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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Hypothalamic radial glia function as self-renewing neural progenitors in the absence of Wnt/β-catenin signaling

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ABSTRACT

The vertebrate hypothalamus contains persistent radial glia that have been proposed to function as neural progenitors. In zebrafish, a high level of postembryonic hypothalamic neurogenesis has been observed, but the role of radial glia in generating these new neurons is unclear. We have used inducible Cre-mediated lineage labeling to show that a population of hypothalamic radial glia undergoes self-renewal and generates multiple neuronal subtypes at larval stages. Whereas Wnt/β-catenin signaling has been demonstrated to promote the expansion of other stem and progenitor cell populations, we find that Wnt/β-catenin pathway activity inhibits this process in hypothalamic radial glia and is not required for their self-renewal. By contrast, Wnt/β-catenin signaling is required for the differentiation of a specific subset of radial glial neuronal progeny residing along the ventricular surface. We also show that partial genetic ablation of hypothalamic radial glia or their progeny causes a net increase in their proliferation, which is also independent of Wnt/β-catenin signaling. Hypothalamic radial glia in the zebrafish larva thus exhibit several key characteristics of a neural stem cell population, and our data support the idea that Wnt pathway function may not be homogeneous in all stem or progenitor cells.

KEY WORDS: Wnt signaling, Radial glia, Neural progenitors, Hypothalamus, Zebrafish

INTRODUCTION

The postembryonic zebrafish brain is highly proliferative and regenerative, characteristics that have been attributed to the presence of radial glia that persist throughout the central nervous system (CNS) and generate neurons (Kizil et al., 2012; Than-Trong and Bally-Cuif, 2015). We previously characterized a population of neural progenitors in the postembryonic zebrafish hypothalamus, which produces multiple neuronal subtypes through adulthood (Wang et al., 2012). A similar process also occurs in the mammalian hypothalamus, where adult neurogenesis contributes to reproductive and feeding behaviors (Kokoeva et al., 2005; Lee et al., 2012; Cheng, 2013). However, the underlying progenitor cell populations supporting hypothalamic neurogenesis remain poorly characterized. Although radial glia have been proposed to fulfill this role in both zebrafish and mouse (Lee et al., 2012; Wang et al., 2012; Haan et al., 2013; Robins et al., 2013), their capacity for self-renewal and differentiation have not been comprehensively tested.

In addition, the molecular pathways regulating radial glial self-renewal, expansion and neurogenesis in the hypothalamus are poorly understood. Previous work from our laboratory showed that Wnt/β-catenin signaling is required for postembryonic hypothalamic neurogenesis (Wang et al., 2012), and other studies have also led to the hypothesis that pathway activity promotes radial glial differentiation (Lee et al., 2006; Wang et al., 2011, 2012; Choe and Pleasure, 2012; Varela-Nallar and Inestrosa, 2013). By contrast, Wnt/β-catenin signaling has also been shown to promote the self-renewal and expansion of neural stem cells in the mammalian telencephalic subventricular zone and dentate gyrus (Qu et al., 2010). The specific function of Wnt/β-catenin activity in hypothalamic radial glia is therefore unclear, leaving an open question as to whether a general role for the pathway exists for all neural stem and progenitor cell populations.

Here we take a genetic approach to identify the neural progenitor cell population in the larval zebrafish hypothalamus, and to characterize the response and regulation of hypothalamic radial glia during tissue growth and regeneration. Our data show that the radial glial population is both self-renewing and multipotent, and exhibits a proliferative response to partial ablation or to ablation of their neuronal progeny. In addition, we use multiple perturbations of Wnt/β-catenin signaling to test the necessity and sufficiency of pathway activity for radial glial self-renewal, expansion and neuronal differentiation. Consistent with studies of non-neural stem cells (Lowry et al., 2005; Blanpain and Fuchs, 2009; Farin et al., 2012), our data show that Wnt/β-catenin signaling is only necessary for the terminal differentiation of specific neuronal progeny. Furthermore, and as shown for radial glia in other brain regions (Wang et al., 2011), we find that ectopic Wnt/β-catenin activity inhibits the expansion of neurogenic radial glia in the hypothalamus. Together, these data suggest that the most generally conserved role for Wnt pathway activity in neural progenitors is in promoting neurogenesis, and that other functions may differ between diverse stem and progenitor cell populations.

RESULTS

Radial glia are multipotent neural progenitors in the postembryonic hypothalamus

In an effort to identify molecular markers of radial glia in the zebrafish hypothalamic posterior recess (Fig. 1A), we found that at 5 days post-fertilization (dpf), Glutamine synthetase (GS) and a *her4.3:EGFP* transgene (Fig. 1B) (Yeo, et al., 2007) both label radially oriented cells contacting the ventricle along with an *Et(Gal4-VP16;myl7:gfp)*^{zc1066a} enhancer trap line that we previously showed to be expressed in radial glia of the posterior recess (Fig. 1C) (Wang et al., 2012). Although there was not complete co-expression, probably owing to transgene mosaicism, the majority of cells expressing GS also expressed *her4.3:EGFP* (Fig. 1D). A consistent minority of cells labeled by

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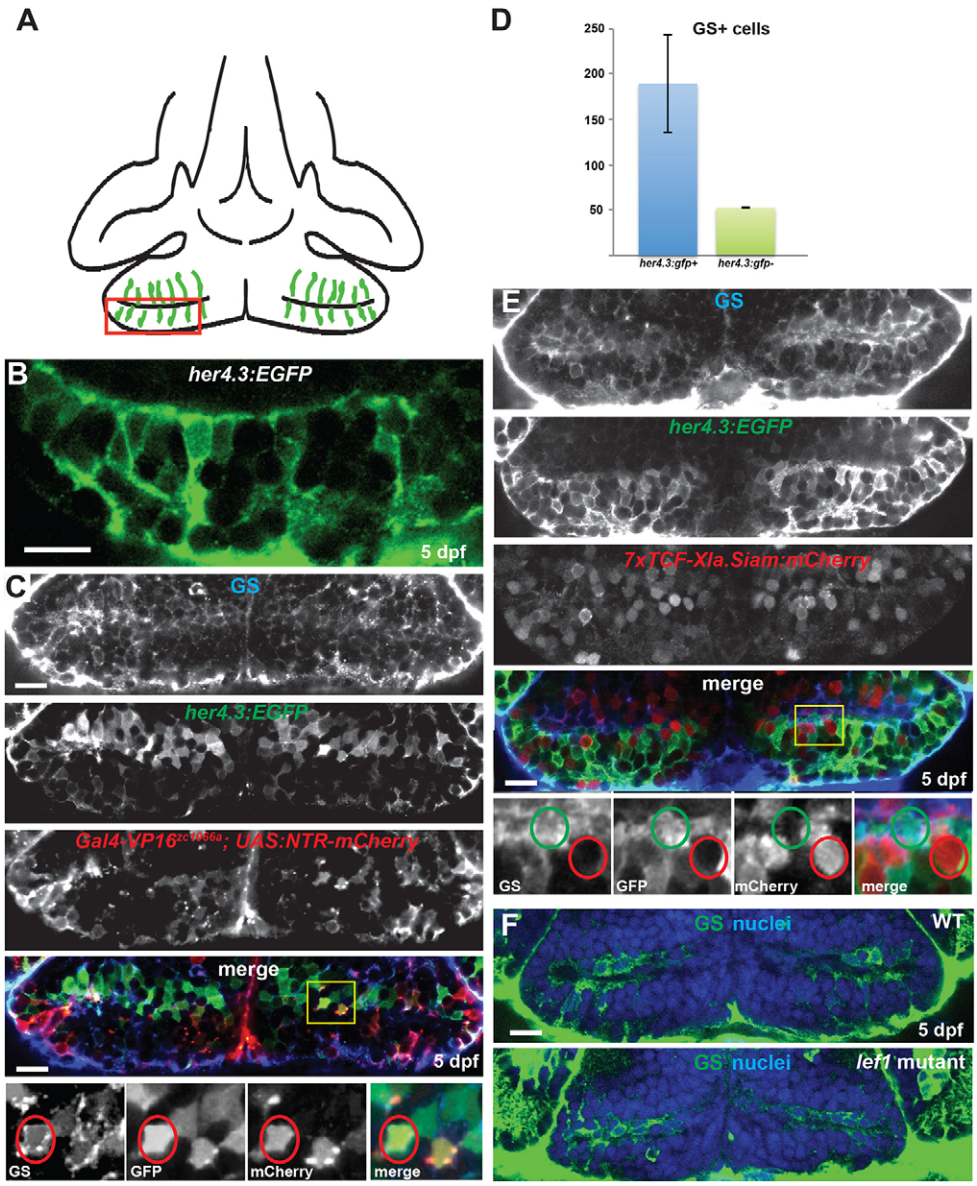

Fig. 1. Radial glia in the hypothalamic posterior recess are not Wnt-responsive. (A) Diagram of the hypothalamic posterior recess from a ventral view of a 5 dpf zebrafish brain. Radial glia are in green; the red box indicates the area depicted in B. (B) High-magnification optical section of *her4.3:EGFP* expression, showing the position of cell soma and radial processes. (C) Radial glial cells in the 5 dpf posterior recess can be labeled with anti-Glutamine synthetase (GS, blue), a *her4.3:EGFP* transgene (green), and a *Gal4* enhancer trap line (red). Yellow box indicates the region shown beneath. A triple-labeled cell is indicated by red circles. (D) Most cells expressing GS or *her4.3:EGFP* also express the other marker. Error bars indicate s.e.m.; *n*=22 optical sections from two brains. (E) Most GS^+ and *her4.3:EGFP*$^+$ radial glia do not express the Wnt reporter transgene *7xTCF-Xla.Siam:GFP*. Yellow box indicates region shown beneath. A reporter-negative radial glial cell is indicated by green circles, and a reporter-expressing non-glial cell is indicated by red circles. (F) GS^+ cells are not reduced in number in *lef1* mutants at 5 dpf. Images are single optical sections from ventral views of whole-mount brains. WT, wild type; nuclei are stained with Hoechst 33342 (blue). Scale bars: 10 µm.

her4.3:EGFP were also GS^-, potentially representing non-glial progeny due to GFP perdurance (Briona and Dorsky, 2014). By contrast, we did not observe expression of either Gfap (Wang et al., 2012) or S100β (data not shown) in this region. Combined with previous evidence supporting the specificity of GS and *her4.3:EGFP* in labeling radial glia throughout the zebrafish brain (Ganz et al., 2010; Grupp et al., 2010; Kroehne et al., 2011), we concluded that both markers also effectively label this population in the posterior recess.

Consistent with our previous work (Wang et al., 2012), we found that only 5.5±2.5% (s.e.m., *n*=11 optical sections from two brains) of GS^+ cells expressed the Wnt reporter transgene *7xTCF-Xla.Siam:mCherry* (Moro et al., 2012) (Fig. 1E). Furthermore, analysis of embryos homozygous for a null mutation in the Wnt effector *lef1* (Wang et al., 2012) at 5 dpf (Fig. 1F) showed that the number of GS^+ cells was in fact increased in the posterior recess relative to tissue size (29.5±1.4 in wild type versus 32.0±2.7 in *lef1* mutants; s.e.m., *n*=3 equatorial optical sections each from four brains). These data, along with observations that *her4.3:EGFP*$^+$ cells are also not decreased in *lef1* mutants (data not shown), support our prior conclusion that hypothalamic radial glia do not require Wnt/β-catenin activity for their formation or maintenance.

To determine the lineage of the radial glial population, we took advantage of an existing transgenic line that uses the *her4.3* promoter/enhancer to drive the expression of tamoxifen-inducible Cre recombinase (Boniface et al., 2009). By crossing this line to the Cre-inducible *ubi:switch* reporter (Mosimann et al., 2011), which expresses mCherry following recombination, we were able to permanently label all progeny (Fig. 2A). Addition of 5 µM 4-hydroxytamoxifen (4-OHT) from 5-6 dpf resulted in the conversion of 1-5 cells per posterior recess, and we did not observe any mCherry expression in untreated larvae. Analysis at 6 dpf showed that 97±3% of $mCherry^+$ cells (s.e.m., *n*=30 optical sections from three brains) were co-labeled by GS (Fig. 2B, Fig. S1A). Six days after recombination, radial chains of labeled progeny were visible extending from the ventricle, including both GS^+ and GS^- cells (Fig. 2C). By 6 weeks post-fertilization (wpf), labeled progeny had expanded into large radial clones (Fig. 2D), which contained only 8.7±1.5% GS^+ cells (s.e.m., *n*=30 optical sections from three brains; Fig. 2E, Fig. S1B). Using markers for

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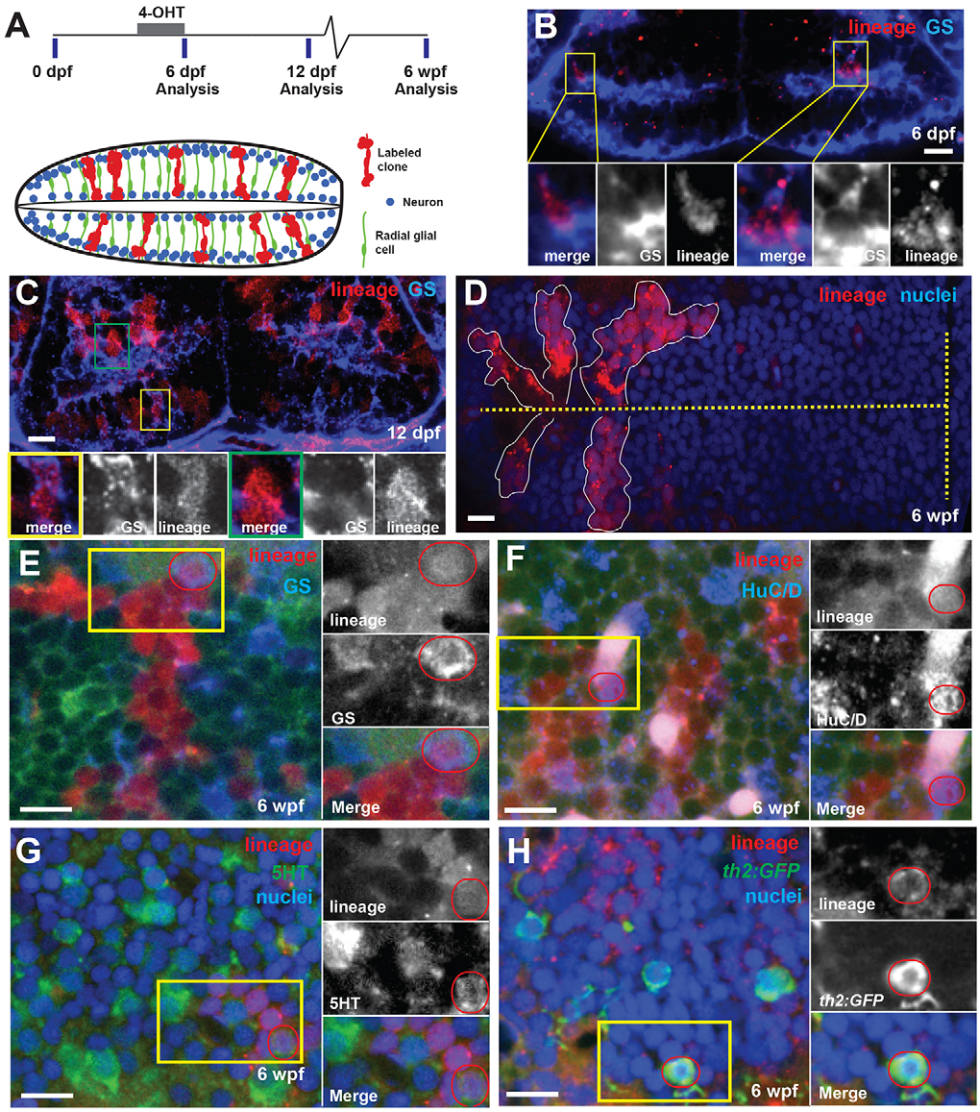

Fig. 2. Hypothalamic radial glia are self-renewing neural progenitors. (A) Timeline and schematic of experiments. Cells expressing *–3her4.1:ERT2-Cre-ERT* and *ubi:loxP-eGFP-loxP-mCherry* were genetically labeled by the addition of 5 µM 4-OHT from 5-6 dpf. Labeled progeny (red) comprise expanding radial units spanning hemispheres of the posterior recess. Processes of GS$^+$ radial glia (green) span the tissue, and nuclei occupy variable positions from the ventricle to the outer edge. Neurons (blue) are located either adjacent to the ventricle or at the outer edge. (B) Immediately following conversion, the few labeled mCherry$^+$ cells are also GS$^+$. Yellow boxes indicate regions shown beneath. (C) Six days after conversion, labeled cells extend radially and include GS$^+$ (yellow box) and GS$^-$ (green box) cells. (D) Five weeks after 4-OHT addition, discrete groups of mCherry$^+$ cells extend from the ventricle (dashed line). (E-H) mCherry$^+$ progeny 5 weeks after recombination include GS$^+$ radial glia (E), HuC/D$^+$ neurons (F), 5HT$^+$ neurons (G) and *th2:gfp*$^+$ dopaminergic neurons (H). Yellow boxes indicate regions shown beneath, and double-labeled cells are indicated by red circles. Green signal in E,F is *ubi:GFP* from unconverted cells. Images are single optical sections from ventral views of whole-mount brains. Scale bars: 10 µm.

differentiated neuronal cell types, we found that at 6 wpf the lineage included neurons labeled by HuC/D (Elavl3/4) (Fig. 2F, Fig. S1C), serotonin (Fig. 2G) (Perez et al., 2013), and a transgenic marker of dopaminergic fate [*Tg(th2:Gal-VP16)*^{zd202}; McPherson et al., 2016] (Fig. 2H). These data indicate that postembryonic neurons in the zebrafish hypothalamus arise from a radial glial population that can self-renew, expand, and generate multiple types of progeny.

Wnt/β-catenin signaling is only required for the differentiation of a specific subset of neuronal progeny

We next tested whether Wnt/β-catenin signaling is necessary for the self-renewal, expansion or neuronal differentiation of radial glia. Using heat shock-mediated expression of the secreted Wnt signaling inhibitor Dkk1 (Stoick-Cooper et al., 2007), following conversion of the Cre-labeled population with 4-OHT, we examined the effects on lineage size and composition. After conversion from 5-6 dpf, *Tg(hsp701:dkk1-GFP)*^{w32} embryos were heat shocked once daily and fixed at 9 dpf. Although Dkk1 expression effectively inhibited Wnt signaling, as determined by *in situ* hybridization of the Wnt/β-catenin target *sp5l* (Weidinger et al., 2005) (Fig. S2), it did not result in a significant difference in the total number of labeled cells (Fig. 3A,D) or in the percentage of GS$^+$ radial glia (Fig. 3B,D) in the lineage. Dkk1 expression also did not inhibit the differentiation of HuC/D$^+$ neurons from labeled radial glia (Fig. 3C) and, in fact, caused a small but statistically insignificant increase in neurogenesis. These results suggest that radial glia can divide and produce neuronal progeny in the absence of Wnt pathway activity.

As an alternative method to inhibit Wnt signaling we examined the radial glial lineage in *lef1* mutants. After 4-OHT-mediated conversion from 5-6 dpf and lineage analysis at 9 dpf, we found that loss of *lef1* also did not significantly change the percentage of GS$^+$ radial glia (Fig. 3E) within mCherry-labeled progeny. As we reported previously, *lef1* is required to generate a subset of HuC/D$^+$ neurons in the posterior recess (Wang et al., 2012). Our lineage analysis confirmed that these *lef1*-dependent neurons arise from radial glia and showed that they specifically reside within two cell diameters of the ventricle (Fig. 3F,G). However, they comprise only a small portion of radial glial progeny, and the number of non-ventricular neurons was not decreased (Fig. 3F). Combined with the results of Dkk1 overexpression, these data led us to conclude that Wnt/β-catenin signaling is not necessary for radial glial self-renewal or expansion. In addition, whereas Lef1-mediated Wnt activity is required for the differentiation of ventricular neurons, it is not required for the majority of neurogenesis in the hypothalamic posterior recess.

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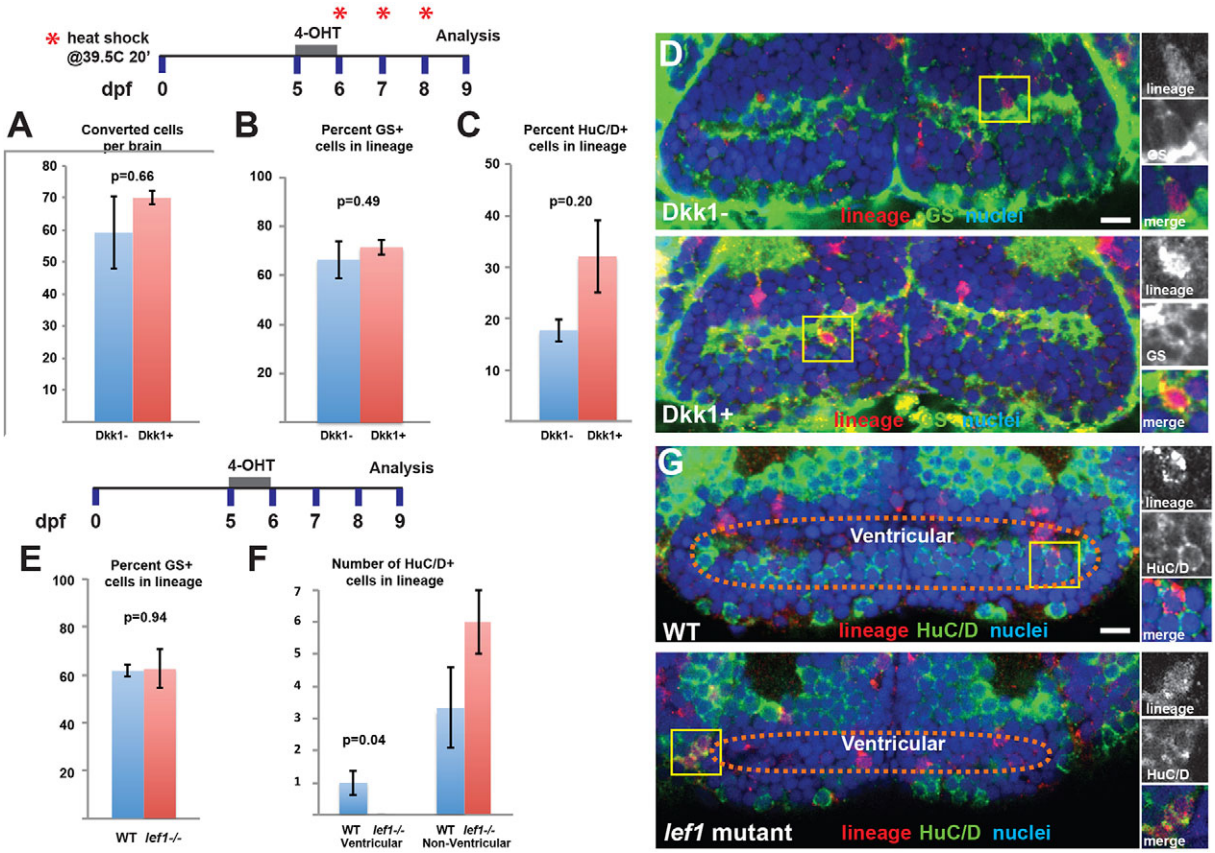

Fig. 3. Wnt/β-catenin signaling is only required for the differentiation of a specific subset of ventricular neurons. (A,B) Following recombination of *–3her4.1:ERT2-Cre-ERT*$^{+}$ progeny from 5-6 dpf, expression of Dkk1 from 6-9 dpf does not affect the average number of labeled cells (A) or the percentage of GS^{+} cells (B) in the lineage. (C) The differentiation of HuC/D^{+} neurons from labeled radial glia is not inhibited by Dkk1 expression. (D) Representative images of labeling for lineage and GS in control and Dkk1-expressing larvae. (E) In *lef1* mutants there is no change in the percentage of GS^{+} cells after conversion from 5-6 dpf and analysis at 9 dpf. (F,G) *lef1*-dependent ventricular neurons fail to arise from the radial glial lineage, whereas other non-ventricular neurons are not significantly affected. Yellow boxes indicate areas shown to the right. Images are single optical sections from ventral views of whole-mount brains. Error bars indicate s.e.m.; *n*=50 confocal slices from five brains for each experiment. Scale bars: 10 µm.

Partial genetic ablation of radial glia leads to increased proliferation of GS^{+} cells

To test whether hypothalamic radial glia show a regenerative response similar to other neural stem cell populations, we used the *Et(Gal4-VP16,myl7:gfp)*^{zc1066a} enhancer trap line in combination with the *Tg(UAS-E1b:NTR-mCherry)*^{jh17} effector line to express Nitroreductase (NTR) specifically in radial glia of the posterior recess (Otsuna et al., 2015), and thus ablate cells using metronidazole (MTZ) (Davison et al., 2007; Pisharath et al., 2007). Consistent with previous studies, incubation of non-transgenic larvae in 1 mM MTZ did not significantly affect cell proliferation or cell death (data not shown). By contrast, after incubation of NTR-expressing larvae in 1 mM MTZ from 5-6 dpf we observed partial ablation of radial glia (Fig. 4A,B, Fig. S3), and the remaining radial glia, which were labeled either by GS (Fig. 4C,D) or *her4.3:EGFP*, showed variable but significantly increased BrdU labeling from 7-8 dpf (Fig. 4C-F). This result suggested that radial glia can react to a decrease in their own population with a corresponding increase in self-renewal.

To determine if the proliferative response of radial glia to partial ablation requires Wnt/β-catenin signaling, we repeated our experiments in the presence of Dkk1 overexpression and in *lef1* mutants. In both cases we observed a similar increase in BrdU incorporation within GS^{+} cells, as in control animals (Fig. 4G,H). These data indicate that, just as during normal growth, the regenerative expansion of hypothalamic radial glia is also Wnt/β-catenin independent.

Radial glia proliferate in response to the genetic ablation of progeny

We next investigated whether hypothalamic radial glia exhibit a proliferative response to the loss of a progeny cell type. Since we had observed that the lineage included dopaminergic neurons labeled by the *th2* enhancer/promoter (Fig. 2F), and we found that the enhancer was not expressed in GS^{+} cells (Fig. 5A), we used a transgenic line [*Tg(th2:Gal-VP16)*^{zd20}; McPherson et al., 2016] to drive *UAS:NTR-mCherry* expression (Fig. 5B). Following incubation in a high dose (2.5 mM) of MTZ from 5-6 dpf to maximize the level of ablation, we observed a significant increase in BrdU labeling within the GS^{+} population at 8-9 dpf (Fig. 5C-E) but not 1 day earlier or later (Fig. 5C). The proliferative response coincided with a decrease in the overall number of GS^{+} cells at 9 dpf (Fig. 5F). Along with an increase in BrdU-labeled GS^{-} cells (Fig. 5D), which are likely to be non-glial Sox3^{+} neural progenitors (Wang et al., 2012), these data are consistent with the depletion of radial glia observed during regeneration in the adult zebrafish telencephalon (Barbosa et al., 2015).

Wnt activation blocks expansion of the radial glial population

Based on evidence that ectopic Wnt/β-catenin signaling leads to a decrease in the number of radial glia (Wang et al., 2011, 2012), we

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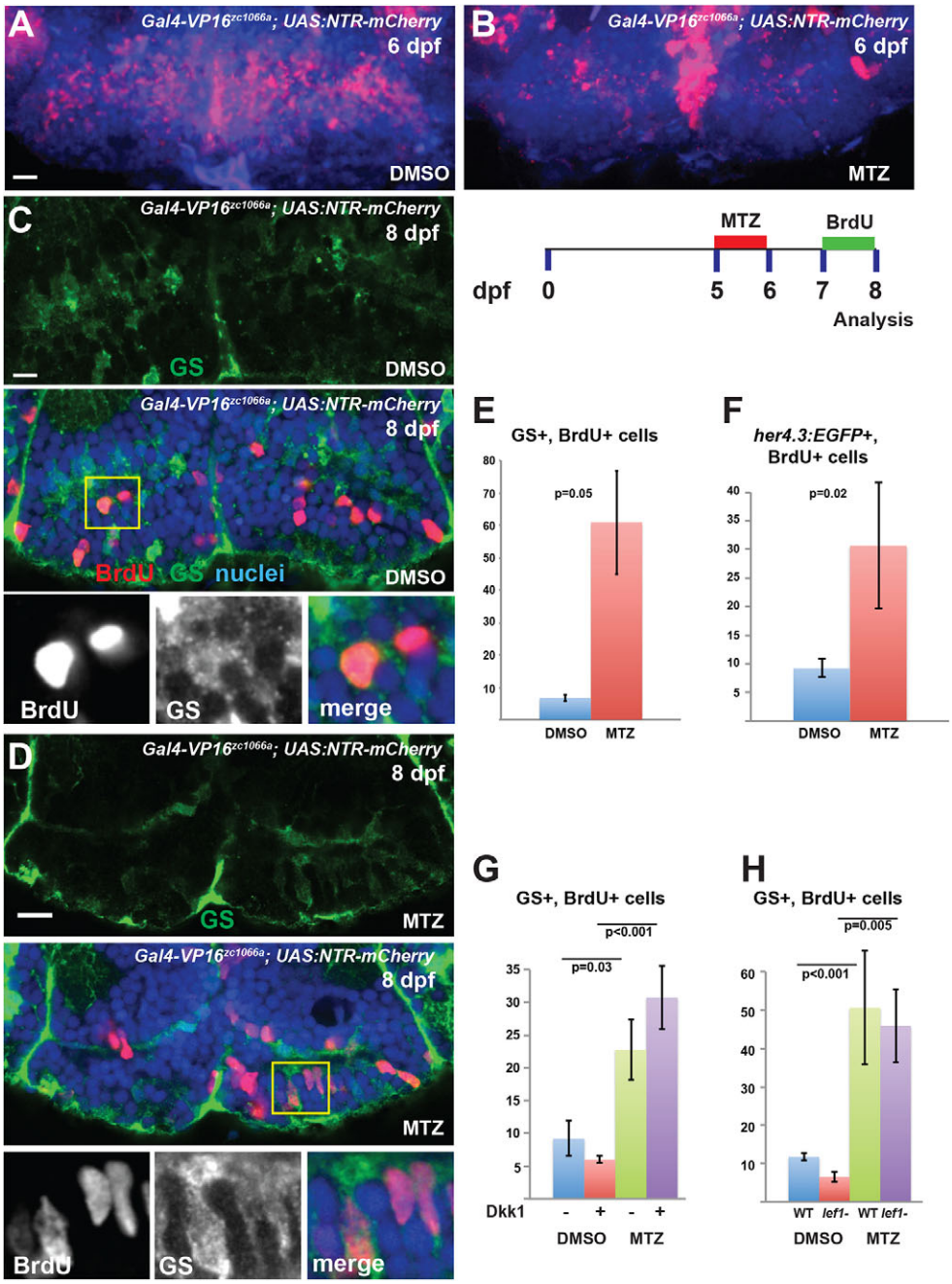

Fig. 4. Hypothalamic radial glia respond to partial ablation by increasing proliferative activity. (A,B) Partial ablation of NTR-mCherry-expressing radial glia (red) by incubation in 1 mM MTZ from 5-6 dpf; DMSO provides a control. (C-F) After partial ablation from 5-6 dpf and BrdU labeling from 7-8 dpf (C,D; yellow box indicates region shown beneath), there is a significant increase in the number of BrdU$^+$ radial glia labeled either by GS (C-E) or by *her4.3:EGFP* (F) expression. (G,H) Inhibition of Wnt signaling by Dkk1 expression (G), or *lef1* mutation (H), does not block the increase in BrdU labeling following partial ablation. Images are maximum-intensity *z*-projections (A,B) or single optical sections (C,D) from ventral views of whole-mount brains. Error bars indicate s.e.m.; *n*=40 optical sections from four brains for each experiment. Scale bars: 10 µm.

examined whether pathway activity specifically inhibits the normal expansion of their progeny. Following induction of *wnt8a* at 5 dpf using the heat shock-inducible transgenic line *Tg(hsp70l:wnt8a-GFP)*^{w34} (Weidinger et al., 2005), we observed a significant decrease in the number of GS$^+$ cells at 6 dpf compared with controls (Fig. 6A). Continuous *wnt8a* expression over multiple days resulted in lethality, so to test the longer term consequences of pathway activation we incubated animals in 4 µM BIO, a pharmacological activator of Wnt/β-catenin signaling (Sato et al., 2004; Shimizu et al., 2012; Lush and Piotrowski, 2014) from 6-9 dpf. This experiment also produced a small but significant decrease in the number of GS$^+$ cells compared with controls (Fig. 6B,C), suggesting that Wnt signaling either inhibits radial glial expansion or causes the loss of GS expression.

To specifically test these possibilities, we next performed lineage analysis in the presence of BIO from 6-9 dpf after recombination from 5-6 dpf. We found that the total number of mCherry$^+$ cells in animals treated with BIO was significantly decreased compared with controls (Fig. 6D), coupled with a relative increase in the proportion of GS$^+$ cells within the labeled population (Fig. 6E). The smaller number of progeny was not due to cell death, as neither *wnt8a* induction (Wang et al., 2012) nor BIO treatment (data not shown) caused a significant increase in apoptosis. Our results could therefore by explained by a decrease in the number of radial glia undergoing amplifying divisions, combined with the decrease in neurogenesis that we previously demonstrated to result from constitutive Wnt activation (Wang et al., 2012).

DISCUSSION

Hypothalamic radial glia exhibit multiple features of neural stem cells

Our results demonstrate that hypothalamic radial glia in zebrafish are self-renewing neural progenitors that can undergo a regenerative response, characteristics that are hallmarks of a stem cell population.

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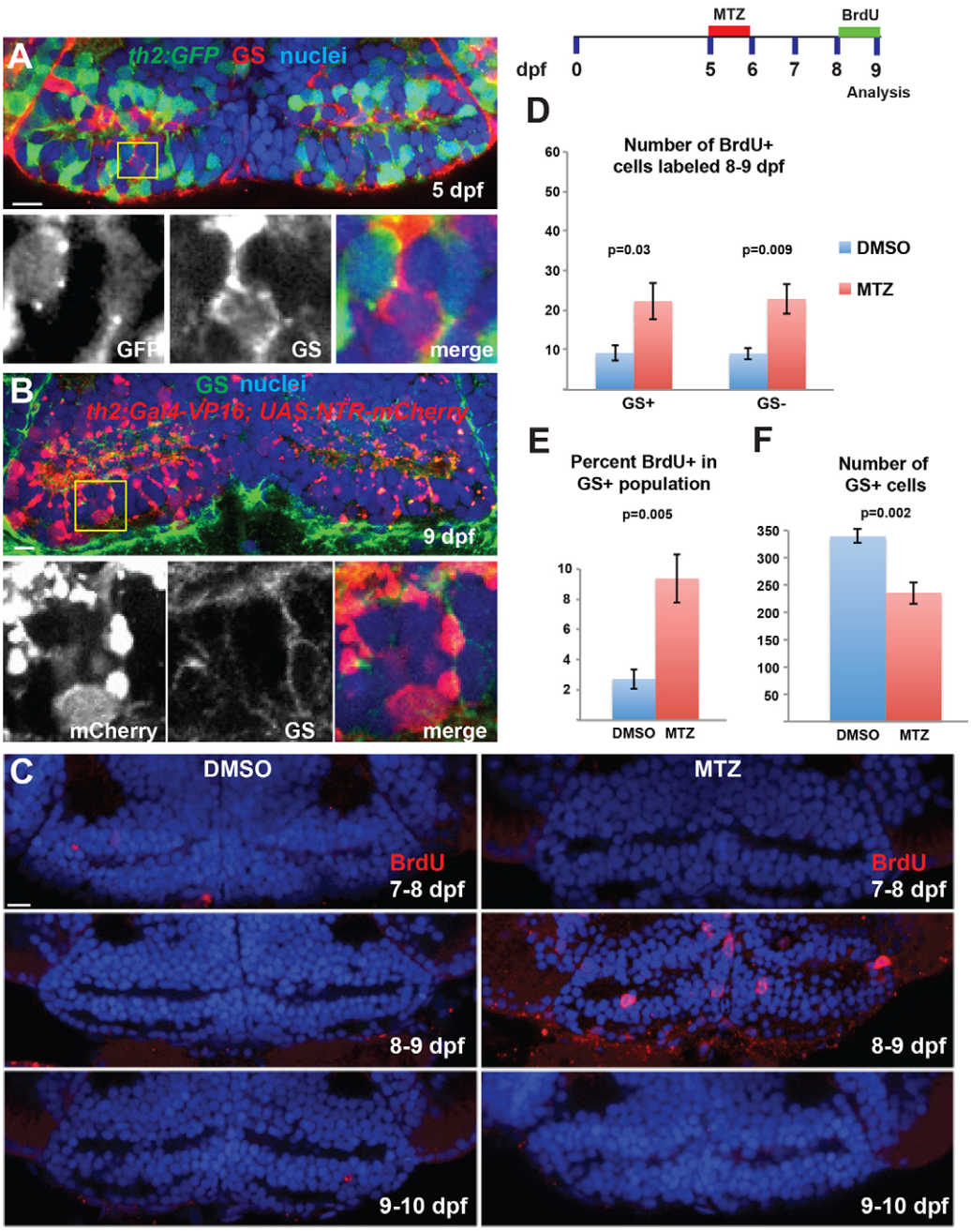

Fig. 5. Hypothalamic radial glia proliferate in response to ablation of dopaminergic progeny. (A,B) The *th2:GFP* (5 dpf, A) and *th2:Gal4* (9 dpf, B) transgenes do not label GS$^+$ radial glia. Yellow boxes indicate regions shown beneath. (C) Ablation of *th2:Gal4*$^+$ cells from 5-6 dpf leads to increased BrdU labeling only at 8-9 dpf. (D,E) Ablation of *th2:Gal4*$^+$ cells increases the number and percentage of GS$^+$ radial glia, as well as the number of GS$^-$ cells, labeled with BrdU from 8-9 dpf. (F) The overall number of GS$^+$ radial glia is decreased at 9 dpf following ablation of *th2:Gal4*$^+$ cells. Images are single optical sections from ventral views of whole-mount brains. Error bars indicate s.e.m.; *n*=50 optical sections from five brains for each experiment. Scale bars: 10 µm.

Because we were not able to follow the lineage of single cells, we cannot determine whether individual radial glia are multipotent with respect to neuronal fate. However, the expansion that we observe in the lineage over a 5-week labeling period indicates that radial glia contribute significantly to the growth in size of the posterior recess, and our marker analysis shows that the population as a whole generates several neuronal subtypes.

While previous studies from our laboratory and others have observed the presence of proliferating neural progenitors in the adult zebrafish hypothalamus (Wang et al., 2012; Perez et al., 2013), the work described here focused on an earlier period of larval development. The behavior of radial glia during this period is therefore not strictly equivalent to that of other adult stem cell populations, which are typically quiescent or support tissue homeostasis rather than growth. Future studies testing the lineage and injury response of radial glia in the adult zebrafish posterior recess will provide more insight into whether they function as true neural stem cells.

Wnt/β-catenin signaling is not necessary for hypothalamic radial glial self-renewal or expansion

Studies in the CNS (Piccin and Morshead, 2011) and other tissues (Nusse, 2008; Holland et al., 2013) have resulted in the hypothesis

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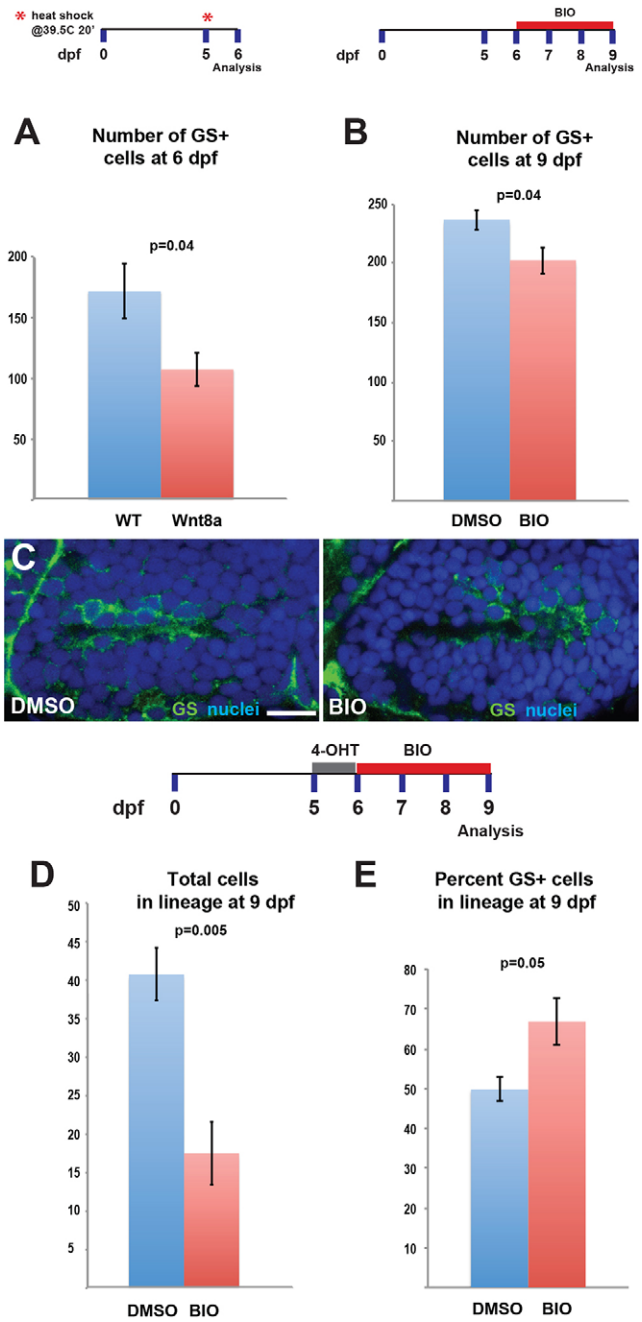

Fig. 6. Wnt/β-catenin signaling activation blocks expansion of the radial glial population. (A) Induction of Wnt8a at 5 dpf leads to a decrease in the number of GS^+ radial glia at 6 dpf. (B) Addition of 4 µM BIO, a Gsk3β inhibitor, daily from 6-9 dpf leads to a decrease in GS^+ radial glia. (C) Representative images of labeling for GS in control and BIO-treated larvae. Images are single optical sections from ventral views of whole-mount brains. Scale bar: 10 µm. (D) Following recombination from 5-6 dpf, incubation in BIO causes a significant decrease in the number of labeled progeny at 9 dpf. (E) Incubation in BIO causes a significant increase in the percentage of GS^+ cells within the labeled lineage. Error bars indicate s.e.m.; *n*=40 optical sections from four brains for each experiment.

that Wnt/β-catenin signaling might function generally to promote stem and progenitor cell proliferation. In order to achieve the increase in population size that we observe in our lineage analysis, radial glia must undergo amplifying self-renewing divisions, and, as has been shown in the telencephalon (Barbosa et al., 2015), regeneration may require these divisions at an even higher frequency. However, our data indicate that ectopic Wnt activity in fact inhibits expansion of the hypothalamic radial glia population, while other studies suggest that signals such as FGF (Kaslin et al., 2009; Robins et al., 2013) and Sonic hedgehog (Dave et al., 2011; Shikata et al., 2011; Komada, 2012) are likely to promote this process.

We found that a specific subset of *lef1*-dependent neurons located near the hypothalamic ventricle arise from the radial glial lineage (Wang et al., 2012). Combined with other studies in the retina (Agathocleous et al., 2009), cerebral cortex (Munji et al., 2011; Zhang et al., 2014), hippocampus (Seib et al., 2013) and midbrain (Castelo-Branco et al., 2003), our data suggest that the most widely conserved role for Wnt/β-catenin signaling in the CNS might be to regulate the differentiation of specific subsets of committed neural progenitors.

Wnt/β-catenin activity does not act identically in all neural stem and progenitor cells

Our experiments support the idea that diverse neural stem and progenitor cell populations are likely to exhibit different responses to Wnt/β-catenin signaling. Although Wnt ligands and reporters are expressed at high levels in the hypothalamic ventricular zone (Wang et al., 2012), radial glia largely fail to respond to these signals. This low activity state could be regulated by extracellular or intracellular pathway antagonists, or radial glia might simply fail to express the appropriate receptors to transduce Wnt signals. Regardless of the mechanism, it appears that this characteristic of hypothalamic radial glia is similar to other radial glial populations in the zebrafish retina and spinal cord (Goldman, 2014; Briona et al., 2015), but differs from radial glia in the mammalian dentate gyrus (Qu et al., 2010). Other studies have similarly shown that neural progenitor populations vary dramatically in their interpretation of pathway activity (Poschl et al., 2013). Understanding these differences might help provide insight into the basis of radial glial, and neural stem/progenitor cell, heterogeneity.

MATERIALS AND METHODS

Zebrafish

Embryos were obtained from the following zebrafish lines: *Tg(her4.3:EGFP)*^{y83} (Yeo et al., 2007), *Tg(ubi:loxP-eGFP-loxP-mCherry)*^{cz1701} (Mosimann et al., 2011), *Tg(−3her4.1:ERT2-Cre-ERT2)*^{vu298} (Boniface et al., 2009; Mosimann et al., 2011), *Et(Gal4-VP16,myl7:gfp)*^{zc1066a} (Wang et al., 2012), *Tg(UAS-E1b:NTR-mCherry)*^{ih17} (Davison et al., 2007; Pisharath et al., 2007), *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} (Moro et al., 2012), *Tg(hsp701:dkk1-GFP)*^{w32} (Stoick-Cooper et al., 2007), *Tg(hsp70l:wnt8a-GFP)*^{w34} (Weidinger et al., 2005), *lef1*^{zd11} (Wang et al., 2012), *Tg(th2:GFP-aequorin)*^{zd201} and *Tg(th2:Gal-VP16)*^{zd202} (McPherson et al., 2016). Embryos were staged according to Kimmel et al. (1995). All experiments were approved by the University of Utah Institutional Animal Care and Use Committee.

Transgenic embryos were identified by GFP tag expression following heat shock induction of *wnt8* or *dkk1*, or by PCR amplification of trunk tissue for *dkk1* induction in the presence of the *–3.5ubi:loxP-EGFP-loxP-mCherry* reporter using the following primers (5′-3′): *dkk1* forward, TCGACTCAAGGATCACCACA; *gfp* reverse, TCCCTCAAACTTGACT-TCAGC. *lef1* mutant animals were identified by the absence of posterior neuromasts as labeled with DASPEI (Invitrogen) (McGraw et al., 2011; Wang et al., 2012).

Treatment of embryos and larvae

Cre-mediated recombination was performed by incubation in 5 µM 4-hydroxytamoxifen (4-OHT; Sigma, CAS RN 68047-06-3) in 1% DMSO from 5-6 dpf. Ablations were performed by incubation in 1 mM

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metronidazole (MTZ; Fluka, 46461) from 5-6 dpf. To activate Wnt/β-catenin signaling, larvae were incubated in 4 µM BIO (Sigma, B1686) from 6-9 dpf, with fresh solution added each day. For BrdU labeling, larvae were incubated in 10 mM BrdU for 1 day prior to fixation for all experiments. Heat shock experiments were performed by incubating larvae in 50 ml conical tubes in a 39.5°C water bath for 20 min. For experiments from 5-9 dpf, larvae were not fed.

Immunohistochemistry and *in situ* hybridization

Embryos were fixed in 4% paraformaldehyde with 5% sucrose overnight at 4°C. Brains were then dissected for immunohistochemistry and trunks were placed in PCR tubes for genotyping. Whole brains were washed in water, incubated in 2 M HCl for 20 min at room temperature (for BrdU detection), washed, and permeabilized with one unit of dispase (Gibco, 17105-041) for 90 min at room temperature. Primary antibodies were all used at 1:500 dilution and incubated overnight at 4°C: mouse anti-Glutamine synthetase (Millipore, MAB302), rabbit anti-DsRed (Clontech, 632496), chicken anti-GFP (Aves Labs, GFP-1020), chicken anti-BrdU (Immunology Consultants Laboratory, CBDU-65A-Z), rabbit anti-5-HT (ImmunoStar, 541016), mouse anti-HuC/D (Molecular Probes, A21271), goat anti-L-Plastin (Santa Cruz Biotechnology, sc-16657). Following washes, fluorescent secondary antibodies (Invitrogen; diluted 1:500 in solution containing Hoechst 33342) were incubated overnight at 4°C. Brains were imaged on a Nikon A1 confocal microscope with a 60× oil objective. The entire posterior recess was imaged using 3 µm steps encompassing roughly 40 µm total, cell counting was performed, and images were exported to Photoshop (Adobe), Illustrator (Adobe) and ImageJ (NIH) for figure generation.

In situ hybridization was performed as described previously (Wang et al., 2012) using an antisense probe for *sp5l* generated from a PCR-amplified cDNA template. A T7 RNA polymerase initiation sequence was added to the 5′ end of the reverse primer. *sp5l* F, GTTTCCCAGCCACATGCAAC; *sp5l* R, CCAAGCTTCTAATACGACTCACTATAGGGAGAATGCTCC-CATCGCAACCATT.

Statistical analyses

Excel (Microsoft) was used to perform two-tailed equal variance *t*-tests; $P<0.05$ was interpreted as statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Experiments were performed by R.N.D., Y.X., A.D.M. and A.V.T. Transgenic lines were made by A.D.D. [*Tg(th2:GFP-aequorin)*^{zd201}] and by J.L.B. [*Tg(th2:Gal-VP16)*^{zd202}]. R.N.D. wrote the manuscript. R.I.D. supervised all the experiments and edited the manuscript.

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Supplementary information

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