Role of the IgG Superfamily Receptor Neogenin in the Development of the Mammalian Olfactory System

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Papel da Neogenina, Receptor da Superfamília das Imunogolbulinas, no Desenvolvimento do Sistema Olfactivo dos Mamíferos

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"There is nothing in a caterpillar that tells you it's going to be a butterfly."

- R. Buckminster Fuller

À Andréane et Gabriel.

Aos meus pais.

À minha irmã.

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ActRIIA - Activin receptor type II A

AOB – accessory olfactory bulb

BCIP – 5-Bromo-4-chloro-3-indolyl phosphate

bHLH – basic helix-loop-helix

β3GnTI - β1,3-N-acetylglucosaminyltransferase I

BMP – bone morphogenic protein

BMPR – bone morphogenic protein receptor

BrdU – bromodeoxyuridine

cAMP – cyclic adenosine monophoshpate

Cdc42 – cell division cycle 42

cDNA – complementary deoxyribonucleic acid

CNS – central nervous system

Cre – Cre recombinase

CREB – cAMP responsive element binding protein

cRNA – complementary ribonucleic acid

D-CDK – Cyclin-dependent kinase for Cyclin D

DCC – deleted in colorectal cancer

DEPC – diethyl pyrocarbamate

Dll – Delta-like

E-CDK – Cyclin-dependent kinase for Cyclin E

Ebf – early B-cell factor

EGF – epidermal growth factor

EGTA – ethylene glycol tetraacetic acid

ERK – extracellular-signal-regulated kinase

FAK – focal adhesion kinase

FBS – fetal bovine serum

FGF – fibroblast growth factor

Foxg1 – Forkhead box g1

Gap43 – growth-associated protein 43

GBC – globose basal cell

Gdf – growth-differentiation factor

GFP – green fluorescent protein

Gt – gene-trap

GTP – guanosine triphosphate

HBC – horizontal basal cell

HEK – human embryonic kidney

Hes – Hairy and enhancer of Split

HFE – High Fe

IgG – immunoglobulin G

INP – immediate neuronal precursor

IRES – internal ribosome entry site

ISH – in situ hybridization

LARG – leukemia-associated guanine nucleotide exchange factor

Lhx – LIM homeobox

Mash1 – mammalian Achaete-Scute homolog 1

NBT – nitro blue tetrazolium chloride

NCAM – neural cell adhesion molecule

NeuroD – neurogenic differentiation

NFAT – Nuclear factor of activated T-cells

Ngn1 – Neurogenin 1

Npn – Neuropilin

O/E – Olf/Ebf

OAZ – O/E associated zing finger protein

OB (MOB) – (main) olfactory bulb

OE (MOE) – (main) olfactory epithelium

Olf – olfactory neuron-specific transcription factor

OMP – olfactory marker protein

OP – olfactory placode

OR – olfactory receptor

OSN – olfactory sensory neuron

P21(Cip1) – CDK-interacting protein 1

P27(Kip1) – Cyclin-dependent kinase inhibitor 1B

PBS – phosphate-buffered saline

PFA - paraformaldehyde

Phd – HIF prolyl-hydroxylase

PIP – phosphatidylinositol-phosphate

PITP – phosphatidylinositol transfer protein

PKA – protein kinase A

PKC – protein kinase C

RGC – retinal ganglion cell

RGD – arginine-glycine-asparagine

RGM – repulsive guidance molecule

RhoA – Ras homolog family, member A

RNA – ribonucleic acid

Roaz – rat O/E associated zing finger protein

Robo – Roundabout

ROCK – Rho Kinase

RT-PCR – reverse-transcriptase polymerase chain reaction

Runx – Runt-related transcription factor

Sema – Semaphorin

Ser – Serrate

SHIP – Src homology-2 containing inositol 5-phosphatase

Six – SIX homeobox

Slit – Slit homolog

SMAD – Sma- and Mad-related proteins

So – Sine oculis

Sox – sex determining region Y box

TBS – Tris-borate saline

TGFß – transforming growth factor beta

tRNA – transfer RNA

TUNEL – terminal deoxynucleotidyl transferase dUTP nick end-labeling

Unc5 – Unc-5 homolog

VN – vomeronasal

VNO – vomeronasal organ

VR – vomeronasal receptor

vWF – von Willebrandt factor

WT – wild-type

X-Gal – bromo-chloro-indolyl-galactopyranoside

Zfp423 – zinc finger protein 423

Resumo

A investigação recente sobre a Neogenina, um receptor da superfamília das imunoglobulinas, tem implicado esta proteína na regulação de processos ligados ao desenvolvimento em contextos tão diversos como a formação das glândulas mamárias e o direccionamento axonal em vertebrados. Homóloga do receptor de Netrinas DCC (Deleted in Colorectal Cancer), a Neogenina tem afinidade para as Netrinas assim como para uma segunda família de ligandos, as RGM (Repulsive Guidance Molecules). Descrita inicialmente no contexto do direccionamento axonal no tracto visual da galinha, a Neogenina provou, neste sistema, ser um receptor para sinais repulsivos levando os axónios que exprimem Neogenina a ser repelidos quando em contacto com o ligando RGM. Foi demonstrado que a Neogenina também promove atracção axonal na presença de Netrina. Outras actividades da Neogenina têm que ver com morfogénese e proliferação e diferenciação celular. São de especial relevância para este trabalho os papéis deste receptor na regulação da proliferação e diferenciação neuronal assim como da sobrevivência neuronal em diferentes modelos vertebrados.

Neste trabalho determinámos que a *neogenina* e o seu ligando *rgmB* são ambos exprimidos pelo epitélio olfactivo embrionário assim como pós-natal, em padrões complementares que sugerem interacções entre estas moléculas em diferentes estádios do desenvolvimento dos neurónios sensoriais olfactivos (NSO). A *neogenina* tem a sua expressão intensificada em duas fases temporalmente distintas do desenvolvimento dos NSO, com níveis de expressão elevados nos progenitores dos OSN e nos OSN diferenciados maturos, apresentando baixos níveis de expressão nos OSN imaturos. Tendo em consideração as actividades descritas para a Neogenina no desenvolvimento neuronal, este padrão de expressão sugere que esta proteína pode exercer funções diferentes em diferentes fases do desenvolvimento de um OSN. Nomeadamente, a expressão em células progenitoras sugere actividades relacionadas com proliferação/diferenciação, enquanto que a expressão nos NSO maturos sugere actividades relacionadas com sobrevivência/direccionamento axonal nestes neurónios.

Adicionalmente, determinámos que a expressão de *rgmB* também é modulada ao longo do desenvolvimento do OSN de um modo pronunciadamente complementar ao da *neogenina*, com níveis de expressão baixos ou nulos ao nível das células progenitoras e dos NSO maturos e com elevados níveis de expressão nos NSO imaturos.

Através de experiências de genética em ratinhos determinámos que a expressão da Neogenina é necessária para a produção de quantidades normais de NSO maturos. A nossa análise da proliferação e da apoptose no epitélio olfactivo sugere fortemente que a redução no número de NSO maturos observada numa linhagem de ratinhos deficiente para a *neogenina* tem a sua origem numa capacidade reduzida das células progenitoras de sair do ciclo celular e de se diferenciar e não num aumento da morte celular.

Embora os nossos resultados quanto à expressão da Neogenina na população de NSO maturos sugiram um papel para este receptor no direccionamento axonal nestes neurónios, não encontrámos defeitos severos nas projecções do epitélio olfactivo de ratinhos deficientes para este receptor. No entanto, dados recentemente publicados sobre a linhagem de ratinhos analisada neste trabalho permitem-nos não pôr de lado desde já a possibilidade de que a Neogenina tem um papel relevante no direccionamento dos axónios dos NSO no seu trajecto para o bolbo olfactivo.

Abstract

The IgG superfamily receptor Neogenin has been shown in the last years to be implicated in developmental processes as diverse as mammary gland formation and axon guidance in vertebrates. A homologue of the Netrin receptor Deleted in Colorectal Cancer (DCC), Neogenin binds Netrins as well as a second family of ligands, the repulsive guidance molecules (RGMs). Initially described in the context of axon guidance in the chick optic tract, Neogenin was found in this system to act as a repulsive cue receptor, leading to growth cone collapse and repulsion of Neogenin-expressing axons when presented with RGM ligand. Netrin-elicited axon attraction through Neogenin has since also been described. Other activities pertain to morphogenesis and cell proliferation and differentiation. Of special relevance for this work are the roles shown for Neogenin in regulating neuron proliferation and differentiation as well as neuronal survival in diverse vertebrate model organisms.

We have found that *neogenin* and its ligand *rgmB* are both expressed in the developing and the postnatal olfactory epithelium in complementary patterns suggestive of interplay at different stages of olfactory sensory neuron (OSN) development. *Neogenin* was found to be upregulated at different stages of OSN development, with high levels of expression in OSN progenitor cells, low levels in immature OSNs and high levels in differentiated mature OSNs. In the light of the known roles described for Neogenin in neuronal development, this pattern of expression suggests that Neogenin might fulfil different functions in the same OSN-lineage cell at different phases of its development. Namely, expression in OSN progenitor cells suggests proliferation-/differentiation-related activity, whereas expression in mature OSNs suggests survival-/axon guidance-activity. *RgmB* was found to also have its expression modulated during OSN development in striking complementarity with Neogenin, with low or absent expression in progenitor cells of the OE and mature OSNs and high levels of expression in the immature OSN population.

Through mouse genetics experiments we have found that Neogenin expression is necessary for generation of normal numbers of mature OSNs. Our investigation of proliferation and cell death in the OE strongly suggests that the reduction in mature

OSN numbers in a mouse model deficient in Neogenin expression stems from decreased ability of progenitor cells to exit the cell cycle and differentiate, rather than from increased cell death.

Although our results pertaining to the fine expression pattern of Neogenin in mature OSNs suggest that this receptor may function in the context of axon guidance in these cells, we have not found major olfactory axon targeting defects in the gene-trap mouse line analyzed. Recent data published on this mouse line allows us to still consider a relevant role for Neogenin in OSN axon guidance a likely possibility.

CHAPTER 1

INTRODUCTION

1   INTRODUCTION

Vertebrate sensory systems are responsible for accurately gathering and interpreting information from the outside world and ultimately eliciting appropriate behaviour leading to successful adaptation to and survival in the environment. The development of a nervous system is an extremely complex event depending on the concerted action of multiple developmental processes that are tightly regulated through intrinsic and extrinsic cellular mechanisms. In mammals, a number of neurons ranging in the trillions are generated that must differentiate in the right place and at the right time to selectively form synaptic connections. Achievement and maintenance of such a massive level of complexity depends on tight regulation of processes such as progenitor cell migration and exit from the cell cycle, acquisition of required neuronal morphologies, development of axons and dendrites and establishment of synapses. The field of neural patterning has changed drastically in the last few years with the finding that molecules previously believed to exclusively play roles in morphogenesis, like Wnts and Sonic hedgehog, play a dual role in the nervous system, first in neuronal differentiation and, later on, on actual axon guidance, using different receptors to exert their different functions (Charron and Tessier-Lavigne, 2007). In contrast, other molecules like the transmembrane receptor Neogenin have been proposed to play a role in these two functions in different contexts, through binding of different ligands (Wilson and Key, 2007). Although these findings have broadened the knowledge on neurogenesis, researchers have been hard pressed to uncover the details of the signaling events responsible for mediating such diverse developmental activities. Understanding the intimate mechanisms underlying the capacity of these proteins to perform complementary roles in different contexts of neuronal development will be key to furthering the advance towards understanding and treating neural degeneration and neural injury.

Being responsible for detecting odours and consequently for eliciting behaviours related to feeding and foraging as well as social behaviours in vertebrates, the olfactory system has a particularity which makes it a model of choice for studying the

development and regeneration of sensory neurons. Being directly exposed to the air in the nasal cavity and only protected by mucus produced by neighbouring epithelial cells, mature olfactory sensory neurons (OSNs) are vulnerable and constant victims to insult by volatile or airborne particles. To guarantee constant amounts of functioning OSNs in the olfactory epithelium (OE), neurogenesis is ongoing in this tissue throughout the life of the animal (Calof et al., 2002, Murdoch et al., 2007). Understanding how neurogenesis and neuroregeneration are regulated in this system will contribute towards answering questions and finding solutions to the problems posed by severe nerve injury as well as neurodegenerative disease.

1.1 THE MAIN AND ACCESSORY OLFACTORY SYSTEMS

Two olfactory systems can be found in the mouse, both comprised of sensory epithelia located in the nose and central nervous system target fields located on the olfactory bulbs (OBs). The main olfactory system is responsible for the sensing of odorants leading to complex behaviours including feeding and foraging as well as social behaviours. In this system the olfactory epithelium (OE) produces olfactory sensory neurons that express olfactory receptors (ORs) and project axons to establish stereotypic connections with second order neurons in the glomerular layer of OBs. The mature olfactory sensory neurons possess a specialized ciliated structure where high concentrations of ORs are present at the cell membrane, and which is directly exposed to the lumen of the nasal cavity (Fig. 1).

The accessory olfactory system is responsible for the detection of pheromones and odorants leading to social and sexual behaviours in the mouse. The sensory epithelium for this system is located in the vomeronasal organ (VNO) where vomeronasal sensory neurons expressing putative pheromone receptors (VRs) stereotypically project axons towards a substructure of the OBs, the accessory olfactory bulbs (AOBs), where they establish connections with second order neurons, which in their turn relay VNO input to the amygdaloid complex (Fig. 1; Reviewed in Halpern and Martinez-Marcos, 2003).
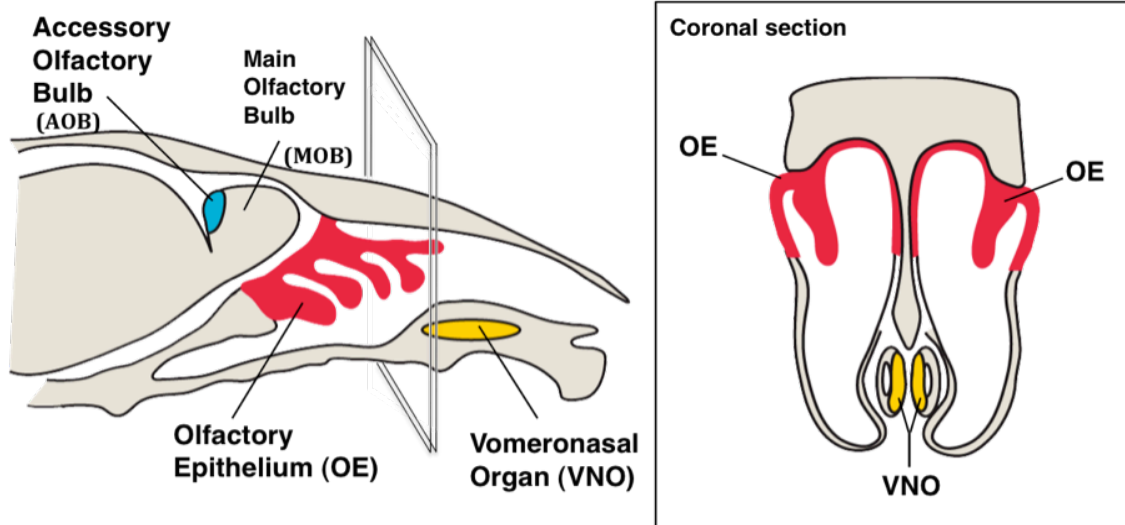
Figure 1. The murine olfactory system. The main olfactory system is responsible for eliciting feeding and foraging as well as social behaviors in response to odors in rodents. Its sensory organ is the main olfactory epithelium (MOE), a pseudo-stratified neuroepithelium lining the nasal cavity in which olfactory sensory neurons (OSNs) express olfactory receptors and detect airborne odorants. OSNs project axons and establish synapses at the level of the main olfactory bulbs (MOBs) where odorant-elicited signals are relayed to the olfactory cortex. The accessory olfactory system is responsible for the detection of pheromones controlling sexual and aggressive behaviors in the rodent. Sensory neurons in the vomeronasal organ (VNO) express putative pheromone receptors and project axons to the accessory olfactory bulb (AOB), a structure localized in the dorsal aspect of the MOB. Inset shows a coronal view of the MOE and the VNO.

1.2 NEURONAL PROGENITORS OF THE OLFACTORY EPITHELIUM

A defining characteristic of the OE lies in the organization of the OSN lineage itself. In the murine OE basally located progenitor cells – globose basal cells (GBCs) – divide replenishing their own population and giving rise to two stages of transit-amplifying cells – immediate neuronal precursors (INPs) – that divide a limited number of times to give rise to immature OSNs located between progenitor cells and mature OSNs (Fig. 2). Immature OSNs then undergo maturation and project axons towards the olfactory bulbs while their cell bodies acquire more apical positions in the OE. This chain of proliferation events gives rise to a pseudo-stratified epithelium

and guarantees the maintenance of OSN numbers in the OE, throughout life (Calof et al., 2002; Beites et al., 2005).

The OE has its origin in the olfactory placode (OP), a thickening of the surface ectoderm visible at embryonic day 9 (E9). At E10 the placode starts invaginating, giving place to the nasal pit. At E11 the first OE axons extend towards the OB primordia and first OSN dendrites appear. At E12 olfactory pits are deeply invaginated and the lateral and media nasal processes fuse giving rise to the nasal septum and two separate nasal cavities. At E13-E14 turbinates are formed and OE stratification is established. At E16.5 OE axons reaching the OB form the first protoglomerular structures (Cuschieri and Bannister, 1974). At E18 the OE turbinates are fully formed and the pseudo-stratified OE is actively projecting axons to the OB establishing the first synapses and forming the first glomeruli.

1.3 REGULATION OF NEUROGENESIS IN THE OE

1.3.1 TRANSCRIPTIONAL REGULATION IN THE OE

Although the precise transcriptional regulation events driving transition between proliferation, differentiation and maturation phases in the OSN lineage are yet to be fully understood, a fair amount of knowledge has accumulated about the transcription factors present in the different cell populations of this lineage.

Work by Cau et al. (1997, 2000, 2002) has thoroughly dissected the basic helix-loop-helix (bHLH) transcription factor cascades driving commitment of the multipotent progenitor cells and differentiation of newly committed neuronal progenitors in the OSN lineage. This work has shown that in the E10 OP both Mammalian Achaete/Scute Homolog 1 (Mash1) and Neurogenin1 (Ngn1) have a role in cell fate determination, being able to compensate for each other's ablation. Later on, at E12.5 these two bHLH transcription factors seem to have separate and sequential roles in the developing OE, Mash1 keeping its role in fate determination, whereas Ngn1 restricts its activity to promoting differentiation of committed OSN progenitors. Characterization of Mash1/Ngn1 double mutants also sowed these

transcription factors to be required for neurogenesis in the OE, as these animals were totally devoid of OSNs.
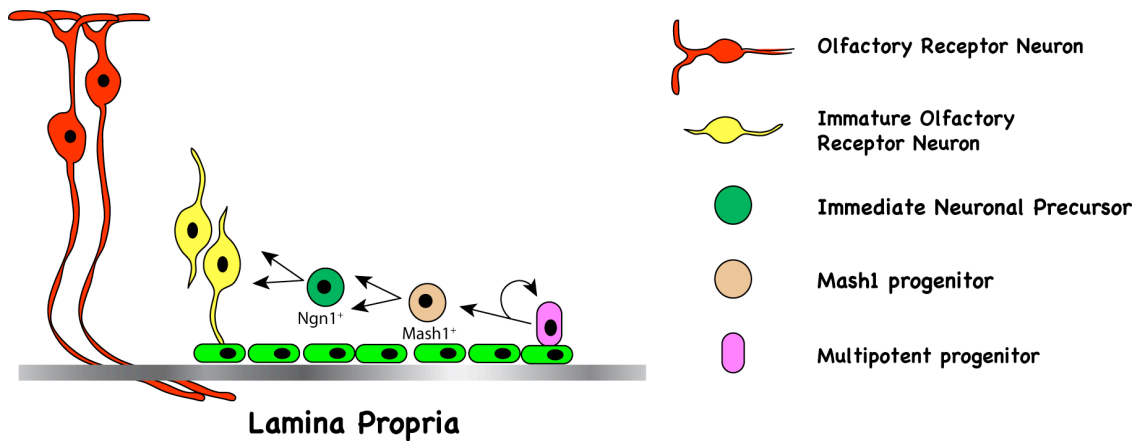
Figure 2. The olfactory sensory neuron lineage. Multipotent progenitor GBCs in the most basal aspect of the OE constantly divide to replenish their own population and to give rise to transit-amplifying Mash1-expressing precursor cells. Mash1-positive precursors then undergo a limited number of division cycles before further differentiating into Ngn1-expressing neuronal precursor cells, which, after a small number of divisions, exit the cell cycle and differentiate into immature OSNs. Bipolar immature OSNs then develop a dendrite and start projecting their axon towards the lamina propria, then to the OBs. Mature OSNs develop a dendritic knob with a ciliated structure exposed into the nasal cavity and project their axon to a specific glomerulus in the OB. This process starts during embryonic development leading to the formation of a pseudo-stratified olfactory epithelium and takes place throughout life guaranteeing the maintenance of appropriate number of OSNs in the OE in normal conditions or following injury.

Loss of Mash1 leads to absence of basal progenitor cells as early as 12.5, indicating that these cells fail to be generated in the absence of Mash1. Placement of Serrate (Ser) and Enhancer of Split (Hes) downstream of Mash1 in this system, implicating concomitant Notch signaling events, is highly indicative of neural determination mechanisms well documented across species (Chitnis et al,. 1995, Chitnis and Kintner, 1996; Henrique et al., 1997), making Mash1 a bona fide candidate for proneural activity in the OSN lineage.

While not precluding generation of basal cells with all the hallmarks of OSN progenitors (Mash1, Dll3, Hes5), loss of Ngn1 leads to absence of differentiated OSNs in the OE, presumably caused by failure to express transcriptional regulators

responsible for specification and differentiation of OSNs (Phd1, NeuroD) leading to progenitor cells not exiting the cell cycle (Cau et al., 2002). These authors further determined that transcription factors known to play roles in specification of neuronal phenotypes after differentiation were not all downstream of Ngn1 in the basal compartment of the OE. Interestingly, they found Phd1 of the paired-homeodomain transcription factor family to be downstream of Ngn1 but in parallel Lhx2 was found to depend directly on Mash1 expression, suggesting a combinatorial network of general and OSN-specific genes leading to the overall OSN phenotype.

The same group also reported a small subpopulation of Ngn1-positive basal progenitor cells consistently present in Mash1 null mice (Cau et al., 1997), indicating an alternate pathway to determination at work in these cells. This question remains to be addressed.

bHLH transcriptional repressors Hes1 and Hes5 have also be shown to play diverse roles in OE neurogenesis regulation (Cau et al., 2000). Hes1 has been shown to play two sequential roles in OE development, first, as soon as 24-26 somite stage, as a pre-pattern gene, in a way analogous to its related *Drosophila* gene hairy, by circumscribing Mash1 expression to a delimited area of the OP. Hes1 mutants show expanded Mash1 expression domains. Because this pattern of expression is set up even before any neuronal precursor cells are present in the OP this activity can not be ascribed to lateral inhibition, a cell-cell signalling process where, in a cluster of neuronal progenitor cells, one cell expressing slightly more of a Notch ligand activates Notch in the adjacent cells, effectively inhibiting their proneural genes while activating its own, thus limiting the number of nascent neuroblasts and guaranteeing the maintenance of a pool of progenitor cells in the tissue. At E10.5 loss of Hes1 leads to formation of clusters of Mash1-positive precursor cells in the OP, suggesting a neurogenic role implicating lateral inhibition (Chitnis et al., 1995, Henrique et al., 1997, Simpson, 1997). Considering that Mash1 has been previously shown to drive expression of Serrate ligands in the OP (Cau et al., 1997) and that it has been proposed that Hes1 expression is dependent on Notch in neural tissues (Ohtsuka et al., 1999), the working model is that Hes1 participates in a feed-back loop responsible for preventing adjacent cells to express Mash1. In contrast Hes5 is

described as being expressed exclusively under control of Notch signaling in neural tissues, where its role is limited to lateral inhibition (Ohtsuka et al., 1999). Although these properties remain to be confirmed in OE development, the fact that absence of Mash1 leads to almost complete absence of Hes5 expression in the OP, suggesting its dependence on Notch signaling in this tissue, as well as the synergic effect observed in Hes1/Hes5 double mutants, where numbers of OSNs as well as numbers of OSN clusters were sharply increased, are strong indicators that they do translate to this neural tissue. Because absence of Hes1 leads to an increase in Mash1 expression yet leaves Ngn1 levels mostly unaltered, it was considered likely that Ngn1 was also negatively regulated. Analysis of the Hes1/Hes5 double mutant showed a synergic effect on the increase in Ngn1 expression without leading to an increase in Mash1 expression (Cau et al., 1997), suggesting that Hes5 regulates OSN development through repressing Ngn1 expression. Although the mechanisms through which Hes1 and Hes5 regulate Mash1 and Ngn1 expression remain to be explained, their temporal and physical patterns of expression as well as similar reports in other systems (Nakao and Campos-Ortega, 1996; Jennings et al., 1999; Castella et al., 1999) place them in good standing as key players in regulation of development in the OSN lineage.

In a recent publication, Chen et al. (2009) described two new players in the transcriptional regulation of OSN lineage development. Six1 and Six4 are mammalian homologs of the *Drosophila* sine oculis (*so*) transcription factor, part of a network regulating eye development in the fly. Containing a Six domain and a six-homeodomain for DNA binding domain these two transcription factors are coexpressed in the preplacodal, domain through to embryonic age E14, with Six becoming gradually restricted to the apical and basal compartments of the OE. Following OP formation Six1 and Six4 have been shown to be necessary for neurogenesis in the developing OE. While not affecting progenitor cell determination in the OP at E12-E12.5, loss of Six1 leads to a failure in neurogenesis from these progenitors, as assessed through the absence of neuronal lineage markers Mash1, Ngn1, NeuroD, Phd1, Lhx2, Ebf1 and Gap43. As apoptosis is increased and proliferation is decreased in Six1 mutant mice, phenotypes that are exacerbated by the additional loss of Six4, it has been proposed that these transcription factors are

necessary for expansion and differentiation of early progenitors in the OP. As other transcriptions implicated in placode formation and progenitor specification (namely Sox2) are not affected, it is likely that they work in conjunction with Six1 and Six4 to trigger the neuronal lineage program leading to Mash1 expression. The particular details of these regulation mechanisms remain to be uncovered.

The Forkhead winged-helix transcription factor Foxg1, which plays important roles in regulating anterior neural structure development, has been recently shown to promote neurogenesis in the OE through modulation of TGFß signaling in this tissue (Kawauchi et al., 2009a). Ablation of Foxg1 leads to drastic reduction in Mash1-positive progenitors and to and even stronger decrease in Ngn1-expressing INPs at E11. This decrease does not stem form increased apoptosis but rather from interaction with growth differentiation factor 11 (Gdf11) signaling. Gdf11 is responsible for promoting cell-cycle arrest in OSN progenitors by inducing the cyclin-dependent kinase inhibitors p21^{Cip1} and p27^{Kip1}, inhibitors of cyclins E-CDK2 and D-CDK4/6 (Wade-Harper et al., 1993; Koff et al., 1994; Polyak et al., 1994; Wu et al., 2003; Kawauchi et al., 2009). Foxg1 antagonizes Gdf11 activity, presumably through binding with SMAD complexes downstream of Gdf11, in a dose-dependent manner, with Foxg1$^{-/-}$; Gdf11$^{-/+}$ presenting a light rescue phenotype and Foxg1$^{-/-}$; Gdf11$^{-/-}$ showing close to normal OE morphogenesis.

Olf1/EBF (O/E) transcription factors are part of the repeated helix-loop-helix (rHLH) family and have been described as regulating gene expression and OSN development in the OE (Wang et al., 1993, 1997, 2004; Cheng and Reed, 2007). O/E-1, O/E-2 and O/E-3 have been shown to be expressed in the basal as well as the neuronal compartment of the OE and to bind promoters for a set of genes participating in the olfactory transduction pathway being capable of activating reporter genes in vitro (Wang and Reed, 1993; Wang et al., 1997). Individual and combined loss of two members of this family, O/E-2 and O/E-3, in the developing OE leads to increasingly severe targeting defects in dorsal OB innervation by incoming OSN axons (Wang et al., 2004), indicating a role in promoting terminal differentiation of OSNs. The multiple zinc finger transcription factor Zfp423/OAZ is also expressed in the OE and binds O/E transcription factors, effectively inhibiting their activity (Tsai

and Reed, 1997; Cheng and Reed, 2007). OAZ is expressed downstream of Mash1 and spans the E18 OE acquiring a pattern of expression limited to recently differentiated OSNs in young and adult postnatal mice, where it partially overlaps Ngn1 expression. Expression of OAZ was shown to be required in this precise time frame of the OSN lineage and elegant gain-of-function assays showed that OMP-driven OAZ expression in mature OSNs is sufficient to arrest their maturation and maintain them in an immature state, as assessed by olfactory marker protein (OMP) suppression and Gap43 (an immature neuron marker) upregulation, concomitantly leading to increased apoptosis (Cheng and Reed, 2007). Gain of function in these mice also engendered a failure in dorsal OB innervation by OSN axons. Axons of OSNs expressing ORs normally located in this area showed reduced numbers as well as widespread presence in the OE and weak OR expression in this paradigm. Mutant axons also showed inability to converge onto glomeruli. OAZ expression late in OSN maturation, acting as a dominant suppressor of the O/E transcription factors, clearly precludes the accomplishment of final steps of the O/E transcription program required for terminal differentiation of the OSNs. This striking observation is indicative of the significance of the temporal delimitation of OAZ expression on the OSN development timeline suggesting a role in immediate neuronal precursor (INP) to early OSN differentiation. Another interesting possibility arising from this data is that OAZ may be promoting maintenance of the immature state in the neurons where it is expressed, possibly driving expression of immature neuron marker genes like Gap43 and contributing to prevent OR-gene choice in cells at this stage. These hypotheses remain to be confirmed.

The Runt-related transcription factor (Runx) family is composed of homologs to Drosophila Runt. Similarly to their invertebrate homolog Runt, Runx transcription factors are implicated in regulating various developmental processes in vertebrates, being associated with various forms of cancer in humans (Ito, 2004). Of the three existing mammalian Runx genes, Runx1 is required for definitive haematopoiesis (Speck and Gilliland, 2002), Runx2 for osteogenesis (Komori, 2006) and Runx1 and Runx3 play a key role in dorsal root ganglion (DRG) proprioceptive neuron development. Runx1 has been shown to be expressed in a portion of the Mash1-positive progenitors, in most of the NeuroD-positive precursor cells as well as

in a small fraction of b-III-tubulin-expressing OSNs in the developing murine OE (Thériault et al., 2005). In contrast, no overlapping was found with neural cell adhesion molecule (NCAM), a marker for mature OSNs. Loss of Runx1 function in the OE led to an accelerated differentiation of progenitor cells, giving rise to an OE with normal overall cell numbers but with cell type proportions tilted towards increased mature OSN and decreased basal NeuroD-positive precursor cell numbers. This, associated with gain-of-function results showing increased numbers of proliferating cells in olfactory neurospheres infected with Runx1-encoding adenovirus, is strongly indicative of a role for Runx1 regulating the transition from precursor cell to OSN by promoting OSN precursor cell proliferation. Functional assays performed by the same group on cortical primary cultures confirm these results and further indicate that this effect requires the DNA-binding activity of Runx1 and that the observed increase in precursor cells in the gain-of-function context is not conducing to cell-cycle arrest, rather leading to increased mature neuron numbers. Also in primary cortical cultures, Thériault et al. (2005) showed that these effects are most surely derived of transcriptional repression by Runx1 and further showed that Runx1 successfully suppressed expression of a reporter gene under control of the p21^{Cip1} promoter in transfected cortical progenitors. These results make a strong case for Runx1 regulating precursor to neuron transition yet they remain to be reproduced in the OE.

1.3.2 CYTOKINE REGULATION IN OE DEVELOPMENT

Regulation of OSN development has been shown to also involve signaling by cytokines present in the OE. Members of the fibroblast growth factor (FGF) and the bone morphogenetic protein (BMP) families of cytokines are expressed in the OE and have been shown to play roles in promoting survival and proliferation of OSN lineage cells. FGF family members play important roles in vertebrate development by regulating cell proliferation, cell growth and developmental patterning in diverse tissues (Osnitz, 2000). FGFs have been shown to promote proliferation in progenitor cells of the OE in vitro (de Hamer et al., 1994) and FGF8, a member of this

superfamily that has been described as a mitogen in neural tissues (Lee et al., 1997; Xu et al., 2000) and as promoting the survival of specific cell populations of neurons (Mathis et al., 2001; Chi et al., 2003; Storm et al., 2003), has been shown to be necessary for neurogenesis in the OE (Kawauchi et al., 2005). Although its mode of action remains to be uncovered FGF8 expression in the OE has been shown to be necessary for OE morphogenesis and for the generation of the OSN lineage. Based on the location of FGF8 expression in Sox2-positive basal cells in the postnatal OE and on the severe apoptotic phenotype found in *fgf8* null OEs, FGF8 is postulated to engender its proneurogenic effect by promoting the survival of progenitor cells at the stem of the OSN lineage.

1.3.3   ROLE OF BMPs IN EARLY STAGES OF THE OSN LINEAGE

In vitro studies have also shown members of the bone morphogenetic protein (BMP) family of cytokines to contribute to the regulation of neurogenesis in the OE. BMP2, BMP4 and BMP7 are all expressed in the OE and have been shown to inhibit neurogenesis in OE explant cultures (Shou et al., 1999), an effect reminiscent of earlier results showing an inhibitory effect of excess OSNs on in vitro OSN neurogenesis (Mumm et al., 1996). BMP4 and BMP7 have been shown to be expressed in the OE proper in the developing as well as in the adult, with BMP being restricted to basal cell patches and BMP4 being widely expressed in immature and mature OSNs. High concentrations of both BMP4 and BMP7 were shown to strongly inhibit neurogenesis in OE neuronal colony forming assays. Conversely, low concentrations of BMP4 were shown to robustly promote significant increased neurogenesis in these assays. This dual function for BMP4 is further supported by the observation that the BMP inhibitor Noggin, rather than increasing neurogenesis in these assays, induces a strong inhibition of neurogenesis leading to complete blocking of colony formation (Shou et al., 2000). The inhibitory action of BMPs on OE neurogenesis has been shown to be achieved by targeting the Mash1-positive population of OSN progenitors in a process leading to proteolytic degradation of the Mash1 transcription by targeting to the proteasome (Shou et al., 1999). The

mechanism by which low BMP4 concentrations promote OSN neurogenesis was shown to consist in supporting the survival of newly born OSNs rather than stimulating proliferation in progenitor cells of the OSN lineage (Shou et al., 2000) yet the cellular mechanisms responsible for this effect remain to be known.

1.3.4 AXON GUIDANCE IN THE FORMATION OF A TOPOGRAPHIC OLFACTORY MAP

One of the hallmarks of the vertebrate olfactory system resides in the pattern of expression of different ORs across the OSN population. Each mature OSN expresses one of ~1000 OR genes in a monoallelic manner (Chess et al., 1994, Malnic et al., 1999) and all OSNs expressing the same OR project axons to the same glomeruli in the OB. But it has been shown that OR choice is not stochastic all across the OE. Each OR gene is only expressed with high frequency in one of four partially overlapping zones along the dorsomedial to ventrolateral axis of the OE and is stochastically expressed by OSNs within its respective zone. This zonal organization of OR expression can be seen as soon as age E13 and all the way into adulthood (Ressler et al., 1993, Strotmann et al., 1995; Sullivan et al., 1995; Miyamichi et al., 2005). Concomitantly, these four OE zones project OSN axons that establish synapses and form glomeruli stereotypically in four zones distributed along the dorsal to ventral axis of the OB surface, respectively, forming a topographic olfactory map (Fig. 3).

The first molecules shown to play a role in the targeting of OSN projections were the ORs themselves. Work by Mombaerts et al. (1996) and Wang et al. (1998), where chromosomal loci coding for different ORs were removed or substituted with cassettes containing coding regions of other ORs, showed that ORs themselves play an instructive role in OSN axon targeting to the OB, with the deletion of the coding sequence led to failure of the incoming axons to converge at the glomerulus and substitution of the coding sequence led to the formation of an ectopic glomerulus in the vicinity of the one corresponding to the new coding sequence.
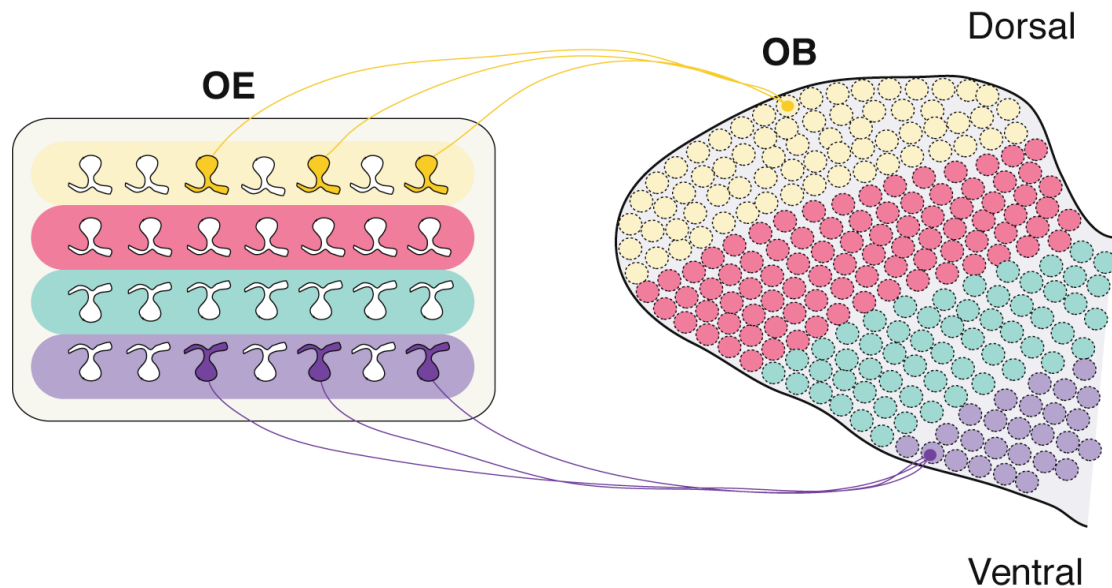
Figure 3. Zonal organization of the olfactory system. Olfactory sensory neurons express only one of ~1000 OR genes in the mouse. Rather than being stochastically expressed across the OE, four sub-groups of ORs are preferentially expressed in four zones along the dorsoventral axis of the OE. Within each zone, each OSN expresses one of their sub-group ORs at random. OSNs expressing the same OR then project their axons to the same glomerulus on the OB where they establish synapses with second-order neurons. The zonal organization of the OE translates into the glomerular map in the OB with dorsal zones of the OE projecting to the dorsal OB and ventral zones of the OB project to the ventral OB.

ORs have since been shown to be present at the OSN axon termini (Strotmann et al., 2004; Barnea et al., 2004) and have been proposed to exert their instructive role through axon-axon and growth cone-growth cone interactions (Feinstein et al., 2004; Mombaerts, 2006). This hypothesis remains to be proven. More recent work suggests that OR-evoked cAMP levels are responsible for the OSN axon targeting phenotypes observed. ORs are seven-pass transmembrane G-protein coupled receptors that generate cAMP through activation of the olfactory-specific heterotrimeric G-protein G_{olf}. Activation of G_{olf} induces adenylyl cyclase type III activity leading to cAMP production and consequently to opening of cyclic nucleotide gated channels.

Although previous work had shown that G_{olf} null mice were anosmic but presented no glomerular map defects (Belluscio et al., 1998; Lin et al., 2000), Imai et al. (2006) and Chesler et al. (2007) have shown that ORs modified to be deficient in G-protein coupling do fail to converge into glomeruli and to establish synapses in the OB, a

phenotype rescued by expression of constitutively active G-proteins or constitutively active protein kinase A (PKA), which is downstream of cAMP signaling. Additionally they found that OSNs producing different levels of cAMP form glomeruli that are shifted along the rostro-caudal axis of the OB. A different G-protein, G_s, expressed in OSNs but not participating in odorant-evoked signaling, is postulated to drive OR-dependent cAMP synthesis at varying intensities in different OE axons. Differences in cAMP levels are then believed to lead to differential expression of axon guidance genes under the control of the PKA-regulated cAMP response element-binding transcription factor (CREB), and consequently to targeting of incoming OE axons to different coordinates along the OB rostro-caudal axis. This has been shown for the Semaphorin receptor Neuropilin1, with high expression in OSNs expressing a high cAMP OR transgene and absence in OSNs expressing a G-protein-coupling deficient OR.

Members of the Semaphorin (Sema) family and their receptors, the Neuropilins (Npn) (Castro et al., 1999; Pasterkamp et al., 1998; Renzi et al., 2000; Schwarting et al., 2000; Walz et al., 2002; Cloutier et al., 2004; Schwarting et al., 2004) were the first guidance molecules shown to play a role in the establishment of the olfactory glomerular map. The repulsive guidance molecule Sema3A is expressed by glial cells in the ventral midline of the OB and repels incoming Npn1 positive axons, promoting their targeting to the lateral or medial OB (Pasterkamp et al., 1998; Schwarting et al., 2000, 2004).

Eph and Ephrin expression has also been shown to play a role in olfactory axon guidance and to be modulated by cAMP signaling (Cutforth et al., 2003; Serizawa et al., 2006). Different levels of Ephrin-A expression on OSN axons expressing different ORs modulates targeting of these axons along the rostro-caudal axis of the OB, where Eph-A3 and Eph-A5 are expressed. Namely, overexpression of Ephrin-A5 was shown to lead to an anterior shift in the glomeruli of the OSN populations tested whereas ablation of Ephrin-A3 and Ephrin-A5 led to a shift towards the caudal aspect of the OB.

Recent work by Cho et al. (2007) has shown the implication of a third family of classical guidance cues in the formation of the olfactory glomerular map.

Roundabout (Robo) receptors are a family of receptors for repulsive guidance molecules of the Slit family initially described in *Drosophila* as preventing ipsilateral axons from crossing the midline and commissural axons from recrossing it (Seeger et al., 1993; Battye et al., 1999; Kidd et al., 1999) and that have since been shown to be implicated in OSN axon targeting in *Drosophila* and zebrafish (Jhaveri et al., 2004; Cho et al., 2007). A graded pattern of Robo2 expression in the OE associated with expression of the repulsive ligand Slit1 in the ventral aspect of the OB has been shown to be necessary for the targeting of zone I axons to the dorsal OB. Ablation of Robo2 or Slit1 led to mistargeting of a subset of zone I axons to ventral regions of the OB. These results are the first to show that the dorsoventral targeting of OSN axons responds to guidance cues on the OB, rather than just being a reflex of the OR zonal organization in the OE.

Molecules not associated to classical axon guidance have also been shown to play instructive roles in the establishment of the olfactory glomerular map. Lactosamines are carbohydrates expressed by OSNs located dorsomedially in the OE that are necessary for the successful steering of axons towards their cognate glomeruli (Schwarting et al., 1991, 1992). Ablation of β1,3-N-acetylglucosaminyltransferase I (β3GnTI), an enzyme necessary for addition of N-acetylglucosamine to growing carbohydrate chains leads to inability of lactosamine expressing axons to innervate their glomeruli and eventually to the loss of these OSN populations (Henion et al., 2005, Schwarting et al., 2005, 2007). Members of the galectin family of carbohydrate-binding proteins expressed in the extracellular matrix have been proposed to contribute to the targeting of axons expressing lactosamines. Zones of high Galectin expression are believed to delineate restricted paths of navigation for lactosamine-expressing axons by promoting axonal adhesion along these tracts and to promote fasciculation of these axons (Mahanthappa et al., 1994; Tenne-Brown et al., 1998; Crandall et al., 2000).

Additionally to these mechanisms affecting navigation and targeting of OE axons to the OB other molecules are under study that affect olfactory map formation through establishing OSN axonal identity through axon-axon interactions leading to fasciculation of related axons and mutual repulsion of unrelated axons. Kirrel2 and

Kirrel3, two immunoglobulin superfamily proteins, were shown by Serizawa et al. (2006) to be expressed at complementary levels on OSN axons in a manner dependent on the specific OR expressed. Kirrel2 and Kirrel3 are capable of homophilic but not heterophilic interaction and the graded high-Kirrel2/low-Kirrel3 to low-Kirrel2/high-Kirrel3 expression across the OSN population is proposed to act in concert with Ephs and Ephrins to bring related axons together and unrelated axons apart. Modification of the OSN 'fingerprint' by overexpression of Kirrel2 in half of the OSN population expressing a given OR was shown to lead to formation of a second glomerulus for this OR.

Although a broader picture of the mechanisms underlying coarse navigation of incoming OSN axons to the OB is now available, the knowledge accumulated still does not account for the complexity of the glomerular organization found at the OB.

1.4 NEOGENIN AND ITS LIGANDS

Neogenin is a single pass transmembrane receptor of the immunoglobulin-g(IgG)-like family, presenting 53% amino acid identity and 70% amino acid similarity with the Netrin receptor Deleted in Colorectal Cancer (DCC), containing four IgG-like domains, six fibronectin-III domains (Fig. 4; Vielmetter et al.; 1994). Neogenin has been shown to play different roles in multiple developmental processes in vertebrates, having as ligands members of the Netrin and the repulsive guidance molecule (RGM) families (deVries and Cooper, 2008). The Netrin family of diffusible guidance molecules has been extensively studied in the context of axon guidance but has, in recent years been found to regulate cell migration and to participate in multiple developmental processes, particularly in laying out the patterning of branching organs and systems (reviewed in Cirulli and Yebra, 2007). RGMs are glycosylphosphatidylinositol (GPI)-linked proteins with no homology to Netrins that, beyond their original identification as repulsive guidance cues, have been shown to take part in processes as diverse as neural tube closure, BMP signalling and iron metabolism (reviewed in Corradini et al., 2009). Neogenin was initially found to act in the chick temporal retinal ganglion cell (RGC) axons repelling them away from the
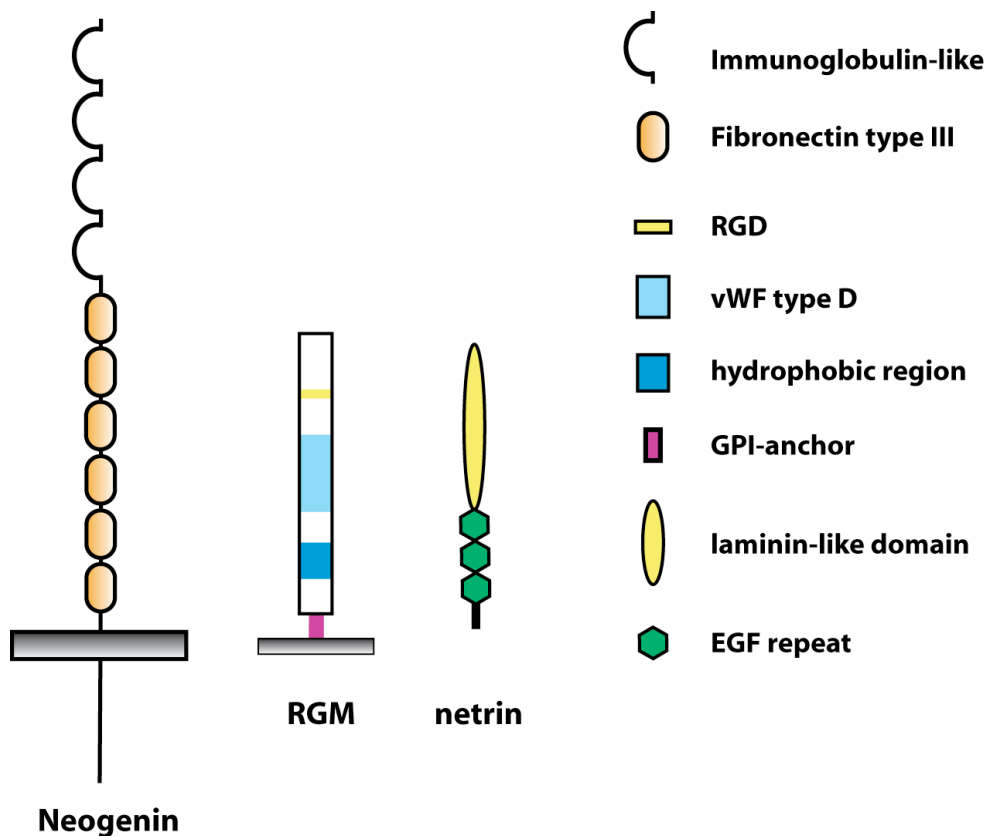
Figure 4. Structure of Neogenin and its ligands. Neogenin is an IgG-like family transmembrane receptor that binds to two families of molecules, RGMs and Netrins. The extracellular region of Neogenin contains four immunoglobulin-like and six fibronectin type III domains. RGMs are GPI-anchored proteins containing an RGD motif, a von Willebrand factor type D domain, and a hydrophobic stretch. Netrins are secreted molecules containing a laminin-like domain and epidermal growth factor (EGF) repeats.

high-RGM posterior optic tectum to establish connections in the low-RGM anterior optic tectum (Monnier et al., 2002; Rajagopalan et al., 2004).

Neogenin has since been shown to regulate several other processes including cell proliferation and differentiation in the chick and mouse (Matsunaga et al., 2004, 2006; Conrad et al., 2009), early axon guidance events in Xenopus supraoptic tract (Wilson and Key, 2006), cell-cell adhesion and epithelial organization and polarity in Xenopus and mouse (Srinivasan et al., 2003; Mawdsley et al., 2004; Kee et al., 2008). Some of the data pertaining to regulation of cell death have been contradictory and further work remains to be done to shed light on these functions. Namely, knockdown of the Neogenin ligand RGMa or overexpression of Neogenin have been shown to lead to increased cell death in the chick spinal cord (Matsunaga et al., 2004) whereas overexpression of RGMa in Xenopus has been shown to elicit

increased cell death through Neogenin (Shin and Wilson, 2007) and to have no effect on mouse DRG cell death (Conrad et al., 2007).

In parallel, loss of Neogenin has been shown to lead to increased cell death in the zebrafish neural tube and the mouse mammary gland (Srinivasan et al., 2003; Mawdsley et al., 2004), indicating that rather than absence of the ligand it's the absence of Neogenin signal that is related to cell death phenotypes.

Unlike its homolog DCC, signaling downstream of Neogenin is still poorly understood (Fig. 5). Three intra-cellular domains, P1, P2 and P3, are conserved in Neogenin, presenting 70% to 90% amino acid sequence similarity to the DCC sequences (Ren et al., 2004; deVries and Cooper, 2008). The Neogenin P3 domain has been shown to bind focal adhesion kinase (FAK), a key component of Netrin1-mediated DCC signaling, and to be associated with FAK in the developing CNS (Li et al., 2004, Liu et al., 2004; Ren et al., 2004). Neogenin expression was shown to lead to increased FAK phosphorylation in HEK293 cells. Additionally, Netrin was shown to induce tyrosine phosphorylation of Neogenin through an unknown mechanism in cultured rat neurons and phosphorylated Neogenin showed binding to the Src homology 2 (SH2) domain of Fyn and Lck Src-family kinases and Src homology-2 containing inositol-5-phosphatase 1 (SHIP1) (Ren et al., 2008). Interestingly, the major site of Fyn phosphorylation of DCC – tyrosine Y1418 – is conserved in the Neogenin cytoplasmic domain (Y1467), suggesting Fyn binding might have biological significance (Meriane et al., 2004). Both Fyn and FAK can phosphorylate Cdc42 and Rac, constituting a likely pathway for Netrin-Neogenin elicited axon attraction. The tyrosine phosphatase SHP2 was also shown to bind phosphorylated Neogenin opening the possibility for a deactivation mechanism for this signaling pathway.

RGM-Neogenin signaling in the context of axon repulsion has recently been shown to implicate the Rho family of GTPases (Hata et al., 2006; Conrad et al., 2007). RGMa signaling through Neogenin was shown to activate RhoA and protein kinase C (PKC) but not Cdc42 or Rac1 in PC12 cells, an effect blocked by RhoA and PKC inhibitors as well as by Rho kinase (ROCK) inhibitor and Netrin. In agreement with these results, Kubo et al. (2008) have shown that Myosin IIA mediates RGMa-induced outgrowth inhibition in granular neuron cultures, leading to reduced F-actin
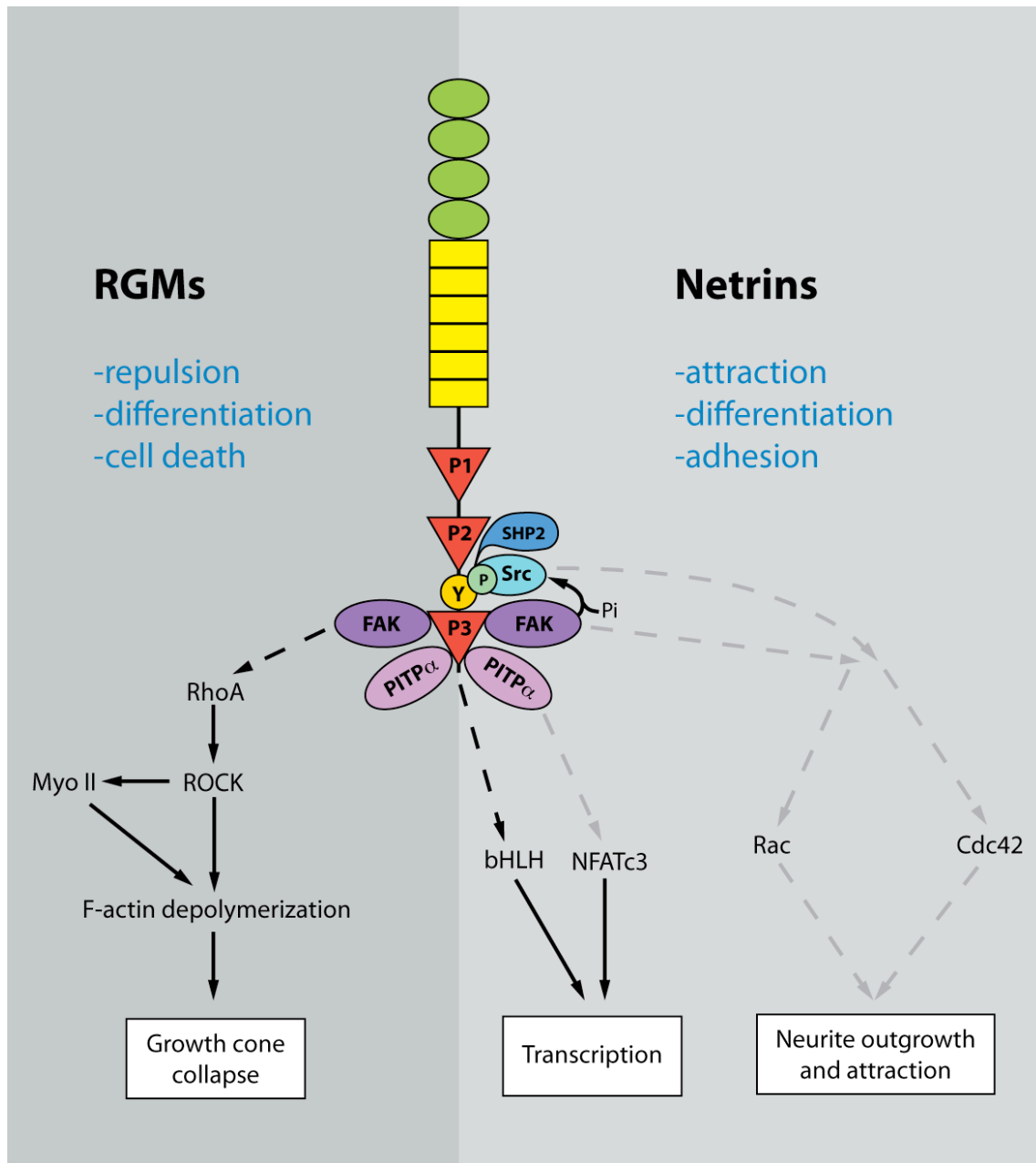
Figure 5. Signaling downstream of Neogenin. Modified from deVries and Cooper, J Neurochem 2008. Various binding partners have been found for the P3 intracellular domain of Neogenin. Both focal adhesion kinase (FAK) and PITPα bind to the P3 domain constitutively. Following Netrin-1-elicited tyrosine phosphorylation Src kinases bind to phosphotyrosine 1467 and are phosphorylated by FAK leading to a positive feedback cycle predicted to activate Rac and Cdc42 in a manner analogous to netrin/DCC signaling. Additionally, tyrosine phosphorylation leads to binding of the phosphatase SHP2 suggesting regulation of Netrin-Neogenin signalling. Conversely, RGM/Neogenin signaling activates RhoA and ROCK leading to growth cone collapse involving MyosinIIA. Netrin2/Neogenin binding induces activation of promyogenic bHLH and NFATc3 transcription factors and leads to upregulation of promyogenic genes. Black arrows indicate pathways known to be activated by Neogenin. Grey arrows indicate pathways yet to be verified for Neogenin but predicted based on Netrin/DCC signaling.

in the growth cone, in a RhoA/ROCK-dependent manner. An *in vivo* interaction between Neogenin and a Myosin X splice variant was described in the chick where it is postulated to block Netrin-elicited axon outgrowth by inhibiting DCC-Myosin X interactions (Zhu et al., 2007).

The constitutive binding of the Neogenin intracellular P3 domain to phosphatidylinositol transfer protein alpha (PITPα), an enzyme that promotes hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) leading to neurite outgrowth and attraction following Netrin1 signaling through DCC, indicates a possible path to Neogenin-elicited second messenger activation in the context of axon guidance (Xie et al., 2005). Binding of SHIP1 to the P3 domain of Neogenin as previously referred suggests phosphatidylinositol-3,4,5-triphosphate (PIP_3) is also a likely second messenger downstream of Neogenin with SHIP1 acting as negative regulator of the pathway.

Finally, Neogenin has been shown to play an important role in myogenesis. Neogenin is part of a cell-surface complex of different immunoglobulin superfamily proteins and cadherins playing a promyogenic role in the developing muscle (Kang et al., 2004). Work in the C2C12 myoblast cell line has shown that overexpression of *neogenin* in these cells promotes myotube formation, whereas *neogenin* knockdown inhibits this process. Myotube formation was shown to be increased following Netrin signaling through Neogenin, being accompanied by upregulation of muscle-specific genes. Activation of myogenic NFATc3 transcription factor in C2C12 cells following Netrin treatment suggests this pathway as a participant in the observed promyogenic effect. This promyogenic effect is lost and bHLH- and NFAT-dependent transcription is downregulated in myoblasts mutant for the Ig-family receptor CDO in a mechanism predicted to depend on the cadherin-binding properties of CDO (Kang et al., 1997, 1998, 2003; Cole et al., 2004). The type of multi-receptor complex proposed is reminiscent of cis-interacting axon guidance receptor complexes previously described to arise at the growth cone (Hong et al., 1999; Stein and Tessier-Lavigne, 2001; Rhee et al., 2002; Chang et al., 2004). This model remains to be verified and the signaling pathways implicated to be fully understood. Bae et al. (2009) have added new elements to this model by placing FAK and ERK downstream of Netrin

signaling in C2C12 cells, suggesting that modulation of activity of these kinases is a component of Neogenin-elicited promyogenic activity.

As previously referred, being closely related to DCC, Neogenin has been shown to bind Netrin-family ligands but, unlike DCC, a second, unrelated, family of guidance ligands, the RGMs, have been found to also signal through Neogenin. Members of the Netrin and the RGM guidance cue families have been shown to regulate diverse developmental processes in vertebrates, in particular in neural development, through Neogenin-mediated signaling.

RGMs are GPI-linked proteins that owe their name to their initial identification as a factor responsible for repelling Neogenin-expressing temporal retinal ganglion cell axons away from the posterior and towards the anterior region of the optic tectum in the chick (Monnier et al., 2002; Rajagopalan et al., 2004). RGMs contain an N-terminal signal peptide, a tri-amino acid motif, an RGD site, a partial von Willebrand factor type D domain and a c-terminal GPI anchor (Fig. 4; Monnier et al., 2002). Three RGM family members have been described in the mouse, RGMa and RGMb being expressed in a complementary manner in the nervous system while RGMc is expressed in striated muscle and in liver (Niederkofler et al., 2004; Schmidtmer, 2004; Schmidtmer and Engelkamp, 2004; Oldekamp et al., 2004). All three RGMs have been shown to act as BMP co-receptors in various contexts (Fig. 6; Babitt et al., 2005, 2006, 2007; Samad et al., 2005; Kuns-Hashimoto, 2007; Kanomata et al., 2009).

RGMs have been implicated in very different biological processes in vertebrate development and physiology. As previously referred, axon guidance roles have been described for RGMa in Xenopus (Wilson and Key, 2006) but only *in vitro* evidence has been obtained in mouse (Brinks et al., 2004; Metzger et al., 2007). In compensation, RGMs have been shown to participate in processes ranging from neural tube morphogenesis to Iron metabolism in hepatic cells in the mouse.

Niederkofler et al. (2004) have shown that ablation of RGMa leads to cephalic neural tube closure defects in the mouse. Later work on zebrafish and *Xenopus* embryos (Mawdsley et al., 2004; Gessert et al., 2008; Kee et al., 2008) shows that
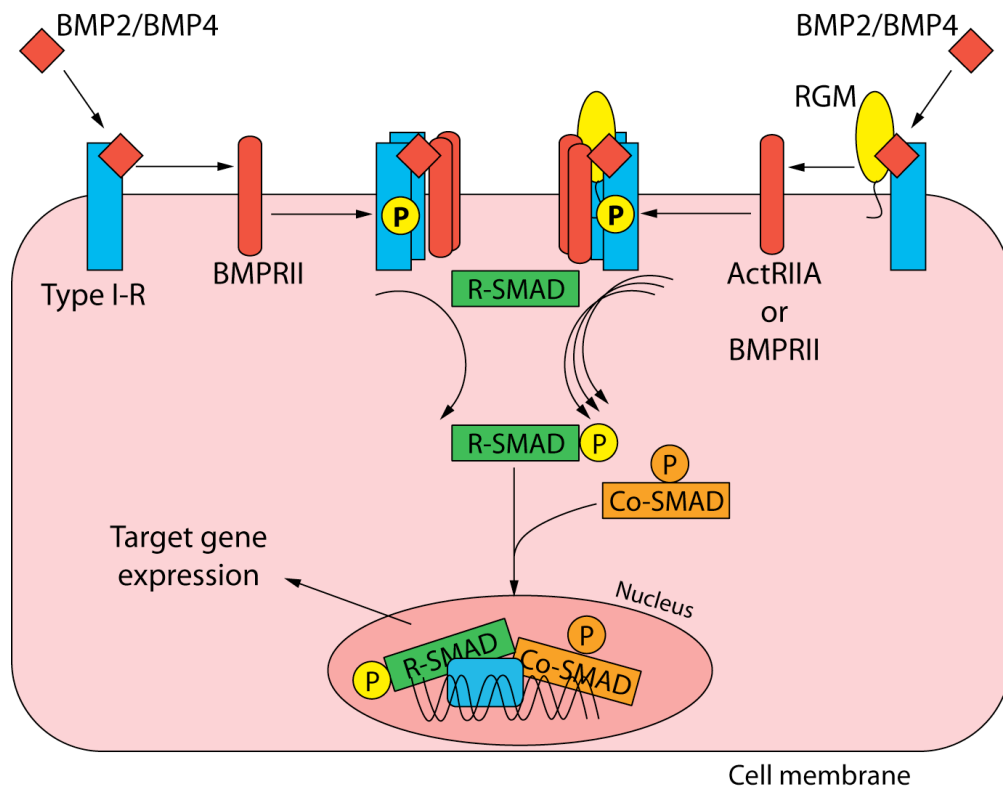
Figure 6. RGMs are BMP co-receptors. Modified from Corradini et al., Cytokine Growth Factor Rev 2009. RGMs have been shown to be co-receptors for BMP4 and BMP2 in different contexts. Primarily, RGMs have been shown to enhance the sensitivity of cells to low dosages of BMPs. An alternative way in which RGMs modulate BMP signals is to expand the BMP receptor pool to receptors that are usually second choice, namely the activin receptor type II A (ActRIIA). This modulation leads to an increased rate of SMAD phosphorylation and concomitantly to increased expression of target genes.

RGMa/Neogenin interaction plays an essential role in establishing cell polarity necessary for successful neural tube morphogenesis. The loss of polarity found in deep layer cells of the neural plate in RGMa and Neogenin morphants was shown to be due to disruption of the microtubule cytoskeleton.

Loss of polarity was shown to lead to an inability of these cells to interact with their neighbour cells and concomitantly to engender the neural fold elevation movements necessary for neural tube formation. Although this has not been shown in the mouse, it is likely that similar events lead to the phenotype found in RGMa null mice. RGMa has also been shown to modulate BMP2- and BMP4-elicited signaling. RGMa expression was shown to expand the range of BMPRs transducing BMP signals leading to increased SMAD activation. In the presence of RGMa activin receptor type II (ActRIIA) presents higher sensitivity BMPs and contributes to the overall BMP

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signaling leading to increased levels of activated SMADs and increased expression of target genes (Xia et al., 2009).

RGMc (also called hemojuvelin or HFE2) has mainly been described as participating in iron metabolism being expressed almost exclusively in striated muscle and the liver (Huang et al., 2005; Babitt et al., 2006; Bae et al., 2008). RGMc has been shown to regulate the expression of the peptide hormone Hepcidin (Lin et al., 2005; Babitt et al., 2006, 2007), a direct inhibitor of Ferroportin and a major regulator of iron homeostasis (Ganz, 2005). Although there are contradictory data as to the effect of BMP signaling through RGMc, the best current model for regulation of Hepcidin expression in hepatocytes proposes a requirement for Neogenin to form a complex with RGMc, BMP receptor I and II homodimers and BMP2 or 4 for effective signaling. Such a complex would then lead to the activation of SMADs and consequently to upregulation of Hepcidin, with the Neogenin intracellular domain possibly contributing to the signaling (Kuns-Hashimoto et al., 2008; Yang et al., 2008; Zhang et al., 2009).

RGMb (also called DRAGON) has mainly been implicated in BMP signaling (Samad et al., 2005; Kanomata et al., 2009) with axon guidance functions only lately uncovered in the context of spinal cord injury (Liu et al., 2009). RGMb binds BMP2 and BMP4 but no other TGFß ligands and has been shown to enhance BMP signaling by associating with BMP receptors (BMPRs) in vitro (Samad et al., 2005). RGMb is expressed in the neural tube in the developing mouse and *Xenopus* in a pattern coinciding with BMP type I and BMP type II receptors and was found to lead to ectopic neuron increase and neural crest cell decrease when overexpressed in *Xenopus* embryos. BMP2 and BMP4 binding to RGMb was shown to be independent of BMPR presence but BMP/RGMB signaling was shown to be dependent on BMPR function leading to activation of the SMAD1 pathway in presence of BMPR receptors *in vitro* and *in vivo*. Concordant with these results, microinjection of RGMb into two-cell stage *Xenopus* embryos leads to upregulation of a set of anterior markers and a decrease in neural crest cell markers when compared to the uninjected tissues. When expressed at the cell surface RGMb is postulated to increase the sensitivity of cells to low concentrations of BMPs in this context, a mechanism with likely implications in tissue patterning. These results indicate that RGMb, by modulating

BMP sensitivity in the developing *Xenopus* neural tube, acts on patterning in this tissue by promoting a neural fate rather than a neural crest cell fate when expressed. Although the details of the signaling implicated remain to be uncovered, it is likely that formation of a heteromer with Neogenin, as described for RGMc, is involved, as Neogenin has been shown to be expressed in the developing neural tube (Gessert et al., 2008; Kee et al., 2008). Representative of the multiplicity of roles played by RGM family members in different tissues and different species, a recent publication reports RGMb as inhibiting, rather than modulating or enhancing, BMP signaling in the C2C12 myoblast cell line (Kanomata et al., 2009). In these cells RGMb was shown to actually suppress BMP-elicited SMAD transcriptional activity in a process independent of Neogenin and implicating a yet to be defined binding partner for RGMb at the cell surface. Additionally, Samad et al. (2004) found RGMb expressed in DRGs playing a role in neuron adhesion in this tissue, through homophilic interactions and Conrad et al. (2009) showed a role for RGMb in guiding Neogenin-expressing granule precursor cells in the dentate gyrus.

More recently, both RGMa and RGMb have been found to be upregulated at the lesion site after spinal cord injury preventing axon regrowth (Hata et al., 2006; Liu et al., 2009). Blocking of RGMa activity by intrathecal administration of a function-blocking antibody has proven effective in restoring axon growth with concomitant enhanced locomotor recovery in hemisectioned rats (Hata et al., 2006). In parallel, RGMb was shown to be expressed in neurons and oligodendrocytes in the spinal cord and to be part of the myelin-derived inhibitor signal, being upregulated at the site of injury after spinal cord hemisection (Liu et al., 2009).

Interestingly, recent work by Hata et al. (2009) has for the first time shown Neogenin to be joined by the Netrin receptor Unc5B as an RGMa co-receptor as well as the implication of leukemia-associated guanine nucleotide exchange factor (LARG) in RhoA signaling by this complex, bringing new insight on previously described signaling mechanisms as well as on a molecular basis for the multiplicity of functions as yet described for Neogenin and its RGM ligands. Unc5B was found to be constitutively bound to Neogenin and to be required for RGMa-elicited RhoA activation and growth cone collapse in cultured rat cortical neurons. The PDZ

domain-containing Rho guanine nucleotide exchange factor (RhoGEF) LARG was found to associate with Unc5B but not Neogenin in rat cortical neurons. LARG is a substrate of FAK, constituting a good candidate for phosphorylation by FAK bound to the Neogenin P3 intracellular domain in an Unc5B/Neogenin/LARG complex. Indeed LARG was shown to be required for RhoA activation and growth cone collapse downstream of RGMa and FAK was shown to participate in RGMa-elicited RhoA activation *in vitro* as well as *in vivo*.

Although a fair amount of knowledge has accumulated as to the role of Netrins at the level of OB projections to the olfactory cortex, this family of Neogenin ligands has only been found to participate in early events of OE development. Work in the rat has shown Netrin-1 to be evenly expressed in the mesenchyme of the OE lamina propria contiguous to the basal OE, with an peak of expression around embryonic age E13 and downregulation at E19 with low post-natal expression (Astic et al., 2002). Concomitantly, DCC is transiently expressed in OSNs in a time window corresponding to the peak in Netrin-1 expression. High DCC expression was found at E13 and expression decreased at E15, suggesting a role in initiation of OSN axon projection leading to but stopping at the stage when OSN axons are robustly reaching the OB nerve layer. Interestingly, although both Netrin-1 and DCC are downregulated before birth, they were shown to be upregulated following bulbectomy. This suggests a recapitulation of embryonic conditions to establish an attractive/permissive environment promoting new axon growth in an extensive regeneration context where massive OSN death has left the OE devoid of pioneer axons leading the way to the OB. This time-course study hasn't been performed in the mouse, but both Netrin-1 and DCC have been shown to be expressed at E12 in the murine OE and to regulate the migration of luteinizing-hormone releasing-hormone (LHRH) neurons from the VNO to the basal forebrain (Schwarting et al., 2001, 2004). Although these studies have shown defects in vomeronasal nerve targeting in Netrin mutant mice, no OE axon targeting penotypes have been reported in these mice.

1.5 PROJECT AIM

In light of the involvement of the Neogenin receptor and its RGM ligands in neuronal development and regeneration, we have examined their role in the development of the olfactory system. The olfactory system is a model system of choice for the study of neurogenesis and axon guidance, in normal as well as in regeneration paradigms. We examined the role of Neogenin in the development of the olfactory system by

1) Determining the detailed patterns of expression of Neogenin and its RGM ligands in the olfactory system, with particular attention to developmentally regulated expression in the OSN lineage. We have assessed general expression patterns by *in situ* hybridization and have made use of mouse genetics tools to further elucidate finer details of expression in different cell populations in the OE.

2) Examining the requirement of Neogenin for the generation of mature OSNs *in vivo*. In light of the expression patterns we have observed for Neogenin and RGM-B in the olfactory epithelium, we explored possible functions for Neogenin in the context of OSN development by analyzing phenotypes affecting different populations of OSN lineage cells in a *neogenin* gene-trap mouse line.

3) Investigating the role of Neogenin in axon guidance of olfactory projections *in vivo*. Our observation that RGM ligands are expressed in the OB suggested they may regulate the targeting of axons through interactions with Neogenin. We therefore assessed the targeting accuracy of OSN projections to the OB in a *neogenin* gene-trap mouse line.

CHAPTER 2

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 TISSUE HARVEST AND CONSERVATION

MOR-174-9-IRES-tau-GFP, SP1-IRES-tau-GFP, P2-IRES-tau-LacZ (Mombaerts et al., 1996) transgenic mouse lines used in this study were graciously provided by Dr. Tyler Cutforth.

ES cells containing the Neogenin gene-trapped allele (Mitchell et al., 2001; Leighton et al., 2001) were obtained from Bay Genomics (ES cell line KST265) and were expanded, introduced in C57BL/6 mouse blastocysts that were then re-implanted in oestrous females. Chimeric mice were then selected in the progeny and bred. Progeny from the chimeras was then genotyped using primers against Neomycin. Chimeric mice where germ line transmission was verified were then used as founders for the colony. Genotyping of these mice for distinction between those carrying one copy and those two copies of the gene-trap allele was achieved by performing an X-Gal staining on 1mm long tail snips, for post-natal mice, and on a forelimb for embryonic ages.

For all mouse work at embryonic ages, crosses were set up with one male and two females per cage and embryonic day 0 (E0) was considered to be the date of vaginal plug.

2.1.1 FOR *IN SITU* HYBRIDIZATION

To prevent RNA degradation tissues used exclusively for in situ hybridization were frozen shortly after harvest. E18 embryos and post-natal day 10 (P10) pups were dissected, washed in ice-cool PBS, covered in OCT (Tissue Tek Optimal Cutting Temperature – Sakura Finetek) and immediately flash frozen without fixation in 2-methyl butane cooled with dry ice. Frozen tissue was then kept at -80°C until used.

E18 mouse embryos were fixed by immersion for 1h in ice-cold 4% PFA in PBS then cryoprotected in 30% sucrose in PBS at 4°C. After anaesthesia, P10 and adult mice were transcardially perfused with ice-cold 4% PFA in PBS, post-fixed 1h in fixation solution. Tissue was then cryoprotected in 30% sucrose in PBS at 4°C, covered in OCT compound, flash frozen in a mix of 2-methylbutane and dry ice and kept at -80°C.

2.2 *IN SITU* HYBRIDIZATION

2.2.1 PROBES

RgmA and *rgmB* probe constructs were obtained from Dr. Dieter Engelkamp (Schmidter and Engelkamp, 2004) and Neogenin probe construct was obtained from Dr. Jeroen Pasterkamp. Non-radioactive in situ hybridization probes were synthesized using the Roche Dig-labelling kit. Following single endonuclease digestion immediately upstream (antisense probe) and downstream (sense probe) of the cDNA sequences we then made use of promoters included upstream and downstream of these sequences to synthesize riboprobes. Appropriate RNA polymerases were used to transcribe sense and antisense cRNA probes. Inclusion of modified 2' deoxyuridine- 5' triphosphate containing a digoxigenin group (Dig-dUTP) in the transcription mix leads to synthesis of Dig-marked riboprobes.

Riboprobe synthesis Protocol: 10 μg of plasmid containing the template cDNA were digested for 2h at 37°C with 30 units of a single restriction enzyme in a 20 μl reaction. One enzyme was used to cleave the plasmid immediately upstream of the cDNA sequence and a different one immediately downstream, to allow for synthesis of a sense (control) and an antisense cRNA riboprobe, respectively. Digests were cleaned twice by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, precipitated by addition of 1:10 vol:vol of 3M sodium acetate pH 5.2, and 2.5 volumes of 100% ethanol. After 5 min centrifugation at 8000 rpm, pellet was washed with 70% ethanol

(prepared with diethylpyrocarbonate (DEPC) treated dH$_2$O), air dried 15 min and resuspended in 20 μl DEPC-treated dH$_2$O. Digestion efficiency was assessed by agarose gel electrophoresis of purified digests alongside intact plasmid. In vitro transcription followed using 2 μl of the purified digests. The Roche Dig RNA labelling protocol was used. In vitro transcription occurred in 20 μl in 1x Transcription buffer (Roche) with 2 μl of template digest, 2 μl Dig-RNA labelling mix (Roche #11277073910), 1 μl Protector RNase inhibitor (Roche #03335399001) and 2 μl of T3 or T7 RNA polymerase (Roche # 11031163001, #10881767001) in DEPC-treated dH$_2$0, during 2h at 37°C. Transcription mixes were then subjected to acid hydrolysis for a duration that was subject to optimization in order to obtain average probe sizes around 300 bp, enhancing tissue penetration while maintaining specificity. Hydrolyzed probes were run alongside non-hydrolyzed in agarose gel electrophoresis to assess size and amount. Probes were then precipitated by adding 1:10 vol:vol of 4 M lithium chloride and 2.5 volumes of 100% ethanol to the probe mix. Riboprobes were allowed to precipitate overnight at -80°C. Precipitate was then centrifuged 20 min at 14000 rpm 4°C, pellet was washed with 70% ethanol (DEPC-dH$_2$O) and finally resuspended in 20 μl DEPC-treated dH$_2$O and kept at –80°C until used.

2.2.2   IN SITU HYBRIDIZATION PROTOCOL

Fresh frozen tissue was sectioned at 20 μm and slides allowed to air-dry for 1h. Sections were then fixed for 20 min in PBS with 4% PFA, washed three times with PBS and acetylated for 10 min. After 2 washes with PBS and one wash with 2x SSC sections were prehybridized 3h at 60°C in hybridization solution (5x Denhardt's, 250 μg/mL baker's yeast tRNA, 5x SSC, 50% formamide). Hybridization was performed overnight at 60°C with probes diluted in hybridization solution. Stringency washes were then performed at 60°C and consisted in 5 min in 5x SSC, 1 min in 2x SSC and30 min in 0.2x SCC in 50% formamide. All solutions up to this point were prepared with DEPC-treated ddH$_2$O. Sections were then washed in 0.2x SCC at room temperature and immunological detection of the digoxigenin-marked probes

was performed. Sections were washed 5 min in buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl), incubated 1h in 1% blocking reagent (ROCHE), washed 5 min in buffer 1, then incubated 3h with a 1:3000 dilution of anti-Dig-AP Fab fragments in buffer 1. Excess antibody was removed by washing twice with buffer 1. Colour reaction was prepared with a 5 min wash with buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl2). Sections were then incubated with NBT/BCIP reagent diluted 1:50 in buffer 2 and colour reaction allowed to occur until optimal staining was obtained. Reaction was stopped by extensive wash in PBS and coverslips were mounted on slides with a mounting medium containing 25% w/vol Glycerol and 10% w/vol MOWIOL 4-88 compound (Calbiochem #475904) in 0.1 M Tris-HCl pH8.5. Stained tissue was imaged with a Zeiss Axio Imager M1 microscope coupled with a Qimaging Retiga EXi CCD camera through the Northern Eclipse software (Empix Imaging).

2.3 IMMUNOHISTOCHEMISTRY

2.3.1 ANTIBODIES

Primary antibodies used were as follows. Goat anti-OMP antibody (WAKO #544-10001) was used at a dilution of 1:5000. Mouse anti-ß-III tubulin antibody (Promega #9PIG712) was used at 1:6000 dilution. Mouse anti-Ki-67 antibody (BD Pharmingen #556003) was used at 1:300 dilution. Rat anti-BrdU antibody (Abcam # Ab6326) was used at 1:250 dilution. Rabbit anti-GFP antibody (Invitrogen, Carlsbad, CA #A6455) was used at 1:250 dilution.

Secondary antibodies were Donkey anti-goat Alexa 546, Donkey anti-mouse Alexa 488, Donkey anti-rabbit Alexa 488, Donkey anti-Rat Alexa 488 (Invitrogen, Carlsbad, CA) and were all used at a 1:500 dilution.

2.3.2 IMMUNOHISTOCHEMISTRY PROTOCOL

10 μm or 20 μm cryosections of fixed tissue were set onto superfrost plus slides (VWR) and allowed to air-dry for 1h. Sections were then washed 2x5 min with TBS (150 mM NaCl, 50 mM Tris:HCl pH 7.6). For anti-Ki67 immunostaining, sections were subjected to antigen retrieval by microwaving 10 times 30 s in 100mM Tris:Cl, pH 10. Sections were then blocked 1h at room temperature with TBS, pH 7.5, 10% foetal bovine serum (FBS), 0.5% Triton X-100. Primary antibodies were diluted in blocking solution and incubated with sections overnight at 4°C in a humidified chamber. Sections were washed three times 5 min with TBS before addition of the appropriate secondary antibodies diluted in blocking solution and incubated for 1h at room temperature in a humidified chamber. Sections were then washed once with TBS, once with Hoechst 1:4000 in TBS, twice with TBS, then coverslips were mounted with Fluoromount G (Southern Biotech, Birmingham, AL). All immunohistochemistry was imaged with a Zeiss Axio Imager M1 microscope coupled to a CCD camera (Qimaging Retiga EXi) and a fluorescence and using the Northern Eclipse software.

2.4 X-GAL STAINING

Sections were washed 5 min in buffer A (0.1 M PO_4 buffer, pH 7.4, 2 mM $MgSO_4$, 5 mM EGTA) then 10 min in buffer A and twice 5 min in buffer B (0.1 M PO_4 buffer, pH 7.4, 2 mM $MgSO_4$, 0,01% sodium deoxycholate, 0.02% NP-40). Sections were then incubated in buffer C (0.1 M PO_4 buffer, pH 7.4, 2 mM $MgSO_4$, 0,01% sodium deoxycholate, 0.02% NP-40, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/mL X-Gal), which contains X-Gal, a substrate of ß-galactosidase that produces blue colour when cleaved by this enzyme. Staining was allowed to develop until colour intensity was optimal. Slides were then washed in 1x PBS and coverslips mounted with a mounting medium containing 25% w/vol Glycerol and 10% w/vol MOWIOL 4-88

compound in 0.1 M Tris-HCl pH8.5. Stained tissue was imaged with a Zeiss Axio Imager M1 microscope coupled with a Qimaging Retiga EXi CCD camera through the Northern Eclipse software (Empix Imaging).

2.5 BRDU INCORPORATION / QUANTIFICATION

Pregnant females and post-natal mice were injected intra-peritoneally with 50 μg BrdU per gram of body weight. Animals were sacrificed 3h post-injection. Fixation was performed by 1h immersion in ice-cold 4% PFA in PBS for embryos and by transcardial perfusion followed by 1h post-fix for post-natal mice. Tissue was then cryoprotected in 30% sucrose in PBS and flash frozen in a mix of 2-methylbutane and dry ice and kept at -80°C. 10 μm Sections were rehydrated with PBS, washed with ddH20 then subjected to antigen retrieval by 20 min incubation with 10 mM sodium citrate, pH 6.0, at 90°C and denaturation, by incubation with 2N HCl, 10 min on ice, 10 min at room temperature and 20 min at 37°C. Sections were then sequentially washed with TBS, pH 8.3 and TBS, pH 7.5. Immunohistochemistry was performed as described above, using as primary antibody the Rat anti-BrdU (Abcam # Ab6326) and as secondary antibody Donkey anti-Rat Alexa 488 (Invitrogen, Carlsbad, CA).

For embryonic age tissue septal OE was imaged under the fluorescence microscope and BrdU positive cells in the basal aspect of the OE on both sides of the OE septum were counted using the Northern Eclipse 7.0 software (Empix Imaging). Additionally the length of the analyzed OE was measured with the same software. For P10 mice counts were performed in a rostral area of the turbinates where two straight stretches of OE are found. One complete slide was counted per animal taking care of selecting sets of sections in the same rostro-caudal position in the OE. Counts were normalized to total length counted and averaged between mice.

2.6 TUNEL STAINING

TUNEL staining was performed on 10 μm coronal sections of E18 OE, using the TACS 2 TdT-Fluor in situ apoptosis detection kit (R&D Systems # 4182-30-K). Sections were rehydrated by washing in 100%, 90% and 70% ethanol, 5 min each. Rehydrated sections were washed 10 min in 1x PBS, then incubated 15 min with 50 μl of proteinase K solution, covering the slide with parafilm. After two 2 min washes in deionized water, slides were immersed in 1x Terminal deoxynucleotidyl transferase (TdT) labelling buffer, then covered with 50 μl labelling reaction mix containing biotinylated dNTPs, TdT enzyme, 1x Mn^{2+}, in 1x TdT labelling buffer. TUNEL reaction took place during 1 h at 37°C in a humidified chamber. Slides were then immersed in 1x TdT Stop buffer for 5 min and washed twice for 2 min with deionized water. Slides were then covered with 50 μl of Streptavidin-Fluor solution with parafilm and incubated 20 min at room temperature in the dark. Slides were then washed two times 2 min with 1x PBS and mounted with Fluoromount G.

OE sections were imaged under the fluorescence microscope and TUNEL-positive cells were counted using the Northern Eclipse 7.0 software (Empix Imaging).

2.7 WHOLE MOUNT OLFACTORY BULB PREPARATIONS

2.7.1 X-GAL STAINING

Adult mice of the desired genotype were anaesthetized with 2x avertin (2,4% w/v 2, 2, 2-tribromoehtanol, 1.55% vol/vol tertamyl alcohol), euthanized, and their crania dissected to reveal the olfactory bulbs and olfactory epithelium. Tissue was then fixed 20 min in ice-cold 4% paraformaldehyde (PFA) then washed 5 min in buffer A (0.1 M PO_4 buffer, pH 7.4, 2 mM $MgSO_4$, 5 mM EGTA) then 25 min in buffer A and twice 5 min in buffer B (0.1 M PO_4 buffer, pH 7.4, 2 mM $MgSO_4$, 0,01% sodium deoxycholate, 0.02% NP-40), all at room temperature. Colour reaction was then performed in pre-warmed buffer C (0.1 M PO_4 buffer, pH 7.4, 2 mM $MgSO_4$, 0,01%

sodium deoxycholate, 0.02% NP-40, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/mL X-Gal) at 37°C until optimal staining was attained. Photos were taken using a Zeiss Stemi 2000 dissection microscope coupled to a Qimaging Retiga EXi CCD camera, through the Northern Eclipse software (Empix).

2.7.2 EPIFLUORESCENCE

Adult mice of the desired genotype were anaesthetized with 2x avertin, euthanized, and their crania dissected to reveal the olfactory bulbs and olfactory epithelium. The tissues thus dissected were placed under an epifluorescence microscope and imaged to reveal lateral and medial glomeruli. Angles and distances were measured in the Northern Eclipse 7.0 (Empix) software.

2.8 STATISTICAL TREATMENT OF DATA

In all quantification results presented, averages were compared by two-tailed unpaired t-test. Statistical treatment of the data was performed with the InStat program (Graphpad Software).

CHAPTER 3

RESULTS

3 RESULTS

3.1 EXPRESSION OF *NEOGENIN* AND ITS *RGM* LIGANDS IN THE MURINE OE

3.1.1 EXPRESSION OF *NEOGENIN* IN THE OE

In order to asses the possible role of Neogenin in the development of the mammalian olfactory system, we investigated the pattern of expression of this axon guidance receptor and of its RGM ligands in the murine OE and OBs.

Expression studies for these molecules were performed at two ages - embryonic age E18, when OE axons are actively projecting towards their OB target fields and the first glomerular structures are being formed, and postnatal age P10 when the olfactory system has reached maturity, normal OSN turnover is under way and the OB glomerular layer is well defined. Importantly, at both stages studied OE stratification is established and well detectable.

We initially assessed the pattern of expression of *neogenin* (neo) in the OE by in situ hybridization on coronal OE sections of fresh frozen E18 and P10 CD1 mice. To further characterize the subpopulations of cells expressing Neogenin we used a mouse line expressing lacZ in cells where *neogenin* is transcribed. Further description of this mouse line is found in section 3.2. Both approaches revealed Neogenin is strongly expressed in the OE in more apical layers of the OE and in the basal-most layers of this tissue while presenting weak expression in intermediary layers of the OE (Fig. 7). In situ and X-Gal signals are found in basal layers of the OE adjacent but not contiguous to the OE lamina propria, placing Neogenin as highly expressed in an area populated by the GBC population, the progenitor cells at the origin of the OSN lineage. Immunohistochemistry against Ki67 (Fig. 7 F), a cell cycle marker, performed on consecutive sections to the ones stained with X-Gal, confirms that the basal layers expressing Neogenin correspond to basal dividing cells in the OE.
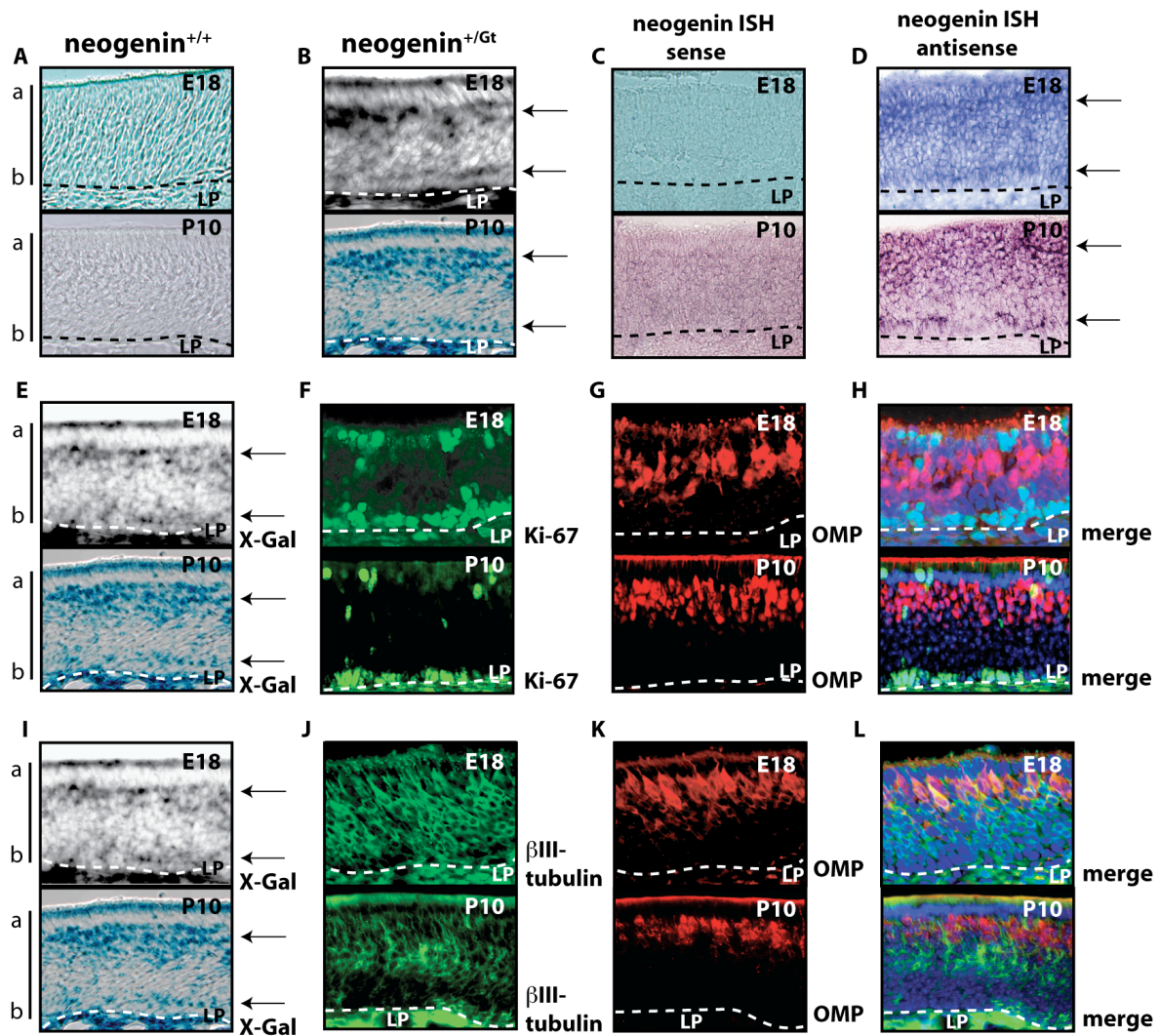
Figure 7. Expression of *neogenin* in the olfactory epithelium. ***A,B,E,I***, X-Gal staining on sections of OE from *neogenin* wild-type (***A***) and *neo*[+/Gt] (***B***) E18 embryos and P10 pups. ***C,D***, *In situ* hybridization with *neogenin* sense (***C***) and antisense (***D***) Dig-labeled cRNA probes on sections of OE from *neogenin* wild-type E18 mouse embryos and P10 pups. Both at embryonic and post-natal ages *neogenin* transcript is found in cells located in the apical and basal regions of the OE (arrows), as well as Neogenin protein expression as assessed by ß-galactosidase activity in mice carrying a gene-trap allele leading to expression of *lacZ* in cells where *neogenin is transcribed*. ***E-L***, X–Gal staining (***E,I***) and immunostaining with Ki-67 (***F***), ßIII-tubulin (***J***) and OMP (***G, K***) on adjacent sections of *neo*[+/Gt] OE (***H, L*** – merged). Panels ***E*** and ***I*** show the same two E18, and P10 X-Gal stained sections. Sections shown on panels ***F–H***, and ***J–L*** are sections immediately adjacent to the ones on ***E*** and ***I***. High Neogenin expression is detected in layers populated by precursor and mature OSNs of the OE while low expression levels are found in intermediary layers populated by immature OSNs. LP: Lamina propria.

Additionally, the globous morphology of the cells where *neogenin* expression is found is characteristic of globous basal cells (GBCs) rather than horizontal basal cells (HBCs), which are closely apposed to the lamina propria and present a flattened morphology. Immediately apical and adjacent to the GBCs, as a result of cell cycle exit of recently differentiated INPs, starts the portion of the OE containing the differentiated cells of the OSN lineage. As the pseudo-stratified structure of the OE reflects the OSN lineage timeline, early progenitor stage GBCs are found in the basal aspect with terminally differentiated OSNs at the apical-most aspect of the OE, below the sustentacular cell layer. These cells can be distinguished from immature OSNs through their expression of OMP. Although some co-immunostaining can occur in OSNs having recently achieved terminal differentiation, the intermediate layers of the OE populated by immature OSNs can be visualised by ßIII-tubulin immunoreactivity associated with absence of OMP immunoreactivity. X-Gal stained OE sections compared to consecutive sections immunostained with OMP (Fig. 7 G, K) and ßIII-tubulin (Fig. 7 J) antibodies place the apical region of high Neogenin expression at the level of the mature OSN domain and the intermediary region of low Neogenin expression at the level of the immature OSN domain of the OE.

3.1.2 EXPRESSION PATTERNS OF *RGMA* AND *RGMB* IN THE OLFACTORY SYSTEM

To assess the pattern of expression of the Neogenin ligands RGMa and RGMb in the OE we performed in situ hybridization in parallel with immunohistochemistry against Ki67, ßIII-tubulin and OMP on consecutive coronal OE sections from E18 and P10 CD1 mice. (Fig. 8). In agreement with the literature that states that RGMa and RGMb are present in complementary and seldom overlapping patterns of expression in the central nervous system, we found only one of these two family members expressed in the OE. Our in situ results (Fig. 8 C, E), when compared with the Ki67 immunohistochemistry (Fig. 8 D), reveal that, at their basal-most, the cells expressing high levels of *rgmB* in the OE are located adjacent and apically to the basal GBC layers. The apical-most *rgmB* in situ signal, when compared to OMP immunostaining (Fig. 8 F), appears to constitute a boundary adjacent but

immediately basal to the OMP-positive mature OSN layers. This positions the domain of high *rgmB* expression in the immature OSN population of the OE.
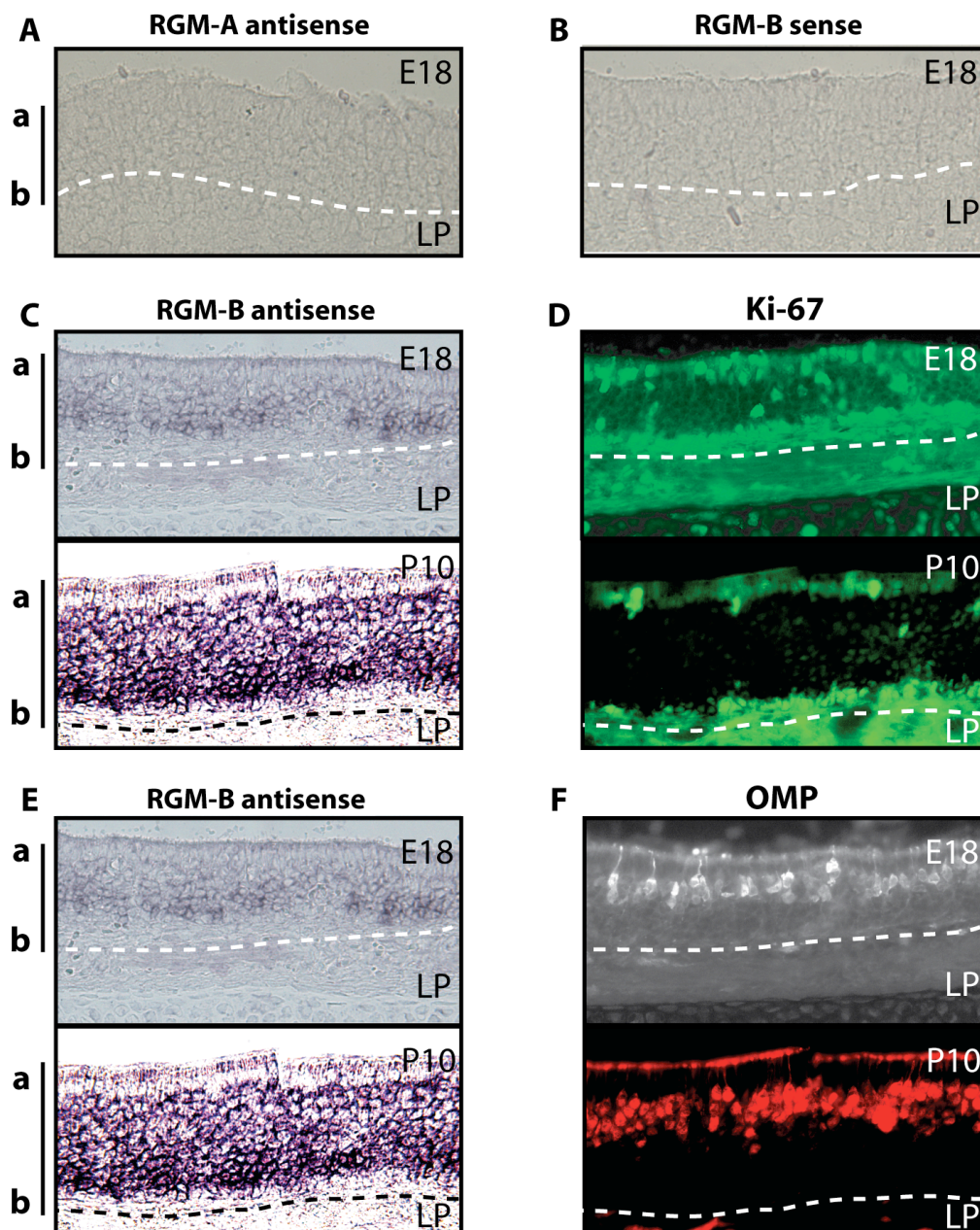
Figure 8. Expression of *rgmA* and *rgmB* in the olfactory epithelium. **A-C, E**, *In situ* hybridization with Dig-labeled cRNA probes and immuno-histochemistry (**D, F**) on sections of OE from wild-type E18 mouse embryos and P10 mouse pups. *RgmA* is not detected in the OE (**A**), while high *rgmB* expression is detected in cells located in the basal region of the OE (**C, E**). Panels **C** and **E** show the same two E18, and P10 sections. Sections shown on panels **D**, and **F** are sections immediately adjacent to the ones on **C** and **E**. Adjacent sections were immunostained with Ki-67 (**D**), and OMP (**F**). No *rgmB* is detected in the most basal (b) OE layers that contain ONS precursor cells and very low expression levels are detected in the most apical (a) region that contains mature OSNs. LP: Lamina Propria.

The patterns of expression observed for *neogenin* and *rgmB* in the murine OE are particularly interesting in that they appear to be complementary in the three separate domains across the apicobasal axis of the OE. While Neogenin is highly expressed in basally located progenitor cells and in apically located mature OSNs, RGMb is most highly expressed in immature OSNs located in the middle layer of the OE (Fig. 9 inset). This pattern of expression is rather interesting as the boundaries where high expression of *neogenin* and *rgmB* overlap correspond to transition steps in the OSN lineage, namely the initial differentiation from immediate neuronal precursor (INP) to immature OSN, in the basal boundary, and the terminal differentiation from immature OSN to mature OSN in the apical boundary. It is therefore possible that RGMb/Neogenin interactions may regulate the development of OSNs in the OE. Furthermore, the high levels of expression of Neogenin in mature OSNs suggests that Neogenin may additionally play a role in the guidance of projections from these neurons towards the OBs. The possibilities posed by the pattern of expression found in the OE are simplified in figure 9.

3.2 GENERATION AND CHARACTERIZATION OF *NEOGENIN* GENE-TRAP MICE

To examine the role of Neogenin during development of the nervous system in vivo we took a mouse genetics approach taking advantage of a Neogenin gene-trap ES cell line available commercially.

The KST-265 mouse ES cells, which have previously been shown to generate chimeric mice with efficient germ-line transmission, were acquired from Bay Genomics and pronuclear injections were performed at the transgenic facility of the Centre de Recherches du CHUM (Centre Hospitalier de l'Université de Montréal). Chimeric mice were obtained and bred to generate heterozygous animals through germ-line transmission. This ES cell line contains a gene-trapped Neogenin allele which leads to the retention of a truncated form of Neogenin in the ER. In addition, the cassette inserted in an intron of the neogenin allele drives expression of beta-galactosidase (ß-gal) and placental alkaline phosphatase (PLAP).
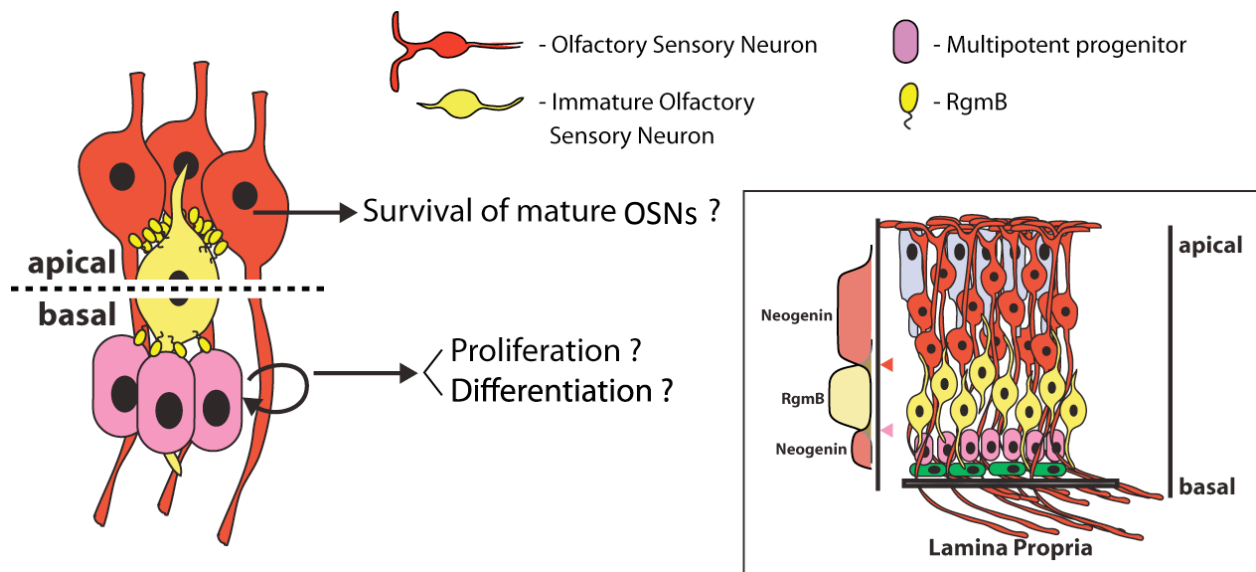
Figure 9. Model for Neogenin and RGMb function in the OE. The patterns of expression found for Neogenin and its ligand RgmB in the OE engenders two boundary regions where high RgmB/Neogenin expression overlaps (inset, arrowheads). These boundaries correspond to the layers in the OE where INPs are differentiating into immature OSNs (pink arrowhead) and where immature OSNs are going through terminal differentiation into mature OSNs (red arrowhead). The organization of the OE as a pseudo-stratified epithelium allows for an inter-mingling of cells at different stages of the OSN lineage. Namely, immature OSNs may be in contact with basal progenitor cells and with apical mature OSNs either independently or simultaneously. The simplified diagram on the left represents the signaling possibilities for Neogenin and RgmB in this tissue, according to what has been described as roles for Neogenin in other tissues. Note: The immature OSN (yellow) is represented as being adjacent to progenitor as well as mature OSNs for ease of representation.

We characterized the Neogenin gene-trap mouse line to determine if Neogenin expression is fully ablated in this mouse. Three sets of primers (Fig. 10 B - A, B, C) were used for reverse-transcriptase polymerase chain reaction (RT-PCR) on RNA extracts of wild-type (WT), *neo*[+/Gt] and *neo*[Gt/Gt] mice. Primers A target a sequence upstream of intron 7 and should lead to amplification in all genotypes. Primers B target a sequence straddling the gene-trap cassette insertion point and should lead to amplification in RNA from tissues carrying at least one copy of the Neo[Gt] allele and to no amplification in the WT. Primers C target a sequence downstream of the gene-trap cassette and should lead to amplification in RNA from tissues expressing at least one WT allele and to no amplification in the *neo*[Gt/Gt] RNA.

Figure 10. Characterization of the *neogenin* gene-trap allele from BayGenomics KST265 ES cells (Leighton et al, 2001). **A**, The gene-trap DNA construct is inserted in intron 7 of the *neogenin* locus. Splicing of the gene-trap cassette with coding sequences upstream of the insertion site leads to a fusion transcript containing the coding region of ß-geo (a fusion between the ß-galactosidase and neomycin phosphotransferase), an internal ribosomal entry site (IRES), and the coding region of human placental alkaline phosphatase (PLAP). As a result a ß-geo fusion protein is expressed and retained within an intracellular compartment in the cell body, and PLAP is expressed and present along the axons at the cell surface. The expression of the ß-geo fusion protein provides a convenient way to identify neogenin-expressing neurons in heterozygous mice. Furthermore, axonal projections of neogenin-expressing neurons can be visualized by alkaline phosphatase staining in heterozygous mice. However, this approach presents the caveat that axonal projections observed in heterozygous mice may not be completely representative of projections normally observed in WT mice. (Continued on next page)
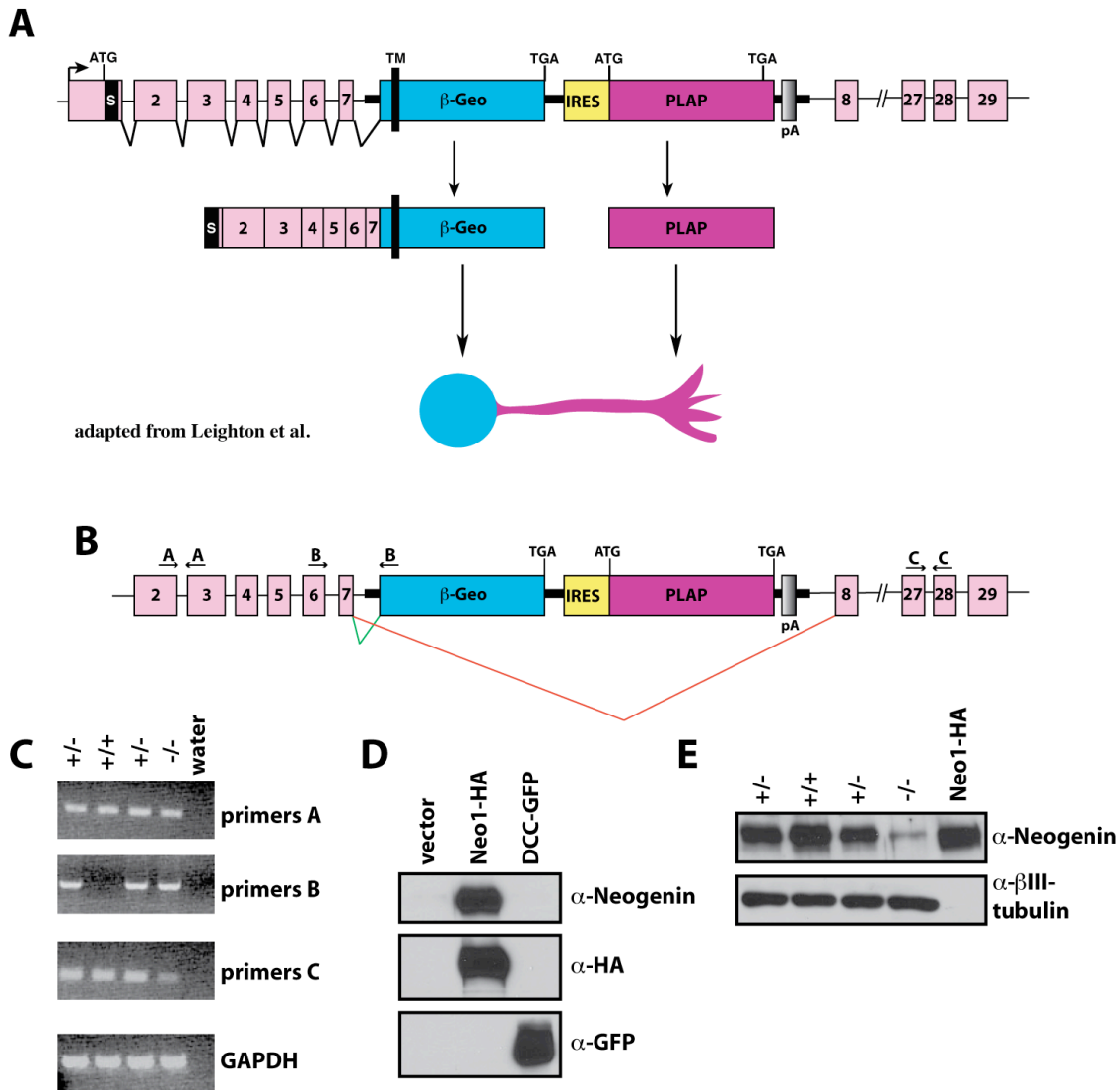
Figure 10 (cont.). Characterization of the *neogenin* gene-trap allele from BayGenomics KST265 ES cells (Leighton et al, 2001). ***B***, Diagram of the site of insertion of the gene-trap cassette inside the *neogenin* genomic locus. The positions of three sets of primers used for RT-PCR analysis are also indicated. Aberrant splicing around the gene-trap cassette (red) may lead to expression of full-length neogenin protein. The sources of two possible splice products are shown in green and red. ***C***, RT-PCR analysis using primers in the 5' end (primers A) to detect all mRNA products, using a set of primers that include one primer in the lacZ gene (primers B) to detect fusion mRNA products, and using primers in the 3'-most region (primers C) to detect only WT mRNA products. A signal is observed using primers C suggesting that some amount of WT mRNA is produced in the $neo^{Gt/Gt}$ mouse. ***D***, Immunoblots using a commercially available neogenin antibody (Santa Cruz H175) on lysates of 293T cells expressing either HA-tagged neogenin or GFP-tagged DCC. The H175 antibody specifically recognizes Neogenin and does not recognize DCC. ***E***, Immunoblotting using the H175 Neogenin antibody on brain lysates from WT, $neo^{+/Gt}$ and $neo^{Gt/Gt}$ E18 embryos from a single litter. In homozygous embryos, there is a severe yet incomplete decrease in the amount of full-length neogenin protein produced.

RT-PCR results were as expected in WT and heterozygous RNA extracts but amplification with primer set C in $neo^{Gt/Gt}$ RNA extract indicated the presence of full length neogenin mRNA (Fig. 10 C). This result was confirmed by western-blot for Neogenin on protein extracts of WT, $neo^{+/Gt}$ and $neo^{Gt/Gt}$ mice (Fig. 10 D, E). After verifying antibody specificity by assessing cross-reactivity with DCC (Fig. 10 D), western blot was performed against protein extracts. Confirming RT-PCR results, a small amount of full length Neogenin protein was detected in $neo^{Gt/Gt}$ mice (approximately 10% of WT). Full-length Neogenin expression detected by RT-PCR and Western-blot is presumably the consequence of a splicing event leading to the excision of the gene-trap cassette (Fig. 10 B). Such splice-around events have previously been described (McClive et al., 1998; Stanford, Cohn & Cordes, 2001) and, depending on their frequency, may lead to hypomorphic phenotypes or to the virtual absence of detectable phenotypes. Our results were recently confirmed in an article by Bae et al. (2009) discussing finer details of the role of Neogenin signaling in myogenesis, where the hypomorph character of this allele is shown as well as a dependence of the splicing-around frequency on the stage of development of the mice as well as the tissue analyzed.

3.2.1   REDUCED NUMBERS OF MATURE OSNS IN *NEO*^{GT/GT} MICE

Based on the growing body of publications describing Neogenin as acting as a regulator of cell proliferation and differentiation in different tissues and systems, the first analysis we performed on the *neogenin*$^{Gt/Gt}$ mice was at the level of the cells of the OSN lineage populating the OE proper, looking for phenotypes suggestive of defects in OSN development.

In the light of the results obtained as to the expression pattern of *neogenin* and *rgmB* in the OE we first proceeded to assess whether *neo*$^{Gt/Gt}$ mice presented any phenotype in the mature OSN population, the end-point of the OSN lineage. To this effect, we performed immunohistochemistry against the olfactory marker protein (OMP), a marker of mature OSNs, on coronal cryosections of E18 OE (Fig. 11). Counting of OMP-positive cells in WT and *neo*$^{Gt/Gt}$ OEs revealed a 30% decrease in the mature OSN population.

To examine in more detail the reduction in the number of mature OSNs in *neo*$^{Gt/Gt}$ mice and assess whether reduced expression of Neogenin differentially affects OSNs in different regions of the OE, we crossed the *neogenin* gene trap mouse line with two mouse lines expressing markers in specific subpopulations of OSNs, namely P2-IRES-tau-LacZ and SP1-IRES-tau-GFP (Mombaerts et al., 1996). These mouse lines are extremely useful as both Tau-LacZ and GFP proteins diffuse throughout the expressing neurons allowing for straightforward visualization of cell bodies and axons through X-Gal staining and immunochemistry against GFP, respectively.

Counting of these OSN populations was performed on coronal sections of the complete OE of WT and *neo*$^{Gt/Gt}$ littermates at postnatal age P10, following staining for the respective reporter genes (Fig. 12). Although the two mature OSN populations analyzed showed reduced numbers in the *neo*$^{Gt/Gt}$ mice, the P2 OSNs (Zone 2 – intermediate) showed the strongest reduction in numbers with 55% less

OSNs in the *neo*^{Gt/Gt} mouse (Fig. 12 B). The SP1 OSN population (Zone 4 – ventral) showed a less severe phenotype with a reduction in OSNs of 26%.
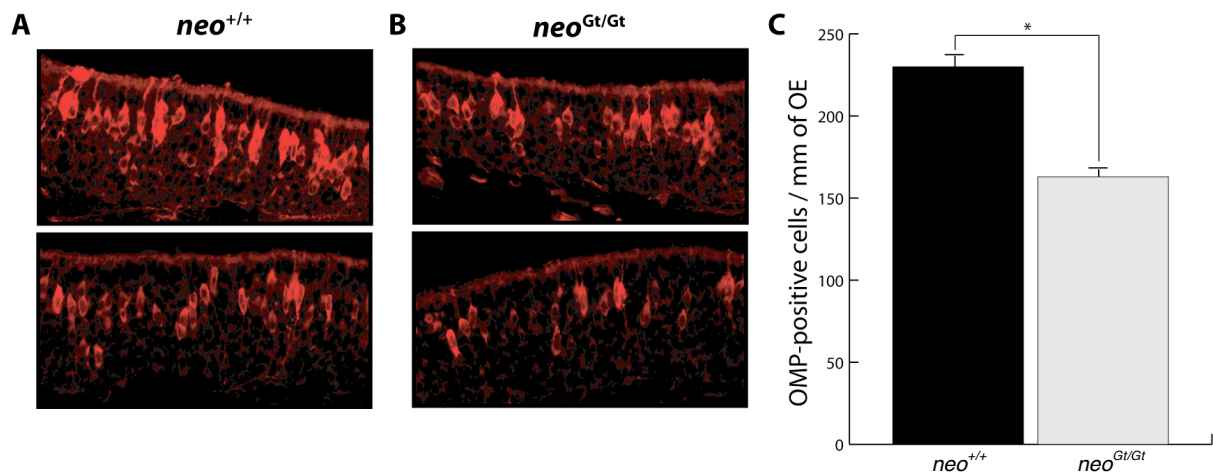
Figure 11. Mature OSN numbers are reduced in *neogenin*^{Gt/Gt} OE. ***A,B***, Anti-OMP immunostaining on coronal sections of OE from two WT (***A***) and two *neo*^{Gt/Gt} (***B***) E18 neogenin mice. ***C***, Graphical representation of the number of OMP-positive neurons per mm of OE in WT and *neo*^{Gt/Gt} embryos. A significant 29% decrease in the number of OMP-positive mature OSNs is observed in *neo*^{Gt/Gt} mice when compared to WT (p=0.017; N= 4 for *neo*^{+/+}; N=4 for *neo*^{Gt/Gt}).

3.2.2 *NEOGENIN*^{GT/GT} MICE PRESENT A TREND TOWARDS ENHANCED PROLIFERATION AND REDUCED APOPTOSIS IN THE OE

Given that Neogenin expression is found in GBCs and in mature OSNs, the reduction in mature ONS numbers in the *neo*^{Gt/Gt} OE might arise from aberrant regulation of Neogenin signaling either in mature OSNs or in progenitor cells. To address this question we investigated proliferation in the basal compartment of the *neo*^{Gt/Gt} OE and apoptosis in the mature OSN layer.

Since Neogenin/RGM signaling has been shown to be involved in regulating apoptosis in other vertebrates (Matsunaga et al., 2004; Shin and Wilson, 2008), we first addressed the question of whether the reduction in mature OSN numbers in the *neo*^{Gt/Gt} mice was accountable to an increase in OE apoptosis. We performed TUNEL staining on OE of WT and *neo*^{Gt/Gt} embryos at age E18, a developmental

stage where extensive apoptosis of OSNs at different degrees of maturation is occurring (Voyron et al., 1999). Counting of apoptotic cells (Fig. 13 A, B) showed a trend towards a decrease in apoptotic cells although the difference found between WT and $neo^{Gt/Gt}$ mice is not statistically significant (p=0.176). More animals will need to be added to the study to ascertain the accuracy of this result.

Figure 12. $Neogenin^{Gt/Gt}$ mice show diversely reduced OSN numbers in different regions of the OE. **A-D**, X-Gal staining (**A, B**) and anti-ß-galactosidase immunohistochemistry (**C, D**) on coronal sections of OE from P10 Neogenin WT and $neo^{Gt/Gt}$ mice carrying either a modified allele of the P2 OR (IRES-tau-LacZ) or a modified allele of the 174 OR (IRES-GFP). Arrowheads indicate stained OSN cell bodies in different regions of the OE. **E**, Average OSN numbers are reduced by 55.2% (**p=0.002) in P2 OSNs and by 26.0% (*p=0.038) in SP1 OSNs in the $neo^{Gt/G}$ mice (P2$^{+/lacZ}$:$neo^{+/+}$ - Average OSNs per OE: 10241.0, SE=341.79, N=3; P2$^{+/lacZ}$:$neo^{Gt/Gt}$ - Average OSNs per OE: 4582.3, SE=811.92, N=4; SP1$^{+/GFP}$:$neo^{+/+}$ - Average OSNs per OE: 8344.8, SE=541.81, N=4; SP1$^{+/GFP}$:$neo^{Gt/Gt}$ - Average OSNs per OE: 6178.8, SE=463.99, N=6). **F**, Diagram representing the OE zones spanned by the OSNs of the two modified OR lines used. D: Dorsal, L: Lateral, M: Medial, V: Ventral.

As Neogenin has also been shown to promote differentiation of neurons from progenitor cells in chick (Matsunaga et al., 2006) we examined whether any phenotype could be found in the progenitor cells of the *neo*^Gt/Gt OE. We performed BrdU pulse assays in E18 WT and *neo*^Gt/Gt embryos in order to assess whether the reduction in mature OSN numbers observed could be explained by changes in the proliferation rate of progenitor cells in the *neo*^Gt/Gt OE. Following immunohistochemistry on coronal sections of WT and *neo*^Gt/Gt OE, BrdU-positive cells located in the basal aspect of the epithelium were scored and counts compared between genotypes (Fig 13 B, C).

Quantification of BrdU-positive cells in the basal aspect of the OE revealed an increase in dividing cells in this area in the *neo*^Gt/Gt (7.1 cells per micron; SE: 1.26, N=3) when compared to the WT (3.9 cells per micron; SE: 0.28, N=3), indicative of heightened progenitor cell numbers in the absence of Neogenin. Although only scoring as not quite significant with a two-tailed p-value of 0.069 the difference found is an indication that Neogenin may me implicated in regulating early steps in the OSN lineage.

The heightened BrdU incorporation seems to suggest an arrest of GBCs in their proliferative state or an impaired capacity to exit cell cycle and differentiate into OSNs. This is an interesting possibility that fits with the roles described for Neogenin in regulating proliferation and differentiation in other systems.

3.3   NEOGENIN IN THE GUIDANCE OF OE AXONS TO THE OB

In addition to playing a role in the generation of OSNs the expression of Neogenin in mature OSNs suggests it may also contribute to the guidance of OSN axons on their path to the OBs.

To address the possibility that Neogenin expression in mature OSNs participates in the guidance events leading projections from the OE to their target fields in the OB, we went on to assess expression of Neogenin RGM ligands in the OB outer layers (Fig. 14).
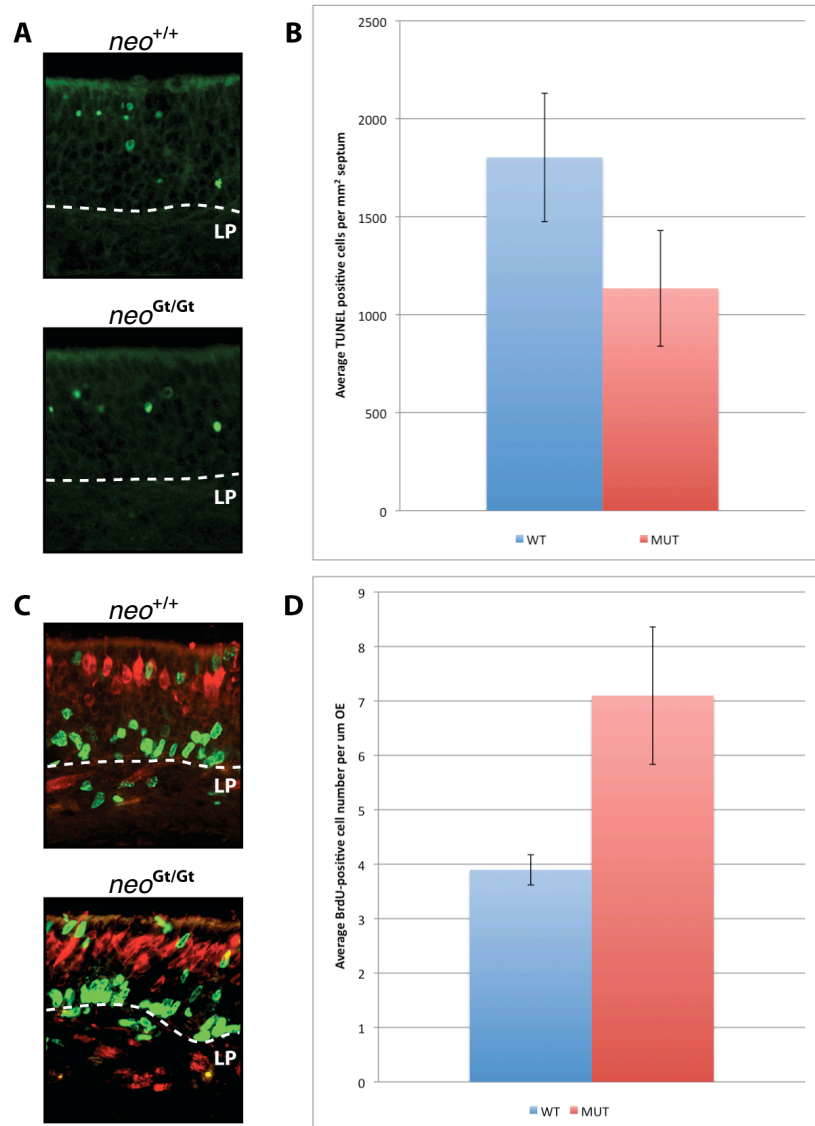
Figure 13. Apoptosis is reduced and proliferating cell numbers are augmented in *neogenin*$^{Gt/Gt}$ OE. **A**, TUNEL staining on cryosections of E18 WT and *neo*$^{Gt/Gt}$ littermates. **B**, Quantification of TUNEL-positive cell counts averaged 1802.8 cells per mm^2 in the WT (SE – 327.65, N=4) and of 1134.1 cells per micron in the *neo*$^{Gt/Gt}$ mice (SE – 295.11, N=5). Student's t-test confers no statistical significance to the reduction in apoptotic cell numbers in the *neo*$^{Gt/Gt}$ OE at this stage of development (p = 0.176) **C**, Immunostaining against BrdU on cryosections of E18 WT and *neo*$^{Gt/Gt}$ littermates reveals an increase in the number of proliferating cells in the *neo*$^{Gt/Gt}$ OE at this stage of development. OMP immunostaining is shown in red. **D**, Quantification of BrdU-positive cell counts averaged 3.90 cells per micron in the WT (SE - 0.277, N=3) and of 7.10 cells per micron in the Neogenin mutant (SE - 1.263, N=3), with the difference scoring not quite significant in the Student's t-test (p=0.069).

Figure 14. RGM ligands are expressed in a graded manner in the murine olfactory bulbs. **A-D**, In situ hybridization against *rgmA* and *rgmB* was per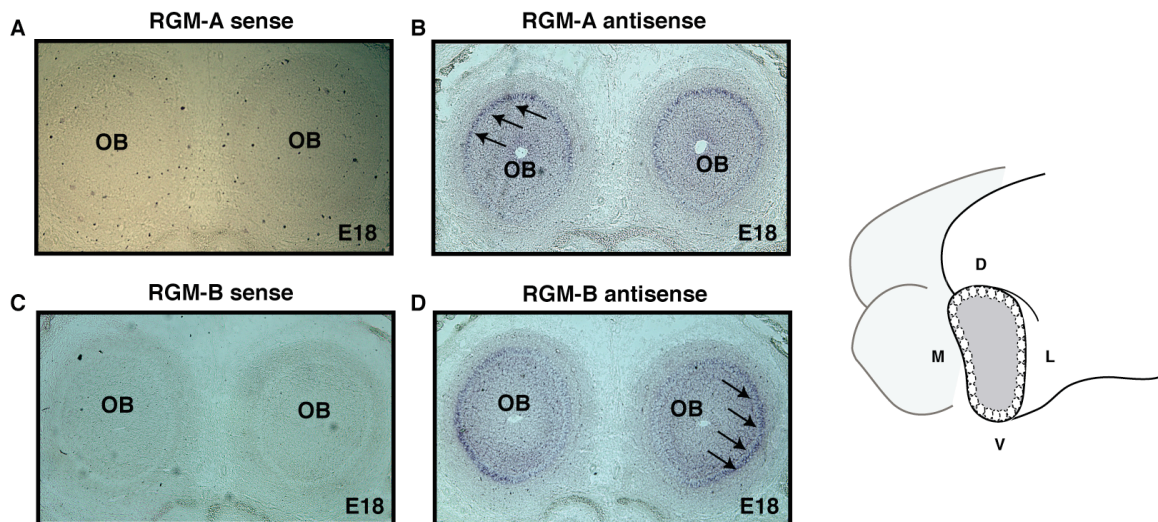formed on coronal sections of E18 CD1 mouse embryo OBs using sense (**A, C**) and antisense (**B, D**) cRNA probes. While *rgmA* is expressed at high levels in the dorso-lateral aspect of the OBs, high *rgmB* expression is restricted to the lateral aspect (arrows). Low levels of expression of *rgm* mRNA are found in the medioventral aspect of the OBs. D: Dorsal, L: Lateral, M: Medial, V: Ventral.

The approach taken was to perform in situ hybridization for *rgmA* and *rgmB* on coronal sections of the OBs from CD1 mouse embryos at E18, a stage of active projection of OE axons to the OB. In situ signals in the mitral cell layer of the OB, where the cell bodies of the second order neurons that synapse with incoming OSN axons are located, showed a graded expression of these Neogenin ligands in the OB with RGMa expression high in the dorsal aspect of the OBs and low at the ventral aspect, and *rgmB* expression high in the lateral aspect and low in the medial aspect of the OBs (Fig 14 C, D). This pattern of expression could indicate a role in the guidance of incoming axons from the OE by repulsion of Neogenin-expressing axons away from regions of high RGM expression. The results presented so far as to Neogenin expression in the OE don't allow us to say whether different populations of mature OSNs present different levels of Neogenin expression yet the presence of repulsive ligands for this receptor in such an extensive area as described suggests that this be the case, or that, if all OSNs express Neogenin, RGM-elicited signaling is silenced allowing for the establishment of connections all around the OB.

3.3.1 Neogenin is expressed in a "salt-and-pepper" manner in the OE

Having found RGM ligands extensively expressed in the OB at a developmental stage where OE axons are actively projecting toward their target fields in this tissue, we went back to analyzing Neogenin expression in the OE. To look in more detail at the pattern of expression of Neogenin in the mature OSN population, we again made use of the secretory trap reporter genes. We performed double immunostaining with anti-b-galactosidase and anti-OMP on sections of *neogenin*$^{+/Gt}$ OE from E18 embryos (Fig. 15). As expected, b-galactosidase-positive OSNs were found in the apical-most layers of the OE, but careful comparison with OMP fluorescence reveals that not all mature OSNs express Neogenin. This "salt-and-pepper" expression of Neogenin in OSNs projecting axons to the OB allows for the possibility that the graded expression of *rgmA* and *rgmB* found in the OBs may play an informative role in guidance of these axons towards their cognate target glomeruli. Previous studies have uncovered guidance cues expressed in gradients in the OB and responsible for steering incoming axons to their cognate glomeruli (Castro et al., 1999; Pasterkamp et al., 1999; Renzi et al., 2000; Schwarting et al., 2000; Walz et al., 2002; Cutforth et al. 2003; Cloutier et al., 2004; Schwarting et al., 2004; Cho et al., 2007) but possibility that Neogenin might regulate OSN development and differentiation early on to later act as a guidance receptor in the same cell is a novel and interesting one.

3.3.2 Glomerular phenotypes in the *Neogenin*$^{Gt/Gt}$ mice

While reduced numbers of mature OSNs are observed in *neo*$^{Gt/Gt}$ mice, a large proportion of OSNs are generated and express ORs. This made it possible for us to investigate whether Neogenin expression in mature OSNs is required for accurate targeting of OSN axons in the OB.

The assessment of targeting phenotype in the *neo*$^{Gt/Gt}$ mice was done making use of the OR-reporter mouse lines previously described (Fig. 16). To assess
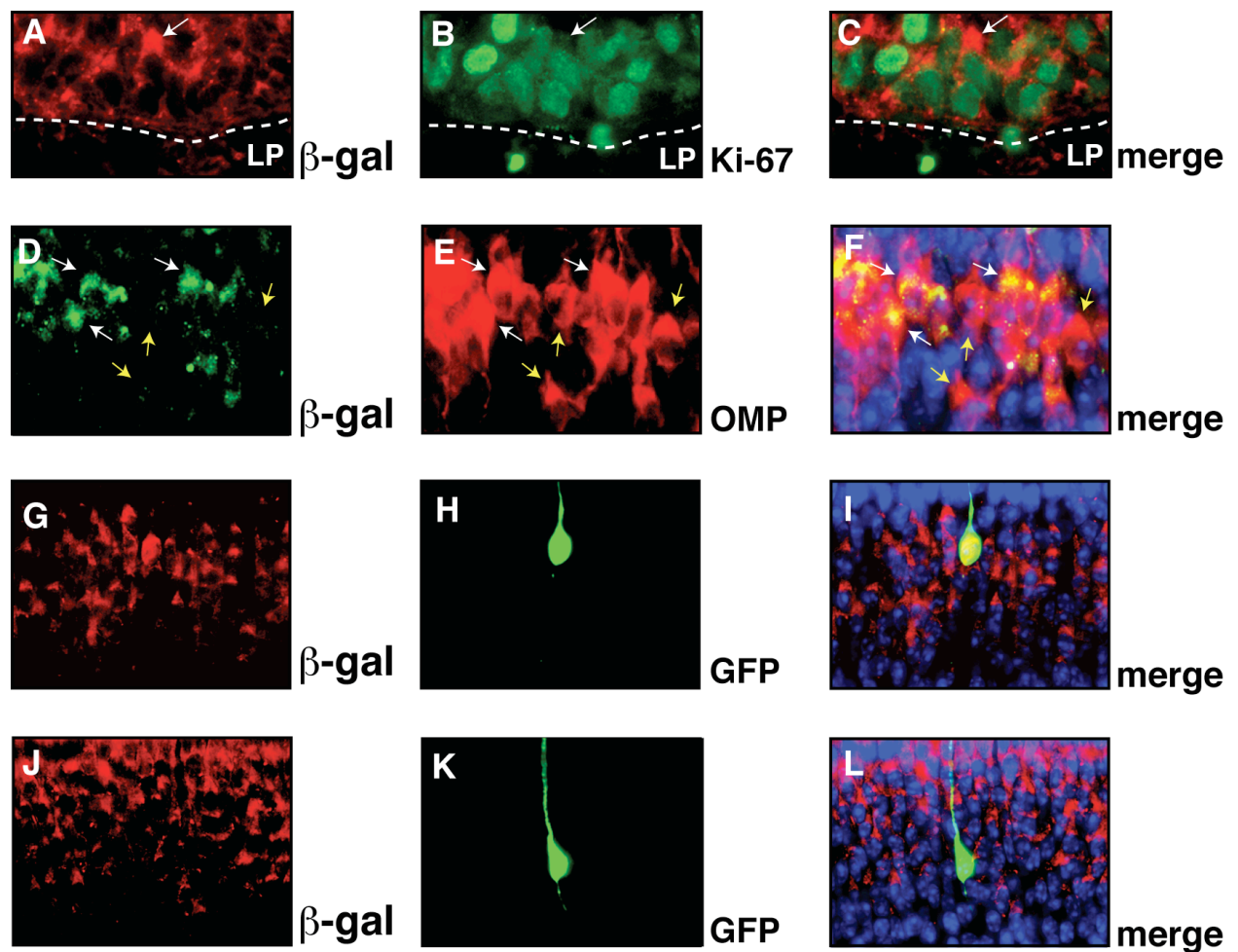
Figure 15. Neogenin expression in different OE cell populations. ***A-C***, Immunostaining with ß-galactosidase (***A, C***) and Ki67 (***B, C***) antibodies on a section of OE from an E18 heterozygous Neogenin embryo. ß-galactosidase, the reporter for Neogenin expression, is detected in the cytoplasm (arrow) of Ki-67-positive precursor cells. Ki-67 immunoreactivity is observed in the nucleus. ***D-F***, Immunostaining with ß-galactosidase (***D, F***) and OMP (***E, F***) antibodies on a section from an E18 heterozygous Neogenin embryo. ß-galactosidase, the reporter for Neogenin expression, is detected in a subset of OMP-positive mature OSNs (white arrows) but not in others (yellow arrows). ***G-L***, Immunostaining with ß-galactosidase and GFP antibodies on a section of OE from a P15 neogenin heterozygous mouse expressing tau-GFP under the control of the MOR174-9 olfactory receptor promoter. While some MOR174-9-positive neurons express ß-galactosidase (***G-I***), others do not (***J-L***). This suggests that Neogenin is only expressed in a subset of MOR174-9 positive mature OSNs. LP: Lamina Propria.

whether the observed Neogenin pattern of expression in the OE and of RGM ligands in the OB play a role in OSN axon guidance, we performed whole mount epifluorescence on dissected heads of Neogenin WT and mutant adult mice carrying either the SP1-IRES-tau-GFP or the MOR-174-9-IRES-tau-GFP allele. Results and

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Figure 16. *Neogenin*$^{Gt/Gt}$ mice do not present severe olfactory axon targeting defects. ***A-D***, Whole mount epifluorescence of olfactory bulbs from adult *neo*$^{Gt/Gt}$ and *neo*$^{+/+}$ mice. ***A, B***, *neo*$^{+/+}$ and *neo*$^{Gt/Gt}$ olfactory bulbs of mice carrying a modified MOR174-9 allele expressing the reporter gene GFP. ***C, D***, *neo*$^{+/+}$ and *neo*$^{Gt/Gt}$ olfactory bulbs of mice carrying a modified SP1 OR allele expressing the reporter gene GFP (arrowheads: medial glomeruli). (***E***) Diagram of measurement method used for lateral and medial glomeruli. The green circle represents the glomerulus. α represents angles measured with origin O. %dist = g / t x 100. (***F***) Table of measurements and standard errors for %distance and angle of analyzed glomeruli. Apart from a very significant (p= 0.0054) yet small difference between distances in *neogenin* WT and *neo*$^{Gt/Gt}$ MOR 174-9 lateral glomeruli (Blue bordered cells) and a significant (p=0.043) yet small difference between angles in SP1 *neogenin* WT and *neo*$^{Gt/Gt}$ glomeruli, no severe defects were found in axon targeting of these OSN populations in the *neo*$^{Gt/Gt}$ mice (MOR 174-9/*neo*$^{+/+}$, N=6 bulbs; MOR174-9/*neo*$^{Gt/Gt}$, N=8 bulbs; SP1/*neo*$^{+/+}$ lateral, N=8 bulbs; SP1 *neo*$^{+/+}$ medial, N=6 bulbs: SP1 *neo*$^{Gt/Gt}$ lateral, N=10 bulbs; SP1 *neo*$^{Gt/Gt}$ medial, N=6 bulbs). A: anterior, L: Lateral, M: Medial, P: posterior.

MOR 174-9	%Dist	SEM	Angle	SEM
WT Lateral	95.63	1.317	23.02	2.305
neo$^{Gt/Gt}$ Lat.	87.29	1.960	28.85	1.576
WT Medial	62.25	2.848	35.35	3.574
neo$^{Gt/Gt}$ Med.	57.60	2.182	27.83	3.364

SP1	%Dist	SEM	Angle	SEM
WT Lateral	84.39	2.044	51.03	2.299
neo$^{Gt/Gt}$ Lat.	86.14	2.133	43.58	2.249
WT Medial	18.72	2.890	38.65	2.996
neo$^{Gt/Gt}$ Med.	22.33	3.022	44.29	3.552

measurement methods are presented in figure 16 (E, F). Due to variability in OB size between all mice analyzed (MOR174-9 WT: 728.4±32.77; MOR174-9 *neo*$^{Gt/Gt}$: 763.2±34.79; SP1 WT: 877.1±42.45; SP1 *neo*$^{Gt/Gt}$: 900.7±66.21; unit – pixels) distances measured are presented as ratios to OB length. The only differences found in the *neo*$^{Gt/Gt}$ mice compared to WT were a small shift towards a more caudal/dorsal position (8.37%) in the MOR174-9 lateral glomerulus distance measurement, considered very significant, and a 7.45° dorsal shift in the SP1 lateral glomerulus angle measurement, considered significant. The mild phenotypes observed and the absence of severe aberrant glomerular positioning in the *neo*$^{Gt/Gt}$ mice may indicate the lack of an important axon guidance role for Neogenin in the OSNs yet the fact that the mouse line used in this study is a confirmed hypomorph (Bae et al., 2009) raises the possibility that a reduced amount of full length Neogenin present at the cell surface may be allowing for the accurate targeting of the majority these axons.

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CHAPTER 4

DISCUSSION
AND
FUTURE DIRECTIONS

4   DISCUSSION AND FUTURE DIRECTIONS

In searching for proteins that might play key roles in developmental processes in the murine olfactory epithelium we have found that Neogenin, a transmembrane receptor of the IgG-like family, as well as its ligand RGMb, a GPI-linked protein, are indeed expressed in this tissue in manned and at time points suggestive of such roles.

In situ hybridization, as well as X-Gal staining using a transgenic mouse line expressing ß-galactosidase under control of the *neogenin* promoter, reveal that, at embryonary as well as post-natal stages, expression of *neogenin* is found in the OE. Immunostaining of consecutive sections against Ki67, a marker for cells undergoing division, and OMP, a marker for mature OSNs, combined with X-Gal staining of adjacent sections, places the higher levels of *neogenin* expression in layers of the OE corresponding to these two cell populations, with an interceding gap of lower expression in intermediary layers populated mainly by immature OSNs. This pattern of expression suggests that not only may Neogenin play a role in axon guidance events along OSN axon paths towards the OBs but that it may also participate in early stages of the OSN lineage.

Probing for Neogenin ligands at these same time points revealed that one of the two Repulsive Guidance Molecule ligands found in the murine nervous system is indeed expressed in the OE. ISH for *rgmB* paired with consecutive section immunostaining against Ki67and OMP shows an expression pattern somewhat complementary to the one found for *neogenin*, with low levels of expression in apical and basal layers of the OE but high levels in the intermediary layers composed mostly of immature OSNs. This pattern of expression associated with the pseudo-stratified structure of the OE suggests that RGMb molecules found on immature OSNs may at different stages of the cells development signal through Neogenin to the progenitor cells as well as mature OSNs.

To analyze the *neogenin* gene-trap mouse we first assessed whether the numbers of mature OSNs were maintained compared to WT, through immunohistochemistry against OMP on cryosections of E18 OE. OMP-positive cell counts revealed

statistically significant reduction of 29% in the mutant, a result in accordance with the possibility of Neogenin participating in regulation of OSN development.

Based on the patterns of expression found for *neogenin* and *rgmB* in the OE, the reduction in OSN numbers found in the *neo*^{Gt/Gt} mice may be ascribed to the absence of Neogenin signaling events that in the WT take place either at the level of the mature OSNs or of the progenitor cells. Namely, reduced mature OSN numbers in the mutant may be brought on by increased death in these cells or by an event taking place in the basal progenitor cell stage of the lineage.

Being expressed in actively projecting mature OSNs, Neogenin remains a prime candidate for a role in mediating axon guidance events in the murine OS. Investigation of the expression of the pattern of expression of *rgmA* and *rgmB* by ISH on coronal sections of the E18 mouse OB shows that both are expressed in the mitral cell layer in gradients and are thus well placed to constitute a guidance cue for incoming OSN axons. Namely, RGMa is expressed in a high dorsal to low ventral gradient whereas *rgmB* presents a high lateral to low medial gradient. The existence of this quite large domain of repulsive cues in the OB led us to the question of whether all mature OSNs expressed *neogenin*.

Taking advantage of the reporter genes carried by cells expressing the *neogenin* gene-trap cassette we performed double immunohistochemistry against beta-galactosidase and OMP. This assay clearly showed that not all OMP-positive OSNs are beta-galactosidase positive indicating than Neogenin is not ubiquitous but rather expressed in a salt-and-pepper manner in this population of neurons. The existence of two such populations of mature OSNs forms an attractive basis for sorting between medial and lateral glomeruli upon arrival at the OB when combined to the gradients of *rgmA* and *rgmB* described.

Nevertheless analysis of OSN axon targeting by whole mount epifluorescence showed no phenotype in the *neo*^{Gt/Gt} mouse, allowing for the possibility that Neogenin might play a role earlier on in OSN axon projection or that other, redundant, guidance cues compensate for the absence of Neogenin.

Initially identified as a repulsive guidance cue receptor, Neogenin has since been shown to be implicated in regulation of cell proliferation and differentiation in the vertebrate nervous system. The results described here suggest that Neogenin might be switching roles in the OSN lineage, being highly expressed at early progenitor stages, where it is implicated in the regulation of proliferative rates, then down-regulated at immature OSN stages to finally be up-regulated at mature OSN stages where it may promote differentiation or survival, but also play its classical role of guidance cue receptor.

Consistent with these hypotheses, $neo^{Gt/Gt}$ mice present heightened proliferative rates in the globose basal cell layer and reduced numbers of mature OSNs. Although no targeting defects were found in the adult mutant, residual amounts of full-length Neogenin may be responsible for the absence of a phenotype.

The multi-faceted nature of the Neogenin receptor makes it an attractive candidate for an elegant means of accomplishing regulation at different stages in neuroepithelial development.

Although it remains unclear whether Neogenin regulates proliferation at an earlier stage or in differentiation at a later progenitor stage in the basal OE, the results described suggest that the reduction in mature OSN numbers found in the mutant may be brought on by the lengthening of the proliferative state in these cells. Taking in consideration that in a healthy situation in the WT a basal level of Neogenin signaling may be elicited in the GBCs by the abundant RGMb expression in the adjacent immature OSNs, the possibility of a feedback loop contributing to the conservation of OSN numbers arises. In the presented model a basal level of RGMb signaling from immature OSNs to GBCs may be responsible for: 1 - restraining proliferation rates at basal levels in GBC. Death or maturation of immature OSNs may then, through lowering of Neogenin signaling, lead to heightened proliferation rates in order to replenish the OSN population; 2 – promoting a basal rate of differentiation in the GBC precursor population. GBCs migrating apically following their limited division cycles may then be triggered for differentiation upon contact with young immature OSNs.

As not all of the mature OSN population expresses Neogenin, a role for this receptor in promoting survival of these cells seems unlikely. Although our data do not allow for the distinction between early and late phases of mature OSN differentiation, the possibility that the distinction between Neogenin expressing and non-expressing neurons may be representative of neurons at different stages of differentiation rather than belonging to distinct populations is an interesting one. In this model, *neogenin* expression could possibly be turned on after initial differentiation to reinforce the differentiation process upon RGMb binding. It could then be turned off once OSNs have reached complete maturity, which would agree with the observation that no targeting defects are found in the mutant.

Close analysis of the *neogenin* gene-trap mutant used in this research has revealed that rather than a knockout this line is hypomorphic for *neogenin*. This has recently been confirmed by Bae et al. (2009), whose analysis of the role of Neogenin in skeletal myogenesis regulation reveals phenotypes in the same order of severity than the ones described in this thesis. Hypomorphism for *neogenin* in this line of mice may explain the lesser severity of some of the phenotypes described and the absence of targeting defects in the mice analyzed, as the Neogenin protein still present may suffice to compensate for the reduction in protein level.

4.1.1 NEOGENIN IN OE DEVELOPMENT

In recent years a considerable amount of work has been dedicated to identifying molecules that might be implicated in regulating proliferation and differentiation in the OE, at cell-autonomous as well as non cell-autonomous levels. Resulting from these investigations an ever more complete picture of who the players are in this system has arisen. Yet the exact manner in which these interact and coordinate to give rise to the lifelong maintenance of mature OSN levels has remained unclear. Moreover none of the mentioned research has approached the question of whether cell-cell interaction events are implicated in regulating OE development in developmental as well as regeneration paradigms.

The results we have presented, taken in conjunction with the fact that Neogenin has proven to be more than a guidance receptor assuming varied roles in the context of neuron development and tissue morphogenesis, place this receptor at an excellent position, temporal and spatial, to be displaying different aspects of its multifunctional nature along the OSN lineage.

Neogenin expression in the basal-most layers of the OE detectable at E18, embryonic age at which the OE's pseudo-stratified organization has already been established and OSNs are actively projecting towards the OBs, as well as in the post-natal mouse combined with the concomitant expression of the Neogenin ligand RGMb in the adjacent layers of immature OSNs at the same ages made us ask the question of whether this ligand/receptor pair might be contributing towards regulation events in early stages of the OSN lineage.

The fact that *neogenin* is expressed in GBCs and then downregulated in immature OSNs may suggest the participation in a developmental switch at this transition stage.

The initial finding that the overall mature OSN numbers were reduced in the *neo*$^{Gt/Gt}$ mouse substantiated our supposition that Neogenin might be acting as more than a guidance cue receptor in the OE, and that it might instead or in parallel be regulating cell proliferation, differentiation or survival in this system.

Considering the results found in the chick embryo spinal cord by Matsunaga et al. (2004) implicating Neogenin in promoting the survival of neurons, a straightforward mechanism leading to the reduction of mature OSN numbers in the *neo*$^{Gt/Gt}$ mouse might have been an increase of cell death in this cell population. TUNEL assays proved this possibility to be wrong as a trend towards decrease in apoptotic cell numbers was found in the OE of *neo*$^{Gt/Gt}$ mice.

Another way in which Neogenin might be promoting the normal development of OSNs is that it might be part of a developmental switch – being down-regulated at the INP/immature OSN transition and then up-regulated at the immature/mature OSN transition. Although no concrete evidence for this hypothesis is at hand, we have

found that after a pulse of BrdU at E18, a 78% increase in basal proliferating cells was present in *neo*[Gt/Gt] embryos when compared to their WT littermates.

In the light of the different mechanisms in which Neogenin has been shown to participate to mediate epithelial and neuroepithelial development, different possibilities arise as to how the strong reduction in *neogenin* expression leads to the phenotypes observed.

According to results published by Matsunaga et al. (2004) in the chick embryo, where RGMa signaling through Neogenin is shown to promote neuron differentiation, the increase in of proliferating cells in the *neo*[Gt/Gt] mouse as well as the reduction in mature OSNs observed may arise from the strong reduction of Neogenin signaling in the GBCs, namely the INPs which, in the GBC population, represent the proliferative stage closest to differentiation.

Additionally, the OE being a pseudo-stratified neuroepithelium, and under the light of the results shown by Kee et al. (2008), the Neogenin/RGMb/Neogenin expression pattern may contribute towards the establishment of cell polarity at the key points represented by the INP to immature OSN differentiation as well as the immature to mature OSN transition, time points at which extensive polarized changes occur in the differentiating and maturing cells.

As confirmed by the recent publication of Bae et al. (2009) the *neogenin* gene-trap mouse used in this work is a hypomorph for Neogenin, rather than leading to complete ablation of expression. This might mean that the observed reduction was the result of varying degrees of reduction in different OSN populations.

The first evidences pointing towards the details of Neogenin signaling underlying the multitude of activities described for this IgG family receptor in vertebrates have only recently begun to be uncovered, as reviewed in De Vries and Cooper (2008). Most of the downstream effectors of Neogenin signaling as yet identified are related to actin dynamics regulation in the growth cone, namely RhoA and ROCK leading to f-actin depolymerization following RGMb signaling, and possibly Src kinase and Focal Adhesion Kinase (FAK) leading to neurite outgrowth following Netrin binding.

As to Neogenin signaling leading to cell proliferation/cell differentiation the little evidence that has emerged has been obtained through work on myogenesis. Kang et al. (2004) first determined in vitro, using different luciferase reporter transgenes, that in C1C12 myoblast cells Neogenin activated myogenic bHLH factor-dependent and NFATc3-dependent reporter genes and that in the case of NFATc3 this could be enhanced by Netrin-2. Subsequent work from Xie et al. (2005) shows that phosphatidylinositol transfer protein-α (PITPα) interacts constitutively with Neogenin in cultured cortical neurons, an interaction that is enhanced by Netrin signaling through Neogenin and which requires the presence of the P3 domain in Neogenin and of the 20 C-terminal amino acids of PITPα. Although in a not very convincing manner, the authors of this article attempted placing PITPα downstream of Neogenin in the context of neurogenesis by reporting a close phenocopy of Neogenin knockdown in zebrafish (Mawdsley et al., 2004) by expression of PITPα morpholinos in zebrafish embryos. Additionally, later work from the same group (Xie et al., 2006) places phospholipase C gamma (PLCγ) downstream of Netrin-1/DCC signaling, leading to Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) hydrolysis which leads to enhanced production of inositol triphosphate (IP$_3$) and concomitantly to calcium release from intracellular stores. This is a plausible link to the Neogenin-dependent NFATc3 signaling mentioned above, as NFAT signaling is calcium dependent. Although it has not been shown, PITPα signaling may lead to the same pathway in the case of Neogenin, which, in the same article, is shown not to interact with PLC γ. Interestingly, Ren et al. (2008) showed that Src homology-2 containing inositol-5-

phosphatase 1 (SHIP1), an enzyme central for IP_3 control interacts with Neogenin at the receptor's phosphotyrosine 1467 residue while it does not bind DCC. This could be indicative of an alternative pathway leading to NFAT dependent gene expression downstream of Neogenin when compared to DCC.

Additionally to the PITP pathway, Neogenin-associated kinase activation may also participate in promoting neurogenesis in the OE. In a recent article Bae et al. (2009) have determined that in C1C12 myoblasts FAK and Extracellular signal-Regulated Kinase (ERK) are activated downstream of Neogenin in vivo and that they appear to be involved in Neogenin's promyogenic function. Although the contribution of FAK and ERK activation towards Neogenin-dependent promyogenic signaling remains to be assessed, partly due to the promiscuous character of these kinases, it constitutes a plausible player in regulation of myogenesis and could be playing a role in olfactory neurogenesis as well.

4.1.3   RGMB AS A BMP CO-RECEPTOR IN THE OE

In parallel with Neogenin signaling, a plausible role for the RGMb pattern of expression found in the OE involves BMP signaling. BMP4 has been shown to play a dose-dependent role in OE development, causing cell death at high concentrations and promoting survival of recently differentiated immature OSN at low concentrations (Shou et al., 2000). Interestingly, this young OSN population is the one expressing the highest levels of *rgmB* in the OE during development and postnatally and RGMb has been shown to bind DMP4, but not BMP7, the other BMP expressed in the OE (Samad et al., 2005). In the light of the roles shown for RGMs in modulation of BMP signaling in other tissues and given that two BMP receptors are expressed in the OE (BmprIA and BmprIB; Dewulf et al., 1995; Zhang et al., 1998) it is likely that RGMb promotes OSN survival by modulating BMP4 signaling in these cells, either by enhancing receptor affinity to BMP4 or by recruiting more receptor from the available pool.

4.1.4 NEOGENIN IN OSN AXON GUIDANCE

Neogenin being an axon guidance receptor that has been shown to direct targeting by repulsion as well as attraction in diverse vertebrate systems (Monnier et al., 2002, Rajagopalan et al., 2004, Wilson and Key, 2006), we were initially inclined to focus on uncovering such a role in OE axon guidance for this receptor. Our results after analysis of $neo^{Gt/Gt}$ mice only revealed very mild defects in the positioning of SP1 or MOR174-9 glomeruli on the OB. This counterintuitive result may indicate that the expression of Neogenin in mature OSNs mostly contributes towards processes other than axon guidance in these cells.

Although the results obtained through whole-mount analysis of MOR174-9-GFP and SP1-GFP olfactory bulbs revealed only small differences between the $neo^{+/+}$ and the $neo^{Gt/Gt}$ mice in terms of OSN axon targeting, it is possible that the amount of Neogenin reaching the cell surface in the $neo^{Gt/Gt}$ mouse , though strongly reduced, suffices for accurate guidance of these axons toward their cognate target fields in the OB. Thorough characterization of the hypomorph mouse line in Bae et al. (2009) shows that the efficiency of the gene-trap varies at different time points and in different tissues in these mice. Taking in consideration the high levels of expression found for *neogenin* by ISH as well as X-Gal staining in the mature OSN layer, even when compared with the basal layers, it appears plausible that more of the intact Neogenin protein is found in these cells.

Answering these questions will require further investigation and finer genetic tools. These are discussed in the next section.

4.1.5 FUTURE DIRECTIONS

To circumvent the limitations presented by the hypomorph *neogenin* mouse we are now in the process of generating a mouse line carrying a conditional *neogenin* allele. This approach will allow us to fully excise *neogenin* and assess how the complete ablation impacts the phenotypes found in the $neo^{Gt/Gt}$ mouse at the level of the OE and whether it will engender OE axon targeting defects at the level of the OB.

Furthermore, this approach will allow us to answer questions pertaining to the finer details of the role of Neogenin in the OE. The Cre mouse lines already in our hands will allow us to pinpoint with greater accuracy at which stage or stages Neogenin is necessary to conserve normal OE function. Crosses with the Nestin-Cre line, in which *neogenin* will be excised at an early GBC stage will entail the loss of *neogenin* expression all through the OSN lineage thus yielding a phenotype closer to the knockout than the ones described in this work. In parallel, crossing of the *neogenin* conditional line with OMP-Cre mice will allow us to answer two questions. First it will tell us whether the reduction in mature OSN numbers is recapitulated by removing *neogenin* in the mature cells, while maintaining normal Neogenin signaling at the GBC/immature OSN interface, indicating a role in mature OSN survival, or whether the absence of Neogenin at this stage leads to a normal phenotype. Additionally, efficient excision of *neogenin* in the mature OSNs will be instrumental in ascertaining whether Neogenin does or does not play a targeting role in the guidance of OSN axons to the OBs, with the advantage that normal Neogenin expression in the OB periglomerular and mitral cell layer will not be affected. The conditional *neogenin* line will also be of the utmost interest to determine whether knockout in the developing or the regenerating OE will entail histogenesis deficiencies leading to any degree of stratification failure or cell polarity defects in *neogenin*-expressing cells, which would be in agreement with research of Mawdsley et al. (2004) and Kee et al. (2008).

Because *neogenin* is expressed at different times during progenitor to mature OSN transition and because its pattern of expression closely delineates key moments in differentiation and maturation in these cells, it will also be extremely interesting to determine what transcriptional regulation mechanisms are involved. Some of the transcriptional factors known to regulate OSN development and that are believed to regulate transition steps in OSN development are particularly attractive as candidates for regulation of *neogenin* and *rgmB* in the murine OE. In particular the OAZ - O/E transcription factors which, like *rgmB* and *neogenin*, have their distribution in the OE establishing close to clear-cut frontiers between the basal progenitor/precursor compartment, an intermediary immature OSN compartment and the apical terminally differentiated OSN compartment. With O/E transcription factors expressed throughout the OE and OAZ acting as an O/E repressor in the immature

OSN compartment it is easy to draw a parallel with *neogenin* strongly expressed in progenitor/precursor cells and mature OSNs and *rgmB* highly expressed in immature OSNs. Such a striking overlap in terms of expression pattern is highly suggestive of correlation. These indications are further substantiated by the fact that the OAZ mutant mice present a mature OSN loss phenotype comparable in severity to the one we describe in the *neo*$^{Gt/Gt}$ mice (Cheng and Reed, 2007). An exciting possibility to explore would be that OE transcription factors would be upstream of Neogenin and OAZ upstream of RGMb. OAZ upregulation at the transition from precursor cell to immature OSN would then silence *neogenin* and drive *rgmB* expression in young neurons. Downregulation of OAZ at the transition to terminal differentiation would then silence *rgmB* and allow *neogenin* expression in the mature OSNs.

An *rgmB* conditional mouse line is also in preparation, which will be central in ascertaining the role of this GPI-linked protein in OE development. Besides signaling through Neogenin it is likely that RGMb participates in BMP signaling in this tissue. It will be of major interest to verify whether low concentrations of BMP4 still promote immature OSN survival in OE explants where RGMb has been knocked out in the OSN lineage. This would represent yet another level of complexity in this system, making Neogenin and RGMb important pieces in the cell signaling events regulating OSN lineage development.

CHAPTER 5

REFERENCES

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Astic, L., Pellier-Monnin, V., Saucier, D., Charrier, C. & Mehlen, P. (2002) Expression of netrin-1 and netrin-1 receptor, DCC, in the rat olfactory nerve pathway during development and axonal regeneration. *Neuroscience*, 109 (4), pp. 643-56.

Babitt, J.L., Zhang, Y., Samad, T.A., Xia, Y., Tang, J., Campagna, J.A., Schneyer, A.L., Woolf, C.J. & Lin, H.Y. (2005) Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor. *Journal of Biological Chemistry*, 280 (33), pp. 29820-7.

Babitt, J.L., Huang, F.W., Wrighting, D.M., Xia, Y., Sidis, Y., Samad, T.A., Campagna, J.A., Chung, R.T., Schneyer, A.L., Woolf, C.J., Andrews, N.C. & Lin, H.Y. (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nature Genetics*, 38 (5), pp. 531-9.

Babitt, J., Huang, F., Xia, Y., Sidis, Y., Andrews, N. & Lin, H. (2007) Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. *Journal of Clinical Investigation*, 117 (7), p. 1933–1939.

Bae, G., Yang, Y., Jiang, G., Hong, M., Lee, H., Tessier-Lavigne, M., Kang, J. & Krauss, R.S. (2009) Neogenin regulates skeletal myofiber size and focal adhesion kinase and extracellular signal-regulated kinase activities in vivo and in vitro. *Molecular Biology of the Cell*, 20 (23), pp. 4920-31.

Barnea, G., O'Donnell, S., Mancia, F., Sun, X., Nemes, A., Mendelsohn, M. & Axel, R. (2004) Odorant receptors on axon termini in the brain. *Science*, 304 (5676), p. 1468.

Battye, R., Stevens, A. & Jacobs, J.R. (1999) Axon repulsion from the midline of the Drosophila CNS requires slit function. *Development*, 126 (11), pp. 2475-81.

Beites, C.L., Kawauchi, S., Crocker, C.E. & Calof, A.L. (2005) Identification and molecular regulation of neural stem cells in the olfactory epithelium. *Experimental Cell Research*, 306 (2), pp. 309-16.

Belluscio, L., Nemes, A., Gold, G. & Axel, R. (1998) Mice Deficient in Golf are anosmic. *Neuron*, 20 (1), pp. 69-81.

Brinks, H., Conrad, S., Vogt, J., Oldekamp, J., Sierra, A., Deitinghoff, L., Bechmann, I., Alvarez-Bolado, G., Heimrich, B., Monnier, P.P., Mueller, B.K. & Skutella, T. (2004) The repulsive guidance molecule RGMa is involved in the formation of afferent connections in the dentate gyrus. *Journal of Neuroscience*, 24 (15), pp. 3862-9.

Calof, A.L., Bonnin, A., Crocker, C., Kawauchi, S., Murray, R.C., Shou, J. & Wu, H. (2002) Progenitor cells of the olfactory receptor neuron lineage. *Microscopy Research and Technique*, 58 (3), pp. 176-88.

Castella, P., Wagner, J.A. & Caudy, M. (1999) Regulation of hippocampal neuronal differentiation by the basic helix-loop-helix transcription factors HES-1 and MASH-1. *Journal of Neuroscience Research*, 56 (3), pp. 229-40.

Castro, F., Hu, L., Drabkin, H., Sotelo, C. & Chedotal, A. (1999) Chemoattraction and chemorepulsion of olfactory bulb axons by different secreted semaphorins. *Journal of Neuroscience*, 19 (11), p. 4428.

Cau, E., Gradwohl, G., Fode, C. & Guillemot, F. (1997) Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development*, 124 (8), pp. 1611-21.

Cau, E., Gradwohl, G., Casarosa, S., Kageyama, R. & Guillemot, F. (2000) Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. *Development*, 127 (11), pp. 2323-32.

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 (8), pp. 1871-80.

Chang, C., Yu, T., Bargmann, C. & Tessier-Lavigne, M. (2004) Inhibition of netrin-mediated axon attraction by a receptor protein tyrosine phosphatase. *Science*, 305 (5680), p. 103.

Charron, F. & Tessier-Lavigne, M. (2007) The Hedgehog, TGF-ß/BMP and Wnt Families of Morphogens in Axon Guidance. *Advances in Experimental and Medical Bilogy*, 621, pp. 116-33.

Chen, B., Kim, E. & Xu, P. (2009) Initiation of olfactory placode development and neurogenesis is blocked in mice lacking both Six1 and Six4. *Developmental Biology*, 326 (1), pp. 75-85.

Cheng, L.E. & Reed, R.R. (2007) Zfp423/OAZ participates in a developmental switch during olfactory neurogenesis. *Neuron*, 54 (4), pp. 547-57.

Chesler, A.T., Peterlin, Z.A., Zou, D., Matthews, G.A., Le Pichon, C.E., Pei, X., Miller, M.C. & Firestein, S. (2007) A G protein/cAMP signal cascade is required for axonal convergence into olfactory glomeruli. *Proceedings of the National Academy of Sciences of the United States of America*, 104 (3), pp. 1039-44.

Chess, A., Simon, I., Cedar, H. & Axel, R. (1994) Allelic inactivation regulates olfactory receptor gene expression. *Cell*, 78 (5), p. 823–834.

Chi, C.L. (2003) The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. *Development*, 130 (12), pp. 2633-2644.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. & Kintner, C. (1995) Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature*, 375 (6534), pp. 761-6.

Chitnis, A. & Kintner, C. (1996) Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. *Development*, 122 (7), pp. 2295-301.

Cho, J.H., Lépine, M., Andrews, W., Parnavelas, J. & Cloutier, J. (2007) Requirement for Slit-1 and Robo-2 in zonal segregation of olfactory sensory neuron axons in the main olfactory bulb. *Journal of Neuroscience*, 27 (34), pp. 9094-104.

Cirulli, V. & Yebra, M. (2007) Netrins: beyond the brain. *Nature reviews. Molecular cell biology*, 8 (4), pp. 296-306.

Cloutier, J., Sahay, A., Chang, E.C., Tessier-Lavigne, M., Dulac, C., Kolodkin, A.L. & Ginty, D.D. (2004) Differential requirements for semaphorin 3F and Slit-1 in axonal targeting, fasciculation, and segregation of olfactory sensory neuron projections. *Journal of Neuroscience*, 24 (41), pp. 9087-96.

Cole, F., Zhang, W., Geyra, A., Kang, J. & Krauss, R.S. (2004) Positive regulation of myogenic bHLH factors and skeletal muscle development by the cell surface receptor CDO. *Developmental Cell*, 7 (6), pp. 843-54.

Conrad, S., Genth, H., Hofmann, F., Just, I. & Skutella, T. (2007) Neogenin-RGMa signaling at the growth cone is bone morphogenetic protein-independent and involves RhoA, ROCK, and PKC. *Journal of Biological Chemistry*, 282 (22), pp. 16423-33.

Conrad, S., Stimpfle, F., Montazeri, S., Oldekamp, J., Seid, K., Alvarez-Bolado, G. & Skutella, T. (2009) RGMb controls aggregation and migration of Neogenin-positive cells in vitro and in vivo. *Molecular and Cellular Neurosciences* (in press).

Corradini, E., Babitt, J.L. & Lin, H.Y. (2009) The RGM/DRAGON family of BMP co-receptors. *Cytokine & growth factor reviews*, 20 (5-6), pp. 389-98.

Crandall, J.E., Püschel, A.W., Schwarting, G.A., Dibble, C., Butler, D., Pays, L., Ahmad, N. & Kostek, C. (2000) Patterning of olfactory sensory connections is mediated by extracellular matrix proteins in the nerve layer of the olfactory bulb. *Journal of Neurobiology*, 45 (4), pp. 195-206.

Cuschieri, A. & Bannister, L.H. (1975) The development of the olfactory mucosa in the mouse: electron microscopy. *Journal of Anatomy*, 119 (Pt 3), pp. 471-98.

Cutforth, T., Moring, L., Mendelsohn, M., Nemes, A., Shah, N.M., Kim, M.M., Frisén, J. & Axel, R. (2003) Axonal ephrin-As and odorant receptors: coordinate determination of the olfactory sensory map. *Cell*, 114 (3), pp. 311-22.

DeHamer, M.K., Guevara, J.L., Hannon, K., Olwin, B.B. & Calof, A.L. (1994) Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors. *Neuron*, 13 (5), pp. 1083-97.

DeVries, M. & Cooper, H.M. (2008) Emerging roles for neogenin and its ligands in CNS development. *Journal of Neurochemistry*, 106 (4), pp. 1483-92.

DeWulf, N. Verschueren, K. Lonnoy, O. Moren, S. Gimsby, A. Vande Spiegle, K. Miyazono, K., Huylebroek, D, Ten Dijke, P. (1995) Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology*, 136, pp. 2652-63.

Feinstein, P., Bozza, T., Rodriguez, I., Vassalli, A. & Mombaerts, P. (2004) Axon guidance of mouse olfactory sensory neurons by odorant receptors and the beta2 adrenergic receptor. *Cell*, 117 (6), pp. 833-46.

Ganz, T. (2003) Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*, 102 (3), p. 783.

Gessert, S., Maurus, D. & Kühl, M. (2008) Repulsive guidance molecule A (RGM A) and its receptor neogenin during neural and neural crest cell development of Xenopus laevis. *Biology of the Cell*, 100 (11), pp. 659-73.

Halpern, M. (2003) Structure and function of the vomeronasal system: an update. *Progress in Neurobiology*, 70 (3), pp. 245-318.

Harper, J.W., Adami, G., Wei, N., Keyomarsi, K. & Elledge, S. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, 75 (4), p. 805–816.

Hata, K., Fujitani, M., Yasuda, Y., Doya, H., Saito, T., Yamagishi, S., Mueller, B.K. & Yamashita, T. (2006) RGMa inhibition promotes axonal growth and recovery after spinal cord injury. *Journal of Cell Biology*, 173 (1), pp. 47-58.

Hata, K., Kaibuchi, K., Inagaki, S. & Yamashita, T. (2009) Unc5B associates with LARG to mediate the action of repulsive guidance molecule. *Journal of Cell Biology*, 184 (5), pp. 737-50.

Henion, T.R., Raitcheva, D., Grosholz, R., Biellmann, F., Skarnes, W.C., Hennet, T. & Schwarting, G.A. (2005) Beta1,3-N-acetylglucosaminyltransferase 1 glycosylation is required for axon pathfinding by olfactory sensory neurons. *Journal of Neuroscience*, 25 (8), pp. 1894-903.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquié, O., Ish-Horowicz, D. & Lewis, J. (1997) Maintenance of neuroepithelial progenitor cells by Delta-Notch signaling in the embryonic chick retina. *Current Biology : CB*, 7 (9), pp. 661-70.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M. & Stein, E. (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell*, 97 (7), pp. 927-41.

Huang, F., Pinkus, J., Pinkus, G., Fleming, M. & Andrews, N. (2005) A mouse model of juvenile hemochromatosis. *Journal of Clinical Investigation*, 115 (8), p. 2187–2191.

Imai, T., Suzuki, M. & Sakano, H. (2006) Odorant receptor-derived cAMP signals direct axonal targeting. *Science,* 314 (5799), pp. 657-61.

78

Ito, Y. (2004) Oncogenic potential of the RUNX gene family: 'overview'. *Oncogene*, 23 (24), pp. 4198-208.

Jennings, B., Tyler, D. & Bray, S. (1999) Target specificities of Drosophila enhancer of split basic helix-loop-helix proteins. *Molecular and Cellular Biology*, 19 (7), p. 4600.

Jhaveri, D., Saharan, S., Sen, A. & Rodrigues, V. (2004) Positioning sensory terminals in the olfactory lobe of Drosophila by Robo signaling. *Development*, 131 (9), p. 1903.

Kang, J., Gao, M., Feinleib, J.L., Cotter, P.D., Guadagno, S.N. & Krauss, R.S. (1997) CDO: an oncogene-, serum-, and anchorage-regulated member of the Ig/fibronectin type III repeat family. *Journal of Cell Biology*, 138 (1), pp. 203-13.

Kang, J., Mulieri, P.J., Miller, C., Sassoon, D.A. & Krauss, R.S. (1998) CDO, a robo-related cell surface protein that mediates myogenic differentiation. *Journal of Cell Biology*, 143 (2), pp. 403-13.

Kang, J., Feinleib, J.L., Knox, S., Ketteringham, M.A. & Krauss, R.S. (2003) Promyogenic members of the Ig and cadherin families associate to positively regulate differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, 100 (7), pp. 3989-94.

Kang, J., Yi, M., Zhang, W., Feinleib, J.L., Cole, F. & Krauss, R.S. (2004) Netrins and neogenin promote myotube formation. *Journal of Cell Biology*, 167 (3), pp. 493-504.

Kanomata, K., Kokabu, S., Nojima, J., Fukuda, T. & Katagiri, T. (2009) DRAGON, a GPI-anchored membrane protein, inhibits BMP signaling in C2C12 myoblasts. *Genes to cells*, 14 (6), pp. 695-702.

Kawauchi, S., Shou, J., Santos, R., Hébert, J.M., McConnell, S.K., Mason, I. & Calof, A.L. (2005) Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse. *Development*, 132 (23), pp. 5211-23.

Kawauchi, S., Santos, R., Kim, J., Hollenbeck, P.L., Murray, R.C. & Calof, A.L. (2009a) The role of foxg1 in the development of neural stem cells of the olfactory epithelium. *Annals of the New York Academy of Sciences*, 1170, pp. 21-7.

Kawauchi, S., Kim, J., Santos, R., Wu, H., Lander, A.D. & Calof, A.L. (2009b) Foxg1 promotes olfactory neurogenesis by antagonizing Gdf11. *Development*, 136 (9), pp. 1453-64.

Kee, N., Wilson, N., De Vries, M., Bradford, D., Key, B. & Cooper, H.M. (2008) Neogenin and RGMa control neural tube closure and neuroepithelial morphology by regulating cell polarity. *Journal of Neuroscience*, 28 (48), pp. 12643-53.

Kidd, T., Bland, K.S. & Goodman, C.S. (1999) Slit is the midline repellent for the robo receptor in Drosophila. *Cell*, 96 (6), pp. 785-94.

Koff, A., Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J. & Roberts, J.M. (1994) p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & Development*, 8 (1), pp. 9-22.

Komori, T. (2006) Regulation of osteoblast differentiation by transcription factors. *Journal of Cellular Biochemistry*, 99 (5), p. 1233–1239.

Kubo, T., Endo, M., Hata, K., Taniguchi, J., Kitajo, K., Tomura, S., Yamaguchi, A., Mueller, B.K. & Yamashita, T. (2008) Myosin IIA is required for neurite outgrowth inhibition produced by repulsive guidance molecule. *Journal of Neurochemistry*, 105 (1), pp. 113-26.

Kuns-Hashimoto, R., Kuninger, D., Nili, M. & Rotwein, P. (2008) Selective binding of RGMc/hemojuvelin, a key protein in systemic iron metabolism, to BMP-2 and neogenin. *American journal of physiology. Cell Physiology*, 294 (4), pp. C994-C1003.

Lee, S.M., McMahon, A.P., Danielian, P.S. & Fritzsch, B. (1997) Evidence that FGF8 signaling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development*, 124 (5), pp. 959-69.

Leighton, P.A., Mitchell, K.J., Goodrich, L.V., Lu, X., Pinson, K., Scherz, P., Skarnes, W.C. & Tessier-Lavigne, M. (2001) Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature*, 410 (6825), pp. 174-9.

Li, W., Lee, J., Vikis, H.G., Lee, S., Liu, G., Aurandt, J., Shen, T., Fearon, E.R., Guan, J., Han, M., Rao, Y., Hong, K. & Guan, K. (2004) Activation of FAK and Src are receptor-proximal events required for netrin signaling. *Nature Neuroscience*, 7 (11), pp. 1213-21.

Lin, D.M., Wang, F., Lowe, G., Gold, G.H., Axel, R., Ngai, J. & Brunet, L. (2000) Formation of precise connections in the olfactory bulb occurs in the absence of odorant-evoked neuronal activity. *Neuron*, 26 (1), pp. 69-80.

Lin, L., Goldberg, Y.P. & Ganz, T. (2005) Competitive regulation of hepcidin mRNA by soluble and cell-associated hemojuvelin. *Blood*, 106 (8), pp. 2884-9.

Liu, G., Beggs, H., Jürgensen, C., Park, H., Tang, H., Gorski, J., Jones, K.R., Reichardt, L.F., Wu, J. & Rao, Y. (2004) Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. *Nature Neuroscience*, 7 (11), pp. 1222-32.

Liu, X., Hashimoto, M., Horii, H., Yamaguchi, A., Naito, K. & Yamashita, T. (2009) Repulsive guidance molecule b inhibits neurite growth and is increased after spinal cord injury. *Biochemical and Biophysical Research Communications*, 382 (4), pp. 795-800.

Mahanthappa, N.K., Schwarting, G.A., Cooper, D.N. & Barondes, S.H. (1994) Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. *Development*, 120 (6), pp. 1373-84.

Malnic, B., Hirono, J., Sato, T. & Buck, L. (1999) Combinatorial receptor codes for odors. *Cell*, 96 (7), p. 713–723.

Mathis, L., Kulesa, P.M. & Fraser, S.E. (2001) FGF receptor signaling is required to maintain neural progenitors during Hensen's node progression. *Nature Cell Biology*, 3 (6), pp. 559-66.

Matsunaga, E., Tauszig-Delamasure, S., Monnier, P.P., Mueller, B.K., Strittmatter, S.M., Mehlen, P. & Chédotal, A. (2004) RGM and its receptor neogenin regulate neuronal survival. *Nature Cell Biology*, 6 (8), pp. 749-55.

Matsunaga, E., Nakamura, H. & Chédotal, A. (2006) Repulsive guidance molecule plays multiple roles in neuronal differentiation and axon guidance. *Journal of Neuroscience*, 26 (22), pp. 6082-8.

Mawdsley, D.J., Cooper, H.M., Hogan, B.M., Cody, S.H., Lieschke, G.J. & Heath, J.K. (2004) The Netrin receptor Neogenin is required for neural tube formation and somitogenesis in zebrafish. *Developmental Biology*, 269 (1), pp. 302-15.

Meriane, M., Tcherkezian, J., Webber, C.A., Danek, E.I., Triki, I., McFarlane, S., Bloch-Gallego, E. & Lamarche-Vane, N. (2004) Phosphorylation of DCC by Fyn mediates Netrin-1 signaling in growth cone guidance. *Journal of Cell Biology*, 167 (4), pp. 687-98.

Metzger, M., Conrad, S., Skutella, T. & Just, L. (2007) RGMa inhibits neurite outgrowth of neuronal progenitors from murine enteric nervous system via the neogenin receptor in vitro. *Journal of Neurochemistry*, pp. 2665-2678.

Mitchell, K.J., Leighton, P.A., Pinson, K.I., Kelly, O.G., Brennan, J., Zupicich, J., Scherz, P., Goodrich, L.V., Lu, X., Avery, B.J., Tate, P., Dill, K., Pangilinan, E., Wakenight, P., Tessier-Lavigne, M. & Skarnes, W.C. (2001) Functional analysis of secreted and transmembrane proteins critical to mouse development. *Nature Genetics*, 28 (3), pp. 241-9.

Miyamichi, K., Serizawa, S., Kimura, H.M. & Sakano, H. (2005) Continuous and overlapping expression domains of odorant receptor genes in the olfactory epithelium determine the dorsal/ventral positioning of glomeruli in the olfactory bulb. *Journal of Neuroscience*, 25 (14), pp. 3586-92.

Mombaerts, P., Nemes, A., Wang, F., Dulac, C., Chao, S.K., Mendelsohn, M., Edmondson, J. & Axel, R. (1996) Visualizing an olfactory sensory map. *Cell*, 87 (4), pp. 675-86.

Mombaerts, P. (2006) Axonal wiring in the mouse olfactory system. *Annual Review of Cell and Developmental Biology*, 22 (7), pp. 713-37.

Monnier, P.P., Sierra, A., Macchi, P., Deitinghoff, L., Andersen, J.S., Mann, M., Flad, M., Hosnberger, M.R., Stahl, B., Bonhoeffer, F. & Mueller, B.K. (2002) RGM is a repulsive guidance molecule for retinal axons. *Nature*, 419 (6905), pp. 392-5.

Mumm, J.S., Shou, J. & Calof, A.L. (1996) Colony-forming progenitors from mouse olfactory epithelium: evidence for feedback regulation of neuron production. *Proceedings of the National Academy of Sciences of the United States of America*, 93 (20), pp. 11167-72.

Murdoch, B. & Roskams, A.J. (2007) Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. *Journal of Molecular Histology*, 38 (6), pp. 581-99.

Nakao, K. & Campos-Ortega, J. (1996) Persistent Expression of Genes of the Enhancer of Split Complex Suppresses Neural Development in Drosophila. *Neuron*, 16, pp. 275-286.

Niederkofler, V., Salie, R., Sigrist, M. & Arber, S. (2004) Repulsive guidance molecule (RGM) gene function is required for neural tube closure but not retinal topography in the mouse visual system. *Journal of Neuroscience*, 24 (4), pp. 808-18.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. & Kageyama, R. (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO Journal*, 18 (8), pp. 2196-2207.

Oldekamp, J., Krämer, N., Alvarez-Bolado, G. & Skutella, T. (2004) Expression pattern of the repulsive guidance molecules RGM A, B and C during mouse development. *Gene expression patterns*, 4 (3), pp. 283-8.

Osnitz, D.M. (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *BioEssays*, 22 (2), pp. 108-12.

Pasterkamp, J., De Winter, F., Holtmaat, J. & Verhaagen, J. (1998) Evidence for a Role of the Chemorepellent Semaphorin III and Its Receptor Neuropilin-1 in the Regeneration of Primary Olfactory Axons. *Journal of Neuroscience*, 18 (23), pp. 9962-9976.

Polyak, K., Lee, M., Erdjument-Bromage, H., Koff, A., Roberts, J., Tempst, P. & Massagué, J. (1994) Cloning of p27Kip1, a Cyclin-Dependent Kinase Inhibitor and a Potential Mediator of Extracellular Antimitogenic Signals. *Cell*, 78 (July), pp. 59-66.

Rajagopalan, S., Deitinghoff, L., Davis, D., Conrad, S., Skutella, T., Chedotal, A., Mueller, B.K. & Strittmatter, S.M. (2004) Neogenin mediates the action of repulsive guidance molecule. *Nature Cell Biology*, 6 (8), pp. 756-62.

Ren, X., Ming, G., Xie, Y., Hong, Y., Sun, D., Zhao, Z., Feng, Z., Wang, Q., Shim, S., Chen, Z., Song, H., Mei, L. & Xiong, W. (2004) Focal adhesion kinase in netrin-1 signaling. *Nature Neuroscience*, 7 (11), pp. 1204-12.

Ren, X., Hong, Y., Feng, Z., Yang, H., Mei, L. & Xiong, W. (2008) Tyrosine phosphorylation of netrin receptors in netrin-1 signaling. *Neuro-Signals*, 16 (2-3), pp. 235-45.

Renzi, M., Wexler, T. & Raper, J. (2000) Olfactory Sensory Axons Expressing a Dominant–Negative Semaphorin Receptor Enter the CNS Early and Overshoot Their Target. *Neuron*, 28, pp. 437-447.

Ressler, K.J., Sullivan, S.L. & Buck, L.B. (1993) A zonal organization of odorant receptor gene expression in the olfactory epithelium. *Cell*, 73 (3), pp. 597-609.

Rhee, J., Mahfooz, N.S., Arregui, C., Lilien, J., Balsamo, J. & VanBerkum, M.F. (2002) Activation of the repulsive receptor Roundabout inhibits N-cadherin-mediated cell adhesion. *Nature Cell Biology*, 4 (10), pp. 798-805.

Samad, T.A., Srinivasan, A., Karchewski, L.A., Jeong, S., Campagna, J.A., Ji, R., Fabrizio, D.A., Zhang, Y., Lin, H.Y., Bell, E. & Woolf, C.J. (2004) DRAGON: a member of the repulsive guidance molecule-related family of neuronal- and muscle-expressed membrane proteins is regulated by DRG11 and has neuronal adhesive properties. *Journal of Neuroscience*, 24 (8), pp. 2027-36.

Samad, T.A., Rebbapragada, A., Bell, E., Zhang, Y., Sidis, Y., Jeong, S., Campagna, J.A., Perusini, S., Fabrizio, D.A., Schneyer, A.L., Lin, H.Y., Brivanlou, A.H., Attisano, L. & Woolf, C.J. (2005) DRAGON, a bone morphogenetic protein co-receptor. *Journal of Biological Chemistry*, 280 (14), pp. 14122-9.

Schmidtmer, J. (2004) Isolation and expression pattern of three mouse homologues of chick Rgm. *Gene Expression Patterns*, 4 (1), pp. 105-110.

Schmidtmer, J. & Engelkamp, D. (2004) Isolation and expression pattern of three mouse homologues of chick Rgm. *Gene Expression Patterns*, 4 (1), p. 105–110.

Schwarting, G.A. & Crandall, J.E. (1991) Subsets of olfactory and vomeronasal sensory epithelial cells and axons revealed by monoclonal antibodies to carbohydrate antigens. *Brain Research*, 547 (2), pp. 239-48.

Schwarting, G., Deutsch, G., Gattey, D. & Crandall, J. (1992) Glycoconjugates are Stage- and Position-Specific Cell Surface Molecules in the Developing Olfactory System, 1 : The CC1 Immunoreactive Glycolipid Defines a Rostrocaudal Gradient in the Rat Vomeronasal System. *Journal of Neurobiology*, 23 (2), pp. 120-129.

Schwarting, G., Kostek, C., Ahmad, N., Dibble, C., Pays, L. & Puschel, A. (2000) Semaphorin 3A is required for guidance of olfactory axons in mice. *Journal of Neuroscience*, 20 (20), p. 7691.

Schwarting, G.A., Kostek, C., Bless, E.P., Ahmad, N. & Tobet, S.A. (2001) Deleted in colorectal cancer (DCC) regulates the migration of luteinizing hormone-releasing hormone neurons to the basal forebrain. *Journal of Neuroscience*, 21 (3), pp. 911-9.

Schwarting, G.A., Raitcheva, D., Bless, E.P., Ackerman, S.L. & Tobet, S. (2004) Netrin 1-mediated chemoattraction regulates the migratory pathway of LHRH neurons. *European Journal of Neuroscience*, 19, pp. 11-20.

Schwarting, G.A., Raitcheva, D., Crandall, J.E., Burkhardt, C. & Püschel, A.W. (2004) Semaphorin 3A-mediated axon guidance regulates convergence and targeting of P2 odorant receptor axons. *European Journal of Neuroscience*, 19 (7), pp. 1800-10.

Schwarting, G.A. & Henion, T.R. (2007) Lactosamine differentially affects olfactory sensory neuron projections to the olfactory bulb. *Developmental Neurobiology*, 67 (12), pp. 1627-40.

Seeger, M., Tear, G., Ferres-Marco, D. & Goodman, C.S. (1993) Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron*, 10 (3), pp. 409-26.

Serizawa, S., Miyamichi, K., Takeuchi, H., Yamagishi, Y., Suzuki, M. & Sakano, H. (2006) A neuronal identity code for the odorant receptor-specific and activity-dependent axon sorting. *Cell*, 127 (5), pp. 1057-69.

Shin, G.J. & Wilson, N.H. (2008) Overexpression of repulsive guidance molecule (RGM) a induces cell death through Neogenin in early vertebrate development. *Journal of Molecular Histology*, 39 (1), pp. 105-13.

Shou, J., Calof, A.L., Murray, R.C. & Rim, P.C. (2000) Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. *Development*, 127 (24), pp. 5403-13.

Shou, J., Rim, P.C. & Calof, A.L. (1999) BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor. *Nature Neuroscience*, 2 (4), pp. 339-45.

Simpson, P. (1997) Notch signaling in development: on equivalence groups and asymmetric developmental potential. *Current Opinion in Genetics & Development*, 7 (4), p. 537–542.

Speck, N. & Gilliland, D. (2002) Core-binding factors in haematopoiesis and leukaemia. *Nature Reviews Cancer*, 2 (7), p. 502–513.

Srinivasan, K., Strickland, P., A, V., Shin, G. & Hinck, L. (2003) Netrin-1/Neogenin Interaction Stabilizes Multipotent Progenitor Cap Cells during Mammary Gland Morphogenesis. *Developmental Cell*, 4, pp. 371-382.

Stein, E. & Tessier-Lavigne, M. (2001) Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science,* 291 (5510), pp. 1928-38.

Stevens, A., Battye, R. & Jacobs, J.R. (1999) Axon repulsion from the midline of the Drosophila CNS requires slit function. *Development*, 126 (11), pp. 2475-81.

Storm, E.E., Rubenstein, J.L. & Martin, G.R. (2003) Dosage of Fgf8 determines whether cell survival is positively or negatively regulated in the developing forebrain. *Proceedings of the National Academy of Sciences of the United States of America*, 100 (4), pp. 1757-62.

Strotmann, J., Wanner, I., Helfrich, T. & Breer, H. (1995) Receptor Expression in Olfactory Neurons During Rat Development: In Situ Hybridization Studies. *European Journal of Neuroscience*, 7, pp. 492-500.

Strotmann, J., Levai, O., Fleischer, J., Schwarzenbacher, K. & Breer, H. (2004) Olfactory receptor proteins in axonal processes of chemosensory neurons. *Journal of Neuroscience*, 24 (35), pp. 7754-61.

Sullivan, S.L., Bohm, S., Ressler, K.J., Horowitz, L.F. & Buck, L.B. (1995) Target-independent pattern specification in the olfactory epithelium. *Neuron*, 15 (4), pp. 779-89.

Tenne-Brown, J., Puche, A. & Key, B. (1998) Expression of galectin-1 in the mouse olfactory system. *International Journal of Developmental Biology,* 42 (7), p. 791–799.

Thériault, F.M., Nuthall, H.N., Dong, Z., Lo, R., Barnabé-Heider, F., Miller, F.D. & Stifani, S. (2005) Role for Runx1 in the proliferation and neuronal differentiation of selected progenitor cells in the mammalian nervous system. *Journal of Neuroscience*, 25 (8), pp. 2050-61.

Tsai, R.Y. & Reed, R.R. (1997) Cloning and functional characterization of Roaz, a zinc finger protein that interacts with O/E-1 to regulate gene expression: implications for olfactory neuronal development. *Journal of Neuroscience*, 17 (11), pp. 4159-69.

Vielmetter, J., Kayyem, J.F., Roman, J.M. & Dreyer, W.J. (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. *Journal of Cell Biology*, 127 (6 Pt 2), pp. 2009-20.

Walz, A., Rodriguez, I. & Mombaerts, P. (2002) Aberrant sensory innervation of the olfactory bulb in neuropilin-2 mutant mice. *Journal of Neuroscience*, 22 (10), p. 4025.

Wang, F., Nemes, A., Mendelsohn, M. & Axel, R. (1998) Odorant receptors govern the formation of a precise topographic map. *Cell*, 93 (1), p. 47–60.

Wang, M.M. & Reed, R.R. (1993) Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. *Nature*, 364, pp. 121-6.

Wang, M.M., Tsai, R.Y., Schrader, K.A. & Reed, R.R. (1993) Genes encoding components of the olfactory signal transduction cascade contain a DNA binding site that may direct neuronal expression. *Molecular and Cellular Biology*, 13 (9), pp. 5805-13.

Wang, S.S., Lewcock, J.W., Feinstein, P., Mombaerts, P. & Reed, R.R. (2004) Genetic disruptions of O/E2 and O/E3 genes reveal involvement in olfactory receptor neuron projection. *Development*, 131 (6), pp. 1377-88.

Wang, S.S., Tsai, R.Y. & Reed, R.R. (1997) The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. *Journal of Neuroscience*, 17 (11), pp. 4149-58.

Wilson, N.H. & Key, B. (2006) Neogenin interacts with RGMa and netrin-1 to guide axons within the embryonic vertebrate forebrain. *Developmental Biology*, 296 (2), pp. 485-98.

Wu, H., Ivkovic, S., Murray, R., Jaramillo, S., Lyons, K., Johnson, J. & Calof, A. (2003) Autoregulation of neurogenesis by GDF11. *Neuron*, 37 (2), p. 197–207.

Xia, Y., Yu, P.B., Sidis, Y., Beppu, H., Bloch, K.D., Schneyer, A.L. & Lin, H.Y. (2007) Repulsive guidance molecule RGMa alters utilization of bone morphogenetic protein (BMP) type II receptors by BMP2 and BMP4. *The Journal of biological chemistry*, 282 (25), pp. 18129-40.

Xie, Y., Ding, Y., Hong, Y., Feng, Z., Navarre, S., Xi, C., Zhu, X., Wang, C., Ackerman, S.L., Kozlowski, D., Mei, L. & Xiong, W. (2005) Phosphatidylinositol transfer protein-alpha in netrin-1-induced PLC signaling and neurite outgrowth. *Nature Cell Biology*, 7 (11), pp. 1124-32.

Xie, Y., Hong, Y., Ma, X., Ren, X., Ackerman, S., Mei, L. & Xiong, W. (2006) DCC-dependent phospholipase C signaling in netrin-1-induced neurite elongation. *Journal of Biological Chemistry*, 281 (5), pp. 2605-11.

Xu, J., Liu, Z. & Osnitz, D.M. (2000) Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures. *Development*, 127 (9), pp. 1833-43.

Yang, F., West, A.P., Allendorph, G.P., Choe, S. & Bjorkman, P.J. (2008) Neogenin interacts with hemojuvelin through its two membrane-proximal fibronectin type III domains. *Biochemistry*, 47 (14), pp. 4237-45.

Zhang, D., Mehler, M.F., Song, Q. & Kessler, J.A. (1998) Development of bone morphogenetic protein receptors in the nervous system and possible roles in regulating trkC expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18 (9), pp. 3314-26.

Zhang, A., Yang, F., Wang, J., Tsukamoto, H. & Enns, C.A. (2009) Hemojuvelin-neogenin interaction is required for bone morphogenic protein-4-induced hepcidin expression. *Journal of Biological Chemistry*, 284 (34), pp. 22580-9.

Zhu, X., Wang, C., Dai, P., Xie, Y., Song, N., Liu, Y., Du, Q., Mei, L., Ding, Y. & Xiong, W. (2007) Myosin X regulates netrin receptors and functions in axonal path-finding. *Nature Cell Biology*, 9 (2), pp. 184-92.

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