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bHLH Transcription Factors and Neurogenesis in the Zebrafish Late Embryonic and Adult Central Nervous System

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bHLH Transcription Factors and Neurogenesis in the Late Embryonic and Adult Zebrafish Central Nervous System

Kumulative Arbeit

Birgit Adolf

Abstract

Over the past decades adult neurogenesis, neural stem cells and regeneration potential in adult organisms have got into focus of neuroscience with the aim to develop neuronal replacement therapies based on manipulation of endogenous multipotent precursors. Thus, understanding the molecular mechanisms underlying adult neurogenesis and the survival and integration of new neurons in already existing circuits is of major interest. The present work uses the zebrafish system to identify domains of continuing neurogenesis in the late embryonic, early larval and adult central nervous system and to isolate molecular factors that could be involved in neurogenesis or the maintenance of the neural progenitor state in these domains. The results are summarized below:

(I) Transcription factors of the basic helix-loop-helix (bHLH) class play a crucial role in early neurogenesis. However, whether the same factors are involved in late neurogenic processes as well, and how they act, remains unknown. Thus, we first looked for new genes encoding factors with a bHLH structure from late embryonic tissues in a degenerate PCR approach. We identified two new bHLH transcription factor-encoding genes for the *atonal* subfamily, called *atoh1.2* and a member of a distinct subfamily, called *beta3.1*, both expressed in distinct subpopulations of hindbrain precursor cells at late embryonic stages and therefore likely involved in late neurogenic processes.

In addition we identified a transcription factor-encoding gene belonging to the *Hairy/E(Spl)* family, referred to as *helt*, expressed in domains reminiscent of described proliferation zones in the midbrain of late embryonic stages as well as in midbrain proliferation zones during adulthood. *helt* is therefore a promising candidate to be involved in the control of neurogenesis at these stages.

(II) In contrast to mammals, proliferation and neurogenesis in the adult brain have been demonstrated to be more widespread, although still restricted to discrete foci, in lower vertebrates. Thus, we tested the validity of the zebrafish as a good model system to further study adult neurogenesis by characterizing self-renewing progenitors in the adult zebrafish telencephalon. We showed that these progenitors have neural stem cell-like characteristics and give rise to new neurons. We demonstrated that olfactory bulb neurogenesis in zebrafish shares many remarkable

similarities to that in rodents, giving rise to GABAergic and dopaminergic neurons, and involves an equivalent to the rostral migratory stream. We further identified a new population of stem cells giving rise to new neurons that stay in the telencephalon proper.

(III) Following this demonstration, we aimed to characterize neural stem cells in other areas of the zebrafish adult brain, and we were able to identify two proliferation zones at the junction between the midbrain and the cerebellum, referred to as tectal proliferation zone (TPZ) contributing neurons to the tectum and isthmic proliferation zone (IPZ) contributing neurons to the tegmentum. The IPZ contains a population of cells expressing the gene *her5*, which, in the embryo, actively prevents neurogenesis and keeps cells in a progenitor state. We demonstrated that *her5*-expressing cells in the adult brain display properties of stem cells, suggesting that the function of Hairy/E(Spl) factors in the specification of the neuronal progenitor state might be conserved in adulthood.

Together we validated the zebrafish as a good model system to study adult neurogenesis and identified several new zones and molecular players of late neurogenic processes. Thereby we opened the way to learn from lower vertebrates, like zebrafish to identify adult neural stem cell characteristics and to asses the general molecular mechanisms of late neurogenesis.

Zusammenfassung

In den letzten Jahrzehnten sind adulte Neurogenese, neuronale Stammzellen und das Regenerationspotential in erwachsenen Organismen in den Fokus der Neurowissenschaften gerückt, mit dem Ziel, Therapien für den Ersatz von Nervenzellen zu entwickeln, die darauf basieren, körpereigene multipotente Vorläuferzellen zu manipulieren. Es ist somit von grösstem Interesse, die molekularen Mechanismen zu verstehen, die der adulten Neurogenese, wie auch dem Überleben und der Integration neuer Nervenzellen in bereits existierende Netzwerke zugrunde liegen. In dieser Arbeit wurden im zentralen Nervensystem des Modellorganismus Zebrafisch in spätembryonalen und früher Larvenstadien, sowie in erwachsenen Tieren, Regionen fortlaufender Neurogenese identifiziert und molekulare Faktoren isoliert, die an der Neurogenese oder am Erhalt des neuronalen Vorläuferstatus in diesen Regionen beteiligt sind. Im Folgenden werden die Ergebnisse zusammengefasst:

(I) Transkriptionsfaktoren der basic helix-loop-helix Familie spielen eine bedeutende Rolle in der frühen Neurogenese. Dennoch ist nicht bekannt, ob dieselben Faktoren auch an späteren Prozessen der Neurogenese beteiligt sind, noch, wie sie wirken. Somit suchten wir zunächst mit Hilfe der Methode der degenerierten PCR nach neuen Genen, die Faktoren kodieren, welche eine bHLH Struktur aufweisen und in Geweben spätembryonaler Stadien exprimiert werden. Auf diese Weise fanden wir zwei neue Gene: *atoh1.2*, das zur *atonal*-Familie gehört und *beta3.1*, das einer entfernten Unterfamilie angehört. Beide sind in verschiedenen Populationen von Vorläuferzellen im Hinterhirn spätembryonaler Stadien exprimiert und wahrscheinlich an späten Prozessen der Neurogenese beteiligt.
Außerdem haben wir ein Mitglied der Transkriptionsfaktoren-Familie Hairy/E(Spl) identifiziert. *helt* wird in Domänen exprimiert, die an bereits beschriebene Proliferationszonen im Mittelhirn spätembryonaler Stadien, als auch in Proliferationzonen im Mittelhirn erwachsener Fische erinnern. *helt* stellt somit einen vielversprechenden Kandidaten dar, der an der Kontrolle der Neurogenese in diesen Stadien beteiligt ist.
(II) Es wurde gezeigt, dass Proliferation und Neurogenese in niedrigeren Wirbeltieren, im Gegensatz zu Säugetieren, in zusätzlichen Domänen konzentriert sind. Wir prüften den Zebrafisch auf seine Eignung als Modellorganismus für weitere

Studien der adulten Neurogenese, indem wir selbst-erneuernde Vorläuferzellen im Telencephalon erwachsener Zebrafische charakterisierten. Wie wir zeigen konnten, weisen diese Vorläuferzellen Eigenschaften neuronaler Stammzellen auf und produzieren neue Nervenzellen. Zudem konnten wir demonstrieren, dass die Neurogenese im olfaktorischen Bulbus im Zebrafisch einige bemerkenswerte Ähnlichkeiten mit der olfaktorischen Neurogenese in Nagetieren aufweist, GABAerge und dopaminerge Neuronen produziert und ein Äquivalent zum rostralen Migrationsstrom mit einschließt. Außerdem haben wir eine neue Population von Stammzellen gefunden, die Neuronen hervorbringt, die anschließend im Vorderhirn verbleiben.

(III) Darauf aufbauend beabsichtigten wir, Stammzellen in zusätzlichen Regionen im adulten Zebrafischgehirn zu charakterisieren und waren imstande, zwei Proliferationszonen zu identifizieren: die tectale Proliferationszone (TPZ), die Nervenzellen für das Tektum produziert, und die isthmische Proliferationszone (IPZ), die Nervenzellen für das Tegmentum beisteuert. Beide liegen am Übergang zwischen Mittelhirn und Cerebellum. Die IPZ enthält eine Population von Zellen, die das Gen *her5* exprimieren, das im Embryo aktiv die Neurogenese verhindert und dafür sorgt, dass die Zellen in einem Vorläufer-Status verbleiben. Wir haben gezeigt, dass *her5*-exprimierende Zellen im erwachsenen Gehirn Eigenschaften von Stammzellen besitzen, was darauf schließen lässt, dass die Funktion von Hairy/E(Spl) Faktoren in der Spezifizierung des neuronalen Vorläufer-Status im Erwachsenenalter möglicherweise erhalten bleibt.

Mit dieser Arbeit haben wir den Zebrafish als Modellorganismus für die Erforschung adulter Neurogenese bestätigt und einige neue Zonen und molekulare Faktoren identifiziert, die an späteren neuronalen Prozessen beteiligt sind. Damit wurde der Weg dafür geebnet, von niedrigeren Wirbeltieren, wie dem Zebrafisch, zu lernen, die Eigenschaften adulter neuronaler Stammzellen zu untersuchen und die grundlegenden molekularen Mechanismen später Neurogenese zu verstehen.

Abbreviations

Ara-C	Arabinosyl-Cytosin
BDNF	brain-derived neurotrophic factor
BLBP	Brain lipid-binding protein
bHLH	basic helix-loop-helix
bHLH-O	basic helix-loop-helix-Orange
Bmi-1	B lymphoma Mo-MLV insertion region 1
BMP	bone morphogenic protein
BrdU	5-bromo-2-deoxyuridine
Cb	cerebellar plate
Cce	Corpus cerebellis
Ce	Cerebellum
CNTF	Ciliary neurotrophic factor
CNS	central nervous system
DG	dentate gyrus
Dlx	distal-less homeobox protein
drc	dorsorostral cluster
dSub	dorsal subpallium
DV	dorsoventral
EGL	eminentia granularis
Eph	Ephrin receptor
E(Spl)	Enhancer of split
Fgf	fibroblast growth factor
GABA	gamma-aminobutyric acid
GFAP	glial fibrilary acidic protein
Hairy/E(Spl)	Hairy and enhancer of split
HDAC	Histone deacetylase
Helt	Hey-like transcriptional repressor
Her	Hairy-related
Hes	Hairy and enhancer of split
Hey	Hairy/E(Spl)-related with YRPW motif
Hyp	hypothalamus
hpf	hours-post-fertilization

Id	Inhibitor of differentiation
in 1-2	subpopulation of interneuron precursors
IPZ	isthmic proliferation zone
IZ	intervening zone
LCa	Lobus caudalis cerebelli
lrl	lower rhombic lip
mAbs	monoclonal Antibodies
Mash	Mouse achaete-scute homolog
Atoh	Atonal homolog
Math	Mouse atonal homolog
Mes	mesencephalon
Mgn/Megane	Mesencephalic GABAergic Neurons Enhancer of Split-related
MHB	midbrain hindbrain boundary
MLF	medial longitudinal fascicle
MyoD	myogenic differentiation protein
NeuroD	Neurogenic differentiation protein
Ngn	Neurogenin
nMLF	nucleus of medial longitudinal fascicle
NSC	neural stem cell
OB	olfactory bulb
OPC	oligodendrocyte precursor
OT	optic tract
Pa	pallium
Pax	paired box transcription factor
PCNA	Proliferative Cell Nuclear Antigen
PGZ	periventricular gray zone
Pn	Pons
PSA-NCAM	Polysialylation isoform of neural cell adhesion molecule
r1	rhombomere 1
r2	rhombomere 2
rh	rhombencephalon
RGC	retinal ganlion cells

RMS	rostral migratory stream
SGZ	Subgranular zone
Shh	Sonic hedgehog
SOT	supraoptic tract
Sox	SRY-box containing protein
Sub	subpallium
SVZ	Subventricular zone
Tec	tectum
Te	telencephalon
TeO	optic tectum
TGFα	transforming growth factor α
TH	tyrosine hydroxylase
Thal	Thalamus
TL	torus longitudinalis
TLE	Transducin-like enhancer protein
TLX	T-cell leukemia homeobox
TPOC	tract of the postoptic commissure
TPZ	tectal proliferation zone
url	upper rhombic lip
Val/vc	Valvula cerebelli
vcc	ventral caudal cluster
vrc	ventrorostral cluster
vSub	ventral subpallium
Wnt	Wingless-type
ZLI	zona limitans intrathalamica

Contents

Chapter 1

Introduction

The aim of my PhD project was to study neurogenesis in late embryonic, early larval and adult zebrafish central nervous system (CNS) and to identify the candidate transcription factors involved in controlling late neurogenesis in fish as a basis for further studies in vertebrates.

Neurogenesis in vertebrates starts with the definition of proneural cluster in the embryonic neural plate. Early neurogenesis is tightly regultated in time and space by defined cascades of transcription factors. A set of these factors is encoded by proneural genes forcing the cells to differentiate and become neuronal or glial precursor cells, while the combined expression of gene sets inhibits neurogenesis and keeps the cells in a proliferative undifferentiated state. The factors involved in these early steps of neurogenesis are very well studied and, among them, transcription factors of the basic helix-loop-helix (bHLH) class family play a crucial role.

Until half a century ago, it was believed that the massive neurogenesis taking place during embryonic neurogenesis comes to an end shortly after birth and that the ability for the generation of new nerve cells in vertebrates is definitely arrested from this timepoint on. Today it is known from studies performed in all vertebrates that neurogenesis is still taking place in restricted niches in the CNS of adult vertebrates. But there is only very little known about the regulation of neurogenesis in late embryonic stages and during adulthood. Thus understanding the mechanisms underlying the regulation of ongoing neurogenesis in later stages and in adults is of great importance for developmental neuroscience as well as for therapeutical treatments of brain injury and neurodegenerative deceases.

To address these issues, I have focussed my studies on the proliferation zones in the zebrafish CNS in late embryonic, early larvae and adult stages. I used the zebrafish as model system because it is a well established and well studied model for early neurogenesis. In addition, large numbers of embryos are obtained per cross, and embryos develop externally and are optically clear. Further the zebrafish is accessible to genetic techniques (e.g. transgenesis or mutagenesis) and

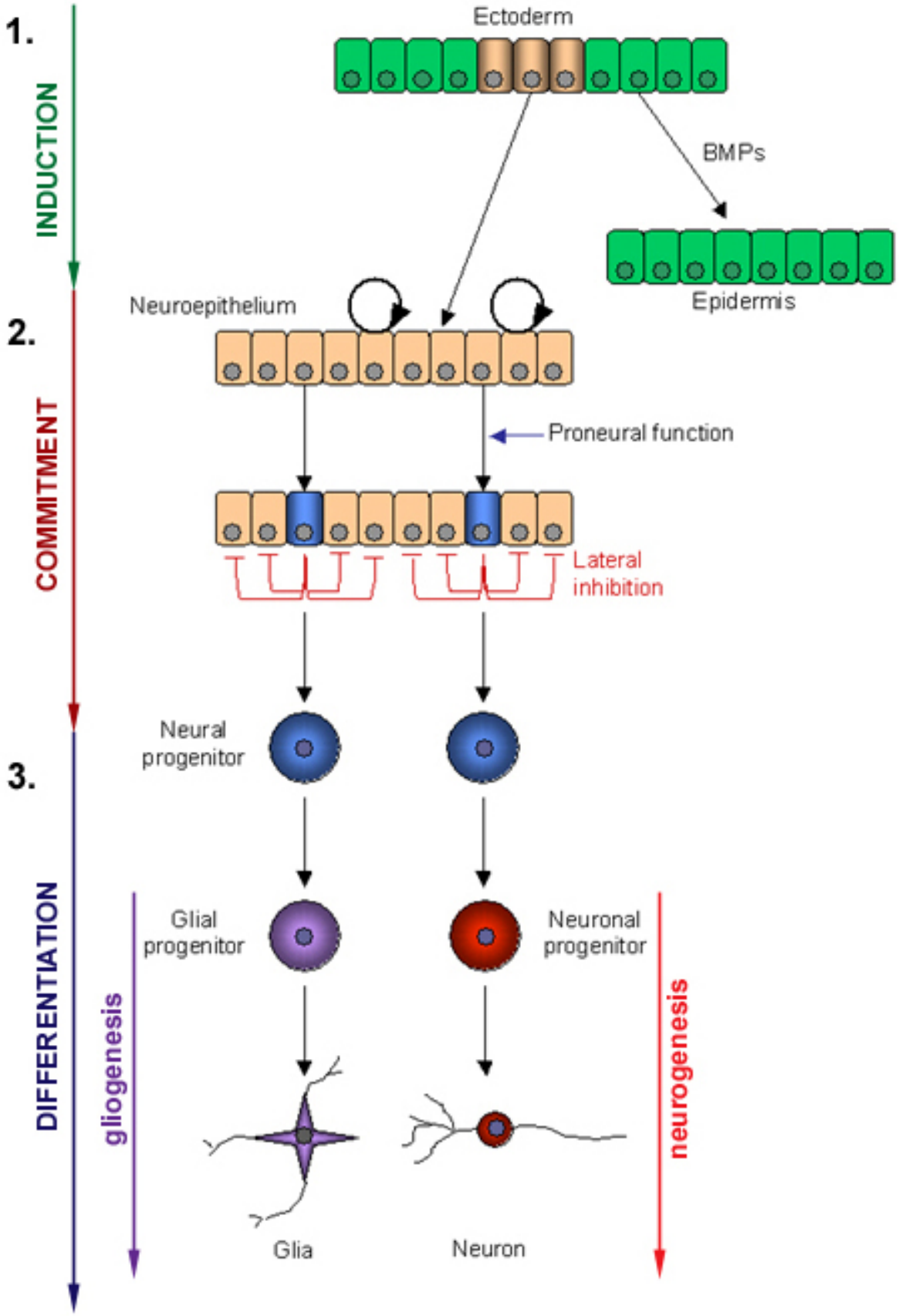
manipulations (e.g. injections of 5-bromo-2-deoxyuridine (BrdU), Morpholinos or RNA).

1.1 Early Neurogenesis

During development a variety of different cell types that compose the vertebrate nervous system have to be generated in correct numbers and at the appropriate places to build up defined networks. The adult vertebrate CNS comprises four major cell types: the neurons, the myeline-forming oligodendrocytes, the astrocytes and the ependymal cells lining ventricles. All these cell types are generated during embryonic development in a process called neurogenesis, which is a multi-step process starting with neural induction and leading to the generation of a vast array of differentiated neurons and glial cells (Bertrand et al., 2002; Kintner et al., 2002; Appel and Chitnis, 2002). Neurogenesis starts during gastrulation with the formation of the neural plate in a process called neural induction when neuroepithelial cells are generated from the ectoderm (Wilson and Edlund, 2001). In a second step, “proneural clusters” are defined within the neural plate by the combined expression of “proneural genes” (promoting neurogenesis and commiting progenitor cells) and of neurogenesis inhibitors (maintaining embryonic neural progenitors). In the last step, committed progenitors differentiate into either neuron or glia (Fig. 1).
All these steps are characterized by the interaction of specific signaling pathways and the expression of various combinations of transcription factors (Bertrand et al., 2002).

Fig. 1: Diagram of multiple steps involved in early neurogenesis in vertebrates:

1) Induction: Neuroepithelial cells are generated from the ectoderm, forming the neural plate, during gastrulation. These cells are generated by the interaction of different signaling pathways inducing the neural fate and inhibiting the epidermal fate (orange cells), whereas in other cells the epidermal fate is induced while the neural fate is inhibited (green cells). **2) Neural Commitment:** Neuroepithelial cells have self-renewing properties. Proneural clusters are defined by the antagonistic expression domains of proneural genes and neurogenesis inhibitors. Progenitors are further selected within the clusters by a process called lateral inhibition. Selected neural progenitors get further committed to a neural lineage and have a limited mitotic potential. **3) Differentiation:** Committed progenitors can give rise to neurons and/or glial cells, generally neurons are produced first and glial cells are generated later.
After Kintner, 2002 and Bertrand et al., 2002


1.
INDUCTION
2.
COMMITMENT
3.
DIFFERENTIATION
Ectoderm
BMPs
Epidermis
Neuroepithelium
Proneural function
Lateral inhibition
Neural progenitor
Glial progenitor
Neuronal progenitor
gliogenesis
neurogenesis
Glia
Neuron

1.1.1. Neural Induction

Neural induction is the first step in neurogenesis and starts during gastrulation by the definition of neuroepithelial cells in the ectoderm leading to the formation of the neural plate. These cells are defined by the interaction of three signaling pathways: Fgf promotes a neural versus epidermal fate, and Wnt and Bmps act antagonistically. It was shown that Fgf signaling plays a crucial role in promoting a neural fate in more than one way. First of all it is involved in the initiation of neural induction already before gastrulation (Streit et al., 2000), second it blocks transcription of *Bmp* and therefore inhibits an epidermal fate (Streit and Stern, 1999) and third it activates a signaling cascade necessary for the progression towards a neural fate (Wilson and Rubenstein, 2000) (Fig. 2A).

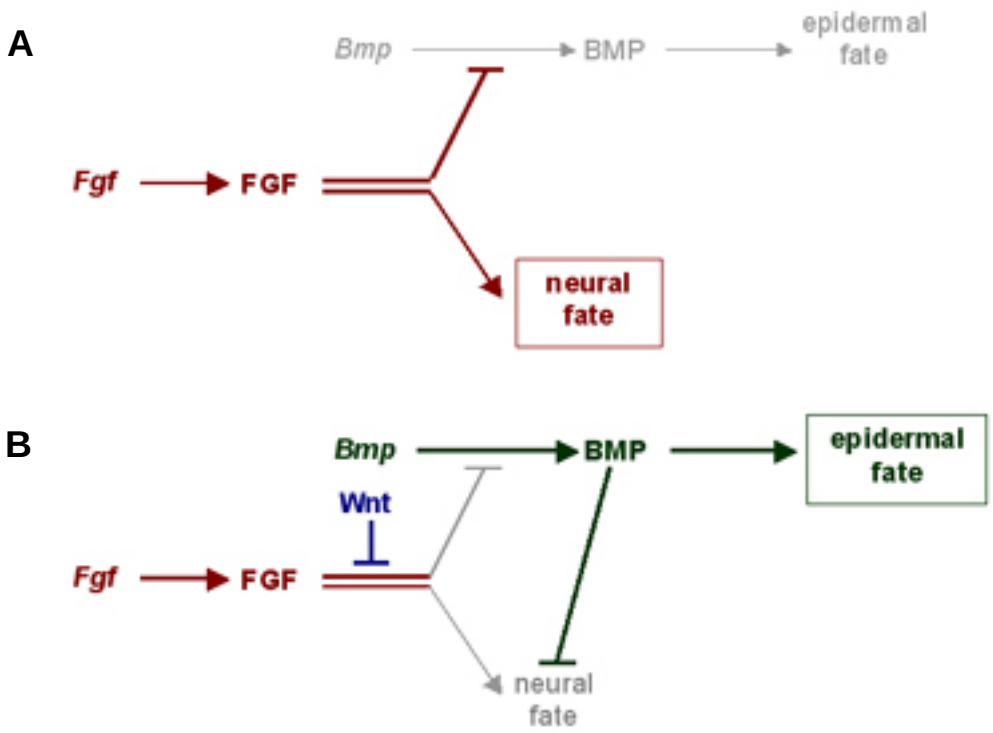


Fig. 2 Interaction of signaling pathways during neural induction in the embryo: (After Lumsden and Krumlauf, 2001)

Neural induction is performed by interaction of Wnt, Fgf and Bmp signaling to promote a neural versus epidermal fate. **(A)** Neural fate is adopted in cells in the medial epiblast by lack of Wnt signaling permitting repression of *Bmp* and the activation of a pathway necessary for the progression to a neural fate by Fgf signaling. **(B)** The high level of Wnt signaling in cells of the lateral epiblast inhibits Fgf signaling and therefore permits Bmp signaling directing the cells to adopt an epidermal fate.

Bmps on the other hand have been shown to promote an epidermal and inhibit a neural fate, but blocking their function is not sufficient to induce a neural fate (Streit and Stern, 1999) (Fig. 2B). Fgf signaling is negatively modulated by Wnt signaling, which thereby favors Bmp action towards an epidermal fate (Wilson et al., 2001) (Fig. 2B). Wnt gradient is highest in cells of the lateral epiblast, which therefore are committed to an epidermal fate. In contrast, cells in the medial epiblast lack Wnt signaling and in response to Fgf signaling are committed to a neural fate. At the end of gastrulation neuroepithelial cells are committed towards a neural lineage and do no longer respond to signals inducing alternative fates (Wilson and Edlund, 2001).

1.1.2 Definition of proneural clusters in vertebrates

The cells of the neural plate are undifferentiated neural progenitors that self-renew by symmetric divisions (Fujita, 2003; Alvarez-Buylla et al., 2003). Next, following a pattern strictly regulated in time and space, so-called “proneural clusters”, i.e. clusters of progenitors that will become committed towards differentiation, appear along the anteroposterior and dorsoventral axes at specific and identical sites in all vertebrates (Bally-Cuif et al., 1993, Easter et al., 1985; Wilson and Easter, 1992). The location and extent of the proneural clusters are defined by the expression of positive and negative regulators establishing competent zones. Among the positive transcription factors defining proneural clusters are zinc-finger proteins, winged helix and proneural bHLH factors, like Achaete-Scute-like, Atonal and Neurogenin proteins. Intervening with proneural clusters are areas that show delayed differentiation, like the “intervening zone” (IZ) at the midbrain hindbrain boundary (MHB), which stays proliferating (Geling et al., 2003, 2004; Ninkovic et al., 2005). In these areas, negative regulators like homeodomain proteins, zinc-finger proteins and bHLH factors of the Hairy/Enhancer of Split family (Hairy/E(Spl)) play an important role (Bally-Cuif and Hammerschmidt, 2003).

It is known from studies in the developing telencephalon of mouse embryos that three proneural bHLH factors are expressed in different domains of the ventricular zone giving rise to different types of neurons (Guillemot and Joyner, 1993; Lo et al., 1991; Sommer et al., 1996): Ngn1 and Ngn2 are expressed in the dorsal telencephalon which gives rise to glutamatergic neurons whereas Mash1 is predominantly expressed in the ventral telencephalon which gives rise to GABAergic and cholinergic

neurons. Loss-of-function studies with these factors have revealed a loss of GABAergic neurons in the ventral telencephalon in Mash1 knockout mice (Casarosa et al., 1999) whereas loss of Ngn2 results in an upregulation of Mash1 in the dorsal telencephalon thus some progentiors differentiate into GABAergic neurons in this region (Fode et al., 2000). Thus different proneural genes are involved in the generation of different types of neurons.

1.1.3 Stereoptypic spatio-temporal development of early neurogenesis in vertebrate embryos

This strictly controlled definition of proneural clusters is followed by the appearance of neuronal clusters at specific and identical sites in embryos of different vertebrate species, establishing a conserved scaffold (Fig. 3). The ventral caudal cluster (vcc) is one of the earliest neuronal clusters observed in all vertebrates in the anterior midbrain and basal diencephalon. Neurons of the vcc are the first to send axons caudally in an axonal tract called medial longitudinal fascicle (MLF). Differentiation of the vcc is followed by that of two telencephalic clusters, the ventrorostral cluster (vrc) and the dorsorostral cluster (drc), sending out axons connecting the different nuclei in transversal and longitudinal tracts (Kimmel, 1993 and Ross et al., 1992). These neuronal clusters are separated by areas of delayed differentiation, like the IZ at the MHB (Geling et al., 2003, 2004; Ninkovic et al., 2005).

During vertebrate embryonic neurogenesis, this scaffold is established temporally and spatially in an identical way in all vertebrate organisms (Vaage, 1969; Bally-Cuif et al., 1993; Easter et al., 1993, 1994; Wilson et al., 1990; Mastick and Easter, 1996). In zebrafish early neurogenesis occurs in two successive waves (Kimmel, 1993): Primary neurogenesis that starts at late gastrulation and continues during embryogenesis to produce early neurons, the Rohon-Beard sensory neurons and the primary spinal motorneurons (Kimmel and Westerfield, 1990) that build, after the formation of axons, the first functional embryonic and early larval neuronal scaffold, allowing the fish larva to move and feed (Easter et al., 1994). Secondary neurogenesis starts at about 48 hours-post-fertilization (hpf) at all rostro-caudal levels and at post-embryonic stages taking over the functions of the primary system by a refined and increasingly complex network (Müller and Wullimann, 2003).

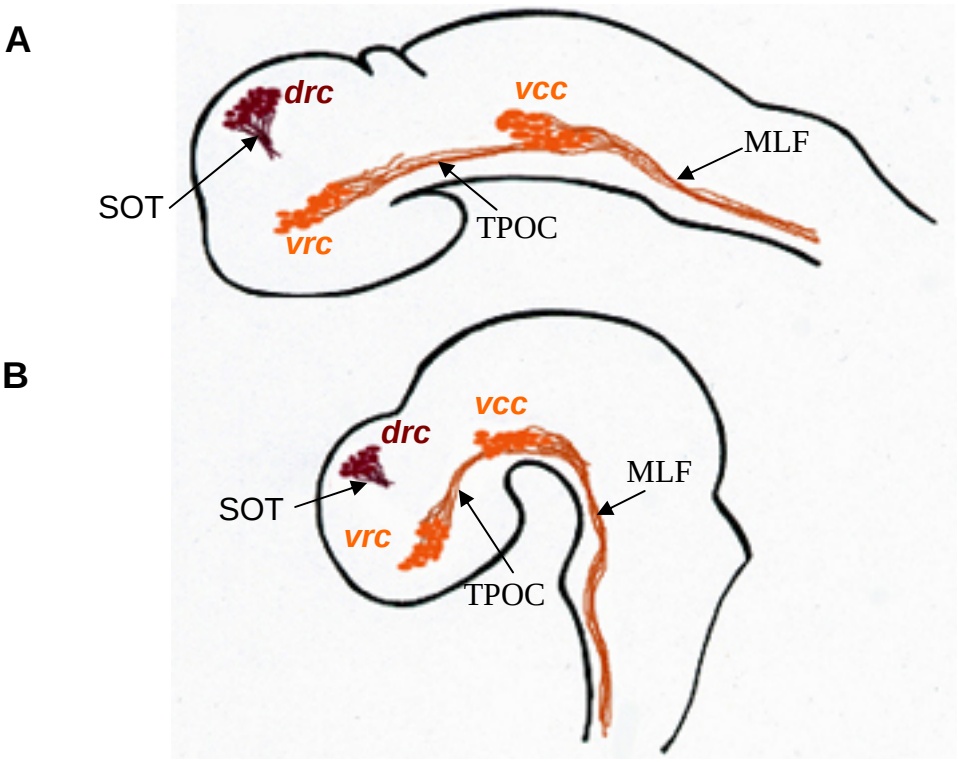


Fig. 3: Schematic drawing of neural clusters and axon tracts in vertebrates at embryonic stages:

Lateral views of the embryonic brain anterior to the left, dorsal up of a zebrafish embryo at about 20 hpf **(A)** and a mouse embryo at an equivalent stage **(B)** in comparison: **Neural clusters**: drc: dorsorostral cluster; vcc: ventrocaudal cluster; vrc: ventrorostral cluster. **Axonal tracts**: MLF: medial longitudinal commissure; SOT: supraoptic tract; TPOC: tract of the postoptic commissure.
(After Ross et al 1992)

1.1.4 Lateral inhibition mechanisms and selection of neural progenitors within the proneural clusters, commitment towards differentiation

Within proneural clusters, expression of proneural genes gets rapidly restricted to a small number of cells that become committed neural progenitors and further enter a neural-differentiation pathway (Artavanis-Tsakonas et al., 1999; Chitnis and Kintner, 1996; Lewis, 1998). The selection of neural progenitors is mediated by a process called “lateral inhibition” that occurs through the activation of the Notch signaling pathway. In the beginning, all cells of a proneural cluster express proneural genes, Notch and the Notch ligands at a roughly similar level but with stochastic variations. Direct transcriptional activation of the Notch ligands Delta/Serrate and Jagged by proneural genes (Casarosa et al., 1999; Fode et al., 1998; Ma et al., 1998, 1999;

Chitnis and Kintner, 1996) in the future selected progenitor cells activates Notch signaling cascade in the neighbouring cells by Notch-Delta interactions. Notch activation results in the upregulation of repressor-type bHLH transcription factors of the Hairy/E(Spl) family, like Hes1 and Hes5 in the mouse (Jarriault et al., 1995; Ohtsuka et al., 1999; Kageyama et al., 2005), which in turn down-regulate expression of the proneural genes, like *Mash1*, *Math3* and *Ngn2*, thereby maintaining the cells in an undifferentiated state (Gaiano et al., 2002). In the future selected progenitors, Delta activation further leads to an upregulation of expression of Delta itself and of proneural genes (Culi and Modolell, 1998; Kintner C., 2002), thereby amplifying the selection process. Upon high level of expression of proneural factors, a cascade of helix-loop-helix factors like Coe2 or Hes6 becomes activated, which in turn turns on a later set of proneural genes like NeuroD (Dubois et al., 1998; Bae et al., 2000; Koyano-Nakagawa et al., 2000). These cells are then irreversibly committed to neuronal differentiation, cell cycle arrest and repression of gliogenesis (reviewed in Kageyama et a., 2005; Bertrand et al., 2002).

1.1.5 Neuronal differentiation

Differentiation starts after a progenitor cell becomes irreversibly committed by expression of proneural genes at high levels. But proneural genes are only transiently expressed and become downregulated before the progenitor cell leaves the proliferation zone and begins to differentiate (Ma et al., 1996; Ben Arie et al., 1996; Gradwohl et al., 1996), thus other genes must be involved in promoting terminal differentiation (Kintner C., 2002). High level of proneural gene expression results in cells cycle exit (Edlund and Jessell,1999; Ohnuma et al., 2001), inhibition of glial differentiation (Fig. 4) and activation of a cascade of neuronal differentiation genes, like members of the *NeuroD* family (Kageyama and Nakanishi,1997; Lee, 1997). Gain-of-function experiments with Ngn1 and Ngn2 in mouse have shown that these two proneural genes are required for induction of Math3 and NeuroD (Fode et al., 1998; Ma et al., 1999) thus neuronal differentiation genes are downstream targets of proneural bHLH proteins. Members of the NeuroD family as well as Math2 and Math3 are no longer expressed in progenitors but are expressed in cells that are (or will become) neurons starting in immature neurons and are maintained during neuronal differentiation (Lee et al., 2000). bHLH proteins NeuroD and NeuroD2 are

required for differentiation and survival of granule cells in cerebellum and hippocampus (Liu et al., 2000; Miyata et al., 1999, Schwab et al., 2000).

1.1.6 Neuronal and glial differentiation are controlled in a temporal manner

During CNS development multipotent progenitor cells that generate both neurons and glia are widespread components in the neuroepithelium. The timing of appearance of neurons and glia is highly reproducible and follows a precise schedule where neurons are generated primarily in the embryonic period, while most of the glia are generated after birth (Jacobson, 1991). There are two controversial models discussed by which the sequential generation of neurons and glia could be achieved.
In the model of switch (Fig. 4A) embryonic progenitors produce different types of progeny in distinct phases (first neuroblasts and then glioblasts), thus the timing underlies changes in the embryonic progenitor. The change from neurons to glial cells is also accompanied by an alteration in the division pattern from an asymmetric mode to a (at least initially) symmetric mode (Qian et al., 1998, 2000). In an alternative model (model of segregation), multipotent embryonic progenitors generate at an early stage restricted neuronal and glial progenitor cells. There, the timing of appearance of differentiated progeny would be determined by regulating the already restricted progenitor cells (Luskin et al., 1993; McCarthy et al.,2001) (Fig. 4B).
But although the mechanisms involved in neuronal differentiation is quite well studied the mechanisms of gliogenesis are not well understood today. The subsequential appearance of neurons and glia can be due to environmental changes, cell-intrinsic changes or a combination of both that could alter the neurogenic and gliogenic potential of the embryonic progenitor cells or regulate the already restricted progenitor cells. During embryonic neurogenesis, neural progenitor cells are maintained by expression of bHLH repressor genes of the Hairy/E(Spl) family, like *Hes1* and *Hes5*, inhibiting neuronal differentiation (Kageyama et al., 2005; Ishibashi et al., 1994; Ohtsuka et al., 2001) by repressing bHLH activator genes (Hatakeyama et al., 2004). This process, in addition, might permit gliogenesis by maintaining neuroepithelial precursors for later differentiation steps, usually gliogenic. At the same time proneural genes have been shown to inhibit gliogenesis during neurogenesis suggesting an important role for proneural genes in the switch between neurogenesis and gliogenesis (Vetter, 2001) (Fig. 4A).

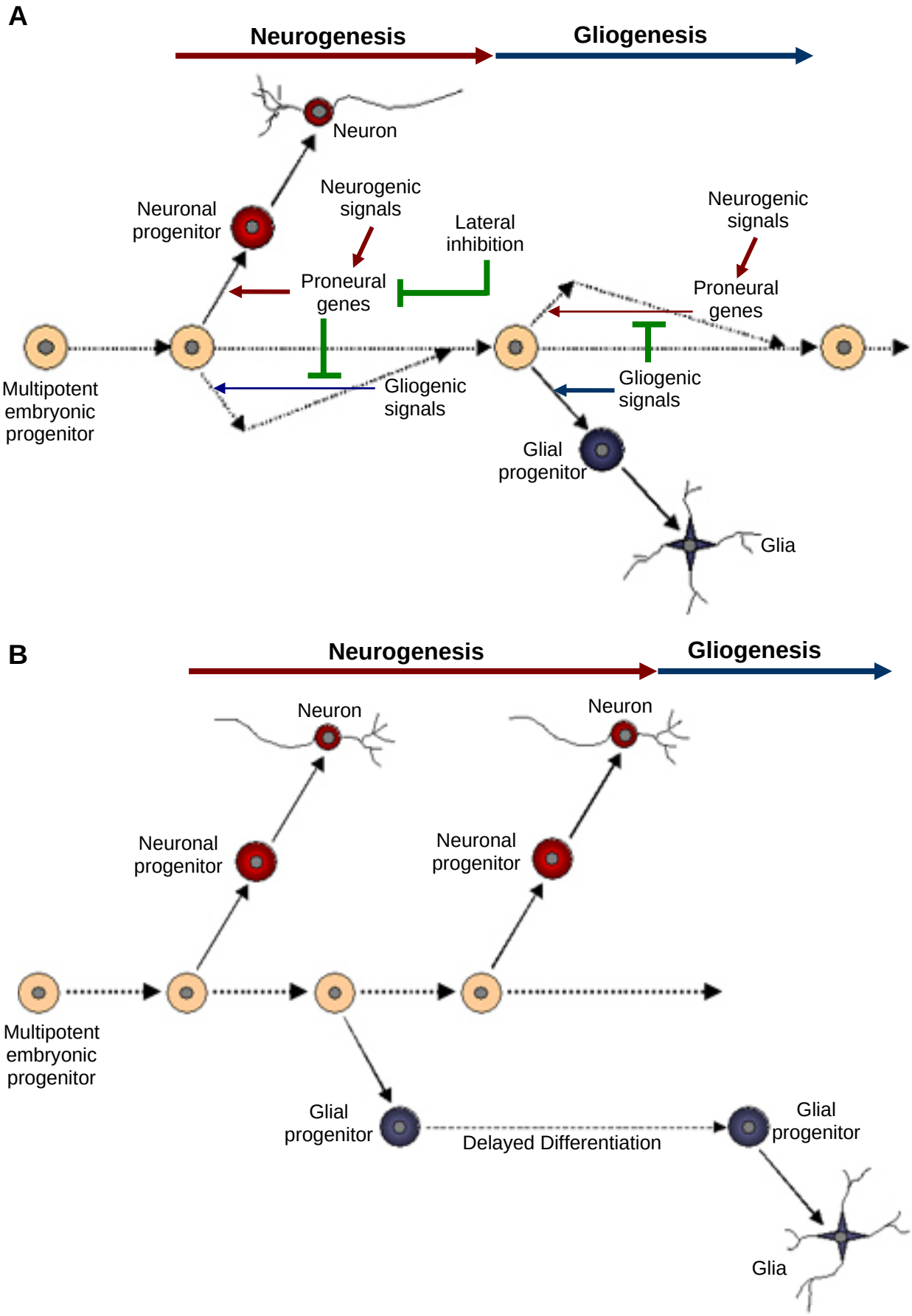

A
Neurogenesis
Gliogenesis
Neuron
Neuronal progenitor
Neurogenic signals
Lateral inhibition
Proneural genes
Neurogenic signals
Proneural genes
Multipotent embryonic progenitor
Gliogenic signals
Gliogenic signals
Glial progenitor
Glia
B
Neurogenesis
Gliogenesis
Neuron
Neuron
Neuronal progenitor
Neuronal progenitor
Multipotent embryonic progenitor
Glial progenitor
Delayed Differentiation
Glial progenitor
Glia

Fig. 4: Neurogenesis and Gliogenesis during embryonic development :

During embryogenesis neurogenesis and gliogenesis are controlled in a temporal manner with generation of neurons first and production of glia later. There are two controversial models: **(A)** The model of switch and a possible way of its regulation: A multipotent embryonic progenitor cell generates neuronal progenitor cells first and glial progenitor cells later. In the first period of differentiation neurogenic factors induce proneural gene expression that in turn activate a neuronal-differentiation pathway downstream while gliogenesis is inhibited. Gliogenic factors activate glial differentiation in a second phase in which neurogenesis is inhibited and glial cells are generated.
(B) The model of segregation: Neuronal and glial progenitor cells are generated early from a multipotent embryonic progenitor cell. Properties of the already restricted progenitor cells and the environment determine the time of appearance of neurons and glia.

Gliogenesis is also activated by gliogenic signals like BMPs or FGF, like FGF2, which might promote the switch from neurogenesis to gliogenesis (Johe et al., 1996; Qian et al., 1997, 2000) and promote glial differentiation pathways (Nakashima et al., 1999, 2001). Further, Notch-Delta signaling has been suggested to play a role in glial specification as progenitors expressing an activated form of Notch promotes the formation of radial glia embryonically (Gaiano et al., 2000) and Notch signaling promotes the formation of Müller glia and Schwann cells as well (Furukawa et al., 2000; Morrison et al., 2000). It is as well possible that differentiation along the astrocyte lineage may be a default state of brain development that occurs in the absence of neuronal and oligodendroglial differentiation signals. Thus, at later stages of development, when neurogenesis is largely over, cells would differentiate along the astrocyte lineage (Zhou and Anderson, 2002; Nieto et al., 2001).

1.2 Neural stem cells

Until the early 1990s the central dogma of neurobiology was that vertebrates are born with a certain number of neurons and that the generation of new nerve cells is definitely arrested after birth. During embryogenesis, neurogenesis takes place massively, continuing for a short time after birth, but after that the nerve paths would be something fixed, ended, and immutable (Ramon y Cajal, 1914). Studies of the last decades however have overturned this long-held dogma and shown that neurogenesis persists throughout adulthood in the vertebrate CNS, although restricted to niches.

The first hint on ongoing neurogenesis in adult mammalian brains came from studies using [^{3}H]-thymidine labeling (Altman and Das, 1965), demonstrating that cells in the dentate gyrus (DG) of the hippocampus are able to incorporate radioactive thymidine, integrating in the DNA of dividing cells during S-phase. New methods for labeling dividing cells, like retroviruses and 5-bromo-2-deoxyuridine (BrdU, a marker of the S-phase of the cell cycle that can be detected with an antibody), came up later and facilitated studies of neurogenesis in the adult CNS (Corotto et al., 1993; Luskin, 1993; Seki and Arai, 1993). The development of the neurospheres technique (Reynolds and Weiss, 1992) and the identification of epigenetic factors stimulating neurogenesis opened a broad field for neural stem cell research and manipulation.

1.2.1 Definition of a neural stem cell

Neural stem cells (NSC) by definition are capable to self-renew and are multipotent within the neural lineage, i.e. can give rise, at least in vitro, to neurons and glia (Gage, 2000). Stem cells show a variety of repertoires and their potentialities become more and more restricted to the generation of a specific cell type (Fig. 5): (I) a totipotent stem cell is capable of producing a complete organism, when implanted into the uterus of a living animal but does not self-renew. (II) pluripotent stem cells, like embryonic stem cells, can give rise to every cells type of an organism, except trophoblasts of the placenta but are unable to give rise to the form and structure of an organism (Thomson et al., 1998).

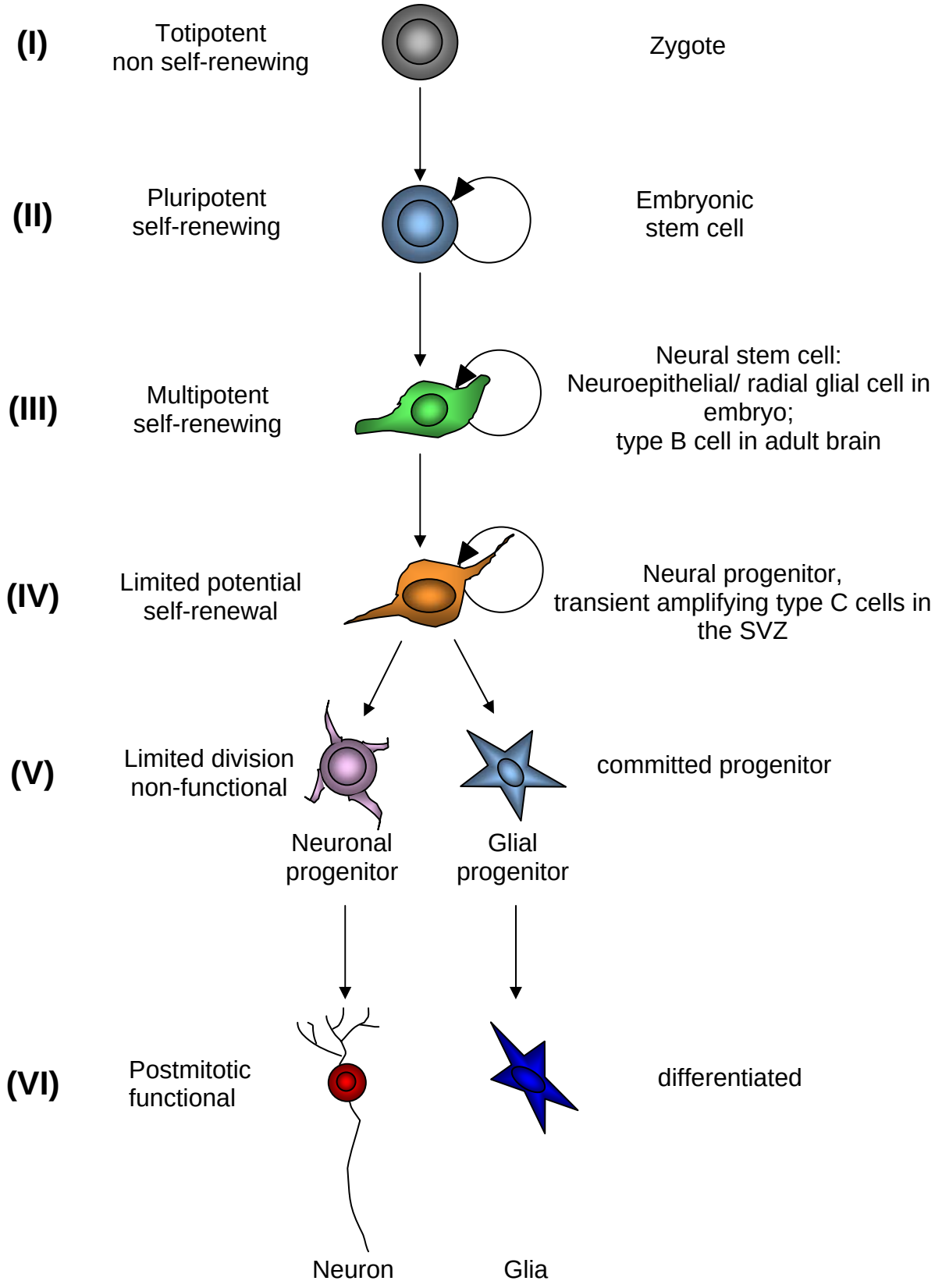

(I)
Totipotent
non self-renewing
Zygote
(II)
Pluripotent
self-renewing
Embryonic
stem cell
(III)
Multipotent
self-renewing
Neural stem cell:
Neuroepithelial/ radial glial cell in embryo;
type B cell in adult brain
(IV)
Limited potential
self-renewal
Neural progenitor,
transient amplifying type C cells in the SVZ
(V)
Limited division
non-functional
committed progenitor
Neuronal
progenitor
Glial
progenitor
(VI)
Postmitotic
functional
differentiated
Neuron
Glia

Fig. 5: Model for stem cell potentials and hierachical specification of precursors during development and adult neurogenesis:

Schematic illustration of a cell lineage diagram and hierarchy of classes of mammalian stem cells that can give rise to neurons starting with the multipotent neural stem cell progressing to progenitors committed to generate only one single cell type. Restriction of fate and the cell types are listed.
After Gage, 2000.

(III) The category of multipotent stem cells contains most types of stem cells. Their potentialities are defined by the organ from which they are derived: these cells are capable of giving rise to every cell type of that organ but only cells of that organ. Regarding the CNS, neuroepithelial cells, radial glial cells (e.g. Frederiksen and McKay, 1988; Malatesta et al., 2000, 2003) as well as type B astrocytes (adult NSCs) (e.g. Doetsch et al., 1997, 1999, 2003) belong to the category of multipotent stem cells and can give rise to undifferentiated precursors, neurons, oligodendrocytes and astrocytes. This potential gets restricted around the onset of neurogenesis and during development they do not generate these cell types directly but rather via precursors that are capable of generating only one single cell type. This restriction might occur stepwise via intermediate precursors that are only oligo- or bipotenial (IV) (e.g. Novitch et al., 2001; Gregori et al., 2002) and that amplify to generate a population of precursors restricted to a sublineage (V) (Weissman et al., 2001). These cells are often referred to as progenitors that still are not postmitotic and proliferate to generate further fate-restricted progenitors. The last progenitors prior to differentiation to a neuron or glia (VI) are sometimes referred to as “-blasts” (Fig. 5).

1.2.2 Adult neural stem cells in vertebrate evolution

Since the discovery of continuing production of new neurons beyond embryonic and early postembryonic stages, adult neurogenesis has been demonstrated in all major vertebrate groups like mammals (e. g. Altman, 1962, 1963, 1969, Altman and Das, 1965, Kaplan and Hinds, 1977; Bayer et al., 1982; Corotto et al., 1993; Lois and Alvarez-Buylla, 1993, 1994; Song et al., 2002), including primates (Gould et al., 1997, 1998, 1999; Eriksson et al., 1998), birds (Goldman and Nottebohm,1983; Burd and Nottebohm, 1985; Paton et al., 1985), reptiles (López-García et al., 1988, García-Verdugo et al., 1989; Pérez-Sánchez et al., 1989; Pérez-Canellas and

Garciá-Verdugo,1996), amphibians (Bernocchi et al., 1990; Chetverukhin and Polenov 1993; Polenov and Chetverukhin, 1993) and bony fish (Johns, 1977; Meyer, 1978; Raymond and Easter, 1983; Zupanc and Zupanc, 1992; Zupanc and Horschke, 1995; Zupanc et al., 1996).

But, although ongoing neurogenesis in adults was found in all vertebrate species there are differences between lower and higher vertebrates. In higher vertebrates like in mammals, adult neurogenesis is restricted to only two regions (Fig. 6A): the subventricular zone (SVZ) in the lateral wall of the lateral ventricle, generating newborn neurons that migrate towards the olfactory bulb along the rostral migratory stream (RMS) (Altman, 1969; Luskin, 1993; Lois and Alvarez-Byulla, 1994), and the subgranular zone (SGZ) of dentate gyrus (DG) in the hippocampus (Altman and Das, 1965; Gould et al., 1998, Eriksson et al., 1998), generating new granule cells that migrate radially and integrate in the CA3 region of the hippocampus (Stanfield and Trice, 1988; Markakis and Gage, 1999). In contrast, in lower vertebrates, like teleosts (Fig. 6B), proliferation zones were found in dozens of regions in the adult brain (Richter and Kranz, 1970; Kranz and Richter, 1970; Zupanc and Horschke, 1995; Ekström et al., 2001).

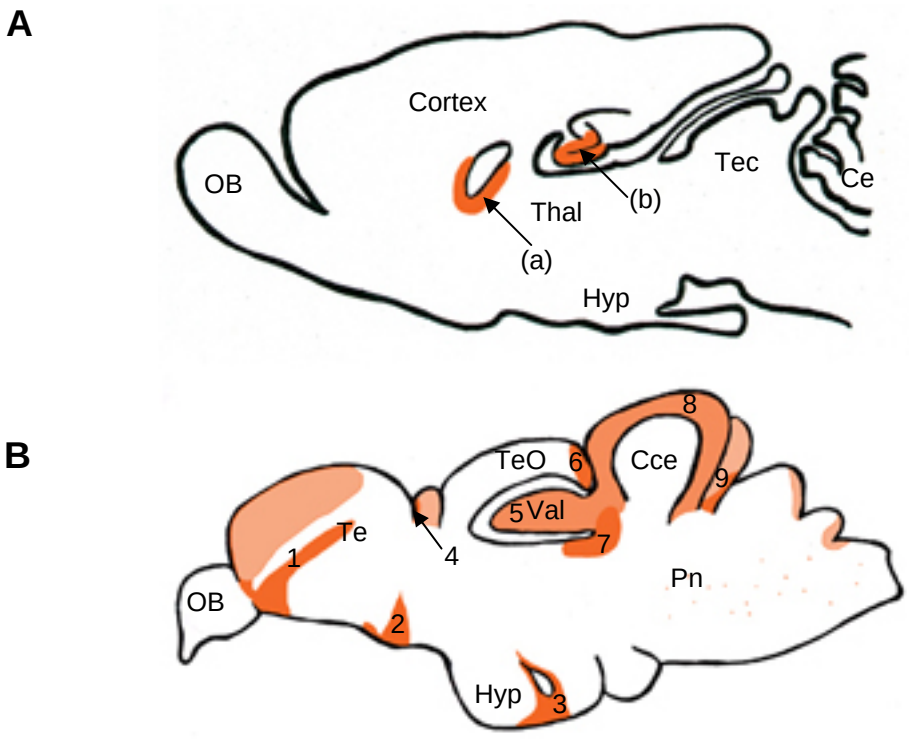


Fig. 6: Schematic drawing of the proliferation zones in and adult mouse and zebrafish brain:

All pictures are sagittal views dorsal up, anterior to the left:
(A) Proliferation zones in the adult mouse brain: (a) subventricular zone (SVZ) of the lateral ventricle; (b) subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. **(B)** Proliferation zones in the adult zebrafish brain: (1) ventricular proliferation zone in the telencephalon, (2) optic tracts, (3) ventricular zone of the hypothalamus, (4) anterior thalamus, (5) molecular layer of the valvula cerebelli, (6) tectal proliferation zone (TPZ), (7) isthmic proliferation zone (IPZ), (8) eminentia granularis (EGL), (9) molecular layer of the lobus caudalis cerebelli.
Abbreviations: Cce: Corpus cerebellis; Ce: Cerebellum; Hyp: Hypothalamus; Pn: Pons; OB: olfactory bulb; Te: telencephalon; Tec: Tectum; TeO: optic tectum; Thal: thalamus; Val: Valvula cerebelli

1.2.3 Description of adult neurogenesis in rodents

As already mentioned above, neurogenesis in adult rodents is restricted to two regions in the brain, namely the SVZ in lateral ventricle and the SGZ in the DG of the hippocampus (Fig. 6A). New neurons are generated in these two regions and migrate to their final position where they get integrated into the already existing neuronal networks.

1.2.3.1. SVZ progenitor cells give rise to OB interneurons

Previous studies in the brain of adult rodents and other mammalian model systems have shown that new neurons in the OB are generated in the SVZ, a layer of dividing cells, in the wall of the lateral ventricle in the forebrain (Doetsch et al., 1997; Luskin, 1993). The SVZ mainly consists of four cell types: neuroblasts or type A cells, stem cell astrocytes or type B cells, immature precursors or type C cells and ependymal cells located between the SVZ and the lateral ventricle as a single layer of cells (Doetsch et al., 1997) (Fig. 7). There have been two controversial theories regarding the cellular origin of NSCs. The first hypothesis claimed that NSCs of the adult SVZ are ependymal cells lining the lateral wall of the ventricle (Johansson et al., 1999). In the second hypothesis NSCs were identified as astrocyte-like cells expressing glial fibrillary acidic protein (GFAP) and nestin (Chiasson et al., 1999; Doetsch et al. 1999; Laywell et al. 2000). This dilemma is not yet solved, but in the following I will focus on the second hypothesis because most arguments point on astroglial-like cells representing NSCs in the adult brain. Strong evidence came from elimination studies

using the anti-mitotic drug Ara-C, which kills fast dividing cells and was observed to spare relatively quiescent SVZ astrocytes, from which the fast dividing pool and newborn neurons were later reconstructed (Doetsch et al., 1999). Tracing experiments, using a retrovirus specifically labeling dividing astrocytes in adults (Holland and Varmus, 1998) further showed that, after some time, neuroblasts were labeled. They migrated to the olfactory bulb, and differentiated there into granule and periglomerular cells (Doetsch et al 1999).

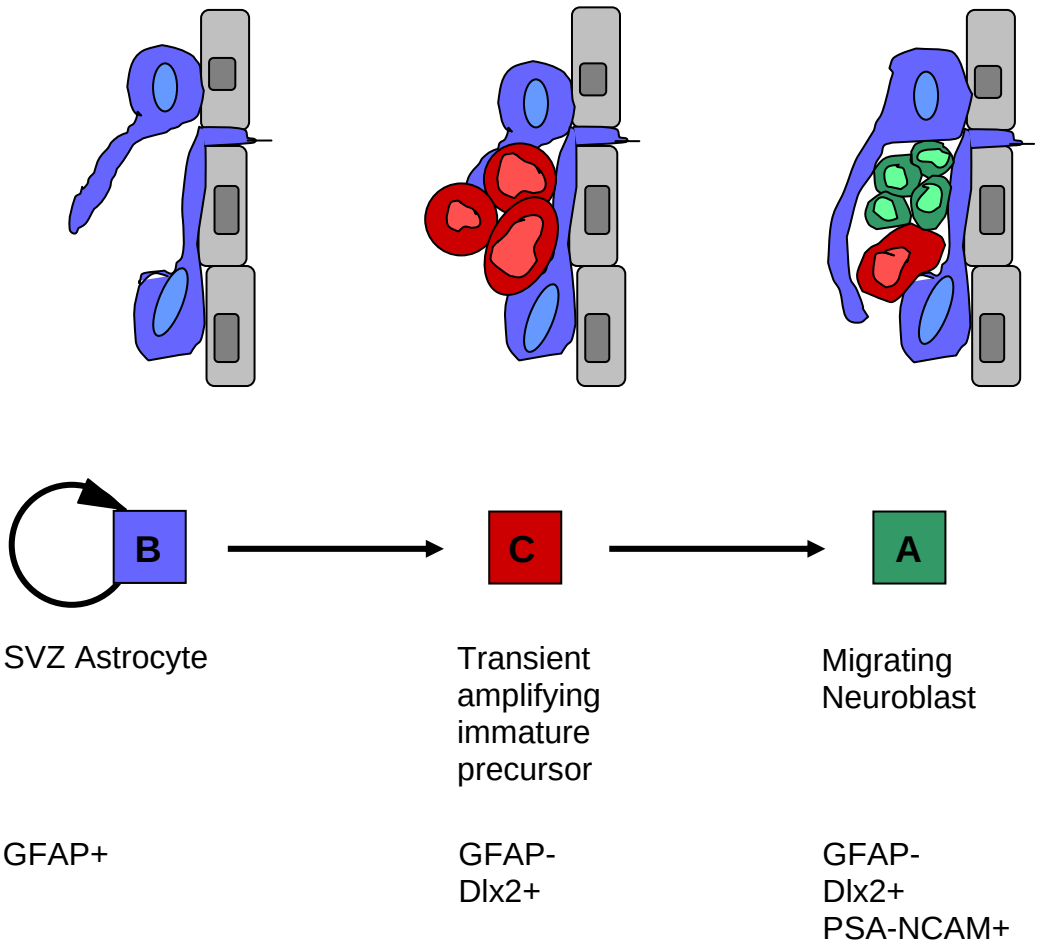


Fig. 7: Model of neurogenesis in the SVZ of an adult rodent brain

Primary precursors in the adult SVZ are the SVZ astrocytes (type B cells, blue) that divide and give rise to immature precursors (type C cells, red) which form clusters and generate migrating neuroblasts (type A cells, green). The lateral wall of the SVZ constitutes of non-dividing ependymal cells (grey). Diagnostic markers indicated at the bottom.
After Doetsch et al., 1999.

Some of the SVZ astrocytes (type B cells), acting as stem cells, contact the lumen of the ventricle and have a single small cilium characteristic of neuroepithelial stem cells in the embryo (Fig. 7) (Doetsch et al., 1999). Dividing SVZ astrocytes give rise to transient amplifying immature precursors (type C cells), which form clusters and in turn divide rapidly, generating neuroblasts (type A cells). Neuroblasts divide as they migrate tangentially in chains along glial tunnels, formed by the processes of type B-cells forming the RMS reaching into the OB where the cells finally differentiate into granule and periglomerular neurons (Doetsch and Scharff, 2001). Although mostly neurons are generated in the adult SVZ, it is also a site for the generation of oligodendrocytes (Nait-Oumesmar et al., 1999).

1.2.3.2 Progenitor cells in the SGZ of the DG in the hippocampus give rise to hippocampal granule cells

Adult hippocampal neurogenesis has been demonstrated in birds (Barnea and Nottebohm, 1994), reptiles (Lopez-Garcia et al., 1988), rodents (Altman and Das, 1965; Kaplan and Bell, 1983, 1984; Seki and Arai, 1993) as well as in primates (Gould et al., 1997, 2001; Eriksson et al., 1998). New neurons and glia in the adult mammalian hippocampus are generated in the SGZ, a germinal layer between the DG and the hilus, and differentiate into hippocampal granule cells (Markakis and Gage, 1999). But compared to the massive migration of newborn neuroblasts observed in the RMS, migration in the hippocampus covers only short distances. The SGZ consists of four cell types: astrocytes (type B cells), dividing immature D cells, newly generated neurons and endothelial cells (Fig. 8). The SGZ astrocytes extend basal processes contacting the basal lamina and an apical process into the granule cell layer. They function as stem cells therefore are self-renewing and give rise to dividing immature type D cells. Type D cells may funtion as transient amplifying precursor, however they do not have a clear homologous cell type in the SVZ, divide less frequently and are more differentiated than transient amplifying cells in the SVZ (Doetsch and Scharff, 2001). These cells generate new granule cells projecting an axon to the CA3 region of the hippocampus (Stanfield and Trice, 1988; Markakis and Gage, 1999).

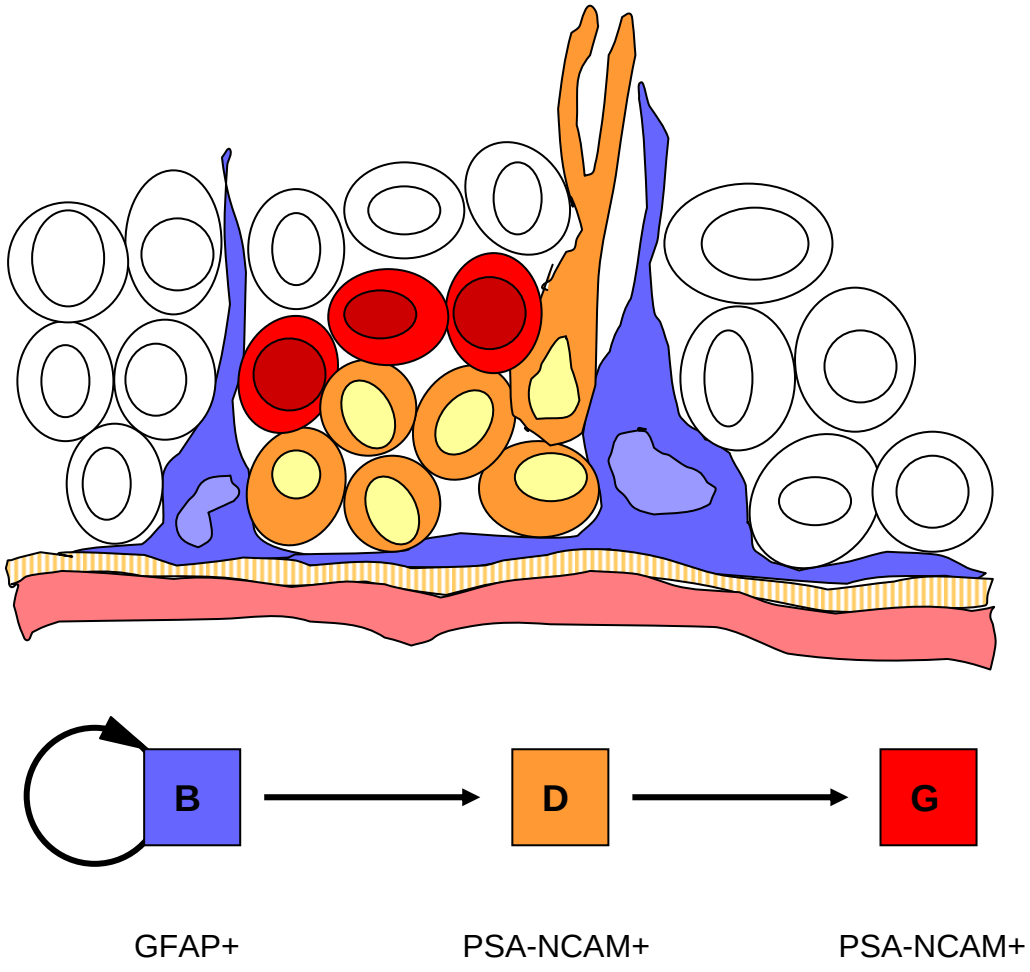


Fig. 8: Model of neurogenesis in the SGZ of an adult rodent brain

SGZ astrocytes (type B cells, blue) self-renew and give rise to clusters of immature dividing progenitors (type D cells, orange), adjacent to the SGZ astrocytes, which generate new granule cells (type G cells, red). These granule cell integrate in turn into the DG granule cell layer.
After Doetsch et al, 2001.

1.2.4 Description of adult neurogenesis in teleost fish.

It is known that lower, non-mammalian vertebrates like teleost fish, harbor an enormous regenerative potential after injury that is consistent with the generation of a larger number of new cells in a larger number of regions in the CNS of adults. Investigations in different teleost organisms prior to our studies have shown that proliferation takes place in dozens of brain regions mainly in ventricular, paraventricular and cisternal systems (Alonso et al., 1989; Huang and Sato, 1998;

Zupanc and Horschke 1997) (Fig. 9). The first studies on regeneration and neurogenesis in adult teleost were done in the spinal cord revealing ^{3}[H]-thymidine-labeled neurons both under normal and regenerating conditions (Anderson and Waxman, 1985). Since then more and more studies in the adult teleost CNS were done mainly based on proliferation and revealed that different teleostean species have an enormous potential to produce new cells in several brain regions like the telencephalon, diencephalon, mesencephalon, rhombencephalon and the cerebellum, where the vast majority of dividing cells are found in high concentrations in small, well-defined areas of the brain ("proliferation zones") (e.g.; Zupanc and Zupanc, 1992; Soutchek and Zupanc, 1995; Zupanc et al., 1998; Zikopoulos et al., 2000; Zupanc, 2001; Ekström et al., 2001; Byrdes and Brunjes, 2001). In the following I would like to highlight a few regions of major interest:

After a short pulse proliferation was found at the telencephalic ventricle and in the OB where new cells are generated in the olfactory nerve layer of adult zebrafish (Byrdes and Brunjes, 2001). Cells in the olfactory nerve layer do not stain with neuronal markers and display a glial morphology. After some time labeled cells have populated all layers of the OB (the olfactory nerve layer, the glomerular layer and the internal layer) with some of the cells in the internal layer showing characteristics of neurons indicating neuronal differentiation (Byrd and Brunjes 1998, 2001). It was hypothesized that most of these cell have migrated into the OB, probably from the telencephalic ventricle, and are not derived from cells generated in the olfactory nerve layer. In the telencephalon (Fig. 9, green box), in addition to proliferating cells localized at the telencephalic ventricle, labeled cells were found along the dorsoventral axis in the subpallium (Fig. 9, red arrow (1)) and the pallium as well as in the dorsolateral telencephalon of different teleostean model systems (Zupanc and Horschke, 1995; Ekström et al., 2001; Byrds and Brunjes, 2001). The dorsal and lateral pallium has been implicated in the control of spatial and emotional memory in goldfish (Rodriguez et al., 2002; Portavella et al., 2002, 2003, 2004). Thus, these domains might be equivalents to the SVZ and SGZ of rodents. Proliferation studies also revealed a zone with dividing cells located in the caudal part of the optic tectum of different teleost fish (Fig. 6B (6)) (Zupanc and Horschke, 1995; Markus et al., 1999; Raymond and Easter, 1983; Zikopoulos et al., 2000; Ekström et al., 2001). It seems that most of the newly generated cells continue to reside in this area

(Raymond and Easter, 1983) raising the hypothesis that the optic tectum grows from its caudal end, contributing new cells to this area.

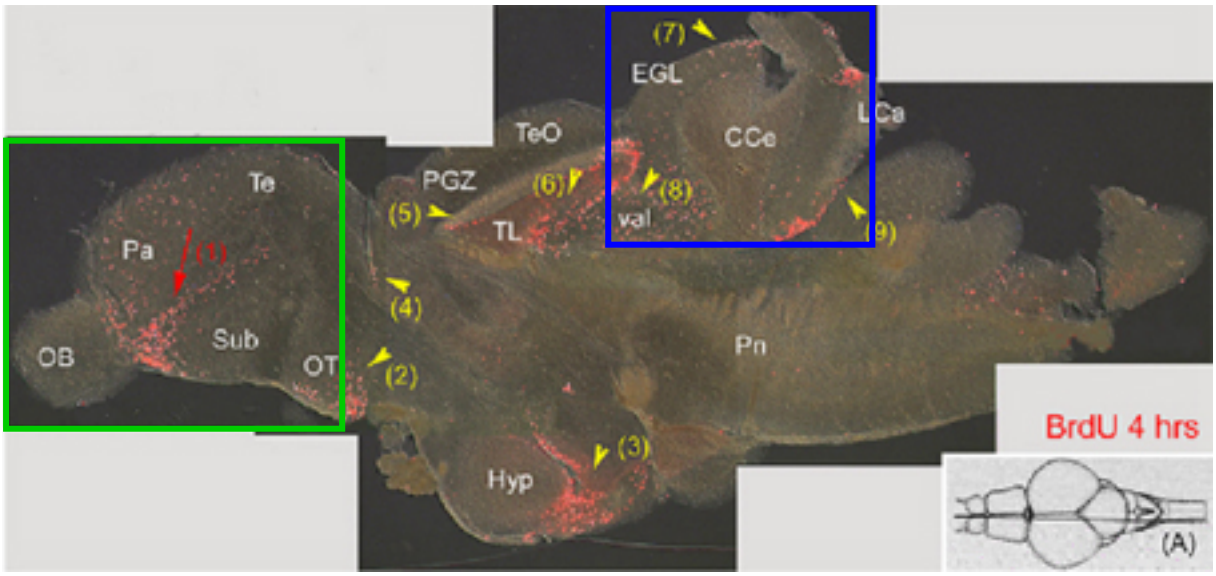


Fig. 9: Proliferation zones in the adult zebrafish brain:

Parasagittal section of an adult zebrafish brain, sacrificed 4 hrs after 2 BrdU injections, at the level indicated in (A), anterior to the left. Intensely labeled sites are numbered (red arrows: telencephalon; yellow arrows: other domains): (1) intense stripe within the ventral subpallium, (2) optic tracts, (3) ventricular zone of the hypothalamus, (4) anterior thalamus, (5) periventricular gray zone, (6) torus longitudinalis, (7) cerebellar external granular layer, (8) valvula cerebelli, (9) lobus caudalis cerebelli.
Abbreviations: CCe: corpus cerebellaris; EGL: external granular layer; Hyp: hypothalamus; LCa: lobus caudalis cerebelli; Pa: pallium; PGZ: periventricular gray zone; OB: olfactory bulb; OT: optict tract; Pn: pons; Sub: subpallium; Te: telencephalon; TeO: tectum opticum; TL: torus longitudinalis; val: valvula cerebelli.

More studies on proliferation and neurogenesis however, have been performed in the cerebellum (Fig. 9, blue box). In *Apteronotus* it was found that about 75% of all newly generated cells are produced in the proliferation zones of the cerebellum (Fig. 9) (Zupanc and Horschke, 1995), a situation that seems to be conserved in other teleosts (Kranz and Richter 1970; Zikopoulos et al., 2000; Ekström et al., 2001). Proliferation takes place in the molecular layers of two major subdivisions of the cerebellum – the valvula cerebelli (Val) (Fig. 9, (8)) and the corpus cerebellis (Fig. 9, (7)) and in addition new cells were produced in the granule cell layer of the eminentia granularis (Zupanc and Horschke, 1995; Zupanc et al., 1996). It was further shown

that the cells migrate, guided by radial glial fibers (Zupanc and Clint, 2003), to specific traget regions, e.g. the granule cell layers in both the corpus cerebellis and the valvula cerebelli, while cells from the eminentia granularis migrate through the adjacent molecular layer into a second granule cell layer (Zupanc et al., 1996). Basically only half of the newly generated cells reaches their target region, due to apoptosis that is consistently higher in the granular layers then in the molecular layers, indicating a selection of the newly generated cells, probably based on activity (Soutschek and Zupanc, 1996). Newborn neurons spared from apoptosis get integrated into the existing networks and at least some of them differentiate into granule cell neurons (Zupanc et al., 1996; Zupanc, 2001). Taken together, teleosts, including zebrafish, are an attractive model to study adult neurogenesis with a number of widespread but still restricted proliferation zones, a large number of newly generated cells that survive over long periods and continuing neurogenesis in adults. But until today the stem cell lineage in teleosts is not known.

1.2.5 Lineage relationship between early embryonic and adult neurogenesis

As discussed earlier, in the early embryonic ventricular zone, most neurons and glia in the brain are generated by neuroepithelial cells that initially are neurogenic. Slightly later, glial precursors appear as the first cells distinguishable from neuroepithelial cells. Radial glial cells reveal remarkable similarities to neuroepithelial cells: they remain part of the neuroepithelium, reach the ventricular (inner) and pial (outer) surface of the neural tube, divide at the ventricular surface but they can be distinguished by glial specific markers (Götz and Barde, 2005). It has been shown by fate mapping studies, that radial glia function as progenitors during development most of them generating only neurons, some are already specified to generate only glial cells both by symmetric divisions and only a few are still multipotent and capable of generating both neurons and glia by asymmetric divisions. (McKay, 1997; Frederiksen and McKay, 1988; Malatesta et al., 2000, 2003; Noctor et al., 2002; 2004; Götz und Barde, 2005). Thus it is possible to describe radial glial cells as the “late” NSCs during embryonic development. The more obvious functions of radial glia however were that of migratory scaffolds guiding migrating neurons to their target

regions and glial progenitors differentiating into astrocytes perinataly after neuronal migration is complete (Schmechel and Rakic, 1979; Voigt, 1989).

In terms of potential, most radial glial cells are restricted to the generation of only one cell type. One outcome is to differentiate into oligodendrocyte precursors (OPCs) promoted by Olig2 (which in early stages is involved in differentiation of motor neurons) and Nkx2.2 (Sun et al., 2001; Zhou et al., 2001). The OPCs then undergo a terminal differentiation into myelinating cells when they reach the white matter. In contrast to OPCs the genetic program involved in the generation of other forms of glia are poorly understood. Another outcome is to differentiate into astrocytes, which represent a fairly plastic and heterogenous population of cells. The molecular mechanisms underlying astrocyte formation are still not clear at the moment but it is likely that a default pathway via inhibition of neurogenesis and oligodendrogenesis via Notch signaling (Tanigaki et al., 2001). A third outcome, much studied to date, is the generation of neurons.

Notch signaling and its effectors have a well-characterized role in the inhibition of neurogenesis and therefore in keeping undifferentiated progenitors. Recently however it was shown that the activity of Notch1 was restricted to one particular progenitor population – the radial glial cells, and, rather than inhibiting neurogenesis, it promoted the acquisition of a radial glial phenotype (Gaiano, 2000; Yoon et al., 2004, 2005; Ever and Gaiano, 2005). Further inactivated Notch3 promotes a radial glial/progenitor character in the embryo and an astrocyte fate after birth (Dang et al., 2006), thus Notch1 and Notch3 seem to function in a similar way. In addition FGF signaling seems to play a role in self-renewal (Hack et al., 2004) and in the induction of a radial glial phenotype as well as the maintenance of progenitors with radial glial morphology interacting with Notch signaling (Yoon et al., 2004).

Introduction of activated Notch into the mouse forebrain using a retrovirus before the onset of neurogenesis revealed that it first promoted a radial glial phenotype and many of the infected cells postnataly became periventricular astrocytes – the cell type that has previously been shown to be neural stem cells in the adult (Gaiano et al., 2000) indicating a linear relationship between radial glia and stem cells in the adult CNS. This was further highlighted labeling radial glial cells in postnatal mice that could be followed into the adult neurogenic regions, where they had transformed into astrocytes. The latter, in turn were capable of producing neurospheres, thus individual radial glia and their progeny in the adult brain act as stem cells (Merkle et

al., 2004; Garcia et al. 2004). Therefore neural stem cells are most likely contained within the lineage: “neuroepithelium - radial glia - astrocytes” (Alvarez-Buylla et al., 2001). In contrast to mammals however, in cold-blooded vertebrates and birds, radial glia persist into adult life (Horstmann, 1954; Stensaas and Stensaas, 1968; Stevensons and Yoon, 1982; Alvarez-Buylla et al., 1987; Connors and Ransom, 1987; Zamora and Mutin, 1988) and studies in adult birds have shown that radial glial cells function as primary precursors for new neurons (Alvarez-Buylla et al., 1998). Thus radial glial cells might have a potential role as neuronal progenitors in continuing neurogenesis in cold-blooded vertebrates – like zebrafish (Zupanc and Clint, 2003).

1.2.6 Regulation of the adult stem cell activity depends on cell-intrinsic signals, on the neurogenic niche and the influence of physiological and environmental signals

1.2.6.1 Molecular processes involved in controlling the stem cell state and fate

It has been shown that neurogenic regions in adult vertebrates are located at ventricular zones in mammals. In teleosts, in which most neurogenic regions are found to be located at subventriclular, paraventricular or cisternal systems, or in domains derived from areas previously located at the ventricle but that were translocated as a result of the eversion of the fish telencephalon. The anatomy of the neurogenic regions (described in 1.2.3) with NSCs in close vicinity to the lumen of the ventricle as well as to blood vessels, the basal lamina and ependymal cells, exposes cells to a specialized microenvironment – or germinal “niche”. These niches are composed of soluble factors, membrane-bound molecules and extracellular matrix (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch, 2003; Alvarez-Buylla and Lim 2004). Cell-cell interactions within the neurogenic regions (Doetsch et al., 1997; Mercier et al., 2002; Palmer et al., 2000; Seri et al., 2001) influence the maintenance, activation and differentiation of stem cells via feedback signaling and impact on the cell cycle or the mode of division. Astrocytes have contact to all cell types as well as to the basal lamina and the blood vessels thus can detect alterations in cell numbers, translate signals from the vasculature and other cells and are able to secrete factors

supporting neurogenesis themselves (Lim and Alvarez-Buylla, 1999; Song et al., 2002) thus they function as support cells as well as NSCs. In addition transplantation experiments of precursors from one neurogenic region into the other resulted in the formation of new neurons specific for their host while transplantation of precursors into non-neurogenic regions failed to regenerate new neurons (Gage et al., 1995; Doetsch and Alvarez-Buylla, 1996; Suhonen et al., 1996; Herrera, 1999). Together this suggests that the niche microenvironment can be instructive in fate specification of neural stem cells.

Bone morphogenic proteins (BMPs), known to inhibit neurogenesis and oligodendrogenesis while promoting astrocyte differentiation, are produced by SVZ astrocytes. Thus, the neurogenic potential of SVZ astrocytes is under constant inhibition by BMPs while ependymal cells secrete noggin, which antagonizes the inhibitory effects of BMPs (Lim et al., 2000) and thereby promotes neurogenesis in the SVZ.

The role of Notch in early development is very well studied. In adults it was shown that Notch1 is expressed in both the SVZ and the SGZ (Stump et al., 2002) but its role in adult neurogenesis is to date only suggestive. Overexpression of activated Notch in embryo promotes radial glial identiy and postnatally the production of dense clusters of SVZ astrocytes (Gaiano et al., 2000) thus its role might be in suppressing neuronal differentiation and maintaining precursor cell properties. Activation of Notch in postnatal SVZ cells prevents migration, suppresses neuronal differentiation, decreases proliferation and thereby creates a more “quiescent” cell type (Chambers et al., 2001). Further it was shown that transient activation of Notch caused a rapid and irreversible loss of neurogenic capacity accompanied by accelerated glial differentiation (Morrison et al., 2000). Since Notch signaling is context dependent, it remains to be clarified whether it promotes terminal glial differentiation (Tanagaki et al., 2001) or a precursor state in adult germinal regions. Thus Notch signaling and BMPs decrease proliferation and induce an “astrocyte” phenotype in the SVZ cells.

Growth factor signaling controls cells division and it was shown that infusion of EGF and FGF expands the SVZ cell population (Gage, 2000; Tropepe et al., 1997). But only FGF2 induces an increase in the number of newborn cells (mainly neurons) in the olfactory bulb (Kuhn et al., 1997). EGF selectively acts on transient amplifying C-cells to arrest neuroblast production, divide rapidly and become highly migratory, invading the adjacent brain (Doetsch et al., 2002) while the more quiescent stem

cells do not seem to be affected. Loss of TGFα (the mitogen usually present in the SVZ) results in decreased proliferation and fewer cells reaching the OB (Tropepe et al., 1997) but it is not clear whether this reveals an effect on the stem cell population, or as it was shown for EGF on transient amplifying cells as well.

Brain-derived neurotrophic factor (BDNF) was shown to play a role in proliferation, as ventricular administration of BDNF results in increased proliferation and an augmented number of neurons in both neurogenic regions (Zigova et al., 1998; Benraiss et al., 2001; Pencea et al., 2001). Ciliary neurotrophic factor (CNTF) injected into the adult mouse forebrain enhances self-renewal of NSCs *in vivo* (Shimazaki et al., 2001) while its administration to NSCs *in vitro* results in an almost exclusive differentiation into astrocytes (Johe et al., 1996).

Eph/ephrin signaling has an effect on proliferation, as SVZ astrocytes express ephrinB2/3 ligands and infusion of EphB2 leads to an increase in astrocyte cell number and induces these cells to contact the ventricle (Conover et al.,2000). Shh on the other hand was shown to be involved in the regulation of stem cells in both neurogenic regions. Overexpression in the DG leads to increased proliferation and neurogenesis of SGZ cells and it maintains proliferation of hippocampal neuronal progenitors in culture (Lai et al., 2003). Conditional removal of the Shh receptor Smoothened (*Smo*) from neural precursors in embryos led to decreased proliferation in the SVZ and SGZ and fewer neurons in their target regions. Along this line, in vitro studies have demonstrated fewer neural stem cells in these animals, implicating Shh as a stem cell maintenance-factor (Machold et al., 2003). Alternatively, Shh may function in promoting proliferation or support the survival of transient amplifying cells. Thus the role of Shh is to date not clear.

In addition neural stem cells require a set of specific cell-intrinsic factors. Two transcriptional regulators, Bmi-1 (Molofsky et al. 2003) and TLX (Shi et al., 2004) have been shown to be involved in maintaining adult neural stem cells in a proliferative state. Sox2 is also expressed in proliferating precursor cells and glial-like cells that are believed to be stem cells. It would be involved in proliferation and/or maintenance of NSCs as well as in neurogenesis and the maintenance of neurons in specific regions (Ferri et al., 2004). Loss of Sox2 results in a reduction of proliferation in both neurogenic regions and leads to severe phenotypes including behavioral defects associated with neurodegeneration.

Very recent studies identified Wnt as a player to regulate neurogenesis in the DG of the hippocampus. Several Wnt-family members are expressed in the DG (Lie et al., 2005; Shimogori et al., 2004) with at least *Wnt3* expressed in hippocampal astrocytes (Lie et al., 2005). *In vitro* studies revealed the stimulation of Wnt-β-catenin signaling in NSCs by astrocyte-derived Wnts. This subsequently had a neurogenic effect as a result of increased neuronal fate commitment and proliferation of neuroblasts. *In vivo* enhanced Wnt signaling promotes the generation of immature neurons in the DG, whereas overexpression of a dominant-negative Wnt leads to an almost complete suppression of neurogenesis in the DG. Mice with Wnt mutations or lacking key components of the canonical signaling cascade show major defects in progenitor cell proliferation and abnormal hippocampal morphogenesis (Li and Pleasure, 2005; Zhou et al., 2004). Wnts are made and secreted by astrocytes in the adult hippocampus and specifically increase the proliferation of neuronally restricted precursors and differentiation. Thus, adult neurogenesis in the DG is regulated by the Wnt pathway, which might be the central pathway in neuronal fate determination.
Taken together the major signaling pathways involved in proliferation, neurogenesis and maintenance of progenitors during development are retained in the adult germinal niche; thus, there are likely many parallels between embryonic development and the adult brain germinal zones.

1.2.6.2 Physiological and environmental signals affect the stem cell niche

Steroid hormones play a role in regulating the survival of new neurons in song control nuclei (Rasika et al., 1994, 1999; Burek et al., 1995; Hidalgo et al., 1995) as well as in the adult hippocampus, where estrogen increases the number of dividing cells in the subgranular layer (Tanapat et al., 1999). Thus, sexual behavior has an influence on neurogenesis. In addition, an increase in glucocorticoid levels suppresses cell proliferation and inhibits neurogenesis in the SGL (Cameron and Gould, 1994; Gould et al., 1997). Neurogenesis and/or neuronal recruitment in the DG can be altered by adrenal steroids, as shown by an increase of proliferation and neuronal birth after adrenalectomy (Gould et al., 1992; Cameron and Gould, 1994; Cameron and McKay, 1999). Thus, glucocorticoids exert an inhibitory role. However, since only very few mitotic cells express glucocorticoid receptors (Cameron et al., 1993), they seem to act indirectly.

Environmental signals seem to have an effect on proliferation and neurogenesis as well. Active learning, an enriched environment as well as voluntary exercise seems to promote neurogenesis in the hippocampus (Gould et al., 1999; Kempermann et al., 1997; van Praag et al., 1999). The higher number of new neurons reached with an enriched environment, however, is due to an increased survival of the new cells rather than to an increased proliferation (Kempermann et al., 1997), while physical exercise promotes proliferation, an increase in survival of new neurons as well as a selective increase of long-term potential of the DG (van Praag et al., 1999). A prolonged physical exercise, however, seem to have a negative effect on progenitor proliferation in the DG (Naylor et al., 2005).

Taken together, the regulation of adult neural stem cells is dependent on the interaction between a vast array of signals intrinsic to the niche environment and macroenvironmental factors acting at the level of the organism as a whole.

1.2.7 Hypothetical roles of adult neurogenesis in vertebrates

Since the discovery of adult neurogenesis the question on the role of continuing generation of new cells is still open. In the adult hippocampus in rodents (Kempermann et al., 1997; Gould et al., 1999) and the song control nuclei in adult song birds (Nottebohm, 1985; Nottebohm et al., 1990; Barnea and Nottebohm, 1994; Alvarez-Buylla et al., 1990), it has been suggested that new neurons play a role in plasticity and learning to adjust to changes in the environment. Thus, neurogenesis may be involved in the modification of brain circuitry involved in sexual behavior. Further it was assumed that the formation of connections between new and existing neurons increases the brain capacity to process and store novel information (Gage et al., 2000, 2002). In addition neuronal replacement is necessary to keep circuits functional and prolong health and reproductive fitness of an animal (Nottebohm, 2002). New hippocampal neurons were also shown to be involved in processing memory as killing of dividing cells and thereby a reduction in the number of new neurons in the hippocampus impairs the hippocampus-dependent memory tasks (Shors et al., 2001).

Less is known about olfactory neurogenesis. It could be that incorporation of new neurons is important when the olfactory circuits are already responding to environmental signals to adjust the olfactory circuitry when the environment or

relevance of odors changes. An increased proliferation in the SVZ and incorporation of new neurons in the OB have been shown for rodent females in certain periods during pregnancy, which may help them to better recognize their offspring after birth (Shingo et al., 2003). It was also shown that a lot of new granule cells soon after their arrival to the OB and developing the first synaptic connections undergo apoptosis (Petreanu and Alvarez-Buylla, 2002), reflecting a possible process of selection maybe based on the level of activity (Cecchi et al., 2001) that could be important to improve olfactory discrimination between similar odors. In mice in which the number of cells reaching the OB is severly reduced, olfactory discrimination between odors is reduced while olfactory sensitivity and olfactory memory appear to be unaffected (Gheusi et al., 2000). Thus new neurons in the olfactory bulb may have a role in olfactory discrimination.

In addition to all the possible roles of adult neurogenesis hypothesized in higher vertebrates, teleostean adult neurogenesis might play specific functions that cannot be generalized to other vertebrate species. It was shown in fish that the brain is growing throughout life, paralleling a growth of the body that results from the addition of new cells to many organs. Body growth therefore might require the establishment of new circuits to read out (in case of sensory information) or stimulate (e.g. for motor control) these new cells.

1.3 The bHLH transcription factors

Transcription factors of the BASIC-HELIX-LOOP-HELIX (bHLH) class have been first identified in *Drosophila melanogaster* in mutants lacking subsets of external sense organs (Ghysen and Dambly-Chaudiere, 1988). By RT-PCR and yeast two hybrid screens, counterparts of these genes have been isolated in vertebrates. The bHLH factors were identified as the key players during the phase of neurogenesis. They act as proneural genes in promoting selection of progenitors committed to a neuronal fate (Guillemot et al., 1993), but also in neuronal subtype specification or in neuronal differentiation by inducing a cascade of neuronal differentiation genes (Blader et al., 1997; Ma et al. 1996). In addition, they have been shown to function in the inhibition of glial fates (Tomita et al., 2000; Nieto et al., 2001). Therefore they do not only play a crucial role in proliferation and neurogenesis but also in gliogenesis.

1.3.1 Molecular structure and DNA-binding capacities of bHLH transcription factors

Although there exists a large number of bHLH transcription factors that are involved in various functions, all show some common structural features. They contain a N-terminal basic domain followed by a HLH domain that consists of two α-helices connected by a variable loop region (Ferre-D'Amare et al. 1993)

Transcription factors of the bHLH class have been shown to dimerize to perform their function as DNA-binding proteins (Murre et al 1989). Homo- and heterodimers are formed by the interaction of the two helices of each dimerization partner forming a four-helix bundle (Fig. 10) (Ferre-D'Amare et al., 1993; Ellenberger et al., 1994; Ma et al., 1994).

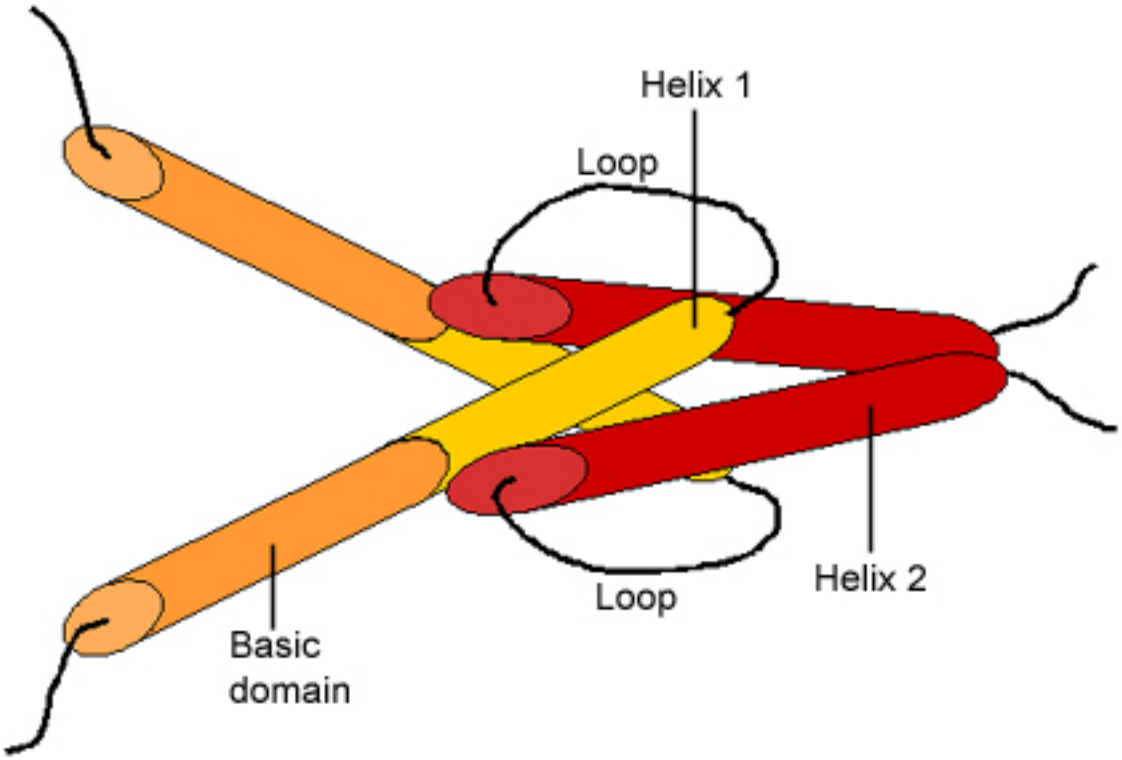


Fig. 10: Structure of a dimer of a bHLH transcription factor

The two α-helices of both bHLH partners form a four-helix bundle (after Ferre D'Amare et al, 1993)
Colour coded: basic domain orange; Helix 1 yellow; Helix 2 red

Specific DNA binding of the bHLH dimers is performed by the basic domain that fits structurally into the main grove of the DNA double helix (Murre et al., 1994). All together, 10 residues of the bHLH domain directly contact the DNA, 7 of which are located in the basic domain, whereas the other three are scattered in the HLH region. 9 of the 10 residues that make the direct contact during DNA-binding are completely conserved among members of the different families of bHLH protein. These residues bind to specific hexanucleotide motifs within the DNA sequence.

bHLH transcription factors can be subdivided according to their structural features, biochemical characteristics and biological functions, into four monophyletic groups A, B, C and D (Table. 1) (eg. Fisher and Caudy, 1998; Iso et al., 2003; Massari and Murre, 2000; Ledent and Vervoort, 2001). Proteins of Group A bind to the consensus sequence CANNTG or 'E boxes' (eg. Murre et al., 1989; Van Doren et al., 1992) on their target gene and thereby lead to their transcritpional activation (Van Doren et al., 1992). Group B proteins are able to bind both, the E-boxes (CANNTG) and the 'N-boxes' (CACNAG) and act as transcriptional repressors (Fisher and Caudy, 1998; Davis and Turner, 2001). This group can be subdivided in two groups according to the presence or absence of an additional functional domain known as the Leucine Zipper domain, involved in dimerization (Henriksson and Luscher, 1996; Facchini and Penn, 1998). Group C consists of transcription factors that belong to the bHLH-PAS family (Crews, 1998), with a PAS domain involved in dimerization. These proteins have an affinity to DNA-binding sites with a consensus sequence consisting of only 5 nucleotides (ACGTG or GCGTG) but their biochemical activity is still unknown. Members of Group D differ from the bHLH proteins since they lack the basic domain and therefore are unable to bind DNA although they are able to form dimers with other bHLH transcription factors which consequently are no longer able to bind to DNA (Benezra et al., 1990; Ellis et al., 1990). Group D members indirectly inhibit neurogenesis as they act as antagonists of Group A proteins (Van Doren et al., 1991; Van Doren et al., 1992). Examples for Group D are the Id proteins in vertebrates or Emc in Drosophila.

Group	Structural features	Biochemical activity	Examples	DNA binding sites
A	bHLH	transcriptional activator	MyoD, Mash	E-box CANNTG
B	bHLH leucine zipper	transcriptional repressor	Myc, Max	E-box CANNTG
	conserved proline in basic domain	transcriptional repressor	Hairy and E(Spl) Hes, Esr, Her	E-box CANNTG N-box CACNAG
C	bHLH-PAS	unknown	Sim, ARNT	ACGTG GCGTG
D	HLH	antagonist of Group A	Id, Emc	No DNA binding

Table 1: Subdivision of the bHLH family in monophyletic groups and their Function

After Fisher and Caudy, 1998.

1.3.2 Transcritpion factors of the Atonal family

In vertebrates, bHLH genes are functionally highly heterogeneous and involved in the regulation of numerous processes. For the Atonal-family, several factors have been identified in vertebrates that have all been classifilk ed as transcriptional activators with a positive function in promoting neurogenesis. All members of this family show molecular characteristics of the Atonal family, like highly conserved basic and HLH domains. Other vertebrate *atonal*-related genes are characterized by the presence of conserved family-specific residues in the bHLH domain and therefore are grouped into distinct subfamilies, such as Neurogenin, NeuroD, Olig and Beta (Fig. 11) (Lee, 1997; Hassan and Bellen, 2000), indicating that they also might have different functions during neurogenesis. Upon homo- or heterodimers formation, all these proteins have been shown to specifically bind the ‘E-box’ (CANNTG) (Murre et al.,1989) in the promoters of various target genes. Binding specificity is determined by the composition of the bHLH dimer and the two central positions of the hexanucleotide, as well as the adjacent positions.

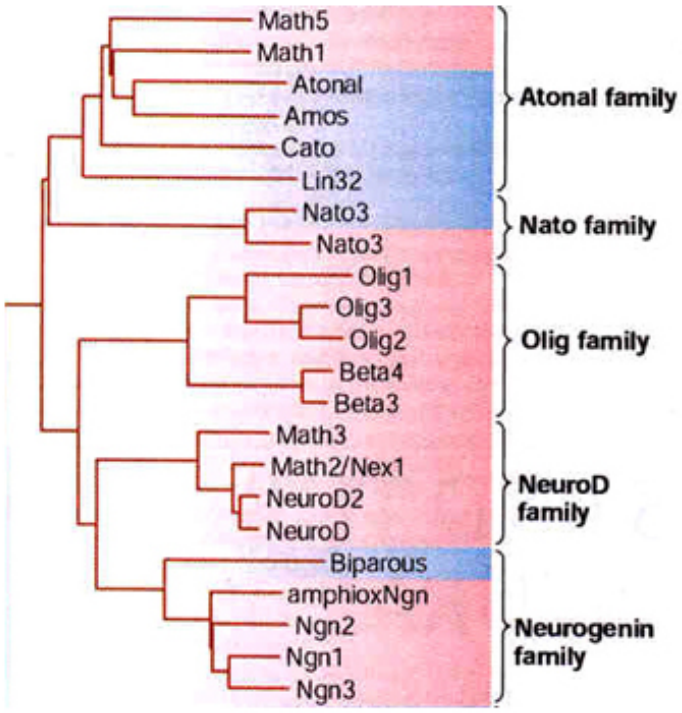


Fig. 11: Phylogenetic tree of Atonal-related subfamilies

Phylogentic tree on the basis of the bHLH domain of Atonal and Atonal-related subfamilies of invertebrates (blue) and vertebrates (red). Proteins have been grouped in distinct families in vertebrates because of the closer sequence similarities in the bHLH domain.
(After Bertrand et al., 2002)

1.3.2.1 Atonal proteins and their proneural function

Until now, six *atonal* transcription factor-encoding genes, isolated in zebrafish, have been published. Although 3 of them (*atoh2a*, *atoh3* and *atoh*4) are today identified as *atonal*-related genes belonging to the *NeuroD* (*atoh2a*/*NeuroD1a*, *atoh3*/*NeuroD4*) and the *Neurogenin* families (*atoh4*/*ngn3*), the other three are identified as *atonal* genes because of their bHLH sequence closer to that of *Drosophila ato*. One of them is *atoh7* (formerly known as *ath5* or *lakritz*) the zebrafish orthologue of *Math5*, which is expressed in retinal ganglion cells (RGCs) (eg. Stenkamp and Frey, 2003; Loosli et al., 2003; Kay et al., 2001; Ryu et al., 2005) and seems to be involved in the specification of the RGC fate in multipotent retinal progenitors (Kay et al, 2005). This is reminiscent to the function of *Math5*. The other two, *atoh1a* and *atoh1b*, are the zebrafish orthologues of *Math1* (following gene duplication in zebrafish). Both are expressed in the hindbrain of the developing zebrafish embryo (Koester et al,2001,

2006; Adolf et al., 2004, Appendix 1) and like in mouse are probably involved in cerebellar granule cell differentiation.

1.3.2.2 The Beta family and their function as transcriptional repressors

Another group of bHLH transcription factor encoding genes belonging to the *atonal*-related subfamilies is distinct from the *atonal*-genes and more closely related to the *olig* (Fig. 11). In mouse this group consists of only 2 genes named *Beta3* (*Bhlhb5)* and *Beta4 (Bhlhb4).* Another *Beta*-gene described in mouse, *Beta*2 is today identified as *NeuroD1.* Interestingly although members of the Atonal related subfamilies usually act as transcriptional activators (which is also true for the Beta2 (NeuroD1)), Beta3 and Beta4 act as transcriptional repressors. These factors are unable to bind to DNA even though they can dimerize. *Beta* genes encode dominant negative forms of Beta protein, lacking a functional basic domain and indirectly inhibit transcriptional activation by a mechanism called squelching (Peyton et al., 1996; Xu et al., 2002). Expression of *Beta3* and *Beta4* in rodents is found in the pancreas and in highly restricted patterns in the developing central nervous system (Bramblett et al., 2002; Kim et al., 2002; Brunelli et al., 2003; Liu et al., 2007). In adult mice *Beta3* is still expressed in low levels in the hippocampal dentate gyrus and CA3 region (Kim et al., 2002). In zebrafish there is to date only one gene isolated from this subfamily, *beta3.1*. It is expressed in various domains in the CNS (olfactory pits, nMLF, retina and hindbrain) (Adolf et al., 2004) in a pattern reminiscent of its mouse orthologue (Brunelli et al., 2003; Liu et al., 2007) and therefore may likewise function in a similar way as a transcriptional repressor.

1.3.3 Hairy and Enhancer of split (Hairy/E(Spl)) bHLH family and their function as transcriptional repressors

Until now numerous proteins related to the Hairy/E(Spl) bHLH family have been identified in vertebrates. Most members of this family are known to act as transcriptional repressors and are involved in various developmental processes such as neurogenesis, gliogenesis, segmentation/somitogenesis or myogenesis (eg. Fisher and Caudy, 1998; Kageyama and Nakanishi, 1997; Damen et al. 2000; Sieger et al. 2004, 2006; Parkhurst et al., 1990). Based on sequence considerations, the

Hairy/E(Spl) family can be subdivided into 5 major subfamilies: Hairy, E(Spl), Hey, Stra13 and Helt (Fig. 12). Although most of these proteins are acting as transcriptional repressors, the conserved differences in the primary sturctures between the factors belonging to these different subfamilies suggest that they might function in distinct ways and/or be differently regulated at the post-translational level (Davis and Turner et al., 2001).

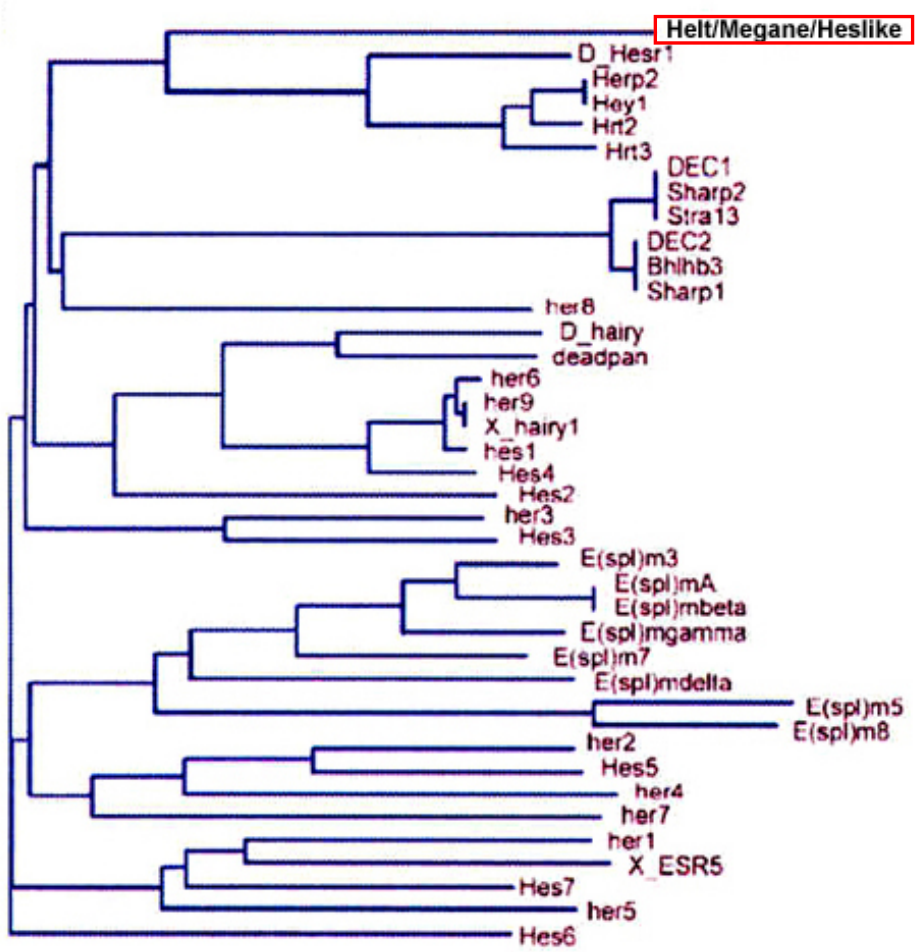


Fig. 12: Phylogenetic tree of the Hairy/E(Spl) family

Phylogenetic tree on the basis of the bHLH domain of Hairy/E(Spl) related subfamilies. Note that Helt /Megane/Heslike (red box) represents a new subfamily besides Hey- and Hes/Her subfamilies.
(Guimera et al., 2006).

A common and specific structural feature for proteins of all subfamilies of the Hairy/E(spl) family is a conserved double helix known as Orange domain (Dawson et al., 1995) or helix3/4 (Knust et al., 1992), C-terminal to the highly conserved bHLH domain (Fig. 13). The molecular function of this domain is currently not very well understood but a possible function could be in specifying protein-protein interaction during dimerization (Leimeister et al., 2000; Naktani et al. 2004) and/or in repression mechanisms (Nakatani et al., 2004). With the exception of the Helt and Stra13 protein, the other subfamilies of Hairy/E(Spl) proteins have a conserved tetrapeptide motif at the C-terminus, either WRPW for the Hairy and E(Spl) proteins or YXXW for the Hey proteins. This tetrapeptide motif functions in the recruitment of the Groucho/TLE co-repressor (Fig. 13) (Davis and Turner, 2001).

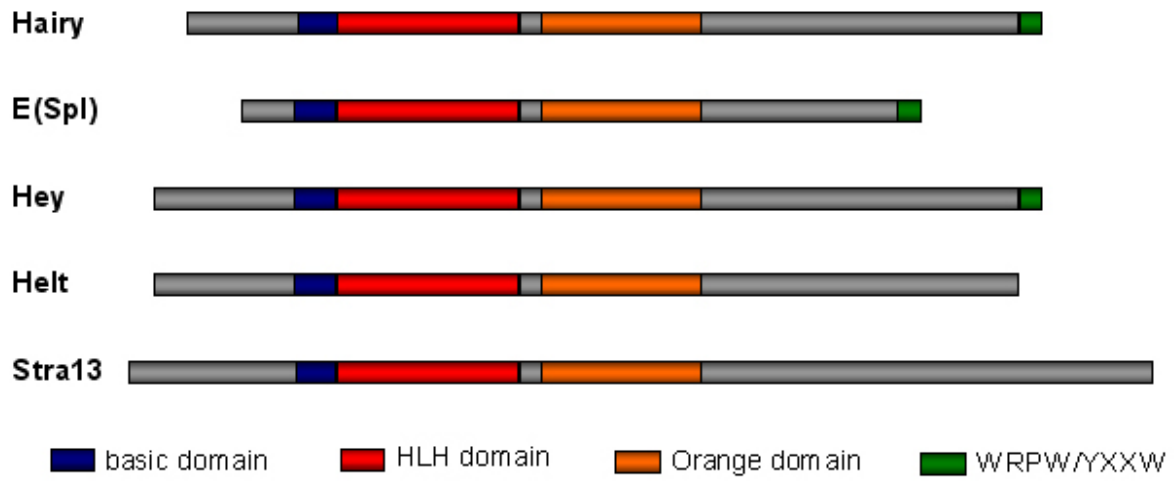


Fig. 13: Schematic representation of Hairy/E(Spl) protein domains:
The bHLH and Orange domains are present in all subfamilies, while the C-terminal tetrapeptide motif is missing in Helt and Stra13 subfamilies. Hairy-like and E(Spl)-like factors usually end with WRPW, while all Hey-like proteins have a YXXW motif near the C-terminus. Conserved domains are colour-coded, grey might contain some motifs shared within a subfamily but are not generally conserved.
Abbreviations: HLH: helix-loop-helix; P: proline; R: asparate; W: tryptophan
After Davis and Turner, 2001

bHLH transcritpion factors of the Hairy/E(Spl) family belong to Group B proteins and therefore can bind to both ‘E-‘ (CANNTG) and ‘N-boxes’ (CACGCG or CACCAG) with a higher affinity to ‘N-boxes’ than to ‘E-boxes’ (Van Doren et al.,1994).

Transcripts are expressed in the developing nervous system (*her3*, *her4*, *her5*, *her6*, *him*, and *her9*) (Hans et al., 2004; Takke et al., 1999; Müller et al., 1996; Pasini et al. 2001; Ninkovic et al., 2005; Sieger et al., 2004) and the presomitic mesoderm (*her1*, *her4*, *her6*, *him* and *her7*), where *her1* and *her7* are cycling and play a critical role (Takke et al., 1999; Holley et al., 2002; Ninkovic et al., 2005; Sieger et al., 2004; Leve et al., 2001). *her5* is additionally expressed in the endoderm (Bally-Cuif et al., 2000).

1.3.3.1 Mechanisms of transcriptional repression through Hairy/E(Spl) proteins

Three possible mechanisms have been proposed in biochemical and genetic studies.
(I) DNA-binding dependent active repression (Kageyama and Nakanishi, 1997; Kageyama et al., 2000) by which Hairy/E(Spl) proteins form homo- or heterodimers binding to the DNA consensus sites. The corepressor Groucho (*Drosophila*) or its vertebrate homolog TLE/Grg interacts with the WRPW-motif and in turn recruits the histone deacetylase (Rpd3 in *Drosophila* or HDAC in vertebrates), which might repress transcription through alteration of the chromatin structure (Paroush et al., 1994; Grabvec et al., 1996).
(II) DNA-binding independent passive repression by protein sequestration. In this case Hairy/E(Spl) proteins form non-functional heterodimers with other bHLH transcription factors (like E47) that usually function as dimerization partners for tissue specific bHLH activator proteins (like Mash1 or MyoD), thereby preventing the formation of functional heterodimers (Sasai et al., 1992; Hirata et al., 2000).
(III) A third possible mechanism of repression is via a putative protein interaction motif, the Orange domain, which is able to repress transcription of its own promoter (Hes1) as well as the *p21* promoter (Castella et al., 2000).

1.3.3.2 The Helt subfamily – transcriptional repressor proteins

Helt proteins constitute a distinct subfamily of the Hairy/E(Spl) proteins (Fig. 12, red box). Helt proteins were identified in different vertebrates but interestingly were not found in invertebrates until now (Miyoshi et al., 2004).
Only one gene belonging to the *Helt* familiy has been published (analysis of Ensembl zebrafish genome release zv7) in zebrafish until now. Like its mammalian

orthologues, Helt shows a conserved lysine residue in the basic domain (in Hairy/E(Spl) proteins there is a proline residue and in Hey proteins a glycine residue conserved in this position) and it lacks the C-terminal tetrapeptide motif. In zebrafish as well as in other vertebrates *helt* is expressed in the midbrain (Miyoshi et al., 2004; Guimera et al., 2006) but, in contrast to mammals, *helt* expression in zebrafish reveals to phases of expression probably indicative of different functions of Helt (see article in preparation appendix 2).

In mouse, Helt was shown to be able to form homo- and heterodimers, and the Orange domain could still form dimers even in the absence of the bHLH domain. Furthermore it was shown for Helt proteins to preferentially bind to E-boxes like the closely related Hey-proteins, but in contrast to this subfamily Helt lacks the C-terminal tetrapeptide motif and in turn it is not able to recruit corepressors. In addition it was shown that the bHLH domain was not required for repression and therefore Helt proteins might use another mechanism of repression than the Hairy or E(Spl) proteins (Nakatani et al., 2004).

CHAPTER 2

Aims and Achievements of the thesis

The main intention of my project was the identification of domains of continuing proliferation and neurogenesis in the late embryonic, early larval and adult central nervous system (CNS) and the isolation of molecular factors that could be involved in the maintenance of the neural progenitor state in these domains. Indeed, although a lot is known of the cascades of factors involved in neurogenesis during early embryonic development, the factors involved in maintenance of neural stem cells and the generation of new neurons at late stages are still poorly understood. Until now, most studies focussing on adult neurogenesis, neural stem cells and regeneration potentials in adult organisms have been performed (at least on the molecular level) in the rodent forebrain. There, adult neurogenesis is however limited to two regions, the SVZ in the lateral wall of the lateral ventricle and the SGZ in the dentate gyrus of the hippocampus (eg. Altman and Das, 1965; Doetsch et al., 1997, 1999, 2001; Seri et al., 2001).
To gain insight in continuing neurogenesis and the identification of factors likely involved in these processes in late embryonic, early larval and adult CNS, I used several approaches in the vertebrate model system zebrafish (*Danio rerio*):

(I) It is known that transcription factors of the bHLH class are involved in early neurogenesis. However, although it is likely that such factors are involved in late neurogenic processes as well, it is not known whether the same factors are at play at late stages, and how they act. Thus I first simply looked for new genes encoding factors with a bHLH structure from late embryonic tissues. Using this approach, in the first part of my work, I identified and characterized the expression of two new bHLH transcription factor-encoding genes for the *atonal* subfamily, called *atoh1.2* and a member of a distinct subfamily, called *beta3.1*. These results are described in Chapter III.1.1 and in appendix 1. In addition I identified a transcription factor-encoding gene belonging to the *hairy/E(Spl)* family, refered to as *helt*. The characterization of its expression pattern and its likely function in neurogenesis at late

embryonic stages and during adulthood are reported in chapter III.1.2 and appendix 2.

(II) It has been shown in most vertebrates that new neurons are generated in adults but our knowledge about continued neurogenesis in the adult vertebrate CNS is mostly derived from investigations in the two rodent neural stem cells models, the SVZ of the lateral wall of the lateral ventricle and SGZ of the dentate gyrus in the hippocampus (e.g. Altman and Das, 1965; Doetsch et al., 1997, 1999, 2001, 2002; Seri et al., 2001). In lower vertebrates however, proliferation and neurogenesis has been demonstrated to be more widespread although still restricted to discrete foci.
In the second part of my work, I tested the validity of the zebrafish as a good model system to further study adult neurogenesis by characterizing self-renewing progenitors in the adult zebrafish telencephalon. I showed that these progenitors have neural stem cell-like characteristics and give rise to new neurons. I observed that OB neurogenesis in zebrafish shares many remarkable similarities to that in rodents and involves a rostral migratory stream. Finally I identified a new population of stem cells giving rise to new neurons that stay in the telencephalon proper. Results on this topic are described in Chapter III.3 and appendix 3.

(III) Following this demonstration, we aimed to characterize neural stem cells in other brain areas, and we were able to identify two proliferation zones at the junction between the midbrain and the cerebellum, refered to as tectal proliferation zone (TPZ) and isthmic proliferation zone (IPZ). This work was conducted by Prisca Chapouton, a postdoctoral fellow in the laboratory, in collaboration with me. The IPZ contains a population of cells expressing the gene *her5*, which, in the embryo, actively prevents neurogenesis and keeps the cells in a progenitor state (Geling et al., 2003, 2004; Ninkovic et al., 2005). *her5*-expressing cells in the adult brain display properties of stem cells, suggesting that the function of Hairy/E(Spl) factors in the specification of the neuronal progenitor state might be conserved in adulthood. The results on this topic are discussed in Chapter III.4 and appendix 4.

Chapter 3

Results

3.1 Cloning of new bHLH transcription factor-encoding genes likely involved in neurogenesis in the zebrafish CNS

3.1.1 *atoh1.2* and *beta3.1* – two new bHLH-encoding genes expressed in selective precursors of the zebrafish anterior hindbrain

Article Appendix 1

Although neurogenesis during early embryonic development and the cascades involved in early neurogenesis are quite well known today, only little is known about late embryonic, early larval and adult neurogenesis and the factors involved in controlling these later neurogenic processes. It is known that transcription factors of the bHLH family play a crucial role in the control of early neurogenesis but it remains unclear whether the same factors or yet unknown factors are involved in late neurogenesis. In order to identify new proneural bHLH transcription factor-encoding genes, we used a degenerate PCR approach using oligonucleotides for different bHLH transcription factor families. For the proneural family *atonal*, we cloned two new genes, *atoh1.2* and *beta3.1*. *atoh1.2* is closely related to *atoh1.1*, another zebrafish *atoh1* gene, and *Ath1* genes in other vertebrates, and its specific expression is found in the lateral subpopulation of *atoh1.1* positive cells (Köster and Fraser, 2001) in the zebrafish hindbrain. Expression arises at the tail-bud stage in two stripes at the edge of the neural plate at the hindbrain level. At 24 hpf *atoh1.2* expression is restricted to the lateral subpopulation of *atoh1.1*-positive cells displaying a characteristic profile at the edge of the cerebellar plate and in the lower rhombic lips. This expression profile is maintained at 48 hpf, dorsomedially with a sharp limit at the MHB, containing precursors of the later valvula cerebelli (Fig. 14, *atoh1.1* in blue, *atoh1.2* in red). At 72 hpf however, expression of *atoh1.2* gets restricted to a group of cells at the junction between the cerebellum and the optic tectum, containing precursors of the future valvula cerebelli. Its expression is reminiscent of the expression pattern of *Ath1* in the mouse (Gazit et al., 2004), where

it was shown to be essential for the genesis of cerebellar granule cells. Further all *atoh1.2* positive domains contain proliferating cells at late embryonic and early larval stages indicating that it might be involved in neurogenesis in late developmental stages.

beta3.1, a member of the distinct subfamily *beta*, is closely related to other *Beta3* genes in other vertebrates. In contrast to the members of the Atonal family, Beta proteins in mouse were shown to act as transcriptional repressors (Peyton et al., 1995; Xu et al., 2002). *beta3.1* expression in zebrafish is first detectable at tail-bud stage in two clusters of the anterior neural plate which can be localized at 3 somites to rhombomere 1 (r1). At 24 hpf expression of *beta3.1* is found in broad dorsolateral stripes of interneuron precursors in the rhombencephalon and the spinal cord starting anteriorly in r1. In addition it gets strongly expressed in the retina, where it is maintained until at least 72 hpf. At 48 hpf *beta3.1* expression in the rhombencephalon and the spinal cord is subdivided into two longitudinal stripes of presumptive interneurons, with the medial populations extending from r2 caudally, while the lateral populations start in r1, reaching the MHB anteriorly (Fig. 14, *beta3.1* in green). Additional expression domains in the forebrain detectable at these later stages are the olfactory pits and the neurons of the medial longitudinal fascicle (nMLF). This expression profile is maintained at 72 hpf without any changes. Zebrafish *beta3.1* is expressed predominantly in the hindbrain and in addition it is strongly expressed in the retina, the olfactory pits and in the interneurons of the MLF.

Expression patterns of zebrafish *beta3.1* during embryonic development are reminiscent of its mouse counterpart *Beta3* (Brunelli et al., 2003; Liu et al., 2007). With exception of the retina and the interneurons of the lateral longitudinal fascicle, *beta3.1* expression contained proliferating cells in all domains suggesting a likely function in neurogenesis late during development. In summary we have identified two new bHLH transcription factor-encoding genes both expressed in two distinct precursor populations in the anterior hindbrain, with a sharp limit at the MHB. Taken together with the expression patterns of *ngn1* (Blader et al., 1997) and *ash1b* (Allende and Weinberg, 1994; Müller and Wullimann, 2003), the anterior hindbrain can therefore be subdivided into subdomains of bHLH expression.

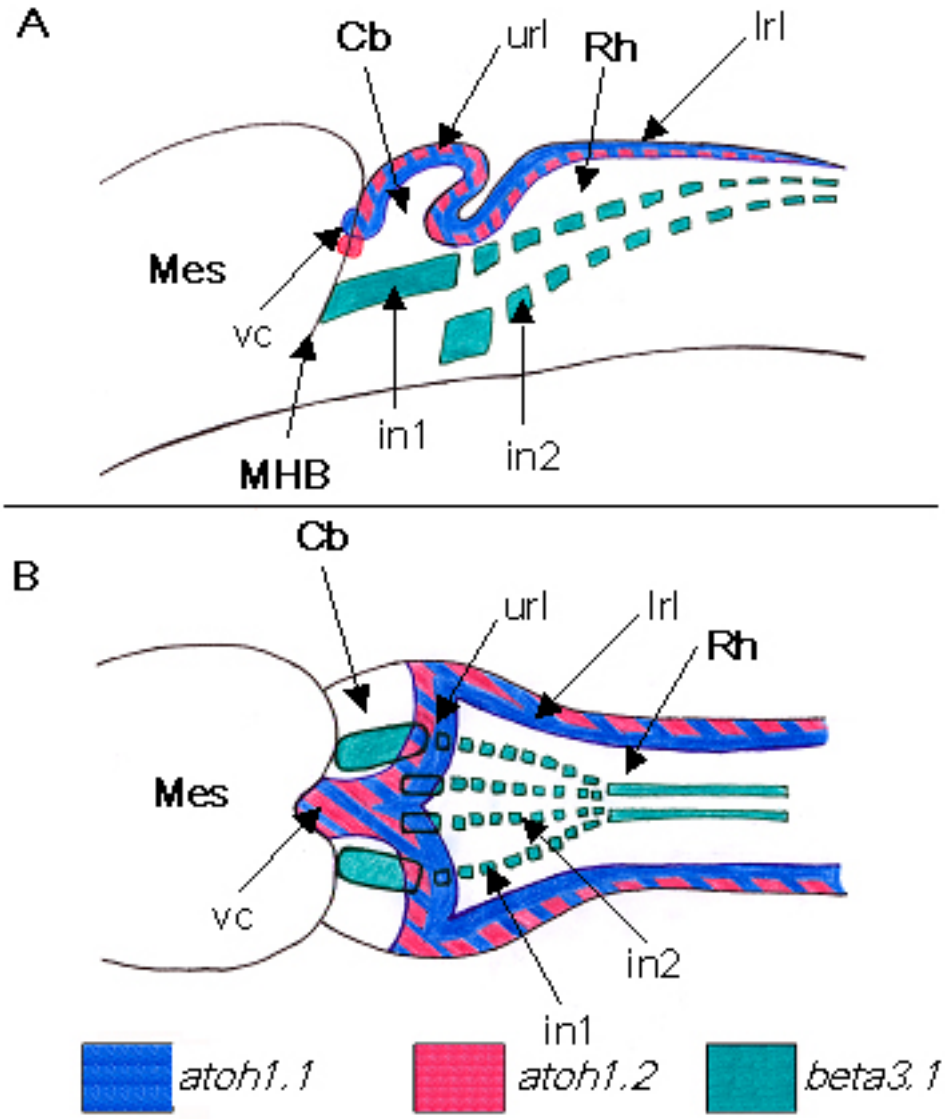


Fig. 14: Expression profiles of *atoh1.1*, *atoh1.2* and *beta3.1* in the zebrafish hindbrain at late embryonic stages:

Schematic lateral (A) and dorsal (B) representation of the relative expression domains of *atoh1.1* (blue), *atoh1.2* (red) and *beta 3.1* (green) in the zebrafish embryonic hindbrain at 48 hpf.
Abbreviations: Cb: cerebellar plate; in1-2: subpopulations of interneuron precursors; lrl: lower rhombic lip; MHB: midbrain-hindbrain boundary; Mes: mesencephalon; rh: rhombencephalon; url: upper rhombic lip; vc: valvula cerebelli

3.1.2 *helt* – a new bHLH-encoding gene expressed in proliferation zones in the CNS of late embryonic, early larval and adult stages

Manuscript in preparation appendix 2

Another bHLH transcription factor-encoding gene was identified using oligonucleotides of the Hairy/E(Spl) family. It is the zebrafish orthologue to mouse Heslike/Helt/Mgn (Miyoshi et al., 2004; Nakatani et al., 2004; Guimera et al., 2006), belonging to the Helt subfamily, which phylogenetically shows a high homology to the subfamily of Hey factors. Special characteristics of the Helt subfamily in comparison to Hes/Her and Hey factors are the lack of a proline-/glycine residue in the basic domain, replaced by a lysine, and the lack of the tetrapeptide motif at the C-terminus. *helt* expression in zebrafish is first detectable in a non-bilateral cluster in the most ventral part of the neural tube at 5 som. At 14 som expression expands dorsally in a separate stripe and broadens along the anteroposterior and mediolateral axes. Expression at that time can be located in the mesencephalon, between but at some distance from the zona limitans intrathalamica (ZLI) and the MHB. At 24 hpf expansion of *helt* expression in the anteroposterior axes progresses with the ventral band reaching the diencephalic-mesencephalic border rostrally and the MHB caudally, whereas the dorsal band can be localized in the pretectum. Until that stage *helt* expression in zebrafish is remarkably similar to the expression pattern of its ortholog in the mouse (Miyoshi et al., 2004; Guimera et al., 2006), with the exception that expression in the zebrafish includes the ventral midline. At late embryonic (48 hpf) and early larvae (72 hpf) stages, the expression pattern changes dramatically. It now lines the edges of the tectum dorsally, ventrally and at the midline, thus is now reminiscent to described proliferation zones (Wullimann et al., 1999, 2000; Müller and Wullimann 2002) (Fig. 15).

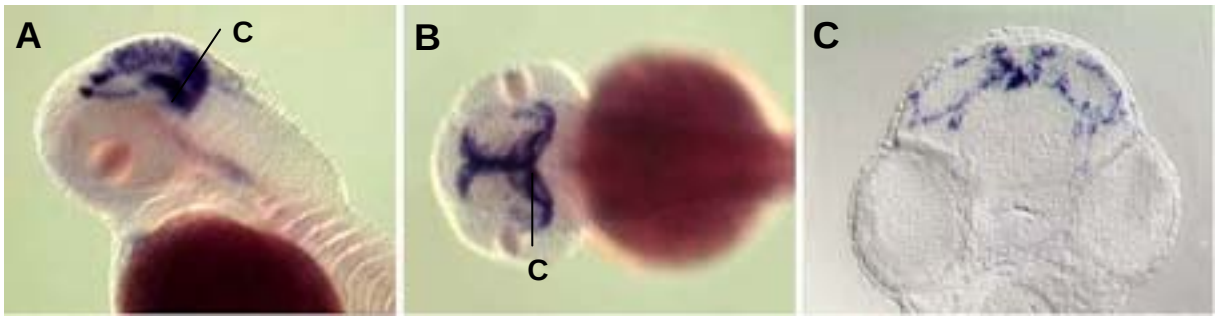


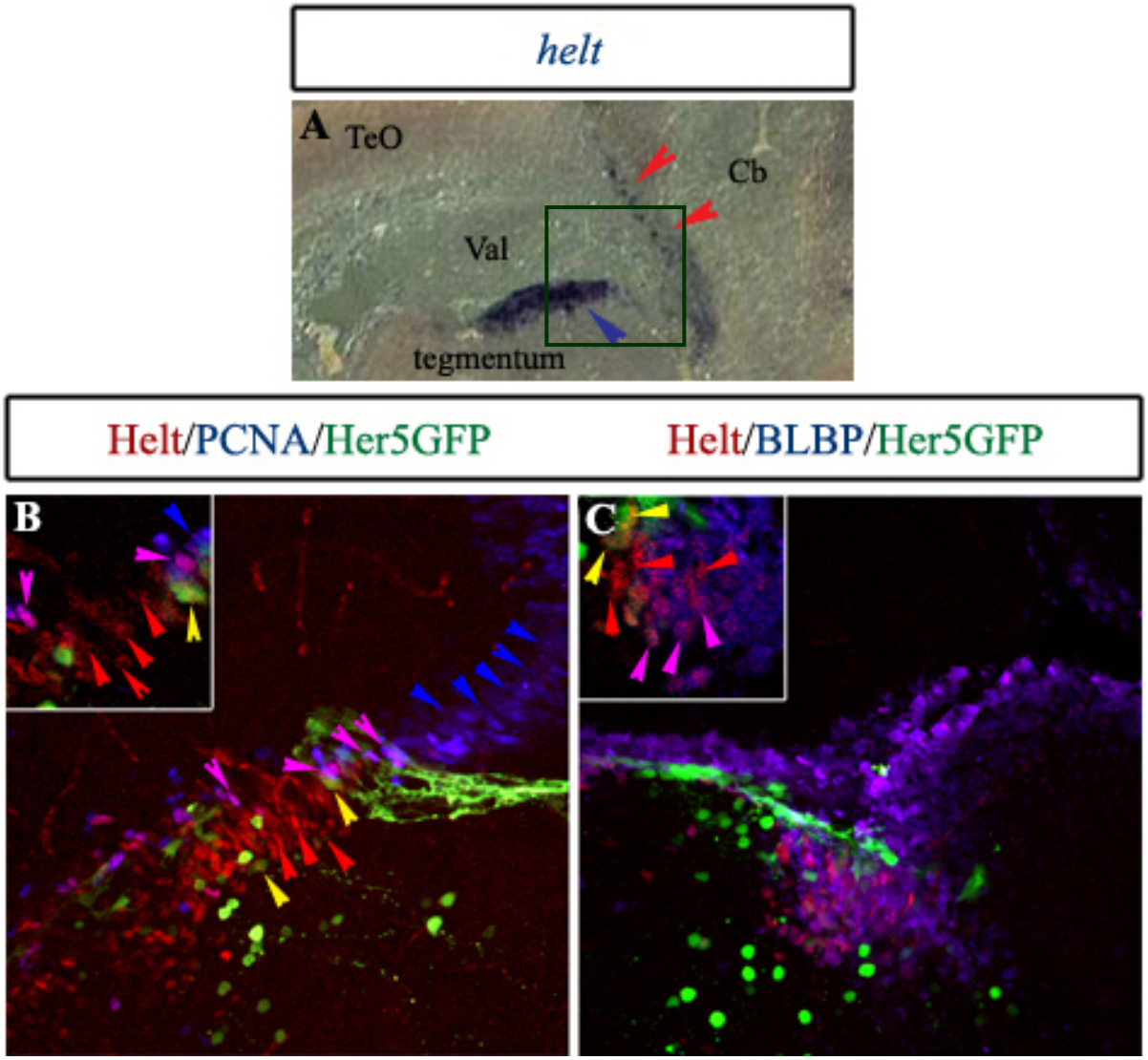
Fig. 15: Expression pattern of *helt* in the zebrafish midbrain at late embryonic stages:

Helt expression in the tectum at 48 hpf in a lateral (A) dorsal (B) view (anterior to the left) and in a cross section (C) at the level indicated.

To improve our detection of Helt at late stages, we raised an anti-Helt antibody. In situ hybridization (Fig. 16 A) and immuno-staining revealed, that *helt* is still expressed in the adult zebrafish midbrain, in a pattern reminiscent to two described proliferation zones: the tectal proliferation zone (TPZ) at the junction between the tectum and the cerebellum (Fig. 16 A, red arrowheads), and the isthmic proliferation zone (IPZ) at the edge of the ventricle at the intersection between the valvula cerebelli, the torus semicircularis and the tegmentum (Fig. 16 A, blue arrowhead) (Chapouton et al., 2006). Detailed immuno-stainings using the Helt antibody and PCNA (a marker for proliferating cells) or BLBP (a marker for radial glial cells) in the *her5pac:egfp* transgenic line (Tallafuss and Bally-Cuif, 2003) revealed that Helt is in fact expressed in a subpopulation of Her5-expressing stem cells (Chapouton et al., 2006) in the IPZ (Fig. 16 B, C), and additionally in a subpopulation of neighbouring fast proliferating cells, while the vast majority of Helt-positive cells found adjacent to the IPZ co-expresses BLBP. In the TPZ however, the Helt domain defines a subpopulation of BLBP-expressing cells, and, the vast majority of Helt-expressing cells is proliferating and expresses PCNA.

Fig. 16: Expression of Helt revealed in the adult zebrafish brain

All views are sagittal sections of the adult brain at the midbrain level, anterior left.
(A) *helt* expression revealed by in situ hybridization is located in the midbrain lining the boarder between the tectum and the cerebellum (red arrowheads) and along the posterior aspects of the tectal ventricle at the level of the valvula cerebelli (blue arrowhead). **(B-C)** Triple immuno stainings (Helt, red; PCNA/BLBP, blue; Her5-GFP, green) on *her5pac:egfp* transgenic brains in projections and single confocal planes (insets) in the area boxed in green in **A** reveal that Helt expression is partially overlapping with the proliferations zone IPZ (pink arrowheads to Helt/PCNA double-positive cells in **B** or Helt/BLBP double-positive cells in **C**, and yellow arrowheads to Helt/Her5-GFP double-positive cells in **B** and **C**).
Abbreviations: Cb: cerebellum; TeO: optic tectum; Val: valvula cerebelli.



Conclusion:

Although a lot is known about the cascades of transcription factors involved in early neurogenesis, little is known whether these factors also play a role in late neurogenic processes and additionally their profiles are not sufficient to describe the profiles of neurogenesis at late embryonic, early larval stages and during adulthood. Thus it is very likely that other, yet unknown factors are involved in neurogenesis at late stages and during adulthood. I report here the identification of three new transcription factor-encoding genes *atoh1.2*, *beta3.1* and *helt*, which define late proliferation zones and are good candidates to mediate late neurogenesis events:

(I) *atoh1.2* and *beta3.1*, highlighting distinct populations of proliferating precursor cells in the anterior hindbrain.

(II) *helt*, expressed in proliferation zones of the tectum at late embryonic and early larval stages, and in the two described proliferation zones at the junction between midbrain and cerebellum during adulthood. There, Helt-positive cells express both, markers for proliferation and markers for glia.

3.2 The study of neurogenesis and factors that control neurogenesis in the adult zebrafish brain

3.2.1 Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon.

Article Appendix 3

It was shown in most vertebrates that the generation of new neurons is continuing in the CNS during adulthood. However, most of the studies concerning adult neurogenesis have been done in the rodent forebrain where neurogenesis is tightly restricted to two small regions, the SVZ in the lateral wall of the lateral ventricle and the SGZ of the DG in the hippocampus (e.g. Altman and Das, 1965; Doetsch et al., 1997, 1999, 2001, 2002; Seri et al., 2001). Neurogenesis appears to be more widespread although still restricted to disinct foci in lower vertebrates (Garcia-Verdugo et al., 2002; Goldman, 1998; Zupanc, 2001). Although a lot was done to identify neural stem cells, their characteristics and their properties, our understanding of the cellular and molecular mechanisms underlying adult neural stem cells remains only fragmentary. In order to provide and validate new models on this issue we have focused our search for stem cells on the adult zebrafish telencephalon in comparison to what is known in rodents. Using BrdU (a marker for the S-phase during cells cycle) to map proliferating cells in the telencephalon, we observed that the ventral subpallium was intensely labelled, especially in a longitudinal stripe of cells joining the posterior telencephalon with the OB, refered to as subpallial stripe (Fig. 17 A).

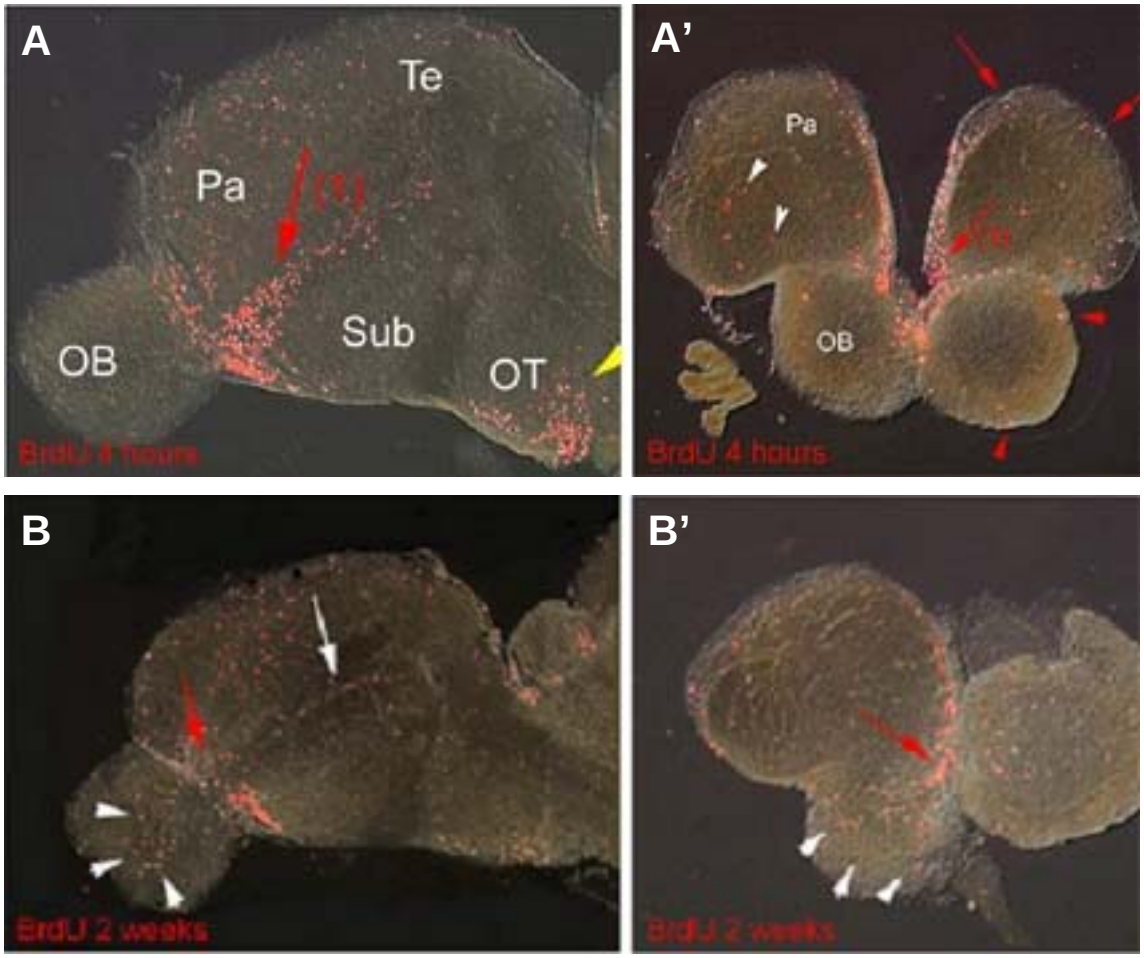


Fig. 17: Proliferation sites in the adult zebrafish telencephalon

BrdU immunohistochemistry (red staining) after a survival time of 4 hours (A, A') and 2 weeks (B, B') on parasagittal sections (A, B, anterior to the left) and cross-sections (A', B'): Intense incorporation of BrdU in the subpallial stripe (red arrow (1)) in the ventral subpallium which decreases after longer survival times (B, white arrow), while cells are migrating into the OB (B, B', white arrowheads) and into the telencephalic parenchyma.
Abbreviations: OB: olfactory bulb; OT: optic tract; Pa: pallium; Sub: subpallium

In cross sections, we could show that these cells are lining the telencephalic ventricle at all dorsoventral (DV) levels (Fig. 17B). Furthermore, we could show that the growth fraction varied along the DV axis, with a high density of BrdU-positive cells and a consistently higher labeling index (proportion of cells in S-phase among the population of dividing cells) in the ventral subpallium and the posterior pallium, indicating that the precursors of these domains are fast proliferating while other ventricular telencephalic precursors divide more slowly (Fig. 18). Tracing the BrdU-labeled cells after different survival times, we found that cells that are born at the telencephalic ventricle are migrating from the ventral subpallium into the OB and from

the dorsal subpallium and pallium into the telencephalic parenchyma to more lateral positions (Fig. 17 B, B'; Fig. 18).

We could show that the cells progressively colonizing the OB likely originate from dividing cells at the ventricle of the ventral subpallium and migrate along the ventral subpallial stripe, a migration pathway that is strongly stained by PSA-NCAM (a marker for migrating neuroblast and newborn neurons), indicating a possible equivalence to the RMS in mammals (Fig. 18). But in contrast to mammals, there is no evidence that migration in zebrafish is routed along organized GFAP-positive tubes within this stripe.

Furthermore we could show that proliferating cells located along the telencephalic ventricle give rise to Hu-positive newborn neurons adjacent to the ventricle within the telencephalon and in the OB with increasing numbers of BrdU/Hu-double positive cells over time. These findings demonstrate that de novo neurogenesis is actively taking place throughout the adult telencephalon from precursors initially located along the ventricle. Like in mammals, adult neurogenesis in the zebrafish contributed to de novo generation of GABAergic and dopaminergic (TH-positive) neurons in the OB (Fig. 18). In mouse, it was demonstrated that Pax6 is both necessary and sufficient for neurogenesis in the OB and instructs a dopaminergic phenotype when maintained in postmitotic cells (Hack et al., 2005; Kohwi et al., 2005). Interestingly, even though we observed Pax6 expression in the OB in both BrdU-labeled cells and TH-positve interneurons we never observed triple labelled cells in the OB of adult zebrafish thus Pax6 is likely not involved in de novo generation of dopaminergic neurons in olfactory bulb neurogenesis in the adult zebrafish. In contrast to mammals, telencephalic ventricular progenitors in the dorsal subpallium and medial pallium contribute to de novo generation of GABAergic and TH-positive neurons in the telencephalon proper as well. Unlike the situation in the granular layer of the OB, where TH-positive neurons also produce GABA, in the telencephalon proper these populations are adjacent to each other and mostly non-overlapping (Fig. 18).

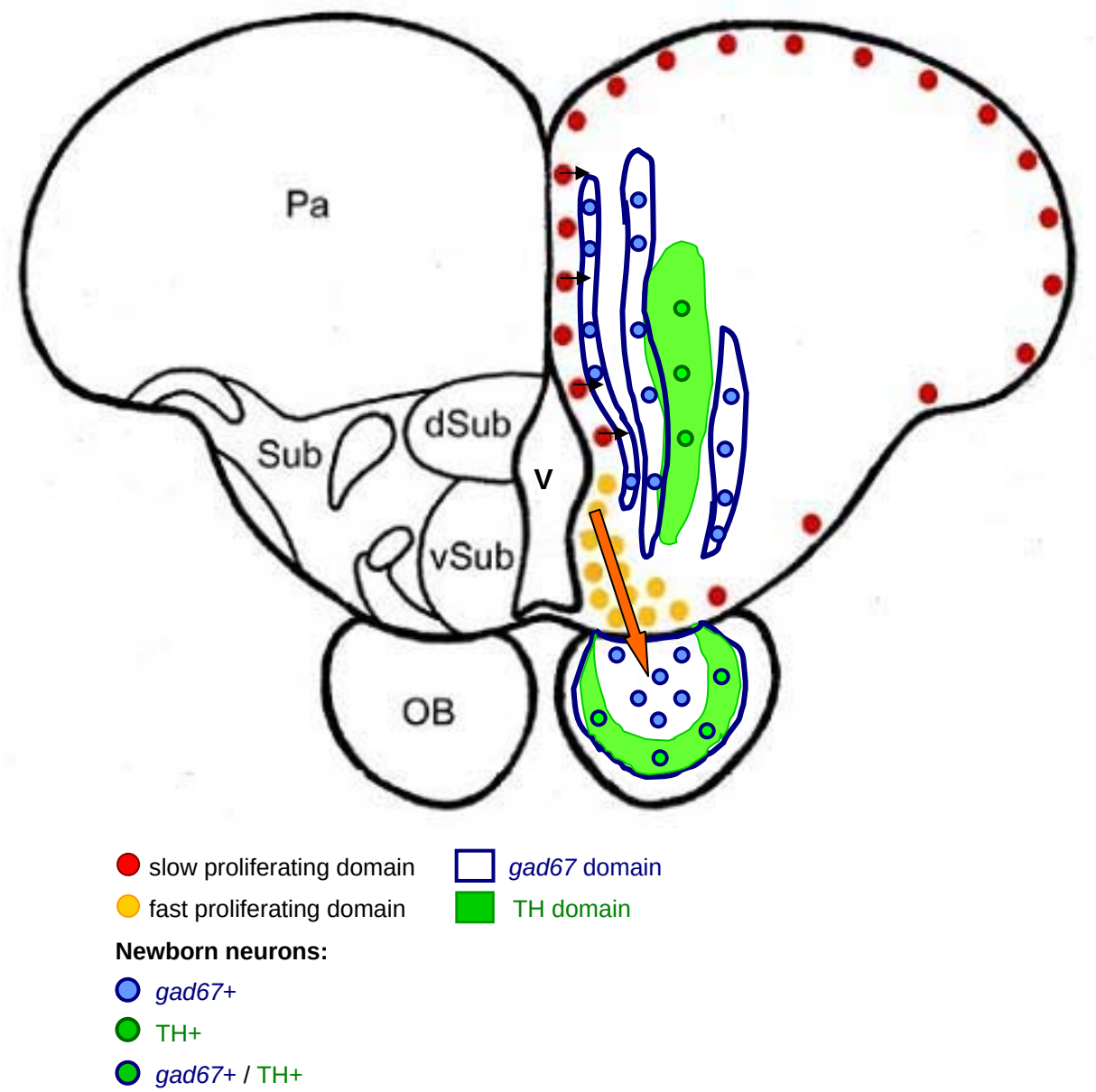


Fig. 18: Zebrafish adult telencephalic progenitors give rise to GABAergic and TH+ neurons in the olfactory bulb and the telencephalon proper:

Schematic summary of adult neurogenesis in the adult zebrafish telencephalon (cross section; color coded): Fast and slow proliferating cells are lining the telencephalic ventricle. Progenitors migrate towards the olfactory bulb via an equivalent to the mammalian rostral migratory stream (orange arrow) and give rise to GABAergic and TH+ neurons. Note that TH+ neurons in the olfactory bulb produce GABA. Progenitors that migrate away from the ventricle into the telencephalic parenchyma give rise to GABAergic and TH+ neurons. Note that in the telencephalon proper GABAergic and TH+ domains are mostly non-overlapping.
Abbreviations: dSub: dorsal subpallium; OB: olfactory bulb; Pa: pallium; Sub: subpallium; V: telencephalic ventricle; vSub: ventral subpallium

We next aimed to characterize the nature of these adult telencephalic progenitors. Neural stem cells in the SVZ and SGZ in the adult mammalian forebrain are characterized as slowly dividing astrocytes or type B cells (Doetsch et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002; Götz et al., 2002; Campbell and Götz, 2002). In zebrafish, BLBP (a marker for radial glial cells) (Anthony et al., 2004) is expressed in cells covering the telencephalic ventricular zone, correlating with the expression of common stem cell markers in mouse and fish, like *sox2*, *sox9* and *sox10* (Ellis et al., 2004; Episkopou, 2005; Ferri et al., 2004; Komitova and Eriksson, 2004), with cells incorporating BrdU. Thus, ventricular progenitors of the adult zebrafish telencephalon show characteristics similar to mammalian neural stem cells. Interestingly, in adult zebrafish, strikingly different to the embryonic situation, progenitors in the ventricular zone of the subpallium and the medial pallium express a similar combination of transcription factors, and this combination also differs with that expressed in the dorsal and lateral pallium. In addition, by making use of *ngn1:gfp* transgenic lines (Blader et al., 2003, 2004) where the expression of *ngn1* is driven by different promoter elements, we found that, in the adult zebrafish telencephalon but not in the embryo, a regulatory element of 3.4 kb is sufficient to drive correct GFP expression. Thus, *ngn1* expression in the telencephalic progenitors in the adult zebrafish telencephalon is not simply inherited from embryonic precursors but seems to be recruited de novo via new enhancer elements.

Conclusion:

I report here the establishment of the zebrafish as a good model system to study adult neurogenesis for the following reasons:

(I) I identified characteristic neural stem cells along the ventricle in the zebrafish adult telencephalon.

(II) Zebrafish adult neurogenesis in the OB proved remarkably similar to the situation in mammals, giving rise to GABAergic and TH-positive neurons and involving an equivalent to the rostral migratory stream.

(III) In addition, I identified a novel population of telencephalic stem cells contributing to the generation of GABAergic and Th-positive neurons in the telencephalon proper as well.

(IV) Dorsal and ventral progenitors in the adult telencephalon express a different combination of transcription factors and like in the case of *ngn1* use different enhancer element than their embryonic counterparts. Thus they are likely recruited de novo.

3.2.2 *her5* expression reveals a pool of neural stem cells in the adult zebrafish midbrain

Contribution to the work of Chapouton et al., 2006

This work was conducted by Prisca Chapouton, a postdoctoral fellow in the laboratory, in collaboration with me. I have contributed to this work with preliminary data on BrdU-positive proliferating cells localized in the two proliferation zones in the midbrain, the tectal proliferation zone (TPZ) and the isthmic proliferation zone (IPZ), and in addition in a subpopulation of Her5-positive cells in the *her5pac:egfp* transgenic line. In addition I provided some preliminary data showing expression of neuronal and glial markers in the proliferating domains of the adult zebrafish midbrain, as well as some in situ hybridizations on sections of the adult brain with factors known to be involved in neurogenesis in early embryonic stages, giving us a hint if any of these factors could also be involved in neurogenesis during adulthood. In addition I frequently discussed the project presented here with the first author.

Article Appendix 4

In zebrafish, like in other lower vertebrates, neurogenesis during adulthood is much more widespread although it is still restricted to discrete regions (Zupanc et al., 2005). In order to identify new stem cell populations in the adult zebrafish brain we took advantage of the *her5pac:gfp* transgenic line (Tallafuss and Bally-Cuif, 2003). It

was shown in the embryo that a pool of progenitors located at the MHB is formed under control of the transcription factors Her5 and Him/Her11 that actively inhibit neurogenesis (Geling et al., 2003; Geling et al., 2004; Ninkovic et al., 2005). Expression of Her5-GFP is maintained in the adult zebrafish midbrain in two spots, one in each hemisphere at the intersection between the valvula cerebelli, the torus semicircularis and the tegmentum at the tectal ventricle. Endogenous *her5* is expressed in the ventricular subset of Her5-GFP expression, in cells that display an undifferentiated morphology. Her5-GFP can be found additionally within the tegmentum in cells with a differentiated morphology, due to the stability of the Her5-GFP protein, still present in the cells long after *her5* RNA is switched off. BrdU and PCNA stainings showed, that *her5*-positive cells are located within the IPZ, a novel proliferation zone neighbouring the TPZ. The TPZ generates cells populating the optic tectum and was already described in juveniles in zebrafish and stickleback (Müller and Wullimann, 2002; Ekström et al., 2001) as well as in the juvenile and adult medaka fish (Nguyen et al., 1999). The IPZ can be subdivided in two populations with different labelling index: the ventricular *her5*-positive cells are slow-proliferating whereas the neighbouring ventricular domains comprise a population of fast-proliferating cells. Subsets of the slowly dividing *her5*-positive or ventricular Her5-GFP positive cells express neural stem cell markers, like BLBP and the astrocytic and radial glial marker GFAP (Götz and Barde, 2005; Hartfuss et al., 2001), as well as the transcription factor *sox2* (Ferri et al., 2004). Cumulative BrdU-labelling followed by a long survival time revealed the existence of long-lasting progenitors withing the population of ventricular Her5-GFP cells, showing their ability to self-renew. Finally, differentiated Her5-GFP cells within the tegmentum can be oligodendrocytes (O4+ and Quaking+), astroglia (S100β+) or, in a large proportion neurons (Hu+). Thus the adult *her5*-positive population is multipotent.

Conclusion:

Zebrafish neurogenesis is much more widespread although still restricted to discrete foci mostly located at ventricular regions. Here we characterize a novel zone of continuing neurogenesis in the adult zebrafish brain:

(I) *her5*-expressing cells at the junction between the midbrain and the cerebellum, refered to as the IPZ, show characteristics of neural stem cells, like self-renewal and, at the population level, multipotency. Their progeny cells migrate towards the tegmentum where they differentiate into neurons and glia via an intermediate step of neuroblasts.

(II) These results further suggest that transcription factors of the Hairy/E(Spl) family, involved in the maintenance of a progenitor pools within the zebrafish embryonic neural plate can be associated with (a) neural stem cells niche(s) in the adult zebrafish brain.

CHAPTER 4

DISCUSSION AND PERSPECTIVES

The long-held dogma of neurobiology that vertebrates are born with a fixed number of neurons and that the generation of new neurons is definitely arrested after birth (Ramon y Cajal, 1914) has been turned down in the past two decades. Since then, continuing neurogenesis in the central nervous system of adults has been demonstrated in almost all vertebrate organisms (e.g. Altman and Das, 1965, Kaplan and Hinds, 1977; Bayer et al., 1982; Lois and Alvarez Byulla, 1993, 1994; Goldman and Nottebohm, 1983; García-Verdugo et al., 1989), including primates and humans (Gould et al., 1997, 1999; Eriksson et al., 1998). But studies on adult neurogenesis and regenerative potentials have been concentrated mainly on the rodent forebrain, where adult neurogenesis is very limited and restricted to two regions, the SVZ in the lateral ventricle wall and the SGZ in the dentate gyrus of the hippocampus (eg. Altman and Das, 1965; Doetsch et al., 1997, 1999, 2001, 2002; Seri et al., 2001). In lower vertebrates, however, it was shown that adult neurogenesis is much more abundant although still restricted to discrete foci (Garcia-Verdugo et al., 2002; Goldman et al., 1998; Zupanc, 2001).

Zebrafish neural stem cell zones

My findings in the adult zebrafish demonstrate remarkable similarities in olfactory bulb neurogenesis to the rodent situation (Appendix 3). We have further observed a domain of fast proliferating progenitors in the posterior lateral pallium in the zebrafish telencephalon, suggested, based on molecular markers and functional test in goldfish (Rodriguez et al., 2002; Portavella et al., 2002, 2003, 2004) to be the equivalent to the mouse hippocampus (Wullimann and Rink, 2002). Together, these findings validate the zebrafish as a good model system to study adult neurogenesis in a genetically tractable animal and create a basis to gain insight in cellular and molecular mechanisms underlying adult neurogenesis by detailed comparative studies and forward genetic approaches.

Most interestingly, my results further identify novel zones of telencephalic adult neural stem cells, with ventral precursors generating new neurons (including GABAergic and dopaminergic neurons) within the ventral telencephalon proper, and

a novel population of neural stem cells located in the dorsal telencephalon that has no equivalent in the telencephalon of rodents and generates neurons of a type that we currently did not identify. In the telencephalon of reptiles newly generated neurons migrate from the ventricular zone in the dorsal telencephalon to populate major subdivisions of the cortex (Perez-Canellas and Garcia-Verdugo, 1996; Garcia-Verdugo et al., 2002; Font et al., 2001), and adult neurogenesis in the ventral telencephalon was observed in birds and reptiles (Goldman, 1998; Alvarez-Buylla and Kirn, 1997; Font et al., 2001; Garcia-Verdugo et al., 2002). It would be of high interest to study how telencephalic neurogenesis in these other vertebrates relates to that in zebrafish, both functionally and molecularly. In mammals, however, no equivalent populations of progenitors exist neither in the cortex, nor in the striatum and septum which were proposed to be homologous to the teleostean subpallium (Wullimann and Rink, 2002) and neurons generated in the SVZ migrate solely towards the OB. However, reports have shown that neurogenesis can be induced in situ following damage from local astrocytes that replace lost and damaged neurons in the neocortex of adult mice (Magavi et al, 2000; Chen et al., 2004).

Thus, the generation of neurons might not be restricted to the two neurogenic regions in the rodent forebrain. Therefore, it is of great interest to understand why neurogenesis in other telencephalic regions observed in lower vertebrates has come to an end in higher vertebrates and can be induced in rodents only after injury, as well as to identify the microenvironment and molecular mechanisms underlying telencephalic neurogenesis in lower vertebrates or that might be reactivated after injury to promote de novo neurogenesis in the mouse neocortex.

In addition to neurogenesis in the adult zebrafish telencephalon, we have identified and characterized a novel zone of adult neurogenesis, refered to as isthmic proliferation zone (IPZ), located at the border between the midbrain and the cerebellum, and characterized by the expression of *her5* (Appendix 4). This zone contributes new neurons and glial cells to the tegmentum, while the tectal proliferation zone (TPZ) contributes neurons to the tectum.

The identification of novel zones of continuing neurogenesis in the CNS of adult zebrafish is in agreement with the findings that in lower vertebrates, in contrast to mammals, adult neurogenesis is much more widespread (Garcia-Verdugo et al., 2002; Goldman et al., 1998; Zupanc, 2001). It is still not clear whether all characteristics and properties of rodent adult neural stem cells can be generalized to

other vertebrates, but our results make it now possible to conduct focused comparative studies.

To date, only a handful of factors are known that drive adult neurogenesis. Given our findings at the IPZ, a hint might be to look at Hairy/E(Spl) factors which are known to inhibit neurogenesis and maintain progenitor pools in the embryo. Indeed, the expression of Her5, a transcription factor responsible for the maintenance of the embryonic MHB progenitor pool (Geling et al., 2003, 2004; Ninkovic et al., 2005) is selectively associated with adult neural stem cells at the border between midbrain and cerebellum. Although the embryonic MHB pool (IZ) is conserved among all vertebrates, until now no equivalent for either zone of adult neurogenesis, the TPZ or the IPZ, was found in any other vertebrate than fish. Thus it would be important to search for counterparts of these two zones in other vertebrate organisms, especially in mammals. This can be done by making use of the genes that show a function similar to *her5* in the embryo, like for example *Her1* and *Her3* in the mouse (Sasai, et al., 1992). If similar zones cannot be identified in other vertebrates it would be interesting to discover what the mechanisms are that maintain this zone in fish. To this aim, one could conduct conditional gain- and loss-of-function studies of Her5, by making use of already existing transgenic lines where *her5* or a dominat-negative version of *her5* are driven by the zebrafish heatshock promoter. Further investigations should also aim to determine the identity of neurons encoded by the her5 zone, and the potentialities of Her5-GFP positive progenitors upon transplantation.

More generally, Hairy/E(Spl) factors could highlight other stem cell zones in the adult brain, even perhaps in the telencephalon like Hes5 in mouse which was shown to be expressed at high levels in both the adult SVZ and in the dentate gyrus (Elliott et al., 2001;Stump et al., 2002) but it would be important to define whether this gene is also really expressed in type-B cells and not only in the transient amplifying type-C cells.

Function of adult neurogenesis

It is known for teleosts that the brain is growing throughout life, paralleling the growth of the body by addition of new cells. Brain growth could be in agreement with our findings of telencephalic progenitors contributing new neurons to the telencephalon proper, and with the two midbrain proliferation zones TPZ and IPZ contributing new neurons to the tectum and the tegmentum. In addition proliferation and neurogenesis

were also found in the cerebellum, the medulla oblongata and the hypothalamus (Zupanc and Horschke, 1995; Zupanc et al., 1998, 2005; Byrd and Brunjes, 1998; Ekström et al., 2001), likely also contributing to the growth of the zebrafish brain throughout life. But the findings that neurogenesis happens in different restricted niches in the brain and the new cells do not migrate over long distances suggest the existence of additional mechanisms contributing to brain growth. In addition to brain growth, adult neurogenesis might be involved in behavioral plasticity and learning. Along this line, it was shown that the environment has a high effect on the rate of neurogenesis in adult vertebrates: in songbirds, proliferation and neurogenesis increase seasonally and seem to contribute to song learning (Nottebohm et al., 1990; Barnea and Nottebohm, 1994, Alvarez-Buylla et al., 1997), in rodent females, olfactory neurogenesis increases during the period of pregnancy contributing new neurons to the olfactory bulb which may help the mothers to improve recognition of their babies by smell (Shingo et al., 2003). Further it was shown that an enriched environment or voluntary exercise has an effect on proliferation, neurogenesis and the survival of new neurons in the mouse as well (Kempermann et al., 1997; van Praag et al., 1999). Thus it is very likely that adult neurogenesis allows existing circuits to adapt to environmental changes. Therefore it would be of interest to study changes in proliferation and neurogenesis in the adult zebrafish following changes in the environment, and to study if different changes in the environment could lead to an effect in the different resticted niches in the whole brain. For example, one could think of modifying the acoustic, olfactory or visual stimuli on our fish, and measure whether this is followed by increased proliferation in progenitor zones or survival of the newborn neurons.

Lineage of adult stem cells

We have identified the IPZ via the expression of *her5*, also defining the embryonic MHB progenitor pool. Thus it is possible that adult IPZ neural stem cells develop from long-lasting embryonic MHB progenitors. This would be in agreement with findings in rodents, where astrocytes functioning as neural stem cells in the adult SVZ are directly related in lineage to radial glial cells from the embryonic telencephalic ventricle, the neuronal progenitors in the embryo (Frederiksen and McKay, 1988; McKay, 1997; Malatesta et al., 2000; 2003; Noktor et al., 2002, 2004; Götz and Barde, 2005). It is also striking that, like in adult birds, zebrafish telencephalic

progenitors display characteristics of glia (Alvarez-Buylla et al., 1998; Zupanc and Clint, 2003). In spite of this likely lineage relationship, adult precursor cells in fish, as already shown in the mouse, show characteristics different to those in the embryo. One major difference is that they proliferate slowly while embryonic neural stem cells are characterized by a fast-proliferation mode. Furthermore, our results highlight for telencephalic progenitors that the combination of transcription factors expressed in adult progenitors is distinct to those expressed in embryonic progenitors or that other enhancer elements are used to drive expression of the same genes. For instance, the acquisition of radial glial characteristics that appear only late in zebrafish embryos (Marcus and Easter, 1995) and the expression of *sox2*, a marker for neural stem cells, in both telencephalic and the midbrain proliferation zone, while in embryos its expression is rather late and non ubiquitous. In addition the combinatorial expression of transcription factors known to play key roles in early neurogenesis are not sufficient to describe the profiles of neurogenesis detected in late embryonic and adult stages. Thus it is likely that additional factors are involved in the regulation of late embryonic and adult neurogenesis. Therefore it would be important to identify these transcription factors in order to understand the molecular mechanisms underlying adult neurogenesis in vertebrates, and more generally to understand how the switch in proliferation properties is achieved within a common lineage between embryogenesis and adulthood.

Molecular effectors of late neurogenesis events

To add further data on this issue, we have used a degenerate RT-PCR approach and have cloned three novel bHLH-encoding genes. Two genes were cloned using oligonucleotides for the Atonal family, *atoh1.2* and a gene belonging to a distinct subfamily *beta3.1* (Appendix 1). Both genes are expressed in distinct populations of hindbrain precursors some of which are still proliferating in late embryonic stages and as observed for *atoh1.2* at the very tip of the valvula cerebelli in adults (data not shown) correlating with cells incorporating BrdU. Thus it would be interesting to test both genes for their likely function in proliferation and or neurogenesis in late embryonic and adult stages by conditional gain- and loss of function studies.

The third transcription factor was cloned using degenerate oligonucleotides of the Hairy/E(Spl) family and identified to be the zebrafish orthologue to mouse Heslike/Helt or Megane (Miyoshi et al, 2004; Nakatani et al., 2004; Guimera et al.,

2006) (Appendix 2). In mouse it was shown to be involved in the generation of midbrain GABAergic neurons. Zebrafish *helt* expression is largely similar to that in mouse of *Helt* at early embryonic stages suggesting a likely function in the generation of midbrain GABAergic neurons in the zebrafish as well. Surprisingly however zebrafish *helt* revealed a second phase of expression at late embryonic stages, at the edges of the tectum, reminiscent to described proliferation zones (Wullimann et al., 1999, 2000, 2002). This expression pattern differs from that of Heslike/Helt or Megane in the mouse and zebrafish Helt might have an additional or a different function in neurogenesis compared to its mouse orthologue. *Helt* expression is maintained in proliferation zones in the adult zebrafish midbrain as well, where it is expressed in proliferating cells (double-labeling with PCNA) in both zones, in glial cells (double-labeling with BLBP) predominantly neighbouring the IPZ, as well as in a subpopulation of neural stem cells (double-labeling with Her5-GFP) (Chapouton et al., 2006) in the IPZ. Thus *helt* seems to be the most promissing factor involved in neurogenesis in late embryonic, early larval and adult stages of the three new factors described in my project. The concrete function of Helt both during development and during adulthood still has to be determined. I have already establised a transgenic line (*8xHSEhelt::gfp*) in which helt is expressed under control of a heatshock promoter that can be used for overexpression studies. Preliminary data using this line show that Helt expression can be efficiently induced upon heatshock. Taken together our findings on the expression profile of *helt* in late embryonic and adult stages suggest that it is likely that zebrafish *helt* functions in a different way than its mouse orthologue. Its role is most likely in proliferation, neurogenesis and/or gliogenesis starting late during embryogenesis and perhaps persisting until adulthood. This hypothesis should be tested using conditional functional approaches, e.g. the administration of Grip- or Morpholino antisense oligonucleotides onto brain slices in culture, or transgenic lines where wild-type or dominant-negative versions of *helt* are driven by the zebrafish heatshock promoter. Further, it would be interesting to determine whether the *helt* expressing cells derive from cells expressing *helt* during embryogenesis, and we are currently setting up the CreERT system in zebrafish to address such lineage relationship issues.

Conclusion

Taken together, we have not only validated the zebrafish as a good model system to study adult neurogenesis, but we also identified several new zones and molecular players of adult neurogenesis that could be tested functionally in the adult brain using genetic approaches. A further advantage of zebrafish will be the amenability to focused genetic screens to recover mutants affected in late embryonic or adult neurogenesis. We are also taking advantage of gene or enhancer trap screens, to recover new genes expressed in adult precursors. This should be helpful to better understand the molecular mechanisms underlying adult neurogenesis and for the long run create therapies by manipulating these molecular mechanisms in order to regenerate tissue to help patients suffering of injury or neurodegenerative deseases.

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Appendix 1

Article in *Gene Expression Pattern*

***atoh1.2* and *beta3.1* are two new bHLH-encoding genes expressed in selective precursor cells of the zebrafish anterior hindbrain**

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atoh1.2 and *beta3.1* are two new bHLH-encoding genes expressed in selective precursor cells of the zebrafish anterior hindbrain

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Abstract

Transcription factors of the bHLH class play crucial roles in neurogenesis by controlling the location and timing of neuronal commitment and differentiation, as well as influencing neuronal identity. Proneural bHLH factors belong to the Olig, Neurogenin, NeuroD, Achaete-scute and Atonal subfamilies, and are expressed in partially overlapping or complementary patterns within the vertebrate embryonic neural tube. The combinatorial expression of these factors likely drives the generic and cell type-specific properties of neurogenesis throughout the nervous system. As an approach towards identifying a complete set of vertebrate proneural factors, we report here the isolation of two new zebrafish neural bHLH gene members, *atoh1.2* and *beta3.1*. Among other sites, both are expressed in the late embryonic and early larval anterior hindbrain. In this territory we demonstrate that *atoh1.2* and *beta3.1* are transcribed in distinct precursors, further highlighting the subdivision of anterior zebrafish hindbrain into subdomains of bHLH expression.

Keywords: bHLH; Atonal; atoh1.2; beta3.1; Zebrafish; Central nervous system

1. Results and discussion

1.1. Cloning of zebrafish atoh1.2 and beta3.1

To isolate new zebrafish proneural bHLH-encoding genes, we focused on the late embryonic and early larval midbrain-hindbrain domain. We followed a 3′RACE-RT-PCR approach on cDNA from embryos at 48 and 72 hours-post-fertilization (hpf), using degenerate oligonucleotides designed from the bHLH domain of Atonal-related transcription factors. cDNAs corresponding to two new bHLH genes were recovered. Blast analysis of the deducted full-length protein sequences revealed that these genes belong to two distinct bHLH subfamilies and encode a new Atoh1 (previously Ath1) protein, which we named Atoh1.2 (GeneBank AY581210), and a factor closely related to mammalian Beta3, which we named Beta3.1 (GeneBank AY581209) (Fig. 1A).

Atoh1.2 is highly related to Ath1 proteins of other vertebrates (82% identity in the bHLH domain, Fig. 1A,B), and most significantly to zebrafish Atoh1 (80.4% identity in the bHLH domain), renamed here Atoh1.1 (Fig. 1A,B).

Beta 3.1 is more closely related to Beta3 factors of other vertebrates (98% identity in the bHLH domain) (Fig. 1C) than to another Beta3 protein that we identified in the zebrafish genome (Ensembl Gene ID ENSDARG00000021061) (91.7% identity in the bHLH domain); this second zebrafish Beta3 protein is referred to as Beta3.2 (Fig. 1A,C).

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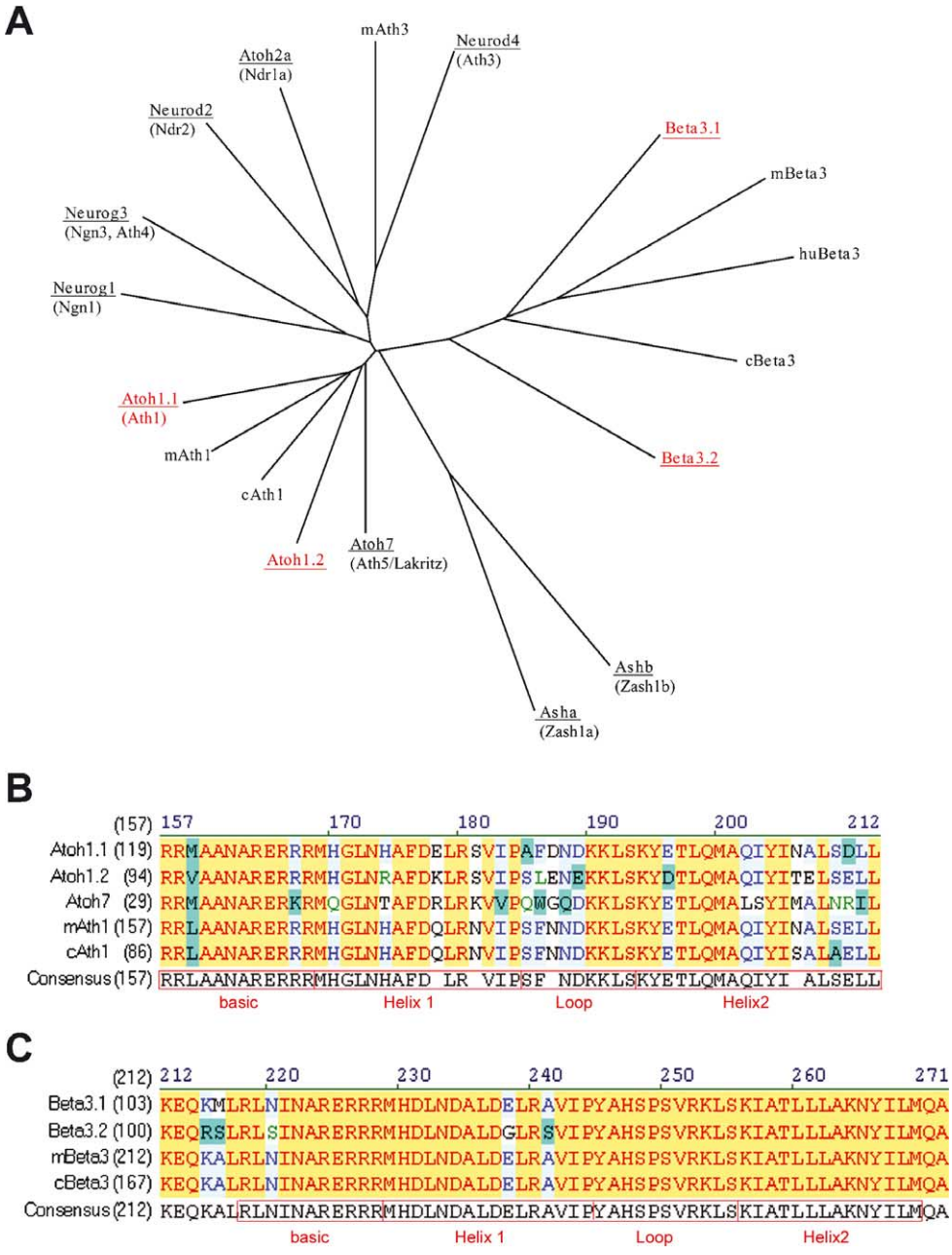

Fig. 1. (A) Unrooted tree (Vector NTI) established from the full length protein sequences of bHLH transcription factor from the Neurogenin (top left corner), Atonal (bottom left), Beta3 (top right) and Achaete-Scute (bottom right) families in different vertebrates. Zebrafish proteins are underlined (with previous nomenclature in brackets), and the factors of interest to this study are in red. Note that Atoh1.2 is a divergent member of the Atoh1 family, and that Beta3.1 is closer to Beta3 of other vertebrates than to zebrafish Beta3.2. The following sequences were used: Atoh1.1 (Genbank AAB82272), Atoh1.2 (GenBank AY591210 linked to Ensembl Sequence ID NA17.1.8369-8989), Atoh7 (Genbank NP571707) Atoh2a (Genbank NP571891), mouse Ath1 (Genbank BAA07791), chicken Ath1 (Genbank AAB41304), mouse Ath3 (Genbank O09105), Neurog1 (Genbank AAH66427), Neurog3 (Genbank NP571890), Neurod2 (Genbank NP571157), Neurod4 (Genbank AAH59424), Beta 3.1 (Genbank AY591209 linked to Ensembl Gene ID ENSDARG00000025861), Beta3.2 (Ensembl Gene ID ENSDARG00000021061), mouse Beta3 (Genbank NP067535), human Beta3 (Genbank AAM28881), chicken Beta3 (Genbank AAQ14265), Asha (Genbank NP571294), Ashb (Genbank NP571306). (B) Aligned sequences (Vector NTI) of the bHLH domain of zebrafish Atoh1.1, Atoh1.2, Atoh7, mouse Ath1 and chicken Ath1. Amino acids (aa) of each protein are numbered and the functional domains are boxed in red. Color code: yellow background: identical aa, green and light blue backgrounds: similar and conservative aa, green letters: weakly similar aa. (C). Aligned sequences (Vector NTI) of the bHLH domain of zebrafish Beta3.1, Beta3.2, mouse Beta3, chicken Beta3. Same code as in (B).

1.2. Expression of beta3.1 at embryonic and early post-embryonic stages

Mouse beta3 is exclusively expressed in highly restricted domains of the developing forebrain (Kim et al., 2002). In contrast, expression of zebrafish *beta3.1* was first detected at the tail-bud stage in two cell clusters of the anterior neural plate (data not shown). At 3 somites, these two clusters (Fig. 2A, blue staining, arrowhead) can be

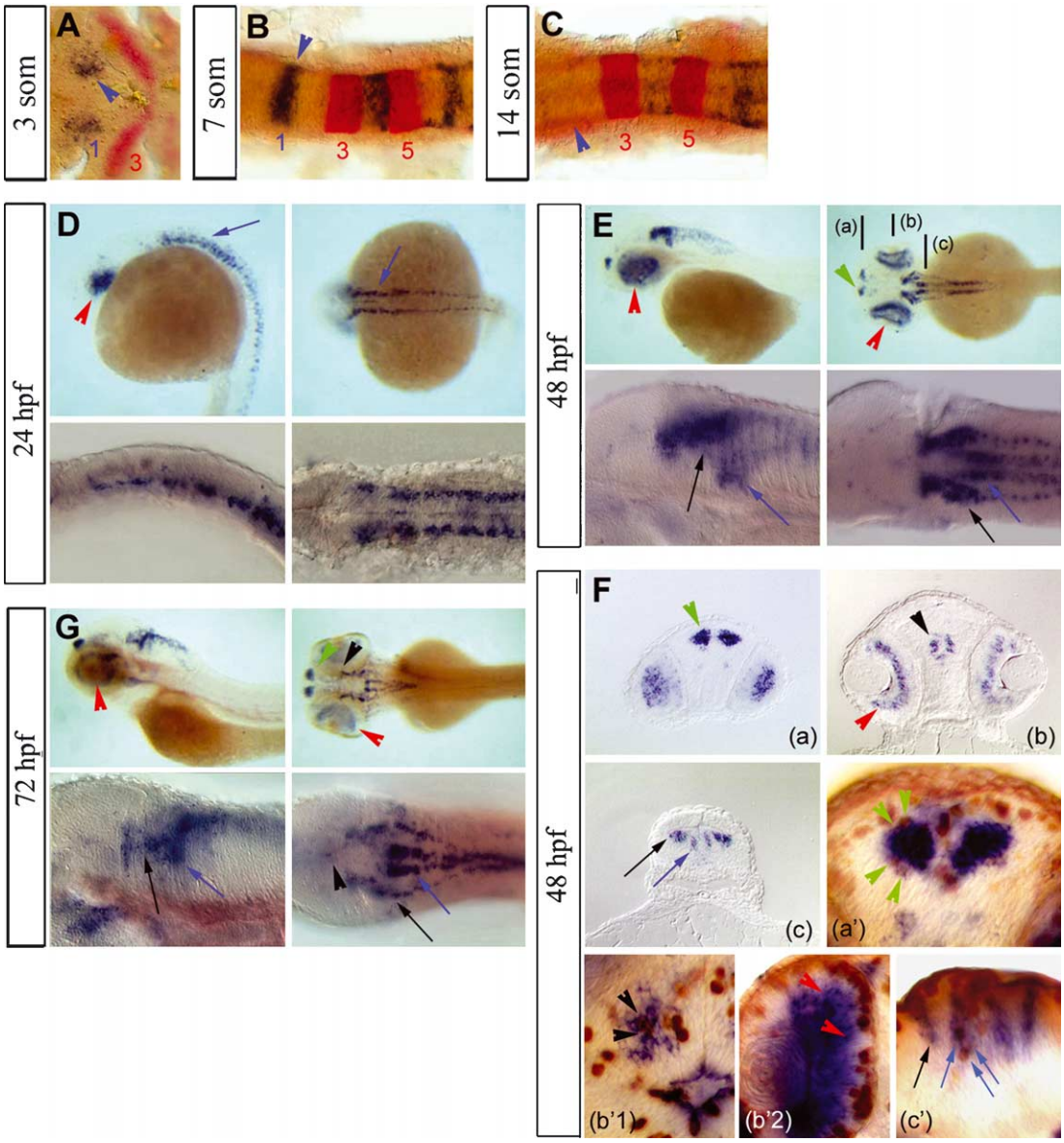

Fig. 2. Expression of beta3.1 revealed by in situ hybridization in zebrafish embryos and early larvae. (A–C) Compared expression of *beta3.1* (blue staining, blue arrowhead) and *krox20* (red) at the stages indicated, dorsal views of flat-mounted embryos, anterior left, locates earliest beta3.1 expression to rhombomere 1 (1). Rhombomeres 3 and 5, revealed by krox20 expression, are indicated (3,5). (D,E,G) Expression of *beta3.1* at the stages indicated (upper panels: whole-mount lateral and dorsal views, anterior left; lower panels: high magnification of the midbrain-hindbrain area, same orientation as in upper panels, flat-mounted embryos). (F) a–c: Frontal sections of the embryo showed in E at the levels indicated. a′–c′: frontal sections at the same levels processed for the co-detection of *beta3.1* RNAs (blue) and phospho-histone H3 protein (brown). Expression domains are color-coded: blue and black arrows: hindbrain and spinal cord interneurons, red arrowhead: retina, green arrowhead: olfactory pits, black arrowhead: nMLF. In a′–c′, arrowheads and arrows point to double-positive cells, except for the retina (red arrowheads) and lateral hindbrain stripe (black arrow), where no double staining was found.

localized to rhombomere 1 by comparison with *krox20*, restricted at that stage to rhombomere 3 (Fig. 2A, red staining). *beta3.1* expression in these clusters merges at the midline at 7 somites (Fig. 2B, arrowhead) and is switched off by 14 somites (Fig. 2C, arrowhead), by which stage prominent *beta3.1* expression is found in the more posterior hindbrain and spinal cord (Fig. 2C and data not shown).

At 24 hpf, posterior expression of *beta3.1* has resolved into broad dorso-lateral stripes of interneuron precursors in the rhombencephalon and spinal cord (Fig. 2D, blue arrow), starting from rhombomere 1 towards posterior. In addition, *beta3.1* is strongly expressed in the retina (Fig. 2D, red arrowhead). The latter expression is maintained until at least 72 hpf (Fig. 2E–G, red arrowheads), while new forebrain transcription domains arise at 48 hpf in the olfactory pits (green arrowheads) and the neurons of the medial longitudinal fascicle (nMLF) (black arrowheads) (Fig. 2E,G and sections Fig. 2Fa and b).

At 48 hpf, *beta3.1* expression in the rhombencephalon and spinal cord has subdivided into two longitudinal stripes of presumptive interneurons. The ventral interneuron population (Fig. 2E–G, blue arrow) extends

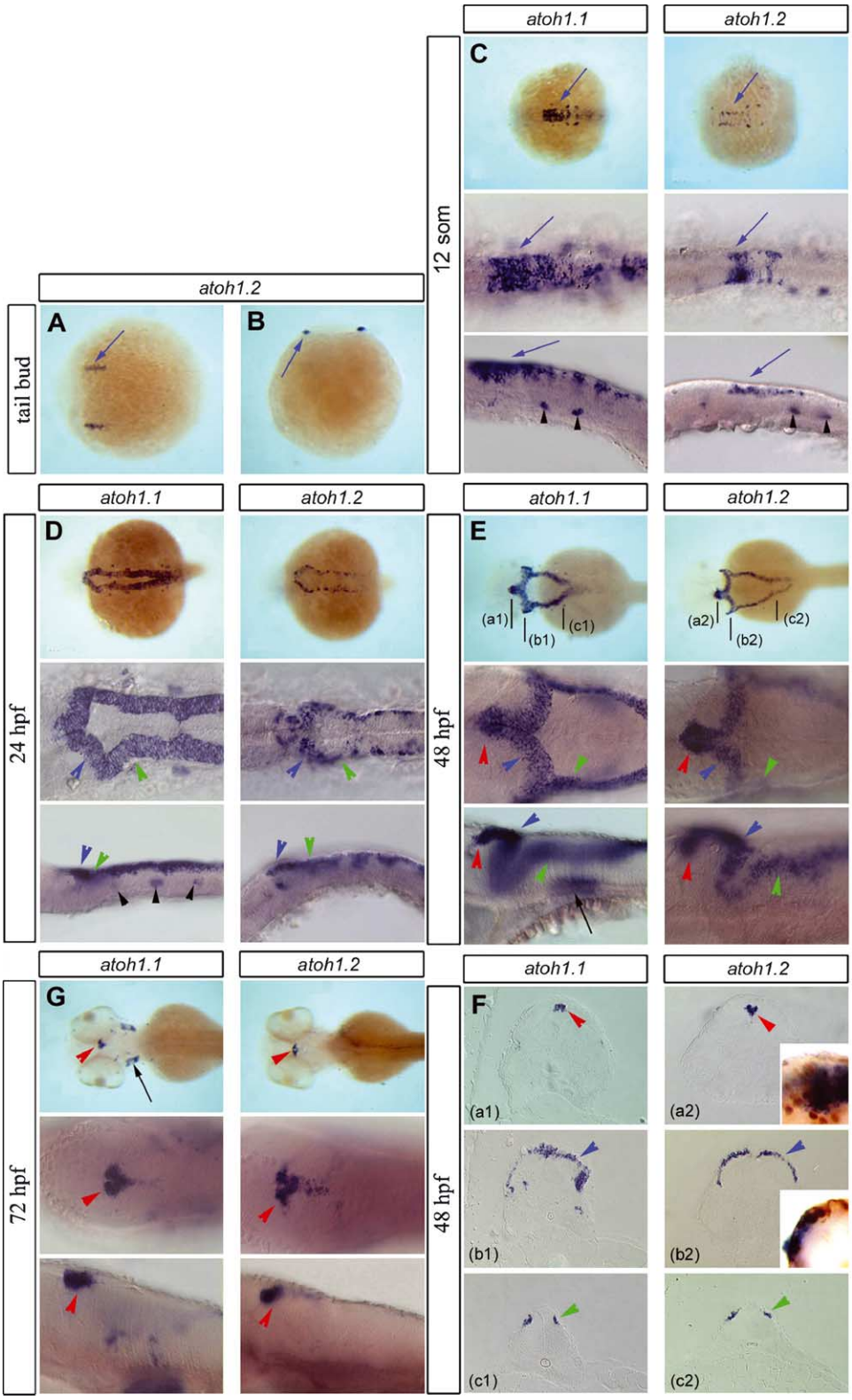

Fig. 3. Compared expression of *atoh1.2* and *atoh1.1* (probes indicated at the top of each panel) revealed by in situ hybridization at the stages indicated. (A,B). *ath1.2* expression precedes atoh1.1 and is found at tail-bud in two lateral cell groups (blue arrows) at hindbrain levels (A: dorsal view, anterior left; (B): frontal view, dorsal up). (C–E,G). Upper panels: whole-mount dorsal views, middle and bottom panels: dorsal and lateral high magnifications views, respectively, of the midbrain–hindbrain area in flat-mounted embryos. Expression domains are color-coded: blue arrow: dorsal hindbrain expression, which resumes from 48 hpf onwards into the upper rhombic lip (blue arrowhead), lower rhombic lip (green arrowhead) and presumptive valvula cerebelli (red arrowhead); black arrowheads: extremities of the otic epithelium (see Supplementary material); black arrow: otic vesicle (see Supplementary material). Note that after 24 hpf, *atoh1.2* is always expressed in a subset of *atoh1.1*-expressing cells. In addition, expression of *atoh1.2* in the presumptive valvula cerebelli extends further

from rhombomere 2 caudalwards, while the lateral stripe (Fig. 2E–G, black arrow) begins in rhombomere 1, reaching the midbrain-hindbrain boundary. Co-detection with phospho-histone H3 at 48 hpf reveals proliferating cells in all domains except the lateral interneurons stripe and the retina (Fig. 2a′–c′).

Together, *beta3.1* expression in the zebrafish embryo and early larva is mostly confined to the hindbrain precursors thus appears radically different from embryonic expression of mouse *Beta3* (Kim et al., 2002).

1.3. Compared expression of atoh1.2 and atoh1.1

Mouse *ath1* and zebrafish *atoh1* expression delineate the edge of the cerebellar plate and the rhombic lips (Koster and Fraser, 2001), and mouse Ath1 is essential for the genesis of cerebellar granule cells (Gazit et al., 2004). Expression of zebrafish *atoh1.2* largely precedes *atho1.1* and is first detected at the tail-bud stage in two stripes at the edge of the neural plate at the hindbrain level (Fig. 3A,B, blue arrow). These stripes resume at the 12-somite stage in scattered expression in the dorsal hindbrain (Fig. 3C, blue arrow) and at the anterior and posterior ends of the otic epithelium (Fig. 3C, black arrowheads) (see Supplementary material), at both sites as a subset of *atoh1.1*-expressing cells. At 24 hpf, *atoh1.1* displays its characteristic profile at the edge of the cerebellar plate (Fig. 3D, upper rhombic lips, blue arrowhead) and in the lower rhombic lips (Fig. 3D, green arrowhead). Expression of *atoh1.2* is restricted to the lateral subpopulation of *atoh1.1*-positive cells, leaving a few rows of unstained cells at the dorsal edge of the neural tube. These relative expression profiles are maintained at 48 hpf (Fig. 3E + sections Fig. 3F). Anteriormost expression of both *atoh1.1* and *atoh1.2* reaches the MHB in a dorsomedial cluster that contains precursors of the valvula cerebelli (Fig. 3E, red arrowhead). In the presumptive valvula, *atoh1.2* expression extends to a sightly deeper layer than *atoh1.1* (Fig. 3E + sections 3F and scheme Fig. 4A). In addition, no staining was detected for *atoh1.2* in the otic vescile, which strongly expresses *atoh1.1* (Fig. 3E, black arrow) (see Supplementary material). At this stage, all *atoh1.2*-positive domains contain proliferating precursors (insets in Fig. 3Fa2 and b2). At 72 hpf, expression of both genes in the central nervous system is exclusively maintained in a restricted group of cells containing valvula cerebelli precursors, at the junction of the cerebellum and the otic tectum (Fig. 3G, blue arrowhead). Again, the expression of *atoh1.2* encompasses deeper cells than *atoh1.1* (Fig. 3G). The absence of *atoh1.2* in the presumptive ear is maintained at that stage (Fig. 3G, black arrow).

1.4. Conclusions

The profiles of *beta3.1*, *atoh1.1* and *atoh1.2* are remarkable in that they all display a sharp limit at the MHB, staining anterior hindbrain but not posterior midbrain precursors. In addition, they highlight distinct and/or only partially overlapping precursor cell populations in the anterior hindbrain (Fig. 4), most of which are still proliferating. Together with the combinatorial expression of ngn1 (Blader et al., 1997) and ash1b (Allende and Weinberg, 1994; Mueller and Wullimann, 2003), these results permit to subdivide the anterior hindbrain into distinct subdomains of bHLH expression (Bertrand et al., 2002; Mueller and Wullimann, 2003).

2. Experimental procedures

2.1. Cloning of atoh1.2 and beta3.1

cDNA was reserved from total zebrafish RNA isolated from embryos of 2 dpf and 3 dpf (Chomczynski and Sacchi, 1987) using the Fermentas RevertAid kit. For the amplification of the two genes *ath1.2* and *beta3.1* the following oligonucleotides and conditions were used following the protocols of Frohman (Frohman, 1993): For the first PCR: 25 pmol primer Q_0: CCA GTG AGC AGA GTG ACG and the first degenerate primer for the Atonal family Ath F1: 5′ ATG CAY GGI YTI AAY CAY GCI TT 3′ (Y: C/T) 25 pmol in a final volume of 50 μl with two cycles at an annealing temperature of 43 °C and 28 cycles with an annealing temperature of 46 °C. For the nested PCR: 25 pmol primer Q_1: 5′GAG GAC TCG AGC TCA AGC 3′ and the second degenerated primer for the Atonal family Ath F2: 5′ AAY CAY GCI TTY GAY CAR YTI MG 3′ (Y: C/T; R: A/G; M: A/C) 25 pmol in a final volume of 50 μl with two cycles with an annealing temperature of 45 °C and 28 cycles with an annealing temperature of 47 °C. The resulting fragments of different sizes were subcloned in the pTopo vector and transformed in DH5α bacteria. The DNA of the different clones was sequenced on both strands and the sequences were analyzed using the NCBI Blast program.

2.2. In situ hybridization and immunocytochemistry

Whole-mount in situ hybridization was done on albino (*alb*/*alb*) embryos, all staged according to Kimmel et al. (1995). In situ hybridizations were done following standard protocols (Hauptmann and Gerster, 1994). For double stainings with the anti-phospho-histone H3 antibody (Upstate Biotechnology) the embryos were embedded in

ventrally than *atoh1.1*. (F). Frontal sections of the embryos showed in E at the levels indicated (a–c). Insets in a2 and b2 are high magnifications of sections at identical levels from embryos stained for *atoh1.2* (blue) and phospho-histone H3 (brown), demonstrating that the *atoh1.2*-positive domain contains proliferating cells.

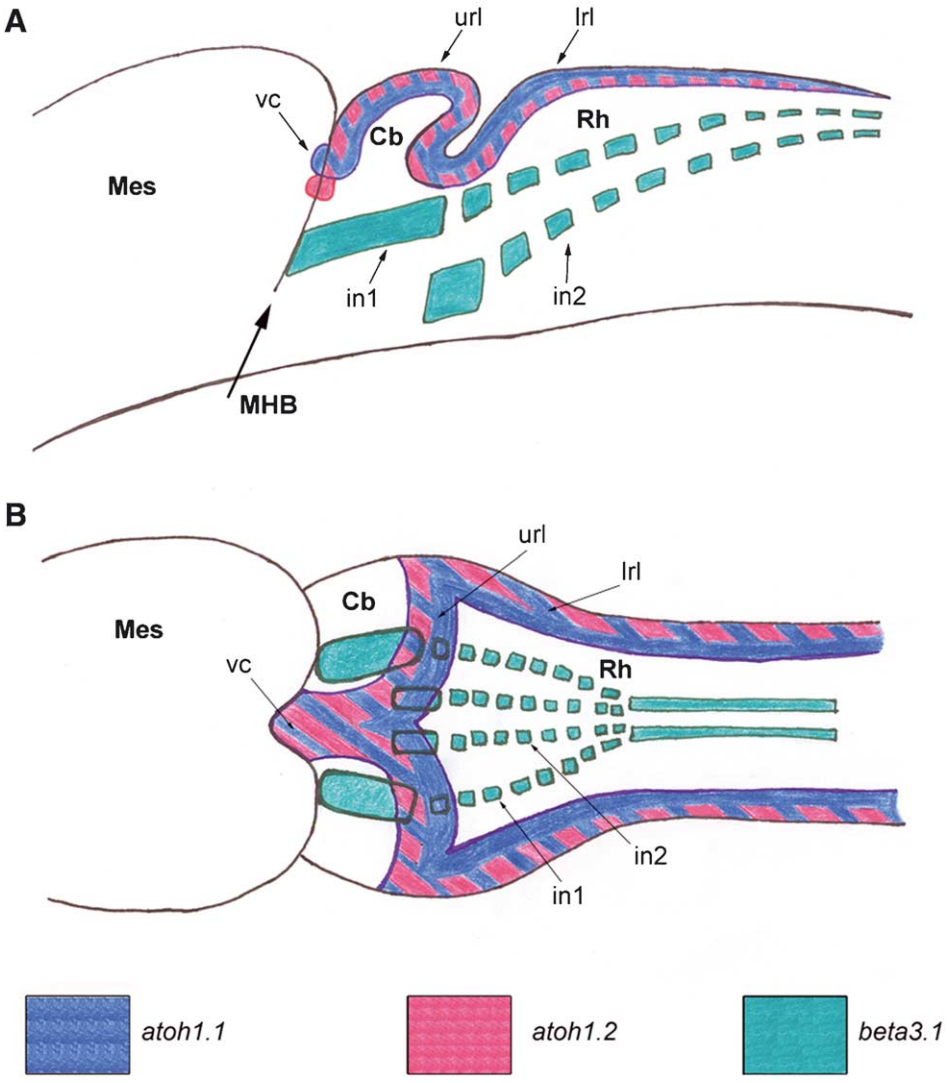

Fig. 4. Schematic lateral (A) and dorsal (B) representations of the relative expression domains of *beta3.1* (cyan), *atoh1.1* (blue) and *atoh1.2* (pink) in a 48-hour old hindbrain (anterior left). Abbreviations: Cb: cerebellar plate, in1-2: subpopulations of interneuron precursors, lrl: lower rhombic lip, MHB: midbrain-hindbrain boundary, Mes: mesencephalon, Rh: rhombencephalon, url: upper rhombic lip, vc: valvula cerebelli.

agarose following ISH, cut at 100 μm on a vibratome and processed for immunocytochemistry. Sections of 2 day-old embryos were done after in situ hybridization by embedding them in JB4 resin and cutting with a glass knife on an ultramicrotome. Sections and flat-mounted embryos were photographed under a Zeiss Axioplan microscope.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.modgep.2004.06.009

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Appendix 2

Manuscript in preparation

helt – a new bHLH-encoding gene expressed in proliferation zones in the central nervous system of late embryonic, early larval and adult stages

Birgit Adolf, Gianfranco Bellipanni, Stefanie Topp, Christian Stigloher, Kremmer Elisabeth and Laure Bally-Cuif

Abstract

bHLH transcription factors play a crucial role in the regulation of neuronal differentiation and neuronal or glial subtype specification. Here we report the molecular characterization and expression analysis of the zebrafish bHLH-encoding gene *helt*. Helt belongs to the Hairy/Enhancer of Split (Hairy/E(Spl)) family of which it shows the conserved characteristics of the bHLH and Orange domains. However, in contrast to other Hairy/E(Spl) factors, it harbors a lysine instead of a proline residue in its basic domain and lacks a C-terminal WRPW motif. These features identify Helt proteins to a distinct subfamily of Hairy/E(Spl) factors. *helt* expression is first detectable in the ventral part of the developing midbrain as well as in the pretectum, mimicking expression of mouse *Helt* at equivalent stages. Later however a new phase of expression is established in proliferation zones of the midbrain at late embryonic, early larval and adult stages. This expression suggests specific roles for zebrafish Helt compared to its mouse counterpart.

Introduction

Neurogenesis is tightly regulated in time and space by the expression of transcription factors of the bHLH class that play a crucial role in differentiation and cell fate decision in vertebrates and invertebrates (Bertrand et al., 2002; Kageyama et al., 1997, Tomita et al. 2000). At the onset of neurogenesis in the neural plate, proneural clusters become defined by a combination of positive and negative cues (Fisher and Caudy, 1998). Positive regulation of neurogenesis is characterized by the expression of proneural factors of the bHLH subfamilies Atonal and Neurogenin, acting as activators and thereby forcing the cells to commit to differentiation and becoming different types of neurons. In contrast, negative cues are encoded by bHLH factors of the Hairy/Enhancer of Split (Hairy/E(Spl)) family acting as transcriptional repressors that inhibit neuronal differentiation and keep the cells in a proliferative state. It has been shown that a number of Hairy/E(Spl) factors are involved in a process called “lateral inhibition” acting as downstream effectors for Notch signaling (Jarriault et al., 1995; Ohtsuka et al., 1999, 2001) by maintaining the number of undifferentiated neural progenitors and neural stem cells in the developing and adult central nervous

system (CNS) (Kageyama et al. 1999; Chapouton et al., 2006). Furthermore, combinations of different bHLH genes have been shown to increase the diversity of neuronal and glial subtypes.

In addition to the bHLH domain, factors of Hairy/E(Spl) subfamily are characterized by an Orange domain, two additional partly conserved helices just downstream of the bHLH domain, and by a C-terminal WRPW motif shown to recruit the TLE/Groucho corepressor mediating transcriptional repression activity. The function of the Orange domain is up to date poorly understood, but it is thought to function in subfamily specificity and/or in transcriptional repression and dimerization. The closely related Hey factors display a YRPW motif instead of WRPW motif. Together, these characteristics define the bHLH-O family.

Here we report the identification of a novel zebrafish bHLH-O transcription factor, called Helt, the zebrafish orthologue to mouse Heslike/Helt or Megane (Mgn) (Miyoshi et al., 2004; Nakatani et al., 2004; Guimera et al., 2006), defining a new distinct subfamily within the Hairy/E(Spl) family. We show that Helt is expressed in the midbrain region of the zebrafish CNS from early embryonic stages on to adulthood. Remarkably, expression of zebrafish *helt* is not identical to that of mouse *Helt* but displays an additional phase of expression in described midbrain proliferation zones at late embryonic / early larval stages (Wullimann et al., 1999, 2000; Müller and Wullimann, 2002) as well as in the adult (Chapouton et al., 2006).

Material and Methods

Cloning of zebrafish helt

cDNA was reserved from Poly(A)$^+$RNA isolated from wildtype zebrafish embryos at a stage of 48 hpf and 72 hpf (Chomczynski and Sacchi, 1987) using the Fermentas RevertAid kit. Following the protocols of Frohman (Frohman et al., 1993) PCR was performed on the cDNA template using degenerate primers designed from the conserved amino acidic sequence of Helix-2 in the bHLH domain of Hairy/Enhancer of split factors:

For the first PCR: 25pmol primer Q_0: 5’ CCA GTG AGC AGA GTG ACG 3’ and 25pmol of the first degenerate primer for the Hairy/E(Spl) family Hes F1: 5’ AAR YTI

GAR AAR GCI GAI ATH YTI G 3' (R: A/G;Y: C/T; I: A/T/C/G; H: A/T/C) in a final volume of 50µl with two cycles at an annealing temperature of 44°C and 28 cycles with an annealing temperature of 47°C. A nested PCR was performed using 25pmol of primer Q_1: 5' GAG GAC TCG AGC TCA AGC 3' and 25pmol of the second degenerate primer for the Hairy/E(Spl) family Hes F2: 5' ARG CIG AIA THY TIG ARH TIR CIG T 3' (R: A/G; Y: C/T; I: A/T/C/G; H: A/T/C) in a final volume of 50µl with two cycles with an annealing temperature of 45°C and 28 cycles with an annealing temperature of 48°C.

The resulting fragments of different sizes ranging from 200bp to 1000bp were subcloned into the pCR II TOPO vector (INVITROGEN) and transfromed in E. coli TOP 10 F' (INVITROGEN). Clones containing different DNA recognized by colony and restriction screen were sequenced on both strands. Sequence analysis were performed using the BLAST service of the NCBI (National Center for Biotechnology Information) and Ensembl.

Full-length cDNA of helt gene

Full-length cDNA of the *helt* gene was obtained using cDNA reserved from total RNA isolated from wildtype zebrafish embryos at stages when the gene is expressed. PCR was performed using primers designed for the C-terminal and N-terminal region of the gene (GenBank NM_207065.2; Ensembl ENSDARG00000056400). The resulting fragment was subcloned in the pCR II TOPO vector (INVITROGEN) and transformed into E. coli DH5α

Generation of Monoclonal Antibodies (mAbs) against Helt:

An internal peptide at the N-terminus of Helt (211RQSPPGHFSWHSSTRRPTC229) was synthesized and coupled to KLH or ovalbumin (PSL, Heidelberg). Rats were immunized with 50µg peptide-KLH using CPG 2006 (Tip Molbiol, Berlin) and IFA as adjuvant. After a six-week interval a final boost without adjuvant was given three days before fusion of the rat spleen cells with the murin myeloma cell line P3X63-Ag8.653. Hybridoma supernatants were tested in a differential ELISA with the specific peptide or an irrelevant peptide coupled to ovalbumin. Positive reacting hybridomas (IgG2a)

were further analysed by immunohistochemistry, and mABs 5D4 and 4A8 were found to specifically recognize Helt.

Fish strains

Embryos were obtained from wild-type zebrafish of the AB strain or Albino (*alb*/*alb*). and staged according to Kimmel et al. (1995). Adult brains were dissected from wild-type AB strain at an age of 5-7 months.

In situ hybridization

Whole-mount in situ hybridization (ISH) was done following the standard protocols (Hauptmann and Gerster, 1994) using digoxigenin- or fluorescein-labeled antisense RNA probes. Sections of two day old embryos were done after in situ hybridization by embedding in JB4 resin and cutting with a glass knife on an ultramicrotome.
ISH on adult brains was done as for whole-mount embryos, starting with whole-mount brains. Following hybridization and rinses the brains were embedded in 3% agarose in PBS and cut serially using a vibrating microtome (HM 650V, Microm) at 100µm before blocking and incubation in anti-digoxigenin antibody, as described for embryos.
For double ISH/immunodetections, embryos and brains were first processed for ISH, then for immunocytochemistry.
The following ISH probes were used: *helt*, *gad67* (Martin et al., 1998), *lim5* (Toyama et al., 1995) and *pax2.1* (Krauss et al., 1996).

Immunocytochemistry

Immunostainings were done on whole-mount or sectioned embryos and adult brain sections. The whole brains and embryos were embedded in 3% agarose in PBS and cut serially using a vibrating microtome (HM 650V, Microm) at 50µm in terms of embryos and 100µm in terms of adult brains. The sections were blocked with 0.5% Triton X-100 and 10% normal goat or normal chicken serum in PBS for 1 h at RT, then were incubated in the primary antibodies diluted in the block buffer at 4°C overnight. Primary antibodies were detected by subclass-specified secondary

antibodies diluted in the block buffer for 45’ at RT. The sections were then embedded in Aqua Polymount (Polyscience).
Primary antibodies used in this study: rat anti-Helt (1:2), rabbit anti-BLBP (1:1500; Feng et al.,1994), mouse anti-PCNA (1:250; DAKO), chicken anti-GFP (1:1000; aveslab). Secondary antibodies used to detect the primary antibodies mentioned above were labeled with: Cy2, Cy3, Cy5 or Alexafluo488, Alexafluo555, Alexafluo647.

Results

Cloning of zebrafish helt

To identify bHLH-encoding genes regulating neurogenesis late in zebrafish development we performed 3’RACE-RT-PCR on cDNA obtained from embryos of late embryonic (48 hpf) and early larval (72 hpf) stages. We used degenerate oligonucleotide primers designed from the bHLH domain of the Hairy/E(Spl) transcription factors and obtained one clone corresponding to a new zebrafish gene of this family expressed in the CNS.
This gene is annotated on chromosome 1 (GenBank NM_207065.2; Ensembl ENSDARG00000056400), and we could amplify a full length cDNA by RT-PCR using specific primers covering the first ATG codon and the Stop-codon as predicted. This cDNA contains a 813 bp open reading frame coding for 271 amino acids. It is distributed over 4 exons with the bHLH-encoding domain split between exon 2 and 3 and the two additional helices of the Orange domain encoded by exon 4.
Blast analysis of the deduced amino acidic sequence shows significant similarities to the vertebrate Hes/Her and Hey factors containing a bHLH and an Orange domain typical of the Hairy/E(Spl) subfamily (Fig. 1B). A phylogenetic tree based on the amino acidic sequences of these domains (Fig. 1A) reveals, within this subfamily, a higher homology to the Hey factors (Fig. 1A) and direct orthology to mouse Heslike/Helt/Mgn (Miyoshi et al., 2004; Nakatani et al., 2004; Guimera et al., 2006), thus we will refer to this factor as Helt. The sequence of the bHLH domain is highly conserved among the different subgroups of Hairy/E(Spl) factors (Fig. 1B) but the Helt proteins lack the conserved proline or glycine residue of the Hes/Her and Hey

factors in the basic region and, instead, harbor a lysine residue in this position (Fig 1B, asterisk). Since the basic domain is for a large part responsible for the specificity of DNA binding (Davis and Turner, 2001), Helt might bind to different gene regulatory sequences, thereby having different functions than Hes/Her and Hey factors. In addition, like its orthologues in other vertebrates, Helt lacks a C-terminal WRPW or YRPW sequence, conserved in Hes/Her and Hey factors (Fig. 1C). The WRPW motif recruits the TLE/Groucho corepressor (Paroush et al.,1994; Grbavec and Stifani, 1996) to mediate transcriptional repression from Hes/Her factors; therefore, Helt might show a different transcriptional activity. Along this line, it was shown that mouse Helt does not interact with any corepressor, thus, functions through a distinct mechanism than Hes/Her and Hey factors (Nakatani et al., 2006).

Spatio-temporal expression of Helt during embryogenesis and late larval stages

helt expression was first detectable by in situ hybridization at the stage of 5 somites (som) in a small, non-bilateral ventral cluster within the anterior neural tube (Fig. 2A). The onset of *helt* expression revealed by RT-PCR is first detectable at tailbud stage (data not shown). This expression domain rapidly expands along the anteroposterior and mediolateral axes. At 14 som, it can be localized to the ventral mesencephalon (presumptive tegmentum reaching into the alar-basal boundary), as shown by double in situ hybridization with *lim5* (a marker for the zona limitans intrathalamica (ZLI)) (Fig. 2C; green arrow) and *pax2.1* (which stains the midbrain hindbrain boundary (MHB)) (Fig. 2D; red arrow). Expression in this location is maintained at least until 72 hours post fertilization (hpf) (Fig. 2E-H, blue arrowheads, domain number 1), reaching the ZLI anteriorly and the MHB posteriorly at 24 hpf (Fig. 2F). Starting at 14 som, a more dorsal stripe of expression becomes visible (Fig. 2B, domain number 2), reaching neither the ZLI rostrally nor the MHB caudally (Fig. 2C, D). It is maintained until 48 hpf (Fig. 2F, G, red arrowheads) and can be localized to the pretectum. Starting at 48 hpf, a third expression domain is established (Fig. 2G, green arrowheads), which persists at later stages (Fig. 2H) and highlights the posterior, medial and lateral edges of the tectum. Cross sections through an embryonic brain at 48 hpf reveal that this expression domain is reminiscent to described tectal proliferation zones (Wullimann et al., 1999, 2000; Müller and Wullimann, 2002) (Fig.

2G,a-d, green arrowheads) this interpretation was confirmed by co-expression of *helt* revealed by in situ hybridization and PCNA (a marker for proliferating cells) revealed by Immunostaining on cross sections through an embryonic brain at 72 hpf in the tectal proliferation zones (data not shown).

Together, these results demonstrate two spatially distinct phases in *helt* expression. In a first phase (establishment of the ventral midbrain and pretectal *helt*-positive domains), *helt* expression follows a profile strongly reminiscent to the embryonic expression of mouse *Helt*. A difference lies however in the strong tegmental expression of zebrafish *helt* while mouse *Helt* transcripts in the ventral midbrain are restricted to the alar-basal boundary (Guimera et al., 2006; Miyoshi et al., 2004). During a second and persistent phase, an additional domain is established at the posterior, lateral and medial edges of the tectum (domain 3), which is not found for mouse *Helt* and suggests an additional role for zebrafish *helt* in tectal proliferation at late embryonic and early larval stages.

Expression of helt during adulthood

In situ hybridization with *helt* on sagittal sections of an adult zebrafish brain shows expression at the junction between the midbrain and the hindbrain (Fig. 3A). Along the anteroposterior axis, expression borders the edge between the caudal midbrain (torus semi-circularis and the posterior edge of the optic tectum) and the cerebellum (red arrowheads), and along the dorsoventral axis it lines the tectal ventricle underneath the torus and the valvula cerebelli (blue arrowhead). Both expression domains located within or adjacent to described proliferation zones: the tectal proliferation zone (TPZ), located at the junction between tectum and cerebellum and the isthmic proliferation zone (IPZ) along the tectal ventricle between the valvula cerebelli and the torus semi-circularis (Chapouton et al., 2006). To facilitate double-labeling for Helt expression and markers of cell proliferation or identity, we raised an antibody against zebrafish Helt. Specificity of this antibody for immunohistochemistry was verified by probing embryos overexpressing Helt upon *helt* RNA injection or upon activation of a *hsp:helt* transgene (not shown). Immunostaining using the Helt antibody shows the same expression domains as the RNA probe (Fig 3B, C, white and yellow arrowheads) but in addition reveals expression of Helt in the roof of the tectal ventricle (Fig 3C, green arrowhead). Taking advantage of the *her5pac:egfp*

transgenic line (Tallafuss and Bally-Cuif, 2003), which expresses Her5-GFP in slow proliferating cells in the IPZ in adults (Chapouton et al., 2006), we tested the relationship between Helt expression and proliferation. We performed triple stainings with Helt, the proliferation marker PCNA and Her5-GFP and observed a restricted overlap between expression of Helt and Her5-GFP (Fig. 3D, yellow arrowheads) or proliferating cells in the IPZ (Fig. 3D, pink arrowheads). In contrast, the vast majority of Helt-expressing cells in the TPZ co-expresses PCNA (Fig. 3E, pink arrowheads). Triple stainings with Helt, Her5-GFP and the radial glial marker BLBP (Feng et al., 1994) further revealed that the vast majority of Helt-expressing cells neighbouring the IPZ express BLBP (Fig. 3F). In this location, it remains possible however that only a subpopulation of BLBP-expressing cells is positive for Helt (Fig 3G, pink arrowheads and data not shown).

Together, these observations suggest a role for zebrafish Helt within two proliferation zones of the adult midbrain, as well as in adjacent and partially overlapping populations of radial glia. These results contrast again with expression of mouse *Helt*, restricted to the subependymal zone and rostral migratory stream of the adult telencephalon (Guimera et al., 2006).

Conclusion and Discussion

Here we report about the identification and isolation of a novel zebrafish bHLH transcription factor-encoding gene of the Hairy/E(Spl) family, named *helt.* The protein sequence of the gene unambigously identifies the new factor as a direct orthologue to mouse Helt/Heslike or Mgn (Naktani et al., 2004; Miyoshi et al., 2004; Guimera et al., 2006) and together with other vertebrate Helt-factors it can be grouped to a distinct subfamily to the Hes/Her and Hey factors, refered to as Helt proteins. Like in the mouse and also in other vertebrate organisms it seems that there exists only one *helt* gene in the zebrafish genome (analysis of Ensembl zebrafish genome release zv7).

Expression analysis of *helt* mRNA demonstrates two spatially distinct phases in *helt* expression. In a first phase, during early embryonic development, *helt* expression is initiated in the ventral part of the neural tube and later establishes 2 domains, one in the ventral midbrain and one in the pretectum. During this first phase *helt* expression is reminiscent of the expression profile of its mouse orthologue *Helt*/*Heslike* or *Mgn*.

In mouse it was shown that *Helt* and *Mash1* are coexpressed in regions giving rise to GABAergic neurons and that the combinatorial expression of both genes is required to promote the formation of GABAergic neurons in the midbrain (Miyoshi et al., 2004). Along this line, lack of *Helt* expression triggers complete loss of GABAergic neurons in the superior colliculus in mutant mice whereas in other regions GABAergic neurons are generated normally, further suggesting that Helt is a key regulator for the proper specification of GABAergic neurons in the dorsal midbrain (Guimera et al., 2006). Although *helt* expression in zebrafish at early embryonic stages is very similar to the expression profiles of *Helt* in the mouse, there is one differences that lie, on the one hand, in the strong expression of zebrafish *helt* in the tegmentum while *Helt* expression in the mouse is restricted to the alar-basal boundary (Guimera et al., 2006; Miyoshi et al., 2004) and, on the other hand, in the absence of coexpression of *helt* and *Gad67* at early embryonic stages. Together this suggests that zebrafish Helt might function in a distinct way than its mouse counterpart. It would be of great interest to test the function of Helt during early embryonic development using genetic approaches. Gain-of-function experiments could be done via injection of *helt* mRNA. Complementarily, the early function of Helt should be assessed in loss-of-function experiments via Grip- or Morpholino knock-down.

A striking difference with the mouse situation arises later during zebrafish embryonic development, when a second and persistent phase of *helt* expression is established at the posterior, lateral and medial edges of the tectum, reminiscent of described proliferation zones (Wullimann et al., 1999, 2000; Müller and Wullimann, 2002). This expression was not reported for mouse *Helt*. In addition we could show that *helt* expression is still detectable within the two midbrain proliferation zones even in the adult brain (Chapouton et al., 2006) in contrast to its mouse counterpart that is restricted to the subependymal zone and rostral migratory stream of the adult telencephalon (Guimera et al., 2006). Using ISH and Immunostaining we could not detect *helt* expression in the telencephalon of adult zebrafish. In the TPZ Helt is mostly expressed in proliferating cells while in the IPZ it is detectable in a small fraction of slowly dividing stem cells or proliferating cells whereas the vast majority of Helt-expressing cells is neighbouring the IPZ and co-expresses BLBP. Taken together these results suggest an additional likely function of *helt* in tectal proliferation and neurogenesis and/or gliogenesis, starting at late embryonic stages and perhaps persisting until adulthood. This hypothesis should be tested using

conditional functional approaches, e.g. the administration of Grip- or Morpholino antisense oligonucleotides onto brain slices in culture, or transgenic lines where wild-type or dominant-negative versions of *helt* are driven by the zebrafish heatshock promoter. Further, it would be interesting to determine whether the *helt* expressing cells in the adult derive from cells expressing *helt* during embryogenesis, and we are currently setting up the CreERT system in zebrafish to address such lineage relationship issues.

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Legends to Figures

Figure 1. Sequence comparison of Helt and related bHLH transcription factors of the Hairy/Enhancer of Split family:

(A) Unrooted phylogenetic tree (PAUP, Parsimony) of Hairy/E(Spl) proteins on the basis of the protein sequences of the bHLH and Orange domains of the Hes/Her (left side), Hey (top right corner) and Helt subfamilies (bottom right corner) in zebrafish and mouse. Zebrafish proteins are underlined and the factor of interest in this study is labeled and boxed in red. Note that Helt belongs to a distinct subfamily from the Hes/Her and Hey factors. **(B)** Aligned protein sequences of the bHLH and Orange domain of Helt and its related factors (Alignment ClustalW, modified by MacClade and visualized on the basis of similarity by Jalview). Note that the proline and glycine residues conserved among Hes/Her and Hey factors within the basic region are altered to a lysine residue in zebrafish Helt (indicated in red) and its mouse orthologue (asterisk). Alignment of the protein sequences of the Orange domains shows a lower sequence homology of Helt, Hes/Her and Hey factors. **(C)** Comparison of the protein sequences of vertebrate Helt factors shows a very high conservation in the bHLH and Orange domains in vertebrates like zebrafish, fugu,

mouse, rat and human. Note that the C-terminal tetrapeptide WRPW-YRPW of Hes/Her and Hey factors is missing within the Helt subfamily.

The following sequences were used for the phylogenetic tree (A) and the alignments (B/C): DrHelt (GenBank NP_996948; Ensembl ENSDARP00000073348), fuguHelt (Ensembl NEWSINFRUG00000151870), mHelt/Heslike/Megane (Ensembl ENSMUSP00000054823), rHeslike (Ensembl ENSRNOP00000014360), hHeslike (Ensembl ENSP00000343464), DrHer8a (GenBank NP_955918), DrHer9 (GenBank NP_571948), mHes1 (GenBank NP_032261.1), mHes2 (GenBank NP_032262.2), DrHeyL (GenBank NP_859425.1), DrHey2 (GenBank NP_571697), mHey1 (GenBank NP_034553), mHey2 (GenBank NP_038932).

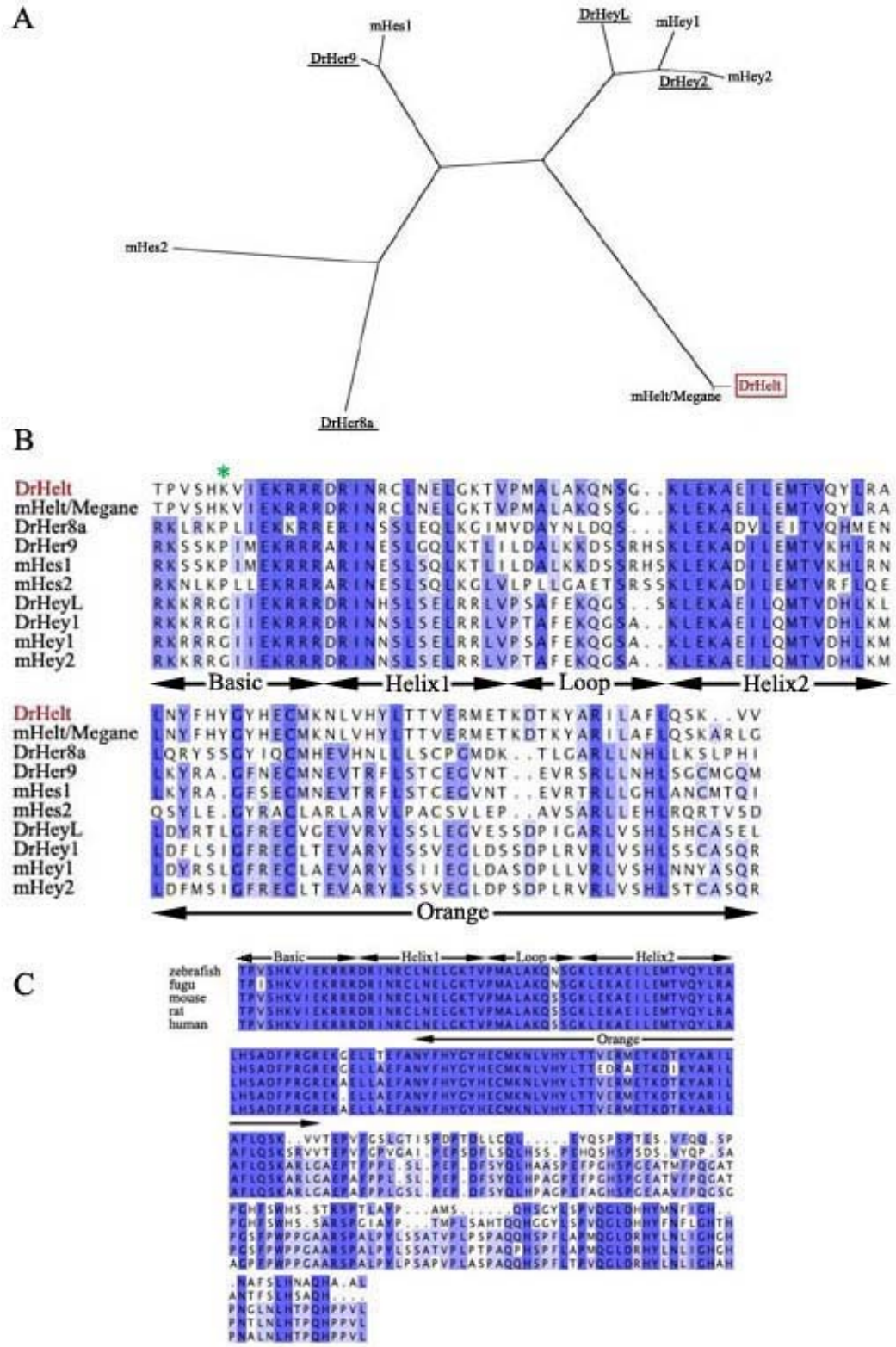
Figure 2. **Expression of *helt* revealed by in situ hybridization in zebrafish embryos and early larvae:**

All panels except a-d depict whole-mount embryos, anterior left, in lateral (A-E, and F-H left panels) or dorsal (F-H, right panels) views. a-d are cross sections at the level indicated in G, dorsal up.

(A, B) Expression of *helt* (blue staining) is first detectable in the basal plate of the anterior neural tube at 5 som **(A)** and expands along the anterorposterior axis at 14 som **(B,** domain 1**)**. At that stage, a second domain becomes established (2). **(C, D)** Double in situ stainings with *helt* (blue arrow) in comparison to *lim5* (**C**, green arrow) and pax2.1 (**D**, red arrow) at 14 som. The major domain of *helt* expression is located within the mesencephalon between the ZLI and the MHB. **(E, F)** At 20 som and 24 hpf, *helt* expression in domains 1 and 2 intensifies and expands. Domain (1) reaches the ZLI rostrally (blue arrowhead) and the MHB caudally (green arrowhead), domain (2) reaches the pretectum (red arrowhead). **(G, H)** After 48 hpf, a third domain is established (green arrowhead) along the posterior, lateral and medial edges of the tectum. Expression reaches the ZLI rostrally (blue arrowhead) and the MHB caudally (green arrowhead). Corresponding arrowheads are used in sections a-d. Abbreviations: teg: tegmentum; TeO: optic tectum

Figure 3. **Expression of Helt revealed in the adult zebrafish brain**

All views are from sagittal sections of the adult brain at midbrain level, anterior left.
(A) *helt* expression revealed by in situ hybridization is located in the midbrain lining the boarder between the tectum and the cerebellum (red arrowheads) and along the posterior aspects of the tectal ventricle at the level of the valvula cerebelli (blue arrowhead). **(B, C)** The same domains are labeled by the Helt antibody (white and yellow arrowheads), but in addition, likely due to better sensitivity of the immunohisotchemistry, Helt expression is detectable in the roof of the tectal ventricle (green arrowhead). **(D-G)** Triple immuno stainings (Helt, red; PCNA/BLBP, blue; Her5-GFP, green) on *her5pac:egfp* transgenic brains in projections and single confocal planes (insets) reveal that Helt expression is partially overlapping with the proliferations zones IPZ and the TPZ (pink arrowheads to double Helt/PCNA-positive cells in **D**, **E**, and yellow arrowheads to Helt/Her5-GFP-positive cells in **D**). Next to the IPZ, however, in a zone that is mostly PCNA-negative, Helt is largely coexpressed with BLBP (pink arrowheads to double-labeled cells in **F**, **G**).
Abbreviations: Cb: cerebellum; IPZ: isthmic proliferation zone; TeO: optic tectum; TPZ: tectal proliferation zones; Val: valvula cerebelli.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mHes2
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DrHelt
mHelt/Megane
DrHer8a
DrHer9
mHes1
mHes2
DrHeyL
DrHey1
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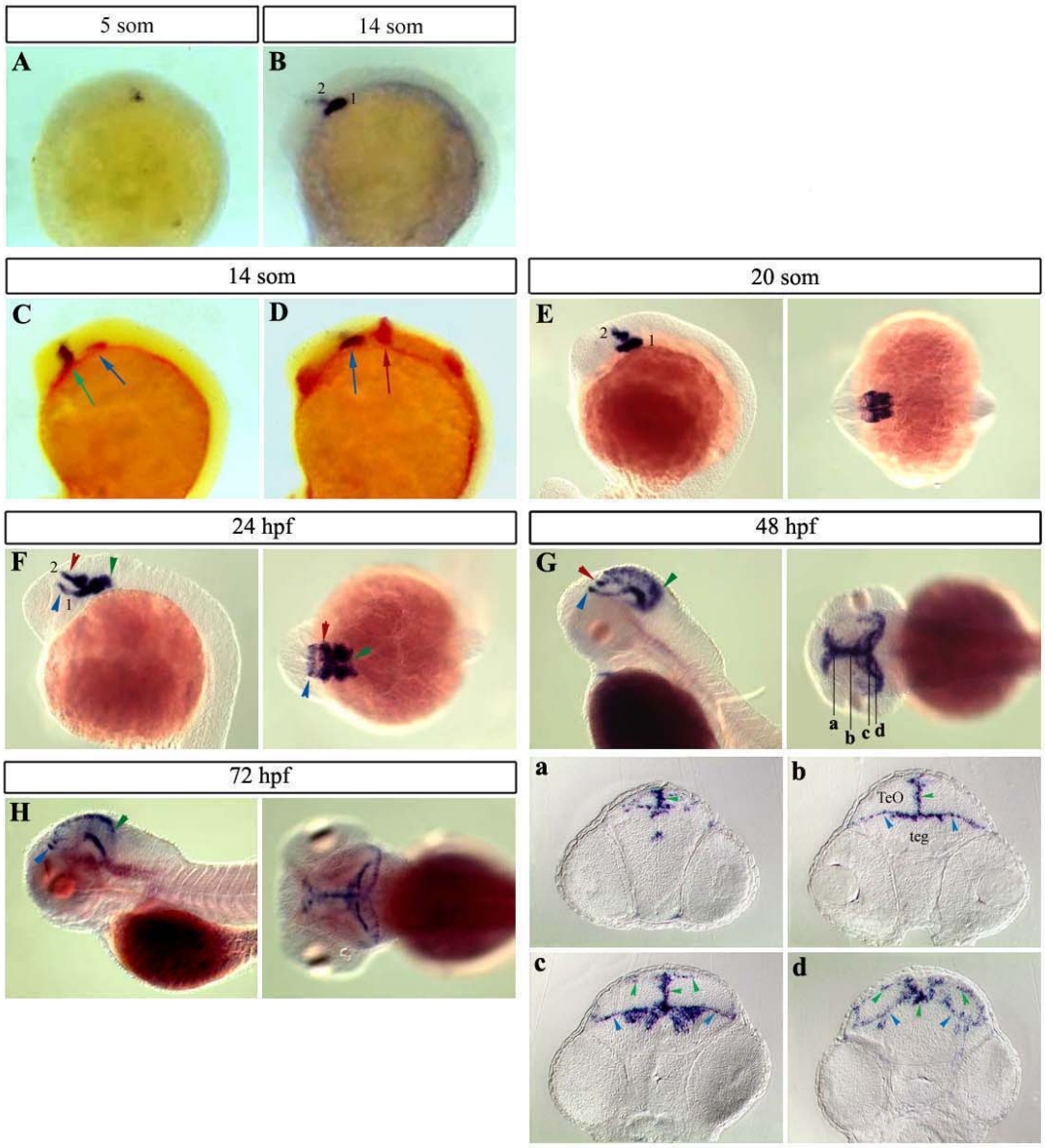
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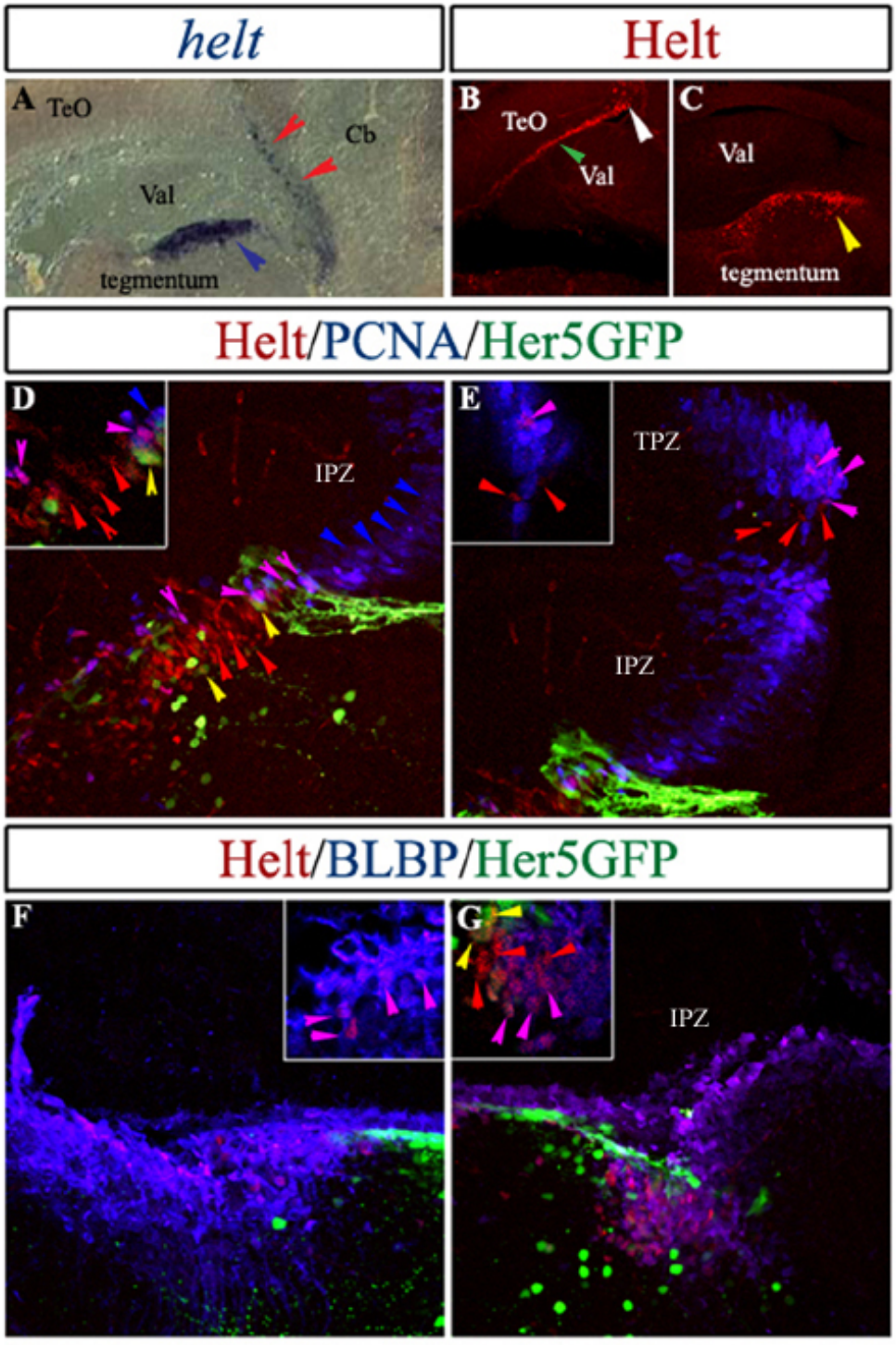
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Helt/PCNA/Her5GFP
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Helt/BLBP/Her5GFP
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Appendix 3

Article in *Developmental Biology*

Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon

Birgit Adolf, Prisca Chapouton, Chen Sok Lam, Stefanie Topp,
Birgit Tannhäuser, Uwe Strähle, Magdalena Götz and Laure Bally-Cuif

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Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon

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Abstract

Our understanding of the cellular and molecular mechanisms underlying the adult neural stem cell state remains fragmentary. To provide new models on this issue, we searched for stem cells in the adult brain of the zebrafish. Using BrdU tracing and immunodetection of cell-type-specific markers, we demonstrate that the adult zebrafish telencephalon contains self-renewing progenitors, which show features of adult mammalian neural stem cells but distribute along the entire dorso-ventral extent of the telencephalic ventricular zone. These progenitors give rise to newborn neurons settling close to the ventricular zone within the telencephalon proper. They have no equivalent in mammals and therefore constitute a new model of adult telencephalic neural stem cells. In addition, progenitors from the ventral subpallium generate rapidly dividing progenitors and neuroblasts that reach the olfactory bulb (OB) via a rostral migratory stream and differentiate into GABAergic and TH-positive neurons. These ventral progenitors are comparable to the mammalian neural stem cells of the subependymal zone. Interestingly, dorsal and ventral progenitors in the adult telencephalon express a different combination of transcription factors than their embryonic counterparts. In the case of *neurogenin1*, this is due to the usage of different enhancer elements. Together, our results highlight the conserved and unique phylogenic and ontogenic features of adult neurogenesis in the zebrafish telencephalon and open the way to the identification of adult neural stem cell characters in cross-species comparative studies.

Keywords: Zebrafish; Adult neurogenesis; Neural stem cell; Telencephalon; Olfactory bulb; Radial glia

Introduction

The continued production of neurons at adulthood has been observed in most vertebrates. However, our knowledge is mostly derived from two mammalian neural stem cell models, the subependymal zone (SEZ) of the lateral wall of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus, where slow-dividing long-lasting progenitors with astrocytic characteristics give rise to newborn neurons (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch and Hen, 2005; Garcia et al., 2004; Goldman, 2003; Kempermann, 2002; Lledo and Saghatelyan, 2005; Merkle et al., 2004; Taupin and Gage, 2002). Neuronal differentiation from the SEZ is preceded by a transient amplification phase and by the migration of committed neuronal progenitors towards the olfactory bulb (OB) along the rostral migratory stream (RMS). Newborn OB neurons, mostly GABAergic interneurons, might help discriminate odorant stimuli (Lledo and Saghatelyan, 2005).

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Many open questions remain on the biology of adult neural stem cells, for example, the characteristics of their "niche" of origin (Alvarez-Buylla and Lim, 2004) or the cell-intrinsic factors maintaining their undifferentiated character or orienting their commitment. Among other transcription factors (Molofsky et al., 2003, 2005; Shi et al., 2004), Sox2 was implicated in attributing stem cell properties, both in the SEZ and SGZ (Episkopou, 2005; Ferri et al., 2004). Also, Olig2 promotes the transient-amplifying precursor state in the SEZ and RMS, and Pax6 the commitment towards a TH-positive neuronal fate in the OB (Hack et al., 2005; Kohwi et al., 2005).

Adult neurogenesis is tightly restricted to the SEZ and SGZ in mammals but appears more widespread in other vertebrates like reptiles (Garcia-Verdugo et al., 2002), birds (Goldman, 1998), and fish (Zupanc, 2001). These models may yield insight into the mechanisms generating other neuronal subtypes than OB or hippocampal granule interneurons in the adult brain. Moreover, by comparison with mammals, they should highlight general versus derived properties of adult neurogenesis, thus crucial elements of neural stem cell maintenance. In teleost fish, proliferation and neurogenesis occur throughout life, correlating with long-lasting brain growth and a high capacity for regeneration (Zupanc, 2001). Proliferation sites are restricted to discrete foci, suggesting the existence of niches. In the zebrafish adult brain, newborn neurons form in the OB, dorsal telencephalon, hypothalamus, preoptic area, optic tectum, and cerebellum (Byrd and Brunjes, 2001; Cameron, 2000; Goldman et al., 2001; Zupanc et al., 2005). However, the location, identity and molecular characteristics of the progenitors involved remain unresolved. Here, we characterize self-renewing progenitors in the adult zebrafish telencephalon. We show that these progenitors are located both in dorsal and ventral telencephalic domains and express neural stem cell-like characteristics and a unique combination of molecular markers. We demonstrate that OB neurogenesis in zebrafish shares many features with that in mammals, validating the zebrafish model to study vertebrate adult neurogenesis. Finally, we identify a novel stem cell population in the dorsal telencephalon, providing a new niche to examine. Our findings establish new neural stem cell models in a genetically tractable animal, setting the stage for detailed comparative studies and forward genetics approaches on vertebrate adult neurogenesis.

Materials and methods

Fish strains

5- to 9-month-old wild-type zebrafish (*Danio rerio*) of the AB strain, or transgenic zebrafish (*-3.4ngn1:gfp*, *-3.1ngn1:gfp*) (Blader et al., 2003; Shin et al., 2003) in AB or ABO background were used.

BrdU labeling

To label cells in S phase, the fish were injected intraperitoneally with 50 µl/g body weight of the thymidine analog bromo-deoxy-uridine (BrdU) diluted in 110 mM NaCl pH 7.0 twice within 2 h. BrdU is taken up by cells in the S phase of mitosis and detectable by immunocytochemistry (Gratzner, 1982). The clearance time of BrdU has been estimated around 4 h in adult fish (Zupanc and Ott, 1999); thus, we injected BrdU twice with a 2-h interval followed by a survival time of 2 h after the last injection, to map cells undergoing division at the time of BrdU exposure. Other survival times ranged between 4 h and 8 weeks after the first pulse. Then the fish were anesthetized with Tricaine before they were killed in ice water. The brains were dissected out and fixed in 4% paraformaldehyde solution at 4°C for 4 h, then progressively dehydrated in MeOH and stored in 100% MeOH at −20°C.

Immunohistochemistry

Immunostainings were performed on 100-µm sections. The whole brains were embedded in 3% agarose in PBS and cut serially using a vibrating microtome (HM 650 V, Microm). The sections were blocked with 0.5%Triton X-100 and 10% normal goat serum in PBS for 1 h at RT, then were incubated in the primary antibodies diluted in the block buffer at 4°C overnight. Primary antibodies used in this study were rabbit anti-BLBP (1:1500; Feng et al., 1994), mouse anti-BrdU (1:100; Roche), rat anti-BrdU (1:200; abcam), rabbit anti-GFAP (1:100; DAKO), rabbit anti-GFP TP401 (1:500; ams), mouse anti-Hu C/D (1: 300; Molecular Probes), rabbit anti-Pax6 (1:300; Babco) mouse anti-PCNA (1:250; DAKO), mouse anti-PSA-NCAM (1:600; Chemicon). Primary antibodies were detected by subclass-specific secondary antibodies labeled with Cy2, Cy3, or Cy5 or enhanced with the tyramide amplification kit (PerkinElmer). The sections were then embedded in Aqua Polymount (Polyscience). Immunodetection of BrdU required a pretreatment with 2 M HCl followed by washes with borate buffer and PBS before the sections were incubated in anti-BrdU antibody. Double immunodetection with rabbit anti-Pax6 and rabbit anti-TH antibodies was performed following the protocol of the tyramide amplification kit (PerkinElmer), as follows. The primary antibody against Pax6 was diluted in blocking buffer to 1:5000 and amplified with the secondary antibody solution (anti-rabbit biotinylated 1:250 in block buffer). Following incubation in streptavidin–HRP (1:100 in PBS), the sections were incubated in tyramide solution coupled with either FITC or TRITC (1:30) for 15 min. Staining for TH was then performed following the standard protocol.

In situ hybridization

In situ hybridization was performed as for whole-mount embryos (Hauptmann and Gerster, 1994), starting with whole-mount brains. Following hybridization and rinses, the brains were then embedded in 3% agarose in PBS and cut serially using a vibrating microtome (HM 650 V, Microm) at 100 µm before blocking and incubation in anti-digoxigenin antibody, as described for embryos. For double ISH/immunodetections, the brains were first processed for ISH, then for immunocytochemistry. The following ISH probes were used: *dlx2a* (former *dlx2*) (Akimenko et al., 1994), *emx3* (former *emx1*) (Morita et al., 1995), *fabp7a* (Liu et al., 2003a), *gad67* (Martin et al., 1998), *ngn1* (Blader et al., 1997), *olig2* (Park et al., 2002), *pax6a* and *pax6b* (Nornes et al., 1998), *ash1a* (Allende and Weinberg, 1994), and *sox2* (PCR-amplified from GeneBank CF416982). All sections were photographed and analyzed under a Zeiss Axioplan microscope or a Zeiss confocal microscope (LSM 510 META).

Results

The zebrafish adult telencephalon contains distinct subpopulations of proliferating cells

Distinct proliferation sites have been described in the teleost adult brain (Ekstrom et al., 2001; Zupanc, 1999; Zupanc and Horschke, 1995), such as, in zebrafish, the retina (Cameron, 2000; Goldman et al., 2001), olfactory bulb (Byrd and Brunjes, 1998), telencephalon and cerebellum (Zupanc et al., 2005), hindbrain or spinal cord (Goldman et al., 2001). However, with the exception of the cerebellum (Zupanc et al., 2005), it has not been determined which of these sites give rise to neurons and/or glia. Here, we used BrdU-birth-dating to map the sites of proliferating cells and the fate of their progeny.

Sacrifice of animals 2 h after two times BrdU injections (see Materials and methods) allows detection of actively proliferating cells. Consistent with previous data (Zupanc et al., 2005), we observed that cell division was abundant in the adult zebrafish brain, with several prominent sites (Fig. 1A). The zebrafish telencephalon is commonly divided into the dorsally located pallium and the basally located subpallium; the pallium is itself subdivided into posterior, lateral, dorsal and medial domains, and the subpallium into dorsal and ventral components (Wullimann et al., 1996) (see Fig. 2F). In the telencephalon, the ventral subpallium was intensely labeled, appearing as a longitudinal stripe (indicated as (1) on Fig. 1) of BrdU-positive cells joining the posterior telencephalon with the olfactory bulb (OB). No staining was observed inside the OB itself, but a few positive cells, interpreted as glia (Byrd and Brunjes, 2001), were encountered in the superficial OB olfactory nerve layer (see Fig. 1B). On cross and horizontal sections (Figs. 1B–E), we observed that most proliferating cells were adjacent to the telencephalic ventricle at all dorsoventral (DV) levels (Figs. 1B–D). Dorsally, they line the outer surface of the telencephalon, in agreement with the interpretation that most of the ventricular surface of the pallium in teleosts is everted (red arrows in Figs. 1B–D) (Wullimann and Rink, 2002). Additionally, a prominent site of cell proliferation was detectable on posterior sections at the junction between the pallium and ventral telencephalic domains overlying the optic tract (Fig. 1D). Finally, a few positive cells could also consistently be observed in nonventricular locations, within the parenchyma (white arrowheads, Figs. 1B, C).

We next addressed whether all these cells displayed similar proliferation characteristics. Although BrdU-positive cells were found at all DV levels, we found that the growth fraction varied between these areas. Strikingly, at anterior levels, the highest density of BrdU-positive cells was observed in the ventral subpallium (numbered (1) on Figs. 1B, C), while it was decreased to a fourth in the dorsal subpallium and to a tenth in the medial or dorsal pallium (Fig. 1F). These observations suggest that the ventral subpallium contains either more or faster dividing proliferating cells than further dorsally located ventricular areas. To resolve this issue, we assessed the labeling index of each domain by calculating the number of BrdU-incorporating cells (number of cells in S phase) within the actively dividing cell population expressing the proliferating cell nuclear antigen PCNA (Figs. 2A–E) (Bravo et al., 1987; Mathews et al., 1984). In addition to a higher density of PCNA-positive cells along the ventricle in the ventral subpallium (Fig. 2B), we observed that the proportion of BrdU/PCNA double-labeled cells was consistently increased (two times) in this domain compared other DV levels (Figs. 1D–E). A high density of BrdU-positive cells and a high labeling index also characterized the ventricular surface of the posterior pallium (numbered 1p on Fig. 1D, and see Figs. 2E, F). Thus, most precursors in the ventral subpallium and posterior pallium are fast proliferating, while other ventricular telencephalic precursors proliferate more slowly. In agreement with a slower cell cycle at the ventricle of the dorsal subpallium, medial, dorsal and lateral pallium, we observed that the number of BrdU-incorporating cells in the latter domains continuously increased over 10 days in cumulative BrdU labeling experiments (not shown). We conclude that proliferating telencephalic cells contain several subpopulations that differ in their speed of division (summarized in Fig. 2F).

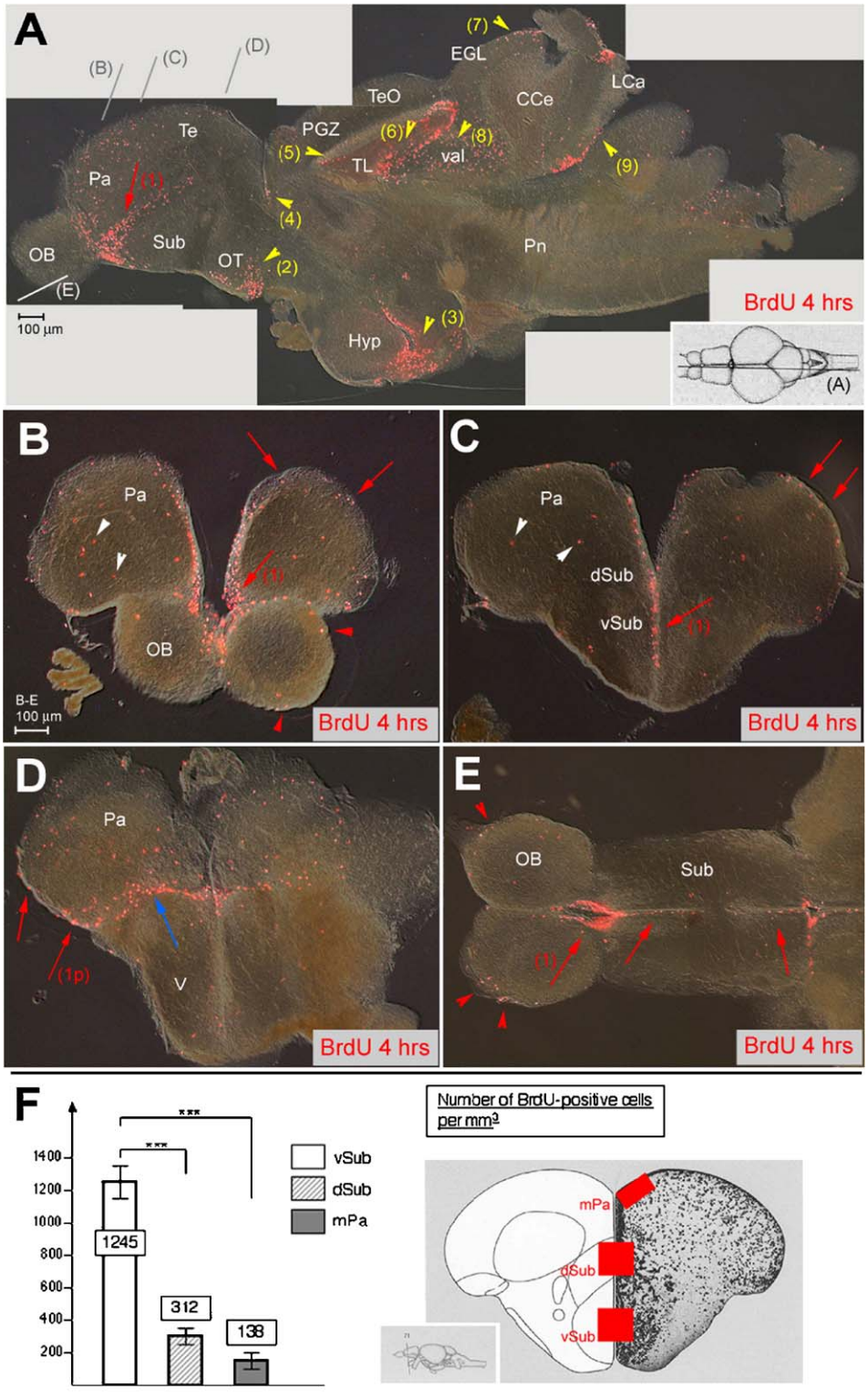
Migration of the progeny of adult telencephalic precursors

We next studied the progeny of ventricular proliferating cells by following BrdU-labeled cells 3 days, and 2, 3, 4, and 8 weeks after injection. In contrast to previous studies (Zupanc et al., 2005), this close spacing of time points allows monitoring cell migration as well as following fast proliferating cells.

Three days after BrdU injection, no major differences were observed in the telencephalon proper (Figs. 3A–C), but a few BrdU-labeled cells could now be detected within the OB (white arrowheads on Figs. 3A, B). A more obvious alteration in the location of BrdU-positive cells was then detectable 2 weeks after the BrdU pulse in the telencephalon and OB (Figs. 3D–F). For example, after 2 weeks, numerous positive cells were found inside the OB (white arrowheads in Figs. 3D, E). The zebrafish OB is organized in three layers (from external to internal: the olfactory nerve layer—ON, the glomerular layer—GL, and the internal layer—IL) (Byrd and Brunjes, 1998). BrdU-positive cells were predominantly found within the IL after 2 weeks, but they also populated the GL 4 to 8 weeks after injection (see below). Thus, cells generated outside the OB appear to migrate into the OB within 2 weeks. In parallel, the total number of positive cells in the ventral subpallial stripe had

Fig. 1. Proliferation sites in the zebrafish adult telencephalon. (A–E) BrdU immunocytochemistry (red staining) on brain sections of 6-month-old adult zebrafish sacrificed 4 h after 2 BrdU injections. Vibratome sections are observed under conventional fluorescence microscopy, (A) parasagittal section, anterior left, (B–D) cross-sections at increasingly posterior levels (indicated in panel A), dorsal up, (E) horizontal section (level indicated in panel A), anterior left. In panel A, intensely labeled sites are numbered (red arrows: telencephalon, yellow arrowheads: other domains). Note the stripe of intense staining within the ventral subpallium (numbered 1 in all sections). Other sites: (2) optic tracts, (3) ventricular zone of the hypothalamus, (4) anterior thalamus, (5) periventricular gray zone, (6) torus longitudinalis, (7) cerebellar external granular layer, (8) valvula cerebelli, (9) lobus caudalis cerebelli. Nonventricular proliferation is also visible in all cross-sections and indicated by white arrowheads. The only staining visible in the OB (E, red arrowheads) likely corresponds to glia in the superficial olfactory nerve layer (Byrd and Brunjes, 2001). In the posterior telencephalon, prominent proliferation is observed at the junction between the dorsal and ventral domains (blue arrow in panel D) and in the posterior pallium (red arrow numbered 1p in panel D). (F) Number of BrdU-positive cells in the telencephalon along the DV axis, counted in the areas boxed in red in the scheme on the right panel (Wullimann et al., 1996). Ventral subpallium: 1245 ± 245 BrdU-positive cells/mm^3, dorsal subpallium: 312 ± 136/mm^3, medial pallium: 138 ± 37/mm^3 (on a total of 9 sections from 3 different fish at anterior telencephalic levels, all areas containing a comparable total number of cells, statistical analysis performed using independent samples Student's *t* test). Abbreviations, after Wullimann et al. (1996): CCe: corpus cerebellaris, EGL: external granular layer, Hyp: hypothalamus, IGL: internal granular layer, LCa: lobus caudalis cerebelli, Pa: pallium, PGZ: periventricular gray zone, OB: olfactory bulb, OT: optic tract, Pn: pons, Sub: subpallium, Te: telencephalon, TeO: tectum opticum, TL: torus longitudinalis, V: posterior domain of the ventral telencephalon, val: valvula cerebelli.

decreased (white arrow in Figs. 3D, F, compare to red arrow in panels A, C), and most cells that had retained the BrdU label in this stripe were now clustered anteriorly, still along the ventricle (yellow arrow in Figs. 3D, E). In addition, BrdU-positive cells located along the ventricle in the dorsal subpallium and pallium shortly after BrdU injection had migrated into the parenchyma to a slightly more lateral position 2 weeks after BrdU labeling (Fig. 3F arrowheads, compare the position of labeled cells in the insets in panels C and F, yellow arrows to the ventricular surface). This redistribution continued over a longer time after BrdU injections, as visible after 19 days (Figs. 7A, B).



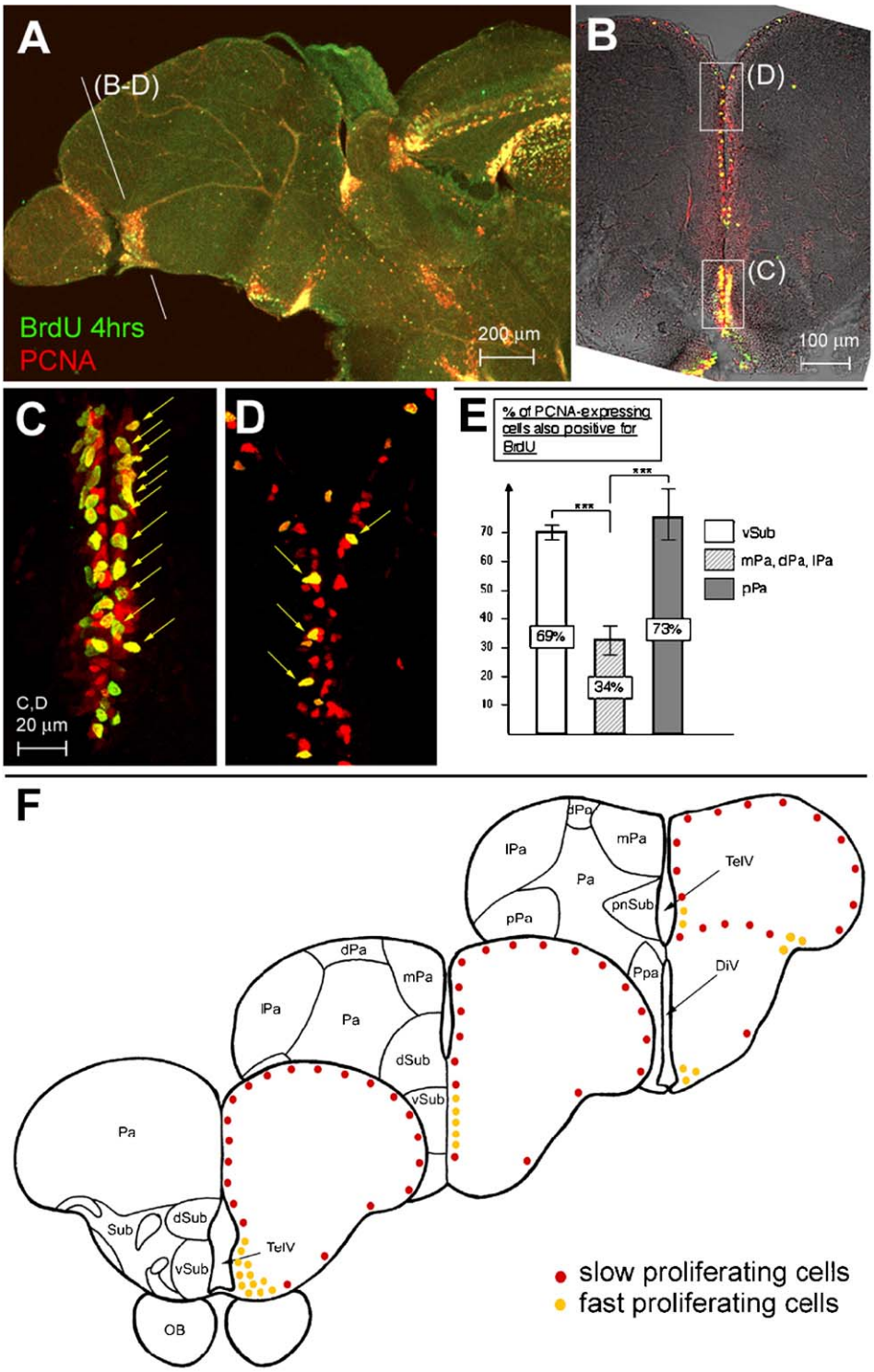


Fig. 2. Different cell cycle characteristics between proliferation zones in the adult zebrafish telencephalon. (A–D) Double immunolabeling for PCNA (red) and BrdU (green) on sagital (A, anterior left) and cross (panels B–D, dorsal up) vibratome sections observed under confocal microscopy; panels C, D are the areas boxed in panel B observed at higher magnification and on an optic section of 1.5 µm. PCNA expression and BrdU-positive cells are concentrated ventrally (compare the density of PCNA-only (red) and double-labeled (yellow arrows) cells in B–D). (E) Labeling index (number of PCNA-positive cells that are also BrdU-positive) in the telencephalon along the DV axis. In the anterior telencephalon: 69% in the ventral subpallium, $n = 115$ PCNA-positive cells counted, against 33.7% in the dorsal subpallium, medial, dorsal, and lateral pallium, $n = 327$; at the surface of the posterior pallium: 73%, $n = 26$. (F) Schematic representation of the zones of fast (yellow dots) and slow (red dots) proliferation in the zebrafish adult telencephalon, as identified in panels A–E. Cross-sections are oriented along the AP axis. Abbreviations: see Fig. 1, and DiV: diencephalic ventricle, dPa: dorsal pallium, dSub: dorsal subpallium, lPa: lateral pallium, mPa: medial pallium, pnSub: postcommisural nucleus of the ventral subpallium, Ppa: preoptic nucleus, pPa: posterior pallium, TelV: telencephalic ventricle, vSub: ventral subpallium.

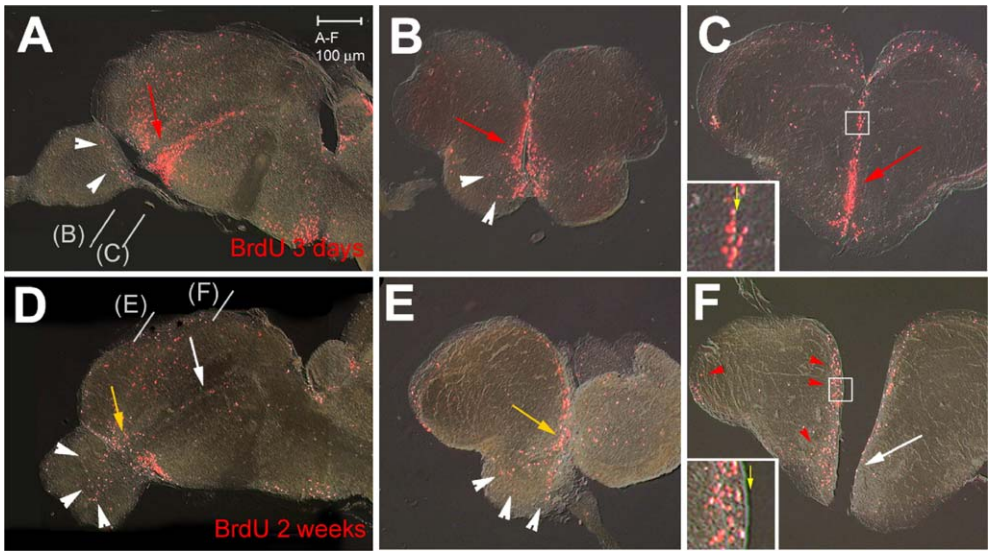


Fig. 3. BrdU-labeled cells (red staining) in the zebrafish adult telencephalon 3 days (A–C) or 2 weeks (D–F) after BrdU injection. All views are vibratome sections observed under conventional fluorescence microscopy (A,D: parasagittal sections, anterior left; B, C, E, F: cross-sections at the levels indicated in panels A, D, dorsal up). Note major changes after 2 weeks: (i) the ventral subpallial stripe is diminished caudally (white arrows in panels D, F) and mostly maintained anteriorly (yellow arrows in panels D, E), (ii) labeled cells are observed in the OB (white arrowheads in panels D, E), and (iii) labeled cells have left the ventricle in the pallium and dorsal subpallium (red arrowheads in panel F, compare insets in panels panels C, F, yellow arrow to the ventricle).

Together, these results indicate that within 2 weeks, most progeny arising from ventricular BrdU-positive cells relocalizes to populate the anterior domain of the ventral subpallial ventricular stripe and the OB, or the telencephalic parenchyma.

Adult telencephalic precursors generate newborn neurons

These results prompted us to address whether the different subpopulations of ventricular telencephalic dividing cells were neurogenic progenitors. We first studied expression of Hu proteins, markers for young postmitotic neurons (Mueller and Wullimann, 2002). We observed that Hu-positive cells were detectable in domains immediately adjacent to the proliferation zones along the telencephalic ventricle and were also abundant in the OB (Fig. 4 and not shown). Together with the results above, this is compatible with a model where ventricular telencephalic proliferating cells give rise to neurons throughout the telencephalon including the OB.

We next examined whether and when ventricular BrdU labeled cells acquire expression of Hu, and we obtained similar results at all telencephalic AP levels. Within the telencephalon proper 4 h after BrdU injection, none of the BrdU-positive cells co-expressed Hu (Fig. 4A), while double-labeled cells appeared starting 3 days after BrdU injection (e.g., Figs. 4B, D), and their proportion increased over 2 weeks to 74% ($n = 406$ BrdU-positive cells) (Figs. 4C, E). Thus, three quarters of all proliferating cells in the telencephalon had acquired a neuronal identity 2 weeks after BrdU incorporation (Fig. 4G). Contrasting with these populations within proximity of the ventricle, proliferating cells detected in the parenchyma (Figs. 1B, C) never expressed Hu (not shown). Interestingly, neuronal differentiation proceeded at a slower rate in the OB where only 41% of all BrdU-positive cells were Hu-positive 2 weeks after BrdU injection (Figs. 4F, H).

Together, these results demonstrate that de novo neurogenesis is taking place throughout the adult telencephalon, from precursors initially located along the ventricle that give rise to Hu-positive newborn neurons close to the ventricular surface and in the OB.

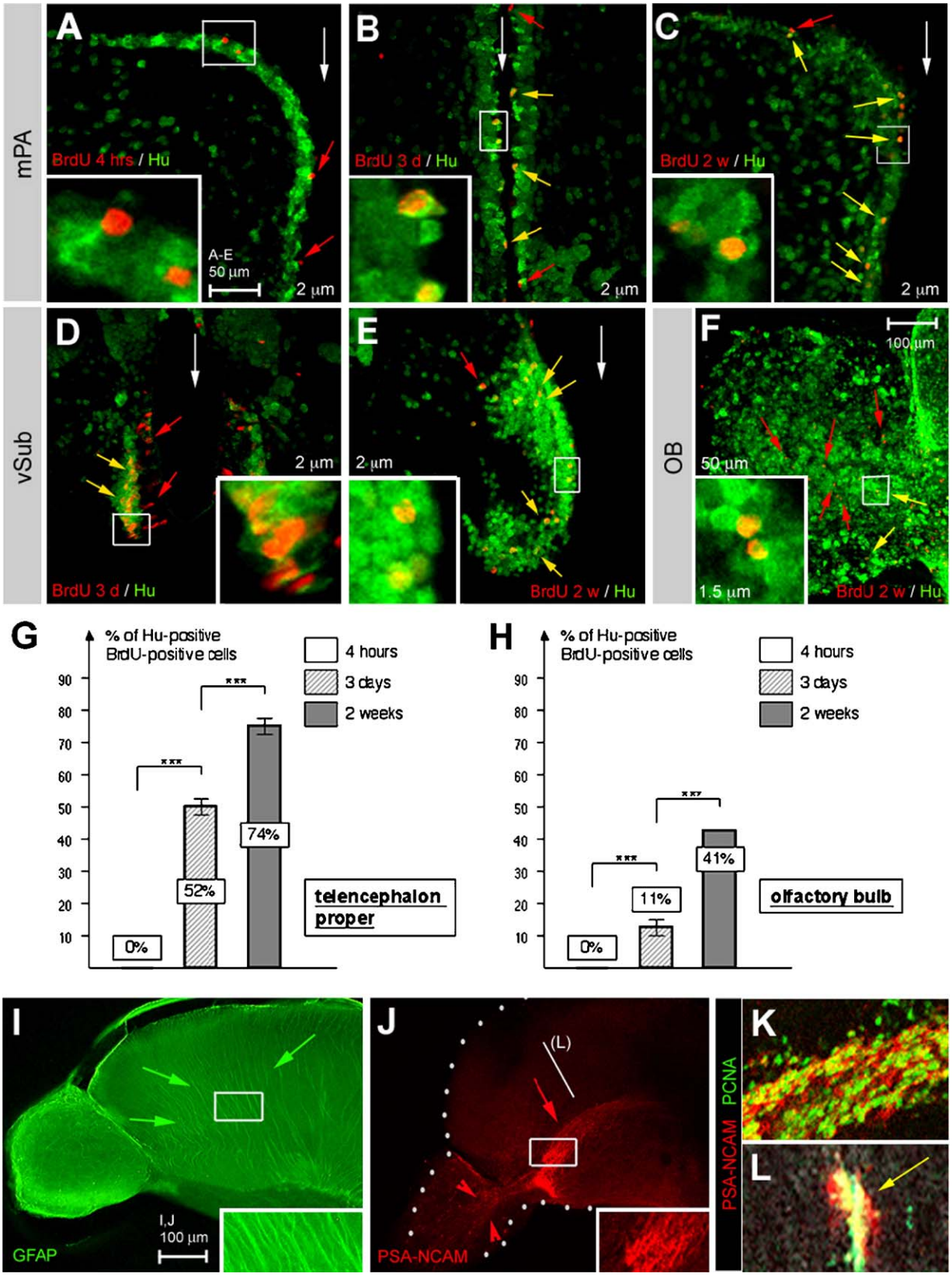
Ventral telencephalic precursors form a RMS-like migration route of neuronal progenitors towards the olfactory bulb

The progressive colonization of the OB by the progeny of dividing cells has been previously documented, but the origin of these cells was not determined (Byrd and Brunjes, 1998; Zupanc et al., 2005). We showed above that no BrdU-positive cells could be detected within the OB shortly after BrdU labeling, suggesting that newly generated neurons must originate somewhere else. Moreover, the number of BrdU-labeled cells in the ventral subpallial stripe decreases over time, paralleled by an increase in the number of BrdU-positive cells in the OB. These observations suggest that OB neurons might originate from dividing cells located at the ventricle of the ventral subpallium. To confirm this interpretation, we aimed at identifying the migratory path and characteristics of the cells en route to the OB. Unlike in mammals, we found no evidence for organized GFAP-positive tubes in the adult zebrafish telencephalon; GFAP-positive cells were present but rather loosely oriented tangentially (Fig. 4I), in agreement with a recent report mapping radial cells in the adult zebrafish brain using the marker AroB (Menuet et al., 2005). However, we observed intense PSA-NCAM staining along the entire subpallial stripe (Figs. 4J–L), reaching into the OB (Fig. 4J, red arrowheads), comparable to the organization of young migrating neuroblasts labeled by PSA-NCAM in the mammalian brain (Doetsch et al., 1997; Jankovski and Sotelo,

1996; Peretto et al., 1997). These results strongly suggest that the ventral subpallial stripe is a migration pathway for telencephalic neuronal progenitors and neurons fated to the OB, thus a possible functional equivalent to the mammalian RMS.

Ventricular telencephalic progenitors give rise to GABAergic and TH-positive neurons in the olfactory bulb

To determine whether adult neurogenesis contributed to the GABAergic population in the OB (Fig. 5A) (Kim et al., 2004;



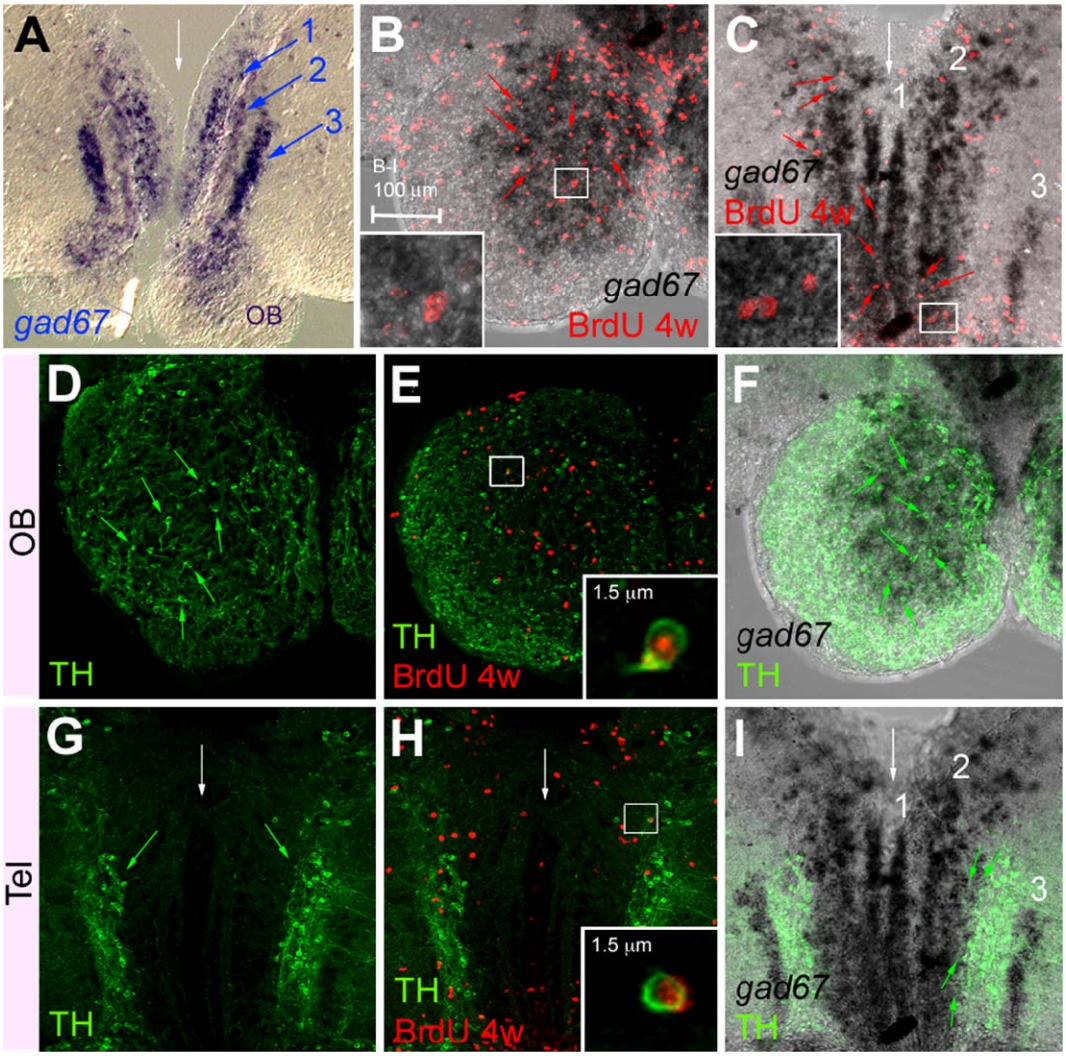


Fig. 5. Telencephalic progenitors give rise to *gad67*- and/or TH-positive neurons in the OB and in the anterior subpallium and medial pallium. Localization of GABA-ergic neurons, revealed by *gad67* (A–C, F, I) (ISH, blue or black) expression and compared with the expression of TH (D–I) (immunodetection, green) and/or the localization of BrdU-positive cells after 4 weeks of tracing (B, C, E, H) (red). Panels B and D–F are focussed on the OB, panels C and G–I on the subpallium and medial pallium, white arrows to the midline. All views are cross-sections, anterior up, panels B–I are confocal photomicrographs. In the OB, newborn neurons in the IL largely express *gad67* (B, red arrows to examples of double-positive cells, one example is magnified in inset). Most TH-positive neurons in the GL (D) are *gad67*-positive (F, green arrows to examples of double-positive cells) and a subset of these neurons are newborn (E). In the subpallium and medial pallium, *gad67* expression is organized in three bilateral columns (numbered 1–3 in A, C, I), which largely contain newborn neurons (C red arrows to examples of double-positive cells, one example is magnified in inset). Some newborn neurons also express TH (H), but these are mostly not GABAergic (I, green arrow to the restricted subpopulation of *gad67*/TH double-positive cells).

Martin et al., 1998), as is the case in mammals, we analyzed whether BrdU-positive cells turned on expression of the GABA synthesizing enzyme Gad67 (now Gad1, zebrafish nomenclature committee) after long-term tracing. After 4 weeks, a large proportion of the BrdU-positive population in the OB expressed *gad67* (Fig. 5B), demonstrating de novo GABAergic

Fig. 4. Adult neurogenesis and neuroblast migration in the zebrafish telencephalon. (A–F) Double immunodetection of Hu proteins (green) and BrdU (red) after increasing tracing times (4 h, 3 days—3d, 2 weeks—2w, as indicated). Panels A–E are cross-sections, dorsal up, and panel F is a sagittal section, anterior left, all observed under confocal microscopy with focus on the medial pallium (mPa) (A–C), ventral subpallium (vSub) (D, E) or OB (F). The position of the midline is indicated by white arrows. Examples of cells positive for BrdU but negative for Hu are indicated by red arrowheads, and examples of double positive cells by yellow arrowheads. All insets are high magnifications of the boxed areas. BrdU/Hu double positive cells can be found along the entire DV extent of the telencephalic ventricle, and to a lesser extent in the OB, after 3 days of tracing. G,H: Percentage of BrdU-positive cells also expressing Hu in the anterior telencephalon in the vicinity of the ventricular zone (G) and in the OB (H). Percentage of double-labeled cells for *n* BrdU-positive cells counted in the telencephalon proper: 0%, $n > 500$ at 4 h, 52%, $n = 942$ at 3 days, 74%, $n = 406$ at 2 weeks. Percentage in the OB: 0%, $n > 200$ at 4 h, 11.2%, $n = 269$ at 3 days, 41.5%, $n = 458$ at 2 weeks. Statistical analysis performed using independent samples Student's *t* test. I: Immunodetection of glia using an anti-GFAP antibody (green staining) (sagittal view, anterior left). Unlike in the rodent telencephalon, no longitudinally oriented fibers are visible. Rather, the projections of the detected glial cells (green arrows, see also inset) are arranged radially. (J–L) Immunodetection of PSA-NCAM (red staining) and PCNA (in panels K–L, green staining) (J: sagittal view, anterior left, OB and telencephalon delimited by the white dots; (K) sagittal view, anterior left, high magnification of a double-labeled preparation at the same level as boxed in panel J; (L) cross-section, dorsal up, at the level indicated in panel J). Note the prominent stripe of PSA-NCAM (red arrow in panel J) in the location of proliferating cells in the ventral subpallium (yellow staining in the overlay with PCNA expression in panels K–L). PSA-NCAM-positive cells reach into the olfactory bulb (red arrowheads in panel J).

neurogenesis in the adult zebrafish OB. Further consistent with adult neurogenesis of TH-positive neurons in the mammalian OB (Kosaka et al., 1995), we observed a low proportion of BrdU-positive cells expressing TH 4 or 8 weeks following BrdU injection (Figs. 5D, E and not shown), demonstrating that some TH-positive neurons in the adult OB (Byrd and Brunjes, 1995; Edwards and Michel, 2002) are newborn. Within 4 weeks, these account for less than 5% of the total TH-positive population, consistent with the lower proportion of newly generated TH-positive neurons in the mammalian OB (Hack et al., 2005). It is likely that these neurons also belong to the newborn *gad67*-positive population since, both in rodents and zebrafish, TH-positive neurons were reported to produce GABA in the OB (Edwards and Michel, 2002; Hack et al., 2005; Kohwi et al., 2005) (see also Fig. 5F). Together, these results demonstrate that de novo neurogenesis in the adult zebrafish contributes to the generation of several types of OB neurons, which include GABAergic interneurons in the IL and TH-positive interneurons in the GL (see Fig. 6H), comparable to the situation in mammals.

Recent results in the mouse highlight a bias for adult telencephalic Pax6-positive progenitors to give rise to TH-positive neurons of the OB GL (Hack et al., 2005; Kohwi et al., 2005). Thus, to further compare the zebrafish model to mammalian models of adult OB neurogenesis, we tested the relation between newborn TH neurons and the expression of *pax6* genes. Two *pax6* genes have been identified in zebrafish, with largely overlapping expression in embryos (Nornes et al., 1998). Most unexpectedly, we observed that the situation in adults was different: while *pax6b* expression was restricted to

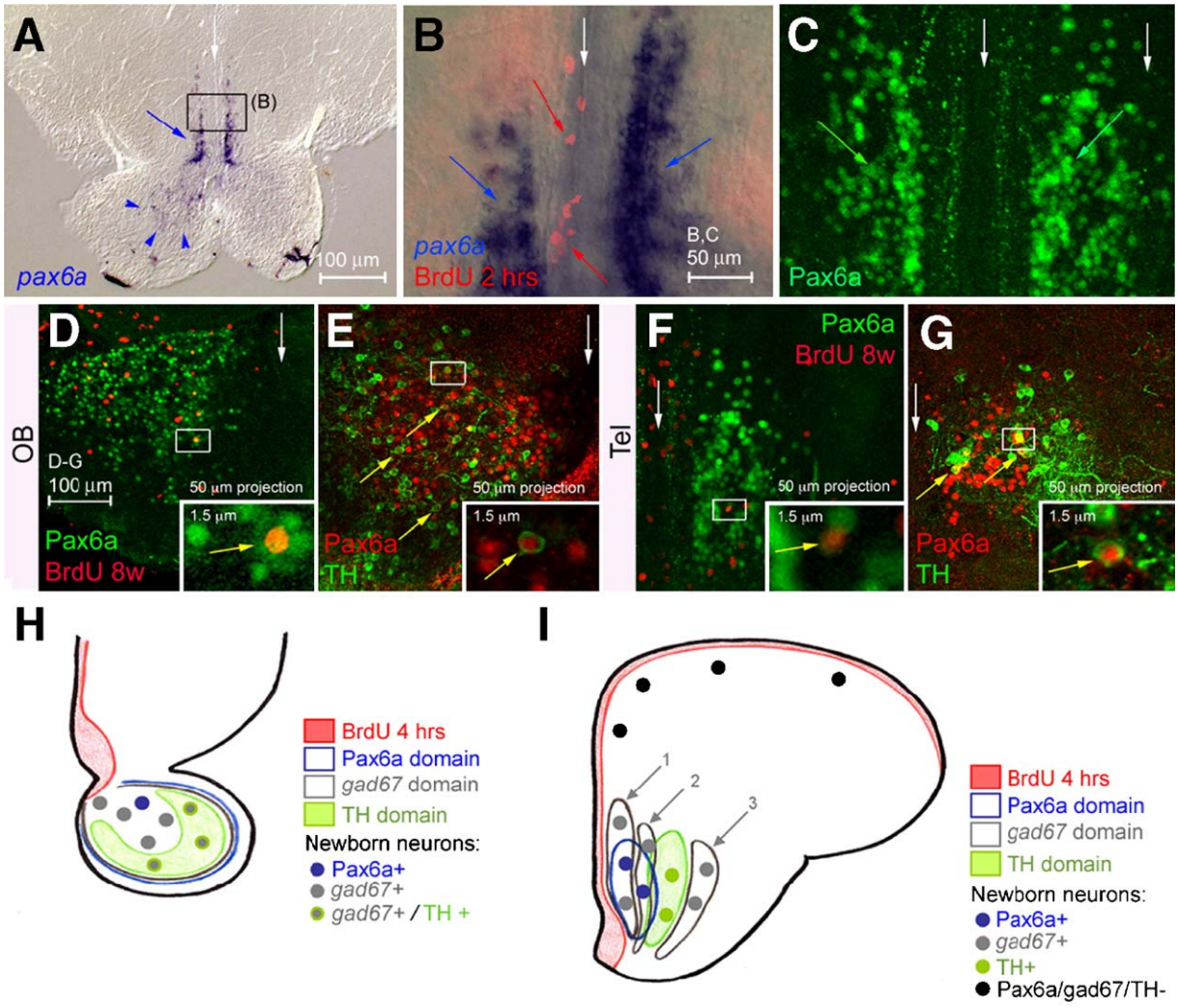


Fig. 6. Pax6a is expressed in neurons of the OB and anterior telencephalon. (A–G) Comparison of *pax6a* expression (A, B: in situ hybridization, blue; C–G: immunodetection, green or red) with BrdU incorporation (red) after 4 h (B) or 8 weeks (D, F) or with TH (E, G, green). All views are cross-sections, dorsal up, with white arrows to the midline; insets are high magnifications of the boxed areas; panel B is an overlay between bright field and fluorescence. Pax6a is expressed in the OB (A, arrowheads, D,E) and in bilateral telencephalic longitudinal stripes (A, B blue arrows, C green arrows, F, G), but not in proliferating cells (red arrows in panel B, see also C). After 8 weeks of tracing, cells expressing *pax6a* RNA (not shown) or protein and doubly positive for BrdU can be observed in both the Ob and telencephalon (D, F) (double labeled cells in insets). Coexpression of Pax6a and TH can also be detected in these domains (E, G) (examples of double positive cells are indicated by arrows and some are magnified in insets). (H, I) Schematics summarizing *gad67*, TH and Pax6a expression in the OB (H) and in the anterior subpallium and pallium (I) as well as the identity of newborn neurons 2 months after BrdU incorporation (color-coded).

telencephalic progenitors and was absent from the OB (see Figs. 9D, D′), *pax6a* expression was complementary. *pax6a* transcripts were found in cells adjacent to but nonoverlapping with ventricular progenitors in the telencephalon (Figs. 6A, B), as well as in numerous cells in the OB (Fig. 6A). None of the cells expressing *pax6a* was PCNA-positive (not shown) or had incorporated BrdU after a 4-h pulse (Fig. 6B). These findings were confirmed using a polyclonal antibody directed against mouse Pax6 that we found selectively labeling regions of zebrafish Pax6a (but not Pax6b) expression (Fig. 6C): positive cell bodies were strikingly absent from the ventricular zone. In the OB, BrdU/Pax6a-double positive cells were observed after 8 weeks of tracing (Fig. 6D), and few TH/Pax6a-double positive cells were also found (Fig. 6E). Thus, the Pax6a- and TH-positive populations overlap, and newborn OB neurons express Pax6a (see Fig. 6H).

Ventricular telencephalic progenitors give rise to GABAergic and TH-positive neurons in the telencephalon proper

As described above (Figs. 4, 5A), ventricular progenitors in the adult zebrafish telencephalon also contribute post-mitotic neurons to the telencephalon proper. In the subpallium and medial pallium, after a few weeks of tracing, these neurons appear aligned as bilateral dorsoventral columns adjacent to the ventricular zone. Strikingly, we observed that *gad67* (Figs. 5A, C), TH (Figs. 5G, I), and Pax6a (Figs. 6A–C) displayed similarly organized expression domains in this location. *gad67* is transcribed in three parallel columns (numbered 1 to 3 in order from medial to lateral, Fig. 5A). TH-positive cell bodies were mostly distributed between stripes 2 and 3, with a small overlap into stripe 2 (Fig. 5I), while Pax6a expression was medial, covering stripes 1 and 2 (compare Figs. 5A and 6A) and partially overlapping with TH expression laterally (Fig. 6G) (see schematic in Fig. 6I). The latter observation contrasts with the situation in juveniles, where TH and Pax6a expression in the telencephalon proper are exclusive (Wullimann and Rink, 2001). All domains are located adjacent to, but not within, the PSA-NCAM stripe (not shown), suggesting that these cells are not fated to the OB but rather overlap with the population described above of newborn neurons fated to the telencephalon proper. Together, these observations suggest the exciting possibility that newborn telencephalic neurons might share subtype identity and/or molecular attributes with newborn OB neurons.

To address this issue, we next determined the identity of newborn telencephalic neurons in the anterior dorsal subpallium and medial pallium by comparing BrdU-positivity in long-term tracings with the expression of the above markers. We found that, within 4 weeks, newborn neurons distribute over the three *gad67* stripes and that a large proportion of these neurons express *gad67* (see Fig. 5C after 8 weeks, and not shown). Newborn neurons positive for Pax6a (Fig. 6F) or TH (Fig. 5H) were also found. Within the time-frame of our experiments, none of the TH-positive newborn neurons in the telencephalon proper coexpressed Pax6a or *gad67* (not shown), in contrast to the OB. Together, these findings demonstrate that the ventricular zone of the subpallium and ventral pallium contribute newborn GABA or TH-positive neurons to the telencephalon proper, but that, unlike in the GL of the OB, these populations are mostly nonoverlapping.

Self-renewing progenitors are located in the dorsal and ventral ventricular zone of the zebrafish adult telencephalon

We demonstrated above that the zebrafish adult telencephalic ventricular zone serves as a source of progenitors for de novo neuronal differentiation in the adult OB and telencephalon proper. This prompted us to analyze the location, cellular, and molecular characteristics of these progenitors. To locate long-lasting progenitors, we analyzed after long-term BrdU tracing which cells that had retained the label were still cycling. To this aim, we traced ventricular BrdU-positive cells for 19 days and double-stained for PCNA expression. While, as described above, many BrdU cells during this time exited the proliferation zones, a few BrdU/PCNA double-labeled cells could still be found along the telencephalic ventricle (including its everted dorsal part) (2.2% of the PCNA-positive cells, $n = 180$) (Figs. 7A–C). Such cells are also observable anteriorly in the ventral subpallium, in a higher proportion (12.5% of the PCNA-positive cells, $n = 112$) (Figs. 7A, D, D′). These results were confirmed after 2 months of tracing (not shown). Thus, self-renewing, label-retaining progenitors are located both within the dorsal and ventral telencephalic ventricular zone. Their proportion among the proliferating cells is however five times higher ventrally than dorsally.

Ventricular telencephalic progenitors have molecular characteristics of glia

Neural stem cells in the adult mammalian brain are also slow dividing and label-retaining and are further characterized by their astroglia-like identity (Alvarez-Buylla and Garcia-Verdugo, 2002; Campbell and Gotz, 2002; Goldman, 2003; Gotz et al., 2002). To determine if this was the case in the adult fish telencephalon, we studied expression of the brain–lipid-binding protein BLBP that labels glial cells in mammals and birds (Anthony et al., 2004; Rousselot et al., 1997). Of the two zebrafish *blbp* genes, *fabp7a* and *b* (Denovan-Wright et al., 2000; Liu et al., 2003b), we found that *fabp7a* was strongly expressed in the telencephalic ventricular zone (Fig. 8B), in a manner identical to *sox2* (Figs. 8A–A″), *sox9a* and *sox10* (not shown), other common markers of adult neural precursors in mouse and fish (Ellis et al., 2004; Episkopou, 2005; Ferri et al., 2004; Komitova and Eriksson, 2004; Pevny and Placzek, 2005). FABP7a-positive cell bodies were observed to cover the ventricular zone, extending radial processes towards the pial surface of the brain (Figs. 8C–E). Two hours after BrdU incorporation, 42% of the BrdU-labeled progenitors were also positive for FABP7a (from 224 counted cells) (Figs. 8C, F). Importantly, however, the percentage of FABP7a-positive BrdU-positive cells decreased over time, as BrdU-labeled cells exited the ventricular zone (27% of co-labeling after 3 days, $n > 500$ counted cells, and 14% after 2 weeks, $n > 500$

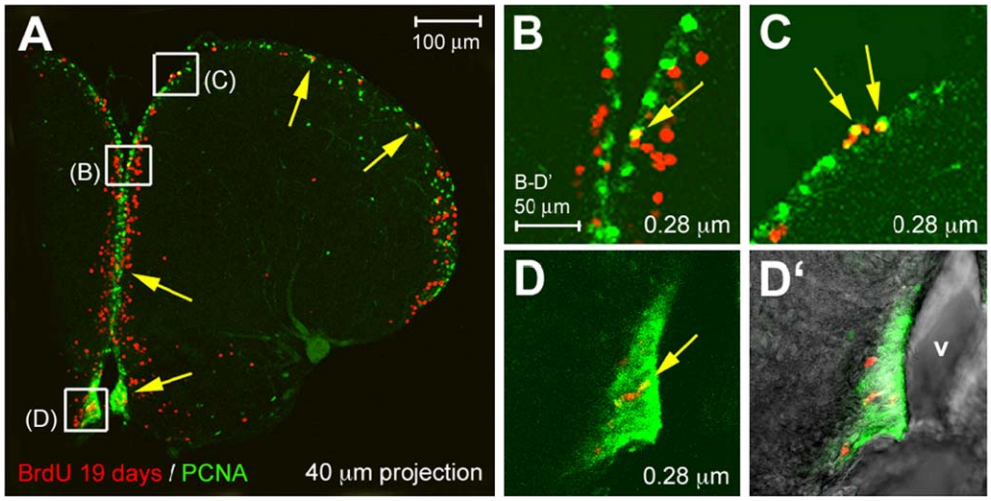


Fig. 7. Long-lasting neuronal progenitors in the zebrafish adult telencephalic ventricular zone. Co-immunodetection of PCNA (green) and BrdU (red) after long-term tracing (19 days), observed under confocal microscopy on cross-sections (thickness indicated, dorsal up). Panels B–D are high magnifications of the areas boxed in panel A, panel D′ is a brightfield and fluorescence view of panel D to locate the ventricle (v). While most labeled cells have exited the ventricular zone, a few BrdU-positive cells are retained in cycle (yellow arrows); these cells are located throughout the DV extent of the telencephalic ventricular zone.

counted cells) (Figs. 6D–F), consistent with the acquisition of a neuronal fate by most of the progeny. We conclude that the ventricular progenitors of the adult zebrafish telencephalon share classical characteristics of mammalian neural stem cells, in particular the expression of cellular and molecular glial characters.

Molecular code characterizing zebrafish adult telencephalic neuronal progenitors

Besides a few cases (Doetsch et al., 2002; Hack et al., 2005; Kohwi et al., 2005; Parras et al., 2004), the transcription factors attributing progenitor properties and fate of SEZ neuronal precursors in the rodent adult brain remain largely unknown.

We found that *emx3* (previously *emx1*, zebrafish nomenclature committee) (Morita et al., 1995), *dlx2a* (previously *dlx2*) (Akimenko et al., 1994), *olig2* (Park et al., 2002), *pax6b* (Nornes et al., 1998), *ash1a* (Allende and Weinberg, 1994) and *ngn1* (now *neurog1*) (Blader et al., 1997) were expressed in BrdU-positive ventricular progenitors of the zebrafish adult telencephalon (Figs. 9A–F′ and not shown). Unexpectedly, in striking contrast to the embryonic situation, the expression domains of these markers were largely overlapping and encompassed the entire ventricular progenitor zone in the subpallium and medial pallium (blue arrows in Figs. 9A–F′). However, the everted, dorsal, and lateral parts of the pallium progenitor zone expressed *pax6b* and *ash1a* (blue arrowheads in Figs. 9D, E) but not *emx3*, *dlx2*, *olig2*, and *ngn1* (white arrowheads in Figs. 9A–C, F). Interestingly, these observations suggest that progenitors located along the ventricular zone of the subpallium and medial pallium in the adult express a similar combination of transcription factors, unlike embryonic neuronal progenitors, and this combination differs with that expressed by adult progenitors in the dorsal and lateral pallium. Thus, the combination of factors expressed in zebrafish adult telencephalic progenitors might not be inherited from embryonic stages but might be recruited de novo to mediate adult neurogenesis.

To examine this idea, we tested whether the telencephalic expression of *ngn1* was controlled by distinct regulatory elements in the adult and embryo. The elements driving *ngn1* expression in the embryonic telencephalon consist of 212-bp-long regulatory region named “lateral stripe element” (LSE) in position −6702 to −6490 of the *ngn1* 5′ noncoding sequence. When this element is deleted, e.g. in the transgenic line *-3.4ngn1:gfp*, which contains only 3.4 kb upstream sequence, no telencephalic expression is observed in the embryo (Blader et al., 2003, 2004). In striking contrast, we found that, in the telencephalon of adult *-3.4ngn1:gfp* transgenic fish, GFP was a faithful reporter of endogenous *ngn1* expression: GFP expression was prominent in cell bodies along the ventricle of the subpallium (Figs. 9G, G′) and medial pallium (Figs. 9H, H′) and partially overlapped with BrdU-incorporating cells (Figs. 9G–H′, yellow arrows). Only one out of three transgenes containing the 3.4 kb upstream sequence of *ngn1* reproduced this pattern, suggesting that additional elements are required for robust expression (not shown). We conclude that *ngn1* expression in zebrafish adult telencephalic progenitors is not simply inherited from embryonic precursors but corresponds to a de novo recruitment of *ngn1* via new enhancer elements. Together, these results argue for a specific combination of factors mediating adult versus embryonic telencephalic neurogenesis in zebrafish and suggest that Pax6b (but not Pax6a), Ash1a, Olig2, Emx3, Dlx2, and Ngn1 might be instructive components of this combination.

Discussion

Our analysis of neurogenesis and neural precursor proliferation in the adult zebrafish telencephalon revealed several key insights. First, we identified, in the dorsal zebrafish telencephalon, a novel zone of adult neurogenesis that has apparently no

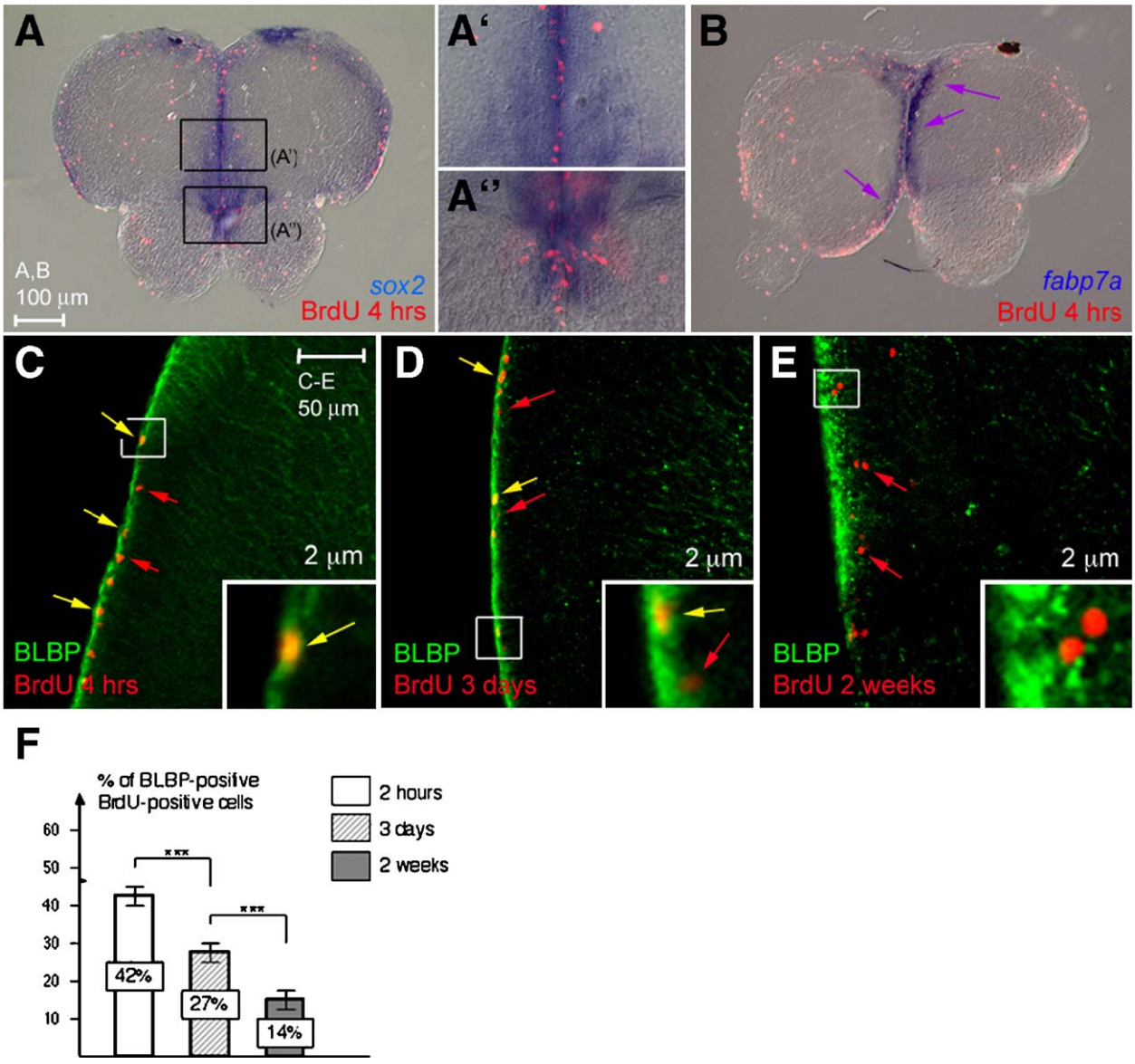


Fig. 8. Telencephalic progenitors express *sox2* and have molecular characteristics of glia. (A–B) Comparison of BrdU incorporation (red) after 4 h tracing with the expression of *sox2* (A–A″) or *fabp7a* (B) (blue, in situ hybridization). Cross-sections, dorsal up. Telencephalic progenitors are found in the expression domain of both markers. (C–E) Analysis in confocal microscopy (thickness 2 µm) of BLBP protein (green) and BrdU incoporation (red) after 4 h (C), 3 days (D) or 2 weeks (E). Examples of cells positive only for BrdU are indicated by red arrows, examples of cells doubly positive by yellow arrows. (F) Percentage of BLBP-positive cells that are also BrdU-positive after progressively longer tracing times. Note that this number strikingly decreases over time.

equivalent in the mouse telencephalon. As this domain also contains long-term label-retaining precursors, it may represent a novel zone of adult neural stem cells. Second, we describe for the first time that adult neurogenesis of olfactory bulb neurons in the fish closely resembles the mammalian counterpart: it also originates in the ventral telencephalon with PSA-NCAM-positive neuroblasts migrating towards the OB where GABAergic and dopaminergic neurons are generated life-long. However, in contrast to mammals, precursors in the ventral telencephalon also generate new neurons destined to the ventral telencephalon proper, notably including TH-positive neurons. Thirdly, we demonstrate that adult neural precursors in the fish telencephalon occur at all dorso-ventral levels but are distinct in their transcriptional code from those present at these positions during embryonic development. Thus, adult and embryonic neurogenesis differ in their molecular terms. Taken together, these data therefore provide not only insights into the phylogenetic but also the ontogenetic differences of neurogenesis in vertebrates.

Novel zones of adult neurogenesis in the dorsal and ventral telencephalon

BrdU-labeling in the adult fish revealed not only proliferation all along the ventricle in the dorsal telencephalon as previously shown (Zupanc et al., 2005) but also that the progeny of these cells differentiates into neurons. These neurons settle at just a short distance from the ventricular zone within the parenchyma.

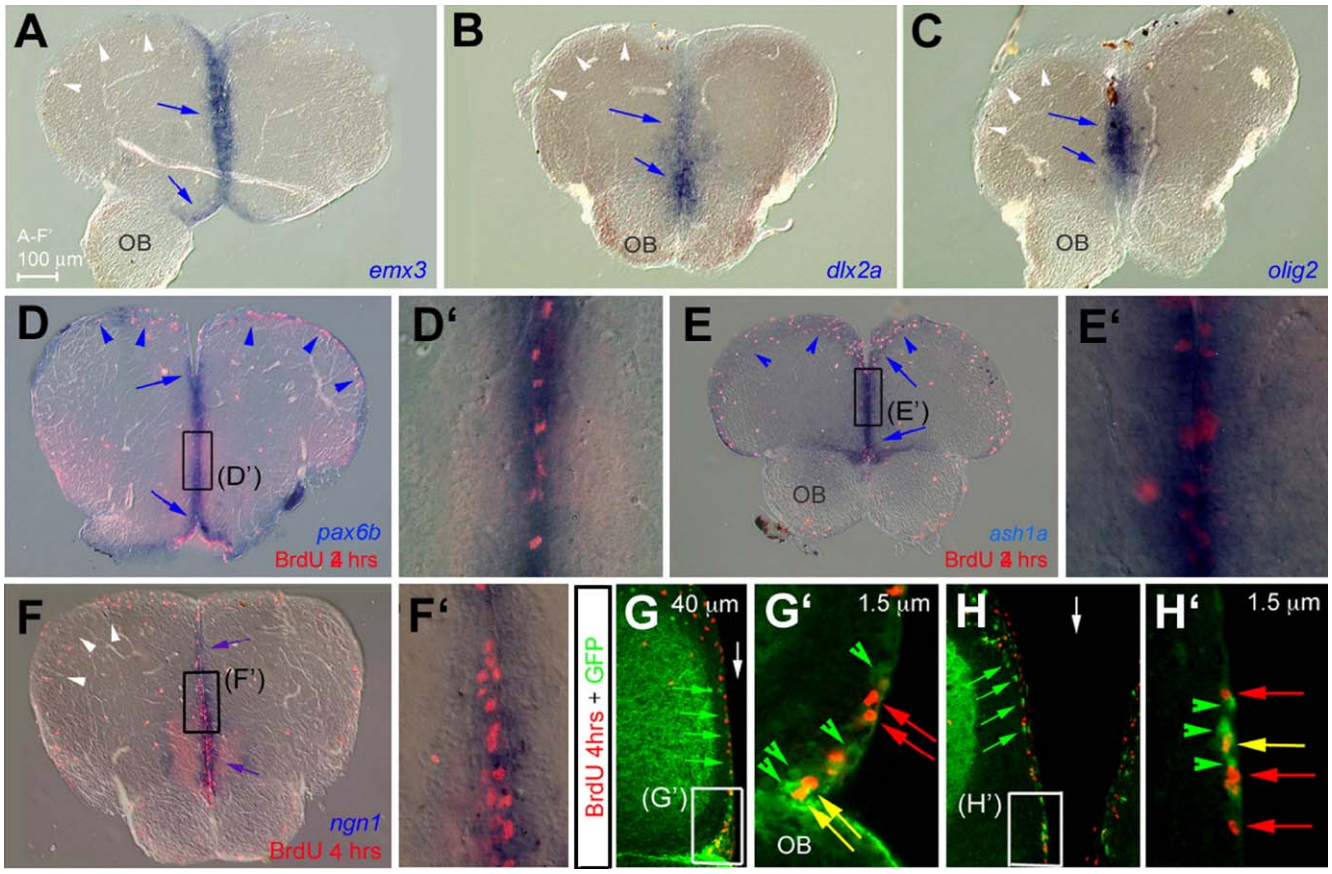


Fig. 9. Unique transcription factors code and promoter usage in adult zebrafish telencephalic progenitors. (A–F′) Compared expression of *emx3* (A), *dlx2a* (B), *olig2* (C), *pax6b* (D, D′), *ash1a* (E, E′), and *ngn1* (F, F′), together with BrdU incorporation after 4 h survival (red staining in panels D–F′), on anterior cross-sections (dorsal up). All markers are expressed in progenitors along the telencephalic ventricle at the midline (blue arrows, note the co-labeling with BrdU in panels D–F′). In addition, *pax6b* and *ash1a* are expressed in progenitors along the dorsally everted telencephalic ventricle (blue arrowheads in panels D, E), while other markers are not (white arrowheads in panels A–C, F). (G–H′) Determination of the enhancer fragment driving *ngn1* expression in adult ventricular telencephalic progenitors by comparing BrdU incorporation (red) and GFP protein (green) in the *-3.4ngn1:gfp* transgenic line (Blader et al., 2004; Blader et al., 2003). Cross-sections observed in confocal microscopy, dorsal up, green arrowheads and arrows point to cells expressing GFP only, red arrows to cells positive for BrdU only, and yellow arrows to double-labeled cells. Panels G and G′ focus on the ventral subpallium, panels H and H′ on the medial pallium, white arrows to the midline. Note that GFP distribution in *-3.4ngn1:gfp* is in agreement with endogenous *ngn1* expression (compare with F). OB: olfactory bulb.

Since the posterior pallium in zebrafish has been suggested as a functional equivalent of the hippocampus in mammals (Wullimann and Rink, 2002) (see Fig. 1D blue arrow), the fast proliferating cells in this region (see Fig. 1D and Zupanc et al., 2005) may correspond to adult neurogenesis in the hippocampal region in mammals (Gage, 2000). However, neurogenesis in the mammalian hippocampus is restricted to the dentate gyrus by nonventricular precursors (Gage, 2000, Taupin and Gage, 2002), an obvious difference to the location of adult precursors all along the telencephalic ventricle in fish.

In the dorsal telencephalon of mammals, adult neurogenesis is restricted to the dentate gyrus and does not occur in the cerebral cortex, while it is widespread throughout the dorsal telencephalon in zebrafish. Interestingly, also in reptiles newborn neurons populate all major subdivisions of the cortex (Font et al., 2001; Garcia-Verdugo et al., 2002) by a short migration to the parenchyma close to the VZ of the dorsal telencephalon (Perez-Canellas and Garcia-Verdugo, 1996). The identity and functional significance of these neurons have not been determined, and it will be of great interest to understand whether dorsally born neurons in reptiles and zebrafish are functionally equivalent.

Adult newborn neurons populate also the ventral domains of the adult fish telencephalon proper. As in the dorsal telencephalon, proliferation occurs along the ventricle, and newborn neurons that acquire GAD and TH settle very close to their site of origin. This pattern of neuron generation in the dorsal and ventral telencephalon suggests that the telencephalon of fish grows by addition of new neurons at the outside, similar to the retina and the midbrain (Marcus et al., 1999; Nguyen et al., 1999). While adult neurogenesis in the ventral telencephalon was also observed in reptiles and birds (Alvarez-Buylla and Kirn, 1997; Font et al., 2001; Garcia-Verdugo et al., 2002; Goldman, 1998; Solis, 2000), in mammals, all neurons generated along the ventro-lateral wall of the telencephalon migrate towards the olfactory bulb. New striatal neurons can be added from the underlying precursor zone in the mouse only after lesion in the overlying striatum (Arvidsson et al., 2002; Parent et al., 2002). As the teleostean subpallium has been proposed to be homologous to the mammalian striatum and septum (Wullimann and Rink,

2002), the molecular cues governing adult neurogenesis of TH-positive neurons in this region will be of particular interest.

Adult generation of olfactory bulb neurons in the zebrafish resembles mammalian olfactory bulb neurogenesis

The addition of newborn neurons to the zebrafish adult OB has been demonstrated previously, but the origin and fate of these neurons was not determined (Byrd and Brunjes, 1998; Zupanc et al., 2005). Following progenitor cell fate over closely spaced time points revealed that BrdU-labelled cells leave the ventral subpallial ventricle and enter the OB. We further discovered the likely migration route of newborn neurons as a stripe of PSA-NCAM-immunoreactive cells reaching into the OB. Indeed, PSA-NCAM regulates neuronal migration to the bulb in the mouse (Chazal et al., 2000) and chains of migrating PSA-NCAM-positive neuroblasts have been observed throughout vertebrates (Doetsch and Scharff, 2001). Moreover, the heterogeneity among the precursors in the subpallium comprising long-term label-retaining progenitors, fast dividing precursors and PSA-NCAM-expressing neuroblasts closely resembles the precursor types observed in the mammalian SEZ (Alvarez-Buylla and Garcia-Verdugo, 2002). Finally, the identity of the newly generated neuronal subtypes in the OB shows close similarities between mouse and zebrafish with *gad67*+/TH− neurons added to the IL and *gad67*+/TH+ neurons settling in the outer part of the zebrafish OB. Interestingly, we also observed Pax6 expression in the adult OB in both the GL and IL (for comparison see, Hack et al., 2005; Kohwi et al., 2005). Pax6 is necessary and sufficient for adult neurogenesis in the mouse OB, and instructs a dopaminergic phenotype when maintained in postmitotic neurons (Hack et al., 2005; Kohwi et al., 2005). Taken together, adult neurogenesis of GABAergic and dopaminergic neurons in the OB may be a conserved feature among most vertebrates, while neurogenesis present in other telencephalic regions of the zebrafish has come to an end in some vertebrate classes.

Identification of neural stem and progenitor cells in the adult zebrafish telencephalon

Our results further provide evidence for the existence of label-retaining precursors, a feature of stem cells, within the ventricular zones of the adult zebrafish telencephalon. BrdU-labeling was retained for weeks by a subset of precursors lining the ventricle and that remained proliferating. Since these cells were still detectable in animals over 2 years of age (data not shown), they likely exist throughout adulthood, a further feature of adult tissue stem cells. These data demonstrate that a subset of adult telencephalic precursors in the zebrafish is slow-dividing and self-renewing. Notably, these label-retaining cells express glial characteristics similar to neural stem cells in the two unique sites of adult neurogenesis in mammals (Doetsch et al., 1999; Seri et al., 2001). Taken together with some molecular expression characteristics (e.g. *sox2*) these findings strongly suggest that the telencephalic ventricular zone of the zebrafish contains long-lasting neural progenitors with stem cell-like properties. Importantly, these label-retaining progenitors are present throughout the dorso-ventral extent of the telencephalic ventricle, in contrast to mammals.

Interestingly, adult neural precursors in the fish telencephalon differed significantly from their embryonic counterparts. For example, one main distinguishing feature between adult and embryonic precursors is the acquisition of glial characteristics that appear only late in zebrafish embryos (Marcus and Easter, 1995). Along the same line, *sox2* that is highly expressed in adult telencephalic precursors is not expressed ubiquitously in the embryonic neural plate of zebrafish (www.zfin.org). Moreover, we discovered that the elements required for *ngn1* expression in adult telencephalic progenitors are distinct from those regulating expression in the telencephalon at embryonic stages (Blader et al., 2004). This provides the first evidence that a molecular mechanism specifies adult neural precursors differently from those during development. In fact, the gene expression domains that we examined here in the adult zebrafish telencephalon differ considerably from those at embryonic ages. For example, *emx3* and *ngn1* are expressed in the dorsal telencephalon of zebrafish embryos, and *dlx*, *ash1* and *olig2* are expressed ventrally (Akimenko et al., 1994; Allende and Weinberg, 1994; Blader et al., 2004; Kawahara and Dawid, 2002; Nornes et al., 1998; Park et al., 2002). In addition, zebrafish *pax6a* transcripts are restricted to the ventral telencephalon and *pax6b* transcripts are absent from the telencephalon at the 24 h post-fertilization (hpf) stage (see Blader et al., 2004 and our unpublished observations). This is notably different from their expression pattern in the ventricular zone of the adult fish, where most of these genes (including *pax6b*, but not *pax6a*) were expressed all along the dorso-ventral axis. Similarly, Pax6 and Emx2 are expressed dorsally while Dlx1,2,5, Mash1 and Olig2 are expressed ventrally in the telencephalon of mouse embryos (Campbell, 2005, and references therein), but these transcription factors are expressed within the same region in the adult ventral telencephalon, the zone of OB neurogenesis (Galli et al., 2002; Hack et al., 2005; Kohwi et al., 2005; Parras et al., 2004). While it is not clear whether this change is due to cell migration or transcriptional regulation, it points to the unique composition of precursors in adult neurogenic zones, both in zebrafish and in mice. Live imaging analyses in zebrafish as well as genetic screens will help to better understand how the distinguishing features between embryonic and adult precursors come about. This should provide direct insight into the mechanisms regulating adult stem cell hallmarks, such as those characterizing the slow-dividing self-renewing neural stem cells that are not present during development.

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Appendix 4

Article in *Development*

***her5* expression reveals a pool of neural stem cells in the adult zebrafish midbrain**

Prisca Chapouton, Birgit Adolf, Christoph Leucht, Birgit Tannhäuser, Soojin Ryu, Wolfgang Driever and Laure Bally-Cuif

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her5 expression reveals a pool of neural stem cells in the adult zebrafish midbrain

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Current models of vertebrate adult neural stem cells are largely restricted to the rodent forebrain. To extract the general mechanisms of neural stem cell biology, we sought to identify new adult stem cell populations, in other model systems and/or brain areas. The teleost zebrafish appears to be an ideal system, as cell proliferation in the adult zebrafish brain is found in many more niches than in the mammalian brain. As a starting point towards identifying stem cell populations in this system, we used an embryonic neural stem cell marker, the E(spl) bHLH transcription factor Her5. We demonstrate that *her5* expression is not restricted to embryonic neural progenitors, but also defines in the adult zebrafish brain a new proliferation zone at the junction between the mid- and hindbrain. We show that adult *her5*-expressing cells proliferate slowly, self-renew and express neural stem cell markers. Finally, using in vivo lineage tracing in *her5:gfp* transgenic animals, we demonstrate that the *her5*-positive population is multipotent, giving rise in situ to differentiated neurons and glia that populate the basal midbrain. Our findings conclusively identify a new population of adult neural stem cells, as well as their fate and their endogenous environment, in the intact vertebrate brain. This cell population, located outside the forebrain, provides a powerful model to assess the general mechanisms of vertebrate neural stem cell biology. In addition, the first transcription factor characteristic of this cell population, Her5, points to the E(Spl) as a promising family of candidate adult neural stem cell regulators.

KEY WORDS: Neural stem cell, Zebrafish, *her5*, E(Spl), Midbrain-hindbrain boundary

INTRODUCTION

Neurogenesis in the adult vertebrate brain is a fascinating but very restricted phenomenon that, despite intense investigation, remains incompletely understood. The capacity to renew neuronal populations after the embryonic waves of neurogenesis have taken place is very limited, and the factors allowing the maintenance of a stem cell state in a fully differentiated environment are poorly known (Alvarez-Buylla and Lim, 2004). Understanding the mechanisms underlying the survival and the regenerative properties of neural stem cells would, however, be of tremendous value.

To date, our knowledge on adult neurogenesis is largely derived from analyses of the vertebrate forebrain. In birds and reptiles, proliferation takes place continuously over the lifetime within the telencephalon, and newly arising neurons survive and are integrated into functional circuits seasonally (Garcia-Verdugo et al., 2002). In rodents, many studies have concentrated on the existence of stem cells and the formation of new neurons in the subventricular zone and in the dentate gyrus of the hippocampus (Doetsch et al., 1999; Seri et al., 2001). In both areas, crucial determinants of neural stem cell maintenance have been found to lie in the so-called niche, a microenvironment that provides extrinsic cues driving stem cell proliferation and survival (for a review, see Alvarez-Buylla and Lim, 2004). However, the essential molecular combination underlying the adult neural stem cell state, and whether this combination also includes factors promoting the progenitor state in the embryonic brain, remain unknown.

To determine the essential mechanisms responsible for the maintenance of neurogenesis within an adult environment, we need to compare the data accumulated on the rodent forebrain to other regions of adult neurogenesis and/or additional vertebrate animal models. To this aim, we focussed our attention on the zebrafish adult brain, a model system highly appropriate for the analysis of stem cell maintenance, but for which very few studies have been conducted. The zebrafish adult brain retains abundant proliferation activity, and several observations demonstrate that adult neurogenesis in zebrafish is a true adult phenomenon rather than a persistent embryonic process. For instance, adult proliferation zones are concentrated in discrete, usually ventricular, regions (Ekström et al., 2001; Zupanc et al., 2005), which suggests the existence of niches as opposed to an interstitial growth mode. Also, we demonstrated striking molecular and lineage similarities between adult neurogenesis events in the zebrafish and mouse adult telencephalon (Adolf et al., 2006).

In order to identify new stem cell populations using the zebrafish model system, we considered the mechanisms acting during embryonic development. In the zebrafish embryo, we previously characterized a pool of progenitor cells located at the midbrain-hindbrain boundary (MHB). Formation of this pool of progenitor cells crucially depends on the Hairy/Enhancer of Split [H/E(Spl)] transcription factors Her5 and Him/Her11, selectively expressed at the MHB and actively preventing neurogenesis: loss of Her5 or Him/Her11 function leads to a premature differentiation of primary neurons across the MHB, and overexpression of *her5* inhibits expression of the proneural gene *neurogenin* (*neurog1*/*ngn1*) and prevents differentiation in neural plate territories adjacent to the MHB (Geling et al., 2003; Geling et al., 2004; Ninkovic et al., 2005). In cell lineage tracing experiments, the MHB progenitor pool further proved

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to be a dynamic population: over time, cells stop expressing *her5* and *him/her11* and exit the pool to enter the adjacent neurogenic regions, and contribute neurons to the whole midbrain-hindbrain territory (Tallafuss and Bally-Cuif, 2003). Together, these data implicated Her5 and Him/Her11 as the key regulators keeping cells in a progenitor state at the embryonic MHB. We reasoned that such crucial control processes might be reiteratively used over time to maintain adult neural stem cells, and we searched for the existence of *her5*-expressing cells within the adult central nervous system (CNS).

We show here that *her5* is expressed in the adult brain in a restricted ventricular cluster of cells at the MHB, and we demonstrate that these *her5*-positive cells have all the required properties of stem cells. Together, our results provide, in the genetically tractable animal zebrafish, a new model system for the study of adult neural stem cell biology. They also suggest that the function of H/E(Spl) factors in specifying a neuronal progenitor state might be conserved in adulthood.

MATERIALS AND METHODS

Fish strains

Two- or 5-9-month-old transgenic fish carrying *her5pac:egfp* [Tg(*her5PAC:EGFP*)^{ne1939}] or *3.4her5:egfp* (Tg(*3.4her5:EGFP*)^{ne1911} (Tallafuss and Bally-Cuif, 2003), or wild-type zebrafish (*Danio rerio*) of the AB strain, were used.

BrdU administration

Fish were injected intraperitoneally with 5 μl/0.1 g body weight of a freshly prepared bromodeoxyuridine (BrdU) solution at a concentration of 16 mmol/l in 110 mmol/l NaCl pH 7.2. Time to analysis ranged between 3 hours and 8 weeks post-injections. Fish were anaesthetized in tricaine, placed in ice water for 5 minutes and subsequently decapitated. Brains were dissected and fixed in 4% paraformaldehyde solution at 4°C for 4 hours, then progressively dehydrated in methanol and stored in 100% methanol at –20°C.

Immunohistochemistry

After rehydration of the brains, immunostainings were performed on vibratome sections (vibrating microtome HM 650 V, Microm): whole brains were embedded in 3% agarose in PBS and cut serially at 100 μm thickness. Sections were blocked in PBS with 0.5% Triton X-100 and 10% normal goat serum for half an hour at room temperature, then incubated in the primary antibodies at 4°C overnight or 2 hours at room temperature. Primary antibodies used in this study were rabbit anti-BLBP (1:1500) (Feng et al., 1994), mouse anti-BrdU (1:100; Roche), rat anti-BrdU (1:200; abcam), rabbit anti-GFAP (1:100; DAKO), rabbit anti-GFP TP401 (1:500; ams), mouse anti-GFP (1:200; BD Bioscience), mouse anti-Hu C/D (1:300; Molecular Probes), mouse anti-PCNA (1:250; DAKO), mouse anti-PSA-NCAM (1:600; Chemicon), mouse anti-Numb (BD Biosciences), rabbit anti-MCM5 (1:600, see below). Rb anti-Quacking6 was kindly provided by Stephane Richard, and rat anti-Musashi was kindly provided by Hideyuki Okano. Secondary antibodies labelled with Cy2, Cy3 or Cy5 (Jackson Laboratories) were used at a 1:500 dilution and incubated for 45 minutes at room temperature. The sections were embedded in Aqua Polymount (Polyscience). Immunodetection of BrdU required a pretreatment with 2 mol/l HCl followed by quick washes with borate buffer and PBS. Sections were photographed and analysed under a Zeiss confocal microscope (LSM 510 META).

Generation of anti-MCM5 antibody

The N-terminal 725 bp of the *mcm5* ORF was cloned into the pTRC-hisB vector (Invitrogen). Proteins were overexpressed in *Escherichia coli* using standard procedures and purified using an Ni^{+}NTA column (Qiagen) under denaturing conditions. Polyclonal antibodies were produced in rabbits according to standard procedures.

In situ hybridization

In situ hybridization was performed as for whole-mount embryos (Hauptmann and Gerster, 1994), starting with whole-mount brains. After hybridization at 70°C and rinses, brains were embedded in agarose and cut at the vibratome as described above. Subsequently, blocking, anti-dig incubation and further steps were performed on free-floating sections. The following ISH probes were used: *ash1a* (Allende and Weinberg, 1994), *her5* (Muller et al., 1996), *sox2* [cDNA clone cb236 (B. Thisse, S. Pflumio, M. Fürthauer, B. Loppin, V. Heyer, A. Degrave, R. Woehl, A. Lux, T. Steffan, X. Q. Charbonnier and C. Thisse, unpublished)], *her4* (Takke et al., 1999), *deltaA* (Appel and Eisen, 1998), *notch1a* (Bierkamp and Campos-Ortega, 1993). Sections were photographed and analysed under a Zeiss Axioplan microscope or a Zeiss confocal microscope (LSM 510 META).

RESULTS

her5 expression is maintained in the adult zebrafish brain

Because *her5* characterizes a progenitor pool in the embryonic CNS, we examined whether it remained expressed in the adult brain. We made use of the *her5pac:egfp* transgenic line (later referred to as *her5:gfp*), where a 40 kb upstream sequence of *her5* drives the expression of a fusion protein containing the first 33 N-terminal amino acids of Her5 and an enhanced green fluorescent protein (eGFP) (Tallafuss and Bally-Cuif, 2003). Because of the brightness of eGFP, this line allows us to identify even minute expression zones. We observed two concentrated spots of Her5-GFP expression in the adult brain, one in each hemisphere (Fig. 1A,B, arrows), corresponding to restricted clusters of cells at the intersection between the valvula cerebelli, torus semicircularis and tegmentum (Fig. 1D). This location approximately correlates to the nucleus lateralis valvulae (Wullimann et al., 1996). Her5-GFP-positive cells are thus confined to the intersection of two transition zones: (1) the anterior-posterior (AP) transition separating cerebellar (valvula cerebelli) from midbrain structures (torus semicircularis, the equivalent to the mouse inferior colliculus); this location is reminiscent of the embryonic position of *her5*-positive cells at the MHB; and (2) the dorsoventral (DV) boundary between the alar torus semicircularis and the basal tegmentum, along the tectal ventricle (dotted line in Fig. 1E-G). Her5-GFP was still strongly expressed in 2-year-old fish (although in a decreasing number of cells, data not shown), suggesting that it is in fact maintained over the animal's lifetime.

Two sets of arguments rule out a potential transgenic artefact and confirm that the presence of Her5-GFP in the adult brain is the result of *her5* expression at adult stages. First, we examined another independent transgenic line in which Her5-GFP expression is driven under a 3.4 kb of *her5* upstream sequence (*–3.4her5:egfp*) (Tallafuss and Bally-Cuif, 2003). Her5-GFP expression was observed at the border between midbrain and cerebellum in this line as well (Fig. 1H). Second, we performed in situ hybridization for the endogenous *her5* RNA, and found *her5*-expressing cells within the Her5-GFP-positive population (Fig. 1C-F). Thus, along the tectal ventricle, Her5-GFP correlates with endogenous *her5* expression and can be used as a valid reporter of *her5* gene activation. Together, these results demonstrate that *her5* is expressed throughout adulthood and characterizes a restricted ventricular cell population at the adult MHB.

To approach the dynamics of the adult Her5-GFP population, we further performed a detailed comparison of the expression domains of Her5-GFP and endogenous *her5* RNA on double-labelled preparations. Indeed, while endogenous *her5* and transgenic *her5-gfp* RNA have an identical distribution (Tallafuss and Bally-Cuif, 2003) (data not shown), the stability of Her5-GFP protein allows its persistence in the cell long after *her5* expression is switched off, and thus permits its use as a tracer of cells that previously expressed *her5*. As described, *her5* RNA-expressing cells in the adult brain were concentrated in a cluster directly apposing the ventricular zone (Fig. 1C,E). These cells displayed an undifferentiated morphology,

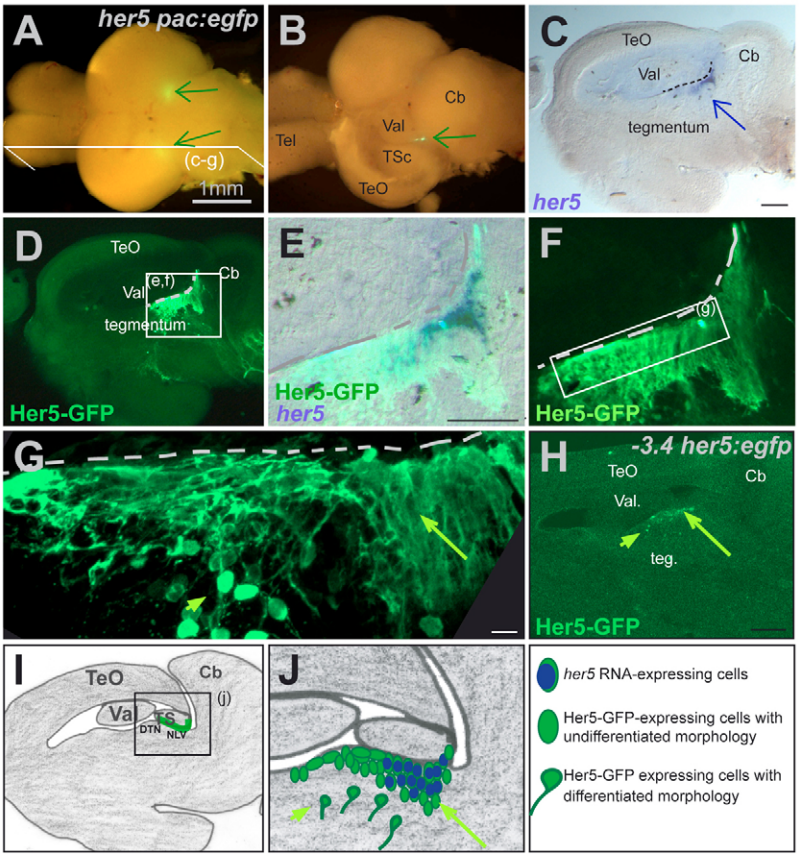

Fig. 1. *her5* expression defines an MHB cluster in the adult brain. (**A**) Whole brain from a 4-month-old *her5pac:egfp* transgenic fish viewed from the top. Two spots of GFP expression (green arrows) are visible between the hindbrain and the midbrain hemispheres. (**B**) Same brain following unilateral removal of the tectum. On the dissected side, the cluster of GFP-positive cells (arrow) is visible between the valvula cerebelli and torus semicircularis. (**C-F**) Sagittal section of a 2.5-month-old *her5pac:egfp* fish depicting the expression of endogenous *her5* RNA (blue staining) in a group of cells included within the cluster of Her5-GFP-expressing cells (green staining). (C) Brightfield view: *her5* is expressed in a cluster of cells lining the ventricle (dotted line) between tegmentum, torus semicircularis and valvula cerebelli (arrow), like Her5-GFP (D,F, fluorescence view). (E) *her5*-expressing cells are indeed located within the Her5-GFP-positive domain (concomitant bright and fluorescence fields). Note also, in E, that GFP-positive cells are located ventrally outside the her5-positive zone. (**G**) High magnification of the Her5-GFP-positive area (dotted line to the ventricle), highlighting the neuroepithelial morphology of ventricular *her5* RNA- and Her5-GFP-positive cells (long arrow), contrasting with the differentiated morphology of their more ventrally located *her5*-negative, Her5-GFP-positive descendants (short arrow). (**H**) Her5-GFP expression in a sagittal section of a 2.5-month-old –3,*4her5:egfp* transgenic brain. Her5-GFP expression highlights the same territory as in *her5pac:egfp* transgenics. In particular, positive cells of neuroepithelial morphology lie at the ventricle (long arrow), while cells of differentiated morphology are found deeper within the tegmentum (short arrow). (**I**,**J**) Schematic representation of a sagittal section at the same medio-lateral position as the sections shown in C-H. The green line in I depicts the *her5*-expressing area, and *her5*- or Her5-GFP-positive cells are colour-coded in J (long arrow to the her5- and Her5-GFP-positive domain, short arrow to the *her5*-negative, Her5-GFP-positive domain, as in G,H). Scale bars: 1 mm in A,B; 100 μm in C,D,H; 10 μm in G. Cb, cerebellum; DTN, dorsal thalamic nucleus; NLV, nucleus lateralis valvulae; Teg, tegmentum; Tel, telencephalon; TeO, optic tectum; TSc, torus semi-circularis; Val, valvula cerebelli lateralis.

with slightly elongated cell bodies and short processes (Fig. 1G, long arrow, Fig. 2B,C). In addition, we observed that Her5-GFP-positive cells were present further ventrally, within the tegmentum. The latter cells displayed a differentiated morphology with rounded cell bodies and long processes (Fig. 1G-J, small arrows, Fig. 2F). We conclude that the Her5-GFP population is dynamic and consists of ventricular cells, located at the MHB and expressing *her5* RNA (and Her5-GFP) (Fig. 1I,J long arrow), which give rise to cells located deeper into the tegmentum and maintain only Her5-GFP (Fig. 1I,J short arrow).

Adult *her5* expression defines a new proliferation zone

During embryonic development, Her5 promotes a progenitor state (Geling et al., 2003; Tallafuss and Bally-Cuif, 2003). Thus, we asked whether *her5*-positive cells are also progenitors in the adult brain. To answer this question, we first assessed whether *her5*-positive cells proliferate. The proliferation markers PCNA and MCM5, which are implicated in the DNA replication machinery (Maga and Hubscher, 2003; Ryu et al., 2005) and are expressed at all phases of the cell cycle, were co-expressed in the same cells and revealed several proliferation zones in the adult zebrafish brain, in agreement with previous maps (Adolf et al., 2006; Zupanc et al., 2005) (Fig. 2A-F). Strikingly, however, we found that *her5*-positive cells were located within a novel zone of proliferation, which we will refer to as the isthmic proliferation zone (IPZ). This proliferation zone has also been observed in another teleost, the stickleback (Ekström et al., 2001). The IPZ (Fig. 2A,F-H, yellow arrows) is neighbouring the tectal proliferation zone (Fig. 2A,F-H, red arrows), which has been described in the juvenile and adult medaka fish (Nguyen et al., 1999), the juvenile zebrafish (Mueller and Wullimann, 2002) and the stickleback (Ekström et al., 2001) and generates cells populating the optic tectum. The two zones are spatially distinct, and are linked by a narrow ribbon of proliferating cells (Fig. 2F, white arrow, and not shown).

To determine whether Her5-GFP cells themselves proliferate, we used confocal microscopy to compare the expression of Her5-GFP protein and endogenous *her5* RNA with that of MCM5 and PCNA. We observed that in young adults 14% of the Her5-GFP cells located close to the ventricle (n=1175 cells out of eight brains of 2-3 months of age) expressed PCNA. This proportion decreased with age (data not shown). Within the more restricted *her5* RNA-positive population, 27% of cells (n=33 cells, two hemispheres) expressed MCM5 (Fig. 2B-E, summarized in Fig. 2H). Hence, about one-third of the *her5*-RNA-expressing population proliferates, suggesting that these cells might be progenitors.

Adult *her5*-positive cells are slow proliferating

Because the population of *her5*-positive cells is located within a larger population of proliferating cells along the AP axis (Fig. 2A-E,H), we wondered whether differences in proliferation rates would define several subgroups of cells in the IPZ area. In order to answer

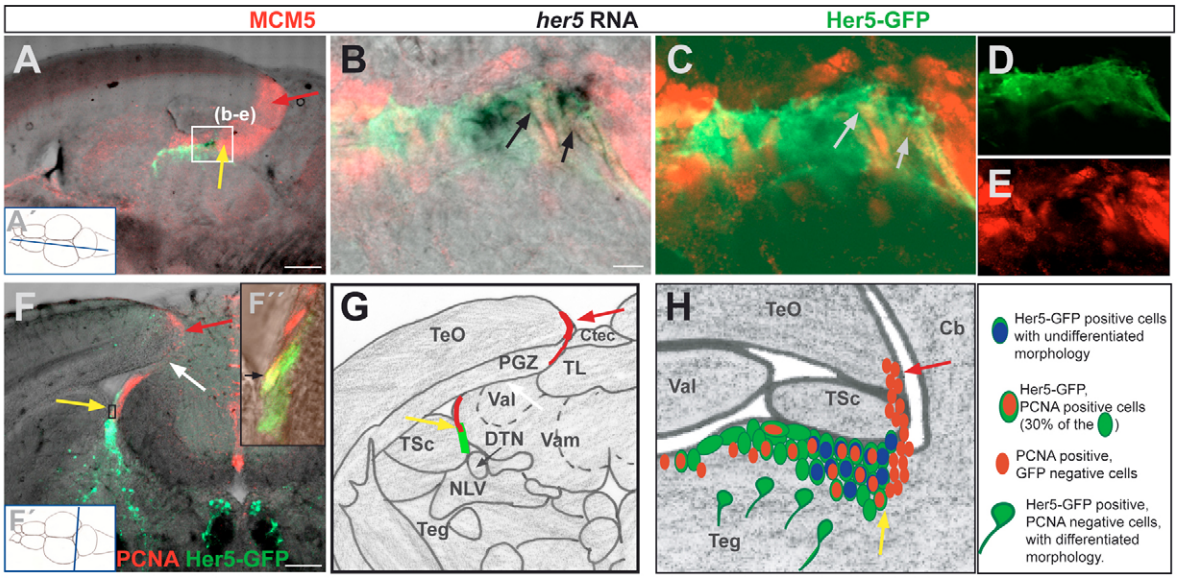

Fig. 2. *her5*-positive cells proliferate and demarcate a new proliferation zone within the adult midbrain. (**A-E**) Saggital section through a 3-month-old *her5:gfp* transgenic brain (at the medio-lateral level indicated in A′, anterior left), displaying the tectal proliferation zone (red arrow) and the IPZ (yellow arrow) labelled by the cell cycle marker MCM5 (red). *her5* RNA-expressing cells (black) and Her5-GFP cells (green) are located in the IPZ. B-E show a higher magnification (single confocal plane) of the section in A showing the expression of the proliferation marker MCM5 (red) in *her5*-RNA- and Her5-GFP-expressing cells, as pointed by the arrows (B: overlay of bright field and fluorescence views, C: overlay of green and red fluorescence channels; single channels are shown in D and E, respectively). (**F**) Cross-section through a 3-month-old *her5:egfp* transgenic brain, at the rostro-caudal level indicated in **F′**, displaying Her5-GFP (green) and proliferating cells stained by PCNA (red). Yellow arrow to the IPZ, red arrow to the tectal proliferation zone, the location of the connecting ribbon of proliferating cells is indicated by the white arrow. (**F″**) confocal plane of the area boxed in F showing two Her5-GFP cells that are PCNA-positive (black arrow). (**G**) Schematic representation of the cross-section shown in F (same colour code for arrows and stainings). (**H**) Same schematic representation of the IPZ area as in Fig. 1J (sagittal view) depicting the location of PCNA- (or MCM5)-positive cells (red) within the Her5-GFP population. Scale bars: 100 μm in A,F; 10 μm in B. Cb, cerebellum; Ctec, tectal commissure; DTN, dorsal thalamic nucleus; NLV, nucleus lateralis valvulae; PGZ, periventricular grey zone; Teg, tegmentum; TL, torus longitudinalis; TeO, optic tectum; TPZ, tectal proliferation zone; TSc, torus semi-circularis; Val, valvula cerebelli lateralis; Vam, valvula cerebelli medialis.

this question, we assessed the rates of proliferation in this domain by calculating the labelling index, defined as the proportion of cells in S-phase within the total proliferating population. Cells undergoing S-phase were marked by two intraperitoneal injections of BrdU within a 2-hour interval between injections, followed by the assay after 2 hours. The whole proliferating population was revealed by the expression of PCNA. The distribution of BrdU- and PCNA-double-positive cells was then assessed by confocal microscopy and compared to the location of GFP-positive cells in triple-labelled preparations (Fig. 3). We found that, within the population of ventricular Her5-GFP-positive cells (Fig. 3A,D green label), only a small proportion of the PCNA-positive cells (Fig. 3A,C red label) were in S-phase (Fig. 3B blue label) at a particular time point (14%, *n*=390 cells counted in two brains) (see Fig. 3E, triple-labelled cells appear white on the merged image, grey arrow). This is indicative of a long cell cycle for Her5-GFP-positive cells. In striking contrast, the ventricular domains neighbouring the Her5-GFP zone along the AP contained a much higher proportion of cells undergoing S-phase (30%, *n*=617 cells counted in two brains) (Fig. 3B,C, examples indicated by short white arrows). Hence, these domains mostly comprise cells displaying a shorter cell cycle. These results were confirmed by demonstrating that the number of cells co-labelled for Her5-GFP and BrdU gradually increased upon cumulative BrdU labelling (not shown). We conclude that *her5*-positive cells are a slow-proliferating population (summarized on Fig. 3F). The long cell cycle of *her5*-positive cells suggests that they could be stem cells in that region of the brain.

her5-positive cells express stem cell markers

To add support to the hypothesis of a stem cell nature for *her5*-positive cells, we analysed whether these cells expressed characteristic neural stem cell markers. In mammals, the expression of the antigens Nestin or RC2 are diagnostic of the neural progenitor state (Götz and Barde, 2005); however, available antibodies against these antigens failed to cross-react with zebrafish. We found several other neural stem cell markers to be expressed in a subset of *her5*-positive or ventricular Her5-GFP-positive cells. For instance, these cells expressed the radial glial marker BLBP, or the astrocytic and radial glia marker GFAP (Fig. 4A, parts a-h′), which usually reflect a neural stem cell state (Götz and Barde, 2005; Hartfuss et al., 2001). We also found a subset of Her5-GFP-positive cells expressing the transcription factor Sox2 (Ferri et al., 2004) (Fig. 4A, parts i-l′), and the asymmetrically inherited cell fate determinants Musashi (Kaneko et al., 2000; Okano et al., 2002; Sakakibara et al., 2002) and Numb (Petersen et al., 2002) (Fig. 4B, parts a-h). These factors have been associated with neural progenitor potential in mammals. Thus, *her5*-positive and ventricular Her5-GFP-positive cells possess molecular attributes of neural stem cells.

her5-positive cells are long-lasting progenitors

These results prompted us to determine whether *her5*-positive cells possess the most crucial property of neural stem cells, self-renewal. To do so, we first labelled S-phase cells with BrdU in a cumulative manner, twice a day over a time course of 9 days, to increase the number of slow proliferating cells labelled. This procedure results

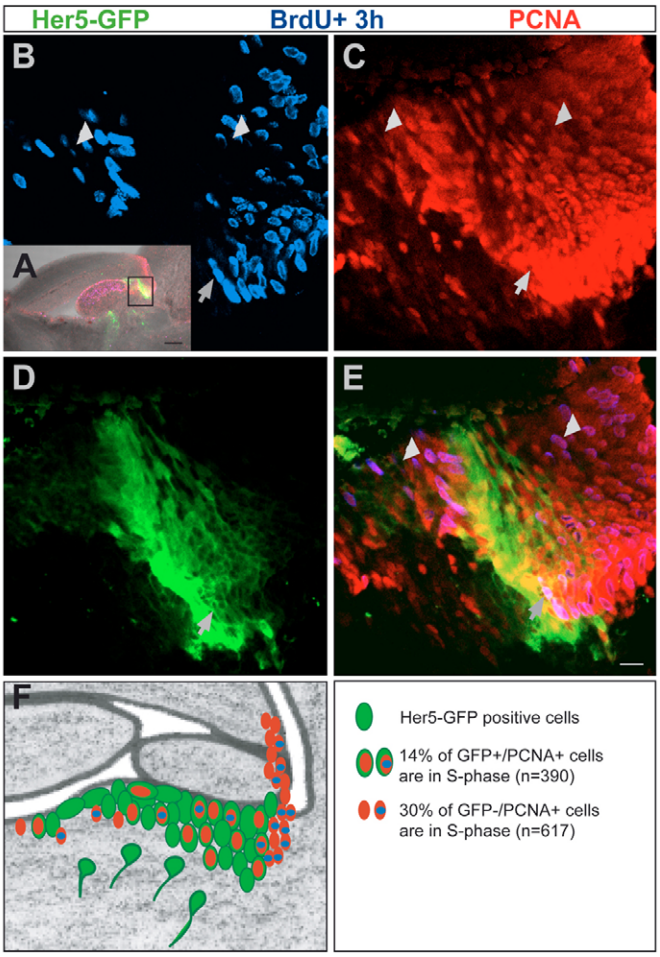

Fig. 3. Her5-GFP-positive cells are slow proliferating. Sagittal section (anterior left) through a 3-month-old *her5pac:egfp* transgenic brain 4 hours after BrdU incorporation. BrdU, PCNA and Her5-GFP are co-detected by immunocytochemistry (BrdU, blue; PCNA, red; Her5-GFP, green). (**A**) Overview of the midbrain-hindbrain area (anterior left), showing the area analysed in B-E (boxed). (**B-E**) Close up on the IPZ, single confocal plane. Her5-GFP-positive cells express PCNA (compare C with D) but few are BrdU-positive. One of these triple-labelled cells is shown by the arrow. This indicates the long cell cycle of Her5-GFP-positive cells. By contrast, a high proportion of the neighbouring Her5-GFP-negative, PCNA-positive population incorporated BrdU (short arrows point to such cells), indicating their short cell cycle. (**F**) Summary drawing of the IPZ area (as in Fig. 1J, Fig. 2H), depicting BrdU labelling (blue) within the Her5-GFP ventricular population and within the neighbouring PCNA-positive, Her5-GFP-negative population. The slow-proliferating Her5-GFP-positive population is juxtaposed along the AP to fast-proliferating domains, which are Her5-GFP-negative. The labelling indexes of *her5*-positive versus *her5*-negative domains are indicated on the bottom right. Scale bars: 100 μm in A; 10 μm in B-E.

in labelling roughly 80% of the PCNA-positive population (not shown). Given that globally 14% of Her5-GFP-positive cells express PCNA, we estimate that our cumulative BrdU experiments labelled approximately 12% of the ventricular Her5-GFP-positive population. Two months after BrdU labelling, the animals were sacrificed and immunostained for PCNA, BrdU and Her5-GFP, and we searched for long-lasting, or self-renewing, progenitors (i.e. BrdU-positive cells that maintained PCNA expression after 2 months). We found that such long-lasting progenitors were present within the ventricular Her5-GFP-positive population, as shown in Fig. 5 [the triple-labelled cell (arrowhead) positive for PCNA (red), BrdU (blue) and Her5-GFP (green) appears white on the merged image (c); two brains were analysed and seven and one triple-labelled cells were found, respectively]. More would probably be revealed if it was technically possible to label the entire slow-proliferating population. In more frequent cases, we found Her5-GFP-positive cells that had incorporated BrdU and remained located in the ventricular cell cluster of neuroepithelial morphology, without expressing PCNA (not shown). These cells, which do not display a differentiated morphology and are outside the region expressing differentiation markers (see below), could have entered a quiescent state. Together, these results show that a proportion of *her5*-positive cells possesses the ability to self-renew.

her5-positive cells are multipotent and contribute to de novo neurogenesis

Finally, the ultimate trait of a stem cell is its multipotency, and we tested whether *her5*-positive cells shared this property. We first used long-term tracing following cumulative BrdU labelling to establish the global evolution of the progeny of *her5*-positive cells in space. By analysing the location of BrdU-positive cells right after the last injection and 2 months later, we observed a prominent redistribution of BrdU-labelled cells towards more ventral domains over time [see Fig. 5B, compare the location of Her5-GFP-positive cells (green) with BrdU-positive cells (blue) below (blue arrow)]. This result is in agreement with the location of Her5-GFP-positive, *her5*-RNA-negative cells of differentiated morphology (Fig. 1G,I, small arrows) and suggests that the progeny of *her5*-positive cells contributes to the tegmentum. By contrast, cells originating from the tectal proliferation zone (Fig. 5B, red stating, red arrow), which do not express Her5-GFP, redistribute towards the optic tectum (Fig. 5B, black arrow).

To determine whether de novo neurogenesis is taking place in the IPZ, we used two approaches. First, we examined the expression of several proneural genes involved in the embryonic neurogenic cascade (Bertrand et al., 2002; Chapouton and Bally-Cuif, 2004). We observed that *deltaA*, *deltaB*, *her4*, *ngn1* and *ash1a* were all expressed within or immediately below the IPZ (Fig. 6A-D, and not shown). At the cellular level, there was a partial overlap between Her5-GFP and *ash1a* or *her4* expression (see Fig. 6C,D). Thus, the molecular factors required for neuronal differentiation are present in the IPZ area. Second, we tested whether newborn cells in the tegmentum acquire a neuronal identity, as revealed by expression of the postmitotic neuronal marker Hu (Barami et al., 1995; Mueller and Wullimann, 2002). To this aim, we labelled progenitors with two pulses of BrdU, and analysed the identity of their progeny 2 weeks later. We found that, below the IPZ, many BrdU-labelled cells expressed Hu 2 weeks after their generation (Fig. 6E-H). Together, these results demonstrate that de novo neurogenesis is ongoing in the adult IPZ area.

We next addressed directly the fate of the progeny of the *her5*-positive cells, tracing their fate in vivo, using Her5-GFP as a lineage marker. As described above, Her5-GFP-positive cells that no longer express *her5* transcripts and that display a differentiated morphology are found within the tegmentum (see Fig. 1G,J, small arrows, Fig. 6L,P,T) after having left the ventricular proliferating pool. To define the identity of these differentiated Her5-GFP-positive cells, we studied expression of the neuronal precursor marker PSA-NCAM (a polysialylated form of the cell surface protein NCAM expressed on young migrating neuroblasts), the neuron-specific marker Hu, the oligodendrocyte precursor markers

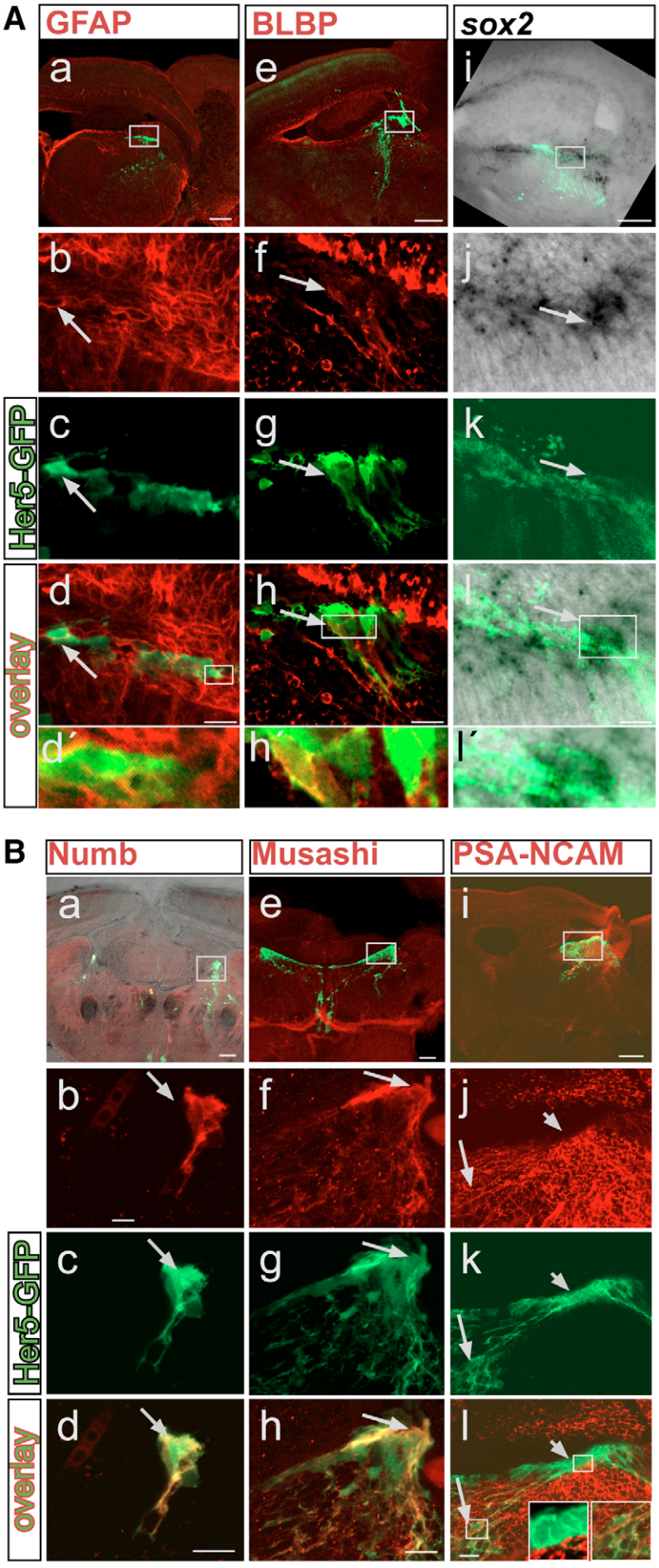

Fig. 4. Her5-GFP-positive cells lining the tectal ventricle express stem cell markers. (**A**) Co-localization of Her5-GFP (green) with GFAP (a-d′), BLBP (e-h′), *sox2* (i-l′). (**B**) Co-localization of Her5-GFP (green) with Numb (a-d), Musashi (e-h) and PSA-NCAM (i-l). Sagittal (GFAP, BLBP, *sox2*, PSA-NCAM) or cross-sections (Numb and Musashi) are shown. *sox2* is detected by in situ hybridization (black signal), while all other markers are detected by immunohistochemistry (red signal). (A, parts i-l′; B, part a) An overlay with the brightfield view in addition to fluorescence. All long arrows point to cells that co-express Her5-GFP and the respective markers. (B, parts j-l) Her5-GFP-positive cells located close to the ventricle, which also express *her5* RNA (Fig. 1C,E), are PSA-NCAM-negative (short arrow, left inset) while Her5-GFP-positive cells located further ventrally do express PSA-NCAM (long arrow, right inset), indicating their differentiation into a neuronal fate. Scale bars: 100 μm in a,e,i; 10 μm in b-d,f-h,j-l.

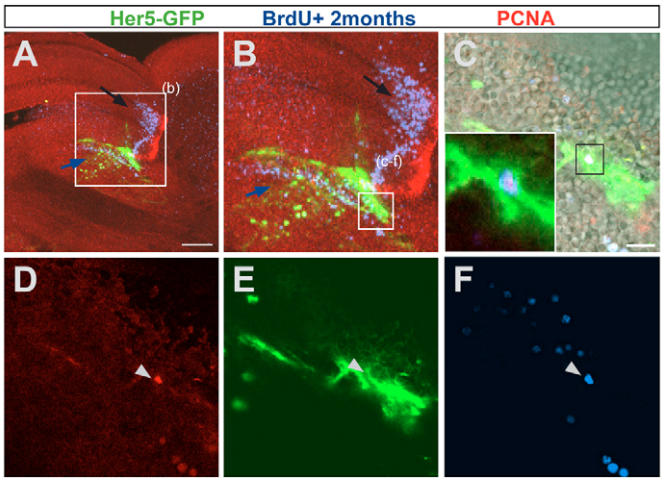

Fig. 5. her5-positive cells are self-renewing. A 3-month-old *her5:gfp* transgenic brain was injected cumulatively over 9 days with BrdU and sacrificed 2 months later. The sagittal section is seen as an overview in **A**, and magnified in **B-F**, as single confocal planes. (C-F) Enlargement on an Her5-GFP cell (green, pointed by an arrowhead) that has incorporated BrdU 2 months earlier (blue) and is PCNA-positive (red), indicating that it has remained in a proliferative state after division, i.e. it is a self-renewing progenitor. Note that most cells of the IPZ that have incorporated BrdU 2 months earlier move towards ventral positions into the tegmentum (see in B the location of blue cells below the Her5-GFP domain, blue arrow), while cells originating from the tectal proliferation zone are displaced towards anterior into the optic tectum (B, black arrow). Scale bars: 100 μm in A; 10 μm in C.

O4 and Quaking (QKI) (Chen and Richard, 1998; Larocque et al., 2005) and the astrocytic markers GFAP and S100β. We found that few Her5-GFP-positive, *her5*-RNA-negative cells expressed O4 and Quaking (Fig. 6I-P, mean of eight O4-positive cells per brain, analysed in three brains, and five QKI-positive cells counted in one brain), and thus had acquired an oligodendroglial fate. These cells were located close to the ventricle in the anteriormost position within the Her5-GFP cluster. In that same area, we found few GFP-positive cells expressing S100β (six cells in one hemisphere of one brain analysed, data not shown); thus, these cells acquired an astrocytic fate. In addition, a large proportion of Her5-GFP-positive, *her5*-negative cells expressed the neuronal marker Hu (Fig. 6Q-T). In fact, 100% of the cells that left the progenitor cluster, displayed long processes and were located in the tegmentum ventrally to the progenitor pool were Hu-positive (100 cells per brain, analysed in two brains). Thus, *her5*-positive cells also prominently differentiate into neurons in situ. Finally, we also observed that Her5-GFP-expressing cells located between the ventricular surface and the Hu-positive neurons expressed PSA-NCAM (Fig. 4B, parts i-l). In the mammalian adult forebrain, PSA-NCAM characterizes cells that are still proliferating but have recently exited the stem cell and transit amplifying pools to enter the neuronal commitment pathway, and is generally found in close association with neural stem cell pools (Doetsch et al., 2002a). Thus, the co-expression of Her5-GFP and PSA-NCAM demonstrates the existence of the intermediate, neuroblast step between the *her5*-positive stem/progenitor cells and the Her5-GFP-positive, *her5*-negative, postmitotic neurons. Together these results demonstrate the multipotentiality of adult *her5*-positive cells in their original environment, where they give rise to differentiated neurons and glia.

Finally, to prove directly that Her5-GFP-positive newborn neurons in the adult tegmentum originate from *her5*-expressing cells that were proliferating during adulthood, we traced newly born cells using both BrdU and the stability of GFP. Cells labelled by cumulative BrdU administration over 9 days were analysed after 2 months of survival in triple-stained preparations for Her5-GFP (Fig. 7A-C,E, green), BrdU (Fig. 7A-D, blue) and the neuronal marker Hu (Fig. 7A-C,F, red). We observed that triple-labelled cells were present below the IPZ area (Fig. 7A-C, these cells appear white on the merged image; mean of eight cells per brain, four brains were injected at 3 months and analysed at 5 months). This result indicates that progenitors proliferating 2 months earlier still express Her5-GFP in the vicinity of the IPZ and have given rise to Hu-expressing newborn neurons. Together these results demonstrate that the *her5*-expressing population contains dividing progenitors that give rise to newborn tegmental neurons (Fig. 7G).

DISCUSSION

In spite of the abundant literature on vertebrate adult neural stem cells, it remains largely unknown whether, and to what extent, the properties defined in the adult rodent telencephalon can be generalized. We identify here a novel zone of adult neurogenesis in situ, the IPZ, at the border between midbrain and hindbrain structures. We demonstrate that the H/E(Spl) transcription factor Her5 is selectively expressed in this domain, and we further provide evidence that *her5*-positive cells, but not their immediate neighbors, display a combination of neural stem cell attributes. Finally, direct tracing in vivo demonstrates that the progeny of these cells reach ventral positions within the tegmentum, where they acquire a neuronal or glial identity. Together, our findings identify in vivo a new neural stem cell population. Further, our results show that, as in the embryo, expression of some *e(spl)* genes is associated with the neural stem cell state in the adult brain. Thus, the examination of E(Spl) expression and function in a systematic manner in the vertebrate adult brain might reveal new selective markers and control mechanisms of the adult neural stem cell state.

A new neural stem cell model in the vertebrate adult brain

The most important finding of the present work is the identification of a new population of neural stem-cell-like progenitors in the zebrafish adult brain. Our arguments supporting this conclusion are based on the observations that cells within the Her5-GFP-positive population: (1) are slow-proliferating (Fig. 3); (2) are positive for the expression of the neural stem cell markers BLBP, GFAP, *sox2*, Musashi and Numb (Fig. 4); (3) are self-renewing progenitors (Fig. 5); and (4) give rise to differentiated neurons and glia (Figs 6, 7). These properties together fulfil the requirements defining the neural stem cell state. Further, we observed that this population persists throughout life, although, as for mammalian neural stem cells (Kippin et al., 2005), the number of *her5*-positive cells and the proportion of PCNA-positive cells within the Her5-positive cells tend to decrease with age (fewer positive cells are found in a 1-year-old brain than in a 3-month-old brain).

These results are important for several reasons. First, they provide us with a new model to be compared with known mammalian neural stem cell populations. Second, this stem cell population is located outside the telencephalon, further broadening the field of investigation for neural stem cell characteristics within the adult vertebrate brain. Third, this new model is established in the zebrafish, a species directly amenable to the study of gene

function by means of transgenes or mutations. Finally, this stem-cell-like population is readily accessible for experimentation via Her5-GFP expression in our transgenic model. Together, our findings provide the stem field with a most promising model to help characterize the general principles of adult neural stem cell biology. The division and differentiation potentialities of neural progenitor cells are often addressed in vitro, using the neurosphere assay (Reynolds et al., 1992). Using this assay, one conclusive example of non-telencephalic neural stem cells was recently revealed in mammals at postnatal stages (Lee et al., 2005). Because we are working here with a completely new set of potential stem cells in a new vertebrate model, we were concerned that alterations in cell potentialities in such culture systems might bias our findings. Thus we opted for direct, Her5-GFP-mediated tracing of the IPZ stem cell pool as a more reliable test, providing concomitantly the first demonstration of a non-telencephalic population of neural stem cells generating neurons and glia *in situ* in the vertebrate adult brain.

Current knowledge about the mammalian telencephalon led to a model (Doetsch et al., 2002b) in which stem cells are slow-proliferating (the 'B' state), and give rise to committed precursors (the 'A' state) via a quickly dividing, transiently amplifying population (the 'C' state). By combining a short BrdU pulse with PCNA or MCM5 expression studies (Fig. 3), or using cumulative BrdU labelling (not shown), we found, indeed, that her5-positive cells proliferate slowly. Thus, they probably represent the 'B', true stem cell state. However, it remains unclear whether a transiently amplifying population originates from the Her5-GFP-positive domain. We observed that cells adjacent to the her5-positive population along the AP axis do divide quickly. This is particularly

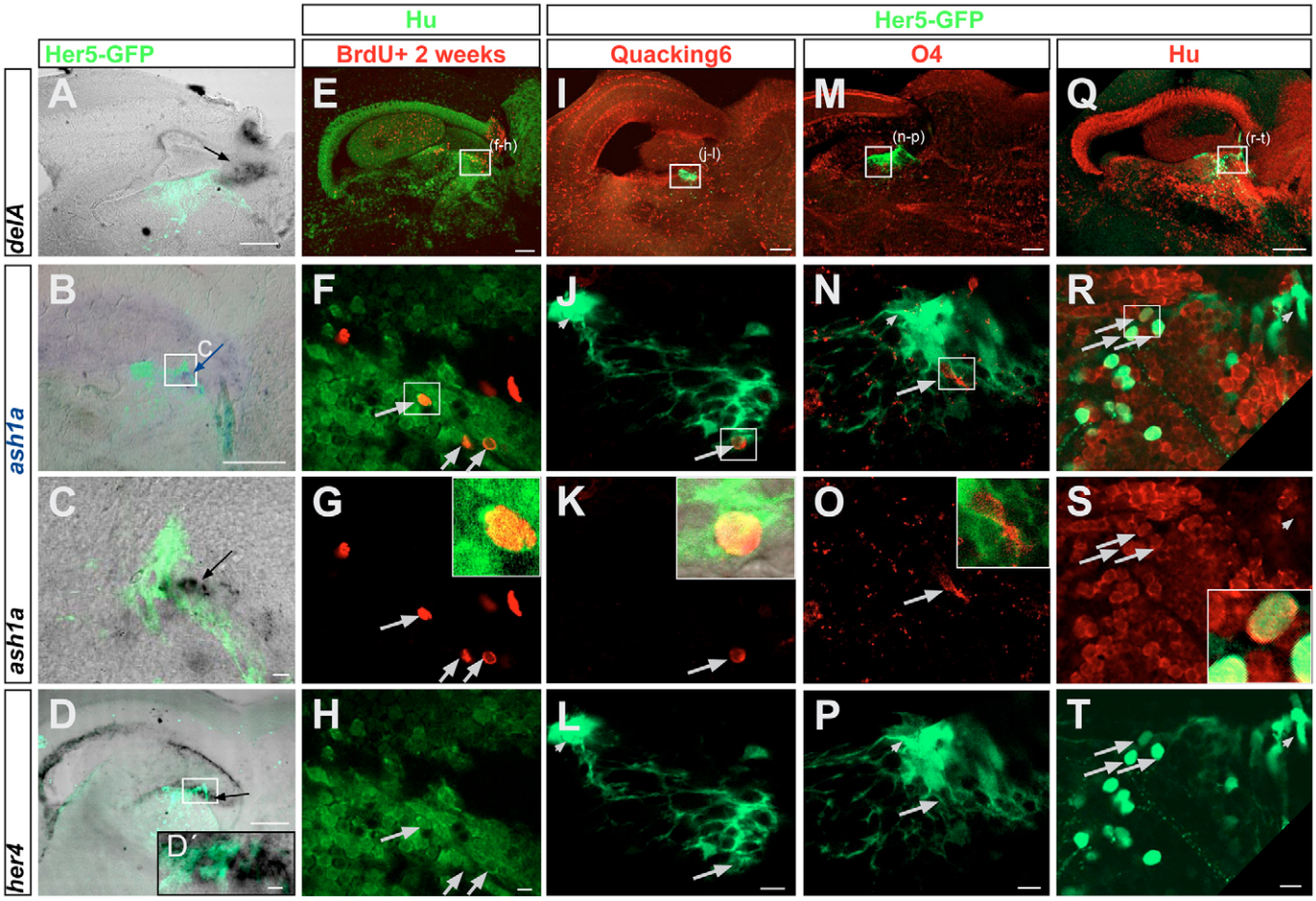

Fig. 6. Her5-positive cells differentiate into neurons and oligodendroglia in vivo. (**A-D**) Expression of proneural genes within or near the IPZ, indicating an ongoing neurogenesis in that area. *deltaA* (A), *ash1a* (B,C) and *her4* (D,D′) are detected by in situ hybridization on sagittal sections of *her5:gfp* brains (anterior left) and visible as a black or blue (in B) signal (indicated by the arrows). All panels are an overlay of brightfield and fluorescence exposures, also revealing Her5-GFP protein in green. Note some overlap between Her5-GFP and *ash1a* (C) or *her4* (D′). (**E-H**) Generation of neurons (Hu-positive cells in green) 2 weeks after BrdU injection (red) next to the IPZ, in a 6-month-old non-transgenic brain. Confocal planes of a sagittal section (anterior left) seen as an overview in E, and magnified in F-H. The arrows point to BrdU/Hu double-positive cells. The inset in G depicts an enlarged double-labelled cell boxed in F. (**I-P**) Differentiation of Her5-GFP-positive cells (green) into oligodendrocytes. Confocal planes of a sagittal section (anterior left) as an overview in I and M, and magnified in the panels below. Some Her5-GFP-positive cells pointed to by the long arrows co-express QKI-6 (J-L, enlarged in the inset in K) or O4 (N-P, inset in O). Small arrows in J,L,N,P point to the ventricular Her5-GFP-positive cells, which are negative for QKI-6 or O4. (**Q-T**) Differentiation of Her5-GFP-positive cells into neurons. Confocal planes of a sagittal section (anterior left) seen as an overview in Q, and magnified in R-T. All Her5-GFP-positive cells located away from the ventricular surface express the neuronal marker Hu (long arrows in R-T to some of them, enlargement of one double-labelled cell in the inset in S). The small arrows in R-T point to the ventricular Her5-GFP-positive population, which is Hu-negative. Scale bars: 100 μm in A,B,D and in the upper row; 10 μm in C,F-H,J-L,N-P,R-T.

obvious for progenitors contributing to the growth of the optic tectum (Fig. 3). However, we could not detect Her5-GFP expression in these fast proliferating cells. Of particular importance, within the tegmentum, newborn cells having already acquired the neuroblast fate (PSA-NCAM) (Fig. 4) or differentiation markers (Hu, Quaking, O4) (Fig. 6) still express Her5-GFP. Thus, if progeny cells exit the her5-positive pool along the AP axis to contribute to the fast-proliferating domain, then their elimination of the Her5-GFP label must be much faster than that in ventral populations. Consequently, an abrupt transition would be created from Her5-GFP-positive to Her5-GFP-negative domains along the AP axis, strikingly different from the gradual transition observed along the DV axis, where Her5-GFP expression progressively diminishes towards the ventral tegmentum. Because long-term BrdU tracing also highlights a ventral migration of ventricular cells of the IPZ area (Fig. 5B), we currently favour a model in which ventricular her5-positive progenitors primarily generate ventral progeny, without a prominent intermediate fast-proliferating state. This interpretation will, however, need to be tested using direct cell tracing, independent of the Her5-GFP label.

Another intriguing aspect of the IPZ is the combination of its location at the MHB and its expression of Her5. We have not directly addressed the origin of the adult her5-positive pool, and cannot exclude that these cells migrate into the IPZ from another, possibly neighbouring, source and initiate her5 expression de novo. However, both their final location and their molecular profile are reminiscent of the embryonic intervening zone (IZ), suggesting that the IZ and IPZ progenitor pools, in addition to sharing molecular components, might be related in lineage. In embryos, her5 is expressed throughout the DV axis; its restriction to the alar-basal boundary in adults might result from the growth of the dorsally located tectum and torus semicircularis and the ventrally located tegmentum, leaving a cluster of expressing cells in between, adjacent to the tectal ventricle. Mammalian adult neural stem cells of the subventricular zone have also been demonstrated to originate from a remnant of the embryonic telencephalic ventricular zone, and their astrocytic nature is also seen as a late derivative of the radial glia, which serve as neuronal progenitors in the embryo (Götz and Barde, 2005; Merkle et al., 2004). Thus, our observations reinforce the notion that adult neural stem cells might develop from long-lasting embryonic progenitors. It is clear, however, that these acquire at adulthood typical characters that contrast them from embryonic progenitors, such as, at the IPZ, the loss of expression of some embryonic IZ genes (e.g. him, not shown), the initiation of expression of glial markers (which are absent from the IZ), and the acquisition of a slow-proliferation mode.

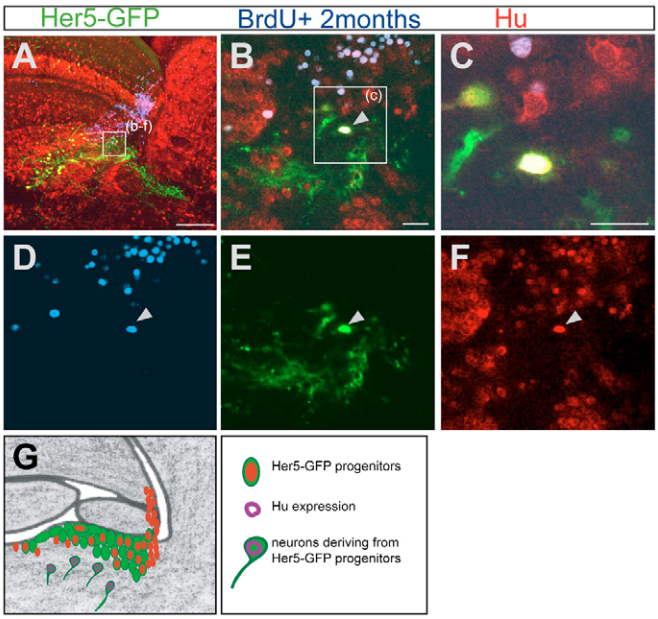

Fig. 7. Her5-GFP-positive newborn tegmental neurons originate from cells proliferating at adulthood. (**A-F**) Sagittal section (anterior is to the left) of a 5-month-old brain stained for Her5-GFP (green), Hu (red) and BrdU (blue), 2 months after a cumulative BrdU labelling. (A) Overview of the tegmental region containing differentiated Her5-GFP-positive cells. (B-F) Higher magnifications of the same section. The arrow in B points to a cell in the tegmentum triply labelled for Hu, BrdU and GFP, indicating that this neuron is deriving from a Her5-expressing cell and was generated 2 months earlier. The single channels of this confocal plane are depicted in D-F. (C) Higher magnification of the same triple-labelled cell. (**G**) Summary scheme of the IPZ area (as in Fig. 1J, Fig. 2H, Fig. 3F), depicting the tegmental neurons (Hu staining, purple) generated by Her5-expressing progenitor cells. Scale bars: 100 μm in A; 10 μm in B,C.

Significance of de novo neurogenesis in the adult midbrain

In the embryo, the IZ gives rise to the entire midbrain-hindbrain domain (Tallafuss and Bally-Cuif, 2003) and thus probably to the entire panel of neuronal types in this region. Whether the adult IPZ is of such functional relevance remains to be tested, but such experiments are rendered difficult by the fact that the Her5-GFP label is not permanent. It is lost over time in differentiating cells, leading to an underestimation of the number of newborn neurons that actually form from the Her5-GFP-positive zone. Nevertheless, we counted an average of 100 Hu-positive neurons carrying the transient marker Her5-GFP at a given time point in the adult tegmentum, and the significance of this value will be further increased when put in perspective with the size of the specific neuronal population(s) contributed to by the *her5*-positive pool. We have not detected specific cell death in the IPZ area and surrounding domains within the adult brain (not shown), suggesting that the differentiated neurons originating from the IPZ are not immediately eliminated on site. The identification of their neurotransmitter phenotype, as well as of the networks into which they might be integrated, will await the development of techniques allowing permanent tracing of *her5*-positive cells in adulthood.

An important question for future studies will also be to determine whether the zebrafish IPZ discovered here has a counterpart in adult mammals. Adult neurogenesis in the mammalian adult substantia nigra was proposed in one study (Zhao et al., 2003), but these findings remain controversial (Lie et al., 2002). Although the substantia nigra belongs to the midbrain, we believe that it cannot be compared to the zebrafish IPZ, first because these regions are anatomically distinct (the substantia nigra is located far too ventrally), and secondly because the zebrafish midbrain is devoid of dopaminergic differentiation (Rink and Wullimann, 2001). Because the progenitor properties of the embryonic IZ have been documented in all vertebrate embryos (Bally-Cuif et al., 1993; Hirata et al., 2001; Palmgren, 1921), and because it relies, in mammals as well as in zebrafish, on the expression of E(Spl) factors (Hirata et al., 2001), our findings here suggest the exciting possibility that a domain equivalent to the IPZ might also not be restricted to teleosts. Thus it would be most interesting to re-examine whether the junction between midbrain and hindbrain in the adult mammalian brain

contains stem cells, perhaps using as tools *e(spl)* probes. Should this domain in other vertebrates not display a neurogenic potential, then comparing the developmental steps and the differential expression genes in the zebrafish and mammalian MHB could also give crucial insight into the factors involved in the maintenance or disappearance of this germinal zone.

her5 expression is associated with the maintenance of a stem cell state in the adult brain

We previously demonstrated an active role of Her5 in maintaining the neural progenitor state during embryonic development. In this study, we now document expression of *her5* in a cluster of progenitors with neural-stem-cell-like properties in the adult midbrain. These results suggest that the active role of Her5 in preventing neural differentiation might be maintained in adulthood, and we are currently manipulating *her5* expression within the adult brain to test this hypothesis. A puzzling aspect of the *her5*-positive population is its heterogeneity. In particular, we found within the IPZ *her5*-positive cells that displayed neuroepithelial morphology but were PCNA- (or MCM5)-negative (Fig. 2E,H). Some of them had incorporated BrdU at an earlier stage (not shown). These cells do not express differentiation markers; however, they might be in an intermediate state of commitment towards the differentiated state. Alternatively, and perhaps more likely as they are located at the ventricle, these cells might have entered a quiescent state. This interpretation is supported by the fact that PCNA expression is often shut down or undetectable in quiescent cells (Maga and Hubscher, 2003), but will need to be verified once markers of the quiescent state are identified. Another interesting issue is to determine whether single *her5*-positive cells, as opposed to the *her5*-positive population, are multipotent in vivo. Such analyses will await the development of single cell long-lasting tracing techniques in the adult zebrafish brain. Notably, however, for technical reasons the demonstration of a multipotent fate at the single cell level in vivo is also still pending for adult mammalian SVZ stem cells.

A specific class of Notch-independent E(Spl) factors, which act upstream of the lateral inhibition cascade, is involved in maintaining progenitor pools within the embryonic neuroepithelium (Bae et al., 2005; Baek et al., 2006; Geling et al., 2003; Hans et al., 2004; Hatakeyama et al., 2004; Kageyama et al., 2005; Ohtsuka et al., 2006) and our findings suggest that this is extended to the adult neurogenesis. Interestingly, using the *her5pac:gfp* transgenic line, we observed Her5-GFP protein expression in several other very restricted clusters within the zebrafish adult brain (data not shown). These clusters were always located in the immediate vicinity of, or within, a proliferating zone, as for example at the margin of the retina, in the ventricular zone of the subpallium, in the habenula, or in the hypothalamus. We repeated these observations in several independent lines (e.g. in *–3.4her5:egfp*). Her5-GFP-positive cells were in most regions early postmitotic, and *her5* RNA could not be detected. However, we detected endogenous *her5* expression and the co-expression of PCNA in Her5-GFP-positive cells within the hypothalamus (not shown). It will now be an important issue to assess the function(s) of the Notch-independent H/E(Spl) transcription factor family in adulthood, and determine whether it plays a general role in maintaining neural stem cells in regenerative regions of adult brains.

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PUBLICATIONS

1. **A. Tallafuss, B. Adolf and L. Bally-Cuif:**
   Selective control of neuronal cluster size at the forebrain / midbrain boundary by signalling from the prechordal plate.
   Dev. Dyn. 227: 524-535 (2003).

2. **B. Adolf, G. Bellipanni, V. Huber and L. Bally-Cuif:** *atoh1.2* and *beta3.1* are two new bHLH-encoding genes expressed in selective precursor cells of the zebrafish anterior hindbrain.
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3. **B. Adolf, P. Chapouton, C. S. Lam, S. Topp, B. Tannhäuser, U. Strähle, M. Götz and L. Bally-Cuif:** Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon.
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4. **P. Chapouton, B. Adolf, C. Leucht, S. Ryu, W. Driever and L. Bally-Cuif.**
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POSTERS AT INTERNATIONAL MEETINGS

1. **B. Adolf, P. Chapouton, M. Götz, and L. Bally-Cuif:** Proliferation and neurogenesis in the adult zebrafish brain
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