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Analysis of neocortical neurogenesis and oligodendrogenesis regulation by the Wnt-beta-catenin pathway

by

Roeben N. Munji

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Developmental Biology

in the

[redacted]

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by

Roeben N. Munji

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stress-filled times throughout my undergraduate and graduate years. I must also thank all the friends I've made during my undergraduate and graduate years. All of you have, in some way, contributed to my success but especially my sanity (LOL). Thank you all for helping me balance work and play.

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Analysis of neocortical neurogenesis and oligodendrogenesis regulation by the Wnt-β-catenin pathway

Roeben N. Munji

Abstract

To understand how the complex architecture of the brain is formed during development, it is essential to identify the signals that regulate the behaviors of neural progenitor cells. To date, many of the signals that regulate cell proliferation and differentiation of neuronal and glial progenitors of the neocortex are unknown. For my primary thesis work, I investigated the role of the Wnt-β-catenin intercellular signaling pathway in the behaviors of neural progenitors of the mouse neocortex. In this study, we show that the Wnt-β-catenin pathway (WBP) regulates the differentiation of intermediate progenitors (IPs; also known as basal progenitors) into neurons. Upregulation of the WBP by overexpression of Wnt3a secreted agonist in the neocortex induced early differentiation of IPs into neurons and the accumulation of these newly born neurons at the border of the subventricular zone and intermediate zone. Long-term overexpression of Wnt3a led to cortical dysplasia associated with the formation of large neuronal heterotopias. Conversely, downregulation of the WBP with Dkk1, a secreted Wnt antagonist, during mid and late stages of neurogenesis inhibited neuronal production. Lastly, consistent with previous reports, we also show that the WBP also promotes radial glia (RG) expansion. Thus, our findings show differential effects of the WBP on distinct groups of cortical neuronal progenitors: RG expansion and IP differentiation. Moreover, these findings suggest that dysregulation of the WBP can lead to developmental defects similar to human cortical malformation disorders. In a collaborative project, I contributed

to address the role of the WBP in oligodendrogenesis. The WBP has previously been shown to inhibit the maturation of oligodendrocyte precursor cells (OPCs). Here, we show that the WBP also inhibits the production of OPCs using both *in vitro* and *in vivo* approaches. Our in vivo results show that downregulation of the WBP with a dominant negative form of LEF1 transcription factor or Dkk1 promoted early production of OPCs in the neocortex. Together with previous work, these findings identify multiple roles for the WBP in both neurogenesis and oligodendrogenesis.

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Wnts influence the timing and efficiency of OPC generation in the telencephalon

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Analysis of neocortical neurogenesis and oligodendrogenesis regulation by the Wnt-β-catenin pathway

Chapter 1: Overview

The formation of the mammalian neocortex is a tightly regulated process that requires the coordination of progenitor cell proliferation and differentiation. Defects in signaling pathways that result in aberrant progenitor cell proliferation (or differentiation) often have detrimental effects, causing malformations in the neocortical architecture that lead to impairment of function. Thus, it is necessary to identify the molecular signals that govern the behaviors of progenitor cells. The Wnt-β-catenin intercellular signaling pathway has been implicated in the regulation of neocortical progenitor behaviors (Woodhead et al., 2006; Zhou et al., 2006; Freese et al., 2010). Its role in promoting neurogenic progenitor proliferation has been established (Chenn and Walsh, 2002; Machon et al., 2007; Wrobel et al., 2007). However, in vitro-based studies suggests that the Wnt-β-catenin pathway (WBP) might also function to promote differentiation of neurogenic progenitors (Hirabayashi et al., 2004; Israsena et al., 2004; Kuwahara et al., 2010). In chapters 2-5 of this dissertation, I present my primary thesis project addressing the possibility that the WBP regulates both cell proliferation and differentiation in neocortical neurogenesis.

The WBP has also been implicated in the regulation of oligodendrogenesis both by inhibiting production of oligodendrocyte precursor cells (OPCs) and differentiation of OPCs into oligodendrocytes (Shimizu et al., 2005; Kessaris et al., 2008; Fancy et al., 2009; Ye et al., 2009). To further dissect the functions of the WBP in neocortical development, we tested whether the WBP inhibits the onset of production of neocortex-derived OPCs. A summary of this work and the manuscript are presented in Chapter 6 of this dissertation.

The ability of one signaling pathway to regulate different cellular processes in different cell types is a common theme in biology. Our findings further provide examples of how the WBP has been adapted to control multiple steps in the organization of the

neocortex. We show that the WBP regulates neurogenesis by regulating both the proliferative expansion of radial glia (RG) and differentiation of intermediate progenitors (IPs). We also provide further evidence that the WBP specifically inhibits the timing of production of neocortex-derived OPCs. Thus, the WBP regulates neocortical progenitor cells to influence both the generation of neurons and oligodendrocytes.

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Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors

Chapter 2: Introduction

Neocortical neurogenesis

The neocortex begins as a single layer of neuroepithelial progenitor cells. Over the course of neurogenesis, the size and complexity of the neocortex dramatically changes (Fig. 2.1A) (Dehay and Kennedy, 2007). At E10.5 the single layered neocortex consists of the ventricular zone (VZ) where radial glia (RG) cells, the primary neurogenic progenitors of the neocortex, reside. During early neurogenesis, RG cells undergo symmetric divisions that give rise to two daughter RG cells (yellow layer, Fig. 2.2A). These self-renewing symmetric divisions expand and establish the RG population but also, due to the epithelial nature of RG cells, elongate the neocortical epithelium (Fig. 2.1B). RG cells also undergo asymmetric divisions that either generate one RG and one neuron or one RG and one intermediate progenitors (IPs) (Fig. 2.2B-C). IPs comprise the secondary progenitor pool of the neocortex that form the subventricular zone (SVZ) (blue layer, Fig. 2.1A). IPs contribute to neuron production through symmetric terminal divisions that produce two daughter neurons (Fig. 2.2E). The thickening of the neocortex results from the production of projection neurons by both RG and IPs (Fig. 2.1C) (Dehay and Kennedy, 2007; Molyneaux et al., 2007; Noctor et al., 2007, 2008).

Newly born projection neurons migrate in the basal direction to the cortical plate where they form the molecularly defined layers termed Layer VI, V, IV and II/III (Fig. 2.1A and C). These layers are formed sequentially in an inside-out (apical-basal) manner with Layer VI composing neurons with the oldest birthdates and Layer II/III composing neurons with the youngest birthdates. In this study, we use molecular markers that specifically identify Layer VI and V (green layers, Fig. 2.1A) as one group and Layer IV-II (red layers, Fig. 2.1A) as another group. Due to relative birth order and layer location, Layer VI-V is termed early-born/deep layer neurons while Layer IV-II is termed late-born/upper layer neurons (Molyneaux et al. 2007).

In mid neurogenesis the dynamics of RG and IP behaviors change, leading to the decline of the RG pool and the rapid expansion of the IP pool. This event coincides with decreased numbers of RG symmetric self-renewing divisions and increased numbers of RG IP-producing divisions. IPs also undergo symmetric self-renewing divisions that generate two daughter IPs that contribute to the expansion of the IP pool (Fig. 2.2D). (Dehay and Kennedy, 2007; Noctor et al., 2007, 2008).

The dynamic changes in RG and IP numbers have layer specific consequences. Since the RG pool is largest in early neurogenesis and smallest in late neurogenesis, RG cells generate the majority of early-born/deep layer neurons. Conversely, since the IP pool is smallest in early neurogenesis and largest in late neurogenesis, IPs generate the majority of late-born/upper layer neurons (Molyneaux et al., 2007; Noctor et al., 2007, 2008).

The Wnt-β-catenin pathway (WBP)

The WBP has been implicated in the regulation of neocortical neurogenesis (Chenn and Walsh, 2002; Machon et al., 2007; Wrobel et al., 2007). The WBP is an intercellular signaling pathway that regulates many developmental processes including cell proliferation, specification, differentiation and migration. The pathway is initiated in receiving cells by binding of secreted Wnt ligands with Frizzled-LRP5/6 receptor complexes at the plasma membrane (Fig. 2.3A). This ligand-receptor interaction promotes stabilization of β-catenin levels and translocation of β-catenin into the nucleus where it acts as a transcription factor. β-catenin binds to LEF/Tcf transcription factors and activate transcription of genes with LEF/Tcf promoter elements (Fig. 2.3A). In the absence of Wnt ligand, β-catenin levels are kept to a minimum through continued proteasome degradation, blocking transduction of the pathway (Fig. 2.3B) (Logan and Nusse, 2004; Clevers, 2006).

The WBP promotes expansion of RG *in vivo*

The Wnt-β-catenin pathway is believed to be a key regulator of RG behavior. Loss-of-function studies in LRP6 and β-catenin decrease cortical neuron production (Woodhead et al., 2006; Zhou et al., 2006). This phenotype is supported by gain-of-function studies that suggest that the pathway promotes self-renewal of neuroepithelial progenitors during early neurogenesis (Chenn and Walsh, 2002; Machon et al., 2007; Wrobel et al., 2007). For instance, expression of a dominant active form of β-catenin promotes RG expansion and the elongation of the neocortical epithelium (Wrobel et al., 2007). These phenotypes are consistent with the induction of RG symmetric self-renewing cell divisions. Moreover, this experiment also resulted in the inhibition of neuron and IP production, suggesting that induction of the WBP inhibits RG asymmetric divisions (Wrobel et al., 2007).

The WBP promotes differentiation of neurogenic progenitors *in vitro*

Other researchers have suggested a role for the WBP in promoting differentiation of neurogenic progenitors. Induction of the WBP in primary cultures of neocortical neural progenitors (termed primary neural precursor cells) promotes increased neuronal differentiation. Furthermore, these studies show that the promoters of Ngn1 and Ngn2, transcription factors that specify neuronal lineages, contain LEF/Tcf elements that can be induced with β-catenin in vitro (Hirabayashi et al., 2004; Israsena et al., 2004). Thus, these studies bring to question whether the WBP regulates differentiation of neocortical neurogenic progenitors *in vivo*.

A possible role in intermediate progenitor regulation

Some studies suggest a role for the WBP in the production of IPs. Expression of dominant active β-catenin in RG cells promoted the cells expressing ectopic β-catenin to localize more in the SVZ and express an IP molecular marker (Kuwahara et al., 2010). A

second study had similar findings, also showing increased SVZ localization of β-catenin-transfected RG cells (Hirabayashi et al., 2004). These findings contradict the studies that show a specific role for the WBP in promoting RG expansion and inhibiting IP production (Chenn and Walsh, 2002; Machon et al., 2007; Wrobel et al., 2007). The difference in these results might be due to the time point of manipulation. These studies manipulated RG cells at E12.5 and E13.5 (time points near the transition between early and mid neurogenesis) while the studies that argue for a role in RG expansion manipulated RG cells at E8.5 (pre neurogenesis) and E11 (early neurogenesis). Finally, a third study suggests that Wnt7a promotes maintenance of IP fate. They found that expression of Wnt7a in RG cells promoted cells expressing ectopic Wnt7a to arrest in the SVZ while control cells continued to migrate to the cortical plate (Viti et al., 2003).

Summary

Previous studies implicate multiple roles for the WBP in neocortical neurogenesis but also reveal that further investigations are necessary to reach a clearer understanding of this process. It is our hypothesis that the WBP regulates both proliferation and differentiation *in vivo* in a context dependent manner, likely separated by either progenitor cell type or progenitor cell state. Importantly, some previous experiments suggest that the WBP specifically regulates IPs, but there has been little direct consideration of this question (Viti et al., 2003; Hirabayashi et al., 2004; Kuwahara et al., 2010).

We set out to determine whether the WBP regulates RG and IPs independently. We utilized secreted factors of the pathway, Wnt3a ligand (Fig. 2.3A) or Wnt inhibitor Dkk1 (Fig. 2.3C), to avoid the caveats of transcription factor manipulation including activation of signaling in cells not normally responsive to Wnt ligands and the cell

adhesion consequences of β-catenin. Our study uncovered a novel role for the WBP in promoting IP differentiation and the formation of neuronal heterotopias.

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Figures of Chapter 2: Introduction

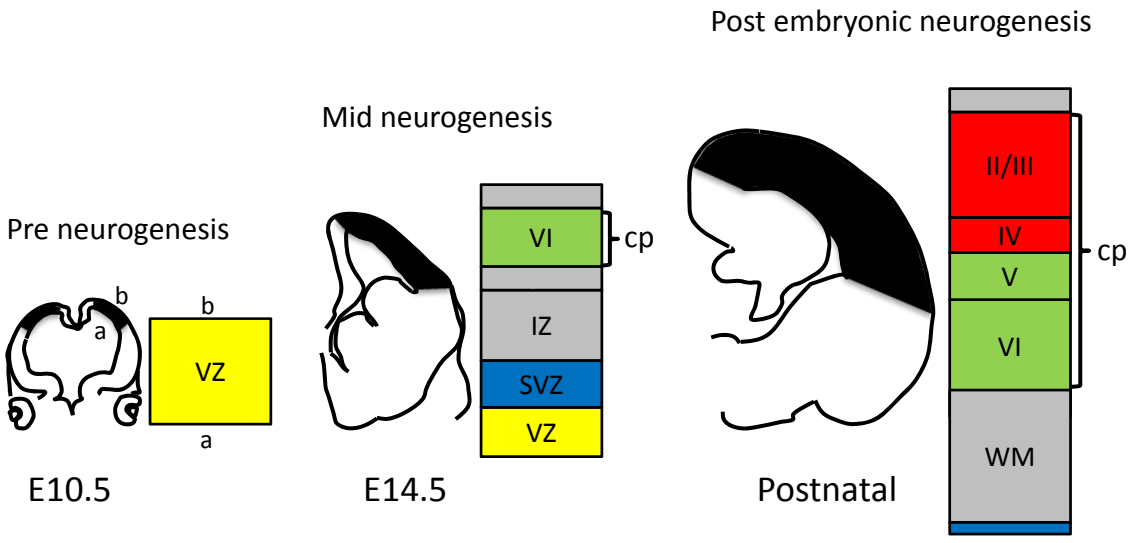
Figure 2.1. Development of the neocortex. (A) The neocortex begins as a single layered epithelium that develops into a multilayered structure. Depicted are coronal sections of mouse neocortices (black region of cerebral cortex) during pre neurogenesis (E10.5, whole head), mid neurogenesis (E14.5, right side of cerebral cortex) and post embryonic neurogenesis (postnatal, right side of cerebral cortex). Along each coronal section is a diagram depicting the layers of the neocortex at the corresponding stage of development. E10.5 neocortex consists of the ventricular zone (VZ, yellow box) where radial glia (RG) progenitor cells reside. RG cells give rise to intermediate progenitors (IPs) that make up the subventricular zone (SVZ, blue box, shown in E14.5 and postnatal). Both RG and IPs produce projection neurons that make up the layers of the cortical plate (cp) (shown in E14.5 and Postnatal diagrams). These layers are formed sequentially in an inside-out (apical-basal) manner with Layer VI composing neurons with the oldest birthdates and Layer II/III (composing neurons with the youngest birthdates. (B) RG cells undergo symmetric self-renewing divisions that expand the RG pool and elongate the neocortex. The left diagram depicts RG cells of the VZ during pre neurogenesis. The middle diagram depicts the progression of a RG cell undergoing symmetric cell division that generates two RG daughter cells. The right diagram depicts a neocortical epithelium that has increased in length due to multiple RG symmetric self-renewing divisions. (C) RG (depicted here) and IPs generate projection neurons that migrate in the basal (b) direction to superficial regions. The left diagram depicts a neocortex with one layer of neurons. The center diagram depicts a RG cell undergoing asymmetric cell division that generates one RG and one neuron. The right diagram depicts a timepoint after a second layer of neurons have been generated. Migration of newly-born neurons passed older neurons generates new cp layers and thickens the neocortex. a-apical surface, WM-white matter.

Figure 2.2. Cell division types of neurogenic progenitors. The progression of cell divions that radial glia (RG) and intermediate progenitors (IPs) undertake are depicted in (A)-(E). (A) RG symmetric self-renewing division that generates two daughter RG cells. (B) RG asymmetric division that generates one RG and one neuron daughter cells. (C) RG asymmetric division that generates one RG and one IP daughter cells. (D) IP symmetric division that generates two daughter IPs. (E) IP symmetric division that generates two daughter neurons.

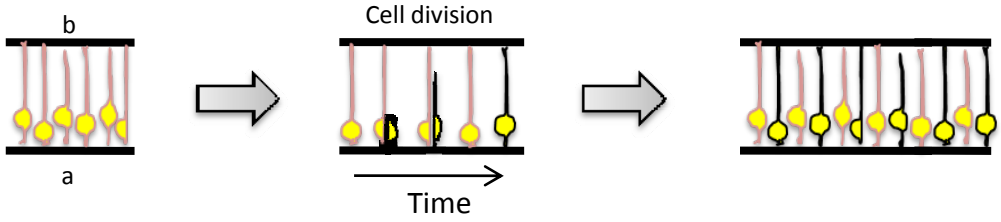
Figure 2.3. The Wnt-β-catenin pathway. (A)The Wnt-β-catenin pathway (WBP) is initiated in receiving cells by binding of secreted Wnt ligands with Frizzled-LRP5/6 receptor complexes at the plasma membrane. This ligand-receptor interaction promotes stabilization of β-catenin levels and translocation of β-catenin into the nucleus where it acts as a transcription factor. β-catenin binds to LEF/Tcf transcription factors and activate transcription of genes with LEF/Tcf promoter elements. (B) In the absence of Wnt ligand, β-catenin levels are kept to a minimum through continued proteasome degradation, blocking transduction of the pathway. (C) Binding of secreted Dkk1 antagonist of the WBP to LRP5/6 Wnt-coreceptor inhibits transduction of the WBP (Logan and Nusse, 2004; Clevers, 2006).

Figure 2.1. Development of the neocortex

A. Dramatic enlargement and layering of the neocortex



B. Proliferative expansion of RG expands the RG pool and elongates the neocortex



C. Production of projection neurons generates layers and thickens the neocortex

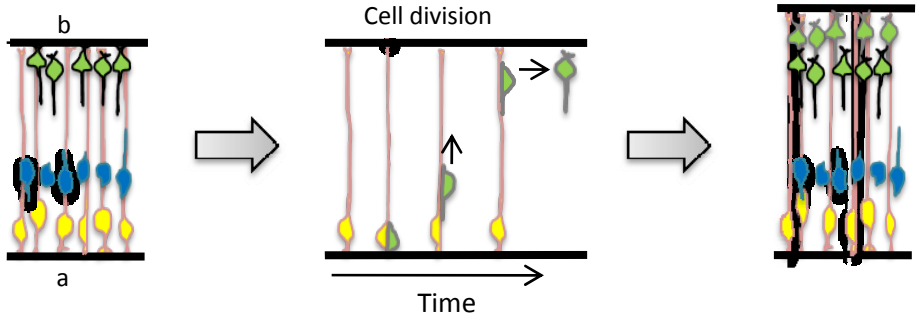
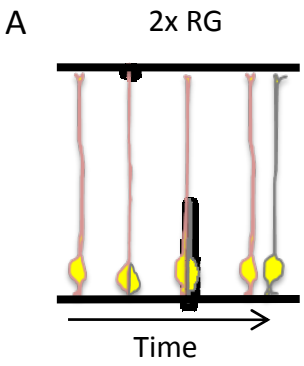
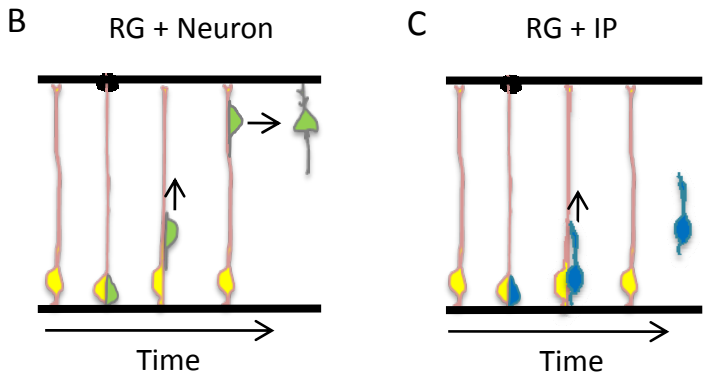


Figure 2.2. Cell division types of neurogenic progenitors

Radial glia symmetric self-renewing divisions



Radial glia asymmetric divisions



Intermediate progenitor symmetric divisions

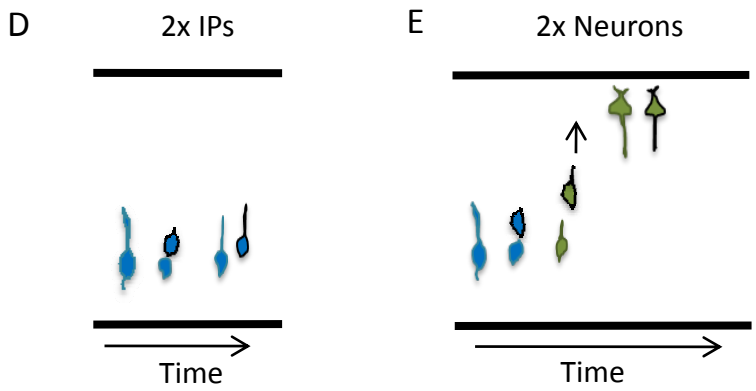
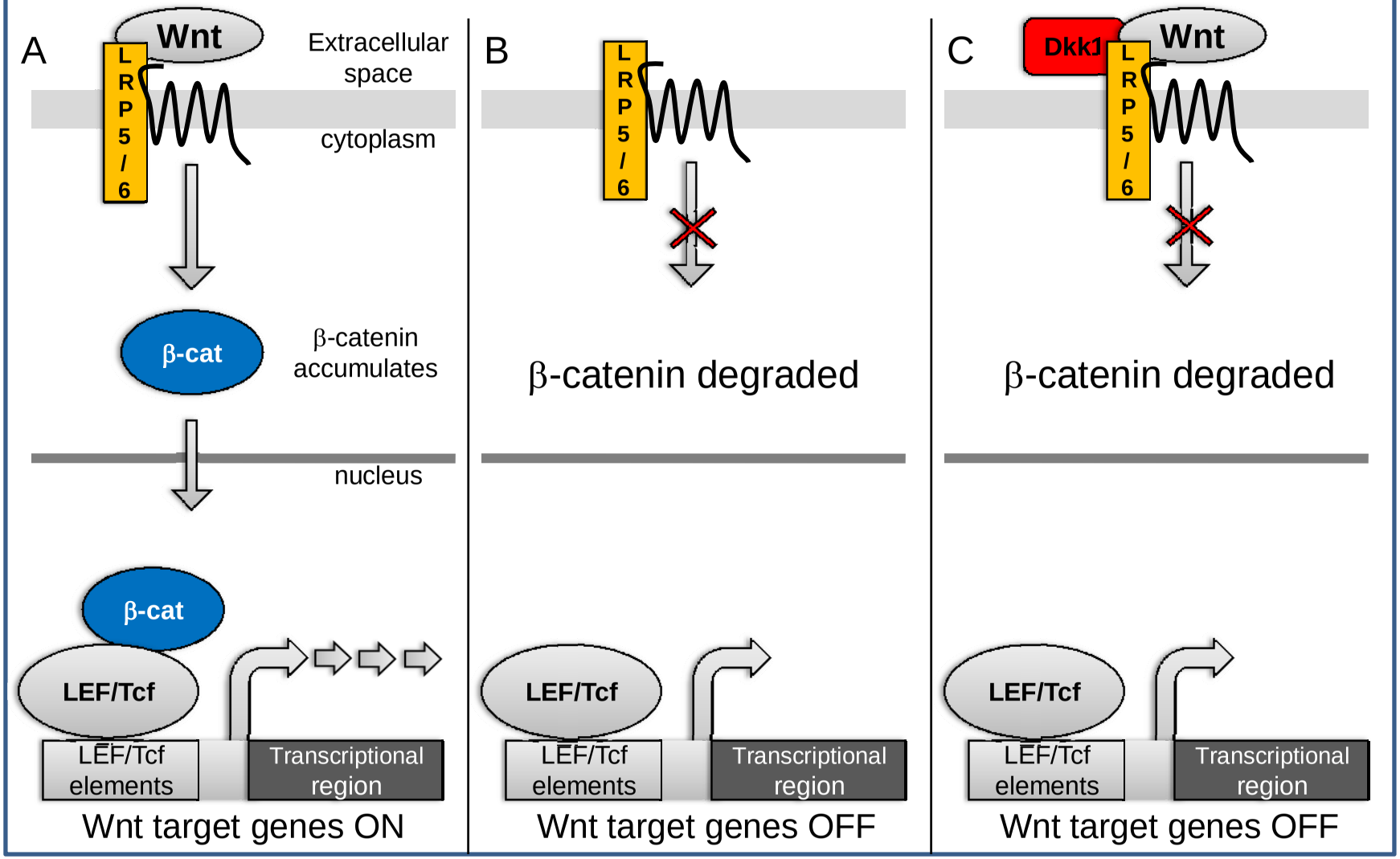


Figure 2.3. The Wnt-β -catenin pathway



Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors

Chapter 3: Materials & Methods

Animals. In utero electroporations were performed on CD1 wildtype (Charles River Laboratories) and BAT-gal transgenic heterozygous (Maretto et al., 2003) mice. RNA *in situ* hybridization and X-gal reaction were performed on tissue obtained from CD1 and BAT-gal mice, respectively. All animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

***In utero* electroporation, expression plasmids and BrdU labeling.** Electroporations were performed as previously described in Li et al. (2008). Electroporations were conducted on E13.5 or E15.5 embryos, allowed to continue gestation *in utero* and harvested either at E14.5, E16.5, E18.5 or P2. DNA solution (100-300nl of 2-3μg/μl plasmid/10%Trypan Blue/10mTris-HCl pH8.0) was injected in the left lateral ventricles of cerebral cortices (Fig. 3.1A-B). DNA solution was allowed to diffuse throughout the ventricle prior to exposing ventricles to electric field at 33V for E13.5 and 37V for E15.5 (Fig. 3.1C-D). The following plasmids were used: M38 TOP-dGFP (R. Moon, Addgene plasmid 17114); pCIG (GFP) (Megason and McMahon, 2002); pCIG2mouseWnt3a (Wnt3a), subcloned with pCIG2 (Hand et al., 2005) and mouse Wnt3a cDNA cloned from Ambion cDNA library; pCIGchickenWnt3a (Wnt3a) (Galli et al., 2004); pCIGLEF1-VP16, subcloned with pCIG and LEF1-VP16 (LEF1 fused to the herpes simplex virus VP16 transactivation domain-contact Guangnan Li for info, grant.li@ucsf.edu); pCIGmouseDkk1 and pCIGΔaa29-48humanβ-catenin (provided by L.W. Burrus, SFSU) (Δaa29-48humanβ-catenin (Tetsu and McCormick, 1999)); pCImRFP (provided by J.L.R Rubenstein, UCSF). Water-solubilized BrdU was injected into the peritoneal cavity to a final dose of 50μg BrdU/g bodyweight.

Immunohistochemistry and X-gal reaction. Mouse brains were collected, fixed,

cryoprotected, and coronally cryosectioned at 20μm using standard methods. Sections were immunolabeled and stained with X-gal using standard methods. The following primary antibodies were used: chicken anti-GFP (1/800, Aves Labs), mouse anti-BrdU (B44, 1/50, BD Biosciences), mouse anti-MAP2 (2a and 2b, 1/500, BD Pharmigen), mouse anti-NF-M (1/1000, Zymed), rabbit anti-Active Caspase3 (1/500, BD Pharmigen), rabbit anti-CDP/Cux1 (M-222, 1/200, Santa Cruz Biotechnology), rabbit anti-Tbr2 (1/500, Chemicon/Millipore), rabbit anti-Pax6 (1/200, Covance), rat anti-Ctip2 (25B6, 1/300-1/800, Abcam), rat anti-Ki67 (TEC-3, 1/300, Dako), Primary antibodies were detected with goat secondary antibodies conjugated to Alexa fluorophores (Invitrogen). β-galactosidase enzymatic activity was detected with X-gal substrate (Invitrogen).

***In situ* hybridization.** Mouse brain sections were prepared as described for immunohistochemistry. RNA *in situ* hybridization was performed as described in Li et al. (2008). Axin2 DIG-labeled RNA probe was generated from pYX-Asc-mouseAxin2 (Open Biosystems cat. EMM1002-7496341). Axin2 expression pattern was verified by comparison to published expression pattern in the Atlas of Gene Expressions, www.genepaint.com.

Image analysis, quantification and statistical analysis. Images were captured with QI imaging CCD camera and QCapture Pro software (Burnaby, British Columbia Canada). For each separate experiment, three or more embryos were used for qualitative analyses (n≥3) and five or more embryos were used for quantitative analyses (n≥5). Embryos from two or more litters were used for each separate experiment. For cell counts at E16.5, BrdU+ or Tbr2+ cells within a 100μm wide neocortical column were counted. For cell counts at P2, Cux1+ or Ctip2+ cells within a 300μm wide neocortical column were counted. Quantified results were expressed as the mean±SEM for *n* given samples. Two-tailed Student's *t* test with unequal variance was used to analyze data

except for Dkk1 electroporations in which one-tailed tests were used. A value of $p<0.05$ was considered statistically significant.

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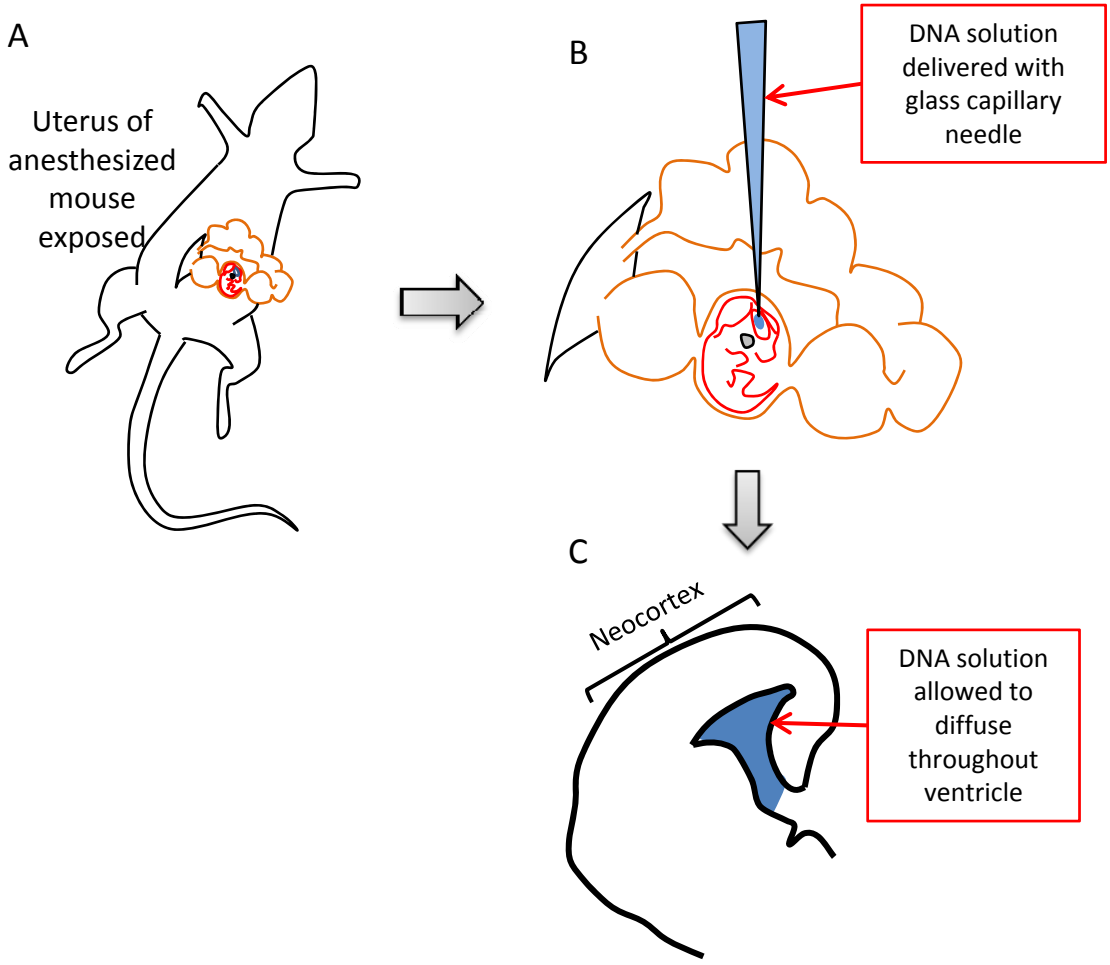
Figure of Chapter 3: Materials & Methods

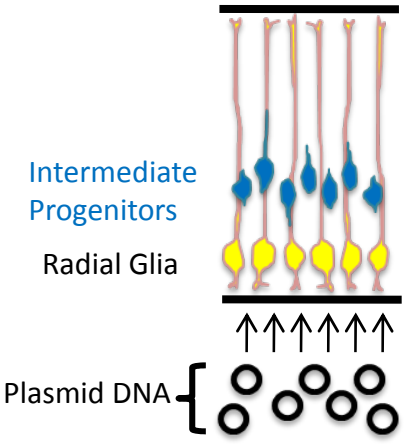
Figure 3.1. *in utero* electroporation. To ectopically express cDNA of interest into neocortical cells, we used the *in utero* electroporation technique. (A) The embryos of an anesthesized mouse are made accessible by extending a uterus from the body cavity. (B) The uterus is illuminated to visualize the embryo inside. Then plasmid DNA solution colored with trypan blue is injected into a lateral ventricle of the embryo with a glass capillary needle. (C) A coronal section of the cerebral cortex depicting the DNA solution inside the ventricle. (D) The cortical area of the embryo head is exposed to an electric field that drives DNA into neocortical cells. By default, cells that are localized at the apical/ventricular surface, radial glia cells, take up the DNA. (E) Expressed soluble factors such as Wnt3a or Dkk1 are secreted into the environment.

Figure 3.1. *in utero* electroporation

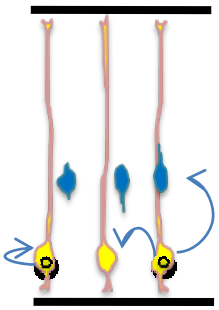
Plasmid DNA solution is injected into lateral ventricle of the embryo



D. Electric field drives DNA into radial glia cells



E. Wnt or Dkk1 sercreted into the environment



Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors

Chapter 4: Results

Ectopic Wnt3a causes cortical dysplasia and neuronal heterotopias

We analyzed WBP function by expressing Wnt3a in the neocortex at E13.5, a time-point when RG symmetric self-renewal has already slowed to avoid strong effects on this process, and analyzed the results at P2 when neurogenesis and migration have primarily subsided. We expressed Wnt3a for three reasons: (1) Wnt3a, Wnt2b and Wnt8b are thought to generate the medial-high to lateral-low gradient of Wnt-β-catenin activity in the neocortex. (2) Wnt3a has been shown to effectively activate the WBP. (3) Expression of a secreted ligand of the Wnt pathway incorporates regulation from the extracellular space to the nucleus and should elicit effects on cells normally responsive to Wnt ligands unlike expression of transcription factors of the Wnt pathway.

We utilized *in utero* electroporation of plasmids to ectopically express GFP, as a control, or Wnt3a, in the developing mouse neocortex. We first tested whether electroporated Wnt3a upregulated the WBP in BAT-gal transgenic and wildtype embryos. The BAT-gal transgene is a WBP reporter allele with β-galactosidase under the control of LEF/Tcf responsive elements (Maretto et al., 2003). We detected electroporated regions by immunolabeling for GFP and the expression of the BAT-gal transgene by X-gal colorimetric reaction with β-galactosidase in coronal sections of mouse brains. Wnt3a, but not GFP, strongly upregulated the expression of the BAT-gal transgene (Fig. 4.1A-D). Moreover, it is clear that Wnt3a elicits a non-cell autonomous effect. Increased β-galactosidase activity was seen beyond electroporated regions (solid lines-electroporated region in Fig. 4.1C,D; dashed lines-non-cell autonomous activity in Fig. 4.1D). To confirm that Wnt3a upregulates endogenous signaling as well, we analyzed Axin2 mRNA expression, a direct target of the WBP, by RNA *in situ* hybridization. Wnt3a electroporation at E13.5, but not GFP, increased and laterally

extended Axin2 mRNA expression by E14.5 (Fig. 4.S1A,B). Thus, ectopic expression of Wnt3a in the neocortex upregulates the WBP within 24 hours of electroporation.

To test the ultimate effects of Wnt3a overexpression, we analyzed electroporated brains at P2, 8 days post electroporation. The GFP expressing cells in control brains were distributed throughout the cortical plate whereas many GFP+ cells in Wnt3a-electroporated brains were clustered in a mass adjacent to the ventricle (cortical plate (cp) GFP, cp/white brackets; ectopic mass GFP, heterotopia (ht)/red brackets; Fig. 4.1E,I). To investigate the nature of these cell clusters, we analyzed the expression of the neuronal markers Ctip2 and Cux1 by immunolabeling. Ctip2 is a transcription factor expressed moderately by Layer VI and strongly by Layer V early-born/deep layer neurons (Fig. 4.1F,H). Cux1 is a transcription factor expressed strongly by layer IV-II late-born/upper layer neurons (Fig. 4.1G,H). Labeling with Ctip2 and Cux1 showed that Wnt3a-induced cell masses were predominantly made up of neuronal cells (ht, Fig. 4.1J-L).

To determine how long-term induction of Wnt signaling affects the organization of IPs remaining in the cortex by P2, we analyzed the expression of Tbr2 by immunolabeling. In control brains, there were few remaining Tbr2+ cells found in the SVZ (arrows, Fig. 4.1M-O). However, in Wnt3a-electroporated brains there were many more Tbr2+ cells in disorganized clumps or organized in rosettes in an expanded SVZ adjacent to the heterotopic neuronal mass (rosettes, arrowheads, Fig. 4.1P-R). Thus, long-term upregulation of the WBP expands the population and dramatically alters the organization of these cells. These factors are consistent with characteristics that would lead to production of excess neurons. Furthermore, disorganization and rosette reorganization of IPs could cause newly born neurons to migrate incorrectly, which could lead to the disorganized cortical plate and periventricular heterotopia seen at P2.

The formation of an extra-neocortical neuronal mass larger than the normal epithelium suggests that Wnt3a ectopic expression induces excess progenitor proliferation but does not ultimately hinder neuronal differentiation. This result confirms a function for the WBP in promoting progenitor proliferation as previously been described (Chenn and Walsh, 2002; Logan and Nusse, 2004; Woodhead et al., 2006; Zhou et al., 2006; Wrobel et al., 2007). Moreover, it potentially implicates dysregulation of Wnt-β-catenin signaling pathway in the occurrence of neuronal migration disorders, as previously suggested (Zhou et al., 2006; Freese et al., 2010).

Wnt3a promotes expansion of RG and differentiation of IPs.

To begin to determine the mechanism of Wnt3a effects that lead to the severe phenotype seen at P2, we examined the distribution of neuronal progenitors 3 days post electroporation, at E16.5. We immunolabeled for Pax6 to label RG and Tbr2 to label IPs. The density of cells and thickness of Pax6+ ventricular zone (VZ) is much greater after Wnt3a-electroporation compared to control (yellow brackets, Fig. 4.2A,C), This is consistent with Wnt3a inducing RG to undergo self-renewing divisions, an established function of the WBP. Next we analyzed the distribution of Tbr2+ cells. We demarcated the expression pattern of Tbr2 into two domains: the “deep Tbr2 domain” (DTD), consisting of Tbr2+ cells within the VZ and SVZ (Tbr2 domain below the arrowheads in Fig. 4.2B), and the “upper Tbr2 domain” (UTD) consisting of the more sparsely distributed Tbr2+ cells in the IZ (Tbr2 domain above the arrowheads in Fig. 4.2B). Strikingly, Wnt3a electroporation led to a dramatic depletion of UTD IPs compared to control (Fig. 4.2B,D). There are several potential cellular mechanisms for this change: Wnt3a may 1) block production of IPs from RG; 2) decrease proliferation of IPs; 3) block transition of IPs from the DTD to UTD; 4) induce early differentiation of IPs into neurons; 5) force IPs to become a non-neuronal Tbr2- cell population.

Normally, an increase in RG proliferation and population would be expected to lead to increased production of IPs. However, we observed a clear decrease of IPs in the UTD of experimental samples (Fig. 4.2B,D). It seems likely that induction of RG proliferation by Wnt3a might also be associated with inhibiting transition of RG into the IP state, thus reducing the total IP population. Previous work by Wrobel and colleagues (2007) has shown that genetically inducing RG proliferation with a dominant active allele of β-catenin (ΔEx3β-catenin) inhibits the production of IPs. To test whether this is the explanation for loss of IPs after Wnt3a electroporation, we quantified the number of Tbr2+ cells in the total and Tbr2 subdomains (DTD and UTD) (Fig. 4.2E,F; quantification: Control N=7, Wnt3a N=9, Fig. 4.2G). Since IPs should be generated and seen first in the DTD (at the VZ-SVZ border), the numbers of cells in the DTD should be reduced if there is a block in IP production from RG. Indeed, the total number of Tbr2+ cells is significantly decreased in Wnt3a-electroporated brains compared to controls (Total, Control: avg. 207 cells/100μm column, SEM+/-11.6; Wnt3a: avg. 172 cells/100μm column, SEM+/-9.7, Pvalue 0.037; Fig. 4.2G), but the number of Tbr2+ cells in the DTD is unchanged (DTD, Control: avg. 139 cells/100μm column, SEM+/-5.6; Wnt3a: avg. 151 cells/100μm column, SEM+/-7.8, Pvalue 0.219; Fig. 4.2G). Instead, there is a dramatic decrease in UTD IPs after Wnt3a electroporation compared to control (UTD, Control: avg. 69 cells/100μm column, SEM+/-7.6; Wnt3a: avg. 21 cells/100μm column, SEM+/-5.6, Pvalue 0.0003; Fig. 4.2G). Thus, the decrease in total Tbr2+ cells is more likely due to the depletion of UTD Tbr2+ cells rather than a failure to generate DTD Tbr2+ cells from RG.

The loss of Tbr2+ cells in the UTD could be due to a failure of IPs to continue to proliferate in this zone. To assess this, we calculated the mitotic fraction of Tbr2+ cells in the DTD and UTD by acute BrdU labeling (50μg BrdU/g body weight 1hr before harvest

at E16.5). We quantified the total, DTD and UTD fraction of Tbr2+ cells that are BrdU+ (Fig. 4.2E,F; quantification: Control N=7, Wnt3a N=6, Fig. 4.2H). The S-phase fraction of DTD Tbr2+ cells in Wnt3a-electroporated brains is similar to controls (DTD, Control: avg. 0.086/100μm column, SEM+/-0.0077; Wnt3a: avg. 0.084/100μm column, SEM+/-0.0062, Pvalue 0.828; Fig. 4.2H). Interestingly, although the number of UTD Tbr2+ cells is dramatically decreased, the S-phase fraction of UTD Tbr2+ cells after Wnt3a electroporation is also similar to controls (UTD, Control: avg. 0.347/100μm column, SEM+/-0.052; Wnt3a, avg. 0.335/100μm column, SEM+/-0.094, Pvalue 0.913; Fig. 4.2H). Thus, although there is a dramatic loss of UTD IPs, the level of proliferation of IPs is maintained.

To test if UTD IPs are absent because they have stopped expressing Tbr2, we traced the lineage of IPs by pulsing with BrdU 24hrs before harvesting embryos. This method labeled progenitors that are in the S-phase at E15.5 and allowed us to visualize their location at E16.5. If the absence of UTD IPs is not due to failed production of this population but is due to downregulation of Tbr2 expression, we would see an extra pool of BrdU+ cells that are Tbr2- in Wnt3a-electroporated regions but not in controls. Indeed, we observed such a large pool of BrdU+, Tbr2- cells in the apical-most region of the UTD of Wnt3a-electroporated brains but not in controls (Fig. 4.2I,L). To test if the BrdU+, Tbr2- pool of cells are no longer dividing or have just lost Tbr2 expression, we immunolabeled with Ki67, an active-cell cycle marker. This confirmed that the BrdU+, Tbr2- cells found in the UTD are also Ki67- and, thus, are no longer proliferative (Fig. 4.2J,K,M,N). The disappearance of UTD IPs and the concomitant appearance of BrdU+, Tbr2- post mitotic cells in the UTD suggest that ectopic Wnt3a induces UTD IPs to become post mitotic.

The IP pool is critical for generating the correct number of cortical plate neurons. Intermediate progenitors amplify the neuronal output of RG by providing 1 or 2 extra steps of self-renewing divisions prior to producing a pair of neurons on its last symmetric division. We therefore hypothesized that premature differentiation of IPs and accumulation of the resulting neurons in the UTD in Wnt3a-electroporated regions would lead to a reduction in the number of neurons reaching the cortical plate during this period. To address this hypothesis, we examined Ctip2 and Cux1 expression by immunolabeling at E16.5, 3 days post electroporation. Within the cortical plate, the expression patterns of both markers were reduced in Wnt3a-electroporated regions compared to controls (white dashed lines, Fig. 4.2O-V). The population of late-born/upper layers neurons labeled by Cux1 was more strongly affected, being either absent or thinner in Wnt3a-eletroporated regions relative to the unaffected regions and controls (white dashed lines, Fig. 4.3Q,R,U,V). To rule out a significant contribution of cell death to this effect, we immunolabeled for active Caspase3, a marker of cell death, and observed no significant increase of apoptosis in Wnt3a-electroporated brains compared to controls (Fig. 4.S2A-D). These results suggest that premature differentiation of IPs and accumulation of these newly born cells in the UTD in Wnt3a-electroporated brains lead to reduction in the number of neurons reaching the cortical plate.

Lateral expansion of the neocortical epithelium is achieved by self-renewal of RG. At E16.5, the regions electroporated with Wnt3a are frequently thinner than regions distant from the site of electroporation and in controls (double-ended arrows, Fig. 4.2O,S; solid line, Fig. 4.1A-D;). This morphology is consistent with the abnormal expansion of the RG pool observed at E16.5 (Fig. 4.2A,C) in Wnt3a-electroporated brains and may ultimately contribute to the production of excess neurons observed at P2 once this increased cohort of RG produces neurons.

Intermediate progenitors can be targets of Wnt signaling *in vivo.*

If the WBP is physiologically important for IP differentiation, then endogenous Wnt-β-catenin signaling must be active in IPs. To test whether IPs are responding to endogenous Wnts, we electroporated a TOP-destablizedEGFP (TOPdGFP) WBP reporter plasmid into E13.5 brains and analyzed expression of dGFP at E14.5. First, to test the relative expression of dGFP in expressing cells we co-electroporated TOPdGFP with a control plasmid ubiquitously expressing RFP, (pCImRFP). Since RFP is under the control of a constitutive promoter, the level of RFP expression serves as a visual measure of the relative electroporation efficiency. We observed cells strongly expressing RFP that were either weakly or strongly expressing dGFP (Fig 3A1,A4). Similarly, we observed cells weakly expressing RFP that were either weakly or strongly expressing dGFP (Fig 3A2,A3). This expression heterogeneity suggests that the Wnt reporter expression differences that we observe are not solely due to differences in plasmid number but rather probably readout of endogenous levels of Wnt signaling. Thus, as others have also concluded (Woodhead et al., 2006), the TOPdGFP plasmid introduced into the neocortex by electroporation can be used to identify the presence of endogenous Wnt-β-catenin signaling.

We next analyzed the colocalization of Tbr2 and dGFP to determine if IPs are included among the cells responsive to endogenous Wnt-β-catenin signaling. Many Tbr2 expressing cells were dGFP+ (arrows, Fig. 4.3B). In the VZ few Tbr2+ cells were also dGFP+ (Fig. 4.3B1), while in the most basal area of the DTD many more are doubly expressing Tbr2 and dGFP (Fig. 4.3B2). By E14.5 some Tbr2+ cells have emerged from the DTD and migrated into the UTD (Fig. 4.3B). These UTD Tbr2+ cells also express dGFP (Fig. 4.3B3). These observations indicate that Tbr2-expressing IPs respond to endogenous Wnt-β-catenin signaling.

Wnt3a promotes differentiation of neurons in progenitor domains and disorganization of RG and IP distribution.

The BrdU+, Tbr2- post mitotic cells that accumulated in the UTD in Wnt3a-electroporated regions are likely immature neurons born from the missing UTD IP population. To determine if these cells will mature into the neurons that later comprise the heterotopia seen at P2, we looked for ectopic expression of neuronal markers at E18.5 (5 days post electroporation). Normally Ctip2 expression is only strong in layers VI and V of the cortical plate at E18.5 (Fig. 4.4B,D). However, in Wnt3a-electroporated regions there was ectopic expression of Ctip2 in progenitor domains (ht, Fig. 4.4B,D,F,H). Cux1 is typically expressed both in progenitor domains and in layer IV-II of the cortical plate at E18.5 (Fig. 4.4C,D). However, in Wnt3a-electroporated regions Cux1 expression was dramatically expanded in the progenitor/IZ domains and decreased in the cortical plate ((Fig. 4.4C,D,G,H). Together with our previous findings, our results suggest that ectopic Wnt3a induces premature differentiation of UTD IPs and accumulation of these cells in progenitor domains. This hypothesis is consistent with the decreased thickness of the cortical plate and increased thickness of the heterotopic region (cp/white double-ended arrows, ht/yellow double-ended arrows, Fig. 4.4A-H; cp/white brackets, ht/red brackets, Fig. 4.1E,I).

To further understand the progression of heterotopia formation, we analyzed the distribution of RG and IPs at E18.5, 5 days post Wnt3a-electroporation. As before, we used Pax6 to label RG and Tbr2 to label IPs. Using these markers we noted scattering of progenitors and reorganization of progenitors into rosettes (scattered cells-arrows; rosettes-arrowheads, Pax6, Fig. 4.4I-L, Tbr2 Fig. 4.4M-P). Thus, Wnt3a overexpression leads to premature differentiation of IPs to neurons, disorganization of progenitor distribution and formation of periventricular neuronal heterotopias.

Dominant activation of Wnt signaling drives ectopic neuronal differentiation.

To determine whether cell autonomous activation of Wnt signaling leads to similar effects as ectopic Wnt3a expression, we tested whether a strong dominant active form of LEF1 could elicit the same effects. The LEF/Tcf family of transcription factors transduce the transcriptional output of the WBP. We used a dominant active form of LEF1 fused to the transactivation domain of the VP16 protein from the herpes simplex virus (LEF1-VP16). Consistent with the effects of Wnt3a electroporation, LEF1-VP16 promoted the differentiation of Tbr2+ IPs in the UTD 3 days post-electroporation (Fig. 4.5A-P). The cohort of LEF1-VP16-electroporated cells did not express Tbr2 (arrowsheads, Fig. 4.5B-D,F-H) or Ki67 but strongly expressed Ctip2 (arrows, Fig. 4. 5I-P). We saw similar effects after expression of a dominant active form of β-catenin (Δ29-48βcat) (arrows, Fig. 4.S3A-H).

Dkk1 inhibits the production of neurons during mid and late neurogenesis

To determine whether Wnt signaling is required for the normal production of neurons in the neocotex, we ectopically expressed Dkk1, a secreted antagonist of the WBP coreceptor LRP6, and analyzed the production of neocortical neurons. We used Ctip2 to label early-born/deep layer neurons and Cux1 to label late-born/upper layer neurons. We then counted the numbers of neurons of Layer V and Layer IV-II, neurons primarily born after the time-point of electroporation at E13.5. Ectopic expression of Dkk1 at E13.5 (Fig. 4.6A-H) inhibited the production of Layer V neurons but not the production of Layer IV-II neurons (Ctip2, Control: N=6 avg. 92 cells/300μm column, SEM+/-7.06; Dkk1: N=5, avg. 69 cells/300μm column, SEM+/-7.37, Pvalue 0.027, Fig. 4.6I; Cux1, Control: N=6 avg. 792 cells/300μm column, SEM+/-23.45; Dkk1: N=5, avg. 744 cells/300μm column, SEM+/-48.55, Pvalue 0.186; Fig. 4.6J). This result suggests that Dkk1 might selectively regulate deep layer neuron production or it may indicate a more general inhibition to neuron production that is just transient. To test this specificity

we expressed Dkk1 at E15.5 (Fig. 4.6K-R) and found that it specifically inhibited production of Layer IV-II (Ctip2, Control: N=6 avg. cells/300μm column, SEM+/-5.20; Dkk1: N=6, avg. cells/300μm column, SEM+/-3.80, Pvalue 0.487, Fig. 4.6S; Cux1, Control: N=6 avg. 595 cells/300μm column, SEM+/-35.09; Dkk1: N=6, avg. 514 cells/300μm column, SEM+/-21.86, Pvalue 0.043; Fig. 4.6T). These results suggest that inhibition of Wnt signaling by Dkk1 generally inhibits neurogenesis without specifically regulating different layer specificities and are consistent with the phenotype of the Wnt3a electroporation experiments. The agonist Wnt3a generates extra neurons while the antagonist Dkk1 inhibits the production of neurons. Moreover, since IPs generate the majority of neurons after E15.5, our results implies a role for the WBP in the regulation of IP behavior.

References of Chapter 4: Results

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Figures of Chapter 4: Results

Figure 4.1. Ectopic Wnt3a causes cortical dysplasia and neuronal heterotopias. (A-D) The capacity of ectopic Wnt3a to induce Wnt-β-catenin signaling was tested by electroporating E13.5 BATgal transgenic embryos with control or Wnt3a expression plasmids. Electroporated embryos were analyzed at E16.5 for GFP expression of electroporation plasmids and β-galactosidase expression of the BATgal transgene. (A,C) Immunolabeling for GFP. (B,D) Xgal staining for β-galactosidase activity. Black solid line in (D) demarcates the region similar to the electroporated region in (C) (white solid line). Dashed lines in (D) demarcate Xgal staining beyond electroporated region. (E-R) Characterization of P2 brains electroporated at E13.5 by immunolabeling for GFP, Ctip2 and Cux1 layer neuron markers and Tbr2 IP marker. (E,I) White brackets indicate region of GFP localization in the cortical plate (cp) while red brackets indicate localization of GFP in a heterotopic structure apical to the cp. Yellow arrowheads in (E-H) indicate the medial boundary of GFP electroporation. (F-H,J-L) Immunolabeling for Layer VI-V and Layer IV-II neurons with Ctip2 (F,H,J,L) and Cux1 (G,H,K,L) antibodies, respectively. (M-R) Analysis of IP distribution with Tbr2 immunolabeling. Arrows in (M-O) indicate IPs in control sample while white arrowheads in (P-R) indicate IPs in rosette formation in the experimental sample. Scale bars: (A,E) 500μm, (F,M) 200μm. hc-hippocampus, ht-heterotopia, nc-neocortex.

Figure 4.2. Wnt3a promotes expansion of RG and differentiation of IPs. Brains electroporated at E13.5 were analyzed at E16.5 for the expression of progenitors and neurons by immunolabeling. (A,C) Analysis of RG distribution with Pax6 immunolabeling. Yellow bracket indicates the thickness of the ventricular zone (vz). In (B), (D-F) and (I-N), the boundary between the UTD and DTD subpopulations of Tbr2+

IPs is demarcated by the pairs of yellow or white arrowheads. (B,D) Examination of IP distribution with Tbr2 immunolabeling. (E-H) Analysis of the numbers and S-phase fraction of IPs. BrdU was injected 1hr before harvest for S-phase fraction analysis. (E,F) Representative samples used for quantification of Tbr2+ cells numbers in (G) and the S-phase fraction of Tbr2+ cell populations in (H) with Tbr2 and BrdU immunolabeling. (I-N) Lineage tracing of progenitors in S-phase with BrdU injected at E15.5, 24hr before harvest and analysis at E16.5. (I,L) Co-immunolabeling for BrdU and Tbr2 to determine the localization of IPs born at E15.5. (J,K and M,N) Triple immunolabeling for BrdU, Ki67 and Tbr2 to analyze the cell cycle state of BrdU-labeled cells. (O-V) Immunolabeling for Ctip2 and Cux1 to determine the effect of Wnt3a on neuron production. Yellow dashed lines in (O-V) demarcate the medial boundary of electroporation. White dashed lines in (S-V) indicate the region in which Ctip2 and Cux1 expression are most strongly affected. Nodules positive for ectopic Ctip2 is indicated with a white (*) in (S-V). Scale bars: (A,E,I) 50μm, (O) 200μm. UTD-upper Tbr2 domain, DTD-deep Tbr2 domain. Error bars represent SEM, black (*) and (**) in (G) indicate $p<0.05$ and $p<0.001$, respectively.

Figure 4.3. Intermediate progenitors can be targets of Wnt signaling *in vivo.*

Analysis of Wnt-β-catenin activity in IPs. Embryos were electroporated with the TOPdGFP Wnt-β-catenin signaling reporter at E13.5 and analyzed at E14.5 for dGFP expression to visualize Wnt-β-catenin signaling activity. (A) Co-electroporation of pCImRFP and TOPdGFP plasmids to determine if dGFP expression is affected by electroporation efficiency. Electroporation efficiency is measured by the fluorescence level of RFP, which is expressed under a ubiquitous promoter. (A1) Cells expressing strong RFP and weak dGFP. (A2) A cell expressing weak RFP and dGFP. (A3) Cells expressing weak RFP and strong dGFP. (A4) Cells expressing strong RFP and dGFP.

(B) Immunolabeling for Tbr2 in brains electroporated with TOPdGFP alone to determine if Wnt-β-catenin signaling is active in IPs. Arrowheads indicate cells co-expressing Tbr2 and dGFP. Examples of Tbr2+, dGFP+ cells in the ventricular zone (vz) (B1), basal DTD (B2) and UTD (B3). Scale bars: (A,B) 25μm, (A1) 3.125μm, (B3) 6.25μm. DTD-deep Tbr2 domain, UTD-upper Tbr2 domain.

Figure 4.4. Wnt3a promotes differentiation of neurons in progenitor domains and disorganization of RG and IP distribution. Characterization of E18.5 brains electroporated at E13.5 by immunolabeling for neuron and progenitor markers. (A-H) White dashed lines demarcate the boundaries between the cortical plate (cp), heterotopia (ht), neocortex (nc), choroid plexus (p) and hippocampus (hc). White double-headed arrows indicate the thickness of the cp while yellow double-headed arrows indicate the thickness of the ht. (A,E) Immunolabeling for GFP show distribution of GFP in the cp and ht. Immunolabeling for Ctip2 (B,D,F,H) and Cux1 (C,D,G,H) show expression of layer neurons in the cp and ht. (I-P) Immunolabeling of Pax6+ RG, Tbr2+ IPs and active cell cycle marker, Ki67, to show the distribution and cell cycle state of progenitors. (K,L,O,P) Arrows indicate examples of scattered progenitors while arrowheads indicate examples of progenitors in rosette formations. Scale bars: (A,I) 200μm. (IV-II)-Layer IV-II, (V)-Layer V, (VI)-Layer VI.

Figure 4.5. Dominant activation of Wnt signaling drives ectopic neuronal differentiation. Embryos electroporated at E13.5 with a dominant active form of LEF1 (LEF1-VP16) were analyzed at E16.5 to test the effect of dominant activation of the WBP on the distribution and molecular identity of IPs. (A,E) Immunolabeling for GFP and Tbr2 IP marker to determine the localization of electroporated cells. (B-D,F-H) Higher

magnification images of samples (A) and (E). Arrowheads in (F-H) indicate the positions of cell cohorts electroporated with LEF1-VP16 expressing GFP but not Tbr2. (I-P) Immunolabeling for GFP, Ki67 active cell cycle marker and Ctip2 neuronal marker. Arrows in (M-P) indicate cell cohorts electroporated with LEF1-VP16 co-expressing GFP and Ctip2 but not Ki67. Scale bars: (A) 200μm, (B,I) 50μm.

Figure 4.6. Dkk1 inhibits the production of neurons during mid and late neurogenesis. (A-J) Brains electroporated at E13.5 with Dkk1 WBP inhibitor were analyzed at P2 to test the effect of Dkk1 starting at mid neurogenesis. (A,E) Immunolabeling for Ctip2 and GFP shows distribution of electroporated cells. (B-D,F-H) Higher magnification of samples (A) and (E) immunolabeled with Ctip2 and Cux1. (I) Quantification of the numbers of Ctip2+ Layer V neurons. (J) Quantification of the numbers of Cux1+ Layer IV-II neurons. (K-T) Brains electroporated at E15.5 with Dkk1 were analyzed at P2 to test the effect of Dkk1 on late neurogenesis. (K,O) Immunolabeling for Ctip2 and GFP shows distribution of electroporated cells. (L-N,P-R) Higher magnification of samples (K) and (O) immunolabeled with Ctip2 and Cux1. (S) Quantification of the numbers of Ctip2+ Layer V neurons. (T) Quantification of the numbers of Cux1+ Layer IV-II neurons. Scale bars: (A,K) 200μm, (B,L) 50μm. Error bars represent SEM, (*) indicate $p<0.05$.

Figure 4.S1. Wnt3a upregulates Axin2 mRNA expression. (A,B) Wildtype embryos were electroporated in utero with Wnt3a and analyzed at E14.5 for GFP expression by immunolabeling (A) and Axin 2 expression by RNA *in situ* hybridization (B). Scale bar: (A) 500μm.

Figure 4.S2. Ectopic Wnt3a does not affect levels of cell death. (A,B) Embryos electroporated at E13.5 and injected with BrdU at E15.5 were analyzed at E16.5 for cell death by immunolabeling for Active Caspase 3 (AcCasp3) and BrdU. Ectopic BrdU in (B) indicates region affected by Wnt3a overexpression. (C,D) Boxed regions in (A) and (B). Arrows in (C,D) indicate cells positive for AcCasp3. Scale bars: (A) 100μm, (C) 25μm.

Figure 4.S3. Dominant active β-catenin drives ectopic neuronal differentiation. Embryos electroporated at E13.5 with a dominant active form of β-catenin (Δ29-48βcat) were analyzed at E16.5 with Ctip2 Layer VI-V neuron marker. (A-H) Immunolabeling for GFP and Ctip2 show localization of electroporated cells at low magnification (A,E) and high magnification (B-D,F-H). Arrows in (F-H) indicate region of GFP+ and Ctip2+ cohort of cells. Scale bars: (A) 200μm, (C) 100μm.

Figure 4.1

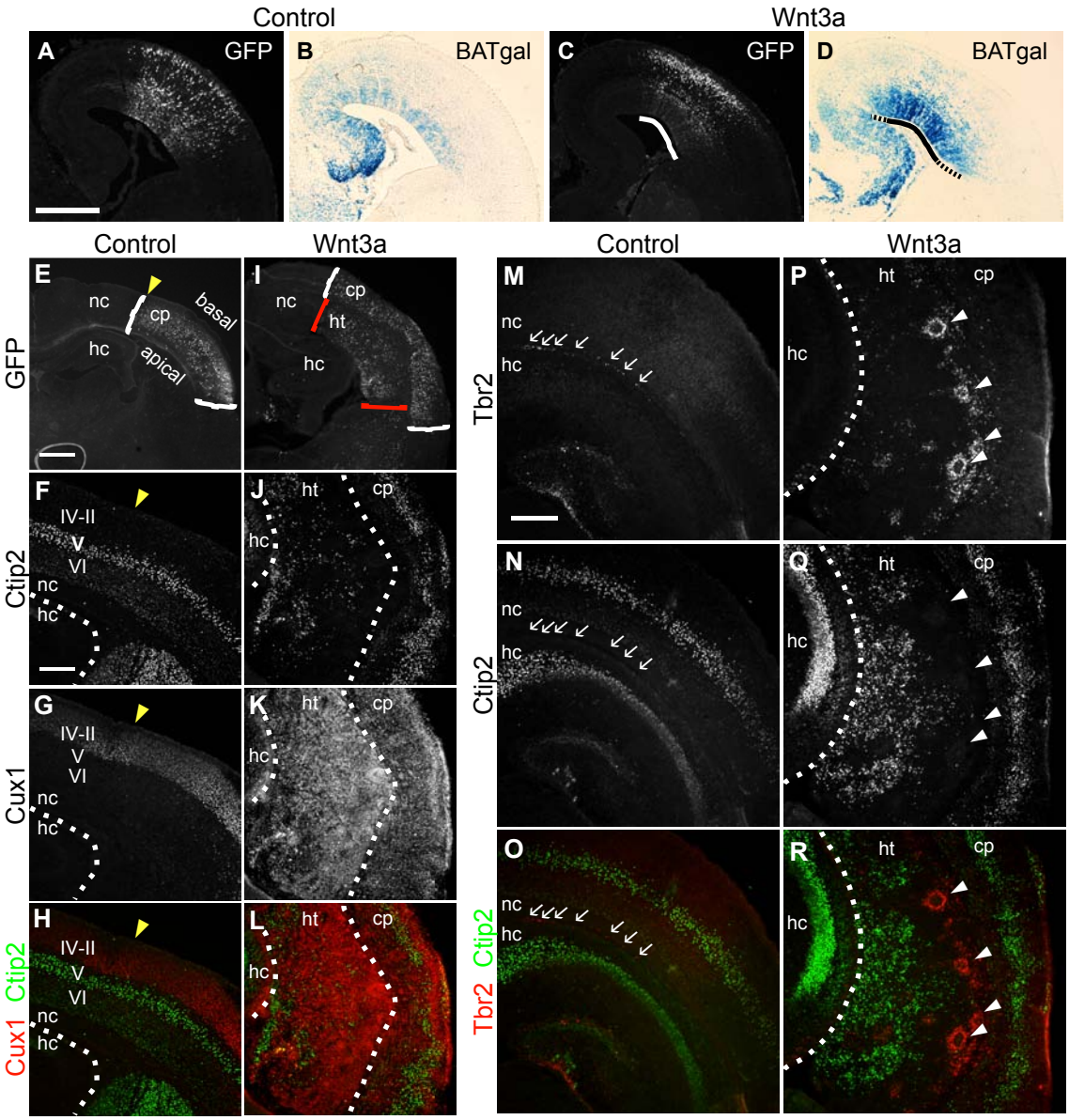
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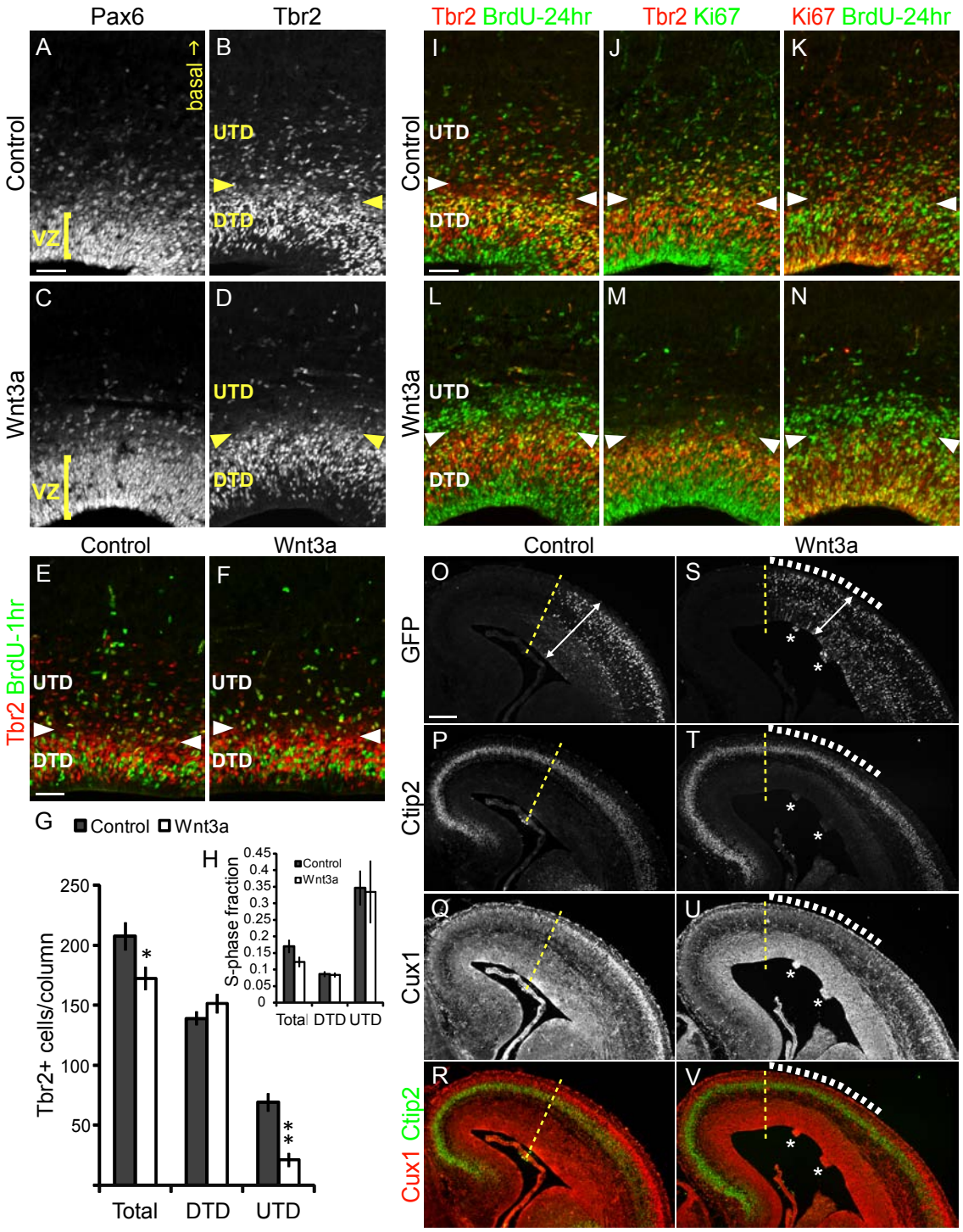
Figure 4.2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Tbr2 BrdU-1hr
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Ctip2
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Figure 4.3

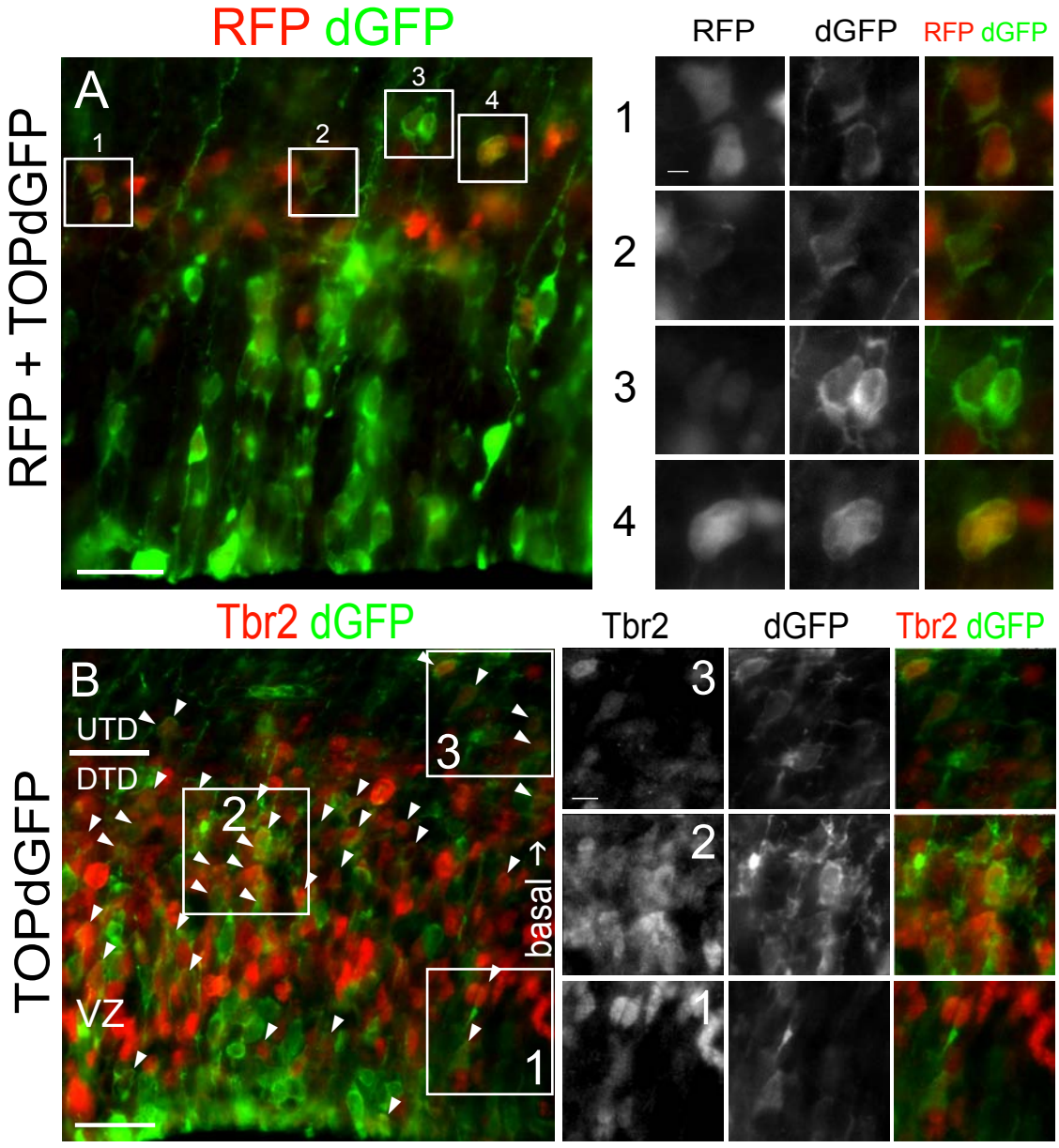
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Figure 4.4

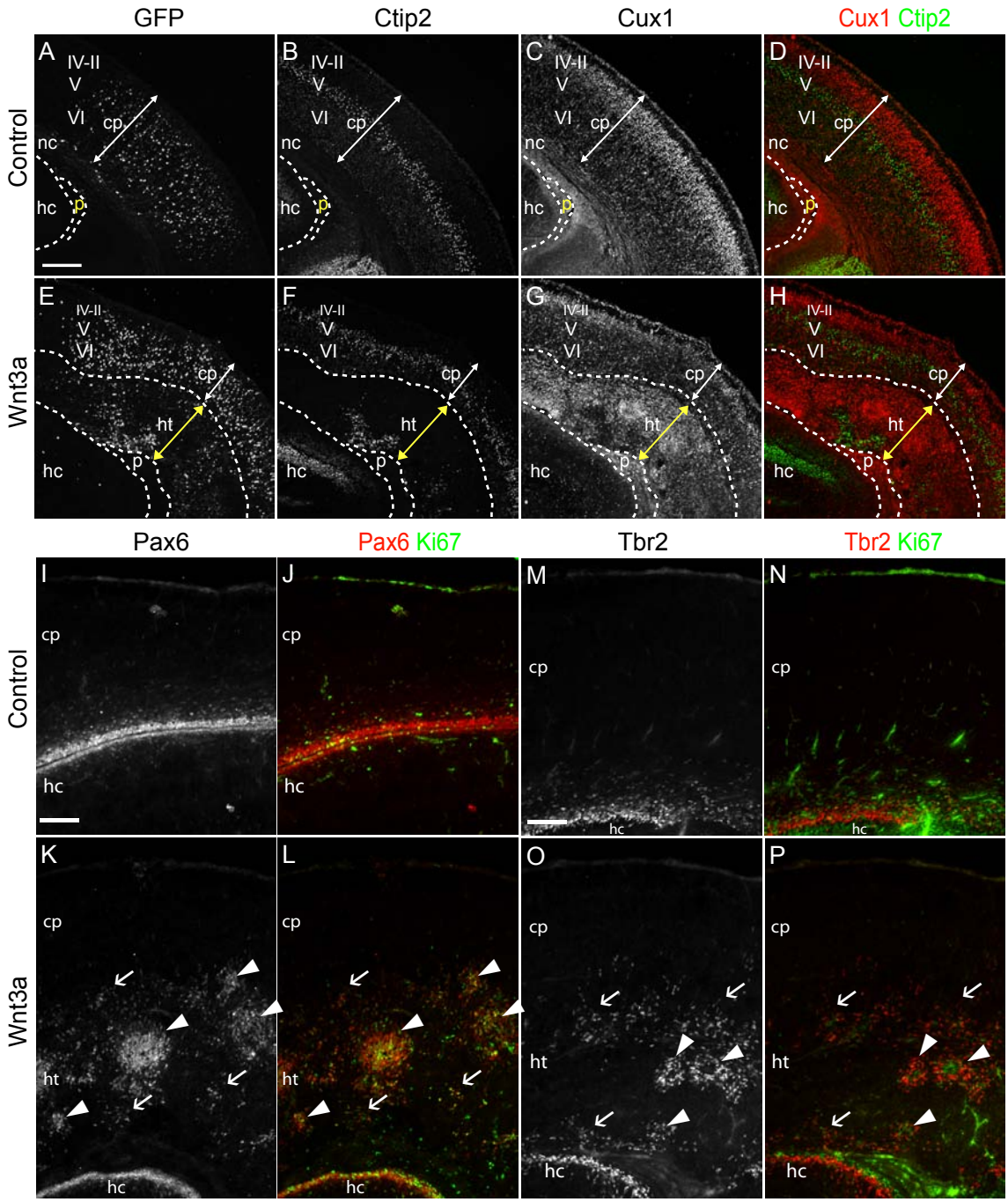
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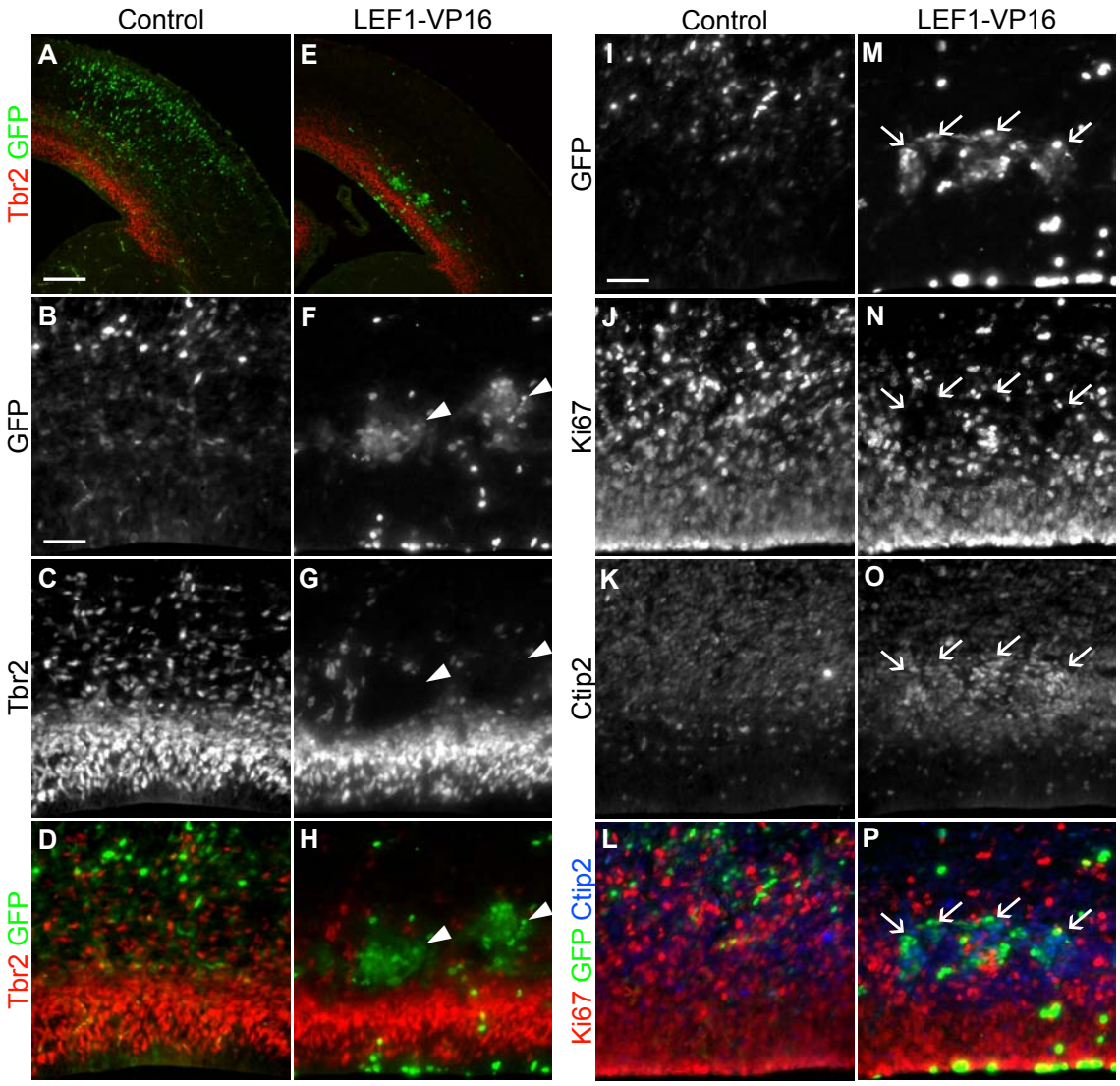
Figure 4.5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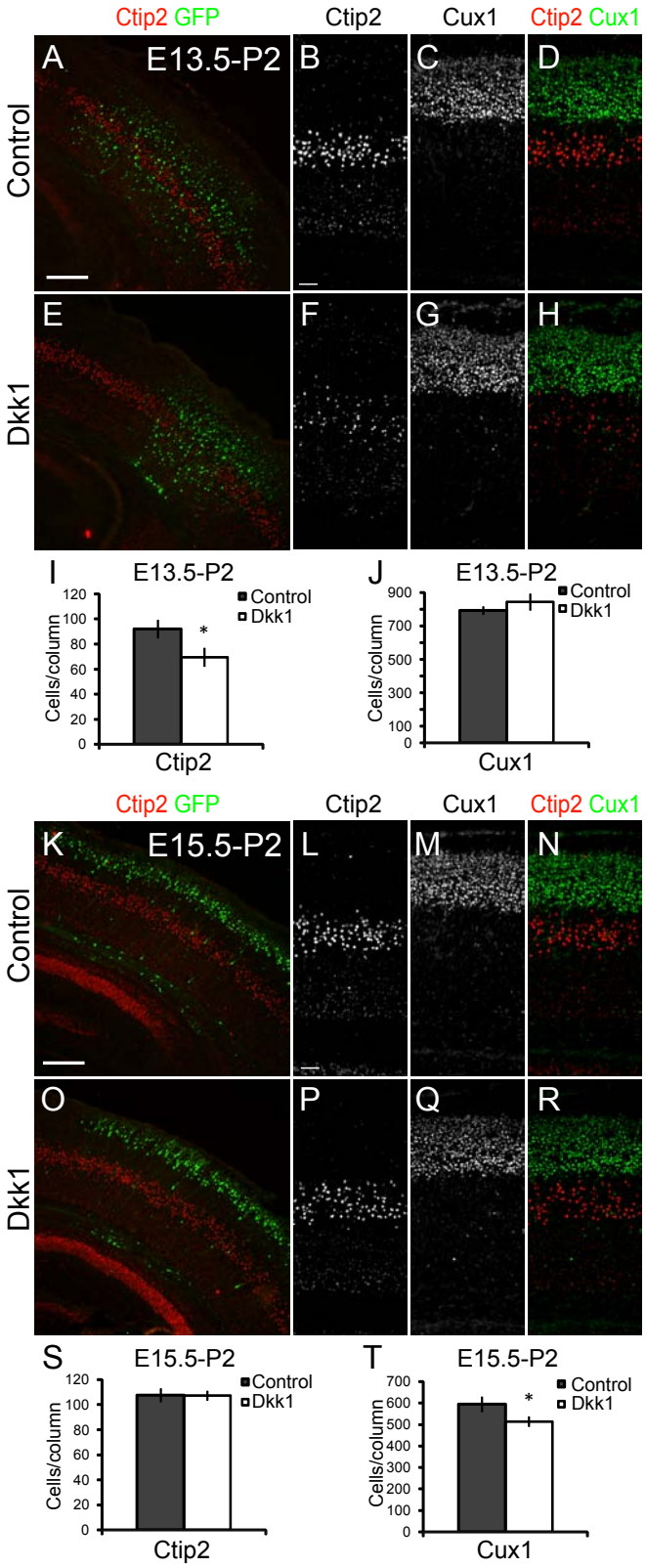
Ki67 GFP Ctip2

Figure 4.6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E13.5-P2
Control
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Cux1
Ctip2 Cux1
K
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L
M
N
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O
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R
Dkk1
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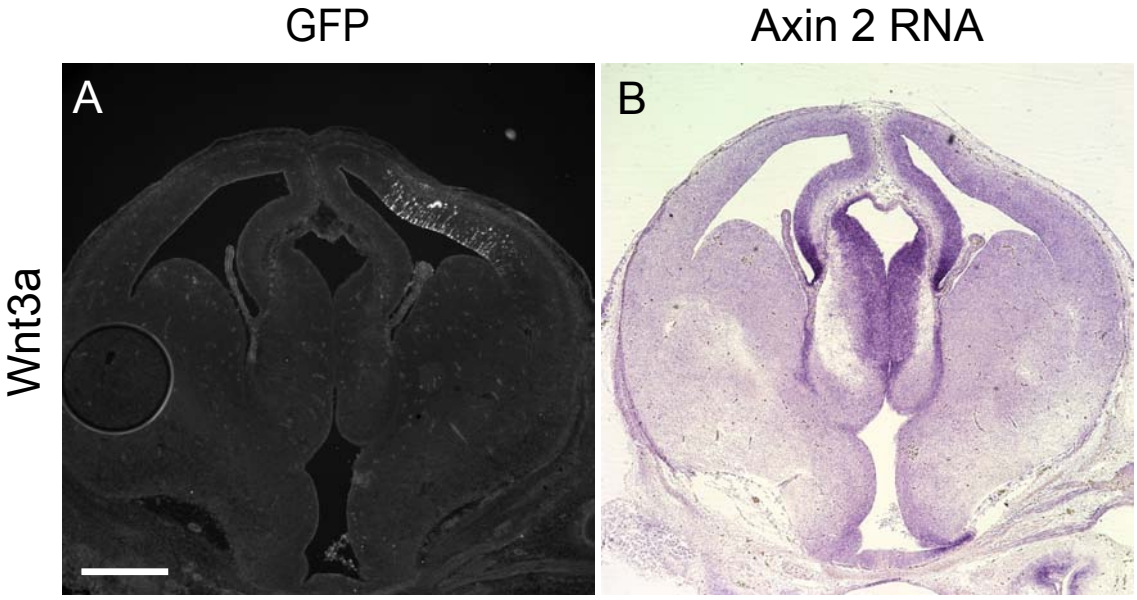
Figure 4.S1



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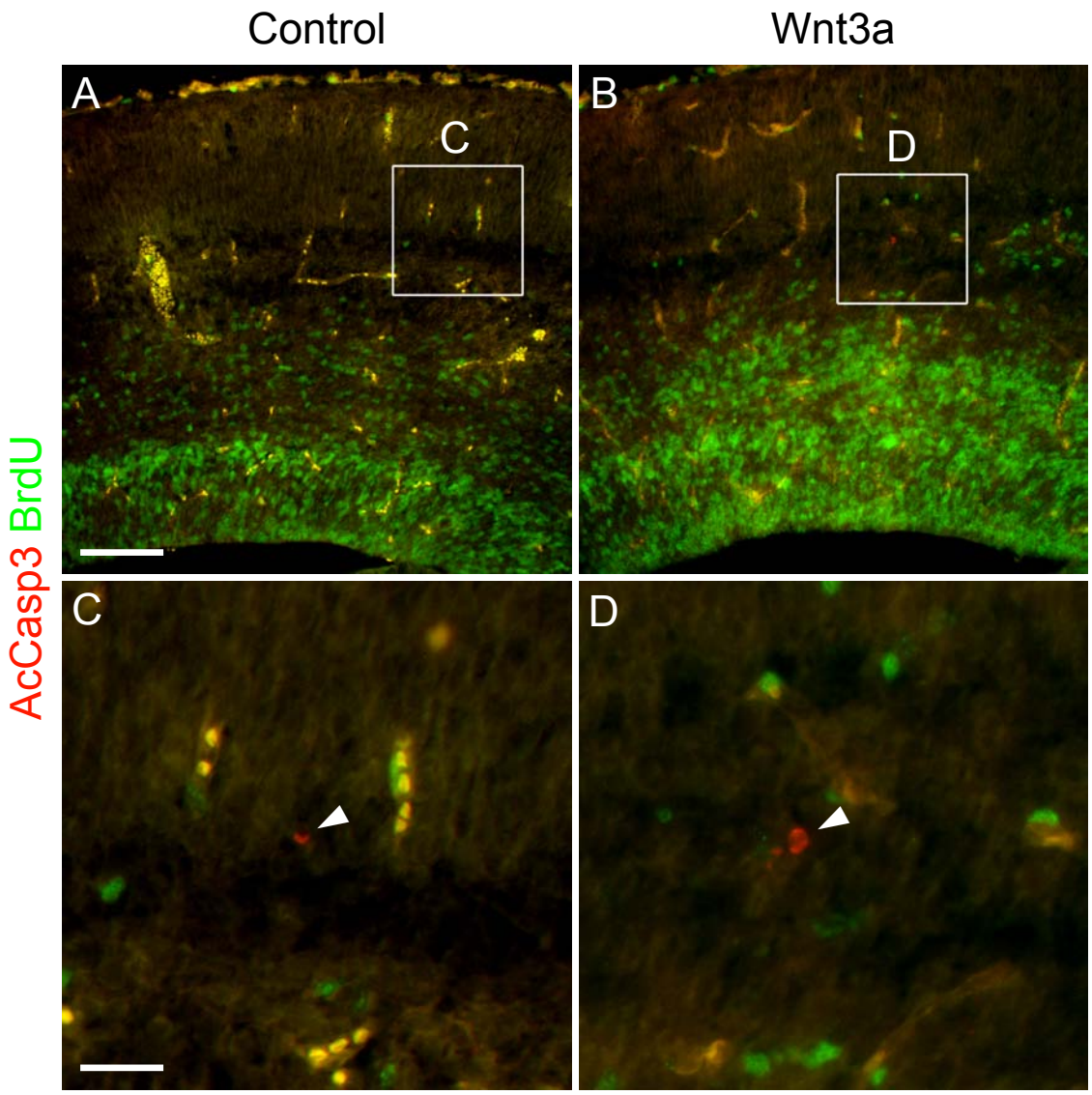

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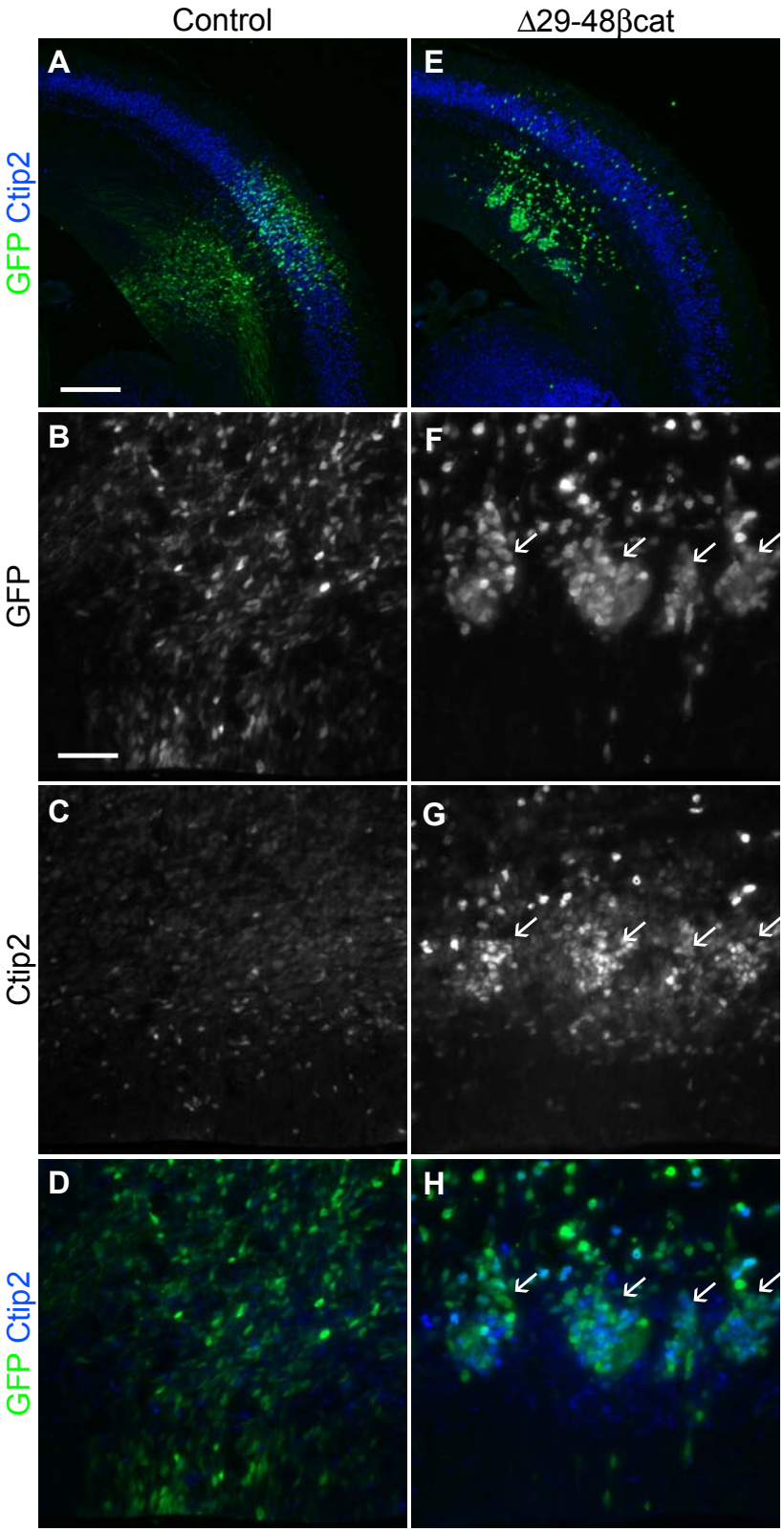

Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors

Chapter 5: Discussion

The IP stem cell pool is critical for the generation of the correct numbers and subtypes of neurons during mammalian neurogenesis. In this study we show that the WBP is active in neocortical IPs and required for production of projection neurons during mid and late stages of neurogenesis. We further show that the WBP plays a pivotal role in regulating IP differentiation and that dysregulation of the pathway can lead to cortical dysplasia and the formation of neuronal heterotopias.

The WBP regulates IP differentiation

Our data shows that induction of the WBP with excess ligand leads to early differentiation of IPs into neurons. These neurons fail to migrate to the cortical plate and accumulate within the UTD (Fig. 4.7A). Together these findings indicate that the WBP regulates the timing or progression of IP differentiation. These findings have not previously been described by other *in vivo* studies, perhaps because other studies relied solely on expression of intracellular signaling molecules that may have additional cell-autonomous consequences. Like our results, previous *in vitro* studies did show that Wnt-β-catenin signaling induces neuronal differentiation in neural precursor cell cultures (Hirabayashi et al., 2004). Moreover, the promoters of the proneuronal bHLH transcription factors, neurogenin1 (Ngn1) and neurogenin2 (Ngn2) contain LEF/Tcf elements and can be activated by β-catenin *in vitro* (Hirabayashi et al., 2004; Israsena et al., 2004), and loss-of-function mutation of β-catenin leads to loss of Ngn2 expression (Maretto et al., 2003). In accord with these previous findings, our data further implicates the WBP in specifically regulating the timing or progression of differentiation of IPs into projection neurons.

Superficially there appears to be a contradiction between the ample evidence that the WBP promotes symmetric progenitor proliferation during early and mid neurogenesis (Chenn and Walsh, 2002, 2003; Zechner et al., 2003; Woodhead et al., 2006; Wrobel et

al., 2007) and the studies showing that, beginning at mid neurogenesis, the WBP promotes differentiation of neuronal progenitors (Hirabayashi et al., 2004; Israsena et al., 2004; Hirabayashi and Gotoh, 2005; Guillemot, 2007). Retroviral infection of RG with either S33Yβcat or Wnt7a led to enrichment of infected cells in the SVZ (Viti et al., 2003; Kuwahara et al., 2010). These affected cells could be arrested in the SVZ due to early differentiation of IPs, similar to what we have observed with our results. Thus, our results along with other studies suggest that the WBP regulates both self-renewal and differentiation but in different pools of progenitors: self-renewal in RG and differentiation in IPs (Fig. 4.7B).

Complementing our findings using excess Wnt ligand, we found that downregulation of endogenous Wnt-β-catenin signaling with Dkk1 inhibits neuronal production during mid and late stages of neurogenesis. This implies a required role for the WBP in the regulation of IP behavior, since IPs generate a large proportion of neurons during mid neurogenesis and may function as the only neuron-generating progenitor pool during late neurogenesis (Noctor et al., 2007, 2008). Downregulation of endogenous Wnt-β-catenin signaling with Dkk1 likely reduces the number of IPs that differentiate over a given time period, leading to less neurons produced over the course of neurogenesis.

The differential effects of Wnt3a and LEF1-VP16 on the UTD subpopulation of IPs suggest that the DTD and UTD IP subpopulations have some distinctions. A dramatic loss of UTD IPs that coincided with the formation of ectopic neurons in the UTD was observed in Wnt3a-electroporated brains, but the size and proliferation level of DTD IPs were unaffected. Thus, it appears that there is specificity in the response of UTD IPs to Wnt-β-catenin signaling. We defined the subpopulations of Tbr2+ IPs into the DTD and UTD domains in this study based on the Wnt3a phenotype we observed as there are

currently no known molecular markers that define the DTD and UTD IP subpopulations. However, based on our results there must be some cellular distinctions between these subpopulations of IPs, although they may have a precursor-product relationship.

Excess Wnt signaling leads to cortical dysplasia and neuronal heterotopias.

Long-term upregulation of the WBP with Wnt3a resulted in cortical dysplasia associated with the formation of large neuronal heterotopias. The heterotopias are composed of both early-born/deep layer and late-born/upper layer projection neurons and contained extensive axonal and dendritic processes (data not shown). These features are similar to those found in cortical malformations of patients (Fox and Walsh, 1999; Lu and Sheen, 2005; Lian and Sheen, 2006). Our findings indicate possible roles for the WBP in cortical malformation disorders.

We observed several factors that likely contributed to these findings in the Wnt3a-electroporated brains. Ectopic differentiation of IPs in the UTD is most likely the direct factor for the formation of ectopic neurons in progenitor domains. We observed the stepwise expansion of the pool of ectopic neurons in progenitor domains from E16.5 to E18.5 to P2. Secondly, the abnormal horizontal expansion of RG, observed at E16.5, would eventually lead to extra IPs and neurons. To this end, a dramatically larger population of Tbr2+ IPs is present at P2 in Wnt3a-electroporated brains compared to controls. Third, we observed in some samples Ctip2+ nodules protruding at the ventricular surface of the neocortex of Wnt3a-electroporated brains at E16.5 (asterisks, Fig. 4.2S-V). These are likely RG that have directly differentiated into neurons within the VZ. Lastly, disorganization and reorganization of RG and IPs into rosettes, which we observed at E18.5 and P2, could lead to disruption of migration of daughter neurons to the cortical plate.

In conclusion, we've defined new roles for the Wnt signaling pathway in controlling the terminal differentiation of IPs and shown that dysregulation of this signaling axis can

lead to cortical malformations similar to those seen in some patients. This will make it attractive in the future to consider the Wnt signaling pathway as a target for mutations in the inherited disorders of cortical migration.

References of Chapter 5: Discussion

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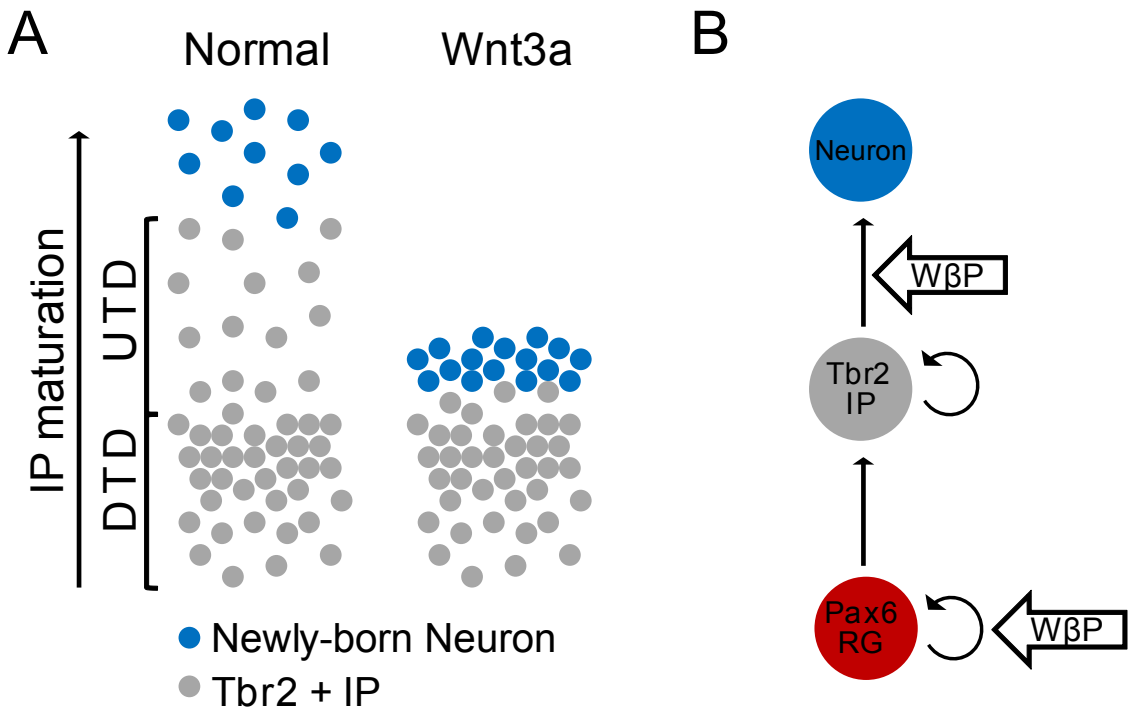
Zechner D, Fujita Y, Hulsken J, Muller T, Walther I, Taketo MM, Crenshaw EB, 3rd, Birchmeier W, Birchmeier C (2003) beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev Biol 258:406-418.

Figure 5.1. The WBP regulates RG self-renewal and IP differentiation. (A) Schematic comparing the effect of ectopic Wnt3a to the normal distribution of IPs and newly-born IP-derived neurons. The current understanding of IP behavior indicates that IPs differentiate as they migrate from the ventricular zone towards the cortical plate. Our results indicate that excess Wnt3a advances the maturation process of IPs, causing IPs to ectopically differentiate into neurons in the UTD. The resulting neurons fail to migrate to the cortical plate and accumulate in the UTD to form a neuronal heterotopia. (B) Our results also show that the WBP (WβP) plays a dual role in neocortical neurogenesis, promoting RG self-renewal and IP differentitation. DTD-deep Tbr2 domain, UTD-upper Tbr2 domain.

Figure 5.1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IP maturation
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DTD
Newly-born Neuron
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IP
Pax6
RG
WβP

Wnts influence the timing and efficiency of OPC generation in the telencephalon

Chapter 6: Summary of Secondary Project

Overview

The WBP has previously been shown to regulate different aspects of oligodendrogenesis (Kessaris et al., 2008). Here, we show that the WBP inhibits the production of oligodendrocyte precursor cells (OPCs) using both *in vitro* and *in vivo* approaches. Our in vivo results show that downregulation of the WBP with a dominant negative form of LEF1 transcription factor or Dkk1 secreted antagonist promoted early production of OPCs in the neocortex. Supplementary background information, a summary of this work and the manuscript are presented below.

Neocortical oligodendrocyte precursor cells (OPCs)

The glial cells of the neocortex, astrocytes and oligodendrocytes, arise from different immediate progenitors. At the end of neurogenesis, RG cells undergo their last terminal cell divisions that generate astrocytes (Noctor et al., 2002; Anthony et al., 2004). Neocortex-derived OPCs begin to be produced at birth (Postnatal day zero/P0), robustly produced by P5 and mature into oligodendrocytes in later postnatal stages. The precursor cells of neocortex-derived OPCs are ultimately derived from the neuroepithelial progenitor cells that also give rise to RG cells. However, these OPC progenitors appear to remain dormant until postnatal stages (Kessaris et al., 2006; Kessaris et al., 2008).It is important to note that OPCs that are detected in the neocortex are not all generated from neocortical progenitors. At embryonic day 13 (E13), OPCs begin to be generated from the medial glanglionic eminence (MGE) and anterior entopenduncular area (AEP). Additionally at E15, OPCs begin to be generated from the lateral ganglionic eminence (LGE) and caudal ganglionic eminence (CGE). Both populations of OPCs migrate to populate ventral and dorsal domains of the forebrain, including the neocortex (Kessaris et al., 2008).

The WBP inhibits multiple steps of oligodendrogenesis

Current data suggests the WBP inhibits generation of OPC production and differentiation (Kessaris et al., 2008). Induction of the WBP in spinal cord explants, promotes differentiation of OPCs into mature oligodendrocytes (Shimizu et al., 2005). This finding is further supported by a study that genetically induced the WBP with dominant active β-catenin in the oligodendrocyte lineage. Dominant activation of the WBP inhibited the production of OPCs and oligodendrocytes of the spinal cord and forebrain (Ye et al., 2009). However, the genetic method used to express dominant active β-catenin utilized the Olig1-cre, a genetic line that expresses very early in forebrain development. It is unclear whether this study tested the role of the WBP in the specification of an early precursor of the oligodendrocyte lineage or the production of OPCs at P0. Therefore, the question whether the WBP inhibits the production of neocortex-derived OPC remains open.

Summary

Neocortex-derived OPCs begin to be generated at birth. Strong WBP activity in the neocortex that weakens as development completes (Machon et al., 2007) appears to coincide with the onset of production of neocortex-derived OPCs. Moreover, continued activation of the WBP in the oligodendrocyte lineage of the forebrain from early development to postnatal ages completely blocks the production of OPCs and oligodendrocytes (Ye et al., 2009). In accord with these data, we hypothesized that the WBP might function to inhibit the onset of production of neocortex-derived OPCs till birth. We first characterized the effects of perturbing the WBP in cultured cells. We show that soluble Wnt3a protein inhibits the production of OPCs in primary cultures of perinatal neocortical progenitors (termed neural precursor cells or neural progenitor cells (NPCs)). Conversely, soluble Dkk1 protein or transfected dominant negative LEF1 promote production of OPCs from NPCs. These results indicate that neocortical OPC

production is influenced by the WBP. We further analyzed the role of the WBP *in vivo*. We specifically tested whether blocking the WBP 3.5 days before birth will prematurely initiate OPC production in the neocortex. We discovered that downregulation of the WBP with a dominant negative form of LEF1 transcription factor or Dkk1 secreted antagonist promotes early production of OPCs in the neocortex. Thus, endogenous WBP activity in the neocortex likely functions to regulate the timing of production of neocortex-derived OPCs.

Manuscript:

Wnts influence the timing and efficiency of OPC generation in the telencephalon

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Abstract

Oligodendrocyte precursor cells (OPCs) are generated from multiple progenitor domains in the telencephalon in developmental succession from ventral to dorsal. Previous studies showed that Wnt signaling inhibits the differentiation of OPCs into mature oligodendrocytes. Here we explored the hypothesis that Wnt signaling limits the generation of OPCs from neural progenitors during forebrain development. We manipulated Wnt signaling in mouse neural progenitor cultures and found that Wnt signaling influences progenitors cell autonomously to alter the production of OPCs, and that endogenous Wnt signaling in these cultures limits the efficiency of generating OPCs from neural progenitors. To examine these events *in vivo,* we electroporated a soluble Wnt inhibitor or a dominant-negative transcriptional regulator into embryonic mouse neocortical ventricular zone before the usual onset of OPC production and showed that decreasing Wnt signaling in the cortical progenitor domain results in early production of OPCs. Our studies indicate that Wnt signaling influences the timing and extent of OPC production in the developing mammalian telencephalon.

Introduction

Oligodendrocyte precursor cells (OPCs) are generated from distinct progenitor zones in the forebrain beginning at various times during embryonic development (Kessaris et al., 2006). The mechanisms regulating the spatial and temporal production of OPCs have not been clearly elucidated; in particular, it is not clear why OPCs appear at later stages dorsally than ventrally. Sonic Hedgehog regulates the production of OPCs from ventral progenitor zones, but the signaling pathways that regulate the dorsal generation of OPCs are only beginning to be elucidated (Richardson et al., 2006).

Wnts are secreted glycoprotein ligands that regulate development of dorsal CNS structures (Lee and Jessell, 1999). Wnts have prominent roles in progenitor cell

proliferation, cell lineage and differentiation decisions (Freese et al., 2009). In the telencephalon Wnt signaling plays a prominent dorsalizing role (Lee et al., 2000; Kim et al., 2001; Backman et al., 2005; Zhou et al., 2006; Machon et al., 2007; Chenn, 2008; Gulacsi and Anderson, 2008; Freese et al., 2009). Some have suggested that the high-low, dorsal-ventral gradient of Wnt signaling in the cortex is related to the gradient of cortical neuronal differentiation (Machon et al., 2007). Studies have shown that Wnts inhibit the differentiation of OPCs into oligodendrocytes and thereby regulate myelination and remyelination following injury (Shimizu et al., 2005; Fancy et al., 2009; Ye et al., 2009).

These factors all raise the possibility that Wnt ligands in the dorsal forebrain influence the generation of OPCs from neural progenitors, thereby controlling the timing of OPC production. A recent report (Ye et al., 2009) demonstrated that disrupting β-catenin changes the number of OPCs in the developing CNS and that Wnts work with histone deacetylases to regulate oligodendrocyte differentiation. However, this study leaves a number of unanswered questions since it relied on the Olig1-credriver line expressed very early in the forebrain and on manipulating β-catenin directly. Reliance on loss-of-function alleles of β-catenin does not allow exclusion of non-Wnt functions of β-catenin in the ventricular zone such as cell adhesion. Also, reliance on the dominantly active β-catenin can lead to effects that are not normally regulated by Wnt signaling *in vivo*.

We wanted to determine whether Wnt signaling influences the generation of OPCs from neural progenitor cells and whether it plays a role in the delayed appearance of OPCs from dorsal telencephalic regions. We regulated the levels of extracellular Wnt ligand and cell-autonomously altered Wnt signaling. We show that Wnt signaling negatively regulates the specification of OPCs from neural progenitors and that inhibition of Wnt signaling drives a robust pre-scheduled increase in the production of OPCs in the

cortex. This indicates that Wnts influence the timing and balance of OPC production from ventricular zone progenitors.

Methods

Animals. All animal protocols were approved by the UCSF IACUC. P5 CD1 pups (Charles River) were used for cultures. BAT-gal mice (Maretto et al., 2003) were used for β-galactosidase (β-gal) expression analysis and for DNLef1 electroporations. C57Bl6 mice (Jackson) were used for Dkk1 electroporations. Male and female mice were used for all experiments.

Retroviral production. pLNCX2 (Clontech) was modified to include a new multiple cloning site and histone H2B fused to monomeric red fluorescent protein (pL*H2BmRFP*). A dominant-negative Lef1 (DNLef1) construct (Lef1 (Clontech) with a truncated N-terminus) was inserted. Retrovirus was produced in GP2-293 cells (Clontech) and concentrated using standard methods.

In utero electroporations (EP). See (Li et al., 2008) for method. EPs were targeted to the lateral cortex at E13.5 or E15.5 and collected 4 or 6 days later. The DNLef1 construct was inserted into pCAGGS (chicken β-actin cytomegalovirus promoter-driven expression). pCIG (GFP) (from A. McMahon (Megason and McMahon, 2002)) was used as a control. pCIG-Dickkopf-1 was provided by L.W. Burrus.

Immunohistochemistry and X-Gal staining. Brains were processed using standard methods. Cells and tissue sections were stained using standard protocols with: rat anti-PDGFRα (BD Biosciences Pharmingen,1:500 for tissue and 1:800 for cells), rabbit anti-Olig2 (Millipore, 1:1000 for tissue), mouse anti-β-gal (Promega, 1:2000 for tissue and 1:6000 for cells), and mouse anti-O4 (Sigma, 1:30 for cells). Fluorescent secondary antibodies (1:1000, Invitrogen) and DAPI (1:3000, Sigma) were used. For X-gal staining, floating sections were stained with X-gal substrate (Invitrogen).

Cell Culture. Neural progenitor cells (NPCs) were from lateral ventricle SVZ tissue of P5 CD1 mice. Tissue was dissociated with 0.1% Trypsin (Worthington) and 0.1% DNAse1 (Roche). DMEM/F12 (50:50, Gibco) was supplemented with 10X hormone mix (40 mg transferrin, 10 mg insulin, 3.86 mg putrescine, 4.0 ml 3mM selenium, 4.0 ml 2mM progesterone, 10 ml 2 mg/ml Heparin [all from Sigma]), 0.8 ml 30% glucose, 0.6 ml 7.5% $NaHCO_3$, 10 ml 30% glucose, 7.5 ml 7.5% $NaHCO_3$, 2.5 ml 1 M HEPES, 5 ml 200 mM glutamine, 5 ml Pencillin-Streptomycin, 2 ml Fungizone). Media containing EGF (Sigma, 10 ng/ml), FGF (Sigma, 20 ng/ml) and B27 (Invitrogen) was added. Cells were plated at 80,000 cells per 25 cm^2 flask (Corning), grown in 5 ml of complete media plus growth factors and B27. Passage 1 or 2 NPCs were used.

Cell culture – Wnt treatments. NPCs were dissociated with 0.05% trypsin-EDTA, washed with PBS, pelleted and resuspended in 500μl of complete media with 2% FBS. The cells were plated on laminin-coated (1 mg/ml, Invitrogen) chamber slides (Nunc) at 37,500 cells/well. Mouse Wnt3a (0.15ng/μL), Dickkopf1 (Dkk1, 0.03ng/μL), or an equivalent volume of PBS were added to each well. One half of the media was replaced each day. The cultures were analyzed after 4 days *in vitro* (DIV).

Cell culture – Retroviral infection. NPCs were dissociated, washed with PBS, pelleted and resuspended in 500μl complete media containing EGF, FGF, B27 and 5 μg/ml polybrene (Sigma). Concentrated virus (50-100 μl; approx $1x10^5$ transducing units) was added to approximately 300,000 cells, which were spin infected for 90-120 min at 170 x *g*. The cells were plated in complete media containing growth factors in a 25cm^2 flask for 24 h to allow integration and expression. Cells were resuspended in complete media with 2%FBS and plated on chamber slides at approximately 37,500 cells/well and analyzed after 4 DIV.

Image analysis and quantification. Three to four biological replicates of each experiment were performed. Ten 20X magnification images were quantified for each replicate of each cell culture condition. Cell counts from 3-4 coronal sections of each EP brain were averaged.

Statistical analysis. Results are expressed as mean ± SEM. Data were analyzed using two-tailed Student's *t* test with unequal variance. Multiple comparisons were made using ANOVA with a post-hoc Holm-Sidac test. Any value of $p \leq 0.05$ was considered significant.

Results

Wnt signaling regulates OPC generation from telencephalic progenitor cells

To test the role of Wnt signaling in the generation of OPCs, we cultured neural progenitors from the telencephalons of perinatal mice. We plated the progenitors and exposed them to FBS to induce differentiation along with soluble mouse Wnt3a ligand (Fig. 6.1*C,D*), soluble mouse Dickkopf1 (Dkk1) Wnt inhibitor (Fig. 6.1*E,F*), or an equivalent volume of PBS as a control (Fig. 6.1*A,B*) for 4 days *in vitro* (DIV) and then labeled the cells with the OPC markers PDGFRα and O4. Treatment with Wnt3a caused a 25% decrease in the percentage of OPCs and inhibition of Wnt signaling by Dkk1 caused a 54% increase in the percentage of OPCs compared to controls (Fig. 6.1*K*). This was not a result of changes in the proliferation or apoptosis of OPCs (Fig. 6.1*L*). These results indicate that Wnt signaling influences the generation of OPCs from progenitor cells.

To determine whether this is a cell-autonomous effect on the progenitor cells, we infected progenitors with a retrovirus encoding a dominant-negative form of Lef1 (DNLef1-mRFP) (Fig. 6.1*I,J*, arrowheads indicate infected OPCs) or a nuclear mRFP control vector (Fig. 6.1*G, H*). PDGFRα and O4 labeling revealed a doubling in the

percentage of infected OPCs 4 days post infection (Fig. 6.1*M*). To ensure that this effect was actually autonomous, we counted the OPCs among uninfected cells in these cultures and found no difference between control and experimental conditions (Supplemental Fig. 6.S1).

Our data indicate that Wnt signaling influences the production of OPCs from neural progenitors, but we wanted to determine whether high levels of Wnt signaling are inconsistent with the decision of neural progenitors to generate OPCs. To examine this, we cultured neural progenitors from a Wnt signaling reporter mouse (BAT-gal). These cultures were differentiated for 4 DIV and then labeled with antibodies for β-gal (Fig. 6.1*N*, arrows) and PDGFRα (arrowheads). Similar to previously published reports (Fancy et al., 2009; White et al., 2009), we found that β-gal was expressed in approximately 6% of the OPCs in our cultures. This indicates that a part of the normal differentiation program for OPCs involves a lack of significant Wnt signaling at the time of OPC specification.

Wnt signaling regulates the timing of OPC generation during development

A previous report (Kessaris et al., 2006) showed that there are multiple waves of OPC production beginning ventrally and that cortical production does not begin until ~P0. We wanted to assess global levels of Wnt signaling in the cortical progenitor zone before and after the onset of OPC production from the cortex. X-gal staining of BAT-gal brains revealed that Wnt signaling is very high in cortical progenitors at E17.5 (before cortical OPC production) and decreased by P5 when OPC production is prominent (Fig. 6.2*A,B*). Labeling these brains with antibodies for β-gal and PDGFRα (Fig. 6.2*C,F*) showed that at E17.5 there are many cells in the cortical VZ/SVZ/IZ and many presumptive neurons in the cortex that have strong Wnt signaling (Fig. 6.2*D*,*E*), but that at P5 the number of cells near the ventricle that show strong Wnt signaling was decreased and that the OPCs in the cortex do not show evidence of strong Wnt

signaling (Fig. 6.2*G,H*). It should be noted that at E17.5 many OPCs that were generated from ventral progenitor domains had migrated to the cortex and were within the region of strong cortical Wnt signaling (Fig. 6.2*C,D*) (Kessaris et al., 2006), but no OPCs are generated from the cortical progenitor zone at this age (Fig. 6.2*E*) (Kessaris et al., 2006). We have replicated the lineage tracing of cortically derived OPCs performed previously (Kessaris et al., 2006) using Emx1-Cre and found that few if any OPCs in the cortex at P0 are derived from the cortical progenitor domain at P0 but that by P4 cortically derived OPCs were abundant (data not shown). At P5 OPCs were throughout the cortex (Fig. 6.2*F*), including in the progenitor zone, which showed reduced Wnt signaling (Fig. 6.2*H*). At this stage, many of the cells that showed the strongest Wnt signaling are associated with presumptive blood vessels (Fig. 6.2*G* arrowhead) (Franco et al., 2009).

Manipulation of Wnt signaling in progenitors changes the timing of OPC generation

The timing of decreasing Wnt signaling in cortical progenitors raises the possibility that OPC generation from the cortex is limited by the strong Wnt signaling present prenatally and that this is a factor in determining the onset of OPC production. To test this idea we performed *in utero* electroporations (EP) of a DNLef1 or control mRFP construct into neocortical progenitors at E15.5 and collected the embryos 4 days later at P0.5 (Fig. 6.3*A,B*, arrowheads indicate EP OPCs). This approach alters the level of Wnt signaling in primary multipotent cortical progenitors and addresses whether Wnt signaling influences production of OPCs from these cells. PDGFRα and Olig2 antibody labeling showed that there was a 66% increase in the percentage of EP OPCs after DNLef1 electroporation compared to controls (Fig. 6.3*E*). No changes in the mitotic fraction or apoptosis of EP OPCs were observed (data not shown). This indicates that

shutting off the transcriptional output of the Wnt pathway in progenitors drives an increase in the production of OPCs.

To determine whether the endogenous levels of Wnt ligand present in the brain limit early OPC production, we performed EPs of Dkk1 or control GFP (Fig. 6.3*C, D*, white boxes indicate region of quantification) at E15.5 and collected the brains 6 days later. PDGFRα and Olig2 antibody labeling showed a 61% increase in the number of OPCs in the EP region (Fig. 6.3*F*). This manipulation is not cell-autonomous and influences both the neural progenitors and the OPCs already present in the cortex. Since others have shown that Wnt signaling is observed in a subset of cortical OPCs and may drive their proliferation (Fancy et al., 2009; White et al., 2009), the Dkk1 EP may decrease proliferation of OPCs already present in the cortex. Therefore, the decrease in Wnt signaling in the progenitor domain must cause an increase in the number of OPCs produced which overcomes their decreased drive to proliferate. This indicates that decreasing Wnt signaling in progenitors or decreasing their exposure to endogenous levels of Wnt ligand in the environment leads to an early onset of cortical OPC generation.

Discussion

Our study shows that Wnt signaling in progenitor cells influences the generation of OPCs in a cell-autonomous manner. Strong Wnt signaling inhibits OPC generation and low levels of Wnt signaling both accompany and drive OPC generation. This mechanism is used during development to influence the timing of OPC generation and is a significant factor influencing the efficiency of OPC production from neural precursor cells.

Decreased Wnt signaling accompanies the appearance of and drives production of OPCs

We have shown that Wnt signaling in the cortical progenitor domain is strong at embryonic ages when mostly neurons are produced and that there is a significant decrease in Wnt signaling when OPCs are generated. The addition of Wnt3a ligand to neural progenitor cultures causes a significant decrease in the percentage of OPCs produced from these progenitors, whereas addition of Dkk1 strongly increases the percentage of OPCs produced. This indicates that Wnt ligands and inhibitors are capable of acting directly on neural progenitors and that changes in the extracellular concentrations of Wnts and inhibitors can significantly influence the lineage decisions of progenitors. The lesser effect of Wnt3a compared to Dkk1 implies that significant Wnt ligand levels natively found in these culture systems are one inhibitor of OPC production.

Retroviral infection of DNLef1 into neural progenitors drove a significant increase in the percentage of OPCs produced from these infected progenitors without influencing the uninfected cells. This indicates that Wnt signaling acts autonomously to influence lineage decisions of individual progenitor cells, and that lower Wnt signaling levels favor the production of OPCs. Previous reports indicate that Wnt signaling drives proliferation in OPCs (Fancy et al., 2009; White et al., 2009) and blocks terminal differentiation during development and remyelination (Shimizu et al., 2005; Fancy et al., 2009; Ye et al., 2009). We did not see these effects in our neuronal progenitor cultures when exposed to Wnt ligand or inhibitors for short periods of time. However, when we exposed our cultures to FBS plus Wnt3a ligand for 8 days we observed a 46% increase in the percentage of OPCs in the Wnt3a condition caused by a 257% increase in the proportion of proliferating OPCs (apoptosis of OPCs does not explain these changes) (Supplemental Fig. 6.S2).

To test the influence of Wnt signaling on the timing of OPC production from cortical progenitors, we electroporated a Dkk1-expressing construct into the progenitor zone of the lateral embryonic cortex at E15.5, before the time of cortical OPC production. Dkk1 is a secreted protein which prevents activation of the Wnt pathway by binding to the Wnt receptor LRP5/6 (Kawano and Kypta, 2003). At P2 the number of OPCs observed in the electroporated region was increased despite the potential for decreased proliferation of the earlier born OPCs caused by decreased Wnt signaling (Fancy et al., 2009; White et al., 2009). This indicates that Wnts act on the cortical progenitors and that decreasing Wnt signaling in a physiologically relevant manner, without manipulating intracellular Wnt signaling components, influences the cell types produced from cortical progenitors.

We also determined that Wnt signaling acts on the cortical progenitors in a cell-autonomous manner by electroporating a DNLef1-expressing construct into the lateral cortical progenitor zone at E15.5 and examining OPC production 4 days later, at P0.5 when cortical production of OPCs normally begins (Kessaris et al., 2006). These studies show that shutting off the transcriptional output of the Wnt pathway in the primary progenitors of the embryonic cortex and their progeny significantly increases the percentage of electroporated cells becoming OPCs. This indicates that Wnt signaling acts cell-autonomously on cortical progenitors to influence the production of OPCs.

Wnt signaling is not the only mechanism influencing the production of OPCs. We electroporated DNLef1 into the lateral cortical progenitor zone at E13.5 and analyzed the brains at E17.5, several days before the reported onset of cortical OPC production (Kessaris et al., 2006). We observed no significant difference in the percentage of electroporated cells positive for OPC markers (data not shown). This indicates that there are additional mechanisms inhibiting OPC production at earlier stages in development.

Our studies indicate that high Wnt signaling has contrasting effects on the oligodendrocyte lineage at different developmental stages – blocking specification of OPCs but driving the proliferation of already established OPCs (this latter finding in agreement with previous publications (Fancy et al., 2009; White et al., 2009)). In addition, Wnt signaling is known to inhibit OPC differentiation during development and remyelination (Shimizu et al., 2005; Fancy et al., 2009; Ye et al., 2009). Thus Wnt signaling plays multiple roles at distinct points in the oligodendrocyte lineage. Possible mechanisms for the effects of Wnt signaling on OPC specification come from previous studies showing that Wnt signaling down regulates Olig2 gene expression in HCN cells (Ye et al., 2009) and induces the expression of the neurogenic gene NeuroD1 in adult hippocampal sub-granular zone progenitors (Gao et al., 2009; Kuwabara et al., 2009). If a similar mechanism exists in the SVZ during development, it could strongly favor neuronal production.

References of Chapter 6: Wnts influence the timing and efficiency of OPC generation in the telencephalon

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Figures of Chapter 6: Wnts influence the timing and efficiency of OPC generation in the telencephalon

Figure 6.1. High Wnt tone decreases OPC production from neural progenitors while low Wnt tone enhances production. Application of Wnt3a to neural progenitor cultures for 4 days decreased the number of PDGFRα- (***C***, 11.34±0.93%, mean percent of total cells±standard error) and O4-positive cells (***D***, 8.40±0.53%) and application of Dkk1 antagonist increased the number of OPCs (***E***, 23.14±1.88%***; F*** 15.48±1.56%) compared to control cultures (***A***, 15.06±0.89%***, B***, 11.38±0.75%). This is graphed in (***K***). This is not a result of proliferation (Ki-67) or apoptosis (actived Caspase3) of OPCs as the percentage of proliferating OPCs was greatest in the Wnt3a group (3.19±0.65%, Control = 1.89±0.46%, Dkk1 = 2.30±0.27%) and the percentage of apoptotic OPCs was greatest in the Dkk1 group and significantly greater than control (Dkk1 = 1.78±0.49%, Control = 0.15±0.08% Wnt3a = 0.20±0.09%) (***L***). Retroviral infection of neural progenitors with DNLef1-mRFP results in a significant increase in PDGFRα-positive cells (***I***, 23.74±3.26%) and O4-positive cells (***J***, 20.07±1.99%) by 4 days post infection compared to mRFP control infection (***G***, 8.18±1.18%; ***H***, 10.12±1.62%). Arrowheads indicate infected OPCs. This is graphed in (***M***). N = 3 for all groups. Neural progenitor cells were cultured from BAT-gal mice. (***N***) 6.32±1.13% of PDGFRα-positive BAT-gal cells (arrowheads) show detectable Wnt signaling (arrows). Scale bars = 10μm. (*****) indicate $p \leq 0.05$ for ANOVA in (***K***) and Student's *t* test in (***L*** and ***M***).

Figure 6.2. Wnt tone decreases in progenitor zones before they produce OPCs. ***A, B***, BAT-gal sections were treated with Xgal. ***A***, Cortical cells have high Wnt tone during neurogenesis (E17.5). ***B***, Wnt tone in the cortex and SVZ is significantly decreased by the time of OPC production (P5). ***C-H***, β-galactosidase and PDGFRα immunohistochemistry of BAT-gal forebrain. ***C***, At E17.5, cells near the ventricle and in

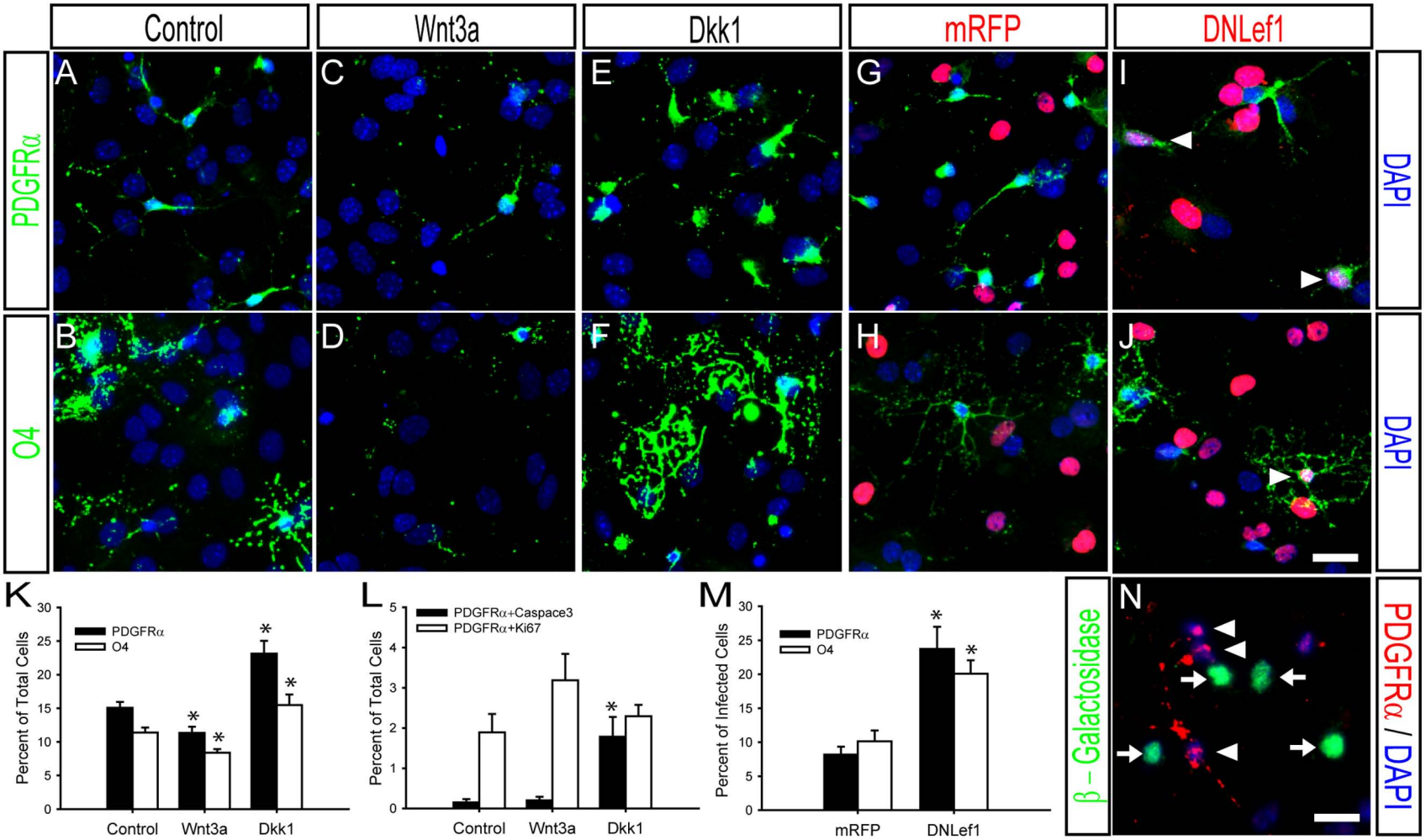
the cortex show strong Wnt signaling. Higher magnification images (***D***, ***E***). ***F***, By P5 only cortical blood vessels and scattered cortical cells show strong Wnt tone. Higher magnification images (***G***, ***H***). Most PDGFRα-positive cells do not have strong Wnt signaling. Lines indicate lateral ventricle. Arrowhead indicates blood vessel. Scale bars = 500μm.

Figure 6.3. Decreasing Wnt tone in cortical progenitors results in a pre-scheduled increase in cortical OPC production. E15.5 EP of mRFP control (***A***) or DNLef1-mRFP (***B***) into the lateral cortex were collected at P0. DNLef1 EP caused an increase in the percentage of EP PDGFRα (DNLef1 = 9.87±1.66%, RFP = 4.86±0.75%) and Olig2-positive cells (not shown, DNLef1 = 9.62±1.13%, RFP = 3.24±1.11%). Arrowheads indicate EP cells that are PDGFRα-positive. This is graphed in ***E***. E15.5 EPs of GFP control (***C***) or Dkk1-GFP (***D***) into the lateral cortex were collected at P2. A region of interest was selected in the center of the EP area (white box) and the total number of PDGFRα or Olig2-positive cells within the region were quantified. Scale bar = 500μm. ***F***, Dkk1 EP resulted in a significant increase in the number of PDGFRα (205.25±3.26) or Olig2-positive cells (346.50±28.83) in the EP region compared to control (116.75±9.92 PDGFRα and 215.50±16.25 Olig2). N = 3-4 for all groups. (*****) indicates $p \leq 0.05$ for Student's *t* test in (***E*** and ***F***).

Supplemental Figure 6.S1. DNLef1 infection of neural progenitors does not alter OPC production from uninfected cells. Uninfected cells from DNLef1 or mRFP infected cultures were quantified for PDGFRα expression at 4 DIV. There was no difference in the percentage of uninfected OPCs (mRFP = 8.56±4.23%, DNLef1 = 10.13±1.01%).

Supplemental Figure 6.S2. Wnts induce OPC proliferation. Application of Wnt3a to neural progenitor cultures for 8 days significantly increased the number of PDGFRα-positive cells (27.28±2.09%) compared to control cultures (***A***, 18.66±1.19%). This is a result of increased proliferation of OPCs as the percentage of proliferating OPCs was greatest in the Wnt3a group (13.15±3.11%, Control = 3.68±0.38%) and was not a result of apoptosis (***B***, Wnt3a = 3.11±0.79%, Control = 0.35±0.13%). (*****) indicate $p \leq 0.05$ for Student's *t* test.

Figure 6.1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I
J
K
L
M
N
PDGFRα
O4
Percent of Total Cells
Control
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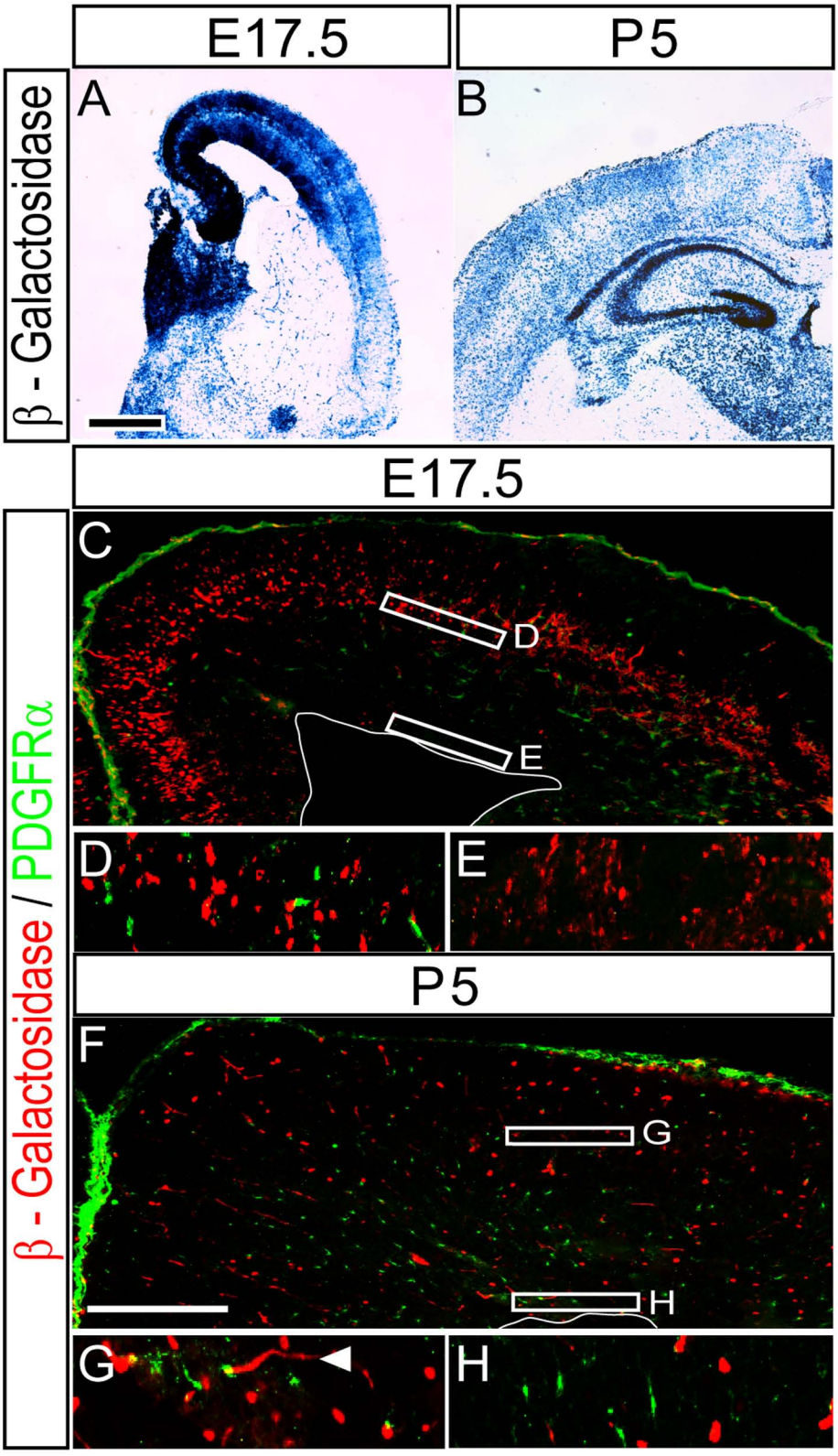
PDGFRα / DAPI

Figure 6.2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β - Galactosidase
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B
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G
H
G
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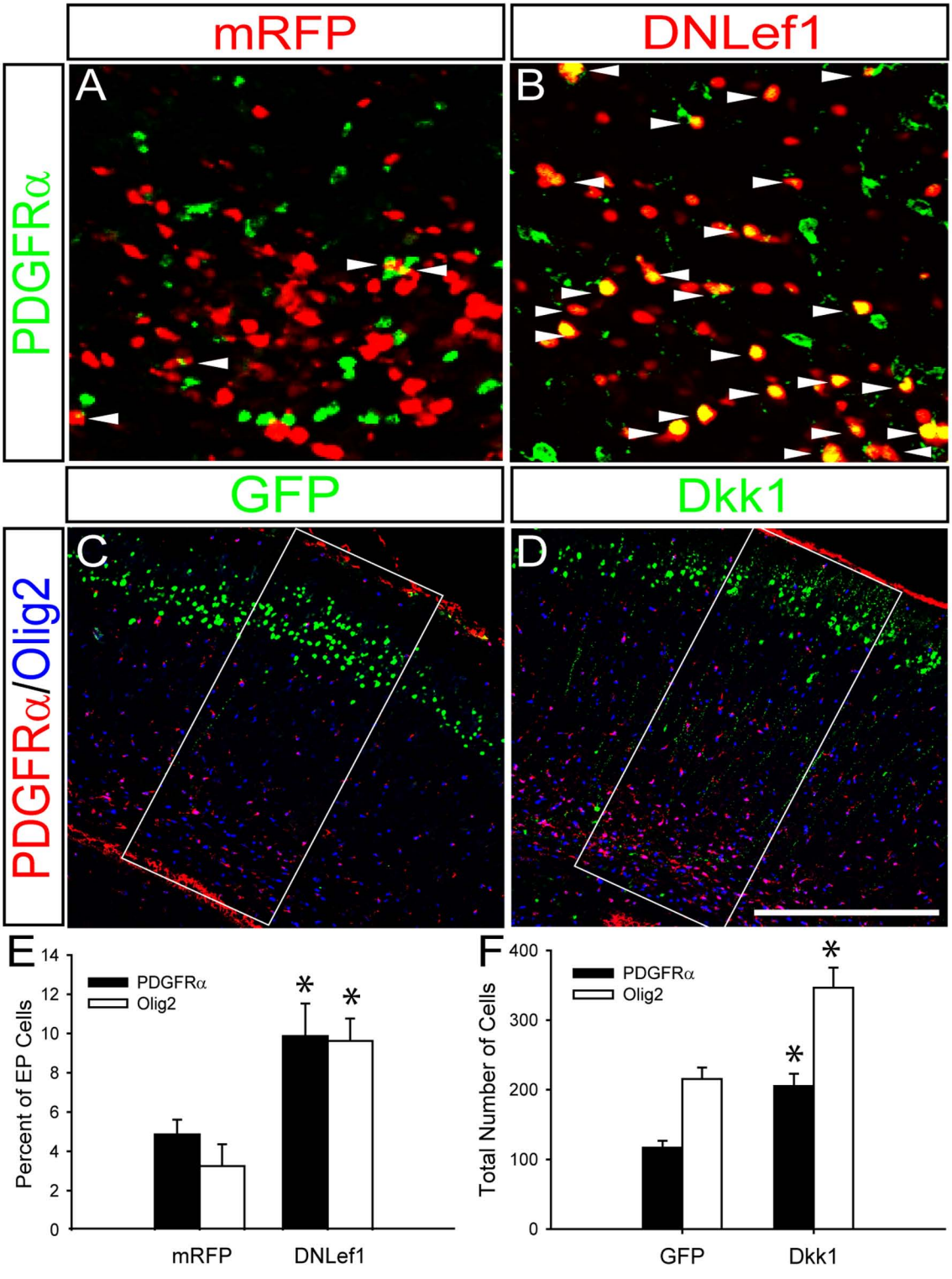
Figure 6.3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D
E
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Total Number of Cells
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Olig2
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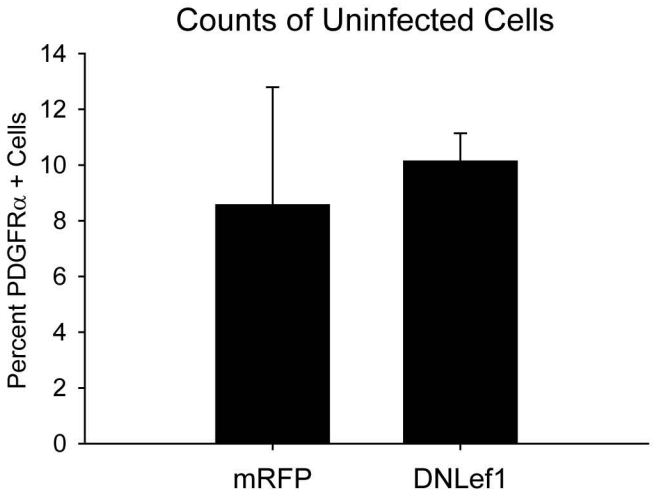
Dkk1

Figure 6.S1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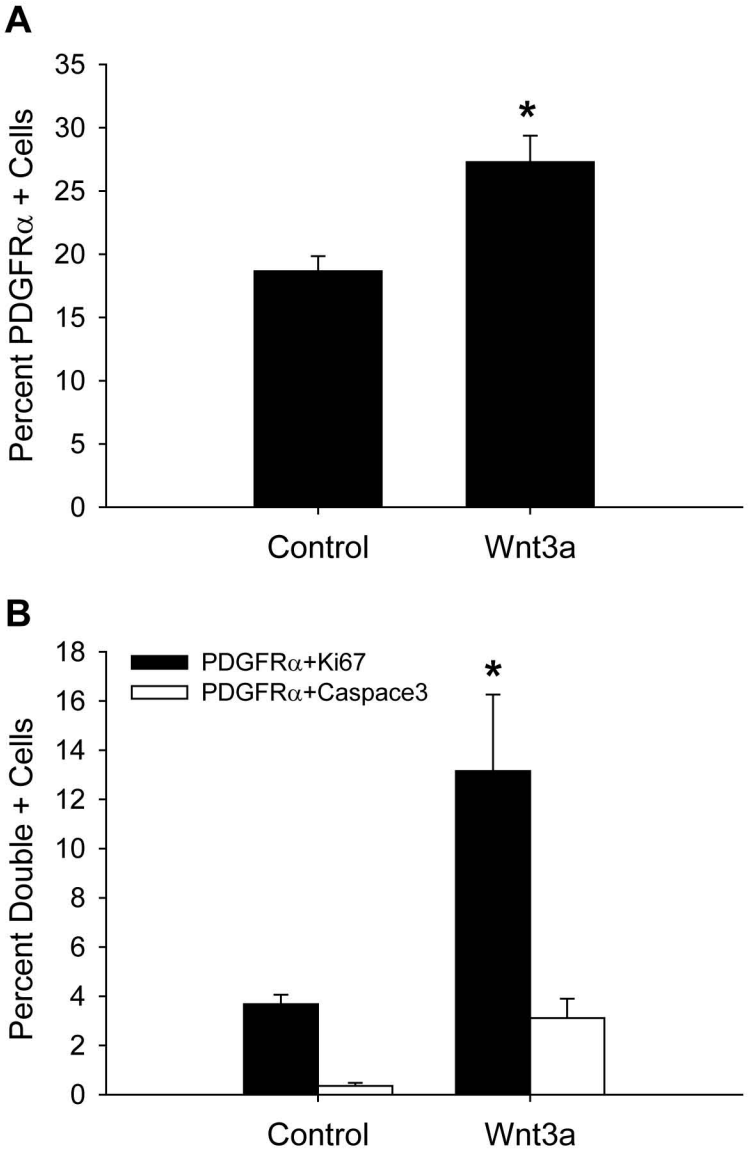
DNLef1

Figure 6.S2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PDGFRα+Caspace3
*
Control
Wnt3a

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