Distinct roles of neuroepithelial-like and radial glia-like progenitor cells in cerebellar regeneration

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Summary

Zebrafish can regenerate brain injury, and the regenerative process is driven by resident stem cells. Stem cells are heterogeneous in the vertebrate brain, but the significance of having heterogeneous stem cells in regeneration is not understood. Limited availability of specific stem cells might impair the regeneration of particular cell lineages. We studied regeneration ability of the adult zebrafish cerebellum. The zebrafish cerebellum contains two major stem and progenitor cell types, ventricular zone and neuroepithelial cells. Using conditional lineage tracing, we demonstrate that cerebellar regeneration depends on availability of specific stem cells. Radial glia-like cells are thought to be the predominating stem cell type in homeostasis and after injury. However, we find that radial glia-like cells play a minor part in adult cerebellar neurogenesis and in recovery after injury. Instead, we find that neuroepithelial cells are the predominant stem cell type supporting cerebellar regeneration after injury. Zebrafish are able to regenerate many, but not all cell types in the cerebellum, which emphasizes the need to understand the contribution of different adult neural stem and progenitor cell subtypes in the vertebrate CNS.

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Introduction

Neural stem and progenitor cell populations are heterogeneous and biased in their ability to produce specific cell types in the vertebrate brain (Shen et al., 2006; Merkle et al., 2007; Kaslin et al., 2009; Ganz et al., 2010; Marz et al., 2010). The implication of heterogeneous brain stem cells for tissue regeneration is not well understood. However, the limited availability or depletion of specific stem and progenitor cells could impair the regeneration of specific brain cell lineages (Liu et al., 2009; Encinas et al., 2011; Barbosa et al., 2015). Teleost fish have a much greater ability to regenerate CNS injuries than mammals (Becker et al., 1997; Kaslin et al., 2008; Reimer et al., 2008; Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Kizil et al., 2012). Neural regeneration in the zebrafish telencephalon is driven by distinct tissue resident stem and progenitor cells (Reimer et al., 2008; Kroehne et al., 2011; Barbosa et al., 2015), but cellular regeneration has not been studied in detail in other brain parts. However, zebrafish maintain widespread neural stem cell activity along the neural axis throughout life suggesting that stem cells could potentially repair injury in any brain region (Adolf et al., 2006; Grandel et al., 2006; Kaslin et al., 2008). The current consensus drawn from relatively few studies in one brain region, the telencephalon, suggest that the teleost brain can repair most if not all cell types (Reimer et al., 2008; Kroehne et al., 2011; Barbosa et al., 2015). However, it is unclear whether all cell types can be regenerated after brain injury in every part of the brain. We previously demonstrated that the zebrafish cerebellum contains stem and progenitor cells with different capacities to generate neurons and glia over their life span (Kaslin et al., 2009; Kaslin et al., 2013). Two main types of stem and progenitor cells give rise to the different cells in the juvenile and adult cerebellum. Neuroepithelial-like stem and progenitor cells in the upper rhombic lip continuously gives rise to granule cells in adult and aging zebrafish (Kaslin et al., 2009; Kaslin et al., 2013). Progenitor cells in the ventricular zone (VZ) give rise to the other cell lineages, such as Purkinje Cells, Golgi, stellate and eurydendroid cells and the VZ progenitor cell activity and production nearly ceases in the adult zebrafish cerebellum (Kaslin et al., 2009; Kaslin et al., 2013). Given these differences in stem and progenitor cell behaviour we sought to determine which stem and progenitor cell population(s) respond to injury and contribute to cellular regeneration in the zebrafish cerebellum. The zebrafish cerebellum is ideally suited for this because it has well defined cellular architecture and cell types (Bae et al., 2009; Kaslin et al., 2009; Kaslin and Brand, 2012). In addition, the architecture and cell types have direct counter parts to the mammalian cerebellum (Kaslin and Brand, 2012). We performed a unilateral

surgical injury that removed all cerebellar cell types to determine the adult zebrafish capacity to regenerate these diverse cell types. By using histological analysis, BrdU pulse chases and genetic lineage tracing we found that only specific cell lineages regenerate well after injury in the adult cerebellum. To confirm the role of different stem and progenitor cell populations in recovery we used stem cell specific reporters and proliferation markers. Stem and progenitor cell activity is significantly upregulated after injury and the capacity to generate specific cell lineages directly correlates with the presence of particular stem and progenitor cell types in the adult cerebellum. We found that the population of neuroepithelial-like stem cells declined in the juvenile fish but remained stable in the aging cerebellum. Contrary to the behaviour of the neuroepithelial-like stem cells, the number and activity of VZ progenitor cells was nearly exhausted. However, juvenile fish up to one month demonstrate activity of both stem and progenitor cell types and production of all major cerebellar cell types. By performing injury experiments in the juvenile zebrafish we found that the juvenile fish can regenerate all cell lineages. Taken together, our data demonstrate that only cell types that are homeostatically produced in the adult zebrafish cerebellum regenerate well, and that cerebellar stem and progenitor cells keep to their specified cell lineages even after injury. In contrast to the zebrafish telencephalon, radial glia-like cells play only a minor part in the adult cerebellum during homeostasis or after injury. Our findings highlight the importance of understanding heterogeneity of stem cells *in vivo*. Importantly, our data brings significant contrast to the predominating view that zebrafish can regenerate all cell types in the CNS.

Material and Methods

Zebrafish

Zebrafish were bred and maintained according to standard procedures (ZFIN.org). All animal procedures were approved by the Regierungspräsidium Dresden (permit AZ 24D-9168.11-1/2008-1 and -4) and Monash Animal Ethics Committee (MARP/2013/096), in accordance with the requirements of the National Health & Medical Research Council of Australia. Wild type experimental fish were from the *gol-b1* line in the AB background. Fish were raised at a density of 50-60 fish/tank. The larvae/juveniles were kept in 7 l mouse cages and moved to 11 l glass tanks at 1 month of age. Fish of either sex were used. For the quantifications and lesion experiments: 1 month old juvenile fish had a body length of 9 mm (+/- 0.5 mm) , 3 month old young adults were 19 mm (+/- 1 mm) and 6 month old adult fish 30 mm (+/- 2 mm).

The cerebellar injury assay

Cerebellar lesions were made under anesthesia by drilling a hole in the neurocranium (Trepanation) with a vibration free dental drill (WPI), and then careful removal of cerebellar tissue. In the 6 month old adult fish, the lesion results in unilateral removal of a 350µm wide and approx. 500µm deep column of cerebellar tissue. In the 1 month old juveniles a 100µm wide and approx. 200µm deep column of cerebellar tissue was removed.

Transgenic lines

The Tg(*-5.5ptf1a:DsRed), Tg(nestin:GFP)* and *Tg(hsp70l:DsRed2(floxed)EGFP)* lines have been described previously (Kaslin et al., 2009; Kroehne et al., 2011; Kaslin et al., 2013). The *Tg(ptf1a:cherryT2aCreERT2)* line was made by cloning the cherryT2aCreERT2 in place of dsred in the previously used *-5.5ptf1a:DsRed* construct (Kaslin et al., 2013). Stable transgenic lines were created by Tol2 mediated transgenesis and the pT2AL200R150G vector (Kawakami et al., 2004; Urasaki et al., 2006). 25pg of vector DNA and 50 pg of Tol2 transposase mRNA were injected into fertilized eggs at one cell stage. F0 were raised and in-crossed. F1 progeny were identified by screening for cherry expression.

Tamoxifen induced lineage tracing

Recombination in zebrafish embryos was achieved by incubating the fish for 24-72 hours in E3 containing 5µM 4-OH and 0.1% DMSO. Recombination in juvenile fish was induced by incubating the fish three times overnight (14 hours) in 5µM Tamoxifen in E3 containing 0.1% DMSO. In adult zebrafish the recombination efficacy of immersed fish was compared to intraperitoneal or intraventricular injection of Tamoxifen or 4-OH. The immersion protocol resulted in more robust and a higher recombination rate. The lesions were performed one day after recombination. Unconditional recombination (leakiness) was tested by heatshocking *Tg(ptf1a:cherryT2aCreERT2)1; Tg(hsp70l:DsRed2(floxed)EGFP)* fish for three days. No unconditional recombination was detected in one month old fish (n=15).

BrdU labelling

To label cycling cells, zebrafish were immersed in 7.5 mM BrdU (Sigma) solution (Grandel et al., 2006). The BrdU was dissolved in E3 medium and the pH adjusted to 7.5.

Tissue preparation

Brains were exposed in situ and fixed at 4°C overnight in 2-4% paraformaldehyde/0.1 M PB (pH 7.5). They were washed twice with PB and transferred for decalcification and cryoprotection to 20% sucrose/20% EDTA in 0.1M PB (pH7.5). Brains were frozen in 7.5% gelatine/20% sucrose and cut at 16µm. Sections were stored at -20°C.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Kaslin et al., 2004). Briefly, primary and secondary antibodies were incubated in PBS with 0.3% TX. Tissue sections were incubated in primary antibodies overnight at 4 C° and secondary antibodies for 1 h RT. The slides were then washed in PBS TX and mounted. We used primary antibodies to Parvalbumin (mouse, Chemicon, 1/5000), L-plastin (rabbit, kindly provided by Michael Redd, 1/5000), NeuroD1 (mouse, Abcam, 1/2000), HU-C/D/Elavl3 (mouse, Invitrogen, 1/250), PCNA (mouse, Dako cyto, 1/1000), S100β (rabbit, Dako cyto, 1/2000), GABA (rabbit, Sigma, 1/10000), Zebrin II/Aldolase C (mouse, kindly provided by Richard Hawkes, 1/250), Pax2 (rabbit, Covance, 1/750), GFP (chick, mouse, Invitrogen, 1/1000), DsRed (rabbit, Clontech, 1/1000), BrdU (rat, Serotec, 1/500). For detection Alexa 488, 555, 633 conjugated secondary antibodies were used (Life sciences).

Image acquisition and processing

Live embryos or zebrafish were anesthetized with 0.1 % Tricaine (Sigma), mounted in 1.5% LMP agarose in embryo medium 3 (E3) and imaged with a Leica TCS SP5 confocal microscope using HCX APO L 20/0,5 NA, HCX APO L 40/0,8 NA, HCX APO L 63/0,9 NA dipping objectives. Other images were taken with a Leica TCS-SP5 confocal microscope using HC PL APO CS 20x/0,7 NA, HCX PL APO 40/1,25 NA and HCX PL APO 63x/1,2 NA objectives. To minimize cross-talk between the channels in multi colored specimens

sequential image acquisition was performed. The images were processed using ImageJ v.1.44 (http://rsb.info.nih.gov/ij/), Volocity and Adobe Photoshop CS2. Figures were assembled using Adobe Photoshop CS2 and Corel Draw X3.

Behavioral analysis

Automated video tracking of swimming performance was done by using a zebra tower setup and zebra lab software (Viewpoint, France).The swimming arena was 15 cm in diameter and filled with a 10 cm water column. Fish were accommodating 1min. in the arena prior to start of recording. Swimming behavior was recorded for 5 minutes at distance, speed and pattern was analysed using the Zebra lab software (Viewpoint, France).

Quantification and statistical analysis

We assumed normal distribution of variance among animals used in the experiments. To minimize variation fish raised from the same cohort were used (siblings) with size difference <10% in length. A minimum of 5 animals were analysed / experimental condition. The exact number of n's analysed is stated for each experiment in the results section. We quantified the number of labelled cells in every fourth section at 16 μm throughout the entire anterior-posterior length of the cerebellar corpus (8-9 sections analysed in adult fish). The cell size is small in adult zebrafish (typically 6-12 μm) and section interval (48 μm) makes counting of the same cell in adjacent section in a series unlikely, and therefore no-post correction method was used to correct for over-representation of cell number. For co-localization studies with cellular markers, co-localization was verified by analyzing high resolution confocal stacks. The optical sections were taken with 0.5-1μm intervals using 40x (1,25 NA) or 63x (1.2 NA) objectives. For all analyses a normal distribution of values was assumed. Using power calculations for 80% power at 5% significance level and S.D. values measured from experiments show requirement of 4-6 animals/group. Comparison between the groups was made by unpaired two-tailed Student's t-test or one-way ANOVA analysis with Tukey's Multiple comparisons post-test. Data are presented as mean +/- standard deviation. Statistical significance was established at *p< 0.05, **p< 0.01 and ***p< 0.001, **** = <0.0001. α=0.5, using Prism (v.4.03 and 6, GraphPad).

Results

To address if all cerebellar cell types can be regenerated after injury we performed a surgical lesion paradigm ('Trepanation') where all cerebellar cell types are removed from the adult zebrafish (≥6 months old, Figure 1 A). The surgery unilaterally removed 29% of the *corpus cerebelli* volume (+/-6%, n=7, measured one day after injury, Figure 1 B-C). Importantly, the lesion spared the cerebellar recessus and the associated stem cell niche (Figure 1 B-C, Supplementary Figure 1 A, (Kaslin et al., 2009; Kaslin et al., 2013). Rapid wound healing, regeneration of skin and bone was detected within days after the lesion (Supplementary Figure 1 B). During the first weeks after injury, postural and locomotory behavioural deficits characterised by erratic rapid swimming were detected in the injured fish, but these deficits gradually disappeared within four weeks (Figure 1 D, $p<0.001$ 1, 3 and 7 days post injury, $p<0.05$ 14 days post injury, n=11 for sham injured, n=11 for injured group). To analyse if the functional recovery was accompanied by morphological regeneration after injury we quantified tissue recovery 1, 3, 6 months after injury (Figure 1 E). Morphological quantifications of the lesion size showed that the granule cell layer area significantly increased one month after injury and very significantly increased three to six months after the lesion compared to three days after injury (Figure 1 E, n≥5/timepoint, 1m $p<0.05$, 3 and 6 m <0.001). In contrast, the Purkinje cell layer and the molecular layer were not significantly increased after the injury (Figure 1 E n≥5/timepoint, 1m p=0,835, 3m p=0,678, 6m p=0,754). The Purkinje cell layer and the molecular layer remained noticeably perturbed twelve months after injury indicating that the cell types in these layers regenerated poorly (n=5, Supplementary Figure 1 C). However, we detected restoration of the granule cell layer. The recovery of the tissue could be caused by general tissue movements and cell migration. To determine if cells are produced after injury we performed a series of 5-bromo-2-deoxyuridine (BrdU) pulses and chase experiments in combination with immunohistochemical stainings for cell type specific markers. To detect cells that were proliferating after injury we performed consecutive 24h long continuous pulses of BrdU given on day three, seven, ten and fourteen after the injury (Figure 1 F). In agreement with the morphological analysis, the BrdU pulse chase experiments showed that the vast majority of cells produced after injury were located in the granule cell layer (Figure 1 F-G). Newly produced cells may not survive for longer times in the adult brain and this is particularly noticeable after injury (Kernie and Parent, 2010; Magnusson et al., 2014). However, in agreement with the morphological analysis an over two-fold increased number of granule cells were produced *de novo* and maintained at the

lesioned hemisphere three to six months after the lesion, as compared to the unlesioned hemisphere or sham control (n≥5/timepoint, p<0,001, Figure 1 H). No difference in the number of BrdU labelled cells were detected between 3 and 6 months suggesting that the produced cells are stably maintained over time (n≥5/timepoint, p>0.05, Figure 1 H). To determine if inhibitory cell lineages were produced we used γ-aminobutyric acid (GABA) and parvalbumin (PV), Zebrin II (ZII) and Pax2 to further identify Purkinje and Golgi neurons, respectively. GABA was used to identify all inhibitory neurons, while parvalbumin (PV) and ZebrinII (ZII) were used to label Purkinje cells (Bae et al., 2009; Kaslin et al., 2013). Pax2 is required for the differentiation Golgi and stellate cells in the mouse cerebellum (Maricich and Herrup, 1999; Kaslin et al., 2013). In agreement with the morphological analysis only a very small number of GABA/BrdU immune positive inhibitory neurons were detected three months after the injury (n=5, 16.74 cells/brain +/- 7.33 cells, Figure 1 I-J, Supplementary Figure 1 E). A similar number of Pax2/BrdU immune positive cells were detected three months after injury suggesting that primarily Golgi cell were produced (n=5, 11.23 cells/brain +/- 9.67 cells, Supplementary Figure 1 D). No BrdU labelled PV or ZII+ Purkinje neurons were detected at any of the analysed time points after the injury (n=5/each time point, 1.5 months PL, 3 months PL, 6 months PL, Figure 1 F-J, Figure 1-supplement 4). Eurydendroid cells were identified by using the *olig2:egfp* transgenic line that labels most eurydendroid cells and oligodendrocytes in the corpus cerebelli (Bae et al., 2009; Kaslin et al., 2013). Similarly, no BrdU labelled *olig2:egfp* positive cells (eurydendroid cells) were detected three months after injury (n=5, Figure 1 K), but rare BrdU and *olig2:egfp* double positive and neuronal marker negative cells (oligodendrocytes) were observed in the granule cell layer (n=5, 5.3, +/- 2.6 cells/brain, Figure 1 L). Gluthamine synthetase (GS) or S100β labeling was used to identified Bergmann glia (Kaslin et al., 2009; Kaslin et al., 2013). A small number of BrdU and GS/ S100β positive glial cells were detected three month after injury (n=5, 24.6, +/- 6.4 cells/brain, data not shown, Figure 3 C-E). Furthermore, we performed unilateral needle stick injury to restrict damage because injury size could hamper the regenerative response. In agreement, with the surgical injury no BrdU labelled PV or ZII+ Purkinje neurons were detected four weeks after injury (n=5, data not shown). Taken together, our data demonstrate that granule cells readily regenerate after injury, but regeneration of other cell types is limited in the adult cerebellum.

Neural regeneration in the zebrafish brain is driven by distinct tissue resident stem and progenitor cells (Kroehne et al., 2011; Barbosa et al., 2015). However, it is known that stem

and progenitor cells are heterogeneous in the mammalian and zebrafish brain (Merkle et al., 2007; Kaslin et al., 2009; Ganz et al., 2010; Marz et al., 2010). The implication of heterogeneous stem cell populations in tissue regeneration continuous to be unclear. However, limited availability or depletion of specific stem cells could impair the regeneration of specific cell lineages. In the cerebellum, two distinct populations of neural stem and progenitor cells are established early during development, but only one of the populations remains active in the adult (Kaslin et al., 2009; Kaslin et al., 2013). The maintained and actively cycling stem cell population displays neuroepithelial-like characteristics and primarily produces granule neurons (Kaslin et al., 2009; Kaslin et al., 2013). The other population with stem cell and progenitor cell characteristics is located at the VZ and display radial glia-like characteristics, and produces inhibitory neuronal cell lineages and glia in the juvenile cerebellum. This population gradually becomes quiescent, or alternatively exhausted, in the adult and aligns with the ceased production of most inhibitory cell lineages in the cerebellum (Kaslin et al., 2013). Exhaustion of neural stem and progenitor cells over time may be a general trait of vertebrate brains, since recent studies have highlighted the possibility that the neural stem pool gradually is depleted over time in the zebrafish and in the rodent brain (Barbosa et al., 2015). We therefore sought to determine which cell types respond to injury and contribute to cellular regeneration. The transgenes *ptf1a:DsRed* and *nestin:gfp* distinctly label VZ progenitor cells and neuroepithelial-like progenitor cells, respectively, in the zebrafish cerebellum, and they can be used to monitor stem and progenitor cell activity (Kani et al., 2010; Kaslin et al., 2013). Furthermore, ptf1a is a required factor for the generation of all inhibitory neuronal lineages in the cerebellum (Hoshino et al., 2005). To determine if stem and progenitor cell activity declines with age, we quantified the number of *nestin:gfp*+ and *ptf1a:dsred*+ cells in the young and aging zebrafish cerebellum (3, 6, 14 and 22 months). The number of *nestin:gfp*+ neuroepithelial-like cells very significantly declined over time in the aging cerebellum (Figure 2 A, $n \geq 5$/each time point, $p<0.001$). However, no significant decline in cell number was detected between 14 and 22 months suggesting that the active cerebellar stem cell pool remain stable in the aging cerebellum (Figure 2 A). The number of *ptf1a:dsred*+ cells significantly declined in the aging cerebellum and was very low in the adult (Figure 2 B, $n \geq 5$ for each time point, $p<0.05$). However, no difference in *ptf1a:dsred*+ cell number was detect between 6 months and later time points suggesting that the population remains stable and largely quiescent from 6 months onwards (Figure 2 B). To determine if stem and progenitor cells can be activated after injury, we performed a quantitative co-localization analysis with the cell proliferation marker proliferating cell nuclear antigen

(PCNA) in the lesioned cerebellum of six months old *ptf1a:DsRed*+ and *nestin:gfp*+ fish. PCNA is expressed during G1 to G2 phase of the cell cycle and is thus a broad cell proliferation marker. The number of *ptf1a:DsRed/PCNA*+ and *nestin:gfp/PCNA*+ cells significantly increased during the first two weeks after the lesion ($p<0.01$, Figure 2 C-F). A five-fold and eleven-fold increase in the number of proliferating *ptf1a:DsRed*+ and *nestin:gfp*+ stem cells was seen in lesioned hemispheres between 7 days and 14 days post lesion (Figure 2 C-F, Supplementary Figure 2 A-B). The number of proliferating *ptf1a:DsRed/PCNA*+ cells returned to uninjured control levels 30 days after the lesion (Figure 2 F). We previously showed that the majority of *Dsred*+ cells are non-cycling in the adult cerebellum, and they display morphological molecular characteristics of differentiating neurons or glia (Kaslin et al., 2013). In agreement with this, the proportion of persisting *Dsred*+, but non-cycling cells (PCNA-), increased 7 days after injury, suggesting that the *Dsred*+ cells exited the cell cycle and are differentiating (Supplementary Figure 2 C). The number of proliferating *nestin:gfp*+ stem cells was still elevated compared to the uninjured control 30 days after the lesion, showing that neuroepithelial-like stem cell activity is augmented a long time after injury (Figure 2 C, Supplementary Figure 2 A). Taken together, our data demonstrate that both neuroepithelial-like cells and VZ progenitors are activated and proliferate after injury. However, the relative number of activated VZ progenitors is very low even at peak of activation (<5 cells/section, Figure 2 F). These findings are in agreement with the BrdU pulse chase experiments that demonstrated that very few inhibitory neurons were produced (Figure 1).

In addition to the *ptf1a:DsRed* or *nestin:gfp* cells at the lesion site, there was an increase of proliferating cells in the cerebellar parenchyma. In agreement with previous studies (Kroehne et al., 2011; Kyritsis et al., 2012), the majority of proliferating parenchymal cells co-labelled with the leukocyte marker L-plastin that labels macrophages and microglia (Figure 3 A-B). A small subpopulation of proliferating S100β+ Bergmann glia was also detected in the cerebellar parenchyma (Figure 3 C-D). Proliferating leukocytes and glia are prerequisites for glial scarring and chronic inflammation, known key factors that impair neural regeneration in mammals (Fitch and Silver, 2008). The number of proliferating S100β+ and L-plastin+ cells in the parenchyma returned to uninjured levels 30 days after the lesion (Figure 3 A, C). There was no accumulation of the glial proteins GS/S100β+ 6 months after the lesion (Figure 3 E). Furthermore, we could not detect signs of fibrotic scarring using acid fuchsin orange G and hematoxylin/eosin staining 6 and 12 months after the lesion (Supplemental Figure 1 C, and

data not show). The resolution of inflammation and the lack of significant glial or fibrotic scaring during recovery is in line with previous findings in zebrafish brain and retina (Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Kyritsis et al., 2012).

Our data suggested that there may be a lack of specific stem or progenitor cells in the adult cerebellum to replace specific cell lineages such as the Purkinje cells. We previously found that both neuroepithelial- and VZ progenitor cells are active in the juvenile zebrafish cerebellum and have the capacity to produce all cell lineages, including Purkinje cells up to one month of age (Kaslin et al., 2013). To determine if juvenile fish can regenerate Purkinje cells after injury, we performed lesion experiments similar to the ones in the adults (Figure 4 A). To label the cells that were produced after injury, fish were given a 24h long BrdU pulse at day 5, 7 and 10 after the lesion. The fish were then sacrificed at 1.5 and 3 months after the lesion, and examined for co-localization with BrdU and Purkinje cell markers. Three months after injury we detected BrdU and ZII double positive Purkinje cells at the lesion site (Figure 4 B-C, n=6, 3.23, +/- 2.42 BrdU/ZII+ cells). To identify the contribution of the ptf1a lineage after injury and further examine the cellular behavior of the VZ progenitor population we performed conditional genetic lineage tracing experiments in juvenile and adult zebrafish. We created a driver line with cherry and the tamoxifen inducible Cre^{ERT2} recombinase driven by the 5.5 kb 5' fragment of the ptf1a promoter that we previously used to drive dsred expression, *Tg(ptf1a:cherryT2aCreERT2)*[1] (Hans et al., 2009; Kaslin et al., 2013), Supplementary Figure 3 A-C). As a reporter, we used a temperature inducible line that drives EGFP after excision of a loxP-flanked dsred stop sequence, *Tg(hsp70l:DsRed2(floxed)EGFP)* (Hans et al., 2011; Kroehne et al., 2011). Recombination in the *Tg(ptf1a:cherryT2aCreERT2)*[1] x *Tg(hsp70l:DsRed2(floxed)EGFP)* line was induced by incubating embryos in 4-hydroxytamoxifen (4-OH) and adults with tamoxifen. No recombination was detected in the absence of tamoxifen in embryonic or adult zebrafish (Supplementary Figure 3 D-E). In agreement with previous studies on cerebellar neurogenesis (Volkmann et al., 2008; Bae et al., 2009; Kaslin et al., 2013) recombination between one and three days post-fertilization resulted in a multitude of GFP labeled cells in all cell layers of the cerebellum, including PV+ positive Purkinje cells, Golgi cells and granule cells (n=12 fish, 10/12 recombined fish, Figure 4 D-E). Furthermore, early recombination resulted in complete unilateral labeling of the neuroepithelial stem cell domain and granule cells (detected in 3/12 recombined fish, Figure 4 E), suggesting that cells produced in one hemisphere do not cross the midline. Later recombination, from day 5 to 6 or at one month and analysis one or two

months later, resulted in sparse mosaic labelling of radial-glia like cells, inter-neurons and Purkinje cells (Figure 4 F-G, Supplementary Figure 4 A). In fish recombined from day 5 to 6, clones of PV negative inter-neurons were found around the radial glia-like cells at the VZ (6 clones consisting of 9.2 ± 3.7 cells, n=10, Figure 4 F). In contrast, no clones of cells were detected in proximity to labelled radial glia-like cells in fish that were recombined at one month and analysed three months later, suggesting that the cells were relatively quiescent (4 clones analysed, n=11 , Figure 4 G). Tamoxifen induction resulted in very low recombination rates in adult zebrafish in agreement with the very low number Ptf1a expressing cells (<1 cell/section, Figure 2 F). One month after recombination, scarce PV- cells with stellate and Golgi cell-like morphologies were the only recombined cell type detected in the cerebellum (4.7 ± 2.3 recombined cells/brain, n=24, Supplementary Figure 4 B). In summary, the lineage tracing experiments demonstrated that production of cells from the ptf1a cell lineage is rare and limited to inter-neurons beyond one month of age.

To identify the contribution of the ptf1a lineage after injury in juvenile zebrafish (1 month), recombination was induced by three consecutive tamoxifen incubations one day before the lesion. One month after the injury and recombination the fish were heat shocked for three days to reveal the recombined cells and their progeny. In agreement with the BrdU pulse-chase experiments, GFP/PV+ Purkinje cells, and GFP+/PV- Golgi and stellate cells were detected in the cerebellum of tamoxifen treated fish (Figure 4 H, n=14, 0.33, +/- 0.56 GFP/PV+ cells, 1.64, +/- 2.34 GFP+/PV- cells). To confirm the results obtained from BrdU pulse chase experiments in the adult cerebellum (e.g. inability to regenerate Purkinje cells), we performed recombination experiments in three month old zebrafish after injury. Similar to uninjured adult fish, the recombination rate was low in the injured adult fish (1-3/cells brain, n=30 fish). We only detected Purkinje cell negative (PV/ZFII) GFP+ recombined cells (37 recombined cells analysed, n=30, Supplementary Figure 4 C), demonstrating that inter-neurons recover successfully. The number of analysed cells was relatively low but the findings are consistent with the morphological analysis, BrdU pulse chase experiments, abundance and activity of VZ progenitor cells.

Discussion

Our findings bring significant contrast and implications to the assumption that the highly regenerative zebrafish model may be able to regenerate all cell types in the CNS. It is well established that stem cells are heterogeneous in the vertebrate brain (Shen et al., 2006; Merkle et al., 2007; Kaslin et al., 2009; Ganz et al., 2010; Marz et al., 2010) but the implications of this have yet to be uncovered. The zebrafish telencephalon regenerates well and the cerebellum can regenerate many but not all cell types. This implies that there may be differences in the regenerative potential between the telencephalon and cerebellum. The circuitry and different cell subtypes of the zebrafish telencephalon are not known in detail and therefore we cannot rule out the possibility that some cell types regenerate poorly in the telencephalon. Another possibility is that, in contrast to the cerebellum, all stem/progenitor cell types are maintained in the adult telencephalon and consequently they are able to produce all the cell lineages required after injury. Future studies revealing new neuronal sub-populations and connections in the telencephalon will support either of these alternatives. A possible difference between the telencephalon and cerebellum is that early born long projecting neurons (eurydendroid cells) were injured in the cerebellum. In contrast, in the telencephalon mostly if not only inter-neurons were injured. Inter-neurons regenerate well in both the cerebellum and telencephalon. It is not clear if projecting neurons regenerate in the telencephalon. Typically projecting neurons are produced early during development in vertebrates and inter-neurons later highlighting the possibility that it is more challenging to regenerate early produced cell lineages (Carletti and Rossi, 2008; Gage and Temple, 2013). This aligns well with the post-embryonic and adult neurogenesis in vertebrates where only inter-neurons are produced in the adult.

In the zebrafish brain, radial glia-like stem and progenitor cells are thought to be the predominating stem cell type in homeostasis and after injury (Kroehne et al., 2011; Kizil et al., 2012; Barbosa et al., 2015; Becker and Becker, 2015; Than-Trong and Bally-Cuif, 2015). Here, we report that radial glia-like cells play only a minor part in adult cerebellar neurogenesis and in recovery after injury. In contrast, we find that the neuroepithelial–like stem cells are the main stem cell type in the adult cerebellum. The cerebellar stem cells arise early during embryonic development from a common ventricular domain in the hindbrain (Kaslin et al., 2013). The neuroepithelial-like stem cells are derived from the dorsal part of the ventricular zone and the VZ progenitor cells are derived from the ventral part of the ventricle.

The neuroepithelial-like stem cells remain active in the aging cerebellum where they continuously produce substantial amounts of granule cells throughout life. In contrast, the activity of ptf1a expressing VZ progenitor cells is largely exhausted in the adult cerebellum, and the loss correlates with the very rare genesis of inter-neurons, Bergmann glia and oligodendrocytes. The post embryonic VZ progenitor cells in the zebrafish cerebellum display many similar characteristics with the recently identified bi-potent astroglial progenitors in the mouse cerebellar white matter (Parmigiani et al., 2015). Furthermore, other VZ progenitor cell derived cell types, such as Purkinje and eurydendroid cells are not produced beyond juvenile stages. The juvenile-to-adult shift in repertoire of cells produced is accompanied by expression of Pax2 by the VZ progenitor cells at late juvenile stages in zebrafish (Kaslin et al., 2013). Pax2 is required for the differentiation Golgi and stellate cells in the mouse cerebellum (Maricich and Herrup, 1999). This implies that the potential of the VZ progenitor cells gets more restricted over time, and is intrinsically controlled by expression of transcription factors that defines the output. Consistent with this notion, the capacity to regenerate granule cells is high in adult zebrafish, while the capacity to regenerate inter-neurons or Purkinje and eurydendriod is very low or absent following injury. In contrast, the juvenile cerebellum can regenerate Purkinje cells after injury. A plausible explanation for the ability of juvenile fish to regenerate Purkinje cells is the abundance of VZ progenitors and the capability to produce Purkinje cells during homeostasis (Kaslin et al., 2009; Kaslin et al., 2013). Given the closely related shared developmental origin, it is remarkable that the adult cerebellar stem and progenitor cells appear to only be able to contribute to their homeostatic cell lineages after injury (e.g. granule cells or inhibitory inter-neurons) suggesting that the injury induced signals are not sufficient to re-program the stem cells or the environment to enable regeneration of all cerebellar cell types. Taken together, the regenerative potential in the adult cerebellum largely reflects adult cerebellar neurogenesis.

We previously found that VZ progenitor cells become quiescent, or alternatively exhausted (terminally differentiate or die), in the adult cerebellum (Kaslin et al., 2013). In agreement, recent imaging and lineage tracing experiment studies in the telencephalon of rodents and zebrafish suggest that the neural stem and progenitor cell pool is gradually exhausted over time and that this may impact neural regeneration (Encinas et al., 2011; Rothenaigner et al., 2011; Barbosa et al., 2015; Calzolari et al., 2015). In the adult zebrafish cerebellum, we detected several-fold activation of VZ progenitors after injury, suggesting that quiescent cells are activated. However, the available pool of cells may be limiting because the total number

of activated cells was very low (<5 cells/section), indicating that the population is largely exhausted in the adult. In agreement with the limited number of activated VZ progenitors, we only detected modest regeneration of inhibitory inter-neurons. Taken together, our data suggests that the neural stem cell pool is irreversibly depleted over time in the zebrafish cerebellum, and that this has significant consequences for tissue repair.

The widespread adult neurogenesis along the brain axis in zebrafish provides exciting potential as a new powerful model for studying brain regeneration and neuronal stem cell diversity. The intriguing finding that the zebrafish can regenerate some neuronal types, but not others, opens up new possibilities to tease out the molecular mechanisms that allow and restrict brain regeneration and neurogenesis in the vertebrate brain. Moreover, our results illustrate the importance of studying the role of stem cell heterogeneity in the brain. Understanding the molecular basis of *in vivo* neural stem cell heterogeneity may be of great relevance for future therapeutical approaches.

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Figures

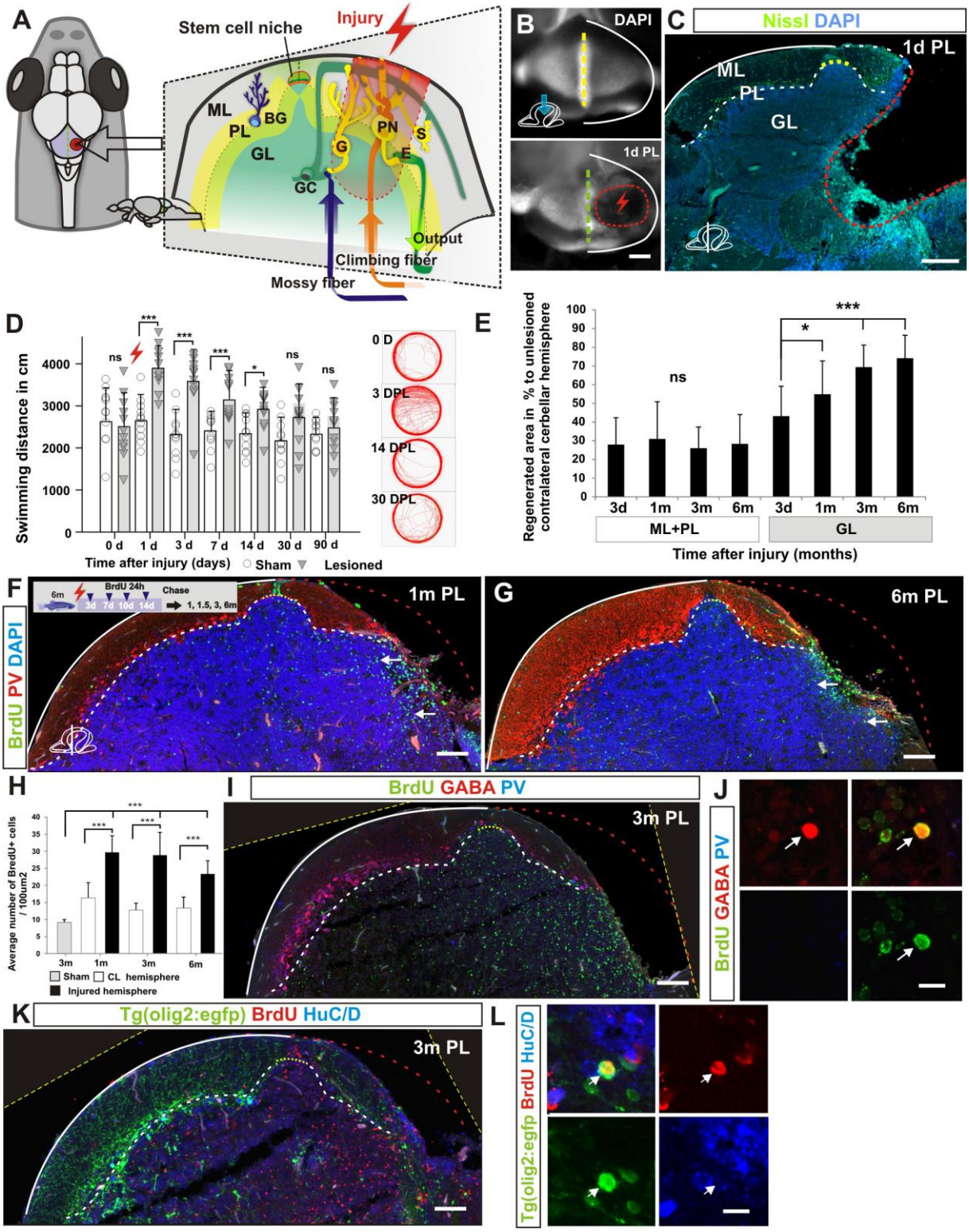


Figure 1. A. Overview of the cerebellar architecture in zebrafish and injury model. The cerebellum contains few cell types with distinct morphological. The cerebellum has a simple laminar three layered architecture consisting of a molecular layer (ML), Purkinje cell layer (PL) and a granule cell layer (GL). The unilateral injury removes cells from all three cell layers. Importantly, the lesion spares the stem cell niche. The granule layer consists of densely packed excitatory granule cells and inhibitory Golgi neurons. The PL contains Purkinje neurons (PN), a specialized glia type, Bergmann glia (G), and excitatory eurydendroid cells (E). The ML consists of nerve fibers and scattered inhibitory stellate cells (S). Neural stem cells are maintained in the dorsomedial part of the cerebellum around a remnant of the IVth ventricle. **B.** A piece of the corpus cerebelli, including all cell layers is unilaterally removed (red lightning). A confocal maximum projection showing a dorsal view of an unlesioned and a lesioned cerebellum shortly after the injury (3 hours). The stem cell niche is indicated with a yellow hatched line. The extent of the lesion indicated with a red hatched line. **C.** A cross section of the corpus cerebelli showing the lesion one day after injury. **D**. Automatic tracking of swimming distance and pattern during five minutes. Cerebellar injury induced significantly increased swimming activity from one to fourteen days after injury (n=11 for sham and injured group, 1, 3, 7 DPL $p<0.001$, 14 DPL $p<0.05$, error bars show as standard deviation). **E.** Morphological quantification of the lesioned area after injury. Lesioned area compared to lesion size measured three days after injury. $n\geq5$/timepoint, 1m $p<0.05$, 3 and 3, 6 m <0.001, error bars shown as standard deviation. **F-G.** Cross section of the corpus cerbelli showing that BrdU stained cells (green) are recruited and maintained (arrows) in the GL of the lesioned hemisphere one and six months after injury. Few BrdU stained cells are detected in the ML and PL. Purkinje cells stained with parvalbumin (red). **H.** Quantification of BrdU positive cells in the granule cell layer in the injured and contralateral hemisphere 1 month, 3 months and 6 months after injury (n=5/timepoint, $p<0,001$, error bars shown as standard deviation). Sham control animal is included as reference point for normal granule cell production rate (3 months after BrdU pulse). CL=contra lateral uninjured hemisphere. **I-J.** Cerebellar cross section showing BrdU (green), GABA (Red) and PV (Blue) staining three months after injury. Very few BrdU/GABA positive cells are detected (arrow). **K-L**. Cerebellar cross section showing olig2:gfp (green), BrdU (red) and HuC/D (Blue) staining three months after injury. BrdU/olig2:gfp positive cells are very rarely detected (arrow). Yellow hatched line show original picture border in rotated in images.

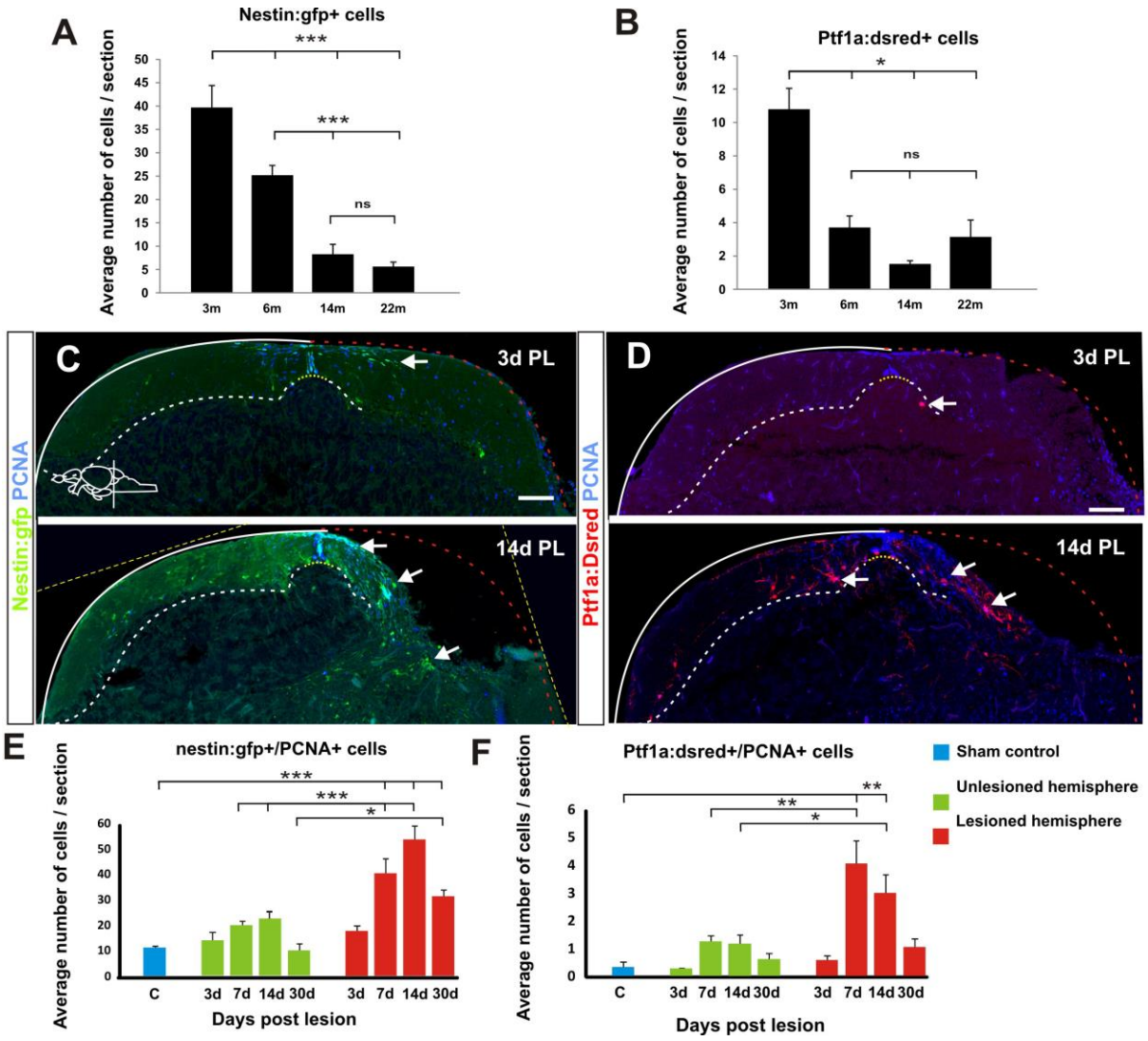


Figure 2. A-B. Quantification of nestin:gfp and ptf1a:Dsred cells in the aging cerebellum during homeostasis (*nestin:gfp*: n=5 at 3, 6 and 22 months, n=6 at 14 months, $p<0.001$; *Ptf1a:dsred*: n=7 at 3, 6 months, n=6 at 14 months, n=5 at 22 months, $p<0.05$, error bars shown as standard error of mean). **C-D**. Maximum projections of cerebellar cross sections showing stem cell activation after injury. Initially the tissue morphology is severely altered at the level of the injury therefore cross sections from the very rostral end of the lesion was chosen to show the stem cell response**.** Three days after the lesion both *ptf1a:DsRed*+ (red) *nestin:gfp*+ (green) stem cell activity (white arrows) is unaltered. Stem cell activity (white arrows) is significantly increased seven day after injury and peak around two weeks after injury. Proliferating cells are labeled with PCNA (blue). **E-F**. Quantification of cerebellar stem cell activity after injury. A significant increase in number of *nestin:gfp*+ cells is seen

from 7 to 30 days after the lesion (Sham control n=5, 3DPL n=4, 7DPL n=6, 14DPL n=5, 30 DPL n=5, $p<0.05$-0.001, error bars shown as standard error of mean). A significant increase in *ptf1a:DsRed*+ stem cells is detected 7 and 14 days after injury (Sham control n=7, 3DPL n=4, 7DPL n=5, 14DPL n=5, 30 DPL n=5, $p<0.05$-0.001, error bars shown as standard error of mean). Note 10 x differences in scale on Y axis between E and F (60 versus 6 cells). Yellow hatched line show original picture border in rotated in images.

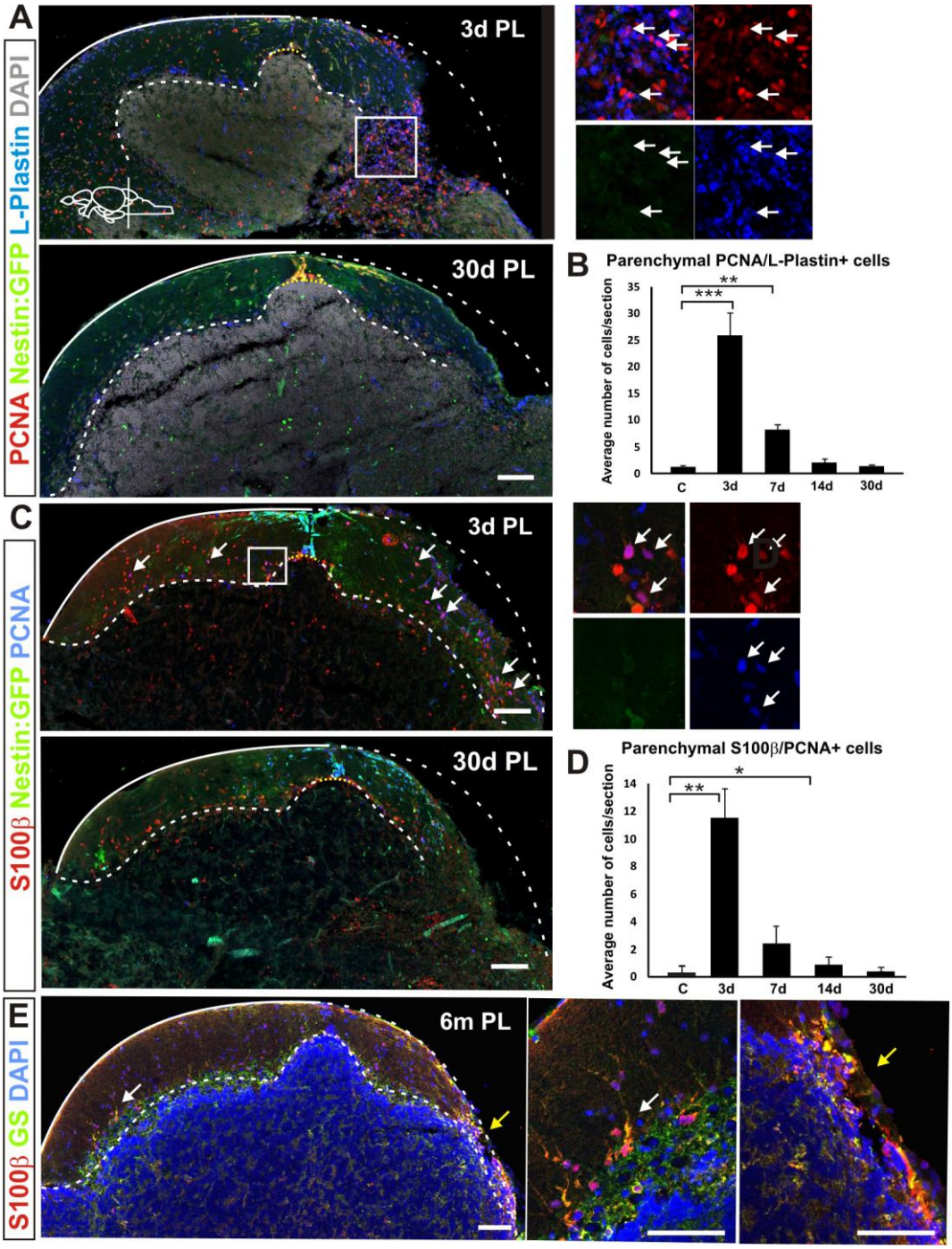


Figure 3. A. Cerebellar cross section showing proliferating (PCNA+) *nestin:gfp*-parenchymal cells (green) co-localizing with the leukocyte marker L-plastin (white arrows) three days after injury. Parenchymal proliferation and leukocytes numbers restored towards homeostatic levels thirty days after injury. **B.** Quantification of proliferating PCNA/L-plastin positive cells in the cerebellar parenchyma after injury. A significant increase in the number of proliferating L-plastin cells are detected during the first week. The number of proliferating

L-plastin cells are back to control levels 14 days after injury ($p<0.001$, n=5/each time point). **C.** Proliferating Bergmann glia (PCNA/S100β+) are also detect in the parenchyma after injury (white arrows). **D.** Quantification of proliferating Bergmann glia in the cerebellum after injury. A significant number of proliferating Bergmann glia are detected in the cerebellar parenchyma three days after injury ($p<0.01$, n=55/each time point). The number of proliferating Bergmann glia decreases the first week after injury and is back to homeostatic levels thirty days after injury. **E.** The glial proliferation do not result in glial scarring. No notable accumulation of the glial markers S100β (red) and GS (green) can be detected at the injury site six months after injury (n=5).

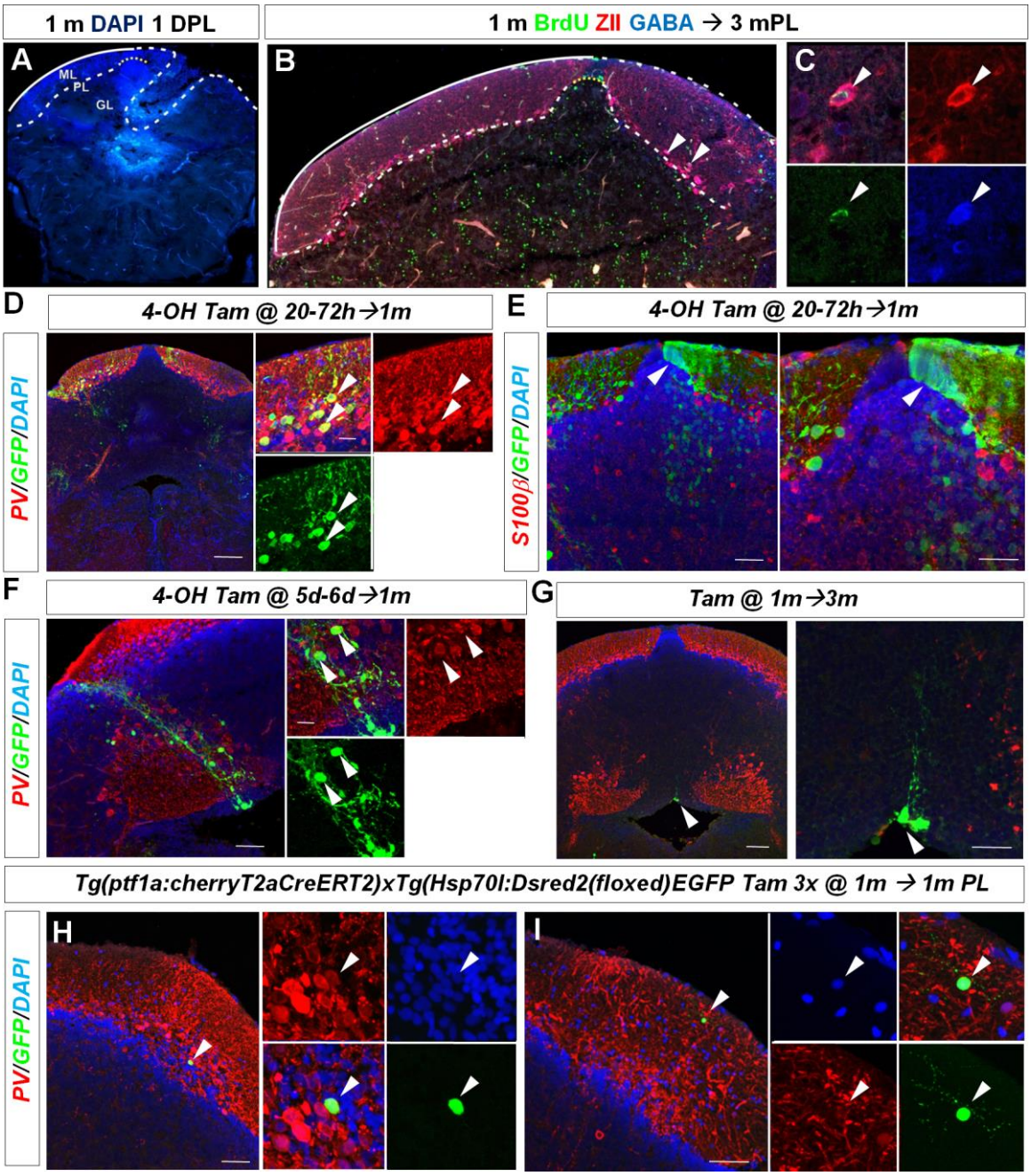


Figure 4. A. Representative cerebellar cross section in a juvenile one month old zebrafish showing the lesion area one day after injury (red arrow). **B-C.** BrdU/ZII/GABA+ Purkinje neurons are found at the lesion site three month after the injury (white arrows). **D.** Cerebellar cross section showing recombined GFP/PV+ Purkinje cells in *Tg(ptf1a:cherryT2aCreERT2)1; Tg(hsp70l:DsRed2(floxed)EGFP)* fish one month after recombination. **E.** Cerebellar cross section showing unilateral GFP labeling of neuroepithelial-like stem cells (white arrow) and granule cells. **F.** GFP labelled radial glia-like

cells and a clone of PV- inter-neurons (white arrows) recombined between 5-6 dpf. **G**. Quiescent GFP labelled radial glia-like cells recombined at one month and analysed three months later. **H.** Recombined GFP/PV+ Purkinje cell in the one month after tamoxifen treatment and injury of a juvenile zebrafish. **I.** A recombined GFP+ and PV- stellate cell one month after tamoxifen treatment and injury.

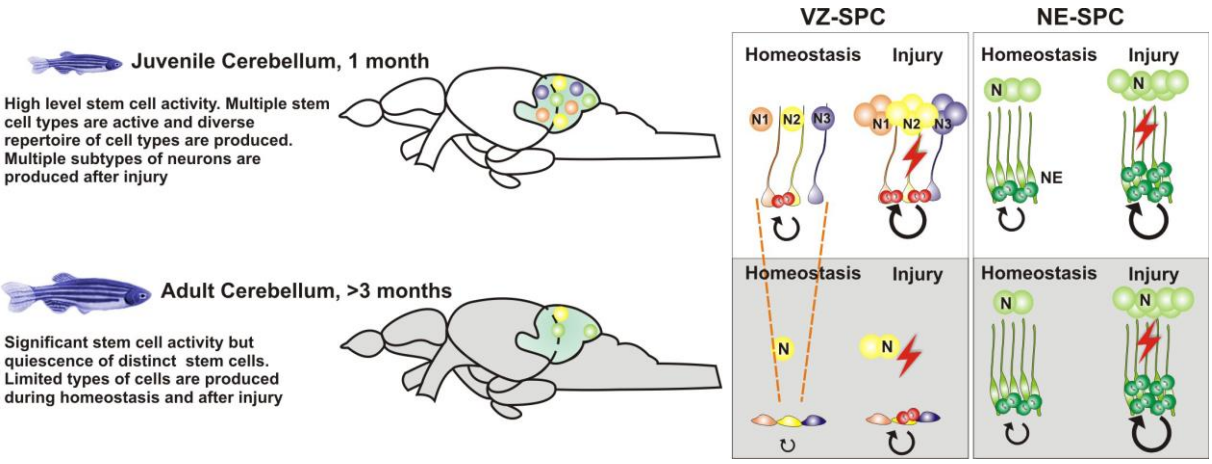
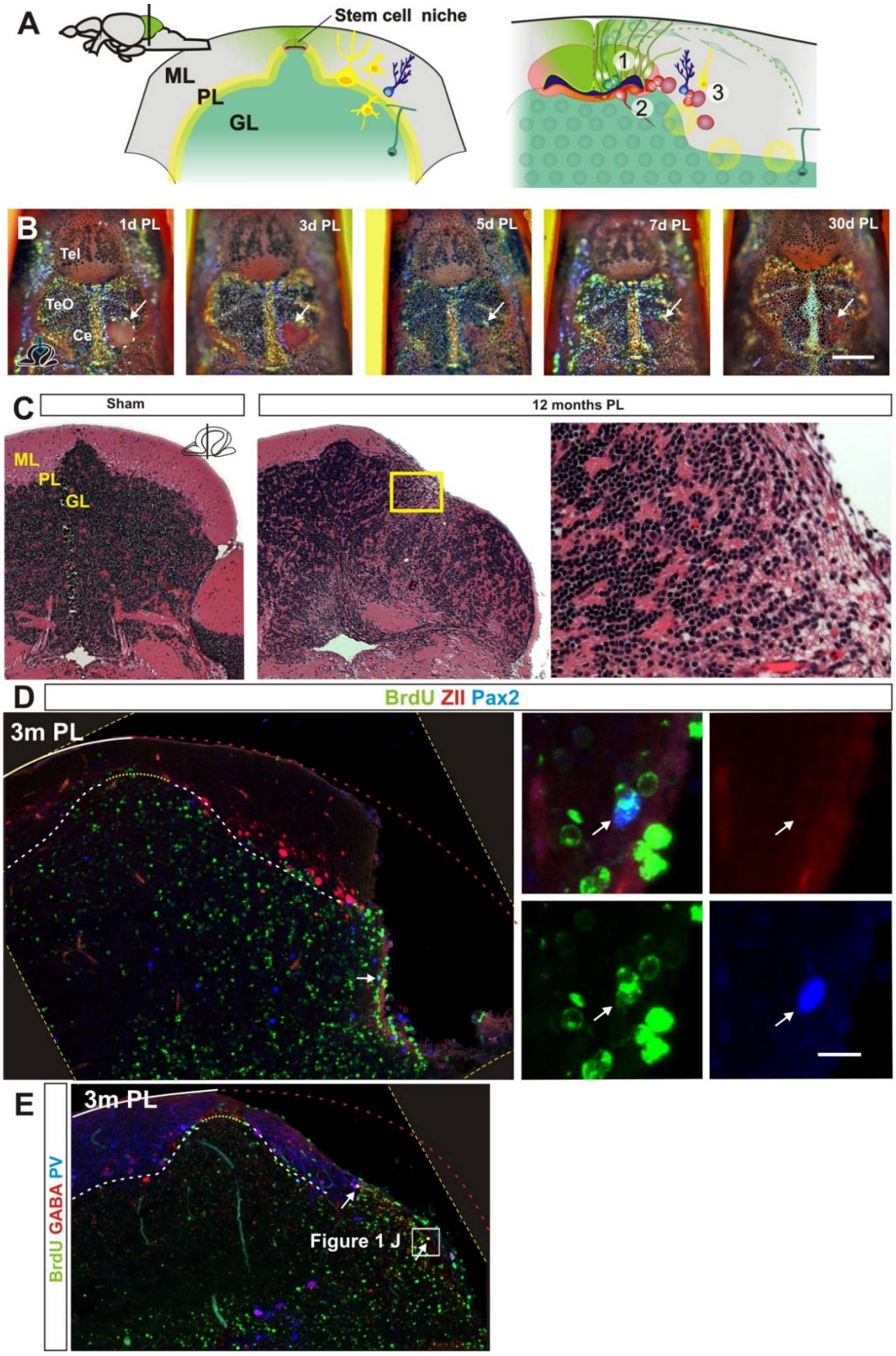
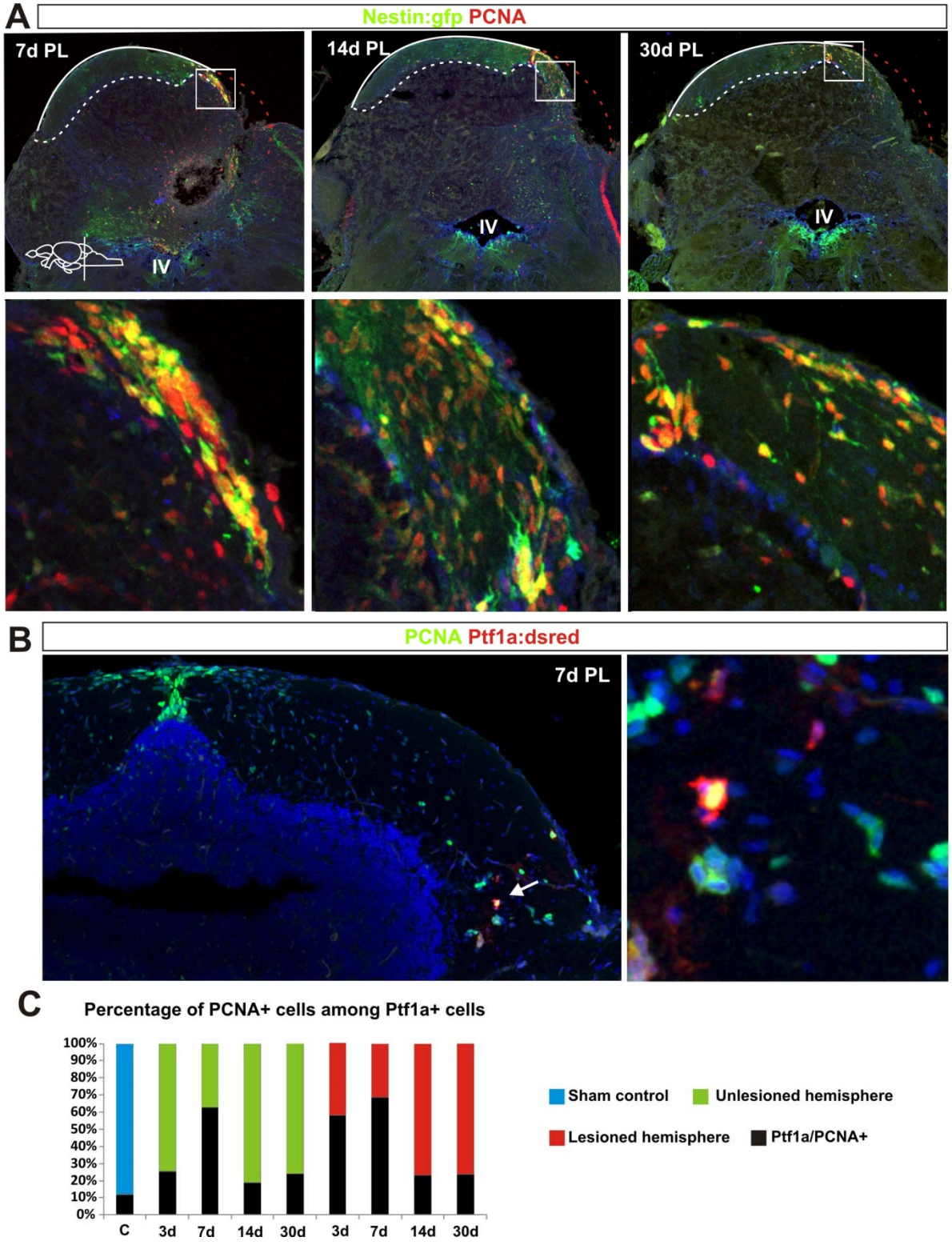


Figure 5. The diversity of stem cells is lost in the cerebellum during the transition from juvenile to adult and has an impact for homeostatic and regenerative neurogenesis. The juvenile maintains radial glia-like VZ and neuropithelial-like stem and progenitor cells (VZ-SPC and NE-SPC) and is able to produce all major cell types during homeostasis and after injury. In contrast, the adult cerebellum does not produce all cell lineages and is not able to produce all cell types after injury. In the adult cerebellum the VZ-SPC's become quiescent while NE-SPC's are maintained continuously and contribute to granule cell neurogenesis. N=neurons, NE=Neuroepithelial-like cell.

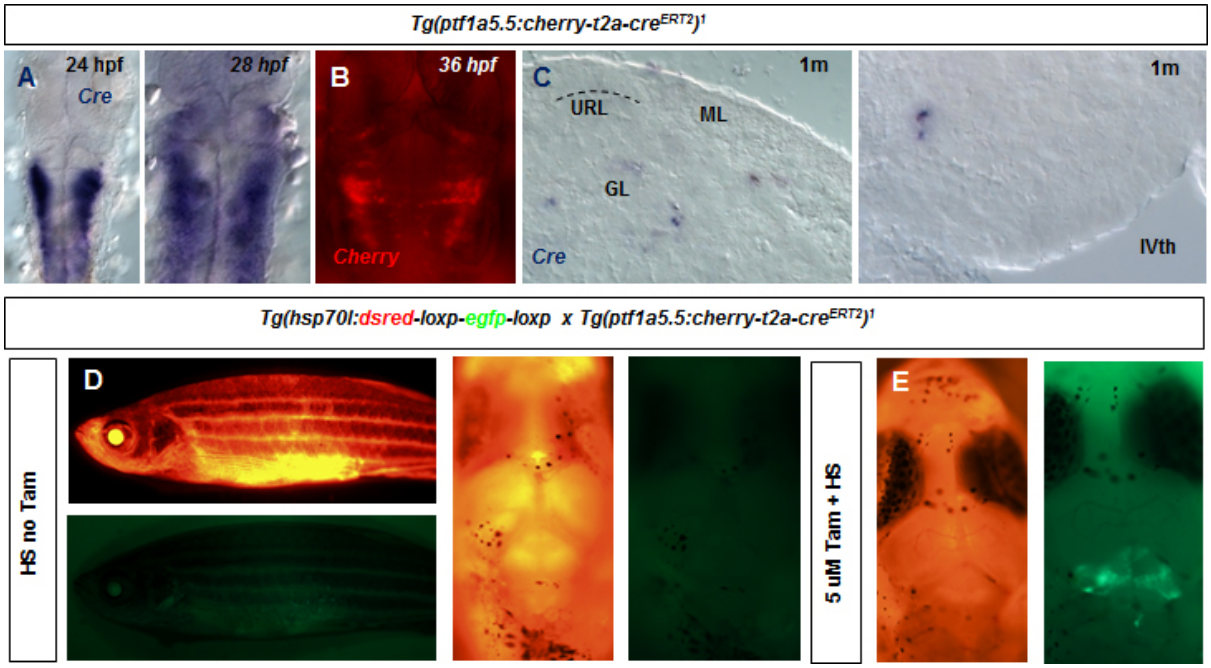
Supplementary figures



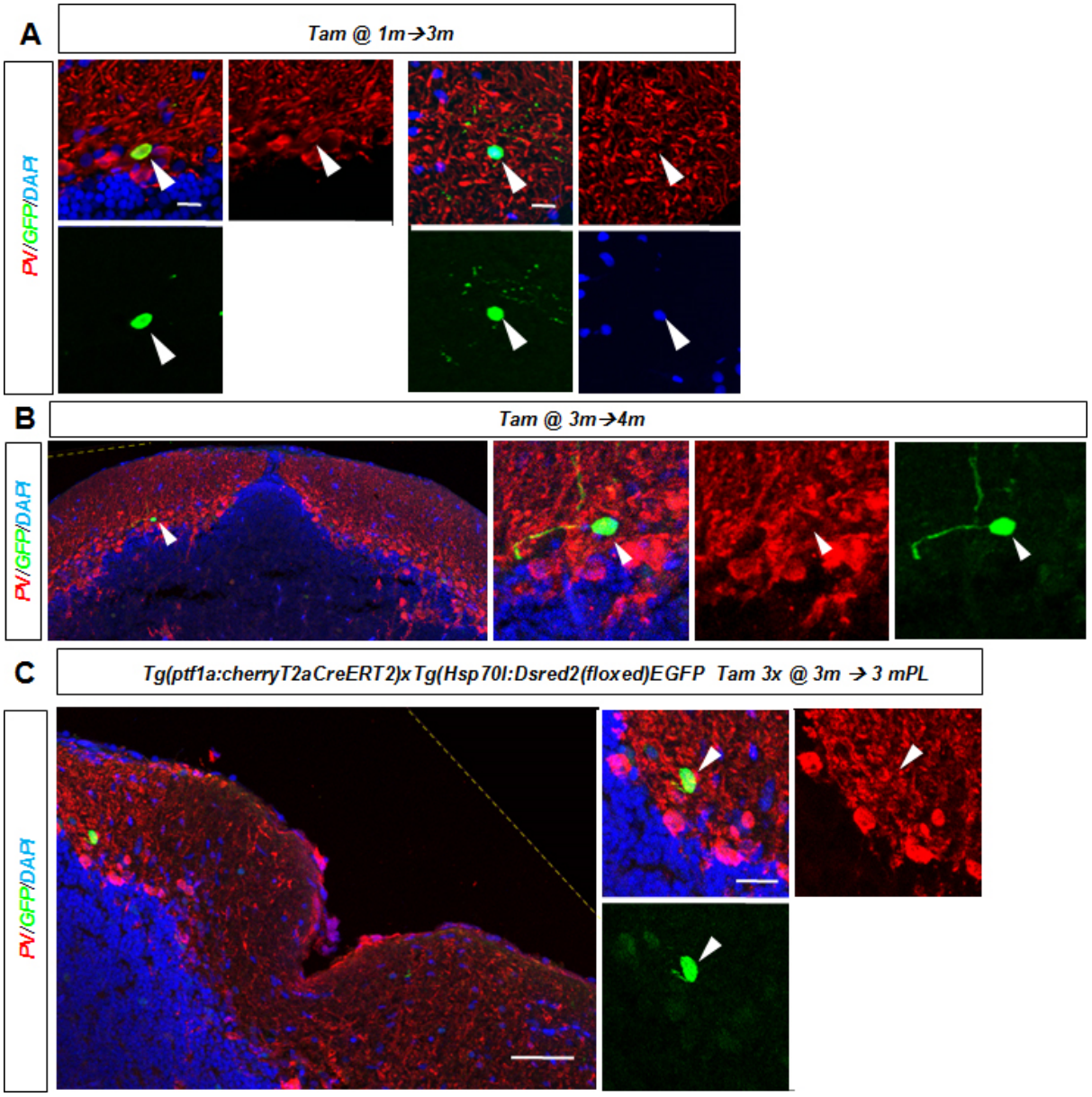
Supplementary Figure 1. A. Overview of the adult stem cell niche in the cerebellum. Stem cells are located around a small out pocketing of the fourth ventricle. 1. Polarized neuroepithelial-like stem cells (green) are restricted to the midline of the dorsal cerebellum. The stem cells give rise to rapidly migrating granule precursors (dark green) that initially migrate dorsolaterally and give rise to granule neurons in a distinct outside-in fashion. 2. Radial glia-like stem and progenitor cells are located ventral to the neuroepithelial-like stem cells and largely quiescent in the adult. 3. Bergmann glia and inhibitory inter-neurons are produced at a low level at the lateral margin of the ventricle. In addition, rare stem cells reside in the cerebellar parenchyma. GL=granule cell layer, ML=molecular cell layer, PL=Purkinje cell layer. **B.** Representative timelapse of a lesioned fish showing rapid wound healing after injury. Dorsal view of the lesion site (hatched white line and white arrow). The injury is fully covered three days after injury and at five days after injury pigment cells are readily detected at the injury site. Ce=Cerebellum, PL=post lesion, Tel=Telencephalon, TeO=Optic tectum. **C.** Hematoxylin and Eosin stained cerebellar cross section from sham injured and injured fish twelve month after injury (n=5/condition). The Purkinje and molecular cell layer has recovered very poorly. No notable fibrotic or glial scarring is detected. GL=granule cell layer, ML=molecular cell layer, PL=Purkinje cell layer. **D.** Cerebellar cross section showing BrdU (green), ZebrinII (Red) and Pax2 (Blue) staining three months after injury. No BrdU/ZII positive cells are detected and very few BrdU/Pax2 positive cells are detected (arrow). E. Cerebellar cross section showing BrdU (green), GABA (Red) and Parvalbumin (Blue) staining three months after injury. No BrdU/PV positive cells are detected and very few BrdU/GABA positive cells are detected (arrows). Yellow hatched line show original picture border in rotated in images.



Supplementary Figure 2. A. Confocal maximum projections of cerebellar cross sections showing stem cell activation after injury. *nestin:gfp*+ (green) neuroepihelial-like stem cells and proliferating cells labelled with PCNA (red). **B.** Confocal maximum projections of cerebellar cross sections showing a proliferating (green) *ptf1a:Dsred*+ (red) VZ progenitor in the parenchyma. Proliferating cells labelled with PCNA (green), DAPI (blue). **C.** Quantification of proliferating cells among the pool of Dsred+ cells. Dsred is very stable (many days) in the cells and persist in differentiating cells. The proportion of PCNA+ cells is reduced notably 14 days after injury suggesting that majority of Dsred+ cells are differentiating. (Sham control n=7, 3DPL n=4, 7DPL n=5, 14DPL n=5, 30 DPL n=5).

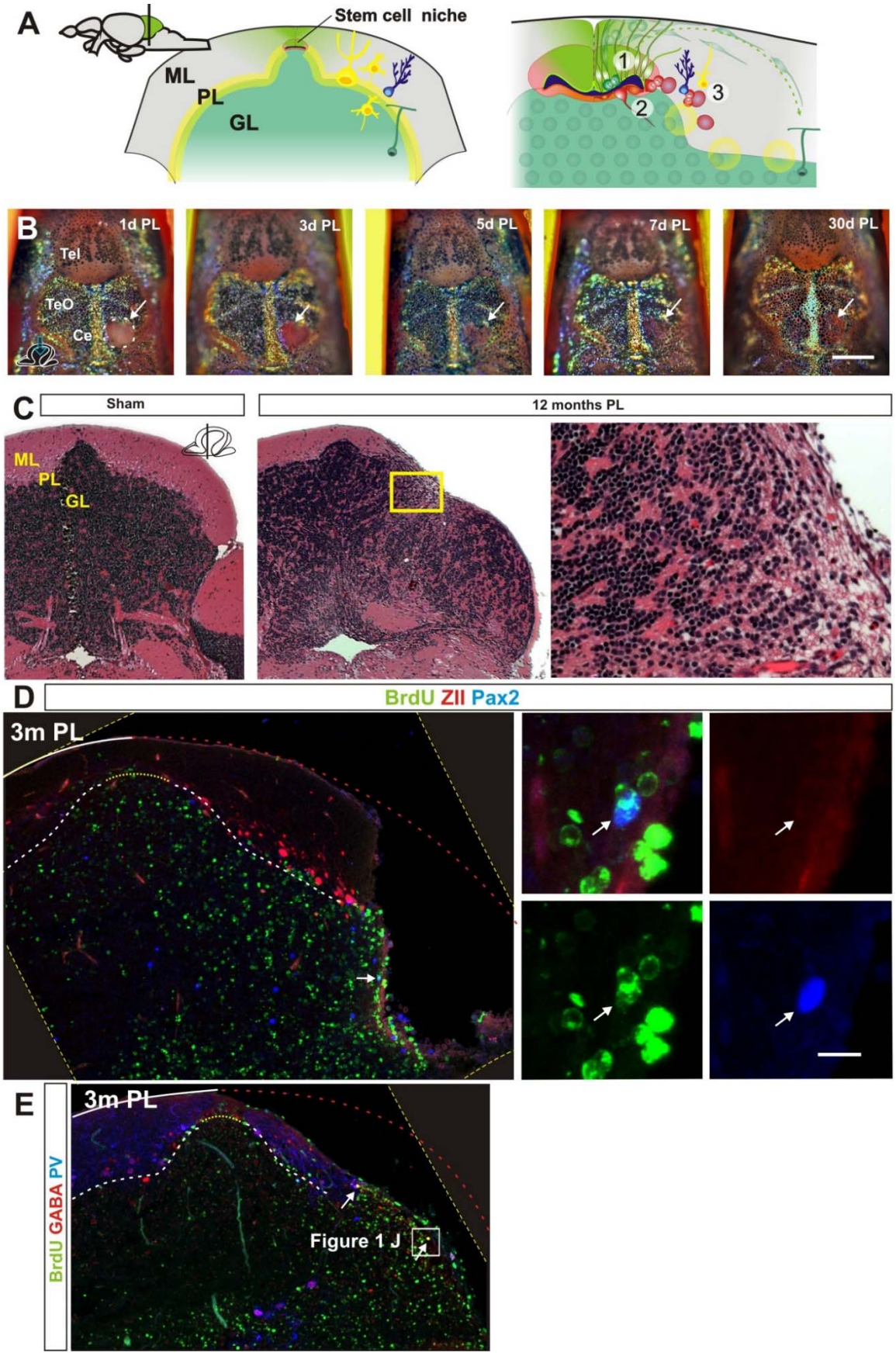


Supplementary Figure 3. A. *In situ hybridization* and analysis of the expression of the *Cre* transgene in the the *Tg(ptf1a:cherryT2aCreERT2)1* fish line. *Cre* is expressed in the ventricular zone of the embryonic hindbrain and cerebellar primordium. **B**. Expression of cherry in the embryonic hindbrain and cerebellum in the *Tg(ptf1a:cherryT2aCreERT2)1* fish. **C.** Expression of *Cre* in cross section of the cerebellum of a one month old zebrafish. **D**. Heatshocking of *Tg(ptf1a:cherryT2aCreERT2)1; Tg(hsp70l:DsRed2(floxed)EGFP)* juvenile zebrafish fish does not result in unconditional recombination (n=12). **E.** Heatshocked tamoxifen treated juvenile fish shows recombined cells (green) in the cerebellum.

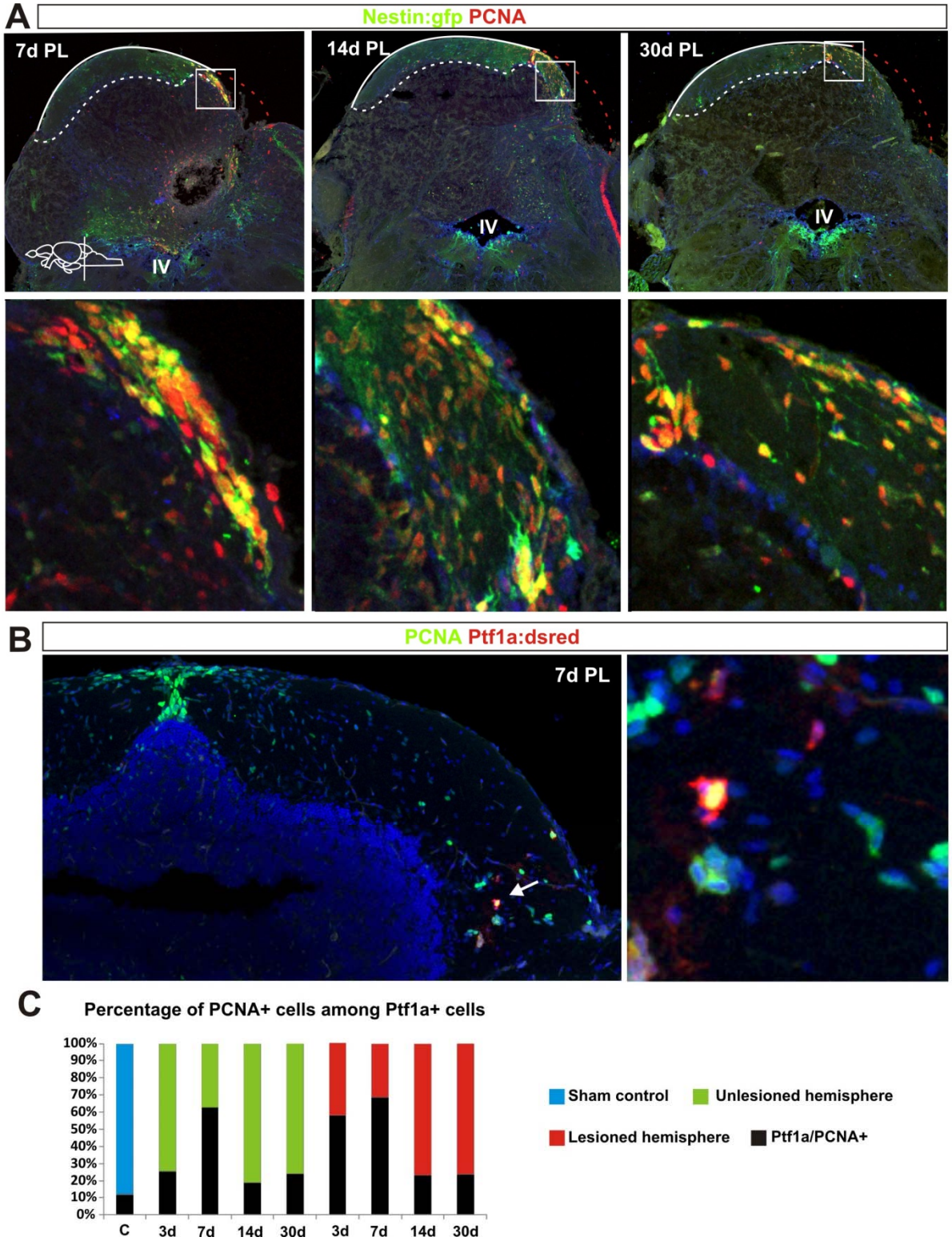


Supplementary Figure 4. A. Recombined GFP/PV+ Purkinje cell (left) and PV- negative inter-neuron (right) with stellate morphology in the cerebellum 3 months after recombination of a one month old juvenile fish. **B.** A recombined GFP/PV- inter-neuron in the adult cerebellum one month after tamoxifen treatment. **C.** A recombined GFP+ and PV- inter-neuron in the cerebellum three months after injury and tamoxifen treatment of an adult zebrafish. Yellow hatched line show original picture border in rotated in images.

Supplementary figures



Supplementary Figure 1. A. Overview of the adult stem cell niche in the cerebellum. Stem cells are located around a small out pocketing of the fourth ventricle. 1. Polarized neuroepithelial-like stem cells (green) are restricted to the midline of the dorsal cerebellum. The stem cells give rise to rapidly migrating granule precursors (dark green) that initially migrate dorsolaterally and give rise to granule neurons in a distinct outside-in fashion. 2. Radial glia-like stem and progenitor cells are located ventral to the neuroepithelial-like stem cells and largely quiescent in the adult. 3. Bergmann glia and inhibitory inter-neurons are produced at a low level at the lateral margin of the ventricle. In addition, rare stem cells reside in the cerebellar parenchyma. GL=granule cell layer, ML=molecular cell layer, PL=Purkinje cell layer. **B.** Representative timelapse of a lesioned fish showing rapid wound healing after injury. Dorsal view of the lesion site (hatched white line and white arrow). The injury is fully covered three days after injury and at five days after injury pigment cells are readily detected at the injury site. Ce=Cerebellum, PL=post lesion, Tel=Telencephalon, TeO=Optic tectum. **C.** Hematoxylin and Eosin stained cerebellar cross section from sham injured and injured fish twelve month after injury (n=5/condition). The Purkinje and molecular cell layer has recovered very poorly. No notable fibrotic or glial scarring is detected. GL=granule cell layer, ML=molecular cell layer, PL=Purkinje cell layer. **D.** Cerebellar cross section showing BrdU (green), ZebrinII (Red) and Pax2 (Blue) staining three months after injury. No BrdU/ZII positive cells are detected and very few BrdU/Pax2 positive cells are detected (arrow). E. Cerebellar cross section showing BrdU (green), GABA (Red) and Parvalbumin (Blue) staining three months after injury. No BrdU/PV positive cells are detected and very few BrdU/GABA positive cells are detected (arrows). Yellow hatched line show original picture border in rotated in images.



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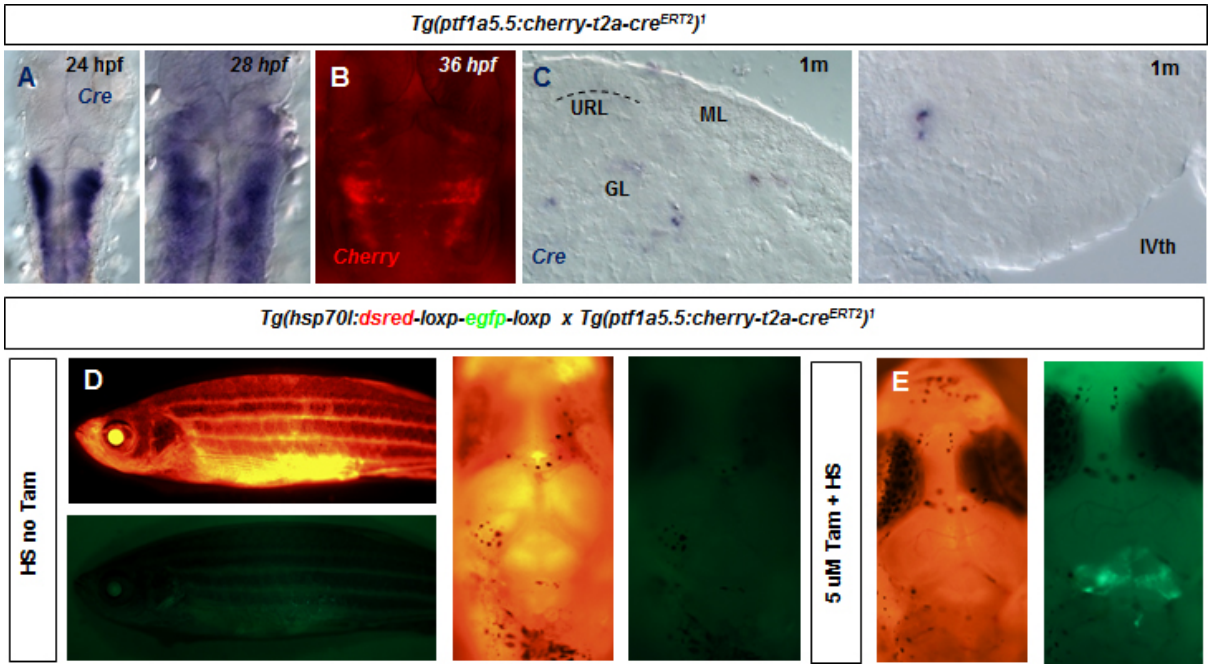

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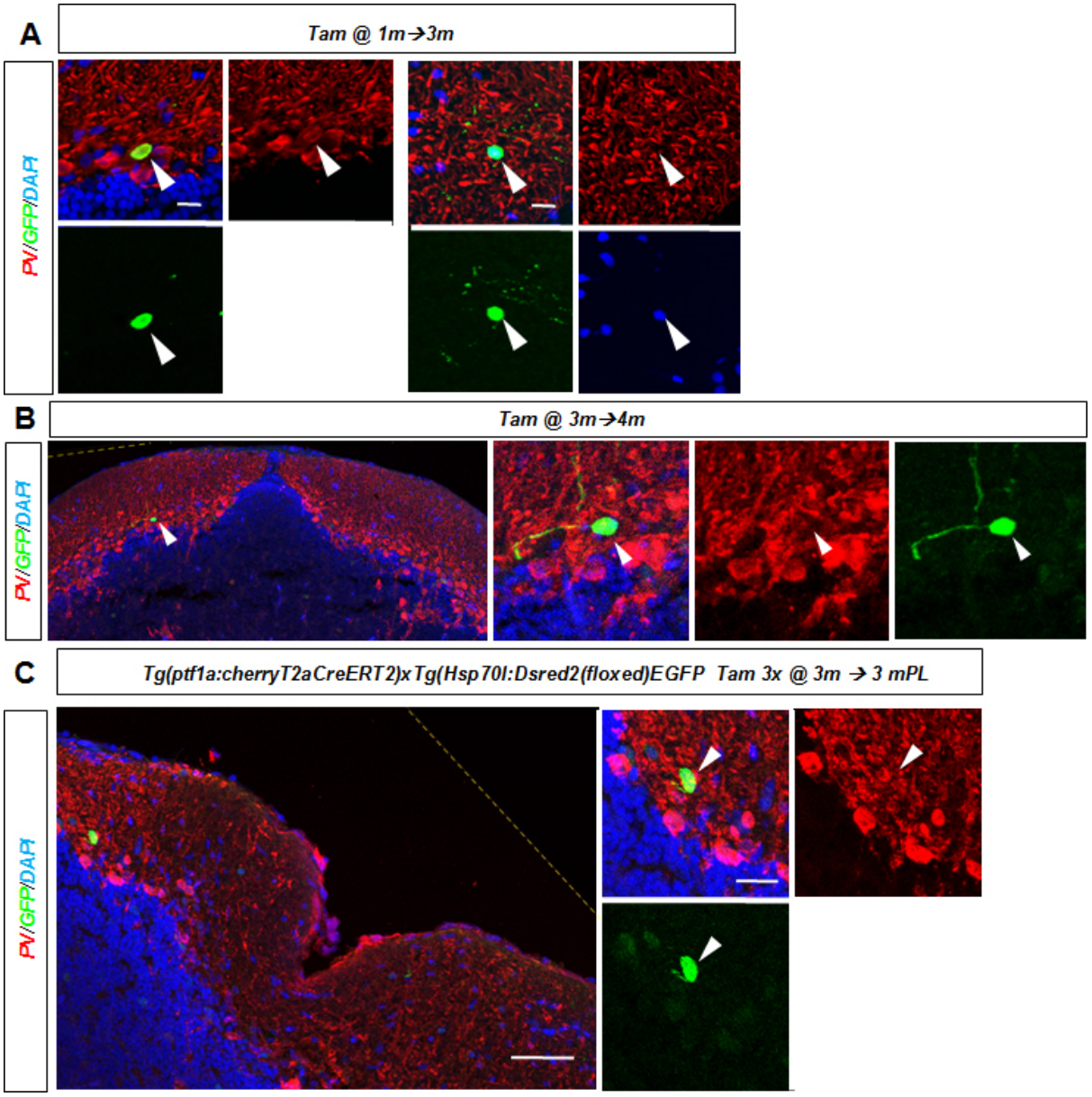

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