

Wnt Signaling Regulates Postembryonic Hypothalamic Progenitor Differentiation

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SUMMARY

Previous studies have raised the possibility that Wnt signaling may regulate both neural progenitor maintenance and neuronal differentiation within a single population. Here we investigate the role of Wnt/ β -catenin activity in the zebrafish hypothalamus and find that the pathway is first required for the proliferation of unspecified hypothalamic progenitors in the embryo. At later stages, including adulthood, sequential activation and inhibition of Wnt activity is required for the differentiation of neural progenitors and negatively regulates radial glia differentiation. The presence of Wnt activity is conserved in hypothalamic progenitors of the adult mouse, where it plays a conserved role in inhibiting the differentiation of radial glia. This study establishes the vertebrate hypothalamus as a model for Wnt-regulated postembryonic neural progenitor differentiation and defines specific roles for Wnt signaling in neurogenesis.

INTRODUCTION

Although neurogenesis was originally defined as a phenomenon exclusive to developing brains, it has now been identified in the adult CNS of mammals (Ming and Song, 2011) and non-mammalian vertebrates (Kizil et al., 2012). The regulation of post-embryonic neurogenesis is thus a critical modulator of CNS homeostasis and plasticity, and the molecular mechanisms underlying this process are of obvious intense interest. One of the best-characterized signaling pathways involved in developmental neurogenesis is the regulation of target gene transcription by Wnt/ β -catenin signaling. For example, the hippocampus of null mutants for *Wnt3a* (Lee et al., 2000), *LRP6* (Zhou et al., 2004), and *Lef1* (Galceran et al., 2000), contains a smaller dentate gyrus, with reduced production of granule neurons and abnormalities in radial glial scaffolding due to proliferation and patterning defects. These results suggest that Wnt activity may

be required for the proliferation and normal differentiation of embryonic neural progenitors at early stages.

Recently, several studies with conditional approaches have revealed new aspects of Wnt signaling in postembryonic neurogenesis. One prevailing model suggests that Wnt activity is required to keep neural progenitors undifferentiated (Machon et al., 2007; Mutch et al., 2010). However, other data indicate that ectopic Wnt activity can both inhibit and promote neuronal differentiation (Tang et al., 2010). Consistent with these conflicting outcomes, hippocampal progenitors persist as GFAP⁺ radial stem-like cells when Wnt function is lost (Kuwabara et al., 2009), whereas activation of the pathway in the rostral migratory stream, by *Apc* deletion, results in developmental arrest of *Ascl1*⁺ transit amplifying cells (Imura et al., 2010). Together, all these studies suggest an untested unifying model in which Wnt activity is required for an early step of the neurogenesis pathway, but must be inactivated for differentiation to proceed.

We have previously shown that Wnt signaling through *Lef1* is required for neurogenesis in the embryonic zebrafish hypothalamus (Lee et al., 2006). Subsequently, we found that both Wnt signaling and neurogenesis continue in the zebrafish hypothalamus through adult stages (Wang et al., 2009). Compared with other forebrain regions, the hypothalamus is relatively unstudied as a model of postembryonic neurogenesis. Although the hypothalamus has been identified as a region with proliferation and neurogenesis in adult mammals (Kokoeva et al., 2007; Lee et al., 2012; Migaud et al., 2010; Pérez-Martin et al., 2010a), the regulation and function of this regional activity are poorly understood. Hypothalamic neurogenesis could be significant in the regulation of multiple autonomic and endocrine pathways, as already demonstrated with feeding behavior (Kokoeva et al., 2005). The presence and gene expression profiles of specific neuronal lineages, such as *Dlx*⁺ GABAergic precursors, are similar to those of other brain regions (Yee et al., 2009). In addition, the hypothalamus contains persistent radial glial tanycytes that are essential for endocrine function and can also serve as a neural progenitor population (Lee et al., 2012; Rodríguez et al., 2005). However, the regulation of tanycyte differentiation has remained uncharacterized.

Here we identify Wnt-responsive cells in the embryonic and postembryonic zebrafish hypothalamus, and find evidence for

pathway activation first in unspecified progenitors, and again later in postmitotic neural progenitors. Consistent with the profile of pathway activity, we find that Wnt signaling is first required for proliferative expansion of unspecified progenitors in the embryo and later regulates the differentiation of GABAergic and serotonergic lineages. Significantly, our data indicate that Wnt signaling must be inactivated in order for neuronal differentiation to occur. In contrast, Wnt activity is not required for the differentiation of radial glia, and ectopic pathway activation disrupts the generation of these cells. Finally, we show that Wnt activity is present in ventricular and parenchymal cells of the adult mouse mediobasal hypothalamus that express neural progenitor markers. In this system, we find that the role of Wnt signaling in radial glial development is evolutionarily conserved. Together, these data lead to a general model for Wnt function in postembryonic hypothalamic progenitor differentiation.

RESULTS

Wnt Activity Is Present in Unspecified Hypothalamic Progenitors at Early Embryonic Stages

We previously showed that Wnt signaling and the transcriptional mediator Lef1 regulate embryonic hypothalamic neurogenesis (Lee et al., 2006). In subsequent work, we found that Wnt pathway activity is present in the hypothalamus throughout the life of the animal (Wang et al., 2009). To more completely characterize Wnt-responsive cells in the zebrafish hypothalamus, here we employed a combination of transcriptional reporter lines. We used both *TOP:GFP*, a Wnt reporter containing 4 TCF binding sites upstream of a destabilized GFP transgene (Dorsky et al., 2002) as well as *TCFSiam:GFP*, a Wnt reporter containing the *Xenopus siamensis* promoter upstream of *eGFP* (Moro et al., 2012), to label Wnt-responsive cells and their progeny. At 32 hr postfertilization (hpf), we found that Wnt-responsive cells were present in the presumptive lateral and posterior recess regions (Figure 1A). At this embryonic stage, almost all *TCFSiam:GFP*⁺ and *TOP:GFP*⁺ cells were PCNA⁺ (Figure 1B; Table 1). In contrast, few *TCFSiam:GFP*⁺ and *TOP:GFP*⁺ cells expressed Sox3 (Figure 1B; Table 1), a marker and direct activator of neural progenitor gene expression (Bergslund et al., 2011), and colocalization of both reporters with the neuronal marker HuC/D was very low (Figure 1B; Table 1). Together, these data show that the majority of Wnt-responsive cells and their progeny in the 32 hpf zebrafish hypothalamus are proliferating unspecified progenitors.

Wnt Signaling Is Active in Hypothalamic Neural Progenitors at Postembryonic Stages

At 4 days postfertilization (dpf), the third ventricle and its associated posterior recess serve as reliable hypothalamic landmarks. At this stage expression of *TCFSiam:GFP* and *TOP:GFP* suggested that most Wnt-responsive cells are located in the posterior recess (red oval in Figure 1C). At this stage, we found that Wnt-responsive cells were primarily PCNA⁻/Sox3⁺ neural progenitors, although a few were PCNA⁺/Sox3⁺, indicating a transition through cell cycle exit (Figure 1D; Table 1). BrdU labeling also showed that the majority of 4 dpf Wnt-responsive cells were not proliferating (Figure S1 available online).

At 4 dpf, we used GFP perdurance in the two reporter lines to determine which lineages derive from the Wnt-responsive cells. To confirm the difference in reporter responsiveness, we expressed the *hs:dkk1* transgene (Stoick-Cooper et al., 2007) to conditionally inhibit canonical Wnt signaling in both reporter lines and monitored the decrease in GFP expression following heat shock. We found that it took 24 hr to achieve an 80% reduction in *TCFSiam:GFP*⁺ cells, compared to only 8 hr for *TOP:GFP* (Figure S1). Both Wnt reporters were coexpressed with the pan-neuronal marker HuC/D, the GABAergic lineage markers *dlx5/6:mCherry* and GABA, and serotonin. However, coexpression was much higher in cells expressing the stable reporter *TCFSiam:GFP* (Figures 1D and 1E; Table 1), and relatively low in cells expressing the destabilized reporter *TOP:GFP* (Figure S2; Table 1). Because we did not observe any overlap between markers for the GABAergic and serotonergic lineages (Figure S2; Table 1), these experiments suggest that Wnt-responsive cells generate at least two different neuronal lineages. Furthermore, the different compositions of the destabilized and stable GFP reporter populations suggest that Wnt activity is rapidly silenced as neuronal differentiation proceeds.

In the adult hypothalamus, analysis of *TCFSiam:GFP* and *TOP:GFP* expression confirmed a similar distribution of Wnt-responsive cells in the posterior recess region. We previously reported that *TOP:GFP*⁺ cells in the adult hypothalamic lateral recess did not express the proliferation marker PCNA, the postmitotic neuronal marker HuC/D, or GFAP (Wang et al., 2009). To determine the identity of GFP⁺ cells in the posterior recess, we performed whole-mount sagittal analysis of this region using 150 μm confocal projections taken from the midline. We found that Sox3 continues to be expressed in the majority of Wnt-responsive cells in the posterior recess (Figure 1G; Table 1). The expression of neuronal markers still distinguished the destabilized and stable reporters, as many *TCFSiam:GFP*⁺ cells, but very few *TOP:GFP*⁺ cells, coexpressed HuC/D (Figures 1G and S2; Table 1). In addition, we found that adult hypothalamic Wnt-responsive cells continue to contribute to the GABAergic and serotonergic lineages identified in 4 dpf hypothalamus (Figure 1G; Table 1).

Together, these analyses show that in the posterior recess region of the 4 dpf and adult zebrafish hypothalamus, Wnt activity is present in Sox3⁺ GABAergic and serotonergic progenitors as they become postmitotic, but is then lost as they undergo further differentiation.

lef1 Is Required for Postembryonic Hypothalamic Neurogenesis

By analyzing the expression of multiple pathway components, we found that along with the Wnt8b ligand, the two transcriptional mediators Lef1 and Tcf7 were the best candidates to mediate Wnt signaling in the posterior hypothalamus. In particular, expression of Lef1 protein and of a *tcf7:GFP* reporter (Nagayoshi et al., 2008) mirrored that of *TCFSiam:GFP* (Figure S3; Table 1). Although Lef1 and *tcf7:GFP* were coexpressed in some cells (Figure S3), nonoverlapping expression in other cells suggested nonredundant roles in the hypothalamus.

Identified mutations in individual zebrafish *Tcf* genes produce relatively minor defects (Muncan et al., 2007; Nagayoshi et al., 2008), allowing us to investigate the role of Wnt activity in

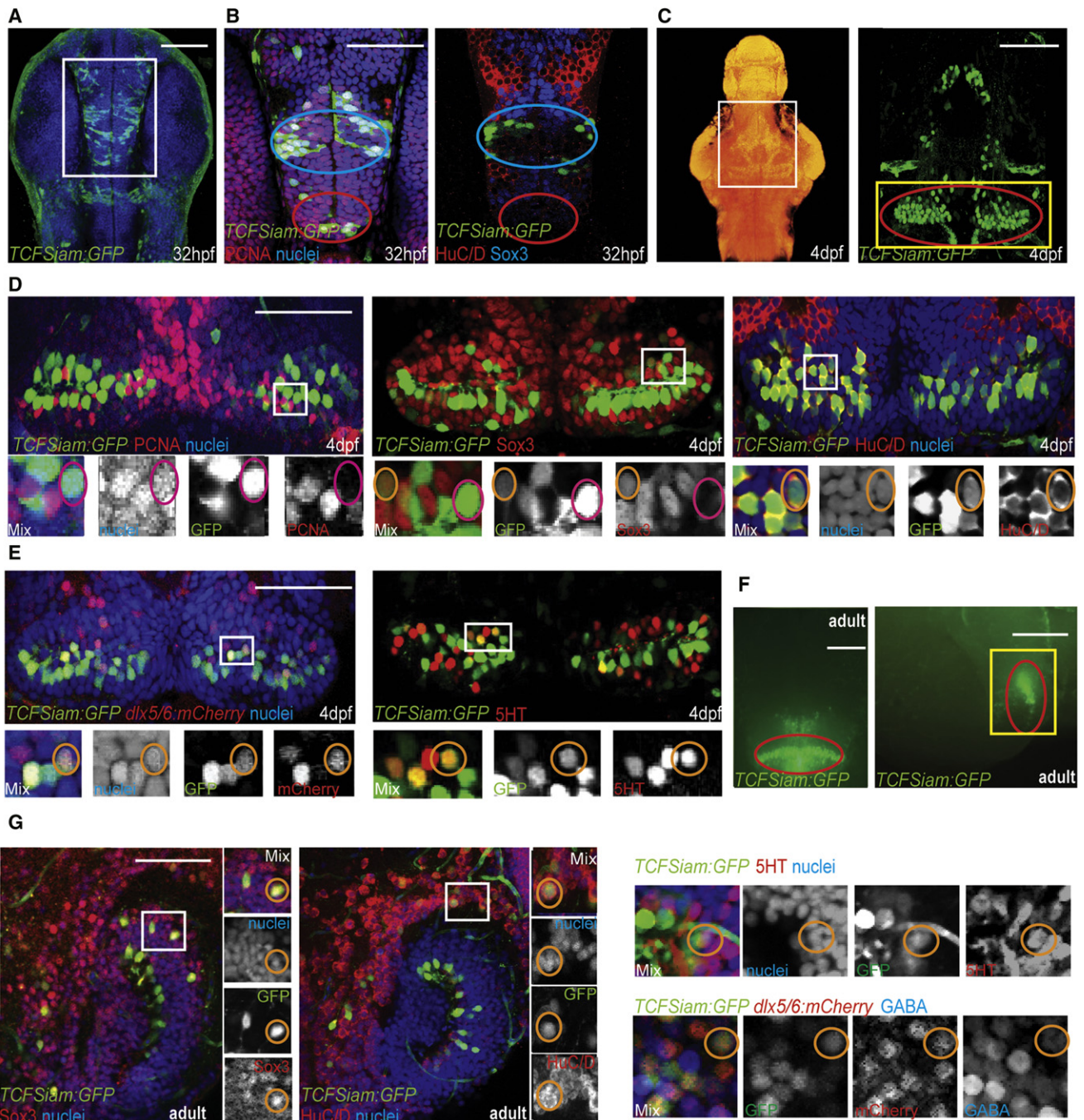


Figure 1. Identification of Wnt-Responsive Cells in the Zebrafish Hypothalamus

(A) Ventral view of *TCFSiam:GFP*.

(B) Costaining with PCNA, Sox3, and Hu. Blue ovals label the presumptive lateral recess, and red ovals label the presumptive posterior recess. White box in (A) marks the area shown.

(C) Maximum intensity confocal Z-projection of *TCFSiam:GFP* at 4 dpf. In nuclear stained ventral view of brain on left, white box marks hypothalamic area shown on right. Red oval marks the posterior recess.

(D) Costaining of *TCFSiam:GFP* with PCNA, Sox3, and HuC/D. Yellow box in (C) marks the area shown.

(E) Costaining of *TCFSiam:GFP* with *dlx5/6:gfp* and serotonin. Yellow box in (C) marks the area shown.

(F) Dissection microscope ventral and sagittal views of *TCFSiam:GFP* in the adult hypothalamus. Red oval labels the presumptive posterior recess.

(G) Costaining of *TCFSiam:GFP* with Sox3, Hu, 5HT, *dlx5/6:mCherry*, and GABA in the adult posterior hypothalamus. Yellow box in (F) marks the area shown.

Single optical sections from ventral views are shown in all panels, unless otherwise indicated. Small orange circles label cells with colocalization, and small magenta circles label cells without colocalization. Scale bars represent 80 μm (A–E, G), and 250 μm (F).

See also Figure S1.

Table 1. Coexpression Percentage of Cell Type Markers with Reporters

	PCNA	Sox3	HuC/D	<i>dlx5/6:mCherry</i>	GABA	5HT
32 hpf						
<i>TOP:GFP</i>	97.3 ± 1.5	13.1 ± 3.5	1.3 ± 0.5	–	–	–
<i>TCFsiam:GFP</i>	96.2 ± 1.2	7.2 ± 2.3	2.1 ± 0.8	–	–	–
4 dpf						
<i>TOP:GFP</i>	5.2 ± 1.3	89.2 ± 7.1	5.2 ± 2.1	7.1 ± 3.2	1.7 ± 1.1	2.9 ± 0.5
<i>TCFsiam:GFP</i>	9.3 ± 1.7	40.3 ± 9.9	78.2 ± 9.3	48.1 ± 9.9	31.2 ± 5.0	17.2 ± 4.3
<i>tcf7:GFP</i>	4.3 ± 2.3	68.1 ± 11.7	81.2 ± 6.8	26.1 ± 7.2	16.1 ± 4.1	30.1 ± 3.2
<i>dlx5/6:GFP</i>	9.2 ± 1.2	39.2 ± 7.1	61.3 ± 5.4	–	73.1 ± 6.1	0
Adult						
<i>TOP:GFP</i>	<1	98.1 ± 1.1	3.1 ± 2.4	<1	<1	1.1 ± 0.7
<i>TCFsiam:GFP</i>	<1	47.3 ± 3.4	66.3 ± 5.3	37.0 ± 4.9	7.1 ± 1.8	5.2 ± 1.5
<i>tcf7:GFP</i>	<1	59.1 ± 4.2	73.1 ± 4.1	–	9.1 ± 1.6	7.3 ± 2.5

Cells were counted from maximum intensity confocal Z projections of at least three individual brains for each set of markers. The entire hypothalamus (32 hpf) or posterior recess of whole (4 dpf) and half (adult) brains, defined by anatomical landmarks, was counted. Error = ±SD. dpf, days postfertilization; hpf, hours postfertilization. See also Figure S2.

neurogenesis in the context of grossly normal morphology. We chose to focus on *lef1* for several reasons. First, zebrafish *tcf7* null mutants are viable and fertile, suggesting a minor required function in development (Nagayoshi et al., 2008). Second, mouse *Lef1* mutants exhibit a smaller dentate gyrus with neurogenesis defects in the hippocampus (Galceran et al., 2000; Zhou et al., 2004), but hypothalamic phenotypes have not been explored. Finally, in zebrafish *lef1* morpholino knock-down produces neurogenesis defects in the hypothalamic GABAergic lineage (Lee et al., 2006).

To explore the later functions of *lef1* in the hypothalamus, we generated a *lef1* mutant using zinc finger nuclease-mediated gene targeting (Meng et al., 2008). Genomic (Figure 2A) and immunohistochemical (not shown) analyses indicated that this allele is a functional null, and we observed similar phenotypes to two other *lef1* null alleles generated by ENU mutagenesis (McGraw et al., 2011; Valdivia et al., 2011). At 15 dpf, *lef1* mutants can be identified by their smaller fins. Although mutant fish exhibit similar body mass and brain size as their wild-type siblings (Figures 2B and 2C), we observed a significant decrease in the size of the posterior hypothalamus. Using confocal volume reconstruction, we found the mutant posterior hypothalamus is 50% of wild-type size (Figures 2C and 2E), suggesting a specific proliferation defect in this tissue.

At 1 month postfertilization (mpf), the *lef1* mutant posterior hypothalamus is only 40% of wild-type size (Figure 2E), and 1 week BrdU tracing showed few proliferating cells in this region, with most of the remaining BrdU⁺ cells located near the midline (Figure 2D). In addition, the HuC/D⁺ neuronal population in the paraventricular posterior recess region is almost completely absent in *lef1* mutants (Figure 2D). Although the Sox3⁺ neural progenitor pool is also smaller, BrdU labeling suggested that the remaining proliferating cells are arrested in the Sox3⁺ state and unable to progress into HuC/D⁺ neurons of either the GABAergic or serotonergic lineages (Figures 2D and 2F).

Although our previous data suggested a more significant role for *lef1* in early hypothalamic development, morpholino-based studies can be subject to off-target effects or targeting of mater-

nally deposited pre-mRNA. Our analysis of zygotic mutants suggests that at postembryonic stages through adulthood, cells in the *lef1* mutant hypothalamus fail to proliferate or differentiate normally and are arrested as Sox3⁺ neural progenitors.

Wnt Signaling Is Required for Hypothalamic Neurogenesis throughout Life

The phenotype of *lef1* mutants suggested a continuous requirement for Wnt signaling in hypothalamic neurogenesis, but the lack of conditional mutagenesis approaches in zebrafish precluded us from fully testing this hypothesis. We therefore used transgenic lines to perform conditional gain- and loss-of-function assays at early embryonic (24–32 hpf), later embryonic (3–4 dpf), and adult (6 mpf) stages. We used three different heat-shock lines to manipulate Wnt/β-catenin signaling: *hs:wnt8a* to activate the pathway (Weidinger et al., 2005), and *hs:dkk1* (Stoick-Cooper et al., 2007) and *hs:axin1* to inhibit the pathway. Neither heat shock alone nor transgene expression caused significant apoptosis, except for a small elevation following *hs:axin1* expression from 3–4 dpf (Figure S4). In addition, activation and inhibition of Wnt signaling caused the predicted effects on the target gene *axin2*, as assayed by in situ hybridization and RT-PCR (Figure S4).

Because our data suggested a role for Wnt signaling in unspecified proliferating progenitors at 32 hpf, we first analyzed the effects on proliferation following heat shock. A 2 hr BrdU pulse was performed at 22 hpf, followed by heat shock at 24 hpf, and fixation at 32 hpf. We found that the number of pH3⁺ cells in the hypothalamic region was significantly increased following Wnt activation and decreased following Wnt inhibition (Figures 3A and S4), suggesting that Wnt signaling regulates overall proliferation levels. Following Wnt activation the hypothalamus was also noticeably larger with a folded ventricle and pH3⁺ cells outside the midline (Figure S4). We found similar effects on the number of BrdU⁺/Sox3⁺ neural progenitors (Figure 3A), suggesting canonical Wnt signaling is also necessary and sufficient for the expansion of this population. In contrast, we did not observe any precocious expression of neuronal

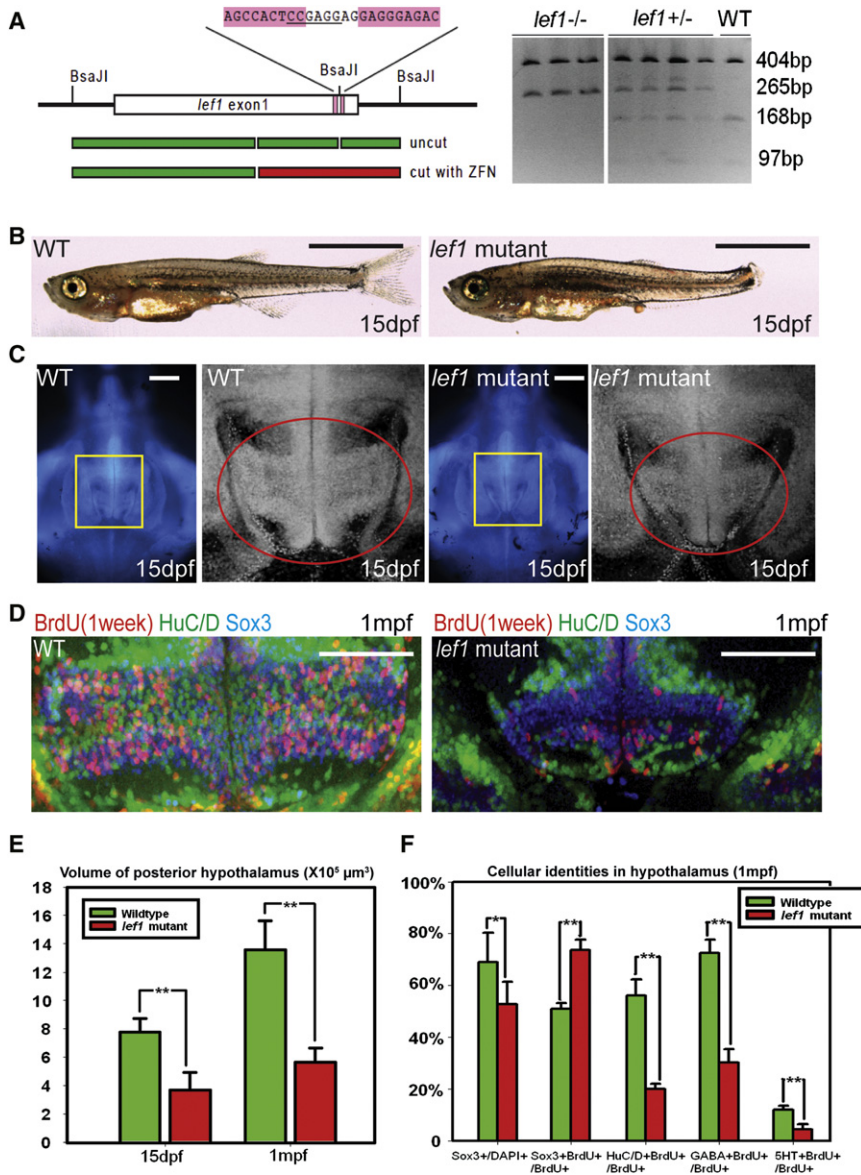


Figure 2. *lef1* Is Required for Proliferation and Neurogenesis in the Postembryonic Hypothalamus

(A) *lef1* ZFN target region and genotyping. (B and C) Whole fish (B) and brain size (C) comparisons of *lef1* mutant to wild-type at 15 dpf. Boxed region in left panel is enlarged on right, posterior recess is circled. (D) Expression of HuC/D, Sox3, and 7-day BrdU labeling in posterior recess of wild-type and *lef1* mutant hypothalamus. (E) Quantification of posterior hypothalamus size. (F) Tracing of proliferating cells. *lef1* mutants have a smaller Sox3⁺ progenitor pool, but a higher percentage of BrdU⁺ cells express Sox3 and fewer produce HuC/D⁺, GABA⁺, or 5HT⁺ neurons. Single optical sections from ventral views are shown in (C) and (D). Scale bars represent 2 mm (B), 200 μm (C), and 100 μm (D). Brain volumes were calculated using Amira software. Cell counts were collected from maximum intensity Z projections through the posterior recess of three individual samples for each genotype and calculated using Velocity software. *p < 0.05, **p < 0.005. Error = ±SD. See also Figure S3.

percentage of BrdU⁺ cells to remain as undifferentiated Sox3⁺ progenitors, and a lower percentage to differentiate into GABAergic and serotonergic progeny (Figures 3C and S4). The proportion of BrdU⁺/Sox3⁺ cells that were still mitotic (PCNA⁺) at 4 dpf was increased following Wnt activation and decreased following Wnt inhibition (Figure 3C), indicating a continuing role for the pathway in maintaining the mitotic state. However, a decrease in overall *dlx5/6:gfp* expression (Figure S4) following Wnt activation, coupled with the lack of an overall increase in BrdU⁺ cells (most of which are Sox3⁺), strongly suggest a specific effect on differentiation. We therefore conclude

differentiation markers such as *Dlx* genes or HuC/D following heat shock (not shown). These data are consistent with existing models of Wnt as a mitogen in progenitor cells (Megason and McMahon, 2002).

Our analysis of Wnt activity at 4 dpf suggested a potential role for the pathway in neuronal differentiation. We therefore analyzed the effects on proliferation and differentiation following heat shock at this stage. A 2 hr BrdU pulse was performed at 70 hpf, followed by multiple heat shocks every 8 hr, and fixation at 96 hpf. In control embryos, we found that the number of BrdU⁺ cells in the posterior recess increased ~3-fold over 24 hr, but we observed no statistically significant changes in overall BrdU labeling following expression of *hs:wnt8a*, *hs:dkk1*, or *hs:axin1* (Figure 3B). We then analyzed the BrdU⁺ population to determine whether Wnt signaling was necessary or sufficient to promote differentiation of neural progenitors. Surprisingly, we found that both activation and inhibition of Wnt signaling caused a higher

that both activation and inhibition of Wnt signaling lead to a significant failure of progenitors to differentiate, independent from any effect on cell cycle exit.

Considering our observation of Wnt activity only prior to differentiation, these data suggested that Wnt signaling is required for progenitors to commit to neurogenesis but must be inhibited for the process to continue. We therefore tested whether progenitors would eventually differentiate following transient Wnt activation. The same experimental paradigm described above was used, except BrdU⁺ cells were followed for two additional days. In this case, we found that the block in differentiation was indeed temporary, as by 6 dpf the number of BrdU⁺ GABAergic precursors had recovered to control levels (Figure 3D).

To test whether the function of Wnt signaling in neurogenesis was conserved at adult stages, we analyzed 6 mpf zebrafish using *hs:wnt8a* and *hs:dkk1*. Because of the slower rate of proliferation at this stage, fish were incubated in BrdU for

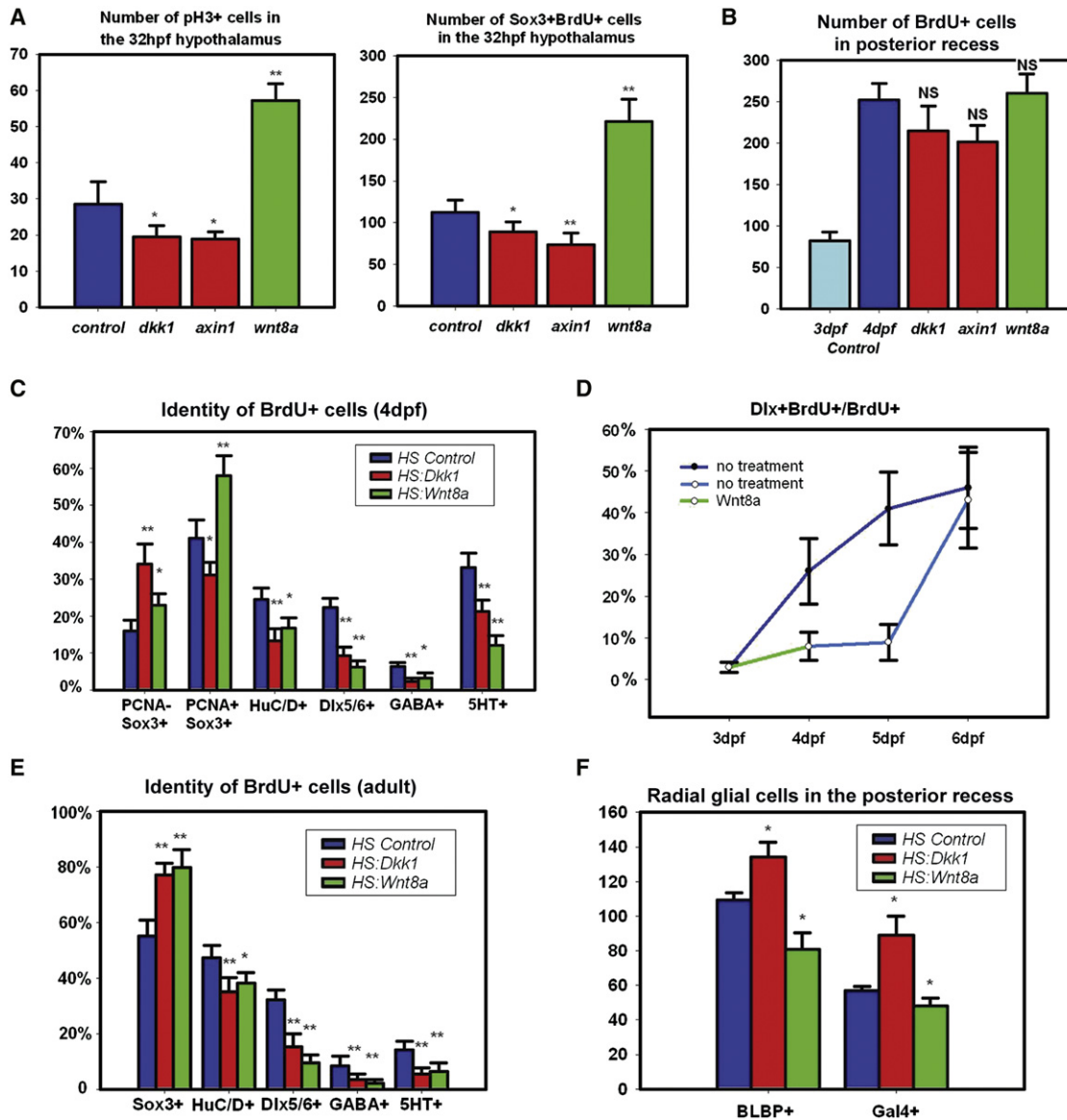


Figure 3. Wnt Signaling Regulates Hypothalamic Progenitor Proliferation and Differentiation

(A) pH3⁺ and BrdU⁺/Sox3⁺ cell numbers in the 32hpf hypothalamus following 2 hr labeling and Wnt pathway inhibition or activation at 24 hpf. (B) BrdU⁺ cell numbers in the 4 dpf posterior recess following 2 hr labeling and Wnt pathway inhibition or activation at 3 dpf. (C) BrdU⁺ cell fates in the 4 dpf hypothalamus following labeling and Wnt pathway inhibition or activation at 3 dpf. (D) Percentage of BrdU⁺ cells expressing *dlx5/6:gfp* following Wnt pathway activation at 3 dpf. (E) BrdU⁺ cell fates in the adult hypothalamus following 2-day labeling and Wnt pathway inhibition or activation for 15 days. (F) Number of Gal4⁺ and BLBP⁺ cells in the 4 dpf posterior recess following Wnt pathway inhibition or activation at 3 dpf. All cell counts were collected from ventral maximum intensity confocal Z projections through five individual brains. The entire hypothalamus was counted at 32 hpf, the entire posterior recess was counted at 4 dpf, and a hemisphere of the posterior recess was counted in adults. * p < 0.05, ** p < 0.005. Error = ±SD. See also Figure S4.

2 days, followed by 15 daily heat-shocks. We then traced the BrdU⁺ cells in the posterior recess region and found that as at 4 dpf, an increased percentage remained as Sox3⁺ neural progenitors, and fewer differentiated into mature neurons following both Wnt activation and inhibition (Figures 3E and S4). These data suggest a constant postembryonic requirement for sequential activation and inhibition of Wnt signaling in the differentiation of hypothalamic neural progenitors.

Wnt Signaling Inhibits Hypothalamic Radial Gliogenesis

Previous reports have implicated negative regulation of Wnt signaling in the maintenance of radial glia (Wang et al., 2011), a cell type that has been identified as a potential neural progenitor in the hypothalamus (Lee et al., 2012; Xu et al., 2005). We therefore asked whether the Wnt pathway was active and functional in this cell population in zebrafish. Although we did not observe any expression of GFAP in the posterior recess (not

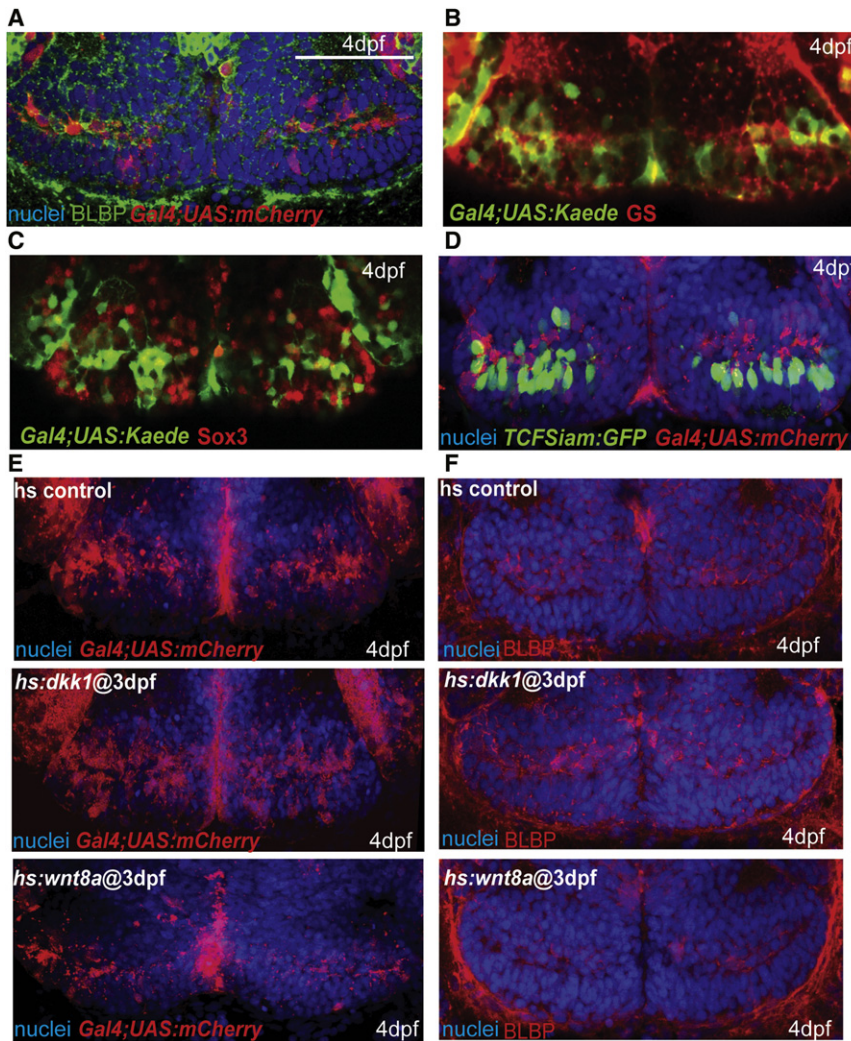


Figure 4. Wnt Signaling Inhibits the Formation of Radial Glia in the Posterior Recess at 4 dpf

(A) Coexpression of mCherry driven by the Gal4 *zc1066a* insertion with the radial glial marker BLBP.

(B) Coexpression of Gal4-driven Kaede with the radial glial marker glutamine synthetase.

(C) Costaining for Sox3 and Gal4-driven Kaede. Most Gal4⁺ cells have low or absent Sox3 expression.

(D) Costaining for *TCFSiam:GFP* and Gal4-driven mCherry. Few Gal4⁺ cells show Wnt reporter activity.

(E and F) Gal4 *zc1066a*-driven mCherry (E) and BLBP (F) expression in the posterior recess of 4 dpf embryos following Wnt pathway inhibition or activation at 3 dpf. Single ventral confocal optical sections are shown in all panels. Scale bar represents 80 μm.

shown), we took advantage of a Gal4 insertion that labels radial glia expressing Brain lipid-binding protein (BLBP) and glutamine synthetase, (GS) (Figures 4A and 4B). The level of Sox3 immunoreactivity in labeled cells was variable (Figure 4C), suggesting that a subset of them might be neural progenitors. However, few Gal4⁺ cells showed evidence of Wnt reporter expression at 4 dpf (Figure 4D).

To test the role of Wnt signaling in radial glial development, we performed functional assays using *hs:dkk1* and *hs:wnt8a*. We heat shocked 3 dpf embryos every 8 hr and fixed at 4 dpf. We observed a higher number of Gal4⁺ and BLBP⁺ cells following Wnt inhibition and a lower number following Wnt activation (Figures 3F, 4E, and 4F), suggesting that Wnt activity is not required for the formation of radial glia, and ectopic Wnt activity results in a loss of this cell type.

Wnt Activity Is Present in the Adult Mouse Hypothalamus

In contrast to zebrafish, which have widespread proliferation throughout the adult CNS (Chapouton et al., 2007), adult neurogenesis is much more limited in mammals. However, prolifer-

ating progenitors have been identified in the rodent hypothalamus, where adult neurogenesis can influence feeding behavior (Kokoeva et al., 2005; Lee et al., 2012; Pierce and Xu, 2010). To determine whether the presence of Wnt signaling is conserved in the adult mouse hypothalamus, we used the reporter *BAT-LacZ* (Maretto et al., 2003) to identify Wnt-responsive cells. From sagittal sections, we observed abundant β-gal⁺ cells in the adult cerebral cortex, hippocampus, olfactory epithelium, and cerebellum, as well as the posterior hypothalamus (Figure 5A). From coronal sections through the hypothalamus, we determined that the paraventricular and

arcuate nuclei have the highest density of Wnt-responsive cells in this region (Figure 5B).

To determine the identity of hypothalamic Wnt-responsive cells we performed double immunohistochemistry for β-galactosidase and markers of either progenitors or differentiated cell types. We found that β-gal⁺ cells exist in the ventricular and parenchymal zones of the mediobasal hypothalamus (Figure 5C). In the ventricular zone, β-gal⁺ cells were primarily Sox2⁺ ependymal cells and Sox2⁺, Sox3⁺, and GFAP⁺ subependymal cells (Figures 5C–5E). Although the majority of β-gal⁺ cells in the parenchymal zone were HuC/D⁺ and NeuN⁺ (Figures 5C–5E), some expressed Doublecortin, a marker of differentiating neurons, and many expressed the GABAergic precursor marker *Dlx2* (Figures 5C–5E). Together, these results suggest that neural progenitors in the adult mammalian hypothalamus could be Wnt-responsive, as in zebrafish.

β-Catenin Negatively Regulates the Production of Ventricular Hypothalamic Tanycytes in the Mouse

To determine the function of Wnt signaling in adult mouse hypothalamic progenitors, we took a conditional genetic

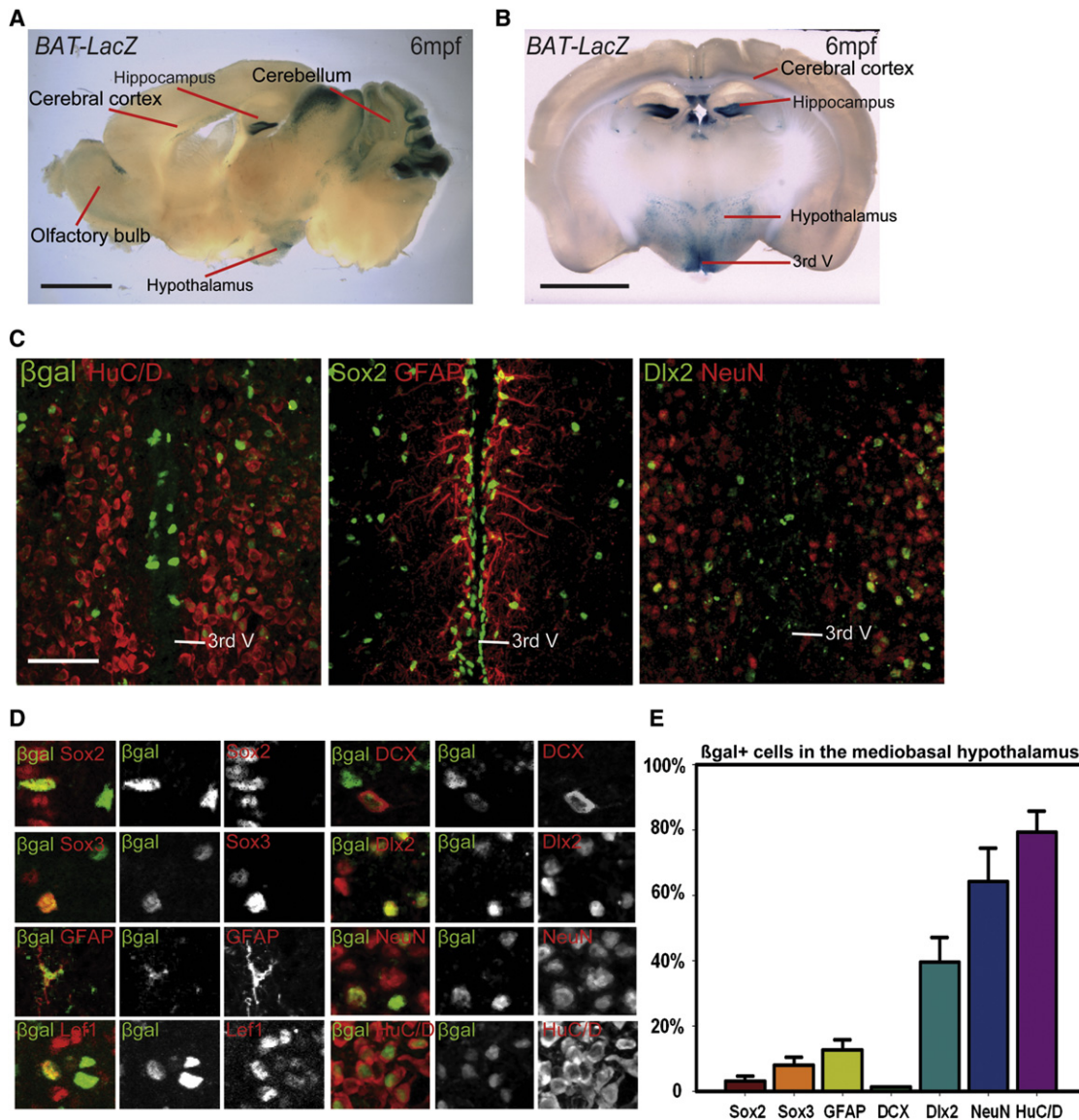


Figure 5. The Adult Mouse Hypothalamus Has a Wnt-Responsive Cell Population

(A and B) Sagittal (A) and coronal (B) sections of adult *Bat-LacZ* mouse brains.

(C) β -gal⁺ cells are distributed in both the ventricular zone (where Sox2⁺ and GFAP⁺ cells reside) and the parenchymal zone (where Sox2, HuC/D⁺, NeuN⁺, and Dlx2⁺ cells reside). Coronal 40 μ m cryosections are shown.

(D) Colocalization of β -gal with specific markers in the hypothalamus.

(E) Percentage of marker coexpression within the β -gal⁺ population. Single confocal optical sections are shown in (C) and (D). Scale bars represent 2 mm (A and B), and 80 μ m (C). Cell counts were collected from the mediobasal hypothalamus, using six 40 μ m cryosections each from three mice. Error = \pm SD.

approach to remove and hyperactivate β -catenin. Because the expression of Notch pathway effectors has been observed in both the adult zebrafish and mouse hypothalamic ventricular zones (Chapouton et al., 2011; Lee et al., 2012), we chose to trace and manipulate cells expressing the Notch effector gene *Hes1*. We observed that knock-in mice expressing tamoxifen (TM)-dependent CreERT2 recombinase from the *Hes1* locus (*Hes1*^{C2}) (Kopinke et al., 2011) drive efficient and inducible recombination in hypothalamic ventricular progenitors (Figures 6A and 6B). Lineage tracing experiments with *R26R*^{LacZ}

reporter mice (Soriano, 1999) showed that *Hes1*⁺ cells are present in the ventricular zone of the adult hypothalamus 5 days after tamoxifen administration (Figure 6A), followed by expansion into the parenchymal zone 9 months posttamoxifen (Figure 6B).

To further characterize the *Hes1*-expressing population, we utilized the *R26R*^{EYFP} reporter (Srinivas et al., 2001) in mice that received tamoxifen at P60. The majority of EYFP⁺ cells after a 5-day chase were located at the ventricular surface, having a cellular morphology without radial processes

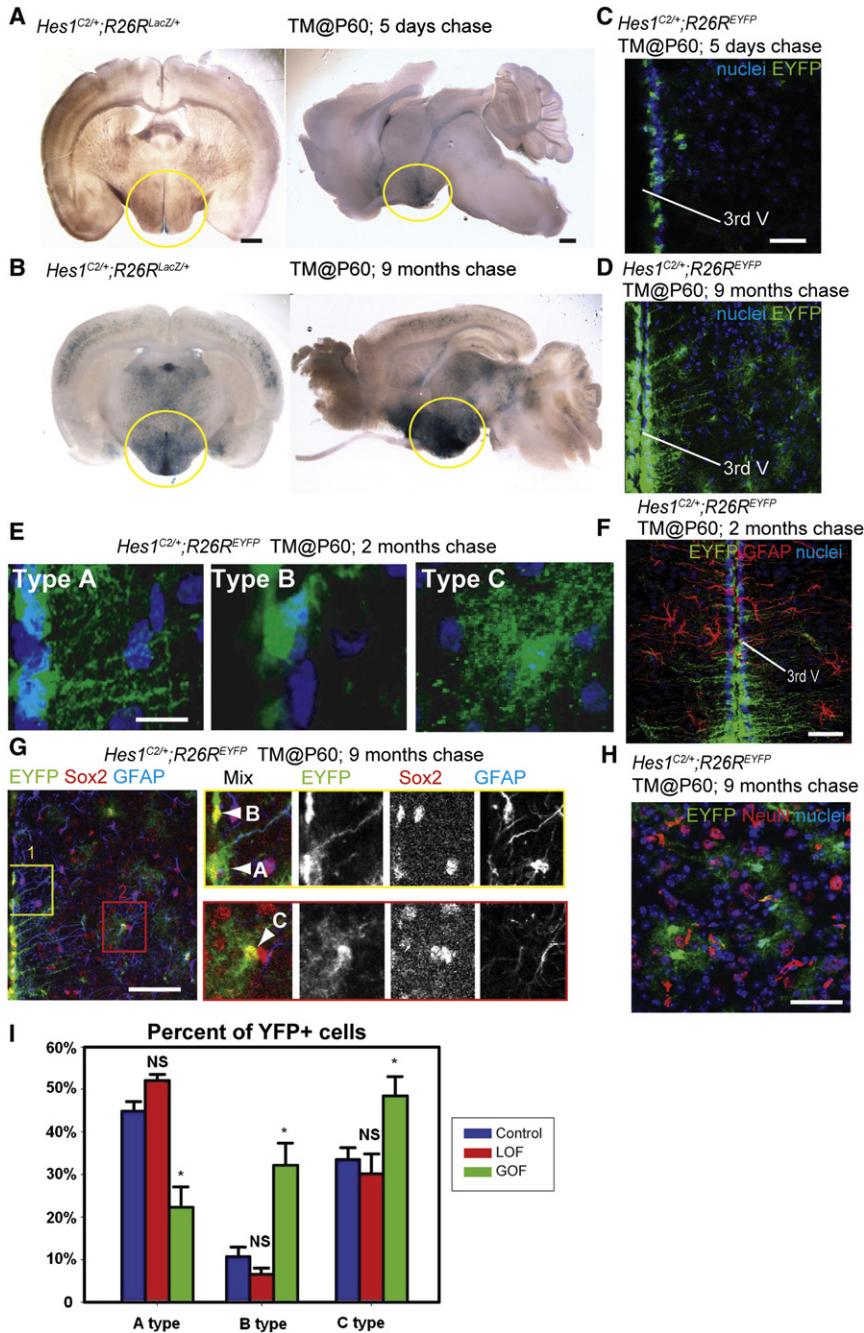


Figure 6. Wnt Signaling Inhibits the Production of Tanycytes from Adult *Hes1*⁺ Progenitors

(A and B) Coronal and sagittal sections of adult *Hes1^{C2/+}; R26R^{LacZ/+}* mouse brains, 5 days and 9 months after TM administration at P60.

(C and D) Coronal cryosections through the hypothalamus of adult *Hes1^{C2/+}; R26R^{EYFP/+}* mouse brains, 5 days and 9 months after TM administration at P60.

(E) The three types of EYFP⁺ cells observed at 2 months post-TM.

(F) Expression of EYFP and GFAP in *Hes1^{C2/+}; R26R^{EYFP/+}* mice 2 months post-TM.

(G and H) Marker analysis of *Hes1^{C2/+}; R26R^{EYFP/+}* mice 9 months post-TM. Boxes in (G) show enlarged ventricular (yellow) and parenchymal (red) zones in which cell types are indicated by arrowheads. All EYFP⁺ cells are Sox2⁺, and some ventricular Type A cells are also GFAP⁺.

(H) Expression of EYFP and NeuN⁺ in the parenchymal zone, where no colabeled cells are observed.

(I) Percentage of Type A, B, and C EYFP⁺ cells 2 months following β -catenin inactivation or activation. Single confocal optical sections are shown in (C–H). Scale bars represent 1 mm (A and B), 80 μ m (C, D, F, G, and H), and 10 μ m (E). Cell counts were collected from the mediobasal hypothalamus, using six 40 μ m sections from three mice for each genotype. **p* < 0.05. Error = \pm SD.

(Figure 6C). After a 2-month or 9-month chase, EYFP⁺ cells comprised three classes. The most predominant class (“Type A”) was located at the ventricular surface and had long radial processes (Figures 6D and 6E). All of these cells were Sox2⁺ (Figure 6G) and BLBP⁺ (not shown), but only the most dorsal were GFAP⁺ (Figures 6F and 6G). This combination of morphology and marker expression closely matches a profile previously described for radial glial tanycytes (Lee et al., 2012; Pérez-Martín et al., 2010b; Rodríguez et al., 2005). A second class (“Type B”) was similar to the ventricular cells observed after a 5-day chase, lacking radial processes (Figures 6E and

6G). These cells were all Sox2⁺ (Figure 6G), BLBP⁺ (not shown), and GFAP[−] (Figure 6G). A third class (“Type C”) was located in the parenchymal zone, and was Sox2⁺ with stellate morphology (Figures 6E and 6G). Based on these characteristics, we conclude that Type C cells are most likely astrocytes. Notably, we never observed any Sox10 (not shown) HuC/D (not shown) or NeuN (Figure 6H) expression in Type C cells, or in the entire *Hes1* lineage. Together, these data indicate that *Hes1*⁺ progenitors do not produce neurons in the adult mouse hypothalamus, but do produce multiple classes of glia.

To investigate the role of Wnt/ β -catenin signaling in the differentiation of *Hes1*⁺ progenitors, we crossed the *Hes1^{C2}* line with *Catnb^{lox(ex2-6)}* (Brault et al., 2001) and *Catnb^{lox(ex3)}* (Harada et al., 1999) mice allowing for β -catenin loss- and gain-of-function experiments, respectively. In addition, all mice carried one copy of the *R26R^{EYFP}* reporter allele, enabling us to determine the fate of recombined *Hes1*⁺ cells. All mice were genotyped and treated with 2 mg TM at P60, and EYFP-expressing cells in the mediobasal hypothalamus were analyzed 2 months later. This dose of TM was used to avoid lethal effects of recombination in the intestinal *Hes1*⁺ lineage (Kopinke et al., 2011).

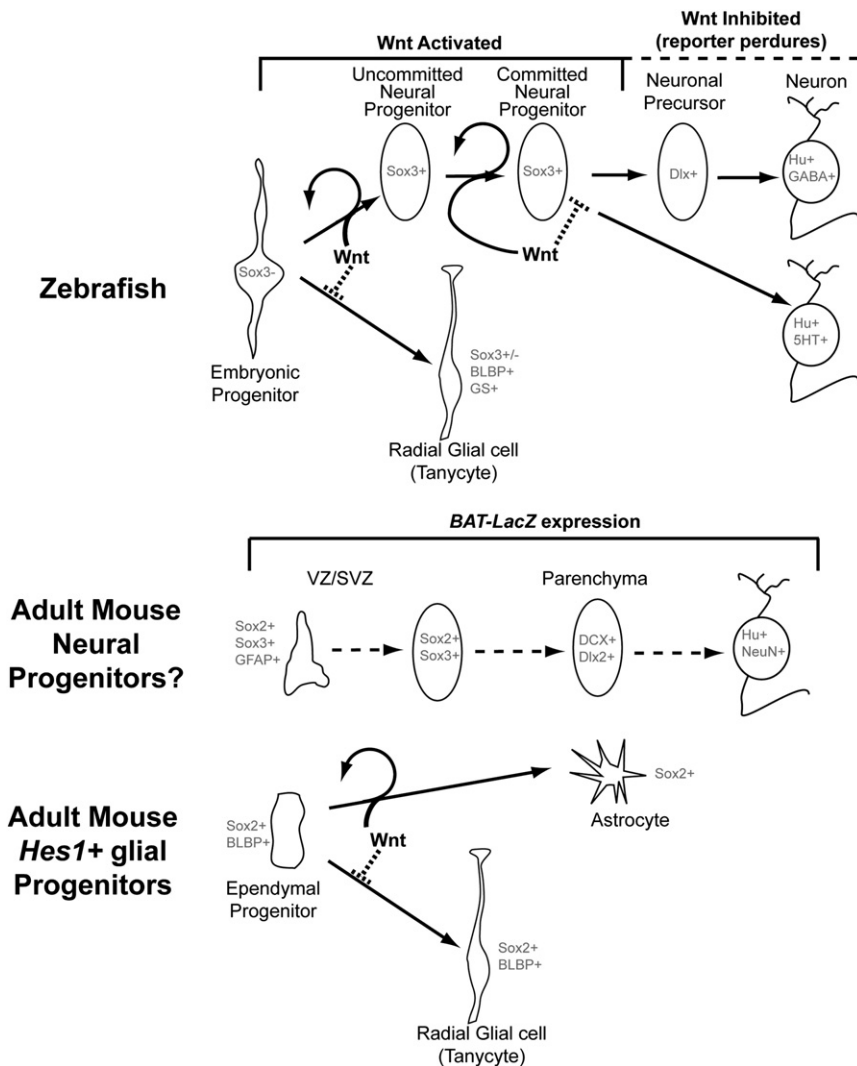


Figure 7. Model for the Role of Wnt Signaling in Hypothalamic Progenitor Differentiation

In zebrafish (top), Wnt signaling is active in unspecified embryonic progenitors and a neural progenitor, but is lost as these cells undergo neurogenesis. Wnt signaling promotes mitotic activity in progenitors, and is also required for their ability to undergo neuronal differentiation, which underlies the transition from an “uncommitted” to a “committed” progenitor state. Finally, Wnt signaling must be inhibited for differentiation to proceed. In contrast the formation of radial glia is inhibited by Wnt activity. In the adult mouse (bottom), Wnt signaling is active in ventricular/subventricular zone cells (VZ/SVZ) that express neural progenitor markers, and in parenchymal zone cells that express neuronal and neuronal precursor markers. Ventricular *Hes1*⁺ progenitors do not require Wnt activity to make radial glial tanycytes, and ectopic pathway activity inhibits radial glial formation at the expense of other fates.

DISCUSSION

Wnt Signaling Regulates Hypothalamic Progenitor Differentiation in Zebrafish and Mouse

In this study, we have shown that both the postembryonic zebrafish and adult mouse hypothalamus contain Wnt-responsive cells (Figure 7). In zebrafish, hypothalamic Wnt-responsive cells are unspecified progenitors at early embryonic stages and Sox3⁺ neural progenitors at postembryonic stages, contributing to GABAergic and serotonergic neuronal lineages. In the adult mouse, both ventricular and parenchymal cells expressing

neural progenitor and GABAergic precursor markers also exhibit Wnt activity. In addition, we have identified radial glial tanycyte lineages in both species, marked by a Gal4 transgene in zebrafish and by *Hes1* in mouse. Our functional analyses demonstrate that Wnt/ β -catenin signaling regulates progenitor proliferation in the early zebrafish embryo, but primarily regulates neuronal differentiation at postembryonic stages. In contrast, in both zebrafish and mouse Wnt signaling is not required for the formation of radial glia, and ectopic Wnt activity disrupts the formation of these cells. Together, this work suggests a conserved role for Wnt signaling in postembryonic hypothalamic progenitor differentiation in vertebrates.

Following both gain and loss of β -catenin function, we observed numerous EYFP⁺ cells in the ventricular and parenchymal zones, as in controls. Analysis of HuC/D and NeuN expression indicated that no EYFP⁺ cells of any genotype were neurons (data not shown). Because we used a low TM dose, recombination was mosaic and each animal had a variable number of EYFP⁺ cells, thus we were unable to statistically analyze the size of the lineage. Despite this, we did not observe a qualitative difference in number of EYFP⁺ cells between different genotypes, suggesting there was no major effect on proliferation during the 2-month period. When we analyzed the proportion of Type A, B, and C EYFP⁺ cells, we found that β -catenin activity was not required for radial glial differentiation (Figure 6). In contrast, β -catenin activation led to a significant increase in the proportion of radial glia, with a corresponding increase in the proportion of Type B ventricular cells without processes, and Type C astrocyte-like parenchymal cells (Figure 6). These results suggest that Wnt/ β -catenin activity negatively regulates the production of mouse ventricular hypothalamic tanycytes, consistent with our findings in zebrafish.

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Sequential Activation and Inhibition of Wnt Signaling Is Required for GABAergic Neurogenesis

Surprisingly, we found that both activation and inhibition of Wnt signaling led to arrest in the Sox3⁺ progenitor state, and failure of further differentiation. Our data support a model proposed previously in retinal development (Agathocleous et al., 2009), in which Wnt signaling must be active in order for

uncommitted progenitors to acquire the competence to differentiate (Figure 7), but must then be inhibited for cells to complete the differentiation process. Consistent with this model, we find that Wnt signaling is active in postmitotic progenitors before they express *Dlx* genes, but is absent as they differentiate (Figure 7). A recent study examining constitutive activation of Wnt signaling in adult neural stem cells also supports this model (Imura et al., 2010). In our study, BrdU tracing only allowed us to follow proliferating cells, leaving open the possibility that once committed progenitors have already received the necessary Wnt signal, pathway inhibition will promote their differentiation.

Consistent with other work suggesting that Wnts generally act as mitogens (Megason and McMahon, 2002), we found that Wnt activity is associated with progenitor proliferation at embryonic and postembryonic stages. However, we also showed a clear role for Wnt activity in postmitotic neural progenitors, as *Sox3+PCNA-* cells still fail to differentiate following Wnt pathway inhibition (Figure 3C). Even following Wnt pathway activation, our data support a specific effect on neuronal differentiation in addition to any proliferative expansion (Figure S4). Within a single cell population, the function of Wnt signaling may thus change over time in a context-dependent manner. Our data suggest that although Wnt activation promotes the mitotic expansion of progenitors, it also acts to hold cells in the progenitor state once they have become specified. In different cell populations, the specified state may or may not be proliferative, leading to seemingly conflicting phenotypes.

Hes1⁺ Ventricular Zone Progenitors Do Not Produce Neurons in the Adult Mouse Hypothalamus

Ventricular zones have been identified as the primary adult neural stem cell niches in the vertebrate brain (García-Verdugo et al., 2002). Using *Hes1^{C2}* lineage tracing, we observed a significant number of cells expanding outside the ventricular zone, but these cells did not become neurons. Adult hypothalamic neurogenesis in mammals has only been characterized relatively recently (Yuan and Arias-Carrión, 2011), partly because very few neurons are produced at any time. Consistent with the idea that the mammalian hypothalamus contains a functionally significant level of adult neurogenesis during normal homeostasis (Lee et al., 2012; Yuan and Arias-Carrión, 2011), we observed numerous *Dlx2⁺* cells in the parenchymal zone. Although recent work has identified ventricular *Nestin⁺* tanycytes of the hypothalamic medial eminence as neural progenitors in the postnatal mouse (Lee et al., 2012), it is not clear whether they contribute to homeostatic adult neurogenesis as the lineage was only analyzed until P75. Furthermore, this study described additional neurogenesis that did not arise from *Nestin⁺* cells. It is possible, therefore, that a progenitor niche separate from the ventricular zone contributes to neurogenesis, or that *Nestin⁻/Hes1⁻* neural progenitors exist in the ventricular zone and subsequently migrate to the parenchyma (Migaud et al., 2010). To test the specific role of Wnt signaling in adult mouse hypothalamic neurogenesis, it will be necessary to employ conditional approaches using markers identified by our *BAT-LacZ* analysis, such as *Sox2*, *GFAP*, and *Dlx2*, in addition to *Nestin*.

The Role of Wnt Signaling in Hypothalamic Radial Glia

Our data show that tanycyte formation and maintenance does not require Wnt signaling. In contrast, we find that Wnt pathway activation leads to decreased tanycyte numbers at the expense of neurons in zebrafish, and other glial cell types in mouse. Other work has revealed a similar role for β -catenin in Bergmann glia, which are transformed into astrocyte-like cells following *Apc* mutation (Wang et al., 2011). Interestingly, zebrafish do not contain true astrocytes but instead have radial astroglial-like cells throughout their brain (Grupp et al., 2010). Combined with the known neurogenic capacity of radial glia in the adult zebrafish brain (Kroehne et al., 2011), this raises the interesting possibility that the alternative fates promoted by Wnt signaling differ between zebrafish and mouse due to the different developmental potential of glial progenitors.

In summary, this work resolves some of the seemingly conflicting conclusions regarding the role of Wnt signaling in neurogenesis, from studies in different systems using different manipulations. We believe that many of these results can be explained by a dual role for the pathway, first in expansion of unspecified progenitors, then later as a required inducer of neuronal differentiation that must subsequently be inhibited for neurogenesis to occur. Furthermore, we have established the vertebrate hypothalamus as a model system for Wnt function in postembryonic progenitor differentiation, and opened the field to future studies examining the role of this process in animal behavior.

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Zebrafish

Zebrafish embryos were obtained from natural spawning of wild-type (AB^{*}), transgenic, and mutant adult fish. A detailed description of the strains used can be found in the Supplemental Experimental Procedures. Adult brains were dissected from anesthetized fish fixed in 4% paraformaldehyde for 2 days. Fish received BrdU pulses by immersion in E3 media with 10 mM BrdU, and received heat shock by immersion in prewarmed E3 media.

Mice

A detailed listing of mouse strains used can be found in the Supplemental Experimental Procedures. Tamoxifen (Sigma T-5648) was dissolved in corn oil and administered by oral gavage at doses of 10 mg (*Hes1* lineage) or 2 mg (β -catenin loss and gain of function experiments) per mouse between 6–8 weeks of age.

X-gal Staining, In Situ Hybridization, and Immunohistochemistry

For X-gal staining in mice, fixed whole brains were sliced at 500 μ m thickness, and staining was performed as described previously (Seymour et al., 2004) followed by clearing in 100% glycerol. Mouse immunostaining and analysis were performed on 40 μ m cryosections as described previously (Kopinke et al., 2011). Zebrafish in situ hybridization and immunohistochemistry were performed as described previously (Oxtoby and Jowett, 1993), except that whole mount brains were first dissected (4 dpf and adult) and sliced longitudinally (adult), followed by collagenase treatment. All mRNA probes have been described previously (Lee et al., 2006; Wang et al., 2009). A detailed list of antibodies used can be found in the Supplemental Experimental Procedures.

Cryosectioning and Microscopy

Cryosections were cut at a thickness of 12 μ m for embryonic zebrafish and 40 μ m for adult mouse brains. All analysis of zebrafish brains was performed on whole-mount samples, except for Lef1 immunohistochemistry. Fluorescent

images of whole mount zebrafish brains were taken using an Olympus FV1000 confocal microscope and an Olympus SZX12 fluorescent dissecting microscope. Maximum intensity Z projections were used for all quantitative analyses. Bright field images were obtained using an Olympus BX51WI compound microscope.

Data Analysis

Colocalization analysis, volume measurements, statistical calculations, and graphs were generated with Image J, Volocity5.4, Amira 5.3.3, Microsoft Excel, and Sigma Plot 10.0. All analyses in zebrafish were performed on whole-mount brains. For analysis at 32 hpf, the entire hypothalamus was counted using the third ventricle and nuclear staining as landmarks. For analysis at 4 dpf–1 mpf, a rectangle defining the entire posterior recess was counted, using the ventricle and nuclear staining as landmarks. For analysis of adults, a rectangle defining the posterior recess in a midsagittal view was counted, using the ventricle and nuclear staining as landmarks. All analyses in mouse were performed on cryosections using morphological landmarks to define the mediobasal hypothalamus. Error bars represent SDs from at least three samples (N is listed in each figure legend), and comparisons of data sets were performed with one-tailed homoscedastic or heteroscedastic t tests.

Quantitative RT-PCR

Total RNA was isolated using an RNeasy extraction kit (QIAGEN) followed by DNase treatment. cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen). mRNA levels were normalized to an average of *beta-actin*. Quantitative realtime PCR using the Sybr Green reagent was performed with an ABI7900, using primers listed below:

axin2: F: TGAAGCGGGAACAGGAAAC; R: AGGAGCAAAGGCAGAGAA,
beta-actin: F: AGGATGCGGAACTGGAAAG; R: GAGGAGGGCAAAGTG
GTAAA.

Isolation of *lef1* Mutants using Engineered Zinc-Finger Nuclease

To introduce mutations in the *lef1* gene, a target site was chosen within the exon 1 using ZiFIT (<http://zifit.partners.org/ZiFIT/>). ZFNs were prepared using a detailed protocol posted elsewhere (<http://wiki.zfin.org/>), in which three-finger array libraries were constructed using OPEN pools (Maeder et al., 2009), but modified to be selectable in bacterial one-hybrid (B1H) system (Maeder et al., 2008). Selected three-finger array sequences were converted to ZFNs by fusion with the FokI nuclease domain. Synthetic mRNAs encoding the ZFNs were injected into one-cell stage zebrafish embryos. Mutations were identified by loss of BsaJI restriction sequence located in the target site. Genomic DNA was extracted from the individual 24 hpf embryos and amplified with the following primers: F: TTGGAGGTGTGCTACTCACG; R: CACTCTCTCCAGCCCAACAT. To isolate germ-line transmitted *lef1* mutations, ZFN-injected embryos were raised to adulthood and progeny were analyzed using PCR and BsaJI digestion.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2012.07.012>.

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