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 telencephalic 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 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 (neurogenin1/ngh1) 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...
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 HE(E) 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)1939] or 3.4her5:egfp (Tg(3.4her5:EGFP)1911 (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 tricine, 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 vibratome sections (vibrating microtome HM 650 V, Microm): whole brains were embedded in 3% agarose in PBS and cut serially at 100 μm.

In situ hybridization was performed as for whole-mount embryos (Hauptmann and Gerster, 1994), starting with whole-mount brains. After the brains were embedded in 3% agarose in PBS and cut serially at 100 μm vibratome sections (vibrating microtome HM 650 V, Microm): whole brains were embedded in 3% agarose in PBS and cut serially at 100 μm.

The sections were dehydrated in methanol and stored in 100% methanol at –20°C.

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:gfpl), 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 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.

**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
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
showing the area analysed in B-E (boxed). (A) Overview of the midbrainhindbrain 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 slowproliferating Her5-GFP-positive cells are 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 triplelabelled cells were found, respectively]. More would probably be revealed if it was technically possible to label the entire slowproliferating population. In more frequent cases, we found Her5GFP-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. 1GJ, 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 ashl1a 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 ashl1a 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 tectum (see Fig. 1GJ, 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.
neuroblast step between the PSA-NCAM demonstrates the existence of the intermediate, (Doetsch et al., 2002a). Thus, the co-expression of Her5-GFP and generally found in close association with neural stem cell pools still proliferating but have recently exited the stem cell and transit mammalian adult forebrain, PSA-NCAM characterizes cells that are positive neurons expressed PSA-NCAM (Fig. 4B, parts i-l). In the into neurons in situ. Finally, we also observed that Her5-GFP-positive, her5-positive stem/progenitor cells in their original environment, where they give rise to differentiated neurons and glia. 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 fulfill 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

| Her5-GFP | BrdU+ 2 weeks | Quacking6 | O4 | Her5-GFP |
|----------|--------------|-----------|----|----------|
| A         | E            | I         | M  | Q         |
| B         | F            | J         | N  | R         |
| C         | G            | K         | O  | S         |
| D         | H            | L         | P  | T         |

**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) seen 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 (I-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.

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 Hubscber, 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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