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# Neogenin function and modulation in spinal motor neuron development

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# Résumé

Le système nerveux est la structure fonctionnelle la plus complexe que l'on connaisse. Cette structure permet aux organismes multicellulaires de percevoir leur existence en tant qu'entités individuelles. Le fonctionnement du système nerveux repose sur l'assemblage précis des circuits neuronaux. Pour ce faire, pendant le développement, les projections axonales des neurones sont guidées par des signaux de guidages. Ceux-ci se trouvent dans l'environnement extracellulaire et permettent aux neurones de trouver leurs cibles respectives. Pour détecter et pouvoir répondre aux signaux de guidage, les cônes de croissance à la pointe des axones expriment des récepteurs de signaux de guidage. Ceux-ci, lorsqu'ils se lient à leurs ligands, induisent des cascades de signalisation en aval, menant à des modulations dans la poussée et la direction des axones. Bien que nous ayons fait de grands progrès dans la compréhension des effets qu'ont les signaux de guidage sur les axones, on en sait beaucoup moins sur la façon dont ces signaux agissent en concert.

Les neurones moteurs qui résident dans la colonne moteur latérale (LMC) de la moelle épinière projettent leurs axones dans les membres, s'appuient sur une multitude de signaux de guidage. Cette thèse démontre que le récepteur transmembranaire Neogenin est impliqué dans plusieurs aspects du développement des neurones LMC, y compris la différenciation et la ségrégation des sous-types de neurones LMC ainsi que le guidage de leurs axones.

Notre laboratoire a précédemment démontré que les axones LMC sont guidés de manière synergique par Netrin-1 et les ephrins. Je vous ferai part de mes

résultats explorant le mécanisme sous-jacent à l'activité synergique de Netrin-1 et d’ephrin-A5 dans le guidage des axones LMC. Je démontre que ephrin-A5 sensibilise un sous-ensemble d'axones LMC à Netrin-1. Je propose que cela est la conséquence d'une augmentation induite par ephrin-A5 dans l'abondance de Neogenin conduisant à une liaison améliorée de Netrin-1 dans les cônes de croissance LMC. En outre, je montre que la modulation de ephrin-A5 des niveaux de Neogenin dépend de l'interaction entre ephrin-A5 et son récepteur EphA4. Je montre également que contrairement à la répulsion des axones de ephrin-A5, la sensibilisation à Netrin-1 se produit indépendamment de la queue cytoplasmique d'EphA4. Ces résultats suggèrent que la répulsion des axones induite par ephrin-A5 et la sensibilisation à Netrin-1 se produisent dans des voies moléculairement distinctes.

Netrin-1, Neogenin et les ephrins sont vastement impliqués lors du développement du système nerveux et au-delà. Notre démonstration que les interactions ephrin-A5 / EphA4 modulent l'abondance de Neogenin et la sensibilité à Netrin-1 ouvre la possibilité a ce que les ephrins puissent avoir un impact profond sur les processus cellulaires dépendants de la signalisation Netrin-1 et Neogenin.

**Mots-clés** : Neogenin, Netrin-1 ephrin, guidage axonal, neurones moteurs

# Abstract

The nervous system is the most exquisitely complex functional structure in the known universe. The nervous system is what enables multicellular organisms to experience life as individual entities. The functionality of the nervous system relies upon the precise assembly of neuronal circuits. To achieve this, during development, the extending axons of neurons are guided by cues in the extracellular environment that enable neurons to find their respective targets. To sense and respond to extracellular cues, growth cones at the tip of axons express guidance cue receptors. The interaction between guidance cues and their receptors induce downstream signaling cascades which lead to modulations in axon outgrowth and directionality. Although we have made great progress in understanding how individual cues guide axons, much less is known about how these cues act in concert.

Motor neurons that reside within the lateral motor column (LMC) of the spinal cord extend axons that innervate the limbs, rely on a multitude of guidance cues. The evidence presented in this thesis shows that the transmembrane receptor Neogenin is implicated in several aspects of LMC neuron development including LMC subtype differentiation and segregation as well as the guidance of their axons.

Our lab has previously shown that LMC axons are guided synergistically by Netrin-1 and ephrins. I will be presenting results exploring the mechanism underlying the synergistic activity of Netrin-1 and ephrin-A5 in LMC axon guidance. I provide evidence that ephrin-A5 sensitizes a subset of LMC axons to Netrin-1. I propose that this is a consequence of an ephrin-A5 induced increase in the abundance of

Neogenin leading to enhanced Netrin-1 binding in LMC growth cones. Furthermore, I show that the ephrin-A5 modulation of Neogenin levels is dependent on the interaction between ephrin-A5 and its receptor EphA4. I also show that contrarily to the repulsion from ephrin-A5, sensitization to Netrin-1 occurs independently from the cytoplasmic tail of EphA4. These results suggest that the ephrin-A5 induced axon repulsion and sensitization to Netrin-1 occur in molecularly distinct pathways.

Netrin-1, Neogenin and ephrins are vastly implicated during the development of the nervous system and beyond. Our demonstration that ephrin-A5/EphA4 interactions modulate the abundance of Neogenin and sensitivity to Netrin-1 brings forth the possibility that ephrins may have a profound impact on cellular processes implicating Netrin-1 and Neogenin.

**Keywords**: Neogenin, Netrin-1, ephrin, axon guidance, motor neuron

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# List of abbreviations

| | |
|---|---|
| ADAM | A disintegrin and metalloproteinase |
| APP | Amyloid precursor protein |
| BC | Boundary cap |
| BMP | Bone morphogenic protein |
| CNS | Central nervous system |
| Dcc | Deleted in colorectal cancer |
| DsCAM | Down syndrome cell adhesion mole |
| E | Chick Embryonic day |
| F-actin | Filamentous actin |
| FAK | Focal adhesion kinase |
| FNIII | Fibronectin type III |
| GAP | GTPase activating protein |
| GDNF | Glial-cell-line-derived neurotrophic f |
| GEF | Guanine nucleotide exchange facto |
| GPI | Glycosylphosphatidylinositol |
| GFRα1 | GDNF receptor alpha-1 |
| HH st. | Hamburger-Hamilton stage |
| HMC | Hypaxial motor column |
| HRP | Horseradish peroxidase |
| HSPG | Heparin sulfate proteoglycan |
| IF | Immunofluorescence |
| ICD | Intracellular domain |
| Ig | Immunoglobulin |
| KD | Knock down |
| LMC | Lateral motor column |

| | |
|---|---|
| LMCl | Lateral LMC |
| LMCm | Medial LMC |
| LMO4 | Lim-only protein 4 |
| MMC | Medial motor column |
| MN | Motor neuron |
| MT | Microtubule |
| Np | Neuropilin |
| OE | Olfactory epithelium |
| PGC | Preganglionic motor column |
| PKA | Protein kinase A |
| PS1 | Presenilin-1 |
| RA | Retinoic acid |
| Raldh2 | Retinaldehyde dehydrogenase-2 |
| RGM | Repulsive guidance molecule |
| RGC | Retinal ganglion cell |
| RTK | Receptor tyrosine kinase |
| Sema | Semaphorin |
| SFK | Src family kinase |
| SpMN | Spinal motor neuron |
| TCA | Thalamocortical axon |
| WNT | Wingless-type MMTV integration sit |
| WRC | Wave regulatory complex |
| WT | Wild type |

# List of figures

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First and foremost, I would like to thank my PhD supervisor, Dr. Artur Kania, for providing me with the opportunity to pursue graduate studies as a member in his lab. Over the years, he has dedicated an enormous amount of time discussing my experiments with me and I am grateful for all the scientific wisdom he has shared. I am also grateful for his encouragement in having high scientific standards and for participating in conferences. Thank you Artur for seeing the opportunity in turning an unexpected electroporation artifact into my first scientific publication. You have also provided me the opportunity of collaborating with several labs, experiences that have enriched my skill set and scientific career. Above all, thank you Artur for listening to what I had to say and taking my opinions into consideration.

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# CHAPTER I - INTRODUCTION

## 1 An introduction to axon guidance

To achieve functional circuitry, neurons must establish precise connections with their cellular targets. To do so, a neuron extends a cellular process termed axon, that navigates through surrounding tissue until the target is reached. Depending on the neuron type and the size of the organism, axons may span just a few micrometers, to several meters, as is the case for some axons in large vertebrates such as whales (Smith, 2009). The field of axon guidance investigates the mechanisms enabling axonal targeting during development. Understanding these mechanisms may be crucial for achieving functional recovery post-injury as well as in the prevention and recovery in neurodegenerative disorders. The father of contemporary neurobiology, Ramon Y Cajal S., first described the specialized cellular structure at the tip of extending axons termed the growth cone (Ramon, 1890). Thought to be inspired by evidence of chemotaxis in leukocytes guided by diffusible bacterial toxins, Cajal postulated the Neurotrophic theory whereby growth cones are proposed to be endowed with chemotactic sensitivities to factors secreted by their cells enabling axons to reach their targets (Metchnikoff, 1892; Ramon, 1892). Strong *in vivo* evidence for the neurotrophic theory came much later with the seminal experiments by Roger Sperry where Xenopus tadpole eyes were surgically reoriented and axonal fibers were able to reach their original targets despite the reorientation (Sperry, 1963). This demonstrated that the target cells were providing positional information rather than this information being intrinsic to the projecting neurons.

The proper targeting of axons is regulated by a multitude of factors that can ultimately be categorized as having attractive or repulsive effects on extending axons (Kolodkin and Tessier-Lavigne, 2011). Different modalities of guidance have been described including chemotaxis, whereby axons are guided towards or away from guidance cue gradients secreted from the target tissue. Guidance cues are integrated into positional information through the expression of guidance cue receptors at the growth cone surface (Kolodkin and Tessier-Lavigne, 2011). Other axon guidance mechanisms have since been described such as haptotaxis, whereby the guidance cue provides adhesion and mechanical traction which promotes the outgrowth of axons along the path of guidance cue expression (Carter, 1965; Varadarajan et al., 2017). Conversely, surround repulsion, whereby the axon outgrowth path is restricted by cells expressing repulsive cues that establish permissive corridors (Keynes et al., 1997). Axon fasciculation also contributes to axon pathfinding, axons often tend to extend as bundles and the impediment of axon fasciculation can result in certain axons to stray away (Landmesser et al., 1988; Landmesser et al., 1990). Furthermore, later born neurons sometimes rely on pre-existing axon tracts to reach their targets (Gallarda et al., 2008; Wang et al., 2011; Wang et al., 2014). The final guidance decision is target recognition and is crucial for axons to know when to stop extending and to begin forming synapses with the target cells (Timofeev et al., 2012).

## 2 The Growth Cone

The growth cone consists of a dynamic subcellular structure located at the tip of axons that is highly sensitive and responsive to the extracellular environment. Growth cones are responsible for guiding axons to their targets by enabling axonal steering, extension as well as retraction (Lowery and Van Vactor, 2009). Its function requires the tight orchestration and crosstalk between different processes such as actin cytoskeleton dynamics, adhesion complex assembly and disassembly as well as membrane trafficking (Vitriol and Zheng, 2012). The growth cone has a complex architecture that can be subdivided into different domains based on its cytoskeletal components (**Figure 1**) (Lowery and Van Vactor, 2009). The peripheral domain (P) is the most dynamic and consists of filopodia which can be described as finger-like projections containing long bundles of actin filaments (F-actin) as well as lamellipodia, located between filopodia, consisting of mesh-like branched F-actin networks (**Figure 1**). Individual microtubules (MT) can also be seen within filipodia. The central (C) domain consists of stable microtubule bundles originating from the axon shaft as well as various vesicles organelles and F-actin bundles (**Figure 1**). Positioned at the interface between the central and peripheral domains is the transition zone where actomyosin contractile structures are formed and allow F-actin retrograde flow, a process required for axon elongation (**Figure 1**) (Lowery and Van Vactor, 2009).

**Figure 1 The structural and spatial divisions in a typical growth cone**

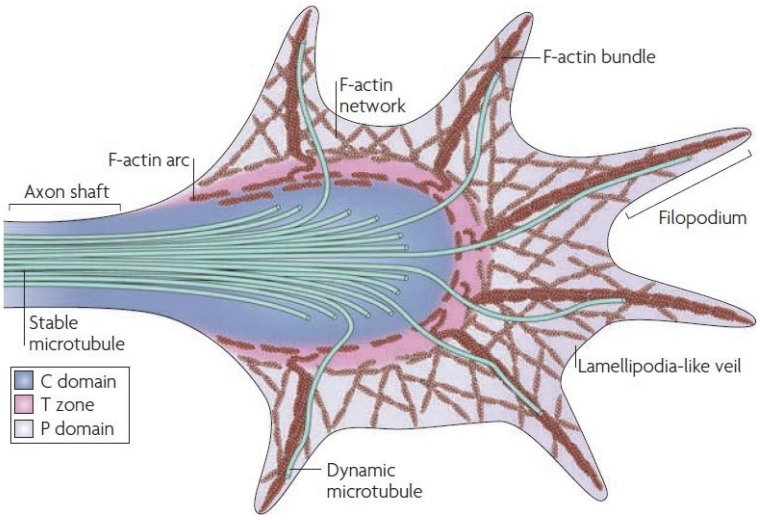



Diagram depicting the spatial subdivisions and structural components of a typical growth cone. (C) central domain, (T) the transition zone and (P) the peripheral domain. Reprinted by permission from Macmillan Publishers Ltd: Nature Publishing Group (Lowery and Van Vactor, 2009)

Growth cone motility and guidance depends largely upon actin dynamics. The spatiotemporal balance between actin polymerization in the P domain and myosin-based actin retrograde flow in the T domain is key in providing directionality to outgrowth (Vitriol and Zheng, 2012).

Rho family GTPases are key regulators of actin dynamics and link surface receptors to actin and microtubule organisation (Stankiewicz and Linseman, 2014). Rho GTPases cycle between their inactive GDP-bound and active GTP-bound states, their active states enable the activation of a variety of downstream regulators and effectors that act upon the actin cytoskeleton. The spatiotemporal regulation of Rho GTPases has been attributed to guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). While the GTP bound state of Rho GTPases is promoted by GEFs, the GDP bound state is promoted by (GAPs) (Stankiewicz and Linseman, 2014). The most extensively studied Rho GTPases members are RhoA,

Rac1 and Cdc42. Rac1/Cdc42 are typically attributed to growth cone development and axon extension while RhoA is typically attributed to axon retraction and growth cone collapse (Stankiewicz and Linseman, 2014). Nevertheless, evidence suggests that the activation balance of Rho GTPases rather than the activation state of a member is required for proper guidance (Barallobre et al., 2005).

## 3 Guidance cues

There are four classical families of guidance cues which comprise of the Netrins, ephrins, semaphorins and slits (Kolodkin and Tessier-Lavigne, 2011). Slits are secreted cues, ephrins are membrane bound and Netrins and semaphorins can be either membrane bound or secreted (Kolodkin and Tessier-Lavigne, 2011; Lai Wing Sun et al., 2011) . Whereas Netrins, ephrins and semaphorins can act as bidirectional cues, capable of eliciting both attraction and repulsion in a context dependent manner, slits have been solely associated with repulsion (Morales and Kania, 2016). More recently, a growing list of morphogens as well as growth factors and cell-adhesion molecules have also been implicated in axon guidance (Kolodkin and Tessier-Lavigne, 2011). Guidance molecules function in other neural functions such as cell migration and synaptogenesis as well as in non-neuronal tissues in processes such as angiogenesis, organogenesis, immune function as well as cancer biology (Hinck, 2004; Kolodkin and Tessier-Lavigne, 2011). The present work will focus on Netrins and ephrins for their relevance in the original work described in chapters III - Results.

## 4 Netrin-1

Netrins are a family of laminin-related extracellular proteins with homologues in all assayed organisms with bilateral symmetry (Lai Wing Sun et al., 2011). Mammals express three secreted netrins (Netrin-1-3) and two GPI-anchored netrins (Netrin-G1, Netrin-G2) (Lai Wing Sun et al., 2011). Netrins have been implicated in a variety of neural processes including cell and axon migration, axon arborisation, synaptogenesis as well as oligodendrocyte development (Lai Wing Sun et al., 2011). Outside the nervous system, Netrins have been implicated in the development of the pancreas, lungs, mammary gland as well as in angiogenesis (Cirulli and Yebra, 2007). Netrin-1, the most extensively studied Netrin member, can illicit axon attraction or repulsion depending on the expression of Netrin-1 receptors by neurons (Colamarino and Tessier-Lavigne, 1995; Hong et al., 1999; Lai Wing Sun et al., 2011; Poliak et al., 2015). The first ascribed function for Netrin-1 was the guidance of commissural axons (Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996).

### 4.1 Netrin-1 in commissural axon guidance

Commissural neurons are responsible for connecting both sides of the CNS. In vertebrates, Netrin-1 was shown to be required for commissural neurons in the developing brain and dorsal spinal cord to extend axons to an intermediate target, the ventral floor-plate (Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996). The guidance effect of Netrin-1 on commissural neurons was originally proposed to occur through long-range chemotaxis whereby secretion of Netrin-1 by floor plate

cells was proposed to establish a gradient of Netrin-1 ranging from high ventral to low dorsal. The long-range chemotaxis theory for Netrin-1 attraction was substantiated by *in vitro* culture experiments showing dorsal commissural axons being attracted towards floor-plate explants, positioned as far as ~250 μm away. This experiment suggested that Netrin-1 could act at a distance by diffusing away from its source cells (Tessier-Lavigne et al., 1988; Serafini et al., 1994). More recently, the requirement for long-range diffusion of Netrin-1 from the floor-plate has been challenged. Evidence in Drosophila shows that in both the midline and the visual system, a tethered Netrin is sufficient to rescue Netrin loss of function phenotypes (Brankatschk and Dickson, 2006; Timofeev et al., 2012). It was also demonstrated that the mechanical attachment of Netrin-1 to a substrate is required for axon outgrowth for mouse commissural axons *in vitro* (Moore et al., 2012). Furthermore, biochemical analysis of Netrin-1 shows that it is tightly bound to the membrane fraction and can only be extracted at very high salt concentrations, making it unlikely to be able to diffuse to long-distance within tightly packed cells, at least in the context of passive diffusion (Serafini et al., 1994). Recently, two independent groups demonstrated that progenitors at the ventricular zone, rather than cells from the floor plate, were the major source of Netrin-1 in guiding commissural axons (Dominici et al., 2017; Varadarajan et al., 2017). Both groups also highlight that Netrin-1 produced by progenitors at the ventricular zone is concentrated at the pial surface that lines the periphery of the spinal cord as well as on the progenitor radial processes encountered by commissural axons when projecting dorsally. Taken together, the evidence suggests that in the context of commissural axon guidance, Netrin-1 acts as a short-

range haptotactic cue rather than through long-range chemotaxis. It remains unresolved whether there is a requirement for the establishment of a Netrin-1 concentration gradient in the guidance of commissural axons in vertebrates, a mouse harbouring a mutation where Netrin-1 is tethered to the membrane may be useful in resolving this question.

### 4.2 Netrin-1 receptors

Netrin-1 receptors are members of the immunoglobulin superfamily (IgSF), proteins that contain domains homologous to immunoglobulins (Lai Wing Sun et al., 2011). There is an abundance of evidence implicating IgSF members in CNS development, a particular subfamily, the IgSF CAMs (cell adhesion molecules) have over 50 members expressed in the mammalian nervous system (Gu et al., 2015). Through homophilic and heterophilic interactions with other CAMs as well as with other receptors and elements of the ECM (extracellular matrix), they influence cell-cell adhesions and regulate cell processes such as cell migration, axon extension, neurite branching, synaptogenesis and plasticity (Leshchyns'ka and Sytnyk, 2016). The receptors identified as eliciting attraction towards Netrin-1 in vertebrates are Dcc, Neogenin and DsCAM (**Figure 3A**) (Lai Wing Sun et al., 2011). The repulsive effects of Netrin-1 are mediated by members of the UNC5 family which include four members in mammals; UNC5A-D (**Figure 3B**) (Leonardo et al., 1997; Hong et al., 1999).

### 4.3 DsCAM

DsCAM (down syndrome cell adhesion molecule) can be alternatively spliced to over 19,000 isoforms. Binding of self-isoforms was shown to elicit repulsion

required for dendrite and axon self-avoidance (Schmucker et al., 2000; Wojtowicz et al., 2007; Hattori et al., 2009). Although there is evidence for DsCAM being implicated in axon attraction to Netrin-1 in *Drosophila* and in chick, complete loss of DsCAM in the mouse does not result in any commissural guidance defects *in vivo,* or alters commissural axon responses to Netrin-1 *in vitro* (Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009; Palmesino et al., 2012). These discrepancies may reflect different requirements for DsCAM among species. It is also possible that the full knock-out of DsCAM in the mouse may bring about compensatory mechanisms not available in the context of chick DsCAM knockdown by shRNA.

### 4.4 Dcc

Unlike DsCAM, loss of Dcc dramatically alters commissural axon guidance, closely phenocopying mice lacking Netrin-1 (Fazeli et al., 1997). Because of defects in commissural axon guidance, human individuals with mutations in *Dcc* display mirror movements whereby contralateral involuntary movements mirror voluntary ones (Srour et al., 2010). Dcc (deleted in colorectal carcinoma) was named for its absence in most colorectal carcinomas and was proposed to be a putative tumor suppressor gene (Fearon et al., 1990). It's role as a tumor suppressor has been substantiated by evidence of Dcc being a death receptor, in prolonged absence of Netrin-1, Dcc initiates a caspase cascade leading to apoptosis (Mehlen et al., 1998; Castets et al., 2011). It's role as a dependence receptor is not ubiquitous, in mice completely lacking Netrin-1, no difference in apoptosis can be seen within the

developing CNS (Bin et al., 2015). It's role as a dependence receptor likely requires specific adaptors as exemplified by DIP13α, proposed to modulate the pro-apoptotic effects of Dcc (Liu et al., 2002).

### 4.5 Neogenin

Closely related to Dcc is Neogenin, first identified in the chicken and named so for its high expression during neural differentiation (Vielmetter et al., 1994). Neogenin is a receptor for members of the repulsive guidance molecule (RGM) family which in vertebrates includes RGMa, RGMb and RGMc, as well as a receptor for Netrins (Keino-Masu et al., 1996; Rajagopalan et al., 2004; Siebold et al., 2017).

### 4.6 Neogenin as a receptor for RGMs

During mouse development, whereas RGMa and RGMb are expressed in and out of the CNS in a rather complementary fashion, RGMc expression is excluded from the CNS (Niederkofler et al., 2004; Oldekamp et al., 2004). RGMs are not only ligands for Neogenin but have also been shown to be BMP co-receptors (Babitt et al., 2005; Samad et al., 2005; Babitt et al., 2006). The binding of RGMa to Neogenin in *cis* is proposed to enhance BMP downstream signaling (Healey et al., 2015). BMP/RGM/Neogenin have been involved in a variety of biological processes such as iron homeostasis, bone formation and astrocyte differentiation (Babitt et al., 2006; Zhou et al., 2010; Huang et al., 2016). RGMc/Neogenin signalling is required for iron

homeostasis, both Neogenin and RGMc null mice suffer from hepatic iron overload, reduced hepcidin expression and defective BMP signaling (Lee et al., 2010).

In the chick optic tectum and the Xenopus forebrain, RGMa acts as a repulsive cue for Neogenin expressing axons (Monnier et al., 2002; Rajagopalan et al., 2004; Wilson and Key, 2006). RGMa induced growth cone collapse is independent of BMP signaling and involves RhoA, the Rho-associated kinase ROCK as well as PKC (Conrad et al., 2007). Furthermore, RGMa induced growth cone collapse was shown to require Unc5b as well as the GEF LARG (Hata et al., 2009). Unc5 proteins are involved in the repulsion from both Netrin-1 and RGMa by associating with Dcc and Neogenin respectively (Hong et al., 1999; Hata et al., 2009). The convergence of Netrin-1 and RGMa signalling pathways at Unc5 receptors suggests a possible enhancement of repulsive signaling where both cues overlap. In mammals, RGMa inhibits axon outgrowth following spinal cord injury, RGMa inhibition using a blocking antibody results in an increase in axon outgrowth and improves functional recovery (Hata et al., 2006). In contrast, RGMa was shown to have neuroprotective capabilities for retinal ganglion cells (RGCs), following optic nerve transection, the intraocular injection of RGMa reduces RGC death (Koeberle et al., 2010). RGMa/Neogenin were also shown to be required for neural tube closure in Xenopus and mice by regulating neuroepithelium morphology (Niederkofler et al., 2004; Kee et al., 2008). Misexpression of RGMa in the developing chick hind-brain was shown to influence neural differentiation through an unknown mechanism (Matsunaga et al., 2006).

RGMb is the least studied member of the RGM family. There is evidence RGMb acting as both a tumor suppressor as well as promoting cancer progressing by regulating BMP signaling(Shi et al., 2015; Li et al., 2016). In the developing olfactory epithelium (OE), loss of RGMb and Neogenin both result in neural differentiation defects (Kam et al., 2016). The authors propose that progenitors expressing Neogenin bind RGMb on neighbouring cells and that these interactions regulate cell cycle kinetics and exit (Kam et al., 2016).

Thus, RGM/Neogenin signaling is involved in a wide variety of processes during development and beyond. RGM/Neogenin signalling outside the CNS often involves the regulation of BMP signaling, which to my knowledge, has not been demonstrated within the CNS. Whereas Netrin-1 binds Neogenin on its fibronectin type III (FNIII) domains 4 and 5, RGMs bind Neogenin on the FNIII domains 5 and 6 (**Figure 3**) (Yang et al., 2008; Xu et al., 2014). *In vitro,* Netrin-1 inhibits RGMa induced growth cone collapse of dorsal root ganglion neurons, suggesting that Netrin-1 and RGMa compete in binding Neogenin (Bell et al., 2013). This provides evidence for the cross-regulation potential of Netrin-1 and RGMa signalling pathways.

### 4.7 Neogenin as a receptor for Netrin-1

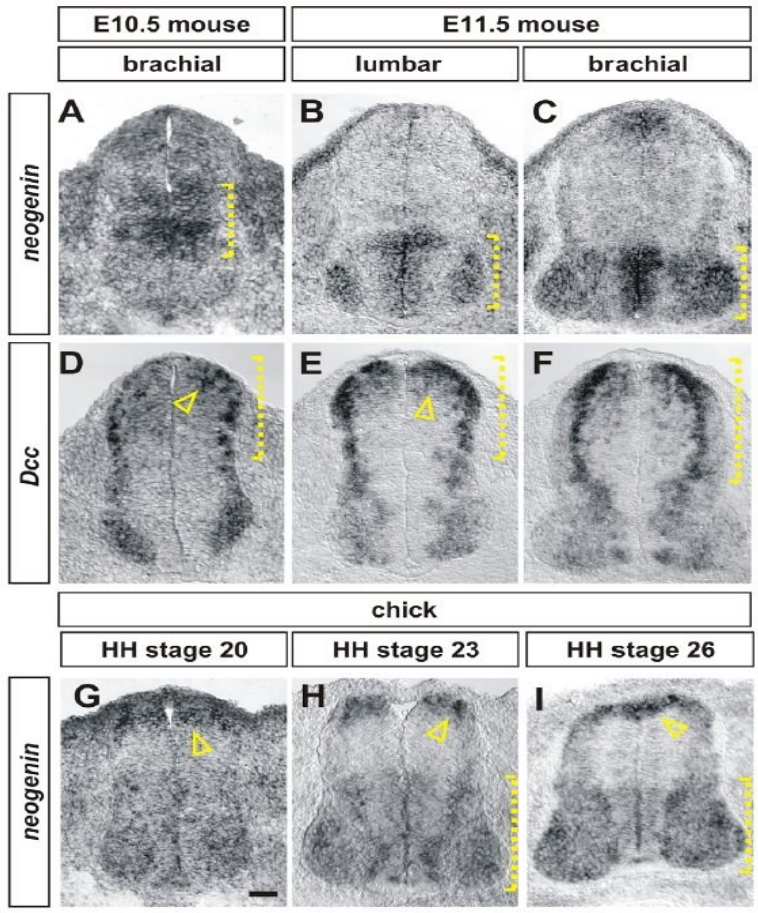
In mice, whereas the loss of Neogenin alone seemingly does not result in spinal commissural guidance defects, the loss of both Neogenin and Dcc exacerbates the guidance phenotype of Dcc mutants to a degree comparable to Netrin-1 mutants. This suggests that both Dcc and Neogenin collaborate in guiding commissural axons

in the mouse (Xu et al., 2014). Neogenin has been shown to be implicated in adult neurogenesis in mice. Loss of Neogenin results in neuroblast migration defects that are presumed to result from a loss of Netrin-1 attraction as well as aberrant differentiation resulting from impaired cell cycle kinetics (O'Leary et al., 2015). In the supraoptic tract of the developing *Xenopus* forebrain, knock down of either Neogenin or Netrin-1 results in similar guidance defects, suggesting they may be acting as a ligand/receptor pair in guiding supraoptic tract axons (Wilson and Key, 2006).

### 4.8 Chick Neogenin

Whereas mammals express both Dcc and Neogenin, the chicken does not appear to have a *Dcc* gene and only expresses Neogenin (Phan et al., 2011). Within the developing chick spinal cord, expression of *Neogenin* closely resembles the combined expression of *Dcc* and *Neogenin* in the mouse spinal cord, suggesting that chick Neogenin may be capable of accounting for the functions of both mouse Dcc and Neogenin (**Figure 2**) (Phan et al., 2011). In the chick spinal cord, knockdown of Neogenin by *in ovo* electroporation of an shRNA construct directed against Neogenin results in commissural axon guidance defects reminiscent of the Netrin1 and Dcc knock-out mice (Phan et al., 2011).

**Figure 2 Chick *Neogenin* expression in the spinal cord closely reassembles the combined expression of *Dcc* and *Neogenin* in the mouse**

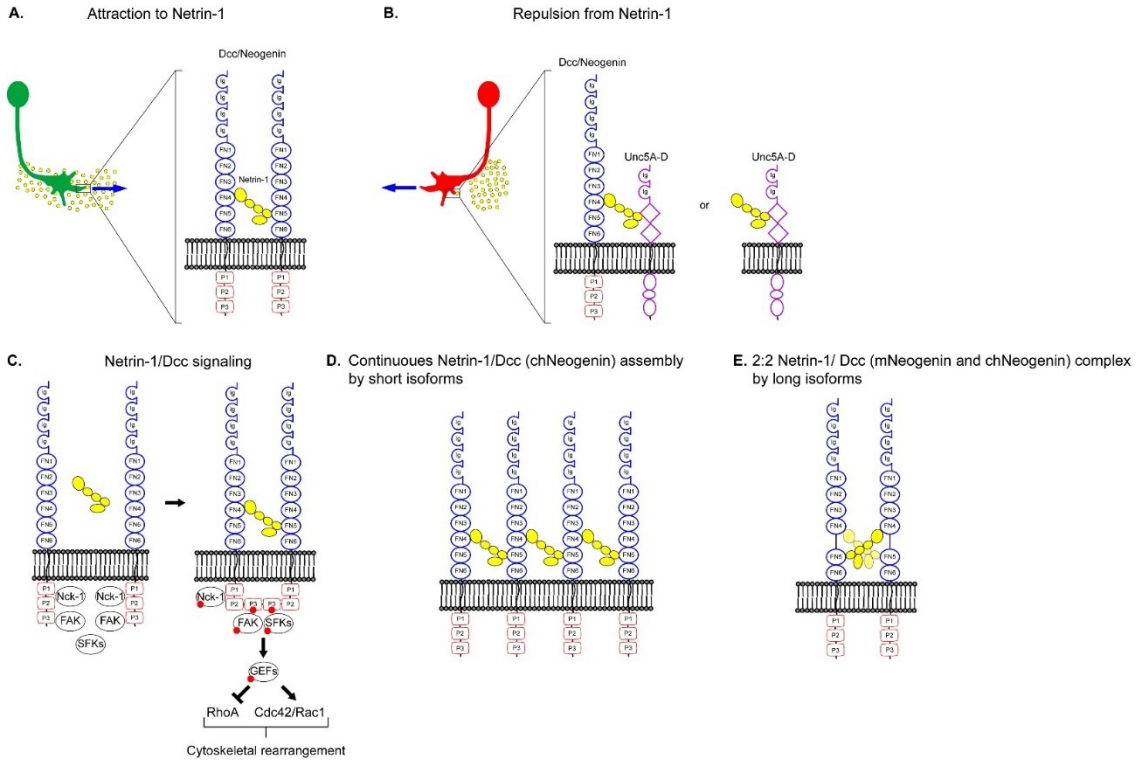



(A–H) In situ hybridization experiments for *Neogenin* (A–C, G–I) and *Dcc* (D–F) on transverse sections of the spinal cord of E10.5 (A, D) and E11.5 (B, C, E, F) mouse embryos and HH stage 20 (G), 23 (E) and 26 (I) chicken embryos. (A–C) In mouse, Neogenin is expressed first in the intermediate spinal cord (bracket, A). By E11.5, *Neogenin* is present at high levels in the ventral ventricular zone as well as broadly in motor neurons (brackets, B, C) (D–F) Mouse *Dcc* is expressed at highest levels in the dorsal-most neural progenitors (arrowhead, D, E) and in post-mitotic neurons (brackets, D–F) throughout the dorsal spinal cord as well as at lower levels in a broad population of motor neurons. (G–I) The distribution of chicken *Neogenin* is a composite of the expression patterns of both mouse *Dcc* and *Neogenin*. At all stages, the highest levels of *Neogenin* expression is in the dorsal-most spinal cord, in dorsal neural progenitors (arrowheads, G, I) and in a population of post-mitotic dorsal neurons whose position is consistent with their being commissural neurons (arrowhead, H). *Neogenin* is also present at lower levels in both the ventral ventricular zone and in motor neurons (brackets, H, I). Scale bar: A, D, G: 30 mm, B, C, E, F, H, I: 40 mm. Taken from (Phan et al., 2011)

### 4.9 Neogenin/Dcc structure

Dcc and Neogenin form a subfamily of IgSFs and share the same overall structure with their extracellular portions containing four immunoglobulin domains followed by six fibronectin type III repeats and three intracellular P domains (P1-3) (**Figure 3**) (Keino-Masu et al., 1996). Whereas the expression of Dcc is mostly restricted to the CNS, Neogenin is highly expressed in the CNS and in mesoderm derived tissue during development as well as in adulthood (Meyerhardt et al., 1997). Netrin-1 has two binding sites for Dcc and Neogenin and both receptors have 2 binding sites for Netrin-1 within their FNIII4 and FNIII5 domains (**Figure 3A**) (Meyerhardt et al., 1997; Xu et al., 2014). The crystal structure of Netrin-1 bound to Dcc and Neogenin reveals that two distinct architectures are possible depending on splice variants, 2:2 heteromers or a continuous ligand/receptor assembly (**Figure 3C, D**) (Xu et al., 2014). The Netrin-1 induced multimerization of Dcc results in the association of Dcc intracellular P3 domains and is required for Netrin-1 induced attraction (Stein et al., 2001).

## Figure 3 Netrin-1 signaling




The vertebrate transmembrane receptors Dcc and Neogenin share the same overall structure consisting of four immunoglobulin domains (Ig), followed by six fibronectin type III (FN1-6) repeats and three intracellular P domains (P1-3). Both receptors have two Netrin-1 (yellow) binding sites within their FN4 and FN5 domains. **A**. The expression of Dcc and Neogenin in growth cones results in Netrin-1 induced axon attraction and growth promotion. **B**. Repulsion from Netrin-1 relies on the binding of Netrin-1 to heteromers of Dcc (Neo) and members of the Unc5 family and may also be possible through Unc5s alone. **C**. Simplified diagram of Netrin-1/Dcc downstream signaling. The binding of Netrin-1 to Dcc induces the phosphorylation (red dots) of the P3 domain leading to receptor multimerization and phosphorylation of kinases such as, FAK and SFKs and association with adaptor proteins such as Nck-1. Activation of SFKs leads to the activation of GEFs which regulate Rho GTPases resulting in cytoskeletal rearrangements. **D, E**. Diagrams depicting the two distinct Netrin-1/Dcc and Netin-1/Neogenin signaling assemblies resulting form differences in linker lengths between FN4 and FN5 domains among Dcc and Neogenin splice variants. **D**. The short isoforms of either Dcc or chNeogenin allow for a continuous assembly of Netrin-1/Dcc (chNeogenin). **E**. The long isoforms of Dcc and chick or mouse Neogenin results in 2:2 heteromers of Netrin-1/Dcc (Neogenin). A-C were adapted from (Lai Wing Sun et al., 2011), D and E were adapted from (Xu et al., 2014)).

## 5 Netrin-1 signaling

Netrin-1 dependent signaling has been extensively studied, mostly focusing on Dcc as its receptor. A thorough review of the literature investigating Netrin-1 downstream signaling events would exceed the scope of this thesis, nevertheless, here are some of the key findings. Stimulation with Netrin-1 induces the tyrosine phosphorylation of Dcc in its P3 domain resulting in receptor multimerization (**Figure 3C**) (Meriane et al., 2004; Lai Wing Sun et al., 2011; Xu et al., 2014). In rodent neuroblastoma cells (NG108-15), ectopic expression of Dcc induces a Netrin-1 dependent increase in surface area and filipodia number. These effects were shown to require the activation of the Rho GTPases Cdc42 and Rac1 (Shekarabi and Kennedy, 2002). Two Rho GEFs, Trio and Dock180, have been implicated in mediating Rac1 activation downstream of Netrin-1/Dcc (Briancon-Marjollet et al., 2008; Li et al., 2008). Furthermore, through a Dcc dependent mechanism, Netrin-1 inhibits RhoA which in turn increases the levels of Dcc at the plasma membrane, thereby sensitizing rat commissural neurons to Netrin-1 outgrowth and guidance *in vitro* (Moore et al., 2008). The tyrosine kinase adaptor protein Nck-1 binds directly to Dcc in rat commissural neurons, the expression of a dominant negative Nck-1 inhibits Dcc's ability to induce Netrin-1 dependent neurite outgrowth and Rac1 activation in N1E-115 cells and fibroblasts respectively (Li et al., 2002).

Three independent groups provided evidence that focal adhesion kinase (FAK), a cytosolic protein tyrosine kinase, binds to Dcc and is phosphorylated upon Netrin-1 treatment and is required for Netrin-1 dependent outgrowth and attraction (Li

et al., 2004; Liu et al., 2004; Ren et al., 2004). FAK has also been implicated in signaling downstream of Neogenin in the context of growth cone collapse and myogenesis (Bae et al., 2009; Endo and Yamashita, 2009). FAK itself is regulated by phosphorylation of multiple tyrosine residues in and outside the nervous system, it is implicated in establishing cell morphology, maturation of adhesive structures and migration (Chacon and Fazzari, 2011). FAK has been implicated in signaling downstream of semaphorins, ephrins as well as Netrins and is involved in both axon attraction and repulsion (Chacon and Fazzari, 2011). Furthermore, FAK has been shown to interact with both activators and inhibitors of Rho GTPases and can phosphorylate as well as be phosphorylated by Src family kinases (SFK) (Mitra et al., 2005).

SFKs have been implicated in multiple aspects of neural development including differentiation, axon outgrowth, fasciculation and guidance (Morse et al., 1998; Hoffman-Kim et al., 2002; Kuo et al., 2005; Robles et al., 2005; Kao et al., 2009). Members of SFKs bind Dcc and are phosphorylated upon Netrin-1 stimulation, and SFK function is required for Netrin-1 dependent commissural axon outgrowth and guidance *in vitro* (Li et al., 2004; Meriane et al., 2004).

To summarize, Netrin-1 binding to Dcc/Neogenin induces the tyrosine phosphorylation within their P3 domains and receptor multimerization. These events enable the activation of adaptor proteins such as Nck-1 and signaling kinases such as FAK and SFKs. Association with FAK promotes traction to the extracellular environment whereas SFKs induce the activation of GEFs such as Trio and Dock180.

The spatiotemporal regulation of GEF activation in turn regulates Rho GTPases and the downstream cytoskeletal remodeling behind growth cone turning and axon outgrowth (**Figure 3C**).

## 6 Modulators of Netrin-1 signaling

### 6.1 Heparin sulfate proteoglycans

Heparin sulfate proteoglycans (HSPG) are polysaccharides of the glycosaminoglycan family and have been shown to bind to hundreds of proteins (Esko and Lindahl, 2001). HSPGs consist of core proteins mainly consisting of syndecans, glypicans, perlecan and agrin, on to which are attached highly charged heparin sulfate side chains (Lee and Chien, 2004). HSPGs can regulate the distribution of growth factors as well as enhance the binding of several ligands implicated in axon guidance (Lee and Chien, 2004). Netrin-1 binds heparin with high affinity and its extraction from the heparin fraction during chromatography requires high salt concentrations. (Serafini et al., 1994). Dcc also interacts with heparin via its fifth fibronectin domain, a domain also implicated in binding Netrin-1(Bennett et al., 1997; Xu et al., 2014). Spinal commissural neurons have a cell-autonomous requirement for heparin sulfate *in vivo* and *in vitro.* Dorsal spinal neurons that are genetically deficient for Heparin sulfate have commissural guidance defects comparable to Netrin-1$^{-/-}$ and Dcc$^{-/-}$ mice *in vivo* and are unresponsive to Netrin-1 dependent outgrowth *in vitro* (Matsumoto et al., 2007). It has been suggested that heparin sulfate could act as a coreceptor for Dcc in binding Netrin-1, therefore the

expression and distribution of HSPGs may play a vital role in modulating the strength of Netrin-1 signaling by promoting Dcc/Netrin-1 interactions (Matsumoto et al., 2007).

### 6.2 Slit/Robo

Once spinal commissural neurons reach the floor-plate, they continue extending contralaterally, away from the floor-plate, before turning rostrally and extend along the rostral-caudal axis of the spinal cord. Post-crossing neurons expressing the receptors Robo1/2 and Neuropilin-2, are repelled from the midline by Slit and Semaphorin3B respectively, expressed by the floor-plate (Zou et al., 2000; Long et al., 2004). To avoid stalling at the midline and recrossing the floor plate, commissural axons must become desensitized to Netrin-1 still expressed at the midline (Stein and Tessier-Lavigne, 2001). *Xenopus* spinal axons in culture are attracted towards a Netrin-1 gradient, when Slit is added to the Netrin-1 gradient, axons become unresponsive to Netrin-1 attraction while retaining the axon outgrowth promoting effect of Netrin-1 (Stein and Tessier-Lavigne, 2001). Furthermore, Xenopus spinal neurons exogenously expressing a truncated Robo receptor, without its intracellular tail, remain attracted towards Netrin-1 in the presence of Slit whereas axons expressing full-length Robo remain unresponsive (Stein and Tessier-Lavigne, 2001) These result suggest that Slit binding to Robo somehow desensitizes axons to Netrin-1 attraction. Experiments where Xenopus axons express chimeric Robo and Dcc receptors, revealed that the cytoplasmic tails of Robo and Dcc were required in Slit dependent desensitization to Netrin-1. Furthermore, co-immunoprecipitation experiments show that Dcc Robo interaction is Slit dependent. Together, these *in*

*vitro* results suggest that spinal commissural axons expressing Robo receptors become insensitive to Netrin-1 attraction when they encounter Slit at the floor-plate (Stein and Tessier-Lavigne, 2001).

*In vitro*, thalamocortical axons (TCAs) are repelled by Slit1 and are unresponsive to Netrin-1 (Bielle et al., 2011). For a subset of TCAs, the rostral TCAs, Slit1 and Netrin-1 provided in combination results in attraction towards Netrin-1, even when Slit1 is homogeneously distributed (Bielle et al., 2011; Dupin et al., 2015). Slit-1 induced attraction to Netrin-1 is PKA dependent and proposed to result from an increase in Dcc surface levels (Leyva-Diaz et al., 2014). The permissive effect of Slit1 on rostral TCA attraction to Netrin-1 requires sub-threshold concentrations of Slit1, insufficient for inducing repulsion (Dupin et al., 2015). This effect requires the expression of the Slit1 receptor Robo1 and its coreceptor FLRT3 by rostral TCAs (Leyva-Diaz et al., 2014).

FLRT proteins can promote cell adhesion through homophilic interactions and promote repulsion by binding Unc5 proteins in *trans* (Karaulanov et al., 2009; Seiradake et al., 2014). They have also been implicated in FGF signaling and synaptogenesis as well as neural migration. (Bottcher et al., 2004; O'Sullivan et al., 2012; Del Toro et al., 2017). Slit1/Robo interactions silence Netrin-1 attraction in commissural axons and allow Netrin-1 attraction in TCAs. The mechanism behind these opposite responses is currently unknown but may by in part due to differences in the expression of coreceptors such as FLRT.

### 6.3 Draxin

Draxin is a secreted protein recently identified as a guidance cue that shares no homology with any other guidance cue families (Islam et al., 2009). *Draxin*$^{-/-}$ mice display a mild fasciculation defects in spinal commissural axons and severe forebrain commissural axon guidance defects comparable to *Dcc*$^{-/-}$ mice (Fazeli et al., 1997; Islam et al., 2009). Draxin was shown to bind to the Netrin-1 receptors Dcc, Neogenin, DSCAM and Unc5A-C as well as to Netrin-1 (Ahmed et al., 2011; Gao et al., 2015). It is also noteworthy that Draxin and Netrin-1 have distinct binding domains on Dcc (Ahmed et al., 2011) *In vitro*, depending on the concentration and neuronal type, Draxin can either stimulate axonal outgrowth or inhibit outgrowth and induce growth cone collapse (Islam et al., 2009; Ahmed et al., 2011; Shinmyo et al., 2015). *Draxin*$^{-/-}$ mice also have thalamocortical projection defects and these defects are comparable in severity to the defects seen in (*Draxin*$^{+/-}$ ; *Dcc*$^{-/-}$ ), (*Draxin*$^{+/-}$ ; *Neo1*$^{GT/G}$), or (*Dcc*$^{-/-}$ ; *Neo1*$^{GT/G}$), suggesting that Draxin genetically interacts with Dcc and Neogenin in the context of axon guidance (Shinmyo et al., 2015). The role of Draxin in thalamocortical projections occurs independently from Netrin-1 since *Netrin-1*$^{-/-}$ mice don't have thalamocortical projections defects (Shinmyo et al., 2015). On the other hand, the similar forebrain commissure phenotype seen in *Netrin-1*$^{-/-}$, *Dcc*$^{-/-}$, and *Draxin*$^{-/-}$ mice raises the possibility that Draxin, by binding to both Netrin-1 and Dcc, may enhance Netrin-1/Dcc interactions (Ahmed et al., 2011; Gao et al., 2015)

### 6.4 Dcc/Neogenin cleavage

Dcc immunoprecipitations done on supernatant collected from cell cultures overexpressing Dcc reveals that Dcc's ectodomain is cleaved near the transmembrane domain (Galko and Tessier-Lavigne, 2000). Furthermore, it was shown that the *in vitro* ectodomain cleavage of Dcc can be blocked by a broad spectrum metalloprotease inhibitor (Galko and Tessier-Lavigne, 2000). The *in vitro* treatment of dorsal spinal explants with metalloprotease inhibitors results in an increase in Dcc expression and a potentiation towards Netrin-1 dependent axonal outgrowth (Galko and Tessier-Lavigne, 2000). It was also demonstrated that Netrin-1 treatment increases the ectodomain cleavage of Dcc (Bai et al., 2011).

The ectodomain of Neogenin can be cleaved by the protease ADAM17 and was proposed to modulate RGMa induced repulsion by regulating surface Neogenin (Okamura et al., 2011). Lrig2, a transmembrane protein that binds Neogenin, can regulate ADAM17 proteolysis of Neogenin (van Erp et al., 2015). The binding of RGMa to Neogenin inhibits Lrig2/Neogenin interactions and allows ADAM17 cleavage of Neogenin. *In vitro*, Lrig2 is required for the RGMa dependent RhoA activation and growth cone collapse. *In vivo*, knockdown of Ligr2 results in the promotion of optic nerve outgrowth following experimental optic nerve crush (van Erp et al., 2015). Considering the homology between Dcc and Neogenin, as well as evidence of Dcc cleavage by metalloproteases, it is likely that Dcc may also undergo ectodomain cleavage by ADAM17 (Galko and Tessier-Lavigne, 2000).

Several transmembrane proteins such as E and N-Cadherin, Notch and amyloid precursor protein (APP), have their ectodomains cleaved, which subsequently leads to the cleavage of their remaining intracellular fragments by γ-secretase (De Strooper et al., 1998; Naruse et al., 1998; De Strooper et al., 1999; Struhl and Greenwald, 1999; Marambaud et al., 2002). Dcc and Neogenin both undergo cleavage by γ-secretase, resulting in the release of their intracellular domains (ICD) (Taniguchi et al., 2003; Goldschneider et al., 2008).The ICDs of Dcc and Neogenin are capable of translocating to the nucleus and have been suggested to act as transcription regulators (Taniguchi et al., 2003; Goldschneider et al., 2008).

In *Drosophila*, the ICD of the Dcc/Neogenin homologue Frazzled, acts as a transcriptional activator of *commissurless*, an antagonist to Slit/Robo midline repulsion (Neuhaus-Follini and Bashaw, 2015). The ICD of Neogenin interacts directly with the transcription coactivator LMO4 (LIM-only protein 4) and can translocate to the nucleus where it can presumably regulate transcription (Goldschneider et al., 2008; Banerjee et al., 2016). RGMa induced outgrowth inhibition of RGC axons is lost when LMO4 is knocked down. This suggests that NeoICD/LMO4 may in part regulate repulsion from RGMa by regulating transcription (Banerjee et al., 2016)

Presenilin-1 (PS1) is an essential catalytic component of the γ-secretase complex, *PS1*$^{-/-}$ mice have a subset of spinal commissural axons that fail to cross the floor-plate, accompanied by spinal motor neurons that aberrantly extend axons to and across the midline (Bai et al., 2011). In early post-mitotic motor neurons, the

coexpression of Slit and Robo by motor neurons is proposed to silence motor neuron axon attraction to Netrin-1 at the midline. In *Robo1/2*$^{-/-}$ mice, a subset of motor neuron axons aberrantly cross the floor-plate as in *PS1*$^{-/-}$ mice (Bai et al., 2011). Co-immunoprecipitation experiments show that Robo1 interacts with full-length but not to a truncated Dcc, lacking its extracellular fragment, referred to as Dcc stub (Bai et al., 2011) In *PS1*$^{-/-}$ mice, the accumulation of Dcc stubs that bind to full-length Dcc at the membrane is thought to inhibit Robo/Dcc interaction, thereby enabling Netrin/Dcc signaling and attraction of motor neuron axons to the midline (Bai et al., 2011)

Thus, the strength and outcome of Netrin-1 signaling can be modulated by cell extrinsic factors, such as the presence of Draxin and Slit in the extracellular environment, as well as cell intrinsic factors, such as the expression of HSPGs, Robo1, FLRT3 and members of the Unc5 protein family. Netrin-1 signaling may also be modulated by the availability of downstream effectors such as Src, FAK, GEFs and GAPs. Exposure to guidance cues that share common signaling components could potentially attenuate Netrin-1 signaling by sequestering common downstream effectors. Conversely, exposure to other cues could potentiate Netrin-1 signaling by bringing downstream effectors in proximity to Netrin-1 signaling complexes.

## 7 An introduction to Eph/ephrin signaling

Eph receptors are a subfamily of the receptor tyrosine kinases (RTKs) that include 14 members sub classified into two groups, A and B, based on their general preference for binding either GPI anchored ligands (ephrin-As) or transmembrane

ligands (ephrin-Bs) (Gale et al., 1996; Kania and Klein, 2016). Exceptions to these classifications are EphB2 which can bind ephrin-A5 and EphA4, capable of binding ephrin-Bs (Gale et al., 1996; Himanen et al., 2004). Eph/ephrin signaling has vast implications in neural and non-neural tissues during development and adulthood as well as in cancer and neurodegenerative diseases (Kania and Klein, 2016)

A particularity of these receptor ligand pairs is that ephrin ligands also act as receptors for Ephs and that Eph/ephrin interactions can elicit downstream signaling cascades in the cells expressing ephrins (Klein, 2004). Forward signaling describes signaling occurring downstream of an Eph subsequent to ephrin binding whereas reverse signaling describes the reciprocal situation. Efficient signaling requires clustering of Ephs and ephrins and activation induces receptor multimerization (Gale et al., 1996; Taylor et al., 2017).

Both forward and reverse signaling involves the phosphorylation of conserved tyrosine residues and the activation of kinases such as SFKs and the modulation of Rho GTPases through GEFs and GAPs (Klein, 2004). Eph/ephrin forward and reverse signaling have been shown to elicit the cleavage of ephrins and Eph by members of the A disintegrin and metalloproteinase (ADAM) and by matrix metalloproteinases (MMPs) respectively (Hattori et al., 2000; Janes et al., 2005; Lin et al., 2008). Eph/ephrin signaling also leads to the trans-endocytosis of Eph/ephrin complexes in both forward and reverse signaling (Zimmer et al., 2003). Eph/ephrin endocytosis regulates cell-cell detachment and in some contexts, is required to terminate signaling (Marston et al., 2003; Zimmer et al., 2003; Janes et al., 2009).

Although most Eph/ephrin interactions result in cellular segregation, axon repulsion and growth cone collapse, there are several Eph/ephrin signaling events resulting in cell adhesion, axon outgrowth and arborisation (Castellani et al., 1998; Holmberg et al., 2000; Kania and Klein, 2016)

Ephs and ephrins are membrane tethered, therefore, their actions are thought to require cell-cell interactions (Kania and Klein, 2016). Recently, a novel Eph/ephrin signaling mechanism was described whereby cells secreting extracellular vesicles containing EphB2 illicit Eph/ephrin reverse signaling in nearby cells expressing ephrins. This raises the possibility that Eph/ephrin signalling may occur at a distance, without the need for direct cell-cell contact (Gong et al., 2016).

### 7.1 Eph/ephrin signaling in axon guidance

Ephs/ephrins are the prototypical example of proteins regulating topographic projections. Many axonal projections form topographic maps whereby the spatial relation among neuron cell bodies is maintained at their axonal targets. Topographic projections enable the efficient processing and integration of spatial relations. Topographic projections are exemplified by the projections of RGC axons from the retina to the optic tectum and the superior colliculus in the chick and mouse respectively (Triplett, 2014). Eph/ephrin signaling plays a crucial role in establishing topography in this system. To briefly summarize, RGCs in the retina display a graded expression of EphAs and EphBs, while the optic tectum and superior colliculus display graded expressions of ephrin-As and ephrin-Bs. These reciprocal expression patterns contribute in RGCs maintaining their relative positions at their target sites

and involves both forward and reverse signaling (Frisen et al., 1998; Thakar et al., 2011; McLaughlin et al., 2014).

Eph/ephrin signaling has also been implicated in establishing the axonal projections required for binocular vision. Whereas most RGCs project contralaterally across the midline at a region where axons from both eyes converge, termed the optic chiasm, a sub-population of RGCs project ipsilaterally in animals endowed with binocular vision (Petros et al., 2008). The expression of EphBs by a subset RGCs allows projecting axons to be repelled by ephrin-Bs at the optic chiasm thereby inhibiting midline crossing and allowing these axons to continue projecting ipsilaterally (Williams et al., 2003).

Like Netrin-1, ephrins have also been implicated in regulating axonal midline crossing in the spinal cord. The brain and spinal cord are connected via ascending and descending spinal tracts. Loss of EphA4 and similarly, loss of ephrin-B3, results in the aberrant crossing of the midline of both ascending and descending axonal tracts (Paixao et al., 2013). The expression of EphA4 on ascending and descending axons allows them to be repelled from ephrin-B3 expressed at the midline (Paixao et al., 2013). Functionally, EphA4 KO mice display a kangaroo-like hopping gait (Dottori et al., 1998). Similarly to guidance defects described for ascending and descending axonal tracts, the hopping phenotype was shown to originate from the aberrant midline crossing of excitatory spinal neurons, no longer repelled by ephrin-B3 expressed at the midline (Kullander et al., 2003; Borgius et al., 2014). The

involvement of Eph/ephrins in LMC axon guidance will be discussed in detail in the sections 10.1-10.3.

## 8. Spinal motor neuron development

Spinal motor neurons (SpMNs) are situated in the ventral portion of the spinal cord and make synaptic connections with muscle fibers in the periphery. The orchestrated activity of spinal motor neurons is behind the muscle contractions required for movement. SpMNs are cholinergic and receive inputs from motor neurons situated in the cortex, sensory neurons as well as by spinal interneurons (Stifani, 2014). SpMNs subtypes are arranged in discrete columns along the rostral/caudal axis of the spinal cord and are positioned vis-à-vis their peripheral targets (Bonanomi and Pfaff, 2010). Medial motor column (MMC) neurons span the entire spinal cord and innervate axial muscles. Hypaxial motor column (HMC) neurons innervate body wall muscles. Preganglionic motor column (PGC) neurons innervate sympathetic ganglia and are mainly restricted to the thoracic spinal cord. Lateral motor column (LMC) neurons innervate the forelimbs and hindlimbs and are positioned at the brachial and lumbar levels respectively (Figure 4 A, B) (Bonanomi and Pfaff, 2010).

During development, the neural tube, derived from endoderm, defines the structure that eventually gives rise to the spinal cord. The neural tube is populated by neural progenitors that differentiate into the diverse neurons that make up the spinal cord (Jessell, 2000). The mechanisms by which early neural subtype specification is

achieved have been studied in great detail (Jessell, 2000; Briscoe and Small, 2015). Within the neural tube, members of the wingless-type MMTV integration site family (WNT) and of the bone morphogenic protein family (BMPs) as well as some of their regulators, are expressed in a decreasing dorsal to ventral gradient. This is accompanied by a decreasing ventral to dorsal gradient of the morphogen sonic hedgehog (shh) expressed by the notochord and neural tube floor plate (Jessell, 2000). Along with retinoic acid (RA) provided by the paraxial mesoderm surrounding the neural tube, exposure to variable concentrations of morphogens results in the differential expression of homeodomain transcription factors by progenitor domains along the dorso-ventral axis of the neural tube (Jessell, 2000). The class identity of post-mitotic neurons is determined by the specific expression of homeodomain transcription factors expressed by the progenitors from which they derive (Jessell, 2000). All SpMNs originate from the same progenitor domain (pMN) defined by the expression of a combination of transcriptions factors including NKX6.1, PAX6, Olig2 and Hb9 (**Figure 4C, D**) (Briscoe and Ericson, 2001; Briscoe and Small, 2015).

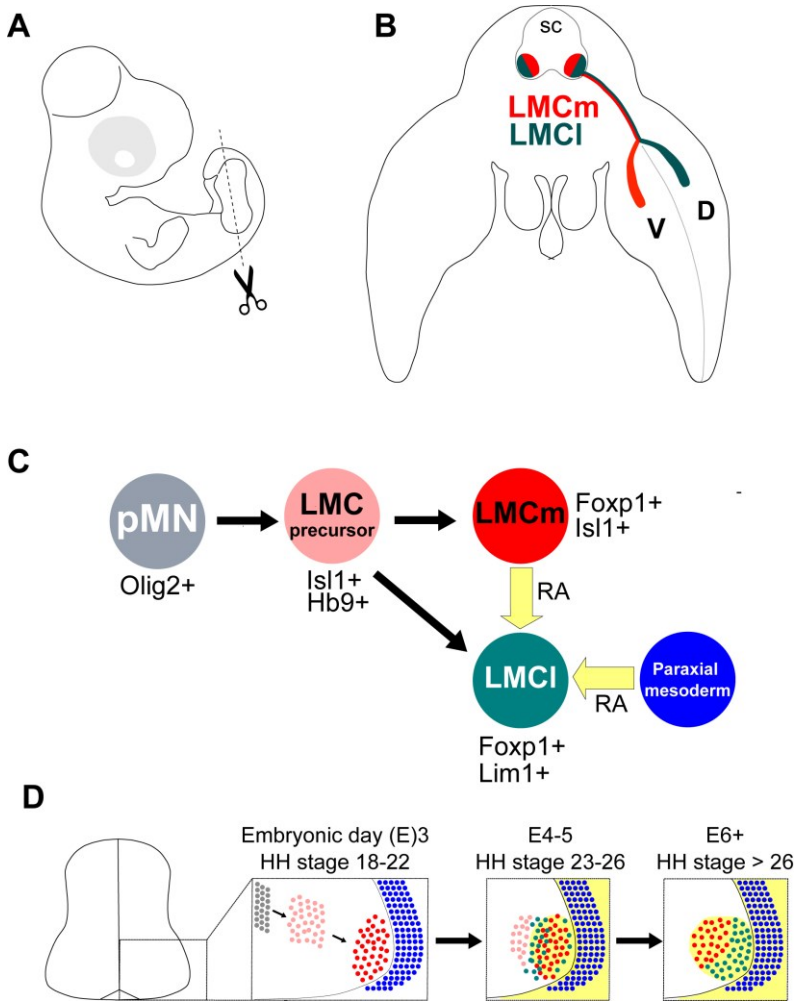
Expression and cross-repression of homeobox (Hox) proteins along the rostral/caudal axis have been implicated in defining the columnar extent of motor neuron subtypes. For example, Hox6 paralogs define the rostral/caudal extent of the brachial LMC, Hox9 paralogs define the PGC at thoracic levels while Hox10 paralogs define the extent of lumbar LMC (Dasen et al., 2003; Bonanomi and Pfaff, 2010).

` Neural progenitors lining the ventricular zone extend radial processes that terminate at the outer limits of the neural tube. At the apical surface of the ventricular

zone, neural progenitors remain adhered through N-Cadherin containing adherens junctions while their end-feet remain adhered to the periphery through integrin-laminin interactions (Meng and Takeichi, 2009; Rousso et al., 2012). Once they exit the cell cycle, new daughter neurons detach from the neuroepithelium at the ventricular zone and differentiate while migrating away perpendicularly from ventricular zone.

The expression of Hox genes leads to the expression of accessory factors by pMNs such as the forkhead transcription factors Foxp2 and Foxp4 (Rousso et al., 2012). In the chick neural tube, premature expression of Foxp2/4 leads to early detachment and differentiation of pMNs, while silencing Foxp2/4 results in a differentiation delay (Rousso et al., 2012). The Foxp2/4 induced detachment of pMNs from the neuroepithelium was shown to occur via the downregulation of N-Cadherin at the ventricular zone through direct binding of Foxp4 to a regulatory region of the N-Cadherin gene (Rousso et al., 2012). N-Cadherin, a homophilic adhesion molecule promoting cell-cell adhesion, was also shown to be downregulated as neural progenitors delaminate from the ventricular zone in the cortex (Zhang et al., 2010) As LMC neurons settle into the most ventrolateral portion of the neural tube, they begin expressing Foxp1 (**Figure 4C**) (Dasen et al., 2008; Rousso et al., 2008). Although LMC neurons maintain a generic motor neuron identity in Foxp1 KO mutants, their axonal projections within the developing limbs appears randomized (Dasen et al., 2008)

## Figure 4 Early development of spinal motor neurons of the LMC




**A**. Depiction of an E5 chick embryo, the dashed line at hindlimb level indicates the location of the embryo cross-section depicted in (B). **B**. LMCm and LMCl neurons reside in the ventral spinal cord (SC) at limb-level with LMCm neurons occupying the medial LMC and projecting axons to the ventral limb and LMCl neurons occupying the lateral LMC and projecting axons to the dorsal limb. **C, D**. LMC neurons arise form the pMN progenitor domain at the ventricular zone that express the transcription factor Olig2. When pMN cells exit the cell cycle, they express HB9 and Isl1 while migrating laterally. Differentiated LMCm neurons occupying the LMC express Foxp1 and Isl1and synthesize retinoic acid (RA, yellow). Later born prospective LMCl neurons express Isl1 and HB9 as they exit the cell cycle. They extinguish Isl1 and begin expressing Lim1 as they migrate in proximity to LMCm neurons. The switch from Isl1 to Lim1 expression is induced and maintained RA provided at first by LMCm neurons and later by all LMC neurons as well as by the paraxial mesoderm adjacent to the ventral spinal cord.

### 8.1 LMC neuron differentiation

LMC neurons differentiate into two subtypes, neurons occupying the medial portion of the LMC (LMCm), project axons to the ventral portion of developing limbs, neurons occupying the lateral portion of the LMC (LMCl), project axons into the dorsal part of the developing limb (**Figure 4B**) (Landmesser, 1978b). LMCm neurons are generated first, the later born LMCl neurons migrate past LMCm neurons and settle at the most ventrolateral position of the neural tube, adjacent to LMCm neurons (**Figure 4C, D**) (Hollyday and Hamburger, 1977). LMC subtype specification involves the differential expression of LIM homeodomain transcription factors. LMCm and LMCl neurons express Isl1 and Isl2 as they exit the cell cycle. While LMCm neurons retain the expression of Isl1/2, LMCl neurons replace expression of Isl1 with Lim1 as LMCl neurons migrate past LMCm neurons (**Figure 4C, D**) (Tsuchida et al., 1994). Retinoic acid signaling provided by LMCm neurons as well as by the paraxial mesoderm adjacent to the neural tube is necessary for LMCl neurons to switch from Isl1 to Lim1 expression (**Figure 4C, D**) (Sockanathan and Jessell, 1998; Ji et al., 2006). Lim1 and Isl1 are cross repressive, overexpression of either in chick LMC neurons leads to the loss of the other (Kania and Jessell, 2003). Lim1 and Isl1 expression contributes to the settling positions of LMC neurons as evident by the medialization and lateralization of LMC neurons overexpressing Isl1 and Lim1 respectively (Kania and Jessell, 2003).

Later in development, LMC neurons become subdivided into motor pools with each pool innervating a specific muscle (Landmesser, 2001). Muscle innervation

occurs in a topographic fashion, the relative position of muscles is mirrored by the relative positions of the motor pools they are innervated by (Landmesser, 2001). The mechanisms behind MN pool specification are still not fully understood but also relies in part on the combinatorial expression of Hox proteins and Cadherins (Dasen et al., 2005; Demireva et al., 2011; Lacombe et al., 2013).

## 9 LMC axon guidance

Evidence that the tissue targeted by LMC axons is instructive in guiding LMC neurons was first provided by transplantation experiments. When short sections of LMC containing neural tube are reversed on their anterior-posterior axis, thereby increasing the distance between LMC neurons and their original targets, LMC axons retain their ability to find their way to their original targets (Lance-Jones and Landmesser, 1981). This ability is lost when longer sections of neural tube are flipped, suggesting that molecular cues in the tissue surrounding the neurons are instructive in guiding LMC axons, although limited in the distance they can do so (Lance-Jones and Landmesser, 1981). Furthermore, when limb buds are rotated on their dorso-ventral axis prior to innervation, LMCm and LMCl axons retain their ability to innervate the ventral and dorsal limb respectively (Ferguson, 1983). These findings were later confirmed through genetic manipulations in mice that alter the dorso-ventral patterning of the limb. In the absence of the LIM homeodomain transcription factor Lmx1b., dorsal limb mesenchyme adopts a ventral identity, in such mutants, LMC axons innervate the limb in a seemingly random fashion (Chen et al., 1998;

Kania et al., 2000; Kania and Jessell, 2003). Conversely, loos of BMP signaling via the loss of bmpr1A, ventral limb mesenchyme adopts a dorsal identity. The dorsalization of the ventral limb compartment results in the innervation of LMCl axons in both dorsal compartments and a failure of LMCm axons to innervate the limbs (Ahn et al., 2001; Luria and Laufer, 2007; Luria et al., 2008). Genetic ablation of Lim1 also results in LMCl axons innervating the limbs seemingly randomly as seen in Lmx1b mutants (Kania et al., 2000). It is therefore apparent that LIM homeodomain transcription factors expressed by LMC neurons as well as by limb mesenchyme is crucial in providing the means by which LMC axons find their targets (Kania et al., 2000).

LMC axon guidance occurs in a stepwise fashion, first, LMC axons must exit the spinal cord through the ventral root. A subset of Boundary cap (BC) cells, derived from neural crest cells, settle adjacent to ventral root (Niederlander and Lumsden, 1996; Golding and Cohen, 1997). BC cells are required for maintaining LMC columnar integrity, in their absence, LMC neurons evade the spinal cord through the ventral root (Vermeren et al., 2003). At the lumbar level of the spinal cord, the expression of Neuropilin-2 and PlexinA1/A2 receptor complexes by motor neurons contributes to their repulsion from BC cells expressing Sema6a (Bron et al., 2007). At the brachial level, loss of Netrin-5 expression by BC cells and loss of Dcc by LMC neurons also results in MNs evading the spinal cord through the ventral root (Garrett et al., 2016).

LMC axons exiting the ventral root extend in tight fascicules, the cell adhesion molecules L1 and NCAM as well as polysialic acid contribute in fasciculating LMC axons (Tang et al., 1992; Tang et al., 1994). Peripheral nerves, including sensory and motor axons, are channeled into the anterior portion of the somites, giving the peripheral nerves a segmented appearance (Keynes and Stern, 1984). Chemorepellents expressed in the posterior and chemoattractant expressed in the anterior portion of the somites have been proposed to restrict nerve outgrowth (Oakley and Tosney, 1991; Fredette and Ranscht, 1994; Krull et al., 1995; Ebens et al., 1996; Wang and Anderson, 1997; Vermeren et al., 2000; Tzarfati-Majar et al., 2001; Roffers-Agarwal and Gammill, 2009). Loss of Sema3A in the forelimb mesenchyme, or loss of its receptor neuropilin-1 (Npn-1) in LMC neurons, results in fasciculation deficiencies as well as premature innervation of the limb (Huber et al., 2005).

**10 Dorso-ventral LMC guidance**

Once LMC axons extend past the somites, axons from a common plexus converge into a tight fascicule that extends to the base of the limbs. At the base of the limb, LMC axons are confronted to project either dorsally (LMCl axons) or ventrally (LMCm axons) **(Figure 4B)**. Several guidance cue families have been implicated in this binary choice, a review of the literature describing the mechanisms underlying this seemingly simple guidance decision will be described below (**Table 1**).

## Table 1 LMC dorso-ventral axon guidance summary

Medial LMC (LMCm) axon guidance to the ventral limb

| Limb mesenchyme expression | LMCm neuron expression | Reference |
|---|---|---|
| Repulsive cues in the dorsal limb: | | |
| Sema3F | Npn-2 | (Huber et al. 2005) |
| ephrin-B2 | EphB1 | (Luria et al. 2008) |
| Netrin-1 | Unc5c (Unc5a, Unc5b) | (Poliak et al. 2015) |
| Attenuation of Eph function in cis | | |
| Desensitization to ephrin-A5 in the ventral limb | ephrin-A5 binding in *cis* to EphAs | (Kao and Kania 2011) |

Lateral LMC (LMCl) axon guidance to the dorsal limb

| Limb mesenchyme expression | LMCl neuron expression | Reference |
|---|---|---|
| Attractive cues in the dorsal limb: | | |
| EphA4 | Ret, ephrin-As | (Marquardt et al. 2005, Bonanom et al. 2012) |
| GDNF | Ret, GFRα1 | (Kramer et al. 2006, Dudanova et al. 2010, Bonanomi et al. 2012) |
| Netrin-1 (*in vitro*) | Neogenin, Dcc | (Poliak et al. 2015) |
| Repulsive cues in the ventral limb: | | |
| ephrin-A5 | EphA4 | (Helmbacher et al. 2000, Eberhart et al. 2002, Eberhart et al. 2004, Bonanomi et al. 2012) |
| Attenuation of Eph function in cis | | |
| Desensitization to ephrin-B2 in the ventral limb | ephrin-B2 binding in *cis* to EphBs | (Kao and Kania 2011) |

### 10.1 Eph/ephrin forward signaling

LMC axons as well as cells from the limb mesenchyme, express several ephrins and Eph receptors as LMC axons are confronted with their dorso-ventral choice (Iwamasa et al., 1999; Eberhart et al., 2000; Luria et al., 2008). Of particular interest, EphA4 and EphB1 are expressed at higher levels in LMCl and LMCm axons respectively while ephrin-A5 and ephrin-B2 are expressed preferentially by ventral and dorsal mesenchyme respectively (Eberhart et al., 2002; Kania and Jessell, 2003; Marquardt et al., 2005; Luria et al., 2008)

In mice lacking EphA4 expression, the peroneal muscles responsible for foot flexion and digit extension are atrophied. Immunohistochemical analysis of hindlimb innervation reveals severe deficiencies in peroneal muscle innervation due to the loss of dorsally projecting neurons within the sciatic plexus (Helmbacher et al., 2000).

Furthermore, overexpression of EphA4 in chick lumbar LMC neurons results in LMCm axons misprojecting into the dorsal limb (Eberhart et al., 2000). As stated previously, the expression of Lim1 by LMCl is required for their guidance, absence of Lim1 in LMCl results in randomized axon projections (Kania and Jessell, 2003). Mice lacking Lim1 expression show lower levels of EphA4 expression in LMCl neurons and overexpression of Lim1 in the chick results in an increase in EphA4 expression in LMC neurons (**Table 1**) (Kania and Jessell, 2003)

Conversely, LMCm neurons expressing EphB1, are repulsed from ephrin-B2 enriched in the dorsal limb (Luria et al., 2008). In ephrin-B2 conditional KO mice, LMCm neurons misproject axons into the dorsal limb and the overexpression of ephrin-B2 in the ventral chick limb bud results in the misprojection of LMCm neurons to the dorsal limb mesenchyme (**Table 1**) (Luria et al., 2008).

### 10.2 Eph/ephrin reverse signaling

The above described a requirement for Eph/ephrin forward signaling, Eph/ephrin reverse signaling has also been described in LMC axon guidance. As previously mentioned, ephrin-As are GPI-anchored and lack an intracellular signaling domain. Therefore, signaling downstream of ephrinAs in *cis*, requires ephrinA coreceptors. In RGCs, the neurotrophic receptors TrkB and p75 can interact with ephrin-As in *cis* and are required for EphA induced reduction in axon branching and axon repulsion respectively (Lim et al., 2008; Marler et al., 2008).As LMC axons are confronted with their dorso-ventral choice, LMC neurons express ephrin-A5/A2 while

EphA4 is expressed in dorsal limb mesenchyme (Iwamasa et al., 1999; Eberhart et al., 2000; Kania and Jessell, 2003). LMCl axons are attracted to EphAs *in vitro* and a subset of LMCl axons misproject ventrally in mice carrying limb-specific EphA4 deletions (**Table 1**) (Marquardt et al., 2005; Dudanova et al., 2012). Although LMCl neurons express p75 and TrkB, theses receptors are not required in conveying Eph/ephrin reverse signaling in LMCl guidance (Bonanomi et al., 2012). The transmembrane tyrosine kinase receptor Ret interacts with ephrin-A5/A2 and was identified as an obligate coreceptor for EphA/ as seen in RGCs ephrin-A reverse signaling in LMCl guidance (**Table 1**) (Bonanomi et al., 2012). Contrary to RGCs, EphA/ephrin-A reverse signaling in LMCl axon guidance is attractive rather than repulsive, this may be the result of ephrin-A5 associating with Ret rather than with p75 or TrkB (Bonanomi et al., 2012). The mechanisms governing the preferential association of ephrin-A5 with specific coreceptors despite their coexpression remains unknown (Bonanomi et al., 2012).

The regulation of both EphA4/ephrin-A forward and reverse signaling, occurring simultaneously in parallel, was proposed to rely on the lateral segregation of EphA and ephrin-As into distinct membrane domains in LMCl growth cones (Dudanova et al., 2012). To my knowledge, no requirement for EphB/ephrinB reverse signaling has been ascribed in LMCm axon guidance to date.

During vertebrate development, sensory neuron axons trail behind MN axons and use them as scaffolds to reach their targets. Mutations causing MN axon guidance defects can result in sensory axons aberrantly extending over misrouted

MN axons (Gallarda et al., 2008; Wang et al., 2011; Wang et al., 2014). The requirement for MN axon tracts for sensory axons may be a consequence of motor circuits arising prior to sensory circuits during evolution (Wang et al., 2014). Sensory axons expressing ephrin-As track along epaxial MN axons expressing EphA3/4. Loss of EphA3/4 in MNs results in sensory axon defects that can be rescued by the expression of truncated EphA4, incapable of forward signaling. These results suggest that sensory axons employ EphA/ephrin-A reverse signaling to navigate over MN axons (Wang et al., 2011)

### 10.3 Eph/ephrin *cis* attenuation

RGC neurons coexpress EphAs and ephrin-As, *in vitro*, overexpression of ephrin-As in RGCs decreases their sensitivity to exogenous ephrin-As. Conversely, the removal of endogenous ephrin-As through PI-PLC treatment sensitizes RGCs to exogenous ephrin-As (Hornberger et al., 1999). Ephrins bind in *cis* and *trans* on different Eph domains. Ephrin-As binding in *cis* inhibits EphA tyrosine phosphorylation, possibly by blocking Eph receptor clustering (Carvalho et al., 2006).

In addition to being expressed in the ventral limb mesenchyme, ephrin-A5 is also expressed by LMCm neurons that navigate within the ventral limb. Conversely, ephrin-B2 expressed by the dorsal limb mesenchyme, is expressed in LMCl neurons (**Table 1**) (Kao and Kania, 2011). Knockdown of ephrin-A5 and ephrin-B2 in chick LMC neurons via *in ovo* electroporation of siRNAs results in misprojections of LMCm and LMCl axons respectively (Kao and Kania, 2011). In LMCm neurons, expression

of ephrin-A5 at the growth cone is thought to desensitize LMCm axons expressing EphA receptors to ephrin-A5 expressed in the ventral limb. Conversely, expression of ephrin-B2 on LMCl growth cones desensitizes them to ephrin-B2 in the dorsal limb (**Table 1**) (Kao and Kania, 2011). Thus Eph/ephrin *cis* attenuation enables LMC axons to fine-tune their responsiveness to exogenous ephrins through the expression of endogenous ephrins.

### 10.4 GDNF / GFRa1/RET

In EphA4 mutant mice, the severity of the peroneal nerve thinning varies depending on the mouse genetic background and *epha4* allele studied, suggesting that their may be other guidance cues instructing LMCl axons (Helmbacher et al., 2000; Kullander et al., 2001; Leighton et al., 2001). Glial-cell-line-derived neurotrophic factor (GDNF) promotes axon outgrowth, synaptogenesis and survival in various neurons (Ibanez and Andressoo, 2017). Secreted GDNF binds to GDNF Family Receptor alpha 1 (GFRα1), a GPI-anchored receptor that like ephrin-A5, requires association with coreceptors such as Ret (Airaksinen and Saarma, 2002). LMC neurons express GFRα1 as well as Ret while GDNF is expressed in the dorsal limb mesenchyme, adjacent to the dorso-ventral branchpoint (Kramer et al., 2006). Mice lacking GDNF have dorsal hindlimb innervation deficiencies with LMCl axons aberrantly innervating the ventral limb, reminiscent of the LMCl guidance defects seen in EphA4 deficient mice (**Table 1**) (Kramer et al., 2006). *In vitro*, GDNF enhances the outgrowth of LMCm and LMCl axons in a concentration dependent

manner, but only LMCl axons show chemotaxis towards GDNF. These results suggest that the LMCl axon guidance defects arise from a lack of attraction rather than resulting from an lack of outgrowth (Dudanova et al., 2010). The requirement for Ret in LMCl guidance is two-fold, it conveys EphA-ephrin-A reverse signaling as well as attraction to GDNF via interactions with GFRα1 (**Table 1**). *In vitro,* exposure to low doses of GDNF, strongly enhances EphA induced LMCl axon outgrowth (Bonanomi et al., 2012). GDNF treatment results in the re-localization of Ret into lipid rafts containing ephrin-A5 , this was proposed to contribute to the GDNF induced sensitization of LMCl axons towards EphAs (Bonanomi et al., 2012).

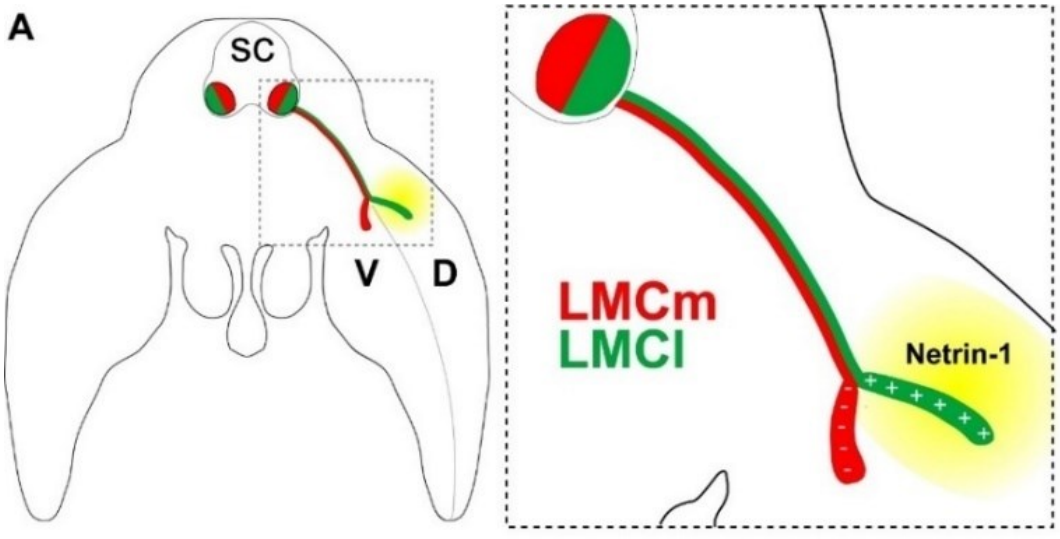
### 10.5 Npn-2/Sema3F

In chick and mice forelimbs, the repulsive cue Sema3F is expressed in the dorsal limb mesenchyme while its receptor Npn-2 is expressed in a subset of LMCm neurons (Huber et al., 2005). In Mice lacking either Npn-2 or Sema3F, a subset of LMCm axons aberrantly innervate the dorsal forelimb (**Table 1**) (Huber et al., 2005). Furthermore, overexpression of Npn-2 in the chick, via *in ovo* electroporation, results in LMCl axons innervating the ventral forelimb (Huber et al., 2005). The expression pattern of Npn-2 and Sema3F in lumbar LMC neurons and in hindlimb mesenchyme suggests that Npn-2/Sema3F are not implicated in guiding lumbar level LMC neurons (A.K. unpublished comment).

## 11 Netrin-1 in LMC guidance

Netrin-1 was identified as a potential guidance cue for LMC axons in a study using microarrays to analyze differential expression of mRNAs in WT vs *Lmx1b*$^{-/-}$ limb buds (Krawchuk and Kania, 2008). At the time of LMC axon outgrowth into the limbs, Netrin-1 is expressed in the dorsal limb mesenchyme, adjacent to the dorso-ventral branchpoint in forelimbs and hindlimbs of mouse and chick. Concomitantly, seemingly all LMC neurons express the attractive Netrin-1 receptors Dcc and Neogenin (just Neogenin in the chick), whereas the repulsive Netrin-1 receptor Unc5c is expressed by LMCm neurons (**Figure 5**). The expression pattern of Netrin-1 and its receptors suggests that Netrin-1 in the dorsal limb could attract LMCl axons through Dcc/Neogenin and repel LMCm axons expressing Unc5c (**Figure 5**).

**Figure 5 Model for the guidance of LMC axons by Netrin-1**




B

| | Netrin-1 receptor expression | Axon response |
|---|---|---|
| LMCm | Dcc, Neogenin, Unc5c | repulsion |
| LMCl | Dcc, Neogenin | attraction |

**A**. Diagram depicting the projection of LMCm and LMCl axons in the limbs of chick or mice embryos. During the dorso-ventral guidance of LMC axons, Netrin-1 (yellow) expressed in the dorsal limb mesenchyme attracts LMCl axons to the dorsal limb and repels LMCm axons into the ventral limb. **B**. Repulsion of LMCm axons from Netrin-1 is dependent on the expression of Unc5c (and possibly Dcc/Neogenin) by LMCm neurons. Attraction of LMCl axons to Netrin-1 is dependent on the expression of Dcc/Neogenin by LMCl neurons. Adapted from (Poliak et al., 2015).

### 11.1 *In vivo* requirement for Netrin-1 in LMC axon guidance

To test the requirement for Netrin-1 in LMC axon guidance, LMC axon projections were analyzed in mice mutant for various Netrin-1 receptors as well as in mice severely hypomorphic for Netrin-1 (*Ntn1*$^{GT}$*). Ntn1*$^{GT}$ mice carry a *Ntn1* gene trap allele expressing beta-galactosidase (Serafini et al., 1996). Guidance defects were assessed by the retrograde labeling of LMC neurons by injecting horseradish peroxidase (HRP) into the dorsal or ventral axon branch, enabling us to asses the extent of LMCm and LMCl guidance defects respectively. The presence of LMC axon guidance defects were ascertained in mice with the following genotypes: *Ntn1* $^{GT/GT}$, *Dcc*$^{-/-}$, *Neo1*$^{GT/GT}$, *Dcc*$^{-/-}$ ; *Neo1*$^{GT/GT}$ double mutants, *Dscam*$^{-/-}$ and *Unc5c*$^{-/-}$ . *Ntn1* $^{GT/GT}$ and *Unc5c*$^{-/-}$ mice show some LMCm axons misprojecting to the dorsal limb with defects much more severe in the *Unc5c*$^{-/-}$ mice (Poliak et al., 2015). This discrepancy in phenotype may be attributable to the remaining Netrin-1 expression in the Netrin-1 hypomorph. It is also possible that Unc5c may have Netrin-1 independent functions in LMCm axon guidance. For instance, FLRT3 can induce repulsion from Unc5 expressing cells and FLRT3 has been implicated in chick limb development (Karaulanov et al., 2009; Tomas et al., 2011). *Neo1*$^{GT/GT}$ is also a hypomorphic gene

trap allele and has variable residual Neogenin expression (Bae et al., 2009). It is therefore possible that the residual Neogenin expression in *Neo1*$^{GT/GT}$ mice is sufficient for attracting LMCl axons towards Netrin-1. Alternatively, Netrin-1 may be required for LMCl guidance events other than the dorso-ventral branch choice, such as the rostral-caudal extend of the dorsal branch. Unlike LMCl axons which are attracted to EphA and GDNF in the dorsal limb, no attractive cue has been attributed to guiding LMCm axons. LMCm axons may therefore by more dependent upon Netrin-1 than LMCl axons (**Table 1**). Since Ret is required for both EphA and GDNF attraction of LMCl axons, perhaps the involvement Netrin-1 in LMCl axon guidance *in vivo* could be revealed by comparing Ret$^{-/-}$; *Ntn1* $^{GT/GT}$ double mutants to Ret$^{-/-}$ single mutants (Bonanomi et al., 2012).

### 11.2 LMCm and LMCl axons respond to Netrin-1 *in vitro*

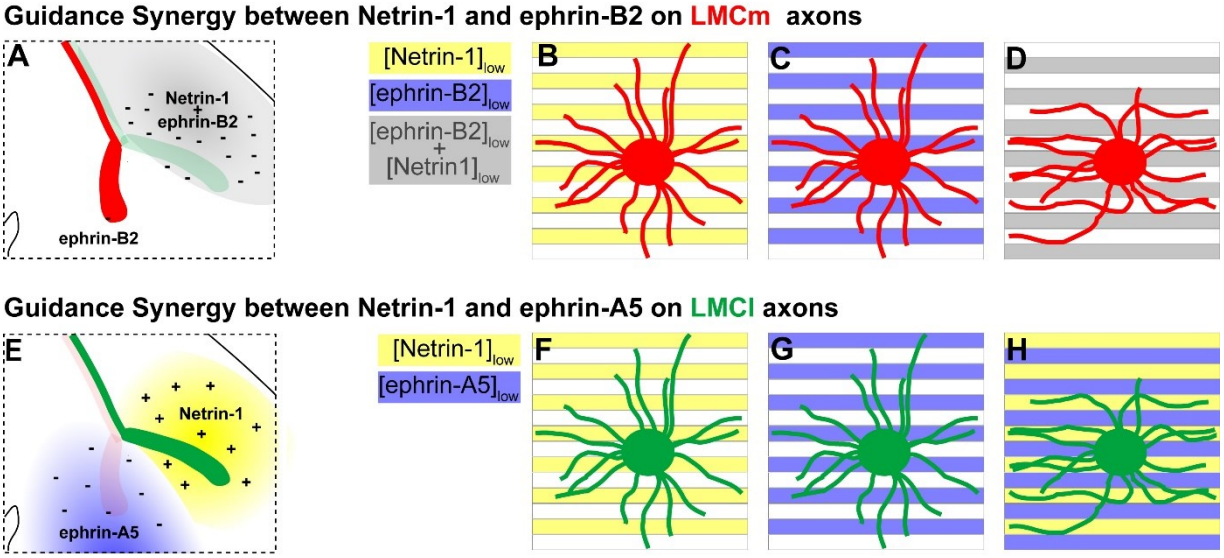
*In vitro* stripe assay experiments were carried out to assess if Netrin-1 can directly influence the behavior of LMC axons. Stripe assays consist of challenging axonal outgrowth of neurons cultured upon alternating stripes containing different proteins, followed by a quantitative assessment of stripe choice (Weschenfelder et al., 2013). When chick LMC explants are challenged to grow over either a control stripe containing the Fc fragment of IgG (Fc) or Netrin-1, LMCl axons show a clear preference for Netrin-1 containing stripes whereas LMCm axons avoid Netrin-1 stripes (Poliak et al., 2015). To assess the requirement for Unc5c in LMCm axons avoidance of Netrin-1 stripes, stripe assays were carried out with LMC explants electroporated with Unc5c siRNAs. Knockdown of Unc5c results in LMCm axons no

longer avoiding Netrin-1 stripes. Avoidance of Netrin-1 stripes can be rescued by overexpressing mUnc5c in conjunction with Unc5c siRNAs, ruling out siRNA induced off target effects in enabling Netrin-1 loss of avoidance (Poliak et al., 2015).

**12 Synergy between Netrin-1 and ephrins in LMC guidance**

We have demonstrated that both LMCm and LMCl axons are responsive to Netrin-1 and ephrins, to determine whether Netrin-1 and ephrin act additively or synergistically on LMC axon guidance, stripe-assay were performed. When LMCm axons are challenged to grow over stripes containing a low concentration of Netrin-1 vs. Fc or a low concentration of ephrin-B2 vs. Fc, no outgrowth preference can be seen (**Figure 8B, C**). Strikingly, when LMCm axons are challenged to grow over stripes containing a combination of low concentrations of (Netrin-1+ephrin-B2) vs. Fc, LMCm axons show a robust preference for Fc stripes (**Figure 8D**) (Poliak et al., 2015) . Theses results suggest that Netrin-1 and ephrin-B2 are integrated synergistically by LMCm axons. Similarly, when LMCl axons are challenged to grow over stripes containing a low concentration of Netrin-1 vs. Fc or a low concentration of ephrin-A5 vs. Fc, no outgrowth preference can be seen (**Figure 8F, G**). When LMCl axons are challenged by alternating stripes containing a low concentration of Netrin-1 vs. a low concentration of ephrin-A5, LMCl axons show a robust preference for Netrin-1 containing stripes, suggesting synergy between Netrin-1 and ephrin-A5 in LMCl guidance (**Figure 8H**) (Poliak et al., 2015) .

## Figure 8 Netrin-1 and ephrins are integrated synergistically by LMCm and LMCl axons




**A**. Diagram depicting LMCm axons *in vivo* being repelled by a combination of Netrin-1 and ephrin-B2 expressed in the dorsal limb. **B-D**. When LMCm axons are challenged with either a control stripe or a strip containing a low concentration of Netrin-1 (B) or ephrin-B2 (C), axons do not have growth preference. When LMCm axons are challenged with a control stripe or a strip containing a combination of a low concentrations of Netrin-1 and ephrin-B2 (D), LMCm axons display a robust avoidance of Netrin-1/ephrin-B2 stripes. **E**. Diagram depicting LMCl axons *in vivo* repelled by ephrin-A5 in the ventral limb and attracted to Netrin-1 in the dorsal limb. **F-H**. When LMCl axons are challenged with either a control stripe or a stripe containing a low concentration of Netrin1(F) or ephrin-A5 (G), axons do not have any growth preference. When the axons are challenged with alternating stripes of low concentrations of Netrin-1 and ephrin-A5 (H) LMCl axons show a pronounced preference for Netrin1 stripes while avoiding ephrin-A5 stripes. Adapted from (Poliak et al., 2015).

Immunodetection of Unc5c and EphB2 on LMC growth cones reveals a high incidence of membrane patches containing Unc5c and EphB2, suggesting possible ephrin/Netrin-1 receptor complexes at the growth-cone. In light of these results, co-immunoprecipitation experiments were carried out on lysates of HEK293 cells co-transfected with EphB2-GFP/ Unc5c-Myc or, EphA3-GFP/ Unc5c-Myc plasmids.

Interestingly, anti-GFP antibody precipitated Un5c when cells were transfected EphB2-GFP but not EphA3-GFP. Furthermore, treatment of HEK293 cells with ephrin-B2 increased the association between Ephb2-GFP and Unc5c-Myc and this association is decreased when cells are transfected with EphB2-KD-GFP, a kinase dead version of EphB2. In line with these results, in a cell line stably expressing EphB2 and transfected with Unc5c-Myc, Netrin-1 treatment together with ephrin-B2 increases the amount phosphorylated EphB2 in comparison with ephrin-B2 treatment alone. Thus, the synergistic activity of Netrin-1 with ephrin-B2 seen in LMCm axons in the context of the stripe-assay and the collapse assay may rely on an ephrin-B2 enhancement of EphB2/Unc5c interaction and the Netrin-1 enhancement of EphB2 phosphorylation.

Considering the evidence for SFKs involvement in both Netrin-1 and Eph downstream signaling, SFKs were considered as a possible molecular link enabling synergy between the two pathways (Zisch et al., 1998; Knoll and Drescher, 2004; Liu et al., 2004; Williams et al., 2006; Kao et al., 2009). To test this idea, levels of SFK-activating phosphorylation (pSFK) were assay in collapsed growth cones of cultured LMC explants that had been exposed to ephrin-B2 and Netrin-1 individually or in combination. The simultaneous treatment with both cues resulted in an increase in pSFK levels in collapsed LMC growth cones. Furthermore, blocking SFK function reduced growth cone collapse of LMC growth cones exposed to low concentrations of Netrin + ephrin-B2. In the context of the stripe assay, blocking SFK function attenuates LMCm axon stripe choice avoidance of stripes containing either cue alone

or in combination. Although these experiments demonstrate a requirement for SFKs in the combined activity of Netrin-1 and ephrin-B2 on LMC neurons, the loss of synergistic responsiveness when blocking SFKs could result from its requirement in individual pathways rather than being at the root of the supra-additive effects.

Our results demonstrate that both LMCm and LMCl neurons respond synergistically to Netrin-1 and ephrins. In LMCl axons, Netrin-1/ephrin-A5 synergize attractive-repulsive (opposing) forces, whereas in LMCm axons, Netrin-1/ephrin-B2 synergize repulsive-repulsive (congruent) forces (Poliak et al., 2015). It is tempting to speculate that Netrin-1/ephrin synergy likely arises from a common mechanism in LMCm and LMCl neurons but our experiments have not addressed this issue directly. It also remains to be determined whether Netrin-1 sensitizes axons to ephrins or if ephrins sensitizes axons to Netrin-1 or rather by mutual sensitization of both pathways. In Chapter III - C, I will provide evidence that furthers our understanding of the mechanism behind Netrin-1/ephrin synergy by focussing on the effects of Netrin-1 and ephrin-A5 on receptor dynamics in LMC growth cones.

# CHAPTER II - MATERIALS AND METHODS

**Chick tissue**

Fertilized eggs (Couvoir Simetin, Mirabel, QC) were incubated (Lyon Technologies, model PRFWD) at 39 ◦C according to standard protocols (Hamburger and Hamilton, 1951). Embryos were cut transversely at approximately 3 segments anterior to the hindlimbs, and fixed in a 4% solution of cold paraformaldehyde (Sigma) in PB (1.0 M phosphate buffer: 20.66% (w/v) Na2HPO4·7H2O, 3.2% (w/v), NaH2PO4·H2O, in H2O) for 75 min at 4 ◦C. Embryos were then washed 3 consecutive times in PBS followed by 3 washes of 5 min at 4 ◦C on a slowly shaking mixer (VMR International), and cryoprotected with 30% sucrose in PB overnight. The next day, embryos were embedded in O.C.T. (Sakura Finetek), and stored at −80 ◦C. 12 µm sections were collected using a Leica cryostat microtome (model CM3050S) and stored at −80 ◦C.

***In situ* mRNA detection**

*In situ* hybridizations were performed as described previously (Law et al., 2016). cRNA probes were generated as follows: target sequence amplification primers were designed using Primer3 version 0.4.0 software (Untergasser et al., 2012) with a probe size set at 600–800 bp. One-step RT-PCR was performed (QIAGEN) using the appropriate primers containing T7 polymerase promoters (Invitrogen) to make and amplify cDNA template from mouse E11.5 pooled brain RNA. The PCR product was purified by gel electrophoresis in 1% agarose gel and gel extraction using QIAquick gel extraction kit (QIAGEN). The purified DNA was then reamplified by PCR. The yield of DNA was estimated using the low DNA mass ladder

(Invitrogen) after gel electrophoresis. DIG-labeled RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase using DIG RNA labeling kit (Roche). All probes were verified by sequencing. Then, 12 µm tissue sections were fixed in 4% PFA, rinsed 3x in PBS, treated with 1 µg/ml proteinase K (in 6.25 mM EDTA, pH 8.0, Invitrogen + 50 mM Tris, pH 7.5; Fisher Scientific) for 15 min, post fixed in 4% PFA, rinsed in PBS, and then acetylated for 10 min in a mixture of 6 ml of triethanolamine (Sigma-Aldrich), 500 ml of distilled H2O, and 1.3 ml of acetic acid (Sigma-Aldrich). Samples were then rinsed and incubated in hybridization buffer containing 50% formamide (Sigma-Aldrich), 5µ SSC (20µSSC is 3 M NaCl, 0.3 M NaAc), 5x Denhardt's (Sigma-Aldrich), and 500 µg/ml salmon sperm DNA (Roche) inH2Ofor 2 h, followed by overnight hybridization at 72°C with DIG-labeled probes at a concentration of 2–5 ng/µl. After hybridization, samples were immersed in 5xSSC at 72°C, followed by 2 45 min washes in 0.2x SSC at 72°C and 1 5 min wash in 0.2x SSC at room temperature. Tissues were then rinsed with B1 buffer containing 0.1 M Tris, pH 7.5, and 0.15 M NaCl (Fisher Scientific) for 5 min, blocked with B2 buffer (10% heat inactivated horse serum in B1) for 1 h at room temperature, and incubated with anti-DIG antibody (1:5000 in B2; Roche) overnight at 4°C. Samples were then rinsed with B1 and equilibrated with B3 buffer containing 0.1 M Tris, pH 9.5, 0.1 M NaCl, and 0.05 M MgCl2 (Fisher Scientific). To detect bound anti-DIG antibodies, samples were incubated with B4 buffer containing [100 mg/ml NBT, 50 mg/ml BCIP (Roche), and 400 mM levamisol (Sigma-Aldrich) in B3 in the dark. The reaction was stopped by immersion in H2O**.**

**Chick and mouse section Immunohistochemistry**

Sectioned tissue was allowed to air dry, and then washed with PBS, incubated in blocking solution (1% heat inactivated horse serum in 0.1% Triton-X/PBS (Sigma)) for 5 min, followed by incubation overnight at 4 ◦C with primary antibodies diluted in blocking solution. The following primary antibodies and dilutions were used: guinea-pig anti-Foxp1 (1:16,000) (Dasen et al., 2008), sheep anti-GFP (1:1000) (Molecular Probes), mouse anti-Neurofilament (3A10) (1:100), anti-Isl1 (39.3f7) (1:100) (Developmental Studies Hybridoma Bank), rabbit anti-Lim1/2 (1:2500) (Tsuchida et al., 1994), Goat anti-Neogenin (R&D). Samples were incubated overnight with primary antibodies followed by three 5 min washes in PBS and 1 h incubation with the appropriate secondary antibody. Slides were then rinsed several times with PBS and mounted with Mowiol mounting medium (9.6%, w/v Mowiol (Calbiochem), 9.6% (v/v) 1 M Tris–HCl (Fisher Scientific), 19.2% (v/v) Glycerol (Fisher Scientific), in H2O). The following secondary antibodies were used: Cy3-conjugated AffiniPure donkey anti-rabbit and anti-mouse IgG (1:1000), Cy5-conjugated AffiniPure donkey anti-guinea-pig IgG (1:500) (Jackson ImmunoResearch Laboratory), Alexa Fluo 488-conjugated donkey anti-sheep and anti-mouse IgG (1:1000, Molecular Probes).

**LMC Explant Immunohistochemistry**

Prior to immunostaining, explants were fixed by replacing half of the culture media with a 37°C solution of 4% PFA, 3% sucrose in PBS for 20 min. at RT and washed repeatedly with PBS. Primary antibodies were incubated in blocking solution (1% heat-inactivated horse serum in 0.1% Triton-X/PBS; Sigma) either 1 hour at 37°C

or overnight at 4°C. The following primary antibodies were used: goat anti-Neogenin (1:300; R&D), rabbit anti-EphA4 (1:1000; Santa Cruz Biotechnology), rabbit anti-Netrin1 (1:1000; Abcam), guinea-pig anti-Unc5c (1:500; Thomas Jessell lab.) , mouse anti-Neuronal Class III β-Tubulin (Tuj1) (1:2000;  Covance), mouse anti-chicken BEN (1:00; Developmental Studies Hybridoma Bank). For surfaced enriched staining, antibodies were added simultaneously with the cue treatment solution.  Explants were washed repeatedly with PBS prior to incubation with appropriate secondary antibodies in blocking solution for 1 h at RT. The following secondary antibodies were used: Cy3- (or Cy-5)-conjugated AffiniPure donkey anti-mouse (rabbit, goat, or guinea pig) IgG (1:1000 for Cy3, 1:500 for Cy5 secondary antibodies; Jackson ImmunoResearch Laboratory), Alexa Fluor 488 donkey anti-mouse (rabbit or sheep) IgG (1:1000; Invitrogen). Explants were then washed repeatedly with PBS and mounted with Mowiol.

**Chick *in ovo* siRNA electroporation**

Chick *in ovo* spinal cord electroporation was performed as described (Croteau and Kania, 2011). For **Figure 11-6**, chick spinal cords were electroporated with the pN2-eGFP expression plasmids and siRNAs (Invitrogen) at a 1:10 molar ratio at HH st.18–19 and incubated at 39°C until harvesting at either E4 (HH st. 23-24) or E5 (HH st. 25–26). For **Figure 25**, Chick embryos were co-electroporated with a pN2-eGFP expression plasmid and pCAGGS-mEphA4 (Gatto, 2014) at a 1:4 molar ratio, and electroporated with pN2-chEphA4-GFP or pN1-chEphA4ΔICD- GFP (lacking intracellular domain) for **Figure 27 and Figure 29**.

**siRNA sequences:**

| | |
|---|---|
| [Neo]siRNA1 | |
| | CACUGGUCCGUAGUGAGAACCUUCATT |
| [Neo]siRNA1 | UGAAGGUUCUCACUACGGACCAGUGTT |
| [Neo]siRNA2 | CAGCCGGAGCUAUCGUCUGUCUAUATT |
| [Neo]siRNA2 | UAUAGACAGACGAUAGCUCCGGCUGTT |
| [Neo]siRNA3 | CAUCCCAACAUUACCUCCCACUUUCATT |
| [Neo]siRNA3 | UGAAGUGGGAGGUAAUGUUGGGAUGTT |
| Control [Neo]siRNA | GGCCUGAAUCCCUAUCUCGAAGUGATT |
| Control [Neo]siRNA | UCACUUCGAGAUAGGGAUUCAGGCCTT |
| | |
| [RGMb]siRNA2 | CCUUAACUGCACACCUAAATT |
| [RGMb]siRNA2 | UUUAGGUGUGCAGUUAAGGTT |
| [RGMb]siRNA3 | GCACCUAUCGCAACUCCAATT |
| [RGMb]siRNA3 | UUGGAGUUGCGAUAGGUGCTT |
| Control [RGMb]siRNA | ACUAUAAGAUACGUAUCGUCGGATT |
| Control [RGMb]siRNA | UCCGACGAUACGUAUCUUAUAGUTT |

***In vivo* LMC axon projection quantification**

Images were acquired using a Leica DM6000 microscope with Volocity imaging software (Improvision). GFP-labeled axonal projections were quantified by combining over threshold pixel counts in limb section images containing limb nerves (10–15 of 12 micrometer limb sections from each embryo) using Photoshop (Adobe). The dorsal or ventral limb nerve was selected by gating on the neurofilament channel and using the Lasso Tool, and pixel counts from the threshold to the maximal level were those indicated in the Histogram window of the GFP channel. Motor neuron numbers were quantified by combining cell counts of a series of spinal cord section images (5–10 of 12 micrometer limb sections from each embryo).

**Explant culture and treatment**

LMC explants were collected from st. 24-25 lumbar spinal cords and incubated for about 18 hours as previously described (Kao and Kania, 2011). For drug treatments, half of the motor neuron media in the explant cultures was replaced with media containing the drug and incubated for 20 min. prior to cue treatment. The following drugs were used: KT5720, KT5823, γ-Secretase Inhibitor IX (DAPT), Anisomycin and SU6656 were purchased from Calbiochem. MG132, Chloroquine diphosphate and the KYL peptide were purchased from Tocris. For cue treatment, half of the culture media was replaced with media alone or media containing recombinant mouse Netrin1, recombinant human ephrin-A5-Fc or Fc (R&D systems). Prior to explant treatment, recombinant human ephrin-A5-Fc was preclustered in a 5:1 molar ratio with either mouse or goat anti-human Fc (Sigma-Aldrich) for 30 min. at 37°C.

***In vitro* stripe assay**

In vitro stripe assay using explants of spinal motor columns were performed as described (Kao and Kania, 2011). In brief, carpets of alternating stripes of Netrin-1, ephrin-A5-Fc, or Fc only as controls were prepared using silicon matrices with micro-well system (provided by Dr. Martin Bastmeyer's laboratory). E5 chick spinal motor column was dissected using sharp tungsten needles (World Precision Instruments) and collected in motor neuron medium [Neurobasal (Invitrogen), B-27 supplement (1:50, GIBCO), L-Glutamate (0.5 mM, Sigma-Aldrich), L-Glutamine (25mM, GIBCO),

and Penicillin-Streptomycin (1:100, Wisent)]. The excised motor column was then trimmed into explants with the size of 1/4 width of motor column, and 20 explants were plated on laminin (20 µg/ml, Invitrogen) coated culture dishes containing different combinations of stripe carpets in motor neuron medium and incubated in 95% air and 5% $CO_2$ at 37ºC overnight. Following incubation, motor column explants were fixed with 1:1 mixture of 4% paraformaldehyde (Sigma) and 30% sucrose in PBS for 5 minutes followed by 4% paraformaldehyde for 5 minutes. After PBS washes, explants were incubated with selected primary antibodies diluted in blocking solution containing 20% serum in 0.3% Triton-X/PBS(Sigma) for 2 hrs at room temperature (RT). Following PBS washes, explants were incubated with secondary antibodies diluted in the same blocking solution for 2 hrs at RT.

**Growth cone image acquisition and quantification***:*

Images were acquired using LSM700 and LSM710 Zeiss confocal microscopes. Regions of interests (ROIs) delimitating growth cones and subsequent analysis was done using Imagej. To assess fluorescence distribution within the growth cones, a Matlab application was designed and programmed by Dr. Dominic Fillion. The application divides growth cones into 100 bins, with bin 1 at the geometric centre and bin 100 following the outer perimeter of the growth cone. The mean fluorescence value for each bin was determined and plotted, bin#100 was omitted from the analysis due to the region of interest (ROI) occasionally slightly over representing the growth cone resulting in the absence of signal in bin#100. To

determine differences in fluorescence distribution regardless of differences in overall fluorescence, the fluorescence value for each bin was divided by the mean fluorescence value of bins 1-99 and termed relative fluorescence distribution. The protein aggregate analysis was done using the particle analysis tool in Imagej. The analysis of fluorescence overlap, the fluorescence signal in individual channels was thresholded and an image representing signal overlap between two channels was generated and quantified. To determine the change in overlap, the data was treated as such ((% signal overlap with cue treatment) / (%signal overlap control treatment).

**Statistical analysis**

Data from the experimental replicate sets were evaluated using Microsoft Excel and Aabel (Gigawiz). Unless stated otherwise, means were compared with Student's unpaired t-test with the threshold for statistical significance set at 0.05, error-bars represent S.E.M. A minimum of three independent experiments were carried out for each treatment condition and between 20 and 35 growth cones were analysed in each experimental replicate

# CHAPTER III - RESULTS

## 1. Neogenin in the responsiveness of LMCl axons to Netrin-1

### Rationale

Upon my arrival in the Kania lab, my initial task was to investigate the requirement for chick Neogenin in LMC axon guidance. By then, most experiments implicating mouse genetics that would eventually be published in Poliak et al. had been completed and the requirement for Netrin-1 in LMC axon guidance had been established. In the chick, Netrin-1 related expression patterns and results obtained from the misexpression of Unc5c in LMC neurons suggested that Netrin-1 could be guiding chick LMC axons as it does in mice. The residual Neogenin expression in *Neogenin*$^{GT/GT}$ mice and the possible redundancy between Dcc and Neogenin in LMC axon guidance in mice prompted us to explore Neogenin function in the chick.

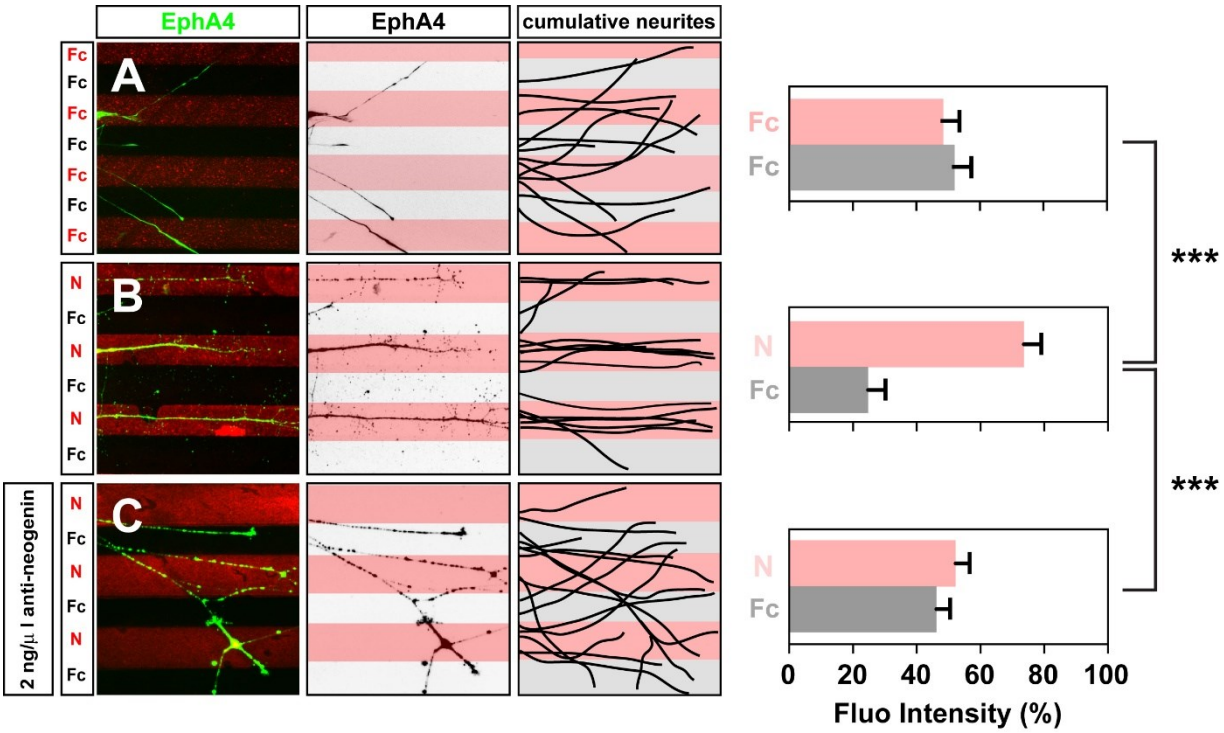
### Neogenin is required for the responsiveness of LMCl axons towards Netrin-1

Through the stripe assay, we have previously demonstrated that chick LMCl axons display attraction towards Netrin-1(Poliak et al., 2015). LMC neurons express the attractive Netrin-1 receptor Neogenin as LMCl axons encounter Netrin-1 expressed in the Dorsal limb. To investigate the requirement for Neogenin in LMCl axon responsiveness towards Netrin-1, a Neogenin antibody that antagonizes the binding of Netrin-1 to Neogenin was added to stripe assays challenging LMCl axons with either a control stripe or Netrin-1 stripes. In the presence of the Neogenin antibody, LMCl axons loose their preference for Netrin-1 stripes, demonstrating the necessity of Neogenin in responsiveness of LMCl axons to Netrin-1 (**Figure 6)**.

**Dcc rescues the loss of LMCl responsiveness to Netrin-1**

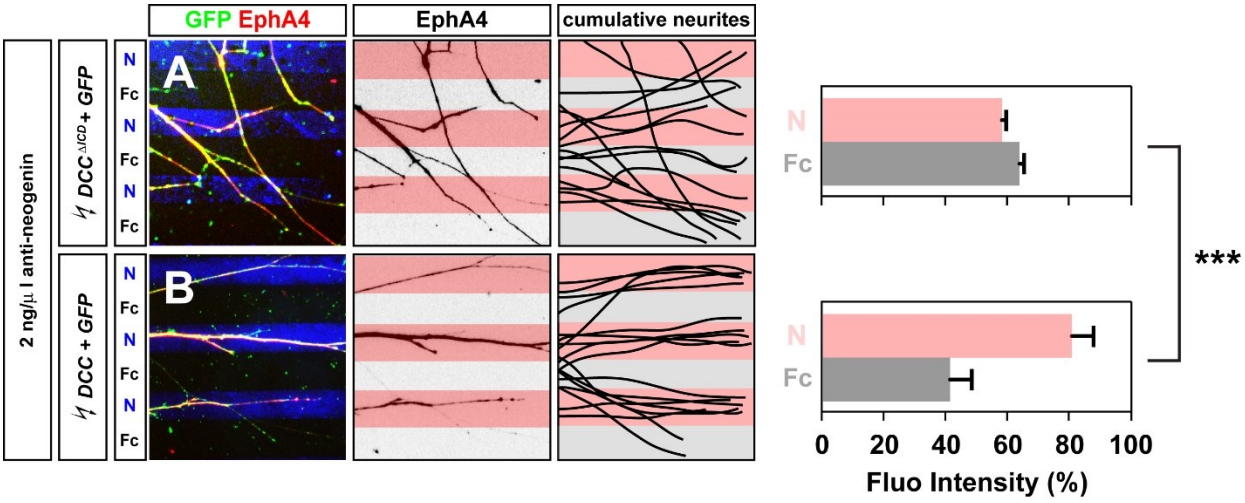
In the chick spinal cord, knockdown of Neogenin by *in ovo* electroporation of an shRNA construct directed against Neogenin results in commissural axon guidance defects reminiscent of the Netrin1 and Dcc knock-out mice suggesting that chick Neogenin can substitute for Dcc in mediating attraction towards Netrin-1 (Phan et al., 2011). To ensure that the loss of LMCl axon responsiveness towards Netrin-1 in the presence of Neogenin antibody was not due to off target effects, I attempted to rescue responsiveness to Netrin-1 by overexpressing Dcc in LMCl axons in the presence of Neogenin antibody. Overexpression of full-length rat Dcc but not a truncated rat Dcc, lacking the cytoplasmic signaling tail ($DCC^{DICD}$), rescued the preference of LMCl axons for Netrin-1 stripes in the presence of Neogenin antibody (**Figure 7**). These results suggest that the loss of LMCl axon responsiveness to Netrin-1 is not the result of off-target effects of the anti-Neogenin antibody. It also confirms previous studies suggesting that chick Neogenin could functionally substitute for Dcc in attraction towards Netrin-1 (Phan et al., 2011).

## Figure 6 Neogenin is required for LMCl axon growth preference for Netrin-1




**A–C**. Left panels: explanted lateral (EphA4+) LMC neurites on Fc/Fc (A) or Netrin-1 (N)/Fc stripes without (B) or with (C) the addition of anti-Neogenin antibody. Middle panels: inverted images where EphA4 signal is depicted as dark pixels overlaid on substrate stripes. Right panels: superimposed images of five representative explants from each experimental group highlighting the distribution of lateral LMC neurites. Bar graphs are a quantification of lateral (EphA4+) LMC neurites on first (pink) and second (pale) stripes expressed as a percentage of total EphA4 signal. N, Netrin-1; error bars = SD; *** = $p<0.001$; statistical significance computed using Mann-Whitney U test; all values (mean ± SD). Adapted from (Poliak et al., 2015) This work was done in collaboration with Tzu-Jen Kao.

**Figure 7 Dcc can functionally substitute for chick Neogenin in LMCl axon preference for Netrin-1**




**A, B**. Left panels: explanted lateral (EphA4+) LMC neurites on Netrin-1 (N)/Fc stripes with the addition of anti-Neogenin antibody. Lateral (GFP+EphA4+) LMC neurites of $Dcc^{DICD}$ and GFP (A) or Dcc and GFP (B) co-electroporated explants. Middle panels: inverted images where EphA4 signal is dark pixels overlaid on substrate. Right panels: superimposed images of five representative explants from each experimental group highlighting the distribution of lateral LMC neurites. Bar graphs are a quantification of lateral (EphA4+) LMC neurites on first (pink) and second (pale) stripes expressed as a percentage of total EphA4 signals. N, Netrin-1; error bars = SD; *** = $p<0.001$; statistical significance computed using Mann-Whitney U test; all values (mean ± SD). Adapted from (Poliak et al., 2015). This work was done in collaboration with Tzu-Jen Kao.

## 2. Netrin-1 and ephrin-B2 collapse LMCm growth cones synergistically

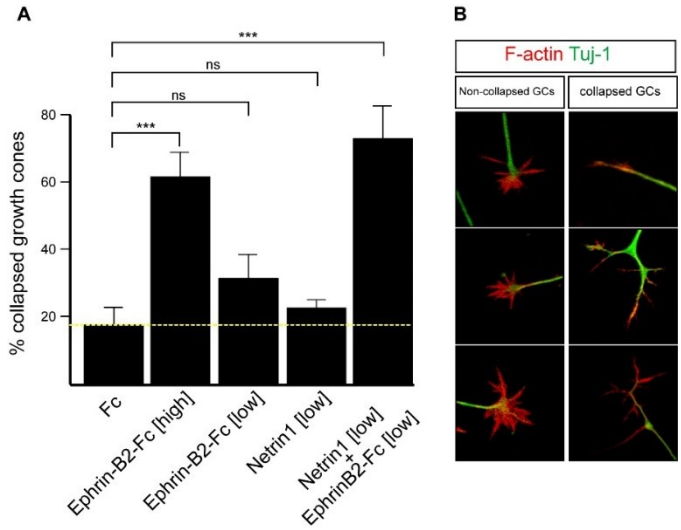
### Rationale

Through the stripe assay, we have previously demonstrated that LMCm axons respond synergistically to Netrin-1 and ephrin-B2 (Poliak et al., 2015). In neurons, guidance decisions are initiated at the growth cone, collapse assays are often used in the field of axon guidance as a measure of the strength of repulsion elicited by

guidance cues. Since LMCm axons avoid both Netrin-1 and ephrin-B2, I performed collapse assay experiments to determine the effects of these cues on LMCm growth cones.

To monitor LMCm growth cones specifically, chicken LMC neurons were electroporated with the medial LMC marker e[Isl1]::GFP prior to culture (Kao et al., 2009). LMC explants were exposed to bath applied Netrin-1 and/or ephrin-B2 for 30 min., followed by a quantification of LMCm growth cone status (**Figure 9**). Whereas exposure to low concentrations of either Netrin-1 or ephrin-B2 fails to induce LMCm growth cone collapse, the simultaneous exposure to both cues results in robust collapse (**Figure 9A**) (Poliak et al., 2015). These results suggest the both cues are integrated synergistically within LMCm growth cones. Since ephrin-A5 and Netrin-1 have opposing effects on LMCl axons, a collapse assay paradigm may not be adequate for investigating Netrin-1/ephrin-A5 LMCl axon guidance synergy.

## Figure 9 Netrin-1 and ephrin-B2 act synergistically on LMCm growth cone collapse




**A**. Percentage of collapsed e[Isl1]::GFP medial LMC growth cones following a 30 min exposure to Fc (10 µg/ml), ephrin-B2-Fc (high: 10 µg/ml; low: 1 µg/ml), Netrin-1 (300 ng/mL) or Netrin-1 and ephrin-B2-Fc (300 ng/mL and 1 mg/ml). Significance computed using Fisher's exact test. **B**. Examples of growth cones quantified in (A) labeled with Tuj1 (green) and F-actin (phalloidin, red). Error bars = SD; *** = $p<0.001$.

### 3. Neogenin in LMC differentiation

### Author contributions

Experiments were designed by Artur Kania and Louis-Philippe Croteau. The *in situ* mRNA detection in sections of chick spinal cords presented in **Figure 10** was done in collaboration with Meirong Liang. All other experiments were entirely performed and analyzed by Louis-Philippe Croteau under the supervision and resources provided by Artur Kania.

## Rationale

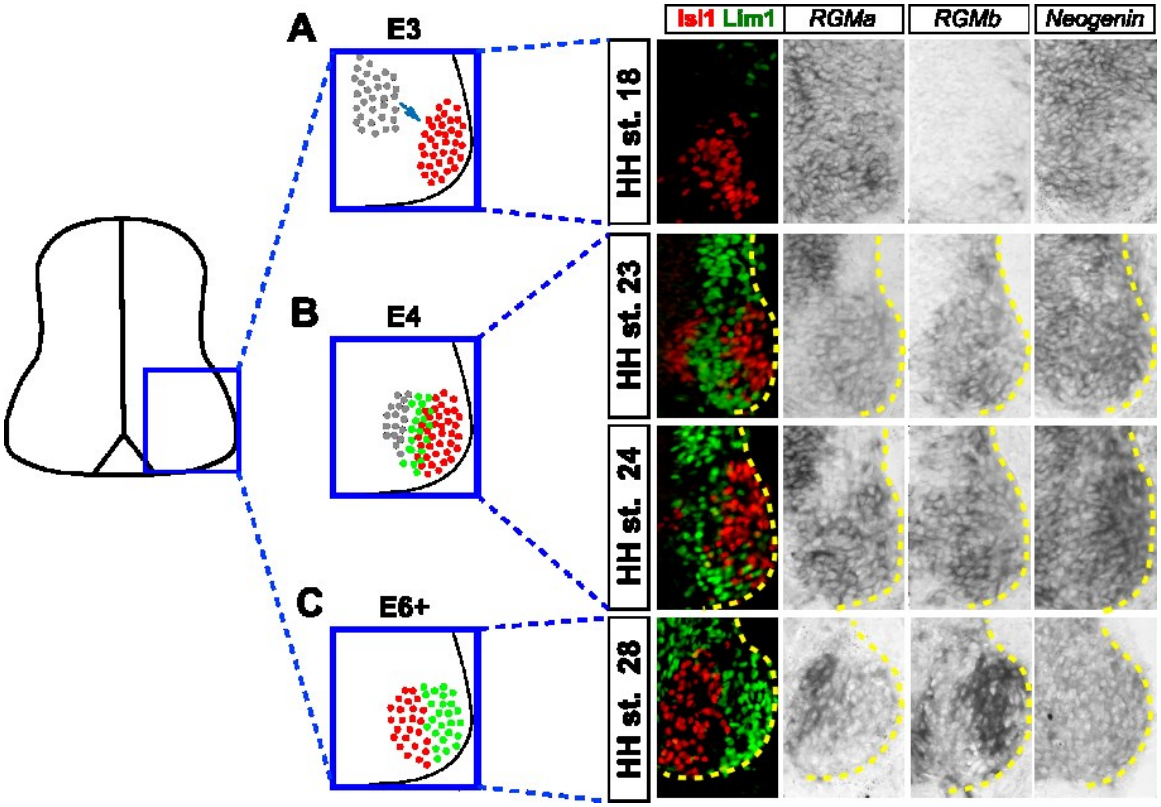
With the objective of revealing an *in vivo* requirement for Neogenin in LMC axon guidance, *Neogenin* was knocked down (KD) in LMC neurons by way of chick *in ovo* electroporation of siRNAs against Neogenin ([Neo]siRNA*)* (Croteau and Kania, 2011). KD of *Neogenin* resulted in alterations in LMC subtype differentiation, a process that occurs prior and during the dorso-ventral LMC axon branch choice (Landmesser, 1978a; Tosney and Landmesser, 1985). Neogenin/RGM signaling has been implicated in neuronal differentiation in various regions of the CNS including the midbrain, hindbrain, cortex and olfactory bulb (Matsunaga et al., 2006; O'Leary et al., 2013; O'Leary et al., 2015; Kam et al., 2016). In the chick, Neogenin, as well as its ligands RGMa and RGMb, are expressed by LMC neurons during their subtype differentiation suggesting that Neogenin/RGM interactions are implicated in differentiation of LMC neurons. We therefore decided to further investigate the potential role of Neogenin in LMC differentiation.

## Expression of *Neogenin*, *RGMa* and *RGMb* during LMC neuron development

To investigate the requirement for Neogenin in LMC development, *in situ* mRNA detection of *Neogenin* as well as its ligands *RGMa* and *RGMb* was performed in chick lumbar spinal cord sections. The developmental window in which LMC neurons become post-mitotic and differentiate into their lateral and medial subtypes occurs between chick embryonic (E) days 3-6 (**Figure 10A**) (Hollyday and Hamburger, 1977; Landmesser, 1978b). At Hamburger-Hamilton stage (HH st.) 18,

MN progenitors as well as newly post-mitotic LMC neurons, identified by the expression of the transcription factor Isl1, express *Neogenin* as well as *RGMa* (**Figure 10A**) (Hamburger and Hamilton, 1951; Tsuchida et al., 1994). At E4 (HH st. 23-24), LMCl neurons extinguish isl1 and express Lim1 as they migrate towards earlier born LMCm neurons (**Figure 10B**) (Landmesser, 1978a; Kania et al., 2000; Sockanathan et al., 2003). During this period, seemingly all LMC neurons express *Neogenin, RGMa* and *RGMb* (**Figure 10B**). At E6, when all LMC neurons have been generated and cell somas have settled into their medial and Lateral positions within the spinal cord, expression of Neogenin prevails. Interestingly, at this stage, *RGMa* seems to be preferentially expressed by LMCm neurons and *RGMb* preferentially expressed by LMCl neurons (**Figure 10C**). Thus, the expression of *Neogenin, RGMa* and *RGMb* by LMC neurons suggests they may be implicated in various processes during LMC neuron development including differentiation and axon guidance.

**Figure 10 Expression of *Neogenin*, *RGMa* and *RGMb* during LMC neuron development**




In *situ* mRNA detection and immunohistochemistry on wild-type chick lumbar-level spinal cord sections. **A**. At E3, (HH st. 18), post-mitotic LMC neurons (Isl1+) express *Neogenin* and *RGMa*. **B**. At E4 (HH st. 23,24), when LMCl neurons begin expressing Lim1, seemingly all LMC neurons express *Neogenin*, *RGMa* and *RGMb*. **C**. At E6, *Neogenin* is expressed by seemingly all LMC neurons, *RGMa* is preferentially expressed by LMCm neurons and *RGMb* is preferentially expressed by LMCl neurons.

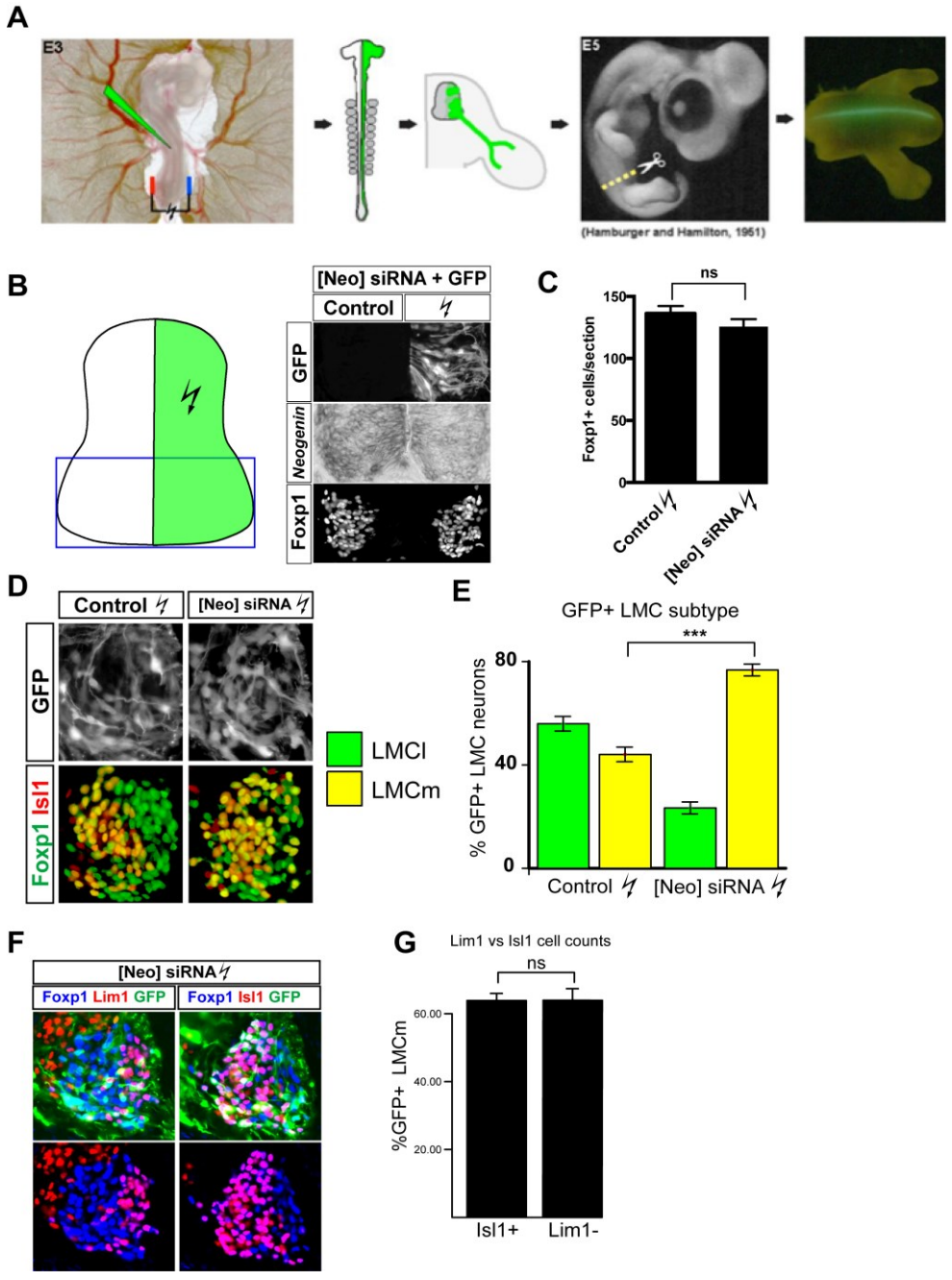
**Knockdown of *Neogenin* alters LMC neuron subtype differentiation**

To investigate the implication of Neogenin in LMC development, HH st. 17-18 embryos were electroporated with a combination of three siRNAs directed against *Neogenin* ([Neo]siRNA), along with a GFP expression plasmid to select for

electroporated cells (**Figure 11A**) (Croteau and Kania, 2011). *In situ* mRNA detection of *Neogenin* in sections of lumbar spinal cord of electroporated embryos shows lower levels of *Neogenin* on the electroporated side of the spinal cord (**Figure 11B**). To determine if the KD of Neogenin affects the total amount of LMC neurons generated, the amount of cells expressing the generic LMC marker Foxp1 were counted and compared to embryos electroporated with a GFP plasmid combined with a scrammbled version of one the Neogenin siRNAs (controls) (Rousso et al., 2008). Results indicate that the KD of Neogenin at E3 does not affect the generation of generic LMC neurons (**Figure 11C**).

To investigate the possible involvement of Neogenin in LMC subtype differentiation, a quantitative comparison of LMC subtype composition between [Neo]siRNA and control embryos was carried out. KD of *Neogenin* results in an increase in the relative proportion LMCm (Isl1+) neurons (**Figure 11D, E**). Furthemore, the increase in Isl1+ neurons is accompanied by an decrease in LMC neurons expressing the LMCl marker Lim1 (**Figure 11F, G**).

## Figure 11 Knockdown of Neogenin alters LMC subtype differentiation



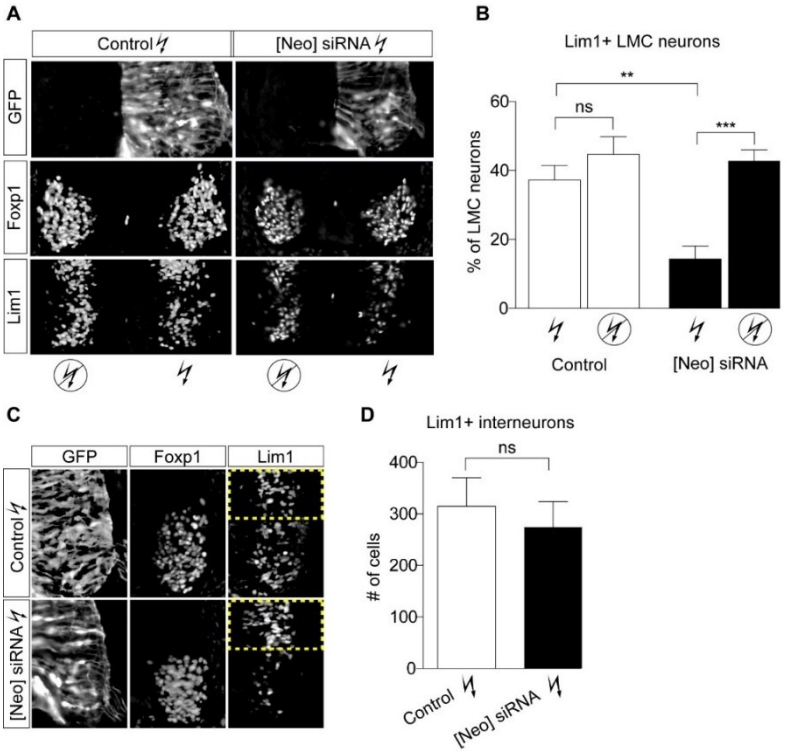

**A**. Explanatory diagram for the knockdown of Neogenin by chick *in ovo* electroporation of siRNAs. A mixture of 3 siRNAs directed against Neogenin and a GFP expression plasmid are injected within the lumen of the an E3 (HH st. 18) chick neural tube. Electrodes are placed at the lumbar level of the spinal cord and current is applied allowing the uptake of the siRNAs and plasmid by neural progenitors lining the ventricular zone of one half of the neural tube. Embryos are then incubated and

harvested at E5 (HH st. 25-26), electroporation efficiency can be monitored through GFP expression. **B**. Electroporation of [Neo]siRNAs results in a decrease in *Neogenin* expression on the electroporated side of the spinal cord while the expression of the LMC marker Foxp1 remains seemingly unchanged. **C**. Quantification of LMC neurons (Foxp1+) in control and [Neo]siRNA embryos. **D**. Detection of Foxp1 and Isl1 in [Neo]siRNA and control embryos. **E** Quantification of the subtype identity of GFP+ LMC neurons showing an increase in Isl1+ LMC neurons in [Neo]siRNA embryos. **F**. Adjacent sections of LMC in a [Neo]siRNA embryo showing the expression of GFP, Foxp1, Lim1 (left panels) or Isl1 (right panel). Quantification of Lim1 and Isl1 positive LMC neurons in [Neo]siRNA embryos shows that the increase in Isl1+ LMC neurons is accompanied by an equivalent decrease in Lim1+ LMC neurons. Error bars represent S.E.M, ns (non-significant), *** $p \leq 0.0001$; unpaired sample t-test.

To distinguish between the requirement for Neogenin in Lim1 expression initiation or Lim1 expression maintenance, a quantitative assessment of Lim1+ LMC neurons was carried out in [Neo]siRNA embryos at E4, at the onset of Lim1 expression in LMC neurons (**Figure 12**). At E4, [Neo]siRNA embryos have significantly less Lim1+ LMC neurons compared to controls (**Figure 12A, B**). Since slight differences in staging between experimental and control embryos could contribute in the differences in Lim1+ LMC neurons, Lim1+ LMC neurons were quantified on the non-electroporated side of the spinal cord. No difference in Lim1 expression between [Neo]siRNA and control embryos occurred within non-electroporated spinal cords halves, ruling out age discrepancies in accounting for differences in Lim1+ LMC neurons (**Figure 12B**). These results suggest that Neogenin is required for prospective LMCl neurons to imitate Lim1 expression but does not address the potential requirement for Neogenin in maintaining Lim1 expression. To test the later would require the knockdown of Neogenin at E4 or latter. This can not be achieved via *in ovo* electroporation due to the increase in vascularization and the thickening of the embryonic tissue.

Lim1 and *Neogenin* are also expressed by spinal interneurons located dorsally to the LMC (**Figure 10B, C**; **Figure 12C**) (Kania et al., 2000). To assess the possible requirement for Neogenin in the expression of Lim1 in interneurons, Lim1 expression by spinal interneurons was evaluated in electroporated embryos. To do so, the number of Lim+ cells were counted in a rectangular selection immediately dorsal to LMC neurons (**Figure 12C**). Interestingly, no difference in Lim1 expression was detected between [Neo]siRNA and control embryos. These results suggest that Lim1 expression occurs independently from Neogenin in spinal interneurons during this developmental period (**Figure 12D**).

### Figure 12 Neogenin is required for establishing Lim1 expression in LMC neurons



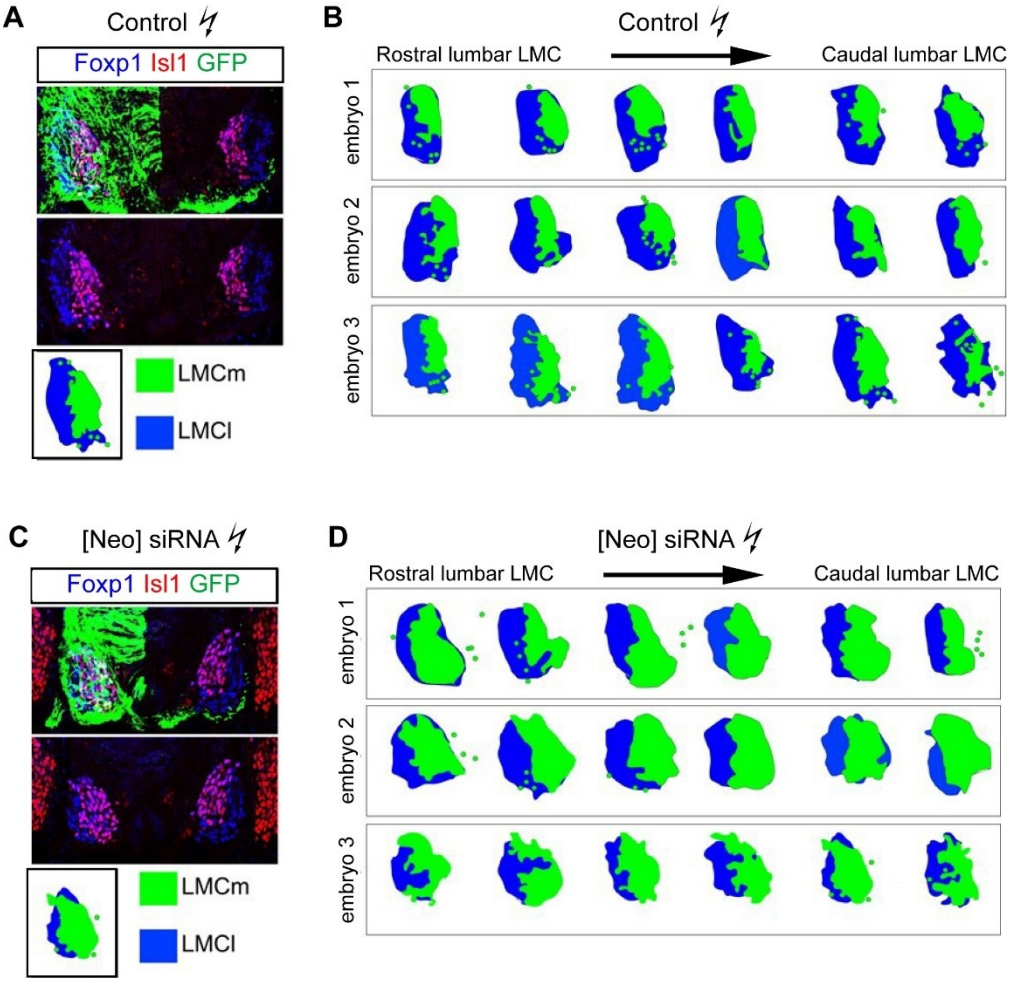

**A**. Detection of GFP, Foxp1 and Lim1 in E4 lumbar spinal cord sections of control and [Neo]siRNA embryos **B**. Quantification of Lim1+, Fxop1+ neurons at E4 in the

electroporated and non-electroporated side of the spinal cord. **C**. Detection of GFP, Foxp1 and Lim1 in E4 lumbar spinal cord sections of control and [Neo]siRNA embryos, the yellow boxes depict the region where Lim1 expression by interneurons was quantified in D. **D**. Quantification of Lim1 expression by spinal interneurons immediately dorsal to LMC neurons. ** p = 0.0038, *** p= 0.0004.

**LMC neuron subtype distribution within the LMC is altered in Neogenin KD embryos**

In [Neo]siRNA embryos, the shift in the ratio of LMCm/LMCl neurons is accompanied by a decrease in LMCm/LMCl segregation and a high occurrence of LMCm neurons occupying areas normally occupied by LMCl neurons (**Figure 11D**). To gain insight in the alteration in LMC subtype distribution in [Neo]siRNA embryos, serial sections spanning the lumbar spinal cord were digitally reproduced to highlight the gross occupancy of LMCm/LMCl subdivisions (**Figure 13**). In control embryos, the LMCl forms an L shape with the most ventral portion of the LMC generally occupied by LMCl neurons (**Figure 13A, B**). In [Neo]siRNA embryos, LMCl is no longer L shaped, while the dorso-lateral portion of the LMCl appears mostly unaffected, the most ventral portion of the LMC is occupied by LMCm neurons (**Figure 13C, D)**. These results suggest that Neogenin may be involved in LMC neuron migration and/or LMC subtype segregation.

## Figure 13 The Distribution of LMCm and LMCl neurons is altered in [Neo]siRNA embryos




**A**, **C**. Detection of Foxp1, Isl1 and GFP in lumbar spinal cord sections of control (A) and [Neo]siRNAs (B) electroporated embryos. **B, D**. LMC subtype occupancy within the lumbar spinal cord of three control (B) and three [Neo]siRNAs (D) electroporated embryos was digitally reproduced based on the gross occupation of LMCm neurons (Isl1+) within the LMC (Foxp1+). KD of Neogenin results in the expansion of the territory occupied by LMCm neurons, particularly in the most ventral portion of the LMC.

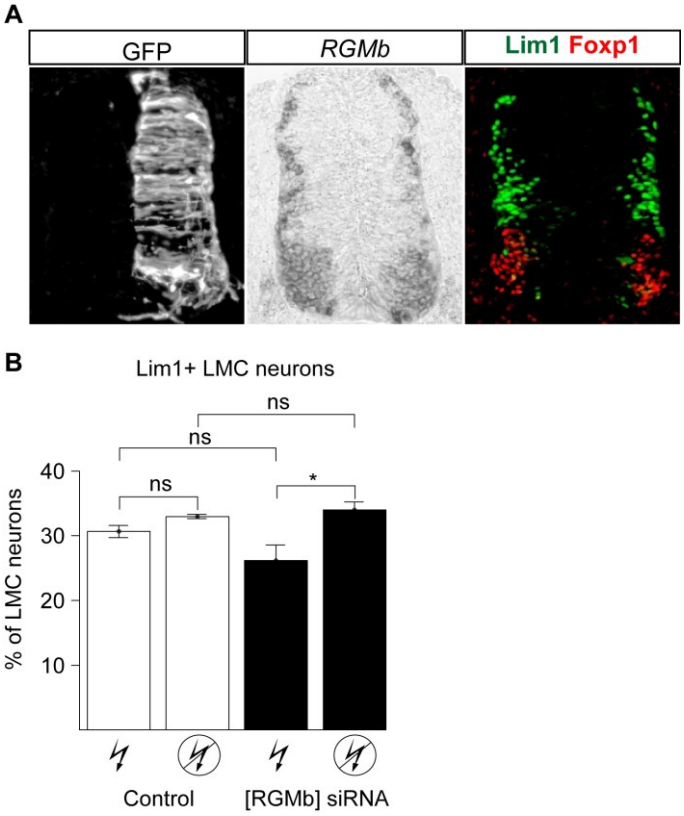
**Investigating the requirement for RGMa and RGMb in LMC subtype differentiation**

The expression of *RGMa* and *RGMb* during LMC neuron development in conjunction with pre-existing evidence of RGMs implicated in neuronal differentiation, suggest that Neogenin may regulate LMC subtype differentiation through interacting with RGMa and/or RGMb (**Figure 10**) (Matsunaga et al., 2006; O'Leary et al., 2013; O'Leary et al., 2015; Kam et al., 2016).

*RGMa* is expressed by MN progenitors as well as differentiated LMC neurons (**Figure 10**), to investigate the possible implication of RGMa in LMC subtype differentiation, a combination of two siRNAs directed against *RGMa* as well as a GFP expression plasmid ([RGMa]siRNA) were electroporated in E3 chick lumbar spinal cords. *RGMa* expression was assessed qualitatively by *in situ* mRNA detection in lumbar spinal cord sections of electroporated embryos. The siRNAs were not effective at knocking-down *RGMa* and LMC neuron differentiation appeared normal in [RGMa]siRNA embryos (data not shown). As an alternative strategy, RGMa was overexpressed in chick lumbar spinal cord through the electroporation of a bi-histrionic plasmid for the expression of RGMa and β-galactosidase. Although the expression of β-galactosidase and increased *RGMa* expression suggest that RGMa was overexpressed, no apparent LMC differentiation defects were observed (data not shown). These results do not rule out an implication of RGMa in LMC subtype differentiation, effective knockdown of *RGMa* using alternative siRNAs or the use of a CRISPR/Cas9 system targeting RGMa could potentially reveal such a role.

*RGMb* is expressed by post-mitotic LMC neurons and by E5-6, *RGMb* appears to be expressed at higher levels by LMCl neurons than by LMCm neurons (**Figure 10B, C**). Furthermore, Lim1+ interneurons also express *RGMb,* suggesting a possible link between RGMb and Lim1 expression in spinal neurons (**Figure 14A**). A combination of two siRNAs directed against *RGMb* with a GFP expression plasmid ([RGMb]siRNA) were electroporated as described previously. Despite no obvious decrease in *RGMb* expression, Lim1+ LMC neurons were quantified in E4 [RGMb]siRNA embryos. Although no significant differences were detected between control and [RGMb]siRNA embryos, a slight but significant difference was obtained when comparing Lim1 expression by LMC neurons between the electroporated and non-electroporated sides of [RGMb]siRNA embryos (**Figure 14B**). To be conclusive, additional [RGMb]siRNA embryos would have to be added to these results. A more effective *RGMb* knockdown using alternative siRNAs or a CRISPR/Cas9 system would potentially result in a more substantial loss in Lim1 expression in LMC neurons.

**Figure 14 A possible implication for RGMb in LMC subtype differentiation**



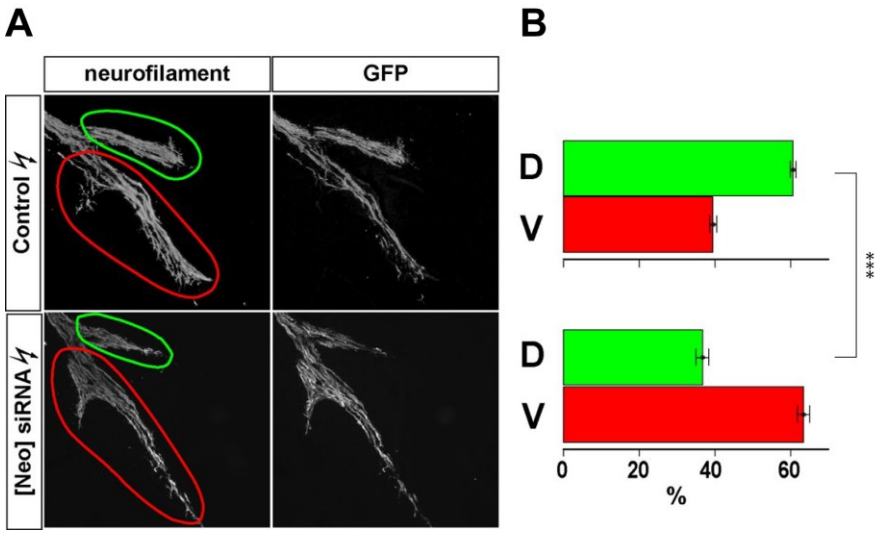

**A.** Detection of GFP, Foxp1, Lim1 and *RGMb* in E4 lumbar chick spinal cord sections from an embryo electroporated with two siRNAs directed against *RGMb* along with a GFP expression plasmid ([RGMb]siRNA)**. B.** Quantification of Lim1+, Fxop1+ neurons at E4 in the electroporated and non-electroporated side of [RGMb]siRNA embryos. * p = 0.044.

### Knockdown of *Neogenin* alters lumbar LMC axon projections

The expression of Lim1 by LMCl neurons is required for their axons to project to the dorsal limb, loss or overexpression of Lim1 in the chick leads to LMCl and LMCm guidance defects respectively (Kania et al., 2000). Furthermore, in mice lacking Lim1 expression, LMCl axons project randomly in the hindlimb (Kania et al., 2000). Considering the downregulation of Lim1 in [Neo]siRNA embryos, it is predicted that the knockdown of Neogenin will result in LMC guidance defects. The requirement

for Neogenin in LMC development may be two-fold, first through the regulation of Lim1 expression and secondly, by enabling LMCl axons to be attracted by Netrin-1 in the dorsal limb (**Figure 5**) (Poliak et al., 2015). To gain insight in the requirement for Neogenin in LMC axon guidance, GFP+ axon projections were quantified in [Neo]siRNA and control embryos by measuring the GFP fluorescence intensities in the dorsal and ventral axonal branches of hindlimbs (**Figure 15**). [Neo]siRNA embryos displayed altered ratios of dorso-ventral axon projections with more ventrally projecting axons, consistent with an increase in the ratio of LMCm/LMCl neurons (**Figure 15B**). To address guidance defects by LMCl or LMCm axons specifically would require the retrograde labeling of LMC neurons by injecting HRP into the ventral and dorsal nerves branches respectively (Poliak et al., 2015).

### Figure 15 Knockdown of Neogenin alters LMC axon projections

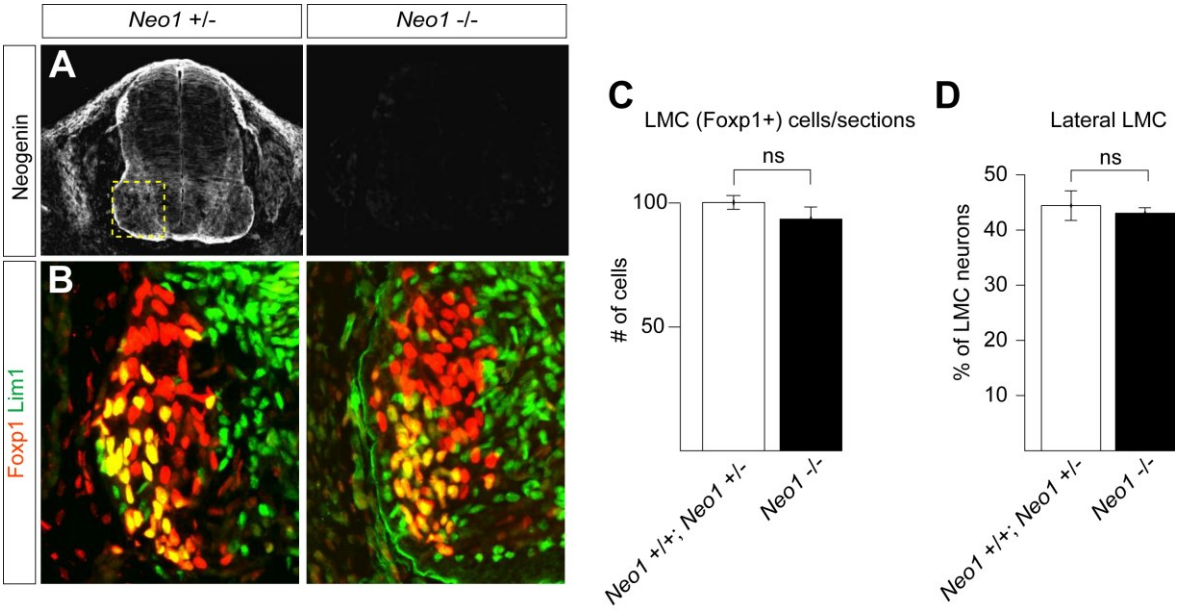



**A**. Detection of neurofilament and GFP in LMC axons in E5 sciatic plexus of control and [Neo]siRNA embryos, the dorsal and ventral branches are circled in green and red respectively. **B**. Quantification of GFP+ LMC axons in the hindlimbs of control and [Neo]siRNA embryos. D: dorsal, V: ventral, *** $p \leq 0.0001$.

**Neogenin may be dispensable for the subtype differentiation of LMC neurons in mice**

*Neogenin* is expressed by developing LMC neurons in the mouse (Poliak et al., 2015). To investigate the requirement for Neogenin in LMC neuron subtype differentiation in mice, the expression of Lim1 by LMC neurons in e11.5 lumbar spinal cord was quantified in *Neo1*$^{-/-}$ mice and compared to WT and *Neo1*$^{+/-}$ littermates, generously provided by the Cloutier laboratory (Kam et al., 2016). Although only a few embryos were analyzed and a thorough analysis would require more embryos, the distribution and total number of lumbar LMC neurons as well as the number of Lim1 expressing LMC neurons was not significantly altered in *Neo1*$^{-/-}$ mice (**Figure 16B, D**). The lack of an LMC differentiation phenotype may reflect a different requirement for Neogenin between chick and mice. It is also possible that the expression of Dcc by LMC neurons in the mouse compensates for the loss of Neogenin. Furthermore, the complete absence of Neogenin at the onset of embryogenesis may allow for the establishment of compensatory mechanisms not available in the context of a partial knockdown of Neogenin via siRNAs.

## Figure 16 LMC neuron subtype differentiation in mice lacking Neogenin appears normal




**A.** Immunostaining for Neogenin in e11.5 lumbar spinal cords of $Neo^{+/-}$ and $Neo^{-/-}$ mice, yellow box represents the approximate region of spinal cord shown in B. **B.** Detection of Foxp1 and Lim1 in e11.5 lumbar LMC of $Neo^{+/-}$ and $Neo^{-/-}$ mice. **C, D**. Quantification of LMC neurons (Foxp1+) (C) and LMCl neurons (Foxp1+, Lim1+) (D) in sections of e11.5 lumbar spinal cords of ($Neo^{+/+}$(n=1); $Neo^{+/-}$(n=1) and $Neo^{-/-}$ (n=3) littermates.

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## 4. Sensitization of spinal motor neurons to Netrin-1 by ephrin-A5

### Author contributions

Experiments were designed by Louis-Philippe Croteau under the supervision and input from Artur Kania. All the *in ovo* electroporations of chick embryos were performed by Meirong Liang. The stripe assay experiments in Figures III-13 and III-14 were performed and analyzed by Tzu-Jen Kao. All other experiments were performed and analyzed by Louis-Philippe Croteau. The MATLAB application used to analyze the immunofluorescence distribution within growth cones was programmed by Dominic Fillion. The ImageJ macro used to define the region of interest within analyzed images was programmed by Chris Law. The results section was partially edited by Artur Kania. Resources were provided by grants obtained by Artur Kania.

### Rational

In the chick, LMCl neurons that innervate the dorsal hindlimb are attracted to Netrin-1 through the expression of the Netrin receptor Neogenin. LMCm neurons that extend axons ventrally avoid Netrin-1 through the expression of the repulsive Netrin receptor Unc5c (Poliak et al., 2015). Furthermore, mice hypomorphic for Netrin-1 as well as Unc5c null mice display LMCm guidance defects (Poliak et al., 2015). LMCm and LMCl axons are also guided by ephrins in the limbs through the expression of Eph receptors (Helmbacher et al., 2000; Eberhart et al., 2002; Eberhart et al., 2004;

Luria et al., 2008; Bonanomi et al., 2012). *In vivo*, LMCl axons expressing the ephrin receptor EphA4, are repelled from ephrin-A5 and ephrin-A2 expressed in the ventral hindlimb (Helmbacher et al., 2000; Eberhart et al., 2004; Bonanomi et al., 2012).

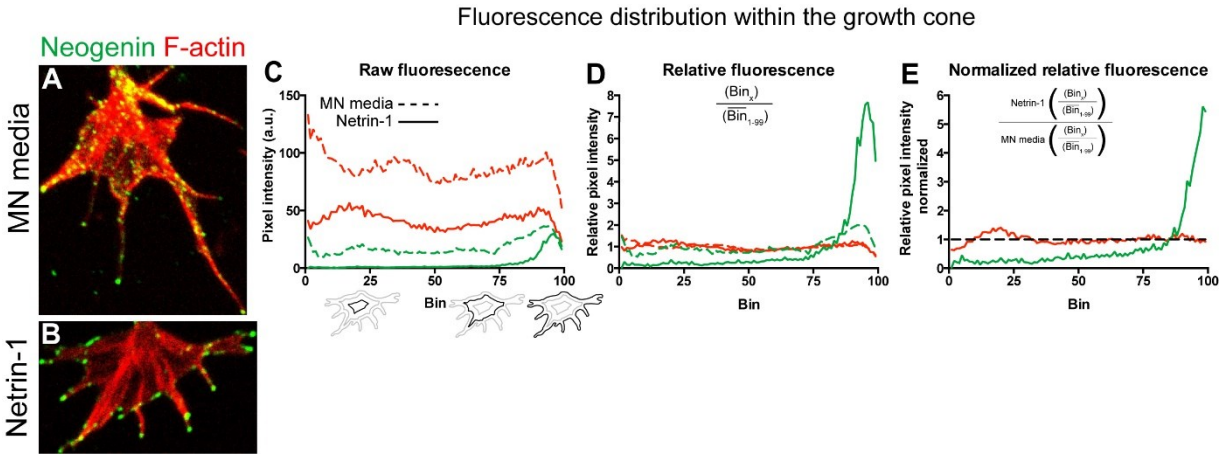
We have previously demonstrated that Netrin-1 acts synergistically with ephrins in LMC axon guidance; *In vitro* assays demonstrate that both LMCm and LMCl axons integrate Netrin-1 and ephrins in a supra-additive fashion (Poliak et al., 2015). To gain insight in the molecular mechanism underlining the synergistic integration of Netrin-1 and ephrins in LMC axons, *in vitro* experiments were carried out focussing on the effects of Netrin-1 and ephrin-A5 on receptor dynamics in LMC growth cones.

**Netrin-1 induces a protein kinase A-dependent re-localization of Neogenin in LMC growth cones**

We reasoned that changes in growth cone receptor dynamics could underlie Netrin-1/ephrin-A5 LMC axon guidance synergy and thus we first assessed the effects of Netrin-1 on Neogenin dynamics in LMC growth cones. To do this, we explanted chick lumbar LMC neurons between Hamburger–Hamilton stages (HH st.) 24 and 26, the developmental period encompassing the dorso-ventral guidance of lumbar LMC axons (Hamburger and Hamilton, 1951; Tosney and Landmesser, 1985; Kania et al., 2000). LMC explants were cultured for ~18 hrs and exposed for 15 minutes to a bath application of motor neuron culture media (MN media) containing Netrin-1 at 300 ng/mL or MN media alone as control, followed by an assessment of F-

actin and Neogenin immunofluorescence (IF) distribution within growth cones. In contrast to MN media alone, Netrin-1 application resulted in the re-localization of Neogenin to the growth cone periphery (**Figure 17A, B**).

### Figure 17 Netrin-1 induces a re-localization of Neogenin in LMC growth cones

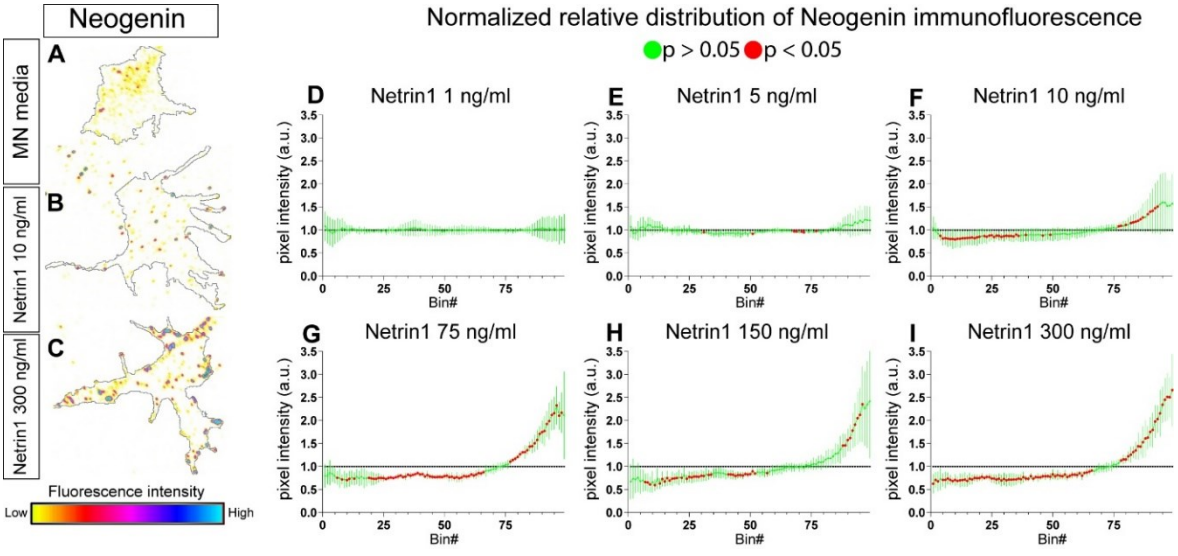



**A, B**. Detection of Neogenin and F-actin IF in cultured LMC neuron growth cones treated with MN media (A) or Netrin-1 300 ng/mL (B) for 15 min. **C**. IF distribution within the growth cone can be assessed by dividing the growth cone into 100 bins (x axis: Bin 0 = geometric centre, Bin 100 = perimeter) and determining the mean fluorescence intensity in each bin (y axis). **D**. Relative fluorescence distribution highlights differences in protein distribution within a growth cone regardless of total fluorescence levels. **E**. Normalized relative fluorescence highlights the influence of Netrin-1 on Neogenin distribution.

To quantify such changes, we plotted Neogenin and F-actin IF intensity in growth cone images that were divided radially into 100 bins, with bin 0 at the geometric centre and bin 100 outlining the growth cone perimeter (**Figure 17C)**. To highlight differences in IF distribution regardless of differences in total the total fluorescence, the mean fluorescence value for each individual bin was divided by the average fluorescence value for bins 1 to 99 (relative fluorescence) (**Figure 17D**) To

highlight Neogenin distribution differences between treatments, relative Neogenin IF values in Netrin-1 treated explants were normalized to MN media treated values (**Figure 17E**). Upon Netrin-1 treatment, Neogenin IF is redistributed towards the growth cone periphery (**Figure 17B, E**). This effect was significant over a range of Netrin-1 concentrations and even as little as 10 ng/mL of it induced Neogenin re-localization (**Figure 18**). Netrin-1 induces re-localization of Neogenin by decreasing levels in bins 1-75 and increasing Neogenin in the most peripheral 25% of the growth cone area (bins 76-100) (**Figure 18F, I**). This may be a consequence of a Netrin-1 induced enrichment of Neogenin in the dynamic lamellipodia and filopodia within the peripheral domain of LMC neuron growth cones (Lowery and Van Vactor, 2009).

### Figure 18 Netrin-1 at 10 ng/mL is sufficient in redistributing Neogenin within LMC growth cones

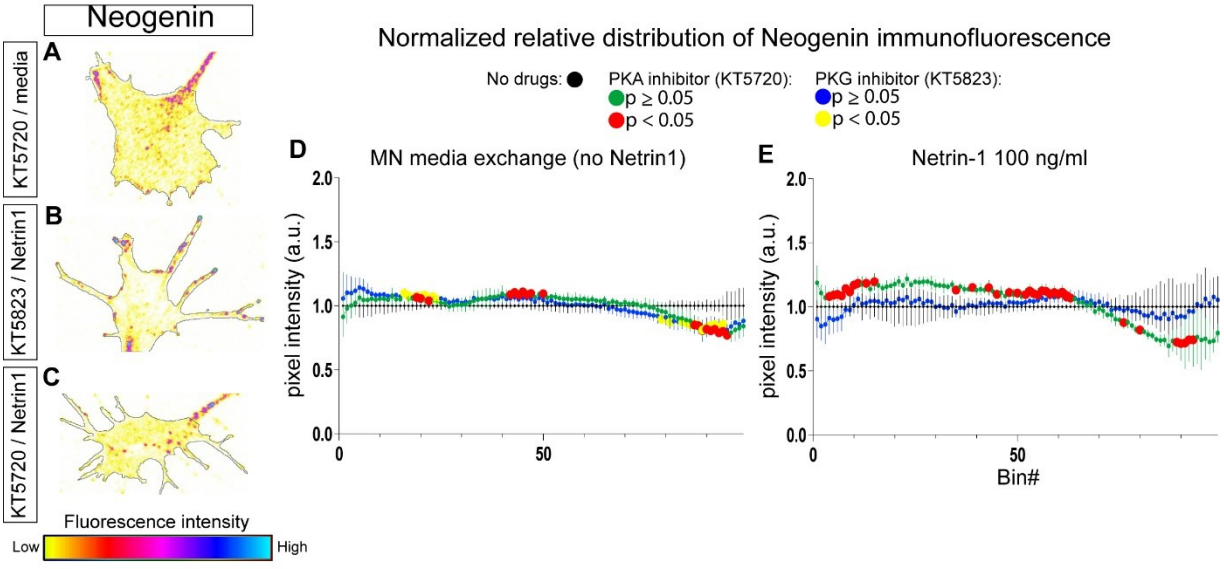



**A, B, C**. Detection of Neogenin in cultured LMC neuron growth cones treated for 15 min. with MN media (A), Netrin-1 10 ng/mL (B) and Netrin-1 300 ng/mL (C), growth cone perimeters are outlined in grey (as well as in all growth cone images in subsequent figures). **D-I**. The normalized relative distribution of Neogenin IF in LMC growth cones subject to different concentrations of Netrin-1. Error-bars represent

SEM. Bins where the relative Neogenin IF differs significantly from MN media treatment are highlighted in red.

In cortical neurons, Netrin-1 induces a protein kinase A (PKA) dependent increase in surface Dcc (Bouchard et al., 2008), we therefore wanted to test whether Netrin-1-induced Neogenin re-localization in LMC growth cones is also PKA dependent. LMC neuron explants were exposed to 100 ng/mL Netrin-1 in the presence of KT5720, a PKA inhibitor or KT5823, a protein kinase G (PKG) inhibitor as control (**Figure 19**). Whereas both KT5720 and KT5823 affect the baseline distribution of Neogenin, only KT5720 attenuated the re-localization of Neogenin induced by Netrin-1 (**Figure 19D, E**). Thus, the Netrin-1 induced, PKA dependent re-localization of Neogenin in LMC growth cones may be analogous to the PKA dependent increase in surface Dcc seen in cortical neurons (Bouchard et al., 2008) .

### Figure 19 Netrin-1 induced re-localization of Neogenin is PKA dependent




**A-C**. Neogenin IF in LMC growth cones pre-treated for 30 min. with either the PKA inhibitor KT5720 (A, C) or with the PKG inhibitor KT5823 (B) at 5μM and subject to a

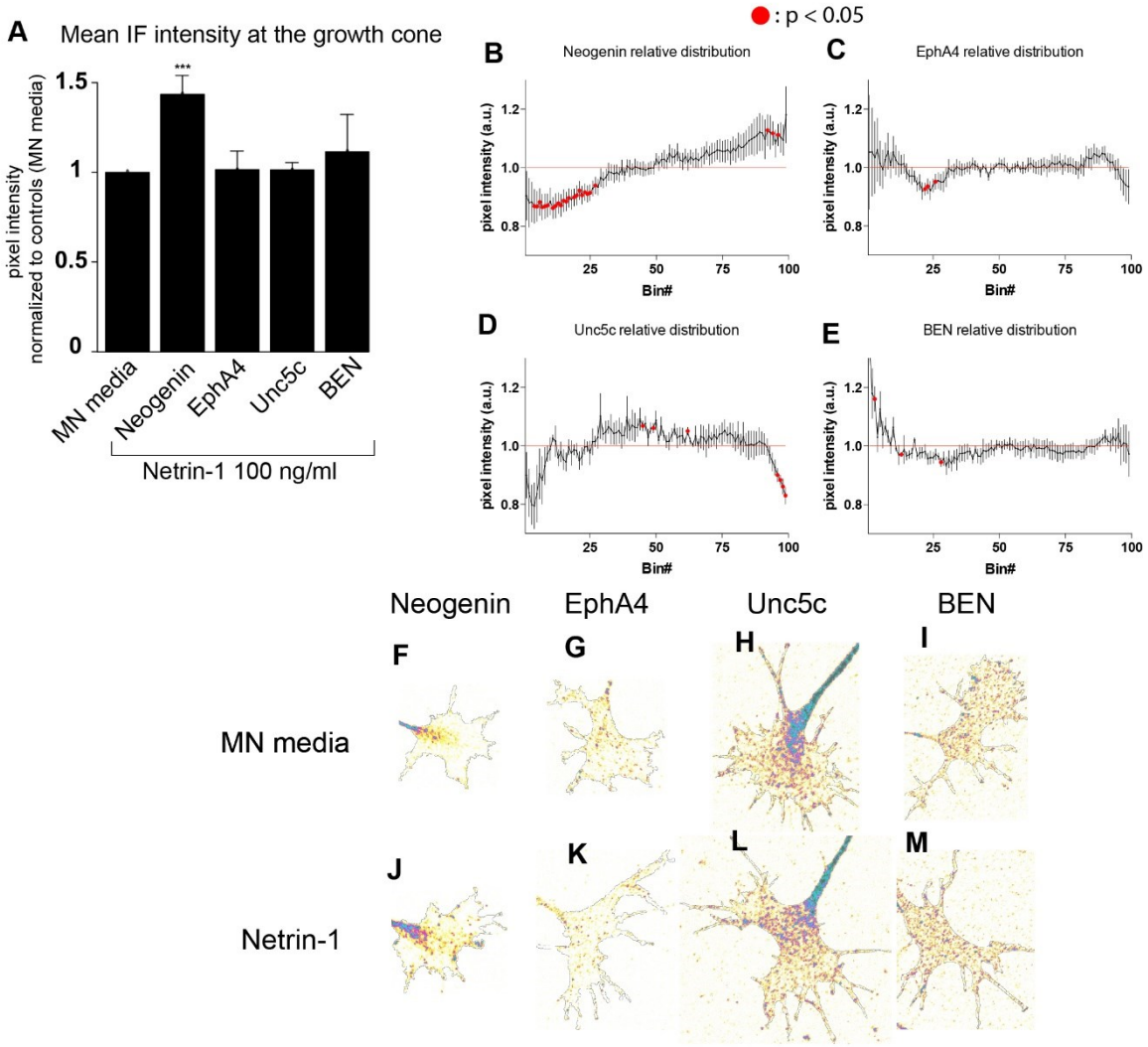
20 min. treatment of either MN media (A) or Netrin-1 100 ng/mL (B, C). **D, E**. Quantification of the relative distribution of Neogenin IF normalized to (No drug) treatment without Netrin-1 (D) or after a 20 min. exposure to Netrin-1 at 100 ng/mL (E).

**The effects of Netrin-1 on protein levels and distribution in LMC neuron growth cones**

We reasoned that Netrin-1 exposure of LMC growth cones might result in wide-spread dynamic changes in cell membrane protein distribution or abundance, and thus contribute to Netrin-1-ephrin-A5 synergistic axon guidance. For example, increased expression of EphA4, the ephrin-A5 receptor required for LMC axon guidance, could result in greater sensitivity to ephrin-A5 (Helmbacher et al., 2000; Eberhart et al., 2002). To explore this possibility, LMC explants where exposed to Netrin-1, and IF levels and distribution within growth cones were determined for Neogenin, the repulsive Netrin-1 receptor Unc5c, EphA4 and BEN, a surface glycoprotein marker of motor neurons (Pourquie et al., 1990) (**Figure 20**). Compared to controls, exposure to Netrin-1 led to a significant increase in total Neogenin signal in LMC growth cones (43% increase; $p<0.001$), without any changes in EphA4, Unc5c or BEN levels (**Figure 20A, F-M*;*** $p>0.5$). Since the IF values quantified are a compilation of the mean IF value in each growth cone, differences in Neogenin IF could be the result of a Netrin-1 induced decrease in growth cone area. To ensure this was not the case, growth cone area measurements were compiled and results show that the average size of the growth cones included in the analysis did not differ between treatments (**Supplementary Figure 1**). Because the Neogenin antibody

antagonizes the binding of Netrin-1 to Neogenin, the 43% increase in Neogenin when subject to Netrin-1 is likely an underestimation of the actual Netrin-1 induced increase. A quantification of the relative distribution of IF revealed that while Netrin-1 induces Neogenin re-localization towards the periphery (**Figure 20B**), Unc5c showed a tendency to be decreased at the periphery, EphA4 and BEN distribution remained largely unaffected (**Figure 20C-E**), suggesting that Netrin-1-mediated Neogenin re-localization is not a consequence of a large-scale protein re-distribution in growth cones.

## Figure 20 Netrin-1 induces specific changes in protein levels and distribution at the growth cone



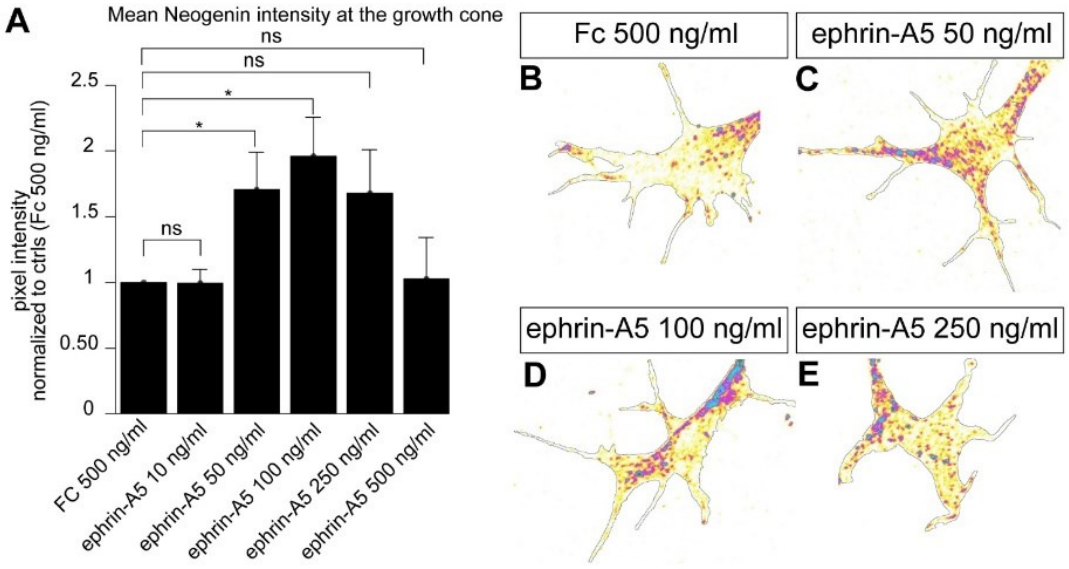

**A-E**. Quantification of Neogenin, EphA4, Unc5c and BEN IF levels (A) and distribution (B-E) in growth cones exposed to either MN media or Netrin-1 100 ng/mL for 20 min. A. Neogenin IF levels increase 43% in the presence of Netrin-1 ($p<0.001$), levels of EphA4, Unc5c and BEN remained unchanged ($p>0.05$) (A). **B-E**. Graphs showing the relative IF distribution within the growth cone of Neogenin (B), EphA4 (C), Unc5c (D) and BEN (E) treated with Netrin-1 and normalized to MN media treatment, Bins with IF values significantly different between treatments are highlighted in red. **F-M**. Examples of LMC neuron growth cones used in the quantifications in A-E.

**Ephrin-A5 increases Neogenin protein levels in LMC growth cones**

Since it is unlikely that Netrin-1-ephrin-A5 synergistic guidance results from changes in growth cone EphA4 distribution or expression levels, we next investigated the possibility that ephrin-A5 may influence Neogenin abundance (**Figure 21A-E**). Compared to Fc control treatment, application of pre-clustered ephrin-A5 at 50 and 100 ng/mL resulted in a significant increase in the levels of Neogenin in LMC growth cones (**Figure 21A**; ephrin-A5 50 ng/mL: 71% increase, p= 0.038; ephrin-A5 100 ng/mL: 96% increase, p=0.011).. Surprisingly, higher levels of ephrin-A5 did not result in increased Neogenin levels (**Figure 21A;** ephrin-A5 250 and 500 ng/mL p>0.5). Exposure to ephrin-A5 at 100 ng/mL did not significantly effect Unc5c, EphA4 or BEN IF levels (**Figure 21F**; p>0.05). The growth cones included in this analysis did not show significant size differences between treatments (**Supplementary Figure 1C**) Whereas the growth cone distribution of Neogenin remained largely unaltered, ephrin-A5 induced a loss of Unc5c, EphA4 and BEN from the growth cone periphery (**Figure 21H-K**). Furthermore, compared to controls, ephrin-A5 also induced a 56% and 18% increase in the size of Neogenin and EphA4 IF clusters, respectively (**Figure 21G*;*** Neogenin: p< 0.001, EphA4 =0.049), while no significant changes in Unc5c and BEN IF cluster size were observed (**Figure 21G**; p>0.05). The ephrin-A5-induced changes in EphA4 IF cluster size is likely due to the oligomerization of EphA4 when bound to ephrin-A5 (Davis et al., 1994). The ephrin-A5 increase in Neogenin IF cluster size may be the result of focal increases in Neogenin within the plasma membrane, possibly occurring adjacent to ephrin-A5/EphA4 interactions. The increased Neogenin

protein levels are in line with increased sensitivity to Netrin-1 seen in LMC growth cones co-exposed to ephrin-A5.

**Figure 21 The effects of ephrin-A5 on protein levels and distribution at the growth cone**

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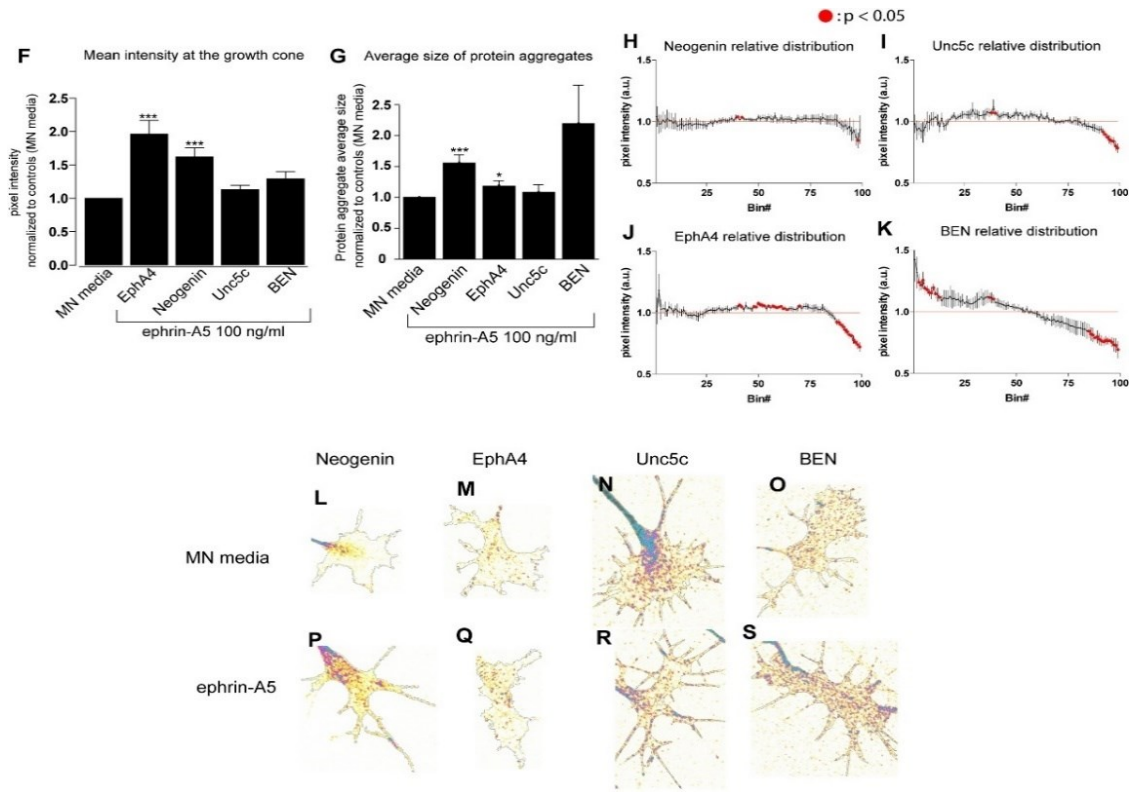



**A**. Cultured LMC explants were subject to a 15 min. exposure to a control solution containing Fc at 500 ng/mL or ephrin-A5 at concentrations ranging from 10 to 500 ng/mL, followed by a quantification of Neogenin IF in growth cones. Ephrin-A5 at 50 and 100 ng/mL results increased levels of Neogenin IF (ephrin-A5 50 ng/mL: 71% increase, p= 0.038; ephrin-A5 100ng/mL: 96% increase, p=0.011). **B-E**. Examples of Neogenin IF in growth cones quantified in A. **F-K**. Quantification of Neogenin, EphA4, Unc5c and BEN IF levels (F), aggregate size (G) and distribution (H-K) in growth cones exposed to either MN media or ephrin-A5 at 100 ng/mL for 20 min. **F**. Ephrin-A5 induced a 64% increase in Neogenin ($p<0.001$), and 94 % increase in EphA4 ($p<0.001$) IF levels while Unc5c and BEN levels remained unchanged. **G.** Ephrin-A5 induced a 56% increase in the average size of Neogenin immunofluorescent aggregates ($p<0.001$) and a 18% increase in the average size of EphA4 immunofluorescent aggregates ($p=0.049$) while Unc5c and BEN average aggregate size did not differ significantly ($p>0.5$ and $p= 0.061$ respectively). **H-K**. Graphs showing the relative IF distribution within the growth cone of Neogenin (H), Unc5c (I), EphA4 (J) and BEN (K) treated with ephnrin-A5 and normalized to MN media treatment, bins with IF values significantly different between treatments are highlighted in red. **L-S**. Examples of LMC neuron growth cones used in the quantifications in F-K.

**Ephrin-A5 induces an increase in the co-localization of Neogenin and EphA4 In LMC growth cones**

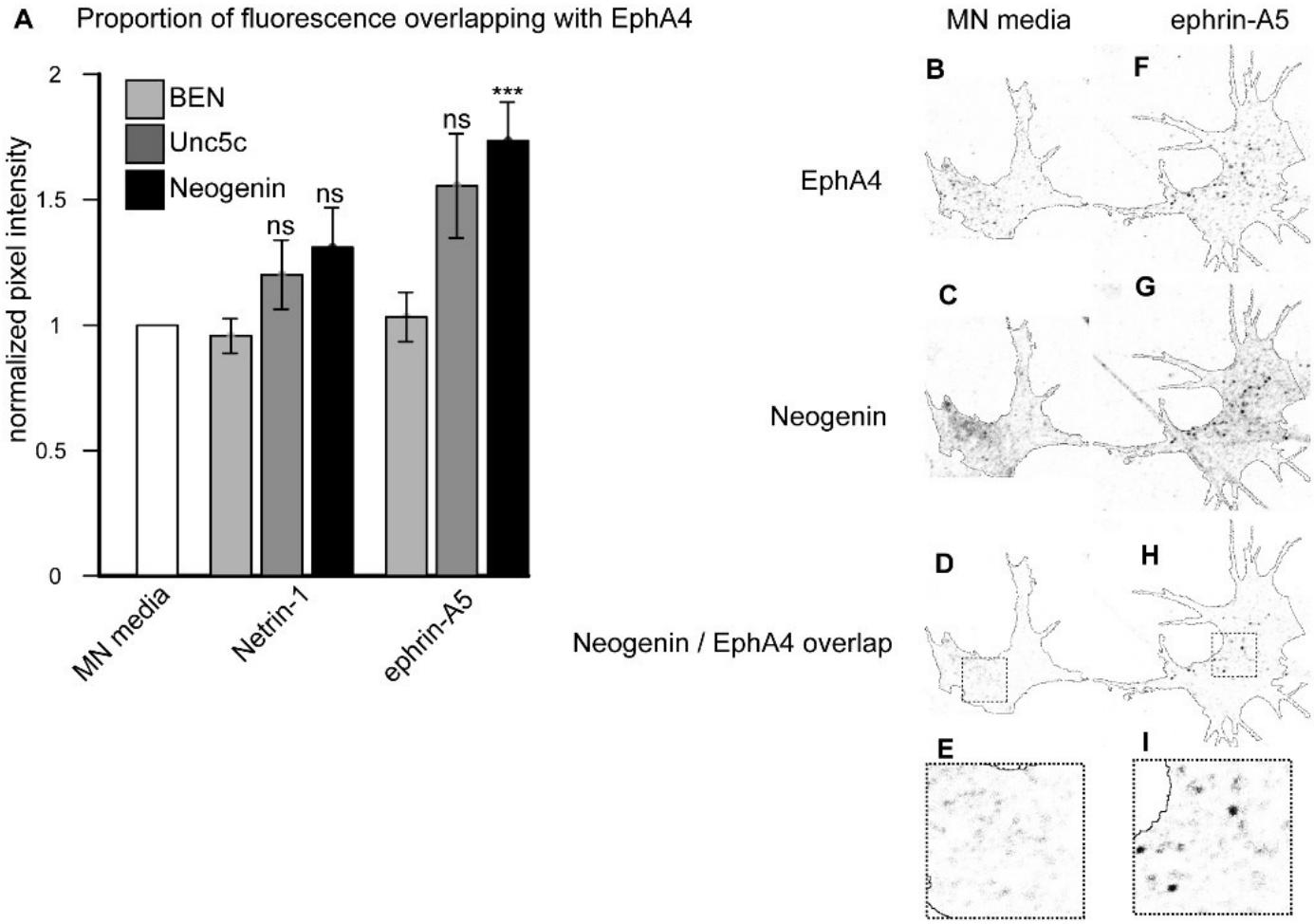
To begin to address the molecular mechanism of how ephrin-A5 may increase Neogenin abundance in LMC growth cones, we first considered the relative dynamics of Neogenin and the ephrin-A5 receptor EphA4 in LMC growth cones. LMC neuron explants were treated with either MN media, ephrin-A5 or Netrin-1 (at 100 ng/mL) followed by immunostaining for EphA4 and either Neogenin, BEN or Unc5C in LMC growth cones. Treatment with ephrin-A5 resulted in an increase in the proportion of Neogenin IF overlapping with EphA4 IF (**Figure 22A**; 73% increase, $p<0.001$). The dense EphA4 IF clusters that appear upon ephrin-A5 treatment often show high levels of Neogenin IF (**Figure 22B-I**), this may reflect an increase in Neogenin levels adjacent to ephrin-A5/EphA4 interactions.

**Ephrin-A5 increases Neogenin growth cone surface levels**

Ephrin-A5 induced LMC axon sensitization to Netrin-1 may result from increased abundance of Neogenin on the surface of growth cones. To test this possibility, we co-applied the extracellular epitope Neogenin antibody and ephrin-A5 at 50 ng/mL or the Neogenin antibody and control medium, to LMC growth cones for 20 minutes, followed by standard fixation. A similar application of an antibody against the intracellular protein beta III tubulin, did not result in any labelling, suggesting enrichment for surface IF (**Figure 23A-H**). A 20 min. treatment with ephrin-A5 resulted in a 56% increase in Neogenin IF (**Figure 23I-K**; $p=0.012$), arguing that ephrin-A5 application results in an enrichment of Neogenin on the surface of LMC

growth cones. Ephrin-A5 treatment also induces a subtle but significant re-localization of surface Neogenin away from the periphery (**Figure 23L, M**).

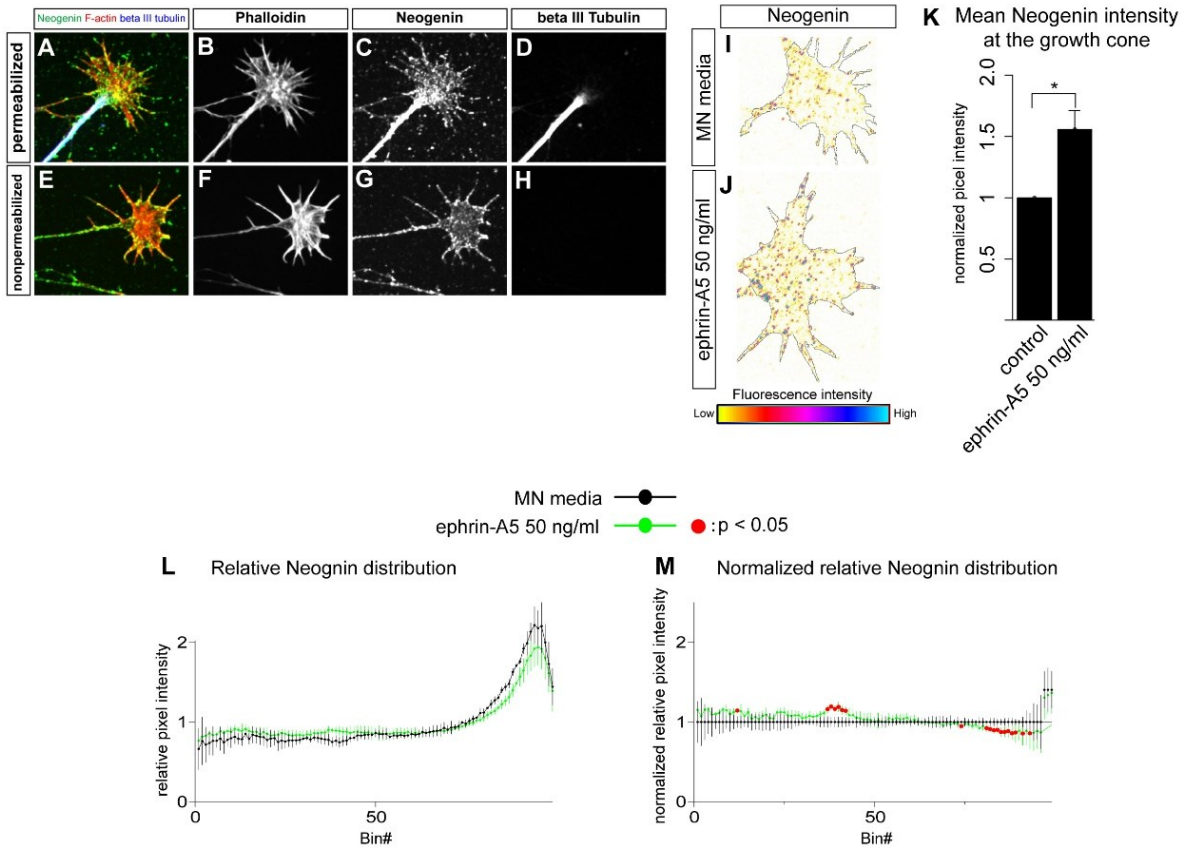
### Figure 22 Ephrin-A5 increases the proportion of fluorescence overlap between Neogenin and EphA4




**A.** Quantification of the proportion of Neogenin, Unc5c and BEN IF overlap with EphA4 IF in thresholded images of growth cones that were treated for 20 min with MN media, Netrin-1 or ephrin-A5 at 100 ng/mL. Ephrin-A5 treatment resulted in a 73% increase in the proportion of Neogenin/EphA4 overlap ($p<0.001$), other tested conditions did not reveal any significance ($p>0.05$). **B-I**. Examples of thresholded images of growth cones immunostained for EphA4 (B, F) and Neogenin (C, G) and treated with MN media (B-E) or ephrin-A5 (E-I). D and H shows the resulting overlap

in Neogenin/EphA4 signal in MN media and ephrin-A5 treated explants respectively, panels E and I are magnified images of boxed regions in D and H.

### Figure 23 Ephrin-A5 increases surface enriched Neogenin in growth cones

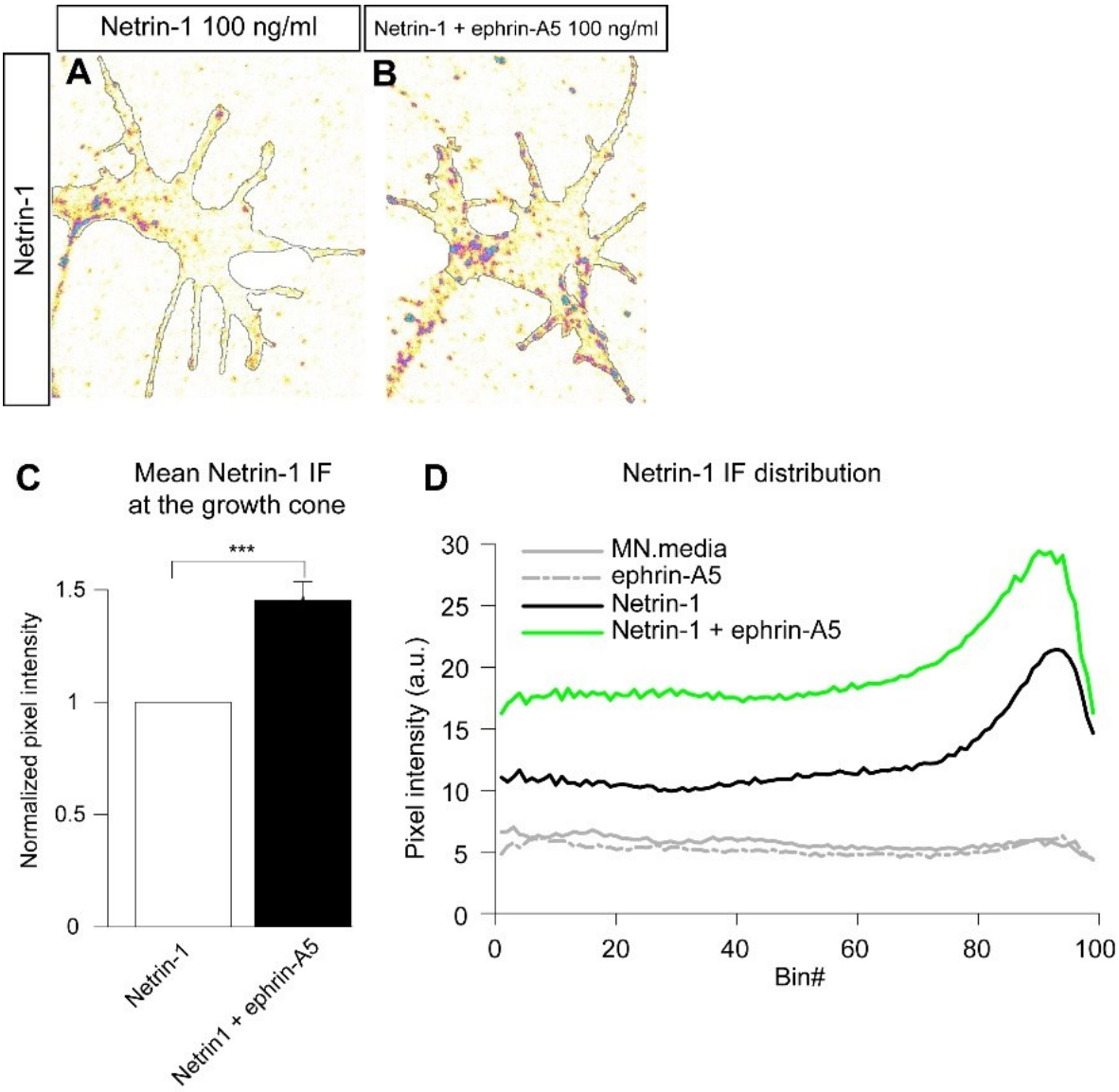



To enrich for surface Neogenin IF, a Neogenin antibody with an extracellular epitope was added to LMC explant cultures during a 20 min treatment with either MN media or ephrin-A5 at 50 ng/mL. **A-H**. To confirm the surface enrichment of Neogenin when the antibody is added to live cultures, an antibody against the intracellular protein beta III tubulin was included in fixed (A-D) and live (E-H) cultures, secondary antibodies and phalloidin to detect F-actin were added post-fix (A-H). **I, J**. Detection of surface enriched Neogenin in growth cones treated with MN media (I) or ephrin-A5 50 ng-mL (J). **K**. Quantification of surface enriched Neogenin IF in MN media or ephrin-A5 50 ng/mL treated growth cones showing a 56% increase in surface enriched Neogenin signal when treated with ephrin-A5 (p=0.012). **L** Quantification of the relative distribution of surface enriched Neogenin fluorescence in MN media and ephrin-A5 treated growth cones **M**. Same as in L but normalized to MN media treatment values, bins with values significantly different between treatments are shown in red.

**Ephrin-A5 enhances Netrin-1 binding in growth cones**

We next sought to determine whether increased LMC growth cone surface Neogenin levels might result in increased Netrin-1 binding to LMC growth cones. LMC explants were incubated with Netrin-1 and ephrin-A5 or Netrin-1 alone as control. After fixation, an antibody against Netrin-1 was used to estimate relative Netrin-1 LMC growth cone binding (**Figure 24**). Compared to a Netrin-1 treatment, the addition of ephrin-A5 resulted in increased levels of Netrin-1 IF in LMC growth cones (**Figure 24A-C**, 45% increase $p < 0.001$). The growth cones included in this analysis did not show significant size differences between treatments (**Supplementary Figure 1E**). In alignment with the enrichment of Neogenin in the periphery of Netrin-1 treated growth cones (**Figure 18**), the distribution of Netrin-1 signal is also enriched at the periphery (**Figure 24D**). Furthermore, the ephrin-A5 induced increase in Netrin-1 signal occurs throughout the growth cone (**Figure 24D**).

## Figure 24 Ephrin-A5 enhances Netrin-1 binding in growth cones

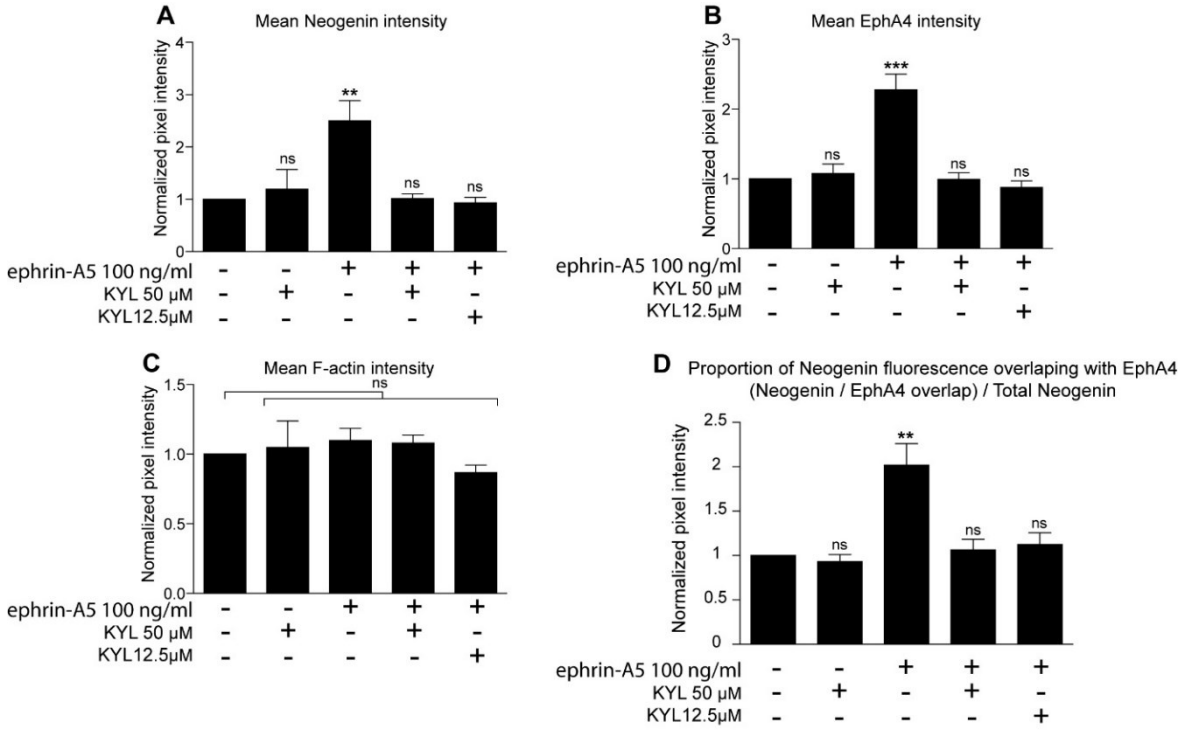



LMC explants were subject to either Netrin-1 or Netrin-1 + ephrin-A5 at 100 ng/mL for 20 min. and immunostained for Netrin-1. **A, B**. Detection of Netrin-1 IF in LMC growth cones treated with Netrin-1 (A) or Netrin-1 + ephrin-A5 (B). **C**. Quantification of Netrin-1 IF in growth cones treated with Netrin-1 or Netrin-1 + ephrin-A5, addition of ephrin-A5 results in a 45% increase in Netrin-1 signal ($p < 0.001$). **D**. Graph depicting the distribution of Netrin-1 IF in growth cones of explants treated with either MN media, ephrin-A5, Netrin-1 or Netrin-1 + ephrin-A5.

**Binding of ephrin-A5 to EphA4 is necessary for the ephrin-A5 induction of Neogenin in growth cones**

The ephrin-A5 induction of Neogenin in LMC neuron growth cones brought us to question the potential involvement of ephrin-A5 receptors for this effect. Chick LMC neurons express EphB2, EphA3 and EphA4 which can bind ephrin-A5 and are therefore potential ephrin-A5 receptor candidates in Neogenin induction (Iwamasa et al., 1999). A 12-amino-acid peptide (KYL peptide) was developed that selectively binds to EphA4 on it's ligand binding domain and inhibits ephrin-A5/EphA4 interactions (Murai et al., 2003). To test the requirement for ephrin-A5/EphA4 interactions in Neogenin upregulation, LMC explants were treated with either MN media, KYL peptide at 50 μM or KYL peptide at 12.5 μM for 20 min. prior to a 20 min. exposure to either MN media or ephrin-A5 100 ng/mL (**Figure 25**). The preincubation of LMC explants with either KYL at 12.5 or 50 μM completely blocked the ephrin-A5 induced increase in Neogenin IF (**Figure 25A**), EphA4 IF (**Figure 25B**) as well as the increase in the proportion of Neogenin/EphA4 fluorescence overlap (**Figure 25D**). These results suggest that ephrin-A5/EphA4 interactions are necessary for inducing an upregulation of Neogenin at the growth cone. These results are in line with previous evidence showing a requirement for EphA4 but not EphA3 in LMC axon guidance (Helmbacher et al., 2000; Vaidya et al., 2003).

## Figure 25 Blocking ephrin-A5/EphA4 interactions inhibits the ephrin-A5 induced increase in Neogenin levels and overlap with EphA4

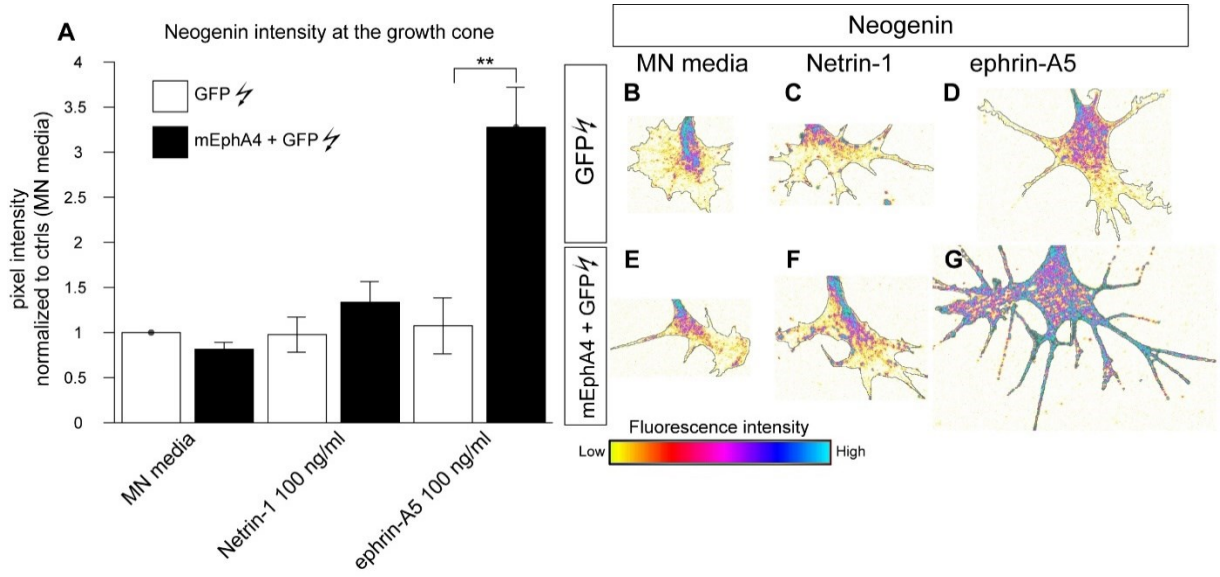



LMC explants were incubated with either MN media, KYL 12.5 µM or KYL 50 µM for 20 min. prior to being treated with either MN media or ephrin-A5 at 100 ng/mL with and without KYL peptide for 20 min. followed by Neogenin, EphA4 and F-actin immunostaining. **A-C.** Quantification of Neogenin (A), EphA4 (B) or F-actin (C) IF in growth cones normalized to MN media treatment D. Quantification of the proportion of Neogenin IF overlapping with EphA4 IF normalized to MN media treatment. A. ** p=0.003, B. ***p<0.001, D. **p=0.009.

**Overexpression of EphA4 potentiates LMC growth cones to the ephrin-A5-dependent increase in Neogenin abundance**

We reasoned that EphA4 could be a critical receptor mediating ephrin-A5-Netrin-1 synergy since it is required for the normal pathfinding of LMC axons (Helmbacher et al., 2000; Eberhart et al., 2002), and that ephrin-A5 exposure results in increased Neogenin abundance and co-localization with EphA4 in LMC growth cones. One prediction of this model is that increasing EphA4 expression levels in LMC growth cones would result in exaggerated increases in Neogenin abundance in response to ephrin-A5. To test this idea, *GFP* expression plasmids alone or together with mouse *EphA4* expression plasmids were electroporated into chick neural tubes at HH st. 18-19 by *in ovo* electroporation (Kania and Jessell, 2003). GFP-expressing LMC neurons were explanted as above, and following treatment with MN media, Netrin-1 or ephrin-A5 (either at 100 mg/mL), Neogenin IF levels were quantified in GFP+ growth cones (**Figure 26A**). Treatment with ephrin-A5 in EphA4 overexpressing LMC neurons led to a threefold increase in Neogenin signal compared to LMC neurons overexpressing GFP alone (**Figure 26A, D, G**), arguing that EphA4 can potentiate the ephrin-A5-induced increase in Neogenin abundance.

## Figure 26 Overexpression of EphA4 enhances the ephrin-A5 induced increase in Neogenin abundance



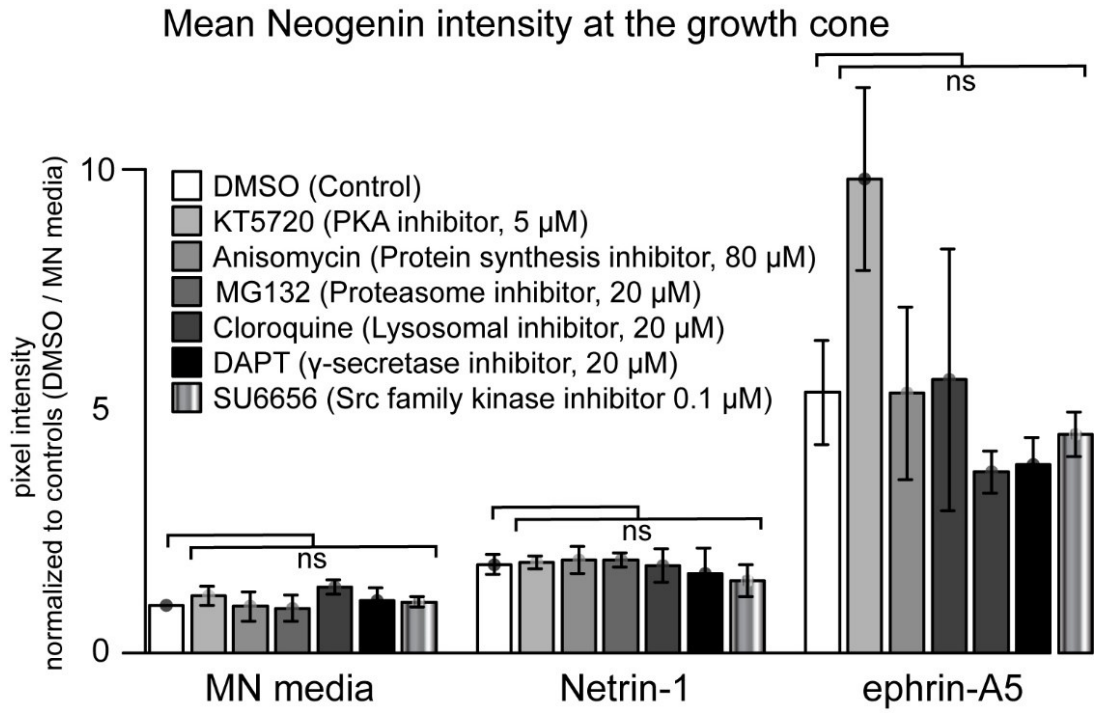

LMC explants from chick spinal cords electroporated with either a GFP expression plasmid alone or in combination with a mEphA4 expression plasmid were subject to a 20 min. treatment of either MN media, Netrin-1 or ephrin-A5 at 100 ng/mL **A**. Quantification of Neogenin IF in growth cones shows that in explants treated with ephrin-A5 and overexpressing EphA4, Neogenin levels in growth cones are threefold higher than in explants expressing GFP alone (** p=0.007). **B-G**. Examples of Neogenin IF in growth cones quantified in A.

### The ephrin-A5-induced Neogenin upregulation is independent of PKA, Src family kinase, protein synthesis, proteosomal or γ-secretase degradation

To gain further insights in the mechanism behind the ephrin-A5 dependent increase in Neogenin, we used pharmacological inhibitors of specific cellular processes, subjecting LMC explants overexpressing EphA4 to these for 20 minutes prior to ephrin-A5 and Netrin-1 treatment, followed by growth cone Neogenin IF quantification (**Figure 27**). To assess the possible role of PKA, we used the PKA inhibitor KT5720 (5 μM, (Kase et al., 1987)), to block protein synthesis we used

anisomycin (80 μM, (Lynch et al., 1954)). Proteasomal and lysosomal Neogenin degradation was blocked by MG132 (20 μM, (Rock et al., 1994)) and chloroquine (20 μM, (Mallucci, 1966)) respectively . Since both EphA4 and Neogenin can be cleaved by γ-secretase (Inoue et al., 2009; Okamura et al., 2011), LMC explants were treated with the γ-secretase inhibitor DAPT (Dovey et al., 2001). Finally, since there is evidence for Src family kinases being activated downstream of both EphA4 and Netrin-1 receptors (Zisch et al., 1998; Knoll and Drescher, 2004; Li et al., 2004; Meriane et al., 2004; Kao et al., 2009), LMC explants were treated with the Src family inhibitor SU6656 (Blake et al., 2000). None of these treatments resulted in significant increase in Neogenin IF when treated with MN media or attenuated the increase in Neogenin levels following ephrin-A5 treatment (**Figure 27**).

**Figure 27 The ephrin-A5 induced Neogenin upregulation prevails despite inhibiting specific cell functions**



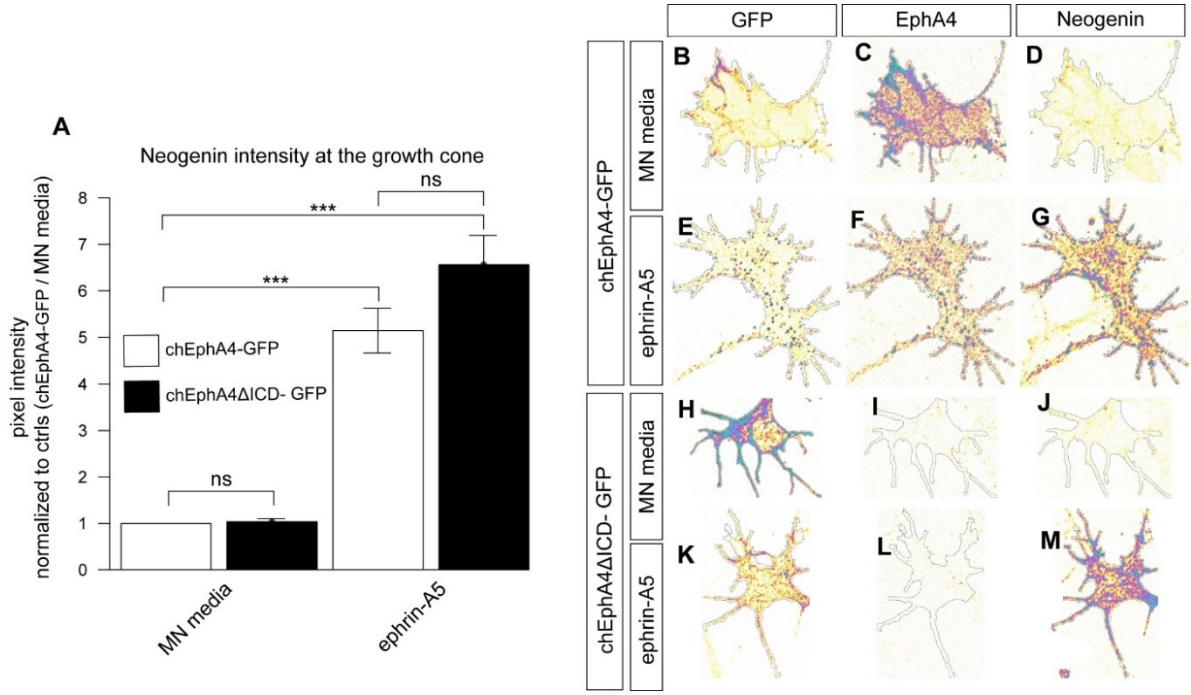

LMC explant cultures were incubated in the presence of various drugs for 20 min. prior to a 20 min. incubation with either MN media, Netrin-1 or ephrin-A5 at 100 ng/mL. followed by immunostaining for Neogenin. A quantification of Neogenin IF in growth cone normalized to DMSO treatment, shows that the drugs did not alter Neogenin levels under assayed conditions.

**The intracellular domain of EphA4 is dispensable for potentiating the ephrin-A5 induced increase in Neogenin abundance**

Next, we asked if the intracellular domain of EphA4, required for the relay of signals from ephrin-As in ephrin/Eph forward signaling (Kullander et al., 2001), is also required for the EphA4-mediated enhancement of ephrin-A5-mediated Neogenin level increase. To do this, we explanted LMC neurons from chick spinal cords electroporated with either expression plasmids encoding an EphA4 and GFP fusion protein (chEphA4-GFP) or a truncated EphA4 missing the intracellular domain and GFP fusion protein (chEphA4ΔICD-GFP). An EphA4 antibody raised against an

intracellular epitope and GFP fluorescence was used to confirm fusion protein expression levels (**Figure 28C**). Both sets of explants were treated with either MN media or ephrin-A5, and Neogenin IF levels were quantified in individual growth cones (**Figure 28A**). Surprisingly, chEphA4ΔICD-GFP expressing growth cones showed a robust upregulation of Neogenin in response to ephrin-A5 when compared to controls (**Figure 28A**, 6.56-fold induction; $p<0.001$), which was indistinguishable from that observed in chEphA4-GFP expressing growth cones (**Figure 28A**, 5.14-fold induction; $p>0.05$). We also noted that in chEphA4-GFP expressing growth cones, in the presence of ephrin-A5, GFP fluorescence becomes punctate and co-localizes with Neogenin (**Figure 28 E-G**). In growth cones expressing chEphA4ΔICD-GFP and treated with ephrin-A5, the appearance of GFP is much less punctate than in chEphA4-GFP expressing growth cones, suggesting a requirement for the intracellular domain of EphA4 for ephrin-A5 induced EphA4 clustering (**Figure 28 E, K**). In line with the pervasiveness of Neogenin induction in chEphA4ΔICD-GFP expressing growth cones, overexpression of an EphA4 with an intracellular mutation blocking the tyrosine kinase activity also enhanced the ephrin-A5-EphA4mediated Neogenin upregulation (data not shown).

**Figure 28 The cytoplasmic tail of EphA4 is dispensable in potentiating the ephrin-A5 induced increase in Neogenin abundance**



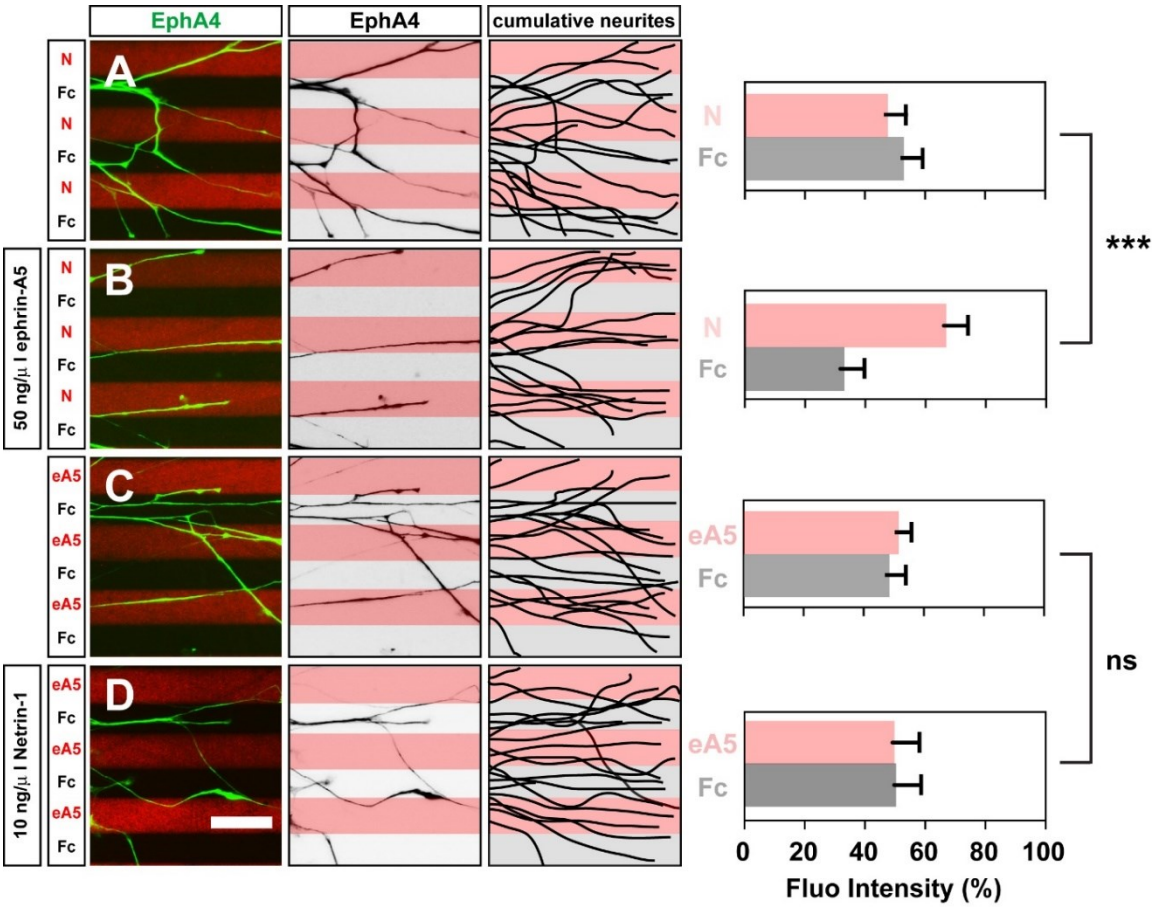

LMC explants from chick spinal cords electroporated with either a chEphA4-GFP or chEphA4ΔICD-GFP expression plasmids were subject to a 20 min. treatment of either MN media or ephrin-A5 at 100 ng/mL and immunostained for Neogenin **A**. Quantification of Neogenin IF in growth cones shows that the ephrin-A5 induced increase in Neogenin signal occurs in growth cones expressing both plasmids (chEphA4-GFP: 5.14-fold induction; $p<0.001$, chEphA4ΔICD-GFP: 6.56-fold induction; $p<0.001$) **B-M**. Examples of GFP, EphA4 and Neogenin IF in growth cones quantified in A.

### Ephrin-A5 sensitization of lateral LMC axons to Netrin-1

When lateral LMC axons are challenged by stripes containing low concentrations of either Netrin-1 or ephrin-A5, they do not exhibit a growth preference, but when challenged with alternating stripes containing Netrin-1 and ephrin-A5 at the same low concentration, they exhibit a robust preference for growth over Netrin-1 stripes (Poliak et al., 2015). We envisaged two possible mechanisms

explaining this behaviour: (1) Netrin-1 sensitizes lateral LMC axons to ephrin-A5 avoidance or (2) ephrin-A5 sensitizes lateral LMC axons to Netrin-1 attraction. To distinguish between these, LMC explants were challenged with stripes containing either ephrin-A5 or Netrin-1 and control stripes containing Fc, with bath application of Netrin-1 or ephrin-A5, respectively (**Figure 29)**. Lateral LMC axons were visualised using EphA4 antibodies, and their stripe preference outgrowth was scored as previously (Poliak et al., 2015). When lateral LMC axons were challenged with alternating ephrin-A5 and Fc stripes (ephrin-A5 / Fc) with or without bath Netrin-1 (10 ng/mL), no avoidance of ephrin-A5 stripes was observed (**Figure 29C, D**, ephrin-A5 51.6 ± 5.0%, ephrin-A5 49.9 ± 8.4% respectively, P=0.5723). Lateral LMC axons challenged with alternating Netrin-1 (10 ng/mL) and Fc stripes (Netrin-1/Fc) show no growth preference (**Figure 29A**, Netrin-1 47.3 ± 6.7%, p>0.05). In contrast, in the presence of bath applied ephrin-A5 (50 ng/mL), lateral LMC axons show preference for Netrin-1 stripes (**Figure 29B,** Netrin-1 66.9 ± 7.8%, P<0.001). Although we cannot rule out the possibility that higher Netrin-1 concentrations could sensitize LMCl axons to ephrin-A5 avoidance, our results suggest that ephrin-A5 sensitizes LMCl axons to Netrin-1 but Netrin-1 does not sensitize LMCl axons to ephrin-A5 avoidance.

## Figure 29 LMCl axons are sensitized to Netrin-1 by ephrin-A5

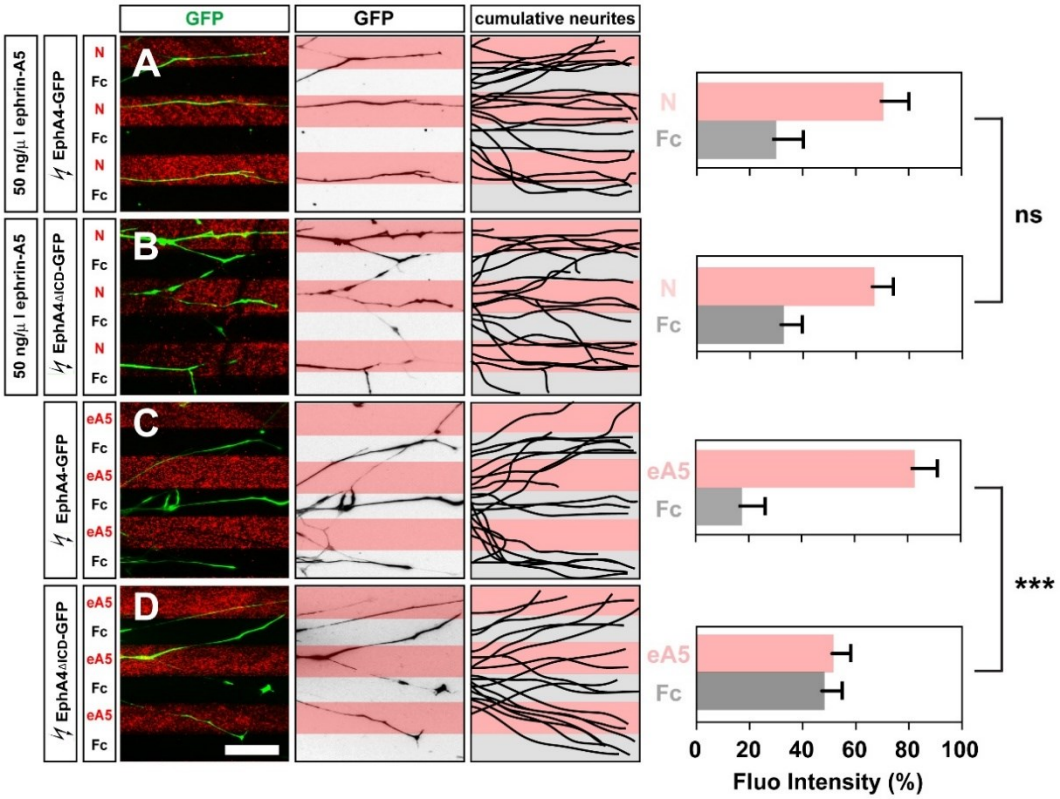



Growth preference on protein stripes exhibited by lateral LMC axons. **A-D**. Left panels: explanted lateral (EphA4$^{+}$) LMC neurites on Netrin-1 (N)/Fc (A and B) or ephrin-A5 (eA5)/Fc stripes with or without bath treatment of ephrin-A5 (B) or Netrin1 (D). Middle panels: inverted images of EphA4 signals shown at left panels. Right panels: superimposed images of five explants from each experimental group representing the distribution of lateral LMC neurites. Quantification of lateral LMC neurites on first (pink) and second (pale) stripes expressed as a percentage of total EphA4 signals. Noted that no preference is detected when lateral LMC neurites are challenged with stripes of low levels of Netrin-1 (10 ng/mL) or ephrin-A5 (50 ng/mL). Minimal number of neurites: 81. Minimal number of explants: 12. N, Netrin-1; eA5: ephrin-A5; error bars = SD; *** = P<0.001; statistical significance computed using Mann-Whitney U test. Scale bar: 50 µm. (These experiments were designed by L.-P.C., executed and analyzed by Tzu-Jen Kao)

**The cytoplasmic tail of EphA4 is dispensable for the sensitization of LMCl axons to Netrin-1 by ephrin-A5**

Our results suggest that the ephrin-A5 dependent upregulation of Neogenin occurs independently of the EphA4 cytoplasmic domain (**Figure 28**). To test whether this also occurs in the context of guidance decisions made by LMC axon, we first challenged LMC explants from chEphA4-GFP or chEphA4ΔICD-GFP electroporated chickens with ephrin-A5/Fc stripes (**Figure 30 C, D**). As anticipated, since the intracellular domain of EphA4 is required for its ability to elicit repulsion from ephrin-A5, LMC axons expressing chEphA4ΔICD-GFP showed less avoidance from ephrin-A5 in contrast to LMC axons expressing chEphA4-GFP (**Figure 30 C, D*;*** chEphA4-GFP: ephrin-A5 stripe = 82.6 ± 9.4%, chEphA4ΔICD-GFP: ephrin-A5 stripe = 51.7 ± 7.4%; p=0.1581). However, expression of chEphA4-GFP and chEphA4ΔICD-GFP resulted in the same enhancement of growth preference over Netrin-1 stripes in the presence of bath-applied ephrin-A5 (**Figure 30 A, B**; chEphA4-GFP: Netrin-1 70.3 ± 10.2%, chEphA4ΔICD-GFP: Netrin-1 67.2 ± 7.7%; p=0.1581). Together, these data argue that EphA4 can promote ephrin-A5-mediated sensitization of LMC axons to Netrin-1 in the absence of its intracellular signal relay.

**Figure 30 The cytoplasmic tail of EphA4 is dispensable in the sensitization of LMC axons to Netrin-1 by ephrin-A5**




Growth preference on protein stripes exhibited by LMC axons. A-D. Left panels: explanted LMC neurites expressing chEphA4-GFP and chEphA4ΔICD-GFP on Netrin-1 (N)/Fc stripes bath treatment of ephrin-A5 (A and B) or ephrin-A5 (eA5)/Fc stripes (C and D). Middle panels: inverted images of GFP signals shown at left panels. Right panels: superimposed images of five explants from each experimental group representing the distribution of $GFP^+$ LMC neurites. Quantification of lateral LMC neurites on first (pink) and second (pale) stripes expressed as a percentage of total GFP signals. Noted that both chEphA4-GFP and chEphA4ΔICD-GFP expressed LMC neurites show preferences over Netrin 1 stripes. Minimal number of neurites: 85. Minimal number of explants: 13. N, Netrin-1; eA5: ephrin-A5; error bars = SD; *** = P<0.001; statistical significance computed using Mann-Whitney U test; scale bar: 50 µm. (These experiments were designed by L.-P.C., executed and analyzed by Tzu-Jen Kao)

# CHAPTER IV - DISCUSSION

## Neogenin in LMC neuron differentiation

Taken together, the experiments presented in this chapter suggest that Neogenin is required in LMC neuron subtype segregation and differentiation in the chick. The formulation of a strong conclusion regarding the requirement for Neogenin in LMC development is hindered by the absence of non-siRNA based experiments. For this reason, we can not rule out the possibility that the phenotype seen in [Neo]siRNA embryos could be the result of siRNA off target effects. Some experiments, such as the analysis of LMC neuron subtype differentiation in [RGMb]siRNA embryos and in *Neo*$^{-/-}$ mice require additional experiments to be conclusive. For these reasons, I have hesitated in including these results in this thesis and had no intention in including work from this chapter in my thesis defence presentation. I will leave it upon my thesis committee to decide whether certain elements of this chapter should be excluded from my final submission. Nonetheless, I will discuss insights in Neogenin function from recent publication that may shed light on my experimental results.

## Neogenin in LMC subtype segregation

In epithelia, cadherin based adherens junctions regulate cell-cell junctional stability (Priya and Yap, 2015). Adherens junctions rely upon the interaction between cadherins and closely opposed actin rings (Priya and Yap, 2015). Actin nucleation via the Arp2/3 enzyme complex is necessary for maintaining epithelial cohesion (Bernadskaya et al., 2011). Arp2/3 dependent actin nucleation is stimulated by the

WAVE regulatory complex (WRC) (Chen et al., 2014). Several surface molecules including proteins involved in adhesion such as protocadherins and axon guidance receptors such as Robo1, Dcc and Neogenin, have WRC binding domains (Chen et al., 2014). In *in vitro* epithelial cell cultures, Neogenin and RGMa are required for adherens junction stability and the spatial control of WRC/Arp2/3 recruitment to adherens junctions (Lee et al., 2016). *Neo*$^{GT/GT}$ mice display a loss of adherens junctions in radial glial cells of the embryonic cortex as well as in ependymal cells in the postnatal ventricular zone (O'Leary et al., 2017). Interestingly, loss of cadherin function through the genetic ablation of N-Cadherin or the combined loss of beta and gamma-catenin in spinal motor neurons, results in the intermingling of LMCm and LMCl neurons (Demireva et al., 2011). Although no change in the generation of LMCl neurons have been reported in mice carrying these mutations, this phenotype is reminiscent of the lack of LMCm/LMCl neuron segregation observed in [Neo]siRNA embryos. Evidence of Neogenin in cadherin based cell adhesion and the requirement for cadherins in LMC subtype segregation suggests that the perturbation in LMCm/LMCl segregation observed in [Neo]siRNA embryos may be attributable to impaired cell adhesion among LMC neurons.

**Neogenin in LMC subtype differentiation**

In [Neo]siRNA embryos, ectopic LMCm neurons more frequently occupy the ventral portion of the LMC while the most lateral population of LMCl neurons remains largely unaltered. These results suggest that the requirement for Neogenin in Lim1 expression may differ among LMCl neurons (**Figure 13**). During LMC neuron development, retinoic acid is generated by the paraxial mesoderm immediately adjacent to the spinal cord (Ji et al., 2006). In mice carrying a conditional mutation that ablates Raldh2 in brachial motor neurons but maintains expression in the paraxial mesoderm, 80% of LMCl neurons are generated (Vermot et al., 2005). Conversely, mice hypomorphic for Raldh2 in the paraxial mesoderm also exhibit a similar decrease in LMCl generation (Ji et al., 2006). This suggests that retinoic acid provided by both LMC neurons and the paraxial mesoderm, contribute to the generation of LMCl neurons (Ji et al., 2006). It is therefore likely that in [Neo]siRNA embryos, the more lateral population of LMCl neurons may acquire sufficient retinoic acid from the paraxial mesoderm whereas the more ventral LMCl neuron subpopulation may be more reliant on retinoic acid derived from LMC neurons.

In the olfactory epithelium of mice, the proper regulation of progenitor cell cycle progression and exit relies on the expression of Neogenin by progenitors and the expression of RGMb by neighbouring immature olfactory receptor neurons (Kam et al., 2016). A partially analogous mechanism may be occurring in LMC subtype differentiation. Prospective LMCl neurons expressing Neogenin may bind RGMb on

earlier born LMCm neurons. These interactions may favor cell-cell contacts that favor retinoic acid uptake by prospective LMCl neurons.

The absence of an obvious LMC subtype differentiation phenotype in *Neo*$^{-/-}$ mice argues that the acquisition of Lim1 expression occurs independently from Neogenin in the mouse (**Figure 16**). We can not rule out the possibility that Dcc expression by LMC neurons could compensate for the loss of Neogenin in Lim1 expression in mice. This would entail a mechanism independent from RGMs since Dcc is not known for binding RGMs. The requirement for Neogenin/RGM in LMC development may differ between chick in mice. Whereas in chick, loss of RGMa results in RGC axon guidance defects, RGC axons appear normal in *RGMa*$^{-/-}$ mice. (Monnier et al., 2002; Niederkofler et al., 2004).

My results suggest that Neogenin is involved in multiple aspects of LMC neuron development in the chick including the acquisition of Lim1 expression by prospective LMCl neurons, the segregation of LMCm/LMCl neurons as well as the guidance of LMC axons. To confirm the involvement of Neogenin in theses aspects of LMC development would require additional experimentation such as the rescue of [Neo]siRNA phenotype by the overexpression mouse Neogenin and or Dcc as well as additional experiments regarding the potential involvement of RGMs

**Sensitization of LMCl axons to Netrin-1 by ephrin-A5**

Through the stripe assay, we have previously demonstrated that ephrin-A5 and Netrin-1 act synergistically on LMCl axons *in vitro* (Poliak et al., 2015). To gain insight in the molecular mechanism underling the synergistic activity of ephrin-A5 and Netrin-1 in LMC neurons, an analysis of receptor dynamics in LMC growth cones subject to Netrin-1 and ephrin-A5 was carried out. Our results demonstrate that ephrin-A5 induces the upregulation of Neogenin in growth cones leading to enhanced Netrin-1 binding. Furthermore, we show that the upregulation of Neogenin is dependent on the interaction between ephrin-A5 and EphA4 and is enhanced by the overexpression of full-length and truncated EphA4. Using stripe assays, we show that bath applied ephrin-A5 sensitizes LMCl axons to Netrin-1 and demonstrate that sensitization occurs independently from the cytoplasmic tail of EphA4. These results demonstrate that ephrin-A5/ EphA4 forward signalling and ephrin-A5/EphA4 sensitization to Netrin-1 occur in molecularly distinct pathways.

**Netrin-1 increases and redistributes Neogenin to the growth cone periphery**

We show that Netrin-1 induces the re-localization of Neogenin towards the growth cone periphery at Netrin-1 concentrations as low as 10 ng/mL in a PKA dependent fashion (**Figure 18, 3**). The observation that surface Neogenin is enriched peripherally in the absence of Netrin-1 treatment (**Figure 23G**) suggests that the Netrin-1- induced peripheral relocalization is likely accompanied by Neogenin insertion into the plasma membrane. The dependence on PKA for membrane

insertion of Dcc has been documented in rat commissural neurons and is likely analogous to the dependence on PKA for Neogenin relocalization (Bouchard et al., 2004; Bouchard et al., 2008). The absence of Netrin-1 induced change in the levels of EphA4 suggests that Netrin-1 unlikely sensitizes LMC growth cones to ephrin-A5 by modulating EphA4. This is substantiated by the results showing that bath application Netrin-1 does not increase the avoidance of LMCl axons to ephrin-A5 stripes (**Figure 29C, D)**. Since the EphA4 antibody used to measure EphA4 IF levels recognizes an intracellular epitope, we can not rule out the possibility that Netrin-1 could increase the membrane insertion of EphA4 without changing total levels. The use of an antibody against the extracellular portion of EphA4 in non-permeabilized, Netrin-1 treated LMC cultures could resolve this query.

**Ephrin-A5 on receptor dynamics in LMC growth cones**

Our results show that a 20 min. exposure to ephrin-A5 at 50 and 100 ng/mL but not at higher concentrations, results in increased growth cone Neogenin levels. *In vitro*, ephrin-A2 can be growth promoting at low concentrations and switch to growth inhibition at higher concentrations in RGCs axons (Hansen et al., 2004). It is proposed that at low ephrin-A2 concentrations, low levels of EphA receptor clustering would favor adhesion and outgrowth whereas high levels of EphA clustering, resulting from higher ephrin-A2 concentrations, would induce repulsive downstream signaling and decreased adhesion through EphA receptor cleavage (Hansen et al., 2004). It should also be considered is that in the experiments presented here, only growth

cones that were non-collapsed and contained lamellipodia were considered for analysis. The higher ephrin-A5 concentrations tested here may have collapsed a subset of LMC growth cones capable of ephrin-A5 induced Neogenin upregulation at lower ephrin-A5 concentrations.

Ephrin-A5 treatment results in a re-localization of EphA4, Unc5c, BEN (**Figure 21H-K**) and surface enriched Neogenin (**Figure 23M**) from the periphery to the growth cone center. This may be the consequence of an ephrin-A5 induced membrane endocytosis in the peripheral domain of the growth cone (Irie et al., 2005; Tojima et al., 2010; Yoo et al., 2011) . The ephrin-A5 induced increase in Neogenin is accompanied by an increase in the average size of Neogenin IF aggregates (**Figure 20 F, G**) and an increase in the proportion of overlap between EphA4 and Neogenin signals (**Figure 21**). Furthermore, when overexpressing chEphA4-GFP, ephrin-A5 induces the appearance of dense GFP+, EphA4+, Neogenin+ aggregates (**Figure 27 B-G**). These results suggest that ephrin-A5/EphA4 interactions increase Neogenin locally. Binding of ephrin-A2 or ephrin-B3 to EphA4 induces EphA4 endocytosis (Zimmer et al., 2003; Deininger et al., 2008). The ephrin-B1 induction of EphB2 endocytosis requires EphB2's cytoplasmic tail and kinase activity (Zimmer et al., 2003). This suggests that in growth cones expressing chEphA4ΔICD-GFP, the ephrin-A5 induction of truncated EphA4 endocytosis is likely impaired. The high level of ephrin-A5 dependent Neogenin induction in chEphA4ΔICD-GFP expressing growth cones (**Figure 27A**) suggest that the increase in Neogenin occurs independently of EphA4 endocytosis and is likely initiated at the membrane surface.

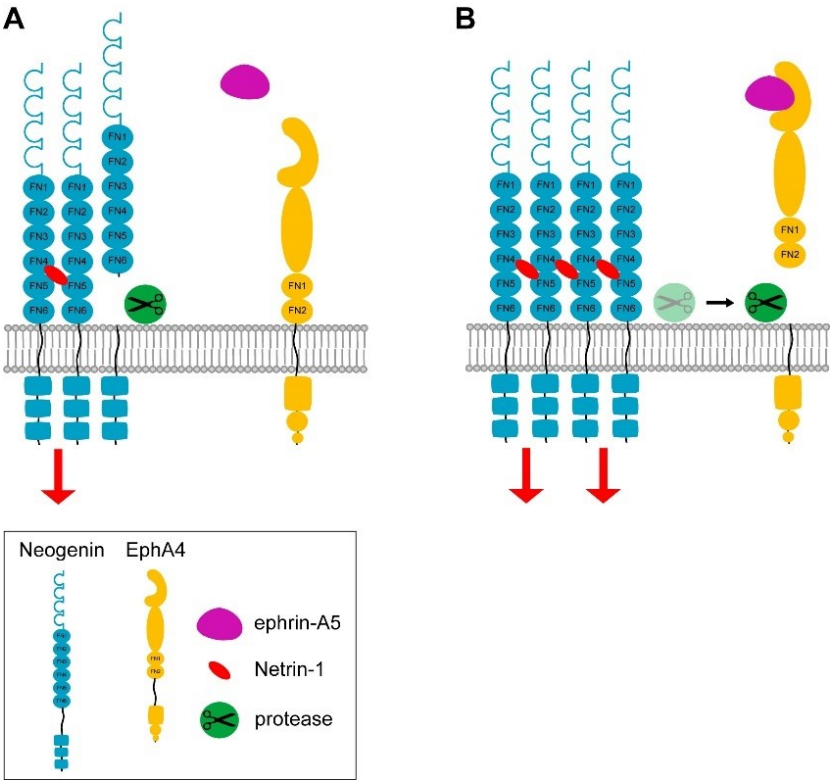
**Ephrin-A5/EphA4 attenuation of Neogenin cleavage as a possible mechanism underlining the sensitization to Netrin-1**

Several transmembrane proteins are regulated by ligand-dependent ectodomain cleavage, followed by a secondary cleavage of the remaining intracellular fragment by γ-secretase (Bai and Pfaff, 2011). The in vitro treatment of dorsal spinal explants with metalloprotease inhibitors results in an increase in Dcc expression and a potentiation towards Netrin-1 dependent axonal outgrowth (Galko and Tessier-Lavigne, 2000). The ectodomain of Neogenin can be cleaved by the metalloprotease ADAM17, and was proposed to modulate RGMa induced repulsion by regulating surface Neogenin (Okamura et al., 2011). Furthermore, cleavage of Neogenin by ADAM17 can be regulated by the transmembrane protein Lrig2 (van Erp et al., 2015). Lrig2 is proposed to shield Neogenin from ADAM17 cleavage and binding of RGMa to Neogenin inhibits Lrig2/Neogenin interactions and allows cleavage by ADAM17 (van Erp et al., 2015). Dcc and Neogenin both undergo cleavage by γ-secretase, resulting in the release of their intracellular domains (ICD) (Taniguchi et al., 2003; Goldschneider et al., 2008). In mice mutant for Presenilin-1, an essential catalytic component of the γ-secretase complex, a subset of spinal commissural axons fail to cross the floor-plate and spinal motor neurons aberrantly extend axons to and across the midline (Bai et al., 2011). These guidance defects are suggested to result from altered sensitivity of spinal neuron axons towards Netrin-1(Bai et al., 2011). Thus, the regulation of Dcc and Neogenin cleavage is a molecular strategy for modulating cellular responses to external cues.

In hippocampal neurons, ephrin-B2 induces the ectodomain cleavage of EphB2 and inhibiting this process by using metalloprotease inhibitors, blocks ephrin-B2 dependent growth cone collapse (Lin et al., 2008). Following ectodomain cleavage, EphB2 is also susceptible to γ-secretase cleavage (Litterst et al., 2007). EphA4 was also shown to undergo extracellular cleavage by metalloproteases and intracellular cleavage by γ-secretase (Inoue et al., 2009). Furthermore, the extracellular cleavage of EphA4 can be regulated by synaptic activity (Inoue et al., 2009). The cleavage of EphB2 by metalloproteases occurs within one of its FNIII domains, a domain shared by Dcc, Neogenin and EphA4 suggesting they may be subject to cleavage by common proteases (Keino-Masu et al., 1996; Lin et al., 2008).

The basal levels of surface Neogenin in LMC growth cones may be regulated by a protease that also cleaves EphA4 upon binding ephrin-A5. If such is the case, the ephrin-A5 induction of EphA4 cleavage may relieve Neogenin proteolysis, resulting in its accumulation adjacent to ephrin-A5/EphA4 interactions (**Figure 31**).

**Figure 31 Tentative model for the sensitization of LMCI axons to Netrin-1**



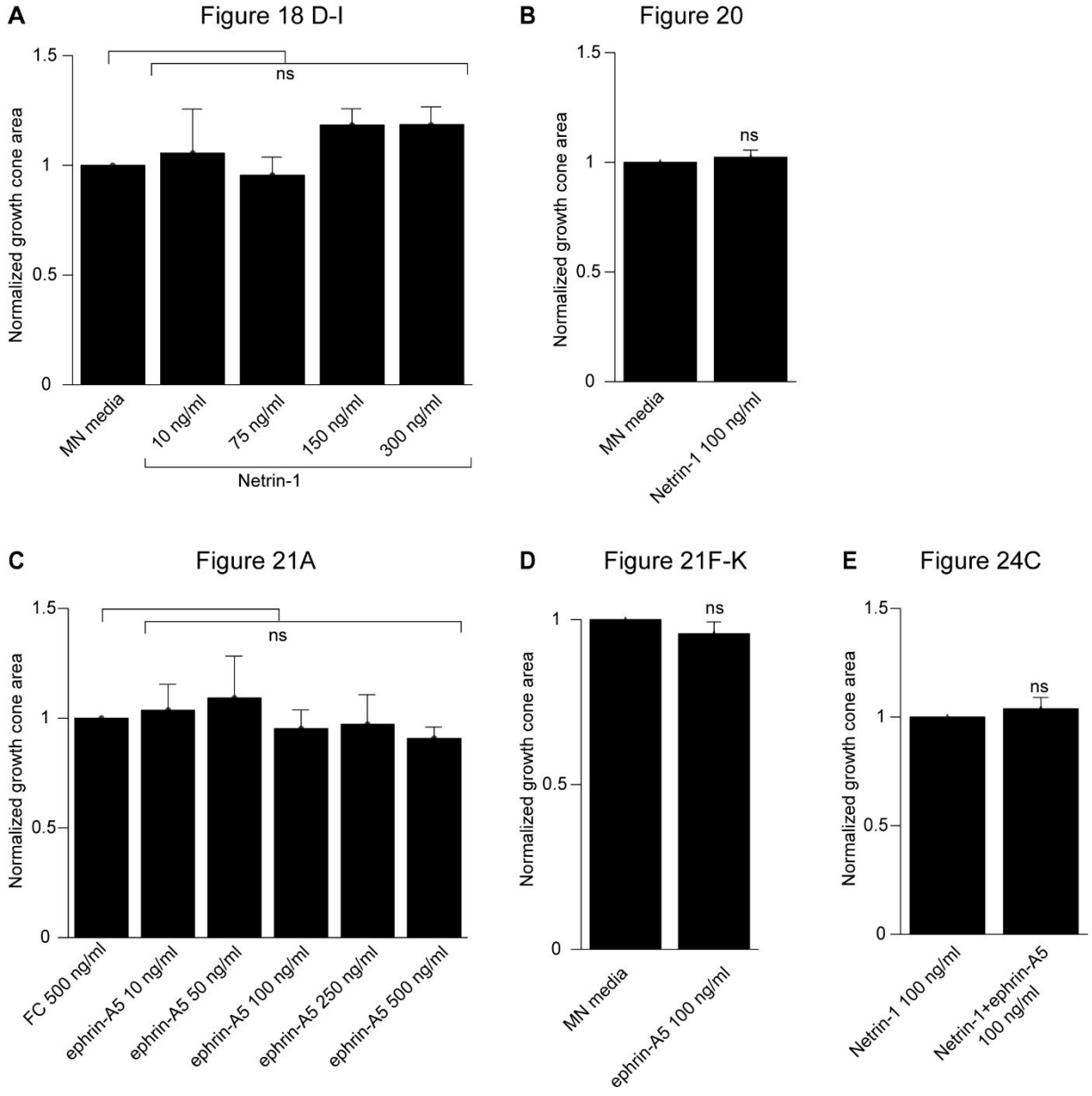

**A**. The basal surface levels of Neogenin at the growth cone may be regulated through the cleavage of its extracellular fragment by a protease. **B.** The binding of ephrin-A5 to EphA4 induces the cleavage of the extracellular fragment of EphA4. If Neogenin and EphA4 are cleaved by a common protease, then the ephrin-A5 induced proteolytic cleavage of EphA4 may attenuate the cleavage of Neogenin in the vicinity of ephrin-A5/EphA4 interactions. This would result in an increase in Netrin-1 binding and a potentiation in Netrin-1 downstream signalling (red arrows).

The increase in the proportion of Neogenin/EphA4 IF overlap upon ephrin-A5 treatment (**Figure 22**) and the co-localization of Neogenin/GFP puncta when overexpressing chEphA4-GFP in the presence of ephrin-A5 (**Figure 28E-G**) suggests that the upregulation of Neogenin is enriched where ephrin-A5/EphA4 interactions occur. The re-localization of surface Neogenin towards the center of the growth cone, where EphA4 is enriched when subject to ephrin-A5 (**Figure 21J**, **Figure 23M**) is also

in line with this possibility. We have recently obtained preliminary data showing that unlike the overexpression of either full-length or truncated EphA4, the overexpression of an non-cleavable EpA4 fails to enhance the increase in Neogenin levels at the growth cone (data not shown). We are now in the process of adding additional experiments implicating non-cleavable EphA4 as well as testing the requirement for proteolytic cleavage in the ephrin-A5/EphA4 dependent upregulation of Neogenin through the use of protease inhibitors. Interestingly, mice in which EphA4 cleavage is abolished, a subset of LMCl axons are misguided into the ventral limb (Gatto et al., 2014). If the ephrin-A5 upregulation of Neogenin requires cleavage of EphA4, the LMCl axons guidance defects seen in the mouse harboring a cleavage resistant EphA4 may be in part due to lower Neogenin/Dcc levels resulting in decreased axon attraction to Netrin-1 in the dorsal limb (Poliak et al., 2015).

EphA4 has been implicated in various neurodegenerative diseases including multiple sclerosis, amyotrophic lateral sclerosis and Alzheimer's disease (Simon et al., 2009; Shen et al., 2010; Van Hoecke et al., 2012; Munro et al., 2013). Interestingly, ephrin-A/EphA4 interactions were shown to regulate the degradation of the Alzheimer's disease related protein amyloid precursor protein (APP) (Lai et al., 2014). Thus, ephrin-A/EphA4 regulation of protein degradation may influence several aspects of neuronal function during development as well as in neurodegenerative diseases.

## Supplementary Figure 1 Growth cone area measurements




To ensure that differences in the mean level of IF among assayed conditions was not due to differences in the size of the growth cones selected for the analysis, the mean growth cone area was determined for each condition and normalized to either MN media treated explants (A, B, D), FC 500 ng/ml (C) or Netrin-1 100 ng/ml (E). The mean growth cone area did not differ significantly in any of the assayed experiments ($p > 0.05$).

# CHAPTER V – CONTRIBUTIONS AND PERSPECTIVES

## Neogenin as a mediator of attractive Netrin-1 signaling in LMC neurons

My thesis project began by investigating the role of chick Neogenin as a Netrin-1 receptor in LMC neurons. Using the stripe assay and a Neogenin function blocking antibody, I demonstrated that Neogenin is required for the responsiveness of LMCl axons towards Netrin-1 in the chick. I also showed that this loss of responsiveness can be rescued through the overexpression of it's closely related homologue Dcc. These results are in line with previous evidence suggesting that chick Neogenin can functionally substitute for Dcc in mediating attraction towards Netrin-1 and establishes chick LMCl neurons as a model system for studying attractive Netrin-1 signaling (Phan et al., 2011; Poliak et al., 2015).

## Speculations on the requirement for Neogenin dependent transcriptional regulation for the expression of Lim1 by LMC neurons

Neogenin was shown to interact directly with the transcription coactivator LMO4 and the intracellular domain (ICD) of Neogenin can translocate to the nucleus where it can presumably regulate transcription (Goldschneider et al., 2008; Banerjee et al., 2016). RGMa induced outgrowth inhibition of RGC axons is lost when LMO4 is knocked down, suggesting that NeoICD/LMO4 complexes may in part regulate RGMa signaling by regulating transcription (Banerjee et al., 2016). Through transcriptional regulation, LMO4 directs the balance between inhibitory and excitatory interneurons in the spinal cord (Joshi et al., 2009). Interestingly, LMO4 is expressed by LMC neurons and was shown to compete with Isl1 in binding LIM homeodomain complexes. Furthermore, LMO4 mRNA is downregulated in cells exposed to retinoic

acid, suggesting a possible role for LMO4 in retinoic acid signalling (Duffy et al., 2017). Taken together, the evidence suggests that Neogenin/RGM interactions in prospective LMCl neurons may induce the nuclear translocation of NeoICD/LMO4 complexes which together with retinoic acid, may induce the switch from Isl1 to Lim1 expression. To assess a potential requirement for LMO4 in Lim1 expression by LMC neurons, LMO4 could be overexpressed or Knocked down in LMC neurons. It would also be interesting to attempt rescuing the LMC differentiation phenotype in [Neo]siRNA embryos by overexpressing LM04.

**Optimization of the chick *in ovo* electroporation protocol**

In my initial *in ovo* electroporation experiments, I noticed that some electroporated embryos displayed a loss in ventricular zone integrity accompanied by ectopic LMC neurons adjacent to the disrupted ventricular zone. These electroporation artifacts led us to optimize the standard protocol for *in ovo* electroporation and to publish our findings (Croteau and Kania, 2011). The revised protocol highlights the importance of the DNA dilution buffer and electrode placement in maintaining the cellular integrity of electroporated spinal cords. We show that the use of TE buffer in the electroporation solution can result in the loss of ventricular zone integrity leading to neuron migration defects. This is likely due to the $Ca^{2+}$ chelating properties of EDTA disrupting Cadherin based adherens junctions at the ventricular zone (Takeichi, 1988; Croteau and Kania, 2011)

## The sensitization of LMC neurons to Netrin-1 by ephrin-A5

My results demonstrate that low concentrations of ephrin-A5 increases the levels of Neogenin and enhances Netrin-1 binding in LMC growth cones. We show that the upregulation of Neogenin is dependent on the interaction between ephrin-A5 and EphA4 and is enhanced by the overexpression of full-length and truncated EphA4. We show that bath applied ephrin-A5 sensitizes LMCl axons to Netrin-1 and demonstrate that sensitization occurs independently from the cytoplasmic tail of EphA4. These results demonstrate that ephrin-A5/ EphA4 forward signalling and ephrin-A5/EphA4 sensitization to Netrin-1 occur in molecularly distinct pathways.

## A potential for ephrin-A5 induced upregulation of Neogenin in various cell types

Although at lower levels than in LMCl neurons, LMCm neurons express EphA4 and encounter ephrin-A5 in the ventral limb (Bonanomi et al., 2012; Gatto et al., 2014). This raises the possibility that ephrin-A5 may also sensitize LMCm axons to Netrin-1. There is evidence demonstrating that repulsion from Netrin-1 requires the interaction between Unc5 receptors and Dcc (Hong et al., 1999). Although ephrin-A5 does not seem to increase Unc5c levels in LMC growth cones, it is possible that higher levels of Neogenin at the plasma membrane may contribute to enhancing Netrin-1/Unc5c signalling. There is also the possibility that other ephrins may induce sensitization to Netrin-1, in mice lacking ephrin-A2, LMCl axons are misguided into

the ventral limb, a phenotype that is exacerbated in *Efna2*$^{-/-;}$*Efna5*$^{-/-}$ double mutants (Bonanomi et al., 2012).

We have yet to investigate the possibility that other neuronal populations may be susceptible to ephrin-A5 induced sensitization to Netrin-1. *In vitro*, In the context of EphA4 overexpression, ephrin-A5 treatment can increase Neogenin levels in cells other than LMC neurons, suggesting that other cell types may be sensitized to Netrin-1 by ephrin-A5 (data not shown). Netrin-1 is required for spinal commissural neurons in the dorsal spinal cord to extend axons ventrally towards the floor plate (Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996). EphA4 and ephrin-A5 are expressed in the dorsal spinal cord as spinal commissural axons extend ventrally (Iwamasa et al., 1999; Eberhart et al., 2004; Kramer et al., 2006). One intriguing possibility is that commissural neurons may also be sensitized to Netrin-1 as growth cones encounter ephrin-A5 expressed by radial glia.

Neogenin is implicated in multiple developmental processes including iron homeostasis, myogenesis and bone formation (Bae et al., 2009; Lee et al., 2010; Zhou et al., 2010). A requirement for Neogenin in adults has also been demonstrated in acute inflammation as well as in adult neurogenesis (Konig et al., 2012; O'Leary et al., 2015). Ephrin-A5 being widely expressed during development and adulthood (Yue et al., 2014) raises the possibility that ephirn-A5 may enhance several aspects of Neogenin function.

**A potential requirement for ephrin-A/EphA induction of Neogenin for neural tube closure**

Lack of either Neogenin, RGMa or Netrin-1 expression have previously been shown to cause neural tube closure defects (Mawdsley et al., 2004; Niederkofler et al., 2004; Kee et al., 2008; Kee et al., 2013). Interestingly, neural tube closure defects are also seen in EphA7 and ephrin-A5 null embryos (Holmberg et al., 2000). During neurulation in mice, three EphA7 splice variants and ephrin-A5 are expressed at the dorsal edges of neural folds (Holmberg et al., 2000). Two EphA7 splice isoforms are truncated and lack kinase domains. It has been proposed that the expression the expression of truncated EphA7 in the neural folds inhibits ephrin-A5/EphA7 repulsion and through an unknown mechanism, promotes adhesion (Holmberg et al., 2000). In light of the evidence provided here whereby ephrin-A5/EphA4 interactions result in Neogenin upregulation, one can imagine a scenario where ephrin-A5/EphA7 interactions in the neural folds would result in increased Neogenin levels thereby increasing Neogenin/RGMa and Neogenin/Netrin-1 interactions required for proper neural tube closure. The prevalence of Neogenin upregulation in the context of overexpressing EphA4 lacking its cytoplasmic tail suggests that the full-length and truncated isoforms of EphA7 could potentially up-regulate Neogenin when bound to ephrin-A5. It would be interesting to determine if the levels of Neogenin expression are diminished in the neural folds of EphA7 and ephrin-A5 null mice.

**Speculations on the requirement for ephrin/EphA4 dependent upregulation of Dcc/Neogenin for LTP**

Post-synaptic EphA4 and ephrin-A3 expressed by astrocytes are required for LTP in the hippocampus (Filosa et al., 2009). The requirement for EphA4 in LTP occurs independently from its cytoplasmic tail. LTP deficits seen in EphA4 null mice are absent in mice expressing EphA4 lacking its cytoplasmic tail (Grunwald et al., 2004). Loss of EphA4 increases the abundance of glial glutamate transporters and LTP deficits can be rescued by blocking glial glutamate transporters (Filosa et al., 2009). The mechanism underlining the requirement for EphA4 in LTP is unknown (Filosa et al., 2009). Interestingly, Dcc null mice also show LTP deficits in the hippocampus, proposed to originate from decreased levels of Dcc-dependent Src activation of NMDA receptors (Horn et al., 2013). Post-synaptic Src activation is necessary and sufficient for inducing LTP (Lu et al., 1998). The overlapping functions between chicken Neogenin and mouse Dcc as well as the expression of Neogenin in the hippocampus in mice raises the possibility that ephrin/EphA4 interactions may in part promote LTP by increasing the abundance of post-synaptic Dcc/Neogenin. The Ephrin-A5 induced increase in post-synaptic Dcc/Neogenin could result in higher levels of Src dependent NMDA receptor phosphorylation an thereby favor LTP (Gad et al., 1997; Phan et al., 2011; Poliak et al., 2015).

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