THE ROLE OF *JAGGED1* IN THE SUBVENTRICULAR ZONE DURING LATE EMBRYONIC DEVELOPMENT

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THE ROLE OF *JAGGED*1 IN THE SUBVENTRICULAR ZONE DURING LATE EMBRYONIC DEVELOPMENT

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Notch signaling plays an important role in regulating olfactory neurogenesis during development of the mammalian subventricular zone. During development, the *Notch* signaling pathway is critical for maintenance of neuronal precursors, cell survival, and for neural stem/progenitor cell self-renewal. *Notch* receptors have been shown to be expressed among the heterogeneous populations of cells in the subventricular zone. However, the regulation of *Notch* remains poorly understood. In the subventricular zone, the *Notch* activator *Jagged1* has been shown to be expressed in cells adjacent to those expressing *Notch* receptors. Moreover, a previous study showed that *Jagged1* is important for self-renewal of neural stem cells in the subventricular zone during postnatal stages. We utilized a conditional *Jagged1* knockout mouse to study the role of *Jagged1* in the embryonic subventricular zone. We found that *Jagged1* is critical for olfactory neurogenesis during development. *Jagged1* mutants exhibited a decrease in the production of neuronal precursors and olfactory interneurons. Additionally, we observed that the loss of *Jagged1* increases cell death in the rostral migratory stream, a specialized migratory stream connecting the subventricular zone and the olfactory bulb. Finally, we show that *Jagged1* is expressed on neural stem cells. Based on these findings, *Jagged1* is proposed as a

critical regulator of neurogenesis in the embryonic subventricular zone.

BIOGRAPHICAL SKETCH

Christopher Blackwood was born in the Bronx, N.Y. on February 21, 1979. He grew up in Harlem, N.Y. and attended Seward Park High School. In 1997, he attended Clark Atlanta University where he earned his Bachelor's degree in Biology in 2001. In 2001, he worked in Susan Sullivan's lab at the NIDCD, identifying novel taste receptors cells in mammals. In 2007, he joined the Ph.D. program in the field of pharmacology within the Department of Biomedical Sciences at Cornell University. He performed his dissertation project in the laboratory of Dr. David Lin, where he focused on understanding the role of *Jagged1* in the subventricular zone. Chris has received several prestigious fellowships to support his dissertation work. He has been awarded The Ford Dissertation, the NRSA National Institute of Health, and the Cornell University Provost's Diversity Fellowships. In 2012, he was inducted into Edward A. Bouchet Graduate Honor Society. While at Cornell he was also a member of the Graduate Diversity Council and the Graduate Student Government Association.

Chris will be joining the BETTR/IRACDA program at Albert Einstein College of Medicine. As a postdoctoral associate, he will be working in Dr. Jean Hebert's lab, which focuses on understanding how the cortex regenerates. As part of the program, he will spend 25% of his teaching duties at Lehman College and Hostos Community College in the Bronx, N.Y.

I dedicate this thesis to my mother Eva Smith-Moree for all her love and support.

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LIST OF ABBREVIATIONS

ALGS	Alagille syndrome
BrdU	Bromodeoxyuridine
CD 133	Neural stem surface antigen AC133
CRE	*Cre recombinase*
CSL	*CBF1, Suppressor of hairless-Lag2*
DSL	*Delta-serrate-lag*
DCX	*Doublecortin*
E17	Embryonic day 17
E18	Embryonic day 18
E19	Embryonic day 19
EGFP	*Enhanced green fluorescent protein*
EGF	*Epidermal growth factor*
ELAV	*Embryonic lethal, abnormal vision*, *Drosophila*
GFAP	*Glial fibrillary acidic protein*
GluR1	*Glutamate receptor 1*
Gsh2	*Glutathione synthase 2*
GR	Granule cells
Hes1	*Hairy and enhancer of split-1*

Jag1	*Jagged1*
Jag2	*Jagged2*
KO	Knockout
dLGE	Dorsal lateral ganglionic eminence
MAP2	*Microtubule-associated protein 2*
Math4A	*Neurogenin2*
Mash-1	*Mammalian achaete-scute homolog 1*
MGE	Medial ganglionic eminence
NCAM	*Neural cell adhesion molecule*
NICD	*Notch intracellular domain*
NeuroD	*Neurogenic differentiation 1*
NSCL-1	*Neuronal stem cell leukemia 1*
NSCs	Neural stem cells
OB	Olfactory bulb
P0	Postnatal day 0
PG	Periglomerular cells
RMS	Rostral migratory stream
SVZ	Subventricular zone
Sox2	*SRY (sex determining region Y)-box 2*
TuJ1	*Beta tubulin 1*
TH	*Tyrosine hydroxylase*
Type A	Neuroblasts
Type B	Neural stem cell

Type C	Transit amplifying progenitors
TUNEL	Terminal Transferase dUTP Nick End Labeling
VZ	Ventricular zone

CHAPTER 1

1.1 Chapter Summary

In this dissertation, I investigate the role of *Jagged1* and *Notch* genes in the subventricular zone during late embryonic stages. In this chapter, I discuss how the subventricular zone is formed and developed. Second, I discuss how the subventricular zone undergoes neurogenesis to produce neurons that will migrate to the olfactory bulbs. Third, I explain how neurogenesis is controlled through the *Notch* signaling pathway. Finally, I discuss how the *Notch* signaling pathway is regulated through *Jagged1* signaling.

1.2. Neurogenesis in the Subventricular Zone

1.2.1 Formation of the Embryonic Subventricular Zone

At embryonic day 10.5 (E10.5), the anterior region of the neural tube forms into three vesicles called the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). The prosencephalon vesicle develops into the lateral ventricle of the forebrain.

By embryonic day 11.5, the lateral ventricle can be characterized into at least three prominent regions: the upper dorsal region, the lateral ganglionic eminence (dLGE), and the medial ganglionic eminence (MGE). The upper most dorsal region gives rise to cortical neurons, the anterior-dorsal region of the dLGE gives rise to

olfactory interneurons, and the MGE, which is positioned ventral to the dLGE, produces GABAergic cortical neurons.

The lateral ventricle outer-most layer consists of neuroepithelial cells, which give rise to neuronal precursors. As development proceeds, a subset of neuroepithelial cells will differentiate into cuboidal epithelial cells. Cuboidal epithelial cell can proliferate to give rise to neural stem/progenitor cells, which divide to form the ventricular zone (VZ) and subventricular zone (Takahashi, 1996) (view section 1.2).

At embryonic day 12.5-18.5, dLGE cells in the ventricular zone give rise to the subventricular zone (view section 1.2), a restricted population of precursors. For example, neural stem cells such as those that express *GFAP*$^{+}$ and *Sox2*$^{+}$ (Kriegstein and Alvarez-Buylla, 2009) may generate specific subtypes of neuronal precursors. These subtypes include: *Emx1, Pax6, EGFR, PSA-NCAM*, *Notch1* and *Mash-1* expressing cells (Young et al., 2007; Kriegstein et al., 2009; Merkle et al. 2007, Kelsch et al. 2007; Ventura & Goldman 2007). Among these neuronal precursor populations are neuroblasts. Neuroblasts undergo tangential migration into the rostral migratory stream (RMS), which is a specialized structure connecting the dLGE and olfactory bulb. Once neuroblasts complete their migration they differentiate into GABAergic interneurons. GABAergic interneurons integrate into either the granule cell layer or periglomerular layer (Wichterle et al., 1999; Young et al., 2007). Hence, the dLGE is critical for olfactory development. As early as E12.5-13.5, a study performed by Vergano-Vera and others (2006) showed that precursor cells from the dLGE labeled

with *enhanced green fluorescent protein* (*EGFP*) that were transplanted into the olfactory bulb of early postnatal mice tested positive for olfactory interneuron markers in the olfactory bulb. In a similar study performed at E14.5-17, cells in the dLGE were labeled with Bromodeoxyuridine (BrdU), a synthetic nucleoside that is used to detect proliferating cells. After 6 hours, BrdU-positive cells were co-labeled with *tyrosine hydroxylase*, a marker for olfactory interneurons. Moreover, they revealed that these labeled cells were highest in the granule and periglomerular layers of the olfactory bulbs (Tuker et al., 2006). Taken together, these studies demonstrated that precursor cells form the dLGE have the ability to migrate and differentiate into olfactory interneurons.

As development proceeds, the VZ begins to regress, and the neural stem/progenitor cells of the subventricular zone become the primary site of neurogenesis for the production of mature interneurons in the olfactory bulbs (Figure 1.1) (Reznikov et al., 1997). However, during development, the signals that regulate olfactory neurogenesis in the subventricular zone are poorly defined. A better understanding of this process has important implications in olfactory development. Therefore, it is critical to elucidate the molecular mechanisms underlying the regulation of neurogenesis in the subventricular zone during late embryonic development.

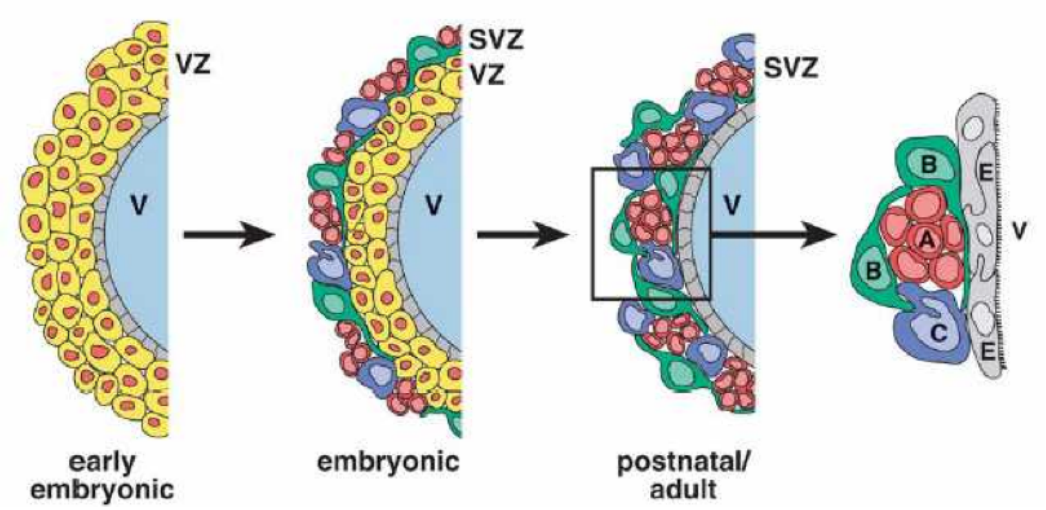

Figure 1.1 Scheme depicting the proliferative ventricular zone (VZ) and subventricular zone through development and into adulthood. The subventricular zone is comprised of multipotent neural stem cells (Type B), transit amplifying progenitor cells (Type C), and neuroblast cells (Type A) that reside along the lateral ventricle (V). Figure modified from Doetsch et al., 1997.

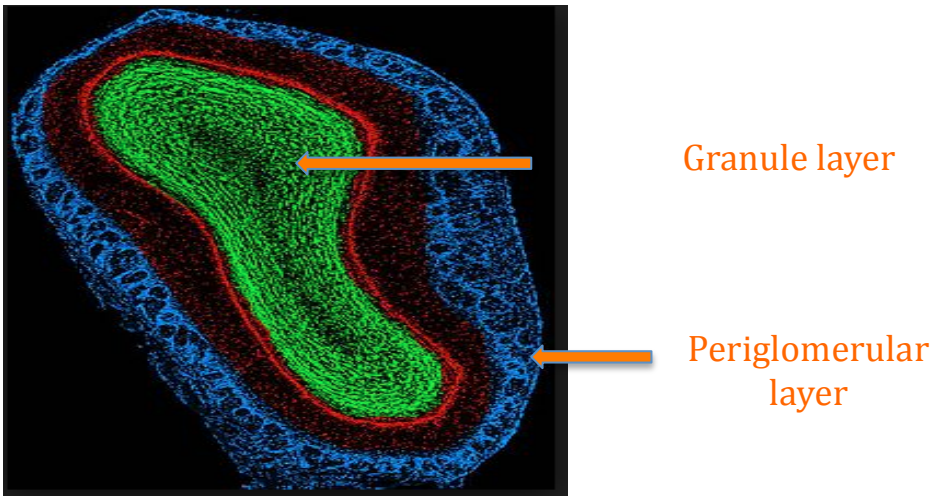

Figure 1.2 Coronal image of mouse olfactory bulb. Top of image is dorsal, right of image is lateral. Periglomerular layer is represented in blue and the granule layer is shown in green. Scale, ventral to dorsal, is approximately 2mm (Matt Valley, 2006)

1.2.2 Neurogenesis in the Subventricular Zone

From late embryonic development to adulthood, the subventricular zone contains a large concentration of multipotent neural stem cells (Type B cells) and transit amplifying progenitor cells (Type C cells) that are responsible for initiating neurogenesis (Doetsch et al., 1997; Doetsch et al., 1999; Conover et al., 2002; Striedter et al., 2009) (figure 1.4). As briefly mentioned in the last section, neural stem cells generate transit amplifying progenitor cells (progenitor/Type C cells), which in turn, produce neuroblasts (Type A cells) (Doetsch et al., 1999; Conover et al., 2002; Striedter et al., 2009). These neuroblasts differentiate into mature interneurons. These interneurons integrate with the existing circuitry and functionally contribute to olfaction (Gheusi et al., 2009; Carleton et al., 2003). Therefore, the neural stem/progenitor cells in the subventricular zone are important contributors to olfactory neurogenesis, and insight into how neurogenesis is regulated is important to understanding how the olfactory system functions.

The subventricular zone provides a suitable model to investigate neurogenesis. First, genes that may disrupt neurogenesis in the subventricular zone are likely to show visible defects in the olfactory neurogenesis pathway (figure 1.3), and markers are available to detect these disruptions. For example, neuroblast marker, *neural cell adhesion molecule* (*NCAM*), can be used to visualize neuroblast defects. Moreover,

neuroblasts are known to migrate tangentially, therefore their pathway can be easily traced to identify disruptions. As a second example, neuroblasts are known to differentiate into granule and periglomerular interneurons; hence any defects in neuroblasts will likely affect these interneurons. These interneurons have been shown to form continuous and well-defined olfactory bulb layers (figure 1.2), and markers can be used to investigate these well-defined layers. Altogether, the subventricular zone is a simple model to detect interruptions in the olfactory neurogenesis pathway.

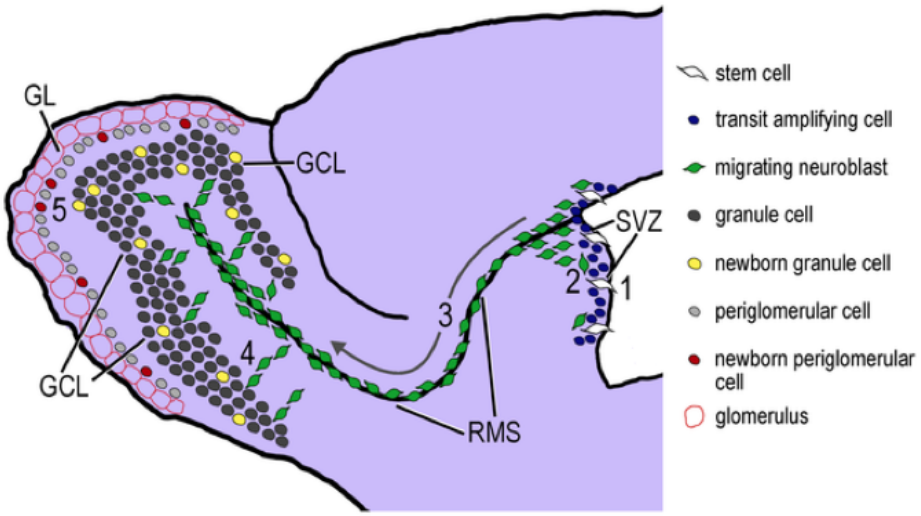

Figure 1.3. Cartoon diagram depicting a sagittal view of the mouse brain during olfactory neurogenesis. Olfactory neuronal precursors (stem cells/transit amplifying cells) proliferate primarily within the subventricular zone (SVZ) where they generate neuroblasts. Arrow indicates the direction of neuronal migration through the rostral migratory stream (RMS). Neuroblasts migrate towards the granular (GCL) and periglomerular (GL) layers of the olfactory bulb (OB). (Sui et al., 2012)

A second advantage of studying the subventricular zone as a model for neurogenesis is that that the subventricular zone contains high numbers of neural stem/progenitor cells that are actively proliferating during development (Striedter et al., 2009). These cells can be isolated and studied *in vitro* using a powerful technique called the neurosphere assay (Reynolds & Weiss, 1992). The neurosphere assay has been one of the most valuable techniques in the study of neurogenesis and stem cell biology (Reynolds and Rietze, 2005; view appendix). Isolation of neural stem/progenitor cells will produce neurospheres in culture (Louis et al., 2013). A neural stem cell, given the appropriate growth conditions, can divide and form into the characteristic 3-D clusters that resemble a free-floating sphere. The number and size of neurospheres produced can provide valuable insights into the molecular mechanisms that control cell division, production of progeny populations, and maintenance of neural/progenitor cells (Reynolds & Ritze, 2005). Therefore, the neurosphere assay provides an accessible tool to study the high numbers of neural stem cells in the subventricular zone.

However, many questions remain about how neural stem/progenitors cells are regulated. For example, what are the signals that maintain neural stem/progenitor populations in an undifferentiated state? Clearly, the proper signals are needed to preserve the pool of neural stem cells, and to prevent premature differentiation (Dang et al., 2006; Mizutani et al., 2007; and Pierfelice et al., 2008). A better understanding

of these processes can have significant implications for elucidating the molecular mechanisms that regulate the embryonic brain.

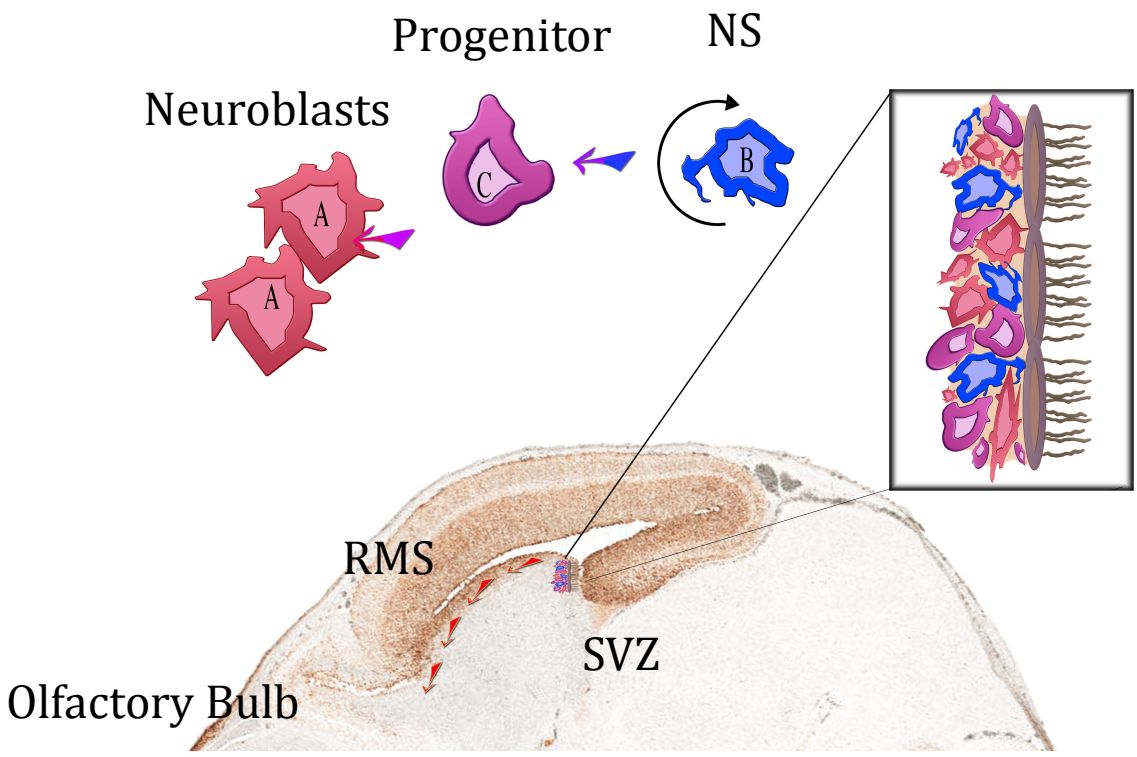

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Figure 1.4 This scheme depicts the stages during olfactory neurogenesis at embryonic day 15.5. In the dorsal lateral ventricle, neural stem cells (blue) generate progenitors (purple), which in turn, produce neuroblasts (red). Neuroblasts migrate through the rostral migratory stream (RMS) (located above red arrows), and into the olfactory bulb to differentiate into mature interneurons. Black box shows the constellation of cells located along the ventricular lining of the lateral ventricle.

1.2.3 The Embryonic and Adult Subventricular Zone are Counterparts

Although this dissertation is focused on the embryonic subventricular zone, it is important to note that their are important similarities between the embryonic and adult subventricular zones. The adult subventricular zone is the primary site of neurogenesis in the central nervous system, producing approximately 30,000 new neurons per day compared to 9,000 in the hippocampus (Cameron and McKay, 2001; Lois and Alvarez-Buylla, 1994). Similar to the embryonic subventricular zone, the adult subventricular zone undergoes tangential migration and gives rise to olfactory interneurons (Wichterle et al., 1999; Parmar et al., 2003; Tucker et al., 2006; Vergano-vera et al., 2006; Young et al., 2007). This process is important to compensate for dying neurons in the forebrain as a result of cerebral ischemia or stroke (Jin et al., 2001; Kadam et al., 2008). Hence, a better understanding of how neurogenesis is regulated during development may provide insight into how a healthy brain is maintained throughout life.

1.3 *Notch* expression and function in the Subventricular Zone

Highly regulated neurogenesis is essential for the development of a healthy brain. At the center of neurogenesis are neural stem cells. A neural stem cell, through its surrounding environment, receives specific instructions to regulate when, how much, and what type of neurons it needs to produce at any given time (Christie et al, 2013). One of the most important receiving components expressed by neural stem

cell populations are the *Notch* receptors (Hitoshi et al., 2002; Alexson et al., 2006; Piccin et al., 2013). Studies have shown that neurogenesis in the subventricular zone is coordinated with the upregulation and activation of the *Notch* signaling pathway (Liu et al., 2007; Wang et al., 2009). Hence, the *Notch* receptors provide coordination with neighboring cells to ensure that the neural stem populations meet the needs of the healthy brain.

1.3.1 Notch expression during late development of mammalian brain

During late embryonic development, *Notch1-3* receptors are expressed among heterogeneous populations of cells in the subventricular zone. Previous studies have found that these receptors are expressed on neural stem/progenitor cells and neuroblasts (Gaiano et al., 2000; Irvin et al., 2001). Interestingly, these expression patterns are observed around the time when the dLGE produces neurons that will undergo tangential migration to the olfactory bulb (Wichterle et al., 2001), leading to the question of whether *Notch* plays a role in the olfactory neurogenesis pathway during development. In support of this model, *Notch1* and *Notch*3 have been shown to promote survival of radial glial cells, which some have classified as part of the neural stem cell populations (Gaiano et al., 2000; Dang et al., 2006). Additionally, *Notch1* has been shown to regulate neuroblasts (Casarosa et al., 1999). Similarly, in postnatal neurospheres, *Notch1* has been shown to be co-expressed with *Nestin*, a marker of neuronal precursor cells (Nyfeler et al., 2005). These findings suggest that *Notch* may play a currently undefined role in neuronal precursor cells.

1.3.2 *Notch* Signaling in the Central Nervous System: An overview

Activation of the *Notch* receptor occurs through physical interaction with a neighboring ligand. Once activated, the *Notch* receptor is cleaved by *metalloproteinase ADAM* and γ-secretase; this then releases the *Notch intracellular domain* (*NICD*). The *NICD* translocates directly to the nucleus, where it forms a transcriptional complex with the DNA-binding protein *CSL* (*CBF1, Suppressor of Hairless-Lag2*), *Mastermind* and transcriptional co-activators, to drive the expression of *Notch* target genes (Bray et al., 2006; Kopan and Ilagan, 2009). In the absence of NICD, *CSL* forms complexes with a variety of co-repressors to suppress the transcription of *Notch* target genes (Bray et al., 2006; Kopan and Ilagan, 2009; Figure 1.5). However, activation of *Notch* results in up-regulation of target genes such as the *Hes* family. *Hes* proteins, in turn, repress the activation of the proneural bHLH factors like *Mash-1*, which is expressed in differentiated cells such as neuroblasts (Guillemot and Joyner, 1993). Thus, activation of *Notch* signaling helps to maintain the *Notch* signaling-receiving cell in an undifferentiated state.

Simplified diagram of the *Notch* pathway

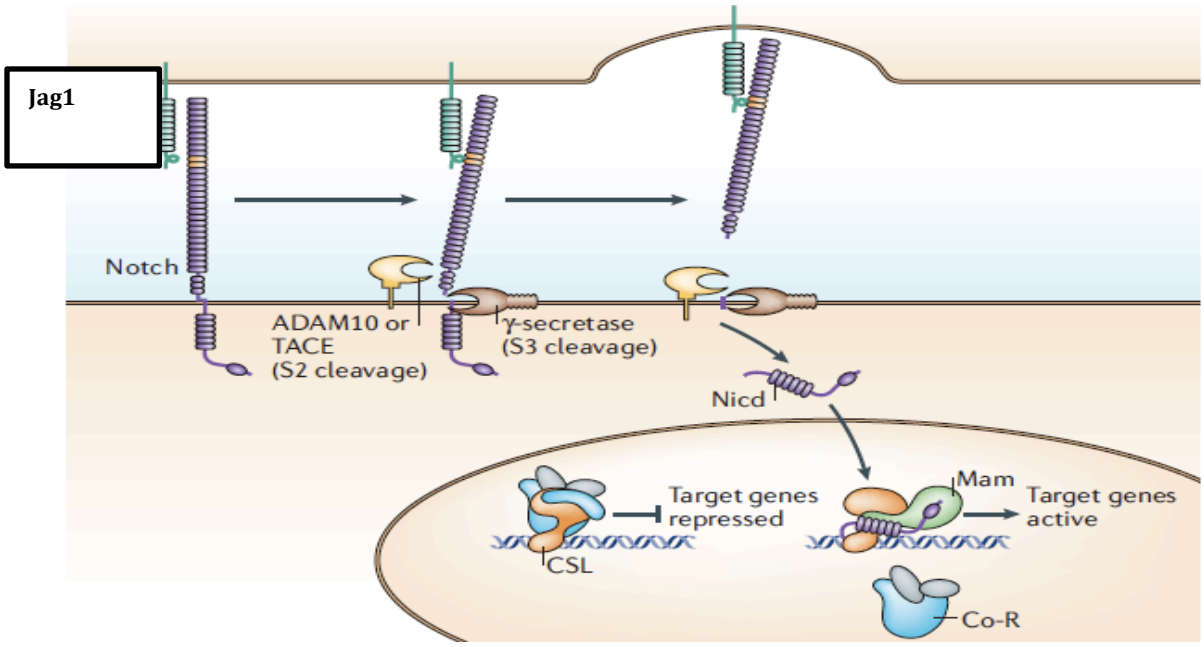

Figure 1.5. Physical interaction of *Jagged1* (green) on one cell with the *Notch* receptor (purple) on another cell results proteolytic cleavages from the *Adam10* (yellow) *and* γ-*secretase* (brown) enzymes. This proteolytic processing mediates release of the *Notch intracellular domain* (NICD), which enters the nucleus and interacts with the DNA-binding *CSL* protein (orange). The co-activator and other transcription factors such as *Mam*, are recruited to the *CSL* complex to displace the Co-repressor (Co-R; Blue). *Notch* activation leads to the upregulation of target genes (modified from Bray, 2009).

1.3.3 Notch Plays Multiple Roles During Development

The *Notch* signaling pathway is well known for its role in cell fate (Weinmaster, 1997; Artavanis-Tsakonas et al., 1999). However, more recent studies have revealed additional functions of *Notch* during embryonic development. These extra roles include neuronal differentiation, cell survival, maintenance of precursor cells, and self-renewal (Wakamatsu et al. 2000, Nakamura et al., 2000, de la Pompa et al. 1997, Hitoshi et al., 2002; Yoon et al., 2008).

Notch has been shown to regulate neuronal differentiation (de la Pompa et al., 1997; Wakamatsu et al. 2000). In chicks, at embryonic day 3, *Notch* signaling was manipulated in neural crest-derived culture by transfecting expression constructs that either activated or inhibited *Notch* function. In cultures transfected with activated *Notch* constructs, neuronal differentiation was examined by immunolabeling for *Elav embryonic lethal, abnormal vision (Elav)* protein, a neural cell marker required for correct differentiation and maintenance of the nervous system. In this study, they found no significant change in neuronal differentiation. Whereas, in cultures transfected with *numb*, an antagonist of *Notch*, they observed a dramatic increase in neuronal differentiated cells. These experiments indicate that inhibition of *Notch* may increase neuronal differentiation in neural crest-derived cultures (Wakamatsu et al., 2000).

Similarly, null mutations in either *Notch1 (Notch1$^{-/-}$)* or *RBP-Jk (RBP-Jk$^{-/-}$)*, a downstream regulator of the *Notch* signaling, showed that inhibition of *Notch* signaling increases neuronal differentiation in mice. In this study, mutant mice were examined for the expression of three early-expressing transcription factors found in differentiating neurons such as *Math4A*, *NeuroD*, and *NSCL-1*. Immunochemistry analysis showed that early-expressing markers were increased in both *Notch1$^{-/-}$* and *RBP-Jk$^{-/-}$* mice (de la Pompa et al., 1997). Consistent with this study, another research group demonstrated that null mutations in the *Notch* downstream effector *Hes1* also exhibited an increase in early-expressing neuronal precursors (Nakamura et al., 2000). During development, persistent expression of *Hes1* prevents migration of neural progenitor cells out of the ventricular zone (Ishibashi et al., 1994). Strikingly, *Hes1* null brains were found to express late neuronal markers (*MAP2* and *neurofilament*) prematurely. Together, these finding suggest that the loss of *Notch* cause an increase in neuronal differentiation.

Notch has also been implicated in cell survival (Nakamura et al., 2000, Theotokis et al., 2006). Previously reported *Notch* mutants (*Notch1$^{-/-}$*, *Hes1$^{-/-}$*, and *RBP-Jk$^{-/-}$*) were found to have an increase in premature neurons. Furthermore, it was shown that the *MAP2* and *neurofilament* expressing neuronal precursors were abnormal, and followed an apoptotic fate (Nakamura et al., 2000). For example, in embryonic day 10.5, *Hes1$^{-/-}$* mice the forebrain was shown to have a significantly high number of apoptotic cells compared to littermate controls (Nakamura et al., 2000).

Moreover, *in vivo,* mutations in the *Notch* signaling pathway led to depletion of neural stem cells and an increase in premature neuronal precursor populations (La Pompa et al., 1997; Hitoshi et al., 2002). Conversely, activation of *Notch* promotes survival of neural stem cells (Theotokis et al. 2006). Therefore, the *Notch* signaling pathway has been demonstrated to maintain neurogenesis; however, the ligand that regulates this process is not fully understood.

Notch has been shown to be critical for neural stem cell survival *in vitro* (Nakamura et al., 2000, Hitoshi et al., 2002, Imayoshi et al., 2010). Using the neurosphere assay, neurospheres generated from *Notch1*$^{-/-}$ or *RBP-Jk*$^{-/-}$, a downstream effector of *Notch* activation, led to depletion of neural stem cells. Conversely, activation of *Notch* promotes survival of neural stem cell *in vitro* (Hitoshi et al., 2002; Theotokis et al., 2006). Similarly, when *Notch1*$^{-/-}$ cultures were infected with virus containing the constitutively active form of *Notch1,* neural stem cell populations were maintained (Hitoshi et al., 2002). These findings suggest that *Notch* is critical for maintaining neural stem cell populations.

Several studies have suggested that *Notch* maintains neural stem cell populations by sustaining its capacity to self-renewal (Nakamura et al., 2000, Hitoshi et al., 2002, Imayoshi et al., 2010). This was determined by using the Colony Forming assay. The Colony Forming assay is similar to the neurosphere assay, and involves isolating chunks of tissue and separating them into individual cells. Neural stem cells undergo unlimited division and differentiation, and under the proper culture conditions

will form neurospheres. The size and number of neurospheres is an indicator of how many neural stem cells are present, but as noted previously, neural stem cells give rise to progenitor cells. Progenitors can also be studied because they too will divide in culture, and will also form neurospheres. However, these neurospheres tend to be smaller in size than those derived from neural stem cells. Moreover, upon repeated passaging progenitors will ultimately disappear. Therefore, one way in which one can study neural stem/progenitor cells in the subventricular zone is to use the Colony Forming Assay. The Colony forming assay enables neurospheres derived from a neural stem cell or from a progenitor cell to be distinguished (Louis et al., 2008).

For instance, in *Notch1*$^{-/-}$ and *Hes1*$^{-/-}$ mutant mice, self-renewal was examined by dissociating primary neurospheres into single cells (Nakamura et al., 2000, Hitoshi et al., 2002, Imayoshi et al., 2010). Then the single cells were evaluated based upon their capacity to self-renewal, and to form secondary neurospheres. They found that both *Notch1*$^{-/-}$ and *Hes1*$^{-/-}$ mutant mice form fewer secondary neurospheres. This suggests the *Notch* signaling pathway may also play a role in self-renewal.

Taken together, these findings suggest that the regulation of *Notch* may play a critical role neuronal differentiation, cell death, maintenance of neural stem cells, self-renewal and cell proliferation.

1.4 The role of *Jagged1* in Mammalian Development

1.4.1 *Jagged1* is a Single-Pass Transmembrane Protein

Five canonical mammalian *Notch* ligands have been described: *Jagged1*, Jagged2, *Delta-1,-3,-4*. The extracellular portion of *Notch* ligands is characterized by their related structural motifs: an N-terminal *Delta-Serrate-Lag2* (DSL) domain, and specialized tandem EGF-like repeats (figure 1.6). *Notch* ligands can be further classified on the basis of the presence or absence of a cysteine-rich domain into the *Jagged* or *Delta*-like group. *Jagged1* and *Jagged*2 groups contain cysteine-rich domains, whereas the *Delta* ligand group does not. Moreover, *Jagged1* and *Jagged*2 have almost twice the number of EGF repeats as *Delta* ligands, but the purpose of these repeats is unknown (Weinmaster et al., 1997). Moreover, the intracellular portion of *Notch* ligands varies in length, and their function remains largely uncharacterized. However, deletion analysis has shown it to be essential for normal function (Hukriede et al., 1997).

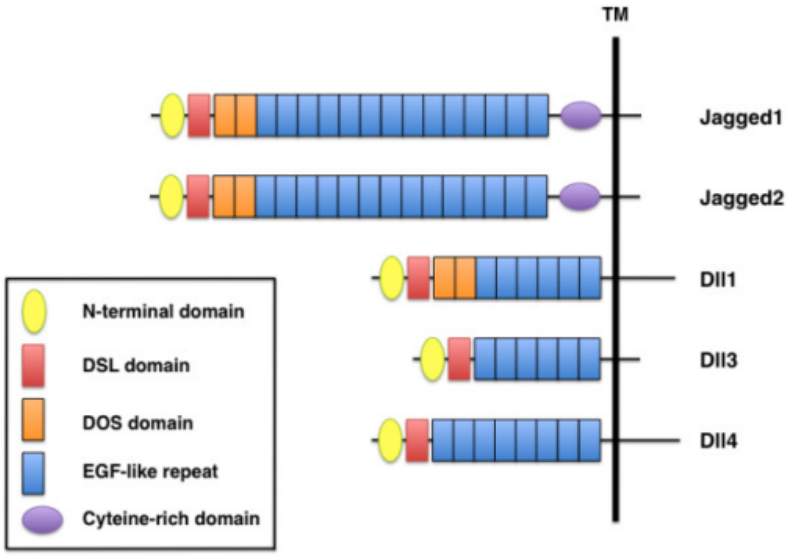

Figure 1.6 *Notch* Receptors and Ligands. Schematic representation of the structural organization of mammalian *Notch* ligands. All *Notch* ligands have an N-terminal domain, a DSL (*Delta*-serrate-*Lag*2) domain, and EGF–like repeats (Kume et al., 2009).

1.4.2 *Jagged1* Expression in the Subventricular Zone

The expression pattern of *Jagged1*, a *Notch* ligand, supports its role as an important activator of *Notch* signaling in the subventricular zone. At embryonic day 17, *Notch* ligands *Jagged1*, *Delta1,* and *Delta*2 are expressed in the subventricular zone (Irvin et al., 2004). Interestingly, at postnatal day 4, *Jagged1* is robustly expressed, whereas *Delta1* and *Delta3* are barely detectable (Stump et al., 2002). Similarly, a recent study performed at the same developmental stage showed that *Jagged1* forms a cluster of expression in the dorsal lateral region of the subventricular zone, whereas *Delta*1 and *Delta*3 form scattered faint patterns around the subventricular lining (Irvin et al., 2004). In postnatal stages, *Jagged1* expressing cells are adjacent to *Notch1* presenting cells (Stump et al., 2002; Nyfeler et al., 2005). This suggests that *Jagged1* presenting cells are positioned to signal to *Notch*-presenting cells in the subventricular zone. Why is *Jagged1* upregulated during late development? Why is the expression of *Jagged1* prominent during the expansion of the subventricular zone? Although *Jagged1*'s expression has been well documented in postnatal and adult stages (Stump et al., 2002; Chojnancki et al., 2003; Irvin et al., 2004; Nyfeler et al., 2005; Givogri et al., 2006; Wang et al., 2009), *Jagged1's role* during embryonic development is less understood.

1.4.3 *Jagged1* Activation of *Notch* in different systems

In vitro, *Jagged1* has been shown to suppress muscle differentiation in rats (Lindsell et al., 1995). In that study, myoblasts expressing *Notch1* could differentiate into myotubes. However, when co-cultured with a cell line expressing *Jagged1*, cells did not align with each other and therefore, could not form myotubes (Lindsell et al., 1995). This study concluded that *Jagged1* prevents muscle cell differentiation.

In addition, *Jagged1* has also been shown to inhibit oligodendrocytes precursor cells from maturing into oligodendrocytes, which is one of the major phenotypes in some cases of multiple sclerosis (John, et al., 2002; Seifert et al. 2007; Wang, et al., 1998). In experiments performed by Seifert et al., they found that activated *Notch* was increased at sites of lesions and plaques where precursor cells are involved in remyelination. Moreover, *Jagged1* also regulates precursor cells in hair cell production. A study performed in *Jagged1* knockout mice ($Pax8^{Cre+/-}$; $Jag1^{f/f}$) showed that deletion of *Jagged1*'s function in otocysts led to progenitor cells' inability to generate mature hair cells. Therefore, *Jagged1* mutants had reduced patches of empty space where hair cells were missing (Hao et al., 2012).

Altogether, *Jagged1* has been shown to be an important regulator in the regulation of precursor cells. Does *Jagged1* regulate precursor cells in the subventricular zone? Understanding the role of *Jagged1* in the subventricular zone may provide insight into how *Notch1* expressing neural stem /progenitor cells are

regulated.

1.4.4 In Humans, JAGGED1 Mutations cause Alagille Syndrome

In humans, most mutations in JAGGED1 (JAG1) cause Alagille Syndrome (ALGS) Krantz et al., 1997, Alagille et al., 1975). ALGS is an autosomal dominant disorder characterized by abnormalities in the central nervous system and other organs (Krantz et al., 1997, Alagille et al., 1975). In the central nervous system, intelligence quotient tests demonstrated significant levels of mental retardation while radiographs of spinal cords showed abnormal growth in patients with this syndrome (Alagille et al., 1975). Due to the decrease in the proliferation of bile ducts, ALGS is also a major cause of chronic liver disease in children (Alagille et al., 1975).

The cause of Alagille syndrome, in most cases, is due to a nonsense or frameshift mutation in the JAG1 gene (Krantz et al., 1997; reviewed by Taxuexda et al., 2012). These mutations result in the generation of abnormal JAG1 protein. Moreover, since JAG1 is a single–pass transmembrane protein, mutations in its N-terminal extracellular domains, the region required for binding and activating NOTCH receptors, may cause harmful effects (Warthen et al., 2006). Furthermore, it has been shown that haploinsufficiency of JAG1 is sufficient to cause ALGS (Taxuexda et al., 2012).

In mice, *Jagged1* null mice phenocopy ALGS syndrome in humans (Xue et al., 1999). *Jagged1* null mice have a reduced diameter of the brain, and vascular defects

in the forebrain (Xue et al., 1999). There is no cure for ALGS, and in *Jagged1* deficient mice, the role in the central nervous system has not been fully investigated due to early embryonic lethality (Xue et al., 1999). *In vitro* studies studying the role of *Jagged1* during embryonic development do not exist. However, postnatal studies using virally infected neural stem cells of *Jagged1* floxed mice have shown that *Jagged1* is required for stem cell renewal (Nyfeler el al., 2005). Hence, *Jagged1* is an important regulator in the central nervous system, and perturbing *Jagged1*-mediated *Notch* signaling may lead to effects on proliferation. Therefore, understanding the molecular mechanism underlying the regulation of *Jagged1* during late embryonic stages has important health implications.

1.4.5 *Jagged1* Mouse Model

Investigating the role of *Jagged1* in the central nervous system has been a challenge due to the lack of an adequate mouse model. *Jagged1* null mice exhibited gross defects in vascular remodeling, which included blood vessels in the cranial regions. Unfortunately, the mice die at E10, which makes studying the role of *Jagged1* during late stages of development impossible (Xue et al., 1999).

A more recent mouse model using a compound reduction of *Jagged1* and *Notch1* (*Jagged1*$^{+/-}$; *Notch*$^{+/-}$) has revealed some information regarding the role of *Jagged1* in the subventricular zone. These mutants showed reduced proliferation in the subventricular zone and rostral migratory stream (RMS) (Nyfeler et al., 2005).

However, due to the absence of specific markers they were unable to investigate whether progenitor cells or neuroblasts were affected by the reduction of *Jagged1* and *Notch1*. Hence, how the loss of *Jagged1* affects cells involved in olfactory neurogenesis such as the neural stem cells, progenitors, neuronal precursors, and mature neuronal cells remains poorly understood. Moreover, Nyfeler et al, 2005 studies in neurospheres suggested that *Jagged1* is required for self-renewal; however, this conclusion requires more supporting evidence. Nyfeler et al. findings were based on evaluating self-renewal abilities of primary and secondary neurospheres using the neurosphere assay. They showed that the loss of *Jagged1* led to reduce formation of secondary neurospheres. However, a previous study determined that any loss of self-renewal at the secondary passage may also affect non-neural stem cell populations (Reynolds and Rietze, 2005). Therefore, it remains to be determined whether the loss of *Jagged1* affects self-renewal of neural stem cells.

1.5. Summary

Due to the lethality associated with complete loss of *Jagged1,* and the lack of mutant mouse models, the role of *Jagged1* in the subventricular zone remains elusive. Much of what is known about *Jagged1* in the subventricular zone has been revealed from studies performed in postnatal heterozygous (*Jagged1*$^{+/-}$; *Notch1*$^{+/-}$) mice. In this mouse model, *Jagged1* has been shown to be important for proliferation and self-renewal of neural stem cells in the subventricular zone (Nyfeler et al., 2005). However, there are many questions that remain unanswered: (1) Does *Jagged1* affect proliferation of neural stem cells or progenitor cells or both during development of the subventricular zone? (2) Does the loss of proliferation affect olfactory development? (3) Does *Jagged1* affect self-renewal of neural stem or progenitor cells?

In this thesis, I investigate the role of *Jagged1* by utilizing a conditional *Jagged1* knockout. *Cre* expression is driven by the promoter elements of the *Foxg1* gene, which is expressed at embryonic day 10 in the forebrain. This *foxg1-cre* driver will delete *Jagged1* function during late embryonic development of the subventricular zone. To further understand the role of *Jagged1* I use the neurosphere assay to evaluate neurogenesis and self-renewal of neural stem and progenitor cells.

Chapter 2 presents the results of my experimentation with *Jagged1 in vivo*. I show that the loss of *Jagged1* dramatically affects proliferation in the subventricular zone. To further understand this, I examined how the loss of *Jagged1* affects neural

stem/progenitor populations, migrating neurons, and mature neurons in the olfactory bulb. This analysis showed that *Jagged1* is a critical regulator of neurogenesis.

Chapter 3 presents the results of my analysis of the role of *Jagged1 in vitro.* To do this, I used the neurosphere assay to study neural stem cells. Using the neurosphere assay, I can isolate large quantities of neural stem cells in the subventricular zone. I also show that the loss of *Jagged1* affects neural stem cells' ability to produce progeny and self-renewal. Moreover, I show that *Jagged1* is necessary for the formation of neurospheres.

Chapter 4 reports the expression analysis of *Jagged1* in subventricular zone. Therefore, I performed double-label *in situ* hybridization of *Jagged1* in neural stem cells. I show that *Jagged1* is co-expressed on different population of neural stem cells in the subventricular zone.

In summary, this thesis provides novel evidence that *Jagged1* is a critical regulator of neurogenesis during late development of the subventricular zone, and provides a better understanding of how *Notch* signaling regulates olfactory neurogenesis.

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CHAPTER 2

JAGGED1-MEDIATED *NOTCH* SIGNALING REGULATES NEUROGENESIS IN THE SUBVENTRICULAR ZONE

2.1 Abstract

Notch signaling is critical for the maintenance of neural stem cells (NSCs) during mammalian neurogenesis. NSCs in the subventricular zone undergo neurogenesis to generate interneurons within the olfactory bulb. *Notch* signaling is controlled by a variety of activators. However, which activator regulates neurogenesis in the subventricular zone is not well understood. Here we show that *Jagged1,* an activator of *Notch,* is critical for neurogenesis in the subventricular zone. In the absence of *Jagged1* the numbers of NSCs, neuronal precursors, and interneurons are reduced. Moreover, we show that *Jagged1* is critical for neuronal precursor survival. These results suggest that *Jagged1* functions to maintain olfactory neurogenesis. This is the first demonstration of *Jagged1* function in the subventricular zone during embryonic development and may provide insight into its role in adult neurogenesis.

2.2 Introduction

In the embryonic mammalian brain, the telencephalon transforms into two highly proliferative vesicles: the ventricular zone (VZ), which consists of a pseudostratified epithelium that lines the lateral ventricle, and the subventricular zone, located adjacent to the VZ (Boulder Committee, 1970). Within the subventricular zone, the dorsal lateral ganglionic eminence (dLGE) contains *GFAP*-expressing neural stem cells (Kriegstein and Alvarez-Buylla, 2009). *GFAP*-expressing neural stem cells may generate into specific subtypes of neuronal precursors. These subtypes include: *EGFR, PSA-NCAM*, *Notch1,* and *Mash-1*-expressing cells (Young et al., 2007; Kriegstein et al., 2009; Merkle et al., 2007, Kelsch et al., 2007; Ventura and Goldman 2007). Among the neuronal precursors are progenitor cells that undergo neurogenesis to generate neuroblasts. However, the genes that regulate this process are not completely clear. Neuroblasts in turn undergo migrate from the subventricular zone through the rostral migratory stream (RMS) into the olfactory bulb (OB), where they differentiate into various types of interneurons). The *Notch* signaling pathway has been demonstrated to regulate neurogenesis in the adult subventricular zone. In contrast, the ligands that regulate the production of newly born neurons during development are not yet fully understood.

The *Notch* receptor family includes four members in mammals: *Notch1*, *Notch2*, *Notch3* and *Notch4* (Artavanis-Tsakonas et al., 1995 and Greenwald et al., 1994).

These receptors are expressed among radial glial, neural stem cell/progenitor, and neuroblast populations in the lateral ventricle of the forebrain (Irvin et al., 2001). During development, *Notch* is essential for the maintenance of neural stem cell/progenitor populations (Imayoshi et al., 2010; Hitoshi et al., 2002), and regulates the production of neuronal precursors (Casarosa et al., 1999). *Notch1* and *Notch3* activation promote radial glial identity (Gaiano et al., 2000; Dang el al., 2006). *In vitro*, *Notch1* activation has also been implicated in neurogenesis (Nye et al., 1994; Morrison et al., 2000). Together, these studies indicate that *Notch* signaling is essential for neurogenesis during embryonic development of the forebrain.

Consistent with the role of *Notch* in the forebrain, *Jagged1*, an activator of *Notch* signaling, is also essential for neurogenesis. During development, *Jagged1* is expressed in the telencephalic vesicles (Yun et al., 2002, Irvin et al., 2004,). In postnatal stages, mice heterozygous for both *Jagged1* and *Notch* (*Jagged1*/+; *Notch*/+) showed a reduction in mitotic cells in the subventricular zone (Nyfeler et al., 2005). In the same study, *Jagged1* is critical for the self-renewal of NSCs, as demonstrated using virally infected neurosphere cultures. These findings suggest that *Jagged1* may be an important mechanism in regulating neurogenesis, yet its role during late development of the lateral ventricle has not been fully investigated. Moreover, the challenge of investigating *Jagged1*'s role in the CNS has been limited, due to early embryonic lethality in *Jagged1*-deficient mice (Xue et al., 1999).

This study investigated the function of *Jagged1* during the development of the

subventricular zone. To circumvent early lethality, a conditional *Jagged1* mutant mouse model was utilized (Gridley et al., 2006). *Jagged1* mutant mice exhibited multiple defects in neurogenesis in the subventricular zone, affecting the generation of NSCs, neuronal precursors, and migrating neurons. The generation of mature interneurons in the olfactory bulb is subsequently affected. This study therefore provides novel evidence that *Jagged1* functions to regulate neurogenesis in the lateral ventricle during development.

2.3 Materials and methods

Mice

All animal protocols were approved by Cornell University's IACUC. *Jagged1* conditional mutants (*Jag1*$^{f/f}$) were generated as previously described (Gridley et al., 2006) and were maintained in a mixed 129Sv/C57BL/6 background. *Jag1*$^{f/f}$ mice (Gridley et al., 2006) were crossed with *Foxg1-cre* mice (Hebert and McConnell, 2000) to generate F1 *Foxg1-cre*; *Jag1*$^{f/+}$ mice. *Foxg1-cre*; *Jag1*$^{f/+}$ mice were also maintained in a mixed 129Sv/C57BL/6 background. These mice were crossed with *Jag1*$^{f/f}$ to generate the controls (+/+; *Jag1*$^{f/f}$ or +/+; *Jag1*$^{f/+}$, heterozygous (*Foxg1-cre*; *Jag1*$^{f/+}$), and mutants (*Foxg1-cre*; *Jag1*$^{f/f)}$. Within-litter, sex matched comparisons were performed for all studies. The day a vaginal plug in a pregnant female was observed was termed day 0.5.

In situ hybridization

In situ hybridization was done as previously described (Rodriguez et al., 2008). In brief, E14.5-P4 mice were euthanized and decapitated. Brains were embedded in OCT (Tissue Tek) and fresh-frozen in liquid nitrogen-cooled isopentane. Subsequently, 20 µm fresh-frozen cryosections were fixed in 4% paraformaldehyde, washed with PBS (phosphate-buffered saline), and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. Slides were washed again and blocked with Blocking Reagent (Roche) for 2hrs. Slides were then hybridized with digoxigenin-labeled antisense RNA probes for 48hrs at 60-65°C and washed with 5x SSC and then with 0.2x SSC. Afterwards slides were cooled to room temperature and blocked with TNB reagent (Perkin Elmer). Alkaline phosphatase conjugated anti-digoxigenin antibody was applied to slides (Roche) in block overnight at 4°C. Slides were washed with B1 buffer (100 mM Tris pH 7.4, 150mM NaCl), followed by B3 buffer (100 mM Tris pH 9.5, 50 mM MgCl, 100 mM NaCl), and reacted in NBT/BCIP (Promega) Each pair (mutants were compared to heterozygous or control samples) was processed simultaneously, and reacted for the same length of time. Probes were cloned by PCR or generated from NIA-BMAP clonesets.

Terminal Transferase dUTP Nick End Labeling (TUNEL) assay for apoptosis

10μm sections were fixed and rinsed with PBS before incubating in pre-cooled ethanol:glacial acetic acid (2:1) for 5 min at -20 °C. After rinsing with PBS, endogenous peroxidase activity was quenched by incubating in .3% hydrogen peroxide for 10 min at room temperature. After washing with PBS, slides were incubated with equilibration buffer (Chemicon) for 10 mins and then incubated with terminal transferase buffer (New England Biolabs (NEB), 1x CoC_{l2} (NEB), 1x restriction buffer 4 (NEB), and 0.5 mm biotin-dUTP (Roche) for 3.5 h at 37 °C. Reactions were quenched with stop buffer (Chemicon), rinsed with PBS, incubated with streptavidin-HRP (Zymed) and reacted using AEC staining protocol (Zymed).

Immunohistochemistry

Telencephalon from matched embryonic stages were fixed in 4% paraformaldehyde (PFA) in phosphate buffer overnight at 4°C. Paraffin samples were washed in 70% ethanol and embedded in wax. 5μm sections were deparaffinized and processed as described previously (Carson et al., 2006) except samples were microwaved in citric acid. Fresh-frozen samples were fixed in 4% PFA, washed in .1M phosphate buffer for 6-12hrs, and placed in 30% sucrose overnight and embedded in O.C.T. GFAP (Millipore; AB5804) and PCNA (Abcam; ab15497) antibodies were applied at a dilution of 1:100, 1:1000 respectively and incubated overnight at 4°C. The bound antibody was detected using FITC goat anti-rabbit secondary antibody (Vector Labs) and an Alexa Fluor-488 anti-FITC tertiary antibody (Molecular Probes). Sections were imaged on a Leica DMRE upright microscope fitted with bandpass filters.

Histology

Samples were embedded in wax and processed for histology by the Cornell Diagnostic Laboratory as described (Luna et al., 1968 and Preece et al., 1972).

Beta-galactosidase activity detection

Sections (10μm) of fresh frozen tissue were fixed in 2% paraformaldehyde/phosphate buffer for 5 min. Subsequently, sections were washed in PBS and incubated in

staining buffer without X-Gal (10 mM PO_4 buffer pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM ferrous cyanide, 3 mM ferric cyanide, and 0.3% Triton X-100) at 37°C. The buffer was then replaced with staining buffer containing 0.2% X-Gal and incubated at 37°C.

Pixel intensity

JPEG images of sections were imported into ImageJ and converted to 8-bit grayscale. 10 to 24 sections from three or more experiments were analyzed for each gene. Thresholds were defined manually but were performed blind to the specific genotype associated with each image. The number of positive pixels was determined using the "measure" function. Intensity values for any given gene were averaged for each animal.

2.4 Results

Expression pattern of *Notch* receptors, *Jagged1*, and *Delta1* signaling in the dorsal lateral ganglionic eminence

Previous studies have shown *Jagged1* and *Delta1* expression in the forebrain at embryonic day 17. However, these expression patterns were only weakly detected, and it was difficult to determine the location of this expression (Irving et al., 2001). We re-examined the expression of *Notch1-3*, *Jagged1,* and *Delta1* using *in situ* hybridization during late embryonic development (defined as embryonic day 15-18; E15-E18). We found that *Notch*1-*3* were expressed in overlapping patterns within the dorsal ventricle of the developing subventricular zone (Fig. 1A-C). Moreover, we detected *Jagged1* and *Delta1* in the same location (Fig. 1D, E). Altogether, these data show that Notch1-3 are expressed in close proximity to their activators, *Jagged1* and *Delta1* at late embryonic stages.

A conditional knockout mutant to delete *Jagged1* in the embryonic subventricular zone

To circumvent early lethality associated with *Jagged1* null mutant mice, we utilized a conditional knockout mouse model to delete *Jagged1.* The *Jag1*$^{f/f}$ mice were created by flanking the *Delta-Serrate-Lag2* (DSL) domain-encoding exon 4 of the *Jagged1*

gene with loxP sites (Gridley et al., 2006) (Fig. 2A). *Foxg1-cre* mice were used to drive the expression of *cre recombinase* activity starting at embryonic day 10 in the forebrain (Hebert and McConnell, 2000). We therefore expected that this would delete *Jagged1* at E10 through E18.5 in the forebrain and developing subventricular zone. The *Foxg1-cre/+* mice were created by means of replacing the intron-less *Foxg1* coding region with *cre.* The J^{flox}/J^{flox} (*Jag1*$^{f/f}$) (site) mice were created by flanking the *Delta-Serrate-Lag2* (DSL) domain-encoding exon 4 of the *Jagged1* gene with loxP sites (Fig. 2A). We crossed *Jagged1* floxed (*Jag1*$^{f/f}$) mice (Gridley et al., 2006) with *Foxg1-cre* (*Foxg1-cre*/+) deleter mice (Herbert and McConnell, 2000) to examine the effects of removing *Jagged1* function. Heterozygous F1 animals were backcrossed with (*Jag1*$^{f/f}$) mice (Fig. 2C) to generate *Jagged1* mutants (*Foxg1-cre*/+; *Jag1*$^{f/f}$), heterozygous (*Foxg1-cre*/+; *Jag1*$^{f/+}$), and controls (+/+; *Jag1*$^{f/+}$ or +/+; *Jag1*$^{f/+}$) offsprings. All offsprings were identified by PCR (Fig. 2D). *Foxg1-cre* deletion of *Jagged*1 protein was confirmed by western blot analysis (Fig. 2B).

Loss of *Jagged1* causes abnormalities in cell division and reduced numbers of astrocytes and neuronal precursors

To evaluate the effect of *Jagged1* on neurogenesis during late development, we investigated proliferation and neuronal cell types generated by neural stem cells. Cell proliferation was examined by immunostaining for proliferating cell nuclear antigen (PCNA). We observed a significant reduction of PCNA labeling in the dorsal ventricle region (Fig. 3A) of the developing subventricular zone of *Jagged*1 mutants relative to

controls at late embryonic stage 17 (E17) (Fig. 3B, C). The quantification of PCNA labeled showed a ~58% reduction in dividing cells (Fig. 3D). Similarly, a significant decrease was also observed in *Jagged1* heterozygous mice. Furthermore, we observed a significant increase in cell apoptosis in heterozygous (~54%) and mutant mice (~75%) in the rostral migratory stream (appendix Fig1). The *Foxg1-cre* mouse itself is known to have effects on proliferation and apoptosis in the cortex and hippocampus (references). To control for potential effects in proliferation and apoptosis that may occur in the subventricular zone, we examined *Foxg1-cre* heterozygous mice that had not been crossed to the *Jagged1* floxed allele. No significant differences were observed between *Foxg1-cre* (*Foxg1-cre*/+) and C57BL/6 (+/+) mice in cell proliferation at P0 (N=3, Fig. 1D) or apoptosis at E17 (appendix Fig1).

Interestingly, the reduction in dividing cells occurs along the ventricular lining where neural stem cell populations reside (Takahashi et al., 1996; Doetsch et al., 1999). We next looked to determine whether the loss of *Jagged1* affects neural stem cells. Sections of the ventricular lining were immunostained by antibodies against *glial fibrillary acidic protein* (*GFAP*), which labels astrocytic neural stem cells (Fig 3L, M; Kornblum, 2007). Sagittal sections showed a ~55% reduction (N=3, Fig. 3ML-N).

The reduction in cell division and the loss of neural stem cells may alter the generation of neuronal precursors derived from neural stem cells (Doetsch, et al. 1999). We therefore examined one population of neuronal precursors using *in situ* hybridization to detect *Mash-1*$^{+}$ progenitors. Strikingly, the mRNA expression of *Mash-1*$^{+}$ progenitors

showed no significant differences (Fig. 3E-G). We then looked to examine one population of neuroblasts known to express *doublecortin (DCX).* Here we observed a dramatic reduction in *DCX*$^{+}$ neuroblasts (Fig. 3I-K), as there were ~50% reductions in mutants (n=3) compared with controls. Taken together, these results suggest that the loss of *Jagged1* at late embryonic stages affects non-*Mash-1*$^{+}$ progenitors but does affect *DCX*$^{+}$ neuroblasts.

Given the dramatic decline in cell division and neuroblasts in the dorsal lateral region (Fig. 3C), we were surprised at the observed expression of *Mash-1*$^{+}$ progenitors. We then asked whether the loss of *Jagged1* affects other populations of neural stem/progenitor cells. To evaluate the effects of *Jagged1* on neural stem/progenitor cells, we investigated the developing subventricular zone and the RMS by *in situ* hybridization using a *Notch1* probe (Gaiano et al., 2000; Irvin et al., 2001). We found that the average numbers of *Notch1* expressing cells were significantly reduced (-109% and -60% respectively compared to control or heterozygous mice) in the subventricular zone at E18 (Fig 4A, 4B, large box; C, D). To better understand how much of these changes are due to the loss of progenitors, we examined *Notch1* expression at the interface between the RMS and the dorsal ventricle, a specialized region where progenitors reside. (Fig. 4A, B; see smaller box). We observed a strong reduction of expression in the mutant (-104%). We next examined the migration of neuronal precursors from the dLGE to the olfactory bulb. We found that average number of *EGFR* expressing cells/mm were significantly reduced in *Jagged1* mutant (-34%) and heterozygous (-37%) mice relative to controls in the rostral migratory stream

(appendix Fig.2).

Jagged1 expression in the RMS

We observed *Jagged1* expression in the junction between the subventricular zone and RMS (data not shown; Nyfeler et al., 2005). These findings suggest that *DCX* and *Jagged1* may be co-expressed within RMS. To more closely examine this expression, we performed a series of double-labeled *in situ* hybridization experiments on E17 mice. We found significant overlap with *Jagged1* and *DCX*$^{+}$ neuroblasts (Fig. 5C; arrows).

Loss of *Jagged1* affects interneurons in the olfactory bulb

Neuroblasts are known to migrate tangentially through the RMS and differentiate into glutamatergic interneurons (Winpenny et al. 2011) within the olfactory bulb. In an effort to determine whether the reduction in *Notch1* and *DCX*$^{+}$ expressing cells within subventricular zone /RMS disrupts mature interneurons in the olfactory bulb, we performed *in situ* hybridization using *tyrosine-hydroxylase*$^{+}$ (*TH*) and *glutamate receptor 1* (*GluR1*) probes. We found *TH*+ expression was reduced in the PG and GR layers (-82%, -68% respectively; Fig. 6A, B, E, F). Similarly, *Jagged1* heterozygous mice also had reduced numbers of interneurons (-41%; Fig. 6E).

As a control, we quantified *gluR1*$^{+}$ cells in the mitral layer of the olfactory bulb, which is not derived from the subventricular zone. We found no significant changes in the mitral cell layer in all mice (Fig 6C, 6D; inside arrows).

Figure 1. Expression of *Notch* receptors and *Notch* ligands in embryonic day 15 lateral ventricle. The first row corresponds to *Notch* receptor expression patterns and the bottom rows are *Notch* ligand expression patterns. The top of each section corresponds to the ventricular region of the cortex. Left is the anterior-dorsal lateral ganglionic eminence (dLGE). (A-G) *Notch1, Notch2, Notch3, Jagged1, Jagged2*, *Delta1, Delta3* are expressed in the dLGE of the developing subventricular zone at embryonic stage 15 (E15) (Fig. A-G).

Figure 1. Expression Pattern of Notch1-3, Jagged1-2, Delta1, and Delta3

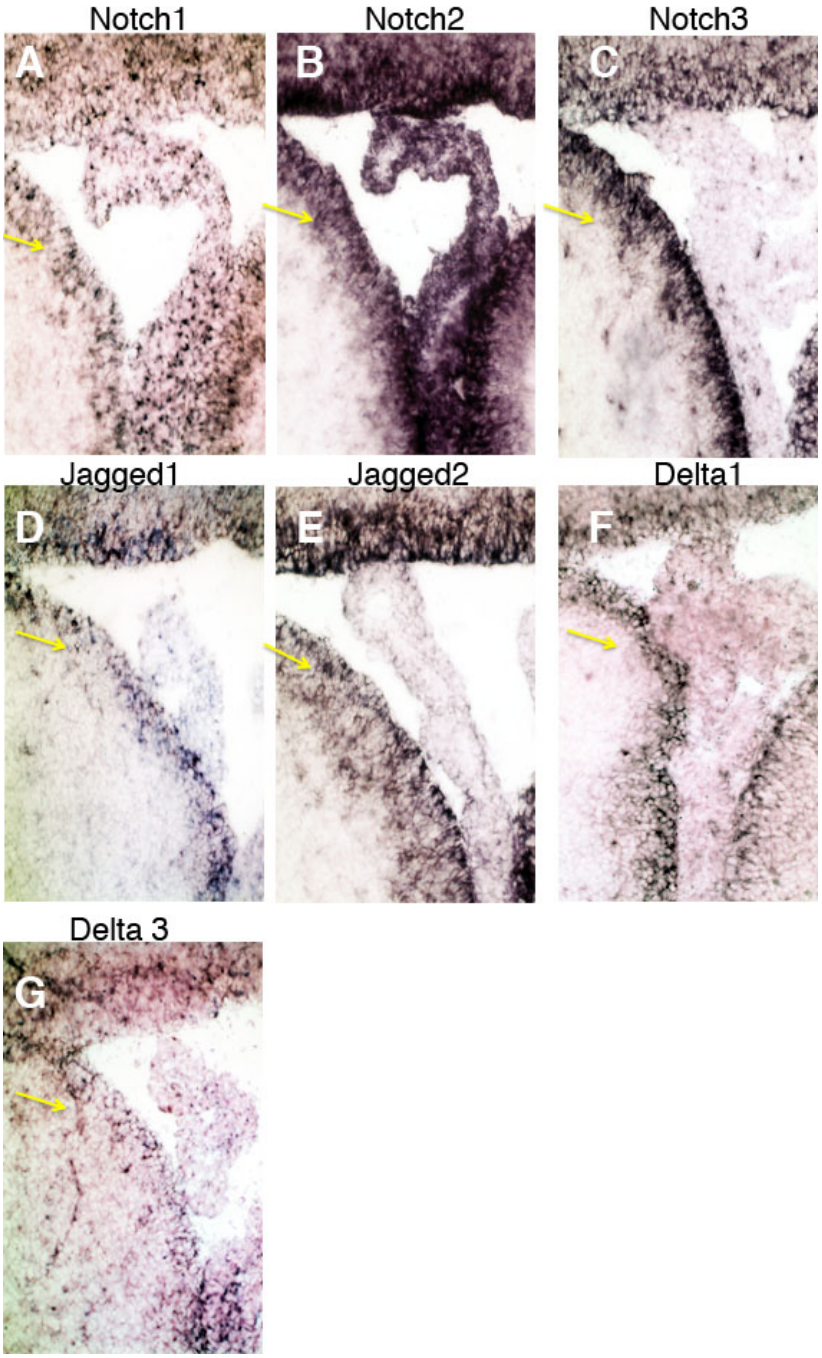


Figure 2. Disruption of *Jagged1* (A) Schematic diagram of targeting construct with loxP sequences flanking exon 4 (Gridley et al., 2006). Construct after *cre recombinase* deletion of exon 4 to represent the nonfunctional *Jagged*1 allele (Foxg1-cre; $Jag1^{f/f}$). (B) F1 mice were crossed to obtain *Jagged*1 control (C), heterozygous (HET), and mutant (MT) mice. (C) PCR was used to identify C, HET, and MT. (D) Western blot analysis with *Jagged1* antibody showing C, HET and MT *Jagged1* protein. *Alpha-Tubulin* was used as a loading control.

Figure 2. Generation of *Jagged1* mutant and control mice

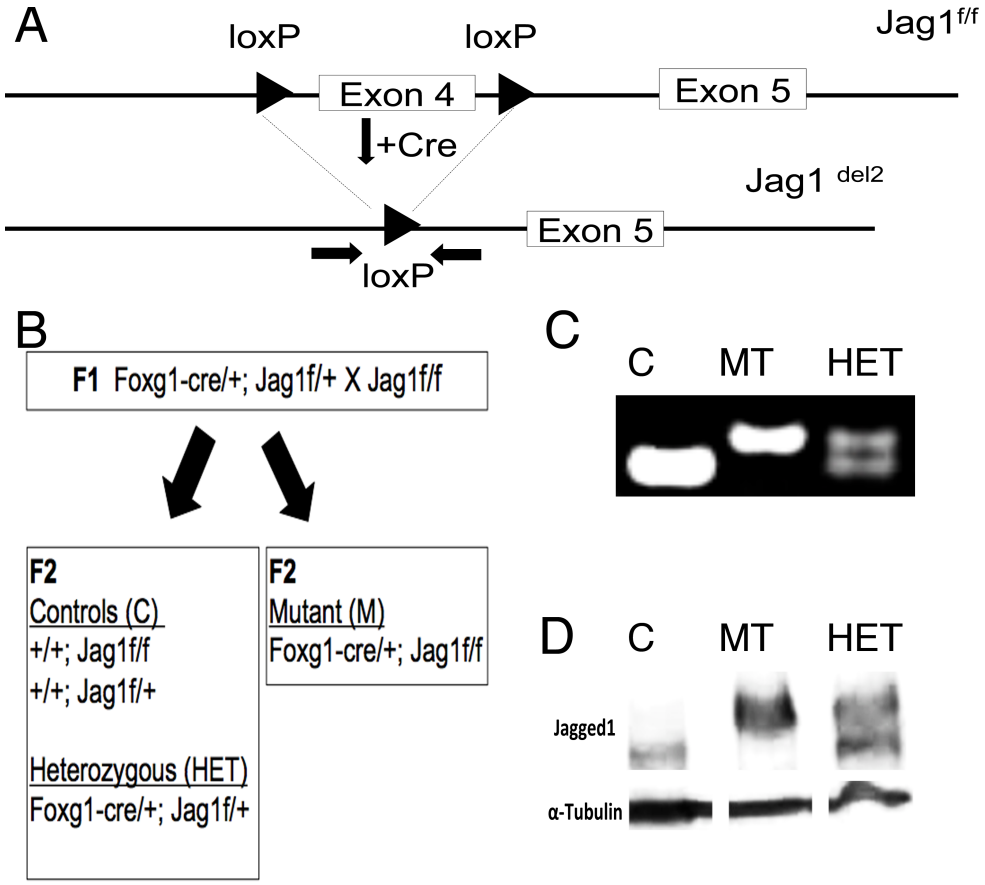


Figure 3. Reduced cell proliferation, neural stem cells and neuronal precursors in the dorsal lateral ventricle. (A-D) Cell division/proliferation, (L-N) astrocytic^{+} neural stem cells, and (E-K) neuronal precursors in the subventricular zone at embryonic day E17. White, grey, and black histograms represent control, heterozygous, and mutant mice respectively. (A) Low magnification view of the lateral ventricle (box represents anterior dorsal region), and B-C higher magnification of the anterior dorsal region. **(**D) the number of PCNA positive cells per section is decreased in mutants (C; N=3; *Foxg1-cre*/+; *Jag1*$^{f/f}$; p=0.004) and heterozygous (D; N=3; *Foxg1-cre*/+; *Jag1*$^{f/+}$, p=0.03) mice compared to control (*Foxg1-cre*/+; *Jag1*$^{+/+}$). (E, F, G) no change was observed in *Mash-1* expression in the dorsal lateral region of the developing subventricular zone.. (H-K) *DCX* expression in the dorsal lateral ventricle is reduced in mutants (K) compared to control p=0.004. (L-N)) *GFAP*$^{+}$ cells are reduced in mutants compared to controls (N) (N=3; p=0.008). Scale bar=100 μm.

Figure 3. *Jagged1* mutant mice exhibit defects in cell division/proliferation and immature neuron production

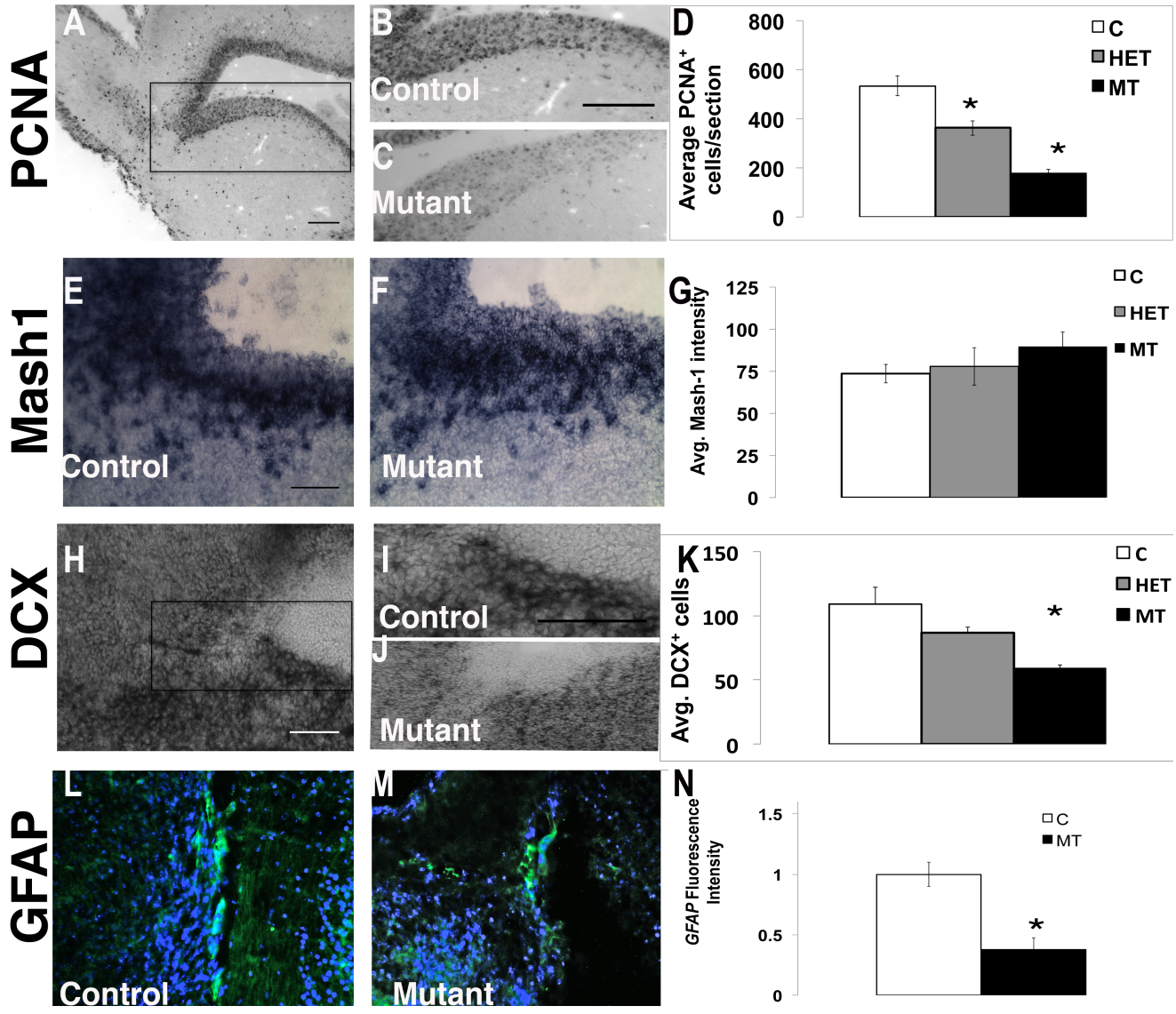


Figure 4. *Notch* Expression is decreased in mutants. White, grey, and black histograms represent control, heterozygous, and mutant mice respectively. **(**A, B) *Notch1* expression in the subventricular zone is significantly reduced in E17 mutants (Foxg1-cre/+; *Jag1*$^{f/f}$, B; larger box) compared to +/+; *Jag1*$^{f/f}$ (control, A). (C) Quantitation showed a significant difference between control, mutant, (n=3; $p<0.001$), and heterozygous mice (n=4; $p=0.05$). (A, B) Similarly, there is a significant difference in mutant (n=5) compared to control (n=3) in the subventricular zone/RMS region (smaller box). Scale bar=100 μm Asterisks in graphs indicate statistical significance using Student's t-Test.

Figure 4. *Notch1* expression is reduced in *Jagged1* mutants

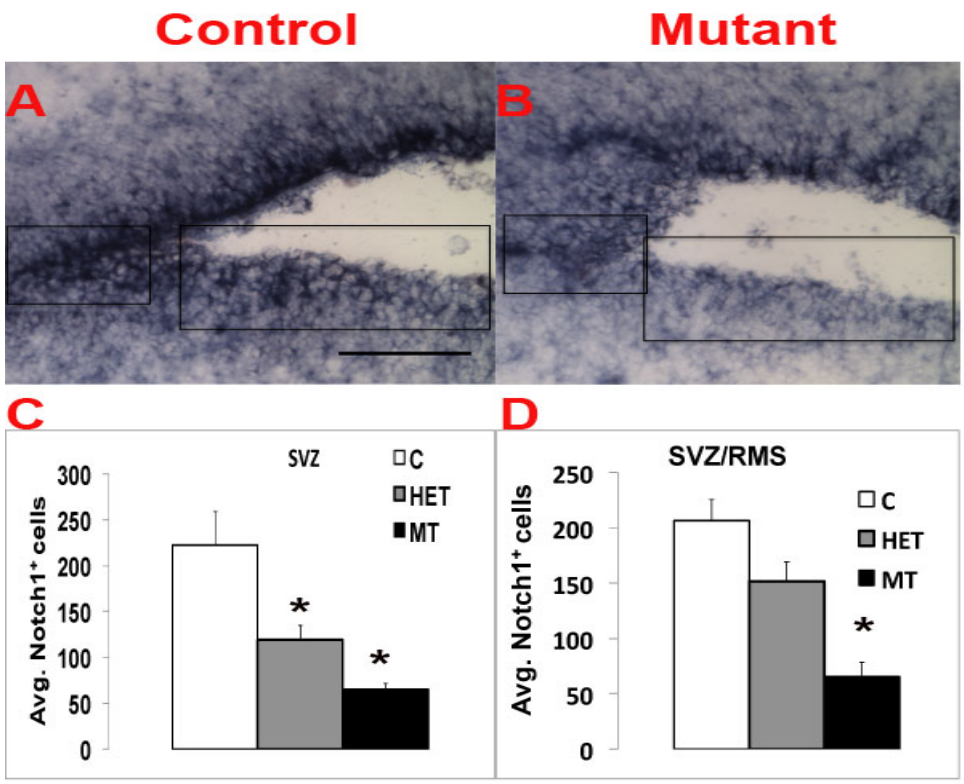


Fig. 5. ***Jagged1 and DCX are co-expressed in the RMS*****.** Double-labeled *in situ* hybridization with mRNA probes for *Jagged1* (A; green) and *DCX* (B; red) in the rostral migratory stream. Merge (C) show that *Jagged1* and *DCX* are co-expressed (arrows) in the same cell. Pictures were taken at 40x magnification using a confocal microscope

Figure 5. *Jagged1* and *DCX* are co-expressed in RMS

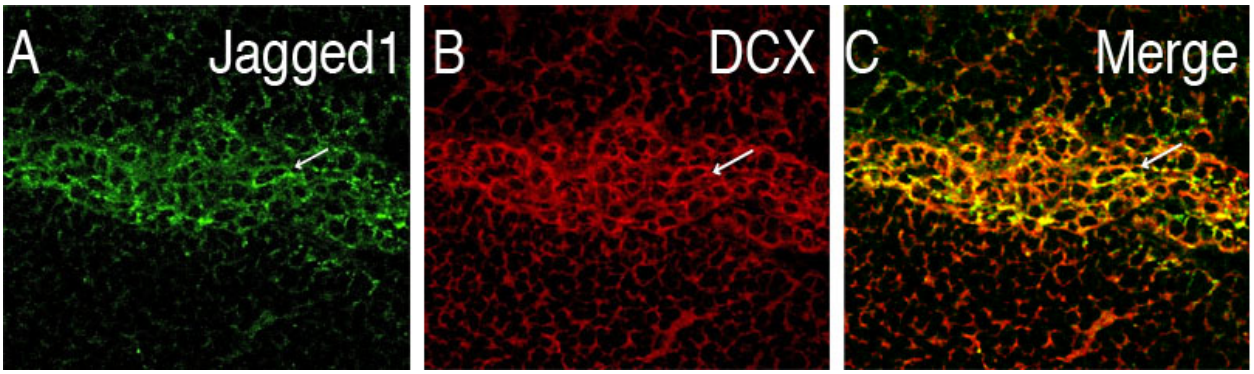
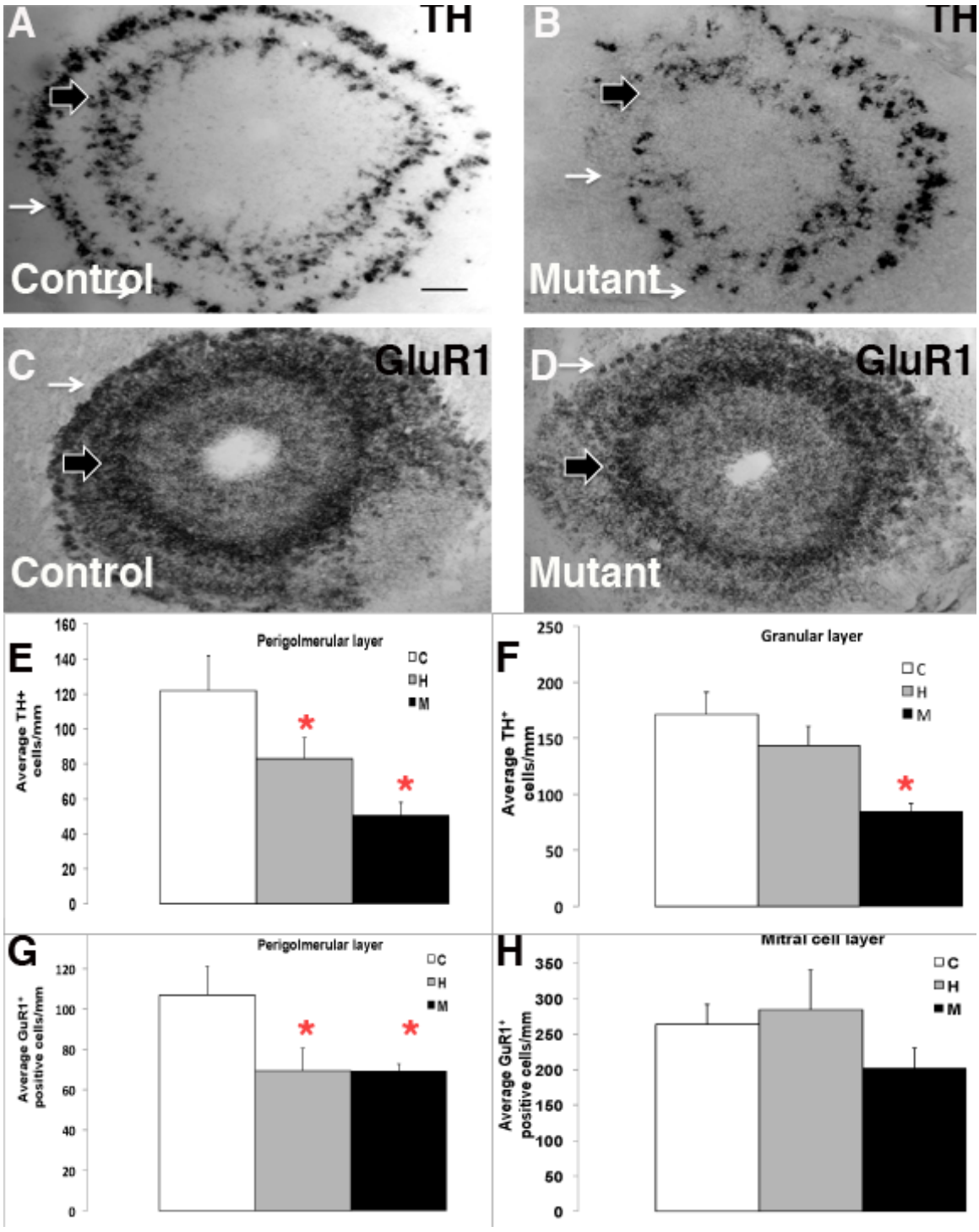


Figure 6. *TH*$^{+}$ and *GluR1*$^{+}$ mature neurons in the periglomerular but not mitral layer are reduced in the olfactory bulb. White, grey, and black histograms represent control, heterozygous and mutant mice respectively. **(**A-B) *Tyrosine hydroxylase (TH) mRNA* expression in the periglomerular layer (outer white arrow) and granule layer (inner black arrow) are reduced in mutant (B; n=4) and heterozygous mice (n=4) compared to control mice (p=0.01; N=3). (E-F) Quantification of the average number of *TH*$^{+}$ cells/mm in periglomerular layer (E) (12mm, 15mm, and 16mm of sections were counted in control, heterozygous, and mutant mice respectively) and granule (F) (15mm, 10mm, and 10mm of sections were counted in control, heterozygous, and mutant mice respectively) were reduced. (C, D,G) *gluR1* mRNA expression is highly reduced in the periglomerular layer (outer arrow) in mutant (n=3) and heterozygous (n=3) mice compared to control (n=5). However, no significant changes were observed in *gluR1*+ cells of the mitral layer (inner arrow) relative to controls. (H) the average numbers of *gluR1*$^{+}$ cells/mm in the mitral layer is unchanged. Scale bar=100 μm Asterisks in graphs indicate statistical significance ($p<0.05$)

Figure 6. Loss of *Jagged1* affects interneurons in the olfactory bulb



2.5 Discussion

We present evidence that *Jagged1* positively regulates neurogenesis during the development of the subventricular zone. In the absence of *Jagged1* function, the generation of neural stem cells, some neuronal precursor cell populations, and migrating neurons is impaired. Furthermore, we show that the production of olfactory interneurons is affected.

Loss of cell proliferation, neural stem cells and neuronal precursors in *Jagged1* mutants

In postnatal stages, *Jagged1* is critical for maintaining NSC populations in the subventricular zone. However, during development *Jagged1*'s function remains elusive. We have found that neurogenesis in the subventricular zone decreased ~60% in *Jagged1* mutants relative to controls. Our findings are consistent with *Jagged1* playing a role in proliferation in adults (Nyfeler et al., 2005) and in various compartments outside of the CNS (Alagille et al., 1975). An alternative interpretation is that the loss of *Foxg1* may have contributed to the decrease in neurogenesis. We observed that *Foxg1-cre* mice exhibited no proliferation defects in the subventricular zone. We have not determined if the loss of *Jagged1* is solely responsible for the ~60% decrease in the subventricular zone or the additive effect of the loss of both *Foxg1* and *Jagged1*. However, previous studies using an alternative approach to delete *Jagged1* have found a similar ~58% reduction in proliferation during postnatal stages (Nyfeler et al., 2005).

These observations support the idea that *Jagged1* is a critical regulator for neurogenesis during the development of the subventricular zone.

NSCs located along the ventricular lining undergo neurogenesis (Doetsch et al., 1999). We found a ~55% reduction of *GFAP*-positive neural stem cells in *Jagged1* mutants compared to controls. These findings confirmed that the loss of *Jagged1* affects NSCs in the subventricular zone. Moreover, we observed that neuronal precursors that are generated from NSCs such as *Notch1, EGFR* and *DCX* are reduced. These findings are consistent with our model that the loss of *Jagged1* signal negatively affects NSCs, and subsequently, neuronal precursors were reduced. An alternative interpretation to our results is that the loss of *Jagged1* may indirectly affect NSCs by affecting *Mash-1* positive cells. *Mash-1* null mice have demonstrated that the loss of *Mash-1* leads to depletion of specific neuronal precursors and reduced neurogenesis specifically in the medial ganglionic eminence (MGE) (Casarosa et al., 1999). Strikingly, these experiments revealed that *Mash-1* has no effect in the LGE where *Jagged1* is expressed. We believed that *Mash-1*$^{+}$ could be derived from a different neural stem cell population. Our model suggests that *Jagged1* is the key regulator of neurogenesis in the subventricular zone, and that the loss of *Jagged1* affects the generation of neuronal precursors.

Mature neurons are affected in *Jagged*1 mutants

Migrating neurons in the RMS eventually differentiate into their final position in the olfactory bulb. We found that *tyrosine hydroxylase* and *glutamate receptor 1* expression is reduced in the periglomerular and granule layers of the olfactory bulb. We interpret our results to indicate that loss of *Jagged1* affects the production of interneurons in the olfactory bulb. This finding is consistent with a model that suggests that defects in the subventricular zone and RMS subsequently affect migrating neurons in the RMS. Furthermore, given that *TH* and *gluR1* is only expressed in a subset of neurons in the periglomerular and granule layer. Examining *calcium binding proteins calbindin* (*CalB*) and calretinin (*CalR*) will provide a better understanding whether the loss of *Jagged*1 severely affects the periglomerular and granule layers of the olfactory bulb.

2.6 Acknowledgements

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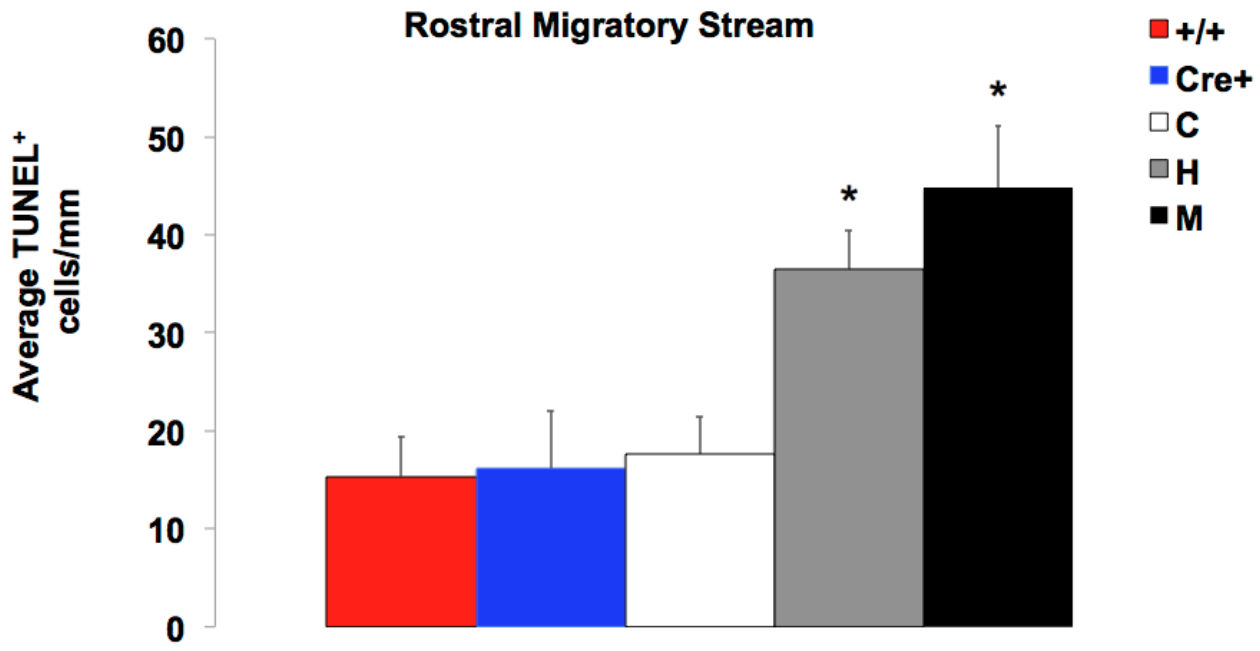
2.7 Appendix

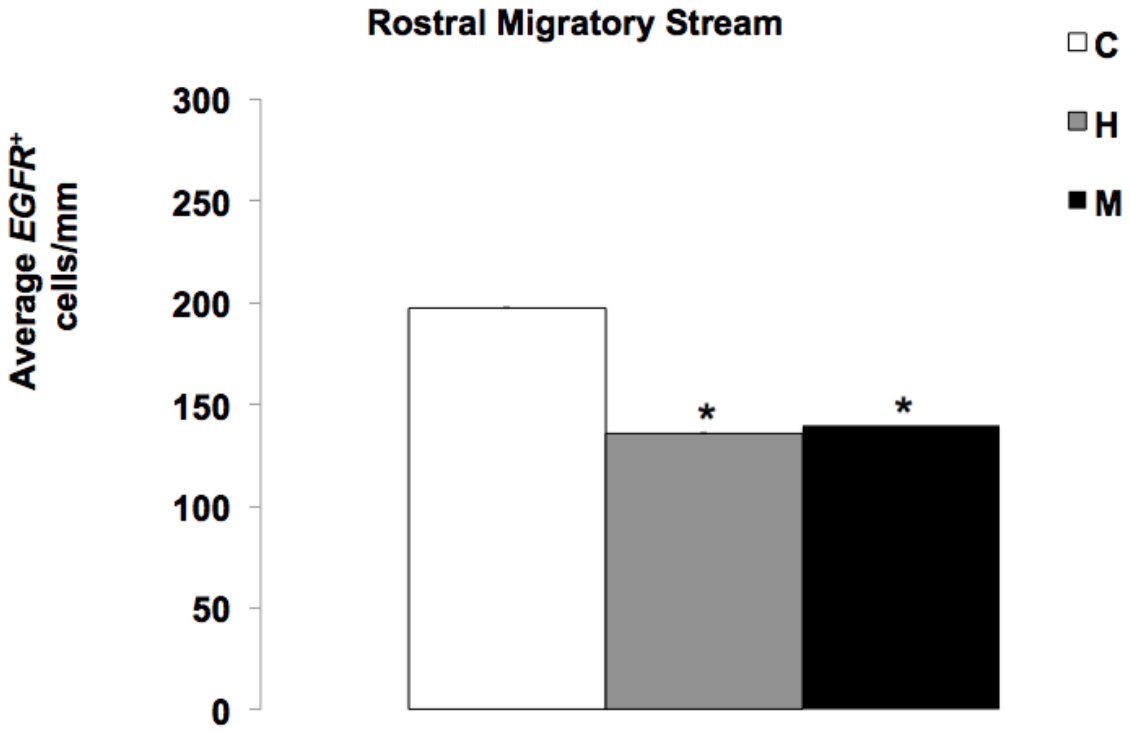
Appendix Fig.1 Loss of *Jagged1* affects cell survival in the RMS. Red and Blue histogram represent *Foxg1-cre (Cre+)* and wild type (+/+) mice respectively. White, grey, and black histograms represent *Jagged1* control, heterozygous, and mutant mice respectively. Quantification of average number of TUNEL-positive cells/mm *Foxg1-cre* (blue) relative to wild type (red) showed no significant changes in cell death. However, heterozygous (N=4; grey) and mutants (N=3; black) relative to controls (N=3; white) showed a dramatic increase. Asterisks in graphs indicate statistical significance ($p<0.05$)

Loss of *Jagged1* affects cell survival in the RMS



Appendix Fig.2 Reduced EGFR expressing neuronal precursors in *Jagged1* mutants RMS. White, grey, and black histograms represent *Jagged1* control, heterozygous, and mutant mice respectively. Quantification of the average number $EGFR^{+}$ expressing cells/mm in the rostral migratory stream (RMS). *EGFR*+ mRNA expression was significantly reduced in the heterozygous (N=2; grey) and mutant (N=3; black) relative to wild type (N=4; white). Asterisks in graphs indicate statistical significance ($p<0.05$)

Reduced EGFR expressing neuronal precursors in *Jagged1* mutants RMS



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CHAPTER 3

In Vitro Effects of the Loss of *Jagged1* on Neural stem/Progenitor Cells

3.1 Abstract

Notch signaling is essential for the maintenance of neural stem/progenitor cells in the subventricular zone during mammalian development. Neural stem/progenitor cells express *Notch* signaling components, however, the *Notch* activator that regulates neural stem/progenitor cells is not well understood. Here we have investigated the effects of the loss of *Jagged1*, a *Notch* activator, on neurogenesis using the neurosphere assay. We found that the loss of *Jagged1* affects cell proliferation, neurosphere formation, and the production of differentiated neurons. Our data indicates that signaling through *Jagged1* is the pivotal mechanism for maintenance of neural stem cell/progenitor cells in the developing subventricular zone.

3.2 Introduction

During embryonic development, neural stem/progenitor populations reside in a highly proliferative microenvironment called the subventricular zone (Takahashi et al. 1996; Striedter et al. 2009). The subventricular zone is the primary site of neurogenesis and is essential for the development of the central nervous system (Reznikov et al. 1997). Moreover, as development proceeds, the neural stem cells that reside in the subventricular zone are critical for the production of mature interneurons in the olfactory bulbs (Doetsch et al. 1996, Reznikov et al. 1997). Furthermore, neural stem/progenitor cells appear to express *Notch* signaling components (Hitoshi et al., 2002, Stump et al., 2002; Irvin et al., 2004; Nyfeler et al., 2005; Wang et al., 2009). However, the *Notch* activator that regulates neural stem/progenitor cell's ability to self-renewal, differentiate, and produce newly born neurons is not well understood. Further investigation into the signals that regulate neural stem/progenitor populations will therefore provide insight into the how these cells are regulated in the subventricular zone.

The *Notch* receptor encodes four transmembrane receptors in mammals: *Notch1*, *Notch2*, *Notch3*, and *Notch4* (Artavanis-Tsakonas et al., 1995). *Notch* receptors are activated upon physical interaction with a membrane-bound ligand

presented on an adjacent cell. Once activated, the *Notch* receptors are cleaved by *metalloproteinases* and γ-secretases. This releases the *Notch intracellular domain* (*NICD*). The *NICD* translocates into the nucleus where it turns on transcriptional repressor genes that help to maintain neural stem/progenitor cells in a proliferative state (Kopan et al., 2009). Gain- and loss-of- function studies have demonstrated that *Notch* plays a critical role in preserving the pool of neural stem cells in the embryonic brain by enhancing self-renewal and inhibiting differentiation (Dang et al., 2006; Mizutani et al., 2007). In culture, cells that are deficient for *γ-secretase* displayed a dramatic decrease in neural stem cells (Hitoshi et al., 2002). Conversely, activation of *Notch* promoted survival of neural stem cells (Theotokis et al., 2006). Together, these observations revealed that *Notch* is essential for maintaining neural stem/progenitor populations. However, the *Notch* ligand required to regulate this process has not been fully investigated.

Jagged1 is a member of the *Serrate/Jagged* family of canonical *Notch* ligands (Lindsell et al., 1995). Early in embryogenesis, *Jagged1* has been implicated in expanding neural stem cells in the neural crest (Nikopoulos et al., 2007). In culture, administering soluble *Jagged1* leads to increase neurogenic potential. Moreover, *Jagged* has been shown to maintain precursor populations from differentiating into astrocytes (Lindsell et al., 1995; Yeo et al., 2007; Kamei et al., 2012). In postnatal mice, *Jagged1* has been demonstrated to be critical for self-renewal of neural stem cells and cell proliferation in the subventricular zone (Nyfeler et al., 2005). Thus, *Jagged1* may be an important regulator of neural

stem cell/progenitor cells during embryonic development of the subventricular zone. However, very little is known about the role of *Jagged1 in vitro*, and it remains to be determined whether *Jagged1* affects neural stem/progenitor populations during embryonic stages.

Here we investigated the function of the loss of *Jagged1* signals on neural stem /progenitor populations *in vitro* in the embryonic mouse subventricular zone. We evaluated the neural stem/progenitors' ability to proliferate into growing spheres using the neurosphere assay. The neurosphere assay is a powerful *in vitro* tool that has been used to study neural stem cells (Reynolds and Louis, 2005; Hassan et al., 2011). The purpose of the neurosphere assay is to produce large number of neurospheres from the lateral ventricle, which can be used to study neurogenesis, self-renewal, and neural stem cell/progenitor populations. However, many of the common neurosphere assays use cells derived from the forebrain that contains mixed populations of cells, including neural stem cells that may reside in the striatum and cortex. To circumvent studying these populations, we developed a neurosphere assay based on a novel isolation approach that generates a high concentration of neurospheres from the subventricular zone (view Chapter 6). Using this novel approach, we assessed proliferation, self-renewal, and differentiation of neurospheres. We found that the loss of *Jagged1* led to reduced numbers of primary neurospheres and also reduced proliferation. Additionally, we confirmed that loss of *Jagged1* does affect neural stem/progenitor cell self-renewal during development. Finally, we show the loss

of *Jagged1* affects neuronal differentiation. Therefore, our findings suggest that *Jagged1* is a critical regulator of neural stem cells and neurogenesis during development of the subventricular zone.

3.3. Materials and methods

Mice

Animals. All animals used in this study were handled in accordance with federal and institutional guidelines, under a protocol (2001-0075) approved by the Cornell University Institutional Animal Care and Use Committee (IACUC). All mice were maintained on the 129Sw/C57Bl/6 background. *Jagged1*$^{flox/flox}$ mice (Gridley et al., 2006) were crossed to *Foxg1-Cre* mice (Hebert and McConnell, 2000), also maintained in a mixed 129Sw/C57Bl/6background, to generate F_1 (*Jagged1*$^{flox/+}$; *Foxg1-cre*) animals. These mice were crossed to generate the mutant (*Jagged1*$^{flox/flox}$ *; Foxg1-cre*), heterozygous (*Jagged1*$^{flox/+}$ *; Foxg1-cre*) and control (*Jagged1*$^{flox/flox}$ *; +/+).* Within-litter, matched comparisons were performed for all studies. The day a vaginal plug was observed was termed day 0.5 or on final day of pregnancy females were observed until vaginal bleeding occurred and was termed E19. Mice were genotyped by PCR analysis of genomic DNA isolated from tail snips biopsies. The PCR primer pairs: (5' cre – CTGACGGTGGGAGAATGTTAAT),

(3' cre-TGATCTCCGGTATTGAAACTCC) was used to monitor cre expression, and primer pair (5' Jag-TCAGGCATGATAAACCCTAGC, 3' Jag-CTACATACAGCATCTACATGC) was used to monitor the *Jagged1* loxp alleles.

Isolation and culture of neurospheres

E17-E19 pregnant mice were euthanized by CO_2. The lateral ventricle region was separated from the rest of the brain under sterile conditions. The tails of the sacrificed animals were used for genomic DNA isolation and the genotype of the animals analyzed by PCR. The lateral ventricle of each individual brain was transferred to 1X Hank's medium, and incubated in 0.25% Trypsin/EDTA. Trypsin was diluted out using 20mls of 1x Hank's. The cells were triturated with 18-gauge, 21-gauge, and 23-gauge needles, collected by centrifugation for 5 mins at 1300rpm. Centrifugation of the cells were then performed using a BSA gradient to used to remove debris and dead cells. The supernatant was removed and replaced with 1x DMEM containing 10% FBS, 1X Penstrep, and incubated for 2-4 hours in a 37° C waterbath. After centrifugation, the pellet was suspended in neurosphere medium containing 1% B27, 10ng/ml EGF solution in DMEM/F12. 10,000 cells were plated per well in a 48 well plate and incubated at 37° C in a humidified incubator with 5% CO_2. Neurospheres formed in 5-7 days and neurosphere medium was added every 3 days to maintain a healthy undifferentiated culture.

Neurosphere differentiation and immunofluorescence

Neurospheres were induced to differentiate by plating on poly-L-lysine-coated coverslips in DMEM/F12 without B27 or EGF. After 2 days the plated

neurospheres were fixed with 4% paraformaldehyde, and blocked in goat serum containing 0.5% Triton or anti-mouse IgG (1:1000). Immunofluorescent analysis of protein expression was performed with antibodies against (*NCAM*) (1:100; Millipore) and (*GFAP*) (1:100; IMGENEX). All of the differentiation experiments were performed at least three independent samples. Bound antibody was detected with Biotinylated goat anti-rabbit (1:1000), Streptavidin Texas Red (1:500), and goat anti-mouse FITC (1:1000).

Self-renewal Assay

Neurospheres were incubated with incubated in 0.25% Trypsin/EDTA for 5-10mins depending on the size. They were then incubated in an equal volume of Trypsin inhibitor and triturated with 21-gauge needles, and plated at 10,000 cell per well into a 48 well plate. DMEM/F12 containing 10% FBS was added every 3 days and neurospheres were passaged on the 6th day.

Quantification and Analysis

Images of neurospheres and differentiated neurospheres were taken using a Cannon EOS Digital Rebel XTi digital camera. Error bars indicate the standard error of the mean.

3.4 Results

Loss of *Jagged1* affects formation of neurospheres and neurogenesis in SVZ-derived cell cultures

To examine whether *Jagged1* is essential for maintaining neural stem/progenitor cells during embryonic development, we investigated how the loss of *Jagged1* affects neural stem/progenitor's ability to form neurospheres at embryonic day 17. We found that *Jagged1* mutants and heterozygous mice displayed >70% reduction in primary neurosphere formation compared to littermate controls (Figure 3.1 A, B, C). We next asked whether the decreased in neurosphere formation may be due to decreased proliferation. Therefore, BrdU incorporation was used to test whether the loss of *Jagged1* affects cell division in SVZ-derived cell cultures. We observed >50% reduction in proliferation between the controls and mutants (Figure 3.1 D). In addition, we found a >30% reduction in proliferation between the heterozygous and mutants (Figure 3.1 D). Collectively, these findings suggest that *Jagged1* is not only critical for neurosphere formation, but it is also important for cell proliferation.

Jagged1 mutants exhibited decrease in highly proliferative neurospheres

Neural stem cell populations have unlimited proliferation and self-renewal capacity. We passaged neurospheres 5-7 times and grew them for 12 days between passages to enrich for putative neural stem cells. Next we examined neurospheres greater than 200μm. We found that the average number of neurospheres generated from *Jagged1* mutant or heterozygous were reduced >47% and >60% (respectively) in comparison to littermate controls (Figure 3.2 A, B, C). This experiment shows that the loss of *Jagged1* decreases the proportion of cells with unlimited proliferation capabilities. (*$p<0.05$)

Jagged1 mutant neurospheres produce reduced numbers of differentiated neurons

Previously, we have shown that the loss of *Jagged1* affects cell division of neural precursors (Figure 3.1). However, *Notch* has been reported to play a role in early differentiation (de la Pompa et al., 1997). To determine whether *Jagged1* affects the production of differentiated neurons, we performed the neurosphere differentiation assay. We found that *Jagged1* mutant neurospheres produced a reduced number of differentiated cells compared to control

neurospheres (Figure 3.3). This result confirms that *Jagged1* may be important for the production of differentiated neurons. We, therefore, immunostained these neurons with *NCAM*, a neuronal marker. We found that *Jagged1* mutant neurospheres displayed decreased number of *NCAM*-expressing cells compared to control neurospheres (Figure 3.4). This suggests that *Jagged1* may be critical for producing the appropriate number of *NCAM*-expressing neurons.

Figure 3.1. Loss of *Jagged1* leads to decreased neurospheres formation and neurogenesis. White histograms represent control (C) mice that have no *Foxg1-cre* in background (*Jag1*$^{f/+}$, *Jag1*$^{f/f}$). Grey and black histograms represent heterozygous (HET) and mutant (MT) mice respectively with *Foxg1-cre* presence in background (*Jag1*$^{f/+}$; *Foxg1-cre (grey), Jag1*$^{f/f}$; *Foxg1-cre (black)*). (A, B) Representative images of neurospheres using the neurosphere assay at embryonic day 17 (E17) showed a dramatically decreased number of primary neurospheres in *Jag1*$^{f/f}$; *Foxg1-cre* (mutant, B) compared to *Jag1*$^{f/f}$; +/+ mice (control, A). (C) Quantitation of the average number of neurospheres formed per 10,000 cells showed ~65% reduction in *Jag1*$^{f/f}$; *Foxg1-cre* (MT, N=3) compared to *Jag1*^{f}/+; +/+ (control, N=5; p=0.015) and *Jag1*$^{f/+}$; *Foxg1-cre* (HET, N=3) compared to *Jag1*$^{f/+}$ (control, N=5; p=0.015). (D) BrdU incorporation in SVZ-derived cells from E17 showed a significant decrease in neurogenesis in *Jag1*$^{f/f}$; *Foxg1-cre* (MT, N=4) compared to *Jag1*$^{f/+}$ (control, N=6; p=0.001). The graph results are shown as S.E.M.

Loss of *Jagged1* leads to decreased neurospheres formation and neurogenesis

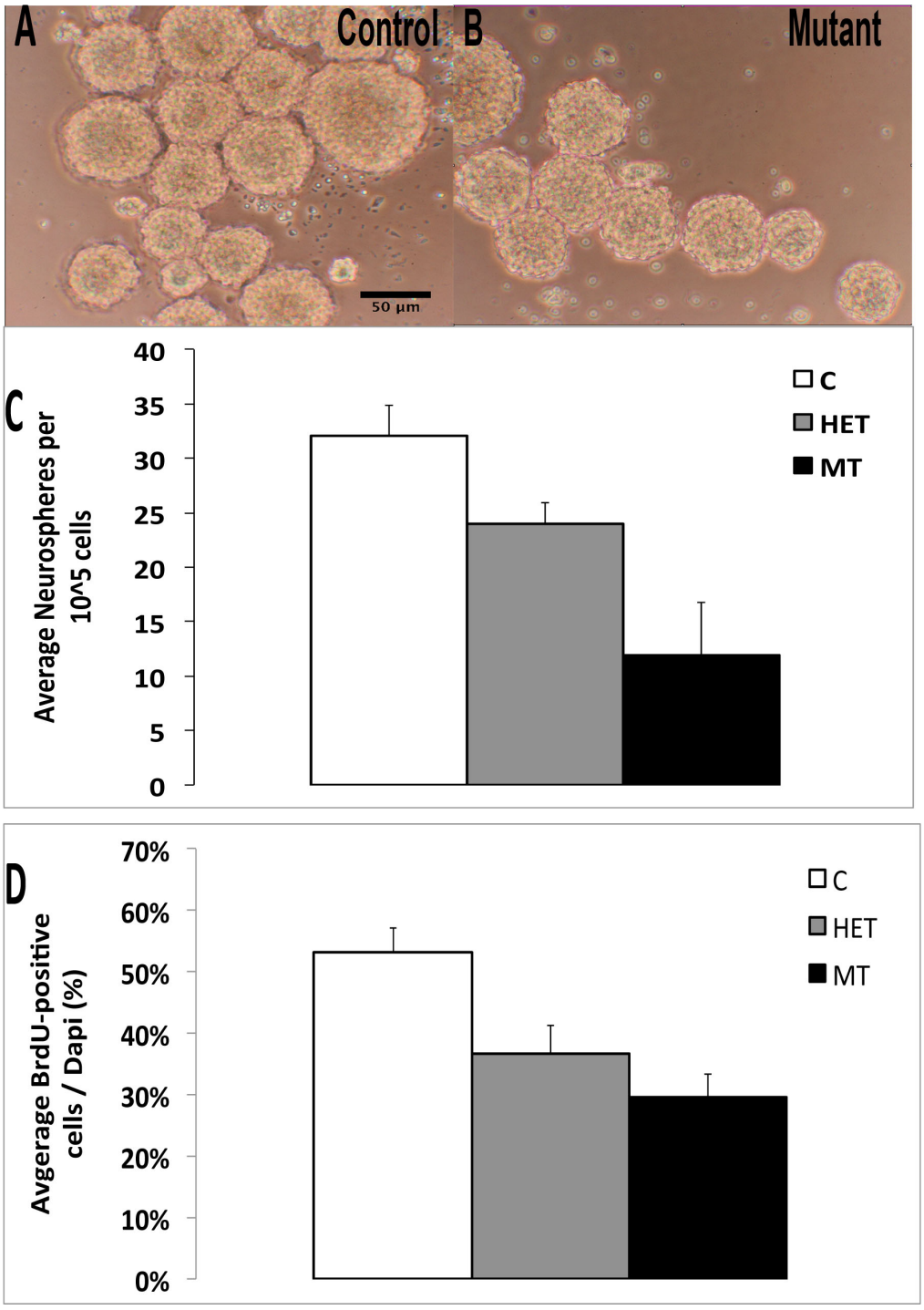


Figure 3.2. *Jagged1* mutants affect putative neural stem cell populations. Representative pictures of neurospheres grown for 12 days *in vitro* from control (A) and mutant (B). (C) Average number of neurospheres per field of view >300 μm after 12 days *in vitro*. Scale bar = 100 μm. The graph results are shown as S.E.M.

***Jagged1* mutants affect putative neural stem cell populations**

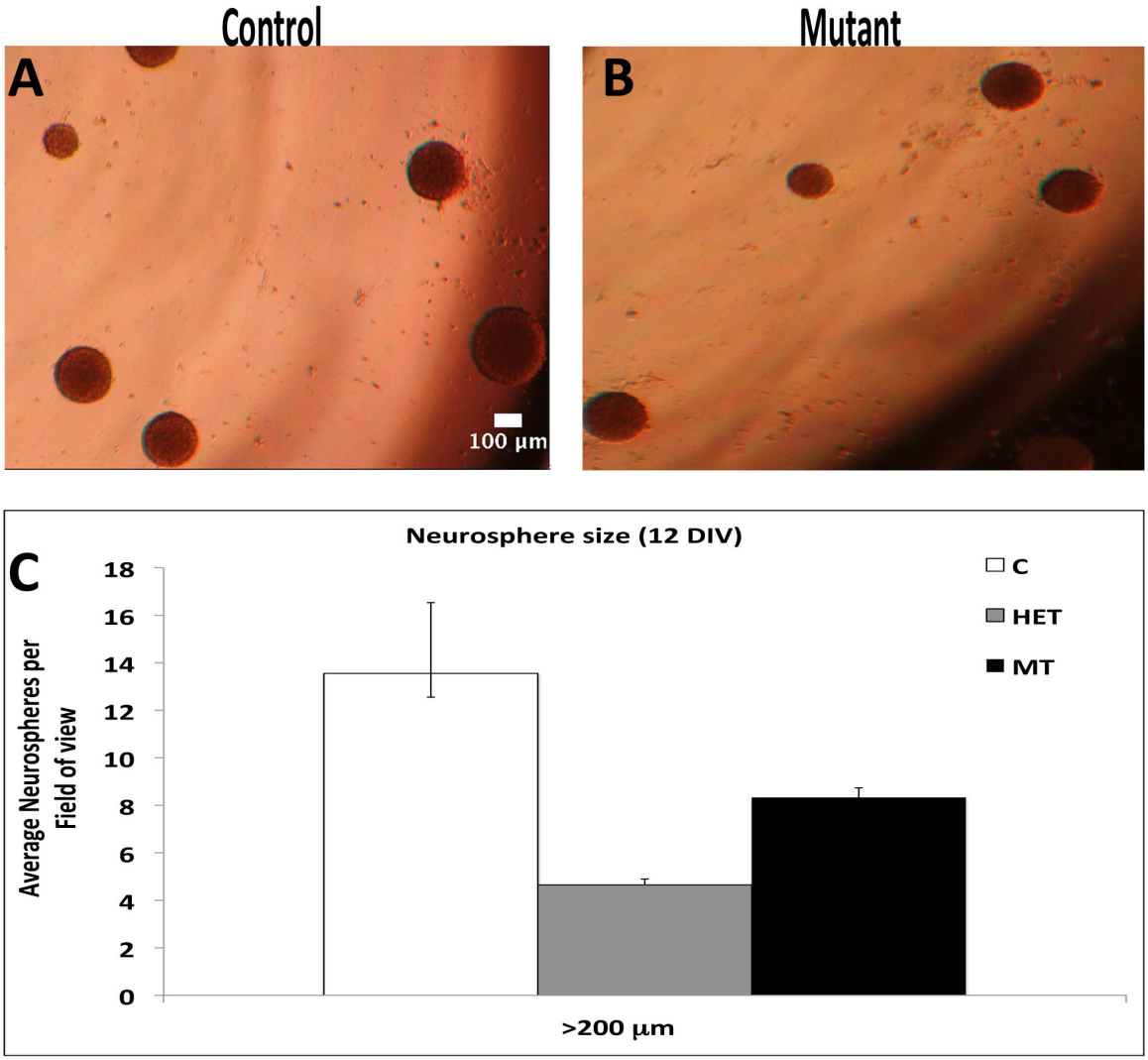


Figure 3.3. *Jagged1* mutant neurospheres exhibited significant decrease in the proportion of differentiated neurons.

Representative pictures of neurospheres gown for 12 days *in vitro* in control (A) and mutant (B). Mutant exhibited a significant decrease in the number differentiated neurons (arrows). Scale bar = 100 μm.

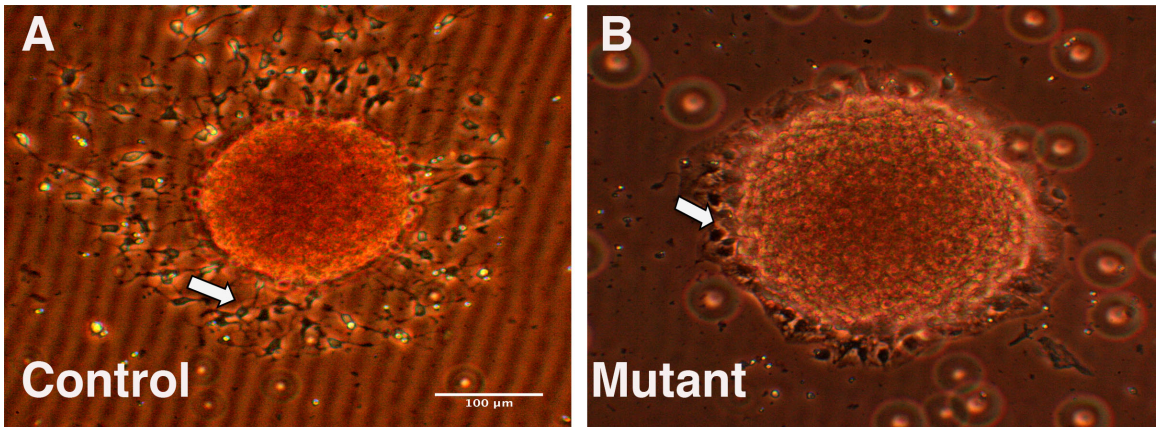
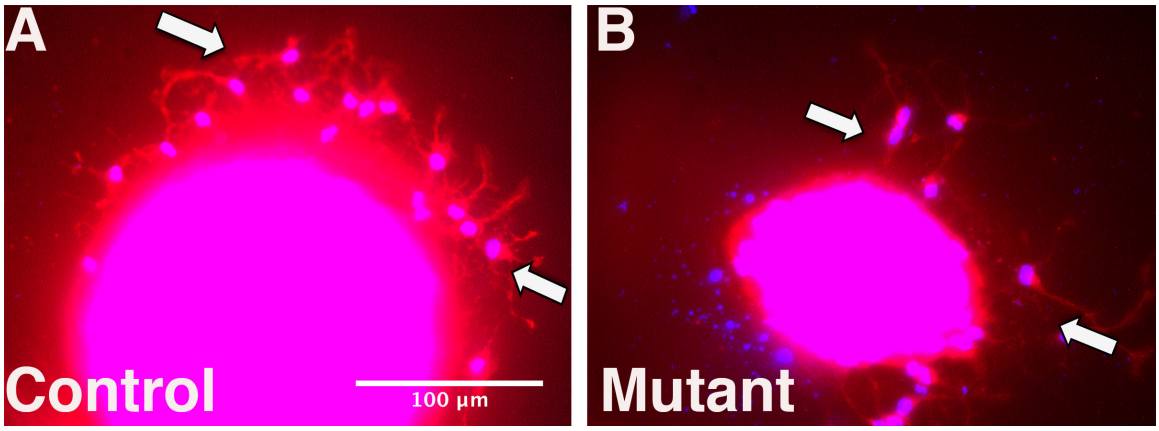

A
Control
100 µm
B
Mutant

Figure 3.4. *Jagged1* mutant neurospheres showed dramatic reduction in *NCAM* positive neurons.

Immunocytochemistry images of E17 neurospheres grown after 12 days *in vitro stained with* anti-*NCAM* (red) and DAPI, a nuclear marker (blue).

Area between arrows shows cluster of *NCAM* positive neurons in control (A) and mutant (B). *Jagged1* mutant showed a dramatic reduction in *NCAM* positive neurons. Scale bar = 100 μm.


A
Control
100 µm
B
Mutant

3.5. Discussion

We present *in vitro* evidence that *Jagged1* is an important regulator of neural stem cell/progenitor populations in the embryonic subventricular zone. In the absence of *Jagged1* function, we show that neurogenesis and neurosphere formation are impaired. Furthermore, we demonstrate that neurosphere self-renewal and putative neural stem cells (sizes >200μm) are decreased. Finally, we reveal that the loss of *Jagged1* is accompanied with a reduction in differentiated cells.

Jagged1 affects neurosphere frequency and size

We have demonstrated that *Jagged1* is critical for neurogenesis *in vitro*. Our results are consistent with a prior postnatal study performed in *Jagged1* and *Notch1* double heterozygous (*Jag1*$^{+/-}$*; Notch1*$^{+/-}$*)* mice that showed that proliferation was significantly decreased in the subventricular zone. (Nyfeler et al., 2005). Similarly, our conditional *Jagged1* mutant mice (view Chapter 2) showed decreased proliferation in the subventricular zone. However, our results are not entirely consistent with other studies showing that loss of *Notch* leads to an increase in premature neurons during early development (E.8) (de la Pompa et al., 1997). We cannot explain why our results differ from de la Pompa (1997), however, it is possible that *Notch* function during early development (E8.5) may

not be regulated in the same manner as late development (E17-E19). An alternative explanation is that the loss of *Jagged1* may initially increase premature neurons to express other ligands to compensate for *Jagged1*. Furthermore, a previous study has shown that early neurons are not viable. Therefore, it may be possible increased numbers of premature neurons are not sustained, and will eventually decrease as they die. This would be more consistent with what we observed *in vivo* in *Jagged1* mutants, as we found a decrease in neuronal precursors and an increase in cell death. Additionally, *Notch* activation has been shown to induce apoptosis in neuronal precursor cells (de la Pompa et al., 1997; Yang et al., 2004). Future experiments using *caspase 3* antibody, or Trypan blue assay may shed some understanding as to whether the loss of *Jagged1* affects cell death *in vitro*.

A priory study in *Notch1*$^{-/-}$ and *RBP-jK*$^{-/-}$ mice demonstrated that loss of *Notch* signaling led to decreases in primary neurosphere number (Hitoshi et al., 2002). Consistent with this findings, we found that the loss of *Jagged1* led to a decrease in the number of primary neurospheres. It is unclear whether the loss of neurosphere are due to losses in neural stem cells, progenitor cells or both. However, *Notch1* has been shown to be important for maintenance of neural stem cells (Hitoshi et al., 2002; Imayoshi et al., 2010). Moreover, activation of *Notch* promotes survival of neural stem cells (Theotokis et al., 2006). Therefore, these findings argue that *Jagged1*-mediated *Notch* signaling may be critical for neural stem cell maintenance. Similar to NSCs, neural progenitor cells have

been shown to transiently express *Notch* (Kageyama et al., 2008). Furthermore, *Notch1* expressing cells on the surface of neurospheres expressed *Nestin* but not *GFAP* (Nyfeler et al., 2005). Therefore, we cannot exclude the possibility that *Jagged1* signals are not affecting progenitor populations. Future experiments using sorted or pure cultures of neural stem or neural progenitor cell may confirm whether *Jagged1* regulates the formation of neurospheres from neural stem or progenitor populations.

Jagged1 affects neural stem cells and self-renewal

A study has shown in postnatal *Jagged1* and *Notch1* hemizygous (*Jagged1*$^{f/x}$*; Notch1*$^{f/x}$) have revealed that J*agged1* does not affect neurosphere size (Nyfeler et al. 2005). It is possible that neurospheres obtained from hemizygous mice, used to make these observations, do not completely delete *Jagged1* function, hence the discrepancy with this study regarding the effects of J*agged1* on neurosphere size. Furthermore, incomplete inactivation of *Jagged1* via viral infectivity, as used in the prior study, has its limitations, and may explain why they did not view a size phenotype. However, our findings are consistent with a study performed in *Notch* mutants that shows that loss of *Notch* signaling decreases neurosphere size (Hitoshi, 2002). We took further steps to verify recombination in our experiments. For example, we confirmed recombination through *Foxg-cre* lac-z experiments (data not shown). We demonstrated that *cre recombinase* activity is expressed in the developing subventricular zone.

Moreover, since previous findings showed that the C57bl/6 background promotes strong recombination by cre (Hebert and McConnell et al. 2000), we backcrossed our mice on to a C57bl/6 background. We showed that there was no difference between the phenotype in the mixed background compared to C57bl/6 background.

Nyfeler and others (2005) have shown that *Jagged1* affects neural stem cells using the neurosphere assay. Briefly, neurospheres were infected with *Cre* to delete *Jagged1's* function in neurospheres. At postnatal stages, they showed *that Jagged1* inhibits neural stem cells' self-renewal. However, there has been been some controversies regarding their experimental approach to establish that neural stem cells were affected and not progenitor cells (Reynolds, 2005). Nyfeler studies concluded that the loss of *Jagged1* effects neurosphere formation. Moreover, they concluded that neural stem cells are the population most affected by the loss of *Jagged1* on neurosphere formation. However, no experiments were performed to verify whether the loss of neurosphere formation was due to its affects on neural stem or progenitor cell. Since it is widely known that both a neural stem cell and a progenitor cell can form neurospheres; therefore, an alternative explanation is that, the loss of *Jagged1* in secondary neurosphere formation may be affecting progenitors. Like NSCs, progenitors are known to undergo self-renewal.

We have taken two approaches to determine whether *Jagged1* affects neural stem cells directly. Our first approach was to examine large classes of neurospheres, it has been demonstrated that large-size neurospheres are consistently derived from a neural stem cell (Louis et al., 2008; Golmohammadi et al., 2008). This is consistent with the notion that neural stem cells have unlimited proliferation capability. We show that *Jagged1* mutants are missing large-size neurospheres. Our second approach was to examine self-renewal after at minimal 5-6 passages. Using these two approaches, we shows that the loss of *Jagged1* leads to the absent of large-size neurospheres. We also found that after five passages the loss of *Jagged1* affects neurospheres' self-renewal capabilities. All together our model favors the *Jagged1* is critical for the maintenance of neural stem cells. Our model is consistent with that of *Notch* function to maintain neural stem cells as previous reported (Piccin et al. 2013).

3.7. Acknowledgements

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CHAPTER 4

Jagged1 Expression on Neural Stem Cells

4.1 Abstract

The *Notch* signaling pathway is essential for maintaining neural stem cell/progenitor cells in the subventricular zone during mammalian development. However, it remains unclear whether *neural stem cell populations express Jagged1*. Here we examine the expression of *Jagged1* during development of the subventricular zone. We found that *Jagged1* is co-expressed in *Sox2*$^{+}$ and *GFAP*$^{+}$ neural stem cells but not expressed in *Vimentin*-expressing neuroepithelial stem cells. Furthermore, we observed that *Jagged1* and *Notch1* might be co-expressed in a subset of cells. Our data indicates that *Jagged1* may be a critical *Notch* component that regulates neural stem cell populations in the developing subventricular zone.

4.2 Introduction

In the embryonic mammalian brain, the telencephalon transforms into two highly regulated vesicles: the ventricular zone (VZ) and the subventricular zone (SVZ). The ventricular zone (VZ) consists of a pseudostratified epithelium that is ~1-2 cells thick, which lines the surface of the lateral ventricle, and consist of *Vimentin*-expressing neuroepithelial cells. These neuroepithelial cells can give rise to *glial fibrillary acidic protein* (*GFAP)* and *sex determining region Y-box2* (*SOX2*)-expressing neural stem cell populations that divide and migrate to form the subventricular zone (4-8 cells thick). The subventricular zone, located next to the VZ, also consist of $GFAP^+$ and $SOX2^+$ neural stem cell populations, and undergo neurogenesis to develop the subventricular and to produce progenitor and neuronal precursor cells (Boulder Committee, 1970). These neuronal precursors interact with $Vimentin^+$ glial cells to support its tangentially migration through the rostral migratory stream, which is a specialized structure connecting cells in the subventricular zone to the olfactory bulb (Doetsch et al., 1997). The *Notch* signaling pathway has been demonstrated to regulate neural stem cell populations in the subventricular zone. However, the signals that regulate neural stem cells during development are not yet fully understood.

The *Notch* receptor family includes four members in mammals: *Notch1*, *Notch2*, *Notch3* and *Notch4* (Artavanis-Tsakonas et al., 1995; Greenwald et al., 1994). These receptors have been shown to be expressed in the embryonic

subventricular zone (Irvin et al., 2001). During development, *Notch* is essential for the maintenance of neural stem cell/progenitor populations (Imayoshi et al., 2010; Hitoshi et al., 2002). *Notch1* and *Notch3* activation promote *GFAP* identity (Gaiano et al., 2000; Dang el al., 2006). Together, these studies indicate that *Notch* signaling is essential for neural stem cell maintenance during embryonic development of the forebrain.

Consistent with the role of *Notch* in the forebrain, *Jagged1*, an activator of *Notch* signaling, is also essential for maintaining neural stem cell populations. During development, *Jagged1* is expressed among neural stem cell populations in the subventricular zone (Yun et al., 2002; Irvin et al., 2004,). In postnatal stages, *Jagged1* has been shown to be critical for the self-renewal of neural stem cells, as demonstrated using virally infected neurosphere cultures (Nyfeler et al., 2005). In the mouse inner ear, *Jagged1* is required to maintain the normal expression levels of Sox2-expressing neural stem cells (Kiernan et al., 2006; Osterele et al., 2008; Neves et al., 2011). However, it is unclear how *Jagged1* regulates neural stem cell populations. These findings suggest that *Jagged1* may be an important mechanism in regulating neural stem cells, yet its role during late development of subventricular has not been fully investigated.

Our report examines the expression of *Jagged1* during the development of the subventricular zone. We use *in situ* hybridization to investigate how *Jagged1* regulates neural stem cells in the subventricular zone. This study, therefore,

provides novel evidence that *Jagged1* functions as a critical receiving molecule expressed on neural stem cells during development of the subventricular zone.

4.3 Material and methods

Mice

All animal protocols were approved by Cornell University's IACUC. *Jagged1* mutants were generated as previously described (Gridley et al., 2006) and were maintained in a mixed 129Sv/C57BL/6 background. *Jag1*$^{f/f}$ (Gridley et al., 2006) mice (Gridley et al., 2006) were crossed with *Foxg1-cre* mice (Hebert and McConnell, 2000) to generate F1 *Foxg1-cre*; *Jag1*$^{f/+}$ mice. *Foxg1-cre*; *Jag1*$^{f/+}$ mice were also maintained in a mixed 129Sv/C57BL/6 background. These mice were crossed with *Jag1*$^{f/f}$ to generate the controls (+/+; *Jag1*$^{f/f}$ or +/+; *Jag1*$^{f/+}$). The day a vaginal plug in a pregnant female was observed was termed day 0.5.

Digoxigenin-labeled *in situ* hybridization

In situ hybridization was done as previously described (Rodriguez et al., 2008). In brief, E17-P0 mice were euthanized and decapitated. Brains were embedded in OCT (Tissue Tek) and fresh-frozen in liquid nitrogen-cooled isopentane. Subsequently, 20 µm fresh-frozen cryosections were fixed in 4% paraformaldehyde, washed with PBS (phosphate-buffered saline), and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. Slides were washed again and blocked with Blocking Reagent (Roche) for 2hrs. Slides were then hybridized with digoxigenin-labeled antisense RNA probes for 48hrs at 60-65°C and washed with 5x SSC and then with 0.2x SSC. Afterwards slides were

cooled to room temperature and blocked with TNB reagent (Perkin Elmer). Alkaline phosphatase conjugated anti-digoxigenin antibody was applied to slides (Roche) in block overnight at 4°C. Slides were washed with B1 buffer (100 mM Tris pH 7.4, 150mM NaCl), followed by B3 buffer (100 mM Tris pH 9.5, 50 mM MgCl, 100 mM NaCl), and reacted in NBT/BCIP (Promega) Each pairs (mutants were compared to heterozygous or controls) and was processed simultaneously, and reacted for the same amount of time prior to the analysis Probes were cloned by PCR or generated from NIA-BMAP clonesets.

Double-label *in situ* hybridization

E17-19 mice were euthanized and decapitated. Brains were embedded in OCT (Tissue Tek) and fresh-frozen in liquid nitrogen-cooled isopentane. Subsequently, 20µm fresh-frozen cryosections were fixed in 4% paraformaldehyde, washed with PBS (phosphate-buffered saline), and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0. Slides were washed again and blocked with Blocking Reagent (Roche) for 2hrs. Slides were then hybridized both digoxigenin-labeled or biotin-labeled antisense RNA probes for 48hrs at 67°C and washed with 5x SSC and then with 0.2x SSC. Afterwards slides were cooled to room temperature and blocked with TNB reagent (Perkin Elmer). Anti-digoxigenin antibody was applied to slides (1:3000; Roche) in block overnight at 4°C. Slides were washed with B1 tween buffer (100 mM Tris pH 7.4, 150mM NaCl, tween .05%), followed by B3 buffer (100 mM Tris pH 9.5, 50 mM

MgCl, 100 mM NaCl). Slides were incubated with 200ul of SA-HRP (1:100; Abcam) in TNB for 30mins at RT. Next slides were washed in B1 buffer with tween (.05%), and incubated in 200 µl of Biotinyl Tryamide (1:50; Perkin Elmer) solution for 10mins. Subsequently, 200 µl of SA-Alexafluor 488 (1:200) in TNB for 30mins at RT, washed B1 with tween, and washed in Fast Red Buffer. Apply 200ul of Fast Red TR/HNPP to slides for 30mins at RT, washed in Fast Red Buffer, and repeated twice. Probes were cloned by PCR or generated from NIA-BMAP clonesets.

4.4 Results

Jagged1 is expressed in *Sox2*-expressing neural stem cells in the ventricular and subventricular zones

Our previous experiments showed that *Jagged1* is critical for olfactory neurogenesis in the subventricular zone (View Chapter 2 and 3). Furthermore, we have shown that *Jagged1* is expressed in the embryonic subventricular zone (View Chapter 3). Moreover, in the subventricular zone, we determined that loss of *Jagged1* negatively regulates neural stem cells' ability to form neurospheres (View Chapter 3). However, it is unclear whether *Jagged1*'s expression overlaps with *Sox2*-expressing neural stem cells in this region. We, therefore, investigated *Jagged1*'s expression on neural stem cells in the developing subventricular zone. To determine whether *Jagged1* is expressing in *Sox2*-expressing neural stem cells, we performed double-label *in situ* hybridization at embryonic day 17 (E17). We found that *Jagged1*-expressing cells overlap with *Sox2*-expressing cells approximately 1-3 cells from the neuroepithelial surface (Figure 4.1; box) in the ventricular zone. Additionally, we detected that *Jagged1* is coexpressed in *Sox2*-expressing neural stem cells that were located approximately 4-8 cells from the neuroepithelial surface within the putative subventricular zone (Figure 4.1; arrows). These observations suggest that *Jagged1* is expressed in *Sox2*-expressing neural stem cells during development of the ventricular and subventricular zones.

Jagged1 is coexpressed with *GFAP*-expressing neural stem cells

Previous studies have shown that astrocytic neural stem cells are located in the subventricular zone. Studies have shown that astrocytic neural stem cells are critical for olfactory neurogenesis. Moreover, at postnatal stages, astrocytic neural stem cells were found in the same cell with *Jagged1* in the neuroepithelial lining (Nyfeler, et al., 2005). We asked whether *Jagged1* is expressed on astrocytic neural stem cells in the embryonic subventricular zone. We performed *in situ* hybridization using probes for *GFAP*, a marker for astrocytic neural stem cells, and *Jagged1* on adjacent sections at E17.5. Consistent with Nyfeler 2005 findings, we found that *Jagged1* is also expressed in *GFAP*-expressing neural stem cells ((A-B) Figure 4.2; red arrow) during development. Furthermore, we found that a subset of *GFAP*-expressing neural stem cells were coexpressed with *Jagged1* approximately 4-8 cells from the neuroepithelial, a region that may putatively be the subventricular zone ((A-B) Figure 4.2; yellow arrow). Taken together, this suggests that *Jagged1* is expressed in a subset of astrocytic neural stem cells.

Jagged1-expressing cells do not express *vimentin* in the ventricular zone or the subventricular zone

Previous studies have shown that neuroepithelial cells, found in the

neuroepithelial lining, can be categorized as a type of neural stem cell (see review from Kriegstein and Alvarez-Buylla, 2009). Moreover, it has been shown that neuroepithelial cells can give rise to neural stem cells that in turn can contribute to olfactory neurogenesis (Kriegstein and Alvarez-Buylla, 2009). We asked whether *Jagged1* is expressed on neuroepithelial cells during development. We preformed a double-label *in situ* hybridization using probes for *vimentin*, a marker for neuroepithelial cells, and *Jagged1*. We found that *Jagged1*-expressing cells do not express *vimentin*. Neuroepithelial cells may also give rise to *vimentin*$^{+}$ lineages that may be found in the subventricular zone. We therefore tested whether *vimentin*-expressing cells are coexpressed with *Jagged1*$^{+}$ cells in the subventricular zone. We did not detect *Jagged1* overlap with *Vimentin* in the neuroepithelial surface (Figure 4.3; white arrows) or subventricular zone (~4-8 cell deep), (Figure 4.3; white box) except in a few cells. However, we found that *Jagged1*-expressing cells co-localized with *Vimentin* within and surrounding the rostral migratory stream (Figure 4.3; purple arrows). These data suggest that *Jagged1*-expressing cells are not expressed on neuroepithelial cells unless they are making their way through the in the rostral migratory stream.

Jagged1 and _Notch1 are_ co-expressed in a subset of cells in the subventricular zone

Previous studies have shown that *Notch1*-expressing cells are expressed

in a subset of neural stem cells. Given that we show that *Jagged1* may also be expressed on neural stem cells (Figure 4.1 and 5.2). We, therefore, investigated whether *Jagged1* and *Notch1* may be presented on the same cell in the embryonic subventricular zone. To address this, we performed a double-label *in situ* hybridization using *Jagged1* and *Notch1* mRNA probes on E17 sections. We found that a subset of *Jagged1*-expressing cells overlays with *Notch1* expression (Figure 4.4; white box) in the embryonic subventricular zone. Taken together, this suggests that *Jagged1* and *Notch1* may coexist on the same cell in the subventricular zone.

Figure 4.1 *Jagged1* is co-expressed with *Sox2*-expressing neural stem cells. Left is the lateral ventricle (LV), top is the dorsal and bottom is ventral. (A) mRNA expression of *Jagged1* (green), and DAPI, a nuclear marker (blue), show that *Jagged1* is predominantly expressed in the subventricular zone (4-8 cells) (arrows), compared to cells along the opening of the neuroepithelial lining (1-2 cells) (white box). (C) Double-label *in situ* hybridization with *Jagged1* (blue) and *Sox2* (red) show co-expression (yellow) in the subventricular zone (arrows). Pictures were taken at 40x magnification using a confocal microscope.

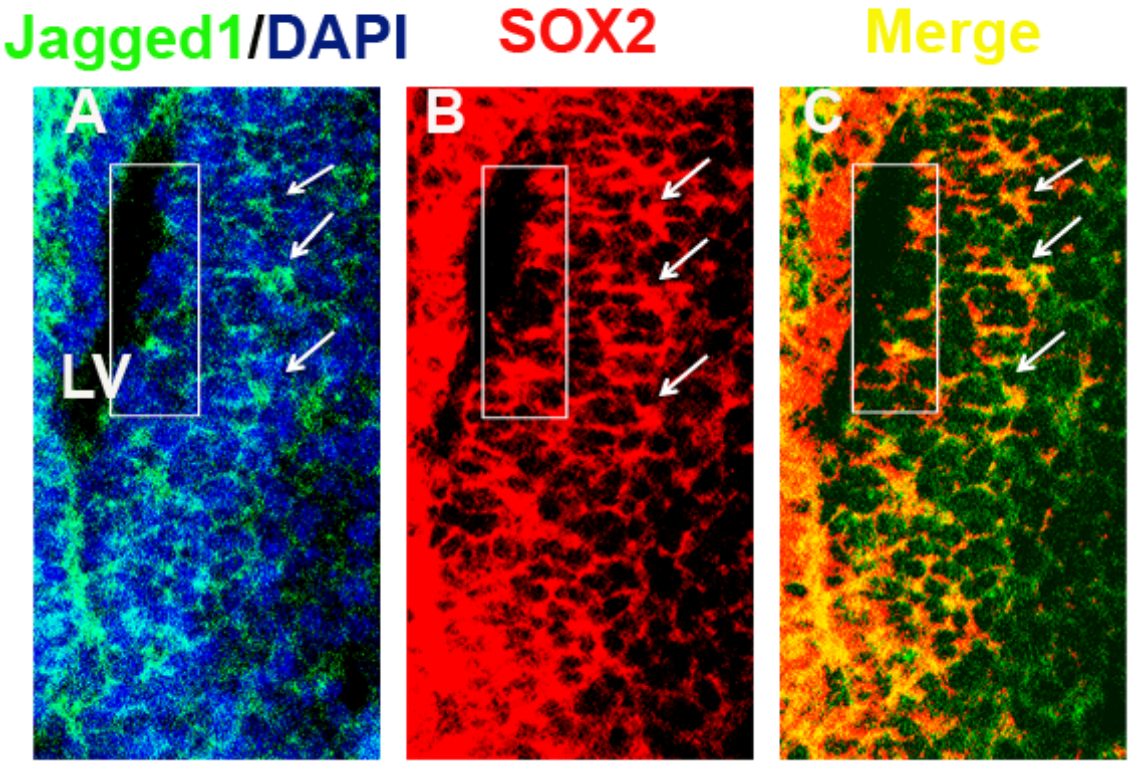

Jagged1/DAPI
SOX2
Merge
A
B
C
LV

Figure 4.2 *Jagged1* is co-expressed with *GFAP*-expressing neural stem cells. Lateral ventricle (LV), top is the dorsal and bottom is ventral. (A-B) *In situ* hybridization using *GFAP* and *Jagged1* probes on 9μm adjacent-sagittal sections. (A-B) *Jagged1* and *GFAP* are expressed on the same cells in the subventricular zone (yellow arrow) and neuroepithelial lining (red arrow). Pictures were taken at 20x magnification.

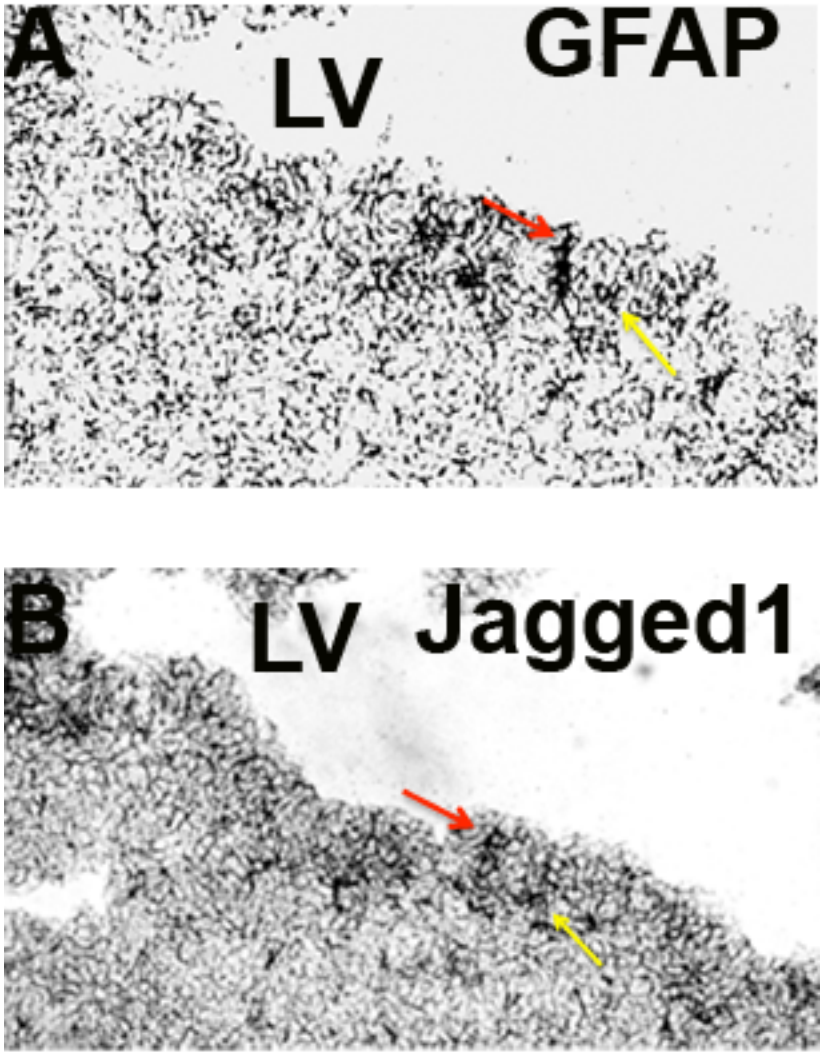

A
LV
GFAP
B
Lv
Jagged1

Figure 4.3 *Jagged1* and *Vimentin* are not co-expressed in the subventricular zone. Left is the open lateral ventricle (LV), and right is the rostral migratory stream (RMS). A sagittal section of dorsal lateral ventricle (dLGE) double-label *in situ* hybridization with mRNA probes for *Jagged1* (green) and *Vimentin* (red) show no co-expressed in the neuroepithelial lining (white arrows) or subventricular zone (small box). However, *Jagged1* is co-localized with *Vimentin* in the rostral migratory (large box; purple arrows) and 20x magnification was taken using a confocal microscope.

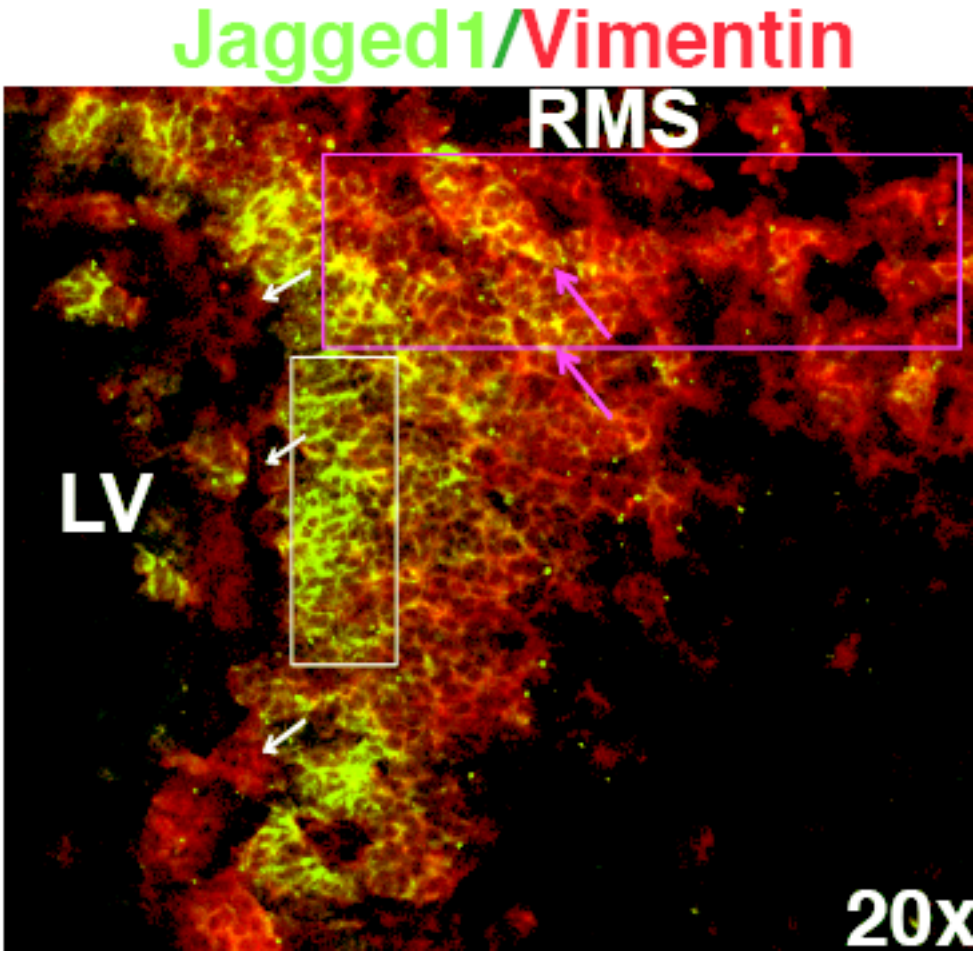
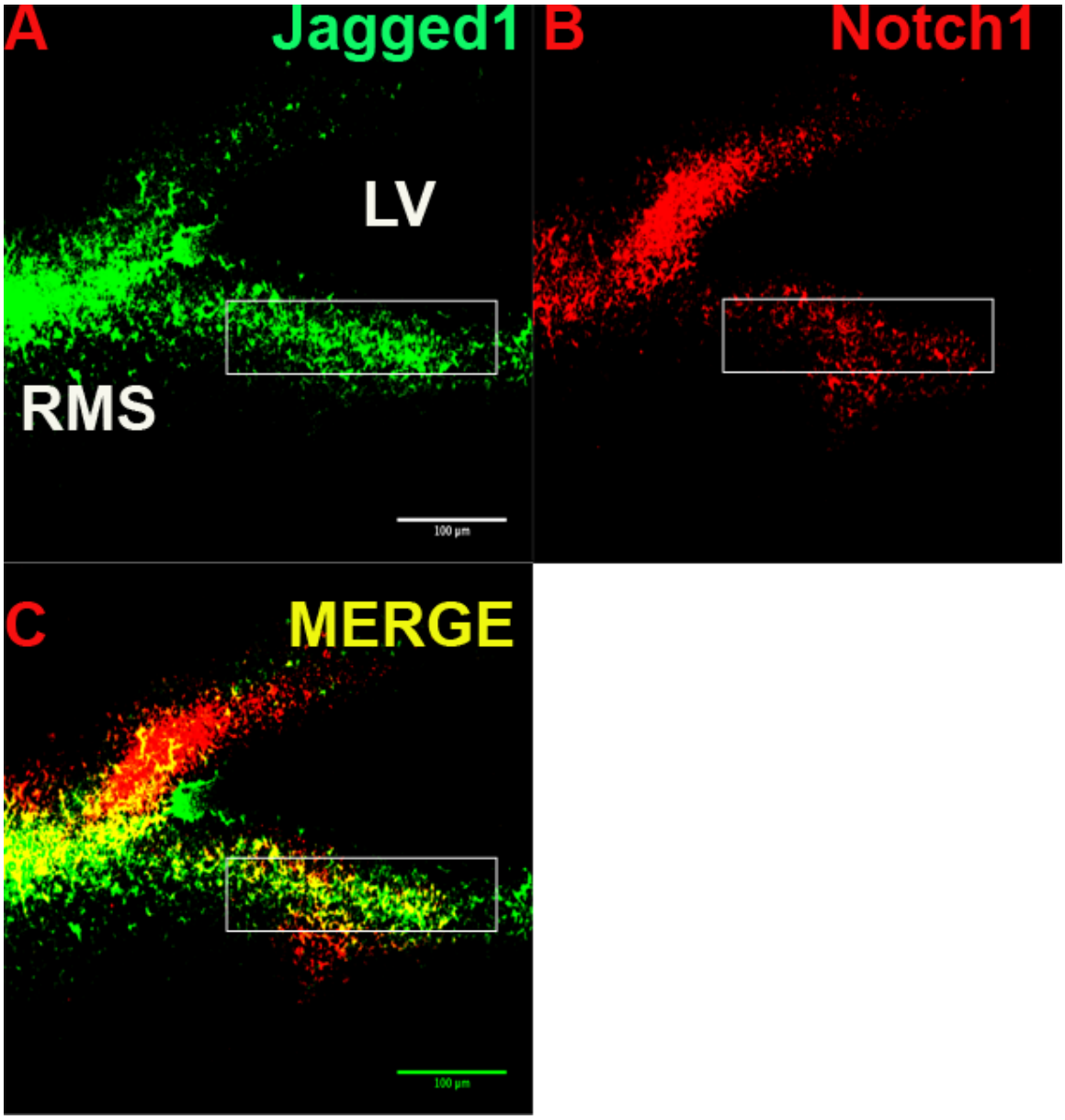

Jagged1/Vimentin
RMS
LV
20x

Figure 4.4. *Jagged1* and *Notch1 are* co-expressed in a subset of cells in the subventricular zone. Left is the open lateral ventricle (LV), top is dorsal, and left is the rostral migratory stream (RMS). (A-C) Double-label *in situ* hybridization with mRNA probes for (A) *Jagged1* (green) and (B) *Notch1* (red) show that they are (C) co-expressed only in a subset of cells in the embryonic subventricular zone (yellow; white box). 20x magnification was taken using a confocal microscope. Scale bar is 100μm.


A
Jagged1
LV
RMS
100 µm
B
Notch1
C
MERGE
100 µm

4.5 Discussion

We present *in vivo* evidence that suggests *Jagged1* may be expressed in neural stem populations in the embryonic subventricular zone. We showed that *Jagged1* is co-expressed in *Sox2*- and *GFAP*-expressing neural stem cells. Furthermore, we show that *Jagged1* is not co-expressed in Vimentin-expressing neuroepithelial cells. Finally, we observed that *Jagged1* and *Notch1* are predominantly located on different cells, however, a subset of cells do express both. Taken together, our findings suggest that *Jagged1* may be a critical signaling molecule in neural stem cells within the subventricular zone during development.

Jagged1 is expressed in *Sox2*-expressing neural stem cells in the subventricular zone

We have demonstrated that *Jagged1* and *Sox2* are expressed on the same cell during the development of the subventricular zone. However, an alternative interpretation is that *Jagged1* and *Sox2* are expressed on separate cells. Hence, co-expression may be conceivable if *Jagged1* is activating *Notch* receptors on Sox2-expressing cells. Our studies cannot confirm whether *Jagged1* is activating or expressed on a *So*x*2*-expressing cell. However, our findings are consistent with prior studies that suggest that *Jagged1* and *Sox2* are co-expressed in the same cell during development in other systems (Neves, et

al., 2011). Future studies might examine sorted *Sox2*-expressing cells for the presence of *Jagged1* protein.

Furthermore, it is unclear what the role of *Jagged1* is in *Sox2*-expressing neural stem cells. However, previous findings (view Chapter 3) have shown that the loss of *Jagged1* using the neurosphere assay affects the production and formation of neural stem/progenitor cells *in vitro* (Nyfeler et al., 2005). Moreover, in *Jagged1* mutants, we found *in vivo* that neurogenesis is severely diminished in the subventricular zone (Nyfeler et al., 2005; View Chapter 2). Thus, a conceivable mechanism could be that the loss of *Jagged1* activation affects the ability to maintain *Sox2*-expressing neural stem cells during development of the subventricular zone. This in turn may cause the reduced olfactory neurogenesis observed in our *Jagged1* mutants. In support of this model, it is widely accepted that *Jagged1* is required to maintain the normal expression levels of *Sox2*-expressing cells in the inner ear (Kiernan et al., 2006; Osterele et al., 2008; Neves et al., 2011). Furthermore, a study has shown that the loss of *Jagged1* causes defects in the production of progenitors in the inner ear (Kiernan et al., 2006). Therefore, *Jagged1's* expression in *Sox2*-expressing cell is suggested to be important for the production of progenitors in the inner ear. Hence, *Jagged1* may play an important role in the maintenance of *Sox2*-expressing neural stem cells during development of the subventricular zone

***Jagged1* is coexpressed with *GFAP*-expressing neural stem cells**

We found that *Jagged1* and *GFAP* are expressed on the same cell in the subventricular zone. We used *in situ* hybridization on adjacent sections to demonstrate that *Jagged1* and *GFAP* are co-expressed. Although an average neuron can vary between 4μm-100μm, we performed this experiment using 9μm sections. The limitations in using 9μm sections is that you get very few cells compared to our standard *in situ* hybridization sections that use 20μm. Therefore, it is possible to expect very few *Jagged1* and *GFAP* cells co-expressed in our experiments. However, a previous study, performed at postnatal stages, using immunohistochemistry has detected *Jagged1* and *GFAP* are co-expressed in larger numbers using thicker sections (50μm) in the subventricular zone (Nyfeler et al., 2005). Moreover, the same study has shown that *Jagged1* is co-expressed with *GFAP*-expressing cells. This finding is consistent with our report that *Jagged1* and *GFAP* are expressed on the same cell in the subventricular zone.

It remains unknown what the role of *Jagged1* is in *GFAP*-expressing neural stem cells. However, our *in vivo* finding (View Chapter 2) has shown that the loss of *Jagged1* affects the production *GFAP*-expressing neural stem cells in the embryonic subventricular zone. Moreover, as mentioned previously, several studies have found that *Jagged1* mutants exhibited reduced neurogenesis in the

subventricular zone (Nyfeler et al, 2005; and View Chapter 2). Furthermore, *in vitro*, we found that the loss of *Jagged1* affects a neural stem/progenitor cell's ability to form neurospheres (view Chapter 3). Interestingly, we observed that neurospheres can be immunostained with *GFAP* antibody (view Chapter 5). This suggest that *GFAP*-expressing cells are important for the formation of neurospheres. Therefore, one of the possible mechanisms could be that the loss of *Jagged1* affects the ability to maintain *GFAP*-expressing neural stem cells. This model would be consistent with a recent study that demonstrated that *GFAP*-expressing cells regulate neurogenesis through the *Jagged1*-mediated *Notch* signaling pathway (Wilhelmsson, et al., 2012). Thus, it may be possible that *Jagged1* on *GFAP*-expressing cells may be important for maintaining neural stem cells in the subventricular zone.

Jagged1-expressing cells do not express _vimentin_ in the ventricular zone or the subventricular zone

Previous studies have shown that neuroepithelial cells generate many neurons, and these neurons seem to express *Notch* ligands in the subventricular zone (Shimojo et al., 2011;[Figure 6]). We labeled neuroepithelial cells with *Vimentin* and *Jagged1* and found that they are not expressed on same cell in the neuroepithelial surface. Furthermore, since neuroepithelial cells generate many neurons, we also examined *Vimentin*-expressing cells in the subventricular zone. In the subventricular zone, we found no *Jagged1/Vimentin* co-expression, except

in a very small subset of cells. These small subset of cells was observed in and surrounding the subventricular zone, but not in the neuroepithelial surface. It is well known that neuroepithelial cells do not migrate outside of the neuroepithelial surface (see reviews: Kazanis et al., 2008; Kriegstein and Alvarez-buylla, 2009). Therefore, we do not believe that they represent *Jagged1*-expressing neuroepithelial cells. However, It would be interesting to confirm that neuroepithelial cells are absent from the subventricular by *in situ* hybridization using probes such as *CD133*.

Furthermore, we found that the small subset of *Jagged*1^{+}/*Vimentin*$^{+}$ cells are numerous as you get closer to the rostral migratory stream. This is consistent with *Vimentin*$^{+}$ cells also paying a role in facilitating neurons through the rostral migratory stream (Doetsch et al., 1997). Therefore it may be possible that the *Jagged1*$^{+}$*/Vimentin*$^{+}$ cells surrounding the subventricular zone are present to support migration into the rostral migratory framework.

Thus *Jagged1*-expressing cells are not expressed on neuroepithelial cells. Furthermore, the majority of *Jagged1* expressing cells do not express *Vimentin* in the subventricular zone, but do so, as the make they way through the in the rostral migratory stream.

***Jagged1* and *Notch1 are* co-expressed in a subset of cells in the subventricular zone**

Several studies have shown that *Jagged1* and *Notch1* are expressed in distinct cells (Lindsell et al., 1996; Irvin et al., 2004; Nyfeler et al., 2005) in the subventricular zone. These findings are consistent with our studies that showed that *Jagged*1 and *Notch*1 are predominately expressed on separate cells in the embryonic subventricular zone.

Although *Notch* is well known to activate *Jagged1* on an adjacent cell, we found that a subset of cells expressed both *Jagged1* and *Notch1*. We cannot explain why a subset of cells coexpressed both *Jagged1* and *Notch1*. A previous study has shown that neural stem/progenitor cells alternate expression between *Delta-like1* and *Hes1* over a 2-3 hour period (Shimojo et al., 2011). This suggest that *Notch* ligands and *Notch*1 signaling components may alternate expression or show co-expression on the same cell. Therefore, an alternative explanation is that *Jagged1* and *Notch1* may also have oscillatory behavior. This would mean that for a short period a given cell might express both *Jagged1* and *Notch1*. A future experiment would be to test for oscillatory function using time-lapse imaging analysis of *Jagged1* and *Notch1* expression in neural stem/progenitor cells.

In conclusion, previous studies have shown the role of *Jagged1* is critical

for neurogenesis in the subventricular (Nyfeler et al., 2005, view Chapter 2). It is not completely understood how *Jagged1* regulates neurogenesis. Our findings reported that *Jagged*1 is likely to be expressed on neural stem cells, specifically *Sox*2- and *GFAP*-expressing neural stem cells. Moreover, we demonstrated that neuroepithelial cell does not express *Jagged1*. Taken together, we believe that *Jagged1* is a critical receiving molecule that regulates neural stem cell population.

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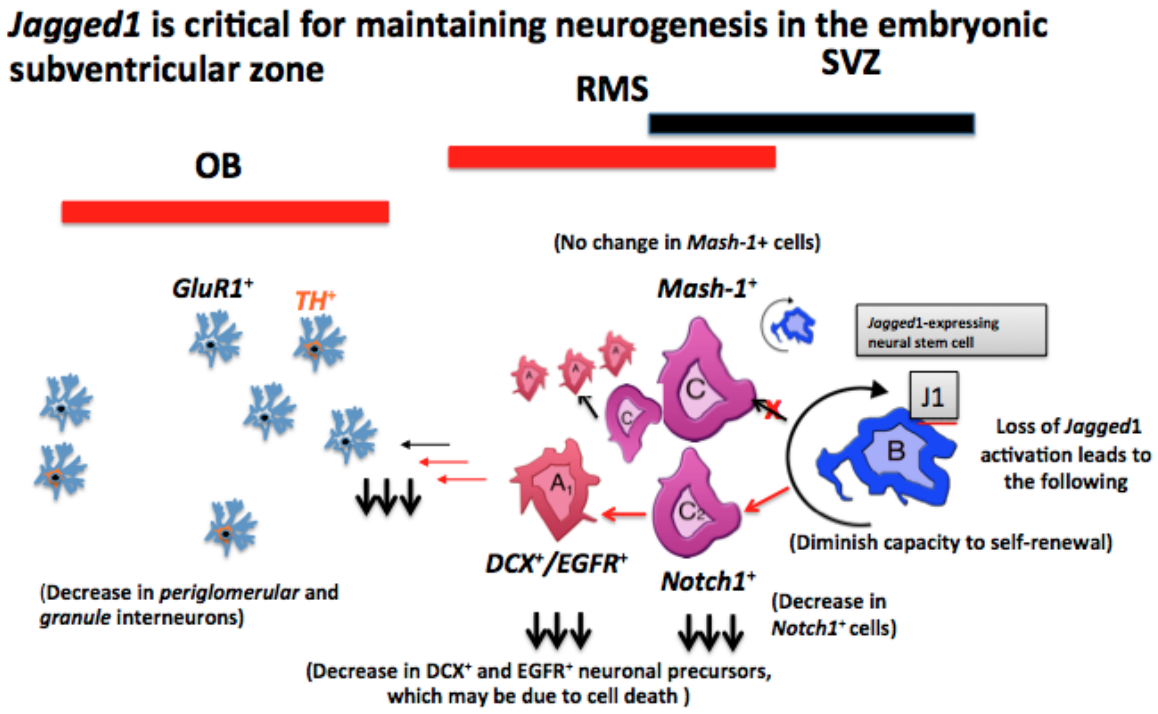
CHAPTER 5

Summary, Observations and Future Directions

5.1 Model system

This dissertation supports the model system that *Jagged1* is critical for maintaining specific types of neurons during olfactory neurogenesis. In the developing subventricular zone, the loss of *Jagged1* activation disrupts neural stem cell's ability to maintain *Notch1*$^{+}$ progenitors. Subsequently, the loss of *Notch1*$^{+}$ progenitor leads to a reduction in *DCX*$^{+}$ neuroblasts. Consequently, the loss of these neuroblasts will lead to reduced production of periglomerular and granule cells in the olfactory bulb.

5.2 Working model system



In this model system, we focused on *Jagged1* function in the subventricular zone. *However, Jagged1* is expressed also in rostral migratory stream (RMS) and olfactory bulb. We are still unclear about the role of J*agged1* elsewhere along our model pathway.

5.3 Summary and future directions

The Notch signaling pathway plays a role in cell fate, neuronal differentiation, cell survival, proliferation, and self-renewal (Weinmaster, 1997; Artavanis-Tsakonas et al., 1999; Wakamatsu et al., 2000; Nakamura et al., 2000, de la Pompa et al. 1997, Hitoshi et al., 2002; Yoon et al., 2008). To date, it is not completely understood which of the *Notch* ligands regulates *Notch* function during the development of the subventricular zone. In this thesis, I set out to expand upon our understanding of how *Jagged1*, a *Notch* ligand, contributes to the *Notch* signaling pathway during development of the subventricular zone.

In the embryonic subventricular zone, a radial glial cell can give rise to different subpopulations of neural stem cells such as those that express *GFAP*$^{+}$ and *Sox2* (Kriegstein and Alvarez-Buylla, 2009). However, it is unclear how many more different subpopulations exist (Kriegstein and Alvarez-Buylla, 2009). *GFAP*$^{+}$ neural stem cells (NSCs) are found along the ventricular lining of the dLGE that give rise to the olfactory bulb interneurons (Doetsch et al., 1999). In *Jagged1* mutants, using immunohistochemistry, we showed that *GFAP*$^{+}$ NSCs were significantly reduced in the ventricular lining of the developing subventricular zone. This suggests that *Jagged1* may be important for maintaining *GFAP*$^{+}$ NSCs in the subventricular zone.

In postnatal studies *in vitro, Jagged1* has been shown to be important for maintaining neural stem cells (Nyfeler et al., 2005). In our *Jagged1* mutants, using our neurosphere assay, we observed that neurospheres were dramatically smaller in size compared to littermate controls. This suggests *Jagged1* may be important for cell division. Previous studies have shown that both neural stem/progenitor cells can form neurospheres (Louis et al., 2008). We found that *Jagged1* mutants formed fewer neurospheres. These findings suggest that *Jagged1* may also be important for maintaining neural stem/progenitor cells. These findings suggest, *in vivo,* that *Jagged1* is not only critical for cell proliferation in neurospheres, but is also important for maintaining neural stem/progenitor populations.

In *Jagged1* mutants, it is conceivable that smaller neurospheres may be the result of growth limitations between a neural stem and a progenitor cell. Neural stem cells have unlimited proliferation ability whereas progenitor cells do not. We therefore should expect differences in size between the two populations over several days *in vitro*. Our data revealed that cell division was dramatically reduced in SVZ-derived cultures from *Jagged1* mutants. This demonstrated that proliferation is affected in neurospheres. This observation is consistent with our *in vivo* finding that revealed proliferation defects in the subventricular zone of *Jagged1* mutants. Furthermore, a study has shown that the loss of *Notch1* and *Jagged1* also affected cell division in the subventricular zone (Nyfeler et al., 2005). Similarly, *in vivo,* we observed that proliferation is dramatically affected in

Jagged1 mutant mice. We reasoned that this might be due to its inability to maintain neural stem cell populations.

An alternative explanation is that the loss of *Jagged1* affects only progenitor populations, and not neural stem cell populations. To address that question, we analyzed neurosphere culture by examining size and capacity for self-renewal. A previous study has shown that neural stem cell-derived neurospheres have unlimited capacity to self-renewal and grow whereas progenitor derived neurospheres do not (Reynolds and Rietze, 2005). If the loss of *Jagged1* negatively affects progenitor populations, we should expect differences in neurosphere formation between the two populations. Serial passages and growth for 12 days *in vitro* (DIV) allowed us to examine the large neurosphere populations, which is likely to represent NSCs. We found that after 12 DIV combined with 4-5 passages, *Jagged1* mutants contained fewer large-size neurospheres, particularly those greater than 200μm in diameter, compared to control. This suggest that loss of *Jagged1* may affects large-size neurospheres (greater than 200μm). Using the neural colony-forming cell assay it has been shown that large neurospheres (greater than >2mm) are derived exclusively from a neural stem cell (Louis et al., 2008). Although we did not grow our neurospheres up to 2mm, we were still able to observe a difference in sizes after 12 DIV. We have not definitively determined whether the large classes of neurospheres are derived from a neural stem cell. Thus, future experiments may included allowing neurospheres to grow on the order of what has been previously

described by Louis et al (2008) as being the hallmark size for identifying neural stem cells-derived neurospheres. Additionally, it may be of interest to use the colony forming assay to determine whether the large class of neurospheres in the controls that are not present in *Jagged1* mutants are indeed neural stem cell-derived. Furthermore, to determine if the large neurosphere originated from neural stem cells, 12 DIV neurospheres from *Jagged1* mutants and controls could be dissociated into single cells then immunostained using *anti-Glial Fibrillary Acidic protein* (GFAP) and Anti-*SRY-related HMG-box* (SOX) to quantify the neural stem cell populations. This may also be performed by qRT-PCR using *GFAP* or *Sox2* to quantify stem cell populations in mutant and control mice.

Furthermore, it is well known that loss of *Notch* decreases neural stem cell populations (Nakamura et al., 2000, Hitoshi et al., 2002, Imayoshi et al., 2010). Previous studies have shown that neurospheres generated from *Notch* mutants failed to maintain neural stem cell populations (Hitoshi et al., 2002, Imayoshi et al., 2010). Additionally, previous studies have shown that *Jagged1* and *Notch* are required for neural stem cells' self-renewal (Hitoshi et al., 2002; Nyfeler et al., 2005). Thus, we reasoned that the loss of *Jagged1* may disrupt *Notch* activation, which in turn negatively affects the *Notch* signaling pathway. Consequently, similar to *Notch* mutants, neural stem populations are dramatically decreased. Therefore, in *Jagged1* mutants, it is possible that the loss of *Notch* activation fails to promote neural stem cell's self-renewal. These studies support that *Jagged1* is critical for maintaining neural stem cells.

Similar to our *in vitro* findings, *in vivo*, we demonstrated that the loss of *Jagged1* reduces cell proliferation (~58%) in the developing subventricular zone. Previous studies have shown that *Notch* is the master regulator of neurogenesis in the subventricular zone (Hitoshi et al., 2002). Therefore, we interpret our findings to suggest that *Jagged1* may be a key regulator of olfactory neurogenesis by activating *Notch* signaling. This explanation is supported by our findings that *Jagged1* mutants also exhibited decreased proliferation along the olfactory migratory pathway. For instance, we found that not only is proliferation decreased in the subventricular zone, but also in the rostral migratory stream (RMS) and olfactory bulbs. In these studies, we revealed defects in proliferation using a PCNA antibody, which recognizes both dividing cells and cells undergoing DNA repair. It would be interesting to determine the contribution for the loss of *Jagged1* effects on proliferation alone. As this may accurately reflect how severe the role of *Jagged1* plays in neurogenesis. This could be performed using BrdU, which labels only proliferating cells.

Furthermore it is well known that neuronal precursors tangentially migrate through the glial tube of the RMS and differentiate into olfactory interneurons. It was later shown that neuronal precursors could be generated from different regionally specialized neural stem cells (Kriegstein and Alvarez-Buylla, 2009 and Doetsch et al., 1999). These neural stem cells may divide into specific subtypes of neuronal precursors: *Emx1-, Pax6-, EGFR-, PSA-NCAM-*, and *Mash-1-*

expressing cells (Young et al., 2007; Kriegstein et al., 2009; Merkle et al. 2007, Kelsch et al. 2007; Ventura & Goldman 2007). In *Jagged1* mutants, using *in situ* hybridization, we found a ~65% decrease in *EGFR*$^{+}$ precursors, but no significant changes in *Mash-1*$^{+}$ precursors in the RMS. This suggests that *Jagged1* selectively affect *EGFR*$^{+}$ neuronal precursors. Thus, the loss of *Jagged1* may affect distinct population of neuronal precursors.

Moreover, It is conceivable that these *Mash-1*$^{+}$ neuronal precursors do not require *Jagged1* signal whereas *EGFR*-expressing precursors do. Previous studies have revealed that there are different subpopulations of neural stem cells (Kriegstein and Alvarez-Buylla, 2009 and Doetsch et al., 1999). As mentioned previously, it well known that these neural stem cell populations are regionally specialized for producing neuronal precursor subtypes (Young et al., 2007; Kriegstein et al., 2009; Merkle et al. 2007, Kelsch et al. 2007; Ventura & Goldman 2007). Therefore, it is possible that *Jagged1* may only affect specific neuronal precursor lineages. Since we only examined a limited number of neuronal precursors, it would be interesting to explore whether the loss of *Jagged1* affects other precursor populations. *In situ* hybridization could be performed using probes that label *polysialylated-neural cell adhesion molecule* (*PSA-NCAM*) and *distal-less homeobox* 2 (*Dlx2*), which are neuronal precursors markers. These markers may provide a more complete picture of the neuronal precursors most affected by the loss of *Jagged1*. Moreover, due to the size and number of the progenitor populations, subtle changes in a single or small

population of cells may be difficult to view using *in situ* hybridization analysis. As an alternative, qRT-PCR may be useful to quantify small changes in neuronal precursor populations.

Furthermore, in *Jagged1* mutants, we observed that the loss in neuronal precursors may have caused a dramatic reduction in the periglomerular and granule neurons in the olfactory bulbs. We found a dramatic reduction in *tyrosine hydroxylase* (*TH*) and *glutamate receptor1* (*gluR1)* mature neurons in the olfactory bulb. These findings suggest that *Jagged1* is important in the production of periglomerular and granule neurons in the olfactory bulbs. Our probes provide a preliminary assay to evaluate *TH+* and *gluR1+* interneurons. However, it is well known that these markers only label a subset of both periglomerular and granule population (Winpenny et al., 2011). For example, in the adult, studies have revealed that periglomerular cells can be subdivided into three nonoverlapping populations based on their immunoreactivity to *tyrosine hydroxylase* (*TH*), calcium binding *proteins calbindin* (*CalB*) or *calretinin* (*CalR*) (Lledo et al., 2006). Given that there are different populations of neuronal precursors that emanate from specialized regions of the developing subventricular zone, it is possible that some populations will be more dramatically affected or less dramatically affected by the loss of *Jagged1*. Furthermore, given that *TH* and *gluR1* is only expressed in a subset of neurons in the periglomerular and granule layer, it would be interesting to survey a greater population of periglomerular or granule neurons. This could be performed by *in situ*

hybridization using probes for calcium binding proteins *calbindin* (*CalB*) or *calretinin* (*CalR*), which label specific subgroups of interneurons in the periglomerular layer in *Jagged1* mutant and control littermates.

In vivo, within the RMS *of Jagged1* mutants, we found an increase in cell death. Previous studies have shown that the loss of *Notch* causes an increase in premature neuronal precursors, which eventually undergo an apoptotic fate (de la Pompa et al., 1997; Nakamura et al., 2000). Additionally, recent studies have reported that *Hes*$^{-/-}$ neuronal precursors are born prematurely and are destined for an apoptotic fate (Nakamura et al., 2000). The loss of *Notch* signaling, as I mentioned in my introduction, is responsible for an increase rate of apoptosis. Therefore, these studies suggest the possibility that the loss of *Jagged1* may fail to activate the *Notch* signaling pathway. This in turn, may prevent neuronal survival. Therefore, this suggests that *Jagged1* is a key regulator in promoting survival, and this may possibly occur through the activation of the *Notch* signaling pathway.

In vitro, we found reduced number of differentiated cells In *Jagged1* mutants. This suggest that *Jagged1* is important for the production of differentiated cells from neurospheres. Similar to the loss of *Notch*, it is possible that the reduction in differentiated cells may be due to cell death. A future experiment would be to examine whether the decrease in differentiated cells from neurospheres was due to an increase in cell death caused by the loss of

Jagged1. This could be determined by staining differentiated neurospheres with *capase3* antibody, which detects cell death. Alternatively, neurospheres can be stained with Trypan blue, which also labels dead cells. In *Jagged1* mutants, an increase in Trypan blue or *caspase3* would indicate an increase in cell death. This may provide an explanation to why *Jagged1* mutants have decreased differentiated cells. An alternative interpretation could be that the loss of *Jagged1* affects early-differentiated neurons. It will be interested to immunostain *Jagged1* mutant neurospheres with early neuronal differentiating markers such as *Math4A*, *NeuroD*, and *NSCL-1*. This will determine whether *Jagged1* affects early-differentiated neurons.

Unexpectedly, although we found that *Jagged1* is expressed in the subventricular zone and RMS, it was surprising that we did not detect cell death in the subventricular zone. We reasoned that it's not until these premature neuronal precursors begin to migrate through the RMS that they undergo apoptosis. However, a few questions arise from this observation. For one, does *Jagged1* have a distinct function in the subventricular zone compared to the RMS? One approach to address this question would be to drive *cre* expression in either the RMS or subventricular zone. If *Jagged1* is deleted in the RMS only, and we find apoptosis is increased, this will support the notion that *Jagged1* serves the same function as in the subventricular zone. Conversely, if we observed no change, this suggests that *Jagged1* may have a distinct function in the RMS compared to its function in subventricular zone.

A previous study has shown that neuronal precursor-glia interaction, as they migrate through the RMS, is critical for repopulation of the olfactory bulbs (Kaneko et al., 2010). It is conceivable that an increase in cell death may not be due to the loss of *Jagged1's* function in the subventricular zone or the RMS. However, it may be due to loss of neuronal precursor and glia interactions that works to sustain tangential migration through the RMS. This inability to migrate may trigger neural precursors' cell death. Therefore, we examined the RMS using a glial marker, but found no dramatic differences, which suggests that there are no major structural defects in the glial tube surrounding neurons. However, one thing we did not examine was whether or not tangential migration was affected in the RMS. Tangential migration involves head to tail movement of neuroblasts through the RMS. Future studies might examine this movement using the Matrigel assay. The Matrigel assay is an *in vitro* technique that can be used to test for tangential migration (Witchterle et al., 1997) using subventricular zone explants taken from *Jagged1* mutants and controls. Matrigel is a three-dimensional extracellular matrix gel composed of collagen IV, laminin, heparan sulfate proteoglycans, and entactin-nidogen (Kleinman et al., 1982). Tangential migration is demonstrated either as cells from the subventricular zone explant form long chains or as cells form a web of interconnected chains. Differential interference contrast (DIC) microscopy can be used to observe the formation of tangential migration. As an alternative, BrdU can be used to visualize neuronal precursor tangential migration.

It may also be possible that the loss of *Jagged1* affects only tangential migration of the neuronal precursors, but not glial tube formation, or vice versa. A recent study has shown that neuronal precursor-glia interaction is critical for tangential migration into the olfactory bulb (Kaneko et al., 2010). Given that neuronal precursors are disrupted in *Jagged1* mutants, it may be possible that the glial tube may also be disrupted. To address this question, the Matrigel Assay could determine if tangential migration is affected independently of tube formation. If the Matrigel Assay shows that chain migration is disrupted, it would eliminate the possibility that chain migration is dependent of tube formation. If chain migration is affected there should be defects in the BrdU labeled neuronal precursors in the RMS. Because the incorporation of BrdU occurs in all dividing cells, distinguishing between neuronal precursors in the RMS and surrounding cells is difficult. However, since the neuronal precursors undergo tangential chain migration, this will make the detection of neuronal precursors based on their organization inside the RMS more obvious. Immunohistochemistry can be performed with *polysialylated neuronal cell adhesion molecule* (*PSA-NCAM*), an antibody, that is used to detect developing and migrating neurons in the RMS (Doetsch and Alvarez-Buylla, 1996, Rougon et al., 1986). This method can be used in conjunction with *TuJ1* (which is an early neural differentiation marker) to confirm that the tangential chain of migrating cells is composed neuronal precursors (Lee et al., 1990).

5.4. Closing remarks

In conclusion, an increasing number of studies have greatly expanded our understanding of the *Notch* signaling pathway. During development, *Notch* signaling was once thought to have a role only in cell fate. However, recently it's now becoming more apparent that *Notch* has multiple functions. In fact, *Notch* plays an important role in neuronal differentiation, cell survival, maintenance of precursors, and self-renewal. During this thesis work, I have built upon these previous findings and examined the role of one *Notch* activator, *Jagged1*, during embryonic development. Similar to *Notch*, my studies have found that *Jagged1* may also plays a role cell proliferation, cell survival, self-renewal, neural stem cell maintenance, and the production of neural precursors and olfactory interneuron. This thesis supports the notion that *Jagged1* is a critical regulator of *Notch* during embryonic development in the subventricular zone. Hopefully, these new insights will help us to better understand the regulation of *Notch*, and provide some future possibilities for targeting aberrant *Jagged1*-mediated *Notch* function for the treatment of neurodegenerative disease.

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Appendix1

CHAPTER 6

Generation of Neurospheres from the Lateral Ventricle of the Late Embryonic Mouse Brain.

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KEYWORDS:

Embryonic, mechanical dissociation, neurosphere, neural stem cell, stem cell biology, progenitor cell

SHORT ABSTRACT:

The neurosphere assay is a powerful tool that has been used to study neural stem cell biology. Here we demonstrate an approach that produces large number

of neurospheres from the lateral ventricle of late embryonic mice.

LONG ABSTRACT:

We describe a protocol for the generation of neurospheres from late embryonic (day 17 or older) mice. An advantage of our approach is that large numbers of neurospheres can be rapidly isolated from a single embryo. Tissue surrounding the lateral ventricle is trimmed away to enrich for neural stem/progenitor populations, and dissociated cells are subsequently cultured for 5-7 days to generate neurospheres. Expected numbers and sizes of neurospheres produced following this protocol are also provided. This protocol can be used for genetic studies, pharmacological manipulation, immunocytochemistry, and self-renewal and differentiation assays.

INTRODUCTION:

Neural stem cells are a tissue-specific subtype of self-renewing and multipotent cells that will produce all neural populations. The neurosphere assay is an important tool that has been extensively employed to study neural stem cell biology[1]. Since its development some 20 years ago[2], neurospheres have been used to study neurogenesis, genes that regulate self-renewal, and molecular mechanisms that control neuronal and glial differentiation[3-5]. For example, the

number and size of neurospheres produced can provide insight into the mechanisms that control cell division and how neural stem/progenitor cells are maintained[6].

We developed a simple dissection technique that helps to maximize the number of neurospheres that can be produced in culture. In some approaches, a brain slicer or other means is used to obtain thick slices of brain tissue from late embryonic stages[7,8]. The area surrounding the ventricle is then microdissected from a given slice to enrich for neural stem/progenitor cells. This approach, while effective, can be painstaking and may require specialized equipment. In contrast, in our approach, the lateral ventricle is visualized with a stereomicroscope, and the surrounding tissue is simply trimmed away using a razor blade or scalpel. This approach requires only half of a single brain, but generates large numbers of healthy neurospheres.

PROTOCOL:

This protocol is designed to generate neurospheres from a single embryo. Multiply all values as needed to generate neurospheres from additional embryos.

1. Set-up prior to tissue dissection

1.1) Establish breeding pairs of mice to obtain embryonic day 17 (E17) embryos. Day 0 is defined as the day a vaginal plug is detected.

1.2) Prepare sterile surgical tools (scissors for decapitation, #5 forceps, razor blades).

1.3) Add 20 mls of Hank's buffer to each of two 10 cm petri plates and place on ice. Add 5 mls Hank's buffer to a 15 ml tube and also place on ice. Reserve another 50 mls of room temperature Hank's buffer.

1.4) Prewarm 20 mls of Hank's-low BSA at 37 °C

1.5) Prewarm 10 mls of Hank's-high BSA at 37 °C.

1.6) Prewarm 10 mls of DMEM/F12 with serum at 37 °C.

1.7) Prewarm 10 mls of neurosphere media at 37 °C.

1.8) Prewarm 2 mls of 0.25% trypsin/EDTA at 37 °C.

2. Tissue dissection

2.1) Euthanize female in accordance with an institutionally approved animal care and use protocol.

2.2) Spray the abdomen with 70% ethanol, and make an incision to expose the uterus. Remove the uterus and transfer it to an empty petri plate.

2.3) Remove embryos from the uterus, spray with 70% ethanol, and decapitate one or more embryos. Rinse each decapitated head in one petri plate containing Hank's buffer, and place in the second petri plate containing Hank's buffer on ice.

2.4) Use forceps to remove the skin and skull. Remove the brain and place into an empty petri dish.

2.5) Use a razor blade to separate the two hemispheres by cutting down the midline of the brain. Place one half of a brain on its lateral surface.

2.6) Identify the location of the lateral ventricle on the medial surface. The ventricle is visible as a T-shaped structure that is slightly darker than the rest of the brain. Using a razor blade or a scalpel, sequentially trim away the brain surrounding the ventricle on all four sides.

2.7) Transfer the dissected tissue into the 15 ml tube on ice.

2.8) If neurospheres are to be isolated from additional embryos (e.g. because of low yield), keep tube on ice until all dissections are complete.

3. Primary neurosphere culture

3.1) Spin sample at 300 RCF in a clinical centrifuge (e.g. IEC) for 3 min. to pellet tissue.

3.2) Aspirate off the supernatant and add 2 mls of pre-warmed Trypsin/EDTA. Incubate at 37 °C for 15 min. with intermittent swirling.

3.3) Spin tube at 300 RCF for 2 min.

3.4) Add 10 mls of room temperature Hank's to trypsin/tissue mixture and incubate at 37 °C for 5 min. with intermittent swirling. Spin culture at 300 RCF for 3 min. and remove the supernatant.

3.5) Repeat wash step 3.4 two additional times.

3.6) Aspirate the supernatant and add 4 mls of Hank's-low BSA.

3.7) Triturate the tissue gently and slowly approximately 10 times with an 18-gauge needle until tissue chunks appear relatively uniform in size. Avoid creating bubbles or foam.

3.8) Triturate the crude cell suspension gently and slowly approximately 7-10 times with a 21-gauge needle until tissue chunks appear relatively uniform in size.

3.9) Triturate the suspension approximately 4-5 times with a 23-gauge needle until suspension appears uniform.

3.10) Add 3 mls of Hank's-high BSA solution to a 15 ml tube. Slowly add the cell suspension to the bottom of the tube underneath the Hank's-high BSA solution using a 23-gauge needle.

3.11) Centrifuge at 300 RCF for 5 min.

3.12) Aspirate supernatant and resuspend cells with 3 mls of prewarmed Hank's-low BSA.

3.13) Centrifuge at 300 RCF for 5 min.

3.14) Aspirate supernatant, and resuspend cells in 5 mls of prewarmed DMEM/F12 with serum.

3.15) Incubate tubes for 2-4 hours at 37 °C to reduce bacterial contamination.

3.16) Centrifuge at 300 RCF for 5 min.

3.17) Resuspend cells in 1 ml of prewarmed neurosphere media.

3.18) Count cells with a hemocytometer. Plate 10,000 cells in a volume of 250 ul in each well of a 48-well plate. Plate at least 8 wells to ensure adequate numbers of neurospheres.

3.19) Incubate at 37 °C in a humidified incubator with 5% CO_2.

3.20) Neurospheres should form within 3-4 days. At day 3, add an additional 100 ml of neurosphere media to each well.

4. Secondary neurosphere culture

After 5-7 days, primary neurospheres should be split to maintain the health of the culture.

4.1) Prewarm 1 ml of Trypsin/EDTA at 37 °C.

4.2) Prewarm 5-10 mls of DMEM/F12 at 37 °C.

4.3) Prewarm 2 mls of neurosphere media at 37 °C.

4.4) Prewarm 1 ml of trypsin inhibitor at 37 °C.

4.5) Collect all neurospheres from at least 8 wells and transfer to a 15 ml tube.

4.6) Spin down neurospheres at 300 RCF for 5 min.

4.7) Remove supernatant and add prewarmed trypsin. Incubate at 37 °C for approximately 5-10 min., depending on the size of neurospheres.

4.8) Add 1 ml of trypsin inhibitor solution and swirl cells for 15 secs to inactivate trypsin.

4.9) Spin down culture at 300 RCF for 5 min.

4.10) Resuspend cells in 4 mls of DMEM/F12.

4.11) Triturate the crude cell suspension gently and slowly approximately 7-10 times with a 21-gauge needle until tissue chunks appear relatively uniform in size.

4.12) Triturate the suspension approximately 4-5 times with a 23-gauge needle until suspension appears uniform.

4.13) Spin down dissociated cells at 300 RCF for 5 min.

4.14) Resuspend cells in 1 ml of DMEM/F12.

4.15) Count cells using a hemocytometer, and plate 10,000 cells in a volume of 250 µl neurosphere media in each well of a 48-well plate. Plate at least 8 wells to ensure adequate numbers of neurospheres.

4.16) Incubate at 37 °C in a humidified incubator with 5% CO_2.

REPRESENTATIVE RESULTS:

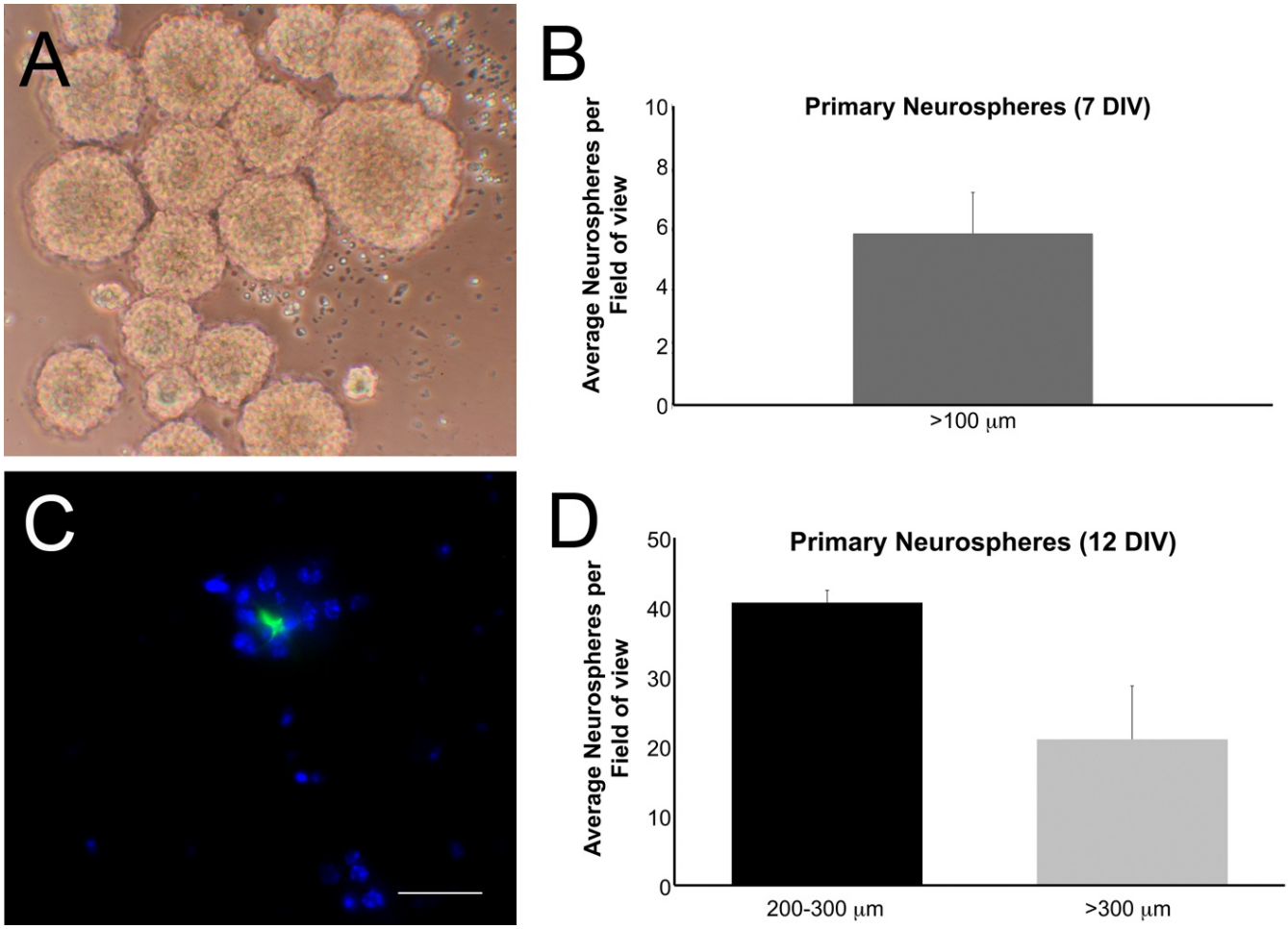

Figure 1. Primary neurospheres generated from the lateral ventricle. (A) Representative picture of neurospheres grown for 7 days *in vitro*. (B) Average number of neurospheres per field of view that are >100 mm after 7 days *in vitro*. (C) Immunocytochemistry of a neurosphere using anti-GFAP antibody (green) and counterstained with DAPI (blue). (D) Average number of neurospheres per field of view between 200-300 mm (grey bar) and >300 mm (black bar) after 12 days *in vitro*. Scale bar = 50 mm in (A) and 100 mm in (C). Error bar represents standard deviation for n=3 separate preparations. Images taken with a EOS Rebel XS (Canon).

Neurospheres should appear around 3-4 DIV. By 7 DIV, neurospheres of varying size (e.g. Fig. 1A) will be apparent, with a subset of these exceeding 100 mm in diameter (Fig. 1B). Neurospheres can be used for a variety of purposes, including immunocytochemistry (e.g. for detection of GFAP-positive cells (Fig. 1C)). Although neurospheres are typically passaged every 5-7 days, they can be cultured for longer, if desired. At 12 DIV, for example, some neurospheres will exceed 300 mm in diameter (Fig. 1D). It has been previously shown neurosphere size and proliferation rate are related[9]. Studies of these larger neurospheres can reveal insight into the mechanisms that control neurosphere growth and division.

DISCUSSION:

There are several key steps that are important to maximize the yield and health of neurospheres. The most important is to incubate the triturated culture in the prewarmed DMEM/F12 with serum for 2-4 hours. This incubation time is necessary in order for the antibiotics in the media to inhibit growth of bacteria, and reduce subsequent contamination. Another essential step is to perform the trituration as gently as possible. Over trituration, or trituration with great force, will result in increased cell death.

Additional modifications to the approach may be required. We have provided general guidelines for the number of cells to be plated per well and the expected number of neurospheres per field of view. If too many cells are plated per well, differentiation may occur. If so, either reduce the number of cells plated per well or increase the concentration of EGF to 20 ng/ml. If desired, trypsin inhibitor can be substituted for the FBS during the generation of primary neurospheres to inactivate proteolytic activity.

Our approach utilizes a mechanical trituration to dissociate cells. By

sequentially processing the cells through successively higher gauge needles, we obtain very few clumps of cells. This eliminates the need for a cell strainer, which can reduce yield, as well as the time needed to perform enzymatic dissociation. Although cell death can be increased with mechanical dissociation, in our hands, this does not seem to affect neurosphere yield or health.

One advantage of our approach is the ease with which tissue surrounding the lateral ventricle can be isolated from the rest of the brain. Although this dissection is relatively crude, it is easier and faster than other approaches which may require a brain slicer and microdissection [7,8]. This eliminates the need for specialized equipment while generating high numbers of neurospheres from a single brain.

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DISCLOSURES:

Experiments on animals were performed in accordance with the guidelines and regulations set for by Cornell University IACUC committee and NIH guidelines.

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