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NEURAL CREST IN DEVELOPMENT AND REPAIR

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In memory of my father and showing gratitude to my mother

“In summary, there are no small problems. Problems that appear small are large problems that are not understood. Nature is a harmonious mechanism where all parts, including those appearing to play a secondary role, cooperate in the functional whole. No one can predict their importance in the future. All natural arrangements, however capricious they may seem, have a function.”

-Santiago Ramón y Cajal, 1897

ABSTRACT

This thesis is focused on several aspects of neural crest biology, largely related to two main issues: 1) understanding its mechanisms of migration and 2) analyzing the potential of neural crest stem cells or their derivatives for nervous system repair. If repair is the aim, knowledge of both timing and events leading to axonal degeneration is required in order to block it and/or increase regeneration outcome. In these studies I have made use of mouse, rat and chicken *in vivo* and *in vitro* models to address specific questions regarding the scope of this thesis.

In **paper I**, changes in distribution of myelin proteins are described after ligation of the sciatic nerve, an injury model which does not allow nerve regeneration to occur. Clustering of MBPs and P_0 (the two most abundant peripheral myelin proteins) was shown to precede their degradation, involving subsequent roles of Schwann cells (SCs) and macrophages in the removal of debris distally to the injury zone. Both clustering and protein degradation steps were found to be accelerated in young rat animals when compared to adult ones.

In **paper II**, similar kind of studies were applied in other two injury models allowing regeneration to occur: a transient compression (crush) of the nerve or an intraneural injection of colchicine, which transiently blocks axonal transport. Changes in distribution of myelin (MBPs) and axonal (PGP9.5) protein immunoreactivities were monitored for up to one month and degeneration/regeneration processes followed and compared in the two models. By applying the trophic factor apotransferrin we were able to block the degeneration produced by colchicine, likely through stabilization of axonal and/or SC cytoskeleton.

In **paper III**, original culture protocols have been applied to efficiently generate highly pure cultures of mature SCs from boundary cap neural crest stem cells (bNCSCs). The *in vitro* SC differentiation from stem cells was found to resemble the *in vivo* process when looking at combinations of markers changing expression during SC lineage. Stem cell-derived SCs were able to myelinate axons both *in vitro* and *in vivo*. These results prompted further work shown in **paper V**, in which the regenerative potential of neural crest stem cell-derived SCs has been tested in the contused spinal cord injury model. Even though in most cases grafted cells died soon after transplantation, when grafted alive they were found to improve locomotor behavior and to enhance endogenous regenerative mechanisms, producing both an increase in axonal re-growth and in recruitment of endogenous glial cells. In this study, evidences are shown suggesting that after injury ependymal cells of the spinal cord central canal can generate endogenous glial cells of myelinating phenotype, likely including some of the Schwann like cells which populate the injury zone and allow axonal re-growth through the injury zone.

In **paper IV**, the scaffolding protein Nedd9 is shown to be expressed by multipotent migrating and post-migratory neural crest cells (NCCs). Nedd9 expression is induced in NCCs at the level of the dermamyotome dorsal lip. Loss-of-function and gain-of-function studies were consistent with a principal role for Nedd9 in NCC migration. Retinoic acid, known to be synthesized by the dermamyotome, was found to induce Nedd9 expression in NCCs. Nedd9 expression pattern in mouse is described in **paper VI**, including mesencephalic ventral midbrain as well as other discrete progenitor populations from diverse tissues and organs.

LIST OF PUBLICATIONS

I. **Setton-Avruj C.P., Aquino J.B., Goedelman C.J., Soto E.F., Villar M.J.** (2002) P_0 and myelin basic protein-like immunoreactivities following ligation of the sciatic nerve in the rat. *Neurochem Res.* 27(11):1293-303.

II. **Aquino J.B., Musolino P.L., Coronel M.F., Villar M.J., Setton-Avruj C.P.** (2006) Nerve degeneration is prevented by a single intraneural apotransferrin injection into colchicine-injured sciatic nerves in the rat. *Brain Res.* 30;1117(1):80-91.

III. **Aquino J.B., Hjerling-Leffler J., Koltzenburg M., Edlund T., Villar M.J., Ernfors P.** (2006) In vitro and *in vivo* differentiation of boundary cap neural crest stem cells into mature Schwann cells. *Exp. Neurol.* 198(2):438-49.

IV. **Aquino J.B., Lallemend F., Marmigère F., Adameyko I., Golemis E.A., Ernfors P.** (2007) Dynamic levels of the signaling molecule Nedd9 functions with integrins to specify cell migration in neural crest cells. *Manuscript.*

V. **Erschbamer M.*, Aquino J.B.*, Hjerling-Leffler J., Ernfors P., Olson L.** (2007) Mouse stem cell-derived Schwann cells promote recovery after spinal cord contusion injury in rats. *Manuscript*.

VI. **Aquino J.B., Marmigère F., Villar M.J., Wagner M., Ernfors P.** (2007) Differential expression and dynamic changes in murine Nedd9 identifies progenitors of diverse tissues. *Manuscript.*

* denotes equal contribution

CONTENTS

LIST OF ABBREVIATIONS

Aa	Amino acids
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
bNCSC	Boundary cap neural crest stem cell
CEE	Chick embryo extract
CNS	Central nervous system
DRG	Dorsal root ganglia
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epidermal-to-mesenchymal transformation/transition
Erk	Extracellular related kinase
FAK	Focal adhesion kinase
Fbn	Fibronectin
GDNF	Glial cell-line derived neurotrophic factor
GPCR	G-protein coupled receptor
HGF	Hepatocyte growth factor
HRG	Heregulin
ISH	In situ hybridization
JNK	Jun-N-terminal kinase
Lam	Laminin
MAPK	P38 mitogen activated protein kinase
MBP	Myelin basic protein
NC	Neural crest
NCC	Neural crest cell
Nedd9	Neural precursor cell expressed, developmentally downregulated 9
NGF	Nerve growth factor
Ngn2	Neurogenin 2
NRG	Neuregulin
NT	Neural tube
P	Postnatal day
PGP 9.5	Protein gene product 9.5
PNS	Peripheral nervous system
RA	Retinoic acid
SC	Schwann cell
SCLC	Schwann cell like cells
Trk	Tropomyosin receptor kinase

1 INTRODUCTION

The aim of this thesis has been to increase our knowledge of neural crest development while trying new strategies for repairing the nervous system. Therefore, it was built at the interface between two leading edge biomedical research fields: Developmental Biology and Regenerative Medicine. A prove for that is the establishment at the Karolinska Institute of its probably two top inter-departmental research centers of these years, the Center of Excellence in Developmental Biology (CEDB) and the subsequent Center of Excellence in Developmental Biology for Regenerative Medicine (DBRM) which I got the fortune to participate in.

Developmental Biology is devoted to study how organisms form and grow, aiming to uncover all different cellular and molecular aspects involved in the emergence of diverse biological structures from a single cell stage to the complex organization found in an adult individual. This discipline has originated from the classical Experimental Embryology and highly evolved thanks to the emergence and developments achieved in Molecular Biology. It includes all aspects of embryonic development (Embryology) but also adds new areas of interest like how organs are regenerated, post-embryonic growth (i.e. metamorphosis, aging) and evolutionary aspects of developmental mechanisms. It aims to study a broad spectrum of processes from large scale events, as morphogenetic changes, to mechanisms at the molecular level driving cell proliferation, differentiation or death, which are often involved in organogenesis. In this field, the neural crest (NC) is a good model system for studying several developmental features like patterning, directed migration, determination and differentiation (Le Douarin and Kalcheim, 1999).

Nervous system repair, within the Regenerative Medicine area, aims to promote recovery after injury. The two main strategies in use to achieve repair consist in: 1) reducing secondary damage due to tissue inflammation, and 2) increasing regeneration, by either cell grafting or enhancement of endogenously occurring mechanisms, which results in replacement of tissue in the injury zone and axonal regrowth.

1.1 THE NEURAL CREST

1.1.1 Origin and general aspects

The development of the NC is one of the most relevant events in vertebrates and it favored their evolutionary successful emergence and irradiation (Glenn Northcutt, 2005). The main property of the NC is their high motility which allows it to populate very distant tissues. The NC is of principal importance since they contribute to the formation of many organs/tissues such as peripheral nervous system (generating derivatives such as neurons and glia), heart, blood vessels, bone, ear, eyes, skin, teeth, meninges, skeletal muscle and adrenal gland. Some connective tissue components of pituitary, lachrymal, salivary, thyroid and parathyroid glands, and thymus are also NC derived (reviewed by Le Douarin and Kalcheim, 1999). After gastrulation, the NC originates from the neural folds and/or the dorsal neural tube in which cells are induced to differentially express proteins driving their epithelial-to-mesenchymal transition

(EMT) and detachment from the neural primordium (delamination). For NC induction both competence of the ectoderm to respond to a neural inducer from the mesoderm (Albers, 1987; Mancilla and Mayor, 1996) and interaction of the neural plate with the non-neural ectoderm (Rollhauser ter Horst, 1979; Moury and Jacobson, 1989, 1990) are required. Regulation of BMP4 expression by signals derived from developing somites and Wnt signaling activation are involved in cell delamination (Sela-Donenfeld and Kalcheim, 2000; Burstyn-Cohen *et al.*, 2004; Taneyhill and Bronner-Fraser, 2005; Shoval *et al.*, 2007). Immediately after delamination, NCCs start migrating either ventrally close to the neural tube or dorso-laterally in proximity to the somatic ectoderm (future skin) (Weston, 1963; Keynes and Stern, 1984; Erickson and Perris, 1993). By largely unknown mechanisms which may depend on their progenitor state, NCCs stop migrating and subsequently differentiate into a variety of cell types.
Some NCCs eventually stop migrating at prospective dorsal and ventral root entry zone levels and become clustered forming the boundary cap. These transient structures were found to be the source of late migrating NCCs shown by lineage tracing to at least produce glial cells and nociceptive sensory neurons *in vivo* (Maro *et al.*, 2004).

1.1.2 Methods for studying the neural crest

The neural crest was first described in chicken by His in 1868 (Hall, 1999). Classical embryology techniques early used to address contribution of NC to tissues/organs consisted in the removal of neural folds, before NC emigration and subsequent analyses of structures that were lacking in those organisms (reviewed by Hörstadius, 1950 and Weston, 1970). In order to establish NC fate maps, several intrinsic and extrinsic molecular markers were used. In amphibian embryos, NCs were identified by cytoplasmic inclusions (Landacre, 1921; Stone, 1932), nuclear staining properties (Milaire, 1959), and cell size differences (Raven, 1937). Le Douarin (1973) has taken advantage of quail nuclear markers to distinguish NCC from chick counterparts and was able to construct NC fate maps in xenografts: antibodies recognizing quail nuclear specific epitopes (monoclonal anti-QPNC, quail non-chick perinuclear antigen, DSHB) were developed afterwards. Other intrinsic markers used were acetylcholinesterase (for identification of presumptive NC within the neuroepithelium; Cochard and Coltey, 1983) and later on, with the emergence of monoclonal antibodies technology, the HNK-1/NC-1 epitope, which is expressed in avian NC progenitors after delamination and in some neural (Vincent and Thiery, 1984) and skin (Erickson *et al.*, 1992; Nataf *et al.*, 1993) lineages. Other antibodies used for NCC identification are: Sox10 (which strongly labels multipotent NCCs but is also expressed by peripheral glia progenitors and oligodendrocytes), Sox2 (which weakly labels NCCs at the boundary cap or at DRG borders), endothelin receptor 3 (EDN3; known to be required for skin pigmentation and gut innervation; Baynash *et al.*, 1994), platelet-derived growth factor receptor alpha (PDGFRα; in cephalic NCCs; Soriano, 1997; Hoch and Soriano, 2003), c-ret (already expressed prior to NCC emigration; Pachnis *et al.*, 1993), p75 (which labels all NCC pathways; Stemple and Anderson, 1992; Bannerman and Pleasure, 1993; Rifkin *et al.*, 2000) and TrkC (for ventrally migrating NCCs but not in melanocytes; Kahane and Kalcheim 1994; Henion *et al.*, 1995; Rifkin *et al.*, 2000).
The use of extrinsic markers opened the possibility to perform NCC potential analyses. First experiments were done by labeling entire embryos and grafting neural folds in

unlabeled embryos (Detwiler, 1937) but lack of specificity and cytotoxicity were among concerns raised against those experiments. The development of vital dyes like DiI (1-1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes), applied either by injection into the neural tube lumen (Serbedzija *et al.*, 1989; 1990) or deposited on cells (Lumsden *et al.*, 1991), permitted more accurate results.
For clonal analyses of single cells different approaches were taken which mainly consisted in application of two different methods: 1) iontophoretic injection of tracer and 2) radiotonic labeling with tritiated thymidine (Weston, 1963). As example of tracer, lysinated rhodamine dextran (LRD; Gimlich and Braun, 1985) has being frequently used in avian neural crest studies (Bronner-Fraser and Fraser, 1988; 1989; Fraser and Bronner-Fraser, 1991; Artinger *et al.*, 1995). A draw-back of these methods is that the labeling gets diluted through cell proliferation. Frank and Sannes (1991) made use of *in vivo* retroviral infection of lacZ which has the advantage of targeting cells in a random manner and of not getting diluted in progeny since the provirus is heritable; however, the probability of infecting more than one cell while using this technique is higher than in tracer injection experimental approach.
First observations of migration of neural crest from neural tube explants were reported by Dorris (1936), in chick. This allowed the performance of further *in vitro* studies on isolated NCCs. Potential of these cells was addressed through two alternative systems: 1) *in vivo* lineage tracing and 2) *in vitro* cloning. *In vivo* approach is indicative of NC potential but it does not give the answer of the entire potential of each individual cell. In vitro approach is permissive to study all potentialities of each individual cell as determined by the spectrum of different cell types it could generate. In vitro models used were: 1) single cell cultures (Baroffio *et al.*, 1988 and 1991; Dupin *et al.*, 1990; Dupin and Le Douarin, 1995; made on feeder layer), 2) low density of cell populations (Cohen and Königsberg, 1975; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992), and 3) labeling of individual cells of a population (Henion and Weston, 1997).
As for other research fields, mouse genetic approach made possible to uncovered several aspects of neural crest biology. The Ret conditional knockout made by Luo *et al.* (2007) confirmed the requirement for this receptor tyrosine kinase in the migration of prospective gut NCCs from the dorsal aorta (Pachnis *et al.* 1993) and in the formation of the enteric nervous system (Durbec *et al.*, 1996; Natarajan *et al.*, 2002). It has also shown that Wnt1 gene is activated in all neural crest derived cells of the DRG and sympathetic ganglia which normally express Ret. Both Noggin and Chordin knockouts were instrumental to address *in vivo* differential requirements for both BMP antagonists in neural crest formation and migration (Anderson *et al.*, 2006). FoxS1 knockout mice even though presenting a very mild phenotype (Heglind *et al.*, 2005) were instrumental to uncover mechanisms of early sensory neurogenesis (Montelius *et al.*, 2007). *In vivo* models for several human pathologies were made by ablating relevant genes functional in neural crest. Conditional deletion of TGFβ receptor type II upon Wnt1 gene activation was able to reproduce several features of the Di George syndrome such as cleft palate, craniofacial dysmorphism, hypoplastic or undetectable parathyroid and thymus glands, and multiple cardiovascular deficiencies due to lack of neural crest-derived smooth muscle cells, likely involving TGFβ signaling components in this human syndrome (Wurdak *et al.*, 2005). Specific requirements for Sox9 and Sox10 expression in NC development were also addressed using mouse genetics (Britsch *et al.*, 2001; Cheung *et al.*, 2005). NCCs die after delamination in Sox9 deficient mice and they were found to only lack from Snail expression while other NC

markers are normally expressed (Cheung *et al*., 2005). Slug/Snail were reported to confer resistance to cell death (Vega *et al*., 2004), and this may provide NCCs capacity for long-distance migration.
An alternative approach for addressing *in vivo* functional role of specific proteins consists in electroporating chick embryos *in ovo*. It can complement mouse genetic models and even serve to address function of certain proteins having highly homologous paralogs, given that knockout mice very often present mild or absent phenotypes due to functional redundancy. The roles of Sox9, RhoB, FoxD3 and Snail/Slug in neural crest EMT and delamination have been addressed by Cheung *et al*. (2005) using this approach. In this very same article, electroporation of FoxD3 construct was shown to: 1) induce Sox10, which in turn initiates HNK-1, and 2) down-regulate N-Cadherins and induce β1 integrin expression in a SoxE independent manner. The role played by Sox10 in the acquisition of neural crest identity and function was studied by McKeown *et al.* (2005), using chick electroporation model.
In ovo electroporation was also instrumental to visualized NCC migratory behavior in whole embryo explants (Teddy and Kulesa, 2004; Kasemeier-Kulesa *et al.*, 2005): NCCs migrate in chain-like formations and show frequent cell-cell interactions likely involving directional guidance cues.

1.1.3 Neural crest motility

NC migration through different pathways depends on the development of somites and it is especially related to the epithelial-mesenchymal conversion of this structure (Loring and Erickson, 1987; Teillet *et al.*, 1987). NCCs start entering the somites at the levels corresponding to 5^{th} to 9^{th} somites, rostrally to the more recently formed somite (Erickson and Perris, 1993). At stage HH15 approximately 65% of NCCs are transversing the rostral sclerotome in chick (Lallier and Bronner-Fraser, 1988). Interestingly, NCCs wait at the staging area (close to the dorsal border of the somite) until the dermamyotome is formed and then progress along the dermamyotome basal lamina, but when they reach its lateral regions lacking from basal lamina depart from the myotome surface and migrate down through the sclerotome (Tosney *et al.*, 1994). The subectodermal/dorsolateral pathway of NCCs is delayed one day in chicken (Serbedzija *et al.*, 1989; 1990; Erickson *et al.*, 1992), maybe due to transient extracellular matrix (ECM) inhibition likely involving ephrinB ligands (Oakley *et al.*, 1994) and/or to the requirement for previous NCC specification into a melanocytic lineage (Erickson and Goins, 1995; Santiago and Erickson, 2002). In mouse, it is known from DiI studies (done by neural tube intraluminal injections at E8.5-E10; Serbedzija *et al.*, 1990) that NCC emigration starts at E8.5. The early migration of NCCs takes place at E8.5-E9.5 and those cells give rise to dorsal aorta and sympathetic ganglia. A second wave of NCCs occurs at E9-E10 and those cells mostly reach the DRG anlage. Slit1-3 were reported to be expressed in the mesenchyme surrounding the aorta and gut, and trunk but not vagal NCCs express Robo1 and 2, suggesting that they may block ventral migration of trunk NCCs (De Bellard *et al*., 2003).
NCCs depend on the availability of adequate extracellular matrix along pathways and on locally expressed ligands and their receptors to migrate. A major role in migration of NCCs is played by short-range signals, mostly through inhibition, which differently affect them according to their cell intrinsic properties.

While injection of antibodies against fibronectin, laminin-heparan sulfate proteoglycans complex or tenascin in cranial NCCs blocks their migration, they do not seem to affect trunk NCCs (Bronner-Fraser, 1986; Bronner-Fraser and Lallier, 1988); nevertheless, it was reported that antibodies against α4 integrins significantly reduce trunk neural crest migration capacity but not delamination (Testaz and Duband, 2001). B_1-integrins, transmembrane receptors known to bind all of ECM molecules mentioned above, are found widespread in NCCs (Duband *et al.*, 1986; Le Douarin and Kalcheim, 1999). Tucker (2004) has shown that electroporation of a β_1-integrin morpholino antisense oligo causes defects in NCC delamination while migration of targeted cells seemed not to be largely affected. No major defects in NCC migration were found in knock-out mice when specific individual integrins were deleted (reviewed by Perris, 1997).

A right balance adhesion-detachment seems to be crucial for NCC delamination and migration (Gurniak *et al.*, 2005) and it is regulated by calcium at the extracellular side of the membrane (Monier-Gavelle and Duband, 1997). Integrin functions in NC development consist in: 1) providing physical anchorage for cell motion; 2) promoting actin assembly to generate tension via focal complexes (Geiger *et al*., 2001), and 3) engaging several tyrosine kinases and GTPases which induce diverse signaling pathways, largely unknown for NCCs (Giancotti and Ruoslahti, 1999; DeMali *et al.*, 2003). Integrins were shown to cooperate with growth factor receptors (ffrench-Constant and Colognato, 2004) and are thought to regulate cell-cell interactions established via cadherins (Monier-Gavelle and Duband, 1997). Intercellular junctions mediated by cadherins (Bronner-Fraser *et al.*, 1992; Monier-Gavelle and Duband, 1995) and N-CAM (Akitaya and Bronner-Fraser, 1992) are still present at neural fold fusion stage but disappear about 5 hours before onset of migration (Newgreen and Gibbins, 1982). Subsets of NCCs were shown to migrate interconnected through their processes by cadherins (Nakagawa and Takeichi, 1995). Cadherin 7 is expressed by NCCs and it does not likely mediate strong cell-cell contacts or interfere with NCC migration (Dufour *et al*., 1999). Cadherins were shown to be required for NCC migration (Xu *et al.*, 2001).

In the trunk, ventral NCC migration follows segmental patterns and it is restricted to the anterior half of the somite, in association with permissive ECM signals (Krull, 2001; Gammill *et al.*, 2006). Fibronectin was early found to be one of such signals (Thiery *et al.*, 1982), since it is expressed *in vivo* in NCC migratory pathways (Duband *et al.*, 1986), and it (or its RGDS constitutive fragment alone) promotes cell attachment, spreading and dispersion *in vitro* (Rovasio *et al.*, 1983; Dufour *et al.*, 1988; Perris *et al.*, 1989). Ephrin ligands (Krull *et al.*, 1997; Wang and Anderson, 1997) and semaphorin 3F (Gammill *et al.*, 2006) and their receptors were shown to determine segmental migration through inhibitory mechanisms.

Motility of NCC was shown to be accelerated by TGF-β1 and 2 (Delannet and Duband, 1992). TGF-β induces Slug expression (Duband *et al.*, 1995), and Slug was involved in NCC EMT since Slug antisense oligonucleotides caused its failure (Nieto *et al.*, 1994). Hepatocyte growth factor/Scatter factor (SF/HGF) and its receptor (c-met) may also be involved in NCC EMT and/or migration. There is overlapping expression of both SF/HGF and c-met in murine NCC (Andermarcher *et al.*, 1996) and HGF over-expression causes skin hyperpigmentation (Takayama *et al.*, 1996). However, its deletion has no apparent phenotype in NCCs (Schmidt *et al.*, 1995). The chemokine stromal cell-derived factor-1 (SDF-1) was shown to regulate migration and to be chemoattractant for trunk NCCs (Belmadani *et al.*, 2005).

A role for Pax3 was also described in NC emigration from the neural tube (Moase and Trasler, 1990) and NC formation was shown to be severely affected in Pax3 mouse mutants (Splotch mutants; Serbedzija and McMahon, 1997). Interestingly, the latter study indirectly showed that a successful migration of NCCs requires the interaction between those cells and the somites and that molecules involved in mechanisms mediating that interaction are lacking in the Splotch mutation. Retinoic acid, of somite origin, was found to regulate Pax3 as well as Wnt1, Wnt3a, BMP4 and Msx2 in dorsal neural tube and/or neural crest of quail embryos at relevant developmental stages, suggesting a role for retinoids in neural crest EMT, delamination and migration (Wilson *et al.*, 2004). Wnt signals are implicated in NCC migration as suggested by migratory deficiency and blocked cell-matrix adhesion when cells were treated with LiCl, an inhibitor of GSK-3, or co-cultured with Wnt1-expressing feeder cells (de Melker *et al.*, 2004).
Mechanisms involved in cessation of migration are largely unknown. In Sema-3A deficient mouse embryos, sympathetic precursors do not coalesce into ganglia; however, when those progenitors were exposed to Sema-3A *in vitro* they clustered and emitted neurites (Kawasaki *et al*., 2002). Shh was reported to inactivate integrins, blocking NCC migration (Testaz *et al*., 2001).

1.1.4 Potential and heterogeneity

As shown by cloning and individual cell labeling experiments, early migrating neural crest consists in a heterogeneous population including multipotent (generating cells of all or many NCC cell lineages), oligopotent (generating few or at least two different cell lineages) and neuronally-lineage restricted progenitors which may respond to different signals to migrate (reviewed by Le Douarin and Kalcheim, 1999).
From *in vitro* clonal studies, the NCCs were found to consist on approximately 80% of multipotent/oligopotent progenitors (Baroffio *et al.*, 1991; Dupin and Le Douarin, 1995). Henion and Weston (1997) found that 44.5% of NCCs that leave the neural tube were monopotent committed precursors just few hours after explanting. In this model, restriction in potential was found to be progressive with time of incubation: at 13-16 hours, 52.2% of NCCs were lineage restricted and this population increased to 77% at 30-36 hours. It still remains unclear which model reflect better the potential of individual NCCs.
In vivo lineage tracing experiments in avian and amphibian embryos resulted in that the majority of NCCs gives rise to cells of multiple lineages in different organs and within each organ (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). Unfortunately, in those studies the identity of cells in each organ was not determined by use of specific cell markers.
Although a major fraction of NCCs *in vivo* are multipotent and are likely dispersed within pathways (Le Douarin and Kalcheim, 1999), over-expression of c-myc in NCCs is followed by preferential migration through the ventral pathway and further neuronal differentiation (Wakamatsu *et al.*, 1997) suggesting a directional migration for sub-populations of NCCs being defined by fate determination. There is still lack of sufficient markers to establish the potential capacity of individual migrating NCCs but much have been improved.

Several factors expressed in the dorsal neural tube may already specify some NCCs before delamination (reviewed by Dorsky *et al.*, 2000). Wnt1 and Wnt3a have being implicated in melanocyte specification and/or expansion (Ikeya *et al.*, 1997; Dorsky *et al.*, 1998; Jin *et al.* 2001; Dunn *et al.*, 2005) as well as sensory neurogenesis (Hari *et al.*, 2002; Lee *et al.*, 2004). BMPs and other TGFβ signaling pathways may also be involved in neural crest specification before emigration (Dorsky *et al.*, 2000).
In addition, NCCs have been shown to present a high degree of plasticity and once differentiated they were found to reverse their phenotype to a multipotent stage or to transdifferentiate (Le Douarin and Dupin, 2003; Real *et al*., 2006). Adult peripheral glial cells have been reported to generate pigment cells after severe peripheral nerve injury in mouse (Rizvi *et al*., 2002).

1.1.5 Neural crest stem cells

A commonly used definition of a stem cell require the fulfillments of the two following conditions: 1) capacity to generate an identical daughter cell (cell renewal) and 2) to give rise to cells more restricted in potential (differentiation). According to this criteria, it has been suggested that a small proportion of NCCs are pluripotent (giving rise to all neural crest derivatives) and self-renew (1-3%) and the great majority of them likely consist in progenitors of different potential capacity (reviewed by Crane and Trainor, 2006). In mammals, neural crest stem cells were first isolated from trunk neural tube explants by flow-cytometry (Stemple and Anderson, 1992): these cells were shown to self renew and to be able to differentiate and therefore deserve the name of NCSCs. Afterwards, they were obtained from embryonic sciatic nerve (Morrison *et al.*, 1999) and mandibular processes (Zhang *et al*., 2006), postnatal gut (Kruger *et al.*, 2002), and adult skin (Sieber-Blum *et al.*, 2004), cardiac tissue (Tomita *et al.*, 2005) and cornea (Yoshida *et al.*, 2006). In avian, two different bipotent precursor cells were shown to self renew in vitro and to produce glia and melanocytes or glia and myofibroblasts (Trentin *et al*., 2004). The boundary cap has been reported to contain stem cells able to self-renew and to generate at least three different lineages in the trunk neural crest progeny (Hjerling-Leffler *et al.*, 2005). NC stem cells are promising tools for cell based therapy.

1.2 NERVOUS SYSTEM REPAIR

1.2.1 Wallerian degeneration and nervous system regeneration.

Peripheral sensory and autonomic neurons reside in metameric structures called ganglia and send projections (axons) to innervate their target tissues. Those neuronal projections are included in structures called nerves in association with glial cells (named as SCs) which are important in diverse neuronal physiological and systemic processes, including isolation of axons and enhancement of transmission speed of electric impulses through neuronal projections. SCs produce myelin, a structure derived from deposition of several layers of cell membrane produced by their turning around axons, consisting therefore mainly in lipids and proteins. Thickness of myelin sheath is

regulated by neuregulin (NRG) levels in the axons to which SCs interact with, it is directly proportional to axonal caliber and the larger the axons are the faster the conduction of nervous impulses is (Michailov *et al*., 2004; Willem *et al*., 2006).

In the Peripheral Nervous System (PNS), myelin protein content consists in 20-30% of Myelin Basic Proteins (MBPs) (Garbay *et al.,* 2000). It was suggested that fragments related to MBPs may triger SC proliferation after injury (Baichwal and DeVries, 1989; Tzeng *et al.,* 1995, 1999). The MBP/P1 family of proteins is composed by 21.5, 18.5, 17 and 14 KDa polypeptides produced by alternative splicing from the same evolutionary highly conserved gene sequence. MBP proteins are hydrophilic, localize to the cytoplasmic side of myelin membranes and are believed to contribute to myelin stabilization through formation of complex structures with negatively charged lipids (Martini *et al.*, 1995). Other MBP, the P2, is present in minor amount than P1 isoforms 18.5 and 21.5 KDa and is complementary to MBP/P1 in the interaction with lipids during compaction of myelin dense lines, but it is only expressed in a selected population of myelin fibers (Garbay *et al.,* 2000).

The P_0, only found in peripheral myelin, represents 50-70% of its total protein content. It is an integral membrane 28KDa glycoprotein also involved in compaction and maintenance of the myelin sheath (Garbay *et al.,* 2000).

When a nerve or the spinal cord of an individual is injured, axons degenerate and myelin brakes-down distally to the lesion site in a process called Wallerian degeneration (Ramón y Cajal, 1928; Stoll *et al.,* 2002). If a fast and efficient removal of debris takes place and signals and physical support promoting axonal re-growth are present, regeneration is allowed (Vargas and Barres, 2007). These characteristics are fulfilled in the PNS, due to the cooperative action of both SCs, which start degrading myelin (Goodrum *et al.,* 1994), and monocytes/macrophages, which rapidly invade, proliferate, produce growth factors and mitogens (inducing SC proliferation) and efficiently clean debris (Lindohlm *et al.*, 1987; Baichwal *et al.,* 1988; Fu and Gordon, 1997; Matsuoka *et al.,* 1997; Stoll and Muller, 1999). It was recently described that after injury SCs (identified by their S100 immunoreactivity) segregate their myelin content by forming ovoid structures within their cytoplasm (Hirata *et al.,* 2000).

In absence of axons, SCs establish an autocrine loop by production of insulin growth factor-1 (IGF-1), neurotrophins-3 (NT3) and platelet-derived growth factor-BB (PDGF-BB), which makes them able to survive (Meier *et al.,* 1999). SCs trigger axonal elongation through: 1) release of neurotrophins (i.e. nerve growth factor, NGF; brain-derived growth factor, BDNF; glial-derived growth factor, GDNF), cytokines and leukemia inhibitory factor (LIF) (Matsuoka *et al.*, 1997; Marcinkiewicz *et al.,* 1999; Zhang *et al.,* 2000; Hoke *et al.*, 2002; Chen *et al.*, 2007); 2) secretion of cell adhesion molecules (N-cadherins and ninjurin) promoting axonal growth (Ide C, 1996; Fu and Gordon, 1997; Luckenbill-Edds, 1997); 3) deposition of basal lamina while proliferating which results in formation of endoneural tubes (also known as SC tubes or "Bungner bands"), physically supporting axonal re-growth (Venstrom and Reichardt, 1993; Ide C, 1996; Fu and Gordon, 1997). Finally, SCs may eventually contribute to redistribution and accumulation of Na^+ and K^+ channels in re-myelinated axons optimizing neuronal conduction properties (Joe and Angelides, 1992; Rasband *et al.,* 1998).

On the contrary, regeneration is very poor after central nervous system injury due to formation of glial scar, proliferation of cells non-permissive to axonal re-growth (which produce and release or expose inhibitory signals) and inefficient mechanisms of debris

removal frequently leading to exacerbated inflammatory reactions causing secondary damage to the tissue.

1.2.2 Trophic factors in maintenance of nervous system mature properties and regeneration.

Trophic factors can be defined as substances stimulating growth, maintenance and survival of cells. It was shown that insults exclusively affecting SCs indirectly cause axonal degeneration (Angevine, 1957; Jacobs *et al.*, 1972; Singer and Steinberg, 1972) and that axonal disintegration produces alterations in SC biology (Meier *et al.,* 1999), arguing for the existence of a broad communication in between both cell types to keep mature nervous system tissue properties and function. Dependence on target-derived signals for peripheral neuron survival was concluded from limb ablation studies (Caldero *et al.*, 1998). The first identified of these signals was Nerve Growth Factor (NGF; reviewed by Levi-Montalcini, 1987) and this finding opened the era of the developmental neurobiology. Other members of the "neurotrophin family" important for survival of specific neuronal populations were subsequently identified, including BDNF, NT-3 and NT-4/5 (Ernfors *et al.*, 1994a; 1994b; Liu *et al.*, 1995b). The first trk receptor was described by Martin-Zanca *et al.* (1986) but it was identified as receptor for NGF in 1991 (Kaplan *et al.*, 1991; Klein *et al.*, 1991). All neurotrophins share low affinity to the p75 NGF-receptor (Barker and Murphy, 1992). In last decades, neurotrophins physiological roles and signaling pathways have being extensively analyzed in development, maintenance and regeneration of the nervous system.

A rapid and transient activation of ErbB2 (receptor for NRGs) has been reported to occur as early as 10 minutes after axotomy, distally to the injury zone, and this was sufficient to cause SC demyelination (Guertin *et al*., 2005). During development and after nerve injury or disease, axons respond to neurotrophins produced by SCs by releasing NRGs (Esper and Loeb, 2004), and reciprocally NRGs also influence glial neurotrophic factor expression (Verdi *et al*., 1996; Hansen *et al*., 2001). NRGs are required for Schwann cell lineage survival, proliferation, differentiation and maturation (reviewed by Jessen and Mirsky, 2005), even though NRG signaling mechanisms after injury may differ from those functional during development (Atanasoski *et al*., 2006).

When axonal transport is blocked, the cell soma of peripheral neurons is deprived from neurotrophins and reacts to increase protein synthesis machinery and promote axonal re-growth. Several nerve injury models were developed to dismantle axonal cytoskeleton based on mechanical or chemical insults. A variety of chemical components are known to either block polymerization (as colchicine; Margolis and Wilson, 1977) or promote de-polymerization (Jordan *et al.*, 1992) of microtubules which are the scaffold required for molecules (including growth factors) to move through axons. Several therapeutic interventions to either promote neuronal regeneration or block cell death induced by injury consist in the application of trophic factors (Muller and Stoll, 1998; Thoenen and Sendtner, 2002), however they have often rendered contradictory results (Kanje *et al.*, 1989; Gold, 1997; Munson and McMahon, 1997; Mohiuddin *et al.*, 1999; Ramer *et al.*, 2000; Boyd and Gordon, 2001; Markus *et al*., 2002).

There is scientific evidence that the trophic factor transferrin (Mescher and Munaim, 1988), a carrier of iron present in blood plasma, may promote neuronal regeneration.

An increased in the endoneural uptake of iron has been described between 2 and 9 days after nerve injury together with up-regulation of transferrin receptor expression, which mediates the transport of saturated transferrin in macrophages and Schwann cells (Raivich *et al.*, 1991; Hirata *et al.,* 2000). Transferrin, as well as other proteins regulating iron levels, has also protective effects against excess of extracellular iron as well as against free radicals of nitric oxide (Aisen *et al.*, 1999; Kaur and Ling, 1999). In the CNS, it is known that a single intracranial injection of apotransferrin (iron-free transferrin) into 3-day-old rats produces an increase in the myelination process (Escobar Cabrera *et al.*, 1994, 1997) and that it stabilizes the microtubular network by increasing the mRNA levels of tubulin, actin and STOP (stable tubule only peptide) proteins (Cabrera *et al.*, 2000). A holotransferrin transgenic mouse was made and its phenotypes in CNS are consistent with a role of this protein in inducing increased myelin protein synthesis and Sox10 and Olig1 expressions, overall suggesting a role for transferrin in oligodendrocyte maturation (Sow *et al.*, 2006). Interestingly, during amphibian limb regeneration presence of axons in blastema is necessary for this process to occur (Geraudie and Singer, 1978; Brockes, 1987) and transferrin has been shown to be required for cell cycling as co-factor of the limiting enzyme for DNA synthesis (Seligman, 1983). In axolotls, by mean of classical experiments of double ligation of the sciatic nerve and prevention of axonal transport through application of colchicine in an *in vitro* multi-compartment system, transferrin was shown to undergo anterograde fast axonal transport (Kiffmeyer *et al.,* 1991). Transferrin was reported to be released distally at growth cones (Meschner and Kiffmeyer, 1992) and its highest levels in sciatic nerves were found when axonal elongation was nearly complete and limb re-differentiation underway (Kiffmeyer *et al.,* 1991).
Knowledge on molecules mediating neuron-glia interaction would likely allow the development of new strategies to increase regeneration outcome, decrease neuropathic pain and protect affected neurons from cell death.

1.2.3 Schwann cells in nervous system repair

Cells presenting SC properties were reported to populate the spinal cord after injury and axons were shown to grow in close apposition with these cells throughout the injury zone (Gilmore and Duncan, 1968; Harrison *et al*., 1975; Blakemore *et al*., 1977). Enhancement of these endogenous mechanisms was mediated by delivery of substances (Kaneko *et al*., 2006; Blesch and Tuszynski, 2007) and likely by cell grafting (Takami *et al*., 2002; Lu *et al*., 2006; Cao *et al*., 2007), with subsequent improvement of locomotor behavioral outcome. Given that SCs are a natural cell type in CNS after injury and that they promote axonal re-growth they are suitable candidates for cell replacement strategies after spinal cord injury. In fact, beneficial effects were abundantly reported after SCs grafting (Honmou *et al*., 1996; Oudega and Xu, 2006; Zujovic *et al.*, 2007). Nevertheless, access to an adequate number of SCs is a limitation for their use in cell based therapy (Mimura *et al*., 2004) which may be eventually overcome by expanding a progenitor pool or stem cell population as a source of SCs.

1.3 NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED 9 (NEDD9)

1.3.1 General description and discovery

Nedd9, also known as HEF1 and Cas-L, is a member of the Cas family of scaffolding proteins described to be associated with cancer metastasis (Minn *et al.*, 2005; Kim *et al.*, 2006), immune system maturation (Seo *et al.*, 2005), leukemia (de Jong *et al.*, 1997), arthritis (Iwata *et al.*, 2005; Miyake-Nishijima *et al.*, 2003) and stroke (Sasaki *et al.*, 2005). It was first reported by Kumar *et al.* (1992) after performing subtraction cloning experiments with the aim of identifying cDNAs for genes highly expressed in the brain during development. In their publication, Kumar and collaborators have described the 3' untranslated region of Nedd9 protein. First complete descriptions of Nedd9 appeared in 1996: published by two independent research groups. One of them named it as HEF1 (Human Enhancer of Filamentation 1), because of the described capacity of its C-terminal domain to induce filamentous yeast budding in *S. cerevisiae*, a property likely related to cell polarization and cell cycle (Law *et al.*, 1996). HEF1 was described to localize in focal adhesions and to associate with FAK and the Abl kinase (Law *et al.*, 1996). Minegishi *et al.* (1996), while trying to clone the gene encoding a protein known to be phosphorylated on tyrosines upon ligation of β1 integrins in T cells, described the same molecule with the name Cas-L (for Crk-associated substrate-related protein, lymphocyte type). The Cas-L protein was also shown to bind FAK and to additionally interact with other integrin effectors like Crk, Nck and SHPTP2 upon adhesion through integrins (Minegishi *et al.*, 1996).

1.3.2 Protein structure, localization and function

Nedd9 has several interaction domains, similar to its two mammalian paralog family members: p130Cas/BCAR1 (Sakai *et al.*, 1994) and Efs/Sin (Ishino *et al.*, 1995; Alexandropoulos and Baltimore, 1996; Alexandropoulos *et al.*, 2003). Only one member of the Cas family of proteins was found in Drosophila (Huang *et al.*, 2007). P130Cas was the first Cas protein described in this family, found to be abundantly expressed in almost all cell lines and tissues and functionally related to cell adhesion and migration (reviewed by Defilippi *et al.*, 2006). Expression of both Nedd9 and Efs/Sin are widespread during embryogenesis but gets restricted to few tissues in adult organisms, and their function is less well known than for p130Cas (Singh *et al.*, 2007).
Nedd9 contains an N-terminal SH3 domain (aa 10-65 in humans; binding to proteins containing poly-proline motif), a domain with large number of SH2 binding sites (aa 90-350 in humans; with several tyrosines; named as "substrate domain"), a domain with large number of serine residues (aa 350-650 in humans) and the C-terminal domain, highly conserved among Cas proteins (Singh *et al.*, 2007). This C-terminal domain may interact with other helix-loop-helix (HLH) proteins and allow homo-/hetero-dimerization of Cas proteins (Law *et al.*, 1999). Interestingly, p130Cas and Efs/Sin but not Nedd9 contain poly-proline residues which make them able to interact with SH3 domains. Nedd9 and p130Cas present DLDV and DDYD caspase cleavage sites (Law *et al.*, 2000; Kook *et al.*, 2000); however, induction of apoptosis mediated by p130Cas was not reported. Both Nedd9 terminal domains were found to interact

with Smad3 (the effector of TGFβ signaling) for protein degradation which suggest a rapid turn-over of this protein (Liu *et al.*, 2000; Nourry *et al.*, 2004).
Nedd9 was described as cytoplasmic but it concentrates in focal adhesions (upon cell adhesiveness to substrate; Law *et al.*, 1996) or in the centrosome or mitotic spindle (during mitosis; Law *et al.*, 1998). Nedd9 structure and sub-cellular localization suggest its involvement in cell adhesion and migration, apoptosis and cell cycle.
Nedd9 full-length over-expression increases cell spreading (Fashena *et al.*, 2002) while forced expression of the Nedd9 C-terminal region results in cell detachment and rounding (O'Neill and Golemis, 2001). Requirement of Nedd9 for migration was shown in glioblastoma (Natarajan *et al.*, 2006) and lymphocytes (Ohashi *et al.*, 1999) and consistently while Nedd9 over-expression promotes cell motility (Fashena *et al.*, 2002) its down-regulation impairs chemotactic responses (Seo *et al.*, 2005), the latter likely through Nedd9 function as component of GPCR signaling pathways (Zhang *et al.* 1999). Signaling through Nedd9 in promotion of cell migration seems to involve phosphorylation mediated by FAK/Src and subsequent interaction with Crk (Minegishi *et al.*, 1996; van Seventer *et al.*, 2001; Fashena *et al.*, 2002; Natarajan *et al.*, 2006) which may lead to GTPase Rac and/or Rap1 activation (Cai *et al.*, 2003b; Tamada *et al.*, 2004). Alternative signaling pathways may involve interaction with BCAR3/AND-34/SHEP2/Nsp2 and Chat and regulation of Cdc42 activation (Sakakibara and Hattori, 2000; Cai *et al.*, 2003a; Regelmann *et al.*, 2006). Rho kinase inhibition (Bargon *et al.*, 2005) and FAK knock-down (van Seventer *et al.*, 2001; Kim *et al.*, 2006) have been reported to block Nedd9-induced increase migration and/or neurite-like extensions. Further downstream, MAPK and Erk1/2 are activated when Nedd9 is over-expressed, but the increase in migration does not involve those pathways (Fashena *et al.*, 2002), and might require JNK activation instead (Dolfi *et al.*, 1998). Nedd9 induced expression results in transcriptional induction of MLCK, p160 ROCK, NIK Ser/Thr kinase, PAKα and several Rho which are functionally related to cell motility (Fashena *et al.*, 2002). In the same study, Nedd9 was reported to cause upregulation of matrix metalloproteinases, ephrins ligands and receptors, and the NRG receptor ErbB2. However, Nedd9 pathway-target specificity for activation of those genes remains to be validated. Cell adhesion regulates conversion of Nedd9 isoforms from p105 into p115; a mobility shift which predominantly reflects serine/threonine phosphorylation state (Zheng and McKeown-Longo, 2002), and it is linked to integrin receptor activation and cytoskeletal organization (O'Neill and Golemis, 2001; Zheng and McKeown-Longo, 2006), and favored Nedd9 proteasomal degradation (Zheng and McKeown-Longo, 2006).
Evidences supporting Nedd9 function in anoikis and apoptosis derive from *in vitro* over-expression experiments. Overexpression of Nedd9 28 KDa C-terminal peptide, similar to the naturally occurring after caspase cleavage, triggers apoptosis (Law *et al.*, 2000). Similar features were observed in weak tumorigenic cell lines after long-term Nedd9 full-length induced expression (Law *et al.*, 2000). Nevertheless, apoptotic mechanisms were not induced when Nedd9 gain-of-function experiments were performed on highly metastatic and invasive cell lines (Kim *et al.*, 2006; Natarajan *et al.*, 2006).
First conclusive experiments showing involvement of Nedd9 and some of its signaling partners in cell cycle progression were conducted by Pugacheva and Golemis (2005). Cells over-expressing Nedd9 present multipolar spindles and supernumerary centrosomes, which overall cause defective cytokinesis (Pugacheva and Golemis, 2005)

through mechanisms involving disruption of RhoA activation cycle (Dadke *et al*., 2006). Consistently, when Nedd9 was knocked-down the centrosome separated prematurely and there was an increase in monopolar and asymmetric spindles (Pugacheva and Golemis, 2005), which resulted in abnormal high numbers of binucleate cells (Dadke *et al*., 2006). Other functions of Nedd9 related to cell cytoskeleton modifications in the formation of cilia were recently reported (Pugacheva *et al.*, 2007).

1.3.3 Control of Nedd9 expression levels.

Several factors have being described that induce Nedd9 expression levels. TGF-β treatment was shown to promote Nedd9 transcriptional activity (Zheng and McKeown-Longo, 2002). Nedd9 expression was induced in neuroblastoma cell lines by retinoic acid [Merrill *et al.*, 2004a; b] and the 5' region of the Nedd9 promoter has been reported to specifically bind a RAR-RXR heterodimer (Merrill *et al.*, 2004a). While over-expression of progesterone receptor-A was found to increase Nedd9 levels (Richer *et al.*, 2002), estrogen receptor-α over-expression and estrogen treatment downregulated Nedd9 (Monroe *et al.*, 2003; Buterin *et al.*, 2006). Nedd9 mRNA was induced after ischemia in rat cortex and hippocampus (Sasaki *et al.*, 2005). Co-occupancy of Nedd9 promoter by Sox2 and Nanog was recently reported (Boyer *et al.*, 2005), hypothetically suggesting its involvement in stem cells. Since Nedd9 expression is known to be induced in cells while cycling, with an expression peak at late G2/M, the increase in levels reported during cancer metastasis (Donninger *et al.*, 2004; Kim *et al.*, 2006) may be associated with high proportion of actively cycling cells. Nedd9 levels are very low in quiescent cells (Law *et al.*, 1998), and in cycling cells after mitosis due to proteasomal degradation (Law *et al.*, 1998; Pugacheva and Golemis, 2005).
Since Nedd9 can be cleaved by caspases, its shorter fragments may eventually function as dominant negatives for the full length active molecule (Law *et al.*, 2000; O'Neill and Golemis, 2001). TGF-β through Smad3 can promote Nedd9 proteolysis and reciprocally Nedd9 can negatively regulate TGF-β signaling (Liu *et al.*, 2000; Nourry *et al.*, 2004; Inamoto *et al.*, 2006).

1.3.4 Nedd9 in development.

A Nedd9 knock-out was made by Seo *et al.* (2005). In contrast to p130Cas knockout, which dies at E11 (Honda *et al.*, 1998), this mouse is viable and fertile as homozygote and does not present gross abnormalities in any tissue. This suggests functional redundancy with other Cas proteins.
Nedd9 involvement in neural processes is suggested by high levels of Nedd9 present in early mouse brains (Kumar *et al.*, 1992) and in the human neuroblastoma SH-SY5Y cells [Merrill *et al.*, 2004a; b]. Nedd9 expression was found to be regulated by retinoic acid in neuroblastoma cells and hindbrain rhombomeres (Merrill *et al.*, 2004a). By performing whole-mount embryo in situ screening in rats, Merrill and colleagues have also reported Nedd9 expression in the neural tube epithelium, DRG, the boundary cap as well as other non-neural tissues (Merrill *et al.*, 2004a).
After global ischemia, Nedd9 is induced in cortical and hippocampal neurons and Nedd9 overexpression was shown to promote neurite elongation in PC12, suggesting

Nedd9 involvement in axonal/dendrite outgrowth (Sasaki *et al.*, 2005). It was recently shown in *Drosophila* that Cas proteins control axonal guidance during development, in an integrin dependent manner (Huang *et al.*, 2007).
In between E10.5 and E13.5 Nedd9 is expressed at higher levels in male gonads in mouse when compared to female ones (Nef *et al.*, 2005), suggesting a role for Nedd9 in sexual differentiation.
The C-terminal domain of Nedd9 was shown to interact with members of the Id family of transcription factors (Law *et al.*, 1999), known to be expressed in the neural tube epithelium (Jen *et al.*, 1997) and in the neural crest (Kee and Bronner-Fraser, 2001), and may contribute to maintaining a progenitor state (Garcia-Campmany and Marti, 2007).

2 AIMS OF STUDY

- Characterize changes in myelin proteins during Wallerian degeneration and establish hallmarks in order to monitor nerve degeneration process.
- Analyze the effect of a single apotransferrin injection on nerve degeneration after mechanical or chemotoxic injuries.
- Generate phenotypically and functionally mature Schwann cells from bNCSCs
- Explore the potential of stem cell-derived Schwann cells in cell replacement strategies for nervous system repair.
- Analyze Nedd9 expression pattern and function, with special focus on neural crest.

3 RESULTS AND DISCUSSION

3.1 PAPER I: CHANGES IN MYELIN AFTER NERVE INJURY

The degenerative processes triggered by nerve injury were first described by Augustus Waller in 1850 (reprinted in Stoll *et al.*, 2002) and exhaustively analyzed by Santiago Ramón y Cajal (1928) in his main research field after gaining the Nobel Prize. In his paper, Ramón y Cajal described the roles of the different cells involved in Wallerian degeneration of transected fibers. As he mentioned, the initial events are accomplished by SCs alone and processes include: 1) increased in SC cytoplasmic volume; 2) interruption of the myelin sheath in the perinuclear region and fragmentation of the sheath into ovoids and ellipsoids; 3) the proliferation of SCs; and 4) the progressive separation of lipoidal ovoids within SC tubes. Then, hematogenous phagocytic cells invade the whole nerve, cluster around the lipoidal masses and become intratubal, accelerating the removal of debris.

Although studies nowadays are seldom done as detailed and meticulously as Ramón y Cajal's, we have now much powerful tools to further uncover such mechanisms. It was the aim of this work to better understand how myelin degradation is achieved after peripheral nerve injury in newborn and adult rats. The main objectives of this work were: 1) to establish myelin protein immunohistochemistry as a procedure to determine hallmarks for monitoring nerve degeneration process, and 2) to temporally relate state of myelin clusters with myelin protein degradation. Since we were interested in analyzing *in vivo* myelin degenerative processes and to avoid confusing effects from newly synthesized myelin (known to occur after axonal re-growth through the distal stump), the ligation of the sciatic nerve was chosen as injury model. Animals were sacrificed at different time intervals in order to cover main steps in myelin processing and degradation. Myelin re-distribution within the nerve was monitored by use of specific myelin protein polyclonal antibodies on tissue sections. In parallel, analysis of myelin proteins integrity within the injured nerve was conducted by mean of SDS page electrophoresis. Protein loss was determined by measuring dry weight of isolated myelin fraction.

In all cases, we have first observed increase accumulation of myelin proteins in clusters (the "coagulated particles" described by Waller) within SC tubes distally to the injury zone, together with increase in P_0 and MBPs immunoreactivities within degenerating nerves mainly due to protein re-distribution. A higher exposure of myelin protein epitopes after partial degradation mediated by SCs may also partially explain our observations.

In adult animals, maximum values of myelin protein immunostained areas were obtained at 7 days after injury, coinciding with peak of abundance of macrophages/monocytes within the nerve. This feature was found to precede the efficient degradation of brokedown myelin proteins. Overall, our results support the use of myelin protein immunohistochemistry as a tool for monitoring changes during nerve degeneration process.

Proximally to the injury zone, alterations in P_0 and MBPs staining intensities were observed at longer periods analyzed without resulting in appearance of myelin clusters within first 2 weeks after injury. These results are consistent with a cellular re-

distribution and/or modification of the myelin protein epitopes allowing higher binding of antibodies within cells morphologically resembling SCs after injury. A decrease in dry weight values of isolated purified myelin fractions normalized to total nerve humid weight was observed in areas proximally to the injury zone, likely due to partial loss of myelin proteins influenced by exacerbated macrophage activity. Alternatively, an increase in humid weight of nerves at regions proximal to the ligature due to macrophage exudation may explain observed changes.

Our results suggest that myelin protein antibodies seem to have very different affinities to their specific epitopes since much higher immunoreactive area values were obtained for MBPs in nerve sections when compared to P_0. Alternatively, epitope modifications produced within SCs may differ in between proteins analyzed. Finally, protein degradation rates mediated by macrophages may differ from cytosolic proteins like MBPs to transmembrane proteins like P_0 (Proost *et al.*, 1993), and the latter may be partially recycled as suggested by results from both electrophoresis and image analyses on nerves distally to the injury zone.

In this study, we also report an increase in the 14 KDa MBP protein isoform. Our preliminary studies of amino acid sequences from peptides at this band suggest presence of fragments with high homology to alpha and beta-globins. Further work conducted in my previous lab showed that these proteins are in fact synthesized in injured nerves by macrophages and hematopoietic progenitors (Setton-Avruj *et al.*, 2007), in opposition to previous reports suggesting repression of alpha-globins at 3 days after sciatic nerve crush (De Leon *et al.*, 1991).

Nerve degeneration was found to be accelerated in newborn rats when compared to adult animals, likely due to higher metabolic rates in the former group, but remained essentially similar in the occurrence of myelin proteins accumulation before their final degradation.

3.2 PAPER II: TRANSFERRIN PREVENTS NERVE DEGENERATION IN A CYTOTOXIC INJURY MODEL

In paper I, we have characterized nerve degeneration process by monitoring changes in distribution of myelin protein immunoreactivities. We were then interested in analyzing the effect of transferrin on nerve degeneration/regeneration and for that purpose we chose two alternative injury models allowing further regeneration: 1) the nerve crush, a traumatic model which involves necrosis, ischemia and blockage of axonal transport, and 2) intraneural colchicine injection, transiently affecting axonal transport.

The development of this project was in fact one of the main purposes for a large collaboration launched (in my previous lab in Austral University, under the supervision of Marcelo J. Villar) with a lab partner, led by Prof. Juana M. Pasquini and Dr. Eduardo F. Soto, at the University of Buenos Aires/IQUIFIB-CONICET (Argentina). Our collaborators have a long history of research focused on the effects mediated by transferrin in oligodendrocyte maturation and CNS myelination (Cabrera *et al.*, 2000; Marta *et al.*, 2002; 2003; Garcia *et al.*, 2004; Paez *et al.*, 2005; 2006; Adamo *et al.*, 2006) and have reported that this protein prevents Schwann cells de-differentiation *in vitro* (Salis *et al.*, 2002).

We have first characterized nerve degeneration process by analyzing myelin and axonal protein immunoreactivities at different time periods in between 1 and 60 days. For

myelin protein characterization we have chosen MBPs as epitope after having seen its great variations over degenerative processes in paper I. In case of axonal markers, PGP9.5 and beta-III-tubulin (also known as Tuj1) were used since both label virtually all neurons in the sciatic nerve. Application of the two injury models used in this paper resulted in a Wallerian degeneration process likely resembling descriptions of paper I with a delayed in myelin protein clustering onset in colchicine model (Singer and Steinberg, 1972). The degree and intensity of the degenerative processes triggered by colchicine were dose-dependent but even at highest colchicine concentrations used, scores of MBP-immunostained area were 50% lower than in crushed or ligation models. First signs of neural alterations after colchicine injection were observed on axonal proteins consistent with that SC de-differentiation distally to the injection site follows loss of healthy axonal contact (Liu *et al.* 1995a). For 10nM colchicine condition, the peak of MBP clustering appearance was observed at 14 days after injury, 7 days later than in traumatic injury models. By 1 month after injury, in both injury models, nerve is already normalized when looking at markers analyzed: axons have regenerated and myelin was re-synthesized.

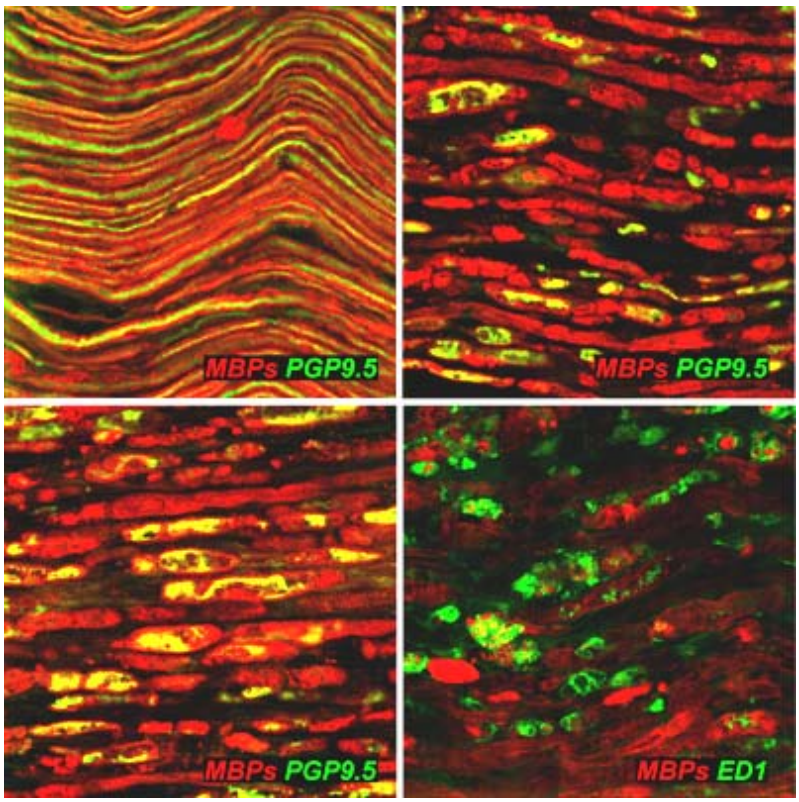

Fig.1: Changes in myelin and axonal proteins during Wallerian degeneration. Upper left: Confocal images showing normal distribution of $MBPs^{+}$ myelin ensheathing $PGP9.5^{+}$ axons. Upper right and lower left: at 7 days after injury, MBP^{+} ovoids included in Schwann cell tubes contains fragments of remaining axonal debris. Lower right: at 14 days, myelin degradation products are very often found included in macrophages which efficiently remove and digest them.

Immunofluorescence studies on transected fibers have revealed presence of both PGP9.5 and MBPs stainings within same ovoid myelin clusters inside SC tubes at 3 and 7 days after injury (unpublished data; Fig.1). At 14 days after injury, these clusters acquire a rounded morphology and are mostly included in $ED1^{+}$ macrophages (Fig.1).

These features suggest that SCs play a role in isolating myelin and axon-derived debris in same lipoidal structures, known to be covered by vimentin layers synthesized by same SCs (Liu *et al.*, 1995a).

Intraneural injection of apotransferrin (0.5 μg/ml to 0.5 mg/ml) simultaneously to colchicine (5mM) prevented Wallerian degeneration, since no features of axonal disruption or clustering of myelin protein signals were observed at 7 days after application. Given that transferrin was unable to prevent crush-induce nerve degeneration we speculate that transferrin works in colchicine injured nerves by stabilizing axonal cytoskeleton but is not able to counteract events triggered by hypoxia/necrosis caused by mechanical injuries likely mediated by mechanisms involving early calcium entry into axons and subsequent proteolytic enzyme activation (Banik, 1992; Martinez and Canavarro, 2000; Wang *et al.*, 2000). Molecular mechanisms mediated by transferrin in prevention of nerve degeneration were not addressed experimentally in our work. Available literature at present mostly highlights effects of transferrin on myelinating glia which are known to express the transferrin receptor (Cho *et al.*, 1997). This would suggest a role of Schwann cell cytoskeleton stability in prevention of axonal degeneration likely involving transcriptional events (Marta *et al.*, 2002; Paez *et al.*, 2006). Alternatively, transferrin may exert direct effects on axonal cytoskeleton (Kiffmeier *et al.*, 1991). Increase in transferrin levels have been reported within motoneurons in models of progressive motor neuronopathy (Moos, 1995). However, transferrin receptors are known to be absent from axons proximally to the injury zone (Raivich *et al.*, 1991), and are only found in the neuronal soma while motoneurons regenerate (Graeber *et al.*, 1989).

It was recently demonstrated that Schwann cells synthesize transferrin during their maturation and after nerve injury (Salis *et al.*, 2007). In the latter condition, it is interesting that SCs express transferrin mRNA in between 3 and 7 days post-injury, too late for being involved in prevention of nerve degeneration but eventually required for myelin protein clustering and partial phagocytosis.

3.3 PAPER III: EFFICIENT GENERATION OF SCHWANN CELLS FROM NEURAL CREST STEM CELLS

Jens Hjerling-Leffler training and expertise in isolating and obtaining highly pure cultures of boundary cap stem cells from E11.5 mouse, under the supervision of Patrik Ernfors, led our lab into the field of neural crest stem cells. In their work, they have characterized bNCSCs and differentiated them into peripheral sensory neurons which were shown to present similar cytological and physiological properties than those of adult DRG (Hjerling-Leffler *et al.*, 2005).

Schwann cell differentiation from NCSCs was first published by David J. Anderson's group (Stemple and Anderson, 1992; Shah *et al.*, 1994). However, they have not expanded these NCCs into formation of neurospheres. Afterwards, Amoh *et al.* (2005) have placed clones of skin-derived NCSCs into the injured area of the rat sciatic nerve and claimed to have obtained Schwann cells which were responsible for behavioral improvements. However, they failed to sufficiently characterize those NCSC-derived $GFAP^+$ cells which present features not corresponding with mature SCs.

We have started by analyzing the fate of bNCSCs when grafted into intact sciatic nerves. In order to identify them in host tissue we have produced NC stem cells from

boundary cap of Rosa26 mice ubiquitously expressing the bacterial beta-galactosidase (lacZ; Zambrowicz *et al.*, 1997). As hosts we used rats, since their larger nerves are easier to handle. Additionally, mouse nuclei differentially present a punctuated pattern of staining after Hoerscht labeling helping in the identification of grafted cells (Dupin *et al.*, 1990; Sextier-Sainte-Claire Deville *et al.*, 1994). As previously shown for NCSCs (Amoh *et al*., 2005), bNCSCs generated $GFAP^+$ glia after being grafted into the rat sciatic nerve. However, no $S100^+$ Schwann cells were found when tissue was analyzed which suggested the need of additional factors, not provided by the intact nerve, to generate them. Consistently, in defined culture medium, bNCSCs were found to become mostly $GFAP^+$ glia and at lower frequency $Tuj1^+$ neurons or smooth muscle cells. Very few cells expressed $S100^+$ at 5 days under these *in vitro* conditions.

We then looked into protocols for priming stem/progenitor cells and designed one based on previously published reports (Wu *et al*., 2002), but removing bFGF since it is known to direct neuronal fate (Whittemore *et al*., 1999). In search for factors promoting a Schwann cell phenotype from cell progenitors, we found abundant literature suggesting that neuregulins may be one of such factors (Shah *et al*., 1994; Meyer and Birchmeier, 1995; Riethmacher *et al.*, 1997; Morris *et al.*, 1999; Woldeyesus *et al.*, 1999). When the EGF domain of heregulin (Neuregulin-1 type I; shared by other neuregulins, Garratt *et al*., 2000) was added to culture medium recipe, bNCSCs differentiated into $S100^+/GFAP^-$ late Schwann cell precursors already at 5.5 days *in vitro*. Those cells presented two or three elongated processes and oval blunt ended nuclei and formed pavement-like arrays, properties already described for Schwann cell precursors (Jessen and Mirsky, 2002). At 5.5 days after plating few cells were found to in addition express GFAP, known to identify the change from a Schwann cell precursor to an immature phenotype (Jessen *et al*., 1990). With time, GFAP expression was induced in all $Sox10^+$ cells *in vitro,* as well as Krox20 in a subpopulation which consists in the myelinating Schwann cell phenotype (Topilko *et al.*, 1994; Parkinson *et al*., 2004). At 30 days *in vitro*, Schwann cells down-regulated Sox2 suggesting that they have adopted a mature phenotype, since both Schwann and satellite glia cells (the two described types of peripheral glia) in adult DRG and/or nerves do not express this marker. As expected, at 3 days after crushing of the rat sciatic nerve Sox2 was up-regulated in Schwann cells (our unpublished results), known to de-differentiate after injury up to at least an immature Schwann cell stage (Jessen and Mirsky, 2005). We have further characterized *in vivo* and *in vitro* the changes in protein expression pattern of Schwann and satellite glia cell lineages (see Figure 2). Most of them were at least partially shared by both glial cell type lineages.

Interestingly, at E10.5-E11.5 Sox2 was found to be expressed at low levels in NCCs at the boundary cap and around the DRG (*in vivo* and *in vitro*) but when cells became glial progenitors (*in vivo*, from E11.5) or Schwann cell precursors (*in vivo* -from E12.5- and *in vitro* -from 5.5 days in culture-) they highly up-regulated Sox2 expression (our unpublished results). Therefore, increase in Sox2 expression levels seems to mark restriction of neural crest to the glial lineage and suggests that Sox2 might be involved in glial restriction in the PNS.

From *in vivo* analysis of mouse embryonic GFAP expression pattern, our results suggest that some Schwann cells are already born at E12.5 in mouse. Interestingly, at this stage GFAP was found to be expressed within nerves at distal regions suggesting that Schwann cell differentiation might proceed in a centripetal way.

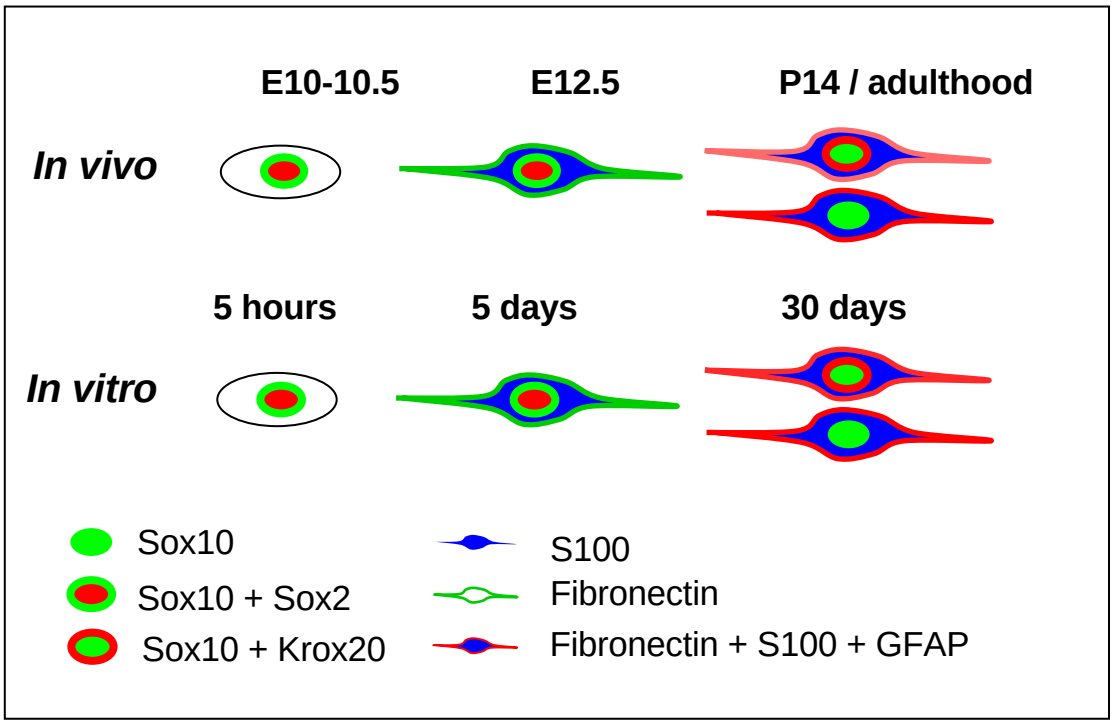

Fig.2: Characterization of *in vivo* and *in vitro* (from boundary cap neural crest stem cells) Schwann cell lineage differentiation.

We have also described for first time expression of Sox10 in adult mouse peripheral glia. S100 expression *in vivo* in some but not all satellite glia cells suggests the existence of different subpopulations of this cell type. Finally, co-expression of Sox10 and Krox20 together with morphological criteria and cell localization helped us to describe presence of Schwann cells within the DRG.

In order to confirm the nature of bNCSC derived SCs and to address their capacity to function as such, we tested their ability to myelinate axons. For that purpose we established a co-culture system. Dissociated bNCSCs were differentiated into Schwann cells for 9 days and C57Bl/6 DRG explants were then added to those cultures. After 31 days of incubation, cultures were fixed and immunostained for Tuj1 and MBP. $Xgal^+$ stem cell-derived SCs were found to ensheath C57Bl/6 axons which nicely responded to signals derived from our differentiated stem cells by defasciculating when entering the bNCSC pool. Furthermore, bNCSC-derived SCs expressed MBP indicative of their capacity to myelinate. This was confirmed *in vivo* by grafting silicone tubes containing bNCSC-derived SCs (pre-differentiated *in vitro* for 25 days, suspended in Matrigel®) into axotomized rat sciatic nerves: $Xgal^+$ SCs were found to ensheath rat axons and express MBP at 90 days after injury. As expected, pre-differentiated SCs were not able to migrate extensively and therefore did not reach proximal or distal stumps of nerves.

After we published these studies, some reports appeared on differentiation of skin-derived neural crest stem cells into Schwann cells also showing that those cells can myelinate (McKenzie *et al*., 2006; Biernaskie *et al*., 2007b). Interestingly, SCs derived from skin NC stem cells were shown to significantly survive after grafting into the injured spinal cord and to produce several beneficial effects including tissue sparing, suppression of reactive gliosis, and promotion of axonal outgrowth and of endogenous SC recruitment within the injury zone. Nevertheless, improvements in locomotor behavior after spinal cord injury were mild when compared to control and not

significant when involving superior central processes (Biernaskie *et al.*, 2007b). *In vitro* protocols used in Freda Miller's group were very similar to the ones we have applied (Biernaskie *et al.*, 2007a).

In an attempt to address regenerative capacity of bNCSCs, we have again placed cells suspended in Matrigel within silicone tubes and after 2 days of SC differentiation treatment in NRGs we grafted them into axotomized rat sciatic nerves. Controls consisted in tubes lacking from stem cells incubated with (NRG) or without (N2) addition of NRGs. Animals were electrophysiologically tested at 90 days after injury and then perfused and tissue analyzed. Interestingly, bNCSCs did not differentiate into Schwann cells and became instead $S100^{-}/GFAP^{+}/Sox2^{+}$ glia, suggesting the requirement of long-term treatment with neuregulins for significantly generating Schwann cells and reducing undesired phenotypes. In bNCSCs condition, slight but not significant improvements in electrophysiological responses were observed considering foot muscle innervation, when compared to N2 control condition (see Figure 3).

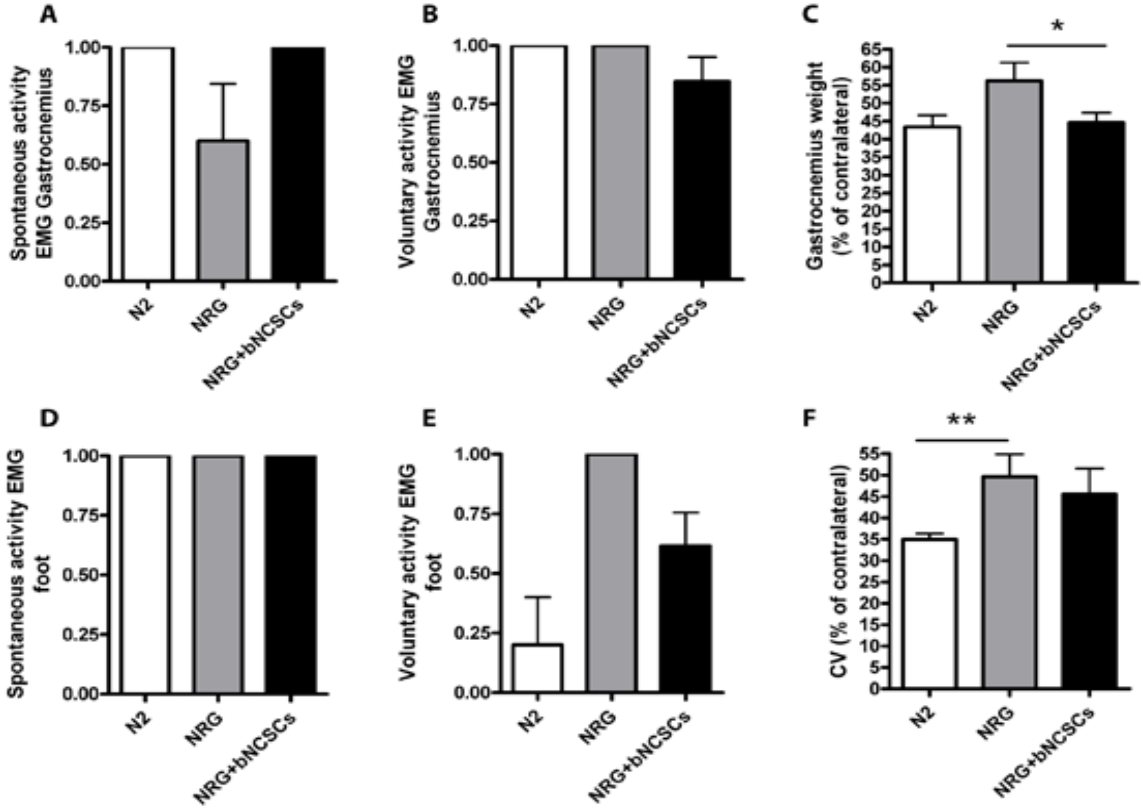

Fig.3: Measurement of parameters suggesting NRG mediated enhancement of axonal regeneration and non-beneficial effect of grafting un-differentiated bNCSCs (A,B,D) Graphs showing electromyogram responses in gastrocnemius (A,B) or foot (D,E) muscles: detection or non-detection of spontaneous (A,D) or voluntary (B,E) responses in each of animals tested were scored as 1 or 0, respectively. (A) Note that while in both N2 and NRG+bNCSCs conditions all animals presented spontaneous activities at 100 days after injury, in 40% of NRG treated animals muscle innervation was fully achieved and therefore no spontaneous activities could be detected. (E) Note that all NRG treated animals showed voluntary peaks of activity in EMG measurements while this was found in 60% of NRG+bNCSCs treated animals and in only 20% of N2 treated animals. (C) Grafting of bNCSCs non-SC pre-differentiated resulted in a minor gain in gastrocnemius weight (measured as percentage of the contralateral gastronemius weight) when compared with NRGs

treated group. (F) Current velocity (as percentage of the contralateral) is increased in NRG treated animals when compared to N2 treated ones. * $p<0.05$. ** $p<0.01$

Interestingly, NRG treatment resulted in: 1) complete innervation of the gastrocnemius muscle in 40% of animals; 2) significant recovery in the gastrocnemius size; 3) appearance of voluntary activity in electromyogram of foot muscles in all animals tested, and 4) significant improvements in current velocity in between sciatic notch and foot muscles (Figure 3). The observed effects likely involves enhance stimulation of SC proliferation and migration (Son and Thompson, 1995; Mahanthappa *et al*., 1996; Trachtenberg and Thompson, 1996).

3.4 PAPER V: STEM CELL-DERIVED SCHWANN CELLS PROMOTE RECOVERY AFTER SPINAL CORD CONTUSION INJURY

Our success in obtaining highly pure cultures of Schwann cells from neural crest stem cells prompted us to study the repair capacity of these cells. In order to pursue this aim we have collaborated with Lars Olson group at the Department of Neuroscience (Karolinska Institute) who have developed tests for performing controlled injuries of the rat spinal cord and tested the effect of different cell types in CNS repair (Hofstetter *et al*., 2002; 2005). Cells were grafted one week after moderate weight-drop spinal cord injury and BBB locomotor tests (Scheff *et al*., 2002) were performed weekly in order to analyze the effect of transplantation. Already one week after grafting animals showed improved locomotor function when compared to control group (on BBB subscore measurements). Our time course analysis of early stages after cell transplantation evidenced existence of axonal re-growth and glial invasion at the injury core suggesting a possible involvement of regeneration in behavioral outcome. Cell transplanted animals reached significantly higher BBB locomotor scores and sub-scores; in the latter, parameters requiring a certain level of coordination are evaluated. Significant improvements in locomotor function were also achieved when a stronger weight drop force was applied. Grafted animals showed significant improvements in grid-walk test at 3 months after injury, suggesting recovery of superior levels of integration.

In order to easily identify grafted cells, Schwann cells were derived from Rosa26 bNCSCs. When tissue was analyzed at 3 months after injury, no $lacZ^+$ grafted cells were observed in sectioned spinal cord in most of animals. In order to draw a time curve of cell death after grafting, groups of 3-4 animals were perfused at 2, 7 and 14 days after transplantation. Surprisingly, no mouse derived Schwann cells were found in any of the experimental rats, not even at 2 days after grafting. Schwann cell death was also previously reported when mature SCs were grafted into injured spinal cord (Hill *et al*., 2006) and it is likely due to absence of axons within the injury zone required for Schwann cells in order to survive. We have then asked ourselves if cells need to be transplanted alive in order to obtain the significant recovery. In order to address this question, cells were freeze-thaw killed before transplantation. Interestingly, the behavioral improvements after grafting of dead cells were not as robust as when cells were placed alive. From previous results, we concluded that endogenous mechanisms mediate the beneficial effects of grafting stem-cell derived Schwann cells.

In transplanted animals, values of total spinal cord area at the injury zone as well as other size parameters measured in the dorsal funiculus area both rostrally and caudally to the injury zone were increased when compared to control condition. This is likely due to reduced atrophy in grafted animals as a result of differential axonal re-growth and endogenous glia recruitment through the injury zone. Consistently, cell transplantation resulted in increase axonal innervation and in density of $Sox10^{+}$ glia not only at the injury zone but also rostrally and caudally to it at three months after injury when compared to controls. These results could also be due in part to neuroprotective effects and in fact at 3 months after injury decreased immunoreactivities to OX42 (a marker for activated microglia and macrophages) were observed in the dorsal funiculus of transplanted animals when compared to controls, proximally to the injury zone.

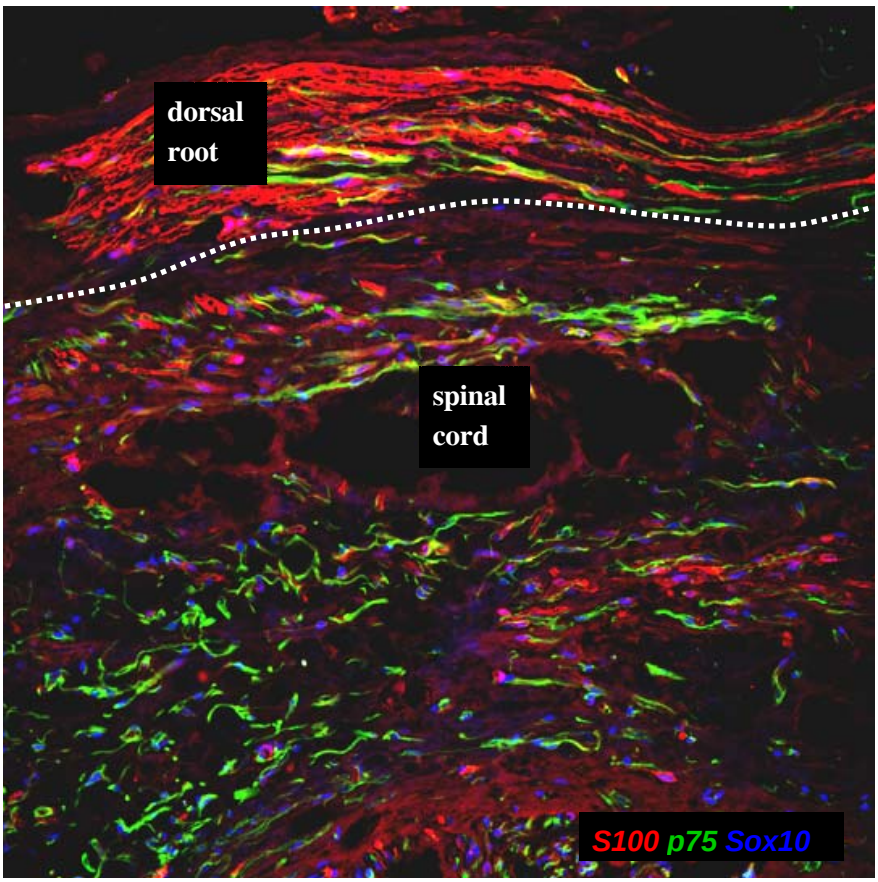

Fig. 4: Presence of p75+/Sox10+ Schwann cell-like cells within the injury zone at 100 days after spinal cord moderate contusion.

The identity of recruited endogenous glia after injury was studied. Interestingly, at areas proximally to and within the injury zone the majority of cells were found to consist in $p75^{+}/S100^{+}/P_0^{+}/Sox10^{+}$ Schwann cell-like cells (SCLCs) which at least in part may derive from progenitors within the CNS (Figure 4). However, distally to the injury zone the recruited cells consisted in $p75^{-}/S100^{-}/ P_0^{-}/Sox10^{+}$ oligodendrocytes, suggesting differential mechanisms of glial cell recruitment at different levels of the injured spinal cord. Number of $p75^{+}$ cells in areas proximal to and within the injury zone was significantly higher in cell grafted animals when compared to control group. Nevertheless since a similar pattern of endogenous glia recruitment was found in both experimental and control conditions when compared to naïve spinal cord, our results

suggest that stem cell-derived Schwann cell transplantation at 7 days after injury enhance naturally occurring endogenous reparative mechanisms after injury.

We have then analyzed the dynamic of changes in cell types within the injury zone and proximally and distally to it in grafted animals (Figure 5). For this characterization we used the combination of four different markers: Sox2 (expressed by the ependymal layer of the spinal cord central canal as well as by mature astrocytes and oligodendrocyte progenitors, and after injury also in different progenitor and immature glial cells), Sox10 (in oligodendrocytes and SCLCs), p75 (in SCLCs) and Rip (glia specified to myelinate, strongly expressed in oligodendrocytes). At two days after injury, very few cells were found to be labeled by these markers within the injury zone, suggesting the loss of most CNS cells at the affected area. Interestingly, many $Sox2^{+}/Sox10^{+}$ oligodendrocyte or SCLC progenitors were found at areas rostrally and caudally to the injury zone and they were more concentrated close to the spinal cord central canal than in its peripheral areas. It is worth noting that few $Sox2^{-}/Sox10^{+}/Rip^{-}/p75^{+}$ apparently mature SCLCs were found in proximal areas of the injury zone. At 7 days after grafting, glia within the injury zone consisted mainly in $Sox2^{-}$ SCLCs which have increased in numbers and reached levels of the distal injury zone. Finally, at 14 days after transplantation SCLC population was even more represented at the injury zone but now $Sox2^{+}/Sox10^{-}$ astrocytes, derived from the ependymal epithelium, reached central areas of the injury and intermingled with SCLCs. Very few $Sox2^{+}/Sox10^{+}$ progenitors were found within the injury zone at all time points analyzed. In conclusion, it seems likely that already differentiated SCLCs invade the injury zone mostly from proximal unaffected areas and this population is expanded over time even though it has down-regulated Sox2 expression. Since SCLC population was found to invade in correlation with axonal re-growth through the injury zone, it is likely that axonally derived signals are required for glial proliferation and survival. And our results also support a role of expansion of endogenous glia population in helping axons to grow within the injury core.

In our studies, we provide for first time evidences that cells within the ependymal epithelium are able to generate *in vivo* oligodendrocyte/SCLCs after injury (Figure 5). At 9 days after injury, several $Sox2^{+}/Sox10^{+}$ cells were found within the disorganized spinal cord central canal epithelium which has become discontinued and opened to the injury zone at this stage. In order to analyze the participation of ependymal cells in generation of recruited endogenous glia after injury, lineage tracing studies using specific inducible mouse strains would be required.

At 3 months after injury, endogenous SCLCs were found to express both P_0 (a myelin protein specific for peripheral glia) and Rip and to myelinate axons. Nevertheless, most of these cells still expressed p75 which is normally down-regulated in mature Schwann cells of the myelinating phenotype (Figure 4). The coexistence of immature features in SCLCs may partially explain why the degree of myelination and functional recovery after injury are minor when compared to normal conditions before injury. Presence of high numbers of macrophages/glia at the injury zone and factors they produce may cause such characteristics in endogenous SCLCs and block their complete maturation.

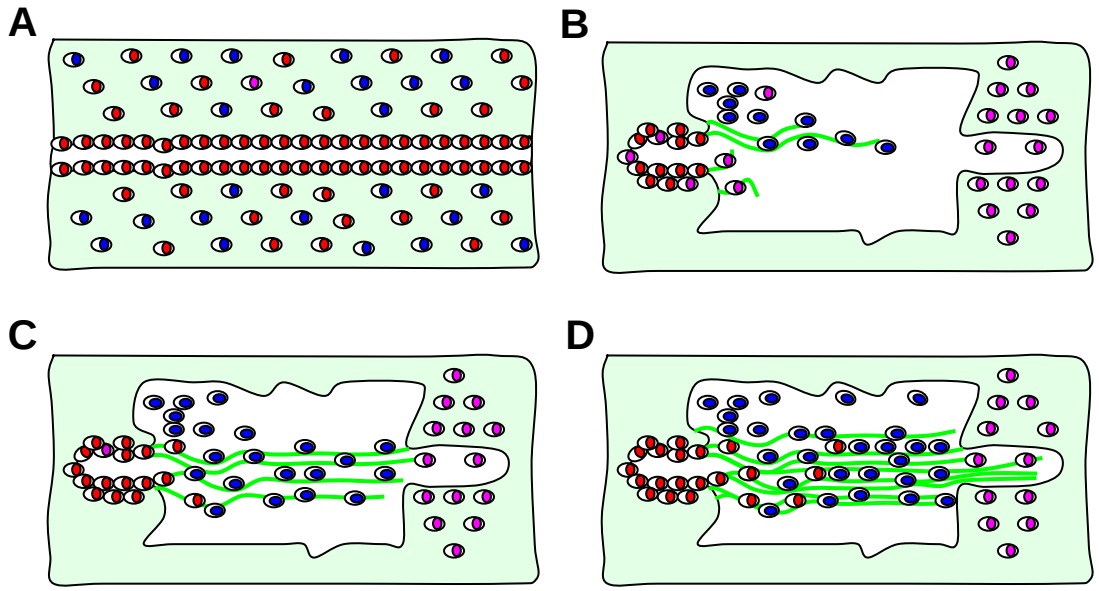

Fig. 5: Changes in glial cell populations and axonal outgrowth in the injured area after spinal cord contusion. Schematics showing dynamic changes within the injured area of the spinal cord at 9 (B), 14 (C) and 21 days (D) after moderate contusion, when compared to un-affected areas (A). Note that after injury most of cells die within the injury zone and this area is filled with microglia and activated macrophages. (A) In healthy spinal cord, ependymal cells lying in central canal wall express Sox2 (red) and constitute a pseudo-stratified epithelium. Glial cells express either Sox2 (astroglia) or Sox10 (blue; oligodendrocytes). (B) Two days after cell transplantation, the ependyma becomes stratified and disorganized and some cells differentiate into $Sox10^{+}$ myelinating glia progenitors (co-expressing Sox2; pink); $p75^{+}/Sox10^{+}/Rip^{-}/Sox2^{-}$ Schwann cell-like cells appeared in proximal areas of the injury zone (cells with oval blue nuclei) and some reach central levels of the injury zone; axons (green) grow in parallel to Schwann cells; abundant oligodendrocyte progenitors appear in spinal cord parenchyma at levels rostral and caudal to the injury zone and the density of these cells decrease from central to peripheral aspects of the spinal cord. (C,D) At 7 days and 14 days after grafting, density of Schwann cell-like cells and axons within the injury zone increase with time.

3.5 PAPER IV: NEDD9 MAKES MULTIPOTENT NEURAL CREST CELLS MOVING

Frédéric Marmigère's findings on homeobox-sequence containing proteins highly enriched in neural tube and DRG at intermediate stages of mouse development by using a degenerative PCR approach has opened interesting lines of research in our lab (Marmigere *et al.*, 2006). One of the mRNA that has been fished was Nedd9. Fred has successfully cloned a probe of 498 bp corresponding to a fragment in the C-terminal domain of Nedd9, including part of a divergent helix-loop-helix motif, which has shown no significant homology with any other paralog member in the Cas protein family. Nedd9 expression pattern was characterized by in situ hybridization (ISH) on sections. Interestingly, Nedd9 expression was observed in multipotent migrating and postmigratory NCCs at E9.5-E10.5 in mouse. Apart from that, its pattern of expression in the nervous system highly resembled the ngn2 one. These two findings prompted further investigations.

To address Nedd9 function in NCCs, the first step we have taken was to obtain Nedd9 full length. Our strategy was to overexpress it in chick by mean of *in ovo* electroporation. Since mouse full-length construct has being published by Erica A. Golemis and colleagues, we contacted her to ask for it and she very generously sent it to us. The construct, originally in pcDNA3, was sub-cloned into a pCA vector, to drive Nedd9 expression under the chick actin promoter. First results suggested a role of Nedd9 in NCC motility since a significant increase in number of Nedd9 targeted cells reaching farther distances in the trunk was seen. *In vitro* experiments, both in mouse bNCSC neurospheres (studies not included in the manuscript) and bNCSC-derived SCs were consistent with that Nedd9 is sufficient to increase migration in NCCs or derivatives. Interestingly, long-term sustained expression of Nedd9 resulted in loss of neural crest cells from all NCC target places, likely due to apoptosis. Since, it seems that normal induction scheme of Nedd9 is sufficient to drive NCC delamination and migration efficiently and that much of phenotype could be due to artificial sustained Nedd9 expression, we concluded that to study its role in a more physiological context loss-of-function models would be more relevant.

We have then designed siRNA naked constructs and repeated in ovo electroporation phenotype studies. To our surprise, a very significant depletion from targeted NCCs at hindlimb levels of the trunk resulted from our first pilot experiment, already at 1.5 days after electroporation. The fact that delamination phenotype was specific for hindlimb levels of the trunk could be due to the fact that an efficient depletion from Nedd9 threshold levels may only be reached at this region. Alternatively, it could be explained by requirement of Nedd9 and β1 integrin signaling for NCC delamination at this specific axial level (Bronner-Fraser, 1986; Tucker, 2004). Apart from that, cells which were able to detach migrated much poorly when compared to controls. These phenotypes were shown to be siRNA dose dependent. We have then made a chick probe for Nedd9 ISH, which show expression of Nedd9 mRNA in NCCs, and confirmed that Nedd9 mRNA is knocked down by our siRNA. Reduction of Nedd9 protein expression levels in NCCs was also observed after Nedd9 siRNA electroporation. Another Nedd9 siRNA construct was designed to confirm Nedd9 knock-down specificity and, as expected, its application resulted in a similar phenotype to the previous one. Interestingly, NCC delamination and migration deficiency was not transient since it was still present and aggravated at 3 days after electroporation. The *in vivo* functional studies were confirmed *in vitro* by explanting neural tubes and analyzing migratory properties in targeted NCCs.

We then turned into investigating whether Nedd9 function in multipotent NCCs motility was dependent on integrin ligands and to address this question NCCs which migrated out from explanted neural tubes were collected, dissociated and re-plated on laminin (ligand of integrins) or poly-D-lysine or heat-denatured laminin (not ligands of integrins). NCCs were only able to efficiently spread in laminin, and Nedd9 siRNA caused a significant reduction in cell spreading area when compared to control. While the majority of scrambled siRNA targeted cells presented a migratory phenotype, Nedd9 depleted NCCs were mostly stationary (Strachan and Condic, 2003).

Finally, since Nedd9 was reported to function in cell lines as scaffolding protein within integrin signalling pathways and it was suggested to regulate focal adhesion and actin cytoskeleton dynamics, properties involved in cell motility, we have analyzed expression of paxillin (a marker of focal complexes) and phalloidin (a marker of actin filaments) in control and Nedd9 siRNA targeted cells. Nedd9 loss-of-function resulted

in decreased levels of focal complexes and actin filaments, a feature that was remarkable in central areas of the cell which almost lack from both sub-cellular components. Stress fibers which are normally found in a fraction of NCCs were rarely seen in Nedd9 deficient cells. The latter phenotype was previously reported in context of p130Cas cell deficiency (Honda *et al.*, 1998).

3.5.1 Nedd9, a marker for multipotency in neural crest cells.

It was reported that the majority of migrating NCCs are likely multipotent (Le Douarin and Kalcheim, 1999; Dupin *et al*., 2007). In our expression pattern analysis we have observed that most of NCCs co-express Nedd9 and high levels of Sox10, known to identify multipotency in migrating NCCs (Kim *et al.*, 2003). Interestingly, while Sox10 expression is kept in NCCs fated to a glial lineage, Nedd9 is not longer expressed in neural crest lineage at E12.5, developmental stage in which most if not all NCCs are *in vivo* lineage committed (our published results; Montelius *et al.*, 2007). This likely suggests that Nedd9 is a marker able to discriminate multipotent NCCs from early neuronal or glial progenitors. Interestingly, the fact that β1 integrin was reported to be a marker for stem cells (Hall *et al*., 2006) and that Nedd9 promoter was found occupied by Sox2 and Nanog in embryonic stem cells (Boyer *et al.*, 2005) suggests a role for Nedd9 in multipotent progenitor cells.
Regarding NCC potential and heterogeneity, in this work we have described different neurogenin 2 (ngn2) mRNA expression levels in the migratory neural crest: while low or no expression of ngn2 was found in different subpopulations of $Sox10^{+}$ NCCs, strong ngn2 expression was mutually exclusive with Sox10 expression. Zirlinger *et al.* (2002) have reported that expression of ngn2 marks a subpopulation of NCCs biased to a sensory but not a neuronal fate. Others have reported that ngns promote neuronal differentiation (Ma *et al.*, 1996; Perez *et al.*, 1999). Our finding of different ngn2 levels of expression in migrating NCCs makes possible therefore to interpret the apparently contradictory results reported by Zirlinger *et al.* (2002), using lineage tracing, and Perez *et al.* (1999) or Ma *et al*. (1996), using gain of function models: as higher levels of ngns expression promoting neuronal specification in progenitor cells. The ngn2 strongly expressing NCCs are likely sensory neuron progenitors reported to have limited proliferative and migratory capacity before they reach their homing place and contribute to the first wave of DRG neurogenesis (Ma *et al.*, 1999). Since Nedd9 is down-regulated in migratory cells already specified to become neurons, this may partly explain why cells expressing high levels of ngn2 stop migrating, triggering DRG condensation.

3.5.2 Nedd9 expression is induced by retinoic acid in neural crest cells.

Our quantification analyses *in vivo* showing changes in Nedd9 expression levels in migrating NCCs suggested that a signal derived from the dermamyotome induces this expression. A peak in intensity of Nedd9 immunoreactivity was seen in NCCs upon dermamyotome dorsal lip proximity and decreased in cells which have passed this landmark downward (Figure 6).
In order to elucidate which molecules induce Nedd9 expression in NCCs, a number of inducible factor candidates and/or of their antagonists/inhibitors were applied for 6 hours on neural tube explant cultures (all-trans retinoic acid, citral, BMP4, Noggin, FGF8, Shh, Wnt3a, Wnt3a plus BMP4, Wnt5a or none –control-). From all factors

tested, retinoic acid is likely the factor inducing Nedd9 expression. Several experimental outcomes support this statement: 1) the highest Nedd9 expression levels were obtained in our *in vitro* model system when explants were cultured in presence of high retinoic acid; 2) upon citral treatment (an inhibitor of retinoic acid biosynthesis; Kikonyogo *et al.*, 1999; Song *et al.*, 2004), Nedd9 was down-regulated in migrated cells with values significantly lower than in control condition; and 3) retinoic acid injection in pregnant mothers up-regulated Nedd9 expression in trunk neural crest of E9.5 mouse embryos after 6 hours of treatment. A minor increase in Nedd9 expression was seen for FGF8, Wnt3a and Shh treatments; however, whether or not this is a consequence of direct or indirect Nedd9 expression regulations remains to be address.

Our results suggesting retinoic acid induction of Nedd9 are consistent with dynamic changes in Nedd9 expression patterns in NCCs. It is known that the expression of Raldh2, the main enzyme synthesizing retinoic acid in the trunk, increases in a posterior-to-anterior fashion in somites and peaks at the level of somite dissociation into dermamyotome and sclerotome (Haselbeck et al., 1999). And consistently, and since Nedd9 is involved in NCC motility, first NCCs migrating down through the rostral half of the somite do so at this very same trunk level (Erickson and Perris, 1993).

Based on our results and on available literature already discussed, we propose that a retinoic acid regulation of Nedd9 expression gives multipotent NCCs competence to respond to extracellular signals and to initiate migration through the sclerotome in an integrin dependent manner (Figure 6). Our data suggests that Nedd9 is implicated in regulation of focal complexes and actin filaments in NCCs, required for cell adhesion and spreading and for the development of traction forces which altogether drive motility. Our results also suggest Nedd9 involvement in neural crest delamination from the neural tube dorsal roof likely through similar mechanisms.

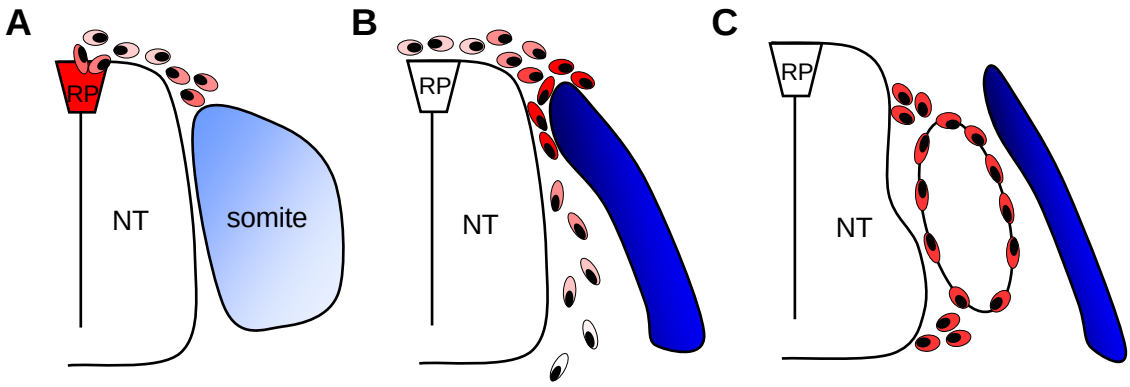

Fig. 6: Nedd9 expression pattern and regulation in neural crest cells. (A) Nedd9 (red) is expressed at high levels in the neural tube roof plate during neural crest delamination. (B) Expression levels of RALDH2 in somites are increased from posterior to anterior regions of the trunk and peaks when they dissociate into dermamyotome (blue) and sclerotome (Haselbeck *et al.*, 1999), and at this axial level neural crest start migrating through the sclerotome. Nedd9 expression in migrating neural crest is induced when they contact the sclerotome and downregulated thereafter. (C) Nedd9 is expressed in postmigratory neural crest at the borders of the DRG and boundary cap an it is downregulated when cells become specified to neuronal lineage. Our functional results indicate that Nedd9 is required for multipotent neural crest cell migration. RP: roof plate. NT: neural tube .

3.6 PAPER VI: NEDD9 IS EXPRESSED IN MULTIPOTENT/OLIGOPOTENT PROGENITORS OF DIVERSE TISSUES

As mentioned before, one of the features that called our attention from Nedd9 in situ hybridization expression patterns was its correlation with ngn2 expression at different levels of the neural tube, from the mesencephalic ventral midbrain up to caudal regions of the trunk. In parallel to characterizing Nedd9 expression in NCCs we decided to map it all through the mouse embryo at intermediate stages of development in which Nedd9 is found. Both in situ hybridization and immunohistochemistry were then performed on sections from E9 to E12.5 embryos.

Nedd9 mRNA expression has already been reported in mammals but this characterization was done at whole rat embryo level (Merrill et al., 2004a). Therefore its characterization at the cellular level during mammalian development was unknown.

We have first studied Nedd9 mRNA expression on trunk sections at E9.5-E10.5 mouse equivalent stages. Nedd9 was expressed at high levels in $Sox2^+$ cells of the neural tube ventricular zone at two dorso-ventral domains corresponding mostly to Ngn2 mRNA expression domains (our results; Scardigli *et al*., 2001; Simmons *et al*., 2001) but extended ventrally reaching the floor plate (Figure 7). Interestingly, it was expressed at much lower levels at intermediate dorso-ventral levels of the neural tube likely corresponding to domains of class B roof plate-independent dorsal progenitors (Figure 7; Helms and Johnson, 2003). It seems likely that Pax7 and/or other transcriptional factors may repress Nedd9 expression. Alternatively, Nedd9 levels would mainly depend on morphogenes originated in both the roof and the ventral plates which may not reach concentrations required for Nedd9 expression at this dorso-ventral level of the neural tube. Apart from that, Nedd9 expression was not found in the roof plate and in dP1 progenitor populations (Figure 7). Nedd9 was absent in the intermediate zone of the neural tube, defined as the region in between the ventricular and the mantle zones (Roztocil *et al*., 1997), in which neuronally committed progenitors presenting low levels of Tuj1 and high levels of Ngn2 mRNA are located (Figure 7).

At E12.5, Nedd9 and Ngn2 mRNAs are homogeneously expressed in the dorso-ventral axis of the neural tube. Nedd9 expression is restricted to the ventricular zone and Ngn2 is mainly found in the intermediate zone (Figure 7).

Interestingly and consistent with previous results, in the mesencephalic ventral midbrain, hypoglossal nucleus and the dorsal motor nucleus of the vagus nerve Nedd9 expression is also downregulated in cells presenting high levels of Ngn2 mRNA expression and upon induction of Nurr1 expression, which identify neuronally committed progenitors (Figure 7; Zetterstrom *et al*., 1997; Wagner *et al*., 1999; Nieto *et al*., 2001; Wallen *et al*., 2001). Pool of $Sox2^+$ progenitors of lateral domains to the ventral midbrain, characterized by their ngn1+ngn2 expression (our unpublished results) which will generate prospective occulomotor neurons express Nedd9 at much lower levels.

We found a remarkable pattern of Nedd9 expression in the cerebellum at E12.5, since it was also present at lower levels in the Nurr1 weakly expressing cells of the mantle zone. Compatibility of Nedd9 expression with low levels of Nurr1 or Nurr1-like proteins was also found in gonads, cartilage primordium and gut epithelium but Nedd9 was downregulated at the DRG when Nurr1-like expression was upregulated.

From previous results, and considering our and others results on Nedd9 induction by retinoic acid (discussed in paper IV section of this introduction), it seems likely that

Nurr1 and Nurr1-like proteins (known receptors for retinoic acid) play a role of negative regulators of Nedd9 expression.

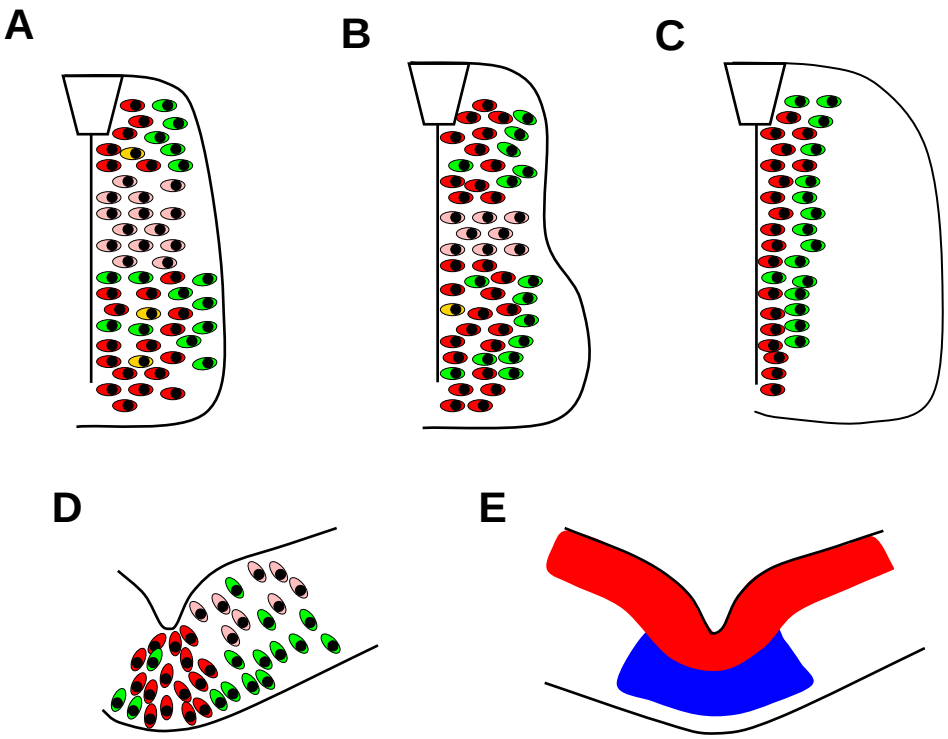

Fig.7: Nedd9 expression pattern in the neural tube. Schematic showing pattern of expression of *Nedd9* (red), *Ngn2* (green) or Nurr1 (blue) in trunk neural tube at E9.5 (A), E10.5 (B) and E12.5 (C) or mesencephalic ventral midbrain at E10.5 (D) and E12.5 (E). Color code pink denotes low levels of *Nedd9* expression. Yellow: possible co-localization of *Nedd9* and *Ngn2*.

Nedd9 was found to be expressed in $Sox2^{+}$ progenitor cells of the retina, olfactory pit, gut epithelium and Rathke pouch. These evidences, together with the expression patterns described for the neural tube, suggest that Nedd9 is restricted to multipotent/oligopotent progenitors of diverse organs and it is therefore downregulated upon lineage commitment (Cai *et al*., 2002; Graham *et al*., 2003).

Other tissues/organs which were found to express Nedd9 are the followings: heart (up to E10.5), respiratory epithelium, mesonephros, blood vessels (early developed limbs), meninges (hindbrain), urinary tract, and boundary cap.

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