Neogenin regulates cell cycle dynamics and differentiation in the developing olfactory epithelium

Christopher Baim

Department of Neurology and Neurosurgery

Faculty of Medicine

McGill University, Montreal

Submitted June, 2017

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science (Neuroscience)

Table of Contents

Abstract iv

Résumé v

Acknowledgements vii

Preface & Contribution of Authors viii

Introduction 1

Introduction to the Olfactory Epithelium 2

Early Olfactory Development 3

OE Neurogenesis 4

Transcriptional Regulation of Neurogenesis 5

Regulation of OE Development 9

The Multi-Ligand Receptor Neogenin 12

Neogenin Ligands 14

Rationale and Aims 17

Materials and Methods 19

Experimental Animals 19

Immunohistochemistry 19

S-phase Duration & Cell Cycle Exit 20

Primary OE Explant Culture 21

Image Capture & Analysis 22

Results 23

Neogenin regulates cell cycle progression in the OE 23

Overproduction of SUS cells by basal progenitors in the OE of $Neo1^{-/-}$ embryos 24

RGMB promotes neuronal differentiation in OE explants 25

Discussion 27
Summary 27
Interpretation and Future Directions 28
Neogenin regulates cell cycle progression and cell fate choice in the OE 29
RGMB-neogenin signaling in progenitor cell differentiation 31
Concluding Remarks 31
Figures 33
Figure 1: Structure, cell types, and cell lineages of the developing murine olfactory epithelium. 33
Figure 2: Detailed cell lineages of the OE and associated proteins. 34
Figure 3: Ablation of neogenin leads to altered cell cycle kinetics and cell cycle exit. 35
Figure 4: Neogenin ablation leads to overproduction of SUS cells. 37
Figure 5: RGMB regulates OE cell differentiation in vitro. 39
References 40

Abstract

The ability to generate a diverse array of cell types is critical for the development of any organism. While many mechanisms underlying this generation of diversity have been explored, a great deal remain to be discovered. Taking advantage of the developing mouse olfactory epithelium (OE) as a model, we investigated the role of the transmembrane receptor neogenin in cellular differentiation. Using an *in vivo* loss of function approach, we identified a role for neogenin in regulating the differentiation of progenitors into glial-like sustentacular (SUS) cells. Mice lacking neogenin were found to have increased numbers of SUS cells and basal progenitor cells. The supernumerary SUS cells observed arose from basal progenitor cells of the OE rather than from self-proliferation. Additionally, we show that neogenin is important for maintaining the size of the OE progenitor pool by regulating cell cycle dynamics of basal progenitors, specifically by promoting cell cycle exit and decreasing S-phase duration. Furthermore, *in vitro* OE explant experiments revealed that the neogenin ligand, repulsive guidance molecule B (RGMB), can promote neuronal differentiation of OE progenitors in a neogenin-dependent manner. Taken together, our results show that neogenin influences cell cycle dynamics as well as cell fate choice in the mammalian nervous system and that this helps to maintain the balance of glial-like and neuronal cells produced.

Résumé

La capacité à générer divers types de cellules est essentielle pour le développement de tout organisme. Tandis que plusieurs mécanismes responsables de la génération de cette diversité cellulaire ont été identifiés, plusieurs demeurent inconnus. Nous avons étudié la contribution du récepteur transmembranaire neogenin dans le processus de différentiation cellulaire, en utilisant comme modèle l'épithélium olfactif chez la souris. À l'aide d'une approche *in vivo*, nous avons identifié un rôle pour neogenin dans la coordination de la différentiation des cellules progénitrices en cellules sustentaculaires (SUS) versus les cellules neuronales de l'épithelium olfactif. En étudiant une souris mutante dans laquelle l'expression de neogenin a été totalement abrogée, nous avons observé une augmentation du nombre de cellules SUS et de cellules basales progénitrices dans l'epithélium olfactif. Nous démontrons que la présence de cellules SUS surnuméraires n'est pas due à leur auto-prolifération mais bien à une augmentation de leur génération par les cellules basales progénitrices de l'épithélium olfactif. Nous démontrons également que neogenin est nécessaire pour contrôler la durée de la phase-S du cycle cellulaire des cellules basales progénitrices et pour promouvoir leur sortie du cycle cellulaire. La régulation du cycle cellulaire par neogenin est importante pour maintenir la banque de cellules progénitrices dans l'épithelium olfactif. De plus, des expériences *in vitro* nous ont permises de démontrer que RGMB, un ligand de neogenin, peut faciliter la différentiation des cellules progénitrices en neurones en activant neogenin. Ensemble, ces résultats démontrent que neogenin influence la dynamique du cycle cellulaire, ainsi que la différentiation cellulaire, dans le système nerveux des mammifères, aidant ainsi à maintenir

l’équilibre qui existe entre la production de neurones et de cellules sustentaculaires dans l’épithélium olfactif.

Acknowledgements

Dr. Jean-François Cloutier for all his expert guidance and support throughout my Master's degree.

My advisory committee, Dr. Stefano Stifani and Dr. Michel Cayouette, and my mentor Dr. Dusica Maysinger for their advice and for challenging me to always improve.

Members of the Cloutier lab, both past and present including: Alex, Allen, Barbara, Candice, Kat, Neelima, Reesha, and Sabrina for their scientific help, but also for keeping me sane(-ish) during my Master's.

Dr. Joseph Kam for his training and without whom this project would not exist.

Emilie Dumontier whose scientific expertise was astonishing and whose patience seemed endless.

Members of the Stifani lab (especially Jae, Karen, Lawrie, Rita, Valerio, and Vince) for all their intellectually stimulating discussions, scientific and otherwise.

Angie Fato for her sage advice and for always making sure I was caffeinated.

Alexandra Tremblay-McLean for always being there for me and for listening to my never-ending thought experiments.

Preface & Contribution of Authors

The results and findings presented in this thesis have been published in Kam et al., 2016 in the journal Development. Excerpts of the results, materials and methods, discussion, and figures from this paper were adapted with permission from the publisher. I have performed the experiments presented in Figures 3E,F, Figure 4D,E, and Figure 5D of this thesis. Dr. Joseph Kam performed the experiments shown in figures 3A-D, 4A-C, and 5A-C. The abstract was translated to French by Alexandra Tremblay-McLean.

Introduction

During development a complex network of coordinated signaling mechanisms is required for the generation of a functioning organism. Such signaling mechanisms are especially important for the development of the nervous system, where they regulate proliferation, survival, differentiation, and axon guidance. A thorough understanding of the mechanisms underlying cell differentiation is crucial for developing new therapies to treat neurodegenerative diseases. This is especially true for the generation of patient derived neuronal cell lines, which can be used for disease modeling, drug screening, and cell therapy (Jung et al. 2012).

One critical component of development is the generation of the different cell types that an organism requires to function. This generation of diversity is regulated by a myriad of both extracellular and intracellular signals. Extracellular cues involved in regulating differentiation often take the form of secreted molecules such as the bone morphogenetic protein (BMP), transforming growth factor beta (TGF-β), and Hedgehog family of ligands. However, increasing evidence has shown the importance of cell-cell signaling in morphogenesis (Perrimon et al. 2012). In the nervous system, for example, N-cadherin interactions in the developing cortex have been shown to stimulate β-catenin signaling, inhibiting differentiation of neural precursors (Zhang et al. 2013). While the evidence for cell-cell interactions in neuronal differentiation has increased, many of the underlying mechanisms involved have yet to be explored.

One protein of particular interest for cell-cell signaling in neuronal differentiation is the transmembrane receptor neogenin. Neogenin is a widely expressed multi-ligand receptor that

has been implicated in an extensive number of developmental functions including axon guidance, neuronal survival, cell adhesion, and differentiation (Wilson and Key 2007). To examine the role of neogenin in regulating neuronal differentiation and fate determination within the nervous system, I have investigated how neogenin influences neurogenesis using the developing murine olfactory epithelium (OE) as a model.

Introduction to the Olfactory Epithelium

Several model systems exist to study the generation of cell diversity during development. One widely used model system is the developing murine OE, in which a single bipotent stem/progenitor population gives rise to both neuronal and glial-like cells. The murine OE serves as an excellent model for studying the molecular mechanisms regulating cell fate choice due to its layered spatial organization, simple cell lineages, and its continuous regeneration throughout life.

In the OE, progenitor and stem cells reside in the basal region adjacent to the basement membrane. Progenitor cells give rise to immature olfactory receptor neurons (iORNs) located in the intermediate region of the OE, which then mature into more apically localized olfactory receptor neurons (ORNs) (Fig. 1A,B). ORNs extend cilia covered apical dendrites into the nasal cavity where they detect odorants binding to olfactory receptors (ORs) on their surface. Following odorant binding, action potentials are relayed via the ORN's axon to the olfactory bulb (OB) of the brain, giving rise to olfactory perception. In addition to ORNs, progenitor cells of the OE also give rise to glial-like sustentacular (SUS) cells. Although SUS cell nuclei reside in the most apical region of the OE, they send protrusions spanning the whole thickness of the OE

(Nomura et al. 2004). In addition to providing structural support to the OE, SUS cells perform multiple glial-like functions, such as providing metabolic support to ORNs.

Early Olfactory Development

The murine olfactory system, comprised of the olfactory epithelium and the OB, begin development as two independent structures. The OB, being a part of the CNS, develops from the germinal zones of the neural tube and can be identified as early as embryonic day 13 (E13) in the rostral telencephalon (López-Mascaraque et al. 1996). In contrast, the nascent OE can initially be identified as an ectodermal thickening on the ventrolateral region of the head, termed the olfactory placode (OP), as early as E9.5 (Cuschieri and Bannister 1975). The OP undergoes thickening until E10, at which point it begins to invaginate forming the olfactory pit, which marks the earliest stage of the nasal cavity. By E10.5, continued invagination deepens the nasal cavity, which is now surrounded by marginal rims that will eventually form the nostrils. This deepening continues through E11.5, resulting in a more elaborate nasal cavity and pronounced nostrils (Cuschieri and Bannister 1975). At this point, the olfactory pit is lined by a pseudostratified epithelium only 5-6 cells deep (Smart 1971). From E11.5 to E13, apically located progenitor cells undergo rapid proliferation, causing progenitor pool expansion and thickening of the OE to 6-8 cells deep (Smart 1971). These progenitors migrate from the apical to basal region of the OE by E13, where they continue to be mitotically active and begin differentiation.

The initial induction of all sensory placodes, including the olfactory placode, is dependent on the expression of a number of transcription factor (TF) families, including Eya, Msx, Pax, Pitx, Six, and Sox (reviewed in Schlosser, 2006). Following olfactory placode induction,

expression of the specific TFs Sox2, Pax6, and Oct-1 can be observed (Grindley et al. 1995; Collinson et al. 2003; Donner et al. 2007). The function of these TFs, and their roles in OE neurogenesis, will be discussed in the next section.

OE Neurogenesis

The OE has long served as an attractive model for studying the molecular mechanisms underlying neurogenesis for several reasons. First, the murine OE has reduced complexity compared to other neurogenic tissues, in that it has a layered spatial organization, produces a readily identifiable population of neurons, and has simple cell lineages. Second, in order to replace dying ORNs or in response to injury, neurogenesis in the OE continues throughout life in rodents (Murray and Calof 1999). These factors, in addition to its higher accessibility compared to other neurogenic structures, has made the OE an ideal model of neurogenesis.

Early in olfactory placode development, cells begin to express a number of transcription factors (TFs) including Oct-1, Pax6, and Pax7 (Grindley et al. 1995; Mansouri et al. 1996; Donner et al. 2007). Additionally, cells of the OP express the TF Sox2, which is known to maintain multipotency and self-renewal (Donner et al. 2007). Shortly after (E10-E11), transit-amplifying cells expressing the basic helix-loop-helix (bHLH) TFs Ascl1 and Neurogenin 1 (Ngn1) can be observed (Guillemot et al. 1993; Cau et al. 1997). By E13.5, most progenitor cells localize to the basal region of the OE, where they remain (Graziadei and Graziadei 1979). These basal progenitors will primarily give rise to ORNs, while the few remaining apical progenitor cells will acquire a glial-like fate becoming SUS cells (Schwob et al. 1994).

The production of ORNs by the now basally located progenitors involves the generation of a number of transient cell types, each expressing a unique combination of TFs and marker proteins (reviewed in Kam et al. 2014) (Fig. 2). Basal progenitors express Sox2, which is required for stem-cell maintenance, supporting the notion that they serve as the neural stem cells of the OE (Wegner and Stolt 2005). These $Sox2^+$ progenitors then give rise to $Ascl1^+$ cells termed committed neural progenitor cells (NPCs), which in turn generate $Ngn1^+$ intermediate neural precursors (INPs). Alternatively, NPCs can give rise to $SUS4^+$ glial-like SUS cells. $Ngn1^+$ cells give rise to neural precursors (NPs) expressing Neuronal Differentiation 1 (NeuroD1), which then undergo differentiation into iORNs that are βIII-tubulin$^+$ (β-III$^+$). Finally, iORNs mature into full-fledged ORNs and begin to express olfactory marker protein (OMP). This coordinated progression from stem/progenitor cells to fully differentiated ORNs is regulated by several intrinsic factors and extrinsic signals that we will examine more closely.

Transcriptional Regulation of Neurogenesis

As mentioned above, Sox2 is essential for both olfactory placode formation and for the maintenance of the OE's stem/progenitor pool. Sox2 is a member of the SRY-related HMG-box family of transcription factors, which are essential regulators of embryonic development, as well as being important for homeostasis and regeneration in adulthood (Avilion et al. 2003; Sarkar and Hochedlinger 2013). Accordingly, homozygous deletion of Sox2 results in abnormal embryogenesis shortly after implantation with 100% lethality by E9.5 (Avilion et al. 2003). Beginning at E9.5, Sox2 is observable in the surface ectoderm of the developing OP (Uwanogho et al. 1995; Wood and Episkopou 1999). Later, Sox2 expressing cells can be found in all layers of the nasal pit (E10-E11.5) and the early OE (E12.5) before the majority settle into the basal layer

(E13.5) for the remainder of OE development and later adulthood (Graziadei and Graziadei 1979; Cau et al. 1997; Donner et al. 2007). Continued Sox2 expression can be observed in apically localized SUS cells (Gokoffski et al. 2011).

Sox genes are capable of binding DNA via their high-mobility group (HMG) domains, acting as transcriptional regulators. However, the specificity of this regulatory ability depends on their binding to cell-specific partner factors (reviewed in Kamachi et al. 2000). Within surface ectodermal cells of the head, Oct-1 acts as the binding partner for Sox2, resulting in the expression of Pax6, which is required for both lens and olfactory placode formation (Donner et al. 2007). Indeed, loss of Oct-1 in combination with heterozygosity for Sox2 results in an absence of Pax6 expression and failed olfactory placode induction (Donner et al. 2007). Later in olfactory development and into adulthood, Sox2 expressing cells serve as a multipotent stem/progenitor population capable of generating both neuronal and SUS cells (Leung et al. 2007; Guo et al. 2010). However, the proper generation of both of these cell types requires the expression of another TF, Ascl1 (Cau et al. 1997; Krolewski et al. 2012).

Ascl1, also known as Mash1, is a bHLH TF that is essential for initiating the transcriptional regulatory cascade required for neuronal development (Guillemot et al. 1993; Cau et al. 1997; Murray et al. 2003). Earliest expression of Ascl1 can be detected in small clusters of cells in the OP at the 24/25 somite stage (~E9.5), before expression expands to the entire periphery of olfactory pit by E10.5 (Cau et al. 1997). During subsequent OE development, Ascl1 positive progenitors migrate to the basal OE, where expression continues into adulthood, with most of these cells being basally localized by E15.5 (Gordon et al. 1995; Cau et al. 1997; Rodriguez et al. 2008).

Ascl1 is required for ORN differentiation during development, as well as for OE maintenance and regeneration in adulthood. Cells expressing Ascl1, commonly referred to as committed neural progenitors, arise from Sox2 positive cells. Expression of Ascl1 in these cells is dependent on decreased Notch1/2 signaling and concomitant downregulation of Hes1, a repressor of Ascl1 (Manglapus et al. 2004; Rodriguez et al. 2008). Interestingly, Ascl1 expression itself is required for the activation of Notch signaling in the OE, indicating a possible homeostatic mechanism regulating neuronal production (Cau et al. 1997). Downstream of Ascl1 are two other bHLH TFs known to promote neuronal fate determination, Ngn1 and NeuroD1 (Cau et al. 1997). Loss of Ascl1 results in significant thinning of the OE and severely reduced neurogenesis due to increased cell death and reduced expression of Ngn1 and NeuroD1 (Cau et al. 1997; Cau et al. 2002; Murray et al. 2003; Krolewski et al. 2012). While neurogenesis is perturbed in Ascl1 mutant mice, SUS generation occurs to the same extent as in wild-type mice and without delay (Krolewski et al. 2012). However, these SUS cells have decreased levels of Hes1, a promotor of SUS cell fate, as well as expression of the Ascl1 3'-UTR, indicating that feedback mechanisms regulating neuronal vs glial-like fate determination are perturbed (Murray et al. 2003; Manglapus et al. 2004; Krolewski et al. 2012).

As mentioned above, downstream of Ascl1 expression lies another neurogenic bHLH TF termed Ngn1. Like Ascl1, Ngn1 expression can first be observed throughout the OP at E9.5, however, unlike Ascl1, which is later restricted to clusters of cells in the periphery, Ngn1 expressing cells are scattered throughout both the periphery and the center of the olfactory pit by E10.5 (Cau et al. 1997). By E12.5, when the OE has begun to develop a more layered structure, Ascl1 and Ngn1 can be observed in discrete cell populations with Ngn1 expressed in

basal progenitors and Ascl1 expressed in progenitors throughout all layers of the OE (Cau et al. 2002). Mice lacking Ngn1 show a severe reduction in OE neurogenesis, specifically due to failed neuronal differentiation as suggested by the decrease in ORNs and that a similar number of progenitor cells are observed in these mice (Cau et al. 2002). Ngn1's role in promoting neuronal differentiation is achieved via its regulation of two genes, Phd1 and NeuroD1 (Cau et al. 2002). Phd1, also known as Uncx, is a paired homeobox TF that is required for proper progenitor cell proliferation and ORN survival in the developing OE (Sammeta et al. 2010). NeuroD1, similar to Ngn1 and Ascl1, is a bHLH TF important for olfactory neurogenesis (Boutin et al. 2010; Packard et al. 2011).

NeuroD1, similarly to Ngn1, is expressed throughout the OP at E9.5 (Cau et al. 1997). However, unlike Ngn1, NeuroD1 expression is restricted to the center of the OP by E10.5 (Cau et al. 1997). Later in OE development, NeuroD1 expression becomes restricted to a basally located subset of cells termed neural precursors (Cau et al. 2002). Ectopic expression of NeuroD1 in the periventricular region has been shown to induce neuronal differentiation while knockdown inhibits terminal differentiation of periglomerular neurons in the OB (Boutin et al. 2010). Interestingly, NeuroD1 does not seem to be essential for production of ORNs in the majority of the OE, as similar numbers of mature olfactory neurons are observed in NeuroD1 null mice (Packard et al. 2011). However, several areas of the OE in these mice, including the tips of some turbinates, lack mature ORNs indicating a region specific requirement for NeuroD1 in ORN maturation (Packard et al. 2011). Together, it seems that NeuroD1 is required for neuronal differentiation in a subset of olfactory neurons of the OE.

Regulation of OE Development

The development of the OE, as with other tissues, requires a fine balance between progenitor cell expansion and differentiation. Due to the regenerative nature of the OE, it is also crucial that this balance is maintained past development, continuing for the duration of an organism's life. As such, a complex network of both cell intrinsic and extrinsic factors has developed to modulate OE growth and development. This includes positive regulators that act to promote proliferation, differentiation, and survival, as well as negative regulators that inhibit these processes.

The fibroblast growth factor (FGF) family is composed of 22 mostly secreted proteins, which are well known for their diverse role in regulating proliferation, migration, differentiation, and survival during development (Ornitz and Itoh 2015). Within the nervous system, FGFs have been shown to be crucial for both neuronal induction and proliferation through interactions with their four receptors (FGFRs) (Ford-Perriss et al. 2001). Multiple FGFs have been shown to be expressed within the olfactory system including FGF-1, -2, -8, and -18 (DeHamer et al. 1994; Key et al. 1996; Kawauchi et al. 2004; Kawauchi et al. 2005). While FGF-2 has been shown to stimulate ORN production and differentiation *in vitro,* only FGF-8 has been shown to play a role in OE development *in vivo* (DeHamer et al. 1994; MacDonald et al. 1996; Kawauchi et al. 2004; Kawauchi et al. 2005).

Expression of FGF-8 can be observed as early as E10.5 within a ring of ectodermal epithelium surrounding the invaginating OP, with some FGF-8^{+} cells coexpressing Sox2 (Kawauchi et al. 2005). FGF-8 expression declines as development progresses, however, basal expression can still be observed in the OEs of adult mice (Kawauchi et al. 2004). Loss of FGF-8

results in severe reductions in the size of the OE, including near absence of neuronal stem and progenitor cells (Kawauchi et al. 2005). While there is no observable change in OE proliferation in these mice, there is a massive increase in apoptosis, particularly around E10.5 (Kawauchi et al. 2005). These findings indicate that FGF-8 is critical for the survival of early stem/progenitor cells in the developing OE.

While positive regulation of neurogenesis is important for proper OE development and maintenance, negative regulation is equally critical for preserving the appropriate balance of neuronal, glial-like, stem, and progenitor cell populations of the OE. This negative regulation of neurogenesis is achieved, in part, by the secretion of factors from maturing ORNs that inhibit proliferation and neuronal differentiation of less mature progenitors. Initial evidence for this homeostatic mechanism included the observation that co-culturing OE neuronal progenitors with an excess of differentiated neurons suppressed neurogenesis (Mumm et al. 1996). At least two factors responsible for this effect have been identified, the TGFβ family members Growth Differentiation Factor 11 (GDF11) and Activin βB (ACTβB) (Wu et al. 2003; Gokoffski et al. 2011).

The TGFβ family is comprised of a wide variety of secreted signaling molecules that have been shown to regulate cellular proliferation and differentiation in an extensive number of developmental tissues, including the nervous system (Hogan 1996; Massagué et al. 2000; Liu and Niswander 2005). GDF11, also known as BMP11, is a member of the activin-like family of TGFβ proteins, which has been shown to inhibit neuron production in the OE (Wu et al. 2003; Gokoffski et al. 2011). GDF11 expression is first observable in epithelial cells of the OP by E10.5 (Kawauchi et al. 2009). From E12.5 onward, GDF11 expression can be found in ORNs and olfactory progenitors in the basal two-thirds of the OE (Nakashima et al. 1999; Wu et al. 2003;

Kawauchi et al. 2009; Gokoffski et al. 2011). Mice lacking GDF11 have increased OE thickness as well as an increase in the number of proliferative cells, especially in the intermediate layer of the OE where $Ngn1^{+}$ INPs are located (Wu et al. 2003; Gokoffski et al. 2011). Analysis of the different cell populations in these mice reveals an increase in INPs, ORNs, and SUS cells, but no increase in $Ascl1^{+}$ NPCs (Wu et al. 2003; Gokoffski et al. 2011). Together, these results indicate that GDF11 acts in an autocrine fashion to negatively regulate OE neurogenesis by inhibiting INP proliferation, likely by inducing reversible cell cycle arrest (Wu et al. 2003; Lander et al. 2009; Gokoffski et al. 2011).

ActβB, another TGFβ family signaling molecule, has also been shown to negatively regulate OE neurogenesis *in vivo* (Gokoffski et al. 2011). Activins are homo- or heterodimers of β-subunits, which have been shown to regulate a diverse array of developmental processes including embryonic induction, fate determination, and tissue morphogenesis (Wijayarathna and de Kretser 2016). While both the ActβA and ActβB subunits are known to be expressed in the nervous system, only ActβB is expressed within the developing OE (Spencer et al. 1992; Feijen et al. 1994; Gokoffski et al. 2011). ActβB is expressed similarly to GDF11, being found in ORNs and olfactory progenitors from E12.5 onward, however ActβB acts in a dissimilar manner (Gokoffski et al. 2011). Mice lacking ActβB have significantly increased numbers of $Ascl1^{+}$ progenitors, but no change in the number of $Ngn1^{+}$ INPs, contrary to the phenotype observed in $GDF11^{-/-}$ mice (Gokoffski et al. 2011). This suggests that while both GDF11 and ActβB inhibit neurogenesis in the OE, they do so through different mechanisms, with ActβB inhibiting proliferation of $Ascl1^{+}$ progenitors and GDF11 inhibiting proliferation of $Ngn1^{+}$ INPs.

While negative regulation of neurogenesis via factors secreted from differentiated neurons, such as GDF11 and ActβB, is crucial for maintaining OE homeostasis, it is also likely that direct cell-cell signaling regulates OE development and cell fate choice. Due to the layered organization of the OE, newly born ORNs lie directly above progenitor cells, giving ORNs the opportunity to influence progenitor cell proliferation and fate choice directly. This type of contact-dependent signaling has been widely investigated within the CNS where, for example, Notch signaling has been shown to regulate neuronal proliferation, survival, and fate choice (reviewed in Lathia et al. 2008). However, similar cell-interaction dependent mechanisms remain to be identified within the developing OE.

The Multi-Ligand Receptor Neogenin

Neogenin is a member of the immunoglobulin superfamily (IgSF) and is structurally homologous to another IgSF member, deleted in colorectal cancer (DCC) (Vielmetter et al. 1994). Neogenin contains an extracellular region composed of 4 immunoglobulin (Ig)-like loops and 6 fibronectin III (FNIII) repeats, followed by a single transmembrane region and a cytoplasmic tail containing three signaling domains (P1, P2, and P3), which are conserved between neogenin and DCC (Vielmetter et al. 1994; Meyerhardt et al. 1997).

Although neogenin is a DCC homologue, their roles during development differ greatly. These differences are highlighted by the dissimilar expression of neogenin and DCC during development. DCC is expressed primarily in the CNS where it is observed in the hindbrain region of the neural tube as early as E9.5. Later, DCC can be observed in several brain regions, including the developing cortex, hippocampus, midbrain, and cerebellum. Restricted DCC

expression is observed outside of the CNS in the mesoderm of developing limbs, the urogenital ridge, and the facial musculature around the developing palate (Gad et al. 1997).

Neogenin is expressed strongly in the CNS, however in contrast to DCC, it is also expressed widely throughout the developing embryo. Neogenin is first observed by E8.5 throughout the endodermal, ectodermal, and mesodermal layers of the embryo. By E11.5, neogenin expression intensifies in connective tissue and expression begins to increase in the developing cortex, while still being expressed at low levels in most embryonic tissue. At E13.5, neogenin expression outside of the CNS becomes localized to many mesoderm derived tissues including the primordial cartilage of the head and face, the vertebral bodies, and cartilaginous condensations of the limbs. At this time, increased expression of neogenin can be observed in the CNS including, but not limited to, the developing cortex, hippocampus, olfactory bulb, and cerebellum. Throughout the rest of embryonic development, neogenin continues to be strongly expressed in the CNS and mesodermal derivatives, especially in bone and cartilage of the head (Gad et al. 1997).

Neogenin is also expressed selectively within the developing OE (Fitzgerald et al. 2006; Kam et al. 2016). Neogenin is observed in the basal region of the OE by E14.5 in cells positive for the proliferative cell marker PCNA, indicating neogenin is expressed by basal progenitors (Fitzgerald et al. 2006). By E16.5, neogenin is found apically in cells co-expressing OMP and basally in cells co-expressing proliferative marker Ki-67, indicating that neogenin is expressed in both mature ORNs and basal progenitors (Kam et al. 2016) (Fig. 1B).

Mirroring its wide expression throughout the embryo, neogenin has been shown to be important for a wide variety of cell functions during development. While better well known for its role in axon guidance in chick and *Xenopus*, neogenin has also been shown to be important for neuronal regeneration, apoptosis, differentiation, and proliferation (Wilson and Key 2007). Neogenin's roles in these cellular processes depend on its binding to three families of ligands: repulsive guidance molecules (RGMs), BMPs, and netrins (De Vries and Cooper 2008; Hagihara et al. 2011).

Neogenin Ligands

The RGM family of glycosylphosphatidylinositol (GPI) anchored glycoproteins consists of three members: RGMA, RGMB, and RGMC (also known as hemojuvelin). All three family members are capable of binding neogenin's extracellular domain with recent crystal structure analysis showing specific interactions with the FNIII-5 and FNIII-6 domains of neogenin (Rajagopalan et al. 2004; Bell et al. 2013). RGMs exist in a membrane-bound state or, through pH dependent autocatalytic cleavage, a soluble form (Bell et al. 2013). While both RGMA and RGMB are expressed in the developing nervous system, RGMC expression is limited to tissue outside of the nervous system including striated muscle and liver (Schmidtmer and Engelkamp 2004; Severyn et al. 2009).

Chick RGM (cRGM), the orthologue of RGMA in mouse, has been shown to promote neuronal differentiation in both midbrain and hindbrain (Matsunaga et al. 2006). *In vitro* experiments using RGMA also support a role for RGMA-neogenin interactions in promoting neuronal differentiation of cortical interneurons (O'Leary et al. 2013). The absence of cRGM results in neogenin mediated apoptosis in embryonic neural tube, indicating neogenin may

function as a death-dependency receptor in some cells (Matsunaga et al. 2004). cRGM has also been shown to mediate repulsion of temporal retinal axons in chick in a neogenin-dependent manner (Rajagopalan et al. 2004; Matsunaga et al. 2006). Experiments in rat models have shown that RGMA inhibits neurite outgrowth via neogenin-mediated activation of the RhoA signaling pathway, suggesting a similar mechanism may be responsible for the repulsive effect of cRGM observed in chick (Hata et al. 2006).

While RGMB is also expressed in the developing CNS, less is known about its function in the nervous system (Severyn et al. 2009). RGMB has been implicated in the migration of neogenin expressing cells within the dentate gyrus *in vivo* (Conrad et al. 2010). Crystal structure analyses of RGMB have revealed that it can form a structural bridge between neogenin and BMP2 (Healey et al. 2015). Addition of BMP2 *in vitro* resulted in RGMB-dependent clustering of neogenin, suggesting that RGMB may be important for regulating the subcellular localization of neogenin (Healey et al. 2015).

BMPs form the largest group of TGFβ proteins and have been shown to regulate numerous developmental processes including neurogenesis and skeletogenesis (Bragdon et al. 2011). Neogenin has been shown to interact directly with BMPs -2, -4, -6, and -7 *in vitro.* BMP-2 binding to neogenin activates RhoA signaling in C2C12 myoblasts, inhibiting p-Smad1/5/8 signaling (Hagihara et al. 2011). Conversely, neogenin is also necessary for the recruitment of BMP receptors (BMPRs) IA and II to lipid rafts, thereby promoting BMPR mediated phosphorylation of Smad1/5/8 (Zhou et al. 2010). Evidence suggests that these opposing roles of neogenin on canonical BMP signaling may take place in a tissue dependent manner, however

additional research must be performed to fully understand neogenin's impact on BMP signaling.

Neogenin binding with the netrin family of secreted proteins has been shown to mediate adhesion, attraction, and differentiation (De Vries and Cooper 2008). Netrin-1-neogenin interactions are involved in promoting cell adhesion between cap cells and the pre-luminal epithelium in the developing mammary gland (Srinivasan et al. 2003). Netrin-1 has also been shown to promote chemoattraction of supraoptic axons via neogenin in *Xenopus* embryos (Wilson and Key 2007). Interestingly, because netrin-1 binds to neogenin's FNIII-4 and FNIII-5 domains, overlapping with RGMs binding site, it is possible that netrins and RGMs compete for neogenin binding (Xu et al. 2014).

Rationale and Aims

Previous research in our lab, recently published in Kam et al. 2016, has shown that the transmembrane receptor neogenin and its ligand RGMB regulate the production of ORNs and SUS cells in the developing OE. Using *in situ* hybridization (ISH) and immunostaining, neogenin was shown to be expressed in ORNs and basal progenitors of the developing murine OE while its ligand RGMB was selectively expressed in iORNs.

To examine the function of neogenin in the development of the OE, we generated a novel *Neo1* null allele (*Neo1*$^{-/-}$) in which excision of the first exon of *Neo1* fully ablates neogenin expression. We examined the development of different cell populations in *Neo1*$^{-/-}$ mice at E16.5. Using immunolabeling, we identified two cell types whose numbers differed significantly from those of littermate controls. First, we observed a 20% increase in the number of basal progenitors, identified by their expression of the proliferative marker Ki-67. Second, we found a 25% increase in the number of Sox2^{+} SUS cells, confirmed by co-expression of the SUS cell marker cytokeratin 18. Interestingly, there was no difference in the number of iORNs as assessed by immunostaining for βIII-tubulin. This increase in progenitors and SUS cells without an increase in iORNs suggested that neogenin may be important for regulating OE progenitor proliferation and cell fate choice in the developing OE.

The selective expression of RGMB observed in iORNs, which lie adjacent to neogenin expressing basal progenitors, raised the possibility that RGMB-neogenin interactions between these two cell populations could be important for progenitor cell differentiation. To examine the effect of RGMB on OE progenitors, we performed an *in vitro* olfactory explant assay (Calof

& Chikaraishi 1989; Calof et al. 1991). Explants from E14-15 *Neo1*$^{+/+}$ embryos were grown under conditions in which the majority of cells migrating away from the explants were neuronal. Under these conditions, 72% of cells surrounding the explants were found to express neuronal marker βIII-tubulin. Interestingly, when explants were grown in media supplemented with recombinant RGMB protein, 84% were found to express βIII-tubulin. In contrast, explants from *Neo1*$^{-/-}$ embryos did not show increased production of βIII-tubulin positive cells. This could be due to a lack of response to RGMB or to an intrinsic poor health of the explants in absence of neogenin. Together, these results indicated that RGMB could promote neuronal differentiation of progenitor cells *in vitro*, possibly in a neogenin-dependent fashion.

While these previous *in vivo* and *in vitro* experiments support the hypothesis that RGMB-neogenin interactions regulate progenitor cell proliferation and differentiation, several questions remained to be answered:

1. How does ablation of neogenin influence progenitor cell division?
2. Does neogenin ablation lead to increased SUS cell proliferation or to a change in cell fate during progenitor differentiation?
3. Does blocking RGMB-neogenin interactions affect RGMB-dependent ORN differentiation *in vitro*?

Materials and Methods

Experimental Animals

We generated *Neo1* mutant mice by standard homologous recombination methods to introduce two loxP sites flanking the first exon of the *Neo1* allele, which encodes the signal sequence. Following germline transmission, heterozygous mice ($Neo1^{+/lox}$) were crossed to FlpE mice to excise the Neomycin cassette used for G-418 selection of targeted ES cell clones. The $Neo1^{+/lox}$ mice were crossed with CMV-Cre mice to obtain germline deletion of the floxed *neogenin* allele ($Neo1^{+/-}$). Those heterozygous mice were bred together to obtain *neogenin* mutant mice.

Mouse embryos were obtained from timed pregnancies in which the morning of vaginal plug was deemed to be E0.5. All animal procedures have been approved by the Montreal Neurological Institute and McGill University, in accordance with the guidelines of the Canadian Council of Animal Care.

Immunohistochemistry

E15.5 and E16.5 heads were fixed in 4% paraformaldehyde (PFA) dissolved in 1x phosphate buffered saline (PBS) for 45 minutes at 4°C. Following fixation, embryos were rinsed with PBS before being cryoprotected overnight in 30% sucrose at 4°C. Once heads sunk to the bottom of the sucrose solution, they were coated with optimal cutting temperature (OCT) compound and flash frozen in 2-methylbutane at -80°C for 10 seconds. Tissue was then stored at -80°C until sectioning.

Heads were cryosectioned at a thickness of 20µm and a temperature of -20°C. Sections were allowed to dry on slides for 30 minutes at room temperature before being used immediately or were stored at -20°C for later use. If slides were stored at -20°C, they were allowed to warm up to room temperature for 20 minutes before staining began. A hydrophobic pen was used to draw a barrier around sections on the slide before sections were rehydrated in PBS for 5 minutes. Sections were then blocked for 1.5 hours in blocking buffer (0.5% Triton X-100 and 10% fetal bovine serum in PBS). Sections were incubated with primary antibodies at their proper dilutions in blocking buffer overnight at 4°C. The following day, this primary antibody solution was removed and sections were washed 3 times for 5 minutes each with PBS before being incubated with fluorescent-labeled secondary antibodies for 1 hour at room temperature. Nuclear counterstaining was then performed with Hoechst (Molecular Probes) at 1:3000 in PBS for 5 minutes before sections were washed 3 times for 5 minutes each with PBS. Slides were mounted with Fluoromount-G (SouthernBiotech) and allowed to dry overnight before being stored at 4°C.

Primary antibodies used and their dilutions were as follows: rat anti-BrdU (Abcam, ab6326; 1:50), rabbit anti-SOX2 (Millipore, AB5603; 1:250), and mouse anti-Ki-67 (BD Pharmingen, clone B56; 1:150). Secondary antibodies used were Alexa 488- and Alexa 546-labeled from Invitrogen (1:500).

S-phase Duration & Cell Cycle Exit

S-phase duration was estimated using dual DNA synthesis labeling, as previously described (Huard and Schwob 1995; Alexiades and Cepko 1996). A solution of 5-ethynyl-2'-deoxyuridine (EdU) in PBS was injected intraperitoneally in E15.5 pregnant dams at a dose of 50

mg/kg. The same pregnant dams were then injected intraperitoneally 2 hours later with a solution of bromodeoxyuridine (BrdU) in PBS at a dose of 50mg/kg. One hour later, embryos were fixed as described above. EdU was detected on sections using a Click-iT EdU Imaging Kit (Invitrogen). To prepare sections for BrdU staining, they were subjected to hot plate antigen retrieval at 90°C with 0.01M Sodium Citrate pH 6 for 10 minutes followed by treatment with 2M HCl for 10 minutes at 4°C, 10 minutes at room temperature, and 10 minutes at 37°C. Sections were then incubated with anti-BrdU antibody and staining proceeded as described above. S-phase length was calculated as 2 hours x (EdU^+ cells / EdU^+ $BrdU^-$ cells).

Basal progenitor cell cycle exit was assessed by injecting E15.5 pregnant dams with EdU, as described above. Embryos were harvested and fixed 24 hours after injection and sections were subjected to EdU Click-It detection and Ki-67 immunohistochemistry. Cells undergoing S-phase shortly following the time of injection were identified as being positive for EdU. Cells that were still in the cell cycle were positive for Ki-67. Therefore, we were able to calculate the fraction of cells that had exited the cell cycle as the fraction of EdU positive cells that were Ki-67 negative (EdU^+ $Ki\text{-}67^-$ cells / total EdU^+ cells).

Primary OE Explant Culture

OE explants were prepared from CD-1 E14.5 and E15.5 embryos (Charles River) and cultured for 18 hours on glass coverslips with or without recombinant RGMB protein (R&D Systems; 1µg/ml). Explants treated with RGMB were cultured in media alone, media supplemented with neogenin antibody (R&D Systems AF1079; 5µg/ml), or media supplemented with control IgG antibody (5µg/ml).

OE explants were obtained from surgically dissected olfactory turbinates. Epithelial tissue was isolated following turbinate digestion in a Trypsin-Pancreatin solution for 50 minutes before being manually triturated into small fragments and plated onto glass coverslips. Glass coverslips (Propper) were acid washed with 0.2M HCl in boiling water for three hours, washed in boiling water 3 times for 5 minutes each, and then rinsed with 95% ethanol which was allowed to dry at 65°C overnight. Coverslips were coated with Poly-D-lysine (Sigma; 1mg/ml) overnight before being sterilized by UV for 1 hour. Finally, coverslips were coated with merosin (Gibco-BRL; 10µg/ml) for 3 hours at 37°C. Explants were cultured, as previously described (DeHamer et al. 1994).

Cells grown on coverslips were fixed in 4% PFA for 10 minutes at room temperature. PFA was quenched in 20mM glycine for 10 minutes and then coverslips were blocked in blocking buffer for 30 minutes at room temperature. Primary staining for Ki-67 (BD Pharmingen, clone B56; 1:200) and βIII- tubulin (Cedarlane, Clone Tuj1 1-15-79; 1:1000) was performed for 1 hour at 4°C. Coverslips were rinsed and stained with fluorescent-labeled secondary antibodies for 1 hour at room temperature. Finally, nuclear counterstaining was performed with Hoechst and coverslips were mounted on slides with Fluoromount-G.

Image Capture & Analysis

Images of fluorescently stained sections and cells were captured using a Carl Zeiss Axio Imager M1 microscope and a QImaging Retiga EXi digital camera. Cell counts were performed using Eclipse (Epix Imaging), ImageJ (NIH), and ICY (Institut Pasteur) software. Cells were counted along 2.5mm of the septal OE. Statistical significance was determined using unpaired two-tailed *t*-tests and one-way ANOVA analyses with Prism software (GraphPad).

Results

Neogenin regulates cell cycle progression in the OE

Since neogenin is highly expressed in the basal progenitors of the OE, and since we observed increased numbers of Ki-67-positive proliferating in the OE of *Neo1*$^{-/-}$ embryos (Kam et al. 2016) (Fig. 3A,C) we hypothesized that neogenin may regulate cell cycle progression of progenitor cells. Indeed, the loss of neogenin from OE progenitor cells may affect cell cycle progression, leading to an accumulation of proliferating progenitor cells. Interestingly, the number of Sox2-positive stem cells in the basal region of the OE was similar in *Neo1*$^{+/+}$ and *Neo1*$^{-/-}$ embryos, and so the generation of stem cells appears unaffected in the absence of neogenin (Fig. 3B,D).

To examine cell cycle progression in the OE, control and *Neo1*$^{-/-}$ embryos were harvested 24 hours after an injection of EdU in pregnant dams at E15.5. OE sections were stained by the Click-iT reaction and with Ki-67 antibodies (see materials and methods). Under these conditions, cells that exited the cell cycle after injection of EdU should be EdU-positive and Ki-67-negative, whereas cells that are still cycling should be positive for both markers. The proportion of cells that exited the cell cycle was reduced in *Neo1*$^{-/-}$ compared to control embryos (Fig. 3F). To assess whether altered cell cycle kinetics may be associated with the decreased cell cycle exit, we determined the length of the S-phase in progenitor cells in the OE of *Neo1*$^{-/-}$ embryos. Pregnant dams were injected with EdU, followed by an injection of BrdU two hours later, as a means of determining S-phase duration (see materials and methods) (Huard and Schwob 1995; Alexiades and Cepko 1996). In control embryos, the length of S-phase was estimated to be 9.03±0.5 hours, which is consistent with a previously reported number in

the adult rat (Huard and Schwob 1995). In contrast, S-phase duration was significantly increased in *Neo1*$^{-/-}$ embryos (12.49±0.5 hours), indicating that cell cycle kinetics are altered in these progenitors (Fig. 3E).

Overproduction of SUS cells by basal progenitors in the OE of *Neo1*$^{-/-}$ embryos

Although the number of dividing cells is increased in the OE, the same number of immature ORNs as in wild-type embryos are produced in *Neo1*$^{-/-}$ embryos (Kam et al. 2016), suggesting these dividing cells may eventually differentiate into another cell type. Since OE progenitor cells can give rise to both ORNs and SUS cells, we examined whether ablation of neogenin expression affects the generation of SUS cells. To assess this in *Neo1*$^{-/-}$ embryos, we counted the number of Sox2-positive cells in the apical region of the OE after confirming that these cells express the SUS cell marker Cytokeratin 18 (CYTK18), as previously described (Fig. 4B) (Gokoffski et al. 2011; Packard et al. 2011). We observed a significant increase in the number of Sox2-positive cells in *Neo1*$^{-/-}$ embryos (Kam et al. 2016) (Fig. 4A,C). Furthermore, the CYTK18 staining signal appeared more intense throughout the OE in *Neo1*$^{-/-}$ embryos, which may reflect an increased number of SUS cell basal processes in the OE of these mice (Fig. 4A).

Even though SUS cells are self-renewing, EdU labeling experiments revealed that the proliferation index of SUS cells is the same in control and *Neo1*$^{-/-}$ embryos, suggesting that neogenin does not control self-renewal of SUS cells (Fig. 4D). We therefore examined whether the supernumerary basal progenitor cells observed in *Neo1*$^{-/-}$ embryos may give rise to SUS cells using an EdU pulse-chase experiment (Fig. 4E). If supernumerary progenitor cells migrate to the apical region to give rise to SUS cells, we would expect to detect an increased number of EdU-labeled SUS cells 24 hours following an EdU pulse. As shown in Figure 4E, a similar number of

EdU labeled SUS cells are observed in the apical region of the OE from control and *Neo1*$^{-/-}$ embryos 2 hours following a pulse of EdU, a time point chosen to exclude EdU-labeled cells migrating from the basal region to the apical membrane. However, 24 hours following an EdU pulse, we observed a 20% increase in the number of EdU-positive SUS cells, indicating that basal progenitor cells have given rise to the supernumerary SUS cells. Taken together, our results indicate that neogenin plays a role in regulating SUS cell production either by inhibiting their generation from progenitor cells or by regulating cell cycle kinetics of these progenitor cells.

RGMB promotes neuronal differentiation in OE explants

Since RGMB and neogenin have complementary patterns of expression in the OE and that ablation of neogenin expression leads to decreased numbers of mature ORNs (Kam et al. 2016), we explored the possibility that RGMB could be the ligand that mediates this effect. To test this, we first examined the effect of RGMB on the differentiation of OE progenitors using an *in vitro* olfactory explant assay (Calof and Chikaraishi 1989; Calof et al. 1991). Explants were prepared from E14-15 embryos and plated on coverslips coated with Merosin. Under these conditions, neuronal cells comprised the majority of migrating cells observed around the explant (Calof and Chikaraishi 1989; DeHamer et al. 1994; Mumm et al. 1996). Indeed, 72% of cells surrounding the explants expressed the neuronal marker βIII-tubulin. In contrast, 84% of migrated cells expressed βIII-tubulin in explants grown in media supplemented with recombinant RGMB protein (Fig. 5A,B). Furthermore, the percentage of migrated cells that expressed the cell division marker Ki-67 is decreased around RGMB-treated explants, suggesting that more progenitor cells underwent differentiation upon RGMB treatment (Fig.

5C). These results indicate that RGMB can promote neuronal differentiation of progenitor cells. The effect of RGMB appeared to be mediated through neogenin, since addition of antibodies against the extracellular region of neogenin to the growth medium significantly attenuated this response (Fig. 5D). Furthermore, treatment of explants from *Neo1*$^{-/-}$ embryos with RGMB did not lead to an increase in the generation of neurons from these explants, suggesting that RGMB-neogenin interactions can promote neuronal differentiation in OE explants (Fig. 5B).

Discussion

Summary

The ability to generate a diverse array of cell types from a single cell is only possible through the extensive cell signaling that takes place during development. Such signaling is especially important for the development of the mammalian nervous system where, depending on the classification method used, there are speculated to be as many as 10^3-10^4 distinct neuronal and glial cell types (Nelson et al. 2006). While many of the signaling mechanisms influencing differentiation within the developing nervous system have been identified, they cannot yet account for the diversity observed. This suggests additional mechanisms remain to be discovered. Additionally, as iPSC treatments for neurodegenerative diseases become a reality, improving our understanding of the mechanisms underlying neuronal differentiation will be essential (Pen and Jensen 2017). In this thesis, I have presented results that support a new role for the transmembrane receptor neogenin in the regulation of cell diversity in the mouse nervous system.

Using the developing murine OE as a model, I identified a role for neogenin in regulating progenitor cell cycle dynamics and promoting a neuronal cell fate. Specifically, an expansion of the OE progenitor pool was observed in *Neo1*$^{-/-}$ embryos with progenitors exhibiting reduced cell cycle exit and increased S-phase duration. In addition, embryos lacking neogenin had supernumerary SUS cells, but no increase in the rate of SUS cell proliferation. Instead, an increase in SUS cell production by basal progenitors is observed, suggesting a shift towards gliogenesis in the absence of neogenin. I also demonstrated that the neogenin ligand, RGMB, can promote neogenin-dependent neuronal differentiation of OE progenitors in an *in vitro* OE

explant assay. My results, therefore, show for the first time using an *in vivo* loss of function approach that neogenin is required for the control of cell division in the mammalian nervous system.

Interpretation and Future Directions

In the OE, bipotent basal progenitor cells contribute to the generation of both ORN and SUS cell populations. The relative levels of expression of the transcription factors Ascl1 and Sox2 appear to underlie cell fate bias with cells that maintain Sox2 expression committing to a glial fate (Gokoffski et al. 2011; Krolewski et al. 2012). Several secreted factors have been identified so far as regulators of cell fate choice in the OE, including Activins, BMPs, GDF-11, and Follistatin (Shou et al. 2000; Wu et al. 2003; Gokoffski et al. 2011; Krolewski et al. 2012). In addition to these proteins, members of the Notch family of receptors and their Delta family transmembrane ligands are expressed in the OE and have been proposed to regulate the development and maintenance of OE cell populations (Cau et al. 2000; Carson et al. 2006; Schwarting et al. 2007). For example, ablation of Notch2 expression is required for the maintenance of SUS cells in adult OE (Rodriguez et al. 2008). My results indicate that neogenin expression in progenitor cells is important for the regulation of their cell division and differentiation. I propose that the activation of the neogenin receptor in progenitor cells regulates cell cycle kinetics and exit. In its absence, dysregulated cell cycle kinetics and a reduction in cell cycle exit lead to increased numbers of progenitor cells that preferentially give rise to SUS cells in the OE. Alternatively, RGMB-neogenin signaling may actively block the differentiation of progenitor cells into SUS cells by inhibiting Sox2 expression, thereby favoring neurogenesis.

Neogenin regulates cell cycle progression and cell fate choice in the OE

The decreased cell cycle exit and extended S-phase observed in progenitor cells of the OE in *Neo1*$^{-/-}$ embryos suggest that OE progenitor cells undergo more self-expanding symmetric divisions as opposed to terminal neurogenic or gliogenic divisions. Indeed, an increase in S-phase length, has been associated with maintained symmetric divisions of progenitors in the subventricular zone of the brain, which ensures minimum DNA replication errors (Arai et al. 2011; Ponti et al. 2013). Interestingly, reduced neogenin expression also leads to decreased cell cycle exit in neuroblasts of the subventricular zone in adult mice (O'Leary et al. 2015). However, it remains unclear whether neogenin is directly responsible for maintaining normal cell cycle dynamics or whether the lack of neogenin is having an indirect effect on OE progenitors.

Ablation of neogenin expression leads to defects in multiple populations of OE cells, including ORN apoptosis, increased numbers of dividing progenitors, and overproduction of SUS cells (Kam et al. 2016). The numerous negative and positive feedback mechanisms that regulate both proliferation and differentiation in the OE make it challenging to distinguish between the direct and indirect phenotypic effects of ablating neogenin expression on the different populations of cells. For example, the increased number of dividing cells in the OE of *Neo1*$^{-/-}$ embryos could be due to an overproliferation of progenitor cells caused by increased apoptosis of ORNs in these mice. Nonetheless, our analyses indicate that an increase in the length of the S-phase and a decrease in cell cycle exit underlie the presence of supernumerary dividing progenitor cells in the *Neo1*$^{-/-}$ embryos. Furthermore, ablation of RGMB led to increased numbers of dividing progenitor cells without having an effect on survival of ORNs (Kam et al. 2016), supporting a direct effect for neogenin signaling in regulating cell cycle progression and

exit.

The increased number of SUS cells observed in *Neo1*$^{-/-}$ embryos are most likely generated through the differentiation of excess progenitor cells in the basal OE. My observations that the proliferative index of SUS cells is unaffected in the OE of these embryos, combined with the EdU pulse-chase experiments, supports this possibility. However, additional experiments aimed at performing lineage tracing of specific OE progenitor cells would be needed to further confirm the origin of the supernumerary SUS cells observed in *Neo1*$^{-/-}$ embryos. This could be achieved by Cre-dependent expression of a reporter protein in progenitor cells using the previously described *Ascl1-CreER*^{TM} mice (Battiste et al. 2007).

The accumulation of dividing progenitor cells in the basal layer of the OE in *Neo1*$^{-/-}$ embryos likely results in the generation of supernumerary SUS cells observed in these embryos. The change in cell cycle kinetics observed in *Neo1*$^{-/-}$ embryos may affect cell fate choice in the progenitors, leading to the overproduction of SUS cells. Alternatively, neogenin signaling may influence progenitor cell fate by regulating the balance of expression of Ascl1 and Sox2 in these cells. Neogenin signaling may restrict expression of Sox2 thereby limiting the production of SUS cells. Ablation of neogenin would lead to an increase in the number of SUS cells generated, as observed in *Neo1*$^{-/-}$ embryos. Neogenin has previously been shown to undergo cleavage of its intracellular domain, which translocates to the nucleus to regulate gene expression (Goldschneider et al. 2008). The cleaved intracellular domain of neogenin may therefore regulate expression of genes that influence cell fate choice, such as Ascl1 or Sox2.

Another possible explanation for the increased number of SUS cells observed in *Neo1*$^{-/-}$

embryos is that this effect is indirectly mediated by the changes observed in the number of dividing progenitor cells. If the excess dividing progenitor cells produce a factor that favors the generation of SUS cells, this could explain the increased number of SUS cells observed in *Neo1*$^{-/-}$ and *Rgmb*$^{-/-}$ embryos.

RGMB-neogenin signaling in progenitor cell differentiation

My *in vitro* studies examining the effect of RGMB on OE progenitor cell differentiation indicate that RGMB can promote the differentiation of these cells into immature OSNs. Both antibody-blocking experiments and loss of function experiments support a role for neogenin in RGMB-induced neuronal differentiation of progenitor cells (Figure 5). However, *in vivo* ablation of either RGMB or neogenin did not affect the number of immature OSNs produced in the OE, suggesting that they are not necessary for the generation of these neurons. In contrast, ablation of either RGMB or neogenin resulted in an increase in the number of SUS cells produced in the OE (Kam et al. 2016). These results suggest that RGMB–neogenin signaling may actually inhibit the production of SUS cells in the OE. It is therefore possible that the increased number of immature OSNs generated from OE explants upon RGMB treatment *in vitro* results from inhibition of the SUS cell fate rather than from the promotion of neuronal fate. Additional *in vitro* experiments examining the ratio of neurons and SUS cells produced in OE explants will be necessary to define a potential inhibitory effect of RGMB on SUS cell production.

Concluding Remarks

Taken together, the experiments I present in this thesis demonstrate that the transmembrane receptor neogenin can regulate cell cycle dynamics and cell fate choice in the mammalian nervous system. Future studies should be aimed at defining the intracellular

pathways and targets that lie downstream of neogenin in cell cycle kinetics and cell fate choice. As neogenin is widely expressed both inside and outside of the nervous system during development, an improved understanding of neogenin's function will greatly increase our knowledge of the cellular processes underlying embryonic development. Additionally, these insights about the mechanisms underlying development will be crucial for developing future treatments for neurodegenerative diseases.

Figures

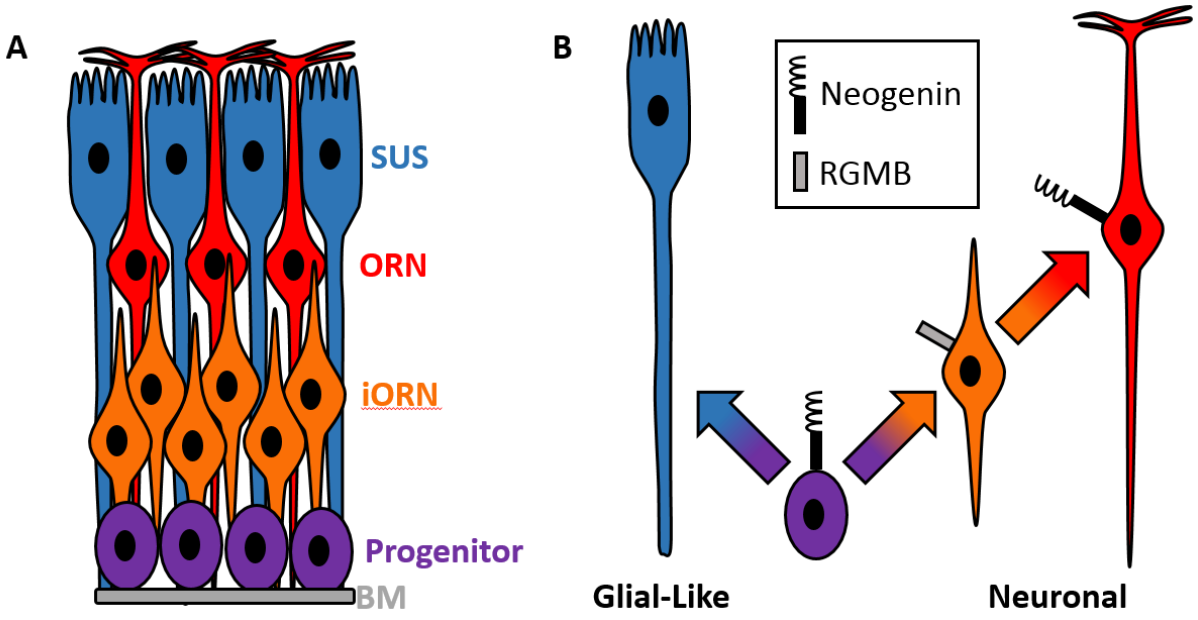

Figure 1: Structure, cell types, and cell lineages of the developing murine olfactory epithelium.

(A) Progenitors and stem cells (purple) are located in the basal region of the olfactory epithelium (OE), adjacent to the basement membrane (BM). Immature olfactory receptor neurons (iORN, orange) reside in the intermediate region. Mature olfactory receptor neurons' (ORNs, red) cell bodies reside in the apical region of the OE; they extend cilia covered dendrites apically into the nasal cavity and axons basally. Sustentacular cells (SUS, blue) reside in the apical region of the OE. (B) Bipotent OE progenitors give rise to both glial-like SUS cells and olfactory receptor neurons. Neogenin is expressed by both OE progenitors and mature ORNs, while its ligand RGMB is expressed by iORNs.

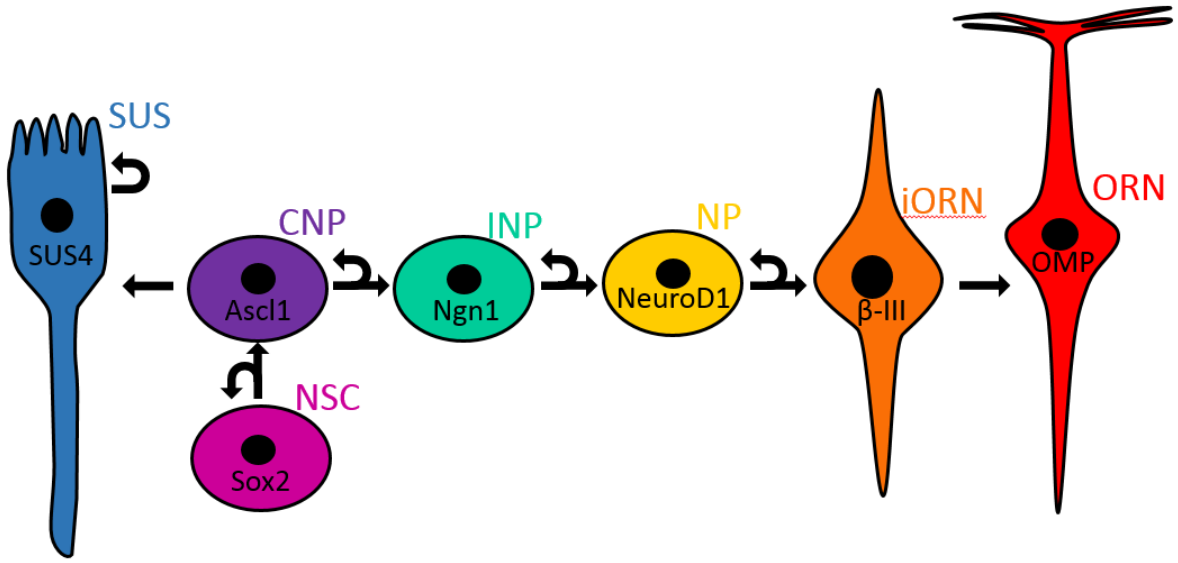

Figure 2: Detailed cell lineages of the OE and associated proteins.

Neural stem cells (NSC, pink) expressing Sox2 give rise to committed neural progenitors (CNP, purple) expressing Ascl1. CNPs can differentiate into either glial-like sustentacular cells (SUS, blue) expressing SUS4 or intermediate neural precursors (INP, teal) expressing Ngn1. INPs give rise to neural precursors (NP, yellow) that express NeuroD1. NPs differentiate into immature olfactory receptor neurons (iORN, orange) expressing β-III tubulin. Finally, iORNs mature into full-fledged olfactory receptor neurons (ORN, red) expressing OMP.

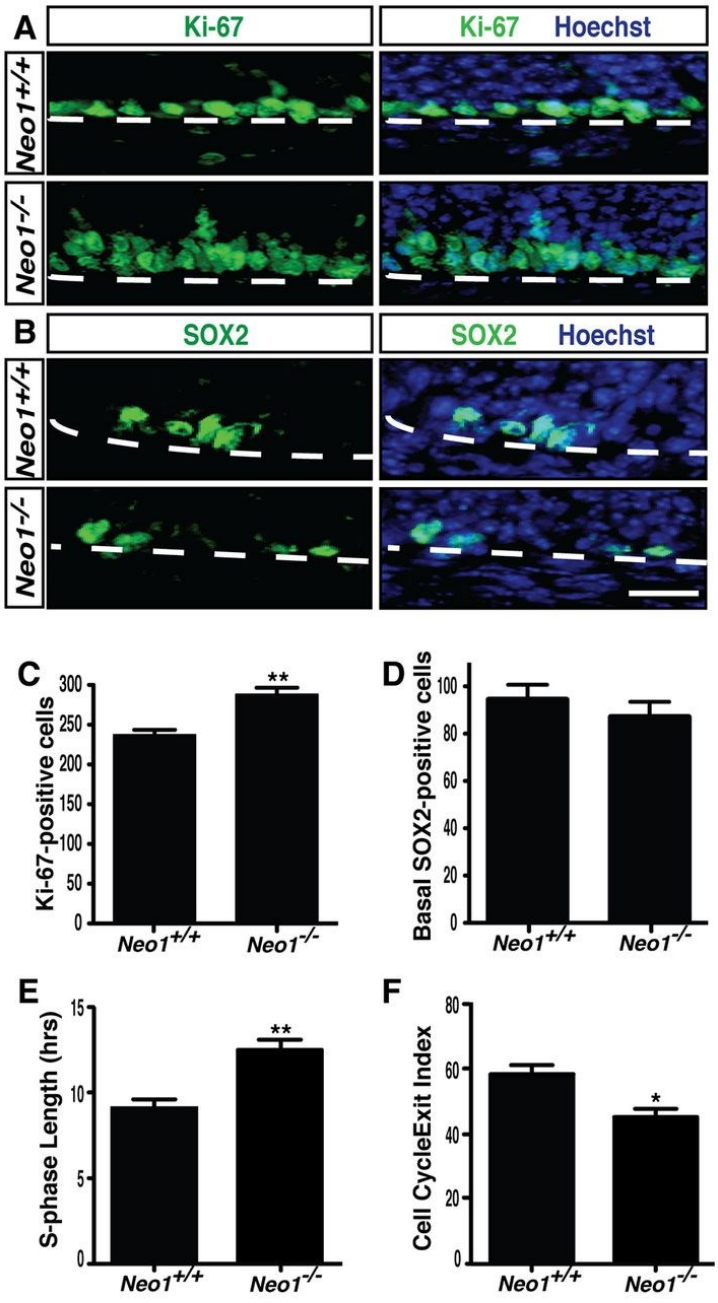

Figure 3: Ablation of neogenin leads to altered cell cycle kinetics and cell cycle exit.

(A-D) Immunolabeling and quantification of Ki-67-positive (A,C) (*Neo1*$^{+/+}$, *n*=5; *Neo1*$^{-/-}$, *n*=8) or SOX2-positive (B,D) (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4) cells in OE sections from E16.5 *Neo1*$^{+/+}$ and *Neo1*$^{-/-}$ embryos. (E) Increased S-phase length in progenitor cells in OE from *Neo1*$^{-/-}$ embryos

(*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). (F) Reduced cell cycle exit index (the percentage of dividing cells exiting the cell cycle over a 3 h period) (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). Counts in C and D represent cell number per 2.5 mm of OE. Values are mean±s.e.m. Student's unpaired *t*-test, **P*<0.05, ***P*<0.01. Scale bar: 100 µm.

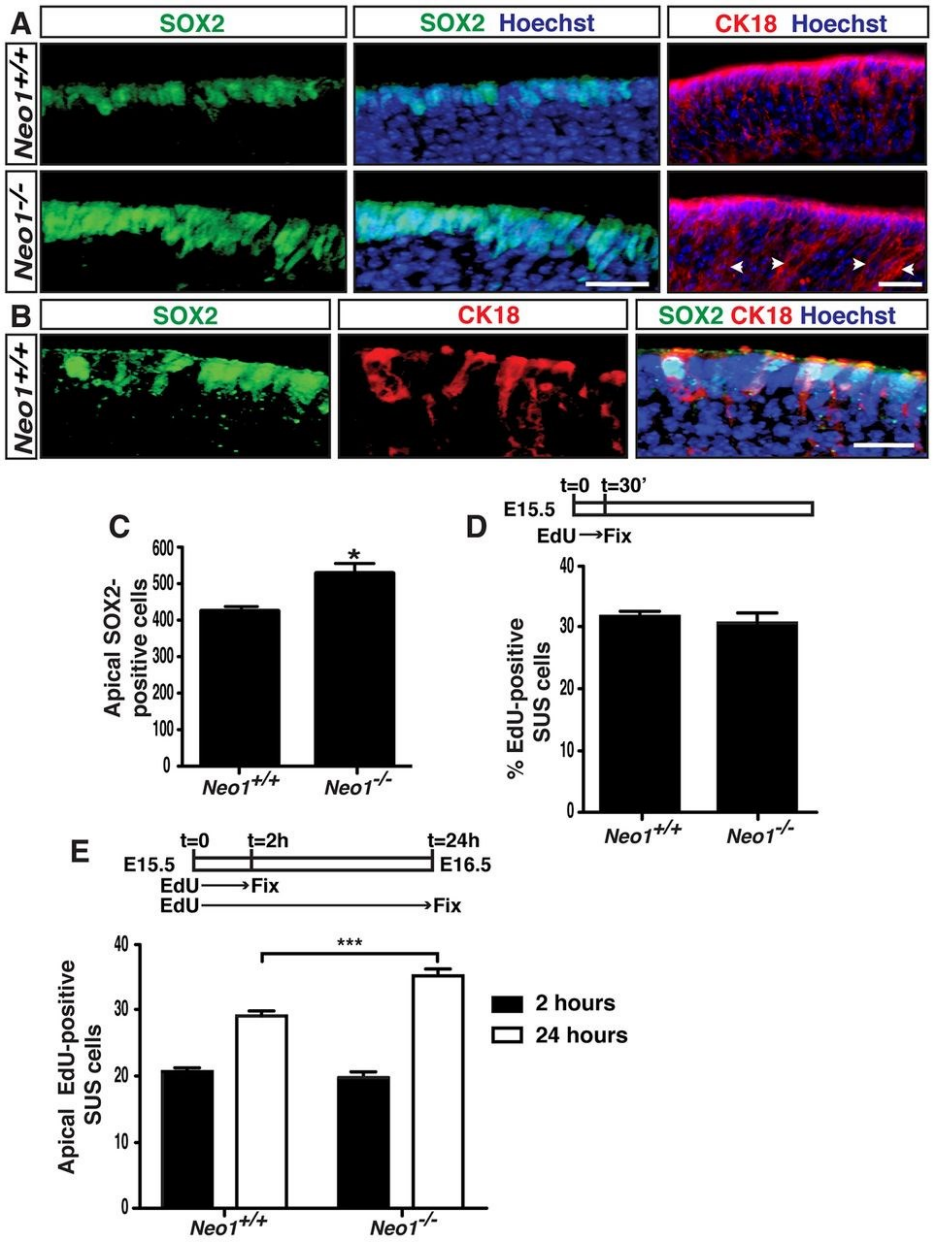

Figure 4: Neogenin ablation leads to overproduction of SUS cells.

(A,C) SOX2-positive and CK18-positive cells in the apical region of the OE in control and *Neo1*$^{-/-}$ embryos. An increased number of SOX2-positive cells (per 2.5 mm of OE) is observed in the apical region of the OE in *Neo1*$^{-/-}$ embryos (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). The CK18 signal is more intense in *Neo1*$^{-/-}$ embryos, with increased labeling of SUS cell basal

processes (arrowheads). (B) Immunolabeling of OE sections with SOX2 and CK18 antibodies shows that most SOX2-expressing cells in the apical region of the OE co-express the SUS cell-specific marker CK18. (D) EdU was administered to pregnant dams and the percentage of SOX2-positive SUS cells that are EdU^+ (proliferation index) was quantified at the time indicated (30 min) (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). (E) EdU was administered to pregnant dams and double $EdU^+/SOX2^+$ cells in the apical compartment were quantified at the times indicated (2 and 24 h) and plotted as cells per 500 µm of OE (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4 for each time point). All values are mean±s.e.m. Student's unpaired *t*-test (C,D) or one-way ANOVA (E) was performed to compare values between groups, **P*<0.05, ****P*<0.001. Scale bars: 100 µm.

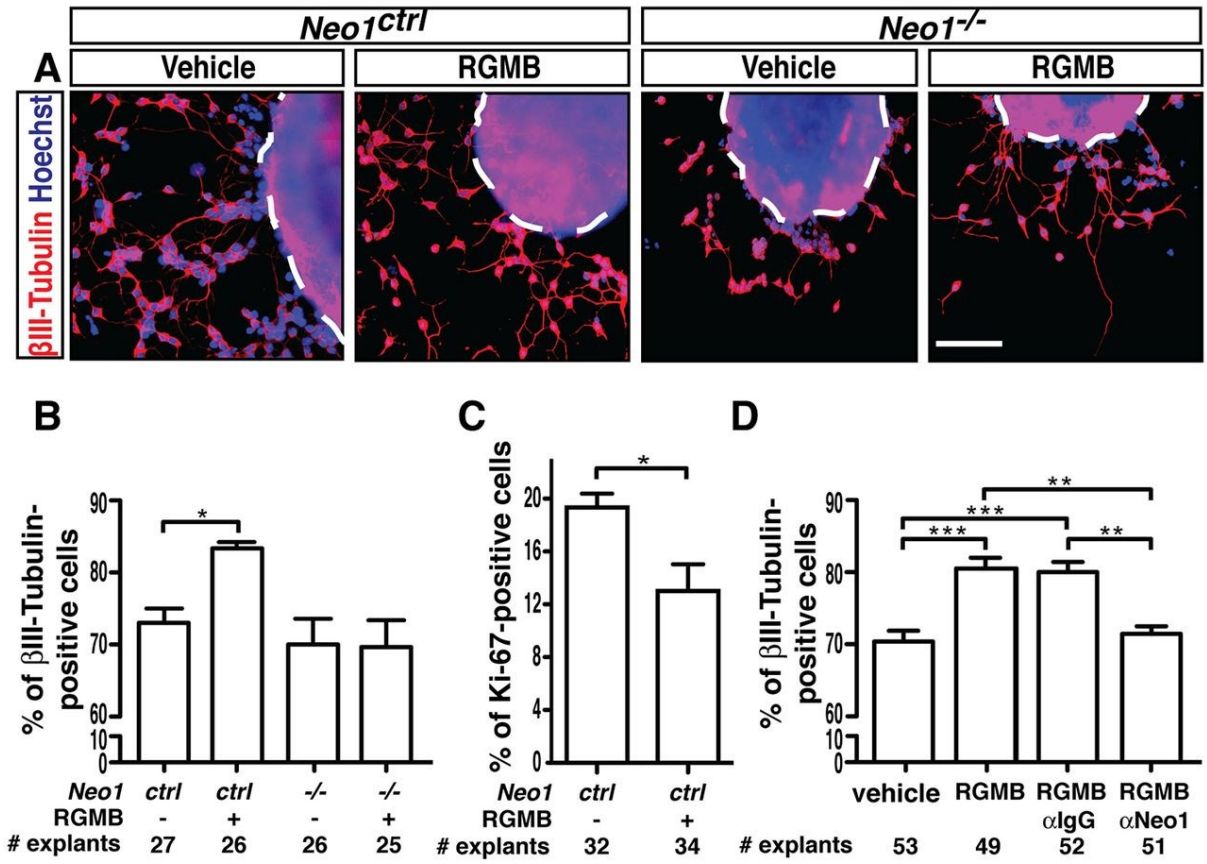

Figure 5: RGMB regulates OE cell differentiation *in vitro*.

(A,B) OE explants were isolated from control (*Neo1*$^{+/+}$ or *Neo1*$^{+/-}$) and *Neo1*$^{-/-}$ E14-E15 embryos and were treated with either vehicle or 1 µg/ml recombinant RGMB. Explants were stained with Hoechst and βIII-tubulin antibodies after 18 h in culture (A), and the percentage of migrated cells around the explants that were βIII-tubulin positive (B) was assessed (*n*=3 experiments). The dashed line (A) delineates the olfactory explants. (C) Percentage of cells that are Ki-67 positive around explants from control mice treated with vehicle or RGMB (*n*=4 experiments). (D) Percentage of migrated cells around OE explants isolated from wild-type embryos treated with RGMB and either a control or neogenin-specific antibody (*n*=4 experiments). Values are mean±s.e.m. One-way ANOVA, **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bar: 100 µm.

References

Alexiades MR, Cepko C. 1996. Quantitative analysis of proliferation and cell cycle length during development of the rat retina. Dev. Dyn. 205:293–307.

Arai Y, Pulvers JN, Haffner C, Schilling B, Nüsslein I, Calegari F, Huttner WB. 2011. Neural stem and progenitor cells shorten S-phase on commitment to neuron production. Nat. Commun. 2:154.

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 17:126–40.

Battiste J, Helms AW, Kim EJ, Savage TK, Lagace DC, Mandyam CD, Eisch AJ, Miyoshi G, Johnson JE. 2007. Ascl1 defines sequentially generated lineage-restricted neuronal and oligodendrocyte precursor cells in the spinal cord. Development 134:285–93.

Bell CH, Healey E, van Erp S, Bishop B, Tang C, Gilbert RJC, Aricescu a R, Pasterkamp RJ, Siebold C. 2013. Structure of the Repulsive Guidance Molecule (RGM)-Neogenin Signaling Hub. Science (80-.). 341:77–80.

Boutin C, Hardt O, de Chevigny A, Coré N, Goebbels S, Seidenfaden R, Bosio A, Cremer H. 2010. NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. Proc. Natl. Acad. Sci. U. S. A. 107:1201–6.

Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. 2011. Bone Morphogenetic Proteins: A critical review. Cell. Signal. 23:609–620.

Calof AL, Chikaraishi DM. 1989. Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. Neuron 3:115–27.

Calof AL, Lander AD, Chikaraishi DM. 1991. Regulation of neurogenesis and neuronal differentiation in primary and immortalized cells from mouse olfactory epithelium. Ciba Found. Symp. 160:249-65-76.

Carson C, Murdoch B, Roskams AJ. 2006. Notch 2 and Notch 1/3 segregate to neuronal and glial lineages of the developing olfactory epithelium. Dev. Dyn. 235:1678–1688.

Cau E, Casarosa S, Guillemot F. 2002. Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871–1880.

Cau E, Gradwohl G, Casarosa S, Kageyama R, Guillemot F. 2000. Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. Development 127:2323–2332.

Cau E, Gradwohl G, Fode C, Guillemot F. 1997. Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124:1611–21.

Collinson JM, Quinn JC, Hill RE, West JD. 2003. The roles of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. Dev. Biol. 255:303–312.

Conrad S, Stimpfle F, Montazeri S, Oldekamp J, Seid K, Alvarez-Bolado G, Skutella T. 2010. RGMB controls aggregation and migration of Neogenin-positive cells in vitro and in vivo. Mol. Cell. Neurosci. 43:222–31.

Cuschieri A, Bannister LH. 1975. The development of the olfactory mucosa in the mouse: light microscopy. J. Anat. 119:277–286.

DeHamer MK, Guevara JL, Hannon K, Olwin BB, Calof a L. 1994. Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors. Neuron 13:1083–97.

Donner AL, Episkopou V, Maas RL. 2007. Sox2 and Pou2f1 interact to control lens and olfactory placode development. Dev. Biol. 303:784–799.

Feijen A, Goumans MJ, van den Eijnden-van Raaij a J. 1994. Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. Development 120:3621–37.

Fitzgerald DP, Seaman C, Cooper HM. 2006. Localization of Neogenin protein during morphogenesis in the mouse embryo. Dev. Dyn. 235:1720–5.

Ford-Perriss M, Abud H, Murphy M. 2001. Fibroblast growth factors in the developing central nervous system. Clin. Exp. Pharmacol. Physiol. 28:493–503.

Gad JM, Keeling SL, Wilks AF, Tan S, Cooper HM. 1997. The Expression Patterns of Guidance Receptors, DCC and Neogenin, Are Spatially and Temporally Distinct throughout Mouse Embryogenesis. Dev. Biol. 192:258–273.

Gokoffski KK, Wu H-H, Beites CL, Kim J, Kim EJ, Matzuk MM, Johnson JE, Lander AD, Calof AL. 2011. Activin and GDF11 collaborate in feedback control of neuroepithelial stem cell proliferation and fate. Development 138:4131–4142.

Goldschneider D, Rama N, Guix C, Mehlen P. 2008. The neogenin intracellular domain regulates gene transcription via nuclear translocation. Mol. Cell. Biol. 28:4068–79.

Gordon MK, Mumm JS, Davis RA, Holcomb JD, Calof AL. 1995. Dynamics of MASH1 Expression in Vitro and in Vivo Suggest a Non-Stem Cell Site of MASH1 Action in the Olfactory Receptor Neuron Lineage. Mol. Cell. Neurosci. 6:363–379.

Graziadei PP, Graziadei GA. 1979. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J. Neurocytol. 8:1–18.

Grindley JC, Davidson DR, Hill RE. 1995. The role of Pax-6 in eye and nasal development. Development 121:1433–42.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL. 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463–476.

Guo Z, Packard A, Krolewski RC, Harris MT, Manglapus GL, Schwob JE. 2010. Expression of Pax6 and Sox2 in adult olfactory epithelium. J. Comp. Neurol. 518:4395–4418.

Hagihara M, Endo M, Hata K, Higuchi C, Takaoka K, Yoshikawa H, Yamashita T. 2011. Neogenin, a Receptor for Bone Morphogenetic Proteins. J. Biol. Chem. 286:5157–5165.

Hata K, Fujitani M, Yasuda Y, Doya H, Saito T, Yamagishi S, Mueller BK, Yamashita T. 2006. RGMa inhibition promotes axonal growth and recovery after spinal cord injury. J. Cell Biol. 173:47–58.

Healey EG, Bishop B, Elegheert J, Bell CH, Padilla-Parra S, Siebold C. 2015. Repulsive guidance molecule is a structural bridge between neogenin and bone morphogenetic protein. Nat. Struct. Mol. Biol. 22:458–465.

Hogan BL. 1996. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10:1580–94.

Huard JMT, Schwob JE. 1995. Cell cycle of globose basal cells in rat olfactory epithelium. Dev. Dyn. 203:17–26.

Jung YW, Hysolli E, Kim K-Y, Tanaka Y, Park I-H. 2012. Human induced pluripotent stem cells and neurodegenerative disease: prospects for novel therapies. Curr. Opin. Neurol. 25:125–30.

Kam JWK, Dumontier E, Baim C, Brignall AC, Mendes da Silva D, Cowan M, Kennedy TE, Cloutier J-F. 2016. RGMB and neogenin control cell differentiation in the developing olfactory epithelium. Development 143:1534–1546.

Kam JWK, Raja R, Cloutier J-F. 2014. Cellular and molecular mechanisms regulating embryonic neurogenesis in the rodent olfactory epithelium. Int. J. Dev. Neurosci. 37:76–86.

Kamachi Y, Uchikawa M, Kondoh H. 2000. Pairing SOX off: With partners in the regulation of embryonic development. Trends Genet. 16:182–187.

Kawauchi S, Beites CL, Crocker CE, Wu H-H, Bonnin A, Murray R, Calof AL. 2004. Molecular Signals Regulating Proliferation of Stem and Progenitor Cells in Mouse Olfactory Epithelium. Dev. Neurosci. 26:166–180.

Kawauchi S, Kim J, Santos R, Wu H-H, Lander AD, Calof AL. 2009. Foxg1 promotes olfactory neurogenesis by antagonizing Gdf11. Development 136:1453–1464.

Kawauchi S, Shou J, Santos R, Hébert JM, McConnell SK, Mason I, Calof AL. 2005. Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse. Development 132:5211–5223.

Key B, Treloar HB, Wangerek L, Ford MD, Nurcombe V. 1996. Expression and localization of FGF-1 in the developing rat olfactory system. J. Comp. Neurol. 366:197–206.

Krolewski RC, Packard A, Jang W, Wildner H, Schwob JE. 2012. Ascl1 (Mash1) Knockout Perturbs Differentiation of Nonneuronal Cells in Olfactory Epithelium. PLoS One 7.

Lander AD, Gokoffski KK, Wan FYM, Nie Q, Calof AL. 2009. Cell lineages and the logic of proliferative control. PLoS Biol. 7:e15.

Lathia JD, Mattson MP, Cheng A. 2008. Notch: from neural development to neurological disorders. J. Neurochem. 107:1471–81.

Leung CT, Coulombe P a, Reed RR. 2007. Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat. Neurosci. 10:720–726.

Liu A, Niswander LA. 2005. Bone morphogenetic protein signalling and vertebrate nervous system development. Nat. Rev. Neurosci. 6:945–54.

López-Mascaraque L, De Carlos J a, Valverde F. 1996. Early onset of the rat olfactory bulb projections. Neuroscience 70:255–266.

MacDonald KPA, Murrell WG, Bartlett PF, Bushell GR, Mackay-Sim A. 1996. FGF2 promotes neuronal differentiation in explant cultures of adult and embryonic mouse olfactory epithelium. J. Neurosci. Res. 44:27–39.

Manglapus GL, Youngentob SL, Schwob JE. 2004. Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. J. Comp. Neurol. 479:216–33.

Mansouri A, Stoykova A, Torres M, Gruss P. 1996. Dysgenesis of cephalic neural crest derivatives in Pax7-/- mutant mice. Development 122:831–8.

Massagué J, Blain SW, Lo RS. 2000. TGFβ Signaling in Growth Control, Cancer, and Heritable Disorders. Cell 103:295–309.

Matsunaga E, Nakamura H, Chédotal A. 2006. Repulsive Guidance Molecule Plays Multiple Roles in Neuronal Differentiation and Axon Guidance. J. Neurosci. 26:6082–6088.

Matsunaga E, Tauszig-Delamasure S, Monnier PP, Mueller BK, Strittmatter SM, Mehlen P, Chédotal A. 2004. RGM and its receptor neogenin regulate neuronal survival. Nat. Cell Biol. 6:749–755.

Meyerhardt JA, Look AT, Bigner SH, Fearon ER. 1997. Identification and characterization of neogenin, a DCC-related gene. Oncogene 14:1129–1136.

Mumm JS, Shou J, Calof AL. 1996. Colony-forming progenitors from mouse olfactory epithelium: evidence for feedback regulation of neuron production. Proc. Natl. Acad. Sci. U. S. A. 93:11167–72.

Murray RC, Calof AL. 1999. Neuronal regeneration: Lessons from the olfactory system. Semin. Cell Dev. Biol. 10:421–431.

Murray RC, Navi D, Fesenko J, Lander AD, Calof AL. 2003. Widespread defects in the primary olfactory pathway caused by loss of Mash1 function. J. Neurosci. 23:1769–80.

Nakashima M, Toyono T, Akamine A, Joyner A. 1999. Expression of growth/differentiation factor 11, a new member of the BMP/TGFbeta superfamily during mouse embryogenesis. Mech. Dev. 80:185–9.

Nelson SB, Sugino K, Hempel CM. 2006. The problem of neuronal cell types: a physiological genomics approach. Trends Neurosci. 29:339–45.

Nomura T, Takahashi S, Ushiki T. 2004. Cytoarchitecture of the normal rat olfactory epithelium: light and scanning electron microscopic studies. Arch. Histol. Cytol. 67:159–70.

O'Leary C, Cole SJ, Langford M, Hewage J, White A, Cooper HM. 2013. RGMa regulates cortical interneuron migration and differentiation. PLoS One 8:e81711.

O'Leary CJ, Bradford D, Chen M, White A, Blackmore DG, Cooper HM. 2015. The Netrin/RGM Receptor, Neogenin, Controls Adult Neurogenesis by Promoting Neuroblast Migration and Cell Cycle Exit. Stem Cells 33:503–514.

Ornitz DM, Itoh N. 2015. The Fibroblast Growth Factor signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 4:215–66.

Packard A, Giel-Moloney M, Leiter A, Schwob JE. 2011. Progenitor cell capacity of NeuroD1-expressing globose basal cells in the mouse olfactory epithelium. J. Comp. Neurol. 519:3580–96.

Pen AE, Jensen UB. 2017. Current status of treating neurodegenerative disease with induced pluripotent stem cells. Acta Neurol. Scand. 135:57–72.

Perrimon N, Pitsouli C, Shilo B-Z. 2012. Signaling Mechanisms Controlling Cell Fate and Embryonic Patterning. Cold Spring Harb. Perspect. Biol. 4:a005975–a005975.

Ponti G, Obernier K, Guinto C, Jose L, Bonfanti L, Alvarez-Buylla A. 2013. Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. Proc. Natl. Acad. Sci. U. S. A. 110:E1045-54.

Rajagopalan S, Deitinghoff L, Davis D, Conrad S, Skutella T, Chedotal A, Mueller BK, Strittmatter SM. 2004. Neogenin mediates the action of repulsive guidance molecule. Nat. Cell Biol. 6:756–762.

Rodriguez S, Sickles HM, DeLeonardis C, Alcaraz A, Gridley T, Lin DM. 2008. Notch2 is required for maintaining sustentacular cell function in the adult mouse main olfactory epithelium. Dev. Biol. 314:40–58.

Sammeta N, Hardin DL, McClintock TS. 2010. Uncx regulates proliferation of neural progenitor cells and neuronal survival in the olfactory epithelium. Mol. Cell. Neurosci. 45:398–407.

Sarkar A, Hochedlinger K. 2013. The Sox Family of Transcription Factors: Versatile Regulators of Stem and Progenitor Cell Fate. Cell Stem Cell 12:15–30.

Schlosser G. 2006. Induction and specification of cranial placodes. Dev. Biol. 294:303–351.

Schmidtmer J, Engelkamp D. 2004. Isolation and expression pattern of three mouse homologues of chick Rgm. Gene Expr. Patterns 4:105–110.

Schwarting GA, Gridley T, Henion TR. 2007. Notch1 expression and ligand interactions in progenitor cells of the mouse olfactory epithelium. J. Mol. Histol. 38:543–553.

Schwob JE, Huard JM, Luskin MB, Youngentob SL. 1994. Retroviral lineage studies of the rat olfactory epithelium. Chem. Senses 19:671–82.

Severyn CJ, Shinde U, Rotwein P. 2009. Molecular biology, genetics and biochemistry of the repulsive guidance molecule family. Biochem. J. 422:393–403.

Shou J, Murray RC, Rim PC, Calof AL. 2000. Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. Development 127:5403–13.

Smart IH. 1971. Location and orientation of mitotic figures in the developing mouse olfactory epithelium. J. Anat. 109:243–51.

Spencer SJ, Rabinovici J, Mesiano S, Goldsmith PC, Jaffe RB. 1992. Activin and inhibin in the human adrenal gland. Regulation and differential effects in fetal and adult cells. J. Clin. Invest. 90:142–9.

Srinivasan K, Strickland P, Valdes A, Shin GC, Hinck L. 2003. Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis. Dev. Cell 4:371–382.

Uwanogho D, Rex M, Cartwright EJ, Pearl G, Healy C, Scotting PJ, Sharpe PT. 1995. Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development. Mech. Dev. 49:23–36.

Vielmetter J, Kayyem JF, Roman JM, Dreyer WJ. 1994. Neogenin, an avian cell surface protein expressed during terminal neuronal differentiation, is closely related to the human tumor suppressor molecule deleted in colorectal cancer. J. Cell Biol. 127:2009–20.

De Vries M, Cooper HM. 2008. Emerging roles for neogenin and its ligands in CNS development. J. Neurochem. 106:1483–1492.

Wegner M, Stolt CC. 2005. From stem cells to neurons and glia: A Soxist's view of neural development. Trends Neurosci. 28:583–588.

Wijayarathna R, de Kretser DM. 2016. Activins in reproductive biology and beyond. Hum. Reprod. Update 22:342–357.

Wilson NH, Key B. 2007. Neogenin: One receptor, many functions. Int. J. Biochem. Cell Biol. 39:874–878.

Wood HB, Episkopou V. 1999. Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech. Dev. 86:197–201.

Wu HH, Ivkovic S, Murray RC, Jaramillo S, Lyons KM, Johnson JE, Calof AL. 2003. Autoregulation of neurogenesis by GDF11. Neuron 37:197–207.

Xu K, Wu Z, Renier N, Antipenko A, Tzvetkova-Robev D, Xu Y, Minchenko M, Nardi-Dei V, Rajashankar KR, Himanen J, et al. 2014. Structures of netrin-1 bound to two receptors provide insight into its axon guidance mechanism. Science (80-.). 344:1275–1279.

Zhang J, Shemezis JR, McQuinn ER, Wang J, Sverdlov M, Chenn A. 2013. AKT activation by N-cadherin regulates beta-catenin signaling and neuronal differentiation during cortical development. Neural Dev. 8:7.

Zhou Z, Xie J, Lee D, Liu Y, Jung J, Zhou L, Xiong S, Mei L, Xiong W-C. 2010. Neogenin Regulation of BMP-Induced Canonical Smad Signaling and Endochondral Bone Formation. Dev. Cell 19:90–102.