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Wnt Signaling and Dendrite Development in Olfactory Interneurons

Author
Pino, Darya

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Wnt Signaling and Dendrite Development In Olfactory Interneurons

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Darya Pino-Dempsey

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Abstract

Neurogenesis occurs in two places in the postnatal brain: the subventricular zone (SVZ) surrounding the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampus. Once cell fate is determined in new neurons, they must begin a process of maturation that allows them to functionally integrate into an existing neural network. An essential aspect of their maturation is their morphological development, as neuronal functionality depends on branched signaling processes called dendrites. Wnt signaling has been implicated in several aspects of postnatal neurogenesis and morphological maturation, but which Wnt pathways are involved has been ambiguous. Moreover, these pathways have only been examined in the dentate gyrus and never *in vivo*. My dissertation project examines the role of Wnt signaling in the morphological maturation of neurons born in the SVZ that eventually become olfactory interneurons. We found that Wnt5a is essential for the morphological maturation of olfactory interneurons *in vitro* and *in vivo*. In our experiments Wnt5a signals through a non-canonical Wnt pathway and works in direct opposition to the actions of canonical Wnt signaling on dendrite development.

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Chapter 1: Introduction

Among the most defining characteristics of neurons is their ability to receive and integrate electrical information across distributed regions of the nervous system. Key to this capacity are specialized processes called dendrites, which utilize a range of properties to assimilate information received from various synaptic inputs. Morphology is arguably the most fundamental component of this signal integration, where size and complexity characteristics convey distinct spatial, temporal and intensity information to the cell body and enable the neuron to decode signals appropriately. Hence, the developmental processes that help bring about these complexities are essential to proper functional development. In the postnatal brain, this process takes on a new dimension, as new neurons must integrate into already established circuitry. Moreover, the discovery of neurogenic niches containing neural stem cells (NSC) in the adult brain opened the doors for a flood of new therapeutic approaches to neurodegenerative disorders, stroke and spinal cord injury. Consequently, much work has gone into the study of postnatal neurogenesis and cell fate determination using these NSCs. The majority of the research, however, has focused on neuronal fate restriction and fewer studies have addressed the maturation process. Extensive research has also focused on the role of activity-dependent control of dendrite morphology, once neurons have established synaptic connections. Further evidence suggests that newly born neurons of the subventricular zone (SVZ), one of the two adult NSC niches, will not survive beyond a two-week period

without appropriate synaptic input (Petreanu and Alvarez-Buylla, 2002). Thus, the cell autonomous program that takes a cell from an immature neuron with few processes to one with a branched, complex dendritic arbor that is capable of receiving synaptic input is fundamental to meaningful neurogenesis, where neurons are born and then successfully integrate into existing circuitry.

In the postnatal brain, neurogenesis is known to occur in the subgranular zone of the dentate gyrus and the SVZ surrounding the lateral ventricles (Altman and Das, 1965; Luskin, 1993). While studies in the dentate have implicated several signaling molecules required for the cell autonomous program of dendrite maturation, they are somewhat limited in that they have only tested these molecules *in vitro* and have not addressed whether or not prolonged signaling is required. Further, their role in the dendritic development of SVZ-derived cells has yet to be explored.

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Chapter 2: Background

Wnt Pathways

Wnts are secreted glycoproteins that play essential roles in embryogenesis and cortical development. As ligands, Wnts interact with 7-transmembrane, G-protein-coupled receptors called Frizzleds (Fz) to initiate several different signaling pathways. The best understood Wnt signaling mechanism—the “canonical” pathway—involves the stabilization and translocation of ß-catenin to the nucleus. There it acts as a component of a transcriptional signaling complex with members of the Tcf/Lef family of transcription factors to activate target genes (Huang and He, 2008). Absent Wnt signaling, ß-catenin is phosphorylated then targeted for degradation in proteosomes via ubiquitization.

Rather than stabilizing ß-catenin, the “Wnt-calcium” pathway utilizes G-protein second messenger systems to mobilize intracellular calcium stores and activate atypical PKC and other calcium responsive pathways (Kuhl et al., 2001; Kuhl et al., 2000a; Kuhl et al., 2000b; Slusarski et al., 1997). A third Wnt pathway couples short-term cytoskeletal reorganization, convergent extension and control of planar polarity through activation of Rho-type GTPases and Jun N-terminal (JNK) serine/threonine kinase (Kohn and Moon, 2005). This is called the “Planar Cell Polarity” pathway, or “PCP.” It is unclear whether the calcium and PCP pathways are truly separable or part of an interconnected signaling web.

Wnt Co-Receptors

While each of these pathways involves the activation of Fz receptors, co-receptors that confer specificity to the different signaling cascades are emerging as critical components of Wnt signaling. For instance, the mammalian lipoprotein receptor related proteins LRP5 and LRP6 and their *Drosophila* homologue *arrow* were shown to be necessary co-receptors for the canonical Wnt signaling pathway (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000; He et al., 2004). LRP6 forms complexes with Frizzled proteins in a Wnt dependent manner and converges intracellularly to regulate ß-catenin signaling (Tamai et al., 2000).

Recently another set of receptor proteins have been implicated in Wnt signaling, particularly in axon guidance in the developing nervous system (Bovolenta et al., 2006). These proteins Ror1, Ror2 and Ryk are receptor tyrosine kinases that also bind Wnt ligands (Liu et al., 2005; Schmitt et al., 2006; Yoshikawa et al., 2003). Recent evidence suggests that the activity of these receptors may antagonize the transcriptional effects of the ß-catenin dependent pathway (Baksh et al., 2007; Li et al., 2008; Medina et al., 2000; Rossol-Allison et al., 2009). Thus an interesting new model is emerging where Wnt signaling specificity is dependent upon receptor context. Indeed, one study has shown that the same ligand, Wnt5a, can be driven to induce either canonical signaling or non-canonical signaling depending on the presence of Ror or Ryk (Mikels and Nusse, 2006). With 19 known Wnt ligands, 10 Fz receptors and at least 5 known co-receptors, there is tremendous potential for a dynamic, specific and tightly regulated Wnt signaling network.

Wnts in Early Development and Patterning

Early in vertebrate development Wnt signaling controls anterior-posterior axis formation and neural patterning (Takahashi and Liu, 2006). Members of the Wnt5a class of proteins, for example, are required for proper convergent extension movements during gastrulation in zebrafish and xenopus (Moon et al., 1993; Ungar et al., 1995). In this process, polarized mesodermal cells intercalate (converge) along the anterior-posterior axis and then extend along and lengthen the neuraxis.

Later in development, anterior inhibition of canonical Wnt signaling is required for forebrain induction. Essential to this process is the establishment of a rostral-caudal Wnt gradient, as the effects of Wnts are highly dose-dependent. Both positive and negative factors are involved in creating the Wnt signaling gradient. Zebrafish *headless (hdl)* mutants lack eyes, forebrain and some midbrain due to a null mutation in Tcf3, a transcription factor that represses Wnt target genes in anterior domains (Kim et al., 2000). Likewise, ectopic expression of some Wnts in posterior regions mimics the *hdl* phenotype. For example, overexpression of Wnt8c in a transgenic mouse model results in the complete loss of anterior forebrain, midbrain and other anterior neural structures (Popperl et al., 1997). Thus in some vertebrates low levels of anterior Wnt activity and high posterior levels are required for proper patterning of neural structures along the A-P axis.

It is less clear if Wnt inhibition is essential for mammalian forebrain development, but evidence from the LRP6 mutant mouse indicates a similar gradient may be operative in at least some subregions of the forebrain (Zhou et al., 2004a). In the cortex, the most prominent loss-of-function phenotypes were first noted in the hippocampus and dentate gyrus, which develop immediately adjacent to the Wnt rich signaling center called the cortical hem (Grove et al., 1998).

In addition to Wnts, fibroblast growth factors (FGFs) FGF8 and FGF17 are important for A-P patterning. FGF8, for example, is expressed in the midbrain primordium and is responsible for midbrain patterning along the anterior-posterior axis (Crossley et al., 1996; Shamim et al., 1999). Overexpression of FGF8 in the anterior cortical primordium increases the size of anterior structures and shrinks posterior regions of the brain. In contrast, suppression of FGFs in anterior regions enhances posterior structures at the expense of anterior structures (Fukuchi-Shimogori and Grove, 2001).

Wnt3a and the cortical hem

The cortical hem is located at the edge of the dorsomedial cortical primordium and establishes medial-lateral patterning of the dorsal telencephalon, including the hippocampus. The cortical hem is a source of both Wnt and BMP proteins (Furuta et al., 1997; Grove et al., 1998; Lee et al., 2000). Wnt3a mutant mice are missing virtually the entire hippocampus and have a transition from laminated neocortex to fimbria with a severe foreshortening of the medial cortical

wall that usually comprises the hippocampal formation (Lee et al., 2000). Molecular analysis of these mutants using markers for cell proliferation shows that they have a dramatic decrease in cell division within the hippocampal neuroepithelium. However there remain small reservoirs of cells expressing cell type specific markers of dentate gyrus, CA3 and CA1 hippocampal subfields. This implies that Wnt3a primarily regulates proliferation of hippocampal neural precursor cells, but not cell type specification.

Lef1 and LRP6 mutant mice have a much more selective hippocampal defect in the dentate neuroepithelium and fail to produce dentate granule neurons (Galceran et al., 2000; Zhou et al., 2004b). Canonical Wnt signaling has also been shown to be important in regulating proliferation of neural precursors in the adult dentate gyrus, suggesting that this function may be the adult equivalent of the embryonic failure in proliferation of dentate precursors (Lie et al., 2005).

Wnts in the developing neocortical ventricular zone

Evidence is also accumulating that Wnt signaling plays an important role in the regulation of proliferation, cell cycle exit and neurogenesis in the developing neocortical ventricular zone (VZ). Overexpression of dominant active ß-catenin in the VZ using the nestin promoter yielded mice with derangements in overall proliferative control of cortical neural precursors (Chenn and Walsh, 2002). It is important to remember, however, that overexpression may not indicate normal function in these processes. Follow up studies examining mice with selective loss of function of ß-catenin in the neocortical VZ may also indicate

a role for Wnts in cortical development (Woodhead et al., 2006), but it is unclear if this effect is specific to Wnt signaling or instead reflects the non Wnt-related role of ß-catenin in the adherens complex (Willert and Jones, 2006).

Data combining analysis of Wnt reporter plasmids and mosaic loss-of-function for ß-catenin adds to our understanding of the role of Wnts in the VZ. These studies indicate that Wnt signaling is strong in the VZ (Noles and Chenn, 2007) and is immediately down-regulated upon the exit of cells to the subventricular zone and intermediate zone (Bonnert et al., 2006). Interestingly, small numbers of neurons reaching the cortical plate reinitiate strong canonical Wnt signaling in neuronal development. LRP6 mutant mice exhibit neocortical defects that are consistent with an essential role for canonical Wnt signaling in the neocortical VZ (Zhou et al., 2004b).

Wnts and Axon Growth, Guidance, and Remodeling

Wnts are involved in other aspects of axon guidance and remodeling. In both the cerebellum and spinal cord, the presence of Wnt7a and Wnt3 (respectively) result in increased growth cone size and complexity, increased axon diameter, and the induction of axon branching (Ciani and Salinas, 2005; Hall et al., 2000). Dorsal root ganglion explants grown in Wnt3a conditioned media display longer, more numerous neurites as compared with those grown in control media (Lu et al., 2004).

The molecular mechanisms for these Wnt effects are still being elucidated. Wnt3a-induced neurite outgrowth is reduced by the addition of Ryk siRNA ,

suggesting Ryk is a required co-receptor for this particular Wnt function (Lu et al., 2004). While the canonical pathway is not directly involved in axon guidance, it is possible that the stabilization of ß-catenin may play a role by indirectly affecting cadherin-mediated adhesion (Zou, 2004). Furthermore, Wnts and Disheveled (DVL) influence microtubule stability, hence changing the dynamics of the cytoskeleton. As this occurs via the canonical pathway through inhibition of GSK3ß, it indicates a potential role for the canonical pathway in axon remodeling (Ciani and Salinas, 2005).

Wnts also play an essential role in guiding commissural axons in the spinal cord. The Wnt4-Fz3 interaction regulates anterior-directed projections once they have crossed the midline, and Wnt4 stimulates the turning of these commissural neurons (Lyuksyutova et al., 2003). The commissural axons of Fz3 mutants project randomly once they have crossed the midline and also exhibit defects in thalamocortical, cortiothalamic, and nigrostriatal axon tracts (Bovolenta et al., 2006). Likewise, the Wnt5-Ryk interaction is important in the establishment of major brain tracts like the corpus callosum and cortico-spinal tract. In Ryk null mice, axons cannot exit the corpus callosum once they reach the contralateral side (Keeble and Cooper, 2006; Keeble et al., 2006). Wnt5a is the demonstrated ligand for Ryk in this role.

Wnts can function as both chemoattractive and chemorepulsive cues in axon guidance, depending on the receptor. Fzs seem to mediate a chemoattractive role for Wnts while Ryk and Derailed (ABB) confer repulsive behaviors (Bovolenta et al., 2006; Ciani and Salinas, 2005; Zou, 2004). For

example, Wnt1 and Wnt5a are attractive to commissural neurons with Fz receptors while these same Wnts are repulsive to cortico-spinal and callosal axons with Ryk receptors (Bovolenta et al., 2006; Liu et al., 2005; Lyuksyutova et al., 2003).

Wnts and Dendrite Development

The complexity of dendritic arbors is directly affected by Wnts. When hippocampal cultures are treated with Wnt7b conditioned media, the neurons exhibit longer dendrites with an increased number of branches. Conversely, the addition of a Wnt inhibitor, sFRP, to these cultures decreases arbor complexity. Moreover, hippocampal cultures taken from Dvl1 deficient mice display a simpler dendritic arbor (Rosso et al., 2005). A similar increase in dendritic branches and length is observed in hippocampal cultures in the presence of Wnt2a conditioned media or when transfected with a Wnt2 expressing construct (Wayman et al., 2006). This enhancement is blocked by Wif, an inhibitor that prevents Wnt from activating its Frizzled receptor, further demonstrating the requirement for Wnt in dendritic outgrowth. Hence it appears that Wnt signaling regulates dendritic development.

Yu and Malenka (2003) found that ß-catenin also influences dendritic complexity of hippocampal neurons *in vitro*. Cells transfected with a stabilized form of GFP-ß-catenin had an increase in the number of dendritic branches that was proportional to the amount of ß-catenin expressed. In contrast, sequestering

ß-catenin by overexpressing N-cadherin significantly decreased dendritic arborization.

Wnt effects on dendrite development are also activity dependent. In a study by Wayman et al. (2006), neuronal activity triggers a calcium signaling pathway that leads to the transcription of Wnt2. Moreover, activity-dependent dendrite growth is blocked by Wnt antagonists, revealing a potential mechanism by which neuronal activity may mediate neurite outgrowth. Similar results were obtained by Yu and Malenka (2003). They determined that high K+ concentrations in hippocampal cultures (which mimics neuronal depolarization) increased the number of dendritic branches, an effect that was enhanced with stabilized ß-catenin. However, sequestering ß-catenin or adding a Wnt antagonist reversed the effects of neuronal activity. Hence Wnts are able to exert effects over dendritic outgrowth and complexity under different circumstances.

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Chapter 3: Manuscript

Wnt5a Controls Neurite Development In Olfactory Bulb Interneurons

Darya Pino[1,2], Youngshik Choe[1,2] and Samuel J. Pleasure[1,2,3,4]

[1]Department of Neurology, Programs in [2]Neuroscience, [3]Developmental Biology, [4]Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California, San Francisco, San Francisco, CA, USA

Abstract

Neurons born in the postnatal subventricular zone (SVZ) must migrate a great distance before becoming mature interneurons of the olfactory bulb (OB). During migration immature OB neurons maintain an immature morphology until they reach their destination. While the morphological development of these cells must be tightly regulated, the cellular pathways responsible are still largely unknown. Our data show that the non-canonical Wnt pathway induced by Wnt5a is important for the morphological development of OB interneurons both *in vitro* and *in vivo*. Additionally we demonstrate that non-canonical Wnt signaling works in opposition to canonical Wnt signaling in neural precursors from the SVZ *in vitro*. This represents a novel role for Wnt5a in the development of OB interneurons and suggests that canonical and non-canonical Wnt pathways dynamically oppose each other in the regulation of dendrite maturation.

Introduction

The subventricular zone (SVZ) surrounding the lateral ventricles is one of two neurogenic niches in the postnatal mouse brain, the other being the subgranular zone of the dentate gyrus. Neurons born in the SVZ migrate along the rostral migratory stream to become olfactory bulb (OB) interneurons. While migrating, immature interneurons maintain a simplified morphology with a single leading process as they migrate in chains (Lois et al., 1996). OB interneurons do not attain full morphological maturity until they reach their appropriate destination within the OB. This entire process from specification to functional integration takes 5-9 days in the adult (Petreanu and Alvarez-Buylla, 2002). Because OB interneuron specification and complete morphological maturity are separated substantially in both space and time, this is an ideal system for studying the morphological development of neurons in the postnatal brain.

Wnt signaling is integral to both neuronal specification and development. In the postnatal brain, Wnts are important in stem cell proliferation (Adachi et al., 2007) and have been shown to be involved in both the differentiation (Lie et al., 2005) and neurite development of hippocampal granule cells (Rosso et al., 2005; Yu and Malenka, 2003). However it is unclear what roles specific Wnt signaling pathways play in the development of these cells. Increased dendritic complexity has been shown to be dependent on β-catenin, however this effect did not require LEF/TCF-mediated transcriptional activation and is therefore unlikely to reflect a role for canonical Wnt signaling (Yu and Malenka, 2003). For instance,

β-catenin could also influence dendritic growth through its role in modulating cell adhesion and the actin cytoskeleton, independent of Wnt signaling (Gavard and Mege, 2005).

Wnt7b, a Wnt ligand associated most prominently with non-canonical Wnt signaling pathways, has also been shown to increase the complexity of hippocampal neurons in culture. The addition of Wnt inhibitor sFRP-1 to these cultures not only blocked the effect, but also simplified the morphology of the neurons beyond the control cultures, indicating an endogenous role for the non-canonical Wnt pathway in the morphological development of hippocampal cultures (Rosso et al., 2005). It is unknown if Wnts play a role in the morphological development of SVZ-derived OB interneurons, though it does appear that canonical Wnt/β-catenin is important for proliferation of progenitor cells in the SVZ (Adachi et al., 2007). Interestingly, traditionally non-canonical Wnt ligands Wnt5a and Wnt7b, but not canonical Wnts, are expressed by OB interneurons themselves (Shimogori et al., 2004).

Using soluble Wnt ligands Wnt5a and Wnt3a, and inhibitors DKK-1 and sFRP-1 on SVZ progenitor cell cultures, we show non-canonical Wnt signaling is necessary for normal morphological maturation of OB interneurons. Interestingly, in our culture system the activation of the non-canonical Wnt pathway acts in opposition to canonical Wnt/β-catenin signaling. Moreover, evidence from the Wnt5a knockout mouse indicates that normal morphological development of OB interneurons is disrupted in the absence of endogenous Wnt5a.

Results

Wnt5a and non-canonical Wnt signaling increase neurite complexity

To study the role of Wnt ligands in regulating the neuronal differentiation of SVZ derived OB interneurons we took advantage of the ease of generating these cells *in vitro* via differentiation from neural precursors. We cultured neural precursor cells from the SVZ of P5 mice, then placed them in serum containing medium for 24 hours to induce differentiation. We then treated the cultures with soluble Wnt ligands and inhibitors for 4 days, changing the medium every 24 hours, and then analyzed the neuritic complexity of the resulting neurons. This experiment is designed to examine the effects of Wnt ligands on differentiation rather than neuronal specification since the ligands were not added until well after the global differentiation stimulus of serum was added to the cells. The largely non-canonical Wnt ligand, Wnt5a, and the largely canonical Wnt ligand, Wnt3a, had markedly different effects on neurite development in these cells (Fig. 1A-C). Wnt5a treatment led to increased neurite growth while Wnt3a either had little effect or inhibited neurite outgrowth. We used a simple measure of neurite complexity, total dendritic branch tip number (TDBTN) (Rosso et al., 2005), to assess these findings in a quantitative way. We found that TDBTN was significantly higher in cultures treated with Wnt5a compared to controls, and cultures treated with Wnt3a had a statistically significantly but modest decrease in TDBTN compared to control cultures treated with vehicle only (Fig. 1F).

Our results imply that activation of non-canonical Wnt signaling in these cultures leads to distinct effects on neuritic maturation compared to activation of canonical Wnt signaling. To tease apart the differences in these effects, we treated the cultures with different families of extracellular Wnt inhibitors to determine pathway specificity. When sFRP-1 was applied to cultures, an inhibitor that blocks both canonical and non-canonical Wnt signaling by sequestering Wnt ligands so they cannot bind their cognate Frizzled receptors (Rattner et al., 1997), the neurite complexity was dramatically decreased (Fig. 1D) and TDBTN was significantly lower than in control cultures (Fig. 1F). In striking contrast, cultures treated with DKK-1, an inhibitor that specifically blocks canonical Wnt signaling by interfering with LRP5/6 co-receptors (Mao et al., 2001), showed a dramatic increase in neurite complexity (Fig. 1E), reflected by a significant increase in TDBTN (Fig. 1E). Our data implies that canonical and non-canonical Wnt signaling have markedly different effects on the morphological maturation of neurons derived from the mouse SVZ. In addition, the prominent effects of inhibitors on cultures without the addition of exogenous Wnts indicate that under normal culture conditions Wnt signaling is probably a potent regulator of neurite outgrowth, both positively and negatively.

Wnt signaling plays a sustained role in neurite differentiation in culture

Wnt signaling appears to be a significant regulator of neurite development in neural precursor cultures during the first few days in culture. However, given the profound roles of canonical Wnt signaling in neural precursor proliferation

and cell fate acquisition, we wanted to determine whether Wnt signaling plays an ongoing role in the growth of neurites or whether the Wnts predominantly act at the stage of initial neurite outgrowth, perhaps by influencing the cell fate acquisition process itself or initial stages of neurite polarization. In addition, if Wnt signaling plays a role in neurite maturation when the interneurons reach the OB, then the effects of Wnts should be ongoing even after several days.

To address this we used neurons differentiated from SVZ cultures that were allowed to grow in serum without additives for 72 hours. We then treated the cells with Wnts and Wnt inhibitors starting at this later time point. It was apparent that neurites continued to extend during this growth period in control cultures (Fig. 2A-B) and this was also apparent when we measured TDBTN (Fig. 2G), which we expressed as a ratio of the TDBTN at the termination of the experiment (6 days post differentiation; 72 hours post addition of vehicle or factors) divided by the TDBTN of sister cultures analyzed at the start of the experiment (3 days post differentiation; t=0 for addition of factors). Like at the earlier stage of neurite outgrowth, we found that Wnt5a increased the complexity of neurite outgrowth significantly over what we observed in controls and Wnt3a inhibited the complexity compared to control situations (Fig. 2C-D;G). The normal increase in neurite complexity was also blocked by sFRP-1 (Fig. 2E;G) and significantly increased when the selective canonical Wnt inhibitor DKK-1 was added to cultures (Fig. 2F-G).

Again the significant effects seen with the inhibitors, particularly the DKK-1 blockade of endogenous canonical Wnt ligand signaling, indicate that this

pathway is an ongoing regulator of neurite maturation in these cultures. The retardation of outgrowth in the sFRP-1 condition compared to the acceleration in the DKK-1 condition suggests an ongoing balance of endogenous non-canonical and canonical Wnt signaling that acts to regulate the rate and extent of neurite growth and complexity throughout the culture period.

Cellular organization of the OB is intact in Wnt5a mutant mice

Since there are many Wnt ligands in the genome, there is extensive redundancy and examples of clear loss-of-function phenotypes for Wnt ligands are not that common. However, we wished to determine if there is a role for Wnt5a in regulating olfactory interneuron neurite development *in vivo*. Previous reports showed that Wnt5a is expressed in the early postnatal mouse OB periglomerular (PGC) and granule (GC) cells (Shimogori et al., 2004). To confirm this, we checked for expression of Wnt5a in the early postnatal mouse by *in situ* hybridization. We found that Wnt5a is highly expressed in GCs and PGCs at both P1 and P5 (Supplemental Fig. 1). Therefore, as immature interneurons reach the OB after migrating through the RMS, they encounter already established neurons expressing Wnt5a as they seek to integrate themselves into the local network. Thus, Wnt5a is present in an appropriate location to influence the neuritic architecture of several classes of olfactory interneurons.

We turned to the previously characterized Wnt5a null mutant mice (Yamaguchi et al., 1999) to determine whether Wnt5a plays a role in OB development. These mice don't survive past birth, so we confined our analysis to

late embryonic stages (E18.5). Since the role of Wnt5a in olfactory interneurons has not been previously examined, we first looked for defects in cell number and organization of PGCs and GCs in the OB of Wnt5a knockout mice. Pax6 is a nuclear marker for subpopulations of both GCs and PGCs in the OB (Kohwi et al., 2005) and allows a straightforward and sensitive measure for the cell density of these groups of neurons. There was no obvious difference between Wnt5a-/- mice and littermate controls in the PGC layer (Fig. 3A-B). Calbindin (CB) is another useful marker expressed in the cytoplasm of subsets of OB interneurons (Kohwi et al., 2007) and we found no clear difference in the numbers of CB+ cells in the OB of mutants and control mice (Fig. 3C-D). To quantify the numbers of Pax6+ and CB+ interneurons in these mice we counted them in standardized counting boxes. For Pax6 we restricted our cell counts to PGCs because of the substantial number of neural progenitor cells that express Pax6 deep in the core of the OB near GCs. This analysis revealed no difference in the numbers of these cells between mutant and control mice (Fig. 3E). We also examined CB+ PGCs and GCs and found no difference between mutants and controls (Fig. 3F-G).

Wnt5a mutant mice have reduced OB interneuron neuropil

Since it is a cytoplasmic marker, CB staining also gave us some information about cellular morphology in the mutant OB interneurons. We noticed that qualitatively it appears that CB+ cells in the Wnt5a-/- OB have more simplified neurite architecture than littermate controls (Fig. 3C-D). The results of our Pax6 and CB analyses indicate that overall organization and cell number of

several groups of OB interneurons are preserved in the OB of Wnt5a-/- mice, but their morphology might be altered.

Given the prominent role of Wnt5a on neurite development in SVZ derived neurons *in vitro* and the suggestion that CB+ neurons might be altered, we wanted to examine structural differences in the OB of Wnt5a-/- mice directly using more sensitive markers of neuropil morphology. Tyrosine hydroxylase (TH) is expressed in a subset of PGCs (Kohwi et al., 2007), staining both their cell bodies and dendrites, and gives a useful view of PGC morphology. Control mice had strong staining for TH in the PGC layer. This marker highlights the cell bodies of these cells, but also stains a reticular network of processes that are distributed throughout the PGC layer forming a meshwork of dendritic processes around the glomeruli (Fig. 4A). In Wnt5a-/- mice the number of PGCs appeared unchanged (similar to our findings with Pax6 and CB) but the density of neuropil staining was dramatically decreased and the reticular network of dendrites was substantially less dense (Fig. 4B). These differences were even more apparent in high power images of the PGCs (Fig. A'-B'). To quantify this difference we measured the width of the TH+ neuropil in multiple places in the PGC layer to establish a measure of PGC layer neurite complexity and found that it was significantly reduced compared to littermate controls (Fig. 4C).

Since periglomerular interneurons are GABAergic, we also stained for VGAT, the vesicular GABA transporter, to further examine the neuropil in the PGC layer in these mutants. This showed that VGAT staining was markedly decreased in the in Wnt5a-/- mice (Fig. 4E). At high power much of the VGAT

staining was punctate in appearance, presumably reflecting its localization to presynaptic GABAergic terminals, and the density of these puncta was apparently decreased in mutants (Fig. 4D'-E'). In addition, as with TH, the thickness of the PGC layer was much reduced in mutants (Fig. 4F).

Discussion

Our results show that the morphological development of OB interneurons derived from the SVZ is dependent on Wnt5a, which likely acts via non-canonical Wnt signaling to increase neurite complexity. Blockade of Wnt signaling in SVZ-derived neural precursor cells *in vitro* using the non-specific Wnt inhibitor sFRP-1 resulted in a simplified neuronal morphology, indicating a role for endogenous Wnts in morphological development. In mice lacking Wnt5a, interneuron morphology is disrupted in the OB, while cell number and OB architecture remain intact. Our findings are reminiscent of those in rat hippocampal cells where sFRP-1 blocks neurite outgrowth and non-canonical Wnt ligand Wnt7b increases dendritic complexity (Rosso et al., 2005). This implies that non-canonical Wnt signaling may be globally important in regulating dendrite maturation and possibly relevant elsewhere in the nervous system.

Wnt5a is able to act as either a canonical or non-canonical Wnt ligand depending on receptor context (Mikels and Nusse, 2006), and previous studies have been ambiguous about which Wnt pathways are important for morphological development in postnatal neurogenesis (Yu and Malenka, 2003; Rosso et al., 2005). In our cell culture experiments, DKK-1, a canonical Wnt

inhibitor acts to increase neurite complexity, and the largely canonical Wnt ligand Wnt3a slows the rate of morphological development. These findings suggest that Wnt5a acts on the non-canonical pathway in this system. It also indicates that canonical Wnt signaling directly suppresses dendrite maturation and acts in opposition to non-canonical signaling in SVZ-derived neurons.

Previous studies have highlighted the role of canonical Wnt signaling in the regulation of neural precursor proliferation and neural cell fate (Yu and Malenka, 2003; Adachi et al., 2007). Our findings suggest that canonical Wnt signaling may have an additional role in maintaining already differentiated OB interneurons in a simplified morphology even after cell fate has been determined (Fig. 2). Coupled with Wnt expression data in the cortex and OB (Shimogori et al., 2004), this leads to an attractive model where neural precursor cells are exposed to canonical Wnt signaling in the SVZ and RMS where they are maintained with a simplified morphology and in a highly motile state. Once the migratory neurons reach the OB and contact mature interneurons in the PGL and GL, they are exposed to Wnt5a via paracrine signaling and complete their morphologic differentiation. This model implicates a novel interaction between canonical and non-canonical Wnt signaling pathways on the regulation of neuronal morphology and may be a general mechanism utilized in other regions of the nervous system.

Methods

Animals. All animal protocols were approved by the UCSF IACUC. P5 CD1s (Charles River) were used for cultures. Wnt5a knockout mice (Yamaguchi et al., 1999) and littermate controls were harvested at E18.5 for staining. Males and females were used for all experiments.

Immunohistochemistry. Brains were processed using standard methods. Cells and tissue sections were stained using standard protocols with: mouse anti-Tuj1 (Covance, cells 1:3000), rabbit anti-MAP2 (Chemicon, cells 1:500), rabbit anti-Pax6 (Covance, tissue 1:150), rabbit anti-CB (Swant, 1:1500 for tissue), rabbit anti-TH (Chemicon, 1:400 for tissue), and rabbit anti-VGAT (Synaptic Systems, 1:200 for cells). Fluorescent secondary antibodies (1:500, Invitrogen) and DAPI (1:1000, Sigma) were used.

Cell Culture. Neural progenitor cells (NPCs) were harvested from lateral ventricle SVZ tissue of P5 CD1s. 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 cm2 flask (Corning), grown in 5 ml of complete media plus growth factors and B27. Passage 2 or 3 NPCs were used.

Cell culture – Wnt treatments. NPCs were dissociated with 0.05% trypsin-EDTA, washed with PBS, pelleted and resuspended in complete media with 2% FBS. The cells were plated on poly-L-lysine (Sigma, MW 70,000-150,000 kD) and laminin-coated (1 mg/mL, Invitrogen) chamber slides (Nunc) at 37,500 cells/well. Plated cells were left to differentiate for 24 hours (Fig. 1) or 96 hours (Fig. 2) before treatment with Wnt factors. At the appropriate time mouse Wnt5a (0.375 ng/μL), Wnt3a (0.15 ng/μL), sFRP-1 (5.0 ng/μL), Dickkopf1 (DKK-1, 0.06 ng/μL) (all from R&D Systems), or an equivalent volume of vehicle were added to each well. One half of the treated media was replaced each day. The cultures were analyzed after 4 days treatment.

Image analysis and quantification. Three biological replicates of each experiment were performed. For *in vitro* experiments, cells from 3-5 20X magnification images were quantified for each replicate of each culture condition, including at least 100 cells from each condition. Cells were excluded from the analysis if all dendrite tips could not be clearly visualized.

One mutant and one littermate control animal were analyzed from three separate litters. Cell counts from standardized counting boxes in 3 coronal

sections of each brain were averaged for both the Pax6 and CB staining. DAPI counterstains were used to confirm the presence of cell bodies. PGL width was measured in 4 places for each TH and VGAT stained section and averaged. These values for each animal were then pooled and compared across genotypes.

Statistical analysis. Results are expressed as mean ± SEM. Data were analyzed using two-tailed Student's t-test with unequal variance. Any value of $p \leq 0.05$ was considered significant.

Figure Legends

Figure 1: Wnt5a and Wnt3a have opposite effects on neurite development. Neurons are identified by staining with Tuj1(red) and MAP2 (green) antibodies. DAPI counterstain is used to show the density of other cells in the culture. **A**) Neurons treated with vehicle for 3 days as a control. **B**) Cultured neurons treated with soluble Wnt5a. **C**) Neurons treated with Wnt3a. **D**) Neurons treated with non-specific Wnt inhibitor sFRP-1. **E**) Neurons treated with canonical inhibitor DKK-1. **F**) Quantification of total neurite branch tip number (TDBTN) for each condition. All p values <0.001. Scale bar = 50 μm.

Figure 2. Effects of Wnts signaling on growth of neurites in later cultures. Wnts and Wnt inhibitors affect neuron development up to 96 hrs after specification. A) Untreated cells after 96 hrs *in vitro* fixed to serve as T=0 for normalization of neurite outgrowth. B) Control neurons treated with vehicle beginning 96 hrs after specification for another 72 hours. C) Wnt5a continues to enhance morphologic development when applied 96 hrs after specification. D) Wnt3a inhibits morphological development when applied 96 hrs after cell specification. E) Neurons treated with Wnt inhibitor sFRP-1 96 hrs after specification continue to develop, though not as rapidly as neurons treated with vehicle alone. F) Canonical Wnt inhibitior DKK-1 enhances morphologic development when

applied 96 hrs after cell specification G) Quantification of TDBTN of treated cells. Numbers are normalized to the TDBTN of untreated cells at start condition. All p-values <0.05. Scale bar = 50 μm.

Figure 3. Interneuron organization and numbers are normal in Wnt5a mutants. A) Wnt5a+/+ and B) Wnt5a-/- OB stained for Pax6. C) Wnt5a+/+ and D) Wnt5a-/- OB stained for CB. E) Number of Pax6+ periglomerular cells is unchanged in mice lacking Wnt5a. F) Number of CB+ periglomerular cells and G) granule cells are unchanged in mice lacking Wnt5a. Scale bar = 50 μm

Figure 4. Reduction of TH+ and VGAT+ neuropil in Wnt5a mutant mice. A) Wnt5a+/+ mice (high power inset A') and B) Wnt5a-/- (high power inset B') mice stained for TH. C) Width of TH+ PGL is reduced in mice lacking Wnt5a ($P<0.01$). D) Wnt5a+/+ mice (high power inset D') and E) Wnt5a-/- mice (high power inset E') OB stained for VGAT. F) Width of VGAT+ PGL is reduced in mutants ($p<0.01$). A, B, D, E scale bar = 50 μm. A', B', D', E' scale bar = 25 μm.

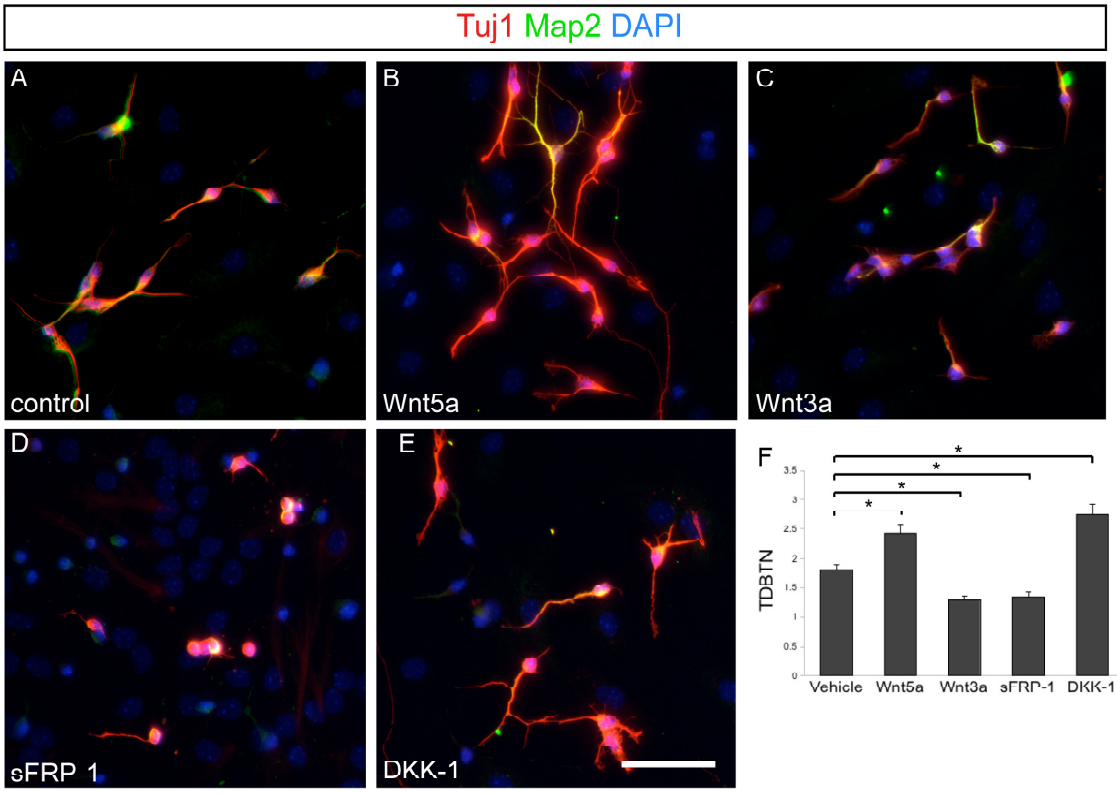
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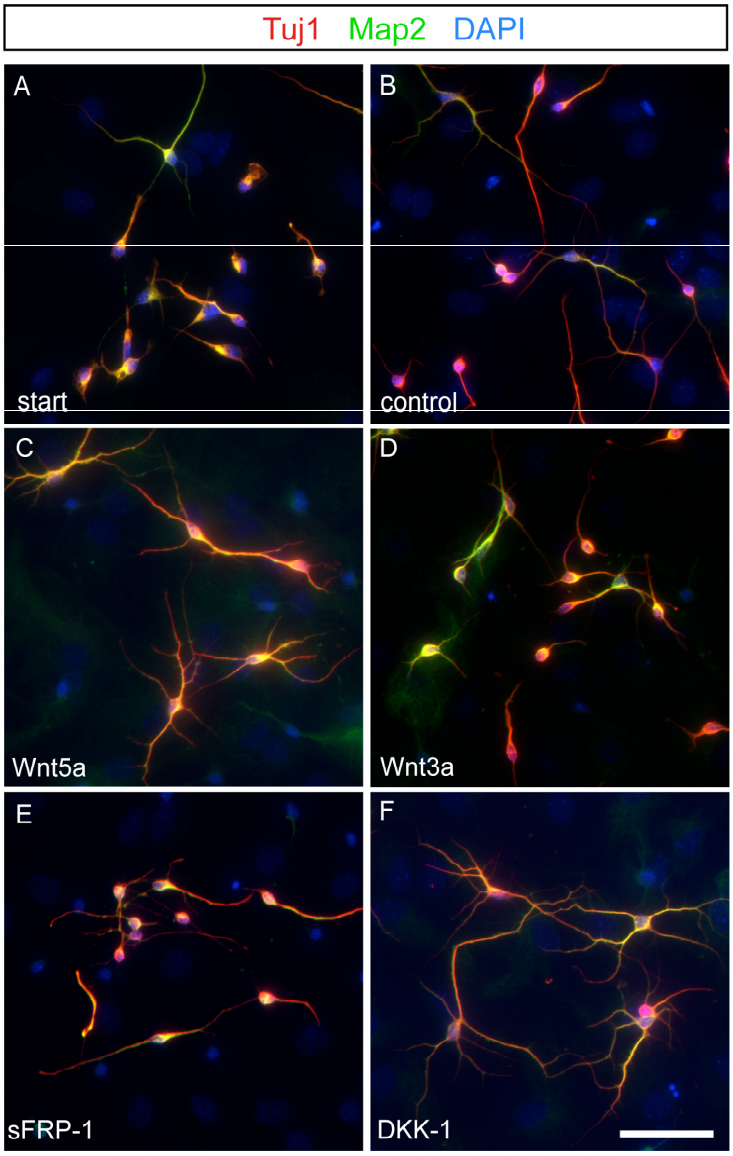
Figure 1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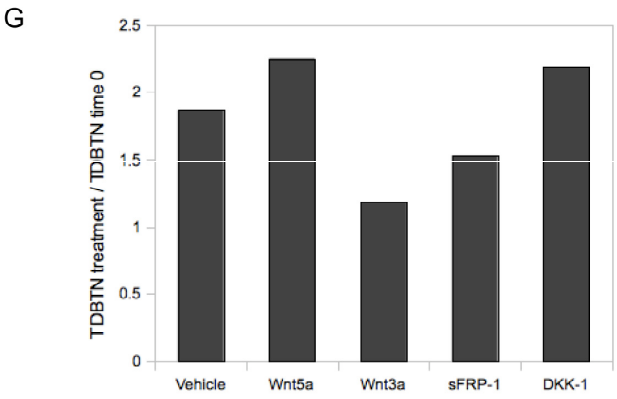
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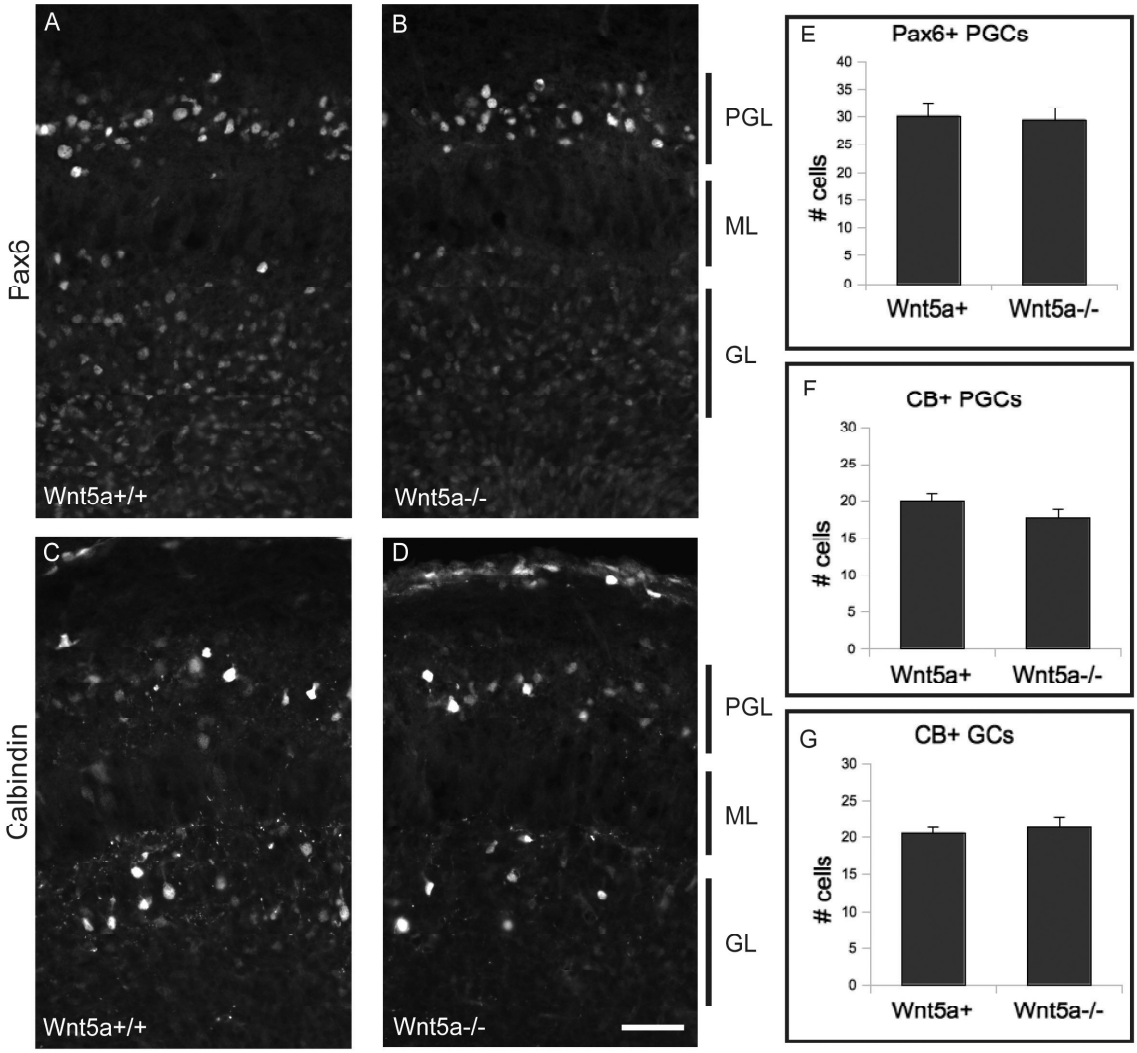
FIgure 2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cells
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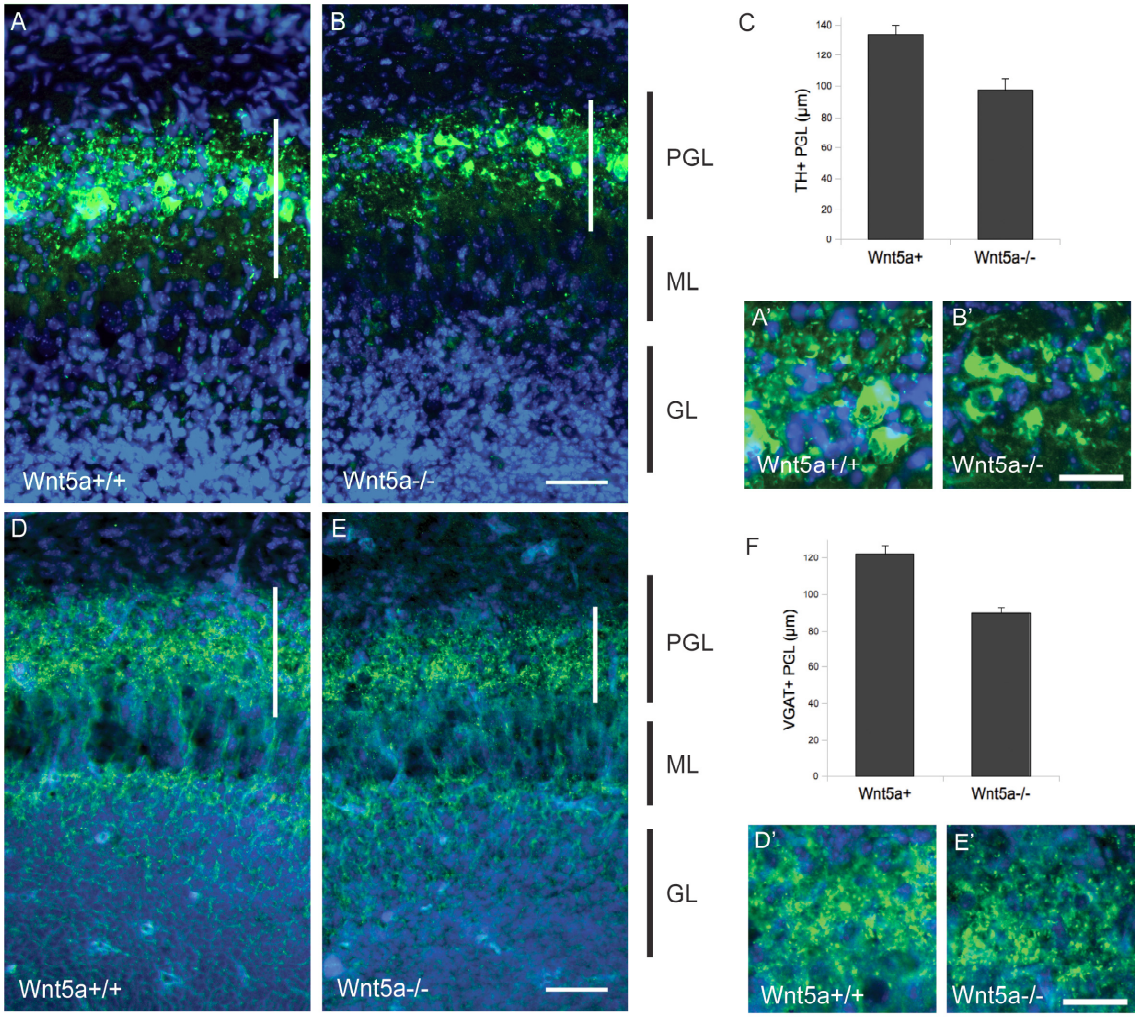
Figure 3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VGAT+ PGL (µm)
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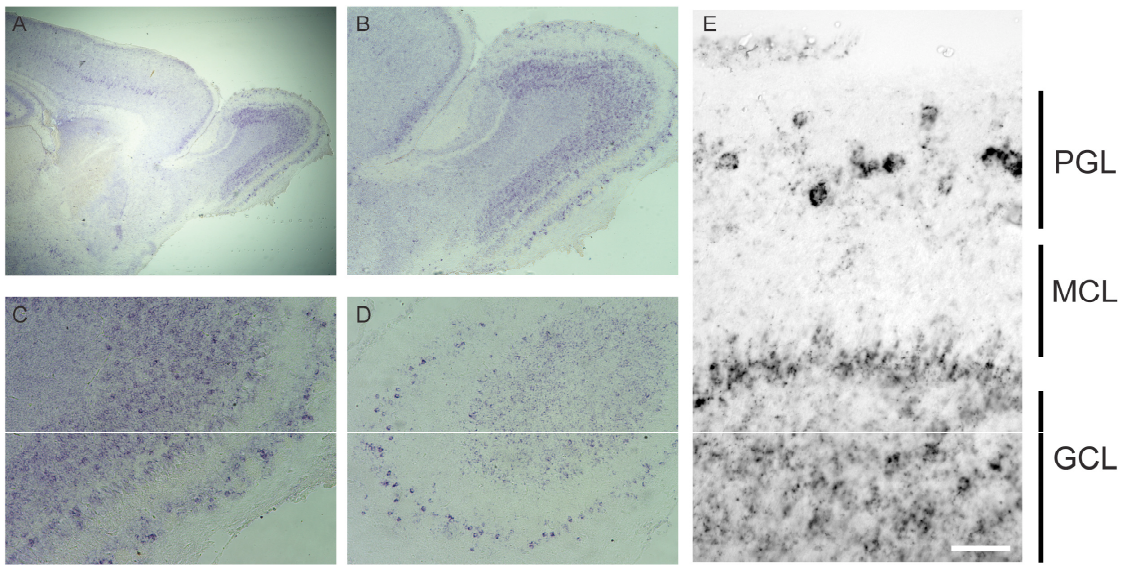
E'

Figure 4



Supplementary Figure 1. Wnt5a is expressed in OB interneurons

In situ hybridizaiton for Wnt5a in P5 and P1 mouse olfactory bulb. A) 2x B) 4x and C) 10x saggital sections showing Wnt5a expression in P1 OB interneurons. D) Wnt5a expression in 10x coronal section of P5 OB. E) High magnification (scale bar = 50 um) of P5 OB shows Wnt5a expression in periglomerular cells and granule cells.

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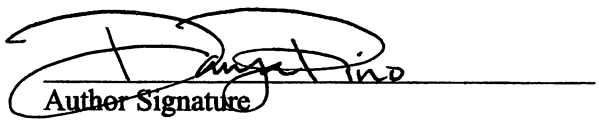

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