic nerves that tend to grow dorsally instead of medially. yot has been found to encode a gene with a mutation in gli2, a transcription factor that acts downstream of hedgehog genes (Karlstrom et al., 1999). The midline defects detected in yot embryos may be due to a loss of Hedgehog signaling through gli2 in the ventral forebrain, which in turn causes a disruption to the induction of cellular guidance cues in the midline.

The forebrain commissures are among the best studied in the zebrafish. Expression of shh, and ephrinA5 (formerly L2 and L4, Wilson et al., 1997; Brennan et al., 1997) is seen in tissue directly ventral to the POC. Pax2.1 protein (as mentioned above) has been reported in the tissue immediately dorsal to the POC (Macdonald et al., 1997) and it is likely that the commissure is formed in the area between these domains of expression. shh has been shown to regulate Pax2.1 protein expression (Macdonald et al., 1997) and may control the exact positioning of the POC. The AC also develops between two domains of gene expression. Tissue ventral to the AC expresses netrin1 and netrin2, whereas tissue dorsal to the AC expresses EphA4, (formerly Rtk1, Wilson et al., 1997). In a similar way to the POC, the AC appears to develop in the area of tissue between two domains of gene expression. In contrast, a direct role for other genes in axon guidance such as pax6 which is expressed in the vicinity of axon tracts, could not be shown so far (Hjorth and Key, 2001).
1.7 Motoneuron specification

Motoneurons are found from caudal midbrain through spinal cord in longitudinal columns on both sides of the ventral midline of the neural tube (reviewed in Eisen, 1999). The subpopulations are subject to combinations of different transcription factors from various sources, such as notochord, paraxial mesoderm and local signals from the neural tube (reviewed in Eisen, 1999). *hedgehog* genes are required for the induction of primary motoneurons in the spinal cord (Lewis and Eisen, 2001), as also shown by use of the *smoothened* (*smu*) mutant, which lacks one of the Hedgehog co-receptors, Smoothened (Varga et al., 2001). Cranial and spinal motoneurons express *islet-1*, which mediates the initiation of differentiation of specific neuronal subtypes (Korzh et al., 1993; reviewed in Eisen, 1998). Later in development, subtypes are defined by the expression of other LIM homeobox genes, *lim3* and *islet-2* (reviewed in Dawid and Chitnis, 2001).

1.8 The structure and development of the epiphysis

In vertebrates, the epithalamus is a distinct neuroanatomical division of the diencephalon (reviewed in Concha and Wilson, 2001). It consists of the habenular complex in the rostral part and the pineal complex in the caudal part. The pineal complex is formed by the epiphysis or pineal organ, a pair of median evaginations situated along the roof plate of the diencephalon, and the asymmetric parapineal in lower vertebrates, an organ whose function still remains unclear.

The epiphysis has been often also called the seat of the soul (Sterne, 1760; reviewed in Natesan et al., 2002) or the ‘third eye’, which refers to the fact that it modulates moody states and its activity is modulated by light. The main function of the pineal gland is the synthesis and release of melatonin, at high levels during the night and low levels during the day (reviewed in Natesan et al., 2002; Moller and Baeres, 2002), therefore providing a system that translates light conditions into the circadian production of endocrine and neuroendocrine signals. By this, the physiology of the body gets prepared in advance for
the period of activity and responds with a rise in heart rate and glucose and cortisol concentrations (Buijs et al., 2003). In mammals, the pineal gland itself is not photoreceptive and receives light information only via the retina and the transmission of information through the suprachiasmatic nucleus (SCN) of the hypothalamus, which works as the circadian oscillator (reviewed in Ganguly et al., 2002). In contrast, in chick light-receptive cells are located also in the pineal, and in addition to the circadian oscillator of the SCN it contains an additional one (Buijs et al., 2003; reviewed in Natesan et al., 2002). In humans, altered levels of melatonin, as well as the ones of its precursors tryptophan and serotonin, are implicated in a number of psychical disorders such as depression, aggression, anxiety and suicide.

Furthermore, in mouse, a new role of serotonin in development could be demonstrated (reviewed in Luo et al., 2003). It functions in the differentiation of neuronal progenitors and morphogenesis of the cranofacial region, the heart and the limbs.

1.9 Structure and function of the epiphysis in the zebrafish

The epiphysis of the zebrafish is one of the first sites in the forebrain where neurons differentiate, therefore providing an excellent model to study mechanisms of early differentiation and axogenesis (Masai et al., 1997). During development, neurons of the epiphysis differentiate into medial photoreceptors and laterally into projection neurons, the factors guiding these process are largely unknown. One of the genes responsible for the requirement of specific identity might be onecut, a cut homeodomain protein with a high homology to human hepatocyte nuclear factor-6 (HNF-6) and Drosophila onecut (Hong et al., 2002), which is expressed in the projection neurons of the epiphysis of the zebrafish from 10 somites on. Since in Drosophila it functions as a differentiation gene during the formation of photoreceptors in the retina, it might play a similar role in differentiation of the zebrafish epiphysis (Nguyen et al., 2000).

Positional identity of the epiphysis is marked and prepatterning is regulated by floating head (flh) (Masai et al., 1997; Cau and Wilson, 2003), a transcription factor that
regulates the proneural genes *ash1a* and *neurogenin1* (Cau and Wilson, 2003), but also the genes exerting lateral inhibition, *delta* and *notch*. These genes show expression in both projecting neurons and photoreceptors and do not contribute to the diversification of these cells.

When specification had occurred, a set of marker genes is expressed in the photoreceptors. First of all, photoreceptors express Opsin, a photoreceptor-specific antigen of the outer segments of photoreceptors in epiphysis and retina (Forsell et al., 2001). Selective markers for the photoreceptors such as *otx5* regulate the transcription of cycling genes, for example serotonin *N-acetyl transferase* (≡arylalkylamine N-acetyltransferase, *aanat*), which is expressed at high levels during the night (Begay et al., 1998; Gamse et al., 2001; Gothilf et al., 2002). The circadian oscillator of the zebrafish is located within the pineal photoreceptors themselves (reviewed in Cahill, 2002), in contrast to the situation in mammals.

### 1.10 The serotonergic system

Serotonin is made from the amino-acid precursor tryptophan in a two step enzymatic catalysis. The rate-limiting enzyme is tryptophan hydroxylase (*tph*), which converts tryptophan into 5-Hydroxytryptophan (serotonin, 5-HT). Serotonin then is converted in two steps into melatonin, beginning with the alkylation of serotonin by the melatonin rhythm enzyme arylalkylamine N-acetyltransferase (*aanat*).

The main source of serotonin is located in the ventral hindbrain in two clusters of cells of which most are situated in the raphe nuclei (reviewed in Goridis and Rohrer, 2002). In mouse, first serotonin positive neurons can be found at E11.5 in rhombomeres 1-3 and more caudal rhombomeres with a gap in rhombomere 4 (reviewed in Goridis and Rohrer, 2002). Studies in tissue culture have shown that FGF8 and SHH in conjunction with FGF4 are inductive signals for serotonergic neurons in isolated tissue explants (Ye et al., 1998). Here, FGF4 is the prepatterning gene of the precursors of serotonergic neurons, by
signaling from the primitive streak, which is adjacent to the posterior neural plate (Ye et al., 1998).

The raphe nucleus of the adult zebrafish is separated into two parts: the dorsal one is homologous to the caudal and rostral part of the mammalian dorsal raphe nucleus and the medial seems to be homologous to the nucleus interpeduncularis and the nucleus centralis superior (Kaslin and Panula, 2001). The factors controlling the development of the raphe nucleus are not known so far.

Three \textit{tph} genes could be cloned so far which can be used to study development of serotonergic neurons in the zebrafish. \textit{tphD1} is expressed in the epiphysis and basal spinal cells from 22 somites onwards (Bellipanni et al., 2002). Expression in the epiphysis persists, and at d 2 and d 3 additional expression domains appear in the retina and diencephalon. The second, \textit{tphD2}, an EST clone, is expressed from 30 hpf onwards in a restricted preoptic cluster in the ventral diencephalon (Bellipanni et al., 2002). Both cannot be found in the raphe nuclei. The third, \textit{tphR}, is expressed in the epiphysis and the raphe nuclei from 35 hpf in a rostral and a caudal cluster (Teraoka et al., 2004). The expression domain in the raphe is located laterally to the \textit{shh}, and adjacent to the \textit{nkh2.2} expression domains, and proceeds the antibody staining with an antibody against serotonin for at least 2 days (Teraoka et al., 2004).

\textbf{1.11 Outcome of this thesis}

The present study is subdivided into three parts. In the first part, a small-scale mutant screen is described in detail and it can be shown that a haploid screen is efficient to generate new mutants defective in the development of the brain.

In the second part, a large-scale diploid screen is described and the mutants obtained are classified and characterised. It can be demonstrated that these mutants are excellent tools to gain more insight into various issues of early development, such as midline formation, cell adhesion, apoptosis and organogenesis. In addition, a large number of brain morphology mutants are described.
In the last part, the mutant *broken heart*, derived from the diploid screen, is analysed in detail. It displays an interesting phenotype in the induction and differentiation of serotonergic neurons and can be used as a model to study the involvement of factors such as Fgf4 and Fgf8 in these processes.
2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from Applichem, Merck, Roth and Sigma, if not stated otherwise. The agarose was bought from Pharmacia and bromphenolblue from Serva.

2.1.2 Equipment

Centrifuges: Beckman Avanti\textsuperscript{TM}J-25
Heraeus Biofuge pico
Heraeus fresco

Tungsten wire: TGW0325, Ø 0.075 mm (WPI)
TGW0515, Ø 0.125 mm (WPI)
TW5-3, Ø 0.120 mm (Clark)

Photometer: Pharmacia Ultrospec 2100 pro

PCR-machine: Stratagene Robocycler Gradient 96
Gene Amp PCR System 9700

Microscopy: Olympus SZX12
Zeiss AxioPhot and Axioskope
SONY 3CCD Color Video Camera mod. by AVT Horn
Zeiss LSM Meta confocal
Materials and Methods

48 well screening plates: manufactured by the workshop of the MPI for Developmental Biology, Tuebingen

Image processing: Apple Power Macintosh G4
NIH Image 1.60
Adobe Photoshop 6.0

Printing: Epson Stylus 700
Fuji Photo Printer LW8.6
Ricoh Aficio

2.1.3 Embryo media and mounts

Ringer:

116.0 mM NaCl
3.0 mM KCl
4.0 mM CaCl₂/6H₂O
1.0 mM MgCl₂/6H₂O
5.0 mM HEPES

10x E2:

300 mM NaCl
10 mM KCl
20 mM CaCl₂/H₂O
20 mM MgSO₄/7H₂O
3 mM KH₂PO₄
0.8 mM Na₂HPO₄/2H₂O

500x NaHCO₃:

357 mM NaHCO₃ in H₂O

E2:

100 ml of 10x E2
2 ml of 500x NaHCO₃
Hanks:
Solution 1:  8.0 g NaCl
          0.4 g KCl in 100ml deionized water
Solution 2:  0.449 g Na$_2$HPO$_4$/H$_2$O
          0.60 g KH$_2$PO$_4$ in 100ml ddH$_2$O
Solution 3:  0.954 g CaCl$_2$ /2H$_2$O in 50ml ddH$_2$O
Solution 4:  1.23 g MgSO$_4$/7H$_2$O in 50ml ddH$_2$O
Solution 5:  0.35 g NaHCO$_3$ in 10ml ddH$_2$O

Solutions 1-4 are combined as Hank’s premix and stored at 4°C:
10ml Solution 1
1ml Solution 2
1ml Solution 3
86ml ddH$_2$O
1ml Solution 4

Hank’s final:  9.9ml Hank’s premix
0.1ml Solution 6

10x Penicillin-Streptomycin solution (PS):  10,000 U penicillin
                                            10mg/ml streptomycin in PBS (Sigma)

E2/PS or Ringer PS:  E2 or Ringer + 10ml 10x PS per liter

100x PTU:  0.3% Phenylthiourea (Sigma) in ddH$_2$O

Tricaine:  0.2% tricaine powder (Sigma) in H$_2$O, pH 7
           working solution: 7ml stock solution per 100ml fish water

Embryo mounting agarose:  1-2% LMP-Agarose (Gibco BRL) in Ringer (live specimen) or PBS
                          (fixed specimen)

Embryo mounting methyl cellulose: 3% methylcellulose (Sigma) in E2 medium

Embryo mounting glycerol:  70% Glycerol in PBS
2.1.4 Fish strains (wildtype)

*Danio rerio*, zebrafish

- Wildtype-strains: Gol, Tue, wik

The Tue strain comes from Tübingen (Haffter et al., 1996), it was inbred for 6 generations to achieve a homogenous, lethal free genetic background. The *golden* line is a viable mutation in the AB background that effects pigmentation and results in reduced pigmentation (Streisinger et al., 1989). The wik strain derives from a wild catch in India and comes from Tübingen.

2.1.5 Antibodies

<table>
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<tr>
<th>name</th>
<th>antigen</th>
<th>dilution</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-acetylated Tubulin</td>
<td>Acetylated Tubulin</td>
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<td>Sigma T-6793</td>
</tr>
<tr>
<td>FITC-coupled anit-mouse Alexa 488</td>
<td>IgG</td>
<td>1:200</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>HRP coupled anti-mouse</td>
<td>IgG</td>
<td>1:200</td>
<td>Sigma A-8924</td>
</tr>
<tr>
<td>HRP-coupled anti-rabbit</td>
<td>IgG</td>
<td>1:200</td>
<td>Sigma A-6154</td>
</tr>
<tr>
<td>Anti-opsin</td>
<td>Opsin</td>
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<td>Wilson lab</td>
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<tr>
<td>Anti-phospho Histone</td>
<td>Phosphorylated Histone H3</td>
<td>1:300</td>
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<tr>
<td>Anti-RM044</td>
<td>Neurofilaments</td>
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<td>Wilson lab</td>
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<tr>
<td>Anti-tyrosine hydroxylase</td>
<td>Tyrosine hydroxylase</td>
<td>1:300</td>
<td>PelFreez</td>
</tr>
</tbody>
</table>
2.1.6 Buffers and stock solutions

10x loading buffer: 50% Glycerol
100 mM EDTA (pH7.5)
1.5 mM Bromphenolblue
1.9 mM Xylenecyanol

TE: 10 mM Tris/HCl, pH 7.4
1mM EDTA pH 8.0

TBE: 890 mM Tris
890 mM boric acid
20 mM EDTA pH 8.0

TAE: 400 mM Tris
10 mM EDTA, pH 8.0

2.1.7 Enzymes

All enzymes were purchased, if not stated otherwise, from MBI Fermentas and New England Biolabs.

2.1.8 Culture media and agar plates

LB-media: 0,5% Yeast extract (Gibco BRL)
1% Trypton (Difco)
200 mM NaCl
to 1000 ml with H2O
20 min autoclaved, stored at 4°C.

LB-plates: 15 g Agar (Difco) with 1000 ml LB-media
20 min autoclaved, cool down to 50°C
add 75 µg/ml ampicillin, 60 µg/ml IPTG and 60 µg/ml X-Gal, pour into petri dishes and store at 4°C.
Materials and Methods

SOC-media:
- 2% Trypton
- 0.5% Yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl$_2$•6H$_2$O

2.1.9 Bacterial strains

*E. coli* XL2-Blue was used as a bacterial stock for plasmid transformation and amplification.

2.1.10 DNA marker

1 kb PLUS DNA ladder: 500 bp - 10 kbp

2.1.11 Plasmids and Constructs

Several constructs used for the generation of ISH antisense RNA probes are based on the PCRII TOPO vector (Invitrogen), the pBluescript II SK+ vector (Stratagene) or the pCS2+ vector (Rupp et al., 1994). Probe synthesis and expression patterns are described: *lim1* (Toyama et al., 1995), *fgf8* (Reifers et al., 1998), *wnt1* (Molven et al., 1991), *eng2* (Ekker et al., 1992), *flh* (Talbot et al., 1995), *ilset-1* (Okamoto et al., 2000), *shh* (Krauss et al., 1993), *twhh* (Ekker et al., 1995), *nkx2.2* (Rohr et al., 2001), *ptc1* (Concordet et al., 1996), *spry4* (Fürthauer et al., 2001), *fgf4* (Grandel et al., 2000), *pax2.1* (Krauss et al., 1992), *her5* (Müller et al., 1996) and *wnt8b* (Kelly et al., 1995). Constructs prepared in the course of this thesis used for ISH antisense RNA probes are listed below. If not noted otherwise, cDNA constructs contain entire open reading frames.
2.1.12 RNA probes and plasmids name and source

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<td>NotI/T7</td>
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<td>BamHI/T7</td>
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</tr>
</tbody>
</table>

2.1.13 Molecular biology kits

DIG RNA labeling kit (Roche Biochemicals)
TOPO TA cloning Kit (Invitrogen)
QIAquick PCR purification kit (Qiagen)
QIAquick gel extraction kit (Qiagen)
QIAprep-Spin-Miniprep-Kit (Qiagen)
QIAprep Midiprep-kit (Qiagen)
2.2 Methods


2.2.1 Fish maintenance

Zebrafish were raised and kept under standard laboratory conditions at about 28.5°C (Brand and Granato, 2002). To accelerate embryonic development, embryos were incubated at 33°C, and to delay embryonic development, embryos were incubated at 18°C from shield stage onwards. Mutant carriers were identified by random intercrosses. To obtain homozygous mutant embryos, two carriers heterozygous for a mutation were crossed to each other. Typically, eggs were spawned synchronously at dawn of the next morning, and embryos were collected, sorted, observed and fixed at different time points of development at 28.5 °C. In addition, morphological features were used to determine the stage of the embryos, as described by Kimmel et al., 1995. In some cases, when fish were kept for longer than 24 hrs, 0.003% phenylthiourea (PTU) was added to prevent melanisation. To dechorionate embryos enzymatically, 15µg/ml Pronase was added to the medium (Sigma).

2.2.2 ENU mutagenesis (Heidelberg haploid screen)

2.2.2.1 Stock solution and ENU neutralising solution

All steps had to be taken out under the hood, since ENU is extremely toxic and cancerogenic. 1g of ENU (N-ethyl-nitroso urea) (Sigma) was dissolved in water and acetic
acid to a final molarity of 10mM acetic acid resulting in a 85.5mM ENU stock. To facilitate the dissolving the solution was stirred for several hours and briefly heated up to 60°C. The final concentration was determined spectrophotometrically. The stock solution was stored in aliquots at -20°C.

To inactivate all ENU containing solutions and materials after treatment, a 5mM NaOH solution was prepared, which was stored under the hood in a 10 l beaker; liquids and material were inactivated in this solution over night.

2.2.2.2 Mutagenesis treatment

Mutagenesis was done in single boxes, with 5 fish per box and a total of 25 fish. Again, all steps had to be done in the hood, and fish had to be handled as gently as possible to avoid stress and additional irritation. Male fish (golden line) were treated in 10mM phosphate buffer pH6.6 including 1mM ENU for 1 hr at 21°C. Then, they were washed in 10mM phosphate buffer pH6.6 in system water for 1 hr at 21°C. Fish were then transferred to another cage filled with phosphate buffer and were left in the buffer over night in the hood. On the next day, the fish were transferred to the fish room, and kept without connection to the system for a few hours to allow equilibration of the temperature. Then, they were fed with Artemia and connected to the system. In total, the treatment was done for 5 times using 4x 1mM ENU and once 2mM ENU.

2.2.2.3 Generation of haploid embryos

This method was based on Pelegri and Schulte-Merker, 1999 (Pelegri and Schulte-Merker, 1999). ENU treated males were outcrossed to wildtype females, and the next generation of females was subjected to the squeezing procedure.

Females were to be separated one night before into single boxes. Best stripping and egg clutch quality were obtained during the first 4 hrs of the light cycle. After preparation, Hank’s working solution was kept on ice. Testes from males were dissected and pooled in the prepared Hank’s solution; per dissected pair of testes 80µl were used. Then, the mixture
was homogenized with a small plastic pestle and the debris was left to settle down for about 5 min on ice. The supernatant was transferred to a new tube; this sperm solution on ice was effective for about 2 hrs.

For UV- inactivation of the sperm, the bottom of a petri dish was filled with ice and placed under the UV lamp. A watch glass was then placed on top and the sperm solution was transferred to the glass. It was irradiated for 2 min with gentle stirring every 30 sec; the distance between the watch glass and the lamp was 38 cm. With a clean pipette tip the solution was transferred to a new Eppendorf tube. UV- treated sperm on ice remained active for about 2 hrs. The females were anesthetised in tricaine solution until no movement of the gills could be observed anymore. The fish were then rinsed in fresh fish water and placed on the bottom half of a petri dish. To avoid premature egg activation, excess water at the anal fin area was dried with a tissue. The fish were squeezed by applying gentle pressure with the index finger onto the belly. Then, females were put into single boxes with fresh fish water for recovery.

2.2.2.4 In vitro fertilisation

Only good egg clutches which looked slightly yellow and stayed together in a compact mass were used for the in vitro fertilisation. At least 30 µl of UV- treated sperm were added to each egg clutch, and sperm and eggs were mixed by moving the pipette tip without lifting it from the petri dish. After 30 seconds 1ml of E3 was added and the petri dish was filled with E3 after 1 additional minute. Petri dishes were transferred immediately to the incubator and eggs were checked after some hours for fertilisation.
2.2.3 Screening

2.2.3.1 Haploid screening

The fertilized eggs were incubated until 28 hrs of development, and then screened for obvious morphological abnormalities. In addition, the quality of eggs was scored as follows: eggs were sorted into A (good quality), B (truncated, distorted body axis but recognisable head and tail), C (only a little bit of tissue on top of yolk) and D (dead) embryos and C and dead ones were removed. The remaining clutch, consisting of A and B embryos, was fixed in 4% PFA and in situ hybridisations with antisense RNA- probes against lim1 and fgf8 transcripts were performed according to the standard protocol.

2.2.3.2 Diploid screening (Tuebingen screen)

Since the whole screen took a year, mutagenesis was done on a monthly basis by co-workers of Artemis pharmaceuticals to ensure that young and healthy fish were available throughout the year. On a weekly basis, fish were paired. Clutches were sorted and eggs were aliquoted into 30 eggs per screen group. Pronase and PTU were added to the dishes, and the embryos were incubated at 33°C o/n. The next day, embryos were screened for morphological abnormalities and transferred into 48 well trays for further development. At 34 hrs of development, they were fixed in 4% PFA and stained with antibodies against Opsin and acetylated-Tubulin using the Tuebingen staining protocol. Phenotypes scored by each screening group had then to be entered into a common database on the basis of a star system. ‘0’ would tell that a phenotype had been found, but this was too unspecific to be of interest to any screening group. One star ‘*’ meant that the mutant is of lower importance and should be kept as a sperm sample only. Two stars ‘**’ indicated that the mutant was of interest to the group and should have been kept as a sperm sample and be outcrossed. Finally, three stars ‘***’ meant highest priority mutant which had to be outcrossed and kept as sperm with first priority.
2.2.3.3 Re-identification of mutants in F3

If a mutant clutch was scored as interesting, the carrier fish (either a squeezed female from the haploid screen or a fish identified in the Tuebingen screen) was outcrossed to a wildtype Tue fish. Resulting families were incrossed randomly and at least 10 clutches per tank were screened for carriers. If no carrier could be found, this procedure was repeated 2 times and families that did not reveal any carriers were then discarded.

2.2.4 Whole mount in situ hybridisation

2.2.4.1 Solutions

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<thead>
<tr>
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2.2.4.2 Preparation of antisense-RNA probes

10 µg of a plasmid containing the cDNA of interest was digested for linearisation. The restriction digest was purified using the QIAquick® PCR purification Kit (Qiagen) and resuspended in 40µl water. 1 µg was used for the transcription reaction using the DIG-RNA-Labelling-Kit (Roche Biochemicals), according to the manufacturers protocol. The RNA probe was purified with mini Quick Spin RNA Columns (Roche Diagnostics), 450µl of Hyb+ were added and the probe was stored at –20°C. Final dilutions for probes were usually 1:50 to 1:500 of the according stock.

2.2.4.3 Experimental procedure for the in situ hybridisation

Dechorionated embryos were fixed overnight at 4°C, washed 2x 5 min in PBSTw, 2x 5 min in 100% methanol and transferred for at least 30 min to 20°C in 100% methanol. The embryos were rehydrated for 5 min in 50% methanol in PBS at RT, washed 2x 5 min in PBSTw, postfixed in 4% PFA for 20 min at RT and washed 2x 5 min in PBSTw at RT. For permeabilisation embryos were then digested with proteinase K (final concentration 10µg /ml in PBSTw) between 1 and 15 min at RT, depending on the developmental stage of the embryos, washed 2x 2 min in 2mg/ml glycine in PBSTw at RT and refixed in 4% PFA for 30 min at RT. Then, embryos were washed 3x 5 min in PBSTw at RT. Embryos were then transferred into prewarmed Hyb+ solution for at least 2 hours at 68°C on a shaker (all subsequent steps at 68°C). Hyb+ was then replaced with the prewarmed RNA probe in Hyb+, and embryos were incubated over night. Then, the probe was taken off (can be stored at -20°C and reused several times), embryos were washed 1x 5 min in Hyb+, 3x 10 min in 25% Hyb+ in 2x SSCT, once 5 min in 2x SSCT and 2x 30 min in 0.2% SSCT. All subsequent steps were performed at RT. Embryos were washed once 5 min in 50% SSCT/50% MABT and once 5 min in MABT, blocked for 1 hr in MABT including 2% DIG block and incubated 2 hrs at RT in anti-DIG AP, 1:4000 in DIG block (Roche Diagnostics). After removal of the antibody solution (can be reused several times) embryos were washed 4x 15 min at RT in MABT and transferred into 24-well plates for detection
with BM purple AP substrate (Roche Diagnostics). The staining was developed to the desired intensity in the dark at RT, stopped with PBSTw and then embryos were postfixed 20 min in 4% PFA. After the staining procedure, the embryos were dissected and sectioned with sharp forceps and tungsten needles, mounted in 70% glycerol and photographed on a Zeiss axiophot. Composites were assembled with Adobe Photoshop.

2.2.5 Bodipy ceramide

Morphology of living embryos was monitored by staining with the green vital dye Bodipy FL-Ceramide (Molecular Probes D-3521). For this, embryos were incubated at 28°C in 100µM Bodipy FL-Ceramide in E3 medium for 45-60 minutes. Stained living embryos were subjected to the confocal microscope for morphological inspection.

2.2.6 Cell death detection

Apoptotic cells were detected in living embryos by the intercalating DNA-stain acridine orange (AO) or, alternatively, in fixed embryos by the TUNEL assay.

2.2.6.1 Acridine Orange

AO (Acridine orange) was applied to dechorionated embryos in E3 medium to a final concentration of 2µg/ml for 1 to 3 hrs. Excess AO was washed off by rinsing the embryos several times in E3 afterwards. Fluorescence was monitored microscopically with a FITC filter set (Hoechst).
2.2.6.2 TUNEL assay

DNA degradation occurring in apoptotic cells yields double stranded DNA fragments as well as single strand breaks. These strand breaks can be detected by enzymatic labelling of the free 3’-OH termini with modified nucleotides, in this case fluorescin-dUTP.

Embryos were fixed over night at 4°C in 4% PFA, then rinsed 2x with PBST, 2x in 100% methanol and incubated in 100% methanol at -20°C (can be stored for several months) for at least 30 min. Then they were washed once with 50% methanol in PBST and 2x in PBST. If embryos were older than 30 hpf, washing steps with 50% methanol and PBST were omitted, and embryos were bleached directly 10 min in 0.3% H₂O₂ in methanol, then the washes were done. Then, embryos were treated with proteinase K (in situ hybridisation- conditions), washed 2x with 2mg/ml glycine, washed 2x in PBST and refixed in 4% PFA for 20 min. After washing the embryos 2x in PBST they were pre-incubated in TUNEL dilution buffer (Roche Diagnostics) for 2x 5 min. The buffer was replaced by the TUNEL reaction containing 5µl TUNEL enzyme (Roche Diagnostics) in 45µl TUNEL label (Roche Diagnostics) per reaction (best in 0.5 ml Eppendorf tubes). All subsequent steps were done in the dark. The reaction was preincubated 1hr on ice and incubated 1hr at 37°C. Then the embryos were washed in PBST and blocked for at least 1hr in 2% DIG block in MABT. They were incubated at 4°C in anti-fluorescein-TUNEL-POD (50µl per reaction, ready to go) (Roche Diagnostics) over night, washed in PBST and stained with DAB Sigma fast tablets (Sigma), according to the manufacturers conditions to desired intensity. The staining was stopped by repeated rinsing in PBST and embryos were then cleared in 70% Glycerol in PBS.
2.2.7 Whole mount immunocytochemistry

wash solution: PBSTx (PBS including 0.8% Triton)
block solution: wash solution including 10% NGS (normal goat serum) (Gibco BRL)
antibody solution: wash solution including 1% NGS

Embryos treated with Pronase and PTU were fixed for 3 hrs at RT in 2% TCA (trichloric acetic acid) and washed at least 2 hrs at RT with washing solution. Embryos older than 30 hpf were permeabilised with 0.005% trypsin in washing solution for 5 min on ice and washed again for 2 hrs in washing solution with repeated changes of the solution. Then, embryos were blocked in block solution for at least 1 hour at RT (can be extended up to 6 hrs) and incubated in primary antibody (anti-acetylated Tubulin, anti-phospho Histone H3, anti-Opsin, anti-RM044 or anti-tyrosine Hydroxylase in antibody solution) over night at 4°C. After removal of the antibody (can be stored in 0.01% NaN₃ at 4°C and reused several times) embryos were washed 2 hrs with several changes of the solution and then incubated in the secondary antibody (goat anti-mouse IgG peroxidase coupled or fluorescent antibody in antibody solution if a monoclonal primary antibody had been used, or goat anti-rabbit IgG peroxidase coupled antibody or fluorescent in antibody solution for a polyclonal primary antibody) for 4 hrs at RT. After removal of the secondary antibody embryos were washed for 2 hrs in washing solution and stained with DAB if a peroxidase coupled secondary antibody had been used. The staining was developed to the desired intensity, stopped with PBST and embryos were cleared with 70% Glycerol.

2.2.8 Anti-acetylated Tubulin staining (Tübingen protocol)

This protocol was used during the screening period in Tübingen. It is identical to the one described above, with the difference that 4% PFA has been used instead of TCA, and the fixation has been done at 4°C over night. In addition, no trypsin digest was used, and the washing steps were done for longer periods.
2.2.9 Inhibition of FGF signaling

FGF-mediated signal transduction was inhibited pharmacologically by SU5402 (Calbiochem). Embryos were dechorionated and incubated in E3 with PTU including a final concentration of 8µm SU5402. Incubation started at 20 somites, 24 hrs and 30 hrs of development and embryos were fixed at 48 hrs of development. SU5402 was stored in a light-protected box as a 8mM stock in DMSO at –20˚C.

2.2.10 Bead implantations

Bead implantation was done after Reifers et al., 2000. Beads coated with recombinant zebrafish Fgf8 (R+D systems), recombinant mouse Fgf4 (R+D systems) or PBS control beads were implanted into the hindbrain ventricle close to the MHB at 24 hrs of development and embryos were fixed at 48 hrs. For implantation, embryos were embedded into agarose and the epidermis was locally opened with a sharp tungsten needle. The bead was implanted with a blunt tungsten needle through the opening in the epidermis.

2.2.11 Coating of beads

600 µl of bead stock solution is pipetted onto a Millipore nylon net (NY 60 04 700, exclusion size 60 µm) that has been attached to the bottom of a cut-off Eppendorf tube. The tube is spun for 2 min at 500 rpm to get a flow through containing small beads. These are transferred to another Eppendorf tube, filled up to 1ml with 100% Ethanol and washed on a shaker at RT for 20 min. After centrifuging for 5 min the supernatant is discarded, and beads are washed 2x with PBS and centrifuged for 5 min each. 15 µl of this bead solution are added to 5 µl of protein solution to obtain a final concentration of 05 µg/µl of protein and incubated o/n at 4˚C. This protein solution and the bead solution can be stored wrapped in parafilm for several weeks at 4˚C.
2.2.12 Complementation assay

If initial morphological screening and/or staining with antibodies or in situ hybridisation revealed a similarity between strains or to already known mutants, carriers of these two lines were crossed against each other. Crossing carriers with recessive mutations in the same gene resulted in a mutant phenotype in a quarter of the embryo clutch, therefore the mutant alleles did not complement each other. If the mutations were introduced in different genes, no mutant phenotype was observed, and the two mutant alleles could complement each other.

2.2.13 Mapping

The mapping of the mutant lines derived from the Tuebingen screen 2000 was performed in collaboration with the groups of Robert Geisler in Tuebingen (initial mapping of \textit{HE409}, \textit{HF028} and \textit{HJ032}) and Will Talbot in Stanford (mapping of the rest and fine mapping of \textit{HE409}). Mutant lines in the Tuebingen background were crossed into the wik-line by outcrossing a Tuebingen carrier with a wik wildtype fish. Embryos of lines to be mapped were fixed in Dresden and sent on dry ice to Tuebingen or Stanford for further processing.

2.2.13.1 Tuebingen fixation protocol

Clutches were sorted morphologically into 48 mutants and 48 siblings at day 1 of development. Embryos were transferred directly into 100\% Methanol and stored until shipment at –20°C.
2.2.13.2 Stanford fixation protocol (Extraction of genomic DNA)

Embryos were dechorionated and sorted individually into 96 well PCR plates, 48 mutants and 48 siblings in total of each clutch. 50 µl of lysis buffer (10mM Tris-HCL pH8.0, 1mM EDTA, 0.3% Tween 20, 0.3% NP40) were added to each well and the plate was incubated for 10 min at 98°C in a PCR machine. Then, 5µl of Proteinase K (form a 10mg/ml stock) was added and the embryos were digested over night at 55°C. The Proteinase was inactivated for 10 min at 98°C and genomic DNA was stored until sending at –80°C.

2.2.14 Mapping procedure

The mapping procedure was carried out as described by Geisler, 2002. A set of 192 SSLPs (simple sequence length polymorphisms) which covered the whole genome of the zebrafish at an equal distance and which revealed a high rate of polymorphisms between the Tuebingen and wik strains were used to carry out PCRs on fixed mutant and wildtype siblings. Products were scored on agarose gels for different band intensities between a Tuebingen- and a wik- derived band. An increased intensity of the Tue-derived band in the mutant embryos in comparison to the siblings indicated potential linkage to the SSLP in question.

2.2.15 RNA isolation from zebrafish

Total RNA isolation was performed using the TRIZOL® reagent from GibcoBRL according to the manufacturer’s protocol. The zebrafish embryos (n=1-100) were collected in a 1.5ml eppendorf cup; after addition of 1ml TRIZOL® reagent, the embryos were homogenized using a small eppendorf pestil. The homogenate was then incubated for 5 min at RT to allow the dissociation of the tissue. After the addition of 200µl chloroform, vigorous shaking and incubation of 3 min at room temperature, the homogenate was
Materials and Methods

centrifuged at 4°C with 13,000 rpm for 15 min. The upper, RNA containing phase was then transferred into a new eppendorf cup and precipitated with 500µl isopropanol for 10 min at room temperature. After centrifugation (4°C, 13,000 rpm, 10 min), the RNA pellet was rinsed with 75% ethanol, dried and resuspended in 20 µl water. The average yield was around 0.5 to 1 µg of total RNA per embryo.

2.2.16 cDNA synthesis

cDNA synthesis was performed according to the manufacturers protocol of SuperScript™II (GibcoBRL) reverse transcriptase. 50 to 500ng of total RNA from zebrafish embryos was used, together with 500 ng oligo(dT)$_{12,18}$ primer (Roche Biochemicals) or with 250 ng of a gene specific primer.

2.2.17 Extraction of DNA from agarose gels

The desired band was cut out of the gel using a sharp razor blade and transferred into a 1.5 ml Eppendorf tube. The DNA was then extracted from the gel using the QIAquick® Gel Extraction Kit (Qiagen). DNA was recovered from the columns with 30µl water and stored at –20°C.

2.2.18 PCR

Polymerase Chain Reaction (PCR) was used to amplify DNA fragments from cDNA in order to produce templates for antisense- in situ probes or templates for cloning. The Advantage Taq Polymerase (Clontech) was used for generation of the onecut probe, the Pfx Polymerase (Stratagene) was used for the other constructs, namely evx1 (even skipped homeobox 1), npy (neuropeptide Y) and n-cad (n-cadherin).
2.2.18.1 Primer design

Primers were designed with the help of Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The internal primers for n-cadherin (primer 2-6) were designed with the help of Zsolt Lele (Wilson lab) and were used exclusively for sequencing.

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2.2.18.2 PCR reaction

For a standard PCR 1 µl cDNA, 1 µl primer forward, 1 µl primer reverse, 1 µl dNTPs, 1 µl MgSO4, 5 µl 10x buffer and 0.5 µl Pfx polymerase were used in total volume of 50 µl. If advantage Taq was used, no MgSO4 was added to the reaction.
Amplification:

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</tr>
<tr>
<td>npy</td>
<td>35 cycles</td>
<td>denature</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>denature</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>annealing</td>
<td>54°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extension</td>
<td>68°C</td>
</tr>
<tr>
<td></td>
<td>last cycle</td>
<td>extension</td>
<td>68°C</td>
</tr>
<tr>
<td>n-cad</td>
<td>35 cycles</td>
<td>denature</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>denature</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>annealing</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extension</td>
<td>68°C</td>
</tr>
<tr>
<td></td>
<td>last cycle</td>
<td>extension</td>
<td>68°C</td>
</tr>
</tbody>
</table>

The PCR fragments of *evx1*, *npy* and *n-cad* were cloned into the pCR4Blunt-TOPO vector (Invitrogen), grown in culture and prepared in mini-preparations (Qiaprep-Spin-Miniprep Kit). The yield of the mini preparations was determined, and the products were given to the sequencing facility for further analysis.

### 2.2.19 Sequencing and analysis

The sequencing was done by the sequencing facility of the MPI-CBG. Sequences were analysed using the program seqMan5.0 by comparing the wildtype sequences with the ones derived from the mutant cDNA.
3 Results

In this part, I will outline the results of the three parts of my work:

(3.1) the results of the haploid screen with examples of the mutants obtained,

(3.2) the results of the diploid screen, with examples for several phenotypic subclasses of mutants,

(3.3) the characterisation of one of the mutants from the diploid screen, *broken heart*.

3.1 Results of the haploid Heidelberg screen

In the first part of the results, I will focus on the outcome of the Heidelberg haploid screen. The screening work was shared between a former post-doc in the laboratory, Noriyuki Morita, and myself. The screen was performed to find new mutants defective in the formation and function of the MHB, which would indicate a non-functional MHB-organiser. Identification of the mutated gene would then contribute to refine the known pathways functioning at the MHB, or even elucidate new. The morphology of the MHB, a neuroepithelial folding, at day 1 of development was one criterion to screen for mutants, since the midbrain and hindbrain are both dependent on a functional organiser.

In addition, a probe cocktail consisting of *fgf8* and *lim1* was used in *in situ* hybridisations for gene expression studies. *fgf8* is expressed from early gastrula stages onwards in a variety of tissues, including the MHB, parts of the forebrain, optic stalk and otic vesicle (Reifers et al., 1998). At the MHB, it functions to maintain marker gene expression and in addition polarises the midbrain.

*lim1* is a homeobox gene and belongs to the LIM homeodomain protein family consisting of two LIM domains. These domains function in protein-protein binding and exert a negative regulatory influence on DNA binding (Toyama and Dawid, 1997). In mice, *lim1* is not expressed in the ectoderm until E10, but knock-out experiments have shown that it is required in primitive streak and visceral endoderm derived tissues to regulate the head organiser and therefore function in head formation (Shawlot and Behringer, 1995; Shawlot et al., 1999). In zebrafish, *lim1* can be found widely expressed in ectodermal structures such as fore-, mid- and hindbrain and spinal cord (Toyama and Dawid, 1997). LIM homeobox
genes in general are implicated in the specification of neuronal identity and axon pathfinding (reviewed in Dawid et al., 1998) and are therefore a good marker for defects in neuronal specification and patterning.

An overview about the complex expression patterns of the two genes, individually and in combination, is shown in Figure 2.

Figure 2: Expression domains of \textit{fgf8} and \textit{lim1} in a diploid embryo. (A-C) lateral views, (D-F) dorsal views. (A,D) Double \textit{in situ} hybridisations with both probes, (B,E) ISH with \textit{fgf8} only and (C,F) ISH with \textit{lim1} only. The expression domains are non-overlapping and represent roughly all parts of the brain at the stage indicated. ep epiphysis, hy hypothalamus, i isthmus, os optic stalk, ov otic vesicle, pt pretectum, tg tegmentum, vt ventral thalamus.

The result of the haploid Heidelberg screen is shown in Table 1. Less than 50% of the mutations discovered in haploid embryos could be re-identified and confirmed in the next generation, of which diploid fish were screened. The reason for this might be that it is extremely difficult to judge in a haploid background whether a phenotype is specific or just related to the strong background. In addition, the staining pattern obtained by antisense \textit{in situ} probes for \textit{lim1} and \textit{fgf8} varied significantly between haploids and diploids, e.g. the size and the shape of individual expression domains of these genes were different depending on the background. Third, the quality of the clutches of different females varied a lot, and embryos had to be compared only within a clutch for gross alteration in both, morphology and \textit{in situ} patterns.
Table 1: Statistics haploid Heidelberg screen.

<table>
<thead>
<tr>
<th></th>
<th>number</th>
<th>percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females squeezed</td>
<td>702</td>
<td>100</td>
</tr>
<tr>
<td>Clutches screened</td>
<td>197</td>
<td>28</td>
</tr>
<tr>
<td>Outcrossed for re-id in diploids</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Recovered phenotypes in diploids</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>Assigned to genes</td>
<td>4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The mutations were assigned to genes by performing genetic complementations with published mutants. For complementation, mutants were chosen with a similar phenotype, suggestive that the same gene was affected. Three of the mutants, e68, 713-07 and 728-04 did not complement spg (spiel ohne grenzen) and were therefore included in the spg analysis done by Gerlinde Reim, a graduate student in the lab. She could show that 713-07 and 728-04 are point mutations in the POU-homeo domain of pou2 (personal communication, and Burgess et al., 2002), and that the e68 allele is not mutated in the coding region of pou2 (Burgess et al., 2002). A 4th mutant, 526-08, did not complement knypek, and was given to the Heisenberg laboratory for further analysis. All other mutants were not included in the complementation analysis, since they displayed novel phenotypes that have not suggested any similarity to any known mutant so far.

The phenotypic subgroups of all 21 mutants kept for re-screening are shown in Table 2. The assignment to a particular group is based on the most prominent phenotype. However, if a mutant showed stronger in situ signals in a specific part of the brain, but weaker signals in another part, e.g. 720-04, it was included in two subgroups and therefore will appear twice in the table.
Table 2: Phenotypic groups haploid Heidelberg screen.

<table>
<thead>
<tr>
<th>Main phenotypic group</th>
<th>Name of mutant</th>
<th>Area affected</th>
<th>Additional phenotypes</th>
<th>Fate of the mutant/Phenotype in diploids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stronger ISH signal in defined areas</strong></td>
<td>713-07</td>
<td>Spinal cord</td>
<td>Degeneration</td>
<td>spg</td>
</tr>
<tr>
<td>720-01</td>
<td>Telencephalon</td>
<td>Degeneration</td>
<td>Lost</td>
<td></td>
</tr>
<tr>
<td>720-04</td>
<td>Telencephalon</td>
<td>see below</td>
<td>Characterisation ongoing</td>
<td></td>
</tr>
<tr>
<td>727-05</td>
<td>MHB and Telencephalon</td>
<td>-</td>
<td>HF028</td>
<td></td>
</tr>
<tr>
<td>804-06</td>
<td>MHB</td>
<td>Split tail and see below</td>
<td>Lost</td>
<td></td>
</tr>
<tr>
<td><strong>Weaker signal in local areas</strong></td>
<td>706-02</td>
<td>Epiphysis</td>
<td>-</td>
<td>No re-id</td>
</tr>
<tr>
<td>706-04</td>
<td>Telencephalon</td>
<td>-</td>
<td>No re-id</td>
<td></td>
</tr>
<tr>
<td>706-05</td>
<td>Hindbrain</td>
<td>Degeneration</td>
<td>Degeneration</td>
<td></td>
</tr>
<tr>
<td>720-04</td>
<td>Pretectum</td>
<td>see above</td>
<td>see above</td>
<td></td>
</tr>
<tr>
<td>727-12</td>
<td>Midbrain/Diencephalon</td>
<td>-</td>
<td>Hindbrain extra fold, blister tail</td>
<td></td>
</tr>
<tr>
<td>728-04</td>
<td>Lim1 domain not present</td>
<td>-</td>
<td>spg</td>
<td></td>
</tr>
<tr>
<td>804-06</td>
<td>Hindbrain</td>
<td>see above</td>
<td>No re-id</td>
<td></td>
</tr>
<tr>
<td>817-01</td>
<td>Not specified</td>
<td>Degeneration</td>
<td>Degeneration</td>
<td></td>
</tr>
<tr>
<td><strong>Local degeneration</strong></td>
<td>623-04</td>
<td>Brain</td>
<td>-</td>
<td>No re-id</td>
</tr>
<tr>
<td>706-05</td>
<td>Brain</td>
<td>see above</td>
<td>Degeneration</td>
<td></td>
</tr>
<tr>
<td>713-07</td>
<td>Brain</td>
<td>see above</td>
<td>spg</td>
<td></td>
</tr>
<tr>
<td>713-10</td>
<td>Eye, Spinal cord</td>
<td>-</td>
<td>Degeneration</td>
<td></td>
</tr>
<tr>
<td>720-01</td>
<td>Brain</td>
<td>see above</td>
<td>No re-id</td>
<td></td>
</tr>
<tr>
<td>721-05</td>
<td>Brain</td>
<td>Hindbrain devoid of staining, delayed</td>
<td>Delayed, Degeneration</td>
<td></td>
</tr>
<tr>
<td>817-01</td>
<td>Brain</td>
<td>see above</td>
<td>Degeneration</td>
<td></td>
</tr>
<tr>
<td>825-01</td>
<td>Brain</td>
<td>see below</td>
<td>No re-id</td>
<td></td>
</tr>
<tr>
<td><strong>ventralised</strong></td>
<td>706-08</td>
<td>Body plan</td>
<td>-</td>
<td>No re-id</td>
</tr>
<tr>
<td>720-10</td>
<td>Body plan</td>
<td>Head and tail reduced</td>
<td>Ventralised</td>
<td></td>
</tr>
<tr>
<td><strong>Body shape</strong></td>
<td>526-08</td>
<td>No tail</td>
<td>-</td>
<td>knypek</td>
</tr>
<tr>
<td>720-11</td>
<td>Curly tail</td>
<td>Body axis short</td>
<td>Degeneration</td>
<td></td>
</tr>
<tr>
<td><strong>others</strong></td>
<td>e68</td>
<td>no description</td>
<td>-</td>
<td>spg</td>
</tr>
<tr>
<td>824-05</td>
<td>No lens</td>
<td>No re-id</td>
<td></td>
<td></td>
</tr>
<tr>
<td>825-01</td>
<td>No lens</td>
<td>Degeneration</td>
<td>Degeneration</td>
<td></td>
</tr>
</tbody>
</table>
3.1.1 Mutants from the haploid screen kept for re-id display specific phenotypes

To be chosen for further characterisation, the mutants had to fulfil different criteria: they had to be specific in a way that only certain domains were affected as judged by morphology, or expression domains in the in situ analysis were specifically lost or gene expression was upregulated. Embryos within the same clutch were compared among each other, and only if a significant difference in morphology and/or expression pattern within the clutch could be found, these embryos were scored as mutants and subjected to outcross and further analysis. 720-04, 706-05 and 721-05 are three examples of mutants in which the expression of fgf8 and/or lim1 is diminished in specific areas (Fig. 3 C,D,G,H) or enhanced (Fig. 3 C). Since none of these mutants displayed any cell death in morphology (data not shown), the difference in staining intensities indicates that either fgf8 or lim1 are regulated differently in the mutant. Indeed, in the re-screen in diploids the phenotype of 720-04 could be detected again, at least partially (Fig. 4). The expression domain of lim1 in the pretectum is still absent, and in addition the staining in the ventral thalamus is diminished (Fig. 3). In contrast, the enhanced expression in the telencephalon cannot be detected anymore (Fig. 4 D’).

Another example of specificity discovered in the haploids is specific cell death, as shown for 713-10 (Fig. 3 J). Here, distinct areas in the brain are filled with dying cells, while the rest of the brain develops normally and without any sign of degeneration. In situ hybridisations on embryos from 713-10 did in contrast show no defects in the expression of lim1 or fgf8 (data not shown).

In the re-screen in diploid clutches, a number of interesting phenotypes arose which had been unexpected, since the original descriptions did not fit the new ones. For example, 727-12 had been described as a mutant with weaker gene expression in the diencephalon and midbrain, but in the next generation and in a diploid background, a hindbrain and tail phenotype evolved instead (Fig. 4 A-C’). Diencephalon and midbrain are completely normal, but an extra fold in the hindbrain is visible (Fig. 4 A’,B’). The epidermis around the tail tip forms blisters, and in addition, blood accumulates ventrally (Fig. 4 C’). This
might indicate an adhesive problem in the mutant (see section on \textit{n-cadherin} mutants), and further studies should test this hypothesis.

\textbf{Figure 3: Mutants from the haploid screen.} Haploids stained with \textit{fgf8} and \textit{lim1} (A-H) and pictures of the morphology of F3 diploids (I,J). Anterior to the left, embryos at 34 hrs (A-H) and 30 hrs (I,J). (A,C) 720-04 as an example of a mutant in which gene expression is stronger in certain domains (arrowhead in C) while others are diminished (arrow in C). (B,D) In 706-05, the expression domain of \textit{lim1} in the hindbrain is completely lost (arrows in D), the MHB domain of \textit{fgf8} is missing in one half of the embryo (white arrowhead in D). (E-H) The \textit{in situ} staining in 721-05 is refined to two broad areas, the MHB (asterisk) and the telencephalon and anterior diencephalon; the rest of the embryo is devoid of any staining. (I,J) Live picture of a degeneration mutant, 713-10. There are two obvious spots of focal cell death in the brain, one in the midbrain and one in the anterior rhombomeres (arrows in J).
Figure 4: Morphology and in situ phenotype of mutants from the haploid screen kept after re-screening diploids. Anterior is left, all embryos are 30 hrs old. (A-C’) 727-12 displays specific phenotypes in the hindbrain and tail tip. In the hindbrain anterior of the level of the ear the neural tissue is folding partially up (arrow in A’) and inwards (arrow in B’). The epidermis at the tail forms blister-like structures (arrowheads in C’), slight ventralisation is apparent through the accumulation of blood posterior to the yolk-extension (arrow in C’). (D,D’) 720-04. In situ hybridisation on diploid embryos, arrows in (D,D’) point to the expression domains in the tegmentum and the ventral thalamus.

3.1.2 Summary of the haploid Heidelberg screen

The haploid Heidelberg screen was performed with the aim to find new mutants defective in the formation of the MHB and therefore allowing further insights in the molecular pathways involved in formation and function of the MHB. I met this goal with three mutants, which turned out to be mutated in the pou2 locus. The other mutants kept from this screen still await further analysis. Possibly, they could help to study different developmental concepts, such as adhesion, focal cell death and convergence-extension.
3.2 Overview on the mutants obtained by the Tuebingen screen 2000

In this section of my thesis, I will give an overview about the general procedure of the screen, the outcome in numbers and show different mutants selected from phenotypic subgroups. In addition, I will present work on one of the mutant groups, which is represented by 3 different alleles of a mutation in the n-cadherin locus.

This screen was performed to find new mutants affecting brain development. Since it was a shared effort between our laboratory and the laboratory of Steve Wilson, London, several brain features were looked at. The group in London is interested in development of the epiphysis and the forebrain, therefore we decided not to do an in situ screen, which would have implied a combination of a range of antisense in situ probes, but rather a live morphology screening and immunostainings with two antibodies, anti-Opsin and anti-acetylated Tubulin, so the goals of us and the other group could be met. Opsin is produced by photoreceptor cells in the epiphysis, and acetylated Tubulin is found in all axon tracts and commissures and their neurons (Wilson et al., 1990).

3.2.1 Major axonal tracts of the brain

First, I will explain the pattern obtained by anti-acetylated Tubulin antibody stainings of wildtype embryos at 34 and 48 hpf. At 34 hpf, the zebrafish brain contains a simple set of connected axon tracts, a sort of "axon scaffold". In the established nomenclature of zebrafish development, a "tract" is described as a bundle of axons that course together in the central nervous system (CNS), without reference to the function, origin or destination of the axons within it (Wilson et al., 1990). These tracts have been formed by axons that have grown between the early primary neuron clusters to either form bundles that connect to one another or to other brain areas. Early axonal scaffold formation acts to make an orthogonal grid, which can be seen best in a dorsal view (Fig. 5 D): two longitudinal tracts are formed along the A-P axis with interconnecting commissures
between them. At 34 hpf a prominent axon bundle has developed along the ventral part of the brain, and is continuous along the whole length of the ventral brain: at the anterior end of the brain it passes around the diencephalon to form the postoptic commissure (POC) (Fig. 5 B). This longitudinal bundle of axons is called the tract of the postoptic commissure (TPOC) (Fig. 5 A). At the level of the midbrain it courses alongside, and slightly dorsal to, the medial longitudinal fasciculus (MLF), an axon pathway which descends the embryo at either side of the developing floor plate in the ventral neural tube (Fig. 5 A,D). Some bundles from the TPOC can be seen to actually join the MLF. Both tracts continue to course longitudinally through the hindbrain and then into the spinal cord. The anterior commissure (AC) also passes around the rostral end of the telencephalon, dorsally to the POC (Fig. 5 B). The AC and POC are connected by a small axon tract called the supraoptic tract (SOT) (Fig. 5 A). The dorsoventral diencephalic tract (DVDT), which is formed by the projecting neurons of the epiphysis, joins the TPOC halfway along the length of the diencephalon (Fig. 5 E). The tract of the posterior commissure (TPC) also joins the TPOC, at the boundary between the forebrain and midbrain (Fig. 5 A). The ventral tegmental commissure (VTC) is found in the floor of the anterior tegmentum and crosses the ventral floorplate in the midbrain (Fig. 5 C). In a similar way to the TPOC, the ventral tegmental commissure is not neatly fasciculated, but instead displays a bundle of fibres crossing the midline. A second longitudinal axon tract that arises in the hindbrain and descends the embryo lying parallel to (but not connecting to) the MLF is the dorsal longitudinal fascicle (DLF) (Fig. 5 D). The DLF is formed by axons of both trigeminal ganglion cells and Rohon-Beard cells. Hindbrain commissural interneurons including the prominent Mauthner Neurons are lined up in a segmented fashion in the hindbrain (Fig. 5 D,H).

A lateral view of the embryo shows the olfactory placodes, and at a different focal plane of the same embryo the trigeminal ganglion caudal to the developing eye (Fig. 5 A’). Rohon-Beard neurons lie in the dorsal spinal cord, and spinal motor neurons descend orthogonally from the ventral spinal cord neurons towards the yolk sac extension (Fig. 5 F). The lateral line descends perpendicular to the spinal cord motor axons down the tail (Fig. 5 F).

By 48 hpf, there is a dramatic increase in the number of axons in the brain. Most of the newly formed axons appear to join pre-existing tracts formed the day before, rather than
making new ones themselves. The tracts have thickened and to project further than in a 34 hour embryo. After two days of development, the optic nerve has formed (Fig. 5 G). In addition, the tectal and cerebellar neuropil has started to form, and cerebellar afferents appear in the isthmic fold, where medial the Isthmic commissure is visible (Fig. 5 H,I). From the hindbrain, branchiomotor neurons innervate muscles arising in the pharyngeal arches (Fig. 5 H).

Figure 5. Anti-acetylated-tubulin labelling of a 34 (A-F) and 48 (G-I) hour wholemount wildtype embryo. (B,C,G) ventral views, (D,E) dorsal views and (A,F,H) lateral views, (I) dorso-lateral view. Anterior is up (B,E,G) or left (A,C,D,F,H,I).

AC anterior commissure, CerA cerebellar afferents, CerN cerebellar neuropile, DLF dorsal longitudinal fascicle, DVDT dorso-ventral diencephalic tract, HCl hindbrain commissural interneurons, HMP hindbrain motor projection, IC isthmic commissure, LL lateral line, MLF medial longitudinal fascicle, Mn Mauthner neuron, Olf olfactory bulb, ON optic nerve, PC posterior commissure, POC post-optic commissure, prEp projecting neurons of the epiphysis, RB Rohon beard neurons, TAC tract of the anterior commissure, TecN tectal neuropile, TPC tract of the post-optic commissure, Trig trigeminus, SMA spinal motor axons, SOT supra-optic tract, VTC ventral tegmental commissure.
3.2.2 Statistical results of the Tuebingen screen 2000

During one year of screening in Tuebingen, 2531 families could be screened by the brain group (Table 3). In total, 220 mutant lines were identified, including those clutches which had been scored as ‘0’ (see Materials and Methods for classification). Of the 98 mutants scored as ‘two’ (**) and ‘three’ (***), 82 could be outcrossed and raised as an F3 family, because the carrier pair of the other 16 lines had died before or did not produce eggs anymore. In the course of re-identification, 47 mutants could not be recovered again, and 13 of the 35 mutants recovered displayed phenotypes different from the ones described originally or revealed two different mutations coming from one carrier fish. The 35 re-identified mutants were assigned to 4 different phenotypic subgroups, as shown in Table 4.

Table 3: Mutants recovered

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total families screened</td>
<td>2531</td>
<td>100</td>
</tr>
<tr>
<td>Total genomes screened</td>
<td>2205.89</td>
<td>-</td>
</tr>
<tr>
<td>Total identified</td>
<td>220</td>
<td>8.6</td>
</tr>
<tr>
<td>** and *** mutants kept for rescreen</td>
<td>98</td>
<td>3.9</td>
</tr>
<tr>
<td>F3 raised</td>
<td>82</td>
<td>3.2</td>
</tr>
<tr>
<td>Not re-identified or lost in F3</td>
<td>47</td>
<td>1.9</td>
</tr>
<tr>
<td>Confirmed and characterised</td>
<td>35</td>
<td>1.4</td>
</tr>
<tr>
<td>New phenotypes in F3 (+ doubles)</td>
<td>13</td>
<td>0.5</td>
</tr>
<tr>
<td>Assigned to genes</td>
<td>8</td>
<td>0.3</td>
</tr>
<tr>
<td>Unresolved</td>
<td>30</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Genomes calculated using the formula $2x(1-0.75^n)$, n is the number of crosses scored per family.
Table 4: Phenotypic groups of the mutants found by the brain group.

<table>
<thead>
<tr>
<th>main group</th>
<th>subgroup</th>
<th>no. of mutant strains</th>
<th>classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain morphology</td>
<td></td>
<td>18</td>
<td>A</td>
</tr>
<tr>
<td>Body shape</td>
<td>Midline</td>
<td>3</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Ventralisation</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Somites/notochord</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curly tail</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Degeneration</td>
<td></td>
<td>8</td>
<td>C</td>
</tr>
<tr>
<td>Organs</td>
<td>Heart</td>
<td>1</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>Ear</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epiphysis</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epidermis</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

An overview of the original phenotypes and the ones appearing in the F3 re-screen is given in Table 5. If a phenotype could be confirmed, no further description was included in the table. Some mutants, e.g. *HE409*, were given to us from a different screening group, because they defined it as a brain mutant with potential interest for our group. Our group did not always have access to all families to be screened, because every screening week a different screening group had first priority for eggs. If clutches with a low amount of eggs were produced, only the groups with highest priority were supplied with eggs from this particular clutch.
Table 5: Phenotypes of the re-identified and characterised mutants from the Tuebingen screen 2000.

<table>
<thead>
<tr>
<th>Name</th>
<th>Original description Tuebingen</th>
<th>Description reid; phenotypic group</th>
</tr>
</thead>
</table>
| **HC006** | large distance between posterior commissure and epiphysis, restriction at the hindbrain, hindbrain malformed, too many cells in the tectal area; retarded eyes; shorter body axis and less Tubulin staining due to delayed growth anterior commissure does not form properly | Two phenotypes:  
**HC006a:** cell death C  
**HC006b:** inflated hindbrain A  
Phenotypes cannot be separated by outcross |
<p>| <strong>HC024</strong> | 4/59 have an <em>oep</em>-like phenotype at 24 hpf. Fused eyes which by 36 hpf have become only one-eye, sometimes on left or right of head, sometimes central. Forebrain structures are lost. It has a larger tegmentum and the MHB is disrupted. It has a curly down tail and a somewhat undifferentiated notochord. The somites are normal but the floor plate is patchy. The fins are reduced. | Confirmed: <em>oep</em> B |
| <strong>HE409</strong> | 6/25 (morphology found by somite screen) no notochord, head strongly reduced by day 3. | New A |
| <strong>HF017</strong> | 11/28 morpholgy at 30 hpf shows disrupted tectum, ill-defined MHB, disrouted cerebellum, undulating hindbrain, a wiggly anterior notochord and a slightly shorter tail. Reminiscent of parachute-like phenotypes. The Tubulin shows no supraoptic tract and a disorganised hindbrain. The spinal cord looks dorso-ventrally expanded and there are perhaps more Rohon Beard neurons. | Confirmed A |
| <strong>HF028</strong> | 7/28 bumpy rostral to hindbrain. Odd shaped. Little or no hindbrain posterior of ear. Expanded ventricles. Small eyes. Laterally expanded neural tube. | Confirmed A |
| <strong>HJ032</strong> | There is overproliferation of post. brain at 24 hpf. The MHB is disrupted. Hindbrain vesicle severly reduced reminiscent of <em>snakehead</em>- class of mutants but the space is filled with supernurnary hindbrain. Bizarre tegmentum, with apparently no tectal vesicle. Cerebellum present but also disrupted. | Confirmed A (<em>n-cadherin</em>) |
| <strong>HJ050</strong> | at 36 hpf: in 4/15 anterior commissure not closed (Tubulin) 5/32 disorganised hindbrain patterning | Confirmed A (<em>n-cadherin</em>) |</p>
<table>
<thead>
<tr>
<th>Name</th>
<th>Original description Tuebingen</th>
<th>Description re-id, phenotypic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK311</td>
<td>5/29 no TPC or epiphysis. Brain appears amorphous in this region. All have curly tail down.</td>
<td>Confirmed B</td>
</tr>
<tr>
<td>HK314</td>
<td>5/18 Amorphous heads and cell death in head. 3 of these when stained with anti Tubulin appear to have cell death specific to hindbrain (r2-r4).</td>
<td>Confirmed C</td>
</tr>
<tr>
<td>HM030</td>
<td>7/30 show reduced/disorganised epiphysis.</td>
<td>Confirmed D (epiphysis)</td>
</tr>
<tr>
<td>HM079</td>
<td>8/30 have incomplete anterior commissure, often too close to postoptic commissure with pathfinding defects.</td>
<td>New A</td>
</tr>
<tr>
<td>HN148</td>
<td>29 hrs morphology: 4/24 flat head, no proper MHB folds (no ventricles and too many cells) at 34 hpf: 3/24 pax2.1 staining at MHB present but folds as structures not visible</td>
<td>Confirmed A</td>
</tr>
<tr>
<td>HS304</td>
<td>8/31 show reduced staining in otic vesicle (one otolith instead of two).</td>
<td>Confirmed D (ear)</td>
</tr>
<tr>
<td>HU005</td>
<td>5/25 smaller forebrain short body; two with split tail retarded (34 hpf)</td>
<td>Two independent phenotypes: HU005a: brain morphology A HU005b: cell death C</td>
</tr>
<tr>
<td>HU012</td>
<td>Mild brain necrosis and curly tail at 28 hpf. Possibly U-shaped somites. Delayed. The Tubulin shows lack of motor axons, the postoptic commissure and the anterior commissure are not closed. The posterior commissure is also absent.</td>
<td>Confirmed C</td>
</tr>
<tr>
<td>HU519</td>
<td>7/24 (36 hpf Tubulin) show rounded hindbrain (lateral view), diffuse staining and scattered Rohon-Beard cells. Hindbrain commissures not formed properly. TPOC is not always visible, often it is weakly stained or diffuse. 24 hpf morphology screen showed a bumpy hindbrain phenotype and bent tail.</td>
<td>Confirmed A</td>
</tr>
<tr>
<td>HX066</td>
<td>4/27 have reduced brain vesicles at 30 hpf. Similar to the snakehead class of mutants. Also have no circulation and mild oedema. The Tubulin was not informative.</td>
<td>Confirmed A</td>
</tr>
<tr>
<td>HX137</td>
<td>6/23 posterior lateral line projection veers ventrally for approx. first 2 somites before resuming normal course. Possible indentation of yolk at hb/spinal cord boundary.</td>
<td>Confirmed B</td>
</tr>
<tr>
<td>Name</td>
<td>Original description Tuebingen</td>
<td>Description re-id, phenotypic group</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HZ378</td>
<td>8/28 curly down tail, anterior commissure not closed. Projections very messy. No epiphysis present with Tubulin or Opsin stain.</td>
<td>Confirmed A</td>
</tr>
<tr>
<td>IA015</td>
<td>7/24 dead head (at 32 hpf morphology) Tubulin (34 hpf): post optic commissure not closed, anterior commissure not formed at all hindbrain disorganised; short body, kinky tail</td>
<td>New: degeneration C</td>
</tr>
<tr>
<td>IF084</td>
<td>4 day morphology: mutants look like strong cyclops phenotype. Also severe curly down tail. Promuding jaw and prospective olfactory bulb from telencephalon (bi-lateral). Fused eyes.</td>
<td>Confirmed: cyclops B</td>
</tr>
<tr>
<td>IM021</td>
<td>30hr morphology screen, 6/25 show small eyes, a flat and small head and missing MHB.</td>
<td>Confirmed A</td>
</tr>
<tr>
<td>IN193</td>
<td>Cartilage Morphology screen 4/20 large somites in D-V axis, short spinal cord, narrow hindbrain. Nervous tissue anterior to cerebellum divided into two compartments (midbrain morphology not matured)</td>
<td>New: D (epidermis)</td>
</tr>
<tr>
<td>IO005</td>
<td>Tubulin Stain 36 hpf: 3/21 Severe anterior truncation. No segmental motor axon projections in the spinal cord. Possibly no somites</td>
<td>New: ventralised B</td>
</tr>
<tr>
<td>IT033</td>
<td>d1 morphology (found in cartilage batch): 6/23 bumpy hindbrain, looks like segmentation problem; gaps (lateral view) midbrain malformed MHB malformed</td>
<td>Confirmed A</td>
</tr>
<tr>
<td>IT419</td>
<td>d1 Tubulin 5/19 embryos had no cerebellum. They had disorganised neural pattern in the anterior hindbrain and in the midbrain.</td>
<td>New: degeneration C</td>
</tr>
<tr>
<td>IY001</td>
<td>d1 morphology: 6/23 inflated hindbrain; constriction at the MHB; small eyes; slightly shorter body; beginning cell death in the head; protruding telencephalon d1 Tubulin: no AC in some cases; present Tubulin is not smooth but lumpy</td>
<td>Confirmed C</td>
</tr>
</tbody>
</table>
### Results

<table>
<thead>
<tr>
<th>Name</th>
<th>Original description Tuebingen</th>
<th>Description re-id, phenotypic group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IY121</strong></td>
<td>d1 morphology: starting necrosis; head with a slight bump at the midbrain; eyes look as if still connected by an optic stalk; hook tail d1 Tubulin: 8/31 retarded; short body; bumpy brain</td>
<td>New D (epidermis)</td>
</tr>
<tr>
<td><strong>JH055</strong></td>
<td>7/36 embryos have commissural defects; TPOC is thicker than normal, possibly with arborisation approaching the midline. AC is delayed, and may not form properly at all. Same embryos also have hindbrain defects - lack of ladder structure, displaced neurons, misrouting axons and spinal cord problems. Some embryos have lateral line which aberrantly migrates onto the yolk.</td>
<td>Confirmed A</td>
</tr>
<tr>
<td><strong>KI104</strong></td>
<td>Tubulin 36 hrs: 2/8 Eyes not obvious. Morphology of brain anterior to MHB very disturbed. Tubulin stain is consequently disorganised. Segmentation of tubulin pattern in hindbrain also absent.</td>
<td>New A (noi)</td>
</tr>
<tr>
<td><strong>KS015</strong></td>
<td>8/29 embryos: Totally disrupted 36 hpf Tubulin pattern. Excess projections all over the brain. General brain morphology disrupted. Excess Mauthner neurons visible? Lateral line seems to originate at midline. Morphology screen at 24 hpf showed a twisted tail and a possible reduced head tissue</td>
<td>New D (heart)</td>
</tr>
<tr>
<td><strong>KW080</strong></td>
<td>8/34 All short embryos. No retardation with respect to axonal scaffold. Variable extra fold in caudal hindbrain.</td>
<td>New A</td>
</tr>
<tr>
<td><strong>KY025</strong></td>
<td>6/28 Tubulin screen: Midbrain and forebrain disrupted, one eye present displaced laterally</td>
<td>Confirmed: oep B</td>
</tr>
</tbody>
</table>

### 3.2.3 Complementation assay

For the establishment of complementation groups, crosses were performed between mutants from the screen and mutants or already described ones with similar phenotypes. The most prominent phenotypic feature of the mutants from the screen was the basis for choice of mutants as crossing partners and mutants with a unique phenotype were not tested. The summary of the results is shown in Table 6.
Mutants that did not complement each other, e.g. IM021 and HN148, were most likely mutated in the same gene and therefore, only one of the alleles was subjected to mapping of the mutant locus. The resulting allele frequencies of the re-identified mutants are shown in Table 7.
Table 7: Allele frequency of re-identified mutants

<table>
<thead>
<tr>
<th>n alleles</th>
<th>n genes</th>
<th>n mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>sum</td>
<td>30</td>
<td>38</td>
</tr>
</tbody>
</table>

The average allele frequency is therefore rather low, at 1.3 alleles/gene. Therefore, the majority of genes is represented by only one allele.

3.2.4 Mapping results

The first step leading to the identification of the gene affected in a mutant is to link the mutated locus onto a genetic map. The rough mapping was done in collaboration with Robert Geisler in Tuebingen (mapping of HE409, HF028 and HJ032) or Will Talbot in Stanford (all others and HE409), and involved assignment to a linkage group (= chromosome). In Dresden, embryos were sorted according to their phenotype, fixed and sent on dry ice to the appropriate lab. Mapping was not done by myself since the labs in collaboration have a long-term experience in the procedure and also the equipment to do high-throughput mapping on several strains at the same time (for reference see Geisler, 2002). The results for the first round of mapping (rough mapping) are shown in Table 8 and 9. HE409 was mapped in both labs, since there was a chance that one of the markers of the Tuebingen mapping could be confirmed and therefore the probability of the linkage could be enhanced. Indeed, Z11403 appears in both trials as the marker the mutation is closest linked to.
Table 8: Results from the Tuebingen mapping.

<table>
<thead>
<tr>
<th>allele</th>
<th>Linkage group</th>
<th>Placement from top</th>
<th>marker</th>
<th>From top</th>
<th>Linkages +/-</th>
<th>LOD score</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE409</td>
<td>LG 19</td>
<td>49.8</td>
<td>Z3816</td>
<td>38.3</td>
<td>21.9</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Z11403</td>
<td>53.5</td>
<td>5.4</td>
<td>27.3</td>
</tr>
<tr>
<td>HF028</td>
<td>LG 24</td>
<td>70.1</td>
<td>Z22375</td>
<td>59.4</td>
<td>10.8</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Z5657</td>
<td>66.3</td>
<td>3.8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Z3901</td>
<td>72.2</td>
<td>4.6</td>
<td>9.5</td>
</tr>
<tr>
<td>HJ032</td>
<td>LG 20</td>
<td>52.7</td>
<td>Z10056</td>
<td>38.9</td>
<td>14.4</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Z11841</td>
<td>49.8</td>
<td>1.7</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Z3964</td>
<td>66.1</td>
<td>7.9</td>
<td>16.4</td>
</tr>
</tbody>
</table>

The possible position of the mutation is shown in “placement from the top”, and the “from top” column shows the position of the Z marker. All distances are in cM (centi Morgan). The mutation is +/- cM away from the indicated marker. The LOD score (logarithm of odds) is the decadic logarithm of the ratio of the likelihoods of whether there is a linkage at the expected map position or not. The score should be over 3, since this determines that a linkage is 1000 times more likely than no linkage.

Table 9: Results from the Stanford mapping.

<table>
<thead>
<tr>
<th>allele</th>
<th>Linkage group</th>
<th>marker</th>
<th>Recombinants/meiosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE022</td>
<td>LG 11</td>
<td>Z13395</td>
<td>3/70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z4190</td>
<td>3/78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z10142</td>
<td>2/72</td>
</tr>
<tr>
<td>HE409</td>
<td>LG 19</td>
<td>Z11403</td>
<td>2/58</td>
</tr>
<tr>
<td>HF028</td>
<td>LG 24</td>
<td>Z11862</td>
<td>0/80</td>
</tr>
<tr>
<td>HK311</td>
<td>LG 24</td>
<td>Z20051</td>
<td>6/48</td>
</tr>
<tr>
<td>HS304</td>
<td>LG 12</td>
<td>Z26301</td>
<td>4/80</td>
</tr>
<tr>
<td>HX066</td>
<td>LG 10</td>
<td>Z22422</td>
<td>4/90</td>
</tr>
<tr>
<td>HX168b</td>
<td>LG 19</td>
<td>Z6589</td>
<td>2/40</td>
</tr>
<tr>
<td>IM021</td>
<td>LG 9</td>
<td>Z20031</td>
<td>3/78</td>
</tr>
</tbody>
</table>

If there are a few recombinants, this means that the mutation is not right on top of the marker, but it is instead somewhere in the same region. If a marker recombines 1 time in 100 meiosis, then on average it will be about 600 kb (about 1cM) away from the mutation. It has to be taken into account that the recombination frequency can vary widely in different regions of the genome, since the recombination frequency is strongly reduced in
centromeric regions, likely due to centromer-specific chromatin characteristics (Will Talbot, personal communication). Some mutants, such as *HK314*, *IY001* and *IY121*, could not be linked to a marker so far.

3.2.5 Phenotypes of the Tuebingen screen 2000

In the next section, I will introduce a variety of phenotypes found in the screen, representing the 4 major phenotypic subgroups defined before, and then in more detail the different types of brain morphology mutants are presented.

3.2.5.1 The Tuebingen screen 2000 revealed two additional *oep* alleles, *HC024* and *KY025* and one new *cyclops* allele, *IF084*

*oep* and *cyclops* are two midline mutants most extensively studied. The *oep* gene is essential for endoderm and anterior axial mesoderm formation as well as for early development of the ventral neuroectoderm (Schier et al., 1997). *cyclops* (*cyc*) mutants fail to develop a floor plate and loss of ventral forebrain results in fusion of the eyes (Hatta et al., 1991). Since the mutants *HC024*, *KY025* and *IF084* display phenotypes reminiscent to *oep* and *cyc* (Fig. 6) and complementation analysis identified them as alleles of the latter (Table 6), no further experiments were planned with these mutants.
Results

3.2.5.2 Mutants affecting neural survival

Mutants displaying cell death in neural tissue have been often found in forward genetic screens and have been described before (Furutani-Seiki et al., 1996; Abdelilah et al., 1996). Although these mutants seem to be affected more unspecifically and therefore less appropriate to study specific developmental aspects, the study of degeneration mutants could help to understand a number of complex and specific cellular mechanisms in development. Cells might undergo programmed cell death because they fail to establish correct synaptic connections and might therefore be depleted of neurotrophins (Abdelilah et al., 1996). In addition, cells simply do not reach their correct stage of differentiation by a certain time of development and are eliminated (Furutani-Seiki et al., 1996).

In this screen, 8 degeneration mutants have been found, and three of them are shown as an example in Figure 7. All these cell death mutants have in common that the cell death, appearing at different timepoints in different mutants, is not restricted to specific brain areas, but rather apparent in most neural tissues, as it can be seen their morphology...
Results

(Fig. 7 A,A’,G,G’,I,I’) or in stainings with either acridine orange, a vital dye that stains apoptotic cells and necrotic cells (Fig. 7 B,B’,H,H’), or with TUNEL staining (Fig. 7 J,J’). TUNEL is specific for staining apoptotic cells, since it labels fragmented DNA characteristic for this kind of cells. When stained with anti-acetylated Tubulin antibodies, the cell death in the whole neural tissue seems to affect only a subset of axonal scaffolds. *HK314* does not have any alteration in the Tubulin pattern and, in addition, seems to recover at day 2 pf morphologically (data not shown). In *HE022* embryos, the projecting neurons of the epiphysis and the posterior commissure are strongly reduced, (Fig. 7 D’) and the anterior commissure is not closed (Fig. 7 E’). Hindbrain commissural interneurons form axon bundles that are not as thick as in the wildtype and are less fasciculated (Fig. 7 F’). One of the alleles of *HC006*, *HC006a*, is affected only in formation of the anterior commissure (Fig. 7 M’), all other axonal scaffolds appear to be normal (although the hindbrain commissural interneurons are generally shorter (Fig. 7 N’).
Figure 7: The degeneration group. (A-C’, G-H’, I-K’) Lateral views, (D,E’, F,F’, L,L’) dorsal views, (E,E’, M,M’) ventral views. Anterior is left (A-C’, F-H’, I-K’, N,N”) or to the top (D-E’, L-M’). Live pictures of HE022 (A,A’), HK314 (G,G’) and HC006a (I,I’) show that the mutants have greyish or brownish cells in the whole brain. These cells are dying, as shown by acridine orange (B’ and H’) or Tunel staining (J’). While HE022 has strong defects in the formation of the epiphysis, posterior commissure (arrowheads in D’), anterior
commissure (arrowhead in E'), and bundled axons in the hindbrain (F'). *HC006a* is more specifically affected in the formation of the anterior commissure (arrowhead in M'). (C-F' and J-N') anti-acetylated Tubulin and anti-Opsin wholemount antibody stainings.

### 3.2.5.3 The ‘skin’ group comprises two mutants with novel phenotypes

*IN193* was given to our group as a potential MHB phenotype mutant and *IY121* was kept as a brain morphology mutant (see Table 5). In the re-screen, both of them failed to show the initial phenotypes, but stably showed new ones. *IN193* is not affected in the morphology of any body part, but displays loose and roundish cells on top of the hindbrain ventricle (Fig. 8 B’). The cells are not dying, as tested with acridine orange (data not shown), excluding the possibility that these cells are dying and therefore losing adhesiveness. This is confirmed by the observation that at 48 hpf loose cells within the hindbrain even form connections (Fig. 8 C’).

*IY121* is also not affected in head and trunk morphology, but the tail is delineated with a rough epidermis, especially around the tail tip (Fig. 8 E’). Loose and roundish cells appear in the dorsal part of the tail and blood cells in the ventral part are not found in blood vessels but rather accumulate in saccule-like structures (Fig. 8 E’). Also in this mutant, the loose cells are not dying (Fig. 8 E’’). Loose cells and blister-like structures at the tail can account for a loss in adhesiveness, as shown for mutants in *n-cadherin* (see section 3.2.5.7). Further studies on *IN193* and *IY121* should therefore take this similarity into account and consider these mutants as candidates of adhesion-defective mutants.
Figure 8: Two new phenotypes of class D mutants, *IN193* and *IY121*. All lateral views, anterior is left. (A-B’) *IN193* displays, compared to its sibling, no morphological differences in the head, but an accumulation of loose cells on top of the epidermis above the hindbrain ventricle (arrowheads in B’). Anti-acetylated Tubulin staining at 48hrs (C,C’) shows that at this stage there are loose cells in the hindbrain ventricle (arrow in C’), which even form axon bundles. The head and trunk morphology of the other mutant, *IY121*, is also not affected (D,D’), but the tail is strongly affected (E’). Blood accumulates in the ventral part of the tail (arrow in E’), and loose cells appear dorsally (arrowheads in E’). In addition, the epidermis bulges around the tail tip and forms blister-like structures. These cells are not dying, as judged by acridine orange staining (E’’).
3.2.5.4 **HS304 is specifically affected in the formation of otoliths in the inner ear**

Development and structure of the inner ear in zebrafish have been studied extensively. Studies in our lab could supply further insight into the mechanisms of ear placode induction, maintenance and inner ear patterning, by analysing the roles of *fgf3* and *fgf8* in these processes (Léger and Brand, 2002). The inner ear forms from an ectodermal thickening, the otic placode, and cavitates at around 18 somites into the otic vesicle. Growth and differentiation of the ear then comprises the formation of three cell types (sensory, nonsensory and neuronal), the membranous labyrinth and the statoacoustic ganglion. The sensory patches of the ear are marked by two otoliths, crystalline deposits of calcium carbonate and proteins. They assist the hair cells in sensing linear acceleration, gravity and sound waves.

The only ear mutant found in the Tuebingen screen 2000, **HS304**, displays a slightly smaller otic vesicle at 24 hpf (Fig. 9 A’), but at 48 hpf this difference in size no longer exists (Fig. 9 B’). In contrast, only one single otolith is visible at 24 (Fig. 9 A’) and 48 hpf (Fig.9 B’), which is located mostly in the posterior part of the ear at the left side of the embryo (Fig. 9 A’,B’), while on the right side it would be located in the anterior part (data not shown). The patterning of the ear is unaffected, since marker genes such as *dlx3*, *pax5* and *otx1* are expressed normally (data not shown). It can be therefore concluded that **HS304** is specifically affected in formation of otoliths, and behavior studies testing deficiencies in balance or hearing could be performed to learn about the effect of one single otolith on the function of the hair cells.

**Figure 9: HS304 is an ear mutant with a specific otolith phenotype.** Morphology of the ear of HS304 at 24 and 48 hpf, anterior is left, lateral views. At 24 hpf, the ear vesicle is smaller in the mutant and the ventral part is slightly thickened (A’). In addition, only one otolith is visible in the posterior part of the vesicle (arrowhead in A’). At 48 hpf, the size of the ear is similar in the wildtype and the mutant (B,B’), but still only one posterior otolith is present (arrowhead in B’).
3.2.5.5 Mutants affecting brain morphology

18 mutants with abnormal brain morphology were isolated in the Tuebingen screen 2000. This class of mutants can be subdivided into snakehead-like mutants, amorphous head mutants, parachute-like mutants, and mutants that display completely novel phenotypes.

4 of them fall into the class of snakehead-like mutants, based on their similarity to the snakehead group described by Jiang et al., 1996 (Jiang et al., 1996). Brain ventricles are collapsed in HN148 (Fig. 10 A’), HX066 (C’) and HU005 (E’). As a consequence, the neural tissue appears to be smaller and more condensed in a dorsal view (Fig. 10 B’, D’, E’). Enlarged heart cavities are obvious in HX066 and HU005 (Fig. 10 C’, E’). HN148 shows the strongest mutant morphology, since structures in the brain such as the MHB are barely visible, if at all (Fig. 10 A’, B’). The expression patterns of genes such as pax2.1, fgf8 and eng2 in HX066 and pax2.1, pax5, pax6, fgf8, eng2 and otx1 in HN148 were examined and showed no detectable differences (data not shown).
Figure 10: The *snakehead*-like class of phenotypes. Lateral (A,A’,C,C’,E,E’) and dorsal (B,B’,D,D’,F,F’) views of the morphology of three different mutants from the *snakehead*-like class, *HN148* (A-B’), *HX066* (C-D’) and *HU005* (E-F’). Anterior is left, stages are indicated. All three show condensed cell mass and reduced ventricles, resulting in a less structured brain, which is most obvious in *HN148* (A’,B’). From dorsal, the neural tube is more narrow (arrows in D’). Arrowheads in A’ and B’ point to the position of the MHB, arrows in C’ and E’ to the heart cavities.
3.2.5.6 Amorphous head mutants

This group of mutants contains 5 different alleles of the same mutation, as shown in the complementation assay (see section 3.2.3). The alleles are HF028, HF017, IT033, KW080, and one of the two HX168 alleles. One additional allele has been found in the Heidelberg haploid screen, 727-05, suggesting that the according gene contains a region that is a hot spot for point mutations inserted by ENU mutagensis. The morphology is dominated by an amorphous tectum and wavy hindbrain (Fig. 11 A’). In a dorsal view it can be seen that the ventricles are smaller (Fig, 11 B’,C’). Anti-acetylated Tubulin staining at 34 hpf reveals specific phenotypes in the formation of the POC and AC (Fig. 11 D’). Both commissures are closed, but the AC appears to be thinner and single axons escape from the axon bundle. The POC is even more defasciculated. The MLF at the hindbrain level is absent, and axons from the hindbrain commissural interneurons are either absent or stumpy (Fig. 11 E’). In summary, mutants of this phenotypic subclass display subtle defects in specific areas of the brain, and gene expression studies of genes involved in fasciculation and axon guidance could help to get further insights into the phenotype.

**Figure 11:** Mutants of the A class display severe morphological alterations and are affected in specific axon scaffolds. (A-C’) Live specimens of HF028 and their siblings. The tectum of HF028 is misshapened, the hindbrain is wavy (arrow in A’) and blood accumulates in the isthmic fold (arrowhead in A’). The ventricles are deflated (B’,C’). (D-E’) anti-acetylated Tubulin staining, (D,D’) frontal views, (E,E’) dorsal views on to the hindbrain, anterior is left. The anterior and post-optic commissures are formed in HF028, but
the AC is slightly (arrow in D') and the post-optic commissure is more severely defasciculated (arrowhead in D'). In the hindbrain, hindbrain commissural interneurons are rudimentary present and these remnants are strongly defasciculated (arrowheads in E'). In addition, no MLF can be detected (in the wildtype arrow in E).

3.2.5.7 A new parachute-like brain mutant, HJ032, has a partially opposite phenotype as compared to cognate N-cadherin mutants and might reveal a new function of the protein

Zebrafish N-Cadherin (Cadherin-2) belongs to Type I Cadherins that consist of 5 extracellular Cadherin-binding domains, a transmembrane domain and an intracellular beta-catenin binding domain (Lele et al., 2002). Cadherins form dimers in cis and interact homophilically with other Cadherin molecules in trans. Cell-cell adhesion is mediated in a Ca\(^{2+}\) dependent manner (reviewed in Redies, 2000; Tepass et al., 2000). In zebrafish, several mutant alleles of n-cad are known, and two of them, parachute (pac) and glass onion, have been described previously (Lele et al., 2002; Malicki et al., 2003). In pac, the adhesion of cells within the neuroectoderm is weak, and convergent movements during neurulation are compromised. The pac alleles described encode a premature stop codon due to incorrect splicing and result in a truncated N-Cadherin only consisting of the first three extracellular domains.

In the new allele HU519, the same phenomenon of loose cell aggregates similar to pac could be observed (Fig. 12 H’, J’). Complementation with the pac allele described above could prove that HU519 is indeed mutated in n-cadherin, as well as HJ032 (Table 6). In contrast, judged by the live phenotypes, HJ032 shows an opposite adhesion phenotype, namely a more dense cell mass and almost no loose cells, except for some loose cells at 15 somites which appear at the dorsal diencephalon (Fig. 12 C’). Careful analysis of the ventricles supports the observation that in HU519 the ventricular space is enlarged (Fig. 13 B), and is filled with loose cells, while in HJ032 the ventricles are collapsed (Fig. 13 D,F,G) and the cell mass appears closely packed. Interestingly, the failure of the neural tissue to converge to the midline is much stronger in HJ032 than in HU519 (Fig. 13 E’, F’). On the other hand, in situ analysis with the MHB markers eng2 and wnt1 shows a similar
Results

broadened neural tube (Fig. 14). Therefore, it can be concluded that both alleles have the same effect on neurulation. The putative difference in cell adhesion could be caused by two mechanisms: either there might be no difference in adhesion, but in HJ032 there might be more cells born and supernumery cells might fill up the ventricular space, or the adhesive properties might be altered in a way that the extracellular domain is functional, but a mutation in the intracellular domain could cause the protein to be dominant active and therefore causes enhanced adhesiveness.

It also has to be mentioned that in the course of re-identification, a third n-cad allele, HJ050, was discovered, which resembles the HJ032 allele but no further studies have been done on it so far.

Figure 12: HJ032 and HU519 show striking differences in their live phenotypes. Live phenotypes of HJ032 and HU519 at indicated stages. Anterior is left, (A-D’, G-H’) lateral views, (E-F’ and I-J’) dorsal views. (A-D’) At 6 som, HJ032 displays a very wavy head and trunk morphology (A’), while HU519 seems to be much milder affected with the same phenotype (B’). At 15 som (C-F’), the lateral view shows the same phenomenon, and from dorsal it can be seen that the tissue is much broader than in the wildtype; again, this is more obvious in HJ032 (E’) than in HU519 (F’). At 24 hrs, the two phenotypes split up (G-J’). The brain of
**Results**

*HJ032* is very condensed (G’), and from dorsal almost no folding MHB structure is visible (I’). In the brain of *HU519*, a lot of loose, rounded-up cells appear in the mid- and hindbrain (arrow in H’ and J’). The asterisks in (G-J’) indicate the position of the MHB fold.

![Figure 13: HJ032 and HU519 display different adhesive properties of neural cells.](image)

(A-C) Live embryos, (E-G) embryos stained with bodipy ceramide. Anterior is left (A-D,G), (E,F) are optical cross sections through the hindbrain at the level of the ears. (A,B) The ventricular space in *HU519* mutants is enlarged as compared to the wildtype (arrows in A and B) and loose cells pile up dorsally (arrowheads in B) as well as posterior to the MHB and also ventrally. In addition, the cell mass of the tectum seems to consist of loose cells. In contrast, in *HJ032* the hindbrain ventricle is very thin (arrows) (C,D,G) and becomes filled with densely packed cells. Also, the midbrain ventricle is strongly reduced and is filled with cells instead. The cross section demonstrates the same observation (E,F).
Results

Figure 14: HJ032 and HU519 have a broadened neural tube. *In situ* analysis of the two new *n-cad* alleles, HJ032 and HU519. Anterior is left, (A’,B’,C’) HJ032, (D’,E’,F’) HU519. The asterisk is located posterior to the MHB. During somitogenesis the widening of the neural tube becomes evident by analysis of the MHB markers, *wnt1* (arrows in A, A’,D,D’) and *eng2* (B,B’,E,E’). At 24 hpf, there is a difference in the lateral extent of *wnt1*, the two *wnt1* stripes which are adjacent at the midline in wt siblings are displaced more laterally in HJ032 (C,C’), but in HU519 these stripes are almost at the lateral edges of the lateral neural border (F,F’).

3.2.5.8 Neither overproliferation nor a mutation in the intracellular domain of N-cadherin account for the phenotype of HJ032.

To test whether overproliferation could be the cause of the dense cell mass, stainings with anti-Phosphohistone H3 antibodies were performed at different stages of development. A similar number of cells was stained with this mitosis marker, suggesting that proliferation is not altered in the mutant (data not shown).

To reveal the site of the point mutation, PCR and sequencing of the PCR products were performed. PCR on cDNA of HJ032, HU519 and wildtype produced bands of 2.3 kbp in size, excluding the possibility that HJ032 or HU519 are splice mutants and the gene product is altered in size due to missplicing (Fig. 15). Sequencing of the alleles was performed using 7 primers which span the whole cds of *zf-n-cadherin* (for details see Materials and Methods). Using the SeqMan program (Version 5.05) to align the sequences
of the mutants with the wildtype one, no point mutation could be detected. This suggests that the mutation is not located inside the coding sequence, but could be possibly upstream.

Figure 15: *N-cadherin* products from mini-preps of wildtype (lane 2), *HJ032* (lane 4) and *HU519* (lane 6) after outcut. Lanes 1, 3 and 5 are marker lanes, the products are about 2.3 kbp in size (band in 2, and lower bands in 4 and 6).
3.3 Detailed analysis of the phenotype of HE409, a novel brain morphology mutant

In the third part of this thesis, I will present a phenotypic analysis of the mutant HE409 as well as functional data revealing some insights into the pathways the mutated gene might be involved in.

HE409 (also termed broken heart, brh) falls into the class of brain morphology mutants. Originally, it had been discovered by the somite screening group as a notochord mutant, but this could not be confirmed in the re-screen. Before 24 hpf, the mutants cannot be distinguished from wildtype siblings morphologically, but by behaviour: they only rarely display spontaneous twitching movements, and when touched with a needle, they do not react (data not shown). At 24 hpf, when the brain normally starts to appear structured, broken heart mutants display a less structured brain as compared to their siblings (Fig. 16 A,A’), while structures like eyes and ears are visible. At 30 hpf, ventricles are smaller in brh, and, as judged from a dorsal view, the folding of the mid-and hindbrain resembles an earlier developmental morphology then embryos at 30 hpf would normally display. No blood circulation can be detected (data not shown), and blood starts to accumulate posterior to the yolk sac extension (arrow in D’) In addition, the heart beat is very slow and not rhythmic, and ventricle and atrium are enlarged (data not shown). At 48 hpf the brain is very condensed (Fig. 16 E’,F’), with the MHB folds barely visible. The oedema is very pronounced (Fig. 16 E’), and the mutants die at day 3 or 4.
Figure 16: Live morphology of broken heart. Anterior is left, (A,B,D,E') lateral views, (C,F,F') dorsal views. The phenotype becomes apparent at 24 hpf, when the folding of the MHB region is obvious in the wildtype (A). In the mutant the folds are barely visible (A'). At 30 hpf, the brain is more condensed, and the ventricles in the fore-, mid- and hindbrain are deflated (arrowheads in B'). At 48 hpf, the oedema, which had formed already before, is very obvious (arrow in E') and the condensation of the brain is even stronger (E',F').
3.3.1 *broken heart* displays delayed axonal outgrowth and an absent posterior commissure and epiphysis at early stages

In order to determine whether *broken heart* is specifically affected in the formation of axon tracts and commissures, anti-acetylated Tubulin staining was performed at 34 and 48 hpf. At 34 hpf, the mutant appears to be delayed in different aspects: The anterior and post-optic commissures are about to form (Fig. 17 C’), but do not close at the same time as in the wildtype (Fig. 17 C). This deficiency recovers at 48 hpf (Fig. 17 E’), but the optic nerve does not form, probably due to the lack of RGC axons leaving the optic cup (Fig. 17 F’). Furthermore, the spinal cord motor axons are not visible (Fig. 17 B’), and do not recover until 48 hpf (data not shown). The posterior commissure and the epiphysial projecting neurons are not detectable at 34 hpf (Fig. 17 A’), but at 48 hpf one single axons forms the PC and also the epiphysis is apparent in a reduced fashion (Fig. 17 D’). Therefore, the *brh* mutant displays a strong delay in the formation of particular commissures (AC, POC, PC), some of which will be formed later (AC, POC), and one, the PC, will be almost lacking. The spinal cord motoraxons are only visible at 72 hpf, and can be taken into account when studying the motionless phenotype of the mutant, since the presence of motoraxons in the spinal cord is one of the prerequisites of movement.
Figure 17: Axonal outgrowth is delayed and the PC and epiphysis are barely formed. Immunostaining with an anti-acetylated Tubulin antibody at 34 (A-C’) and 48 hpf (D-F’). Anterior is left (A-B’) or up (C-F’), (A-B’) lateral views, (C,C’E-F’) ventral views, (D,D’) dorsal views. (A’) The posterior commissure and the epiphysis as well as the spinal cord motoraxons (B’) are not present at 34 hpf (arrows in A’ and B’). Anterior and post-optic commissures have not yet formed (C’), but are delayed and are formed at 48 hpf (E’). At this stage, also one single axon has emerged at the position of the posterior commissure (arrowhead in D’), and a reduced number of projecting neurons in the epiphysis can be detected (arrow in D’). The optic nerve is absent (F’). For abbreviations see Figure 5.

3.3.2 The epiphysis is malformed in broken heart

Since the projecting neurons of the epiphysis are affected in the mutant, also the photoreceptors were analysed for possible defects. Staining with both, anti-acetylated Tubulin as a maker for projecting neurons and anti-Opsin as a marker for photoreceptors, reveals that only a small portion of the epiphysis is present in the mutant (Fig. 18 B). As shown in Figure 17, the mutant embryo is completely devoid of Tubulin in that area, indicating that the remaining cells are photoreceptors (Fig. 18 D). Indeed, staining with Opsin only detects neurons, but the number of neurons is reduced (Fig. 18 F).
3.3.3 The epiphysis of *broken heart* is not defective in neurogenesis

Both photoreceptor neurons and projecting neurons are absent in the epiphysis of *broken heart*. It could be that both types of neurons might be misspecified, or the prospective epiphysial area is already defective in neurogenesis at early stages, and neurons are not born at all. In order to distinguish these possibilities, markers for epiphysial prepatterning and neurogenesis were tested. *flh* is the prepatterning gene in epiphysis, which is required for neuron generation, and *zash1* functions downstream of *flh* to determine neuronal fate (Cau and Wilson, 2003). *Islet-1* functions even further downstream and serves as a marker for early neurons. These markers have in common that they will stain both lineages, projecting neurons and photoreceptors, since these markers do not serve as differentiation cues. Neither at 24, nor at 48 hpf, a difference is visible between the wildtype and the mutant staining of *flh* (Fig. 19 A-B’), *zash1a* (data not shown) and *islet-1* (Fig 19 C-D’). HE409 is not affected in the early steps of epiphysis specification, namely prepatterning of the territory in the roof of the diencephalon and neurogenesis of not yet specified neurons.
Figure 19: Neurogenesis of the epiphysis is normal in broken heart embryos. In situ hybridisations with probes marking neurogenesis in the epiphysis, anterior is up. (A-A’) flh expression at 24 hpf is not altered in the mutant, and this is persistent at 48 hpf (B,B’). The same is true for islet-1, which is also unaffected at 24 hpf (C,C’) and 48 hpf (D,D’).

3.3.4 Neurogenesis is normal, but marker gene expression for differentiated neurons is affected in broken heart

As described before, the neurons of the epiphysis differentiate into projecting neurons, which send down axons that built the DVDT (dorso-ventral diencephalic tract) and photoreceptors, which are responsible for light reception and are part of the circadian clock of the zebrafish. Several marker genes for either of these cell types are available. Expression of these genes was analysed to reveal whether the differentiation was correct and whether the neurons are functional. tphD1 is one of the tph genes of the zebrafish (Bellipanni et al., 2002), that is more closely related to other vertebrate tph genes than tphR (Teraoka et al., 2004). otx5 plays a crucial role in regulating circadian genes in the
zebrafish (Gamse et al., 2001), e.g. *aanat* (serotonin N-acetyl transferase), which is also involved in the production of melatonin (Gothilf et al., 2002). *tphD1*, *otx5* and *aanat* are marker genes for the photoreceptors, while *lim3* and *onecut* are markers for the projecting neurons (Hong et al., 2002). At 24 hpf, the genes marking the projecting neurons, *lim3* and *onecut*, are unaffected (Fig. 20 A’,C’) and their expression resembles that of the wildtype (Fig. 20 A,C). At 48 hpf, however, *lim3* is still unaffected (Fig. 20 B’), but *onecut* expression is already lost at 36 hpf (Fig. 20 D’). In contrast, marker gene expression in the photoreceptors is diminished from 24 hpf onwards. *tphD1* is initiated and expressed at 24 hpf (Fig. 20 E,E’), which is in contrast to the other *tph* gene, *tphR* (Fig. 21). At 48 hrs it is also lost like the *tphR* staining is (Fig. 20 F’ and Fig 21). *otx5* expression is much weaker at early stages (Fig. 20 G’), which persists at least until 48 hpf (Fig. 20 H’). Staining in the parapineal (Fig. 20 H) is not detectable, maybe due to the fact that no parapineal is present in the mutant. *aanat* is also not present at 48 hpf (Fig. 20 I’). It has to be mentioned that *aanat* is dependent on *otx5* function, but may as well be not present or degraded since no serotonin is produced in the epiphysis due to the diminished expression of the tryptophan converting enzymes, *tphD1* and *tphR*.

![Figure 20: Marker gene expression of both photoreceptors and projecting neurons are affected. *In situ* analysis with markers for photoreceptors (E-I’) and projecting neurons (A-D’), anterior is up. *lim3* expression](image)

*Figure 20: Marker gene expression of both photoreceptors and projecting neurons are affected. In situ analysis with markers for photoreceptors (E-I’) and projecting neurons (A-D’), anterior is up. *lim3* expression*
is unaltered in the mutant at 24 and 48 hpf (A-B’), while onecut expression is initially normal at 24 hrs (C,C’), but is lost at 36hpf (D’). (E-F’) tphD1 expression is initiated in brh, but already reduced at 24 hpf (E’). At 48 hpf, it is totally lost in the mutant (F’). (G-H’) The expression of otx5 persists, but is reduced form early on and a parapineal structure cannot be detected. In addition, aanat is lost in the mutant at 48 hpf (I,I’).

3.3.5 The onset and expression of tphR, encoding the rate limiting enzyme for the synthesis of serotonin, is severly affected in broken heart

Since broken heart has a striking epiphysis phenotype, detailed marker gene analysis for the epiphysis had been carried out to get further insights in the mutant phenotype. tphR is a marker for serotonergic neurons, and in the epiphysis it is expressed in the photoreceptors from 30 hpf onwards (Teraoka et al., 2004). Indeed, the onset of expression of tphR in the epiphysis of the mutant is delayed for about 18 hpf as compared to the wildtype (Fig 21 A-H). This long time period might be an indicator that the late onset of expression of tphR is not caused by a general delay in development of the mutant, but rather due to the failure of the epiphysis to differentiate into photoreceptors and projecting neurons or non functional photoreceptors (see section 3.3.4). In the serotonergic neurons of the hindbrain, the raphe nuclei, tphR starts to be expressed at around 35 hpf (Fig 21 I). In the mutant tphR expression is also delayed for about 6 hrs (Fig. 21 J,L,L’). At 48 hpf, three clusters of tphR are obvious: two bilateral longitudinal columns rostrally and two clusters caudally (Fig 21 M). In brh, only the most rostral part of the expression domain is present, but significantly reduced and delocalised to more lateral positions, and the caudal clusters are totally absent (Fig. 21 N). The finding that not only the phenotype and marker gene expression in the epiphysis, but also the expression of tphR in the hindbrain was severely affected, motivated me to study the induction of serotonergic neurons in the hindbrain.
Figure 21: The onset of a marker for serotonergic neurons, tphR, is delayed and expression is severely reduced in broken heart. (A-H) dorsal views of the photoreceptors of the epiphysis, anterior is up, (I-N) dorsal view on the serotonergic neurons in the anterior hindbrain, anterior is left. The onset of tphR in the epiphysis is at about 30 hpf (A), but in the mutant tphR transcript cannot be detected (B). At later stages, at 35 and 40 hrs of development, the expression in the wildtype gets stronger (C,E), but still the epiphysis of the mutant is devoid of any staining (D,F). At 48 hpf, only one or two cells express tphR in the mutant (H). The onset of tphR expression in the hindbrain is slightly later as in the epiphysis, around 35 hpf (I). As in the epiphysis, the hindbrain serotonergic neurons are not stained at this stage (J), and weak expression can be seen at 41 hpf (L’). At 48 hpf, different domains can be distinguished in the wildtype: tow bilateral longitudinal stripes rostrally (arrows in M) and more caudal two separate and much weaker clusters (arrowheads in M). The mutants display a significant reduction of tphR positive neurons (N). Whereas the rostral cluster is strongly reduced, the more caudal clusters are completely absent.
3.3.6 The hindbrain of broken heart embryos is specifically affected

To test whether the loss of caudal serotonergic neurons in the hindbrain of the mutant is specific, or whether the hindbrain is generally degenerated, or onset of specific hindbrain features is generally delayed, antibody stainings and in situ hybridisations with markers for specific hindbrain structures were performed. General segmentation was not affected using krox20 (rh1 and rh3) and rtk1 (EphA 4) (rh1,3 and 5) as markers (data not shown). Reticulospinal interneurons and branchiomotor neurons of the cranial nerves reflect the segmented organisation of the hindbrain (reviewed in Moens and Prince, 2002). Staining with antibodies against acetylated Tubulin (Fig. 22 A,A’) and RM044 (Fig. 22 B,B’) reveal that except for the Mauthner neurons (Fig. 22 B’) all interneurons in the hindbrain are absent (Fig. 22 A’,B’). In contrast, the branchiomotor neurons that express islet-1 are normal (Fig. 22 C,C’). At 48 hpf, however, no axons have grown out of the motoneurons, indicated by the missing facialis, glossopharyngeus and vagus nerves (Fig. 22 D,D’).

Figure 22: The interneurons in the hindbrain of broken heart are affected, while the motoneurons are normal. (A,A’) anti-acetylated Tubulin whole mount staining, dorsal view, and (D,D’) lateral view. The trigeminal nerve (Trig) and the lateral longitudinal fascicle (LLF) are present in broken heart (A’), but the
axons of the commissural interneurons (HCl) are missing. (B,B’) The antibody staining against neurofilaments, RM044, proves that finding, since all interneurons except for the Mauthner neurons and their axons are absent. (C,C’) In situ hybridisation with islet-1. This probe stains motoneurons in the hindbrain, and at 30 hpf there is no difference between the mutant and the wildtype. At 48 hpf, however, motoraxons have not outgrown (D’). White arrowhead indicates hyoid-arch innervating axons (nVII, facialis nerve), black arrowhead indicates first gill arch-innervating axons (nIX, glossopharyngeus nerve), and the arrow indicates second gill-arch–innervating axons (nX, vagus nerve).

3.3.7 The catecholaminergic system is unaltered in broken heart embryos

In addition to the serotonergic system, another aminergic system, the catecholaminergic system, is present in the hindbrain (Guo et al., 1999a). Catecholaminergic neurons use tyrosine as a precursor to subsequently produce Dopamine, Noradrenaline and Adrenaline (reviewed in Goridis and Rohrer, 2002) and the rate-limiting enzyme for this is tyrosine hydroxylase (TH). TH-immunoreactive neurons populate all major brain divisions and most of them are putatively dopaminergic (Kaslin and Panula, 2001). The main source of Noradrenaline is the locus coeruleus in the hindbrain, one of the earliest born neurons in the brain and therefore a good model to study differentiation. At 48 hpf, tyrosine Hydroxylase is expressed normally in the loci coerulei of broken heart (Fig. 23 E,E’), indicating that the differentiation of noradrenergic neurons in the hindbrain is not altered. Indeed, the whole catecholaminergic system is established normally, although the branchial arch-associated catecholaminergic neurons are displaced to more lateral positions (Fig. 23 D,D’).
Figure 23: Immunostaining with TH reveals no defect in the establishment of the catecholaminergic system of brh. Anterior is left, except (D,D’) anterior is up. At 24 hpf, the first TH-positive neurons are visible in the forebrain (A,A’) and lateral to the hindbrain (B,B’). Neither at 24 hpf, nor at 48 hpf, when the locus coeruleus is also immunoreactive (E,E’) there is a difference in staining patterns between mutants and their siblings. aac arch-associated catecholaminergic neurons, e eye, HyDA hypothalamus dopaminergic neurons, LC locus coeruleus.

3.3.8 The hedgehog pathway has no influence on the reduction of tphR positive neurons in the hindbrain of broken heart

Since I could show that the serotonergic phenotype of broken heart is specific, it had to be elucidated which pathways known to be involved in the induction of serotonergic neurons might be affected in broken heart. It has been proposed that diffusible factors emanating from the midline or the MHB can mediate serotonergic neuron development (Hynes et al., 1995; Ye et al., 1998).

A factor emanating from the midline, shh, and one of its target genes, nkm2.2, have been proposed to influence serotonergic neurons (Ye et al., 1998; reviewed in Goridis and
Rohrer, 2002). Shh can induce serotonergic neurons in vitro while nkx2.2 is probably expressed in precursors of 5-HT neurons and is required for caudal 5-HT neurons in the mouse (Pattyn et al., 2003). To test whether the reduction of serotonergic neurons in the hindbrain in brh can be caused by a reduced or altered Hedgehog signaling pathway, in situ hybridisations with an antisense probe against shh, another zebrafish hedgehog, tiggy-winkle hedgehog (twhh) and nkx2.2 have been performed (Figure 24). Neither shh, nor twhh or nkx2.2 show changed expression in the hindbrain at 34 or 30 hpf (Fig. 24 A-F’), a timepoint shortly before the onset of tphR expression in the raphe (Fig. 24). In addition, the receptor of the two hedgehogs, patched1 (ptc1) has been tested whether it might be the signal transduction of the pathway that is affected, and downregulation of the receptor might account for the missing induction. In comparison to the wildtype, expression of ptc1 is not altered in brh (Fig. 24 G,G’). It can be concluded that the phenotype of brh is not due to alterations in the expression of transcripts of the ligands and the receptor of the hedgehog pathway.

Figure 24: Expression of the Hedgehog genes shh and twhh, their downstream target nkx2.2 and their receptor ptc1 is not altered in broken heart.
In situ hybridisation on broken heart embryos and their siblings, anterior is left. (A,A’,C,C’E,E’,G,G’) lateral views, (B,B’,D,D’F,F’) dorsal views at the level of the hindbrain. The distribution of the transcripts in the ventral neural tube is similar in the mutants and wildtype. The expression domain of shh in the zli (zona limitans intrathalamica) seems to be slightly tilted (A,A’), but this is probably a consequence of the altered overall morphological difference between broken heart and wildtype. The width of the expression of shh (B,B’), twhh (D,D’) and nkx2.2 (F,F’) in the ventral neural tube is unchanged.

3.3.9 Determination of the critical timepoint of serotonergic neuron induction by SU5402 inhibition

Candidates for diffusible factors secreted from the MHB to influence the formation of 5-HT neurons are Fgfs. I tested this possibility by loss-of-function, gain-of-function and gene expression studies.

The pharmaceutical reagent SU5402 is an Fgf receptor inhibitor and can be used to block Fgf signaling in the embryo (Mohammadi et al., 1997). In this study, I used it for two purposes: first, to test whether Fgfs play a role in inducing serotonergic fate in the zebrafish, and secondly, to define the possible critical timepoint of an Fgf dependent induction. Wildtype embryos were treated with the inhibitor between 20 som-40 hpf, 24-48 hpf and 30-48 hpf. When treated from 20 som onwards, caudal 5-HT neurons are strongly reduced (Fig. 25 A,B), as compared to the wildtype (Fig. 25 D,E). Inhibition from 24 hpf onwards has a similar effect, also the caudal portion of the raphe nuclei is affected (Fig. 25 G,H). In contrast, inhibition from 30 hpf onwards has no effect anymore and resembles the normal state of tphR expression (Fig. 25 J,K). However, none of the treatments caused a phenotype as severe as the one of broken heart mutants (Fig. 25 O). The inhibition of Fgf signaling obviously affects the caudal serotonergic neurons, while the most rostral ones are not affected and induced even in inhibited embryos. The time window of induction can be determined roughly between 20 som and 30 hpf, since inhibition beyond 30 hpf does not have an effect on tphR expression anymore. spry4 stainings, an immediate target gene of Fgf8 signaling, were done to visualise that the inhibitor treatment was successful (Fig. 25 C,I).
Figure 25: SU 5402 inhibition of wt embryos reveals a role of Fgfs in specifying the caudal tphR positive raphe. (A,C,D,F,G,I,J,L,M) lateral view, (B,E,H,K,N,O) ventral views. All stainings with an antisense probe against tphR, except (C,F,I,L) in situ with a probe against spry4. (A-F) SU inhibition between 20 som and 40 hpf. (A,B) Inhibition causes a loss in tphR staining in the more caudal position in the raphe (arrowhead, control in D and E), while the most rostral portion of the expression domain is unaffected (C,I) Control staining with spry4 shows that the inhibition was successful; the remaining staining is unspecifically trapped substrate. (F) Untreated control embryo stained with spry4 to show the normal expression pattern at 40 hpf and 48 hpf (L). (G-N) SU inhibition between 24-48 hpf (G-H) and 30-48 hpf (J,K). (M,N) Control embryos
stained with tphR at 48 hpf. In embryos treated between 24-48 hpf expression in the caudal raphe is more affected than in embryos treated between 30-48 hpf (arrowheads in G,H and J,K).

3.3.10 Role of Fgf4 in induction of serotonergic neurons in the raphe nuclei

Fgf4 has been described as the mediator of Shh- and Fgf8- signaling in the induction of serotonergic neurons in hindbrain explants by signaling from the primitive streak (Ye et al., 1998). In zebrafish, fgf4 function has been discussed in respect to the promotion of apical fold morphogenesis of the fin in later stages of development (Grandel et al., 2000). Its role in the induction of serotonergic neurons has not been tested in the zebrafish so far. Therefore, I performed in situ hybridisations with an fgf4.1 probe at the stages important for induction of 5-HT neurons (24-30 hpf) to see whether fgf4 expression domains would be located suitably to signal and whether fgf4 expression is affected in broken heart embryos. At 24 hpf, fgf4.1 is expressed in the tailbud, MHB and otic vesicles (Fig 26 A-D’). Expression levels in the otic placodes and in the tailbud are unchanged in broken heart embryos, but drastically lower in the MHB (Fig. 26 B). 6 hours later, the expression in the MHB is still very weak (Fig. 26 E-H), with the medial part of the MHB almost devoid of fgf4.1 staining (Fig. 26 J). It is possible that this medial domain is the source of Fgf4 signaling that induces serotonergic neurons caudally to the MHB in the ventral hindbrain. To test whether ectopic Fgf4 could rescue the phenotype of broken heart, I implanted beads soaked with Fgf4 protein dorsally into the hindbrain ventricle (Figure 27). Using tphR as a readout, the expression of this gene, normally reduced to the most rostral part of the expression domain in the mutant (Fig. 27 A,C) can be expanded more caudally in mutant embryos which have been supplied with an ectopic source of Fgf4 (Fig. 27 B,D). Nevertheless, implantation of the bead cannot restore the expression to the wildtype level (Fig. 27 F). It can be concluded that Fgf4 is able to induce caudal tphR expression in a mutant background.
Figure 26: Expression of *fgf4.1* is altered from 24hrs onwards. (A,B,E,F) lateral views, (C,D) dorsal views, (G,H) coronal sections, (I,J) parasagittal sections at the position of the MHB. At 24 hpf the expression of *fgf4.1* in the MHB is strongly reduced in *brh* (arrowhead in B), while the expression in the ear (D) and tailbud domains (D') are unchanged. At 30 hpf, this reduction is persistent (F), and sections through this area reveal that the overall expression is very weak (H), and especially in the medial part of the MHB, where *fgf4.1* is most strongly expressed in the wildtype, the gene is not present at all (arrows in J).
Figure 27: Implantation of beads soaked with Fgf4 partially rescues the loss of tphR in the hindbrain. *In situ* hybridisations of 48 hrs old embryos, anterior is left. (B,D) A bead coated with Fgf4 and implanted dorsally at 24 hpf can bring back staining of tphR in more caudal positions (arrows in B,D), in comparison with untreated mutants (A,B). Nevertheless, a wildtype staining pattern (F) cannot be restored. Arrowhead in B shows the location of the bead, which was implanted dorsally. (E) spry4 staining is increased around the bead and indicates the biological functionality of the protein.

3.3.11 The loss of medial expression domain of the MHB is observed for a number of genes

The loss of *fgf4.1* expression can be caused by two mechanisms: either *fgf4.1* is lost specifically, or the whole area is misspecified or lost, and then not only *fgf4.1*, but also other MHB markers should be affected. *In situ* hybridisations with *fgf8*, *pax2.1*, *her5* and *wnt8b* suggest that the second possibility is the case (Figure 28). All four genes are significantly weaker in the medial portion of their MHB expression domain, with *her5* being most weakly expressed in the whole MHB territory (Fig. 28 E’,F’), and *wnt8b* almost lost except for the most dorsal part (Fig. 28 J’). From this experiments it can be concluded
that the MHB territory looses identity at 24-30 hpf. Therefore the expression levels of \textit{fgf4.1} and \textit{fgf8} and subsequently the concentration of their proteins is diminished.

\textbf{Figure 28: Marker gene expression at the MHB is changed in \textit{broken heart} embryos.} (A,A’,D,D’,G,G’,J,J’) lateral views, (B,B’,E,E’,H,H’) coronal sections, (C,C’,F,F’,I,I’) parasagittal sections at the level of the MHB. All embryos are 30 hpf. \textit{fgf8} (A-C’), \textit{pax2.1} (G-I’), \textit{her5} (D-F’) and \textit{wnt8b} (J,J’) are strongly reduced at the MHB, with \textit{wnt8b} being most affected since it is only visible in a tiny spot dorsally (arrow in J’). The sections reveal that the overall expression of the genes is reduced, but most strongly at the medial part of the MHB (arrows in B-C’,E-F’,H-I’).
3.3.12 Ectopic Fgf8 can partially rescue the tphR expression

Since fgf8 is one of the key players in 5-HT induction in the hindbrain (Ye et al., 1998) and its expression is reduced in the MHB domain at stages important for induction in the zebrafish, I implanted Fgf8 coated beads into the hindbrain of broken heart embryos to test the possibility whether fgf8 can also restore tphR expression caudally. This time, the bead was implanted laterally to elucidate whether Fgf8, which has been shown to be released from the source and then forming a gradient over the responding tissue (Scholpp and Brand, 2004), can induce tphR in a distance (Fig. 29). Indeed, the bead induces tphR more caudally (Fig. 29 B), but much stronger at the contralateral side of the bead. Compared with the wildtype expression (Fig. 29 C), the contralateral side of the mutant is almost as strongly stained as the wildtype. Fgf8 obviously induces tphR further away from the source, and less efficiently at a position closer to the bead. The conclusion is that in the wildtype situation the caudal raphe, being further away from the source of Fgf8, the MHB, is Fgf-dependent (as also shown in the Fgf4 experiment) and the rostral raphe expression domain is independent of Fgf signalling (or MHB signaling in general) and therefore present in the mutant.

Figure 29: Beads soaked with Fgf8 partially rescue tphR in the hindbrain. In situ hybridisations, anterior is left, embryos are 48 hpf. tphR staining, which is reduced to the most rostral position in the hindbrain in HE409 (A) is expanded caudally in mutants treated with an Fgf8 soaked bead (B). The bead has been implanted laterally (location indicated by the dashed line), and affects the contralateral side more strongly.
Results

(arrowheads in B), than the actual implanted side (arrow in B). The tphR staining on the contralateral side spreads almost as far caudally as in the wildtype (C). (D) Control staining with spry4 of a mutant with bead to show that the protein is functional (arrow shows location of the bead, arrowhead the enhanced expression of spry4), in comparison with (E), showing an untreated mutant embryo.

3.3.13 The ‘educated guess’ or candidate gene approach for HE409

After the map position of a mutant is established, a search for potential candidate genes can be conducted. The only alternative if no candidate gene is available, is positional cloning of the mutation. Since this method is rather time consuming, I decided to put effort into a candidate gene approach. The search would have to be done within several centimorgans of the mapped position, because the accuracy of the map is rather low (Geisler, 2002). Highly problematic are marker positions close to the centromer, but since the marker z11403 maps to position 53.5 cM, and the centromer is at 40.0 cM in LG19 (Robert Geisler, personal communication), the locus of HE409 should be far away from the centromer (see also Table 8). With the help of the ZFIN website (http://zfin.org), genes in the vicinity of the z marker could be identified. Two of them, evx1 (even skipped homeobox 1) and NPY (Neuropeptide Y), were chosen as candidates genes based on their expression patterns and predicted functions. evx1 is expressed in zebrafish in a variety of structures, such as epiphysis and hindbrain and spinal cord interneurons (Thaeron et al., 2000). Since it is not expressed before tailbud stage, a late phenotype would be suggestive. In addition, mutants in the C.elegans homolog of evx1, vab-7, are defective in locomotion (Esmaeili et al., 2002). NPY is a small neuropeptide, which is widespread in the nervous system and has several physiological roles (Soderberg et al., 2000). For example, it is involved in the regulation of circadian rhythm and the control of blood pressure in mammals. Furthermore, in mammals sympathetic nerve fibers innervating the pineal gland use neuropeptide Y as a neurotransmitter (reviewed in Moller and Baeres, 2002).

Both genes were cloned and cDNAs derived from wildtype and mutant embryos were sequenced (see materials and methods section). Unfortunately, the mutant sequences
were identical to the wildtype ones, indicating that no point mutation in the coding sequences of the genes of interest had been introduced (data not shown).
Discussion

The work of my thesis and results obtained have been described in three parts:

(4.1) The Heidelberg haploid screen has produced a small number of mutants specifically affected in the MHB development and development of other brain areas.

(4.2) The large scale diploid Tuebingen 2000 screen has provided 35 new mutant lines defective for genes involved in several steps of early neural development.

(4.3) One of the mutants derived from the Tuebingen screen 2000, broken heart, is specifically affected in the differentiation of brain structures such as epiphysis and hindbrain, and serves as a model to study the development of serotonergic neurons in zebrafish.

4.1 Outcome of the haploid screen

Haploid screens are the method of choice when space and time planned for the screening procedure are limited (Pelegri, 2002). Already the first generation after mutagenesis can be screened for defects, while in F3 screens an additional generation has to be grown up. In addition, the F1 carrier fish can be kept together in a tank and must only be separated into small single boxes after identification. On the other hand, haploidy itself causes phenotypes, which might obscure the mutant phenotype caused by the ENU treatment (Streisinger et al., 1981; Walker, 1999). The embryos are quite short and stocky, although they have the correct number of muscle segments. Haploids have more cells, which in addition are smaller in size. The brain morphology is abnormal, and enhanced cell death is visible. The eyes are incompletely formed at the choroid fissure and otic vesicles are often duplicated at one side of the embryo. Embryos develop a prominent heart oedema and die at day 3-4. The limitations resulting from these haploid phenotypes are obvious: screens designed to detect mutants in somite formation, eye or ear development can only be done in diploid background. This is also the case if phenotypes arising later than day 3 should be screened for. However a haploid screen was our choice for the Heidelberg
screen: the fish facility in Heidelberg was very small, and the whole screen was designed as a small pilot screen rather than a large-scale screen. Furthermore, besides the limitations, there are also advantages in the screening procedure: in a diploid screen, only 25% of one clutch is homozygously mutant in case of a Mendelian recessive mutation. In the haploid progeny, 50% of the clutch should be mutant. Therefore, with this great number of mutant embryos it is feasible to dissect variable haploid phenotypes from the specific mutant ones. Our system to group them into four different groups (A-D) and scoring only A and B embryos also helped to minimise the haploidy-related phenotypes.

The outcome of the screen is satisfying. Roughly 10% of the clutches screened displayed specific phenotypes, and 50% of these could be recovered in re-screening diploids (Table 1). Three of them, e68, 713-07 and 728-04, turned out to be ‘real’ MHB mutants in the re-screen, e.g. the MHB was missing in these embryos (Table 2). All three mutant lines were identified to be defective for pou2, and could contribute to understand the function of pou2 in MHB development (Burgess et al., 2001).

Two other specific haploid mutants, 706-05 and 721-05, unfortunately turned out to be rather unspecifically affected by cell death, which had not been discovered in the initial haploid screen (Fig. 3 and data not shown). On the other hand, phenotypes of the mutations in 713-10, which is specifically affected by cell death in the midbrain- and hindbrain area (Fig. 3), and 720-04, which misses lim1 domains in the pretectum and diencephalon (Fig. 3,4), are fixed in the diploid re-screen and worth of further investigations.
4.2 Limitations of the Tuebingen screen 2000

There are several limitations of screens in general and our brain screen specifically regarding the number of mutations that were identified as being potentially interesting.

The screen for novel brain mutants relied on the ability of the mutated gene to produce an easily detectable phenotype. There may be many developmentally important genes that do not fulfil this and so were not identified during the course of screening. Redundancy of genes during evolution can also mask the actual mutant phenotype resulting from the loss of function of an important gene, and loss of one gene function may facilitate the upregulation of function of another similarly acting gene. Zebrafish *engrailed* genes are important regulators of midbrain-hindbrain boundary development and one example for redundancy. In the fish, there are at least four *engrailed* genes, *eng1, eng1b, eng2* and *eng3* (Scholpp and Brand, 2001). *eng2* and *eng3* are similar to the murine *En-2* in terms of expression (Joyner and Martin, 1987) and chromosomal synteny (Postlethwait et al., 1998). In morpholino knock-down experiments, the strongest phenotype could be obtained by injection of both morpholinos against *eng2* and *eng3* simultaneously into the embryo, and *eng3*-mRNA injection into *eng2* morphants can rescue the *eng2* morpholino phenotype (Scholpp and Brand, 2001). Therefore, it can be imagined that a mutation in *eng3* alone may be phenotypically ‘silent’ and impossible to be detected.

Another example is the redundancy between two *wnt* genes, *wnt1* and *wnt10b* (Lekven et al., 2003). *wnt1* and *wnt10b* are both required for the maintenance of the expression of genes at the MHB and show similar expression patterns in the mid- and hindbrain. Loss-of-function studies could show that the genes are at least partially redundant in function. This was suggested as the reason why a point mutation in *wnt1* could not be detected in large-scale screens so far (Lekven et al., 2003).

The Tubulin pattern is complicated and marks a large number of developing axon tracts and axon related structures (Wilson and Easter, 1991; Fig. 5). It is also stage dependent and, therefore, requires careful staging of embryos to confirm that a difference is not only due to the age variability within a clutch of embryos but is specific for the mutant.
This in practice can be both a hindrance and a benefit. The complexity on the one hand makes screening difficult and slow, but on the other hand allows detection of subtle phenotypes, which may prove to be more interesting in future work. Other groups in the screen 2000 elected simpler screening protocols, which might have been faster, but might have also missed some important mutations to be identified.

Other limiting factors for large-scale genetic screens exist, regardless of the protocol used for the identification of the mutants. The Tuebingen screen appeared to have a high rate of mutation (judged by the number of interesting and specific mutants found) and there was plenty of space for the generation and recovery of novel mutations. Both the amount of aquarium space and the amount of manpower available for setting up fish, collecting embryos, screening for mutations and re-identifying interesting phenotypes did not seem to be limiting. In contrast, the monthly round of mutagenesis was not equally efficient (personal communication).

Problems with the water in the aquarium caused that the fish were not producing as many healthy clutches of embryos as they ideally would do and this may have reduced the numbers of mutants found in the screen. Large numbers of adult fish were produced during the ENU treatment regime, however, any fish that did not lay fertilised embryos at their first or second time of mating had to be discarded. This was necessary to maintain space in the aquarium and nursery, but may have meant that fish carrying interesting mutations were discarded.

4.2.1 The future of genetic screens in the zebrafish: an outlook

Two types of genetic screens performed to date in zebrafish have been carried out and described in this thesis: a haploid one and a three-generation diploid one. For both, the outcome of specific mutations was satisfying, but still on the low side. Although it is much harder to discover and re-identify mutants in haploids, the percentage of mutant lines recovered after re-screening is the same for both screens: about 50% of the lines outcrossed provided re-identified or newly discovered phenotypes (Tables 1 and 3). The reasons for the loss of haploids have been discussed before, the reason why ‘false positives’ had been
discovered in the diploid screen might be that we used 48 well trays to stain 48 different clutches of embryos at the same time. Since the Tubulin staining pattern is dynamic in time (Wilson and Easter, 1991), and the clutches did not develop synchronously, a staining pattern reflecting an earlier state of development might have been misinterpreted as a specific delay in axonal outgrowth. It would be a benefit for future screens if the screening methods would be designed more specifically. The Tubulin staining pattern is not only dynamic, but also very complex and demands highly skilled examinators. Since it stains all axons in the embryo, virtually all areas can be screened for defects, which is very time consuming and tiring. Stainings done either with antisense in situ probes or antibodies, should be focusing on specific structures, e.g. Opsin.

Alternatives to these classical forward genetic screens are reverse genetic morpholino injections and TILLING. Morpholinos block translation of a target gene transiently and often are able to phenocopy the phenotype caused by a mutation in the same locus (Genesis 3, July 2001). Their disadvantage is that the block is only transient, and is not fixed in the germ line. In addition, they would be useless to study later aspects of development, e.g. the differentiation of serotonergic neurons, since a method to apply them later as the 32-cell stage to provide them throughout the embryo has not been established yet. Furthermore, unspecific side effects, for example cell death, are often caused by morpholinos which mask the specific defect in the morphant (personal communication). TILLING is unfortunately still not fully established yet, but in future this method might provide a combination of forward and reverse genetic screens (Wienholds et al., 2002).

Both methods have furthermore in common that they can address only known genes, and unknown genes and their pathways cannot be discovered. For this, the only method is still a classical haploid or diploid screen.

4.2.2 Outcome of the Tuebingen screen 2000: an overview

The 35 mutant lines stably re-identified could be designated to 4 different phenotypic groups: the brain morphology group, consisting of 18 strains, the body shape group (7 mutant lines), the degeneration group (8 lines) and the organ group (5 lines).
(Table 4). Since our screen group was mostly interested in finding mutants to elucidate
gene function in early neuronal development, the brain morphology mutants were given
highest priority in characterisation. Still, the other three groups have high potential in
elucidating specific functions in the pathways affected by the individual mutation.

4.2.3 Mutants affecting other developmental issues than brain morphology

The midline group consists of 7 members, of which three could already be identified
as alleles of oep (HC024 and KY025) and cyc (IF084) (Fig. 6). The allelic strength of each
of these three lines was similar to the ones described before. Moreover, these alleles have
been subject of extensive investigations for years (Schier et al., 1997; Hatta et al., 1991),
therefore we did not further investigate these lines and kept them as stocks in our fish
facility.

The next interesting phenotypic subgroup consists of mutants displaying neural
degeneration. These mutants are quite interesting, since they display a rather general cell
death pattern, as shown with stainings against acridine orange in HE022 and HK314 (Fig. 7
B,B’,H,H’), or anti-TUNEL antibodies, as demonstrated for HC006a (Fig. 7 J,J’). This
might normally indicate a loss of cellular integrity and a dying embryo, but further
stainings with antibodies against acetylated Tubulin could show that this cell death is
obviously affecting only a subset of neuronal cells, while the reminder of neurons is able to
extend axons (Fig. 7 C-F’,K-N’). In the case of HK314, the Tubulin staining pattern does
not even change at all (data not shown), and later no cell death is detectable any more.
What could be the reason for this phenomenon?

Cell death is a normal phenomenon in development, and is necessary since
supernumerous cells are born and only those that eventually make correct connections are
surviving (Wolpert et al., 1998). The cells die by apoptosis, an active process that requires
RNA and protein synthesis, fragmentation of the DNA and phagocytosis. Cells dying
because of pathological reasons are not apoptotic, but necrotic. To explain the phenotype of
HK314, it could be that more cells than normal are made, and these are eliminated
immediately to establish the correct cell number. Studies on cell proliferation should allow
further insight into this issue. In HE022 and HC006a, which are affected in axonal outgrowth, e.g. thinner axonal bundles (Fig. 7 D’) or loss of axonal guidance (Fig. 7 F’), cells needed might have died. The reasons for this are not known yet.

The third group of phenotypes not concerning brain morphology is the organ group. Specific failures in the formation of the heart, the epiphysis (data not shown), the ear (Fig. 9) and the epidermis (Fig. 8) have been reported. The new phenotypes of IN193 and IY121 deserve a few more words of explanation. Why are the cells singled out of their context if they are not dying, but eventually even make connections (Fig. 8 C’)? A difference in cell adhesiveness could be one explanation. Cells adhere to each other and to the extracellular matrix through interactions involving cell-surface proteins, e.g. Cadherins, proteins of the Immunoglobulin superfamily and integrins (reviewed in Gumbiner, 1996). The two mutants might be defective in one of these molecules, which should be expressed rather locally in the hindbrain or the tail, respectively, since the phenotypes are rather specific and affect only a subset of cells in the embryo.

4.2.4 Brain morphology mutants display a range of distinct and novel phenotypes

In the Tuebingen screen 2000 we could discover and re-identify 18 different mutations that display morphological abnormalities in the midbrain, MHB and hindbrain areas.

These mutants have been further subdivided according to their phenotype, and one of these, the snakehead-like class of mutants, has 4 members. All share the feature that their brain ventricles are collapsed (Fig. 10), the heads are relatively flat and the brain structure is very smooth and diffuse. They look quite similar to the group of snakeheads from the first Tuebingen screen (Jiang et al., 1996), and also share with those a normal expression of patterning genes such as pax2.1, fgf8 and eng2 (data not shown). One could conclude that these mutants have been found before and seem not to be affected in patterning, however I think they would be worth a closer examination: how is it possible that the brain morphology is strongly abnormal, but patterning is completely normal? Here, patterning seems to be necessary for morphology, but obviously it is not sufficient.
The next interesting brain morphology subgroup is the one with amorphous head morphology (Fig. 11). On the one hand these mutants, which all are allelic, show specific features of morphology such as a bulky tectum (Fig. 11 A’), a wavy hindbrain (Fig. 11 A’,C’) and specific aberrations in fasciculation and axon outgrowth (Fig. 11 D’,E’). On the other hand, *in situ* hybridisations for patterning genes did not reveal any differences between mutants and their siblings (data not shown). Further studies should consider that the brain phenotype resembles the one of the dwarfs, mutants defective in the formation of notochord caused by mutations in various chains of laminin, that have been discovered in the first Tuebingen screen (Odenthal et al., 1996; Parsons et al., 2002). One of them, *bashful*, could be mapped to linkage group 24, to which HF028 is also mapping (Derek Stemple, personal communication; Table 8). Although an apparent notochord phenotype, the common feature of all dwarfs, has not been found in HF028, complementation studies should be taken out first to exclude that HF028 and the other newly discovered alleles are allelic to *bashful*, since the new alleles could be weaker than the previous discovered ones and only display a very weak, so far undiscovered, notochord phenotype.

4.2.5 Two new alleles of *n-cadherin* reveal a new role of N-cadherin in cell adhesion

In the course of the screen, three *n-cad* alleles have been discovered, one resembling the published phenotype of pac (Lele et al., 2002), *HU519* (Fig. 12 B’-J’), and two new ones, *HJ032* and *HJ050*, which do not resemble the pac morphology (Fig. 12 A’-I’). The difference is, that the cognate *n-cadherin* mutant phenotype consists of loosened, roundish cells that lost contact to their surrounding and are singled out into the ventricular space. Often, these cells are eliminated by apoptosis (Lele et al., 2002). In *HJ032*, the opposite effect on cells can be observed: no single cell can be seen, and the whole brain looks condensed and the ventricles are barely seen (Fig. 13 D-G). Obviously, the loss of a functional protein in *HU519* causes a reduced adhesion, while in *HJ032* the cells might be even more adhesive. Our suspicion was that a point mutation in the intracellular domain of *n-cad* leaves an intact extracellular domain, exerting its function and, moreover, even acting as an enhancer of adhesion. Since no point mutation could be discovered within the
coding region, this cannot be the case. The next step to elucidate the mutation would be to find out more about the nature of the mutation, e.g. whether regions upstream of the coding sequence of \textit{n-cad} are affected by the point mutation.
4.3 Detailed analysis of the *broken heart* mutant

The *broken heart* mutation offers the opportunity to study later events of embryonic development, such as axogenesis and differentiation of neural structures such as the epiphysis and the serotonergic raphe nuclei.

4.3.1 The epiphysis of *broken heart* is defective in photoreceptor function and axonal outgrowth of DVDT axons

The epiphysis of the zebrafish is an interesting organ to study in many respects: in the forebrain, neurons of the epiphysis are the first ones to differentiate and therefore introduce this structure as a model for early differentiation and axogenesis (Masai et al., 1997). Furthermore, the epiphysis of the zebrafish is not only the major site of melatonin production, it also actually senses the input of light and is therefore the regulator of melatonin production itself, in contrast to the situation in mammals (Ganguly et al., 2002; reviewed in Cahill, 2002).

In *brh* embryos, prepatterning of the epiphysial territory is correct: normal expression of *flh* reflects that neurons are born at the normal prospective site of the epiphysis (Fig. 19), and the unchanged expression of the proneural genes *zash1*, *ngn1* and later the marker for early neurons, *islet-1*, demonstrates that neurogenesis takes place at the right time and location (Fig. 19). The signaling events leading to the diversification of the newborn neurons into projecting neurons and photoreceptors are still unknown and have not been addressed in the course of this study. Once the neurons have differentiated, the phenotypes of the two neuron subtypes are rather different: projecting neurons seem to be normal, as judged by the marker gene expression of *lim3* and *onecut* (Fig. 20 A-D’), but fail to project axons at 30 hpf (Fig. 17 A’,D’, 18 B). Only later, at 48 hpf, single axons seem to emerge from the neurons, and the cell bodies are visible by anti-Tubulin antibodies (Fig. 17 D’). In contrast, the expression of *onecut* gets specifically lost from 36 hpf onwards (Fig.
20 D’). The delay in axonal outgrowth has to be put into context with the other outgrowth failures in the embryos: it is not only the DVDT, that is formed too late, but also the spinal cord axons (Fig. 17 B’) as well as different commissures such as the AC, PC and POC (Fig. 17 A’,C’) are not present at the correct time. Therefore, since this outgrowth delay is present in various brain areas, a pan-axonal guidance molecule might be affected.

The situation in photoreceptors differs from that of the projecting neurons, because the photoreceptors seem to be affected from early stages onwards, already at 24 hpf, as demonstrated by diminished in situ hybridisation stainings with tphD1, otx5, aanat (Fig. 20 E-I’) and tphR (Fig. 21 A-H). Interestingly, this is also the stage at which the prepatterning and proneural genes are normally expressed (Fig. 19). Since tphD1, tphR, otx5 and aanat are specific for photoreceptors in the epiphysis (Gamse et al., 2001; Steve Wilson, personal communication), there must be an overlap of expression of early acting genes in both cell types and genes that are exclusively expressed in only one of the cell types. The observation that the expression domains of flh, zash1, ngn1 and islet-1 are the same size in wildtype and mutant embryos, and the domains of the photoreceptor markers are smaller, leads me to ask what the identity of the stained cells might be. One explanation could possibly be that the projecting neurons are favoured at the expense of the photoreceptors, but this is not the case (Fig. 20 A-I’). It might therefore be that cells in the epiphysial area get prepatterned, but fail to differentiate into a photoreceptor fate and remain non-functional.

The epiphysis of broken heart has no functional melatonin production. The circadian gene regulator, otx5, is affected form 24 hpf onwards (Fig. 20 G’,H’). Studies on morphants injected with morpholinos against otx5 have shown that without functional otx5 circadially expressed genes such as aanat, reverβα and irbp are strongly reduced (Gamse et al., 2001). aanat is also absent in broken heart embryos at 48 hpf (Fig. 20 I’). This cannot be explained by the fact that aanat is cycling and, therefore, the embryos may have been fixed at a low aanat expression level, since the cycling expression only starts after day 2 and the gene is uniformly expressed before (Gothilf et al., 2002).

The two tph genes studied, tphD1 and tphR, are both strongly affected in the epiphysis: tphD1 is weakly expressed at 24 hrs, but lost at 48 hrs (Fig. 20 E’,F’), and the onset of tphR is delayed until between 40 and 48 hrs (Fig. 21 A-H). Factors controlling the
expression of tph genes in the epiphysis are not known in the zebrafish. Fgf8, as well as Shh, are likely not involved, since they are expressed normally in the mutant (data not shown).

In mammalian organisms and chick, more is known about signals upstream of tph genes. Intracellular circadian increase of cAMP levels induces the transcription of tph in human and cultured chick pineal cells (Cote et al., 2002; Florez and Takahashi, 1996). By the use of cultured rat pinealocytes, it could be demonstrated that the transcription factors NF-Y and Sp1 bind to the TPH promoter and are essential for the cAMP-mediated transcriptional response (Cote et al., 2002). Furthermore, factors such as BDNF, glucocorticoids and norepinephrine are all involved in the regulation of tph (Siuciak et al., 1998; Clark and Russo, 1997; Florez and Takahashi, 1996).

4.3.2 The role of the floorplate and the MHB in induction of serotonergic neurons in the hindbrain

The floorplate and the midbrain-hindbrain organiser are thought to act as signaling centers in the embryo with a pivotal role in specifying 5-HT neurons in the hindbrain (reviewed in Hynes and Rosenthal, 1999).

In the zebrafish, sonic you (syu) mutants, which are defective for one of the factors emanating from the floorplate, shh, show a severe reduction of 5-HT neurons in the hindbrain (Teraoka et al., 2004). Furthermore, smu (smooth muscle omitted) embryos, lacking the functional Hedgehog co-receptor, Smoothened, are completely devoid of any tphR staining in the hindbrain (Teraoka et al., 2004). In broken heart embryos, which are defective in the formation of caudal 5-HT neurons in the hindbrain (Fig. 21 I-N), the expression levels of shh, twhh and smu have been analysed in in situ hybridisations, but none of these genes was absent or even downregulated (Fig. 24 A-F’). In addition, neither the expression of gata3, nor of nkx2.2, the mediator of Hedgehog signaling, differ between wildtype and mutant embryos (data not shown). The loss of serotonergic neurons in the hindbrain of brh is therefore not due to a misexpression of factors of the Hedgehog pathway. Since the most rostral expression domain of tphR in the hindbrain is present in
brh (Fig. 21 L’,N), it can be speculated whether the signals from the floorplate are responsible for the induction of rostral hindbrain fate, while the more caudal parts are dependent on other factors.

Is it possible that factors from the MHB play a role in specification of caudal raphe in the zebrafish? In explant cultures from rat hindbrain, block of Fgf8 prevented the induction of serotonergic neurons rostrally, but not caudally (Ye et al., 1998). It was therefore concluded that Fgf8 acts as a local cue on the neurons directly next to its source, the MHB, rather than as a long-range molecule on neurons more distant to the source. Recent studies in the zebrafish could show on the other hand that Fgf8 can travel a distance from the MHB and represses pax6 at the diencephalic- mesencephalic border (Scholpp and Brand, 2003). If Fgf8 plays a role in induction of caudal raphe, this group of neurons should be repressed in a loss-of-function situation. Otherwise, Fgf8 should induce them when supplied ectopically. Indeed, I could show that both is the case: to create a loss-of-function situation, SU5402 was applied to wildtype embryos and, therefore, all Fgf signaling, including Fgf8 signaling, was eliminated. In embryos inhibited before 30 hpf, the caudal portion of the tphR staining was absent (Fig. 25 A-I), indicating that a loss in Fgf8 signaling does not influence rostral raphe fate, but the caudal one. In addition, the timepoint of specification of neurons in the hindbrain into serotonergic neurons can roughly be defined between 20 somites and 30 hpf. This rather late time in embryonic development points to a role for Fgfs in determining a specific neuronal fate rather than in induction and patterning. The gain-of-function situation was created by implanting a bead soaked with Fgf8 laterally into the hindbrain. This extra source of Fgf8 was sufficient to bring back the tphR expression in caudal serotonergic neurons, but only at the contralateral side (Fig. 29 B). It has been demonstrated recently that the spreading of Fgf8 is regulated by endocytosis (Scholpp and Brand, 2004), suggesting that the concentration of Fgf8 is more and more diminished the further away it travels form its source. This supported the idea that Fgf8, secreted from the MHB, might not induce 5-HT neurons at a high concentration level, but the more distantly located neurons at a lower dose. In the spinal cord, it could be demonstrated that Shh and Bmp proteins are responsible for patterning neural progenitors along the D-V axis by establishing morphogen gradients from ventral (Shh) and dorsal
(Bmps) sources (Charron et al., 2003). Later, the Shh morphogen gradient and Netrin-1 are required as positive chemoattractants for the guidance of commissural axons.

Alternatively to this concentration-dependent ‘morphogen’ theory, rostral serotonergic neurons might not be able to respond to an Fgf8 signal. This notion is supported by the fact that tphR expression could also be brought back on the side of implantation, however, in a much weaker fashion (Fig. 29 B). In addition, at the level of rhombomere 4, there is a gap in serotonergic neurons in zebrafish (Fig. 21 M) and also in mouse (reviewed in Goridis and Rohrer, 2002). Studies in zebrafish have shown that rhombomere 4 acts as a local signaling center by expressing Fgfs such as Fgf3 and Fgf8 in the early embryo (Maves et al., 2002; Léger and Brand, 2002). Taken together, this would again support the model that high levels of Fgfs do not induce serotonergic neurons (also see below).

Another Fgf discussed as a factor involved in 5-HT neuron induction is Fgf4. In rat, Fgf4 is expressed in the non-ectodermal primitive streak very early in the embryo, and functions as a prepatterning factor along the A-P axis that helps the affected region to respond to later specification cues (Ye et al., 1998). In chick, studies applying ectopic Fgf4 have indicated possible roles for Fgf4 in mesoderm formation, antero-posterior patterning and as a potential inducer of neural tissue acting from competent epiblast (Storey et al., 1998). So far, nothing is known about the expression of Fgf4 in the early zebrafish embryo and its possible role in specification of serotonergic neurons. Between 20 somites and 30 hpf, the possible period of 5-HT induction, fgf4 is expressed in the zebrafish in three domains: in the tailbud, the ear and the MHB (Fig. 26 and data not shown). Therefore, it might very well be that also Fgf4 is acting on hindbrain specification from the MHB. When applied ectopically by a bead implantation into the hindbrain of broken heart, caudal serotonergic neurons were induced in response to Fgf4 (Fig. 27). Therefore, Fgf4 may also act as a planar signal rather than a vertical one coming from underlying tissue.
4.3.3 The MHB territory is not maintained from 24 hpf onwards, resulting in a loss of genes responsible for caudal 5-HT neuron induction

Are Fgf4 and Fgf8 specifically lost, and the mutation has to be searched for in the cascade upstream of Fgfs, or is the whole MHB territory misspecified? In broken heart embryos, the integrity of the MHB is not maintained beyond 24 hpf, as judged by marker gene expression (Fig. 28). Therefore, Fgf4 and Fgf8 are not specifically lost, which can be supported by the finding that the expression of \textit{fgf8} early in the epiphysial territory is completely normal in \textit{broken heart} (data not shown). The complete loss of the MHB could also account for the 5-HT phenotype in \textit{noi} embryos. Here, the expression of \textit{tphR} is also reduced to the rostral portion of the raphe nuclei, although a role for \textit{pax2.1} in the specification of serotonergic hindbrain neurons could not be demonstrated so far (Teraoka et al., 2004).

What can be the reason for the loss of marker gene expression at the MHB? TUNEL stainings performed at different timepoints of development did not reveal any upregulation of cell death in the MHB area (data not shown). Studies with the zebrafish mutant of \textit{fgf8}, \textit{acerebellar}, showed that \textit{fgf8} is required for maintenance of gene expression at the MHB (Brand et al., 1996b; Reifers et al., 1998). Genes such as \textit{her5}, \textit{wnt1}, \textit{pax2.1}, \textit{eng2}, \textit{eng3} and \textit{fgf8} itself are downregulated, and the expression of \textit{her5} is the first to be affected at 5 somites (Reifers et al., 1998). Interestingly, \textit{her5} is also the first gene downregulated in the MHB of \textit{broken heart} embryos, but this becomes apparent as late as 20 somites (data not shown).

In conclusion, the reason for the loss of maintenance of gene expression at the MHB could not be revealed so far. However, the loss of MHB expression might account for the loss of caudal serotonergic hindbrain neurons via reduced levels Fgf4 and Fgf8. A specific role of both Fgfs in differentiation could not be demonstrated, and it has to be taken into consideration that the depletion of Fgfs might leave serotonergic neurons without necessary proliferation or survival signals and therefore causes a more indirect defect on neurons before differentiation can occur.
4.3.4 Are motorneurons in the hindbrain upregulated at the expense of serotonergic neurons in the raphe nuclei?

The induction of hindbrain serotonergic neurons is not only influenced by factors in the Hedgehog and Fgf pathways, but also coordinated by another group of factors, the Homeodomain proteins Phox2a and Phox2b. *phox2* genes are expressed in a variety of neuronal structures and function as neuron-type determinants, e.g. in the promotion of noradrenergic differentiation (Dubreuil et al., 2002; reviewed in Brunet and Pattyn, 2002). In the hindbrain, *phox2b* is expressed in a common precursor pool including visceral motor neuron precursors and precursors for serotonergic neurons (Pattyn et al., 2003). Visceral motorneurons are first produced dorsally, and when in the ventral half the *phox2b* expression is downregulated, serotonergic neurons are produced instead. Interestingly, the expression of *phox2b* is prolonged in time in rhombomere 4, and no serotonergic neurons can therefore be found at this level of the hindbrain (Pattyn et al., 2003). A mutation in the *phox2b* gene leads to an ectopic appearance of serotonergic neurons even in rhombomere 4 (Pattyn et al., 2000).

The zebrafish *phox2a* mutant, *soulless*, displays a loss in trochlear and oculomotor nuclei and fails to develop noradrenergic neurons in the locus coeruleus (Guo et al., 1999a). Is there a relationship between the induction of serotonergic fate on the one hand and generation of hindbrain motor neurons and noradrenergic neurons on the other hand in regard to the *broken heart* phenotype? In case of a relationship, the loss of serotonergic neurons should result in a possible upregulation of motorneurons in the hindbrain area, where the 5-HT neurons would normally be produced, or an upregulation of neurons in the locus coeruleus.

Stainings with anti-tyrosine Hydroxylase could show that the development of the TH-positive neurons, including the locus coeruleus, is completely normal (Fig. 23). In addition, *in situ* hybridisations with an antisense probe against *phox2a* at 48 hpf revealed that *phox2a* is not upregulated, but slightly downregulated in *brh* embryos (data not shown). Both findings indicate that the *broken heart* phenotype is not due to a shift of the balance between 5-HT and motor neuron induction on the one side or 5-HT induction and
noradrenergic neuron specification on the other side, but rather due to a specific failure of induction of caudal serotonergic neurons.

Furthermore, anti-TH stainings in ace revealed that Fgf8 is involved in the specification of catecholaminergic neurons in the locus coeruleus, since these neurons are absent in ace (Guo et al., 1999b; Holzschuh et al., 2003). As mentioned above, serotonergic neurons are severely reduced as well in ace, and locus coeruleus neurons are not affected in brh. An explanation for this might be, that the induction of CA- neurons by Fgf8 in the locus coeruleus happens earlier, at 18-24 hpf, and induction of serotonergic neurons takes place slightly later, between 20 somites and 30 hpf (Fig. 25). Since in brh the fgf8 expression is only reduced after 24 hpf, locus coeruleus neurons are differentiated, but serotonergic neurons are not under the influence of Fgf8.

4.3.5 Axonal outgrowth is affected in the whole embryo

Several bodies of evidence point to the direction that broken heart is unspecifically delayed: the axonal outgrowth in a variety of body parts is delayed significantly (Fig. 17). A closer examination of the hindbrain shows that the development of interneurons is impaired, and in addition, the motorneurons, which are present as judged by the islet-1 marker gene expression, do no extend axons by day 2 (Fig. 22). This phenotype is not based on extensive cell death, since TUNEL staining at different timepoints did not show any significant upregulation of cell death in the hindbrain area (data not shown). The delay in outgrowth of motor axons is not specific for the spinal cord and can be explained by a general failure of the mutant to grow axons at the correct timepoint. The AC and POC are generally delayed as well (Fig. 17 C’). It can be excluded that the AC and POC phenotype is due to a mutation in a gene expressed at the midline, since factors such as gli2 (Karlstrom et al., 1999), shh (Macdonald et al., 1997) and twhh (Ekker et al., 1995), which have been shown to play a role in commissure formation, do not account for the defect in the hindbrain or spinal cord axons, which are not commissural.
4.3.6 The identification of the gene mutated in *broken heart* is a long-term process

The search for a candidate gene started promising, since two candidates, *evx1*, based on its expression pattern (Thaeron et al., 2000), and *npy*, based on its involvement in the regulation of the circadian rhythm (Soderberg et al., 2000), could be detected. Unfortunately, no point mutation could be discovered in the sequences of their coding regions. A mutation giving rise to different splicing like in *ace* (Reifers et al., 1998) can also be excluded, since the PCR products for both genes have the same length as the wildtype ones (data not shown). Unfortunately, no other candidate genes, whose expression domains or signaling pathways would be suggestive for a possible candidate, are found several centimorgans up- and downstream of the mapped mutation. This situation will improve, since the zebrafish genome has not been fully sequenced yet, and once this has been achieved, more and more genes will be identified and placed on the physical map. Furthermore, a great number of ESTs have been assigned to the region around the mutation, but the description of most of them is very poor. The effort to clone and sequence all of them in cDNA derived from wildtype and mutant embryos would be far too time consuming. Here, fine mapping could help to narrow down the region of interest on LG19 by using a different set of SSLP markers for this particular region (Geisler, 2002).

Apart from cloning and the candidate gene approach, another strategy is the careful analysis of the phenotype and an extensive search in the literature for possible effectors of the phenotype. One gene interesting in this context is Pet-1, a member of the ETS domain transcription factors (Fyodorov et al., 1998). In rat, it is expressed in developing and adult 5-HT neurons (Hendricks et al., 1999). In mice lacking Pet-1, the majority of 5-HT neurons fail to differentiate and mice are more anxious and aggressive in their behaviour (Hendricks et al., 2003). It has also been shown that Pet-1 is a transcriptional regulator for genes involved in cholinergic neurotransmission, e.g. the β4 subunit of the neuronal nicotinic acetylcholine receptor (Fyodorov et al., 1998). Since *broken heart* is defective in locomotion, and the expression levels of actylcholine esterase itself is unaltered (data not shown), one could speculate that *brh* could be a point mutation in the *pet-1* gene that leads to the failure of serotonergic neuron induction and could in addition explain the locomotion phenotype. On the other hand, *pet-1* expression is restricted exclusively to serotonergic
neurons (Hendricks et al., 2003), and could therefore not account for the defects in the heart and formation of anterior commissures in $brh, pet-1$ in zebrafish has not been cloned yet, however, it might still be worth a try, because the expression domains in zebrafish could differ from those in mammals. In addition, since a defect in the melatonin synthesis has a lot of implications on various physical and physiological states, it might also be feasible that local gene expression yet causes widespread defects in the whole animal.
5 References


Versicherung zur eingereichten Dissertation

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Dresden, den 07. November 2003

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