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TUM

Zebrafisch Neurogenetik

The induction and early development
of the midbrain-hindbrain in the embryonic zebrafish

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Abstract

The midbrain-hindbrain domain (MH) is an important region of the vertebrate embryonic brain. Indeed, in contrast to other brain domains, it is not formed by segmentation processes, but responds to an organizer activity located at the midbrain-hindbrain boundary (MHB). Thus it is of great interest to understand the factors and mechanisms underlying MH formation. The present work uses the zebrafish system to add important information on the poorly understood steps of early MH development. These results are summarized below:

(i) It is known that MH development is regulated by planar information, however the upstream factors regulating the expression of the known MH markers *her5*, *pax2.1*, *wnt1* and *fgf8* are not known. We identified the zebrafish Btd-related factor Bts1 as a specific regulator of *pax2.1* and its dependent genetic cascade (*pax5*, *eng3*). Because of the crucial function of Bts1 in MH induction, we analysed the regulation of its own expression. We demonstrate that the induction of *bts1* expression likely depends on FGF and Wnt signaling. Thus, we identified one cascade of MH induction through planar signaling.

(ii) Anterior neural plate development is thought to respond to vertical signaling. So far, the precise role of vertical signals in MH development remains unclear. Using a combination of experimental manipulations in mutant lines affected in non-neural tissues, we unravelled the inhibitory influence of a long-range signal, emanating from the prechordal plate, on the refinement of a neural cluster at the forebrain-midbrain boundary. These results give evidence that vertical signals have a precise role in MH development.

(iii) The earliest gene selectively expressed in the prospective MH is *her5*. Using a reporter approach in zebrafish transgenics, we identified *her5* regulatory elements. Further, the *PACher::egfp* transgenic line allows tracing cells of the entire presumptive MH from MH induction onwards. Using this transgenic line in mutant contexts, we demonstrate that in the absence of functional Pax2.1 or FGF8, MH cells partially acquire the fate of neighbouring brain regions, to a different extent depending on the mutant context. Together, these results identify the genomic sequence responding to MH induction factors, and permit to assess the role of early MH factors (such as Fgf8 and Pax2.1) on cell fate.

(iv) We identified a novel Hairy/E(spl) factor, called Him, positioned in an unusual head-to-head orientation close to Her5 genomic locus. Preliminary functional analyses suggest that Him loss-of-function leads, similar to a lack of Her5 function, to a premature differentiation in the normally neuron-free "intervening zone" at the presumptive MHB. Together these results identify a new factor, and potential partner for Her5, during the MH induction phase.

Zusammenfassung

Das Mittelhirn-Hinterhirn (MH) Gebiet ist eine wichtige Region des embryonalen Gehirns, das im Gegensatz zu anderen Hirngebieten nicht durch Segmentation gebildet wird, sondern durch die Aktivität eines Organisationszentrums entsteht, das sich an der MH Grenze befindet. Deshalb ist es von großem Interesse diejenigen Faktoren und Mechanismen zu entdecken, die für die Entstehung des MH notwendig sind. Mit Hilfe des Modellorganismus Zebrafisch erweitert diese Arbeit das Wissen über die bis jetzt nur wenig verstandene Induktion des MH um folgende neue Erkenntnisse:

(i) Es ist allgemein bekannt, dass die Entwicklung des MH Gebietes durch Informationen innerhalb der Neuronalplatte (horizontale Signale) gesteuert wird. Jedoch sind die Faktoren, die eine Induktion der bekannten MH Gene *her5*, *pax2.1*, *wnt1* und *fgf8* steuern, noch nicht entdeckt. Wir haben den zu Btd verwandten Faktor Bts1 aus dem Zebrafischgenom isoliert und ihn als spezifischen Regulator von *pax2.1* und dessen abhängiger genetischen Kaskade charakterisiert. Diese wesentliche Funktion des Faktors Bts1 während der Entstehung des MH hat uns veranlasst dessen eigene Regulation zu untersuchen. Von unseren Ergebnissen schließen wir, dass die Expression von *bts1* wahrscheinlich durch FGF und Wnt Signale ausgelöst wird. Somit haben wir eine Signalkaskade der MH Induktion durch horizontale Signale identifiziert.

(ii) Es wird angenommen, dass die Entwicklung der vorderen Neuronalplatte von vertikalen Signalen beeinflusst wird. Die präzise Rolle dieser vertikalen Signale ist jedoch unklar. Eine Kombination von experimentellen Manipulationen in Fischlinien, die Defekte in nicht-neuronalen Geweben aufweisen, ermöglicht uns den inhibierenden Einfluss von weit wirkenden Signalen, gebildet in der prächordalen Platte, auf die Entwicklung einer neuronalen Gruppe an der Vorderhirn/Mittelhirngrenze aufzuzeigen. Die erzielten Ergebnisse deuten an, dass vertikale Signale während der Entwicklung des MH Gebietes eine eindeutige Rolle spielen.

(iii) *her5* ist das bisher erste bekannte Gen, das im künftigen MH Gebiet spezifisch exprimiert wird. Mit Hilfe der transgenen Fischlinie *her5PAC::egfp* als „Reporter" identifizierten wir die regulierenden Elemente von *her5*. Ferner ermöglicht es diese transgene Linie alle Zellen des MH Gebietes ab seiner Induktion optisch zu verfolgen. Da wir diese transgene Linie auch im Zusammenhang mit der Analyse von mutierten Fischlinien verwendet haben, können wir zeigen, dass MH Zellen durch den Verlust von funktionalem Pax2.1 oder FGF8 Proteinen zu einem gewissen Ausmaß, das von der jeweiligen Mutation abhängig ist, die Identität eines

benachbarten Gebietes übernehmen. Unsere Ergebnisse zeigen somit die Identifizierung der für die Regulation durch induzierende Faktoren notwendige genomische Sequenz und weisen außerdem auf den Einfluss von MH Faktoren wie FGF8 und Pax2.1 auf die Identität einer Zelle hin.

(iv) Wir haben den bisher unbekannten Hairy/E(spl) Faktor Him isoliert, der in einer ungewöhnlichen Kopf zu Kopf Position nahe dem genomischen Lokus von Her5 plaziert ist. Vorläufige Ergebnisse deuten darauf hin, dass ein Ausschalten der Funktion von Him, ähnlich wie das Fehlen des Faktors Her5, zu einer frühzeitigen Differenzierung von Neuronen in der zu diesem Zeitpunkt normalerweise neuronenfreien „intervening zone" („dazwischen liegende" Zone) an der MH Grenze führt. Zusammengefasst zeigen diese Ergebnisse die Identifizierung eines neuen Faktors und potentiellen Partners von Her5 während der Entstehung des MH Gebietes.

Abbreviations used in the text:

AchE   acetylated cholin esterase
ANB   anterior neural border
ANR   anterior neural ridge
AP   anterior-posterior
AVE   anterior visceral endoderm
bHLH   basic helix-loop-helix
bp   base pair(s)
cDNA   complementary DNA
C. elegans   Caenorhabditis elegans
CNS   central nervous system
DNA   deoxyribonucleic acid
DV   dorso-ventral
E   exon
E. coli   Escherichia coli
EGFP   enhanced green fluorescence protein
fig   figure
GFP   green fluorescence protein
h   hours
hpf   hours post fertilization
I   intron
IsO   isthmic organizer
IZ   intervening zone
kb   kilo bases
MBT   mid-blastula transition
mes   mesencephalon
met   metencephalon
MH   midbrain-hindbrain
MHB   midbrain-hindbrain boundary
MLF   medio-longitudinal fascicle
Mo   morpholino
MZ   maternal zygotic
n   number
nMLF   nucleic medio-longitudinal fascicle
nPC   nucleic posterior commissure
nTPOC   nucleic tract of the post optic commissure
mRNA   messenger ribonucleic acid
nls   nuclear located signal
P   prosomere
PAC   phage artificial chromosome
PCP   prechordal plate
PCR   polymerase chain reaction
r   rhombomere
RNA   ribonucleic acid
tg   transgenic
TPOC   tract of the post optic commissure

vcc	ventrocaudal cluster
YSL	yolk syncitial layer
WT	wild-type
zli	zona limitans intrathalamica

Symbols:

::	insertion
R	resistance

Resistance genes:

amp	ampicillin
chm	chloramphenicol
kan	kanamycin
zeo	zeocin

Genes/Proteins:

Asc	Acheate-scute
Bf	Brain factor
BMP	Bone morphogenetic protein
Btd	Buttonhead
Bts	Buttonhead/Sp related protein
Cdk	Cell division protein kinase
E47	Immunoglobulin enhancer binding factor E47
Emc	Extra macrochaetae
En or Eng	Engrailed
E(sp)	Enhancer of split
Esr	Enhancer of split related protein
FGF	Fibroblast growth factor
fgfr	Fibroblast growth factor receptor
Dkk	Dickkopf
Gsc	Goosecoid
Gbx	Gastrulation brain homeobox
Gta	GATA binding factor
Hes	Hairy and Enhancer of split
Her	Hairy and Enhancer of split related
Hgg	Hatching gland cell protein
Him	Her5 image
Hlx	Homeobox gene Hlx
Hox	Homebox protein
HNK	Cell adhesion protein
Id	DNA-binding protein inhibitor ID
krk	Kruppel-type zinc finger protein
L-fng	Lunatic fringe
Lim	Homeodomain factor, name derived from the factors lin-11, Isl-1, mec-3
Mash	Mouse achaete-scute homolog
MyoD	Myogenic differentiation 1
NeuroD	Neurogenic differentiation
Nkx	Homeodomain protein Nkx

Ngn	Neurogenin
Oct	Octamer-binding
Otx	Orthodenticle homebox protein
p-21	Factor P-21
Pax	Paired box protein
Phox	Paired-like homeobox
Pou	POU domain protein
RA	Retionic acid
Raldh	Retinal dehydrogenase
Shh	Sonic hedgehog
Six	Sine oculis homeobox homolog
Sox	HMG-box transcription factor Sox
Stra	Stimulated with retinoic acid
Tar	Type I serine/threonine kinase receptor TARAM-A
Tcf	T-cell-specific transcription factor
Tle	Transducin-like enhancer protein (Groucho-related)
Tlc	T-cell receptor beta chain
Twhh	Tiggy-winkle hedgehog protein
Wnt	Secreted glycoprotein wingless-type protein
Zash	Zebrafish achaete-scute homolog
Zcoe	Zebrafish Olf-1/EBF-like factor

Zebrafish lines:

ace	*acerebellar*
bon	*bonnie-and-clyde*
boz	*bozozok*
cas	*casanova*
cyc	*cyclops*
hdl	*headless*
kny	*knypek*
mbl	*masterblind*
noi	*no-isthmus*
oep	*one-eyed pinhead*
smu	*smoothened*
spg	*spiel-ohne-grenzen*
sqt	*squint*

I. Introduction

The main goal of my Ph.D. project was to obtain information at molecular and cellular levels about early steps of development of the midbrain-hindbrain (MH), using the zebrafish (*Danio rerio*) as a model system.

Why studying the midbrain-hindbrain domain? First, neurons in the MH serve many essential functions: the midbrain controls important aspects in motor function and sensory input and the hindbrain serves as a neuronal connection center controlling mainly sensory and behaviour processes. Thus, it is important to understand how this region is established within the neural tube and how it is patterned. Second, the MH is an interesting territory of brain development as it is not formed by segmentation processes but instead responds to a local organizer activity, the isthmic organizer (IsO), positioned at the midbrain-hindbrain boundary (MHB). Finally, the MH displays an interesting pattern of neurogenesis: the MHB remains, in contrast to other brain areas, undifferentiated until late stages, and this likely permits the MH to grow to one of the largest parts of the adult brain.

During MH development, there are at least two steps involved: an early induction step during late gastrulation, in which the expression of MH genes is initiated in the neural plate, and a later maintenance phase (at about 5-10 somites) in which the expression of these genes becomes refined and starts to depend on each other (maintenance loop). While MH maintenance is well investigated, the previous step, namely the induction of the MH, is less understood. The induction of the MH is believed to depend on planar (within the neural plate itself) as well as vertical signaling (emanating from non-neural tissues underlying the neural plate) and likely occurs during gastrulation.

To study early brain development the zebrafish (*Danio rerio*) is an excellent vertebrate model system. One of the biggest advantages in zebrafish embryos is the optical clarity, which allows the direct visualization of *in vivo* gene expression and cell movements by the use of fluorescent markers, enabling to follow dynamic processes during development (fig1). Further, an external fertilization with access to a big number of eggs and a rapid development makes it ideal for embryological manipulation and genetic approaches.

Although it is believed that a common ground plan for patterning of the neural tube and brain development exists in all vertebrates, there are many differences in detail, which makes it necessary to understand and to compare how the brain in different vertebrates develops.
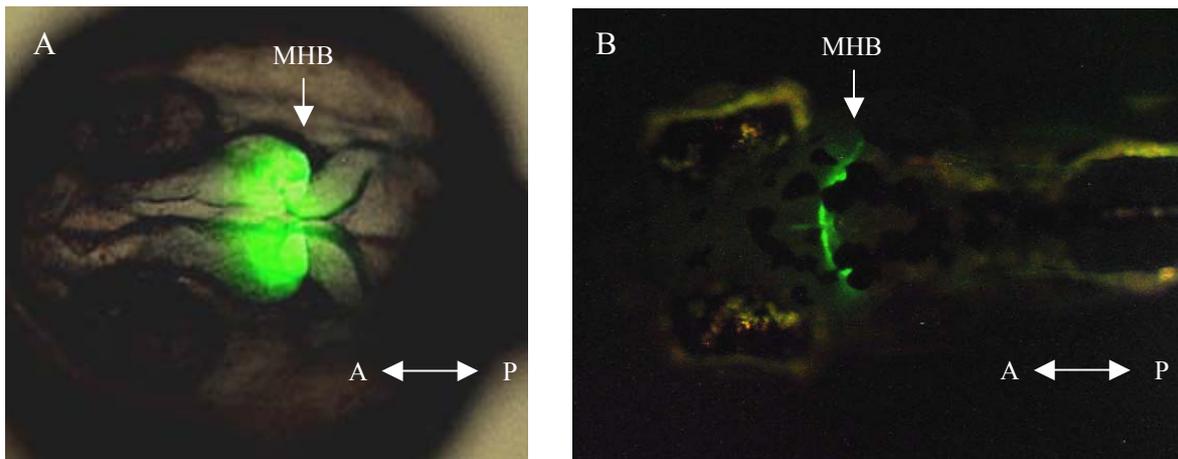
Fig.1. Dorsal view of a living *her5egfp*-transgenic embryo at 24h and 72h (this work).
A. The brain structures with the prominent MHB are morphologically visible. The green fluorescent EGFP protein is expressed in the MH, with the highest intensity at the MHB. B. The expression of EGFP is restricted to the MHB. The embryos are illuminated with bright field and flourescent light, anterior to the left. A, anterior; P, posterior.

I will discuss in the following paragraphs the developmental processes involved in the formation of the brain in vertebrates in general, and in zebrafish in particular, starting with 1. neural plate induction and early neural plate patterning along the anteroposterior (AP) axis, and finally emphasising on 2. midbrain-hindbrain patterning. One further important aspect of brain development, particularly relevant to the MH, is the establishment of neuronal precursor cells and their differentiation, presented in 3. neurogenesis.

1. Neural plate patterning

The expression patterns of a variety of genes suggest a common basis for mechanisms involved in the induction and patterning of the nervous system in anamniotes (zebrafish, *Xenopus*), amniotes (chick, mouse, human) and invertebrates (*Drosophila*). For instance, one important common feature in establishing as well as patterning the neural plate is the involvement of BMP, Wnt and FGF signaling, which are involved in several steps during nervous system development. Further, the discrete role of the organizer in the formation of the neural plate will be discussed briefly.

1.1. Neural plate induction

The embryonic organizer is not primarily involved in neural plate induction

Investigation of the earliest developmental events has focused on the amphibian embryo (*Xenopus*), the first vertebrate system for neuronal induction and axis formation. In the 1920s Mangold and Spemann identified a morphologically distinct group of mesodermal and

endodermal precursor cells (Keller, 1975), which is formed during gastrulation, as a source of neural inducing signals. If the animal cap (ectoderm from the animal hemisphere), including the Spemann organizer, is transplanted heterotopically to a region normally forming the epidermis at an early stage of development, an entire secondary axis and a fully developed secondary nervous system is induced.

When is neural induction initiated and what is the cellular source and molecular nature of the neural inducing signal(s)? Opposite to the former hypothesis that neural cell fate is determined from organizer-secreted proteins, and thus does not occur before the organizer has formed, several studies in *Xenopus* indicate that blastula-stages animal caps contain both prospective neural and epidermal cells. By late blastula-stages, the dorsal ectoderm may be predisposed to neural differentiation, and the border between the future neural and epidermal cells seem to be established before the onset of gastrulation. Consistently, studies in amniote embryos provide evidence that neural induction occurs at the blastula stage, before the organizer region has formed (Wilson and Edlund, 2001). Like the Spemann organizer in *Xenopus*, the node (in mouse and in chick) is sufficient but not necessary to induce ectopic neural cells. Results obtained from mutant mouse embryos failing to develop a functional node and node derivatives (*Hnf3beta*; Ang and Rossant, 1994) form a neural plate with initially correct AP pattern. In addition, genetical (in *MZoep* or *boz* mutants) or surgical ablation of the zebrafish organizer does not prevent the formation of the neural plate (Fekany-Lee et al., 1999; Schier et al., 1997; Shih and Fraser, 1996).

The neural inducing signals
The specific tissues or signals necessary for neural induction at late blastula stages are not completely known. The classical view of early neural patterning, provided from studies in *Xenopus,* suggests in signals emanating from the organizer, such as diffusible BMP inhibitors (chordin, noggin, follistatin), which can bind directly and antagonize BMPs to permit the generation of neural tissue. A number of experiments suggest that ectodermal cells are by default fated to become neural but this process is inhibited by BMP signaling (Wilson and Hemmati-Brivanlou, 1995; Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al. 1995). The ability of BMP signals to block neural and promote epidermal fate in early embryonic cells is conserved among vertebrates. In contrast to results obtained in *Xenopus*, however, in amniote embryos BMP inhibition is not sufficient to induce a neural fate, since a neural plate is formed in mouse mutants lacking functional Follistatin, Noggin and Chordin, (Matzuk et al., 1995; McMahon et al., 1998; Bachiller et al., 2000). Further, BMP inhibitors

alone are not sufficient to induce ectopic neural cells in epidermal ectoderm in chick embryos (e.g. Streit et al., 1998). Instead, only cells at the border region or the neural plate, which are exposed to signals promoting both epidermal and neuronal fate, are sensitive to neuronal fate suppression by BMP signaling (Streit and Stern, 1999). Results in zebrafish mutants suggest that BMP signaling is mainly involved in regulating the size of the neural plate as mutations affected in *chordin/chordino* (or ectopic expression of *bmp4*) show a reduced neural plate (Schulte-Merker et al., 1997; Barth et al., 1999). Conversely, mutations in *bmp2b/swirl* (Nguyen et al., 1998) show the opposite phenotype, an enlarged neural tube. Thus, the current view is that BMP signaling is not required for the induction of neural fate, but instead is necessary for maintaining neural character.

Other signals, distinct from BMP inhibitors, are likely required for the induction of neural fate. There is evidence for the involvement of at least two other signaling pathways, namely FGFs and Wnt (both secreted glycoproteins), in neural induction (fig.2). FGF is necessary for neural induction to proceed and to promote neural fate. Indeed, blocking FGF with FGF receptor antagonist in chick prevents neural fate while *Bmp* expression is maintained (Wilson et al., 2000). The initiation of neural induction by FGF signaling occurs before gastrulation (Streit et al., 2000). Results obtained in chick and mouse embryos further suggest that Wnts induce epidermal fate and repress neural fate by attenuating the response of epiblast cells to FGF signaling (Wilson et al., 2001; Dann et al., 2001).

Model: Interactions between Wnt, FGF and BMP signals expressed in the embryo before the onset of gastrulation generate cells of neural and epidermal fate.

A. The lack of exposure of medial epiblast cells to Wnt proteins permits FGF signaling both to repress *Bmp* and to activate an independent pathway necessary for progression to neural fate.

B. High-level Wnt signaling in lateral epiblast cells inhibits FGF pathway and permits *Bmp* expression and BMP signaling to direct cells to an epidermal fate. In the organizer Wnt, FGF and BMP antagonists are expressed which all seem to be necessary to induce neural character (in chick epidermal or extra-embryonic ectoderm).

Fig.2. Proposed signaling pathway for neural induction in the chick embryo (Lumsden and Krumlauf, 2001).

The medial part of the embryo constitutes therefore a neurogenic region, whereas cells in the lateral region are specified as cells of epidermal character before the onset of gastrulation. At

this stage the character of these cells is still flexible and can be changed if exposed to appropriate signals and may be re-instructed. At the end of gastrulation neural precursor cells no longer respond to signals inducing alternative fates and have thus committed to neural differentiation (Wilson and Edlund, 2001; Pera et al., 1999).

1.2. Common features of early neural plate patterning invertebrates

Posteriorization and anterior maintenance

Posteriorization

Transplantation experiments in *Xenopus* demonstrate the capability of the early organizer to induce a complete secondary axis; if grafted at a later stage, however, only posterior character of the neuroectoderm can be induced. It is believed that during gastrulation a two-step activation-transformation action is mediated by the involuting mesoderm: neural plate cells initially possess an anterior character, while cells of caudal character emerge later through the reprogramming of anterior cells (Nieuwkoop et al., 1952; Doniach, 1993). The signals could pass to the ectoderm either vertically from underlying cells (fig.3, white arrows), or in a planar fashion from organizer cells (fig.3, yellow arrow).
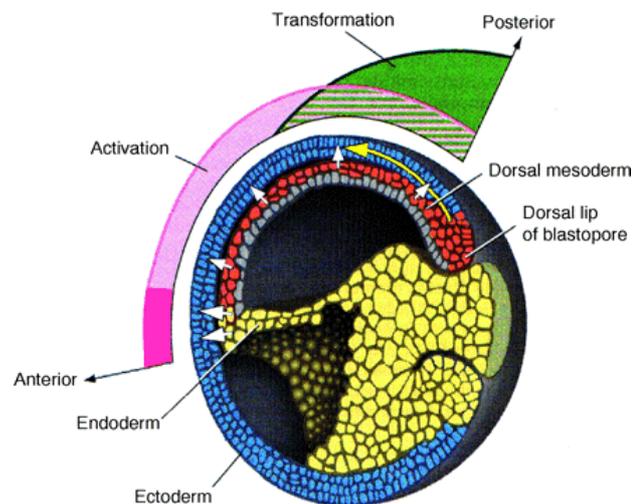
Fig.3. Acquisition of AP pattern during neural induction in *Xenopus* (Lumsden and Krumlauf, 2002).
In the late gastrula, shown in hemisection, involuted cells have reached the anterior pole of the presumptive CNS. Radial signals (white arrows) from the leading-edge endoderm (yellow) and the mesoderm (red) induce neural fate in the overlying ectoderm (blue). Forebrain (dark pink) is induced by leading-edge endoderm and mesoderm. More posterior levels of the ectoderm are activated (light pink) and transformed by a graded posteriorizing activity (green). The yellow arrow shows the route of planar signals.

Fate mapping experiments have substantiated this model by showing directly that cells expressing the forebrain marker *otx2* in the early gastrula down-regulate it by mid-gastrula

stages and instead express midbrain and hindbrain markers (Erter et al., 2001). In addition, signals from adjacent tissues, such as the non-axial mesoderm, are involved at all stages to refine neuraxial patterning (Doniach, 1993 ;Woo and Fraser, 1997). In particular, explant studies in chick and mouse implicate the paraxial mesoderm in posteriorization of the neural tube (Muhr et al., 1997; Ang and Rossant, 1993; Rowan et al. 1999). Prechordal tissues might also confer more anterior character to posterior neuroectoderm (Foley et al., 1997).

Anterior maintenance

In the mouse, grafting experiments (Beddington, 1994), indicate that additional structures than the node are necessary for the induction of anterior structures. The anterior visceral endoderm (AVE), an extraembryonic tissue, is able to induce anterior neural patterning in the overlying ectoderm before the node has formed, implicating the AVE as an organizer of anterior neuroectoderm (Thomas and Beddington, 1996; Tam and Steiner, 1999). Further, inactivation of the homeobox genes *lim1* and *otx2,* which are expressed in the AVE and the anterior mesendoderm in mouse embryos, leads to disturbed rotation of the epiblast and the embryos lack forebrain and midbrain up to the level of the anterior hindbrain (Acampora et al., 1998; Lumsden and Krumlauf, 2002). In contrast, the selective elimination of *otx2* in the neural plate but not in the AVE results in a complete neural plate with a normal induction of *pax2* in the MH, demonstrating that the AVE is necessary to permit at least the induction of a subset of MH markers (Rhinn et al., 1998). Recent results suggest that signals derived from the AVE in fact do not induce but instead protect prospective forebrain cells from posteriorizing signals (Rhinn et al., 1998; Stern, 2001).

General patterning signals

Several protein families have been implicated in establishing AP patterning in the neuroectoderm: (i) posteriorizing signals, such as retinoic acid (RA), FGFs and BMPs and (ii) anteriorizing signals, such as Wnt inhibitors. Their activities are briefly described below.

(i) In mouse and chick the posteriorizing activity of RA is demonstrated by the loss of anterior markers after treatment with RA (Ang et al., 1994; Bally-Cuif et al., 1995). Further, gain-of-function and loss-of-function experiments in *Xenopus* demonstrate a quantitative role for RA in regionalization of the neural plate, as constitutively active RA receptors reduce anterior neural tissue while dominant negative receptors expand anterior neural structures (Blumberg et al., 1997). Recently, a mutation in the RA synthesis enzyme Raldh2/*nkl* indicate the requirement for RA in zebrafish hindbrain formation as well (Begemann et al., 2001). Thus,

RA is required for posteriorizing the neural tube, likely in combination with additional signals. Indeed, gain-of-function analyses in zebrafish show that FGFs and Wnts can suppress anterior genes in an RA-independent pathway (Kudoh et al., 2002).

Likewise, FGF signaling inhibits expression of rostral neural genes and possesses caudalising effect on the early *Xenopus* and chick neural plate (Doniach, 1995; Rodriguez-Gallaro et al., 1996; Storey et al., 1998; Alvarez et al., 1998). However, transplantation experiments in chick and injection of the dominant-negative FGF-R (XFD) in zebrafish suggest that FGFs are not sufficient in themselves to induce caudal character (Muhr et al., 1999; Koshida et al., 1998; Woo and Fraser, 1997). Recent results give evidence that FGF is involved in the formation of the posterior neural region by counteracting BMP signaling within posterior neural cells by inducing the expression of secreted BMP antagonists (Koshida et al., 2002).

(ii) The encoding of anterior neural character requires Wnt inhibition. Gain-of-function experiments in frog embryos showed that Wnt signaling inhibitors, such as dnXWnt8, Frzb, Dkk1 and Cerberus, induce anterior neuroectoderm markers (Glinka et al., 1997). In addition, Wnt3 null-mutant mice lack AP neural patterning although AVE markers are expressed and correctly positioned (Liu et al., 1999). Thus, after establishment of the primary body axis, Wnts play essential roles in patterning the AP axis. Together, it is believed that negative factors secreted from anterior sources, coupled with posteriorly localized expression of Wnts leads to the establishment of a graded Wnt signal along the AP axis. Low levels of Wnt activity lead to the specification of anterior fates, high levels to posterior fate. In fact, the Wnt pathway is suggested as the potentially most critical signaling pathway in influencing early AP patterning (Yanaguchi, 2001; Kiecker and Niehrs, 2001).

1.3. Patterning refinement along AP by local signaling centers

Once early anterior patterning is induced, localized sources of various signaling molecules act as organizing centres that refine the pattern of neighbouring fields to create molecularly distinct domains. These centers (secondary organizer) are believed to act within the neural plate to refine local identities throughout the entire AP axis, mainly based on long-range signaling. So far, two main signaling centres have been identified within the embryonic neural plate. The most anterior center is the 'anterior neural ridge' (ANR), immediately adjacent to the prosencephalon and the anterior ectoderm, which is implicated to be necessary for maintaining forebrain identity (Shimamura and Rubenstein, 1997). Removal of the ANR from explants leads to a failure to express the anterior marker *Bf1*. *Fgf8* is expressed in the ANR and recombinant FGF8 protein is capable of inducing *Bf1*, suggesting that FGF8 regulates the

development of anterolateral neural plate derivatives in chick embryos (Shimamura et al., 1997). Opposing to the anteriorizing properties mediated by FGF signaling, another important and intensely studied organizing center is the 'isthmic organizer', located at the junction between the midbrain and hindbrain, which was shown to be both necessary and sufficient for the development of mesencephalic and metencephalic structures (chapter I.2).

1.4. Early AP neural plate patterning in zebrafish

<u>Zebrafish development at stages of neural plate induction and early patterning</u>

Developmental events

In the following scheme (fig.4) the early stages of zebrafish development during gastrulation, shield formation and the formation of the embryonic axis are shown.

A

Enveloping layer

Deep layer

Yolk syncitial layer

Animal pole

Ventral

Dorsal

Yolk cell

Yolk syncitial nuclei

Vegetal pole

B

blastpore

involuting cells

C

Animal pole

Epiblast

Hypoblast (mesendoderm)

Shield

Ventral

Dorsal

Mesoderm inducing signals

Mesoderm and dorsal inducing signals

Vegetal pole

C'

Cells ingress

Hypoblast

Envelope layer

Epiblast

Involuting cells

Non-involuting cells

YSL

Yolk granule

D   D'

Fig.4. Early structures and cell movements in the zebrafish embryo during gastrulation.
(after Gilbert, 6th edition: A, C, C', D, D' after Driever, 1995; Langeland and Kimerl, 1997; B after Gilbert)
A. In zebrafish, the yolk syncitial layer (YSL) is an extraembrionic territory, derived from deep marginal blastoderm cells which collapse and release their nuclei into underlying yolk cell at mid-blastula transition. B. After cleavage, blastoderm cells become motile and move over the surface of the yolk to envelop it completely (epiboly of blastoderm cells over the yolk). C. The embryonic tissue is then composed of an outer layer, the epiblast and an inner layer, the hypoblast. C'. During gastrulation cells from the marginal zone involute and stream to the animal pole. Because of involution and converging anteriorly and dorsally the embryonic shield is formed at the future dorsal side of the embryo. D. With involution, convergence and extension movements, the endodermal and mesodermal cells move anteriorly, narrowing along the dorsal midline, extending to the animal pole and forming the AP-axis. D'. The entire neural plate (blue) is underlain by axial mesoderm (red) that internalises during gastrulation. Anteriorly, this mesoderm forms the prechordal plate, which produces secreted signals important for induction and patterning of the anterior presumptive brain. Posteriorly the axial mesoderm is called notochord, extending to the tip of the tail.

Zebrafish neurulation process

The neuroectoderm in zebrafish embryos derive, like in other vertebrates, from the dorsal epiblast while cells at the marginal zone (adjacent to the yolk) give rise to the future mesoderm and endoderm. During gastrulation, neural progenitors near the midline move anteriorly toward the animal pole. The more lateral progenitors fill in behind them by moving toward the midline, thereby adopting their appropriate AP positions along the elongating neuraxis (fig.5A). This process, which results in the formation of the neural keel (fig.5B), is equivalent to the more familiar neurulation movements to form the neural tube in other vertebrate embryos. In general, the lateral edges (fig.5, indicated yellow) of the neural plate form dorsal CNS, the medial regions (fig.5, indicated red) become ventral CNS (Goette, 1874; Schmitz et al., 1992). There are two major ways to form the neural tube. In "primary neurulation", which takes place in amniotes, the ectoderm invaginates and pinches off from the surface to form a hollow tube (Schoenwolf, 1991b). In "secondary neurulation", for instance in zebrafish, the

neural tube arises from a solid cord (fig.5C) of cells that sink into the embryo and subsequently hollows out (cavitation) to form a neural tube (Papan and Campos-Ortega, 1994).

A neural plate

B neural keel

C neural rod

midline
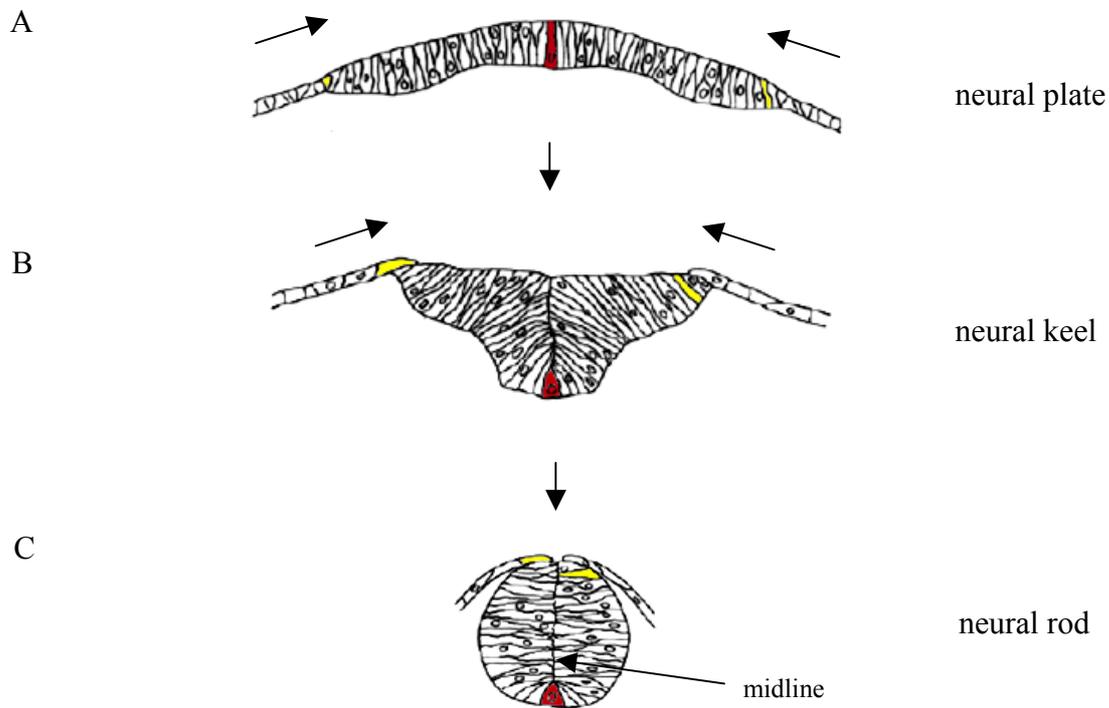
Fig.5. Early morphogenesis of the neural primordium by secondary neurulation.
(after ZFIN/Kimmel et al., 1995, redrawn from Papan and Campos-Ortega, 1994)
Diagramatic transverse sections. The neural plate develops into the neural keel by infolding at the midline. The keel in turn rounds into the cylindrical neural rod. Examples for lateral plate cells are indicated in yellow, medial plate cells are marked red.

Fate map

Neuraxial patterning is thought to be a progressive process. Gastrulation begins at 30% epiboly (5h). The expression of genes regionally restricted to specific subdomains of the presumptive brain is initiated between 80% and 100% epiboly (*pax6, her5, pax2.1, krox20*). They display a predictable organization that reflects the future AP and dorsoventral (DV) order of the central nervous system, though the domains are still overlapping (see fate map in fig.6). The 10h zebrafish neural fate map resembles the neurula fate map of *Xenopus* (Eagleson and Harris, 1990) and chick (Couly and Le Douarin, 1988), in that the brain regions are aligned in the expected AP order of the eventual neural tube, which roughly contribute to the future major brain subdivision: the presumptive forebrain, midbrain and hindbrain. These domains are further reinforced and refined by diverse and locally acting mechanisms and become aligned within the primary axes of the embryo as neurulation proceeds (Woo and Fraser, 1995).
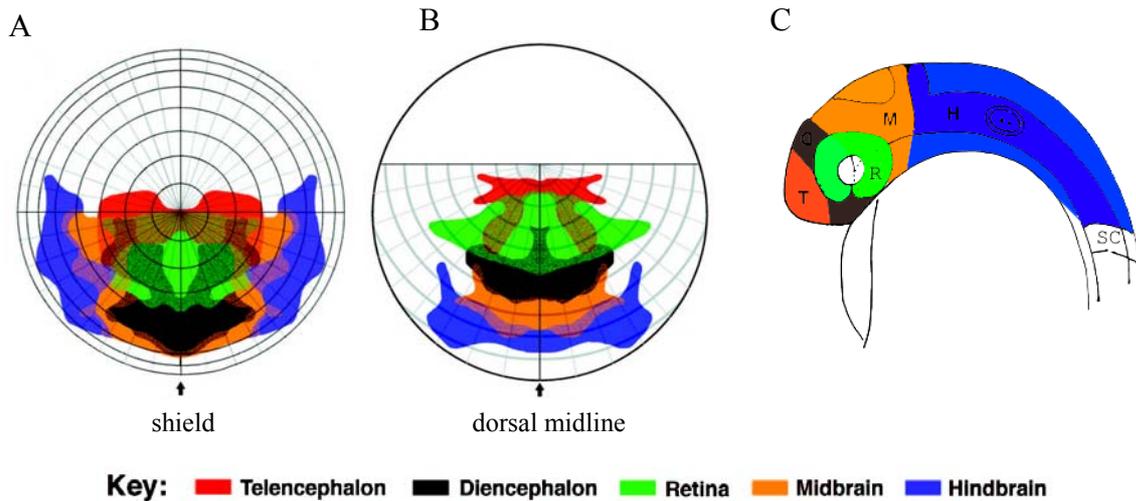
Fig.5.: Organisation of the zebrafish central nervous system (Woo and Fraser, 1997).
Fate map at the beginning (A, 6h) and end of gastrulation (B, 10h), respectively. Domains occupied by progenitors of each brain subdivisions are coded with their representative colours, as shown in the key. Areas of overlap between any two domains are shaded with a mixture of their respective colors. C. Zebrafish embryo at 24h, with morphologically apparent brain region.

Early AP patterning: comparison with other vertebrates

Recent studies in zebrafish have shown that the mechanisms controlling axis formation are largely conserved with amniotes. Similarities and differences in tissue and signals during embryonic development will be described in the following paragraph, with focus on the nervous system.

Like in other vertebrates, neural induction and patterning begin before gastrulation

Consistent with results in other vertebrates, transplantation experiments in zebrafish demonstrate the organizer activity of the embryonic shield. Shield ablation (and adjacent marginal tissue) leads to a loss of axial mesendoderm and floor plate and to defects in the formation of ventral neuroectoderm, but does not significantly alter AP patterning of the neuroectoderm, suggesting that the organizer acts to induce and to pattern the embryonic axis before the embryologically visible shield has formed. Along this line, most organizer genes that inhibit Wnt and BMP signaling, such as *gsc*, *lim1*, *chordino*, and *dickkopf* are expressed before gastrulation begins. A similar conclusion is reached for neural AP patterning. Although perturbation of BMP signaling in live embryos changes the balance between neuroectoderm and epidermis specification and affects DV patterning of the neural plate, the global AP neural pattern is not disturbed (Barth et al., 1999; Nikaido et al., 1999). Further, organizer transplants do not affect AP pattern but instead induce neuroectoderm with an identity that depends on the location of the transplant (Koshida et al., 1998), suggesting that neural AP patterning does not

depend on organizer activity but instead is determined by pre-existing informations before gastrulation or by signals outside the organizer.

Like in other vertebrates, the organizer is progressively posteriorizing

Transplantation experiments in which deep (*gsc*-positive) and superficial regions (*flh*-positive) of the shield were tested (Saúde et al., 2000), demonstrate that deep shield tissue is capable of inducing secondary axes consisting entirely of head structures, while superficial shield tissue induced axes lacking the most anterior structures. Thus, like in *Xenopus*, the zebrafish organizer is regionalized.

In zebrafish, posteriorizing structures include the embryonic margin and PCP

Transplantation of mesendodermal tissue from the lateral or ventral germ ring into the animal pole of an early gastrula embryo induces differentiation of hindbrain structures and expression of the hindbrain marker *krx20* in the forebrain (Woo and Fraser, 1997). In addition, signals emanating from the prechordal plate might transform early neural ectoderm from an anterior to a posterior fate (Koshida et al. 1998). The margin and PCP are equivalent to the amniote non-axial mesoderm, endoderm and PCP, respectively, which exert similar functions (e.g. Woo and Fraser, 1997; Pera and Kessel, 1997; Foley et al., 1997; Camus et al., 2000).

In zebrafish, anterior maintenance might be accomplished by the YSL

The zebrafish YSL (fig.4A) could exert similar function than the chick hypoblast or mouse AVE. Indeed, the genes *hex* and *dkk1* (Ho et al., 1999; Hashimoto et al., 2000) are expressed in the YSL, and their corresponding homologs in mouse embryos are expressed in the AVE. Furthermore, overexpression of *hex* leads to downregulation of *bmp2b* and *wnt8* expression and expansion of *chordin*, indicating a role in regulating early embryonic patterning (Ho et al., 1999).

Comparable mechanisms and factors

Among the candidate poteriorizing factors are Wnts, together with FGFs, RA and the Nodals, which have been shown to be able to posteriorize the neuroectoderm at the expense of more anterior neural plate fates. Analyses of the zebrafish mutants *Tcf3/hdl* and *boz* demonstrate, like for amniotes, an important role of Wnt signaling inhibitors for anterior neural plate development (Kim et al., 2000; Fekany-Lee et al., 2000). In addition to roles in promoting posterior neural fates and regulating development of tissue adjacent to the MHB, the Wnt

pathway may also be involved in regional patterning within the anterior neural plate, emanating from the most anterior region of the neural plate.

Local neural plate organizers

Like in other vertebrates, the zebrafish MHB plays a prominent role in shaping the development of the mes- and metencephalon (chapter II.2). An equivalent of the mouse ANR to date was not demonstrated, but the first neural plate cell row at the end of gastrulation, the anterior neural border (ANB) was identified as having organizing capacities involved in maintenance of the telencephalon (Houart et al., 1998). Recently, one more center with organizer capability was identified in zebrafish, which is located in rhombomere 4 (r4) of the hindbrain (Maves et al., 2002). It was shown that r4 expresses *fgf8* and is necessary for the development of r3 and r5.

A refined role of Wnt inhibition in zebrafish anterior NP patterning

Evidence for implicating the Wnt pathway in zebrafish head development comes from several studies, for instance mutants affected in the Wnt signaling repressor *Tcf3/hdl* (Kim et al., 2000) or affected in *axin1/mbl* (Heisenberg et al., 2001; van de Water et al., 2001), which leads to a higher level of Wnt signaling, causing a deletion of anterior structures. Wnt signaling is thought to be involved in two steps: early Wnt signaling (pre-MBT) mediates the early dorsalizing signal (Moon et al., 1997), required for initiation of the dorsal axis formation, whereas a later phase after MBT appears to be involved in AP patterning of the neural axis (Kim et al., 2002). Recent results show that the rostral and and caudal part of the zebrafish anterior brain are negatively and positively regulated, respectively, by Wnt signals through the Fz8a receptor. Furthermore, different thresholds of Fz8a-mediated Wnt8 signaling in the anterior neural plate during late gastrulation is crucial for the proper patterning of the posterior diencephalon and midbrain (Kim et al., 2002). Indeed, activation of Wnt activity in early gastrula can transform the whole forebrain territory into midbrain while increased Wnt activity after late gastrula stage has limited posteriorizing activity, which can transform rostral fate into more caudal fate only within the anterior brain region (posterior diencephalon expansion). This is in agreement with *mbl/axin* mutants, in which the activity of Axin1, a negative regulator of Wnt signaling is affected and which shows a transformation of telencephalic to a more posterior diencephalic fate, likely caused by overexpression of the Wnt pathway within the neural plate (Heisenberg et al., 2001; van de Water et al., 2001). The induction and partitioning of the anterior forebrain might be accomplished through graded modulation of

Wnt signaling within the anterior neural plate (Houart et al., 2002) (fig.6). Recently, a frizzled-related protein (Tlc) was identified in the ANB, which can bind to and inhibit Wnt proteins (Wang et al., 1997) and which mimics the local signaling properties of the ANB (Houart et al., 2001). In contrast, high levels of Wnt signaling anteriorly, for instance by expressing Wnt proteins in the ANB, show the opposite effect of Tlc, namely inhibiting telencephalic and promoting midbrain-specific gene expression, similar to the results obtained with abrogation of Tlc function. Further, the size of the prospective telencephalon relative to other forebrain domains can be changed by varying the levels of Wnt signaling (by *tlc* expression level) within the anterior neural plate, suggesting that local Wnt agonist/antagonist interactions control the induction and extent of the prospective anterior forebrain. Within the neural plate the source of Wnt inhibitors anteriorly is likely the ANB and the main source of Wnt proteins is the MH, whereas Wnt appears to be involved in patterning the rostral part of the MH.
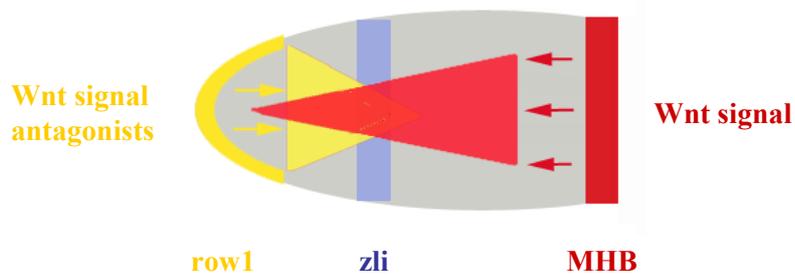
Fig. 6. Conflicting Wnt influences within the anterior neural plate.
Gradients of Wnt (red) and Wnt signaling antagonist (yellow) are established in a gradient along the anterior neural plate. Wnt and Wnt antagonist, emanating from the ANB and MHB, respectively, regulate the induction and extent of the forebrain by local interaction.

2. The midbrain-hindbrain domain

Below, I summarize the current understanding of the mechanisms and factors underlying MH development and detail some major unanswered questions, which were the focus of my Ph.D. work. For recent and more complete reviews, see Lumsden and Krumlauf, 2001; Wurst and Bally-Cuif, 2001; Rhinn and Brand, 2001; Martinez, 2001; Simeone, 2000; Liu and Joyner, 2001.

2.1. Position and functional derivatives

Early subdivisions of the prospective brain

At the end of gastrulation, a series of vesicles form along the anterior neural tube. The presumptive brain can be divided along AP into three domains (fig.7): the forebrain or prosencephalon (blue), which can be divided in six prosomeres (with P1 being the most caudal), the midbrain or mesencephalon (red) and the hindbrain or rhombencephalon (orange and yellow), which is divided in 7-8 rhombomeres. The most anterior part of the hindbrain (r1 and r2) is called metencephalon (orange), rhombomeres r3-r8 myelencephalon (yellow). The MH comprises the mes- and anterior metencephalon (red and orange), that are separated by the isthmus or MHB.

Fig.7. Main regions in the developing brain (modified from Gilbert, 6th edition, after Chiang et al., 1996).

MH derivatives

Once the presumptive brain has formed early subdivision, further regionalization and specification of the future brain regions continue, finally giving rise to the adult brain structures (fig.8). The presumptive MH forms the following structures: (i) the dorsal part of the mesencephalon gives rise the optic tectum and the ventral part forms the tegmentum, mainly controlling sensory input and motor function; (ii) the dorsal part of the metencephalon forms the cerebellum, the ventral part the pons, mainly controlling sensory and behaviour processes.

A   B   C
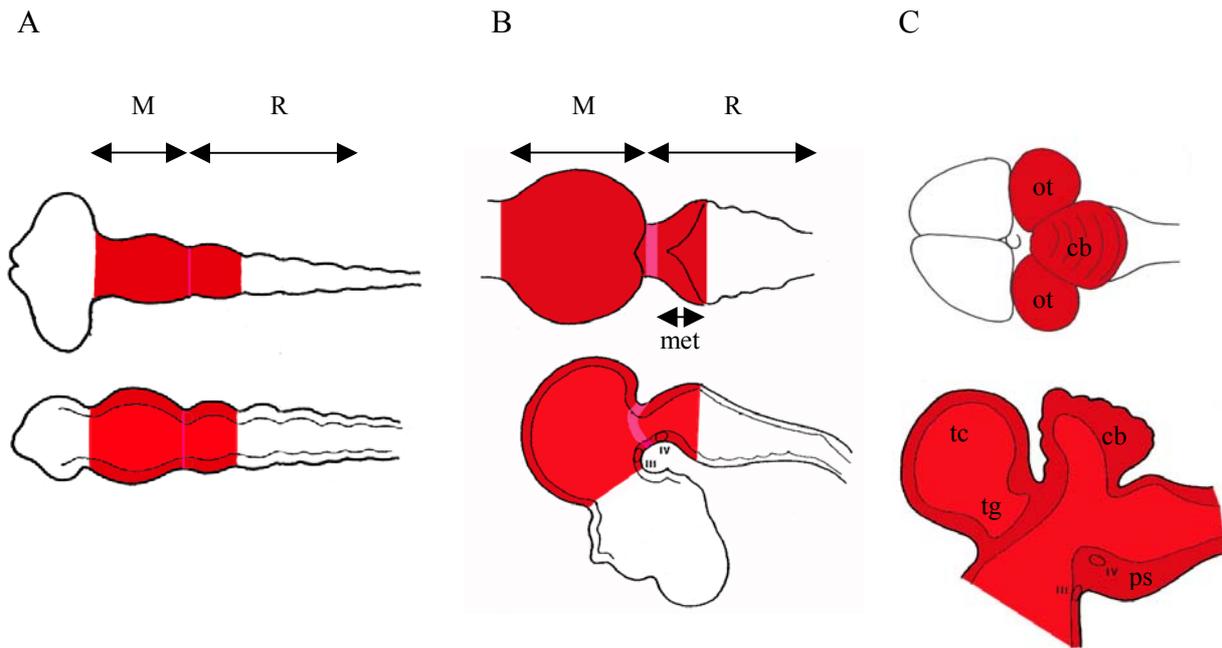
Fig.8. The presumpitve MH and its derivatives in the adult brain (after Vaage, 1969; 1973). Schematic drawings of dorsal (upper row) and lateral or sagittal view (lower row) of the presumptive brain at different developmental stages (A, B) and the resulting adult brain structures (C) in chick. The MH is highlighted in red, the MHB in pink. The arrows indicate the extension of the mesencephalon (M), the rhombencephalon (R), and the metencephalon (met), respectively. cb, cerebellum; tc, tectum; tg, tegmentum; ot, optic tectum; ps, pons; III, occulomotor nerve; IV, trochlear nerve

2.2. The MH follows an atypical mode of development

One clear difference of MH development compared to other prospective brain regions is that it is not built by segmentation but responds to the activity of the IsO, as desribed below.

Other brain territories develop at least in part by segmentation

The formation of the hindbrain with its metameric character and subdivisions into rhombomeres, also revealed by the segmental expression of genes of the *hox* family, depends on segmentation processes. The formation of the forebrain is not fully understood and limited to gene expression patterns and descriptive molecular studies, although there is evidence that the forebrain might share similar mechanisms than the hindbrain. This is true at least for the diencephalon, which is divided in several prosomeres (Lumsden and Krumlauf, 2001). One true compartment without cell mixing is the zona limitans intrathalamica (zli), a boundary between P1 and P2 and that expresses *shh*. Another gene, *Lunatic fringe* (*L-fng*) is expressed in domains flanking the compartment, delineating anterior and posterior borders of the zli (Zeltser et al., 2001).

MH development occurs in a concerted fashion in response to an organizer

The MH does not exhibit internal boundaries to cell migration (Jungbluth et al., 2001), in particular at the MHB, although this border is characterized by a transition in gene expression (*otx2* anteriorly versus *gbx* posteriorly). The order and coherence of the MH are imposed by the activity of a local organizing center, the IsO.

The IsO was first identified in the avian embryo by transplantation experiments at the 10-14 somite stage, in which tissue including the isthmus was placed ectopically to rostral or caudal host territories within the neural tube. The graft induced tectal development in the caudal diencephalon, and cerebellar development in the dorsal hindbrain in the surrounding tissue at the expense of the original diencephalic or rhombencephalic tissue. Conversely, prosencephalic tissue grafted to the isthmus acquired mes-metencephalic fate and gene expression pattern (fig.9).
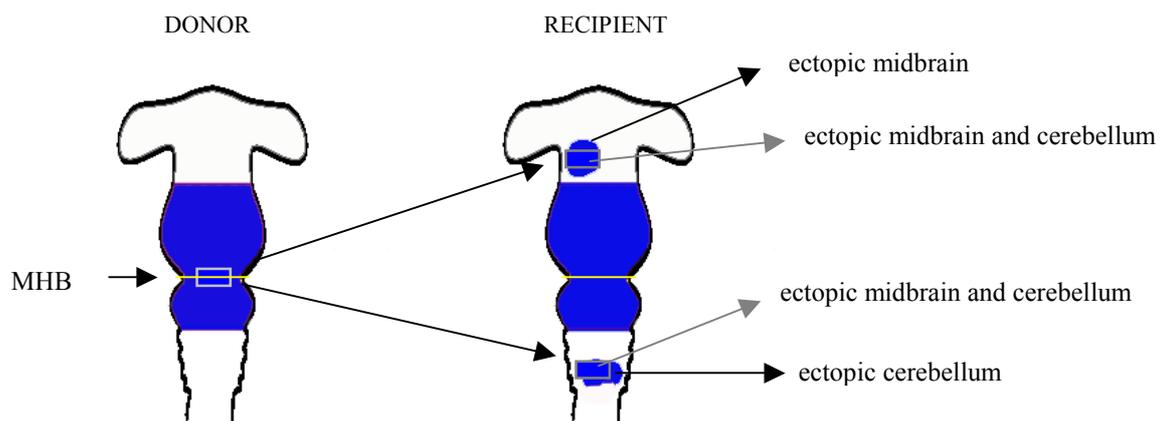
Fig.9. Transplants containing the IsO can induce ectopic MH structures in adjacent brain regions.
Schematic representation of chick transplantation experiments (Liu and Joyner, 2001).
Transplants of the ishtmic tissue (MHB) can induce ectopic expression of MH markers, such as *En2* (blue) as well as ectopic MH development.

Results obtained after bilateral ablation of all or part of the isthmus were also consistent with a role for this structure in the development of the MH. Complete removal of the isthmus led to a loss of the entire midbrain and hindbrain (Wurst and Bally-Cuif, 2001). These experiments have been extended to mouse and zebrafish embryos, indicating that the mes-metencephalic junction is likely to be crucial for neural tube patterning in all vertebrates. Together, these results demonstrate the organizer and growth-promoting capacities of the IsO, and also that the competence of the hindbrain to respond to isthmic signals differs from that of tissue of the posterior forebrain and midbrain, pointing to the importance of additional regional cues.

Further transplantation experiments in chick demonstrate that the IsO controls also the establishment of the rostrocaudal polarity of the MH. Inverted rostral mesencephalic tissue adjusts its polarity according to the new environment. In contrast, when transplanted tissue includes the caudal part of the mesencephalic vesicle (precursors of the isthmic nuclei and cerebellum), it not only maintains its own polarity but can induce the adjacent, more anterior host diencephalic tissue to form caudal mesencephalic structures (fig.10). For more detailed informations see reviews Nakamura et al., 2001; Liu and Joyner, 2001; Joyner et al., 2000; Marin and Puelles, 1994.
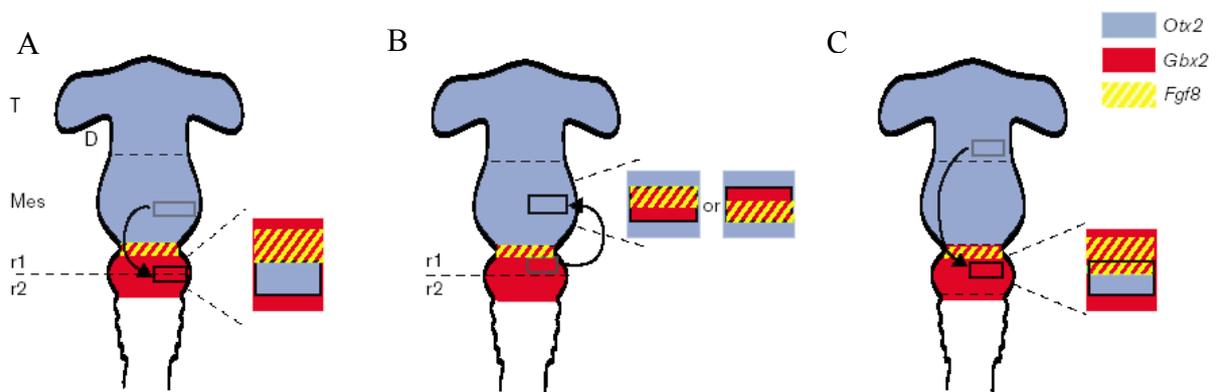
Fig.10. The juxtaposition of mes- and metencephalic structures can induce *Fgf8* expression.
Schematic representation of chick-quail transplantation experiments (Joyner et al., 2000).
A. Midbrain to r1; B. r1 in midbrain; C. caudal diencephalon to r1.The graft taken from the quail donor embryo is represented as grey rectangles and its new ectopic position in a chick host embryo as black rectangles. These transplantations result in a new juxtaposition of *Otx2*+ (grey) and *Gbx2*+ (red) tissues and an induction of *Fgf8* expression (yellow) at the new *Otx2–Gbx2* border 24 hours after the transplantation (shown on the right of each panel). Note that in C. interactions between graft and host result in relocation of this new *Otx2–Gbx2* border within the graft.

2.3. Molecular bases of MH development and IsO activity

Several genes are known to be expressed in a non-ubiquitous expression fashion within the MH, and could mediate the positioning of the IsO and its organizer activities. The spatio-temporal expression profile of these genes and their function are largely conserved throughout vertebrates, although subtle differences exist between species, mostly in the onset of gene expression. Two categories of genes can be distinguished: (i) those expressed at the MHB only (IsO factors), and (ii) those expressed throughout the MH, albeit in a graded manner (MH markers). In a simplistic view, those genes expressed across the entire MH might primarily account for MH identity as a whole, whereas genes expressed asymmetrically within this domain or genes restricted to the MHB might control primarily IsO positioning and/or activity.

MH markers

One of the earliest genes to appear in the presumptive MH encodes the transcription factor Pax2, expressed across the Otx2/Gbx2 boundary. Slightly later *Pax5* is induced in a domain similar to *Pax2*, followed by *En* genes, which are expressed in a graded manner across the entire presumptive MH region (anteriorly decreasing through the mesencephalon and posteriorly through the cerebellum) (fig.11). Gene inactivation and mutation experiments demonstrated that En1, En2, Pax2, Pax5 are necessary, in a redundant manner, for the development of the entire MH, and play a role in early MH specification and polarity.
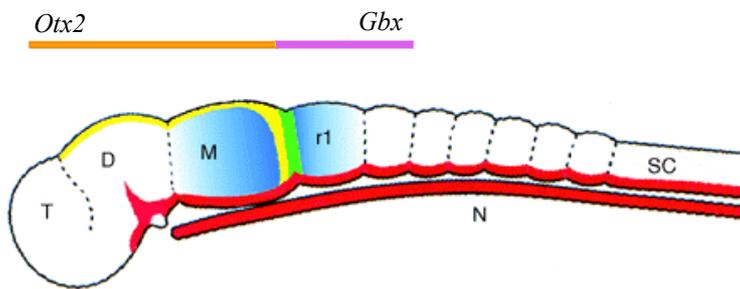
Fig. 11. Dynamics of gene expression patterns at the MHB within the neural tube.
Early midbrain patterning (modified after Lumsden and Krumlauf, 2002).
In an early neural tube stage embryo, *Fgf-8* (green) is expressed in a ring of cells at the isthmus, the constriction between the mesencephalic vesicle (M), and rhombomere 1 (r1). *Wnt-1* (yellow) is expressed in a ring of cells immediately rostral to *Fgf-8* and along the dorsal midline. Both *En-1* and *En-2* (blue) are expressed in gradients that decrease anteriorly and posterior the isthmus. Sonic hedgehog (*Shh*) expression at the ventral midline, is shown in red. T, telencephalon; D, diencephalon; SC, spinal cord; N: notochord. *Otx2* and *Gbx1* are indicated in orange and pink, respectively.

IsO markers and the control of their expression

The glycoprotein Wnt1 is expressed in the presumptive midbrain region, restricted to the Otx2-positive domain, and later to a ring of cells that lies just anterior to the isthmus. Another secreted factor, FGF8, is expressed slightly later. FGF8 starts to be expressed broadly in the Gbx2-positive domain, but is later refined to a ring immediately posterior to Wnt-1 expression. It is believed that the boundary between the midbrain and hindbrain is roughly positioned during late gastrulation and is progressively refined during early somitogenesis. During gastrulation, the two homeodomain proteins Otx2 and Gbx2 (Gbx1 in zebrafish) are expressed in exclusive domains that lie anterior and posterior to the future midbrain-hindbrain junction. At this stage the expression of both genes is still partially overlapping, but will form a sharp boundary at a slightly later stage.

Recent findings demonstrate that the interface between *Otx2* and *Gbx2* expression zones triggers the formation and positioning of the IsO, as revealed by Wnt1 and Fgf8 expression (Broccoli et al., 1999; Millet et al., 1999). The regulation of *Otx2* and *Gbx2* itself, as well as control of Wnt1 or FGF8, are so far only partially understood. In particular, the induction of expression of both *Wnt1* and *Fgf8* are primarily independent of the functions of Gbx2 and Otx2 (Simeone, 2000). For an overview see fig.12.
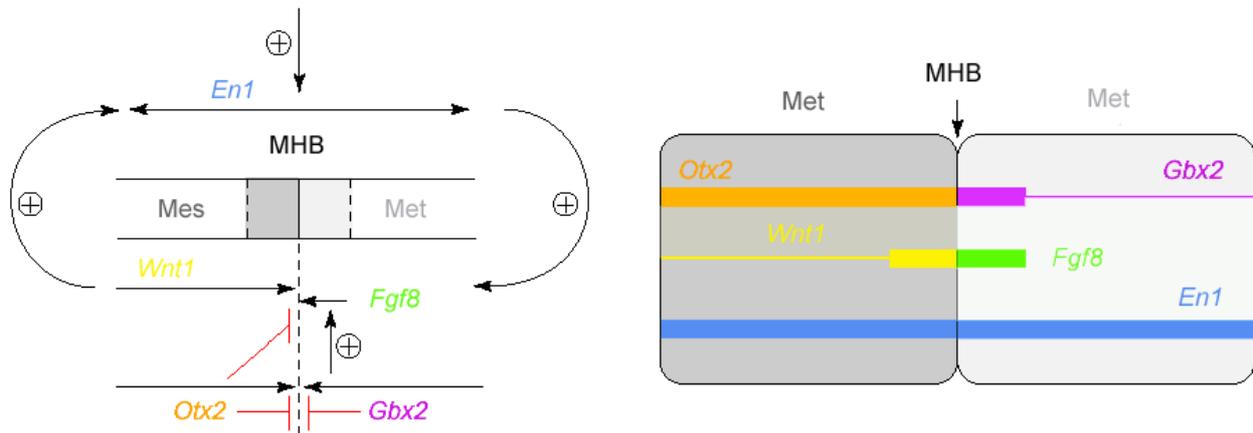
Fig. 12. Genetic cascade that establishes the isthmic organizer (Simeone, 2000).
A signal directed from the anterior notochord to the overlying neuroectoderm activates *En1* expression in the mesencephalon (Mes) and metencephalon (Met). At the same time as *En1* is expressed, *Wnt1* is activated in the Mes and maintains *En1* expression, which, in turn, might be required for *Fgf8* expression in the anterior *Gbx2* domain. Once induced, the early *Fgf8* domain becomes restricted to the Met side of the MHB via a reciprocal negative (red bars) interaction with *Otx2* and a positive interaction with *Gbx2*. At a later stage, the *Otx2*, *Wnt1*, *Gbx2*, *Fgf8* and *En1* domains of expression define a molecular code centred on the MHB and which appears to be maintained by positive and negative genetic interactions. Positive interactions between *Fgf8*, *En1*, *Wnt1* and *Gbx2* maintain their own expression, whereas negative reciprocal interactions between *Otx2* and *Gbx2* or *Fgf8* maintain a sharp *Otx2* posterior border at the mesencephalic side of the MHB. Thick lines indicate expression across most or all of the neural tube and thin lines represent more-restricted expression domains.

The role of the IsO factors FGF8 and Wnt1: the MH maintenance loop

Complete or partial knockout of *Wnt1* or *Fgf8* leads to the gradual disappearance of both mesencephalic and metencephalic structures, indicating that these genes might act to control the maintenance or activity of the IsO. Wnt1 and FGF8 might mediate the long-range organizing activity of the IsO, which permits the induction and maintenance of polarized mesencephalic and metencephalic fates, as demonstrated in ectopic transplants in chick.

Only FGF8 was directly shown to have partially midbrain-inducing and midbrain-polarizing ability (Crossley et al., 1996; Martinez et al., 1999) suggesting that it might be an important mediator of IsO organizing activity. When a bead coated with recombinant FGF8 is implanted in the posterior diencephalon of chick embryos, the expression of *Wnt1*, *Fgf8* and *En2* is

induced in the surrounding cells. These cells later display the character of a complete ectopic midbrain, whose AP polarity is reversed with respect to that of the host midbrain. However, *in vivo*, *Fgf8* is normally expressed after the onset of *Pax* and *En* expression, which suggest that *Fgf8* does not induce but rather maintains IsO activity after its formation by activating the positive feed-back loop that involves *Fgf8*, *Wnt1*, *Pax* and *En*.

If FGF8 is ectopically expressed in the hindbrain, it appears to be insufficient for inducing *En2* expression or cerebellar character in the hindbrain, implicating that the regional competence is an important factor for an inductive effect of FGF8.

The role of Wnt1 remains unclear as there are contradictory results in different genetic manipulation experiments. So far, one hypothesis on the function of Wnt1 on MH development is based on misexpression experiments of *Wnt1* in other neural tube domains than the MH. Wnt1 might regulate primarily regional cell proliferation and midbrain size rather than controlling size and proliferation in the entire MH. In addition, there is indication that *Wnt1* is involved in later processes, such as cell-specification choices at the IsO. More generally, *Wnt1* might maintain cells at the MHB in a proliferate state, keeping them competent to respond to other local factor that control cell specification.

2.4. Major unanswered questions of MH development

The unanswered question of MH induction

Several mutations are known that affect MH development, but all of them have an effect during the maintenance phase, without impairing MH induction, as the initiation of expression of MH markers is not affected. In addition, recent results show that the expression of MH genes is initially induced independently of the IsO (Martinez-Barbera et al., 2001), suggesting that earlier processes are required for the initial induction of the MH. Vertical signaling from the axial mesoderm organizer has been discussed as a primary source of signals, which induces and patterns the pre-specified dorsal ectoderm (Streit et al., 2000). Still, it remains unclear if and to which extent vertical signaling contributes to MH induction.

Neurogenesis

Neurogenesis in the MH shows a non-ubiquitous profile. At the position of the prospective MHB an undifferentiated stripe, the "intervening zone" (IZ), separates the neural differentiation clusters of the forebrain and the midbrain. In mice doubly mutant for *Hes1* and *Hes3*, the IsO cells prematurely differentiate into neurons, demonstrating that Hes1 and Hes3

are involved in IZ maintenance (Hirata et al., 2001). However, the molecular control of IZ formation remains unknown.

2.5. Formation of the MH in zebrafish

Gene expression

By the end of gastrulation, the MH precursors occupy v-shaped domains in the dorsal neuroectoderm. Among the earliest genes expressed in the presumptive MH are, in order of appearance *her5*, *pax2.1, wnt1* in the midbrain domain and *fgf8* in the immediately abutting anterior hindbrain domain at late gastrulation. The factors that induce the expression of these MH markers are not yet identified (fig.12).

Induction   Maintenance
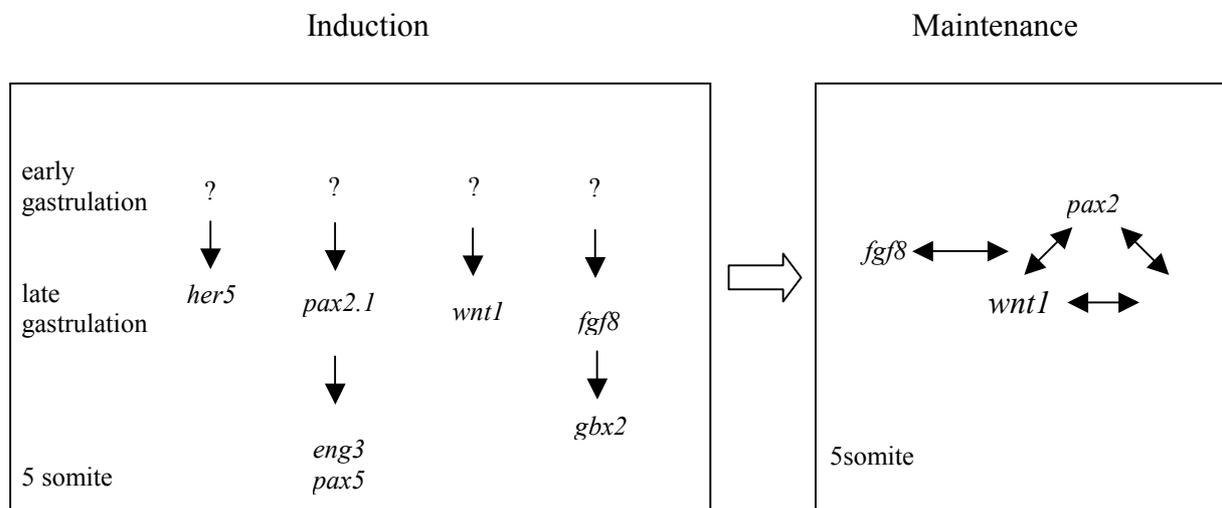
Fig.13. Gene expression in the zebrafish neuroectoderm during early MH development.
The primary signals for induction of the known MH genes, which are at this early stage independent from each other, are mostly unknown. The expression of *Fgf8*, *Pax2*, *Wnt1* and *En* becomes dependent on each other at about the 5-10 somites stage (maintenance).

The MH maintenance process in the zebrafish embryo

Different mutant lines exist with discrete lesions in the MH, allowing to better understand the molecular bases of MH development. *No-isthmus* (*noi*) mutants, disrupted in the function of Pax2.1, have no MHB, tectum and cerebellum. During somitogenesis, the target genes of *pax2.1*, *eng2*, *eng3*, fail to be activated, however *her5, wnt1* and *fgf8* are induced normally. The expression of all MH markers is down-regulated after the 5-7-somite stage (Lun and Brand, 1998).

Acerebellar (*ace*) mutants, deficient in the function of FGF8, lack the MHB and cerebellum (dorsal structures of MH). The expression of *pax2.1* and its target genes, *her5* and *wnt1*, are

normally activated, but are later downregulated (Reifers et al., 1998). Similarly, *fgf8* and *pax2.1* are not affected in a *wnt1* deletion mutant (cited in Wilson et al., 2002), and inactivation of *eng2* and *eng3* by the morpholino technology initially permits the induction of other MH markers but later leads to a severe phenotype accompanied with a complete loss of the midbrain (Scholpp and Brand, 2001). Together, these results suggest that independent signaling pathways are involved in the initiation of expression of the different early MH markers (thus in MH induction), while at later stages, these genes come to depend on each other's expression. In sum, the MH maintenance loop in the zebrafish, which is thought to start at about the 5-somite stage, appears to be similar to other vertebrates.

Factors involved in MH induction in the zebrafish

Timing

 Cell-transplantation experiments indicate that cells from the presumptive midbrain region acquired regional specificity along the AP axis as early as the 55% epiboly (Miyagawa et al., 1996).

 Factors so far isolated

The permissive factor Pou2 is considered to be an ortholog of Oct3/4 in mice, which is involved in controlling stem cell and germ cell differentiation (Burgess et al., 2002). In *spg/pou2* mutants, early development of the MHB is disrupted, further it fails to properly form all of the anterior hindbrain up to rhombomeres 4/5, revealed by an abnormal expression of genes specific to the MH. In contrast, the expression of *otx2* and *gbx1*, whose expression domains meet at the future MHB, is not affected by the disruption of *pou2* protein. Then Pou2 and FGF8 together are necessary to activate *gbx2* and other genes expressed in that hindbrain domain (*spry4*, *fkd3*). Further, Pou2 is thought to control regional competence for FGF8, as injection experiments and Fgf8-soaked beads have revealed that *spg/pou2* mutants are insensitive to FGF8 specifically in the presumptive hindbrain region (Reim and Brand, 2002; Lun and Brand, 1998)

Factors not isolated in other vertebrates

The basic helix-loop-helix transcription factor Her5 in zebrafish, a member of the *Drosophila* Hairy/E(spl) family, has no known homolog in other vertebrates. It is the first gene that appears to be expressed specifically in the MH at 70% epiboly. At later stages it is restricted to the MHB until larval stages, and continues to be expressed in some cells in the midbrain at

adulthood (see chapter II.4., and Chapouton, P., Tallafuß, A. and Bally-Cuif, L., unpublished). As shown in chapter II.5. it is an important regulator of neurogenesis in the MH region.

Instructive factors

So far, the early factors that actively initiate MH development are not known. One major focus of this work was to identify early factors in the zebrafish embryo, either emanating and acting within in the neuroectoderm (planar) or originating from adjacent tissues (vertical), involved in the induction of the MH development.

3. Neurogenesis and bHLH factors

For more detailed reviews see Bertrand et al., 2002; Bally-Cuif and Hammerschmidt, 2003.

Both, neural induction and the initiation of neurogenesis are involved in the partititioning of regional and neural subdivisions in the vertebrate brain; but how is brain morphogenesis related to the arrangements of the earliest neurons? The neuroectoderm is generated during gastrulation and initially consists of undifferentiated dividing cells (prepattern). During development, these cells exit the cell cycle and undergo differentiation to generate the neurons and glia that populate the adult nervous system. This process is temporally controlled so that differentiated cells are generated over a period of time during which other cells continue dividing to build up a large population of progenitor cells to ensure that the final number of neurons and glia can be formed (Chalmers et al., 2002).

3.1. Delimiting neurogenesis sites within the vertebrate NP

3.1.a. Neurogenesis in vertebrates occurs in proneural clusters

Following neural induction, early AP and dorsoventral patterning mechanisms define a reiterated pattern of clusters in the neuroectoderm where neurogenesis is going on, called proneural clusters. Thus, in the vertebrate nervous system neuroblasts are not established in a homogeneous gradient across the neural tube but instead arise in small clusters in a disjoined spatiotemporal pattern.

Stereotyped location of the proneural clusters

The early clusters are present bilaterally, and in the brain they lie near the center of each neuromere in the basal plate (fig.14A). To identify neurons at early stages of differentiation, the enzyme acetylcholinesterase (AchE) (Hannemann and Westerfield, 1989; Wilson et al.,

1990) was used in several studies, showing that this pattern is not random but occurs at specific and identical sites in all vertebrates. Among the earliest neural clusters observed in all vertebrates is the ventrocaudal cluster (vcc), positioned basally at the diencephalic-mesencephalic junction. vcc neurons are the first to send axons caudally, forming a major pathway called the medial longitudinal fascicle (MLF). Slightly later, neurons located more rostrally in the vcc send axons dorsally to form the posterior commissure (fig. 14B) (Kimmel, 1993; Ross et al., 1992). Caudally to this prominent cluster, a stripe of delayed differentiation (intervening zone, IZ), located at the level of the MHB, separates midbrain and anterior hindbrain neurons, leaving an undifferentiated gap between the vcc and presumptive neurons of rhombomere 2 (Geling et al., 2003). This pattern appears to be a common feature during vertebrate neurogenesis (Vaage, 1969; Bally-Cuif et al., 1993; Wullimann and Knipp, 2000), and is likely a zone of extensive growth.
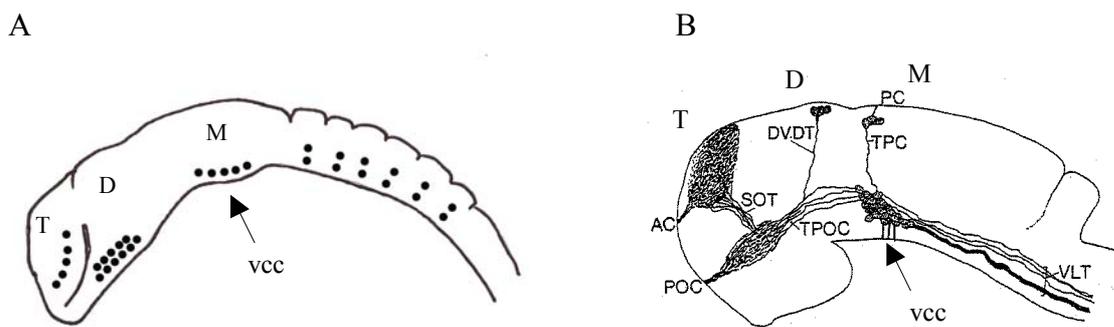
Fig.14. Schematic drawing of neural clusters and axon tracts at different stages.
(Kimmel, 1993; Ross et al., 1992). A. Neural clusters (represented by dots), revealed by AchE, are formed in the center of each neuromere at about 18 somites. B. Axon tracts, labeled by the HNK-1 antibody, in the 24h brain. AC, anterior commissure; DVDT, dorsoventral diencephalic tract; PC, posterior commissure; POC, postoptic commissure; SOT, suproptic tract; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; VLT, ventral longitudinal tract. Dots represent neural cluster. T, telencephalon; D, diencephalon; M, mesencephalon ; vcc, ventro-caudal cluster

Factors involved in positioning the proneural clusters

It is thought that the neurogenesis pattern responds to regional cues, which are transduced through both positive and negative regulators that establish competent zones where the proneural clusters can develop. Several transcription factors are known which positively define proneural clusters. Among those are zinc-finger, winged helix and proneural basic helix-loop-helix (bHLH) factors, which are expressed in broad domains. Local inhibition processes, where factors of the homeobox, zinc finger and bHLH of the Hairy/E(sp1) families play an important role, then refine this broad profile to precise clusters within the neural tube (Bally-

Cuif and Hammerschmidt, 2003). In chapter I.3.3. the structure and characteristics of the bHLH family is described.

3.2. The neurogenesis process and lateral inhibition

Proneural genes

The potential for neural fate is given in each cell of a proneural cluster by the expression of proneural genes. Proneural genes are key regulators of neurogenesis, coordinating the acquisition of a generic neural fate. In the late 1970s, a complex of genes that are involved in regulating the early steps of neural development in *Drosophila* had been identified (*achaete*, *scute*, *lethal of scute*, *asense*), which was the first step to identify the bHLH domain, a structural motif shared by these proteins and responsible for their DNA-binding and dimerization properties. Genetic studies in *Drosophila* and vertebrate models have provided evidence that a small number of 'proneural genes', are both necessary and sufficient, in the context of the ectoderm, to initiate the development of neural lineages and to promote the generation of progenitors that are committed to differentiation. Proneural genes have recently been shown not only to integrate positional information into the neurogenesis process, but also to contribute to the specification of progenitor-cells identity (see below).

Restriction of neural cell number by the lateral inhibition process

As a consequence of the dynamic regulation of proneural gene expression by auto-regulation (where each cell enhances its own expression of proneural genes) and of lateral inhibition (where each cell inhibits proneural expression in neighbouring cells), only a restricted number of cells develops as neuroblasts within each proneural cluster (Culi and Modolell, 1998; Skeath and Carroll, 1992). Lateral inhibition is mediated by the activation of the Notch signaling pathway, which is initiated by the induction of a Notch ligand (Delta, Serrate/Jagged). The expression of Delta in the future progenitor cell activates Notch signaling cascade in the neighbouring cells, resulting in the expression of repressors (Hes/Her/Esr in vertebrates), which in turn directly down-regulate proneural gene expression and prevent cells to become neural. This mechanism ensures that, within a group of equivalent neuroectodermal cells expressing proneural genes, only some can be reinforced to enter a neural fate (committed to differentiation).

Additional functions of proneural genes

The expression of proneural genes in individual neural progenitors is transient. They are down-regulated before the progenitor cells start to differentiate. Distinct genes (e.g. *NeuroD*)

were found which have the ability to promote neural differentiation. More, there is evidence that these factors also promote the arrest of the division of progenitor cells: Indeed, over-expression of *Ngn2* in chick leads to the induction of Cdk inhibitors (p16, 21, 27). Further, in NeuroD loss-of-function mouse embryos, ectopic mitoses are induced (Mutoh et al., 1998). Thus proneural genes might not only coordinate the selection of neuron progenitors but also the expansion of the pool of progenitors or the timing of their differentiation, with consequences on their acquisition of a lineage identity. Further, vertebrate proneural genes have been directly implicated in the specification of some neural subtypes, for instance the role of Mash1 in the specification of noradrenergic neurons. Mash1 acts as a main determinent for the induction of the homeodomain proteins Phox2b and Phox2a in important noradrenergic centers (Lo et al., 1998).

3.3. Main actors of the neurogenesis process: bHLH factors

All known proneural genes belong to the class of bHLH transcription factors, indicating that they have similar biochemical properties. Vertebrate homologs of proneural and neurogenic genes appear to function similarly to their insect counterparts. This means that misexpression of vertebrate neural bHLH genes leads to ectopic neurogenesis, and loss of neural bHLH function leads to failure of formation or differentiation of subsets of neuron (Kageyama and Nakanashi, 1997). However, genetic analyses have revealed that vertebrate neural bHLH genes are functionally highly heterogeneous. Genes of the *asc* and *ngn* family, for instance, have a similar proneural function to that of their *Drosophila* counterparts, whereas other neural bHLH genes are involved in specifying neural differentiation, but have no proneural role, which means promoting a neural versus a glial cell fate. The structure and function of the bHLH factor family are summarized below.

The bHLH family: Molecular structure and DNA-binding capacities
The bHLH transcription factors family consists of a large number of proteins, involved in a variety of different functions. The bHLH domain comprises a DNA-binding basic region (about 60-100 amino acids long), followed by two α-helices separated by a variable loop region (HLH) (Ferre d'Amar et al., 1993) (fig.15). The helix-loop-helix (HLH) domain promotes dimerization, allowing the formation of homodimeric or heterodimeric complexes between different family members (Kadesh, 1993).
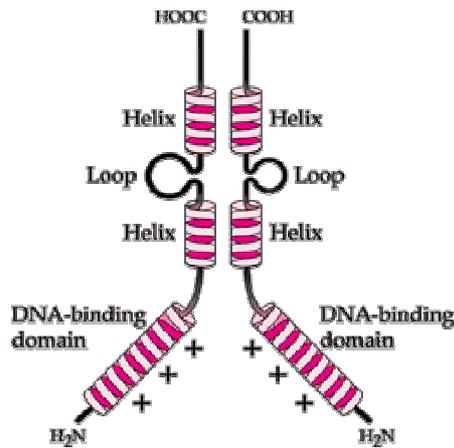
Fig.15. Dimer of bHLH transcription factors (see **www.devbio.com**, after Jones, 1990).
The bHLH proteins bind to DNA through a region of basic amino acids (typically 10-13 residues) that precedes the first α-helix. The helices contain hydrophobic amino acids at every third of fourth position, so that the helix presents a surface of hydrophobic residues to the environment. This enables the protein to pair by hydrophobic interaction with the same protein or with a related protein that display such as surface.

Classification of bHLH proteins

The bHLH family of transcriptional regulators plays crucial roles in the development of various organs and cell types including the nervous system in many animal species (Massari and Murre, 2000). The basic and the HLH domains have distinct functions. The bHLH proteins bind DNA as a dimer via the basic domain, which is the major determinent in DNA binding specifity (Murre et al., 1994). The transcription factors of the basic Helix-Loop-Helix (bHLH) class can be further divided into several groups, according to their structural features, biochemical characteristics and biological functions (e.g. Iso et al., 2003; Fisher and Caudy, 1998; Atchley and Fitch, 1997; Massari and Murre, 2000; Ledent and Vervoort, 2001).

Four monophyletic groups named A, B, C, and D are distinguished (after Fisher and Caudy, 1998) (table 1). Group A and B include bHLH proteins that bind hexameric DNA sequences referred to as 'E-boxes' (CANNTG) and/or N-boxes (CACNAG). Group C corresponds to the family of bHLH proteins known as bHLH-PAS (Crews 1998) involved in a variety of developmental processes. In contrast to all other subfamilies, group D proteins lack a basic domain, for instance Id, Emc and are unable to bind DNA - they act as antagonists of Group A bHLH proteins (Van Doren et al., 1991; Van Doren, 1992). One additional group of putative HLH proteins, the COE family, is characterized by the presence of an additional DNA binding and dimerization domain (COE) (Ledent and Vervoort, 2001).

	Structural features	Biochemical activity	examples	DNA binding site
Group A	bHLH	Transcriptional activators	MyoD, Mash1	Subtype of E-box CANNTG
Group B	bHLH leucine zipper type	Transcriptional repressor	Myc, Max	Subtype of E-box CANNTG
	Conserved proline residue in basic domain	Transcriptional repressors	hairy and E(spl), Hes, Esr, Her	E-box and N-box CANNTG; CACNAG
Group C	bHLH-PAS	?	Sim, ARNT	ACGTG or GCGTG
Group D	HLH	Antagonists of Group A	Id, Emc,	No DNA binding

Table 1. Subdivision of the bHLH family factors in monophyletic groups and their function

The Hairy and Enhancer of split bHLH family

The bHLH proteins are a super-family of DNA-binding transcription factors that regulate numerous processes in both invertebrates and vertebrates. Most members of one family of bHLH factors related to hairy/E(spl) proteins, such as the vertebrate Hes/Esr/Her factors, act as active transcriptional repressors in a variety of developmental processes, for instance neurogenesis, mesoderm segmentation or myogenesis. All members of this family share structural similarities, such as the presence of a proline residue at a conserved position in the basic domain, high conservation within the bHLH domain, conserved amino acids in the Orange domain (Dawson et al., 1995) or helix3/4 (Knust et al., 1992), located C-terminal to the bHLH domain and a strictly conserved WRPW motif at the C-terminus (Davis and Turner, 2001), which allows interaction with the Groucho repressor protein (Fisher and Caudy, 1998). (fig. 16).

basic helix-loop-helix   orange/helix3-4   tettrapeptide

P   WRPW
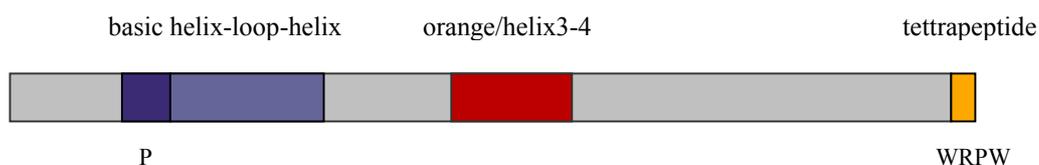
Fig.16. Schematic drawing of hairy/E(spl) family factors (Iso et al., 2003)
Conserved domains are marked in distinct colors. The basic domain is marked in dark blue, the helix-loop-helix domain in light blue, the orange or helix3-4 in red and the C-terminal tetrapeptide motif in orange. Amino acids are abbreviated with P for proline, W for tryptophane and R for aspartate.

Allthough all of these proteins are transcriptional repressors, the conserved differences in the primary structures imply that members of different subfamilies, namely Hairy, E(spl), Hey and Stra 13, might have distinct functions and/or post-transcriptional regulation (Davis and Turner, 2001). DNA-binding site selection and *in vivo* studies have shown that these proteins bind

preferentially to sequences referred to as 'N-boxes' (CACGCG or CACCAG) and have only a low affinity for 'E-boxes' (Ohsake et al., 1994; Van Doren et al., 1994).

Until now, in zebrafish 9 genes belonging to the *hairy/E(spl)*-related genes have been published (Gajewski and Voolstra, 2002). Most of the known *her* genes belong to the E(spl) subfamily (*her1-5*, *her7*), only *her6* belongs to the Hairy subfamily (fig. 16). Among them *her4*, *her6* and *her5* are expressed in the developing nervous system and in addition in the anterior presomitic mesoderm (*her4*, *her6*) or endoderm (*her5*), respectively. In contrast, *her1* and *her7* are both cyclically expressed solely in the presomitic mesoderm but not in the neural plate.
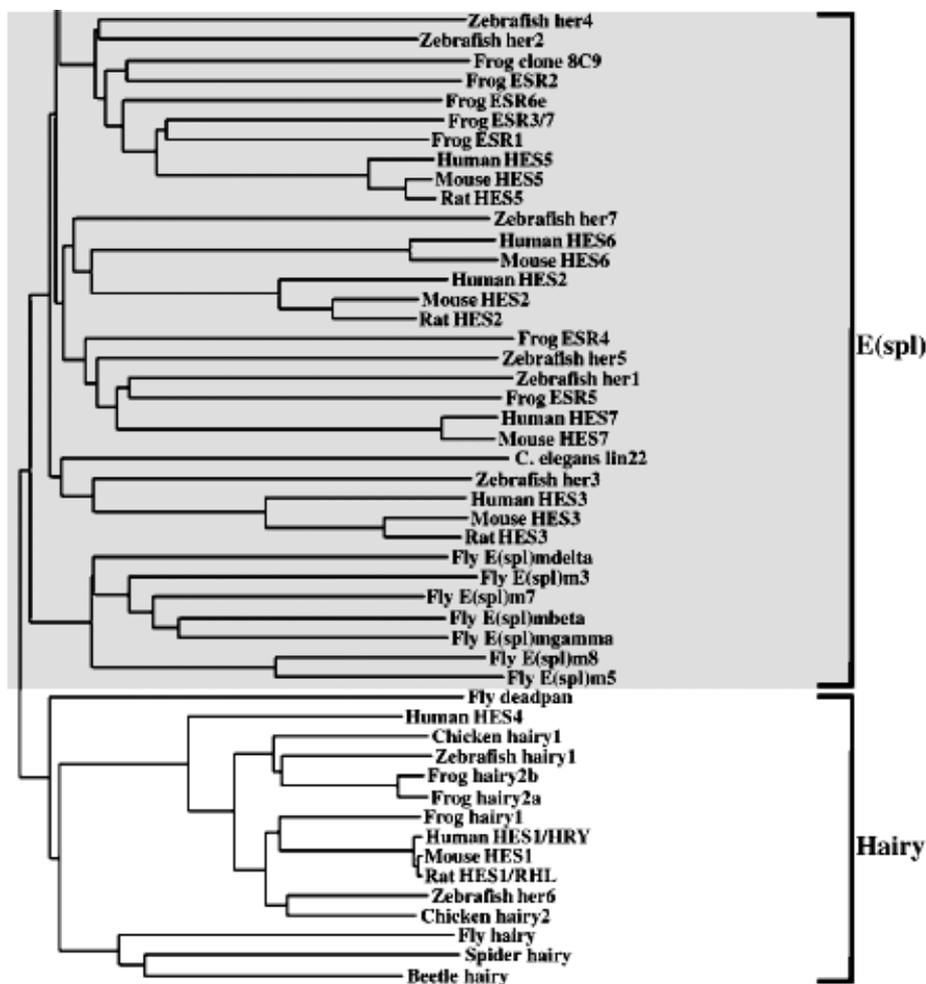
Fig.17. Phylogenetic tree of Hairy and Enhancer of split factors (Davis and Turner, 2003).
Note that Her1-5 and Her7 belong to the E(spl) factors while Her6 is a member of the Hairy subfamily.

The vertebrate Hes/Her/Esr proteins have been shown to act as classical DNA-binding repressors of proneural gene transcription, but they are also thought to inhibit the activity of proneural proteins by interfering with proneural-E-proteins complex formation (Davis and Turner, 2001). Recently it was shown that one member of the Hes family (with an unusually short loop-domain) inhibits other bHLH-factors and acts, unlike all other known member of the Hes/Her/Esr family, as a positive regulator of neurogenesis (Koyano-Nakagawa, 2000).

Mechanisms of transcriptional repression through Hairy/E(spl) proteins

Three mechanisms have been proposed, (i) DNA-binding dependent active repression, (ii) passive repression by protein sequestration and (iii) repression mediated by the Orange-domain (Iso et al., 2003). (i) It is known that Hairy/E(spl) proteins form a homodimer and bind DNA consesus sites. They recruit the corepressor Groucho or its mammalian homolog TLE via the WRPW-motif, which is able to recruit the histone deacetylase Rpd3, which might repress transcription through altering local chromatin structure (Chen et al., 1999). (ii) Passive repression was shown, for instance with Hes1, which can form a non-functional heterodimer with other bHLH factors (e.g. E47, MyoD, Mash1), thereby preventing functional heterodimers such as MyoD-E47 and Mash1-E47 (Sasai et al., 1992; Hirata et al., 2000). HES1 functions as a negative regulator of neurogenesis by directly repressing a proneural gene, *Mash1*. HLH proteins (Drosophila *emc*; vertebrate *Id*) act through passive repression. They have a high affinity for E proteins and therefore compete with proneural proteins by forming heterodimers that cannot bind DNA. Further, (iii) the Orange domain, a putative protein interaction motif, is essential to repress transcription of its own promoter (Hes1) as well as the *p21* promoter (Castella et al., 2000).

II. Aims and achievements of the Thesis

The main intention of my Ph.D. project was to understand the early steps of midbrain-hindbrain (MH) development. Until now, most studies focused on the well understood maintenance phase of MH development, but the early induction remains unclear. To gain insight into the factors and regulatory mechanisms involved in the MH induction step, I used several approaches:

(i) it is known that MH development is regulated by planar signaling (e.g. Simeone, 2000; Bally-Cuif and Wurst, 2001; Liu and Joyner, 2001; Marin and Puelles, 1994). Several MH markers (*her5*, *pax2.1*, *wnt1*, *fgf8*), mainly involved in MH maintenance, are activated in initially independent pathways. It is of great interest to find the factors which induce these MH markers. We based our search for early MH inductive signals on the fact that molecular mechanisms are often conserved among vertebrates and invertebrates. In the *Drosophila* embryo, the zinc finger transcription factor Buttonhead (Btd) is expressed across the head-trunk junction (Vincent et al., 1997) and essential to integrate the head and trunk patterning systems and form of the head/trunk boundary. Because of the known molecular similarities between the *Drosophila* head-trunk junction and the vertebrate midbrain-hindbrain boundary (MHB), homologs of Buttonhead appeared to be good candidates as regulators for MHB development. In the first part of my work, I therefore identified and characterized functionally a zebrafish Btd-related factor, called Bts1. These results are described in chapter III.1. and appendix 1. I discuss the evolutionary implications of this study in chapter III.2 and appendix 2.

(ii) The development of the anterior neural plate is influenced by vertical signaling (e.g. Muhr et al., 1997; Ang and Rossant, 1993; Rowan et al., 1999; Streit et al., 2000), however the precise role of non-neural tissues in MH induction is not clear. As a second part of my work, I used and manipulated several mutant lines with defects in only one or all non-neural tissues to study the direct or indirect effects of these defects on MH induction. Although I failed to find evidence for a necessary contribution of vertical signaling in MH induction in zebrafish, my work unravelled the inhibitory influence of long-range signaling, emanating from the prechordal plate, on a neuronal cluster at the forebrain-midbrain boundary. This work is described in chapter III.3. and appendix 3.

(iii) As an alternative to answer questions that are difficult to solve with general embryological experiments, we used a molecular approach. We reasoned that the identification of the regulatory elements of the first selective marker of the MH in the zebrafish, namely the *her5* gene, should allow to find factors directly inducing *her5* expression and thereby MH development. I therefore used a reporter approach in zebrafish transgenics to isolate the *her5* regulatory elements. This work is described in chapter III.4 and appendix 4. Further applications of this work include *in vivo* tracing the cells of the entire presumptive MH from its induction onwards, in wild-type and mutant contexts, for instance in embryos lacking Pax2.1 or FGF8 function.

(iv) Finally, my study of the genomic *her5* locus allowed me:
First, to identify the exact transcriptional start site of *her5*, thus to permit the design of *her5* morpholinos and the functional study of *her5* (briefly described in III.5 and appendix 5)
Second, to isolate a new gene, named *him*, expressed selectively in the MH at late gastrulation. *him* is a new member of the Hairy/E(spl)-family, located close to the genomic position of *her5* in a head-to-head orientation. Interestingly, it shares a similar spatio-temporal expression pattern in the neural plate as *her5*. I initiated a study of *him* function in MH development, to unravel possible interaction of *her5* and *him*. Preliminary results on Him function suggest a role similar to Her5, in MH neurogenesis. These data are described in III.6.

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III. Results and Discussion

1. Bts1 is the earliest selective regulator of *pax2.1* and its genetic cascade in the MH

The early phase of MH induction and the initiation of expression of the early MH markers (*her5*, *pax2.1*, *wnt1* and *fgf8*) are only incompletely understood. We have addressed the role of planar information in this process, reasoning on the fact that the head-trunk junction in the *Drosophila* embryo and the MH of the vertebrate embryonic neural plate share patterning similarities. The *Drosophila* head gap gene *buttonhead* (*btd*), encoding a zinc finger transcription factor, is expressed across the head-trunk junction and is essential for the formation of the morphological constriction, the cephalic furrow, separating the head from the trunk (Vincent et al., 1997). We used a degenerate PCR approach to clone zebrafish *bts1*, related to the Btd/Sp factor family. In sequence, Bts1 is more closely related to Sp-factors than to Btd, but its specific expression profile in the MH is reminiscent of *btd* at the head-trunk junction in *Drosophila*. Further, Bts1 and Btd act both as transcriptional activators, and their similar expression pattern across boundary regions suggest that both control related developmental processes. During gastrulation *bts1* expression is found in the epiblast up to the level of the presumptive MH, preceding the expression of other MH markers. The expression pattern of *bts1* suggested Bts1 as an important factor in early MH development. Using a combination of gain-of-function (capped RNA injections) and loss-of-function analyses (morpholino injections), we found that Bts1 is both necessary and sufficient for the selective induction of *pax2.1* expression and its dependent genetic cascade, *pax5*, *eng2* and *eng3*, but is not involved in the regulation of *her5*, *wnt1* or *fgf8*. Because of its important function in MH induction, we also studied its own regulation of expression. Using mutant lines affected in *fgf8/ace* (Reifers et al., 1998) or *pax2.1/noi* (Lun and Brand, 1998), we found that *bts1* responds to FGF8 during gastrulation, and later depends on Pax2.1 but no longer on FGF8 function. We conclude that *bts1* might only transiently require Pax2.1/FGF8 function during the MH maintenance loop. In summary, we identified one cascade of MH induction through planar information. These results add an upstream molecular step to our understanding of MH induction (fig.14).
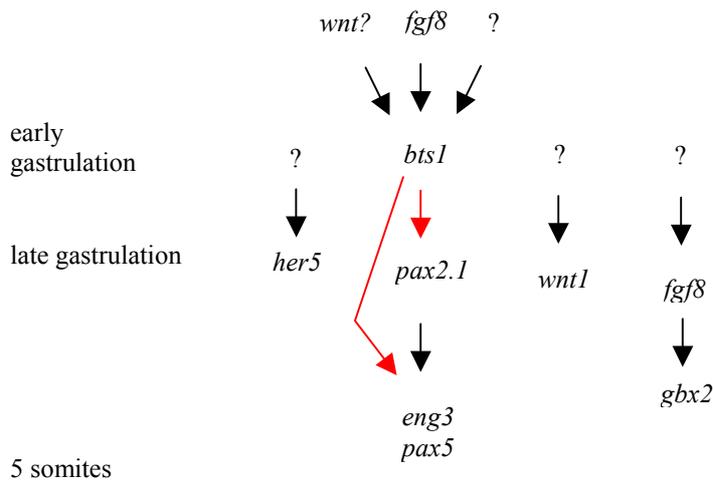
Fig.18. Model of MH induction incorporating *bts1* (Tallafuß et al., 2001).

2. Evolutionary consideration about the vertebrate MH

The results described in II.1 and appendix 1 suggest that insects and vertebrates might have co-opted factors of related families to control the formation of boundary regions, such as the MHB in zebrafish and the head-trunk junction in *Drosophila*. Indeed vertebrates use Bts1, an Sp-like factor in sequence, while *Drosophila* uses Btd. These results also lead us to evolutionary considerations on the existence of a MH-like territory in other chordates, namely ascidians and *Amphioxus* (appendix 2). The question if the vertebrate MH is an ancestral brain region has not been completely answered. The possibility to compare different neural regions by using molecular neural plate markers in addition to morphological studies allows a more complete view of evolutionary conserved regions. Further, we looked in *C. elegans*, *Drosophila*, ascidians, zebrafish and mouse for factors of the Btd/Sp-family. In conclusion, it appears that the MH was elaborated during the vertebrate lineage and a similar set of genes is used to establish related structures.

3. Signals from the prechordal plate control the size of the *six3*-positive neuronal cluster at the di-mesencephalic boundary

It is widely accepted that vertical signaling is involved in brain development. However, its contribution to MH development remains unknown. Using mutant lines primarily affected in non-neural tissues, we have observed an involvement of long-range signaling on development at the forebrain-midbrain boundary. We focused on the zebrafish mutant line *bonnie-and-clyde* (*bon*) (Kikuchi et al., 2000), affected in the gene *mixer*, which exhibits deficiencies in

mesoderm, endoderm and anterior mesendoderm. *mixer* is expressed exclusively in non-neural tissues, namely the presumptive YSL, PCP and endoderm. Thus, observed alteration in brain development can only result from secondary consequences caused from deficiencies in non-neural tissues. Using various markers of the neural tube we found that in *bon* selectively the *six3*-positive neuronal cluster, located at the ventral forebrain-midbrain boundary (future part of the nMLF), is enlarged. Other markers expressed in the prospective brain as well as the formation of axon tracts appeared normal. Thus, in *bon*, neural patterning is not affected by deficiencies in non-neural tissues, but instead the regulation of neuronal cluster size at the forebrain-midbrain boundary is disturbed. As source for the signal normally necessary to restrict the number of cells in the *six3*-positive cluster, the YSL, PCP or endoderm were considered, which are all affected in *bon*. To narrow the number of possible candidates, we tested *casanova* (*cas*) (Alexander et al., 1999) mutants, which only lack endoderm but no other non-neural tissues. We found no alteration in the size of the *six3* neuronal cluster in *cas* mutants, demonstrating that signals from the endoderm alone do not contribute to controlling the size of this cluster. By experimentally rescuing Mixer function selectively in the YSL of *bon* mutants, we further excluded the YSL as source of the signal involved in regulating the *six3* cluster size. In contrast, by selectively rescuing Mixer function in the PCP, we could restore the normal cluster size in *bon*. Thus, our results identify the PCP as the tissue responsible for the neuronal defect in *bon*, and demonstrate the existence of a PCP-derived, long range activity that controls the number of nMLF neurons at the forebrain-midbrain boundary.

4. The transgenic line *her5PAC::egpf* identifies *her5* regulatory elements and allows following the fate of the entire prospective MH

Generation of the transgenic line her5PAC::egfp

her5 (Müller et al., 1996) is the earliest gene expressed specifically in the presumptive MH area (Bally-Cuif et al., 2000). Using homologous recombination in *E. coli* mediated by ET cloning, I engineered a construct based on phage artificial chromosome (PAC) with at least 40kb genomic upstream region of *her5* and thus considered to contain all regulatory elements required for specific regulation, in which *egfp* was inserted in Exon 2 of *her5*. The modified *her5PAC:egfp* construct was then injected into fertilized zebrafish embryos to generate stable transgenic lines. We obtained three independent stable transgenic lines, which reproduced the expected expression pattern in the MH and the pharyngeal endoderm.
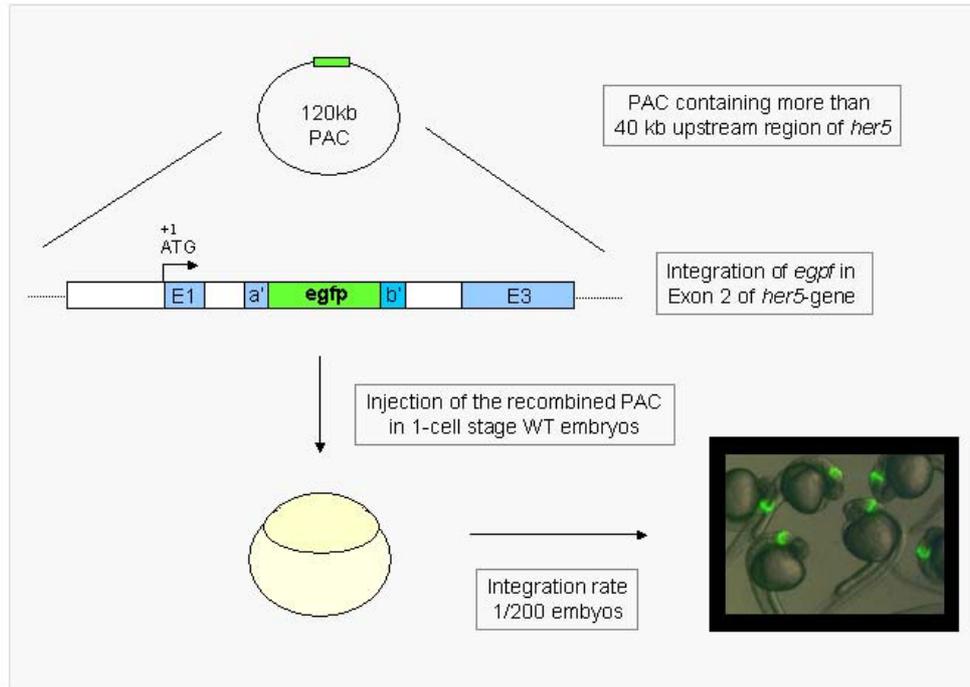
Fig.19. Schematic overview representing the generation of the transgenic line *her5PAC::egpf*.

Identification of the her5 regulatory elements, driving endodermal and neural expression
The *gfp* expression pattern of *her5PAC::egfp* transgenic embryos suggests that all the regulatory elements required for the correct spatiotemporal regulation of *her5* are contained in the construct and were integrated into the genome. To identify the minimal region containing all required regulatory elements we tested *in vivo* various fragments of different length for their ability to drive GFP expression in the two expected regions, the MH and the endoderm. Using this method we identified a fragment of 3.3kb length as minimal regulatory region. As *her5* has two expression domains, in the MH and the endoderm, we identified the elements required for expression in the MH and endoderm, respectively, by subsequently shortening this fragment. From our results we conclude that, on the genomic structure, endoderm regulatory elements are located in a proximal position, while elements required for specific expression in the MH are located distal to the upstream region of *her5* gene. A search for putative binding sites failed to suggest strong candidates for likely upstream factors.

The dynamics of MH development over time
The transgenic lines *her5PAC::egfp* enable to follow the fate of early *her5*-expressing cells and their exact contribution to brain regions from late gastrulation until late larval stages (appendix 4). They further give the possibility to trace cell fate in different mutant contexts.

For instance in the mutants *ace* and *noi*, the MH is initially normally induced but fails to be maintained, and during somitogenesis the MHB and part of the MH are lost (as revealed by morphology and *in-situ* RNA staining), although no dramatic increase in cell death was observed (Lun and Brand, 1998; Reifers et al., 1998). These phenotypes lead to the question what happened to the initially correctly specified cells of the prospective MH. So far, this question could not be answered because of the lack of a stable marker of MH cells. We demonstrated that, in the absence of FGF8 or Pax2.1, MH cells partially acquire the identity of neighbouring territories, to an extent that depends on the mutant context. Our results provide the first direct assessment of MH fate in the absence of IsO activity, and directly support a role for Fgf8 in protecting anterior tectal and metencephalic fates from anteriorization, while Pax2.1 controls the maintenance of MH identity as a whole.

5. Molecular and functional analyses of the bHLH factor *her5*

Her5 is an important factor in regulating neurogenesis in the MH

Vertebrate neurogenesis, namely the process whereby neuronal precursors become selected and ultimately differentiate, is only incompletely understood. The MH displays a neuron-free stripe at the level of the prospective MHB, with delayed differentiation (IZ) compared to other neural plate territories, the neuronal differentiation zones of the forebrain and the hindbrain. Using conditional gain-of-function and morpholino-induced loss-of-function analyses, Geling et al., 2003 (appendix 5) identified Her5 as a crucial inhibitor of neuronal differentiation, acting selectively in the IZ.

Contribution to the work Geling et al., 2003:

One crucial experiment, required for the analysis of Her5 function, was the morpholino-induced loss-of-function study to test if Her5 function is necessary for IZ formation. The design of a functional morpholino required the identification of the functional start codon of *her5* and was essential for blocking endogenous Her5 function completely. The identification of the start codon was permitted by my analysis of the *her5* genomic locus, as described below.

Genomic structure of her5 and identification of the new functional start codon

At the beginning of this work the *her5* cDNA sequence (Müller et al., 1996) was available. Blocking the published *her5* start codon by morpholino-injection could not prevent the translation and function of endogenous Her5, suggesting that the published cDNA sequence

was incomplete and did not include the functional start codon used *in vivo*. To study the requirement of Her5 function on neuronal development, it was necessary to block the endogenous translation of Her5. To this aim I identified the genomic structure of *her5* and identified three additional putative start codons upstream and in reading frame to the published start codon (fig.20). To test which one of the three putative start codons was functional *in vivo*, I used the transgenic line *her5PAC::egfp* (chapter II.4 and appendix 4) as a test system for the ability to block the translation of *egfp* by morpholino-injection. The transgenic line *her5PAC::egfp* contains the intact promoter region and Exon1 of *her5*, that means that the ability to block GFP translation in the transgenic line can be expected to block endogenous Her5 translation. With this system I identified the functional start codon and validated the morpholino chosen. It was then possible to block endogenous Her5 activity and reveal that this leads to a dramatic phenotype in the IZ, where neurogenesis then fails to be inhibited (appendix 5).

Fig.20. Complete sequence of the bHLH-factor Her5.
The identification of the functional start codon leads to 9 additional amino acids (red) in the N-terminus of Her5.

6. Identification of a new *hairy/E(spl)* gene, *him*, and preliminary functional results

The genomic arrangement of him and her5

Upon characterization of the *her5* locus, I identified a new member of the Hairy/E(spl) family, *him* (for *"her5 image"*), located 3.3kb upstream in a head-to-head orientation relative to the genomic locus of *her5* on LG14 (fig.21). This arrangement suggests that both genes share their regulatory elements und thus are likely expressed in a similar spatiotemporal pattern within the zebrafish embryo. Further, they might be involved in related regulatory processes. This would be in agreement with recent findings on the zebrafish genes *her1* and *her7*, which are arranged in a head-to-head orientation. Both are expressed cyclically in the presomitic mesoderm and are involved in regulating mesodermal segmentation (Henry et al., 2002). Identifying the regulatory elements and/or putative binding sites of upstream factors of both genes *him* and *her5* will be an important step in understanding how the regulation of these two genes occurs within a region of only 3.3kb sequence.
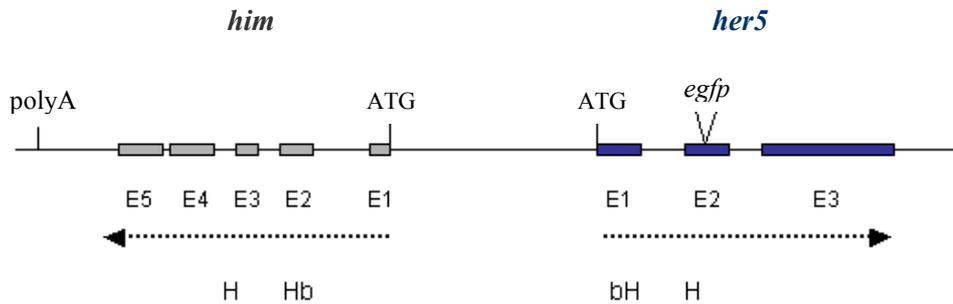
Fig.21. Genomic organisation of the Hairy/E(spl) factors *him* and her5.
Him is organized in 5 exons (grey boxes, E1-E5); the bHLH domain is located in E2 and E3. Her5 consist of 3 exons (blue boxes, E1-E3), with the bHLH domain located in E1 and E2. The ATG indicates the start; the arrows reveal the orientation of *him* and *her5*, respectively.

Isolation and classification of Him

The Hairy/E(spl) factor Him consists of 297 amino acids, determined from full-length cDNA, arranged in 5 Exons on the genome. Sequence comparison between full-length protein sequences of Him and other known Hairy/E(spl) factor shows that Him belongs, according to Davis and Turner (2001), like Her1 and Her5, to the sub-family of E(spl) factors (fig.17). Comparison with zebrafish Her factors further demonstrates that Him is most closely related to Her1 with 33% and Her5 with 28% overall identity. If the sequence comparison is restricted to the functional bHLH domain, required for selective DNA binding capability and dimerization between bHLH factors, Him shows 66% identity to Her1 and 50% to Her5 bHLH domain. We broadened the sequence comparison of Him to all known vertebrate members of the Hairy/E(spl) family. Some of the proteins with the highest conservation level to Him from different vertebrates are represented in fig.22.

A
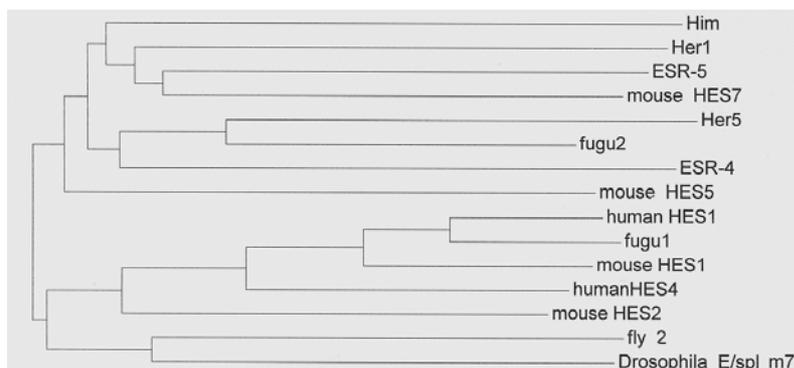
B
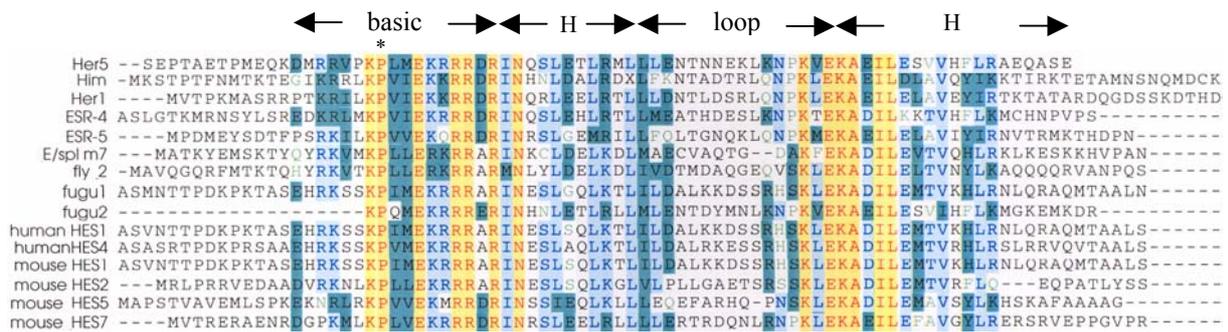
C
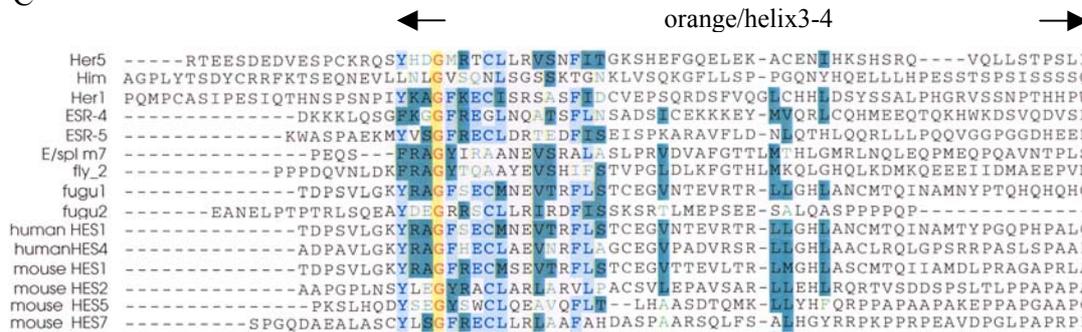
Fig.22. Alignment of members of the Hairy/E(spl) family.
A. Phylogenetic tree showing the relationship of representative members of the Hairy/E(spl) family closely related to zebrafish E(spl) factor Him. Amino acid sequences of the B. bHLH domain and C. orange domain of Hairy/E(spl) factors from *Drosphila*, *Fugu*, zebrafish, mouse and human were aligned by using VectorNTI. Identical residues are in yellow, conserved amino acids in blue. The asterisk indicates the invariant proline residue in the basic domain. All sequences were downloaded from NIH/NCBI: zebrafish Her1 and Her5, fugu1 (lcl|SINFRUP00000088121) and fugu2 (lcl|SINFRUP00000057004) are incomplete amino acid sequences, *Drosophila* E/spl m7 (NP 536753) and fly1, unpublished protein (NP 524503), *Xenopus* ESR-4 and ESR-5, human HES1 and HES4, mouse HES1, HES2, HES5 and HES7.

Expression pattern of him

him starts to be expressed at 30% epiboly in the deep layer of the shield and dorsal margin (fig.23A). From 70% epiboly to 24 hours-post-fertilization (hpf) *him* is expressed in the prospective MH (fig. 23B,C, D), in a spatiotemporal pattern similar to *her5*, as confirmed by double *in-situ* RNA staining for *him* and *her5* (not shown). Because of the selective and early expression of *him* in the prospective MH it is of great interest to unravel Him function during neural development, also in comparison to Her5 function. First preliminary results are described below. In addition to the neural expression domain, *him* is expressed from late gastrulation until late somitogenesis in the presomitic mesoderm, likely in a cyclic fashion (fig.B,C,D). The high percentage of similarity between Him and Her1, together with the expression pattern and function of *her1* in the presomitic mesoderm, suggest that Him is likely involved in mesoderm segmentation.
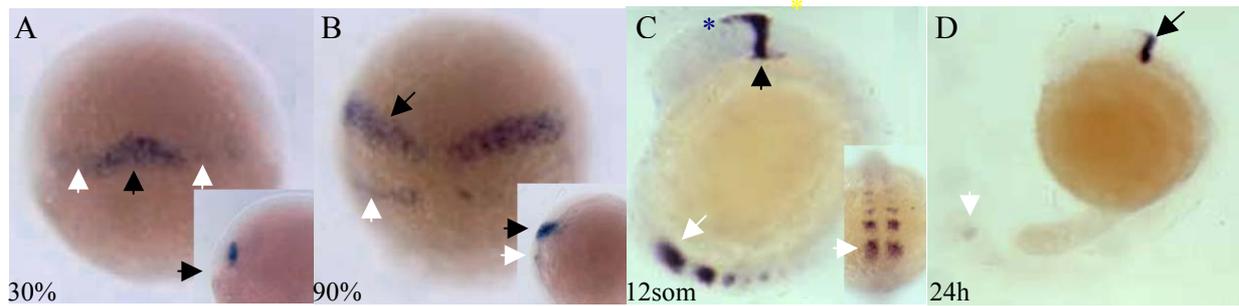
Fig.23.Expression pattern of *him* at different stages.
A. *him* starts to be expressed at 30% epiboly in the deep shield and dorsal margin. The margin at the level of the shield is indicated by black arrows, the expression in some cells in the more lateral dorsal margin are marked by white arrow heads. B. At late gastrulation *him* expression is restricted to the presumptive MH (black arrows) and one stripe in the presomitic mesoderm (white arrows). C. During somitogenesis *him* is expressed at the MHB (black arrow), the roof plate (blue asterisk) and ventrally, in a narrow stripe extending posteriorly (yellow asterisk). In addition *him* is expressed in several stripes in the presomitic mesoderm (the most posterior is indicated in white arrows). D. At 24 h, *him* is mainly expressed at the MHB (black arrow) and a small spot in the tip of the tail (white arrow). All pictures are orientated with anterior to the top. A and B are dorsal views, the inserts are the corresponding lateral views, with dorsal to the left. C and D are dorsal to the right, the insert is a dorsal view of the trunk and tail.

Preliminary results on Him function

We initiated the study Him function by combining gain-of-function, using capped mRNA injection and loss-of-function experiments, using morpholino (MO) injection as well as analysing a zebrafish deletion mutant line, deficient in the three neighbouring genes *knypek*, *him* and *her5* (mutant allele *kny^{b404}*) (kindly provided by Topczewski, J. and Solnica-Krezel, L.). Preliminary results, obtained from loss-of-function experiments suggest that general neural patterning is not affected by Him-Mo injection, as revealed using various neural plate patterning markers and in particular MH markers (*pax2.1, her5, wnt1, eng2*) at different stages (not shown). In contrast, the expression of neurogenesis markers, such as *zcoe2* and *ngn1* was severely affected in the IZ by the loss of Him function (fig.24). The results obtained with Him-Mo show striking similarities to the loss-of-function study with Her5-Mo (Geling et al., 2003 and appendix5), namely the premature differentiation of neurons within the IZ. Given that Him-Mo does not down-regulate *her5* expression, these results suggest that the function of both E(spl) factors is not redundant, at least at early neural development. Rather, both factors appear together necessary for the formation and/or early maintenance of the IZ.
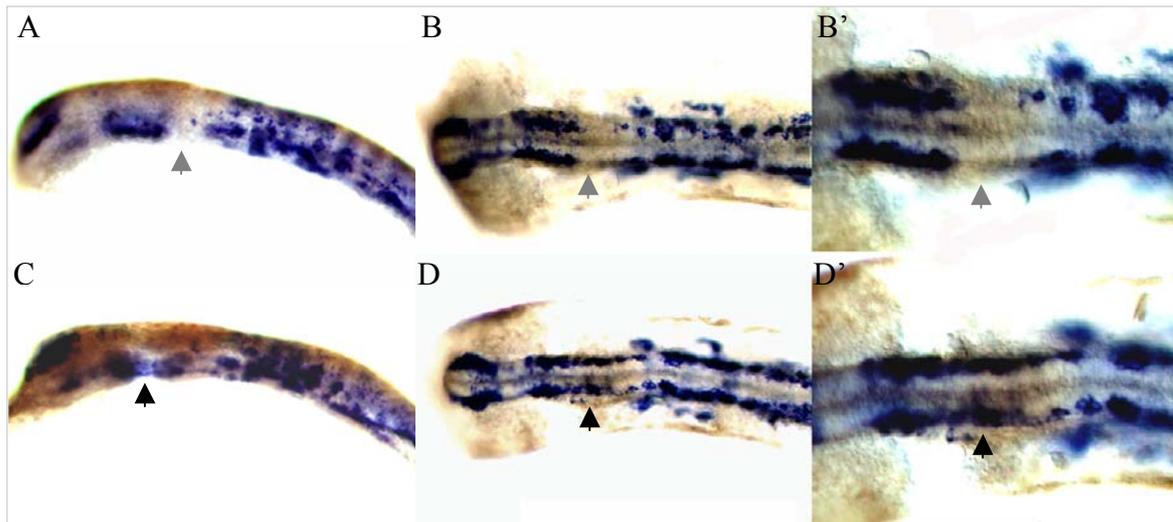
Fig.24. Loss-of-function by Him-MO shows a premature differentiation within the IZ.
At this stage the IZ (grey arrows) is recognizable as a neuron-free region at the level of the prospective MHB, see wild-type embryos in A, B and B'. In contrast, embryos lacking functional Him loose the neuron-free IZ (expected place indicated by black arrows). In this panel all embryos are stained with the neural marker *zcoe2* at the 15 somites stage. As a control wild-type embryos are shown in the top row, the Him-MO injected embryos in the lower row. All embryos are orientated anterior to the left. A and C are lateral view, B, B', D and D' are dorsal views. B' and D' are higher magnifications of B and D, respectively.

7. To do…

To completely understand Him function further experiments have to be done. In addition to the results shown above, several neural markers at different stages (late gastrulation, early and late somitogenesis, 24h and later) have to confirm and to complete the results obtained in Him loss-of-function study. For the loss-of-function study we could use only low concentrations of Him-MO as we observed toxic side-effects if used in a high dose. Thus, we cannot assure if we either decrease or completely knock out Him function by MO injection. Another way to study the lack of Him would be to analyse the deletion mutant line Δ *knypek, him, her5*. As this line not only lacks Him but also Kny and more critically Her5 functions, it would be necessary to rescue both Her5 and Kny in this mutant line. To this purpose we could introduce the genomic sequence containing *her5* and *knypek* by PAC injection (PAC-C8, available in our laboratory). This would allow the study of a complete deficiency of Him function alone without interfering phenotypes caused by the lack of Kny and/or Her5 function.

In sum, loss-of-function analyses will tell which genes require Him function. In addition, we want to analyse Him gain-of-function to study which genes can be induced by Him function. So far, we only focused on early events during neural development and did not include

putative late functions. The expression pattern of *her5* in the adult brain strongly suggests an additional role of *her5* or other Hairy/E(spl) factors at late stages. The close relationship of *her5* and *him*, in particular the similar neural expression pattern, might suggest an additional role of Him during late neural developmental as well.

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Appendix 1

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The zebrafish buttonhead-like factor Bts1 is an early regulator of *pax2.1* expression during mid-hindbrain development

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SUMMARY

Little is known about the factors that control the specification of the mid-hindbrain domain (MHD) within the vertebrate embryonic neural plate. Because the head-trunk junction of the *Drosophila* embryo and the MHD have patterning similarities, we have searched for vertebrate genes related to the *Drosophila* head gap gene *buttonhead* (*btd*), which in the fly specifies the head-trunk junction. We report here the identification of a zebrafish gene which, like *btd*, encodes a zinc-finger transcriptional activator of the Sp-1 family (hence its name, *bts1* for *btd/Sp-related-1*) and shows a restricted expression in the head. During zebrafish gastrulation, *bts1* is transcribed in the posterior epiblast including the presumptive MHD, and precedes in this area the expression of other MHD markers such as *her5*, *pax2.1* and *wnt1*. Ectopic expression of *bts1* combined to knock-down experiments demonstrate that

Bts1 is both necessary and sufficient for the induction of *pax2.1* within the anterior neural plate, but is not involved in regulating *her5*, *wnt1* or *fgf8* expression. Our results confirm that early MHD development involves several genetic cascades that independently lead to the induction of MHD markers, and identify Bts1 as a crucial upstream component of the pathway selectively leading to *pax2.1* induction. In addition, they imply that flies and vertebrates, to control the development of a boundary embryonic region, have probably co-opted a similar strategy: the restriction to this territory of the expression of a Btd/Sp-like factor.

Key words: Zebrafish, Mid-hindbrain, *bts1*, *buttonhead*, Sp factors, *pax2.1*

INTRODUCTION

Neural patterning in vertebrates responds to a combination of planar and vertical inductive signals that progressively subdivide the neural plate into forebrain, midbrain, hindbrain and spinal cord along the anteroposterior axis (Lumsden and Krumlauf, 1996; Appel, 2000). It is a major challenge to understand how this information is encoded at the molecular level, and how the signals are integrated and refined during development to permit the formation of an organized neural plate.

Within the embryonic neural plate, the mid-hindbrain domain (MHD), which comprises the midbrain vesicle and hindbrain rhombomere1 (rh1), follows an interesting mode of patterning. Indeed, a small population of cells located at the junction between midbrain and rh1 ('mid-hindbrain junction'

or 'isthmus') was identified as a source of inductive signals controlling the development of the entire MHD (Martinez et al., 1991; Marin and Puelles, 1994; Martinez et al., 1995; Wurst and Bally-Cuif, 2001). From early somitogenesis stages, the secreted factors Wnt1 and Fgf8 are expressed at the isthmus and are involved in cross-regulatory loops with MHD markers of the *engrailed* and *pax2/5/8* families (Wilkinson et al., 1987; McMahon et al., 1992; Crossley and Martin, 1995; Lun and Brand, 1998; Reifers et al., 1998). These regulatory cascades allow for MHD maintenance at somitogenesis stages. Thus, within the MHD, early signalling events are relayed on-site by the isthmus to maintain MHD specification and achieve short-range patterning. It is of great interest to understand in depth the mechanisms and factors which sustain this mode of patterning.

Accordingly, unravelling the processes of mid-hindbrain

specification remains a major issue. To this aim, the expression of MHD markers was analysed in response to different embryonic manipulations or in mutant contexts in several vertebrates. In the mouse and chick, isthmic organizer formation responds to the confrontation of anterior (*Otx2* positive) and posterior (*Gbx2* positive) identities within the neural plate (Broccoli et al., 2000; Katahira et al., 2000; Millet et al., 2000). However, the expression of *Otx2* and *Gbx2* themselves are probably only involved in the refinement of *Fgf8* and *Wnt1* expression rather than in their induction, as *Fgf8* and *Wnt1* are still expressed in *Otx2*$^{-/-}$ and *Gbx2*$^{-/-}$ mutants (Acampora et al., 1998; Wassarmann et al., 1997). Recent ablation experiments in the mouse also pointed to a role of the axial mesoderm in the regulation of *Fgf8* expression (Camus et al., 2000). Finally, explant cultures in the mouse and *Xenopus*, and transplantations in the zebrafish showed that *engrailed* genes and *pax2.1* expression could be locally induced within the neural plate by non-neural tissues (Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Miyagawa et al., 1996). Thus, MHD specification probably integrates planar and vertical signals, but the factors involved remain unknown.

We were interested in directly identifying factors regulating the initiation of expression of the early mid-hindbrain markers. In the zebrafish embryo, the earliest known mid-hindbrain-specific marker is the gene *her5* (Müller et al., 1996), expressed in the presumptive MHD from mid-gastrulation onwards (70% epiboly) (Bally-Cuif et al., 2000). Shortly afterwards (80-90% epiboly), *pax2.1* expression (Krauss et al., 1991; Lun and Brand, 1998) is induced in a domain mostly overlapping with that of *her5* (this paper). Finally, at the end of gastrulation (tail bud stage), *wnt1* expression is initiated in the same territory (Molven et al., 1991; Lun and Brand, 1998). Late markers such as *eng* genes (Ekker et al., 1992), *fgf8* (Fürthauer et al., 1997; Reifers et al., 1998) and *pax5/8* (Pfeffer et al., 1998) become expressed in the MHD at early somitogenesis stages only. Analyses of *pax2.1/noi* (*no-isthmus*) zebrafish mutants have demonstrated that the induction of *her5*, *wnt1*, *eng2* and *fgf8* expression is independent of Pax2.1 function, while initiation of *eng3* and *pax5/8* expression requires a functional Pax2.1 protein (Lun and Brand, 1998). Conversely, in the mouse, *Pax2* expression is established independently of Wnt1 (McMahon et al., 1992; Rowitch and McMahon, 1995). The early onset of *her5* expression in the zebrafish suggests that it also does not require Wnt1 function. Taken together, these observations suggest that several initially independent pathways lead separately to the activation of *her5*, *pax2.1*, *wnt1* and *eng2*. The expressions of *eng3* and *pax5/8* are initiated subsequently in a Pax2.1-dependent cascade (see Lun and Brand, 1998).

In the *Drosophila* embryo, *buttonhead* (*btd*) is expressed in and necessary for the development of the antennal, intercalary and mandibular head segments (Wimmer et al., 1993). Recently, re-examination of *btd* expression revealed that it covers two rows of cells in the first trunk parasegment, thus crossing the head-trunk junction (Vincent et al., 1997). *btd* mutant embryos fail to activate the expression of *collier* (*col*) in the last head parasegment and *even-skipped* (*eve*) in the first trunk parasegment and do not form a cephalic furrow, the constriction separating the head from the trunk (Vincent et al., 1997). Thus *btd* is essential to integrate the head and trunk

patterning systems and maintain the integrity of the head-trunk junction. Because the MHD also develops in response to the confrontation of anterior and posterior patterning influences, Btd-related factors appeared as good candidate early regulators of mid-hindbrain development in vertebrates, and we initiated a molecular search for zebrafish genes related to *btd*.

btd (Wimmer et al., 1993) encodes a zinc-finger transcription factor of the same family as *Drosophila* and vertebrate Sp factors (Kadonaga et al., 1987; Kingsley and Winoto, 1992; Pieler and Bellefroid, 1994; Supp et al., 1996; Wimmer et al., 1996; Harrison et al., 2000), but has no known vertebrate ortholog at present. We now report the isolation of 11 new zebrafish *btd/Sp1*-related genes (*bts* genes). One of these genes, *bts1*, is transcribed within the presumptive MHD before *her5*, *pax2.1*, *wnt1* and *eng2*. We demonstrate that Bts1 is both necessary and sufficient for the induction of *pax2.1* within the anterior neural plate, but is not involved in regulating *her5*, *wnt1*, *eng2* or *fgf8* expressions. Thus we have identified the earliest known specific regulator of *pax2.1* expression within the embryonic neural plate, and provide further evidence that early specification of the MHD is controlled by several independent genetic cascades. Furthermore, our results imply that flies and vertebrates have likely evolved a similar strategy to cope with the patterning of comparable embryonic regions, by restricting to these regions the expression and function of a Btd/Sp-like factor.

MATERIALS AND METHODS

Fish strains

Embryos were obtained from natural spawning of wild-type (AB), *ace*^{ti282a} or *noi*^{tu29a} (Brand et al., 1996) adults; they were raised and staged according to Kimmel et al. (Kimmel et al., 1995).

Cloning of zebrafish buttonhead/Sp-family members

Random-primed cDNA prepared from tail bud-stage wild-type (AB) zebrafish RNA was amplified using degenerate oligonucleotides directed against the first zinc finger of Btd and Sp1-4 proteins (5′ primers Btd-F1 and Btd-F2) and against their third zinc finger (3′ primer Btd-R):

Btd-F1 5′TG(C/T)CA(C/T)AT(C/T)(C/G)(A/C)IGGITG(C/T)G3′; Btd-F2 5′CICA(C/T)(C/T)TI(A/C)GITGGCA(C/T)ACIG3′; and Btd-R 5′TGIGT(C/T)TTI(A/T)(C/T)(A/G)TG(C/T)TTI(C/G)(C/T)IA(A/G)-(A/G)TG(A/G)TC3′. For cloning of cDNAs *1F*, *2F*, *5F*, *g2*, *g5*, nested PCR-amplification was performed: (1) 100 pmol of each primer Btd-F1 and Btd-R, for 1 minute at 94°C, 1 minute at 42°C, 1 minute at 72°C (two cycles), 1 minute 94°C, 1 minute at 48°C and 1 minute at 72°C (28 cycles); (2) 100 pmol each primer Btd-F2 and Btd-R for 1 minute at 94°C, 1 minute at 46°C, minute at 72°C (2 cycles), minute at 94°C, 1 minute at 50°C and 1 minute at 72°C (28 cycles). For cloning of the cDNAs *bts1*, *G2*, *g5.6*, *G5*, *G1* and *G4*, two rounds of PCR were performed with primers Btd-F1 and Btd-R, using 100 pmol of each primer and 1/100 of the first PCR reaction product (following gel extraction) as template for the second round. Amplification cycles were as follows: 1 minute at 94°C, 1 minute at 42°C, 1 minute at 72°C (2 cycles); 1 minute at 94°C, 1 minute at 48°C, 1 minute at 72°C (28 cycles). PCR products of the appropriate size (160-180 bp) were purified by gel electrophoresis, subcloned and sequenced. The fragment encoding the zinc-finger domain of Bts1 was used for high-stringency screening of a somitogenesis stage cDNA library (kindly provided by Dr B. Appel). Positive clones containing the full-length *bts1* cDNA (3kb) were obtained, one of these clones was sequenced (Fig.1); its GenBank Accession Number is AF388363.

Drosophila stocks and transgenics

To examine the role played by *bts1* in *Drosophila* we used the IT system (immediate and targeted gene expression) developed by Wimmer et al. (Wimmer et al., 1997). In the conditional *btd>AB >bts1* transgene, the *bts1*-coding region is separated from the *btd* promoter by a flp-out cassette containing *lacZ*. *btd >AB>bts1* was constructed by inserting a 2659 bp *Not*I-*Cla*I fragment containing the entire *bts1*-coding region and 1387 bp 3′UTR into the *btd >AB>btd* plasmid (Wimmer et al., 1997) open at *Not*I, and used to generate transgenic fly lines (Rubin and Spradling, 1982). The stock *β2-tub-flp*/Y; *btd >AB >bts1* /TM3, *hb-lacZ* was established and crossed with *btd*^XG81^/FM7, *ftz-lacZ*. To identify embryos mutant for *btd* and expressing *bts1, lacZ* in situ hybridization was performed. RNA labelling and in situ hybridization were performed as described (Crozatier et al., 1996). RNA probes were prepared from *col*, *eve*, *en* and *lacZ*.

Ectopic expression analyses in the zebrafish (constructs and injections)

For ectopic expression of wild-type *bts1*, *pXT7-bts1Δ3′* was constructed which contains the full-length *bts1*-coding region and 23 nucleotides of *bts1* 3′UTR (*Spe*I fragment from *pBS-bts1*) subcloned into pXT7 (Dominguez et al., 1995). Mutant forms *bts1ΔZnF* and *bts1^C->T^* were constructed with the Stratagene Ex Site^TM^ PCR-based site-directed mutagenesis Kit using the following oligonucleotides:

(1) *bts1ΔZnF*, ONbts1ΔZnF1, 5′-*P*-GATGTGCTGTTTCTTCTTT-CCGGGCTC-3′; ONbts1ΔZnF2, 5′-CAGAACAAGAAGAGC-AAAAGTCACGACAAAAC-3′

(2) *bts1^C->T^*, ONbts1^C->T^-1, 5′-*P*-AGTCCGGACACACAAA-GCGTTTTTCGC-3′; ONbts1^C->T^-2, 5′-ACTATAAAAGGTTCAT-GAGGAGCGACCATTTG-3′.

This mutation alters 2 Cys in 2 Tyr in the third zinc finger (TGCTGT→TACTAT). In a null allele of Btd, the second Cys of the third zinc finger is replaced by a Tyr (Wimmer et al., 1993). As Bts1 harbors two adjacent Cys in position 6 and 7 of the third zinc finger, both were mutated. *bts1ΔZnF* and *bts1^C->T^* were subcloned into pXT7. Capped mRNAs were synthesized (Ambion mMessage mMachine kits) and verified by in vitro translation. Injections were carried out at 100 ng/μl into one central blastomere of the 16-cell embryo (10 pl), together with *nls-lacZ* RNA (40 ng/μl) as lineage tracer, and the distribution of the injected progeny was verified a posteriori by anti-β-galactosidase immunocytochemistry (Bally-Cuif et al., 2000). After appropriate staining, embryos were embedded in JB4 resin (Polysciences) and sectioned at 2 μm on an ultramicrotome (Fig. 5D,F).

Design and injections of the *bts1* morpholinos

MO^bts1^ (5′TACCGTCGACACCGACACGACTCCT3′) (Gene Tools LLC, Corvalis, OR) was designed to target positions 1-25 of the *bts1* cDNA. A four bp mismatch morpholino (MO^bts1^Δ4) (5′TACTGTTGACACCGACACAACCCCT3′) was used as control. A morpholino of unrelated sequence (5′CCTCTTACCTCAGTT-ACAATTTATA3′), biotinylated in 3′ and aminated in 5′ to allow for fixation, was used as a lineage tracer when single cell resolution was necessary (Fig. 6C). For detection of the tracer MO (Fig. 6C), embryos were processed first for in situ hybridisation followed by incubation in avidin-biotinylated β-gal complex (Vector, Roche) revealed with X-gal staining. In other cases, *nls-lacZ* RNA was used as tracer (Fig. 6D-H). All MOs were injected at 1-2 mM in H₂O into a central blastomere of 16-cell embryos.

Transplantation experiments

The full-length coding region of mouse *Wnt1* cDNA (van Ooyen and Nusse, 1984) was subcloned into pXT7 and used to generate capped mRNA. *Wnt1* RNA was injected at 10 ng/μl together with *nls-lacZ* RNA (40 ng/μl) at the one-cell stage, and animal pole cells from injected embryos at the sphere stage were homotopically and isochronically transplanted into non-injected recipients.

Inhibition of Fgf signalling by SU5402

Embryos were incubated in 12 μM SU5402 (Calbiochem) in embryo medium from the dome stage until late gastrulation, and then immediately fixed and processed for in situ hybridisation. To control for SU5402 efficiency, embryos similarly treated from the shield stage were verified to develop a phenotype morphologically indistinguishable from *ace* mutants in the MHD area (not shown).

In situ hybridization and immunocytochemistry in the zebrafish

In situ hybridization and immunocytochemistry were carried out according to standard protocols (Thisse et al., 1993; Hauptmann and Gerster, 1994).

RESULTS

Cloning of *buttonhead*-related genes in the zebrafish

We PCR-amplified tail bud stage wild-type zebrafish cDNA using degenerate oligonucleotides directed against the zinc-finger domains of Btd and Sp factors. Eleven partial cDNAs encoding zinc finger domains were obtained (Fig. 1A), each of them from several distinct PCR reactions, suggesting that they correspond to different genes and not to variations due to Taq polymerase errors. All code for triple zinc fingers, 55-85% similar to each other and with the structure Cys₂-His₂ characteristic of Btd and Sp factors (Kadonaga et al., 1987; Kingsley and Winoto, 1992; Wimmer et al., 1993; Pieler and Bellefroid, 1994; Supp et al., 1996; Wimmer et al., 1996; Harrison et al., 2000). They were named *bts* genes (for *btd/Sp*-related). Except in two cases (g5.6 and g5), they are more closely related to the zinc-finger domain of Sp factors (70-94% identity) than to that of Btd (64-80% identity). g5.6 is equally related to Sp and Btd (75% identity), and g5 is more closely related to Btd than to Sp (69% versus 56% identity).

To determine whether one of these factors could be a functional equivalent of Btd at the *Drosophila* head-trunk junction, we examined their expression profiles at the tail bud stage using high-stringency whole-mount in situ hybridization conditions. With the exception of *g5.6* and *G1*, which proved ubiquitously expressed, all other genes tested showed spatially restricted and distinct expression patterns (Fig. 1A), further confirming that they corresponded to different factors. One of them, *bts1*, appeared selectively expressed in the MHD (see Fig. 3), and was therefore selected for further studies. *g5*, the most related in sequence to *btd*, was not expressed in the mid-hindbrain and thus appeared unlikely to be a functional homologue of *btd* in this domain.

High-stringency screening of a zebrafish somitogenesis-stage library with the PCR product of *bts1* produced six positive clones, covering all or part of the same 3 kb cDNA. The longest open reading frame (1102 nucleotides) is preceded by 126 nucleotides of 3′UTR containing a classical Kozak sequence and two in-frame stops upstream of a start methionine (not shown), and predicts a 368 amino acid protein (Fig. 1B). In agreement with these findings, the in vitro translated products of the entire cDNA (containing 1727 nucleotides 5′UTR) and that of its predicted coding region (*bts1Δ3′*, see Materials and Methods) had the same apparent size (40 kDa) (data not shown). The deduced protein Bts1 presents features characteristic of Btd/Sp factors (Pieler and

A.
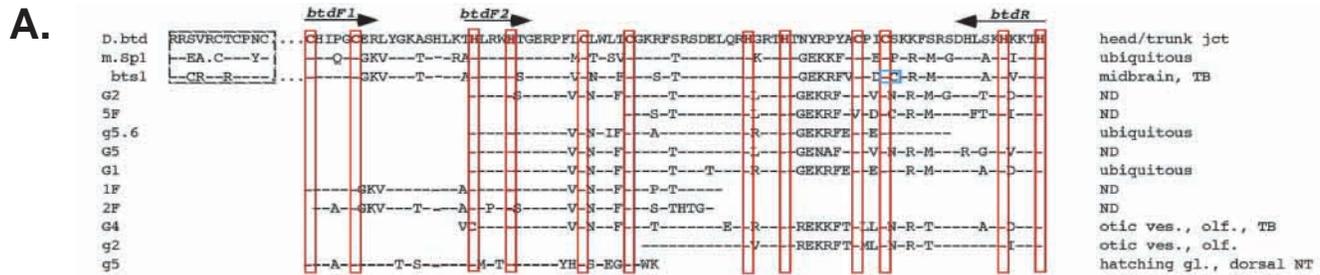
B.

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1 MAAVAVLRNDTLQAFLQDRQPNSSPENSKHSPLALLAATCNRIGHHHGSTPTDFIQVPYDTTLGSPSRIFHPWSNYANHGSTLSSNPSFGLSSKSHLQ
SSYASHHELPLTPPADPTYPMISLRLRCCHVNASLQSTCPPTYVPAVTYAAPAPIPPAMPSFVPEHSELVHQQQRQLSPNPGEDIPWWSLQQGNPVAHSV
HPHRFPIQRGLVLGHTDFAQYQTQIAALLHTKSPLATARRCRRGRCPNCQSSSSSDEPGKKKQHICHIPGCGKVYGKTSHLKAHLRWHSGERPFVCNWLF
CGKSFTRSDELQRHGRTHTGEKRFVCPDCCKRFMRSDHLAKHVKTHQNKKSKSHDKTSDYVKREDLRPRV* 368
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C.
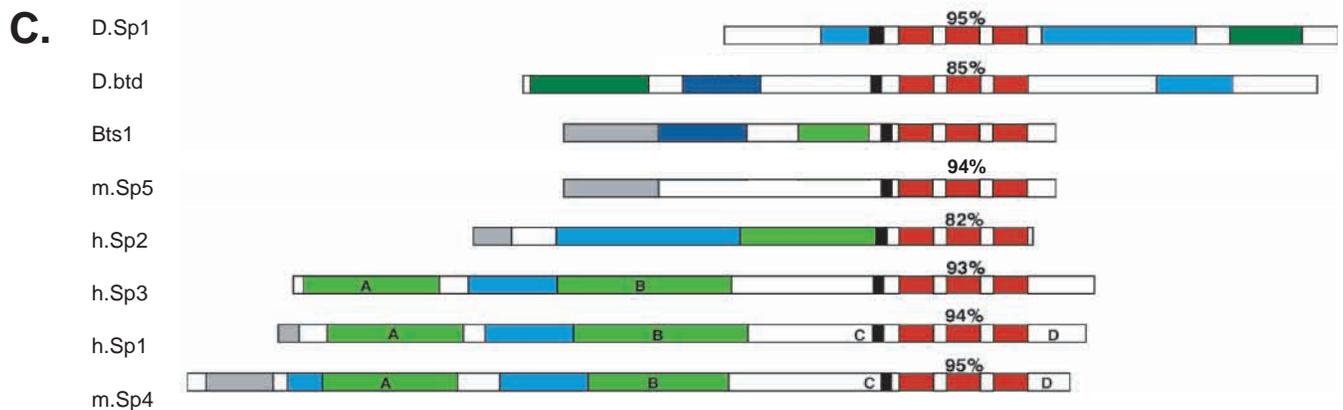
Fig. 1. Structure of the Bts1 protein. (A) Zinc finger domains of the 11 zebrafish Btd/Sp-family members (Bts proteins) isolated, aligned with the corresponding domains of *Drosophila* Btd (Wimmer et al., 1993) and mouse Sp1 (Kadonaga et al., 1987). Positions of the primers used in the degenerate PCR reaction are indicated (arrows). Each zinc finger has the structure $3x(C_2H_2)$ (red boxes highlight Cys and His residues) and is preceded by a 'Btd box' (boxed in black for Btd, mouse Sp1 and Bts1, not indicated for others). The Cys doublet mutated in the negative control-construct Bts1$^{C \rightarrow T}$ (see Fig. 5) is boxed in blue. The expression profile of each *bts* gene at the tail bud stage is summarized in the right column. jct, junction; gl, gland; ND, not determined; NT, neural tube; olf, olfactory placodes; TB, tail bud; ves, vesicle. (B) Sequence of the Bts1 protein. The zinc-finger domains are in red and the Btd-box is boxed in black. S/T and Q-rich, potential transcriptional activation domains are, respectively, in green and blue. The N-terminal domain resembling that of Sp1, Sp2, Sp4 and Sp5 is underlined. (C) Structural alignment of Bts1 and other Btd/Sp proteins (Kadonaga et al., 1987; Hagen et al., 1992; Wimmer et al., 1993; Wimmer et al., 1996; Supp et al., 1996; Harrison et al., 2000). Percentages of similarity between Bts1 and other proteins are given for the zinc finger/Btd box (red/black). Q-rich domains are blue (the Q domain of Bts1 only resembles that of Btd (dark blue) but does not align with others (light blue)). S/T-rich domains are green and the N-terminal domain grey. The transcriptional activation domains identified in Sp1, Sp3 and Sp4 are labelled A-D.

Bellefroid, 1994), such as the triple zinc-finger domain (showing highest homology to those of Sp1, Sp3, Sp4 and the recently isolated Sp5) preceded by an arginine-rich 'Btd box' (Fig. 1B,C), a motif implicated in some cases of transcriptional activation by Sp1 (Athanikar et al., 1997). Outside the zinc fingers and Btd box, recognizable motifs include serine/threonine and glutamine-rich regions in the N-terminal half of Bts1. Such domains have been identified in Btd and Sp factors, and were in most instances shown to mediate transcriptional activation (Courey and Tjian, 1998; Kadonaga et al., 1998). The 43 N-terminal amino acids of Bts1 also show significant similarity to the N termini of Sp1, Sp2, Sp4 and Sp5. Outside these domains, similarity with other Sp-like factors is low. Highest homology is found with Sp5 (52% overall identity) but does not reflect an ungapped alignment (see Fig. 1C). *bts1* was mapped in radiation hybrid panels to linkage group 9,

0.10 cM from marker fb18h07, close to the *hoxd* locus (not shown).

In conclusion, *bts1* shows higher overall sequence similarity with Sp factors than with Btd, but its restricted expression in the mid-hindbrain area at the end of gastrulation, is strongly reminiscent of the local expression of *btd* at the head-trunk junction.

Bts1 binds canonical GC boxes and can act as a transcriptional activator in vivo

The sequence of the zinc-finger domain of Bts1 predicts, in analogy to Sp factors, a DNA recognition sequence of the GC box class (Dynan and Tjian, 1983; Gidoni et al., 1984; Gidoni et al., 1985). To investigate the DNA-binding characteristics of Bts1, in vitro transcribed and translated (rabbit reticulocyte lysate) *bts1* protein product was tested in electromobility shift
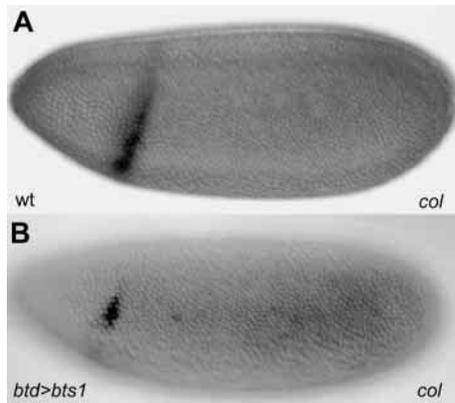
Fig. 2. Bts1 is a transcriptional activator in vivo. Expression of *collier* (*col*) revealed by in situ hybridization at the head-trunk junction of the *Drosophila* blastoderm in wild-type embryos (A) and in *btd* mutant embryos carrying one copy of *bts1* under control of *btd* regulatory elements (B). *btd* mutants show no expression of *col* (not shown). *bts1* can partially rescue *col* expression in *btd* mutants, in a correct spatiotemporal manner.

assay with the zinc-finger binding site of the mouse Pax5 enhancer (Pax5 ZN) (Pfeffer et al., 2000). Bts1 was found to specifically bind to Pax5 ZN but was unable to bind a mutated version of Pax5 ZN in which the zinc-finger binding site has been destroyed (Pfeffer et al., 2000). Thus Bts1 is capable of binding GC boxes in vitro.

Sp factors are highly divergent outside the zinc-finger domain and can act as transcriptional activators or repressors (Majello et al., 1994; Birnbaum et al., 1995; Hagen et al., 1995; Kennett et al., 1997; Kwon et al., 1999; Turner and Crossley, 1999), probably following their interaction with different molecular partners. To determine whether Bts1 behaved as an activator or as a repressor of transcription, we tested whether it could substitute for Btd function in *Drosophila*. Indeed Btd was shown to be a transcriptional activator of the downstream gene *col* (Crozatier et al., 1996), which is necessary for the development of the intercalary and mandibular segments of the head (Crozatier et al., 1999). Transgenic flies were constructed which carry the coding sequence of *bts1* under the control of the *btd* enhancer (Wimmer et al., 1997) (*btd>bts1* flies) and were introduced into a *btd* background. At the blastoderm stage, *btd* embryos completely fail to express *col* (not shown, see Crozatier et al., 1996). We observed that Bts1 was sufficient to partially rescue the expression of *col* in *btd* embryos (Fig. 2B), in a correct spatiotemporal manner along the anteroposterior axis (although in a reduced number of cells, even with two copies of *btd>bts1*; not shown) (compare with Fig. 2A). Thus, at least in this cellular context, Bts1 acts as an activator of transcription.

The similar expression profiles of *bts1* and *btd* at gastrulation, at the junction between anterior and posterior embryonic patterning systems, suggested equivalent developmental functions. However Bts1 and Btd are highly divergent outside the zinc-finger domain, questioning their possible interaction with homologous molecular partners. In addition to *col*, *btd* mutants also fail to express *eve* stripe 1 (Vincent et al., 1997) and *engrailed* (*en*) in the head (Wimmer et al., 1993). Later they lack antennary, intercalary and mandibular head segments. We observed that neither *eve*(1) and *en* expression nor larval head

structures was rescued in *btd>bts1* transgenics (not shown). Thus, our results suggest that the correct spatiotemporal activation of *col* mainly requires the zinc-finger domain of Btd, whereas the enforcement/maintenance of *col* expression, as well as the expression of *eve*(1), *en* and the subsequent development of head segmental derivatives would require stronger activity or additional, non-zinc-finger protein modules that are not present in Bts1.

bts1 expression matches the presumptive mid-hindbrain area from mid-gastrulation stages

The spatiotemporal expression of *bts1* at early developmental stages in the zebrafish was determined by whole-mount in situ hybridization. *bts1* transcripts are first detected at 30% epiboly, in the most marginal cells of the blastoderm and in the yolk syncitial layer, excluding the dorsal embryonic side (Fig. 3A,A′). Expression is maintained in epiblastic cells at the margin during gastrulation, with a broader anteroposterior extent as epiboly progresses (Fig. 3B-E). In addition, a restricted number of cells of the dorsal hypoblast, lining the presumptive prechordal plate and anterior notochord, express *bts1* (Fig. 3C-D′). From 70% epiboly, the anterior limit of *bts1* expression in the dorsolateral epiblast is clearly delimited (Fig. 3D-F, arrows), and lies within the presumptive MHD (see below and Fig. 4). At the end of gastrulation, *bts1* transcription in epiblast cells becomes restricted to the MHD and tail bud. It remains prominent in the MHD until at least 24 hours (Fig. 3F-J and not shown). Additional sites of expression arising during late somitogenesis are the otic vesicles, the somites, and restricted nuclei of the diencephalon (Fig. 3I,J).

To precisely position the domain of *bts1* expression within the presumptive neural plate, we compared its location with known forebrain, MHD or hindbrain markers (Fig. 4). At 75% epiboly, the anterior border of *bts1* expression is located within the posteriormost cell rows of the *otx2*-positive territory, abutting the diencephalic 'wings' of *fkh3* expression (Fig. 4A-C). *bts1* expression overlaps the *her5*-positive domain (Fig. 4D), which slightly crosses the *otx2* border (Fig. 4E). At the tail bud stage, *bts1* expression has acquired a posterior limit (see Fig. 3G). It encompasses the *her5-* and *wnt1*-positive domains (Fig. 4G,I), and largely overlaps *pax2.1* expression, albeit with a slight rostral shift (Fig. 4J). All four domains expressing *bts1*, *her5*, *pax2.1* and *wnt1* extend several cell rows posterior to the caudal limit of *otx2* (Fig. 4H). These spatial relationships were maintained at the five-somite stage (Fig. 4M-R).

The anterior 'wings' of *fkh3* expression have been fate-mapped to the presumptive diencephalon at the 80% epiboly stage (Varga et al., 1999), and *her5* expression to the presumptive midbrain (with a minor contribution to the anterior hindbrain) at 90% epiboly (Müller et al., 1996). Therefore, at 80% epiboly, *bts1* expression in the neural plate comprises the midbrain and more posterior domains, and it is refined to the midbrain and anterior hindbrain from 90% epiboly onwards. These features make *bts1* the earliest known gene expressed across the entire MHD (see Discussion) and suggest that it might be involved in early mid-hindbrain positioning or patterning.

Bts1 is an early regulator of *pax2.1* expression in the zebrafish MHB

We addressed the function of Bts1 within the zebrafish embryonic neural plate using a combination of gain- and loss-
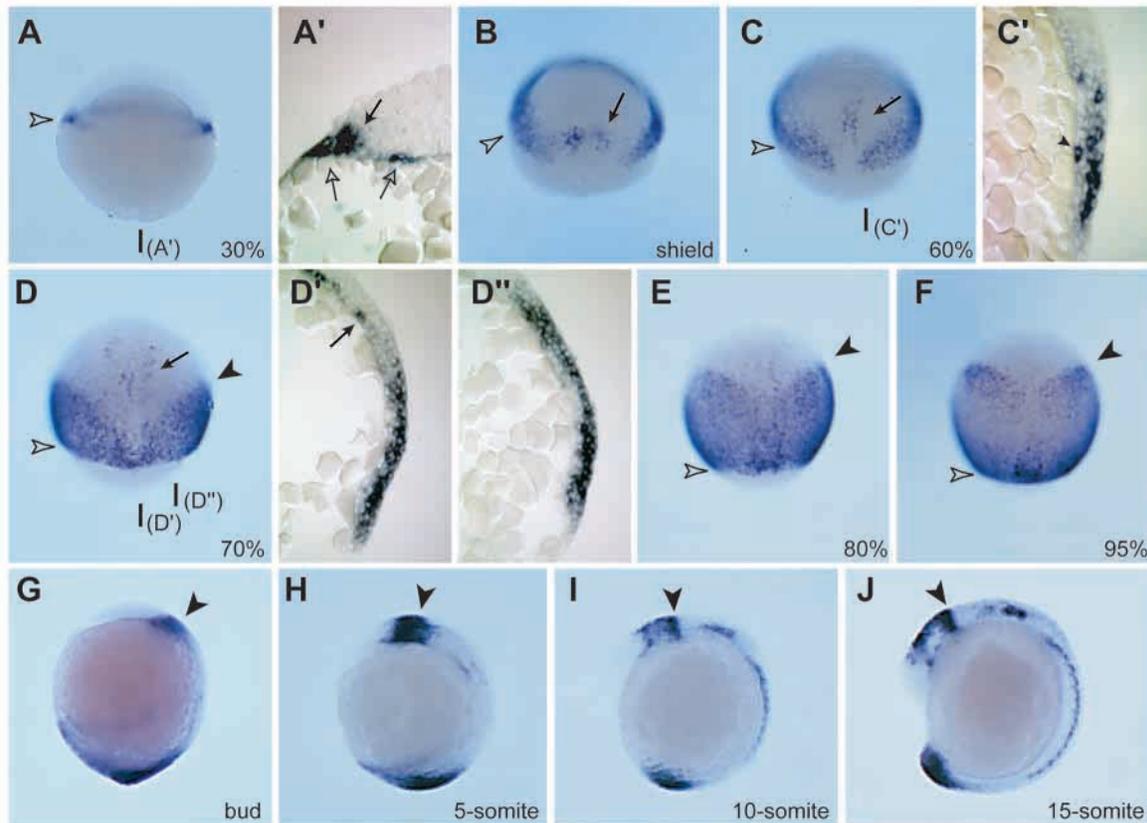
Fig. 3. Expression of *bts1* during gastrulation and early somitogenesis, as revealed by whole-mount in situ hybridization at the stages indicated (% of epiboly). (A-F) Dorsal views, anterior towards the top; (G-J) sagittal views, anterior towards the left. Open arrowheads indicate the blastoderm margin, black arrowheads the mid-hindbrain domain, and small arrows point at hypoblastic expression. (A′,C′,D′,D″) Sagittal sections at the levels indicated, dorsal towards the right, anterior towards the top. *bts1* expression is first detected at 30% epiboly (A,A′) along the ventral and lateral margins of the blastoderm (arrows) and in the yolk syncitial layer (small arrow). During gastrulation (B-F), expression is maintained in the posterior epiblast up to a sharp limit at the mid-hindbrain level, and in hypoblast cells bordering the prechordal plate (small arrow). From the end of gastrulation (F-H), *bts1* expression is confined to the mid-hindbrain level and tail bud and extinguishes from the rest of the epiblast. Additional expression sites during later somitogenesis (I,J) include the otic vesicle, somites and diencephalon.

of-function experiments. To target misexpressions to the neuroectoderm, we injected capped *bts1* mRNA within one central blastomere of the 16-cell blastula. At the 16-cell stage, the four central blastomeres largely contribute to neuroectodermal derivatives (Helde et al., 1994; Wilson et al., 1995). Co-injected *lacZ* RNA served as lineage tracer and we only scored cases where *lacZ*-positive cells were distributed primarily within the neuroectoderm (Fig. 5D,F). Mesodermal markers were unaffected (see *gsc* on Fig. 5E,F; *ntl* and *papc* (data not shown)). Upon misexpression of *bts1*, 50% of embryos injected into regions of the neural plate encompassing the MHD or anterior to it (*n*=72) showed an ectopic expression of *pax2.1* at the tail bud stage (Fig. 5A,B,D-F). By contrast, no induction of *pax2.1* was ever observed in embryos injected only into neural territories posterior to the MHD, or within the epidermis outside the neural plate (*n*=83). Induction of *pax2.1* expression always occurred anterior to the MHD, either in broad patches connected to the MHD (Fig. 5A,D-F) or in scattered cells (Fig. 5B) (at approximately equal frequencies), and in territories showing a high density of injected cells. Within these areas, ectopic *pax2.1* expression appeared restricted to *lacZ*-positive cells (Fig. 5D,F). Notably, no other marker of the early MHD (*otx2*, *her5*, *wnt1*, *eng2*, *pax5*, *pax8*)

proved responsive to *bts1* injections (not shown), thus the effect of Bts1 on *pax2.1* expression appeared highly selective. Finally, no patterning defects of the anterior neural plate were observed at somitogenesis or later stages in *bts1*-injected embryos, suggesting that the maintenance of ectopic *pax2.1* expression requires factors other than Bts1 and/or requires the persistence of Bts1 expression. Two mutant versions of *bts1* were constructed as negative controls. *bts1ΔZnF* is deleted in the entire zinc finger-encoding domain of *bts1* and thus should encode a protein incapable of binding DNA. The second mutant form of *bts1*, *bts1$^{C->T}$*, was designed to mimic the *btd* loss-of-function mutation in *Drosophila* (see Materials and Methods). *bts1ΔZnF-* and *bts1$^{C->T}$*-capped RNAs were injected as described for wild-type *bts1* and at similar concentrations; both proved incapable of inducing *pax2.1* expression (100% of cases, *n*=23 and *n*=29, respectively) (Fig. 5C, and data not shown). Taken together, our results indicate that the ectopic expression of Bts1 is sufficient to induce *pax2.1* expression within neural territories anterior to the MHD during gastrulation.

We next determined whether *bts1* expression was also necessary to MHD development and/or *pax2.1* expression (Fig. 6A-D). Antisense 'morpholino' oligonucleotides have now
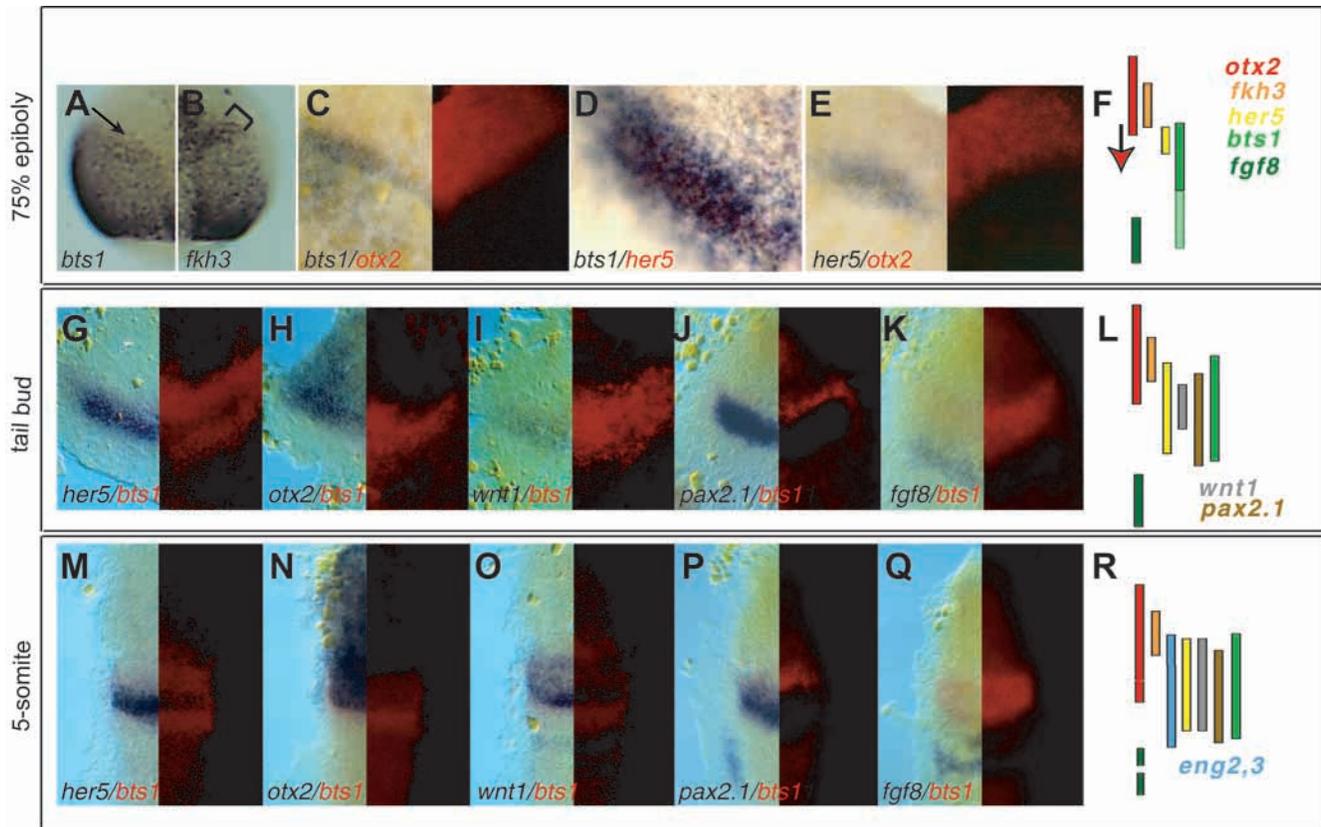
Fig. 4. Comparison of *bts1* expression with other mid-hindbrain markers. Whole-mount in situ hybridization was performed at the 75% epiboly (A-E), tail bud (G-K) and five-somite (M-Q) stages with the probes indicated (colour-coded) (dorsal views, anterior towards the top). (A,B) Single staining for *bts1* and *fkh3*, respectively (whole-mount views of half embryos) (arrow in A indicates anterior limit of *bts1*; bracket in B indicates 'diencephalic wings' of *fkh3* expression). (D) Bright-field view of a flat-mounted MHD, all other panels show a bright field view (left, red and blue labelling) and the contralateral fluorescence view (right, red labelling only) of flat-mounted neural plates. (F,L,R) Corresponding schematics of genes expression profiles (including data not shown) at 75% epiboly, tail bud and five somites, respectively. Note that anteriorly, *bts1* expression never extends to the presumptive diencephalon (compare A with B), and that it crosses the caudal border of *otx2* expression at all stages.

proven to reliably and selectively inhibit RNA translation in many instances in *Xenopus* as well as in the zebrafish embryo (Heasman et al., 2000; Nasevicius and Ekker, 2000; Yang et al., 2001). A morpholino targeting the translation initiation site of *bts1* mRNA was designed (MO^{bts1}) and injected into a central blastomere of the 16-cell zebrafish embryo together with a tracer MO (MO^{ctrl}) (see Material and Methods). At the same concentration, a four base-pair mismatch control MO (MO$^{bts1}\Delta4$) of unrelated sequence had no effect (*n*=32) (Fig. 6D). In all embryos injected with MO^{bts1} across the MHD (*n*=23) and observed at the tail bud stage, a strong reduction of *pax2.1* expression was observed (Fig. 6A) (lineage tracing experiments often revealed a unilateral and patchy distribution of the injected cells; accordingly, *pax2.1* expression was most often diminished on only one side of the neural plate). To determine whether *bts1* expression was necessary to induce and/or maintain *pax2.1* expression, we performed a timecourse analysis of the effect of the MO^{bts1}. We observed that *pax2.1* expression was abolished from its onset (90% epiboly) (*n*=13, Fig. 6B), indicating that *bts1* is necessary for *pax2.1* induction. Some *pax2.1*-expressing cells were always retained. Their varying number and distribution in each embryo (see Fig. 6A,B) suggests that these cells were most likely not or poorly

targeted by the injection. Co-detection of *pax2.1* expression and MO^{ctrl} confirmed this hypothesis as cells maintaining *pax2.1* transcripts do not stain for MO^{ctrl} (Fig. 6C). Therefore, Bts1 appears necessary in all MHD cells for *pax2.1* induction.

However, at the concentrations of MO^{bts1} used, *pax2.1* expression was progressively recovered between the five- and ten-somite stages (*n*=26) (see Fig. 6I,J), and brain development appeared normal at late somitogenesis stages (not shown).

Taken together, our results reveal that *bts1* expression is sufficient to induce ectopic expression of *pax2.1* in the neural plate anterior to the MHD, and is necessary for the induction and early maintenance of *pax2.1* expression in the MHD. Thus endogenous Bts1 may be an early regulator of *pax2.1* expression, a conclusion supported by its expression profile (Fig. 4).

Distinct requirements of mid-hindbrain markers for *bts1* expression

We next examined whether MHD genes other than *pax2.1* require *bts1* for their expression. Upon injection of MO^{bts1} within the embryonic neural plate, the expressions of *her5*, *otx2*, *fgf8*, *wnt1*, *eng2* and *krox20* were never affected (Fig. 6E-H and data not shown). By contrast, expression of *eng3* and

Fig. 5. Bts1 is sufficient to induce *pax2.1* expression in the anterior neural plate. *pax2.1* (A-D) or *pax2.1* and *gsc* (E,F) expression revealed by whole-mount in situ hybridization (blue staining) at the tail bud stage on embryos injected with (A,B,D-F) wild-type *bts1* RNA, or (C) mutant *bts1^{C->T}* RNAs, as indicated (bottom left of each panel). (A-C) High magnifications of the MHD in flat-mounted embryos, anterior towards the top. (E) A whole-mount view, anterior towards the left. (D,F) Sections of the embryos in A,E (respectively) at the levels indicated, anterior towards the left. The red arrows in E,F point to *gsc* expression, and the broken line in F delimits the anterior mesendoderm/neural plate border. All injections were made into in one central blastomere of the 16-cell embryo, leading to a mosaic distribution of the injected RNA in the presumptive neural plate (see

expression of the β-galactosidase tracer (brown nuclei) and in particular D,F). Misexpression of *bts1* induces ectopic *pax2.1* expression (black arrows in A,B,E, black bars in D,F) anterior to the MHD (endogenous *pax2.1* expression is indicated by the white arrowheads or white bars), in broad patches (A,D-F) or in scattered cells (B). Mutant *bts1* RNAs (C, and data not shown) have no effect.
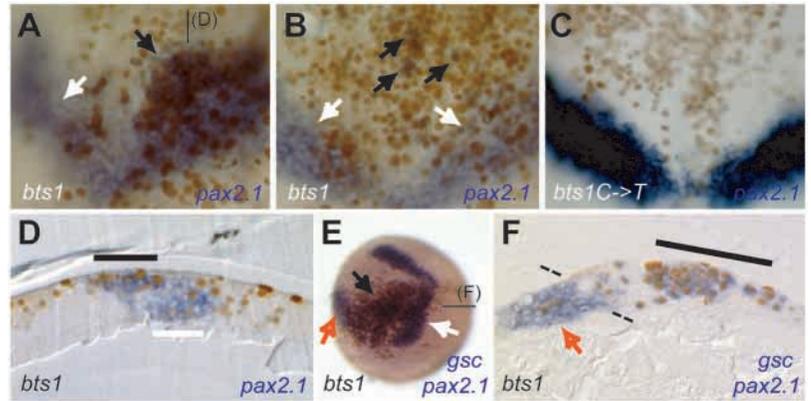
Fig. 6. Bts1 is necessary to the expression of *pax2.1* and its dependent cascade in the MHD. (A-D) *pax2.1* expression revealed by whole-mount in situ hybridisation (purple) at 90% epiboly (B) or tail bud (A,C,D) after injection of MO^bts1 (A-C) or the mismatch control MO^bts1Δ4 (D). All injections were made at the 16-cell stage into one central blastomere. (C) A biotinylated control MO of unrelated sequence co-injected as a tracer (turquoise staining) to monitor the exact distribution of targeted cells (turquoise arrows) compared with *pax2.1*-expressing cells (purple arrows); the area shown is a high magnification of the domain indicated by the black arrow in the inset. (D) *nls-lacZ* RNA used as a tracer to reveal the targeted area (brown staining). (A) Whole-mount views; (B-D) flat-mounts, anterior towards the top; arrows point to injected areas (affected and unaffected expression are indicated by filled and open arrows, respectively). Note that the injection of MO^bts1, but not MO^bts1Δ4, strongly diminishes the number of *pax2.1*-positive cells from the onset of *pax2.1* expression (B), and that cells maintaining *pax2.1* expression have not been targeted by the injection (C). (E-H) Expression of *her5* (E, tail bud), *fgf8* (F, tail bud), *wnt1* (G, one to two

somites) and *eng2* (H, three somites) upon injection of MO^bts1 (conditions as in D). Note that these expression are unaffected. (I-N) Expression of *pax2.1* (I,J), *eng3* (K,L) and *pax5* (M,N) at the five-somite stage upon injection of control MO or MO^bts1, as indicated. (I,J) Dorsal views, anterior towards the top; (K-N) Optical coronal sections, dorsal towards the top. Note that at five somites, the *pax2.1*-dependent markers *eng3* and *pax5* are also affected.
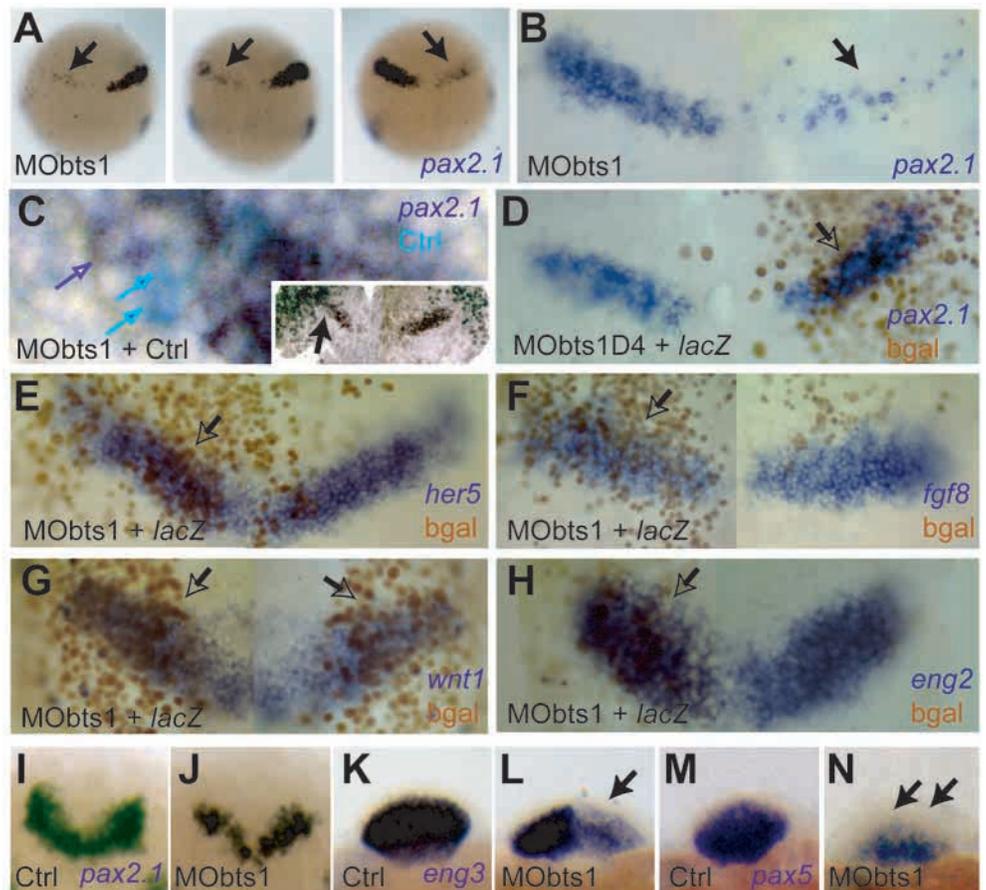
pax5, starting at the three- and five-somite stages, respectively, were transiently inhibited from their onset until approximately the 10-somite stage (Fig. 6K-N). Thus, first, the territories located anterior and posterior to the MHD do not require Bts1 for their early development. Second, at least two initially

independent early gene regulatory pathways operate within the MHD: one requires Bts1 and permits the induction of *pax2.1* expression, and the other is independent of Bts1 and leads to the induction of expression of *her5*, *wnt1*, *eng2* and *fgf8*. Whether *pax5* and *eng3* expressions are directly regulated by
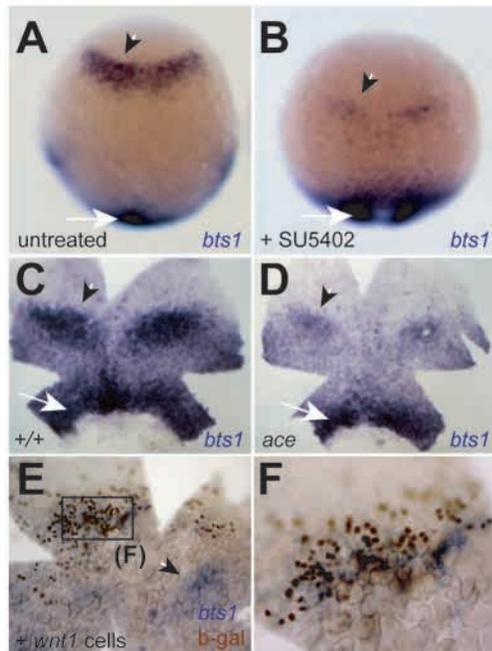
Fig. 7. *bts1* expression at the MHD during gastrulation requires Fgf8 and is activated by Wnt signalling. (A,B) Whole-mount dorsal views of *bts1* expression at the tail bud stage, anterior to the top, without (A) or after (B) treatment with the inhibitor of Fgf signalling SU5402 between the stages dome and tail bud. Note the strong reduction in expression at the mid-hindbrain in B (arrowhead), while expression at the blastoderm margin is not affected (white arrow). (C,D) Flat-mounted views of *bts1* expression in the mid-hindbrain area at the 90% epiboly stage, anterior towards the top, in wild-type (+/+) (C) versus *ace* homozygous mutants (D), as indicated. Mid-hindbrain expression of *bts1* is strongly reduced and maintained only laterally (arrowheads); it remains unperturbed at the blastoderm margin (white arrow). (E,F) *bts1* expression in embryos grafted with *wnt1*-expressing cells within the anterior neural plate. Endogenous *bts1* expression at the MHD is indicated by the arrowhead. Grafted cells were co-injected with *nls-lacZ* RNA and are visualized by anti-β-galactosidase immunocytochemistry (brown nuclei). (F) A high magnification of the grafted area (boxed in E). *bts1* expression is induced around *wnt1*-expressing cells.

Bts1 cannot be immediately concluded from our data, as *pax5* and *eng3* expressions require Pax2.1 at all stages (Lun and Brand, 1998; Pfeffer et al., 1998).

bts1 expression at gastrulation responds to Fgf and Wnt signalling

The crucial role of Bts1 as a selective regulator of *pax2.1* within the neural plate prompted us to investigate the mechanisms regulating its own expression.

Fgf3 and Fgf8 are expressed at the blastoderm margin during gastrulation (Fürthauer et al., 1997; Koshida et al., 1998; Reifers et al., 1998) and the reception of an Fgf signal by marginal cells has been indirectly implicated in the posteriorization of the adjacent neural plate (Koshida et al., 1998). To determine whether *bts1* expression was influenced by Fgfs during gastrulation, we examined its response to SU5402, a general inhibitor of Fgf signalling (Mohammadi et al., 1997). Incubation of embryos in SU5402 from the dome stage onwards lead to a strong reduction of *bts1* expression at
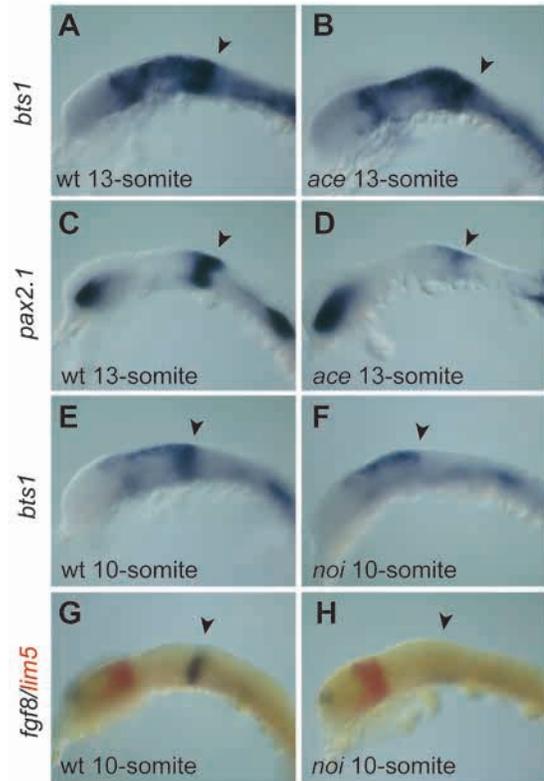
Fig. 8. *bts1* expression during somitogenesis distinguishes Pax2.1 and Fgf8 functions. (A-D) Comparison of *bts1* and *pax2.1* expression in wild-type (left) or *ace* mutant (right) embryos at the 13-somite stage. The MHD is indicated by the arrowhead. *bts1* expression is unperturbed in *ace* (B), when most *pax2.1* expression has already been eliminated (D). (A,B) *ace* embryos identified by their reduced otic vesicles, which also express *bts1* (not visible on the figure). (E-H) Comparison of *bts1* and *fgf8* expression in wild-type (left) or *noi* mutant (right) embryos at the 10-somite stage. *bts1* expression is strongly diminished following the same schedule as other MHD markers (e.g. *fgf8*). *lim5* expression (red) is unperturbed.

the presumptive MHD (Fig. 7A,B). Thus, during gastrulation, *bts1* expression within the neural plate depends on Fgf signalling. By contrast, expression of *bts1* at the blastoderm margin (or later in the tail bud, Fig. 7A,B) remained unaffected by SU5402 treatments. To determine which combination of Fgf3 and Fgf8 might be involved in the early regulation of *bts1* expression in the MHD, we examined *bts1* expression in *acerebellar* (*ace*) mutants, which are solely deficient in Fgf8 function (Reifers et al., 1998). At the 90% epiboly stage, *bts1* expression in the presumptive MHD was severely reduced in 25% of embryos from a cross between two *ace/+* parents (*n*=63) (Fig. 7C,D). Thus, *bts1* expression in the presumptive MHD at gastrulation probably requires Fgf8 signalling, originating from the hindbrain territory or marginal cells (see Reifers et al., 1998). Whether this signal acts directly within the neural plate or via patterning the embryonic margin cannot be ascertained at this point.

bts1 expression was never totally abolished in the absence of Fgf signalling, however, suggesting that additional factors contribute to regulating its expression. As Wnt molecules are produced both at the embryonic margin (Wnt8) (Kelly et al.,

1995) and at the mid-hindbrain junction (Wnt1, Wnt8b) (Molven et al., 1991; Kelly et al., 1995), we tested whether *bts1* expression was also responsive to Wnt signalling. Capped mRNA encoding the mouse Wnt1 protein (van Ooyen and Nusse, 1984) was injected at the one-cell stage into donor embryos, and five to ten cells taken at the sphere stage from the animal pole of these donors were homotopically transplanted into non-injected recipients. At 80% epiboly, 50% of grafted embryos (*n*=38) had received Wnt1-expressing cells within the neural plate anterior to the MHD, i.e. in a region normally not expressing *bts1*. In 30% of these embryos, *bts1* expression was induced around the grafted cells (Fig. 7E,F). Mouse Wnt1 is likely to have the same activity as zebrafish Wnt1, as embryos injected at the one-cell stage displayed a strong headless phenotype (not shown) characteristic of enhanced zebrafish Wnt signalling (Kim et al., 2000). Thus, ectopic Wnt signalling can positively regulate *bts1* expression within the neural plate, and the expression of endogenous *bts1* might also depend on Wnt factors produced at the embryonic margin and/or within the MHD during gastrulation and somitogenesis. Again, this regulation might or not occur directly within the neural plate.

The maintenance of *bts1* expression is differently affected by Pax2.1 and Fgf8 functions

In agreement with the early onset of *bts1* expression in the prospective MHD area, we found that the initiation of *bts1* expression was not affected in *pax2.1/noi* mutant embryos (Lun and Brand, 1998), and thus was independent of Pax2.1 function (not shown). However, the maintenance of *bts1* expression in the MHD during somitogenesis appeared dependent on *pax2.1/noi*: it was gradually lost from the five- to six-somite stage onwards in *noi* homozygous embryos, and disappeared completely by the 10-somite stage (Fig. 8E,F), following the same schedule as other mid-hindbrain markers (see Fgf8 on Fig. 8G,H; Lun and Brand, 1998). The maintenance of expression of all MHD genes studied to date was shown to be also dependent on Fgf8/ace function, within a similar time frame (between the five- and ten-somite stages), suggesting that Fgf8 and Pax2.1 are involved in a common regulatory loop that controls MHD maintenance (Lun and Brand, 1998; Reifers et al., 1998). Thus, surprisingly, we found that following a transient decrease at gastrulation (Fig. 7) *bts1* expression was not affected in *fgf8/ace* mutant embryos at somitogenesis until late stages. At 13 somites, *bts1* expression was normal (Fig. 8A,B), while the lateral and ventral expression domains of other markers were already absent (see *pax2.1* on Fig. 8C,D; Reifers et al., 1998). *bts1* expression started to decline around the 17-somite stage, and was undetectable at 20 somites (not shown). This downregulation might parallel the loss and/or transformation of mid-hindbrain tissue, which is likely to start around that stage. Thus, while *bts1* maintenance depends on Pax2.1, it appears primarily independent of Fgf8 function, suggesting that exit points exist in the Pax2.1/Fgf8 loop to differentially control the expression of some MHD genes.

DISCUSSION

In this study, we relied on the comparable locations of the
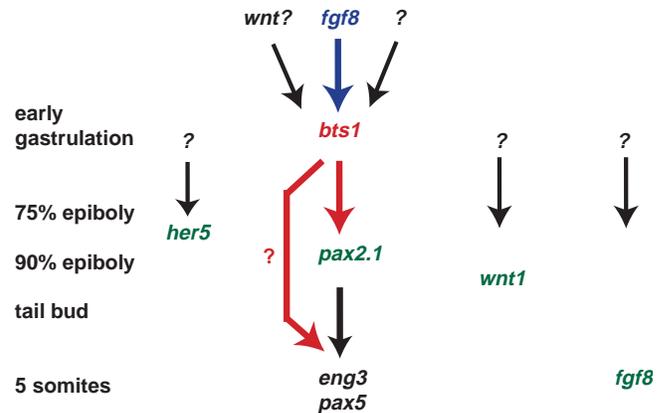
Fig. 9. A model of MHD induction incorporating Bts1 function. Evidence in all vertebrates suggest that the expression of early MHD markers (*her5*, *pax2.1*, *wnt1* and *fgf8*) (green) is established by following independent pathways. Bts1 (red) is a selective inducer of *pax2.1* expression, and its own expression depends on Fgf8 signalling (blue). Other factors regulating *bts1* expression might include Wnt molecules. In turn, Pax2.1 induces *eng3* and *pax5*. Bts1 might also directly regulate the expressions of *eng3* and *pax5* (red arrows).

Drosophila head-trunk junction and of the vertebrate mid-hindbrain within the embryonic body plan to identify candidate regulators of early mid-hindbrain development. In *Drosophila*, Btd is expressed at the head-trunk junction and the zebrafish Btd-related factor Bts1 is an early marker of the MHD. We demonstrate that, in the zebrafish, Bts1 is both necessary and sufficient for the induction of *pax2.1* expression within the anterior neural plate and is expressed at the appropriate time and place during development to exert such a role. We therefore move one step upstream in our understanding of MHD specification by identifying the first known selective and early regulator of *pax2.1* expression (Fig. 9). In addition, our results have important evolutionary implications. They suggest that flies and vertebrates have probably evolved a similar mechanism to cope with the patterning of a hinge region of the embryo, by restricting to these territories the expression of a Btd/Sp factor.

Identification of a large family of *btd-Sp*-related genes in the zebrafish

Our study has revealed the existence of a family of at least eleven zebrafish Bts proteins, related to *Drosophila* Btd and to Sp factors. Stringent in situ hybridisation revealed, for most genes, distinct expression profiles, highly specific of a subset of embryonic structures. Thus, these different Bts factors might take part in a restricted number of non-overlapping developmental processes. Within this family, *Drosophila* Btd and Sp1 and five mammalian Sp factors are known to date. Thus, it is likely that many more members remain to be discovered in mammals. *Drosophila Sp1* and mammalian *Sp1-Sp4* are widely expressed, and Sp1-Sp4 transregulate a multitude of promoters, thereby controlling cellular activities as general as cell cycle progression and growth control (Fridovich-Keil et al., 1991; Kingsley and Winoto, 1992; Hagen et al., 1994; Hagen et al., 1995; Karlseder et al., 1996; Lin et al., 1996; Supp et al., 1996; Zwicker et al., 1996; Jensen et al., 1997) or nuclear architecture (Jongstra et al., 1984;

Philipsen et al., 1993); *Sp5* expression is in contrast very dynamic (Harrison et al., 2000; Treichel et al., 2001). *bts1* is in sequence most closely related to mouse *Sp5*; the two genes also share strong expression in the presumptive midbrain, and a similar map location (*Sp5* lies close to *Hoxd* genes on chromosome 2, a region syntenic to the *hoxd* locus on zebrafish linkage group 9). However, the orthology of *bts1* and *Sp5* is questionable, as outside a few conserved domains, Bts1 and Sp5 sequences are highly divergent (30% deduced amino acid identity). The proline-rich N-terminal half of SP5, proposed to have evolved by domain swapping from BTEB/KLF family members (Treichel et al., 2001), is not identifiable in Bts1. Rather, in Bts1, S/T- and Q-rich domains like in Sp1-4 have been maintained. Further, *bts1* and *Sp5* expressions do not always coincide, and these genes seem to exert different roles during embryogenesis. Indeed the genetic disruption of *Sp5* did not cause brain patterning defects in mouse embryos (Harrison et al., 2000). A definite answer on the possible orthology of *bts1* and *Sp5* will await availability of more sequence information on the zebrafish genome.

Btd and all Sp factors isolated to date bind GC-rich promoter sequences (GC-box; Dynan and Tjian, 1983; Gidoni et al., 1984; Gidoni et al., 1985), and we have shown that Bts1 was capable of recognizing such a motif with an affinity similar to Sp1. The specificity of action of Sp factors has been proposed to arise from the non DNA-binding modules of the proteins, which may interact with different molecular partners (Courey and Tjian, 1988; Kadonaga et al., 1988; Schöck et al., 1999a; Schöck et al., 1999b). In addition, multiple protein isoforms can derive from a single Sp gene and differ in their capacity to activate or repress transcription in a similar cellular context (Kennett et al., 1997). We have used an in vivo system, the *Drosophila* embryo, to determine the properties of Bts1 as a transcriptional regulator. Our results demonstrate that Bts1 is capable of activating the expression of *col*, an immediate downstream target of Btd, suggesting that Bts1, like Btd, acts as an transcriptional activator. This conclusion is in agreement with our finding that in the zebrafish, the initiation of expression of *pax2.1* rapidly follows *bts1* expression at the MHD and is positively dependent upon Bts1 function.

bts1 expression and specification of the mid-hindbrain territory

The earliest known mid-hindbrain-specific markers of the zebrafish neural plate are expressed after mid-gastrulation (75% epiboly). Before that stage, AP regional markers within the neural plate rather cover broad anterior or posterior territories. Until now, the most extended caudal marker was *hoxa-1*, in the spinal cord and rhombencephalon up to the presumptive location of rhombomere 3 (Koshida et al., 1998). This left a gap of more than 10 cell rows between the *otx2*- and *hoxa1*-positive domains (Koshida et al., 1998; A. T. and L. B.-C., unpublished). At 75% epiboly, *bts1* expression overlaps entirely that of *hoxa1* (not shown), and slightly the caudal limit of *otx2* expression. Thus, *bts1* is the first gene expressed in this intermediate territory, which at 75% epiboly would cover most of the presumptive MHD, as it abuts the presumptive diencephalon identified by *fkh3* expression (Varga et al., 1999). In other vertebrates, the anteriormost posterior marker during gastrulation is the homeobox gene *Gbx2* (Wassarman et al., 1997), which precisely abuts *Otx2* from the

end of gastrulation and labels the anterior hindbrain. We found that the rostral limit of *bts1* was at all stages anterior to that of zebrafish *gbx* genes (A. T. and L. B.-C., unpublished).

Our observations further suggest that mid-hindbrain identity is progressively established after mid-gastrulation. Indeed, until late gastrulation, gene expression boundaries in this domain move relative to each other. While newly expressed mid-hindbrain-specific markers align with *bts1*, the caudal limit of *otx2* expression is displaced caudally relative to the *bts1* domain. In the mouse and chick, the caudal border of *Otx2* expression is believed to position the mid-hindbrain junction and to encode midbrain fate. Thus, our expression data suggest that mid- and anterior hindbrain identities are progressively established and refine until late gastrulation. These results are in agreement with the finding that the embryonic margin exerts a posteriorizing activity on hindbrain cells until late gastrulation (Woo and Fraser, 1997; Woo and Fraser, 1998). By contrast, presumptive mid-hindbrain cells transplanted into the prospective forebrain at 55% epiboly are capable of maintaining their fate (Miyagawa et al., 1996).

The factors involved in mid-hindbrain induction remain mostly unknown. In the zebrafish, as in other vertebrates, a combination of vertical and planar signals is likely to operate during gastrulation to specify this territory. The anterior hypoblast of the late zebrafish gastrula has the capacity to induce *pax2.1* expression within the neural plate (Miyagawa et al., 1996). In addition, Fgf signalling received by marginal cells is necessary to posteriorize the neural plate and position the borders of *otx2* and *hoxa1* expressions (Koshida et al., 1998). We extended these findings by showing that the mid-hindbrain component of *bts1* expression at gastrulation is (directly or indirectly) dependent on Fgf8 signalling, originating either from the hindbrain territory or from the embryonic margin (Reifers et al., 1998). However, the role of Fgf8 on *bts1* expression is transient, as *bts1* expression is restored in *ace* mutants from the tail bud stage. Other factors, not affected in *ace*, might relay Fgf8 in its regulation of neural plate patterning at that stage. Given the crucial role of Bts1 in the activation of *pax2.1* expression and of the subsequent Pax2.1-dependent cascade, this rescue of *bts1* expression might explain why early mid-hindbrain development still continues normally in *ace* mutants. Our findings additionally imply that, contrary to previous assumption, early stages of mid-hindbrain development are affected (albeit indirectly) in *ace* mutants. The defects are, however, rapidly compensated for.

Bts1 is an early regulator of *pax2.1* expression and the Pax2.1-dependent molecular cascade

To date, no zebrafish mutants were mapped to the *bts1* locus. We thus addressed Bts1 function by combining gain- and loss-of-function approaches. The specificity of our manipulations is supported by the selective and opposite effects of *bts1* and MO[bts1] injections on *pax2.1* expression. Taken together, our results identify Bts1 as the first known factor that selectively controls *pax2.1* induction and the immediate Pax2.1-dependent cascade at gastrulation and early somitogenesis, and refine our molecular picture of MHD induction (Fig. 9).

It is most probable that, upon MO[bts1] injection, enough non-targeted mid-hindbrain cells remained to progressively

reorganize on-site a complete MHD, after the initial perturbations, which explains our transient phenotypes. A requirement for Bts1 at later stages of mid-hindbrain development, such as during the maintenance phase, is suggested by its persistent expression within the mid-hindbrain territory during somitogenesis. Further analyses will be necessary to directly address this issue.

Our lineage tracings in Bts1 misexpression experiments strongly suggest that Bts1 acts primarily within the neural plate. The fact that *pax2.1* induction is not observed in all ectopic *bts1*-expressing cells in the anterior neural plate, however, might indicate an indirect effect and/or that additional factors or a community phenomenon must reinforce Bts1 activity. It will be most interesting to determine whether Bts1 directly binds and transactivates the *pax2.1* promoter.

Finally, we show that Bts1 can only induce *pax2.1* expression in territories anterior to the MHD. These results suggest that Bts1 needs to act in conjunction with spatially restricted molecular partners to induce *pax2.1* expression, and/or needs to be alleviated from the dominant influence of a posterior inhibitor. It will be of interest to determine which local factors are necessary to potentiate or inhibit Bts1 activity.

bts1 expression and the mid-hindbrain maintenance phase

During mid-hindbrain maintenance, expression of the different mid-hindbrain markers become interdependent. In zebrafish *pax2.1/noi^{tu29a}* mutants, all mid-hindbrain markers, including *fgf8*, are completely downregulated between the 5- and 14-somite stages (Lun and Brand, 1998). In *fgf8/ace* mutants, all markers tested, including *pax2.1*, also begin to be affected at a similar stage (Reifers et al., 1998). These results point to a regulatory loop involving Pax2.1 and Fgf8 functions during mid-hindbrain maintenance. However, the mid-hindbrain phenotypes of *noi* and *ace* mutants are clearly different, in particular as regards *bts1* expression. Indeed in *noi* mutants *bts1* expression is affected and completely downregulated within the same time-frame as other mid-hindbrain markers, whereas it remains unperturbed in *ace* until late somitogenesis. The most likely explanation for this finding is that *bts1* expression is only transiently dependent on Pax2.1, requiring Pax2.1 function at early somitogenesis only but not after the five- to ten-somite stage. Enough Pax2.1 activity would be spared in *ace* mutants until that stage to allow for *bts1* maintenance. Thus, our results highlights the existence of mid-hindbrain markers that only transiently require, and then escape, the Pax2.1/Fgf8 regulatory loop (see also Reifers et al., 1998).

Functional characteristics of Bts1 and their evolutionary implications

Our experiments have allowed us to test the starting hypothesis that factors expressed at the *Drosophila* head-trunk and vertebrate mid-hindbrain junctions would be conserved during evolution. This hypothesis was based on previous reports that documented the expression of homologous genes of the *otd/Otx*, *engrailed/En* and *pax2/5/8* families at equivalent AP levels in urochordate, vertebrate and insect embryos (Wada et al., 1998; Wurst and Bally-Cuif, 2001). We found that Bts1 and Btd do share some functional characteristics, as Bts1 could rescue the expression of *col* in a correct spatiotemporal manner in *btd* mutants. We observed that Bts1 was neither capable of rescuing the expression of *eve* and *en* nor the formation of posterior head structures in *btd* mutants. Under similar conditions, Sp1 could partially restore *en* expression and mandibular derivatives (Wimmer et al., 1993; Schöck et al., 1999a; Schöck et al., 1999b). As a chimeric protein composed only of the SP1 zinc finger fused to the activation domain of VP16 also rescues *en* expression (Schöck et al., 1999b), and given the conservation of Bts1 and Sp1 zinc fingers, Bts1 might simply not have sufficient activity to transactivate the *en* promoter. A similar hypothesis might hold true for the failure of both Bts1 and Sp1 to sustain the development of intercalary and antennal segments (Wimmer et al., 1993; Schöck et al., 1999b). Alternatively, in these processes, Btd might need to interact with cofactors incapable of recognizing the divergent non DNA-binding modules of Bts1 and Sp1.

Taken together, our results indicate that Btd and Bts1 share expression and function characteristics in their control of the development of a comparable boundary region of the embryo. *btd* and *bts1* might have diverged from a common ancestor involved in the development of posterior head territories, or might have been co-opted during evolution in the fly and in vertebrates. We favour the second hypothesis, as Bts1 is more related in sequence to the extant subfamily of Sp factors, including *Drosophila* Sp1, than to the Btd subfamily (which comprises zebrafish members such as our clone g5). Our results therefore have interesting evolutionary implications as they strongly suggest that flies and vertebrates, by restricting to the head-trunk or mid-hindbrain junction the expression and functional domain of a Btd/Sp-family member, have independently developed a similar strategy to pattern comparable territories. Whether Bts1 and Btd are part of a conserved molecular cascade awaits further analysis; we note, for example, that *col* has no vertebrate homologue expressed at the mid-hindbrain junction (Garel et al., 1997; Bally-Cuif et al., 1998; Dubois et al., 1998).

Finally, Bts1 might be an interesting tool to approach other evolutionary questions. For example, the existence or the secondary loss of a MHD-like territory in cephalochordates have been questioned, based on the non-expression of *Pax2/5/8* and on the late onset of expression of *en* homologues at this AP level in *Amphioxus* (Holland et al., 1997; Kozmik et al., 1999). *Amphioxus bts1*, as it acts upstream of the 'traditional' MHD maintenance loop that involves Pax and En, might help resolve this issue.

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Appendix 2

published in *Gene*

Formation of the head–trunk boundary in the animal body plan: an evolutionary perspective

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Abstract

Gene expression analyses and anatomical studies suggest that the body plans of protostomes and deuterostomes are phylogenetically related. In the central nervous system (CNS), arthropods and vertebrates (as well as their closest related phyla the urochordates and cephalochordates) share a nerve cord with rostral specification: the cerebral neuromeres in *Drosophila*, cerebral sensory vesicle of ascidians and lancelets and the large brain of craniates. Homologous genes, in particular of the *otd/Otx* and *Hox* families, are at play in these species to specify the anterior and posterior CNS territories, respectively. In contrast, homologies in the establishment of boundary regions like those separating head and trunk structures in arthropods or mid- and hindbrain domains in chordates are still unclear. We compare in these species the formation, properties and molecular characteristics of these boundaries during embryonic development. We also discuss recent findings suggesting that insects and vertebrates might have co-opted factors of related families to control the formation of these boundary regions, the evolution of which would then appear dramatically different from that of the anterior and posterior CNS domains. © 2002 Elsevier Science B.V. All rights reserved.

1. Introduction

It is now widely accepted that the body plans of insects and vertebrates share a similar anteroposterior (AP) and dorsoventral (DV) organization, albeit with an inversion of the DV axis (reviewed in Arendt and Nübler-Jung, 1996; De Robertis and Sasai, 1996; Ferguson, 1996). Along the AP axis, this unity is particularly apparent for the central nervous system (CNS), which in all cases consists of a posterior nerve cord involved in locomotor control, while anterior 'cerebral' vesicles have specialized sensory functions (see Hartmann and Reichert, 1998; Arendt and Nübler-Jung, 1999; Butler, 2000). The morphological and functional distinctions of the anterior and poster-

ior CNS domains are further underlined at the molecular level, as similar sets of genes are involved in both phyla to control anterior versus posterior CNS development. In *Drosophila*, the homeodomain transcription factor-encoding genes *orthodenticle* (*otd*) and *empty-spiracles* (*ems*), expressed in the labral, antennal and intercalary segments and their neuronal derivatives, are involved in the formation of the anteriormost cerebral neuromeres (proto-, deuto- and tritocerebrum) (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In vertebrates, the *otd* and *ems* homologues *Otx1/2* and *Emx1/2* are generally expressed in the presumptive fore- and midbrain and are necessary for the maintenance of these domains (see review in Acampora et al., 2000, 2001). In both phyla, *otd/Otx* and *ems/Emx* function as 'gap' genes, their absence leading to the deletion of their expression territory rather than to a change in identity. A recent study reports the absence of *Emx* expression from the telencephalon in lamprey, probably reflecting a secondary loss of this gene's function in agnathans (Myojin et al., 2001).

Posteriorly, the combinatorial expression of *Hox*-type genes patterns the nerve cord in both insects and vertebrates (reviewed in Duboule and Morata, 1994; Lumsden and Krumlauf, 1996). These genes share an ordered alignment

along the chromosome in a sequence reflecting their collinear expression along the body AP axis, and their function is ruled by a system of posterior dominance. Further, gain- and loss-of-function analyses indicate that *Hox* genes, in both flies and vertebrates, generally control territorial identity rather than the formation or maintenance of CNS segments. Finally, cross-phylum gene replacement experiments show that invertebrate and vertebrate members of the *Otx* and *Hox* gene families can functionally replace each other (Malicki et al., 1993; Zhao et al., 1993; Lutz et al., 1996; Acampora et al., 1998; Leuzinger et al., 1998; Nagao et al., 1998). Even though this does not constitute proof that these genes (and the structures they control) are homologous across species, it is, combined with expression and sequence data, a strong indication in this direction. Together, the molecular studies described above support the idea that the CNS of insects and vertebrates evolved from a common ancestral ground plan, which relied on the expression of *otd/Otx* and *ems/Emx* genes anteriorly, and *Hox* genes posteriorly (commented on in Sharman and Brand, 1998; Reichert and Simeone, 1999).

It remains unclear, however, whether homologies exist which sustain the development of the intermediate region located at the junction of these anterior and posterior patterning systems. At the gross morphological level, this hinge domain is recognizable as a transition zone in both the arthropod and chordate phyla as it is outlined by constrictions (between the mid- and hindbrain vesicles in the vertebrate CNS – or the cephalic furrow in the *Drosophila* epidermis), or anatomical changes (from a supra- to a subesophageal location of the cerebral neuromeres in the *Drosophila* CNS). However, obvious differences in gene expression and anatomy exist in this area between insects and vertebrates. For example, in the *Drosophila* embryonic brain, but not in the vertebrate CNS, the expression of head gap genes and trunk *Hox* genes overlap at the head–trunk junction (Fig. 1). Indeed, the deuto- and tritocerebrum derive from territories expressing *ems* and the *Hox* genes *proboscipedia* (*pb*), *labial* (*lab*) and *deformed* (*Dfd*), and each of these factors has been demonstrated to be necessary for the development of at least some deuto- and tritocerebral neuronal populations (Hartmann and Reichert, 1998; Hirth et al., 1998). In contrast, in vertebrates, the expression of head gap genes and *Hox* genes does not overlap at the mid–hindbrain junction, rhombomere 1 (r1) expressing neither of these gene categories. Signals emitted from r1 are believed to exert a repulsive influence on *Hox* genes expression (Irving and Mason, 2000). In molecular terms, a territory perhaps more reminiscent of the *Drosophila* head–trunk junction might be located in the vertebrate caudal hindbrain. At this level, gap-like genes such as *krox-20* (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993) and *Hox* genes are co-expressed.

For these and other reasons, summarized below, whether
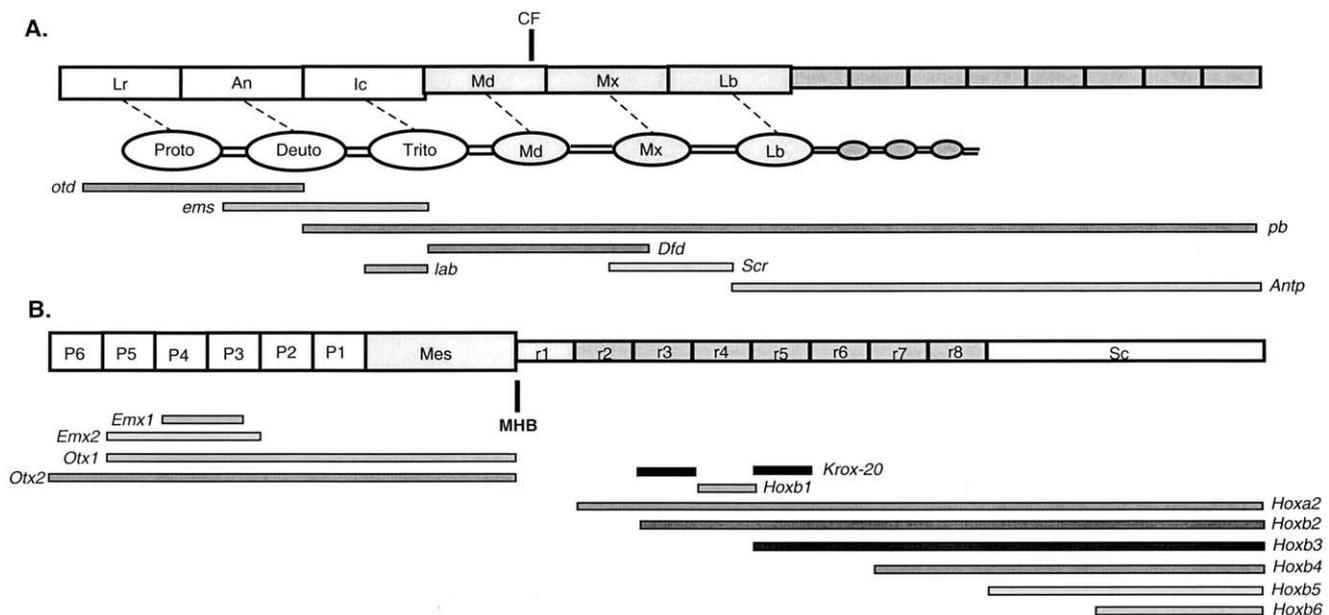
Fig. 1. Comparison of gap and *Hox* genes expression along the animal body plan in the embryonic CNS of *Drosophila* (A) and gnathostome vertebrates (B). Anterior is to the left. In *Drosophila*, the embryo epidermis (top) and CNS ganglia (bottom) are represented; dashed lines indicate the segmental embryonic origin of the ganglia. CF and MHB indicate the positions of the cephalic furrow and mid–hindbrain junction in the embryonic *Drosophila* epidermis and vertebrate CNS, respectively. Note that the expression of *otd/Otx-ems/Emx* and *Hox* genes overlap in *Drosophila* but not in vertebrates, r1 expressing neither type of genes. Overlap between gap and *Hox* genes is observed in a more caudal location in the vertebrate rhombencephalon (*krox-20* expression in r3 and r5). Proto-, deuto- and tritocerebrum: anterior (supraesophageal) cerebral neuromeres, originating respectively from the labrum (Lr), antennal (An) and intercalary (Ic) epidermal segments; Md, Mx, and Lb: mandibular, maxilar and labial posterior (subesophageal) cerebral neuromeres; P1–P6: telencephalic and diencephalic prosomeres; Mes: mesencephalon; r1–r8: rhombomeres 1–8; Sc: spinal cord. (Modified from Hartmann and Reichert (1998) and Sharman and Brand (1998); incorporating Vincent et al. (1997) and Irving and Mason (2000).)

the head–trunk and mid–hindbrain boundary regions are homologous in invertebrates and vertebrates remains subject to debate (see Holland and Holland, 1999). Addressing this question, recent studies demonstrate that transcription factors of the Buttonhead (Btd)/Sp family are used to pattern this hinge territory both in *Drosophila* (Vincent et al., 1997) and in zebrafish (Tallafuß et al., 2001). We discuss here the evolutionary implications of these findings, which suggest that a step of genetic convergence, i.e. the acquisition of a common molecular 'identity', possibly sustains the development of analogous boundary regions within the animal body plan.

2. Anatomical, functional and molecular characteristics of the vertebrate mid–hindbrain territory

The mid–hindbrain domain is a most interesting entity of the vertebrate embryonic neural plate, which differs from anterior and posterior territories in terms of fate, organization, gene expression and developmental properties. We will summarize here its most important characteristics, in the perspective of evolutionary considerations (see also, for recent reviews, Joyner et al., 2000; Simeone, 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

The mid–hindbrain comprises the mesencephalic and first rhombencephalic (or metencephalic) vesicles (Fig. 2). Its anterior half, the mesencephalon, is fated to the tectum (colliculi in mammals) and tegmentum, the first being involved in the reception of ocular and auditory inputs, and the second essentially in behavioral control. Its posterior half, the metencephalon, is fated to the cerebellum, a major motricity center, and to the pons, which receives

and projects complex sensory and motor signals. Between these two domains, the mid–hindbrain junction itself, or isthmus, gives rise to 'isthmic nuclei' which will occupy various final locations within the tectum, tegmentum and pons. Thus, mid–hindbrain derivatives do not exhibit a functional unity. Strikingly, however, the embryonic mid–hindbrain territories develop in a concerted fashion. No compartmental boundary separates the presumptive mes- and metencephalon; moreover, mes- and metencephalic precursors are intermingled (even possibly bipotential) around the mid–hindbrain junction, where extensive cell movements take place (Martinez and Alvarado-Mallart, 1989; Hallonnet et al., 1990; Hallonnet and Le-Douarin, 1993; Millet et al., 1996). Finally, transcription factors of the engrailed and Pax2/5/8 families, expressed across the entire domain, control mid–hindbrain development as a whole (Wurst et al., 1994; Schwarz et al., 1997; Urbanek et al., 1997; Lun and Brand, 1998).

The unified development of the mid- and hindbrain is believed to rely on the presence of a signaling center, the isthmic organizer (IsO), located in the middle of the mid–hindbrain domain. The IsO was initially identified in transplantation experiments in avians: tissue straddling the mid–hindbrain constriction, when transplanted to an ectopic location within the embryonic neural tube, is capable of reconstructing an entire mid–hindbrain at the expense of surrounding host territories (Martinez et al., 1991, 1995). IsO cells are located as a ring straddling the caudal border of *Otx2* expression, at the level of the bipotential territory which contributes both mes- and metencephalic fates. At the molecular level, the organizing capacities of the IsO are outlined by the expression of secreted factors of the Wnt (Wnt1, 8b) and Fgf (Fgf8, 17, 18) families. Of those, at least
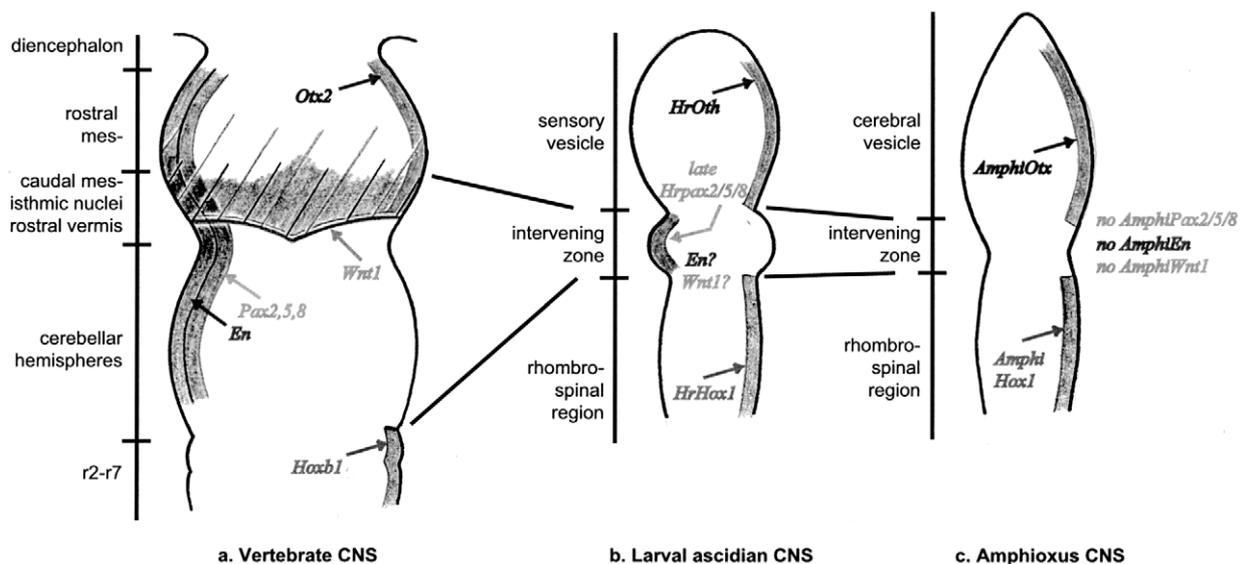
Fig. 2. Fate and gene expression in the intervening zone of the vertebrate (a), urochordate (ascidia, b) and cephalochordate (Amphioxus, c) embryonic or larval CNS. Anterior is to the top. The derivatives of each territory are indicated on the left of each figure. Except for wnt1, gene expression is represented on one side of the neural tube only, but is symmetrical. *En* and *Wnt1* expression have not been studied in ascidia, and the intervening zone of the amphioxus CNS does not express *Pax2/5/8*, *En* and *Wnt1*.

Wnt1 (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and Fgf8 are necessary for mid–hindbrain development as a whole (Meyers et al., 1998; Reifers et al., 1998), and Fgf8 is capable of partly mimicking IsO inductive and organizing activities (Crossley et al., 1996). Expression studies, as well as gain- and loss-of-function experiments, suggest that the IsO exists in all vertebrates.

3. What to expect from an archetypal mid–hindbrain: the IsO might not be an ancestral trait

When looking for structures potentially homologous to the mid–hindbrain domain, it is necessary to determine what characteristics should be considered 'mid–hindbrain-like'. Homologies in the fine structure of the CNS among distantly related animals are often difficult to assess by anatomical studies. Even within the chordate lineage, for instance, the size of the brain varies from around 400 cells in ascidia to several thousands in Amphioxus to billions in the vertebrate brain. This increasing complexity correlates with the adaptation to diversified habitats, the development of a complex motricity, or the response to a more challenging social environment, all of which correspond to functions controlled, at least in part, by mid–hindbrain derivatives. It is therefore perhaps not surprising that the morphology and complexity of the mid–hindbrain structures differ enormously already within the vertebrate lineage. For example, the gross anatomy of the cerebellum varies from that of a single leaf-like structure in amphibians and reptiles to a complex and foliated organ in birds and mammals (Voogd and Glickstein, 1998). Similarly, the mesencephalic auditory center is small in birds (lateral mesencephalic nuclei) but very developed in mammals (inferior colliculus).

The inductive and organizing capacities of the mid–hindbrain boundary, and therefore the presence of an 'IsO-like' domain, also might not be reliable criteria to assess the existence of a territory equivalent to the mid–hindbrain in evolutionary studies. Indeed, it is possible that the IsO of vertebrates is not associated with the formation of mid–hindbrain structures, but rather primarily permits their growth over a long time period. Mid–hindbrain derivatives occupy an important volume of the adult vertebrate brain, proportionally much larger than their representation at embryonic stages. Correlatively, it is very striking that mid–hindbrain structures differentiate relatively late compared to other brain territories. The IsO area itself is one of the latest to mature and contribute post-mitotic neurons. Further, at the molecular level, the factors Wnt1 and/or Fgf8, expressed at the IsO and believed to mediate its activity, are not necessary for the early steps of mid–hindbrain development but are rather required at later stages, for mid–hindbrain maintenance (McMahon et al., 1992; Reifers et al., 1998). Finally, the genetic disruption of IsO function in the mouse and zebrafish can leave intact some mesencephalic and cerebellar structures, namely those originating from the anterior midbrain or from r1 (Thomas and Capecchi, 1990; McMahon et al., 1992; Reifers et al., 1998). Therefore, one might speculate that IsO activity was superimposed during development to a preexisting mid–hindbrain-like territory harboring anterior midbrain and r1 characters, to maintain in this domain a zone of continued growth capacity. Correlatively, the prolonged immaturity of the central mid–hindbrain zone might have permitted a polarized expression of preexisting mid–hindbrain factors and/or the diversification of mesencephalic or metencephalic identities. This hypothesis is also in agreement with the gradient expression of *Engrailed* and *Pax2/5/8* genes, with peak levels at the IsO. Therefore, one might expect to find species developing mid–hindbrain-related structures but no IsO.

Thus, to determine whether and which species harbor homologous structures between the anterior and posterior CNS domains, one is reduced to relying on the existence of a common genetic program supporting the development of this transition zone. Among the best genes to study are probably those expressed across the entire vertebrate mid–hindbrain domain, and in which initiation of expression does not depend on IsO activity, such as *Engrailed* and *Pax2/5/8*.

4. Current evidence argues against the existence of a mid–hindbrain-related territory in invertebrate chordates

The closest relatives of vertebrates are the urochordate ascidia and cephalochordate amphioxus, and gene expression studies have been performed in these species to assess homologous hinge structures in the chordate neural tube.

The ascidian larval neural tube is simple but is formed by neurulation processes similar to those of vertebrates. From both anatomical subdivisions and gene expression studies, the sensory vesicle of ascidia is believed to be homologous to the vertebrate diencephalon, and the posterior visceral ganglion and tail nerve cord to the vertebrate rhombencephalon and spinal cord (see Butler, 2000, and references therein). Between these domains, an intervening zone expressing neither *Hroth* (the ascidian homologue of *Otx* genes) nor *HrHox1* (homologue of vertebrate paralogue group 1 *Hox* genes) exists (Katsuyama et al., 1995, 1996; Wada et al., 1996) (Fig. 2). Expression of a *Pax2/5/8* homologue, *Hrpax2/5/8*, has been reported in this region (Wada et al., 1998); however, it occurs relatively late during development, and is possibly more reminiscent of the late expression of *Pax2* in sensory neurons of the anterior vertebrate hindbrain than of the early mid–hindbrain expression of *Pax2*, *5* and *8*. Expression of ascidian homologues of other mid–hindbrain markers has not been examined to date, and concluding on the homology between the ascidian intervening zone and the vertebrate mid–hindbrain remains premature.

From microanatomical and genetic studies, the amphioxus nerve cord contains anteriorly a region comparable to the vertebrate diencephalon: an unpaired, pigmented frontal organ comparable to an eye, a lamellar body possibly equivalent to the epiphysis, an infundibulum, and a group of ciliated accessory cells assimilated to a hypothalamus (see Butler, 2000, and references therein). Like the vertebrate diencephalon, these structures express Amphioxus homologues of *Otx*, *Nk2* and *Dlx* genes (Holland et al., 1996; Williams and Holland, 1998; Venkatesh et al., 1999). A putative anterior midbrain has been recognized, with a dorsal tectum receiving 'visual' input and a ventral component (part of a ventrally lying population of motor neurons) which may correspond to the midbrain reticulospinal neurons of craniates. Caudally, the existence of hindbrain and spinal cord regions is supported by the expression of *Hox* genes, *AmphiHox1*, *3* and *4* being transcribed in the posterior nerve cord in nested patterns very similar to those of their vertebrate counterparts (Holland et al., 1992; Garcia-Fernandez and Holland, 1994). The anterior border of the anteriormost *Hox* gene, *AmphiHox1*, lies approximately at the level of somite 3, leaving an intervening zone between the *AmphiOtx* and *AmphiHox*-positive domains (Holland and Holland, 1996). However, this territory is not considered equivalent to the vertebrate mid–hindbrain, as it does not express the amphioxus homologues of *Wnt1* and *Pax2/5/8* (see Kozmik et al., 1999). It also does not express *AmphiEn*, in spite of containing structures functionally reminiscent of an anterior midbrain (Holland et al., 1997). Therefore, the arguments in favor of the existence of a mid–hindbrain-like territory in invertebrate chordates remain seldom, suggesting, by lack of evidence, that the mid–hindbrain is a vertebrate character.

However, this conclusion is surprising. Indeed, the developmental processes believed to lead to CNS patterning and/or to the induction of the mid–hindbrain or of the IsO in vertebrates seem phylogenetically conserved in chordates. Mid–hindbrain specification in vertebrates is not well understood, but several studies pointed to a role of non-neural tissues such as the anterior axial meso- or mesendoderm in the 'vertical' induction of *Engrailed*- or *Pax2*-positive territories within the embryonic neural plate (Ang and Rossant, 1993; Miyagawa et al., 1996). It was also recently suggested that the formation of an IsO within the vertebrate embryonic neural plate might be triggered by the juxtaposition of territories of anterior (*Otx2*-positive) and posterior (*Otx2*-negative) identities (Broccoli et al., 1999; Millet, 1999; Katahira et al., 2000). Why these tissues and processes would not lead to the formation of a mid–hindbrain or an IsO in invertebrate chordates is puzzling.

A possible answer to these apparent discrepancies might lie in the genes we have chosen to search for homologies, and their possible acquisition of new functions combined with their loss of ancestral functions between species. Expression studies of *engrailed* homologues across the animal kingdom quite clearly indicate that the ancestral function of *engrailed* was in controlling neurogenesis, and that it was later re-selected to participate in the establishment of body segments (at least in arthropods, annelids, and cephalochordates) or in mid–hindbrain development (in vertebrates) (see Patel et al., 1989; Manzanares et al., 1993, and references therein; Holland et al., 1997). Therefore, the role of *engrailed* in controlling mid–hindbrain development, rather than reflecting an ancestral function, might be a new acquisition, which possibly impinged on an already established and more ancient mid–hindbrain regulatory network. The mutually dependent expression of *Engrailed* and *Pax2* during mid–hindbrain maintenance suggests that this network might concomitantly have been joined by Pax2/5/8 factors. *Pax2/5/8* genes have been isolated in a large variety of species in both protostomes and deuterostomes, and therefore represent an ancient subfamily of highly conserved *Pax* genes (see Czerny et al., 1997; Wada et al., 1998). However, their ancestral function remains enigmatic as they are expressed in very divergent structures in different organisms. Together, this suggests that our search for mid–hindbrain homologies might have relied on recent mid–hindbrain markers, possibly new acquisitions on an ancestral regulatory network. This choice might have hindered extant similarities, and the existence of a mid–hindbrain-like territory in invertebrate chordates deserves further examination.

5. Studies of head–trunk junction formation in the Drosophila embryonic CNS and epidermis suggest new candidates for boundary development in deuterostomes

As mentioned previously, flies obviously do not possess a mid–hindbrain but do exhibit a transition zone between an anterior and a posterior patterning system, both in the embryonic epidermis and larval CNS. Recently, the importance of the transcription factor Btd, expressed across this transition zone, was brought again to attention.

btd encodes a zinc finger transcription factor and was initially identified as a head gap gene in mutant analyses, its loss-of-function mutation causing the absence of antennal, intercalary and mandibular derivatives (Wimmer et al., 1993). *btd* is expressed in the corresponding territories of the embryonic epidermis, as well as in the neuroblasts originating from them. Later studies demonstrated that *btd* mutants also have a severely reduced number of deuterocerebral neuroblasts, and a possible lack of the tritocerebrum (Younossi-Hartenstein et al., 1997). Most interestingly, the expression of *btd* was recently shown to overlap by a few cell rows the first stripe of *evenskipped* (*eve1*) expression in the epidermis of the first trunk parasegment (PS1), and to be necessary for eve1 expression and cephalic furrow formation (Vincent et al., 1997) (Fig. 3). More anteriorly, an immediate target of Btd is the transcription factor-encoding gene *collier* (*col*), necessary for the development of PS0 (Crozatier et al., 1996). The expression of *eve1* antagonizes
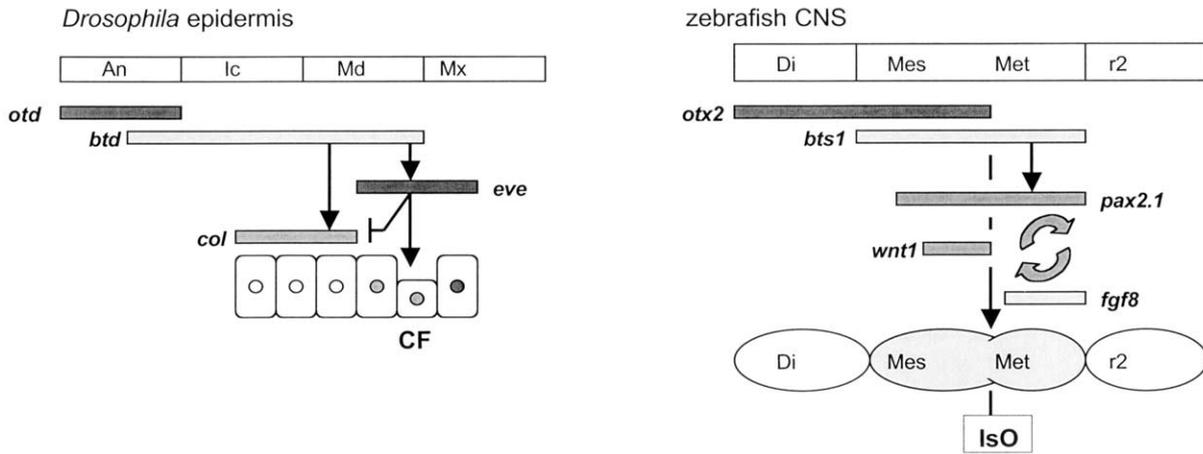
Fig. 3. Function of *btd/bts1* in the embryonic *Drosophila* epidermis (left) and zebrafish CNS (right). Anterior is to the left, and abbreviations are as in Fig. 1. *btd* expression positively regulates the expression of *eve* and *col* (arrows) at the head–trunk junction, which in turn position the cephalic furrow (CF) (redrawn from Vincent et al., 1997). *bts1* is expressed across the entire mid–hindbrain and is both necessary and sufficient for *pax2.1* expression (arrow). *pax2.1*, together with *wnt1* and *fgf8*, is subsequently involved in a regulatory loop maintaining mid–hindbrain identity. Within this territory (Mes Met), the caudal limit of *Otx2* expression positions the Iso (arrow).

that of *col*, setting the PS0/PS1 (or head–trunk) boundary (Crozatier et al., 1999). Importantly, these results highlight a role of Btd in integrating the head and trunk segmentation systems to pattern the head–trunk border in the fly embryo (Vincent et al., 1997).

Btd belongs to a family of transcription factors characterized by a triple zinc finger DNA-binding domain of structure Cys(2)His(2), preceded by an arginine-rich 'buttonhead box' potentially involved in transcriptional regulation (Wimmer et al., 1993). In addition, Ser/Thr or Gln-rich domains have been recognized in some members of this family (including Btd itself) and participate in transcriptional control. Besides Btd, this family comprises all Sp factors (Sp1–5), of which the prototype, human Sp1, was one of the first eukaryotic transcription factors to be identified and cloned (Dynan and Tjian, 1983). Mammalian Sp1–4 are broadly expressed at embryonic stages and adulthood. They recognize a multitude of GC box-containing promoters, thereby controlling general cellular activities as crucial as cell cycle progression, growth or nuclear architecture (Jongstra et al., 1984; Fridovich-Keil et al., 1991; Kingsley and Winoto, 1992; Philipsen et al., 1993; Hagen et al., 1992, 1994; Karlseder et al., 1996; Lin et al., 1996; Supp et al., 1996; Zwicker et al., 1996; Jensen et al., 1997). The expression of mouse Sp5 is more dynamic and has been implicated in the control of somitogenesis in synergy with Brachyury (Harrison et al., 2000; Treichel et al., 2001). A factor closely related to Sp4, named D.Sp1, has also been identified in *Drosophila*. D.Sp1 is located close to *btd* in the fly genome but, like most vertebrate *Sps*, is expressed rather ubiquitously during embryogenesis. It collaborates with Btd in the regulation of mechanosensory organ development, but is not involved at the head–trunk junction (Wimmer et al., 1996; Schöck et al., 1999). An Sp-like protein has also been identified in *Caenorhabditis elegans* (Fig. 4). These data

suggest that Btd/Sps represent an ancient family of transcription factors, of which at least one member, Btd, is involved in controlling head–trunk junction patterning.

One major question arising from these observations was to determine whether Btd, which significantly differs in sequence from all Sp factors including D.Sp1, was a fly specialization, or whether it had direct homologues in other phyla, e.g. chordates. In the latter case, one might obviously want to test whether these homologues are involved in the development of the anterior–posterior patterning transition zone. These questions have been addressed in a recent study reporting the isolation of eleven *btd/Sp*-related genes from zebrafish (Tallafuß et al., 2001). Of these, ten are more closely related in translated sequence to Sps, but one (*g5*) encodes a Btd-like zinc finger domain. In other vertebrates (e.g. the mouse) and chordates (e.g. the urochordates *Oikopleura dioica* and *Ciona savignyi*), factors closely related to g5 have been identified (Fig. 4). They are less related to Btd than g5 and constitute a distinct subfamily. In the absence of more sequence information, the phylogenetic relationship between these factors remains unclear. However, it seems likely that a separation of Btd/Sp-like factors into two distinct Btd versus Sp subfamilies predated the division of protostomes and deuterostomes during evolution.

Interestingly, zebrafish *g5* was found to be expressed ubiquitously during embryogenesis, while one of the ten *Sp* genes, *bts1*, appeared to be the earliest known marker of the presumptive mid–hindbrain. Bts1 is closely related in sequence to mouse Sp5, which is also expressed at the mid–hindbrain junction (Harrison et al., 2000; Treichel et al., 2001). Gain- and loss-of-function analyses further demonstrate that Bts1 is necessary and sufficient to trigger the expression of *pax2.1* and subsequent mid–hindbrain development within the zebrafish anterior neural plate. *bts1*
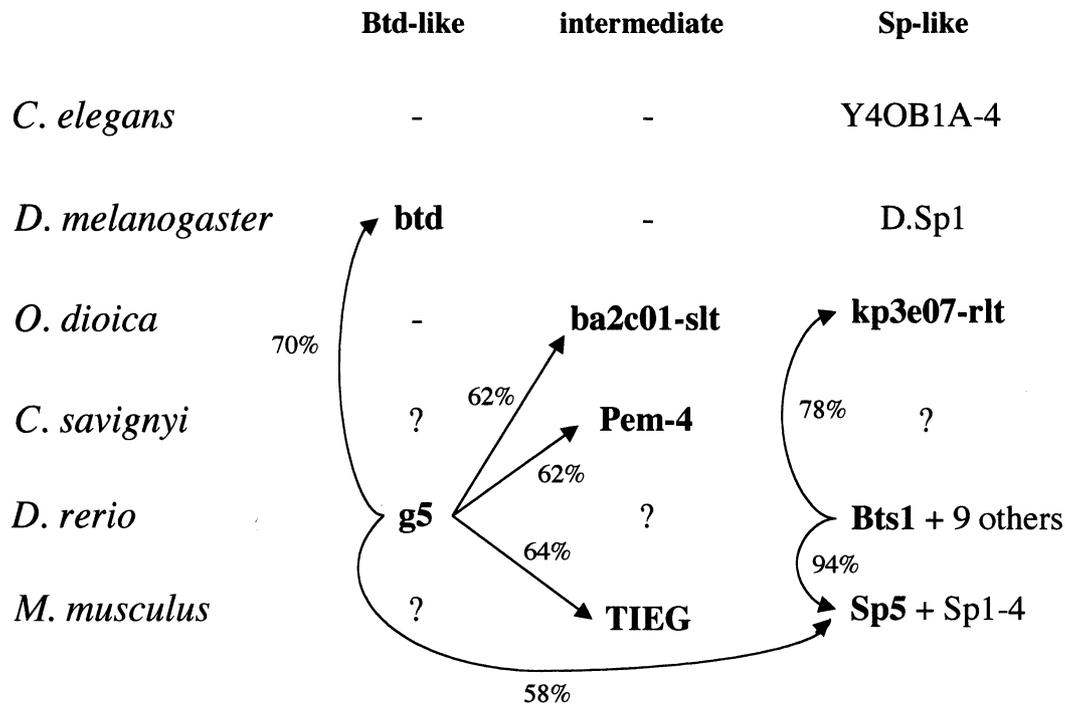
Fig. 4. The Btd/Sp protein family in nematodes (*Caenorhabditis elegans*), dipterans (*Drosophila melanogaster*), urochordates (*Oikopleura dioica* and *Ciona savignyi*) and vertebrates (*Danio rerio* and *Mus musculus*). Proteins with similar triple zinc finger domains from available databases are shown; bold characters highlight the zinc finger domains most related to those of g5 and Bts1; percentages of identity between these domains are indicated. The zinc finger domain of g5 is only distantly related to Sp factors (58% identity, arrow), but close to btd (70%). 'Intermediate' proteins all belong to the sub-family of TGF-beta inducible early gene (TIEG) factors (Fautsch et al., 1998), they are closer in sequence to Sp zinc fingers than to Btd, and do not possess a Btd-box. -: no factor found in the entire genome; ?: no factor found but genome sequencing still pending. Sequences of *O. dioica* are from shot-gun genomic sequences covering only part of a gene (D. Chourrout and R. Reinhardt, pers. commun.; see text for other references).

expression is shown to be dependent on early Fgf8 expression, thus possibly on a neural plate posteriorizing system, and its functional domain is restricted by a posterior factor of dominant activity.

6. Concluding remarks

The results discussed above have several implications.

First, because Btd and Bts1 belong to distinct subfamilies, they suggest that flies and vertebrates may have co-opted Btd/Sp-like transcription factors during evolution to pattern the head–trunk or median–posterior CNS transition zones. In turn, this would indicate that, albeit located at equivalent levels within the body plan, these territories are not homologous. A beautiful example of evolutionary convergence is the formation of ring patterns in the dipteran appendage and in the butterfly wing, both of which rely on the expression of *distal-less-related* genes (Carroll et al., 1994). The formation of head–trunk and median–posterior CNS boundaries in flies and vertebrates might provide an additional example of this phenomenon, if they prove to rely on the co-option of Btd/Sp-like factors. Alternatively, a function in boundary patterning might have already been present in the common ancestor of Btd and Sp factors, and secondarily lost in one of the two subfamilies following the divergence of proto-

stomes and deuterostomes. Expression data from other species will be necessary to help choose between these hypotheses.

Second, Btd and Bts1 might precisely highlight a divergence point between fly and vertebrate regulatory cascades. Indeed, *bts1* expression, like *btd*, responds to a posterior patterning system. The processes limiting the activity of both factors are also similar: in *Drosophila*, ectopic expression of *btd* under control of the *hunchback* promoter does not cause posterior defects, indicating that the functional domain of Btd is, like that of Bts1, limited posteriorly by a factor of dominant activity (Vincent et al., 1997). Further downstream, however, the molecular cascades triggered by these Btd/Sp factors in boundary patterning do not seem conserved: indeed, the major identified targets of Btd at the fly head–trunk junction are *col* and *eve1*, the vertebrate homologues of which are not expressed at the mid–hindbrain junction (Joly et al., 1993; Dole et al., 1994; Garel et al., 1997; Dubois et al., 1998).

Finally, the story of Btd and Bts1 makes Btd/Sp factors interesting candidates to study in invertebrate chordates. Because Bts1 is acting upstream of the well-known mid–hindbrain maintenance loop (which involves *En* and *Pax2/5/8*) in vertebrates, it might reflect a more ancestral regulatory network controlling the development of this territory, and remnants of this network might have been conserved in

invertebrate chordates. Alternatively, since species as divergent as *Drosophila* and the zebrafish rely on factors of this family to pattern this transition zone, Btd/Sps might also have been co-opted in invertebrate chordates. Distinguishing between these two hypotheses might help in elucidating whether CNS transition zones in vertebrates and invertebrate chordates have anything in common at the molecular level, and whether they are more likely homologous or analogous territories. A gene closely related to *Bts1* and to mouse *Sp5* exists in the urochordate *O. dioica* (Fig. 4); it would be most interesting to study its expression pattern during development.

Acknowledgements

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Appendix 3

submitted to *Developmental Dynamics*

Selective control of neuronal cluster size at the di-mesencephalic boundary by long-range signaling from the prechordal plate

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Running title: control of nMLF/nPC size by the prechordal plate

Abstract

Within the vertebrate embryonic neural plate, the first neuronal clusters often differentiate at the intersection of patterning identities, but whether these territorial cues alone control all aspects of neuronal cluster development (location, identity and size) is unknown. Neurons forming the medial longitudinal fascicle (nMLF) and posterior commissure (nPC) are located at the di-mesencephalic (p1/mes) boundary. We report here that expression of the transcription factor Six3 is a common and distinct molecular signature of nMLF and nPC neurons in zebrafish. We demonstrate that different subdomains of *six3* expression individually respond to neural plate patterning according to their location, arguing that intersecting identity cues exert a combinatorial control over the development of early neuronal clusters. Using mutant and manipulated contexts, we further identify a long-range inhibitory influence that selectively limits the number of *six3*-positive neurons identified at the p1/mes boundary, without affecting p1/mes patterning or other neuronal clusters. Selective rescue experiments locate this activity to the prechordal plate. Together, our results highlight the existence of a long-range signaling process that distinguishes between neural plate patterning and the control of neuronal cluster size at the zebrafish di-mesencephalic junction.

Introduction

At early developmental stages, all vertebrates display a mostly similar and highly reproducible neuronal pattern. In all species, neuronal clusters in the basal fore- and midbrain, generating the nucleus of the medial longitudinal fascicle (nMLF), and at the base of the optic stalk, forming the tract of the postoptic commissure (TPOC), are among the first focal sites of differentiation (Wilson et al., 1990; Ross et al., 1992; Easter et al., 1993; Chédotal et al., 1995; Mastick and Easter, 1996; reviewed in Kimmel, 1993; Easter et al., 1994). These clusters have distinct sizes, neurotransmitter phenotypes, axonal routes and targets. How neural tube regionalization, neurogenesis and proliferation events are integrated to achieve this stereotypical pattern of differentiation is not fully understood.

A number of observations highlight that, at early stages, borders of patterning genes expression often prefigure axonal routes (Macdonald et al., 1994; Wilson et al., 1997). Detailed expression studies, and functional analyses in mutant or manipulated contexts support, for some of these patterning markers, a direct or indirect role in the formation (Macdonald et al., 1997; Mastick et al., 1997; Ba-Charvet et al., 1998; Bertuzzi et al., 1999; Hallonet et al., 1999) or the refinement (Hjorth and Key, 2001) of axon trajectories.

The cues controlling the location and size of the first differentiation clusters have been comparably less studied. Transition zones between different combinations of patterning markers also correlate with the position of the first neuronal groups. For example, the differentiating neuron cell bodies of the MLF and posterior commissure (nPC) overlap the anteroposterior (AP) transition between the mesencephalon and caudal prosencephalon (p1) (later on refered to as p1/mes boundary), as defined by anatomical and molecular landmarks (Macdonald et al., 1994; Mastick and Easter, 1996). Similarly, the nMLF lies along the dorsal boundary of Shh expression along the dorsoventral (DV) axis (Macdonald et al., 1994; Barth and Wilson, 1995). In a few cases studied, the development of these neuronal clusters is perturbed when neighboring patterning boundaries are affected. For instance, the perturbation of the p1/mes boundary by lack of the transition factor Pax6, normally expressed in p1, results in the generation of fewer PC neurons in the mouse (Mastick et al., 1997). Based on these observations, it has been proposed that the stereotypical arrangement in time and space of the first neuronal clusters responds to the intersection of regional markers expression.

Within each cluster, the number of differentiating neurons is believed to depend on a lateral inhibition process mediated by Notch/Delta signaling (see for reviews Lewis, 1998; Chitnis, 1999; Blader and Strähle, 2000). Defects in this pathway generally perturb all neuronal groups rather than an individual one, and do not affect the size of the differentiation-competent territories (or proneural clusters). Here again, positional information cues

distributed within the neural tube likely exert the main control over the size of individual proneural clusters, conditioning the relative number of neurons that will differentiate in each cluster.

It remains unclear whether additional signals distinct from local positional information are involved in controlling the sites and extent of neurogenesis within the early neural plate. To approach this question, we have focused on the development of the nMLF and nPC in the embryo of the zebrafish, *Danio rerio*. Because, as described below, the nMLF and nPC neurons are partially intermingled and indistinguishable prior to the extension of processes, we have considered the development of nMLF / nPC neurons as a whole. Our identification of common molecular markers for both neuron types supports this decision (see below). At one day of development, teleost embryos have a relatively simple brain organization, where a schematic scaffold of axon tracts has been built by a small number of neurons (Wilson and Easter, 1991; Ross et al., 1992), facilitating the analysis of developmental abnormalities. Further, in the zebrafish, the nMLF / nPC is one of the first collection of neurons in the brain to appear, free of influence by other neurons or even postmitotic cells. This spatial and temporal isolation from other cells undergoing similar changes also facilitates an analysis of the cues controlling nMLF / nPC development. The ontogeny of the nMLF / nPC has been precisely mapped. AChE activity or immunocytochemistry against HNK1 or acetylated-tubulin revealed the first nMLF cell bodies around 16 hours post-fertilization (hpf), at the junction of the p1 (*pax6.1*-positive) (Kraus et al., 19919; Püschel et al., 1992) and mesencephalic (*eng*-positive) (Ekker et al., 1993) patterning systems along the AP axis, and along the dorsal and ventral edges of *shh* and *nk2.2* expression, respectively, along the DV axis (Macdonald et al., 1994; Barth and Wilson, 1995; Hjorth and Key, 2001). nPC cell bodies are detectable from 18 hpf onwards, they are partially intermingled with the nMLF as well as in an adjacent, more alar location in p1. Thus precise molecular markers are available to assess the influence of both AP and DV cues on neural tube patterning and nMLF / nPC development.

We report here that the transcription factor Six3, in addition to its known expression in the eye field (Kobayashi et al., 1998), is specifically expressed in nMLF / nPC cell bodies from 18 hpf in the zebrafish embryo. Using *six3* expression as a marker, we then analyzed nMLF / nPC development in a number of mutant lines affected in long range patterning cues including planar neural signals and vertical signals from non-neural tissues. Predictably, our results highlight the existence of a number of positive influences commonly necessary for the regulation of neural tube patterning in the p1/mes area and the generation of a normal *six3-*

positive cluster. Surprisingly, however, we also demonstrate that a long-range negative influence, primarily originating from the prechordal plate, is involved (directly or indirectly) in restricting the number of *six3*-positive neurons at the p1/mes border. This inhibitory cue does not correlatively affect AP or DV patterning in a detectable manner in the p1 and mesencephalic territories. Thus, our results demonstrate the existence of a long range signaling activity that distinguishes the regulation of neural tube patterning and the control of neuronal cluster size in a selective location of the anterior neural plate.

Materials and Methods

Fish strains

Embryos were obtained from natural spawning of AB or the following mutant lines: bon^{m425} (Kikuchi et al., 2000), cas^{ta56} (Chen et al., 1996), sqt^{cz35} (Heisenberg and Nüsslein-Volhard, 1997), noi^{tu29a} (Brand et al., 1996), ace^{ti282a} (Brand et al., 1996), smu^{b641} (Barresi et al., 2000). Heterozygous adult carriers were intercrossed to obtain mutant embryos. *MZsqt* embryos, deficient in both the maternal and zygotic contributions of the *sqt* locus, were obtained by raising weakly affected *sqt* homozygous embryos to adulthood. The resulting homozygous *sqt* adults were then intercrossed. All embryos were raised and staged according to Kimmel et al., (1995).

Rescue experiments

To rescue endo- and mesendodermal tissues in the duplicated axes of Nodal mutant embryos (e.g. *MZsqt*), capped *Taram-A** (*Tar**) mRNA (8 pg) (Peyrieras et al., 1998) was injected into one marginal blastomere of 16-celled embryos. For similar rescues in bon^{m425}, because Mixer acts at least in part downstream of Tar* activity, capped *bon/mixer* mRNA (20 pg) was coinjected with *Tar**. All experiments were lineage-traced by coinjecting *nls-lacZ* mRNA (60 pg).

To rescue the function of Bon/Mixer in the YSL of *bon* mutant embryos, capped *bon/mixer* mRNA (20 pg) was injected into the morphologically visible YSL in 1000-celled embryos, together with *nls-lacZ* mRNA as lineage tracer.

In the case of *Tar** injections leading to the formation of a full duplicated axis at 26 hpf, *bon* and *MZsqt* mutant embryos were identified by the characteristic mutant phenotype maintained by their endogenous axis. In all other experiments, to identify *bon* mutant embryos after injection, in situ hybridization and/or cell counts, embryos were *a posteriori* genotyped by PCR as described in Kikuchi et al. (2000).

Staining for markers expression

In situ hybridization, immunocytochemistry and X-Gal stainings were carried-out according to standard protocols (Thisse et al., 1993; Hauptmann and Gerster, 1994). The following probes and antibodies were used: *bon/mixer* (Kikuchi et al., 2000), *bts1* (Tallafuß et al., 2001), gta3 (Neave et al., 1995), her5 (Müller et al., 1996); *hgg1* (Thisse et al., 1994), *hlx1* (Seo et al., 1999), *hoxa1a* (McClintock et al., 2000), *nkx2.2* (Barth and Wilson, 1995), *pax6.1* (Nornes et al., 1998), *six3* (Seo et al., 1998), *sox17* (Alexander and Stainier, 1999), *zash1a, zash1b* (Allende and Weinberg, 1994), *zcoe2* (Bally-Cuif et al., 1998), anti-invected 4D9 antibody (recognizing all zebrafish Eng proteins) (DHSB) (dilution 1/4), anti-HNK1 (DHSB zn12) (dilution 1/500). Flat-mounted embryos were photographed and scored under a Zeiss Axioplan microscope.

Results and discussion

six3 is coexpressed with GATA 3 (gta3) and is an early marker of nMLF/nPC neurons

   nMLF neurons are identifiable from 16hpf onwards (15 somites) by their HNK1 immunoreactivity (Fig.1A-C, E-H). At 18hpf, nPC neurons become detectable along the p1/mes junction (Fig.1A,B). Together, nMLF/nPC neurons organize as two longitudinal stripes, merged at their caudal end and diverging towards anterior in a V shape. To determine whether both neuronal groups could share developing cues, we conducted an in situ

hybridization search for mRNA markers jointly identifying these two neuronal populations between 16 and 36hpf. *gta3* expression was reported in a subpopulation of HNK1-positive neurons in the ventral di- and mesencephalon from 20hpf onwards (Neave et al., 1995), where it was interpreted to transiently label nMLF neurons prior to their differentiation. We found that *gta3* expression is initiated in that location as early as at 17hpf (16-18 somites), where it immediately follows the onset of HNK1 immunoreactivity (not shown). *six3* expression was also described in the ventral midbrain from 24hpf onwards (Kobayashi et al., 1998). We found that *six3* expression is initiated at 18hpf (18 somites) in that location (Fig.1E-H), and that, like *gta3*, it defines a cluster encompassing but slightly more extended than HNK1 in the nMLF (Fig.1G,H, and data not shown). *six3*-positive cells are located away from the ventricular surface, suggesting that they correspond to post-mitotic neurons (Fig.1G). A similar observation was made for *gta3* expression (Neave et al., 1995). At 26hpf, *six3* expression organizes as two branches, of which the ventral branch encompasses the nMLF and the dorsal branch prefigures the nPC (Fig.1A-C). At all stages, *six3* expression appears in exact overlap with *gta3*-positive cells (Fig.1D). nMLF and nPC neurons arise in neighbouring locations, however they develop with a few hours delay, and exhibit distinct projection patterns (nMLF axons being targeted posteriorly, while those of nPC neurons grow towards dorsal). Our identification of *gta3* and *six3* expression as delineating both neuronal populations suggests that the formation of these two clusters however responds to shared developmental cues. Because *six3* only labels the nMLF/nPC territory at this anteroposterior level (in contrast to *gta3*, also expressed in underlying structures), we focused on this marker for subsequent analyses.

The location of the nMLF has been mapped relative to the expression of several molecular markers in the 24hpf zebrafish brain (Macdonald et al., 1994; Barth and Wilson, 1995; Hauptmann and Gerster, 2000; Hjorth and Key, 2001). It lies at the interface of *shh* and *nkx2.2* expression, overlaping the caudal boundary of *pax6.1* expression. To deepen these findings, we positioned *six3* expression relative to a series of additional territorial and neuronal markers at 26hpf, when it defines the nMLF/nPC domain (Fig.1I-P). Along the dorsoventral axis, *six3* expression transects the *nkx2.2*-positive band (Fig.1J). Along the AP axis, it is entirely located anterior to the mesencephalic expression domain of Eng proteins (Fig.1K), and its two branches cross the *pax6.1* expression border (Fig.1I). Finally, it lies mostly outside the expression of markers delimiting proneural fields or newly selected neuroblasts, such as *zcoe2*, *zash1a* and *zash1b* (Fig.1L-N) (Bally-Cuif et al., 1998; Allende and Weinberg, 1994). Its caudalmost cells coexpress *hoxa1a* and lie immediately adjacent to the *bts1*-positive nucleus

of the basal mesencephalon (Fig.1O,P) (McClintock et al., 2000; Shih et al., 2000; Tallafuß et al., 2001) (Results summarized in Fig.1Q).

The position of the *six3*-positive cluster outside AP and DV markers of ongoing neurogenesis further confirms that it is likely mostly composed of post-mitotic neurons. The degree of neuronal commitment of *six3*-positive cells compared to HNK1 expression remains however unclear, as HNK1 expression precedes *six3* in the nMLF, but follows it in the nPC. Because cells positive for *six3* but negative for HNK1 are generally located in a more ventricular location than doubly positive cells (see Fig.1C,G), *six3* expression might identify nMLF/nPC neurons from an earlier differentiation state than HNK1 immunoreactivity. The earliest born nMLF neurons would then follow a different sequence, independent of *six3* expression. Together, our observations demonstrate that *six3* expression distinctly identifies a neuronal cluster, located at the intersection of territorial influences, both along the DV and AP axes. This finding gives molecular support to the postulate of Wilson (1993) that cells at the interface between adjacent expression domains may have an identity distinct from that of either of the neighbouring domains.

We used *six3* expression as a marker to determine which combination of influences conditions the proper development of the nMLF/nPC cluster.

six3 expression at the p1/mesencephalic border depends on positive patterning influences from the midbrain-hindbrain boundary and axial midline structures.

Signaling from the midbrain-hindbrain boundary (MHB) and the axial midline influence neural patterning at a long range. Specifically, the MHB is necessary for mid- and hindbrain patterning and growth (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001), the prechordal plate (PCP)/ventral forebrain is essential to the development of the basal diencephalon (Muenke and Beachy, 2000; Kiecker and Niehrs, 2001), and the notochord/floor plate induces ventral identities in the midbrain, hindbrain and spinal cord (Altmann and Hemmati-Brivanlou, 2001; Poh et al., 2002). Because the di-mesencephalic *six3*-positive cluster is located at the intersection of DV and AP territorial boundaries, we assessed the influence of these patterning cues on its development.

Both *pax2.1/noi* [tu29a] and *fgf8/ace* mutants fail to maintain the MHB organizer and secondarily lack entire dorsal midbrain-hindbrain identity (tectum, isthmus and cerebellum) by 24hpf (Brand et al., 1996; Lun and Brand, 1988; Reifers et al., 1998). Correlatively, the caudal domain of the dorsal branch of *six3*-expression at the p1/mes boundary was significantly reduced in *noi* and *ace* mutants compared to wild-type siblings (Fig.2A-C). These observations

suggest that integrity of the *six3*-positive cluster is, in part, secondarily dependent on MHB activity through its control of midbrain maintenance.

smoothened/slow muscle omitted (*smu^{b641}*) mutants fail to transduce Hh signaling (Barresi et al., 2000; Chen et al., 2001; Varga et al., 2001), they are defective in ventral forebrain development and display strongly downregulated *nkx2.2* expression (Varga et al., 2001). Expectedly, less p1/mes cells expressed *six3* in *smu* at 26hpf, with special reduction of the ventral branch (Fig.2A,D). A stronger phenotype was obtained in *cyclops* (*cyc*), maternal-zygotic *squint* (*MZsqt*) and MZ *one-eyed pinhead* (*MZoep*) mutants, where the p1/mes *six3*-positive cluster generally failed to form (not shown). *cyc*, *MZsqt* and *MZoep* are primarily compromised in Nodal signaling and the formation of all or part of the axial midline (Feldman et al., 1998; Gristman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998; Zhang et al., 1999), and largely fail to specify ventral neural tube identity (Hatta et al., 1001; Krauss et al., 1993). Thus integrity of the *six3*-positive cluster is also, in part, dependent on the specification of ventral neural territories by vertical signaling from axial midline structures.

Importantly, in all mutant contexts studied above, the affected neural domains that normally overlapped with or contacted *six3*-positive cells are mispatterned rather than deleted: MH deletion in *noi* is limited to the Eng-positive domain (Lun and Brand, 1998; our unpublished observations), which is adjacent to but non overlapping with the *six3* cluster (Fig.1K); similarly, *smu* affects *nkx2.2* expression but not the development of a medial floor plate and motorneurons, arguing against a deletion of the ventral neural tube (Chen et al., 2001; Varga et al., 2001). Our findings extend those of Mastick et al. (1997) reporting less nPC neurons in *Pax6* mouse mutants, and together, these results indicate that a combination of positive posterior and ventral influences, each primarily controlling patterning along the AP or DV axes, is integrated to permit the development of an intact *six3*-positive cluster at the p1/mes boundary. Thus, although the *six3*-positive cluster has a unique molecular identity, it is sensitive to identity changes of the different territories that it contacts. This is reminiscent of the behavior of other neuronal clusters located at territorial boundaries within the embryonic brain. For instance the nTPOC, which differentiates along the dorsal boundary of the hypothalamus, fails to form when hypothalamic identity is perturbed (Mathieu et al., 2002). Interestingly, our results also point out that the response of *six3* expression to patterning defects is not all or none, but is limited to the subpopulation of *six3*-posiive cells overlapping the mispatterned territory. Thus, although the nMLF/nPC cluster is characterized as a whole by *six3* expression, its development is controlled in a modular fashion.

The number of *six3*-expressing cells at the p1/mes border depends on the function of Bonnie-and-clyde/Mixer

In contrast to the phenotypes described above, all characterized by a decreased number of *six3*-positive cells, we found that the *six3*-positive cluster was significantly enlarged in the mutant *bonnie-and-clyde*/*mixer* (*bon^{m425}*) (Kikuchi et al., 2000) from 24hpf (80 +/- 5 cells in wt, n=30, 130 +/- 6 cells in *bon* at 26hpf, n=30) (Fig.3A,B). The overall shape of the *six3* cluster was maintained, indicating no anisotropy in the increase in cell number along the AP or DV axes. Similar observations were made looking at *gta3* expression (not shown). To determine whether this phenotype reflected a general enlargement of the p1/mes area, we probed *bon* embryos at 90% epiboly (see *her5* expression in Fig.3E,F), 15 and 24hpf for patterning and neurogenesis markers including the combination described above (Fig.1I-P). None of these profiles showed any detectable size alteration in the p1/mes area at any stage (not shown). Thus we conclude that Bon/Mixer function is selectively involved in limiting the number of *six3*-positive cells in the p1/mes territory in vivo.

The primary phenotype of *bon* mutants is the near complete absence of endodermal precursors and derivatives (Kikuchi et al., 2000). Secondarily, *bon* mutants suffer from cardia bifida and exhibit collapsed brain ventricules. To date, however, no brain patterning or neurogenesis defects have been reported. *bon*/*mixer* encodes a homeodomain protein, the expression of which is restricted to the blastoderm margin, including yolk syncitial layer, at early gastrulation (Alexander et al., 1999; Aoki et al., 2002, and see Fig.3C). These domains are fated to the endoderm proper, the prechordal plate (PCP) and the yolk syncitial layer (YSL), and do not encompass neuroectodermal precursors (Woo and Fraser, 1995; Varga et al., 1999, Warga and Nüsslein-Volhard, 1999). No *bon*/*mixer* expression is detected in the neural plate until at least 36hpf (Fig.3D and data not shown). Thus increased *six3*-positive cell number in *bon* secondarily results from the lack of *bon*/*mixer* expression in precursors of the endoderm, PCP and/or YSL.

bon mutants fail to specify most endodermal precursors (Kikuchi et al., 2000; Aoki et al., 2002, see Fig.3E,F), but a requirement for Bon/Mixer alone in PCP or YSL development was not documented (Poulain and Lepage, 2002). We found that *bon* mutants also exhibit reduced PCP and PCP derivatives such as the hatching gland, identified by *hlx1* and *hgg1* expression at late gastrulation stages (Fig.3G-J). It is unlikely that this phenotype results from the lack of endoderm in *bon*, since the PCP is not affected in *casanova* (*cas*) mutants, which fail to specifiy all endodermal precursors (Alexander et al., 1999). Rather, defective PCP in *bon* likely reflects a direct role of Bon/Mixer in PCP precursors. This requirement must take

place at an early stage, since bon/mixer expression is switched off from the blastoderm margin immediately after the shield stage. *bon* mutants do form a morphological YSL, visible at the dome stage by microscopic inspection (not shown). Because the function and expression profile of the YSL have not been determined, it is however difficult to monitor whether the YSL is fully functional in *bon*. Thus, we conclude that altered p1/mes *six3*-positive cluster in *bon* is caused by defective endoderm, PCP and/or YSL.

Because the *six3* phenotype in *bon* is not accompanied by mispatterning of the p1/mes brain area, our observations highlight for the first time the existence of a (direct or indirect) long-range influence that selectively acts on the development of a given neuronal cluster. Interestingly, this influence also differs from the general patterning signals described above in that it is inhibitory rather than permissive, as it is involved in limiting the number of *six3*-positive neurons developing at the p1/mes boundary in vivo. The cellular process(es) affected by this inhibitory cue remain to be determined. We did not observe specific cell death in the p1/mes area in wild-type embryos at any stage (acridine orange and TUNEL assays, not shown). Likewise, we failed to detect differences in cell proliferation between wild-type and *bon* mutants in this area (anti-phospho-histone H3 immunocytochemistry, not shown). Thus an influence on cell death or cell proliferation in the p1/mes area can likely be excluded. It follows that increased number of *six3*-positive cells in bon rather resembles a local neurogenic phenotype. Further experiments will be required to confirm this hypothesis.

We next attempted to determine which of the tissues primarily affected in *bon* accounts for the *six3* phenotype. We present below an analysis based on selective rescue to dissect the relative role(s) of each structure in the generation of the *six3* expression defect.

Altered *six3* expression in *bon* results from defective *bon* prechordal plate

To determine whether the *bon six3* phenotype was due to the lack of endodermal precursors or derivatives, we monitored *six3* expression in *cas* mutants. *cas* is selectively deficient in endoderm but has normal PCP and YSL (Alexander et al., 1999; Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001) (Fig.4A). Patterning of the anterior neural plate in *cas* appeared normal at all stages (not shown), suggesting that endodermal factors are generally dispensable for brain development in zebrafish. In addition, we found no difference in the number of p1/mes *six3*-positive cells between *cas* and wild-type embryos at 26hpf (n=30) (Fig.4B,C). Thus the lack of endoderm alone can be excluded as causing the *six3* phenotype in *bon*.

To address whether this phenotype was due to the lack of Bon/Mixer expression in the YSL, we selectively rescued Bon/Mixer function in this layer (Fig.4D). Capped mRNA encoding Bon/Mixer (20 pg) was injected into this layer at the dome stage (n>100). Coinjected tracer RNA controlled for the distribution of the injected product throughout the YSL. Injected *bon* mutants displayed reduced cardiac phenotype compared to their non-injected mutant siblings: they developed a heart in medial position, identifiable by morphology and expression of the heart field marker *gata6* (not shown). However this heart was much smaller than wild-type. The injection of a similar dose of *bon/mixer* into the blastoderm of one-celled embryos fully rescued the endoderm deficiency and cardia bifida phenotypes (not shown). These results suggest that the rescue of Bon/Mixer function in the YSL can only very partially compensate for the lack Bon/Mixer activity overall. In addition, we found that *bon* embryos that had selectively inherited *bon/mixer* RNA into the YSL showed a *six3* phenotype identical to that of uninjected *bon* mutants (108 +/- 3 cells in the p1/mes *six3*-positive cluster in injected *bon* at 26hpf, n=15, versus 80 +/- 5 cells in injected wild-types, n=15) (Fig.4E,F). Thus the lack of Bon/Mixer activity in the YSL alone is not sufficient to account for the *six3* phenotype in *bon*.

To test the involvement of the PCP in generating the *six3* phenotype in *bon*, we rescued this tissue (together with the endoderm) by making use of the constitutively active form of the TGF type I receptor Taram-A (Tar*). Tar* drives its expressing cells towards an endo- and mesendodermal fate (Peyrieras et al., 1998; David et al., 2001). When injected into one marginal blastomere at the 16-cell stage, *Tar** mRNA induces the formation of a secondary axis in which the entire endoderm and PCP, and exclusively these structures, derive from the injected cell (see Bally-Cuif et al., 2000; Aoki et al., 2002; David et al., 2002; Mathieu et al., 2002). Thus, when coinjected with *bon/mixer* mRNA into a *bon* embryo, *Tar** induces a secondary axis where Bon/Mixer function in the endoderm and PCP is rescued (Fig.4G). At 26hpf, injected *bon* mutant embryos could be identified by prominent cardia bifida in their endogenous axis, while their secondary axis no longer displayed a characteristic *bon* phenotype. We thus compared the number of p1/mes *six3*-positive cells in the secondary axes of injected *bon* mutants and their injected wild-type siblings. Only embryos displaying a complete secondary axis (i.e. with anterior head and eyes) were considered. We found a comparable number of p1/ mes *six3*-positive cells in the secondary axes of both injected mutant and wild-types (21 +/- 1 cells in *bon*, n=19, versus 21 +/- 1 cells in wild-type, n=21) (Fig.4H,I). Because lack of endoderm alone does not result in a *bon*-like *six3* phenotype (see above), we thus conclude that the increased number of p1/mes *six3*-positive cells in *bon* results from deficient PCP development.

The PCP is remarkable for its influence on forebrain patterning, permitting the development of ventral di- and telencephalic structures at the expense of dorsal identities. Our results provide the first report of an (direct or indirect) activity of the PCP on more posterior brain domains, and selectively targeted to a neuronal cluster. The PCP and at least some of its derivatives (such as the medial aspect of the pharynx) underlie the presumptive midbrain / diencephalic area at all gastrulation and somitogenesis stages. This leaves ample time for the PCP to influence the number of *six3*-posiitve cells at the p1/mes boundary, although our molecular markers only permit a read-out of this effect from 26hpf onwards and do not provide an indication on the timing of this regulatory process.

The PCP factor controlling the number of p1/mes *six3*-positive cells is not a Nodal signal

Bon/Mixer encodes a transcription factor and is thus unlikely to be the direct effector of the PCP in its regulation of *six3* expression. A number of signaling factors originate from the PCP. Among those, Nodal signals have received most attention and are involved in forebrain induction and patterning in zebrafish (Rohr et al., 2001; Mathieu et al., 2002, and references therein). Although the role of Nodal signaling in the development of more posterior brain structures has not been assessed, it is conceivable that deficient Nodal signaling from the impaired PCP in *bon* directly or indirectly influences the number of p1/mes *six3*-positive cells. To test this hypothesis, we studied *six3* expression in *MZsqt, cyc* and *MZoep* mutants where axial midline structures were restored in secondary axes induced by the injection of *Tar** into 16-celled embryos. As above, only embryos displaying a full secondary axis were considered. Injected mutant embryos were identified by the prominent cyclopia of their endogenous axis, while their secondary axis was rescued in this phenotype (Fig.4J). The rescued axial midline structures of injected *MZsqt* and *cyc* mutants are however still deficient in their production of a Sqt or Cyc signal, respectively, while the neuroectoderm of injected *MZoep* mutants is deficient in its processing of Nodal signaling. We found no significant difference in the number of p1/mes *six3*-positive cells between the induced axes of wild-type (20 +/- 1 cells, n=12), *MZsqt* (18 +/- 1 cells, n=6) (Fig.4K,L), *cyc* and *MZoep* embryos (n=3, not shown). Thus the PCP factor limiting the number of p1/mes *six3*-positive cells is unlikely to be a Nodal signal. The PCP expresses a number of signaling factors that, alone or in combination, could account for *six3* regulation. For example, BMP4, 7 and ADMP, Shh and Twhh, Wnts and opponent secreted factors such as Dkk, are, among other expression sites, expressed in the PCP at gastrulation or later stages. The selective role of these factors in mediating PCP development or function remains, with a few exceptions, unexplored. Understanding which

combination of PCP factors is involved in the selective control of *six3* expression will require partial functional rescue of the PCP in *bon* by the coinjection of *Tar** and the relevant morpholinos or dominant-negative mutant forms.

Conclusions

Together, our results demonstrate that nMLF/nPC neurons, which develop at the intersection of AP and DV patterning cues, are identified by their common expression of the transcription factor Six3 (or Gta3). They provide molecular support to the idea that neuronal clusters developing at territorial boundaries display unique molecular identities, but also demonstrate that such clusters can respond to neighboring patterning influences in a modular fashion. Finally, our findings highlight for the first time the existence of a (direct or indirect) long-range inhibitory influence that selectively limits the number of *six3*-positive neurons identified at the p1/mes boundary, and demonstrate that this influence originates from the PCP. To our knowledge, this is the first demonstration of a long-range signaling process that selectively controls the size of a given neuronal cluster without correlatively affecting neural plate patterning.

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Legends to Figures

Figure 1. *six3* expression at the di-mesencephalic border identifies the nMLF/nPC population and lies at the intersection of patterning and neurogenesis markers. Whole-mount embryos were processed for in situ hybridization and/or immunocytochemistry for the markers indicated (colour-coded) and flat-mounted (A,B,D,I-P: sagittal views of the head, anterior left, B and D are high magnifications of the area boxed in A; C,E,F,H: dorsal views of the ventral di- and mesencephalon, anterior left; G:cross section of F at the level indicated, dorsal up). **A-D:** at 30hpf, *six3* expression organizes as two longitudinal branches (blue arrows in B) that encompass the nMLF (brown arrow in B) and nPC (red arrow in B), and exactly coincide with *gta3* expression (D). **E-H:** *six3* expression immediately follows HNK1 immunoreactivity of the nMLF and also labels cells located immediately adjacent to the HNK1-positive cluster in that territory (blue arrows in F,G point to cells only expressing *six3* – also visible in C-, red arrows to doubly labeled cells). **I-P:** *six3* expression (red arrow) overlaps boundaries of territorial (I-K) and neurogenesis (L-P) markers: it crosses the AP boundary of *pax6* expression (I) but is anterior to the midbrain Eng-positive domain (K), it overlaps the *nkx2.2* DV stripe (J), and is located between major sites of ongoing neurogenesis defined by *zcoe2*, *zash1a* and *zash1b* expression (L-N); the ventrocaudalmost *six3*-positive cells coexpress *hoxa1a* (O) but not *bts1* (P). These findings are recapitulated at high magnification in a schematized form in **Q** (color-coded) (red lines to the p1/mes and *shh*/*nkx2.2* boundaries, circle to the projection of the center of the eye).

Figure 2. Distinct subdomains of *six3* expression respond to di- and mesencephalon patterning deficiencies. A-D: expression of *six3* at the p1/mes boundary at 26 hpf in wild-type (A), noi^{tu29a} (B), ace^{ti282a} (C) and smu^{b641} (D) mutants. Arrows point to affected domains of *six3* expression (posterior part of the dorsal branch of in *noi* and *ace*, ventral branch in *smu*). **E:** Schematic representation of the *six3*-positive cluster (blue) and its distinct subdomains affected in *noi* / *ace* versus *smu*.

Figure 3. The number of p1/mes *six3*-positive cells is increased in *bon* mutants, which display molecular alterations in the endoderm and PCP. A,B: high magnification of the p1/mes *six3*-positive cluster in 26hpf wild-type and bon^{m425}, as indicated, anterior left. The number of *six3*-positive cells is 1.5 times increased in bon^{m425}. **C,D:** expression of *bon*/*mixer* in wild-type embryos, sagittal views, anterior left inset: top view) is restricted to precursors of

the endoderm, PCP and YSL at gastrulation and absent from neuroectodemal precursors at all stages. **E-J:** expression of neural plate markers (e.g. *her5*) are not affected in *bon*[m425], but endodermal (*sox17*) and PCP (*hlx1*, *hgg1*) markers are reduced (all views dorsal, anterior up).

Figure 4. The PCP, but not via Nodal signaling, is responsible for the *six3* phenotype in *bon* mutants. A-C: Altered endoderm in *bon* does not cause the *six3* phenotype. **A:** Schematics of the non-neural structures mutant (endoderm, red) and wild-type (PCP derivatives, yellow) in *casanova* (*cas*). **B,C:** high magnifications of the *six3*-positive cluster, and corresponding cell counts, are identical between wild-type (B) and *cas* mutant siblings (C). **D-F:** Lack of YSL Bon/Mixer function does not account for the *six3* phenotype. **D:** Schematics of the non-neural structures mutant (endoderm and PCP, red) and rescued (YSL derivatives, yellow) in *bon*[m425] upon injection of capped *bon*/*mixer* mRNA into the YSL. **E,F:** high magnifications of the *six3*-positive cluster, and corresponding cell counts, reveal the maintenance of a mutant *six3* phenotype upon YSL rescue in *bon*[m425]. **G-I:** Altered PCP causes the *six3* phenotype in *bon*. **G:** Schematics of the non-neural structures mutant (endogenous axis: endoderm, PCP and YSL, red) and rescued (duplicated axis: endoderm and PCP, yellow) in *bon*[m425] upon injection of capped *Tar** mRNA into one marginal blastomere at the 16-cell stage. **H,I:** injected embryos, high magnifications of the *six3*-positive cluster of the secondary axes, and corresponding cell counts, show identical number of *six3*-positive cells upon PCP rescue in *bon*[m425]. **J-L: The PCP signal deficient in *bon* and causing the *six3* phenotpe is not Sqt. J:** Schematics of the structures mutant (endogenous axis: endoderm, PCP and YSL, red) and rescued (duplicated axis: endoderm and PCP, yellow) in *MZsqt* mutants upon injection of capped *Tar** mRNA into one marginal blastomere at the 16-cell stage. **K,L:** Injected embryos, high magnifications of the *six3*-positive cluster of the secondary axes, and corresponding cell counts, show no significant difference in the number of *six3*-positive cells upon PCP rescue in *MZsqt*.

30hpf

A | B | C | D

six3 + HNK1 | *six3* + *gta3*

16 som | **19 som** | **24 som**

E | F | G | H

six3 + HNK1

26hpf

I | J | K | L

six3 + *pax6.1* | *six3* + *nkx2.2* | *six3* + Eng | *six3* + *zcoe2*

M | N | Q

six3 + *zash1a* | *six3* + *zash1b*

O | P

six3 + *hoxa1a* | *six3* + *bts1*

zash1a
pax6
nkx2.2
six3
hoxa1a
bts1

Tallafuß et al., Fig.1

six3

A — wt

B — noi

C — ace

D — smu

E

noi, ace

smu

Tallafuß et al., Fig.2

six3	**A**	**B**
	wt, 26hpf	*bon*, 26hpf
mixer / bon	**C**	**D**
	wt, shield	wt, 10 som
her5 + sox17	**E**	**F**
hgg1	**G**	**H**
hlx1	**I**	**J**
	wt, 90% epiboly	*bon*, 90% epiboly

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Fig.3

A

mutant / wt

B *six3*

wt   80

C

cas   80

D

E *six3*

wt, *bon/mixer-inj.*, YSL   80

F

bon, bon/mixer-inj., YSL   108

G

H *six3*+beta-gal

wt, *Tar*++*bon/mixer*-inj.   20.5

I

bon, Tar++*bon/mixer*-inj.   21

J

mutant / restored

K *six3*

wt, *Tar*+-inj.   20

L

sqt, Tar+-inj.   18

Tallafuß et al., Fig.4

Appendix 4

submitted to *Development*

Tracing of *her5* progeny in zebrafish transgenics reveals the dynamics of midbrain-hindbrain neurogenesis and maintenance.

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Abstract

Within the vertebrate embryonic neural tube, midbrain-hindbrain (MH) development is characterized by an extended maintenance period. During this phase, the continuation of MH growth and the diversification of MH patterning are promoted by the isthmic organizer (IsO), located at the midbrain-hindbrain boundary (MHB). Thus MH development displays long-lasting plasticity; however the potentialities and fate of MH tissue upon impaired IsO activity have not been directly determined. To follow the dynamics of MH maintenance in vivo, we used artificial chromosome transgenesis in zebrafish to construct lines where *egfp* transcription is driven by the complete set of regulatory elements of *her5*, the first known gene expressed in the MH area. In these lines, *egfp* transcription faithfully recapitulates *her5* expression from its induction phase onwards. Using the stability of GFP protein as lineage tracer, we first demonstrate that *her5* expression at gastrulation is indeed a selective marker of MH fate. We show that *her5* expression is subsequently dynamically down-regulated upon cell divisions, revealing a progressive convergence of MH neurogenesis towards the MHB over time. Finally, we trace the molecular identity of GFP-positive cells in the *acerebellar* (*ace*) and *no-isthmus* (*noi*) mutant backgrounds to analyze directly *fgf8* and *pax2.1* mutant gene activities for their ultimate effect on cell fate. We demonstrate that most MH cells are maintained in both mutants but are partially re-specified towards adjacent identities, in a manner that strikingly differs between the *ace* and *noi* contexts. Specifically, our observations directly support a role for Fgf8 in protecting anterior tectal and metencephalic fates from anteriorization, while Pax2.1 controls the maintenance of MH identity as a whole. Together, our results provide the first direct assessment of MH fate in the absence of IsO activity, and shed light on the distinct functions of IsO factors in MH maintenance.

Introduction

Building of the vertebrate embryonic brain is a progressive process that involves a number of consecutive steps controlling patterning and neurogenesis events. Both processes respond to phases of induction and refinement, during which the positional identity and differentiation status of neural cells are specified, maintained or modified in a dynamically controlled manner. Unraveling the dynamics of neural patterning and neurogenesis are crucial steps in our understanding of brain development. Indeed it will highlight the potentialities of given neural territories, thus reveal how their fate and differentiation process are restricted in vivo.

The midbrain-hindbrain (MH) domain of the embryonic neural tube displays extensive plasticity linked to specific ontogenic properties that make it an important model to study developmental dynamics (see (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The MH can be morphologically identified at early somitogenesis stages as comprising the mesencephalic vesicle and the first rhombencephalic vesicle (or metencephalon) (Fig.1); the latter -

also called "rhombomere A" in the chicken embryo (Vaage, 1969)-, will later subdivide into rhombomeres (r) 1 and 2. Detailed fate map analyses in avian embryos demonstrated that the mesencephalon generates all midbrain structures, i.e. essentially an alar visual center, the tectum, and a basal tegmentum, containing cranial motorneuron III (Marin and Puelles, 1994; Martinez and Alvarado-Mallart, 1989). In addition, the caudal third of the alar mesencephalic domain contributes to the dorso-medial part of the cerebellar plate (Hallonet and Le Douarin, 1990; Hallonet et al., 1993; Martinez and Alvarado-Mallart, 1989), while the alar domain of r1 will give rise to remaining, lateral cerebellar structures (Wingate and Hatten, 1999) (Fig.1). Finally, the basal r1 territory will generate the pons, of which a prominent output is cranial motorneuron IV. These distinct fates are prefigured by molecular gradients in the expression of MH genes such as *engrailed-2/3* or *ephrins* (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Key embryology and genetic experiments in all vertebrate models over the last decade highlighted a remarkable feature of MH development: MH structures, although physically and functionally distinct, develop in a concerted fashion. Indeed, their growth and patterning is dependent upon and coordinated by an organizing center (the "isthmic organizer" –IsO-, or "isthmus") located at the midbrain-hindbrain boundary (MHB) (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001) (Fig.1). Among the factors that likely mediate IsO activity are the secreted proteins Fgf8 and Wnt1, expressed on either side of the MHB. Detailed genetic analyses in the mouse, chicken and zebrafish further demonstrated that a positive cross-regulatory loop between the expression of IsO markers, of Pax2/5/8- and of Engrailed-family members was involved in the stabilization and refinement of MH identities from early somitogenesis stages onwards (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Importantly, these studies pointed to the remarkable plasticity of MH identities, the regionalization of which becomes only fixed at a late stage. For instance, in the avian embryo, midbrain AP polarity can be regulated until at least 12-13 somites: at that stage, it is corrected following an experimental rotation of the mesencephalic vesicle in ovo (Marin and Puelles, 1994), or is reorganized around ectopic transplants of MHB tissue (Alvarado-Mallart et al., 1990; Gardner and Barald, 1991; Martinez et al., 1991; Nakamura et al., 1988) or around ectopic foci of Fgf8 expression (Crossley et al., 1996; Irving and Mason, 2000; Lee et al., 1997; Martinez et al., 1999). At the same stage, MH identity can also be changed into a diencephalic or more posterior hindbrain specification in misexpression experiments of diencephalic (Pax6) (Matsunaga et al., 2000a) or r2 (Hoxa2) (Irving and Mason, 2000) factors.

Important insight into the pluripotentialities of MH tissue is likely to be further provided by the analysis of mouse or zebrafish mutants fully or partially deficient in IsO activity. In this respect, the zebrafish mutants for Pax2.1 (*no-isthmus* –*noi*-) and Fgf8 (*acerebellar* –*ace*-) functions are of particular interest, since in these backgrounds the MH is initially properly induced, but its maintenance is deficient (Brand et al., 1996; Lun and Brand, 1998; Reifers et al., 1998). During somitogenesis, strong *noi* alleles progressively cause loss of the tectum, isthmus and cerebellum (Brand et al., 1996;

Lun and Brand, 1998). Similarly, *ace* mutants progressively lack an isthmus and cerebellum (Reifers et al., 1998). *ace* embryos only maintain tectal structures, which express low levels of Eng, *ephrin-A5a* and *–A2*, suggesting that they are of anterior identity (Brand et al., 1996; Picker et al., 1999). In both mutants, differentiation defects are preceded by the progressive loss of expression of markers for the posterior tectum, isthmus or cerebellar territories (Lun and Brand, 1998; Reifers et al., 1998). In addition, in *noi*, posterior expansion of the diencephalic marker *fgfr3* has been reported (Sleptsova-Friedrich et al., 2002). Understanding the fate of initially specified MH cells in these backgrounds would reveal the capacities of MH tissue in the absence of a fully functional IsO. Currently, several (non-exclusive) interpretations can account for the loss of MH identities in *noi* and *ace* mutants, among which the conversion of MH precursors to alternative fates (still to be determined and possibly including cell death), or the increased proliferation of non-MH versus MH precursors, in the absence of a fully functional IsO. Precise tracing of MH cells, initially normally specified at gastrulation in these mutant contexts, would provide invaluable information on the potentialities of MH precursors, thus on the plasticity and dynamics of MH maintenance. Thus, such information would also reveal the exact role(s) of the MH maintenance process in the stabilization, diversification and realization of specific MH identities. This approach requires, however, the previous – and to date lacking - definition of a stable marker selectively identifying all MH precursors.

The MH domain is also characterized by a striking profile of neurogenesis, as neuronal differentiation in the immediate vicinity of the MHB (the so-called "intervening zone", IZ) is much delayed compared to other domains of the neural tube (Bally-Cuif et al., 1993; Palmgren, 1921; Vaage, 1969; Wullimann and Knipp, 2000) (Fig.1). The presence of the IZ is believed to be of key importance to maintain a pool of progenitor cells permitting the extensive growth of MH structures over time (see (Hirata et al., 2001; Tallafuss and Bally-Cuif, 2002)). Recent results suggest that IZ formation is permitted by an active process of neurogenesis inhibition in this location. In zebrafish, the bHLH E(spl)-like factor Her5, selectively expressed at the MHB (Müller et al., 1996), was identified as the crucial element both necessary and sufficient for the formation of the basal IZ domain (Geling et al., 2003). Manipulating *her5* expression until at least 24hpf can alter neurogenesis in the basal MH territory (Geling et al., 2003), suggesting that the MH differentiation profile, like MH patterning, is also subject to dynamic maintenance. These results suggest that, in addition to be required for MH growth, IZ maintenance also plays a crucial role in controlling the extent of neurogenesis over time.

Understanding the dynamics of MH regional specification and neurogenesis are thus important issues since, as described above, sustained MH plasticity correlates with the development of distinct and organized (i) MH derivatives and (ii) neurogenesis domains. To approach this question, we chose to focus on the regulation of *her5* expression. Two main reasons motivated our choice. First, *her5* is the earliest known marker of the MH area (Bally-Cuif et al., 2000; Müller et al., 1996), and as such is the best candidate to label most MH precursors from the moment they are induced within the neural plate. If this proves true, tracing the descendants of cells expressing *her5* at its onset thus should provide the

best available means of assessing the fate of MH precursors in vivo. Second, because *her5* expression within the IZ is precisely complementary to MH neurogenesis sites, and is a crucial factor conditioning the taking place of the neurogenesis process, looking at the regulation of *her5* expression should permit to appreciate, in a complementary manner, the dynamics of MH neurogenesis progression.

We thus embarked on the construction of zebrafish embryos where a stable reporter labels all descendents of *her5*-expressing cells. To maximize our chances of isolating all *her5* regulatory elements, we used in vitro homologous recombination (ET-cloning) (Muyrers et al., 2000; Muyrers et al., 1999; Zhang et al., 1998) to introduce an *egfp* reporter cDNA at the *her5* locus in a PAC containing more than 40 kb of *her5* upstream sequence. We demonstrate in several independent lines that *gfp* expression in transgenic embryos carrying the recombined *her5PAC::egfp*construct faithfully reproduces *her5* transcription at all stages, including the earliest step of *her5* induction. By comparing the distribution of *her5* RNA and GFP protein, we reveal a dynamic restriction of *her5* expression to the MHB over time, and propose that this phenomenon permits the progression of neurogenesis in a converging manner towards the MHB during MH development. Using GFP protein as a stable marker for the descendants of *her5*-expressing cells, we further demonstrate that the earliest *her5*-expression domain at gastrulation encompasses and thus is the first known marker of the whole MH anlage. Thus our lines provide us with the first means to follow MH fate in vivo. We then use GFP protein to follow MH precursors in the *noi* and *ace* backgrounds, and demonstrate that these cells partially turn on a diencephalic fate, albeit with striking spatial and identity differences between the two mutant contexts. Thus our findings, for the first time, directly demonstrate that, in the absence of IsO activity, MH precursors convert to neighbouring fates, and point to differences in the interpretation of MH dynamics between impaired Pax2.1 or Fgf8 activities.

Materials and Methods

Fish strains

Embryos were obtained from natural spawning of AB wild-type or transgenic fish, *ace*[ti282a] or *noi*[tu29a] adults (Brand et al., 1996); they were raised and staged according to Kimmel et al. (Kimmel et al., 1995).

In situ hybridisation and immunocytochemistry

In situ hybridisation and immunocytochemistry were carried out according to standard protocols (Hauptmann and Gerster, 1994). The following in situ antisense RNA probes were used: her5 {Müller, 1996; Thisse et al., 1993); *egfp* (Clontech); *pax6* (Krauss et al., 1991); *fgfr3* (Sleptsova-Friedrich et al., 2002); *otx2* (Li et al., 1994); *hoxa2* (Prince et al., 1998); *krx20* (Oxtoby and Jowett, 1993).

For immunocytochemistry the following antibodies were used: mouse anti-GFP 'JL-8' (Chemicon) used at a dilution of 1/100; mouse anti-invected 4D9 (DHSB) –that recognizes all zebrafish Eng

proteins- used at a dilution of 1/8; revealed using goat-anti-mouse-HRP (Chemicon) (dilution 1/200) followed by DAB/H_2O_2 staining, or goat-anti-mouse-FITC (Dianova) (dilution 1/200). Double in situ hybridisation and immunocytochemistry staining on transgenic embryos were performed as follows: whole-mount embryos were first processed for in situ hybridisation, then cryostat-sectioned at 8µm thickness and the sections were subjected to immunocytochemistry following standard protocols. Embryos were scored and photographed under a Zeiss SV11 stereomicroscope or a Zeiss Axioplan photomicroscope.

Isolation of her5-containing PACs

Two independent PACs containing the genomic *her5* locus were isolated by PCR from pools of library 706 (xx) (RZPD, Berlin) using the following primers: *her5* upstream 5'TAGTAGACCTAGCTGGTCTTTTCAGTCTTTGGAGAGC3', *her5* reverse

5'TAAAAAGGGCACGCACAGAGGAGAGTGATGAGGATGT3', with a 59°C annealing temperature and 30 amplification cycles, producing a specific amplification product of 450 bp. PAC DNA was prepared according to the Qiagen Large Construct kit protocol. Genomic inserts are flanked by NotI sites; digestion with NotI followed by pulse field gel electrophoresis (PFGE) revealed that the inserts of both PACs were above 100 kb. Further restriction analyses and Southern blotting revealed that one of the two PACs contained more than 40kb of upstream *her5* sequence; this Pac was chosen for further experiments.

ET cloning

ET cloning was based on the protocol provided by Stewart, available on the ET cloning web page
http://www.heidelberg.de/ExternalInfo/stewart/ETprotocols.html

1. The following vectors were used:

PEGFP-1 *(Clontech);* PSV40/Zeo *(Invitrogen);* PGlZl3_3 *(modified* PEGFP-1 *with a loxP-flanked* Zeo*-cassette in AflII-site, see below);* pGETrec*, carrying arabinose-inducible* recE *gene (Narayanan et al., 1999);* P705-*Cre (Buchholz et al., 1996);* her5-*containing PAC (*PCYPAC2n *backbone) with a total of about 100kb genomic insert and at least 40kb upstream region of* her5*), further called* her5PAC.

2. Construction of *pGlZl3_3*

pEGFP-1 was digested with AflII, and an insert containing *loxP* and the restriction enzyme site NheI (produced by oligonucleotide annealing) was inserted at this site. Similarly, a *loxP*-NheI was introduced into the vector *pSV40/Zeo* after restriction cutting with BamHI. *pSV40/Zeo::loxP-NheI* was further cut with NheI to release the NheI fragment containing full length of *Zeo^R* and *loxP*, which was inserted into *pEGF::loxP-NheI* open at NheI. This produced *pEGFP::loxP-Zeo^R-loxP*, further refered

to as *pGlZl3_3*. All plasmids containing *Zeo^R* were grown in INFαF' cells. 3. Preparation of the linear fragment *her5a-EGFP::loxP-Zeo^R-loxP-her5b* to homologously recombine into the PAC

Primer design: the fragment for homologous recombination was prepared by PCR using the following primers: primer ET2: 48nt specific to the 5'-sequence of *her5* exon 2 (Fig. 2A, fragment b) and 21nt specific to *pGlZl3_3* (underlined) (sequence: 5'GTC CCC AAG CCT CTC ATG GAG AAA GGG AGG AGA GAT CGC ATT AAT CAA <u>GTC GCC ACC ATG GTG AGC AAG</u>3'), and primer ET1: 47nt specific to the 3'-sequence of *her5* exon 2 (Fig. 2A, fragment b) and 22nt specific to *pGlZl3_3* (underlined) (sequence: 5'CTC ATT GTT TGT GTT CTC AAG TAA AAG CAT CTC AA GGT TTC TAG <u>GCT TAA CGC TTA CAA TTT ACG CCT</u>3').

Oligonucleotide purification: Oligonucleotides ET1 and ET2 were resuspended in water and purified as follows: to 100 µl, 12 µl 3 M Sodium-Acetate (pH 7.5) and 120 µl phenol were added, vortexed and centrifuged for 3 minutes. Then 360 µl Ethanol was added, and the mix was placed 10 sec. at 80°C, washed once with 75% EtOH, dried and finally dissolved in 100 µl water.

PCR amplification of the fragment *her5a-EGFP::loxP-Zeo^R-loxP-her5b*: Template *her5PAC* DNA was denatured for 2 min. at 94°C, followed by two cycles of denaturation at 94°C for 40 seconds. A first annealing was performed at 62°C for 30 seconds, with extension at 72°C for 2 minutes. This was followed by 35 amplification cycles with denaturation at 94°C for 40 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 2 minutes. The reaction was stopped by a final extension at 72°C for 10 minutes and cooled at 4°C. The expected 2kb amplification product was purified using the QIA gel extraction kit (Qiagen) as recommended, and eluted in 50µl water.

4. Preparation of bacterial cells and transformation

The bacterial host cells DH10B containing *her5PAC* were transformed with *pGETrec* and prepared for the recombination with the linear *her5a-EGFP::loxP-Zeo^R-loxP-her5b* fragment as follows: starting from an overnight culture, the cells were grown at 37°C for 90 minutes (to O.D.$_{600}$=0.2-0.3) with shaking. L-arabinose was added to the culture to a final concentration of 0.2% and the culture was grown further until O.D.$_{600}$=0.5 was reached. The cells were then

prepared as electro-competent as described in

http://www.heidelberg.de/ExternalInfo/stewart/ETprotocols.html. Electroporation of 120 ng of *her5a-EGFP::loxP-Zeo^R-loxP-her5b* fragment was performed with 2.5 kV pulses and 25 µF in 100 µl, induced with 0.2% L-arabinose at 37°C for 90 minutes before harvesting and plating twice for selection.

5. Removal of *loxP*-flanked *Zeo^R*-gene by Cre-mediated deletion:

Competent cells carrying the recombined *her5PAC* were transformed with *p705-Cre* using standard protocols. *p705* is based on the *pSC101* temperature-sensitive origin, which maintains a low copy number and replicates at 30°C but not at 40°C. Further, *Cre* is expressed from the *lambdaPR* promoter weakly at 30°C and strongly at 37°C. Finally, these plasmids are lost from cells if incubated at temperatures above 37°C. Thus after transformation the cells were incubated for 2 days at 30°C,

followed by one day incubation at 40°C to give a transient burst of Cre expression after which the plasmids will be eliminated from the cell. The cells were then further grown for 1 day at 37°C, transferred once and finally tested by PCR for excision of the *loxP-Zeo^R-loxP* cassette, generating *her5PAC:egpf*. Because of the presence of a NotI site 3' to the *egfp* gene, digestion of *her5PAC::egfp* with NotI generated two fragments of 45 and 60 kb in addition to the vector backbone. PFGE and Southern blotting with a *her5* probe identified the 45 kb fragment as containing the coding *her5* sequence, thus *her5PAC::egfp* contains more than 40 kb upstream *her5* sequence driving *egfp* expression.

Construction of her5PAC::egfpdeletion fragments

The fragment containing 3650bp of *her5* upstream sequence was obtained by digestion of *her5PAC::egfp* with NotI + BglII followed by pulse field gel electrophoresis, identification by Southern blotting with a probe covering the *her5* 5' region, and gel purification (Qiagen Gel extraction kit). The fragment was subcloned into *pBS(SK)* for amplification, and was repurified by digestion and gel extraction before injection. The upstream fragments of 720 and 980 bp (see Fig.1B) were amplified by PCR from *her5PAC* and subcloned upstream of the xxx-xxx fragment of *pzhsp70-4* (Shoji et al., 1998), serving as a minimal promoter (I. Hokamoto, pers. comm..) and *egfp* (Clontech). The *720-hsp:gfp* and *980-hsp:gfp* inserts were purified by digestion and gel extraction prior to injection. All other constructs were prepared as PCR fragments from *her5PAC::egfp* and purified using the Qiagen PCR purification kit. All fragments were eluted in H_2O (Ambion).

Construction of the transgenic lines

her5PAC::egfp DNA was isolated using the Qiagen Large Construct Kit, eluted in H_2O and injected (in circular form) into fertilized eggs at the 1-cell stage at a concentration of 50 ng/μl. All other constructs were injected as linear fragments at the same concentration. Injected embryos were raised to adulthood and mated to wild-type adults. F1 embryos expressing eGFP were then sorted-out, raised and crossed to wild-type fish to establish the lines. We obtained integration and expression in 3 from 600 injected fish for *her5PAC::egfp* and in average 3 from 50 injected fish for the other fragments. All results presented in this work were verified over at least three generations.

Results

gfp transcription in her5PAC::egfptransgenic lines faithfully reproduces all phases of embryonic her5 expression

Because gene regulatory elements might be located at a distance from the transcriptional start site, we chose to search for *her5* enhancers using a homologous recombination approach in large genomic fragments. Two PACs were isolated that contained the genomic *her5* locus, and the PAC insert containing the longest 5' sequence (over 40 kb, as determined from pulse field gel electrophoresis and Southern blotting) was selected. The genomic structure of *her5* (Fig.2A) was determined by PAC sequencing, and was verified on the endogenous *her5* locus by PCR amplification and sequencing of genomic DNA. The complete *her5* coding sequence overlaps 3 exons, where exon1 contains the transcription start site and encodes the 17 N-terminal Her5 amino acids (Geling et al., 2003). Exon 2 codes for the 32 following amino acids, comprising the basic domain, helix 1 and part of the loop domain of Her5 (Fig.2A). 48 and 47 bp recombination arms overlapping the 5' and 3' halves of exon 2, respectively, were amplified in frame of the *egfp* cDNA and a floxed zeocine-resistance cassette (*zeo*), and the PCR product was recombined into the *her5* locus of the selected PAC using the ET-cloning technology (Muyrers et al., 2000; Muyrers et al., 1999). The resulting recombined PAC contains the *egfp* cDNA in frame after amino acid 33 of Her5 (end of the basic domain) (Fig.2A). The *egfp* cDNA was terminated with a stop codon and polyadenylation signal, thus translation of the recombined mRNA is stopped after a fusion protein that does not comprise the protein interaction motifs of Her5 (HLH and more C-terminal domains). We expected that this fusion protein would not interfere with the activity of other bHLH factors. In line with this prediction, we did not detect any morphological or molecular phenotype in all our transient or stable expression assays (see below, and data not shown).

Three independent transgenic lines were established that carried the recombined *her5* PAC (*her5PAC::egfp*lines). All showed an identical *gfp* RNA expression profile at all embryonic stages examined (data not shown). These lines will be used indiscriminately below. Wild-type *her5* expression is initiated at the 70% epiboly stage in a V-shaped neuroectodermal domain ("MH" in Fig.2C) that was fate-mapped to the midbrain at 90% epiboly (Müller et al., 1996). In addition, *her5* is transcribed in a subset of anterior endodermal precursors at early gastrulation ("e" in Fig.2C) (Bally-Cuif et al., 2000). Accordingly, we detected GFP expression in the MH domain and anterior endoderm at 24 hours post-fertilization (hpf) (Fig.2D) in all *her5PAC::egfp*embryos.

The early control of MH *her5* expression involves two distinct phases: expression is initiated at 70% epiboly by currently unknown regulators, and is maintained and refined after the 5-somite stage by the Pax2.1- and Fgf8-dependent MH regulatory loop (Lun and Brand, 1998; Reifers et al., 1998). To determine whether *egfp* transcription was a faithful reporter of *her5* expression, we performed double in situ hybridization experiments with *gfp* and *her5* probes on *her5PAC::egfp*embryos between 60% epiboly and 24 hpf (Fig.3 and data not shown). *her5PAC*-driven *gfp* transcription faithfully reproduced expression of endogenous *her5* at all embryonic stages tested, both in its onset and spatial extent (Fig.3A,C,D, and data not shown). In particular, *gfp* expression was initiated at 70% epiboly within the neural plate and maintained in the MH domain thereafter, demonstrating that both the initiation and

maintenance phases of *her5* transcription are recapitulated by expression of the transgene. Together, these observations demonstrate that the *her5PAC* construct comprises all the regulatory elements that control endogenous *her5* expression at embryonic stages.

Distinct positive and negative regulatory elements controlling endodermal and neural expression of her5 are organized over 3 kb of upstream sequence

To narrow down the sequences directing MH and/or endodermal expression of *her5*, we performed a deletion analysis series of the *her5PAC::egfp*transgene. A comprehensive series of reporter constructs of varying length encoding the Her5-eGFP fusion protein and comprising between 60 and 3650 bp upstream of the *her5* transcriptional start site (Geling et al., 2003) were amplified by PCR from *her5PAC::egfp*and tested in transient or transgenic assays (black or red lines in Fig.2B, respectively). In the latter case, at least two independent lines were established for each construct. Transient assays generally produced ectopic expression sites compared to transgenic analyses of the same fragments, however comparison of a sufficient number of injected embryos (n>30) allowed to reliably predict the reporter expression profile (not shown). Fragments of upstream sequence smaller than 130 bp produced no expression, probably due to the absence or perturbation of promoter elements (Fig.2B). In transient assays, we observed that all fragments containing 240 bp or more of upstream sequence lead to endodermal (but not neural) expression (Fig.2B). Further, transgenic lines established with 770 bp upstream region *(-0.7her5::egfp)* faithfully recapitulated *her5* endodermal expression, with similar onset and anteroposterior extent (Fig.2D-H and data not shown). All these fragments triggered endodermal GFP expression of similar intensity. These results locate the *her5* endodermal enhancer to the first upstream 240 bp, the first *her5* intron (contained in all constructs), or a combination of both.

We next examined the regulatory elements controlling neural expression of *her5*. We found that all constructs containing more than 770 bp of upstream sequence directed, in addition to endodermal expression, GFP fluorescence within the neural tube (Fig.2D-G). The intensity of neural GFP expression globally increased with fragment's length, the highest intensity being observed in *her5PAC::egfp*transgenics, suggesting that sequences positively controlling expression levels are interspersed over a large upstream genomic fragment. Importantly, MH selectivity was only achieved with upstream sequences of 2.9 kb or more *(-2.9her5::egfp* lines) (Fig.2D,E), while shorter elements invariably triggered GFP expression that overlapped the MH as well as fore- and hindbrain territories (e.g. *–1.3her5::egfp* lines, Fig.2F,G). We conclude that the spatial selectivity of the neural *her5* enhancer is modular. It is likely composed of a combination of regulatory element(s), driving anterior neural expression, and (a) overriding (or negative) element(s), located in a more upstream position and contained within the *–2.9her5:egfp* construct, and that selectively drives (or restricts) this expression to the MH domain.

To determine whether all elements controlling the temporal onset of *her5* expression were also contained within the *–2.9her5* fragment, double in situ hybridization experiments with *gfp* and *her5* probes demonstrated that *gfp* transcription in *–2.9her5::egfp* transgenics faithfully reproduces expression of endogenous *her5*, including its induction and maintenance phases (Fig.3B,E,F, and data not shown). Thus all regulatory elements driving correct MH *her5* both in time and space appear contained within the *–2.9her5::egfp* construct.

To further define the MH-specific element(s), we subcloned short 5' fragments of the *–2.9her5* construct in front of the zebrafish *hsp70* minimal promoter (Shoji et al., 1998) and H. Okamoto, pers. comm.) and *egfp* cDNA (Fig.2B, bottom, 720 and 980 bp fragments). Both minigenes were tested in transient injection assays. While the 980 bp fragment triggered neural expression in a pattern that varied from embryo to embryo and was essentially composed of ectopic, non-MH sites, expression driven by the 720 bp fragment (*720-hsp::egfp* minigene) displayed in most cases (70%, n=60) prominent expression clones within the MH domain (Fig.2I and data not shown). Thus, although we did not analyze in detail the onset of *gfp* transcription in these clones, it appears likely that the sequences directing MH expression of *her5* are at least partially comprised within the 720 bp fragment located between positions –2.9 and –2.2 kb upstream of the *her5* transcriptional start site.

Together, our analysis of the *her5* enhancer more generally demonstrates that spatially distinct and dissociable elements drive endodermal and MH expression of *her5* during embryogenesis. Further, this analysis provides us with several transgenic lines where *gfp* transcription serves as a faithful reporter of endogenous *her5* expression from its onset and throughout embryogenesis.

Endodermal her5 expression at gastrulation is fated to the pharynx

her5 expression is initiated at 30% epiboly in a subpopulation of endo/mesendodermal precursors that transiently overlaps with but is rapidly excluded from the *gsc*-positive population (Bally-Cuif et al., 2000). During gastrulation, endodermal *her5* expression distributes in scattered anterior cells (see Fig.2C); it is extinguished around the tail-bud stage. Within this population, Her5 plays a crucial role in cell fate acquisition, biasing fate choice towards a contribution to the anterior endoderm at the expense of the anteriormost mesendoderm (hatching gland) (Bally-Cuif et al., 2000); however the endodermal *her5*-positive population has not been directly mapped. We have used the stability of the GFP protein to precisely trace the fate of gastrula endo/mesendodermal *her5*-expressing cells in *her5PAC::egfp*embryos. We could detect the GFP protein by fluorescence or immunocytochemistry within the presumptive endoderm from 60% epiboly, i.e. a few hours following the onset of *her5* or *gfp* expression, until 26-30 hpf. This indicates that the GFP protein is stable for approximately 18-20 hours in our lines, and that the GFP protein profile observed at a given time corresponds to all descendants of the cells having expressed *her5* between 18-20 to a few hours before the moment of analysis.

At 60% epiboly and until 24 hpf, GFP protein was detectable in hatching gland precursors (Fig.4A and data not shown), likely descendants of the transiently doubly *her5*- and *gsc*-positive cells noticed at the onset of gastrulation (Bally-Cuif et al., 2000). Mainly, however, GFP protein is found in anterior endodermal cells that distribute from the anteriormost pharyngeal level to about the first somite (Fig.4B,C). At the bud stage, when GFP fluorescence first allows positioning of the MH anlage (Fig.2B), and at subsequent stages (Fig.2C), endodermal GFP clearly extends posterior to the MH level. This location is characteristic of the pharyngeal anlage (Warga and Nusslein-Volhard, 1999; Warga and Stainier, 2002). Cross-sections at somitogenesis stages further indicate that GFP staining meets at the ventral midline (Fig.4C, left inset), thus that the initially *her5*-positive precursors contribute to both medial and lateral pharyngeal structures.

Together, these observations demonstrate that *her5* expression at early gastrulation stages (i.e. between 30 and 60% epiboly) is a selective marker of most, and likely all, precursors of the pharynx. These results make of *her5* the earliest selective pharyngeal marker known to date, and are in line with the proposed role of endodermal Her5 activity in attributing pharyngeal fate (Bally-Cuif et al., 2000).

Neural her5 expression at gastrulation encompasses the entire MH anlage

The MH anlage is composed of precursors for the midbrain, isthmus, r1 and r2 (Fig.1). These domains are together characterized by the expression of Eng2 proteins at somitogenesis stages, but an early molecular marker of the entire presumptive MH remains to be identified. Using single cell labeling in vivo, *her5* expression at 90% epiboly was fate mapped to the presumptive midbrain (Müller et al., 1996), excluding more posterior MH derivatives. The stability of the GFP protein in *her5PAC::egfp* embryos however offers the unique opportunity of following the fate of *her5*-expressing cells from the onset of *her5* expression within the neural plate at 70% epiboly onwards. GFP protein becomes visible in this location at 90% epiboly (not shown), and we then performed a detailed spatiotemporal analysis of its distribution by fluorescence microscopy on live embryos and immunocytochemistry on whole-mount or sectioned specimen (Fig.4B-J). When necessary, GFP protein distribution was compared with the expression of diagnostic molecular markers for diencephalic (Fig.6H,L,O) or hindbrain domains (Fig.4K-O).

The morphological constriction marking the midbrain-hindbrain boundary (MHB) becomes visible from the 10-12-somite stage onwards, and is prominent by 20 somites (Fig.4E, arrow). At the 12- and 20-somite stages, GFP protein clearly distributes over the entire midbrain as well as posterior to the MHB (Fig.4C,E), and a cross-section at the MHB level demonstrates that all neural tube cells are stained (Fig.4C right inset). Whole-mount analyses and lateral sections further reveal intense GFP staining in neural crests streams that exit the midbrain area towards anterior and ventral (Fig.4D, cross section in inset). At 25 somites and later, the isthmic fold has formed and the cerebellar anlage is discernible. GFP protein is detected in the midbrain, isthmus, cerebellar fold and pons (Fig.4F-H, see

also Fig.5A,B). The intensity of GFP staining in the metencephalon is however weak compared to midbrain expression, and becomes undetectable after 26 hpf (Fig.4I). GFP expression at 26 hpf remains prominent in the midbrain, albeit with a clear caudo-rostral decreasing gradient. After 30 hpf, GFP protein is maintained only at the MHB (Fig.4J), in a profile reminiscent of late *her5* RNA transcription (see Fig.5C).

To precisely position the spatial limits of GFP protein distribution, we compared its anterior and posterior borders with the expression of diagnostic markers. *pax6.1*, the zebrafish ortholog of murine and chicken *Pax6*, is expressed within the anterior alar plate with a posterior limit at the di-mesencephalic boundary (Li et al., 1994; Macdonald et al., 1995). From the onset of *pax6.1* expression (12 somites) until at least the 30-somite stage, we found that GFP- and *pax6.1* positive cells precisely abut each other at the di-mesencephalic border (Fig. 6H,J,L,O). We conclude that *her5* expression at its onset within the neural plate comprises all midbrain precursors, with a precise anterior limit abutting and excluding the diencephalon anlage. The posterior extent of GFP protein distribution was determined by comparison with the expression of *hoxa2* from 10 somites onwards, when *hoxa2* exhibits a sharp anterior limit of expression at the r1/r2 boundary (Prince et al., 1998), and with the expression of *krox20* that marks r3 and r5 (Oxtoby and Jowett, 1993). At 10 somites and subsequent stages until at least 30 somites, GFP distribution overlaps r2 (Fig.4K,M,O). A few GFP-positive cells can also transiently be found within r3 and r4 at 10 somites (Fig.4L), but this contribution is marginal and no longer detectable at 20 somites (Fig.4N). Thus, the neural expression domain of *her5* at its onset also comprises all precursors of r1 and r2.

Together, our findings demonstrate that the early neural expression of *her5* is a marker of all mes- and metencephalic derivatives, thus of the entire MH anlage. *her5* expression thus appears as the earliest MH marker known to date.

her5 expression is dynamically regulated and progressively lost upon cell divisions in a converging fashion towards the MHB

The crucial role of Her5 in controlling MH neurogenesis (Geling et al., 2003) prompted us to analyze more directly a potential dynamic regulation of *her5* expression. In 30-somite *her5PAC::egfp*embryos, we observed a dramatic difference in the AP extent of *her5* transcription and GFP protein distribution (Fig.5A-C). Because *egfp* transcription faithfully reflects *her5* expression at all embryonic stages (Fig.3, and data not shown), this observation suggests that *her5* expression is dramatically restricted over cell divisions from a domain covering the entire MH anlage at early gastrulation to be maintained at the MHB only. To confirm this hypothesis, and assess the progression of this phenomenon in time and space, we conducted a precise comparison of *her5* RNA and GFP protein distributions between 90% epiboly (first stage where GFP protein becomes detectable in the MH domain) and 24 hpf. To this aim, double in situ hybridization and immunocytochemical detection

was performed on whole-mount embryos or serial sagittal sections (minimum 3 embryos / stage). At 90% epiboly and until the 1-2 somite stage, the anterior borders of *her5* RNA and GFP protein expression were coincident (Fig.5D, and data not shown). However their posterior limits differed of approximately 1-2 cell rows (Fig.5D, and data no shown). Thus between the onset of *her5* expression in the neural plate (70% epiboly) and 90% epiboly, *her5* transcription becomes restricted of a few cell rows posteriorly, while it is maintained in all its progeny cells anteriorly (Fig.5Pa.b.). At 3 somites, *her5* transcripts distribute over approximately 8 cell rows along the AP axis, while GFP protein covers 15-18 rows (Fig.5E,F). From this stage onwards, prominent differences in the AP extent of *her5* RNA and GFP protein are detectable posteriorly but also anteriorly, on the lateral and basal domains of the midbrain (Fig.5E, black arrows). In contrast, *her5* expression still mostly matches GFP staining along the dorsal midline of the neural tube (Fig.5E, blue arrow). Similar observations can be made until the 12-14-somite stage (Fig.5G,H). At 16 somites, the dorsal expression of *her5* dramatically regresses and *her5* expression is restricted to a band of 4-6 cell rows across the entire DV extent of the neural tube (Fig.5I). At this stage, MH cells have further divided as GFP protein extent now covers approximately 27-30 rows along AP (Fig.5J). This progression is ongoing at least until the 30-somite stage, when GFP protein extends over 45-50 rows, against 3-5 rows for *her5* RNA (Figs. 4I, 5K,L).

To ascertain   the directionality of the progressive restriction of *her5* expression in MH precursors, we revealed *her5* RNA and GFP protein on single sagittal sections in double fluorescence experiments (Fig.5M-O). Such stainings unambiguously located the final *her5* expression domain to the center of the GFP-positive domain, confirming that *her5* expression is lost both anteriorly and posteriorly upon cell divisions. We conclude from these observations that (i) *her5* expression within the MH domain is subject to a highly dynamic regulation and is progressively lost upon cell divisions between 70% epiboly and 24 hpf (Fig.5P), and (ii) more specifically, the restriction of *her5* expression occurs in a centripetal manner towards the MHB but follows a precise spatial sequence: it is initiated posteriorly (in the future metencephalon) before affecting the baso-lateral and finally the dorsal mesencephalic areas. Because *her5* expression, at least in the basal plate, is always adjacent to neurogenesis sites (Geling et al., 2003), these observations lead to the important conclusion that neurogenesis within the MH domain is also a spatially dynamic process that progresses in a converging fashion towards the MHB over time (red arrows in Fig.5Pd.).

Most MH cells are maintained but acquire distinct alternative fates in noi and ace mutant backgrounds

Mutants defective in IsO activity such as *noi* and *ace* induce but later on fail to maintain MH fate, and are deficient in the development of most MH derivatives. The fate of initially specified MH precursors in these contexts is to date unknown. *her5PAC::egfp* embryos provide the first means to

directly assess this fate, since *her5* expression labels all MH precursors, and the elements driving the induction phase of *her5* transcription in wild-type embryos are contained within the transgene.

To fully ascertain that GFP protein could be used as a reliable marker of MH fate in *noi* and *ace*, we also verified that *gfp* transcription faithfully recapitulated *her5* expression in these mutant contexts. Double in situ hybridizations with the *her5* and *gfp* probes were performed on transgenic mutant embryos, and demonstrated an identical initiation (not shown) and later down-regulation of *her5* and *gfp* transcription in these backgrounds (Fig.6A-D). Near-complete down-regulation of *gfp* expression was observable at 24 hpf in *her5PAC:egfp;ace* embryos (Fig.6B) and at the 10-somite stage in *her5PAC:egfp;noi* (Fig.6D), like expression of endogenous *her5*. We conclude that the distribution of GFP protein can be used as a faithful reporter of MH fate in the *ace* and *noi* contexts.

Live observation of 24 hour-old transgenic mutant embryos first revealed that a significant number of GFP-positive fluorescent cells was maintained at that stage in both the *ace* and *noi* backgrounds (Fig.6E-G). These cells distribute over an AP territory that approaches wild-type size (compare Fig.6F,G with E), and throughout the entire DV extent of the neural tube. Paradoxically, dorsal MH identities (Reifers et al., 1998; Lun and Brand, 1998) and basal MH derivatives surrounding the MHB, such as the III and IV cranial nerves (Fig.6E-G, insets) are mostly absent. These results suggest that the cells initially specified as MH have, at least in part, undergone an identity switch in the mutants. We used the co-detection of GFP protein and diagnostic molecular markers expression on single sections to verify this hypothesis and assess the new specification of MH cells.

As described above, in wild-type embryos, the anterior limit of GFP protein abuts at all stages the caudal border of *pax6.1* expression (Fig.6H,J,L,O), a marker for the posterior diencephalic alar plate. Strikingly, however, *ace* mutants showed a significant overlap between these two patterns at the 30-somite stage (Fig.6I,I'), where a large number of cells in the anterior part of the GFP-positive territory co-expressed *pax6.1*. A time-course experiment was performed to demonstrate that GFP-positive cells acquire a *pax6.1*-positive identity at least as early as the 15-somite stages (Fig.6, compare M,M' and L, P,P' and O). In striking contrast to these findings, a distinct *pax6.1*/GFP border was maintained in *noi*, although *pax6.1* expression appeared extended posteriorly compared to its wild-type pattern (compare Fig.6J,K).

Diencephalic cells are also characterized by the expression of *fgfr3* (Fig.6Q). In wild-type transgenic embryos, the GFP-positive territory abuts the caudal border of *fgfr3* expression (Fig.6Q, green arrowheads), which thus shares a common posterior limit with *pax6.1*. As reported previously, we found that *fgfr3* expression extends ectopically towards caudal in *ace* and *noi* (Sleptsova-Friedrich et al., 2002). Double labeling of transgenic mutants reveals, in addition, that the *fgfr3*/GFP border is maintained in the *ace* alar plate, while both markers overlap in the basal plate (Fig.6R). In contrast, the overlap is extensive in *noi*, where all GFP-positive cells co-express *fgfr3* (Fig.6S).

Together, our results have several implications. First, they directly demonstrate that a significant number of cells initially specified as midbrain undergo a partial change towards a

diencephalic identity in the absence of a fully functional IsO. Second, our observations reveal that MH cells change fate to a different extent depending on their alar versus basal location: indeed, basal MH cells in ace turn on *fgfr3* expression while alar cells maintain a MH identity. Finally, our results highlight striking differences between the plasticity of MH identity depending on the mutant backgound considered, as anterior MH cells in the *ace* alar plate switch on a *pax6.1*-positive, *fgfr3*-negative identity, while the opposite is true in *noi*.

Metencephalic derivatives such as the cerebellum fail to develop in both *ace* and *noi* mutant contexts, but the fate of initially specified metencephalic progenitors is unknown. To approach this question, we relied on the expression of *otx2*, a marker of the fore- and midbrain, but not hindbrain territories. In *ace* mutants, we found that the posterior limit of *otx2* expression precisely coincided with the posterior border of GFP protein distribution (Fig.6U). Because no extensive cell death was observed in the mutants (Brand et al., 1996, and data not shown), this result highlights a fate change of initially metencephalic cells towards an *otx2*-positive identity in the absence of Fgf8 function. In contrast, in *noi* mutants, the caudal border of *otx2* expression appeared located half way through the GFP-positive domain, in a manner reminiscent of the wild-type situation (Fig.6T,V). Thus, some AP distinctions related to ante- and post-MHB differences are maintained by the descendents of MH progenitors in *noi*; however whether a subpopulation of metencephalic cells have acquired a more anterior, *otx2*-positive identity cannot be excluded. Posterior GFP-positive, *otx2*-negative cells also express *fgfr3* at high levels (Fig.6S), suggesting that they are of posterior r1 or r2 identity. Because of the dynamic posterior limit of GFP protein distribution in the hindbrain (Fig.4K-O), it was not possible to follow these cells with precision. Nevertheless, these results are sufficient to highlight striking dissimilarities in the fate of metencephalic cells in *ace* and *noi*: all metencephalic descendants acquire, at least in part, a mesencephalic fate in *ace*, while a significant proportion of the metencephalon maintains a hindbrain identity in *noi*. Our results thus reveal distinct functions of Pax2.1 and Fgf8 in the maintenance of MH fate, and an interpretative summary of our results is presented in Fig.7.

Discussion

In this article, we isolate the regulatory elements controlling both the initiation and maintenance aspects of *her5* expression, the earliest marker of the MH area in zebrafish. We construct transgenic tools allowing to precisely trace the progeny of *her5*-expressing cells during zebrafish embryogenesis, and we use these tools in a detailed analysis of the dynamics of MH development. Our fate study of *her5* progeny in wild-type and mutant contexts allows us to make three important conclusions. First, we demonstrate that *her5* expression at its onset defines the MH anlage, making

her5 the first marker of the MH territory. Second, we show that *her5* expression is progressively lost upon cell division in a converging and spatially controlled manner towards the MHB. Because Her5 activity negatively defines neurogenesis sites (Geling et al., 2003), this result implies that MH neurogenesis is dynamically regulated and progresses towards the MHB over time. Finally, we demonstrate that MH cells are mostly maintained but undergo identity changes in noi and ace, and we show that these changes depend on the mutant context. Together, our findings clarify the dynamics of MH neurogenesis and maintenance, and directly determine *pax2.1* and *fgf8* mutant gene activities for their effect on cell fate

Regulatory elements controlling her5 expression

Transgenesis with *gfp* reporters is particularly suited to the study of gene regulatory elements in the zebrafish, since the embryo is optically clear permitting GFP protein distribution to be followed throughout embryogenesis on live specimen. A growing number of reporter lines have been generated in this system that are invaluable tools for cellular and molecular studies of a variety of embryological processes (Shafizadeh et al., 2002); however traditional approaches in the construction of reporter minigenes often lead to incomplete or ectopic expression sites (e.g. Higashijima et al., 2000; Picker et al., 2002). In a pioneering study (Jessen et al., 1999), Jessen et al. used in vitro Chi-mediated recombination in PACs to generate zebrafish embryos where *gfp* expression faithfully reproduces the transcription profile of *rag1*. Because the *her5* enhancer had not been characterized and *her5* expression is complex, we chose here a comparable approach, the ET-cloning in vitro recombination technology (Muyrers et al., 2000; Muyrers et al., 1999; Zhang et al., 1998), to build transgenic lines where *gfp* expression is driven by the complete set of *her5* regulatory elements. During embryogenesis, *her5* expression follows at least three distinct phases, being first transcribed in a subset of endodermal precursors, then induced and maintained within the presumptive MH. In addition, each phase is subject to dynamic regulation, as endodermal expression is transient (Bally-Cuif et al., 2000) and MH expression is drastically downregulated over time (this paper). Precise analysis of *her5PAC::egfp*embryos reveals identical spatiotemporal profiles of *her5* and *gfp* transcription at all stages, demonstrating that our lines indeed fully recapitulate the phases and dynamics of in vivo *her5* expression. Our results confirm the power of artificial chromosome transgenesis in zebrafish to decipher the complexity of developmental gene regulation in vivo.

Although our main goal was to construct a reporter of MH fate and we did not attempt to precisely dissect the *her5* promoter, our deletion analysis (Fig.2) points to distinct elements regulating endodermal versus neural *her5* expression. Sequence comparison of these elements further failed to reveal evident common motifs (not shown). This observation is of interest as these two domains of *her5* expression share a comparable AP level, thus might have been expected to respond to a common subset of inducers. Several other MH markers, including *fgf8* and *gbx2*, are also characterized by

underlying endo- or mesodermal expression, at a stage slightly preceding the onset of transcription in the neural plate (Reim and Brand, 2002) and our unpublished observations). Together these observations suggest that a vertical cross-talk between the endo-mesodermal and neural layers and involving several signals might take part in the initiation or refinement of early MH markers expression.

All early MH markers studied to date, including zebrafish *her5, pax2*.1*, eng2, fgf8* and wnt1, follow a bi-phasic mode of regulation: their expression is induced at late gastrulation, likely by independent pathways, and maintained after the 5-somite stage in a mutually interdependent process (Lun and Brand, 1998; Reifers et al., 1998; Scholpp and Brand, 2001) and see (Wilson et al., 2002). These phases correspond to distinct regulatory elements on the promoters of zebrafish pax2.1 (Picker et al., 2002), mouse Pax2 (Pfeffer et al., 2002; Rowitch et al., 1999) and mouse En2 (Li Song and Joyner, 2000; Song et al., 1996). In contrast to these findings, we have not been able to dissociate initiation and maintenance elements within the *her5* enhancer, suggesting that they are closely linked and/or overlapping at the *her5* locus. The "maintenance" elements of mouse En2 contain and depend upon Pax2/5/8 binding sites (Li Song and Joyner, 2000; Song et al., 1996); those of mouse Pax2 are at least targets for auto- or cross-regulation by Pax2/5/8 proteins (Pfeffer et al., 2002). *her5* expression is clearly dependent upon the presence of Pax2.1 and Fgf8 proteins at somitogenesis (Lun and Brand, 1998; Reifers et al., 1998), however analysis of the *her5* enhancer sequence failed to reveal binding sites for these MH factors of the maintenance loop (not shown). In addition, we showed previously that *her5* expression was not subject to autoregulation (Geling et al., 2003). Maintenance of *her5* expression at somitogenesis thus likely involves relay factors, still to be identified.

The molecular and cellular processes leading to MH induction are currently not understood, but are likely to involve a combination of general planar and vertical signaling mechanisms patterning the gastrula embryo. Only a restricted subset of players involved in the induction phase has been identified: the Oct-like transcription factor Spiel-ohne-Grenzen (Spg) / Pou2, which acts as a general permissive cue to MH induction (Belting et al., 2001; Burgess et al., 2002), and the Btd/Sp1-like zinc finger protein Bts1, a selective inducer of pax2.1 expression (Tallafuss et al., 2001). Accordingly, Oct- and Sp1-binding sites have been identified on the early-acting enhancer of mouse Pax2, and at least the Oct sites are required for enhancer activity (Pfeffer et al., 2002). Similarly, we found that several Oct sites are present on the *her5* MH enhancer (A.T. and L. B-C, unpublished), and it will be important to test their requirement for her5 induction. Whatever the case, however, Spg/Pou2 is expressed and required over a region larger than the MH domain alone (Belting et al., 2001; Burgess et

al., 2002; Hauptmann et al., 2002), and factors restricting *her5* expression to the MH anlage remain crucial components of the MH induction process to be identified. Some of these are likely to bind the distal portion of the *her5* enhancer, as proximal domains drove unrestricted reporter expression to the anterior brain in our transgenic assays (Fig.2F,G).

her5 expression is the earliest marker of MH fate

Our comparison of GFP protein distribution with diagnostic markers of the diencephalon (*pax6.1*, *fgfr3*) positions the early anterior *her5* expression border to the di-mesencephalic boundary. Comparison with rhombencephalic markers (*pax6.1*, *hoxa2*, *krox20*) indicates that the posterior border of *her5* expression is more dynamic and expands, at early stages, a minor contribution into r3 and r4. Soon afterwards, it is limited to the r2/r3 border. GFP-positive cells found in r3 and r4 might be accounted for by a transient overlap of *her5* expression with the r3/r4 anlage at gastrulation. At this stage, *her5* is however not co-expressed with *hoxa1* (A.T. and L. B-C, unpublished observation), interpreted to extend to the r3/r4 boundary (Koshida et al., 1998). Alternatively, the contribution of GFP-positive cells to r3 and r4 might result from the migration of metencephalic cells towards caudal, as documented in the chicken embryo at a later stage (Marin and Puelles, 1995). We cannot formerly exclude either possibility at this point.

Outside this marginal contribution to posterior rhombomeres, the large majority of GFP-positive cells is confined to mesencephalic (midbrain, isthmus) and metencephalic (r1, r2) derivatives. GFP expression encompasses the entire extent of the MH domain, and, as revealed in serial section analyses at representative stages (Fig. 4 and data not shown), displays a ubiquitous distribution within this domain. Thus our results identify *her5* expression at its onset as a comprehensive marker of MH fate. The MH domain is generally considered as an entity because its different sub-territories develop in a concerted fashion (in direct or indirect response to IsO activity), and because it is globally characterized by the expression of molecular markers (such as En2) at somitogenesis stages (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Our results shed ground to these postulates, by conveying the first direct molecular evidence supporting the definition of MH identity as a whole at an early developmental stage. A surprising correlate of this finding is that the earliest *her5* expression domain generally defines MH fate although Her5 function itself does not control the acquisition or maintenance of MH identity. Indeed gain- and loss-of-function experiments revealed that Her5 primarily controls neurogenesis and cell proliferation events, permitting the formation of the IZ at the MHB, without influence on the expression of MH identity or IsO markers (Geling et al., 2003). It is thus likely that *her5* expression is rapidly relayed in time by MH identity factors, possible candidates being *pax2.1* and *eng2/3*.

To our knowledge, *her5* expression is the first among all early neural plate markers to be precisely traced using the sensitive and unbiased approach of a *gfp* reporter in the zebrafish. In the

mouse, tracing of the descendents of *En-2*-positive cells using the Cre-lox system and a β–galactosidase reporter revealed contribution of these cells to the entire AP extent of the MH; however, because a small fragment of the *En-2* enhancer was used, the βgal profile obtained was dependent on the integration context (Zinyk et al., 1998). Zebrafish *pax2.1:gfp* lines have recently been generated (Picker et al., 2002). Although crucial to the understanding of the spatio-temporal regulation of *pax2.1* expression, these lines do not allow tracing of the earliest *pax2.1*-positive domain, since they lack the enhancer element driving initiation of *pax2.1* expression (Picker et al., 2002). Our lineage data will thus also be important to use in perspective to the compared expression pattern of other AP markers to *her5*.

Dynamic regulation of her5 expression and the spatio-temporal progression of MH neurogenesis

An important demonstration of our study is the highly dynamic regulation of *her5* expression over time. Indeed *her5* expression restricts from a domain covering the entire MH anlage at 70% epiboly to a few cell rows at the MHB at late somitogenesis (Fig.5). During this period, the total number of *her5*-expressing cells remains roughly unchanged; in contrast, the number of MH cells greatly increases. This observation demonstrates that *her5* expression is progressively lost upon cell divisions in a converging manner from anterior and posterior towards the MHB. Whether this progressive down-regulation follows an asymmetrical mode of cell division, where *her5* expression is maintained in every other progeny cell at each cellular generation, or rather results from the progression of a maturation gradient within the MH in a manner unrelated to cell cycle events, remains to be determined and will require the tracing of single GFP-positive cells. Several other MH markers, e.g. *pax2.1*, *eng1*, *wnt1* and *fgf8*, display an expression profile that globally compares in extent with *her5* at early and late stages. Thus our results imply that expression of these factors follow a restriction similar to *her5* over time. Together these observations highlight a generally centripetal dynamics of MH development, where the progressive restriction of early markers expression coincides with the initial definition of the MH as a whole and its later maintenance by an organizing activity refined to the MHB.

Within the MH and at least until 24 hpf, *her5* expression spatially defines the IZ and is always adjacent to and non-overlapping with primary neurogenesis sites (Geling et al., 2003). Thus our results on *her5* dynamics correlatively demonstrate that primary neurogenesis progresses from anterior and posterior in a converging manner towards the MHB over time (Fig.5P). Because Her5 function is crucially involved in controlling neurogenesis inhibition within the MH basal plate, our results further suggest that neurogenesis progression is likely permitted by the dynamic down-regulation of *her5* expression over time. Along the DV axis of the neural tube, the combinatorial differentiation-promoting and differentiation-inhibiting activities of Shh and Wnt signaling, respectively, has been

proposed to account for the global ventral to dorsal progression of neuronal maturation (Megason and McMahon, 2002). Thus Her5 might be regarded as a counterpart to Shh and Wnt along DV, which controls the spatial order of neurogenesis progression along AP within the MH domain.

Within the MH basal plate, neuronal identity varies according to, and has been postulated to depend on, the position of the population considered relative to the MHB (Agarwala and Ragsdale, 2002; Broccoli et al., 1999; Wassarman et al., 1997). For instance, nMLF reticulospinal neurons lie at the anterior border of the mesencephalon, while motorneurons (of cranial nerves III and IV) are found adjacent to the MHB. Our results on *her5* and neurogenesis dynamics also imply that these neurons are generated at different times, the former being an early and the latter a late neuronal type. Along this line, the combined action of the two E(spl)-like factors Hes1 and Hes3 is required for IZ maintenance in the E10.5 mouse embryo (Hirata et al., 2001), and premature neurogenesis at the MHB in $Hes1^{-/-}$;$Hes3^{-/-}$ embryos is correlated with the loss of some but not all neuronal identities that normally develop around the MHB after E10.5 (Hirata et al., 2001). Whether the primary determinant of neuronal identity is the AP location of the different populations, or rather is the timing of their engagement into the differentiation process, primarily controlled by *her5* restriction, becomes an important aspect of MH development to address in future studies.

Dynamics of MH fate maintenance

Because *gfp* expression in *her5PAC::egfp* embryos recapitulates the MH induction phase, and is a global marker of MH identity, a major interest of our lines is to permit the direct tracing of MH fate in mutant or manipulated contexts. We focused here on the *noi* and *ace* mutants, which lack the function of the crucial MH players Pax2.1 and Fgf8 and characteristically fail to maintain MH development, but where the fate of MH cells is unknown (Lun and Brand, 1998; Reifers et al., 1998). Our tracings first demonstrate that, in these mutants, a large proportion of the cells initially specified as MH, both in the basal and alar plates, are present. Because none of these cells however maintain molecular characteristics of the posterior midbrain, isthmus or cerebellar/pontine areas (Lun and Brand, 1998; Reifers et al., 1998), a first important conclusion of our data is that these cells must undergo identity changes. Following on this idea, we used double expression analyses to directly assess the alternative fate(s) taken by MH cells in *noi* and *ace*. As discussed below, our results reveal dramatic differences in the dynamics of MH maintenance between the *noi* and *ace* contexts, thereby clarifying a number of important points left hypothetical in MH development.

Ectopic expression and loss-of-function experiments in the mouse, chick and zebrafish demonstrated an antagonism between the expression of Pax6 and En factors, involved in delimiting the di-mesencephalic border. For instance, $En1^{-/-}$;$En2^{-/-}$ mice, which are characterized by a reduced midbrain (Liu and Joyner, 2001), or chicken embryos expressing a dominant-negative form of En-2 (Araki and Nakamura, 1999), correlatively exhibit a posterior expansion of *Pax6* expression.

Conversely, *Pax6⁻ᐟ⁻* mice (Mastick et al., 1997) or chicken embryos expressing a dominant-negative form of Pax6 (Matsunaga et al., 2000b) display expanded expression of midbrain markers. These results have been interpreted as revealing changes in di- or mesencephalic identities at the expense of one-another. In support of these interpretations, we document here that a significant proportion of cells initially specified as MH express *pax6.1* in *ace* (Figs.6,7). A transient overlap in the expression of Pax6 and En has been documented in chicken (Matsunaga et al., 2000a), suggesting that the mixing of GFP and *pax6.1* expression in *ace* might result from the failure to down-regulate *pax6.1* expression in cells that do not confirm MH identity. However, we performed a precise time-course comparison of *pax6.1* and GFP, as well as of *pax6.1* and Eng proteins expression in zebrafish (Fig. 6L,O and data not shown), and failed to observe an overlap of these markers at any stage in this species. Thus, the coexpression of GFP and *pax6.1* in *ace* rather reflects an anteriorization of midbrain cells, and our results for the first time directly demonstrate that mesencephalic to diencephalic identity switches can occur in vivo when IsO activity is impaired.

Importantly, we also show that GFP-positive cells of *noi* mutants do not acquire a *pax6.1*-positive identity. This happens although, in *noi*, *pax6.1* expression is extended posteriorly (this paper Fig.6) and *eng* expression is missing (Lun and Brand, 1998). Thus, at least in the alar plate, diencephalic expansion in *noi* does not occur at the expense of mesencephalic tissue. It is possible that cell death (Brand et al., 1996), lower proliferation rate, or altered influences of midbrain cells on diencephalic development, rather than an identity switch, account for the observed posterior expansion of *pax6.1* expression. These results contrast with the general model described above and stress the importance of direct lineage tracing in the interpretation of patterning phenotypes. Our results also point to the differential regulation of *pax6.1* and *fgfr3* expression in the context of altered MH maintenance, although both markers share an identical expression limit at the di-mesencephalic border in wild-type embryos. This observation more generally demonstrates that factors the expression of which define identical neural plate domains are not necessarily coregulated, and can be differentially sensitive to different degrees of cell commitment revealed in mutant or manipulated backgrounds.

Our results further reveal a similar situation for posterior MH cells. Ectopic expression experiments in chicken demonstrated an antagonism between Fgf8 and Hoxa1 expression to determine r1 versus r2 identities and delimit the r1/r2 boundary (Irving and Mason, 2000). These observations suggested that, in the absence of a functional IsO, r2 identity would expand anteriorly at the expense of r1 (was that shown). Our results indeed strongly suggest that this is the case in *noi*, where an *otx2*-negative domain is maintained in posterior *her5* progeny cells (Fig.6V), while the caudal border of *otx2* expression now abuts *pax6.1* and *fgfr3* expression (not shown, Fig.7). The most likely interpretation of these findings is that r1 cells have turned on an r2 identity. In striking contrast, however, all GFP-positive cells in *ace* are *otx2*-positive. Since no cell death was observed, this demonstrates that metencephalic cells in *ace* mostly shift towards a more anterior identity rather than to r2. These results directly support the early interpretation of Brand et al. (Brand et al., 1996), who proposed the enlarged

tectum of *ace* mutants to partially result from the contribution of posterior cells. Together, our findings thus also highlight striking differences in the response of metencephalic cells to the absence of Fgf8 versus Pax2.1 function.

Finally, another characteristic of MH plasticity is the differential regulation of its alar versus basal development, documented by a number of gene expression analyses in mouse or zebrafish MH mutants or morphants (e.g. Bally-Cuif et al., 1995; Lun and Brand, 1998; Reifers et al., 1998). We show here that MH (GFP-positive) cells of the basal plate ubiquitously turn on *fgfr3* expression in *ace*, while alar MH cells fail to do so. Thus our results extend previous findings by directly demonstrating unequal responses of the MH alar and basal plates to cell identity changes.

It now becomes important to understand the bases for the differential plasticity of *ace* versus *noi* MH cells to cell fate changes. The downregulation of MHB markers occurs generally later in *ace* (completed around the 20-somite stage) than in *noi* (completed around the 10-12-somite stage), making it possible that partial IsO activity is maintained until a later stage in *ace* and prevents, for instance, the invasion of most alar MH tissue by *fgfr3* expression. *fgf8* and *pax2.1* are also expressed in overlapping but non-identical domains, thus their primary and secondary target cells are likely to be distinct. Alternatively (and non-exclusively), the *ace* and *noi* mutations may have different impacts on cell behavior because the Fgf8 and Pax2.1 proteins control distinct cellular processes. Our results permit to analyze directly *fgf8* and *pax2.1* mutant gene activities for their ultimate effect on cell fate, and are in favor of the latter hypothesis. They further allow one to refine current knowledge on Fgf8 and Pax2.1 functions. At least for the alar MH domain, Fgf8 expression prevents anterior mesencephalic cells from acquiring a partial diencephalic identity. Thus we propose that Fgf8, in addition to be necessary for MHB maintenance, is involved, at a distance, in the maintenance of an anterior tectal fate. Further, it protects metencephalic cells from switching to a mesencephalic fate, thus we also demonstrate that it maintains metencephalic versus mesencephalic identity. The latter finding provides direct, lineage-based support to hypotheses raised from loss- and gain-of Fgf8 function in the mouse and chicken (Liu et al., 1999; Martinez et al., 1999; Sato et al., 2001). In these species, Fg8 has also been proposed to control proliferation (Lee et al., 1997). We could not detect, however, gross alterations in the number of *her5* progeny cells between *ace* mutants and wild-type siblings at the stage of our analysis, suggesting that Fgf8 alone, in zebrafish, does not initially play a major role in MH growth. In contrast to Fgf8, Pax2.1 appears ultimately generally required to prevent the MH territory as a whole from acquiring an *fgfr3*-positive fate. *otx2* expression suggests that this transformation reflects an anteriorization of mesencephalic cells and a posteriorization of metencephalic cells, thus a (partial) switch to immediately neighbouring AP fates. These results extend previous findings in the mouse that implied Pax2 (together with Pax5) in the maintenance of MH identity or the IsO as a whole (Schwarz et al., 1997; Urbanek et al., 1997).

Acknowledgements

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Figure Legends

Figure 1. Schematic organization of the MH domain at the 10-somite stage (A,C) and at 24 hpf (B). All views are anterior to the left; A and C are dorsal and ventral views of the alar and basal plates, respectively; B is a sagittal view, the broken line delimiting the alar/basal boundary. The early MH domain comprises the mes- and metencephalic vesicles; the contribution of each vesicle to the late MH derivatives, as demonstrated in transplantation experiments in the avian embryo (Hallonet and Le Douarin, 1990; Hallonet et al., 1993; Martinez and Alvarado-Mallart, 1989) - and without considering the floor and roof plates -, is colour-coded and indicated by the vertical lines: (i) the alar plate of the mesencephalic vesicle contributes to the tectum; (ii) in addition, the caudal third of the mesencephalic

vesicle is at the origin of the alar part of the isthmus and dorso-medial part of the cerebellar plate (future vermis) and alar part of r2; (iii) the alar plate of the metencephalon gives rise to the lateral cerebellum (future hemispheres); (iv) the basal plate of the mesencephalic vesicle gives rise to the tegmentum; (v) the basal plate of the metencephalic vesicle gives rise to the pons (basal r1) and basal plate of r2. The isthmus is colored in yellow. Its basal part has not been precisely mapped and was not studied for its inductive properties of MH fate; it is drawn here based on the expression pattern of isthmic organizer markers such as wnt1 and fgf8. The intervening zone is defined as the territory delayed in neurogenesis (Geling et al., 2003). It is located at the MHB but its spatial relationship with the isthmus has not been established.

Abbreviations: Cb: cerebellum; Di: diencephalons; Is: isthmus; IZ: intervening zone; Mes: mesencephalon; Met: metencephalon; Myel: myelencephalon; Po: pons; r: rhombomere; Tc: tectum opticum; Tg: tegmentum.

Figure 2. Structure of the *her5* genomic locus and reporter constructs, and corresponding GFP expression. A. Construction of *her5PAC::egfp* by ET-cloning-mediated recombination of the *egfp* cDNA within exon 2 of *her5*. The *her5* locus comprises 3 exons (blue), of which exon 2 encodes the basic and first helix domain of the Her5 protein (bHLH domain labeled in red as b, H1, L and H2). Recombination arms (a', b') matching exon2 were amplified in frame with the *egfp* sequence and a floxed zeocine resistance cassette (*zeo*) (top construct). The resulting product was inserted in vitro within a *her5*-containing PAC by ET-mediated homologous recombination (Muyrers et al., 2000; Muyrers et al., 1999). The *zeo* cassette was subsequently deleted by Cre excision in vitro, generating the *herPAC:egfp* construct (bottom line). B. Reporter constructs used to localize *her5* regulatory elements in transient (black lines) or transgenic (red lines) assays. Most constructs were generated from *her5PAC::egfp* (top construct) by PCR amplification and contain *egfp* in frame within *her5* exon 2. Numbering to the left of each fragment refers to the length of upstream sequence from the transcriptional start site, in bp. The 720 and 980 bp upstream fragments of *–2.9her5:egfp* (bottom lines) were subcloned in front of the *hsp* minimal promoter and *egfp*, and do not contain *her5* coding or intronic sequences. The expression profile driven by each construct is written to the right. Note that the enhancer element(s) driving endodermal expression are located within 240 bp of upstream sequence and/or intron 1, and that sequences driving specific MH expression are recovered with 2.9 kb of upstream sequence. The 720 bp most upstream fragment of the *–2.9her5:egfp* construct drives MH expression in transient assays. C. Endogenous *her5* transcription at 70% epiboly (onset of neural *her5* expression) revealed by whole-mount in situ hybridization (blue staining). *her5* is expressed in a V-shaped domain at the AP level of the MH anlage (MH) and in a subset of anterior endodermal precursors (e) (see also). D-I. Selected examples of GFP protein expression driven by representative reporter constructs. Bright field (top) and fluorescent (bottom) views of transgenic (D-H) or injected (I) embryos (constructs as indicated below each panel). All constructs containing more than 720 bp of

upstream sequence (D-H) drive expression to the anterior endoderm. Constructs comprising more than 2.9 kb of upstream sequence (D,E) or the most upstream 720 bp of the –2.9her5:gfp fragment (I) drive selective neural expression to the MH. Intermediate constructs (F,G) drive unrestricted anterior neural expression.

Figure 3. Comparison of endogenous *her5* (blue) and *gfp* (red) RNA transcription profiles in *her5PAC::egfp*(A,C,D) and *–2.9her5:egfp* (B,E,F) transgenic embryos, at the stages indicated. All views are high magnifications of the MH area in flat-mounted embryos, dorsal (A,B,E,F and inset in D) or sagittal (C,D) orientations, anterior to the top (A,B) or left (C-F). Endogenous *her5* and *gfp* expressions exactly coincide at all embryonic stages, including the initiation (A,B) and maintenance (C-F) phases of *her5* transcription, demonstrating that all the regulatory elements driving MH *her5* expression are contained within the *her5PAC::egfp*and *–2.9her5:egfp* constructs.

Figure 4. The distribution of GFP protein in *her5PAC::egfp*embryos reveals the fate of endodermal and neuroectodermal cells expressing *her5* at gastrulation. GFP protein in *her5PAC::egfp*embryos was observed on live specimen (B,J) or revealed by immunocytochemistry (A,C-I: brown DAB staining, and K-O, lower panels: green FITC staining) at the stages indicated (bottom left of each panel). A, B, and H-I are whole-mount views; A,B: dorsal views, anterior up; H,J: dorsal views, anterior left; I: lateral view, anterior left. K-O are sagittal sections, anterior left; the top and bottom panels are bright field and fluorescent views, respectively, of the same sections that were each processed for in situ hybridization (top panels, blue staining, probes indicated bottom right) and immunocytochemistry against GFP protein (bottom panels). Note in A-C that the descendants of endodermal *her5*-expressing cells distribute in part to the hatching gland and mostly to the entire AP and mediolateral extent of the pharynx (delimited along AP by stars in C, and see the cross section of C at hindbrain level, left inset). In E-J, arrows point to the midbrain-hindbrain boundary; note that GFP protein distributes posterior to this level (i.e. to metencephalic derivatives) until 24 hpf, and encompasses r2 (K,M,O; blue and green arrowheads to the anterior limit of *hoxa2* expression; green arrows to GFP-positive cells in r2), with a minor contribution to r3 and r4 before the 20-somite stage (L,N; white brackets to r3 and r5, green arrow in L to GFP cells in r4; green arrowhead in N to the posterior limit of GFP extension at the r2/r3 boundary).

e: endoderm, hg: hatching gland, MH: midbrain-hindbrain domain, MHB: midbrain-hindbrain boundary, nc: neural crests streams.

Figure 5. Dynamic regulation of *her5* expression within the MH domain. A-N. Comparison of *her5* expression (revealed by in situ hybridization, blue staining in C-E,G,I,K, red staining in M,O) and GFP protein distribution (direct visualization under fluorescence microscopy: green in A,B; or revealed by

anti-GFP immunocytochemistry: brown staining in D, green staining in F,H,J,L,N,O) in *her5PAC::egfp*embryos at the stages indicated. A-D are whole-mount views (A: dorsal, anterior left; B,C: lateral, anterior left; D: dorsal view of a hemi-neural plate, anterior up); E-O are sagittal sections, all views focus on the MH domain and are oriented anterior to the left. The MHB is indicated by a red arrow at all stages where it is morphologically visible. E-L are bright field (top panels) and fluorescent (bottom panels) views of the same sections; M-O are red, green or double fluorescent views of the same section. Note the dramatic difference in the extent of *her5* transcripts (C) and GFP protein (A,B) along the AP axis at 24 hpf. Because *egfp* transcription faithfully reproduces *her5* expression in *her5PAC::egfp*embryos (Fig.3) while GFP protein is stable, this demonstrates that *her5* expression is lost from progeny cells over time. This process is progressive (D-L) and sequential: it involves first a restriction of *her5* expression in the posterior aspect of the MH domain (blue and brown arrows to the limits of *her5* RNA and GFP protein staining, respectively, in D, and blue dots to line the posterior limit of *her5* transcription. Note that the two limits coincide anteriorly but differ of 1-2 cell rows posteriorly). At 3 somites *her5* restriction begins in ventral and lateral aspects of the mesencephalon (black arrows in E,G), and continues after 16 somites (I) along the dorsal midline (blue arrows in E,G to maintained dorsal expression of *her5* prior to that stage). Note in M-O that the final *her5* expression domain is located in the center of the GFP-positive territory, demonstratig that *her5* expression gets restricted in a converging manner towards the MHB. P. Resulting model for the regulation of *her5* expression and the progression of neurogenesis between 70% epiboly (a.), 90% epiboly (b.) and 30 somites (c., d.) in the MH domain (combined from the present data and (Geling et al., 2003)). *her5* expression at 70% epiboly (blue), traced using GFP protein in *her5PAC::egfp*embryos, is the entire MH anlage (green lines and labeling, 45-50 cell rows at 30 somites). Between 70 and 90% epiboly (b.), *her5* expression is lost from progeny cells posteriorly (compare green lines and blue colour). At 90% epiboly, *her5* expression is adjacent to the first anterior neurogenesis sites: the ventro-caudal cluster (vcc, pink, precursor of the nucleus of the medial longitudinal fascicle, nMLF) and future motor and sensory neurons of r2 (orange) (see (Geling et al., 2003). At 30 somites (c.), *her5* expression has been dramatically lost upon cell divisions and is restricted to 3-5 cell rows at the MHB. Correlatively (d.), neurogenesis (revealed by *zcoe2* expression) (Bally-Cuif et al., 1998), still adjacent and non-overlapping with *her5* expression (compared c. and d.), progressed towards the MHB (red arrows) (embryo with the same orientation as in c., focus on the basal plate).

Figure 6. Fate of MH cells in *ace* and *noi* mutants. **A-D:** Double ISH for *egfp* (red) and *her5* (blue) in *her5PAC::egfp*transgenic wild-type, *ace* and *noi* siblings at the stages indicated demonstrate that *egfp* transcription also reproduces *her5* expression in *ace* and *noi* and is downregulated following a correct schedule during the MH maintenance phase. **E-G:** Live observation of *her5PAC::egfp*transgenic wild-type, *ace* and *noi* siblings under fluorescence microscopy at 24 hpf

reveals that most cells initially specified as MH (positive for GFP protein, green) are maintained, although MHB identities, such as cranial motorneurons III and IV (revealed using the *isl1:gfp* transgene, insets)(Higashijima et al., 2000) are missing. **H-P':** Comparison of GFP protein (anti-GFP immunocytochemistry, brown staining) and *pax6.1* RNA (ISH, blue staining) at the stages indicated in sagittal sections of *her5PAC::egfp*transgenic wild-type (H,J,L,O), *ace* (I,I',M,M',P,P') and *noi* (K) embryos. I', M' and P' are magnifications of the areas boxed in I,M,P. Note that GFP protein and *pax6.1* expression are never co-expressed anteriorly in wild-type (see H,L,O) and *noi* (see K), while extensive overlap between the two stainings is present in *ace* at the 15, 20 and 30-somite stages (M',P',I'). **Q-V:** Comparison of GFP protein (anti-GFP immunocytochemistry, bottom panels, green staining) and *fgfr3* (Q-S) or *otx2* (T-V) RNAs (ISH, top panels, blue staining) at the stages indicated in *her5PAC::egfp*transgenic wild-type (Q,T), *ace* (R,U) and *noi* (S,V) embryos. Top and bottom panels are bright field and fluorescence views, respectively, of the same sagittal sections. Green arrowheads on the bright field pictures point to the limits of GFP protein distribution. Note in *ace* that anterior GFP-positive cells do not coexpress *fgfr3* (R, compare with Q), and that posterior MH cells are all *otx2*-positive (U, compare with T). In contrast, in *noi*, all MH cells express *fgfr3* (S) but an *otx2*-negative territory is maintained within the caudal GFP-positive population (V).

Figure 7. Schematic representation of the fate of MH cells (green, territory delimited by the green stars) in wild-type embryos (A) or in the absence of Fgf8 (B) or Pax2.1 (C) activities (interpreted from Fig.6, and data not shown). In each drawing, the thin horizontal black line delimits the alar/basal boundary; gene expressions are colour-coded. Pink arrows delimit the population of anterior MH cells that acquires a *pax6.1*-positive identity in *ace*, and blue arrows point to the extension of *fgfr3* expression in *noi*. Note the striking differences in the alternative fates taken by MH cells depending on the mutant context.

Di   Mes   Met   Myel

IZ

Tc   S   Cb

Tg   Po

r1   r2

A

ATG   TAA
a' egfp   zeo   b'
            loxP   loxP

ATG
+1   E2   TAG
    E1 a b   E3
       b H1   L H2

↓ ET-recombination

ATG   ATG   TAA   TAG
    E1 a' egfp   zeo   b'   E3
       b   H1 L H2

↓ Cre-mediated excision

ATG   ATG   TAA   TAG
    E1 a' egfp   b'   E3

C

her5 - 70% epiboly

B

ATG   TAG
+1
    E1   egfp   E3

-60 ⎫
-130 ⎬ no expression
-240 ⎭

-360 ⎫
-420 ⎬ endoderm only
-510 ⎭
(-0.7her5:egfp) -770 ⎭

-950 ⎫
-1150 ⎪
(-1.3her5:egfp) -1350 ⎬ endoderm +
-1760 ⎪ non-restricted expression in
-2100 ⎪ the anterior neural tube
-2670 ⎭

(-2.9her5:egfp) -2900 ⎫
-3420 ⎪
-3650 ⎬ endoderm +
> 40 kb ⎪ specific expression in the MH
(her5PAC:egfp) ⎭

                    ← MH
720 bp   ← ectopic neural
(720-hsp:egfp) 980 bp

D *her5PAC:egfp* — MH, e

E *-2.9her5:egfp* — MH, e

F *-1.3her5:egfp* — e

G *-1.3her5:egfp*

H *-0.7her5:egfp* — e

I *720-hsp:egfp* — MH

A. 90% her5PAC:egfp — her5 (blue), gfp (red)
B. 3-som -2.9her5:egfp
C. 12-som her5PAC:egfp
D. 15-som her5PAC:egfp
E. 20-som -2.9her5:egfp
F. 24 hpf -2.9her5:egfp

A. 60% — hg
B. bud — MH, e
C. 12 som — MH, e, e
D. 12 som — MH, nc
E. 20 som
F. 25 som
G. 30 som
H. 30 som
I. 26 hpf
J. 30 hpf — MHB
K. 10 som — hoxa2, GFP
L. 10 som — krox20, GFP
M. 20 som — hoxa2, GFP
N. 20 som — krox20, GFP
O. 30 som — hoxa2, GFP

A GFP 24 hpf

B GFP 24 hpf

C her5 24 hpf

D her5 GFP 90%

E her5 3 som

G her5 12 som

I her5 16 som

K her5 21 som

F GFP

H GFP

J GFP

L GFP

M her5 12 som

N GFP

O overlay

P
a. 70% epiboly

b. vcc r2 90% epiboly

her5 5-8 rows

c. nMLF Mes her5 r1 her5 r2 30 somites

her5 3-5 rows

GFP 45-50 rows

d. neuro-genesis neuro-genesis zcoe2 30 somites

| ■ GFP | ▨ *otx2* | ▨ *fgfr3* | ▨ *pax6.1* |

⊙ cranial nerves III and IV

Appendix 5

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bHLH transcription factor Her5 links patterning to regional inhibition of neurogenesis at the midbrain-hindbrain boundary

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SUMMARY

The midbrain-hindbrain (MH) domain of the vertebrate embryonic neural plate displays a stereotypical profile of neuronal differentiation, organized around a neuron-free zone ('intervening zone', IZ) at the midbrain-hindbrain boundary (MHB). The mechanisms establishing this early pattern of neurogenesis are unknown. We demonstrate that the MHB is globally refractory to neurogenesis, and that forced neurogenesis in this area interferes with the continued expression of genes defining MHB identity. We further show that expression of the zebrafish bHLH Hairy/E(spl)-related factor Her5 prefigures and then precisely delineates the IZ throughout embryonic development. Using morpholino knock-down and conditional gain-of-function assays, we demonstrate that Her5 is essential to prevent neuronal differentiation and promote cell proliferation in a medial compartment of the IZ. We identify one probable target of this activity, the

zebrafish Cdk inhibitor p27^{Xic1}. Finally, although the *her5* expression domain is determined by anteroposterior patterning cues, we show Her5 does not retroactively influence MH patterning. Together, our results highlight the existence of a mechanism that actively inhibits neurogenesis at the MHB, a process that shapes MH neurogenesis into a pattern of separate neuronal clusters and might ultimately be necessary to maintain MHB integrity. Her5 appears as a partially redundant component of this inhibitory process that helps translate early axial patterning information into a distinct spatiotemporal pattern of neurogenesis and cell proliferation within the MH domain.

Key words: Zebrafish, Midbrain-hindbrain boundary, MHB, Neurogenesis, Her5, bHLH, E(spl), Hairy, Proliferation, Cyclin-dependent kinase inhibitor, p27

INTRODUCTION

A conspicuous feature of the vertebrate embryonic CNS is the absence of a homogeneous gradient of neurogenesis across the neural tube, young post-mitotic neuroblasts arise at discrete patches of the neuroepithelium in a disjoined spatiotemporal pattern. Among the first neurons to differentiate in all species is a basal cluster located at the diencephalic-mesencephalic junction, which projects growth cones caudally to pioneer the medial longitudinal fascicle (MLF) (Puelles et al., 1987; Chitnis and Kuwada, 1990; Metcalfe et al., 1990; Wilson et al., 1990; Ross et al., 1992; Easter et al., 1994; Mastick and Easter, 1996). This neuronal group is known as the ventrocaudal cluster (vcc) or nucleus of the MLF (nMLF). Concomitantly in the hindbrain, motorneurons become identifiable in the center of each even-numbered rhombomere (Lumsden and Keynes, 1989). Molecular markers such as the Atonal-like bHLH transcription factors neurogenins (Gradwohl et al.,

1996; Ma et al., 1996; Blader et al., 1997) confirmed these pioneering studies. These findings demonstrate that the early pattern of neuronal differentiation is established following a highly similar and stereotypical spatiotemporal sequence in all vertebrates, suggesting that it responds to precise and shared patterning cues. How positional identity information and the onset of neurogenesis versus proliferation are integrated in vertebrates is, however, not fully understood.

A crucial and extensively studied domain of the anterior neural plate is the midbrain-hindbrain (MH), which contains at the MH boundary (MHB) the isthmic organizer, a critical regulator of MH growth and patterning (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Strikingly, the MH is also characterized by a distinct pattern of neurogenesis at early stages: mesencephalic and anterior rhombencephalic neurons are separated by a neuron-free, transverse stripe of delayed differentiation (hereafter referred to as 'intervening zone', IZ), precisely located at the level of the MHB. In the

zebrafish, the IZ is identifiable from the onset of neurogenesis, when it separates two of the earliest neuronal clusters, the vcc and the presumptive motorneurons of rhombomere 2 (r2MN). The IZ is conspicuous during neurogenesis of all vertebrates examined (see Palmgren, 1921; Bally-Cuif et al., 1993). According to classical neuroanatomical studies (Vaage, 1969; Vaage, 1973), it corresponds in the chick to a caudal 'mesomere' which initially encompasses half the midbrain but upon regression during development forms a narrow, neuron-free stripe at the junction with the first rhombomere. Lineage analysis in the zebrafish (A.T. and L.B.-C., unpublished) demonstrate that this is a dynamic process where the IZ progressively contributes cells to adjacent territories upon cell divisions. The zebrafish IZ also maintains a large population of proliferating cells at larval stages, long past the time when proliferation has ceased in adjacent neural domains (Wullimann and Knipp, 2000). As such, the IZ has been proposed to play a crucial role in permitting the growth and regionalization of MH structures over a long period (Tallafuß and Bally-Cuif, 2002). Understanding its formation is thus an important issue.

Several factors have been identified that positively define early neurogenesis competence domains and proneural clusters within the embryonic neural plate. Neuronal differentiation-promoting factors include members of the Achate-Scute, Atonal, Gli and Iroquois families (Allende and Weinberg, 1994; Fisher and Caudy, 1998; Cavodeassi et al., 2001; Davis and Turner, 2001). Neuroblasts that engage into the differentiation process are then selected following similar genetic cascades to those originally defined in *Drosophila*. In the zebrafish neurectoderm for example, Neurogenin1 (Ngn1) (Blader et al., 1997; Korzh et al., 1998) drives the expression of *Delta* homologues *delta A (delA)* and *delta D* (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). *delA*, *delD* and *ngn1* transcripts are expressed by engaged but probably still proliferating neuronal precursors. Delta then activates Notch in its neighboring cells, an inhibitory interaction that allows only a subset of precursors within each proneuronal cluster to become neurons. The selected neuronal precursors exit the cell cycle and begin expressing genes characteristic of differentiating neurons, such as *delB*, *zcoe2*, *neuroD* transcripts and Hu proteins, expressed by committed and no longer proliferating cells (Bally-Cuif et al., 1998; Haddon et al., 1998; Korzh et al., 1998; Mueller and Wullimann, 2002).

While a broad network of genes that positively instructs where neurons differentiate has been identified in vertebrates, mechanisms that define where neurons are not permitted to form remain less studied. To date, Hairy/Enhancer of split [E(spl)]-like proteins (Davis and Turner, 2001) such as *Xenopus* ESR6e (Chalmers et al., 2002), and *Xenopus* Zic2 (Brewster et al., 1998), have been identified as inhibitors but the role of their homologs during neural plate development in other species remain unexplored. In *Drosophila*, Hairy has a prominent role in inhibiting neurogenesis. Unlike most transcription factors encoded by the E(spl) Complex, Hairy is a Hairy/E(spl) transcription factor that is not driven by Notch activation, rather it acts as a prepattern gene to define domains in the notum where sensory bristles are not permitted to differentiate (Fischer and Caudy, 1998; Davis and Turner, 2001). A related Hairy/E(spl) factor, Hes1, was shown to be

necessary, together with Hes3, for maintaining a neuron-free zone at the MHB at a relatively late stage (E10.5) in the mouse embryo (Hirata et al., 2001). However these genes did not have an early role in the establishment of the neuron-free zone. Thus, globally, the inhibitory processes regulating neurogenesis in the vertebrate neural plate remain poorly understood.

Using manipulated and mutant contexts in zebrafish, we first demonstrate that the establishment of the neuron-free zone (IZ) at the MHB is crucial to the maintenance of MHB integrity. We next report that expression of the zebrafish *Hairy/E(spl)*-like gene *her5* at late gastrulation precisely prefigures the IZ, separating the vcc from r2MN. By combining knock-down and conditional gain of Her5 function in zebrafish transgenics, we demonstrate that Her5 is essential in vivo for inhibiting neurogenesis and increasing cell proliferation in a medial domain of the IZ, without influencing other aspects of MH patterning. Our results demonstrate that Her5 is part of a key regulatory process that links early axial patterning mechanisms to the spatial pattern of neurogenesis and cell proliferation within the vertebrate anterior neural plate.

MATERIALS AND METHODS

Zebrafish strains

Wild-type embryos were obtained from natural spawning of AB adults, and raised according to Kimmel et al. (Kimmel et al., 1995). *headless* (*hdl*) embryos were obtained by pair-wise mating of heterozygous adult carriers, as described previously (Kim et al., 2000).

hsp-her5 transgenic lines

To construct *hsp-her5* (Fig. 2D), the published coding sequence of *her5* (Müller et al., 1996) flanked by the 5′ and 3′ UTR of *Xenopus* β-globin was extracted from *pXT7-her5Δ3′* (Bally-Cuif et al., 2000) and cloned downstream of *pzhsp70* (Shoji et al., 1996) in pBluescript SK(+). Wild-type *her5* encodes 9 additional N-terminal amino acids (Fig. 2D) (A.T. and L. B-C., unpublished) but both proteins are intact in their bHLH and further C-terminal sequence. The *hsp-5′βglob-her5-3′βglob* insert (2.5 kb) was extracted from the vector backbone by *Sma*I + *Apa*I digestion, resuspended in water and injected at 50 ng/µl into freshly laid AB embryos. Injected embryos were raised to sexual maturity and pair-wise crossed to AB fish. DNA was extracted from pools of 1- to 2-day-old embryos by incubating for 3 hours at 60°C in 250 µl lysis buffer (10 mM Tris-HCl pH 8.3, 1.5 mM MgCl$_2$, 50 mM KCl, 3% Tween-20, 3% NP40; 1.5 mg/ml proteinase K). The samples, complemented with 750 µl H$_2$O, were heated at 95°C for 10 minutes and PCR reactions were carried out using an upstream primer from the zebrafish *hsp70* promoter sequence (5′ GTGGACTGCCT-ATGTTCATCT 3′) and a downstream primer within the *her5* sequence (*her5#2*: 5′ TTTCTCCATGAGAGGCTTGG 3′) that yielded a 900 bp PCR product. For genomic DNA control the following primers were used, which amplified the endogenous *her5* cDNA (*her5#6*: 5′ AGTTCTTGGCACTCAAGCTCAA 3′ and *her5#4AP*: 5′ GCTCTCCAAAGACTGAAAAGAC 3′). PCR was performed with 5 µl of the diluted genomic DNA in 1× PCR buffer with 2.5 mM MgCl$_2$, 2.5 mM of each primer and 0.2 mM dNTPs, for 35 cycles at an annealing temperature of 56°C. Carrier G0 fish were re-crossed to wild-type fish to test for expression of the transgene upon heat-shock: the resulting embryos were submitted to a 1-hour heat-shock pulse before the 24 hpf stage and tested in whole-mount using situ hybridisation for ubiquitous *her5* expression. G0 carriers transmitting inducible *hsp-her5* were then crossed to wild-type fish and the F$_1$ generation was raised. F$_1$ carriers were identified by PCR

on tail genomic DNA. From more than 100 injected embryos, the integration rate in the G0 generation was 15%, of which 50% transmitted the transgene to their progeny. The transgene was inducible in a ubiquitous fashion upon heat-shock in 50% of these families.

Heat-shock induction and time course experiments

50-100 embryos originating from a cross between F_1, F_2 or F_3 heterozygote carriers (to generate both wild-type and transgenic embryos within each pool) were immersed in a 38°C water bath for 1-2 hours from 80% epiboly to the 3-somite stage. The embryos were then fixed in 4% PFA or further incubated at the normal temperature of 28°C before being processed for analysis. All embryos were processed together in blind experiments, the transgenic embryos being identified a posteriori using *her5* in situ hybridization or PCR genotyping as described above. The amount of transgenic *her5* mRNA in the time-course experiment on Fig. 3C was estimated as follows: following heat-shock, embryos were fixed every 0.5 hour and *her5* expression was revealed by whole-mount in situ hybridization using the fluorescent Fast-Red substrate. All embryos were processed in parallel and the color reaction was stopped at the same time. Fluorescence intensities were compared using the linear amplification system of a 3CCD Color Video Camera (Sony MC3255) and the Axiovision Software (Carl Zeiss GmbH).

Antisense experiments

Morpholino antisense oligonucleotides (MOs) were purchased from Gene-Tools, Inc. (Oregon, USA). MOs were dissolved to a stock concentration of 2 mM in H_2O and injected into 1-cell stage embryos at 1 or 2 mM. Sequences were as follows (see also Fig. 2D): MO^{tg}: 5′ CCTTCTCATGTCTTTTTGCTCCATT 3′; MO^{her5}: 5′ TTGGTT-CGCTCATTTTGTGTATTCC 3′. Both MOs were tested for their blocking efficiency by injection into a transgenic line *her5PAC-GFP* (A.T. and L.B.-C., unpublished) which carries an in-frame fusion of Her5 and GFP 3′ to the basic domain of Her5 (thus where endogenous *her5* ATG is used) and more than 40 kb of upstream regulatory sequences; this line faithfully reproduces endogenous *her5* expression. In this line, MO^{her5} fully inhibited the expression of GFP, demonstrating that, in the conditions used, this MO fully blocks the translation of endogenous *her5*. In the same context, MO^{tg} was inefficient at blocking GFP expression.

RNA injections

Capped RNAs were synthesized using Ambion mMessage mMachine kits following the recommended procedure. RNAs were injected at the following concentrations: 25 ng/µl (low dose) or 125 ng/µl (high dose) *ngn1* (Blader et al., 1997), with or without *nls-lacZ* (40 ng/µl) as lineage tracer.

In situ hybridization and immunohistochemistry

Probe synthesis, in situ hybridization and immunohistochemistry were carried out as previously described (Hammerschmidt et al., 1996). The following antibodies were used: mouse anti-myc (Sigma M 5546) (dilution 1:1000), rabbit anti-β-galactosidase (Cappel 55976) (dilution 1:4000), rabbit anti-phosphohistone H3 (Upstate Biotechnology, no. 06-570) (dilution 1:200), mouse anti-HNK1 (DSHB Zn12) (dilution 1:500), mouse anti-human neuronal protein HuC/HuD (MoBiTec A-21271) (dilution 1:300). Secondary antibodies HRP-conjugated goat anti-mouse or goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) diluted to 1:200. The staining was revealed with DAB following standard protocols.

Cloning of zebrafish *Cdk* inhibitor-encoding cDNAs

Random-primed cDNA prepared from 15-somite AB zebrafish RNA was amplified using oligonucleotides directed against cDNA AF398516 (forward primer: 5′ TCCGCTTGTCTAATGGCAGCC 3′; reverse primer: 5′ CACTTCATCCACACAGATGTGC 3′), and EST

BI887574 (forward primer: 5′ CAAGCATCT GGAGCGTCATGTTG 3′; reverse primer: 5′ TAACGGCGTTCATCCTGCTCCG 3′). PCR products were subcloned and sequenced according to standard protocols. EST fx62e01.y1 was obtained from the rzpd (Berlin). All subclones were used for the generation of in situ hybridization probes following standard procedures. Sequence analyses revealed that the three clones encode CDI domain-containing proteins, characteristic of Cdk inhibitors. The CDI domains of BI887574 and fx62e01.y1 are 60% identical to each other and most related to that of *Xenopus* $p27^{XIC1}$ (53-56% identity). They are equally distant from the CDI domain of AF398516 (45% identity). The CDI domain of AF398516 is itself is more related to that of mammalian $p27^{Kip1}$ (56-59% identity) than to *Xenopus* $p27^{XIC1}$ (46% identity). Based on these findings, and on the fact that BI887574 is expressed earlier than fx62e01.y1 (see text), we re-named BI887574 zebrafish $p27^{Xic1}$-a.

Aphidicolin treatments

Embryos were incubated for 2 hours (from 70% epiboly to the 3-somite stage) [compared to an estimated 4-hour cell cycle length at this stage in the neural plate (Kimmel et al., 1994)] in embryo medium containing 1 or 10 µg/ml aphidicolin (Sigma A-9914) at 28.5°C (Marheineke and Hyrien, 2001). The embryos were then washed in embryo medium, fixed and processed for in situ hybridization and immunodetection.

RESULTS

The IZ displays distinct mediolateral restriction to neurogenesis, and results from active inhibition of neuronal differentiation at the MHB

In the zebrafish embryo, the IZ can be visualized as a gap in the expression of markers identifying the first proneural clusters and differentiating neurons. At the tail-bud stage, shortly after the onset of *ngn1* expression, the IZ is clearly visible as a V-shaped *ngn1*-negative area of 6-8 rows of cells that separates the vcc from the early motor neurons of r2 (r2MN) (Fig. 1A). Laterally, in the future alar plate, the IZ abuts the presumptive early sensory neurons of r2 (r2S) (Fig. 1A). This area roughly corresponds to the domain expressing *pax2.1* (red in Fig. 1A), which covers most of the presumptive MH territory at that stage (Lun and Brand, 1998; Picker et al., 2002). By 24 hours post-fertilization (hpf), the IZ appears as a stripe of 3-6 cells wide between neuronal precursors of the basal midbrain and rostral hindbrain (Fig. 1B, bracket). It encompasses the domain of expression of *pax2.1* (Fig. 1C), *wnt1* and *eng1* (not shown), which have narrowed to the MHB at 24 hpf.

Absence of neurons in the MHB domain might reflect the local absence of proneural gene expression at or around the MH junction, the presence of intrinsic or extrinsic cues that actively inhibit proneural function in that location, or both. To discriminate between these possibilities, we determined how the MH domain responds to ectopic expression of the proneural gene *ngn1* within the IZ. One-celled wild-type embryos were injected with capped mRNA encoding Ngn1, and probed at the tail-bud stage for *delA* expression to reveal induction of Ngn1-responsive genes (Fig. 1D-F). A dose of *ngn1* (25 pg) that was sufficient to trigger neurogenesis throughout the neural plate induced *delA* expression within the medial part of the IZ, but not in the lateral or dorsal IZ (88%, *n*=17) (Fig. 1D,E). *delA* expression was effectively induced within the presumptive lateral and dorsal parts of the IZ only upon injection of higher

Fig. 1. The intervening zone (IZ) displays
intrinsic mediolateral differences and is shaped
by antagonistic activities from neurogenesis-
promoting signals and the IsO. Whole-mount in
situ hybridization at the 3-somite stage (A,D-
F,K,L) (dorsal views, anterior to the top) and 24
hours post-fertilization (hpf) (B,C,G-J) (sagittal
views, anterior to the left) with the markers
indicated (bottom left, color-coded).
(A-C) Intervening zone (IZ) location in wild-
type (wt) embryos. At the 3-somite stage, the IZ
separates the ventro-caudal cluster (vcc) from
the r2 motor (r2MN) and sensory neurons (r2S),
and encompasses most of the MH primordium,
as revealed by *pax2.1* expression. By 24 hpf, the
IZ (bracket in B,C) has narrowed to a stripe at
the MHB. (D-F) Intrinsic differences between
the neurogenic capacities of lateral versus
medial domains of the IZ. Upon injection of 25
pg *ngn1* mRNA at the 1-cell stage (*ngn1-inj*, E),
ectopic neurogenesis is induced within the
neural plate outside proneural clusters (arrows
in E) including the basal domain of the IZ
(bracket), while the IZ remains neurons-free in
lateral regions. 125 pg *ngn1* (F) are necessary to
force neurogenesis within the IZ alar domain
(arrow in F; location of the IZ in F is indicated
by the bracket). This phenotype is correlated

with the loss of expression of the MH markers *pax2.1* and *wnt1* at 24 hpf (G-J, red arrows). Note that the profile of *pax2.1* and *wnt1* expression
is otherwise unaltered (*pax2.1*: optic chiasm, hindbrain interneurons: G,H, black arrows. *wnt1*: midbrain dorsal midline, rhombic lips: I,J, black
arrows). (K,L) The anterior-to-posterior extent of the IZ correlates with IsO activity, and is enlarged in *hdl* mutants, which overactivate Wnt
signaling (L compared with K, bracket). Scale bars: 0.1 mm. IZ, intervening zone; vcc, ventrocaudal cluster; r2MN, rhombomere 2
motorneurons; r4MN, rhombomere 4 motorneurons; r2S, rhombomere 2 sensory neurons; *delA*, *delta A*; *ngn1*, *neurogenin 1*.
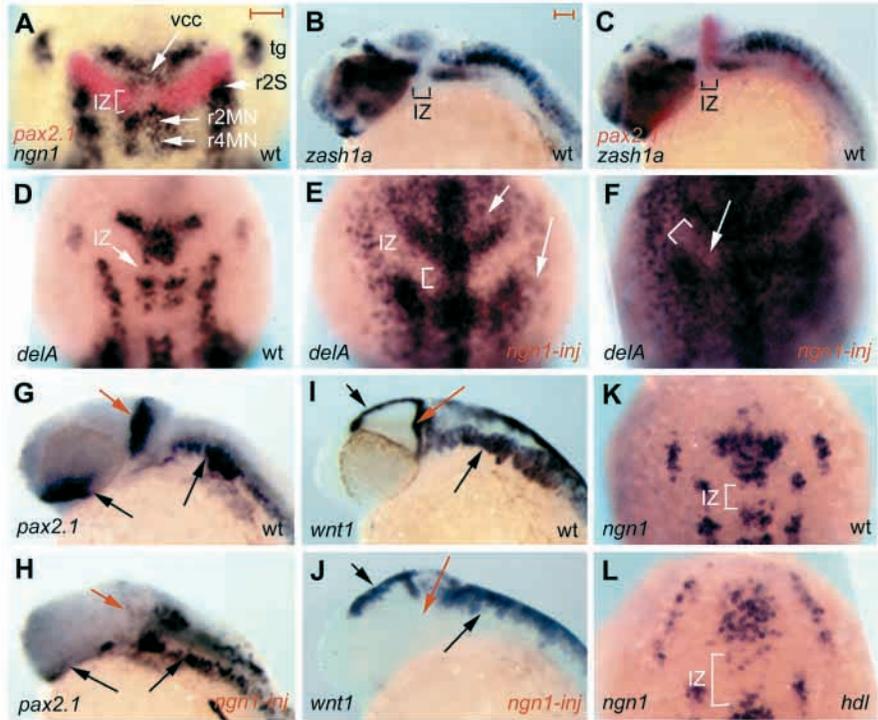
ngn1 doses (125 pg) (Fig. 1F). Even in this case, ectopic *delA*
remained mosaic rather than ubiquitous and the IZ could still
be distinguished (77%, *n*=22) (Fig. 1F, bracket). Similar results
were obtained when probing for the expression of the neuronal
differentiation marker *huC* (*n*=42, data not shown). Thus,
although the IZ is globally non-neurogenic in vivo, there
appears to be intrinsic mediolateral differences in the
mediation of its non-neurogenic character within this domain.
In particular, the lack of expression of neuronal determination
factors such as Ngn1 in the basal IZ domain might solely
account for this region remaining neuron-free, while additional
intrinsic or extrinsic blocks acting downstream or in parallel to
Ngn1 activity are likely involved within the lateral and dorsal
IZ domains.

Because the IZ develops at the MHB, we explored whether
and to what extent IZ formation relates to and/or is required
for isthmic organizer activity. We observed that ectopic
expression of *ngn1* does not generally impair the establishment
of MH identity (as revealed by *eng2*, *her5* or *pax2.1*
expression) at early somitogenesis stages (not shown). At 24
hpf however, the expression of MHB markers such as *wnt1* and
pax2.1 was abolished upon injection of *ngn1* mRNA (Fig. 1G-
J). Thus forced neurogenesis within the IZ eventually
interfered with maintenance of genes that define MHB identity,
suggesting that inhibition of Ngn1 expression and function
may be necessary to maintain MHB identity and/or continued
function of the isthmic organizer.

headless (*hdl*) mutants, characterized by reduced repression
of Wnt target genes by Tcf3, have expanded expression of

genes that define MHB identity (Kim et al., 2000). We
observed that this phenotype correlates with an expansion of
the *ngn1*-free domain in the MH (Fig. 1K,L).

Together, these results suggest that IZ formation depends on
the combination of two antagonistic cues: positive neuronal
differentiation signals, and an opposite inhibitory activity that
is spatially associated with the isthmic organizer. In addition,
they demonstrate that suppression of neurogenesis at the MHB
is crucial to the maintenance of MHB integrity.

her5 expression at the onset of neurogenesis is sufficient to prevent neurogenesis around the MHB

The above results suggest that factors expressed at the MHB
in response to early anteroposterior patterning cues may
actively contribute to the local suppression of neurogenesis.
Among those factors, Her5 appeared to be a good candidate to
encode the anti-neurogenic influence spatially associated with
the MHB. First, it is the earliest selective marker of the MH
domain, and its expression precedes the onset of neurogenesis
(Müller et al., 1996; Bally-Cuif et al., 2000). Second, it belongs
to the Hairy/E(spl) family of bHLH transcription factors,
which generally orient cell fate decisions during development
(Kageyama et al., 1997; Fisher and Caudy, 1998; Guillemot,
1999). In support of our hypothesis, we found that *her5*
expression faithfully outlines the IZ from the onset of
neurogenesis at late gastrulation (Fig. 2A) until at least 24 hpf
(compare Fig. 2B and C).

To examine the potential role of Her5 in IZ formation, we
first used a gain-of-function approach. Ectopic expression of

Fig. 2. Her5 as a candidate to control IZ formation. (A-C) Whole-mount in situ hybridization at the 3-somite stage (A, dorsal view, anterior to the top) and at 24 hpf (B,C, sagittal views, anterior to the left) with the markers indicated (bottom left, color-coded in A). Note that *her5* expression in wild-type embryos delineates the IZ (bracket) from the onset of neurogenesis (A) until at least 24 hpf (IZ identified by the gap in *zcoe2* staining in B). (D) Structures of the wild-type and mutant forms of *her5* cDNA and their encoded proteins used for functional assays. Top: full-length *her5* cDNA as determined from our genomic analyses (A.T. and L.B.-C., unpublished), which starts at ATG1 and encodes nine additional N-terminal amino acids compared to the published sequence (Müller et al., 1996) (see box for protein sequence). Bottom: *hsp-her5*

construct used to generate transgenic lines for conditional misexpression; this construct is built from the clone of Müller et al. (Müller et al., 1996) such that the first ATG is deleted and the second ATG is used for the generation of an otherwise fully functional Her5 protein (see Materials and Methods). As a control, a morpholino directed against ATG2 (MO^tg, inset) inhibits translation of the transgene mRNA but not that of the endogenous *her5* (data not shown, see Materials and Methods). For loss-of-function experiments, a morpholino directed against ATG1 (MO^her5, inset) was used, which inhibits the function of the endogenous Her5 mRNA. Abbreviations as Fig. 1 plus, b, basic DNA-binding motif; HLH, helix-loop-helix dimerization motif; IZ, intervening zone; mes, mesencephalon; r1, rhombomere 1.
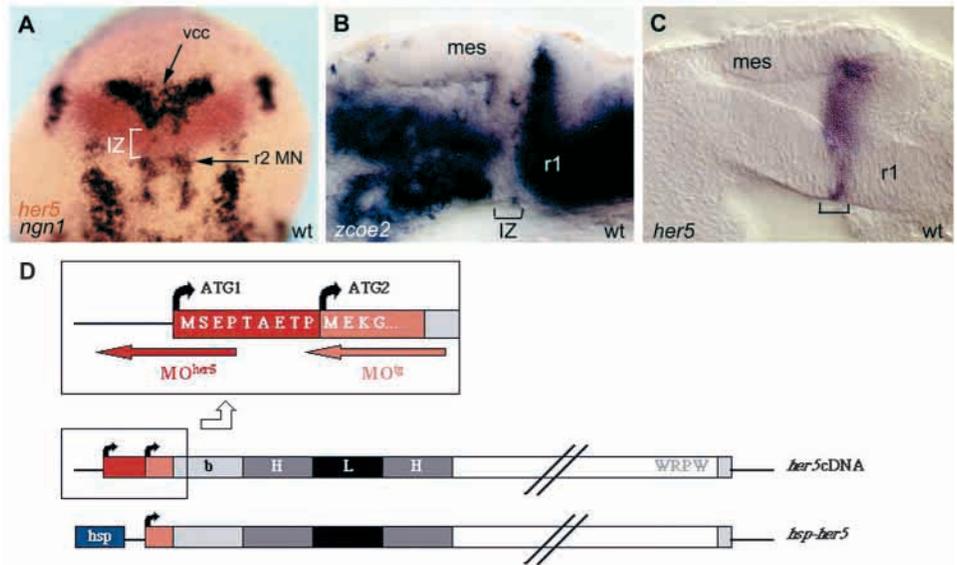
her5 severely perturbs gastrulation (Bally-Cuif et al., 2000), precluding an unambiguous interpretation of a neural phenotype at late stages. To overcome this problem we constructed *hsp-her5* transgenic lines carrying the *her5* cDNA (Müller et al., 1996) under control of the zebrafish heat-shock promoter *zhsp70* (Shoji et al., 1996; Halloran et al., 2000) (Fig. 2D). Three independent *hsp-her5* lines were generated. Because they produced similar results, they are considered together below.

We tested the reliability of *hsp*-driven transcription in these lines by monitoring *her5* expression in transgenic embryos immediately before and after heat-shock. At all stages examined, all embryos originating from a cross between a *hsp-her5* heterozygote and a wild-type fish displayed the endogenous *her5* expression profile (Fig. 3A). Upon heat-shock, strong and ubiquitous expression of *her5* was observed in 50% of the embryos (Fig. 3B), thus *hsp*-driven transcription is only induced upon heat-shock in our lines. In a time-course assay, transgenic *her5* mRNA, revealed by whole-mount in situ hybridization, was detectable as soon as 15 minutes after the beginning of the heat-shock but was gradually lost within the 1.5 hours following its end (Fig. 3C). These results are comparable to those of Scheer et al. (Scheer et al., 2002) and indicate that a heat-shock pulse translates into a narrow time-window when transgene *her5* mRNAs are available for translation.

Heat-shock pulses between 80% epiboly and tail-bud stages resulted in severe defects of *ngn1* expression in most *hsp-her5* transgenic embryos by the 3-somite stage (85% of cases, *n*=30) (Fig. 3E). Strikingly, *ngn1* expression was strongly diminished – in some cases abolished – in territories normally giving rise to the vcc and r2MN, located immediately adjacent to the domain of endogenous *her5* expression (Fig. 2A) (compare Fig. 3E with D). Other sites of neurogenesis, such as the motor, sensory and interneurons

of the developing spinal cord or the trigeminal ganglia, were only marginally affected, if at all. In contrast, *ngn1* expression was not affected when transgenic embryos were injected, prior to heat-shock, with a morpholino selective for the *hsp-her5* transgene (MO^tg) (Fig. 2D). This morpholino has no effect on the translation of endogenous *her5* and does not affect embryonic development (see Materials and Methods). Thus, heat-shocked *hsp-her5* MO^tg-injected transgenic embryos (80% of cases, *n*=20) showed normal *ngn1* expression (Fig. 3F, compare with Fig. 3D and E), demonstrating that the inhibition of *ngn1* expression in the vcc and r2MN areas upon *her5* misexpression (Fig. 3E) is a selective consequence of ectopic Her5 activity.

To test whether ectopic *her5* mRNA provided at late gastrulation is sufficient to permanently inhibit *ngn1* expression in domains adjacent to the IZ, we heat-shocked embryos under the conditions described above, then resumed development at normal temperature and analyzed *ngn1* expression at the 20-somite stage. As *hsp*-driven *her5* mRNA is no longer detectable at this stage, transgenic embryos were identified a posteriori by PCR genotyping (Fig. 3I). We observed long-lasting inhibition of *ngn1* expression, which was still downregulated at the 20-somite stage around and within the MH (83% of cases, *n*=28) (bar in Fig. 3H, compare with G). Later, this phenotype was followed by a lack of neuronal differentiation: at 24 hpf, *hsp-her5* transgenic embryos harbored a significantly reduced number of differentiated vcc-derived nMLF neurons (identified by their HNK1 immunoreactivity) compared to non-transgenic heat-shocked siblings (73% of cases, *n*=15) (brown arrows in Fig. 3O, compare with N). Thus ectopic Her5 activity at the onset of neurogenesis is sufficient to inhibit *ngn1* expression and the subsequent steps of neuronal differentiation around and within the MH domain.

Her5 activity is necessary for IZ formation at early neurogenesis stages

To test whether Her5 activity was necessary for IZ formation, we 'knocked-down' *her5* translation by injecting a morpholino selective for endogenous *her5* (MO[her5]) into wild-type embryos (Fig. 2D, see Materials and Methods). Strikingly, when MO[her5]-injected embryos were assayed at the 3-somite stage for *ngn1* expression, no IZ was discernible in the medial MH domain: the vcc and r2MN clusters were bridged (84% of cases, *n*=19) (compare Fig. 3K with J). TUNEL assays performed between the normal onset of *her5* expression (70%

epiboly) and the 3-somite stage consistently failed to reveal a significant difference in the number of apoptotic cells at any site between wild-type and MO[her5]-injected embryos (Fig. 3L,M, and data not shown) (92% of cases, *n*=25). In contrast, cell counts indicated a large increase in the number of *ngn1*-expressing cells within the medial MH territory in MO[her5]-injected embryos (91 cells ±5) compared to wild-type siblings (48 cells ±4) (90% of cases, *n*=10). Thus, lack of Her5 activity results in the generation of ectopic *ngn1*-positive cells in the territory located between the vcc and r2MN clusters. Importantly, this phenotype was followed by the development

Fig. 3. Her5 is necessary and sufficient to control IZ formation. (A-C) Reliability of the *hsp*-dependent expression system. (A,B) Embryos from a cross between parents heterozygous for the *hsp-her5* transgene probed for *her5* expression (in situ hybridization) before (A) and after (B) a 1-hour heat-shock. While no ectopic expression of *her5* is detected without heat-shock, ectopic *her5* expression is ubiquitously induced upon heat-shock (white arrows indicate endogenous *her5* expression at the MHB, black arrows indicate *hsp*-driven ubiquitous expression). (C) Stability of the induced *her5* mRNA upon heat-shock, determined by whole-mount in situ hybridization (in percentage of the estimated intensity of staining that immediately follows a 0.5-hour heat-shock pulse). Induced mRNAs become undetectable within 1.5 hours following the end of the heat-shock. (D-H,N,O) Ectopic expression of Her5 inhibits *ngn1* expression in the vcc and presumptive r2MN. Whole-mount in situ hybridization or immunocytochemistry with the markers indicated (bottom left, color coded) on transgenic embryos (tg) (E,F,H,O) and their wild-type siblings (wt) (D,G,N) at the 3-somite (D-F), 20-somite (G,H), and 36 hpf (N,O) stages, following a 1-hour heat-shock at late gastrulation (hs). D-F and N,O are dorsal views of the MH area in flat-mounted embryos, anterior to the top; G,H are sagittal views of the head, anterior to left; the insets show unperturbed *ngn1* expression in the spinal cord. The misexpression of *her5* during late gastrulation inhibits *ngn1* expression in the vcc and r2MN at the 3-somite stage (white asterisks in E). Non-heat-shocked transgenics display a *ngn1* profile indistinguishable from non-transgenic controls (not shown). This effect is maintained until at least the 20-somite stage (H), and is rescued upon injection of MO[tg], a morpholino oligonucleotide selective of the transgene (F). At 24 hpf, the number of nMLF neurons (brown arrows), which derive at least in part from the vcc, is also significantly reduced in *hsp-her5* transgenics (O) (red arrow to *her5* expression at the MH junction). (I) Genotyping results to identify transgenic embryos in H (PCR for the transgene). Lane 1: embryo H, lane 2: embryo G, lane 3: negative control, lane 4: positive control. An identical procedure was used to identify embryos in N,O. (J-M,P,Q) The inhibition of Her5 activity leads to the differentiation of ectopic neurons in place of the IZ. J,K: dorsal views of the MH area in flat-mounted embryos at the 3-somite stage, anterior to the top, probed for *ngn1* expression following injection of MO[her5], a morpholino selective of endogenous *her5* (K), compared to non-injected wild-type control embryos (J). Note that the vcc and r2MN clusters are bridged (double arrow), while other neuronal populations (e.g.r4MN, arrowhead) are unaffected. (L,M) TUNEL assay in wild-type (L) and MO[her5]-injected (M) embryos shows that injections are not followed by increased apoptosis in the MH area (bar). (P,Q) At 36 hpf, an ectopic HNK1-positive neuronal cluster (brown arrows) lies across the MH junction (identified by *her5* expression, blue arrow) upon MO[her5] injection. Note reduced *her5* levels at the MHB in Q (compared with P), a late event suggesting indirect positive autoregulation of *her5* expression. IZ, intervening zone; MH, midbrain-hindbrain domain; nMLF, nucleus of the medial longitudinal fascicle; r2MN, motorneurons of rhombomere 2; r4MN, motorneurons of rhombomere 4; vcc, ventrocaudal cluster.

of ectopic differentiated neurons at later stages: in most cases (67% of cases, n=12), bilateral clusters of HNK1-positive neurons formed across the MHB in MO[her5]-injected embryos by 36 hpf, but not wild-type embryos (brown arrows in Fig. 3Q, compare with P and N). Together, our results demonstrate that Her5 is both necessary and sufficient for inhibition of neurogenesis in the medial MHB domain at the onset of neurogenesis, an activity that helps keep the MHB free of differentiated neurons during later development.

Her5 can act in a dose-dependent manner on newly selected neuroblasts to inhibit neurogenesis at least until 24 hpf

Because *her5* expression delineates the IZ until at least 24 hpf, we tested whether it might also be involved in inhibiting neurogenesis at these late stages. When *hsp-her5* transgenic embryos were heat-shocked for 2 hours at the 8- or 15-somite stages, *ngn1* expression was down-regulated across the entire neural plate (Fig. 4A-C,G,I), in a dose-dependent fashion (86% of cases, n=22) (data not shown, and compare Fig. 4B and C). Within and around the MH domain, this phenotype was stable over time (Fig. 4D,E), while in other territories, *ngn1* expression was restored within a few hours of development at normal temperature (data not shown, and blue arrows in Fig. 4E) (87% of cases, n=24). When ectopic Her5 activity was induced at 24 hpf, *ngn1* expression was decreased within the MH domain (77% of cases, n=18) (compare Fig. 4K and J), while other sites remained unaffected (blue arrows in Fig. 4K). Thus Her5 activity is capable of inhibiting neurogenesis throughout somitogenesis.

ngn1 transcripts identify neuronal precursors in which neuronal fate is still being determined and includes cells that might still be proliferating (Ma et al., 1996). To better define the targets of Her5 activity and test whether it could affect cells further engaged in the neuronal differentiation pathway, we monitored the effect of ectopic *her5* expression on the expression of HuC protein. In contrast to the early onset of *huC* RNA expression (Kim et al., 1996), HuC protein immediately labels post-mitotic precursors (Mueller and Wullimann, 2002). In striking contrast to *ngn1* expression, the HuC profile was only moderately affected, if at all, by ectopic Her5 activity (82% of cases, n=11) (Fig. 4F-I). Thus Her5 is sufficient to prevent *ngn1* expression and/or to revert newly selected neuroblasts (*ngn1*-positive but still HuC-negative) to a non-engaged state. However, it does not act on immediately committed precursors such as HuC-positive cells, an observation in line with the idea that the mitotic to post-mitotic transition represents an irreversible commitment.

Her5 activity is not involved in patterning events within the MH domain

Because *her5* expression coincides with a number of markers that define MH identity or the MHB (Lun and Brand, 1998; Reifers et al., 1998; Belting et al., 2001; Reim and Brand, 2002), we asked whether Her5 activity is involved in controlling aspects of MH regionalization. Strikingly, ectopic *her5* expression from the onset of endogenous MH *her5* expression (70% epiboly) in *hsp-her5* transgenic embryos had no detectable effect on the expression of MH patterning markers (*iro1*, *iro7*, *pax2.1*, *eng2*, *eng3*) or IsO activity markers (*wnt1*, *fgf8*) at the 5- and 15-somite stages (n=35; Fig. 5A-C, also data not shown). Because a single short heat-shock pulse

Fig. 4. Her5 activity can inhibit *ngn1* until 24 hpf, in a dose-dependent manner, but does not affect HuC expression. Whole-mount in situ hybridization for *ngn1* (blue) and *her5* (red), with immunocytochemistry for HuC protein expression (brown), at the stages indicated (bottom left) following a two-hour heat-shock (hs) between the 8- and 15-somite stages (A-G), the 15- and 20-somite stages (H,I) and at 24 hpf (J,K) in *hsp-her5* transgenic heterozygotes (+/tg), homozygotes (tg/tg) or their non-transgenic siblings (wt). Dorsal views of flat-mounted anterior neural tubes (A-E,H-K) or tails (F,G), anterior to the top; bracket indicates the IZ, vertical black bar indicates the MH domain. *ngn1* expression is downregulated in a dose-dependent manner immediately after heat-shock at any of these stages (B,C,I). This phenotype is stable in the MH domain (blue arrows to restored *ngn1* expression in the fore- and hindbrain in E). In contrast, neuronal precursors already expressing HuC are only moderately affected (brown arrows in F-I) [HuC is first detectable in vcc neurons at about the 20-somite stage (H,I), thus at the 15-somite stage we focused on HuC expression in the tail (F,G)[. At 24 hpf, only the MH domain is sensitive to ectopic Her5 activity (blue arrows to unaffected *ngn1* expression in the fore- and hindbrain in K). IZ, intervening zone; hs, heat-shock; som, somites.
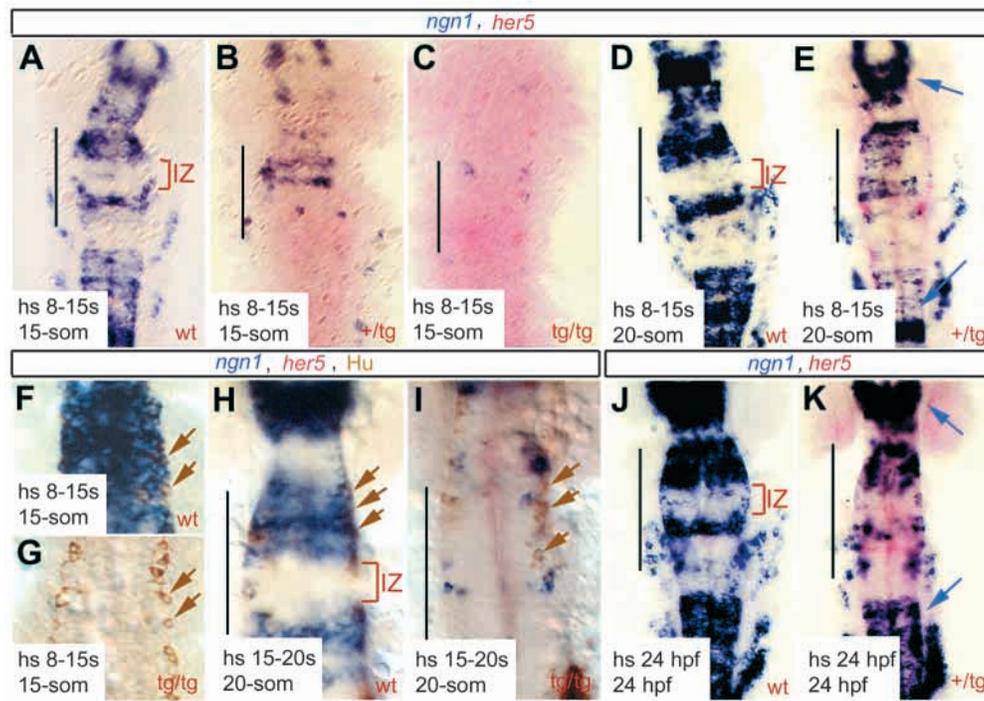
might induce only a transient burst in Her5 activity, insufficient to trigger stable defects, we repeatedly heat-shocked *hsp-her5* embryos until 24 hpf. Again, even in these embryos that received a constant supply of ectopic *her5* mRNAs, no patterning defects were detected (n=25; Fig. 5D, also data not shown), although strong and ubiquitous ectopic expression of *her5* was achieved (Fig. 5D, red staining). These results indicate that ectopic expression of *her5* from late gastrulation onwards is not capable of altering neural patterning.

Similarly, when wild-type embryos were injected with MO[her5], no defects were observed in the induction or maintenance of MH patterning (n=30; Fig. 5E-G, also data not shown). Thus, in contrast to its prominent effect on

Fig. 5. Her5 activity does not control MH regional patterning. Whole-mount in situ hybridization at the 3-somite (E-H), 5-somite (A-C), and 24 hpf (D) stages for the expression of patterning and neurogenesis markers, as indicated (bottom left, color coded), following up- or down-regulation of Her5 activity. All panels are dorsal views of whole-mount (E-G) or flat-mounted (A-D,H) embryos, anterior to the top; arrows point to the MH junction. (A-D) MH patterning is not altered in transgenic *hsp-her5* embryos (right in each panel) compared to non-transgenic siblings (left) by heat-shock during late gastrulation (A-C) or by repetitive heat-shocks (D). (E-G) MH patterning also remains unaltered in MO[her5]-injected embryos (right in each panel) compared to controls (left). (H) Co-expression of *ngn1* and *pax2.1* (see also fluorescent view, inset) across the MHB in a single embryo upon MO[her5] injection demonstrates that neurogenesis and patterning can be uncoupled by Her5 activity.

neurogenesis, Her5 activity is not required for the establishment and early maintenance of MH identities. To ascertain whether Her5 activity could dissociate neurogenesis from MH patterning in a single embryo, we colabeled embryos injected with MO[her5] for MH patterning markers (e.g. *pax2.1*) and neurogenesis markers (e.g. *ngn1*). Both marker types appeared co-expressed across the MH junction (Fig. 5H), a combination never normally observed in vivo (see Fig. 1A). Thus, while *her5* expression in the MH is determined by early patterning cues, its function does not control regional patterning within this domain. Thus Her5 is an essential factor that translates early axial patterning information into a distinct pattern of neurogenesis in the MH domain.

Her5 activity regulates cell proliferation and the expression of the zebrafish cyclin-dependent kinase inhitor-encoding gene $p27^{Xic1}$-a

We next examined the cellular mode of Her5 action. Neuronal differentiation generally correlates with cell cycle exit (Ross, 1996; Ohnuma et al., 2001) suggesting that Her5 activity might be associated with the maintenance of a proliferating state. To test this hypothesis, we counted the number of dividing cells per cell row (phosphohistone H3-immunoreactive, indicating M phase) across the neural plate in wild-type and Her5-manipulated contexts at the onset of neurogenesis (Fig. 6A-F).

Counts of dividing cells in wild-type embryos revealed differences within the IZ. The medial domain (Fig. 6A, A domain) has more cells in M phase than the dorsolateral domain (Fig. 6A, B domains) (n=5) (Fig. 6F, right panel; Fig. 6B,D, brown arrows). Thus intrinsic mediolateral differences in the proliferation status of the IZ in vivo parallel its medially heightened response to ectopic neurogenesis-promoting factors (Fig. 1E) and to lack of Her5 activity (Fig. 3K).

In *hsp-her5* transgenic embryos that were heat-shocked during late gastrulation, the number of cells in M phase was significantly increased throughout the presumptive midbrain and hindbrain regions (Fig. 6A, A-D domains). This included the domain endogenously expressing *her5* (Fig. 6A, A and B domains) as well as the 16 cell rows immediately anterior and posterior to it (Fig. 6A, C and D domains), from which the vcc and r2MN originate (Fig. 6B, C and Fig. 6F, left panel) (n=5). In the presumptive forebrain (Fig. 6A, E domain) the number of dividing cells was not altered, although this area also prominently expressed *her5* (Fig. 6C). Conversely, in MO[her5]-injected embryos, the number of dividing cells was significantly and selectively reduced in domain A (Fig. 6D, E; Fig. 6F, left panel) (n=5). Proliferation in this domain was not abolished but rather brought to a level equivalent to other neural plate territories (Fig. 6F). Together, these results suggest that Her5 activity can influence cell proliferation within and around the MH domain, and that it specifically accounts for the increased number of dividing cells within the medial IZ. In addition, Her5 loss-of-function results point to a strict correlation between domains with a decrease in cell proliferation and an increase in neuronal differentiation.

Cell proliferation involves the tight spatiotemporal control of expression and activity of a number of cellular factors including the cyclin-dependent kinases (Cdk) inhibitors p27 and p57 (O'Farrell, 2001; Ohnuma et al., 2001). Among these, $p27^{Xic1}$ (Bourguignon et al., 1998; Ohnuma et al., 1999), its mammalian relative $p27^{Kip1}$ (Lyden et al., 1999; Levine et al.,

2000; Dyer and Cepko, 2001; Li et al., 2002) and p57[Kip2] (Dyer and Cepko, 2000) play prominent roles in the control of developmental neurogenesis downstream of neurogenic cascades in *Xenopus* and mouse. To identify potential downstream effectors of Her5 proliferative activity, we conducted database searches for zebrafish Cdk inhibitors-encoding genes. Three clones or ESTs encoding probable zebrafish homologs of p27[Kip1] and two closely related forms of p27[Xic1] (-a and -b) were recovered (see Materials and Methods), and the corresponding genes were PCR-amplified from tail bud-stage cDNA. In situ hybridization analyses revealed that only *p27^{Xic1}-a* was expressed in wild-type embryos at the onset of neurogenesis (Fig. 6G,I, also data not shown). Most interestingly, *p27^{Xic1}-a* expression strongly resembles that of *ngn1*, identifying the first primary neurons of the neural plate and avoiding the IZ (Fig. 6G,I). This

expression profile is compatible with a role in linking cell cycle arrest with the differentiation of primary neurons. We thus addressed whether *p27^{Xic1}-a* expression was modulated by Her5 activity. Upon a brief heat-shock at late gastrulation, *p27^{Xic1}-a* expression was severely down-regulated in *hsp-her5* transgenic embryos, while it was unaffected in heat-shocked wild-type siblings (80% of cases, *n*=20; Fig. 6G,H). Conversely, in embryos where Her5 activity was abolished, *p27^{Xic1}-a* expression expanded ectopically across the IZ, overlapping the unaffected expression of *pax2.1* (82% of cases, *n*=22; Fig. 6I,J). Thus, modulating Her5 activity triggers opposite effects on cell proliferation and *p27^{Xic1}-a* expression. This suggests that down-regulation of *p27^{Xic1}-a* expression by Her5 might be involved in mediating the Her5-effected higher cell proliferation of the medial IZ domain in wild-type embryos.

Fig. 6. Her5 activates cell proliferation within the MH domain, but this process is in itself insufficient to account for the regulation of *ngn1* expression. (A,F) *her5* expression (in situ hybridization, blue staining) and density of cells in M phase (brown anti-phosphoH3 immunostaining) (in number of positive cells per cell row) in the anterior neural plate at the 3-somite stage (in territories schematized in A) in *hsp-her5* transgenics (C), their wild-type siblings (B) (both heat-shocked), wild-type (D) and MO^her5-injected embryos (E). A is a schematic representation of the neural plate in B,C; B-E are flat-mounted views of the anterior neural plate (B,C) or the endogenous *her5* domain (D,E), anterior to the top. White arrow in B,C, the endogenous domain of *her5* expression (territories A + B); box in D,E indicates territory A. Proliferation is enhanced in territory A compared to other neural plate domains in wild-type embryos (brown arrows in B,D). Her5 is sufficient to increase proliferation within the MH domain upon ectopic expression (territories A-D) (F, left panel), and is necessary for the increased level of proliferation of territory A (F, right panel). (G-J) Expression of the cyclin-dependent kinase inhibitor-encoding gene *p27^{Xic1}-a* is downregulated by Her5 within the IZ. Expression of *p27^{Xic1}-a* and *pax2.1*, as indicated (bottom left, color-coded), in *hsp-her5* transgenic embryos after heat-shock (H) and MO^her5-injected embryos (J) compared to their wild-type siblings (G,I) at the 3-somite stage. In the vcc and IZ, *p27^{Xic1}-a* expression is strikingly similar to that of *ngn1* (e.g. Fig. 3D). *p27^{Xic1}-a* expression is down-regulated within the neural plate following ectopic *her5* expression (arrow in H), and is activated across the IZ when Her5 activity is blocked (white arrows in J). Concomitantly in the latter case, *p27^{Xic1}-a* expression is partially reduced in the vcc area, a phenomenon at present unexplained but independent of cell migration (A.G. and L.B.-C., unpublished data). (K-N) The direct inhibition of cell proliferation does not affect IZ formation and *her5* expression. Expression of *ngn1* or *her5* (blue in situ hybridization staining) and anti-phosphoH3 immunostaining (brown nuclei) at the 3-somite stage in embryos treated with the cell proliferation inhibitor aphidicolin at the onset of neurogenesis (L,N) compared to mock-treated siblings (K,M). Although phosphoH3 staining is virtually abolished upon aphidicolin treatment, both IZ size (bracket in K,L) and *her5* expression appear normal. Aphi, aphidicholin-treated embryo; hs, heat-shocked embryo; IZ, intervening zone; tg, transgenic; vcc, ventrocaudal cluster.
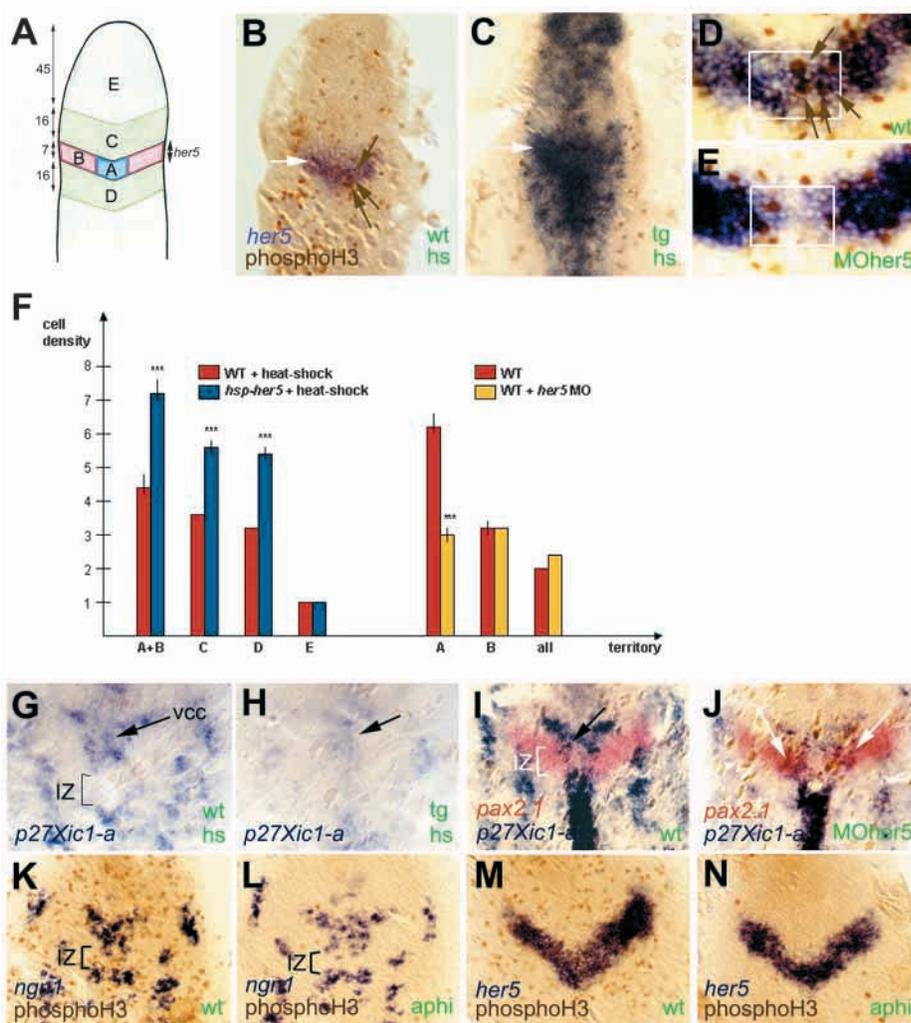
To determine whether Her5-induced effects on neurogenesis and proliferation are causally linked, we assessed neurogenesis in embryos where cell proliferation was blocked. To block cell proliferation, we incubated wild-type embryos in aphidicolin from the onset of *her5* expression until early neurogenesis. Although this treatment virtually abolished cell division (Fig. 6K-N, phosphoH3 staining), it had no effect on *ngn1* (*n*=20; Fig. 6L) or *her5* (*n*=20; Fig. 6N) expression. Thus the activation of cell proliferation by Her5 is not an intermediate step in its inhibition of *ngn1* expression across the IZ. Conversely, our results demonstrate that enhanced Her5 activity in *hsp-her5* transgenics can further upregulate cell proliferation within the endogenous *her5*-positive territory (Fig. 6F, left panel, A+B domain), which is devoid of *ngn1* expression and neurogenesis at all stages. Thus, at least within the IZ, the inhibition of *ngn1* expression by Her5 is unlikely to be an intermediate step in its activation of cell proliferation. Together, these results suggest that the regulation of neurogenesis and cell proliferation across the medial IZ in vivo reflect two parallel but distinct activities of endogenous Her5.

DISCUSSION

In this study, we addressed the mechanisms establishing the pattern of neurogenesis of the vertebrate MH domain. We demonstrated that neuronal differentiation is actively repressed at the MHB, and that this process is necessary for the maintenance of MHB integrity. We provided evidence that the non-differentiation zone (IZ) that splits midbrain from hindbrain neuronal clusters at the MHB consists of a medial and lateral domain with intrinsically different patterns of cell proliferation and potential for neurogenesis. We demonstrated that knock-down of Her5 function uncovers a cryptic proneuronal domain in the medial IZ that is continuous with vcc neurons rostrally and r2 motor neurons caudally. We also demonstrated that Her5 is essential for maintaining relatively high levels of proliferation in this medial domain by a mechanism that may be independent of effects on neurogenesis and involve the Cdk inhibitor p27^{Xic1}-a. Finally, we showed that Her5 activity does not influence MH patterning. Together, our results establish that a local process actively inhibiting neurogenesis at the MHB shapes the MH neuronal differentiation pattern and is essential to MHB maintenance. We identify Her5 as one crucial molecular component of this partially redundant pathway, and demonstrate that Her5 is a key regulator linking early axial patterning information to a distinct pattern of neurogenesis and cell proliferation in the MH domain. These findings more generally shed light on the mechanisms underlying the combinatorial control of patterning, neurogenesis and proliferation events within the vertebrate neural plate.

Differential competence of the MH junction towards neurogenesis

A first conclusion of our findings is that the IZ is not a homogeneous territory but is composed of two subdomains that differ strikingly both in their proliferation properties and in their competence to undergo neurogenesis. The medial IZ exhibits a single block in the differentiation pathway, encoded by Her5 activity, while the dorsolateral IZ likely bears multiple blocks, one operating upstream of *ngn1* expression, and at least one operating downstream or in parallel to this step. These intrinsic differences are unlikely to reflect general lateral versus medial properties of the entire neural plate, since neurons develop elsewhere in lateral domains at the same time as in basal territories (for instance the sensory neurons of r2). They might be due to other local inhibitors redundant to Her5 function in the laterodorsal territory. The transcriptional inhibitors Eng2 and 3 (Ekker et al., 1992), also expressed within the IZ from the onset of neurogenesis (Lun and Brand, 1998), do not appear to be sufficient cofactors. Indeed their ectopic expression is capable of inhibiting *ngn1* expression within the MH, however blocking the activities of Her5, Eng2 and Eng3 together by co-injecting the relevant morpholinos does not extend the neurogenic phenotype triggered by the lack of Her5 activity alone (M.I. and A.C., unpublished). Other candidates might be found within antagonists to neurogenic bHLH proteins, such as Hairy/E(spl) or non-basic HLH factors (A.T. and L.B.-C., unpublished), or among factors related to known neurogenesis inhibitors such as Zic2 (Brewster et al., 1998). The combined use of multiple inhibitors to locally prevent neurogenesis has been postulated to explain the non-differentiation of the superficial ectoderm layer in *Xenopus* (Chalmers et al., 2002). Our results thus provide a new example of this strategy to delimit neuronal differentiation domains during neural plate development.

An intriguing aspect of the phenotype triggered by Her5 gain-of-function is its prominence around and within the MH domain (Fig. 3E,H and Fig. 4), suggesting the presence of local cofactors. These might act on the regulatory elements of *ngn1* or of genes encoding redundant proneural factors to potentiate Her5 activity, or might behave as partners of Her5 to reinforce its activity and/or the stability of the Her5 protein. In favor of these ideas, the *ngn1* enhancer contains an element driving expression preferentially within the MH domain (Blader et al., 2003). In addition, we found that a mutant form of Her5, deleted of its C-terminal Groucho-binding WRPW domain, was inactive in regulating *ngn1* expression (A.G. and L.B.-C., unpublished), suggesting that Groucho-like cofactors are necessary to Her5 function. Along this line, *groucho4* is selectively expressed within the MH domain in the mouse and chick (Sugiyama et al., 2000; Ye et al., 2001).

Her5 activity shapes the midbrain-hindbrain neurogenesis pattern

Her5 acts in vivo as a local inhibitor of neurogenesis at the MHB. Our findings suggest that in the basal MH area, neurogenesis is primarily shaped into a pattern of separate neuronal clusters by a process of local inhibition that likely splits a continuous MH proneural field. Recent studies in *Xenopus* brought to attention the role of neurogenesis inhibitors in organizing zones of differentiation within the neural plate (Bourguignon et al., 1998; Brewster et al., 1998; Chalmers et al., 2002). Our analysis of IZ formation illustrates how neurogenesis inhibitors, superimposed on differentiation-competent territories, are crucial elements in shaping the embryonic neurogenesis pattern in vertebrates.

MO^{her5}-injected embryos display ectopic neurogenesis across the medial IZ from the very onset of *ngn1* expression (Fig. 3K), demonstrating that Her5 activity is essential to the establishment of this neuron-free zone. Whether Her5 is also

involved in medial IZ maintenance at later stages cannot be directly concluded from our loss-of-function data. Such a role, however, would be in line with the observation that *her5* expression continues to define the neuron-free area untill the 24 hpf stage and that ectopic *her5* expression can prevent neurogenesis within the MH domain at least until 24 hpf (Fig. 4). In the mouse, IZ maintenance relies on the combined action of two other Hairy/E(spl) bHLH factors, Hes1 and Hes3 (Hirata et al., 2001), which separately inhibit neurogenesis in a number of instances in vivo (Ishibashi et al., 1994; Ishibashi et al., 1995; Ohtsuka et al., 1999): $Hes1^{-/-};Hes3^{-/-}$ double-mutant embryos display premature neuronal differentiation across the MH junction from late somitogenesis (E10.5) (Hirata et al., 2001). No earlier neurogenic phenotype was detected in these embryos, however, suggesting that Hes1 and Hes3, unlike zebrafish Her5, are not involved in IZ generation. These observations are in keeping with the relatively late onset of *Hes1* and *Hes3* expression within the MH domain (Lobe et al., 1997; Allen and Lobe, 1999; Hirata et al., 2001), and with the observation that Hes1 and Hes3 are more related in sequence to zebrafish Her6 and Her3 than to Her5. Whether Her5 function is, all or in part, relied on by other Her factors at late stages to maintain medial IZ development in the zebrafish will require further study.

Her5 effectors in the control of MH neurogenesis

Her5 belongs to the Hairy/E(spl) class of bHLH transcription factors, generally functioning as transcriptional repressors (see Kageyama et al., 1997; Fischer and Caudy, 1998; Davis and Turner, 2001). Indeed, we demonstrated previously that Her5 functions as an inhibitor of transcription during a first developmental cell fate choice event required for endoderm patterning (Bally-Cuif et al., 2000). The direct targets of Hairy/E(spl) factors remain largely unknown outside of *achate-scute*-related genes (Chen et al., 1997) and some instances of autoregulation (Takebayashi et al., 1994). Our results demonstrate that a rapid response to manipulating Her5 activity is the regulation of *ngn1* expression. Thus the most parsimonious interpretation of Her5 function is that it directly inhibits the transcription of *ngn1*. Alternatively, Her5 might primarily inhibit expression (or activity) of upstream proneural factors such as those belonging to the Ash or Ath bHLH famles. Several such factors have been isolated in the zebrafish (Allende and Weinberg, 1994; Masai et al., 2000; Itoh and Chitnis, 2001), but their expression in the early neural plate was not reported. Addressing this point will be an important issue.

Her5 might also act at other steps of the neurogenic cascade, but our results indicate that the time-window of Her5 action is limited. Upstream of *ngn1* expression are the specification of the MH proneural field (possibly by Iro1 and 7) (Lecaudey et al., 2001; Itoh et al., 2002), the definition of proneural clusters and the singling-out of individual precursors by the Notch-dependent lateral inhibition process (Haddon et al., 1998; Lewis, 1998; Chitnis, 1999; Takke et al., 1999). An action of Her5 at any of these upstream steps is unlikely. First, knocking-down Her5 activity has no effect on the expression of markers of the MH proneural fields or clusters (Fig. 5D and data not shown). In contrast, perturbing Iro function affects *her5* expression (M.I. and A.C., unpublished), placing *her5* downstream of these factors. Second, manipulating the lateral

inhibition machinery, and in particular suppressing Notch signaling, did not affect IZ formation (A.G. and L.B.-C., unpublished), arguing against a role for Her5 upstream of Notch signaling. Further, Her5 does not act far downstream of *ngn1* expression in the neurogenic cascade, as expression of the post-mitotic marker HuC protein (Mueller and Wullimann, 2002) was never reversed upon ectopic Her5 activation (Fig. 4). In the same individuals, *ngn1* expression was virtually abolished. Thus our results support a role for Her5 in regulating the expression (or activity) of proneural factors at a level equivalent to Ngn1 in the neuronal differentiation process.

An important question is, to what extent the mechanism regulating neurogenesis at the MHB differs from those operating elsewhere in the neural plate. All studied bHLH neurogenesis inhibitors in the vertebrate central nervous system act as downstream effectors of Notch activity, with the exception of *Xenopus* HES6 and mouse Hes3. Her5 joins these exceptions as both its expression and activity within the neural plate are independent of Notch signaling in vivo (A.G. and L.B.-C., unpublished). Within the neural plate, Her5 expression and function appear more reminiscent of those of *Drosophila* Hairy than of other vertebrate Hairy/E(Spl) factors known to date. Indeed Hairy operates independently of Notch signaling and is involved in pre-patterning broad non-differentiation zones within the *Drosophila* notum, prior to the onset of neurogenesis (Fischer and Caudy, 1998; Davis and Turner, 2001). Similarly, mouse Hes1 was proposed to negatively delimit neurogenesis domains within the olfactory epithelium (Cau et al., 2000). Her5 appears as the first vertebrate Hairy/E(spl) factor with similar function within the neural plate, and it will be interesting to determine whether our findings can be extended to other family members.

Proliferation and neurogenesis at the MH junction

Two classes of G1 CyclinD:Cdk inhibitors play a prominent role in a developmental context: p16, and the Cip/Kip family members p21, p27 and p57 proteins (O'Farrell, 2001; Ohnuma et al., 2001; Ho and Dowdy, 2002). Zebrafish $p27^{Xic1}$-a expression is negatively regulated by Her5 activity, adding strong support to the idea that Cdk inhibitors control spatiotemporally regulated cell cycle events during embryogenesis and are, at least in part, controlled themselves at the transcriptional level (see Dyer and Cepko, 2000; Dyer and Cepko, 2001; Hardcastle and Papalopulu, 2000; Levine et al., 2000; Ohnuma et al., 1999; Ohnuma et al., 2001). Our findings strongly suggest that the transcriptional inhibition of $p27^{Xic1}$-a is a downstream event of Her5 activity in its activation of cell proliferation within the medial IZ. Her5 thus appears reminiscent of mammalian Hes1 and 3, which inhibit the expression of Cip/Kip family members in vitro (Kabos et al., 2002), and it is possible, like for other Hes factors (Sasai et al., 1992; Kageyama et al., 1997; Hirata et al., 2000; Pagliuca et al., 2000), that $p27^{Xic1}$-a is a direct transcriptional target of Her5. However, our data also suggest that additional cell cycle regulators are responsive to Her5 activity in this domain, since an increased dose of Her5 at the MHB further enhances cell proliferation in *hsp-her5* transgenics compared to wild-type embryos while this domain does not express $p27^{Xic1}$-a.

In its regulation of cell proliferation, Her5 does not appear as an all-or-none switch, but rather as a modulator. Indeed a

basal level of proliferation is maintained in the absence of Her5 activity within the medial IZ. In addition, the laterodorsal IZ, which also expresses *her5*, does not proliferate at a higher rate than other neural plate domains. Several hypotheses might account for these observations. Her5 might act as a permissive factor that enhances the competence of its expressing cells towards extrinsic or intrinsic proliferation triggers. Alternatively, Her5 might alter the length of cell cycle phases to shorten those where cells are responsive to differentiation signals. Finally, Her5 might not act on the cell cycle *per se* but rather orient cell divisions towards a symmetrical mode at the expense of an asymmetrical one.

Finally, our results suggest that the effects of Her5 on cell proliferation and neurogenesis are distinct. In *hsp-her5* transgenics, increased Her5 activity upregulates proliferation even at the MHB, a neurogenesis-free territory. Conversely, blocking cell proliferation does not induce *ngn1* expression at the MHB. Thus, the activation of proliferation by Her5 is not simply a consequence, and is also unlikely to be an upstream step, of its inhibition of neurogenesis. Rather, our results support a model where these two processes are, at least in part, independently regulated by Her5 activity in vivo. Her5 would thus appear as a coordinator of cell division and neuronal differentiation within the MH domain, in a manner reminiscent of the key regulator XBF-1 within the *Xenopus* anterior neural plate (Hardcastle and Paplopulu, 2000). A striking and relevant example is also provided by the bifunctional *Xenopus* protein p27^{Xic1}, which uses separate molecular domains to regulate both cell cycle and cell differentiation in the retina (Ohnuma et al., 1999).

Linking patterning, neurogenesis and proliferation at the MHB

Patterning of the MH domain relies on two series of components, IsO-derived signals (e.g. Wnts and Fgfs) and general MH identity factors (e.g. Pax2/5/8 and Eng proteins) (Martinez et al., 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The expression of markers of both types was reproducibly unaltered at any stage in response to gain- and loss-of-function of Her5, under conditions that influenced MH neurogenesis and proliferation. Her5 activity thus strikingly differs from that of its probable *Xenopus* homolog XHR1 (Shinga et al., 2001) and from mouse Hes1/Hes3 (Hirata et al., 2001), all of which were interpreted as primarily acting on MH patterning. Ectopically expressed *XHR1* markedly enhances *En2* expression, and its dominant-negative forms down-regulated *XPax2* and *En2* in *Xenopus* (Shinga et al., 2001). Similarly, in double *Hes1*$^{-/-}$;*Hes3*$^{-/-}$ mouse mutant embryos, the loss of organizer-specific gene expression such as *Pax2*, *Wnt1* and *Fgf8* precedes neuronal differentiation defects (Hirata et al., 2001). *her5* expression is established by early axial patterning cues, and later responds to isthmic organizer activity (Lun and Brand, 1998; Reifers et al., 1998; Belting et al., 2001; Reim and Brand, 2002). Our results show that Her5 selectively controls neurogenesis and proliferation without retroacting on MH patterning. Thus Her5 is part of a key coupling pathway activated at the MHB to translate early axial patterning and later isthmic organizer information into a local control of neurogenesis and proliferation.

At early somitogenesis stages, the isthmic organizer controls MH patterning and growth (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Later, the MHB remains a prominent source of proliferating cells (Wullimann and Knipp, 2000), proposed to permit the massive and sustained growth of MH structures relative to other neural territories in all vertebrates. Our findings demonstrate that maintenance of an MHB neuron-free zone results from an active mechanism, and further attests the biological significance of this process for MH development, by demonstrating that neurogenesis must be prevented at the MHB to maintain MHB integrity. The inhibitory process involving Her5 might perhaps speculatively be viewed as a self-protective mechanism permitting the maintenance of MHB activity over time, in a manner possibly reminiscent of other signaling boundaries, such as, for instance the *Drosophila* wing margin.

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IV. Materials and Methods

1. Abbreviations and symbols used in the chapter Materials and Methods

A	adenine
AA	amino acid(s)
ab	antibody
amp	ampicillin
bp	base pair(s)
BCIP	5-bromo-4-chloro-3-indolyl-phosphat
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
Chl	chloroform
CNS	central nervous system
d	day
dATP	deoxyadenosine-triphosphate
dCTP	deoxycytosine-triphosphate
dGTP	deoxyguanosin-triphosphate
DAB	3,3'-diaminobenzidine
DIG	digoxigenin
DMF	dimethylformamide
dNTP	deoxyribonucleotid-triphosphate
dpf	days post fertilization
dTTP	deoxythiymidine-triphosphate
EDTA	ethylen-diamin-tetra acidic acid
EtBr	ethidiumbromid
EtOH	ethanol
ferri	potassium ferricyanide
ferro	potassium ferrocyanide trihydrate
G	guanine
IPTG	isopropyl B-D-thiogalactopyranoside
NGS	native goat serum
h	hour
hpf	hours post fertilization

HRP	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranosid
kb	kilo bases
LB	Luria-Bertani-medium
M	molar
MeOH	methanol
mRNA	messenger RNA
NaOAc	potassium acetate
NBT	nitro-blue-tetrazolium
OD_{260}	optical density measured at a wavelength of 260nm
OD_{600}	optical density measured at a wavelength of 600nm
oligo(dT)	oligo-deoxythymidine
ON	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
Phl	phenol
polyA-RNA	polyadenylated RNA
RNA	ribonucleotide
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SSC	disodium citrate buffer
T	thymidine
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Tris	Tris-(hydroxymethyl)-aminomethane
tRNA	transfer-RNA
U	unit
UV	ultraviolet
UTP	uracil-triphosphate
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

Symbols:

$*$	stop-codon in AA-sequences
min	minute
sec	second
x	times

2. Recipes of often used Buffers and Solutions

<u>LB$_0$</u>

1% Bacto Tryptone

0.5% Bacto Yeast extract

1% NaCl

pH7.4

<u>Low salt LB$_0$</u>

1% Bacto Tryptone

0.5% Bacto Yeast extract

0.5% NaCl

pH7.4

<u>LB-plates</u>

LB medium

1.5%Difco agar

<u>10xPBS</u>

8% NaCl

0.2% KCl

0.2M PO$_4$-buffer

pH7.3

<u>PBT</u>

PBS

0.1% Tween-20

<u>20xSSC</u>

0.3M NaCl

0.3M Na-Citrat

<u>10xTBE</u>

0.88M Tris

0.88M boric acid

25 mM EDTA

TE

10mM Tris-HCl, pH8.0

0.5mM EDTA

NTMT

100mM Tris-HCl, pH9.5

50mM $MgCl_2$

100mM NaCl

0.1% Tween-20

<u>In-situ hybridization buffer (ISH-hyb)</u>

65% formamide

5xSSC

50μg/ml heparin

0.5mg/ml Yeast tRNA

0.1% Tween-20

9.2mM citric acid pH 6.0

ad 50ml H_2O (Ampuwa)

<u>PBTBN</u>

PBT

2% NGS

2mg/ml BSA

ad 50ml H_2O

DNA extraction buffer

1.5mM $MgCl_2$

10mM Tris-HCl pH8.3

50mM KCl

3% Tween

3% NP40

Neutralisation buffer

1.5M NaCl

1M Tris-HCl pH 8.0

Denaturation buffer

1.5M NaCl

0.5M NaOH

NBT stock

100mg/ml in 70% dimethyl formamide

BCIP

50mg/ml in 100% DMF

NBT/BCIP revelation solution

225µg/ml NBT

175µg/ml BCIP

fill up to 10ml with NTMT

PT

PBS

0.025% Triton-X100

PTBN

50ml PT

2mg/ml bovine serum albumin (BSA)

2% normal goat serum (NGS)

Embryo medium (EM) (10% Hank's solution)

Full strength Hank's solution:

0.137M NaCl

5.4mM KCl

0.25mM Na_2HPO_4

0.44mM KH_2PO_4

1.3mM $CaCl_2$

1.0mM $MgSO_4$

4.2mM $NaHCO_3$

Glycerin-gelatin

7g gelatin in 42ml water → stirred until solved

50g glycerol

500µl phenol → stirred until solved at 60°C

Sucrose-gelatin

7.5% gelatin in 15% sucrose/phosphate buffer solution

dissolve at 60°C while stirring

Tricaine stock solution

400mg tricaine powder

add 95ml water

bring to pH 7.0 with 1M Tris-HCl, pH9.0

ad 100ml water

3. Animal model organism: Zebrafish

3.1. Description and origin

Zebrafish *(Danio rerio)* belong to the family Cyprinidae, order Cypriniformes, phylum Chordata. They are native to the streams of India. They are easy to raise with a short generation time of about 3 months. The females lay at weekly intervals. As the fertilization is external, it gives easy access to the eggs, which then can be manipulated and observed easily. Another advantage is that they are transparent. Further unlike other fishes, which often have triploid or tetraploid genome, they stay diploid.

3.2. Animal keeping and breeding

The adult fishes were kept in a cycle of 14h light/10h darkness in circulating water (conductivity approx. 600μS reached with sea salt, pH 7.0) with a temperature between 26°C and 28°C.

Embryos/juveniles stayed in Petri dishes for 5 days, then in mouse boxes for 5 days. At this point they were brought into the circulating water system. Juveniles were fed with AZ (powder baby food) until approx. one month of age. Artemia (saline crayfish) were added progressively after day 15 until they reached an age of four weeks, then they were fed with Artemia and dry flakes.

To avoid contamination with pathogens embryos obtained from the quarantine facility, were bleached at a stage of 1-2dpf. To make the stock solution one tablet of bleach was dissolved in 40ml water (30%). For bleaching the embryos 1.5μl of this stock solution was put in 50ml EM and shaken for 5min. The embryos were then rinsed twice for 5min in EM. The bleaching was repeated once and the embryos washed several times in EM, dechorionated and incubated in EM at 28.5°C.

3.3. Embryos

Eggs were obtained from natural spawning. Crosses of adult animals were set up after 3pm in breeding boxes. On the following day the eggs were collected in embryo medium and raised at 28.5°C. Switching on the light in the morning triggered the mating process, and one couple laid about 100-200 eggs. Embryos were staged according to Kimmel et al. (1995). Older stages were determined by counting the number of somites or after the number of hours passed after fertilization (hpf).

4. Molecular Techniques

4.1. Preparation of nucleic acids

4.1.1. Isolation of genomic DNA from embryos

1-100 embryos are transferred into eppendorf tubes and the liquid removed. Then 50μl extraction buffer (see 2) and 10μl proteinase K (2mg/ml) were added, vortexed and incubated for 3h at 60°C and finally heated at 95°C for 3min to inactivate proteinase K activity. Then 100-500μl water were added and vortexed. The DNA could be directly used for pcr reactions or stored at

−20°C.

4.1.2. Isolation of genomic DNA from tail-fins

A small piece of the tail-fin of adult fishes were cut with a scalpel and transferred into an eppendorf tube containing 50µl extraction buffer and 10µl proteinase K (2mg/ml). The following steps were similar as described in 4.1.1.

4.1.3. Isolation of total RNA from embryos

100 embryos were transferred into eppendorf tubes and the liquid removed. For RNA isolation the RNeasy Mini Kit (Qiagen) was used. Thereby the RNA was extracted in 50µl Ampuwa water.

4.1.4. Preparation of cDNA

cDNA was synthesized following the instructions recommended in the Revert Aid First Strand Synthesis Kit (Fermentas) manual. About 1µg total RNA was used for the reverse transcription of the first strand cDNA in a final volume of 20µl. Only the first strand was synthesized, the second strand is synthesized during the PCR amplification by the Taq-Polymerase later on. The synthesis of the first strand cDNA was done by the RevertAid^{TM}H Minus M-MuLV Reverse Transcription Kit (Fermentas) as recommended in the instructions.

4.1.5. Plasmid DNA preparation

To isolate plasmid DNA the Qiagen Mini or Maxi plasmid preparation kit was used, following the recommended protocol. The DNA was eluted in either 50µl (Mini) or 100µl (Maxi) water.

4.2. Preparation of digoxigenin- or fluorescein-labeled anti-sense probes

As template for the *in-vitro* transcription of anti-sense probes linearized plasmid-DNA was used. The reaction contained 10µg DNA, 10U enzyme, 1x restriction enzyme buffer and water ad 50µl and were incubated at 37°C for 1-2h. After inactivation of the enzyme using proteinase K treatment the DNA was purified with phenol/chloroform extraction, followed by chloroform extraction. The DNA was precipitated by adding 0.05M NH_4Ac and 2.5 volumes EtOH. The pellet was washed once with 75% EtOH, dried and resolved in water to a final concentration of 1µg/µl. The digoxigenin- or fluorescein-labeled anti-sense probes were synthesized using 11µl Ampuwa water, 2µl of transcription buffer, 1µl of ATP, CTP, GTP, 0.65 µl UTP and 0.35µl digoxigenin- or fluorescein-bound UTP, 1µl of linearized DNA (as described above), 1µl RNase inhibitor and 1µl RNA polymerase (T3, T7, SP6, respectively). The reaction was incubated at 37°C for 2h. Then 1µl DNase (RNase-free) was added and

incubated for 15min. To precipitate the RNA 80µl TE, 10µl 4M LiCl, 300µl EtOH was added, using an incubation time of 1h at

–20°C. This step was repeated once. All components used were from Fermentas.

4.3. Amplification of DNA by polymerase chain reaction (PCR)

In most PCR reactions the following standard protocol was used. In some cases a modified PCR protocol had to be used to get and/or improve the expected product. The reaction mix contained: 1x reaction buffer, 20mM $MgCl_2$, 20 mM dNTP-mix, sense and antisense primers (25pmol), 150-250ng DNA (if smaller than 10kb), 0.5-1µg DNA (if bigger than 10kb), 2U Taq-polymerase and water to bring the reaction to a final volume of 50µl. As reaction profiles the following temperatures und incubation times were used as described below. To denature the DNA the reaction was incubated at 94°C for 2min, followed by 25-35 cycles under following conditions: denaturation at 92-94°C for 30sec, annealing temperature between 42-68°C (depending on the Tm of the primers) and incubation between 30-90sec; extension at 68°C or 72°C for 30-150sec. Finally to complete the extension the reaction was placed at 68°C or 72°C for 10min and stored at 4°C.

4.4. PCR purification

The DNA fragments from the PCR amplification were purified from primers, nucleotides, polymerases and salts, following the protocol of the QIAquick PCR Purification Kit (Qiagen).

4.5. Isolation and purification of DNA fragments using Gel Extraction

After separation in a 1% agarose gel in 1xTBE, the fragment was cut out of the gel under UV illumination, transferred into an eppendorf tube and purified following the recommended protocol of the QIA Gel Extraction Kit (Qiagen).

4.6. Ligation and Transformation

As vectors for the ligation reaction either pTopo (pTopo system) or a linearized vector treated with alkaline phosphatase (to remove the phosphate and to avoid self-ligation of the plasmid itself), were used. The protocol for the ligation and transformation were mostly similar. To ligate the purified fragments into the prepared vector the ratio of vector (50ng) to fragment (50ng) was chosen to be 1:3 for all ligation reactions. Together with ligation buffer and T4-ligase the reaction was incubated for either 30min at RT or 5min at RT and ON at 14°C. After the ligation the reaction was added to ice-cooled competent bacteria cells *E.coli* TOP10F' (Invitrogen) or DH5α, mixed immediately and incubated for 30min on ice. A heat shock for

45sec at 42°C improves the transformation activity. Then the bacteria are kept for 2min on ice, then 900µl of LB_0 was added and the bacteria were grown, rocking for 1h at 37°C. Finally the bacteria were plated on LB-plates with the appropriate antibiotic and incubated ON at 37°C.

In the case of pTopo vector the colonies could be selected by blue-white screening. Therefore 20µl of IPTG- and 20µl of X-Gal-solution allow a blue-white screening on colonies if the fragment was integrated into the lacZ gene which then was destroyed. Colonies containing a pTopo vector with an inserted PCR fragment appeared white in comparison to colonies which contain the vector alone (without insert), appearing blue. For further analyses the colonies were tested by PCR, restriction pattern analysis or were sequenced.

4.7. Transfer and detection of nucleic acids

4.7.1. Southern Blot

DNA from agarose gels was transferred to nylon membranes (Hybond^{TM} N$^+$) by capillary blot. For that reason the gel was incubated after electrophoresis in 0.25M HCl, then 2x15min in denaturation buffer and finally 2x15min in neutralization buffer. Then the capillary blot was built up (with 10xSSC and 2xSSC as buffers) and left ON at RT. On the following day the filter was washed briefly in 2xSSC and the DNA immobilized using UV cross-linking.

4.7.2. Detection of DNA probes

DNA fragments were labeled using the Megaprime DNA Labeling Kit (Amersham), following the recommended instructions. For the reaction 0.5-1µg DNA was denatured at 95°C for 5-10min and put immediately on ice. The reaction contained 1x reaction buffer, deoxyribonucleotides dATP, dGTP and dTTP (25mM), hexanucleotides as unspecific primers (in a statistical mixture), [α-^{32}-P]-dCTP (50µCi, 3000Ci/mmol) and 1µl Klenow polymerase. The reaction was incubated at 37°C for 30min. Then the incorporated nucleotides were removed using the Nuctrap^{TM} column (Stratagene). The radioactive incorporation ratio was quantified by szint counting.

4.7.3. Hybridization and detection

A nylon membrane containing the immobilized DNA was incubated in Rapid-hyb buffer (Amersham) at 65°C for 1h with gentle rocking to block unspecific binding. For the hybridization the denatured probe (95°C for 5-10min) was added and incubated at 65°C ON with rocking. The concentration of the probe was approximately 1-10x10^{6}cpm/ml hybridization buffer. On the following day the membrane was washed as follows: 20min in 2xSSC + 0.1% SDS at RT, 15min in 2xSSC + 0.1% SDS at 65°C, 15min in 0.2xSSC + 0.1%

SDS at 65°C. Then the membrane was briefly dried on 3MM Whatman paper, sealed in a plastic bag and used for autoradiography. If necessary the membrane could be further washed with higher stringency.

4.7.4 Autoradiography

The membrane was put with the DNA side to the top into a film cassette and covered by a Kodak Biomax^{TM} film (Kodak). Then the film was exposed at −80°C for 1h-1d, developed and analyzed.

4.8. Analysis of sequences

DNA sequences as well as protein sequences and sequence comparisons were analyzed using the NCBI sequence program. Further, the EMBL database was used to obtain sequences from the shotgun sequencing project.

5. Staining of embryos using in situ hybridization (ISH) and immunocytochemistry (IC)

5.1. Preparation of embryos for ISH and IC

The embryos were fixed in 4% PFA and incubated ON at 4°C. Afterwards they were dechorionated and rinsed 3x5min in PBT, followed by a dehydration series in MeOH (each step 2-3 min): 25% MeOH + 75% PBT → 50% MeOH + 50% PBT → 75% MeOH + 25% PBT → 100% MeOH and stored at −20°C.

5.2. Whole-mount ISH on zebrafish embryos

5.2.1. Single-color ISH

After proteinase K treatment, excluding the staining step, the embryos were gently rocked during all steps. Then the embryos were rehydrated through a reverse MeOH/PBT series (see 5.1.) and placed in a 48 well-box. To improve the penetration of the probes the embryos were treated with proteinase K (10μg/ml in PBT), the incubation time was dependent on the age of the embryos. The following incubation times were used: younger than 12hpf: 0min; from 12-22hpf: 3min; older than 22hpf: 15min. To stop the proteinase treatment the embryos were washed 3x5min in PBT, the post-fix 20min in 4% PFA at RT and rinsed 4x5min in PBT. For pre-hybridisation PBT was replaced with 300μl of ISH hybridisation buffer (ISH-hyb) and rocked at 70°C for 2h. For hybridisation the buffer was replaced with 150μl ISH-hyb buffer and 1.5μl digoxigenin- or flourescein-labelled RNA probe was added. The ISH was then incubated ON at 70°C with gentle rocking. On the following day the embryos were rinsed for

10min at 70°C in 75% ISH-hyb buffer + 25% 2xSSC; 10min at 70°C in 50% ISH-hyb buffer + 50% 2xSSC; 10min at 70°C in 25% ISH-hyb buffer + 75% 2xSSC; 10min at 70°C in 100% 2xSSC; and two times for 30min at 70°C in 0.05xSSC. Then the following washing steps were performed: 5min at RT in 75% 0.05xSSC + 25% PBT; 5min at RT in 50% 0.05xSSC + 50% PBT; 5min at RT in 25% 0.05xSSC + 75% PBT; 5min at RT in 100% PBT. Then the embryos were incubated for 1h at RT in ISH block buffer PBTBN, followed by 2h at RT in pre-adsorbed antibody, diluted in PBTBN. Following final dilutions are used: anti-digoxigenin-AP: 1/5000 or anti-fluorescein-AP: 1/2000. After incubation the embryos were rinsed 3x5min and 6x10min in PBT. For revealing the digoxigenin-labeled probe the embryos were rinsed 3x10min in NTMT and revealed in the dark using a solution of NBT and BCIP, diluted in NTMT. For revealing the flourescein-labeled probe the embryos were rinsed 1x10min in 0.1M Tris-HCl, pH8.2 + 0.1% Tween and revealed in the dark with FastRed (Sigma) substrate. After staining the embryos were rinsed 4x10min in PBT and finally ON in PBT/EDTA at 4°C. Finally the embryos were stored in 80% glycerol in PBT at 4°C.

5.2.2. Two-colour ISH

The protocol described in 5.2.1. was used with following modifications:

The embryos were incubated with two RNA probes, one labeled with digoxigenin, the other with fluorescein. For detecting the probes, first the embryos were incubated in anti-flourescein-AP in PBTBN with a final dilution of 1/2000, rinsed in PBT 3x5min, then 6x10min in PBT, followed by 1x10min in 0.1M Tris-HCl, pH8.2 + 0.1% Tween and revealed in the dark with FastRed substrate. After the staining they were rinsed 3x5min in PBT. To detach the anti-flourescein-AP the embryos were incubated in 3x5min in 0.1M Glycin-HCl, pH2.0, rinsed 3x5min in PBT and post-fixed in 4% PFA 20min at RT, rinsed 6x5min in PBT and finally incubated in anti-digoxigenin-AP for 2h at RT. The rinses, revelation and storage were done as described in 5.2.1.

5.3. Immunocytochemistry (IC)

All steps except the staining step were performed at RT, with gentle rocking. For antibody working concentration see Materials and Methods in each specific part of the chapter "appendix".

5.3.1.Whole-mount IC

The embryos were rehydrated in reverse MeOH series (see 5.1.). To increase the permeability they were rinsed up to 1h in water, depending on their age: therefore 24h old embryos were rinsed for 20min, embryos older than 24h for 1h. Then several rinses in acetone (3x5 min) and incubation of the embryos for 10 min at –20°C helped to remove fatty acids. Finally they were rinsed briefly in water and then for 4x5min in PTD. Then they were incubated in ICC block buffer for 1h at RT, followed in primary antibody for 2h at RT or ON at 4°C. To wash the embryos they were rinsed 6x10 min in PTD, then the secondary antibody were incubated for 1-2h at RT, followed by 6x10 min in PTD. For revelation the embryos were first rinsed 10 min in 0.1M Tris-HCl, pH7.5, then incubated 10min in 0.5mg/ml diaminobenzidine (DAB). With adding 0.001% H_2O_2 the revelation process was started. After staining the embryos were rinsed 4x5min in PTD, then ON in PTD at 4°C and stored in 80% glycerol in PBT at 4°C.

5.3.2. IC after ISH

The protocol is identical to 5.3.1., starting from the incubation in the primary antibody, diluted in ICC block buffer. Because of the previous ISH the embryos did not need further treatment with water/acetone or proteinase K to increase the permeabilty.

5.3.3. IC on cryostat sections

After sectioning the slides were rinsed 10min at RT in PT and blocked in PTBN for 1h at RT. The primary antibody was diluted in PTDBN and applied to the sections ON at 4°C. On the following day the slides were rinsed 4x15min in PT with gentle rocking. The diluted secondary antibody was applied onto the sections for 2h at RT, then rinsed 4x15min in PT with gentle rocking. Revelation: for all steps the sections were kept in dark. To the secondary antibody either a. horse radish peroxidase (HRP) or b. FITC were coupled.

a. For revelation of HRP the sections were incubated 10min at RT in 0.5mg/ml DAB. A brown precipitate was visible after adding 0.001% H_2O_2.

After staining the reaction was stopped by rinsing 4x10min in PT at RT, ON in PT at 4°C and mounted in glycerol/gelatine.

b. For monitoring FITC the sections were mounted in glycerol/gelatine and monitored using a FITC-filter.

6. Preparation of flatmounts and sections

6.1. Flatmounts

To get detailed pictures from the staining, the embryos were flatmounted. Therefore the yolk was removed with forceps. The eyes were removed if stained by in situ hybridization and disturbed the view to the brain region.The embryos were then mounted on slides in the appropriate orientation, embedded in glycerol/gelatine and covered with a cover slip.

6.2. Cryostat sections

After ISH the embryos were prepared for sectioning by incubation in 15% sucrose in 0.12M Phosphate buffer ON at 4°C. Then they were imbedded in sucrose-gelatine, orientated and finally the sucrose-gelatine was cut in blocks, frozen in nitrogen-cooled dimethyl-propanol and stored at –20°C. The blocks were cryostat-sectioned at 8μm thickness and the sections were subjected to immunocytochemistry following standard protocols (see 5.3.3.)

6.3. Plastic sections

The embryos were embedded after ISH and/or IC in JB4 resin using the recommended protocol. First the embryos were washed 2x in PBT . Then they were postfixed for 30min in 4% PFA and washed 2x in PBT. The monomere solution A and powder C which were cooled for 30min on ice, mixed together (100ml sol. A + 0,9g of the powder C) and stirred in the dark at 4°C. To 10ml of the monomere solution A+C, 400μl of solution B were added. One embryo was put in one well of a special rubber mold which was then filled with this solution, the embryo was orientated, filled up with the solution and finally covered with parafilm without inclusion of air. The solution was hardened at RT for several h or ON. The block containing the embryo was then cut in sections of 3-6μm on an ultramicrotome and the sections were mounted on a slide.

7. Scoring of the embryos

Embryos were scored and photographed under a Zeiss SV11 stereomicroscope or a Zeiss Axioplan photomicroscope.

8. Additional and more detailed description of methods used in this Ph.D. work

More detailed and specific descriptions of methods (not described in this chapter) can be found in the chapter appendix. The methods listed in the part materials and methods of the work described in chapter III (appendix) are more specific and mostly relevant selectively for each work, respectively.

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	PhD study at the Zebrafish Neurogenetics Group of the Technical University Munich (hosted in the GSF Research Center, Neuherberg), Germany (Dr. Bally-Cuif)
	Techniques: RNA and DNA injections in embryos, in situ hybridisation, immunocytochemistry, molecular cloning, ET-cloning, retrograde

labelling, establishment of zebrafish transgenic lines, classical molecular and cell biological techniques

Oct.-Feb. 99/00   *Interactions of the fungus Venturia inaequalis with the apple host and population analyses of different V. inaequalis populations.*
Pre-doctoral experience at the HortResearch, Mt Albert Research Centre in Auckland, New Zealand (Dr. Plummer)
<u>Techniques:</u> genetic transformation of *Venturia* to express GFP, using *agrobacterium*, establishment of microsatellite marker analyses for molecular identification and population analysis of *Venturia*, construction of a genomic library

Nov.-Sep. 98/99   *The Coprogen Oxidase-gene in Volvox carteri – Structure and Copper-dependent Regulation.*
Masters (Diplom) at the Department of Genetics, Institute of Biochemistry, Microbiology and Genetics, Germany (Prof. Schmitt)
<u>Techniques:</u> classical molecular biology techniques, gene library screening, Southern and Northern Blotting, 3'-5'-RACE, bacterial cloning, gene and protein analyses establishment of copper-free cultures, protein detection

Apr.-Jun. 97   *Components and activity of the ATPase enzyme in Archae bacteria*
internship at the Department of Microbiology, University Regensburg, Germany (Prof. Stetter)
<u>Techniques:</u> basic molecular biology techniques, protein activity tests

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