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# Neuregulin 1 Isoforms as Players in Signaling Networks in Neural Crest Cell Migration, Lineage Determination, and Differentiation

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Wie viele Königreiche wissen nichts von uns!

*Pensées 42/207*, Pascal

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# ZUSAMMENFASSUNG

## *1. Kurze Einführung*

Das periphere Nervensystem (PNS) der Wirbeltiere entsteht aus einer Population von Vorläuferzellen, den Neuralleistenzellen. Während des ersten Drittels der Embryonalentwicklung wird das Neuralrohr durch Einfaltung des Neurektoderms gebildet (**Figur 1**). Durch einen Entwicklungsprozess, Induktion genannt, entsteht im dorsalen Teil des Neuralrohres (Neuralleiste) eine Gruppe von Zellen, die Neuralleistenzellen, welche sich im Laufe der späteren Entwicklung vom Neuralrohr lösen und entlang verschiedener Wege (**Figur 2**) zu unterschiedlichen Zielorten im Embryo wandern. Am Bestimmungsort angekommen, differenzieren die Neuralleistenzellen in verschiedenste Zelltypen aus, wie zum Beispiel Neuronen oder Gliazellen. Ergebnisse aus klonalen *in vitro* Analysen sowie aus *in vivo* Transplantations- und Markierungsexperimenten lassen schliessen, dass dies geschieht, indem Neuralleistenzellen in ihrem Entwicklungspotential sukzessive eingeschränkt werden. In diesem Zusammenhang wurden bereits eine Reihe von Faktoren beschrieben, welche auf die Entwicklung der Neuralleistenzellen einen Einfluss haben. Dabei wird heute davon ausgegangen, dass es ein Zusammenspiel von extra- und intrazellulären Faktoren benötigt, um eine multipotente Neuralleistenzelle ihrer funktionalen Bestimmung zuzuführen. Trotz vieler experimenteller Daten, welche diese Hypothese stützen, ist das

Wissen, wie die Entwicklung der Zellen des peripheren Nervensystems im Einzelnen gesteuert wird, noch lückenhaft. Das Interesse unseres Labors gilt daher der Aufklärung dieser Entwicklungsregulation, welche die Bildung spezialisierter Zelltypen aus multipotenten Vorläufern ermöglicht.

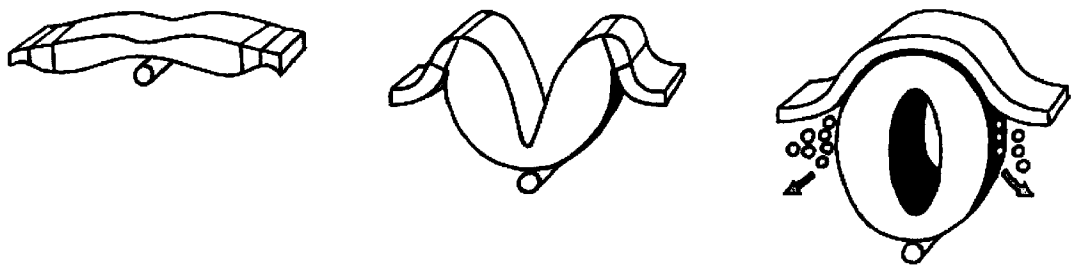


**Figure 1:** Neurulation in amphibians and amniotes

Diagrammatic representation of neural tube formation. The ectodermal cells are represented either as precursors of the neural crest (green) or as precursors of the epidermis (grey). The ectoderm (blue) folds in at the most dorsal point, forming an outer epidermis (grey) and an inner neural tube connected by neural crest cells After neural tube closure, the neural crest cells migrate away from the neural tube to form the peripheral nervous system (adapted from Wolpert, 1999).

## *2. Teil I: Neuregulin 1-Isoformen in der Entwicklung des PNS*

Ein Gen, dessen unterschiedliche Produkte wichtige Schritte in der Entwicklung der Neuralleistenzellen steuern, ist Neuregulin 1 (NRG1). Im ersten Teil dieser Arbeit - einem Review - werden die verschiedenen Funktionen von NRG 1 in der Embryonalentwicklung und Erhaltung des peripheren Nervensystems betrachtet (**Figur 4**), mit dem thematischen Schwerpunkt auf der glialen Linie. Dabei geht es darum, die vielseitigen Funktionen dieses Faktors in ihrem molekularen und zellulären Kontext aufzuzeigen.

*2.1. NRG1-Isoformen vermitteln ihre Signalwirkung über ein erbB-Rezeptorpaar*

NRG1 gehört zur Familie der Neureguline, welche eine Vielzahl von EGF-ähnlichen (engl: epidermal growth factor) Glycoproteinen umfasst. Zwar sind zur Zeit vier Neuregulingene bekannt (NRG1, NRG2, NRG3 und NRG4), die meisten Informationen in Bezug auf deren Funktionen hat man jedoch aus Studien im Zusammenhang mit NRG1 erhalten. Die verschiedenen Isoformen von NRG1, die durch den Gebrauch unterschiedlicher Promotoren und durch differentielles "Splicing" der RNA entstehen, werden in drei Gruppen unterteilt. Deren Hauptvertreter, NRG1 Typ I bis Typ III, sind in **Figur 3** schematisch wiedergegeben. Alle NRG1-Isoformen weisen nebst ihrer spezifischen Proteindomänen die charakteristische EGF-ähnliche Domäne auf. Die Isoformen werden u.a. von sensorischen und motorischen Nerven exprimiert und treten sowohl in membrangebundenem als auch in löslichem Zustand auf. Ihre Signalwirkung wird im sich entwickelnden peripheren Nervensystem hauptsächlich durch ein Rezeptorpaar vermittelt: dem erbB2/erbB3-Dimer. Beide Moleküle sind Tyrosinkinasen, welche an der Zelloberfläche sitzen und sich nach der Bindung von NRG1 an erbB3 zu einem Heterodimer gruppieren, welches spezifische Signalkaskaden innerhalb der Zelle auslöst.

*2.2. Wanderung und Wegfindung der Neuralleistenzellen ist ein NRG1-gesteuerter Prozess*

Durch Studien mit mutanten Tieren, welche entweder kein funktionales NRG1, erbB2 oder erbB3 besitzen, lassen sich einige Aussagen darüber machen, wie die NRG1-Signalwirkungen die Entwicklung der neuralen Zellen im peripheren Nervensystem beeinflussen. Wie in Kapitel 3 des ersten Teils dieser Arbeit dargelegt, konnte gezeigt werden, dass NRG1 Typ I die Wanderung derjenigen Gruppe von Neuralleistenzellen steuert, die später das sympathische Nervensystem bilden wird. Im weiteren scheint NRG1 Typ I einen Einfluss auf die Wanderung und Wegfindung von cranialen Neuralleistenzellen zu haben. Die Art und Weise, wie NRG1 das Wanderungsverhalten der Neuralleistenzellen steuert, ist nicht klar, jedoch lassen *in vitro* Experimente mit Krebszellen die Vermutung zu, dass NRG1

über die Kinasen PAK1 (engl: p21-activated kinase1) und PI-3 (engl: phosphatidylinositol-3 kinase) die Zusammensetzung der Zytoskelett-komponente Aktin verändert, was eine höhere Mobilität der Zellen zur Folge hat.

### *2.3. Unterschiedliche NRG1-Isoformen spezifizieren die Identität der peripheren Gliazellen*

Experimenten mit Neuralleistenzellen, welche in Gegenwart von NRG1 Typ II kultiviert wurden, zeigten, dass Neuregulin die Fähigkeit hat, die Entwicklung der Stammzellen zu Gliazellen zu induzieren. Dieser Vorgang ist abhängig vom zellulären Umfeld der Neuralleistenzelle. Die gebildeten Gliazellen exprimieren den molekularen Satellitenmarker Erm. Dass NRG1 eine prominente Rolle in der Gliogenese spielt, ist von *in vivo*-Daten bestätigt worden. Mutanten des NRG1-Signalsystems, denen die Schwannschen Zellen im peripheren Nerv fehlen, weisen darauf hin, dass die NRG1 Typ III Isoform für die Entwicklung der Schwannschen Zellen von Bedeutung ist. Ich konnte nun zeigen, dass die Isoform NRG1 Typ III - im Gegensatz zur Isoform Typ II - die Bildung von Schwannschen Zellen aus multipotenten Vorläuferzellen fördert, vorausgesetzt, der Faktor ist membrangebunden (**siehe Teil II**). Zusammenfassend lässt sich sagen, dass die Neuregulinisoformen Typ II und Typ III *in vitro* einen unterschiedlichen Einfluss auf die Spezifizierung von Gliazelltypen haben.

### *2.4. Überleben, Proliferation und Myelinisierung der Schwannschen Zellen sind NRG1-abhängige Prozesse*

Bis nach der Geburt sind Schwannsche Zellen von Faktoren abhängig, die deren Überleben garantieren. Nebst der Funktion als Überlebensfaktor hat NRG1 auch einen Einfluss auf die Teilungsrate der Schwannschen Zellen. Somit würde das Axon durch NRG1 über einen Selektionmechanismus verfügen, welcher der Bildung einer korrekten Anzahl von Gliazellen entlang des Nerves dient. Neuere Untersuchungen weisen darauf hin, dass NRG1 auch einen Einfluss auf die Myelinisierung der Nerven hat.

*2.5. Aktivierung unterschiedlicher Signalkaskaden durch verschiedene NRG1-Isoformen*

Die unterschiedlichen Signalwirkungen der Neuregulinisoformen werfen die Frage auf, wie eine differentielle Signalgebung durch NRG1 realisiert wird. Möglicherweise spielen Prozesse eine Rolle, die festlegen, ob der Faktor in membrangebundener oder sekretierter Weise zum Einsatz kommt. Meine Beobachtungen, die ich im zweiten Teil der Arbeit näher beschreibe, scheinen diese Vermutung zu bestätigen. Cofaktoren, wie zum Beispiel das Adhäsionsprotein CD44, scheinen einen Einfluss auf die Verarbeitung der NRG1-abhängigen Signalgebung zu haben. Die Regulation der Empfindlichkeit der Rezeptorzelle auf das NRG1-Signal stellt eine weitere Möglichkeit dar, wie das NRG1-Signalsystem moduliert werden könnte. In diesem Zusammenhang wurde beobachtet, dass der Transkriptionsfaktor Sox10 die Expression des NRG1-Rezeptors erbB3 reguliert. Im Übrigen konnte gezeigt werden, dass die NRG1-Isoformen Typ I und Typ II unterschiedliche Signalkaskaden auslösen können (**Figur 6**).

*2.6. Zusammenfassung*

Zusammenfassend lässt sich sagen, dass NRG1 in der Entwicklung der peripheren Gliazellen eine vielseitige Rolle spielt. Welchen Einfluss NRG1 auf die Entwicklung einer bestimmten Zelle hat, hängt von mehreren Faktoren ab: nebst der unterschiedlichen Wirkungen der Isoformen spielen Rezeptor-Cofaktoren aber auch der zelluläre Kontext eine Rolle in der Entscheidung, welche Signalkaskaden in der Rezeptorzelle ausgelöst werden.

## *3. Teil II: Neuregulin 1 Typ III induziert die Entwicklung von Schwannschen Zellen aus multipotenten Vorläuferzellen*

Frühere Experimente hatten gezeigt, dass NRG1 Typ I in Kulturen von Schwannschen Vorläuferzellen deren längerfristiges Überleben fördert. Zudem stellte man fest, dass diese Zellen in frühe Schwannzellen

differenzierten. Beobachtungen aus Experimenten mit NRG1-mutanten Tieren hingegen lassen den Schluss zu, dass NRG1 Typ III jene Isoform ist, die für die Entwicklung der Schwannschen Zellen von Bedeutung ist: Tiere, denen diese Isoform fehlt, haben keine Schwannschen Vorläuferzellen im sich entwickelnden Nerv. Nahe der Spinalganglien (engl: dorsal root ganglion; DRG), von wo aus der Nerv in die Peripherie projiziert, lassen sich jedoch gliale Zellen finden. Möglicherweise wird deren Entwicklung durch NRG1 Typ II unterstützt. Diese lösliche Isoform wird von den sensorischen Neuronen der Spinalganglien sekretiert.

### *3.1. Neuregulin 1 Typ III fördert das Überleben von Schwannschen Vorläuferzellen*

Im zweiten Teil der Arbeit konnte ich zeigen, dass lösliche Formen der Isoformen NRG1 Typ II und Typ III beide in analoger Weise zum oben beschriebenen *in vitro* Experiment das Überleben und die Differenzierung von Schwannschen Vorläuferzellen fördern (**Figuren 7** bis **9**). Obwohl dies als Hinweis gewertet werden kann, dass NRG1 als Differenzierungssignal wirkt, stellt sich die Frage, ob die beobachtete Entwicklung der Zellen nicht eine Konsequenz des Langzeitüberlebens der Zellen ist und somit einem intrinsischen Programm folgt. Somit lässt sich mit diesen Studien nicht zweifelsfrei klären, ob NRG1 lediglich ein Überlebenssignal ist, oder ob der Faktor auch aktiv die Differenzierung der Schwannschen Zellen fördert. Um dieser Frage nachzugehen wurden die folgenden Studien in einem *in vitro*-Modellsystem durchgeführt, in dem das Überleben der Zellen unabhängig von NRG1 gewährleistet ist.

### *3.2. Membran-gebundenes Neuregulin 1 Typ III induziert Schwannzell-Differenzierung*

Multipotente Vorläuferzellen wurden aus embryonalen Spinalganglien von Ratten isoliert und unter Zugabe von löslichen NRG1 Typ II und Typ III Isoformen kultiviert. Erwartungsgemäss differenzierten NRG1 Typ II behandelte Zellen nicht in Schwannsche Zellen aus. Vorhergehende Studien unseres Labors hatten gezeigt, dass die Typ II Isoform die Entwicklung der

Vorläuferzellen in Satellitenzellen fördert. Wider Erwarten konnte jedoch auch die Typ III Isoform nicht, die Differenzierung in Schwannsche Zellen initiieren (**Figur 10**). Diese Zellen wiesen auch nicht die Merkmale einer Satellitenzelle auf, was nahelegt, dass die Isoformen funktionell intrinsisch unterschiedlich sind. Biochemische Experimente mit NRG1 Typ III deuten darauf hin, dass diese Isoform, wenn sie in Zellen exprimiert wird, mittels einer hydrophoben Domäne in die Zellmembran eingebaut wird. Daher wurde mit Hilfe eines retroviralen Konstruktes NRG1 Typ III in multipotenten Vorläuferzellen exprimiert (**Figur 11**). Interessanterweise zeigten nun jene Zellen, welche in Kontakt mit der NRG1 Typ III-exprimierenden Zellen standen, die molekularen Merkmale einer Schwannschen Zelle, auch in der Gegenwart von löslichem NRG1 Typ II (**Figuren 12** bis **14**). Diese Beobachtungen legten nicht nur nahe, dass NRG1 Typ III eine aktive Rolle in der Differenzierung der Schwannschen Zellen innehat sondern zeigten auch auf, dass die Art und Weise, in der das Signal der Rezeptorzelle präsentiert wird, eine Rolle spielt. Das Modell in **Figur 5** besagt, dass NRG1 Typ III während der Entwicklung die Differenzierung von postmigratorischen Vorläuferzellen in den Spinalganglien initiiert, was nachfolgend deren Auswanderung in den Nerv erlaubt.

# SUMMARY


## *1. Part I: Differential participation of Neuregulin 1 signaling in the development of the peripheral glia*

How a multipotent stem cell gives rise to a variety of different progeny is poorly understood. Even though many factors with an impact on cell fate have been identified in recent years, the precise mechanisms by which cells adopt a specific fate have yet to be determined. The steps leading to a fully differentiated cell are believed to involve sets of extracellular cues and intrinsic programs, which in combination elicit a specific developmental response. Furthermore, diverse proteins with distinct impacts on a receptor cell may be expressed by the same gene. In the first part of my thesis, I have reviewed these issues with a focus on Neuregulin 1, a gene which has been implicated in different developmental events. Neuregulins are a family of epidermal growth factor-like (EGF-like) factors that activate receptor tyrosine kinases of the ErbB type. Neurons produce Neuregulin 1 isoforms, whereas postsynaptic cells and cells associated with neurons (glia or Schwann cell precursors) express the ErbB receptors. The diverging effects of Neuregulin 1 in many different processes during development of the peripheral nervous system (PNS) early on suggested that it might be a key regulator. Neuregulin 1 isoforms were shown to be involved in migration and lineage segregation of neural crest cells. Furthermore, different isoforms of the gene seem to be

responsible for differentiation of distinct glial subtypes. Besides the isoform-specific effects on neural crest cells and their derivatives, the molecular context proved to play a major role in the interpretation of Neuregulin 1 signaling.

## *2. Part II: Membrane-bound Neuregulin 1 type III acitvely promotes Schwann cell differentiation of multipotent progenitor cells*

Many steps of peripheral glia development appear to be regulated by NRG1 signaling but the exact roles of the different NRG1 isoforms in these processes remain to be determined. While GGF2, a NRG1 type II isoform, is able to induce a satellite glial fate in neural crest stem cells, targeted mutations in mice have revealed a prominent role of NRG1 type III isoforms in supporting survival of Schwann cells at early developmental stages. In the second part of my thesis, I present our investigations on the role of NRG1 isoforms in the differentiation of Schwann cells from neural crest-derived progenitor cells. In multipotent cells isolated from dorsal root ganglia, soluble NRG1 isoforms do not promote Schwann cell features, whereas signaling by membrane-associated NRG1 type III induces the expression of the Schwann cell markers Oct-6/SCIP and S100 in neighboring cells, independent of survival. Thus, axon-bound NRG1 might actively promote both Schwann cell survival and differentiation.

# PART I: *NEUREGULIN 1 ISOFORMS AS PLAYERS IN SIGNALING NETWORKS IN NEURAL CREST CELL MIGRATION, LINEAGE DETERMINATION, AND DIFFERENTATION*

## *1. Introduction*

The vertebrate peripheral nervous system (PNS) derives from a transient population of cells called the neural crest cells. These cells detach from the dorsolateral margins of the neural tube and migrate throughout the embryo to distant locations, where they terminally differentiate (Le Douarin and Kalcheim, 1999; **Figure 2**). On the way to fulfilling its distinct functions in a given target structure a neural crest stem cell has to pass through many decisive developmental phases. The processes that underlie this specific development proved to be intricate. Besides the requirement to migrate to the correct target locations in the developing embryo, a crest cell must differentiate into a cell type that displays the characteristics appropriate to a given location and lineage. During the course of many studies it has become clear that an interplay of many different factors is required to fulfill such a complex task (reviewed in Sommer, 2001). Interestingly, some of the factors that were shown to be involved proved to play an important role during distinct

stages of crest development. Here we discuss the diverging activities of Neuregulin 1 during formation and maintenance of the PNS with an emphasis on the glial lineage. In collaboration with many coplayers, Neuregulin 1 has been shown to be active in different developmental steps during the generation of diverse neural structures by neural crest cells.

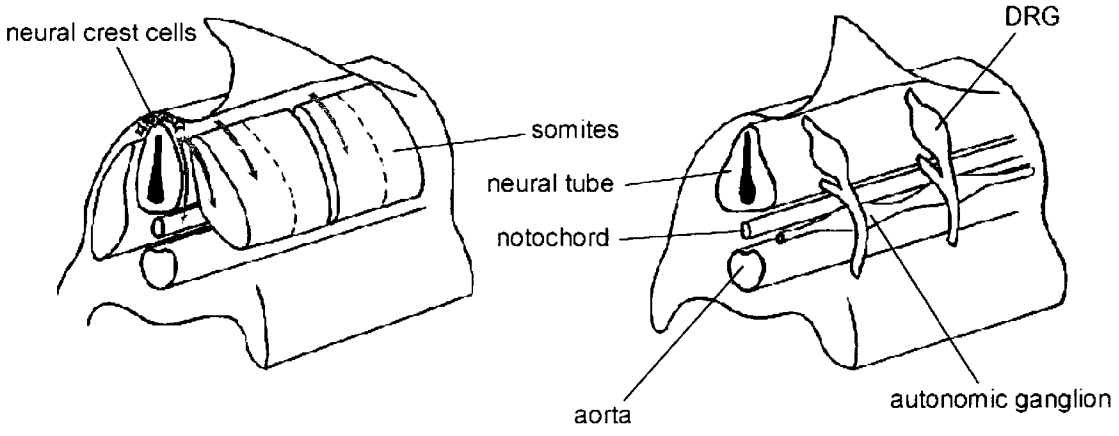



**Figure 2:** Neural crest migration in the trunk of the chick embryo

The cells that take the superficial pathway, just beneath the ectoderm, will form pigment cells of the skin. Those that take the deep pathway via the somites will give rise to a wide variety of crest derivatives (adapted from Kandel, 1991).

In this review, the roles of Neuregulin 1 will be considered in chronological order (**Figure 4**) starting with Neuregulin 1 functions in embryogenesis during emigration of crest cells (*section 3*). In this context, possible mechanisms by which Neuregulin 1 might have an impact on cell motility will be discussed. Fate segregation of postmigratory crest cells in forming ganglia and glial sublineage identity specified by Neuregulin 1 activity is the topic of *section 4*. The role of a distinct Neuregulin 1 isoform promoting early steps of Schwann cell differentiation is considered. *Section 5* deals with the well described Neuregulin 1 activity regulating Schwann cell survival and proliferation. The *in vivo* role in myelination and demyelination, however, is still

elusive. Finally, signaling mechanisms that might distinguish between different Neuregulin 1 isoforms are discussed in *section 6*. Activation of distinct signaling pathways most likely provides the basis for a regulation of PNS development and maintenance by the many Neuregulin 1 isoforms.

## *2. Neuregulin 1 activity is mediated through combinations of ErbB receptors*

The Neuregulins (NRG) comprise a family of epidermal growth factor (EGF)-like glycoproteins which have been implicated in many distinct functions including cell survival, migration, and differentiation. To date four different Neuregulin genes (NRG1, NRG2, NRG3, and NRG4) have been described (reviewed in Adlkofer and Lai, 1999).

### *2.1. The Neuregulin 1 gene encodes a variety of isoforms*

Most is known about the functions of NRG1, which we subsequently will discuss with an emphasis on neural crest development. Usage of different transcription initiation sites and alternate RNA splicing give rise to three types of NRG1 isoforms (reviewed in Garrat et al., 2000a; **Figure 2**). All isoforms share a characteristic EGF-like domain. Even though it has been shown that this domain is sufficient for receptor activation it might not account for all NRG1-mediated biological activities observed (Holmes et al., 1992; this review). EGF-like domains occur as $\alpha$- or as $\beta$-variants which differ in their binding affinities (Marikovsky et al., 1995). In addition, each NRG1 isoform contains characteristic domains: NRG1 type I (also referred to as Neu Differentiating Factor; NDF/heregulin or Acetylcholine Receptor Inducing Activity; ARIA) has an immunoglobulin-like (Ig-like) domain flanking a region rich in glycosylation (Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993). NRG1 type II (primarily identified as Glial Growth Factor, GGF) has a 'kringle' domain and, shares an Ig-like domain with NRG1 type I (Marchionni et al., 1993). Type III, also referred to as Sensory and Motoneuron Derived Factor

(SMDF) or Cysteine-Rich Domain-NRG1 (CRD-NRG1), has an amino-terminal cysteine-rich domain (Ho et al., 1995; Bermingham-McDonogh et al., 1997; Yang et al., 1998). The NRG1 isoforms can either occur as membrane-bound or secreted factors. Isoforms in association with the cell membrane display a transmembrane span, carboxy-terminal to the EGF-like domain, but can also be released to the extracellular space by proteolytic cleavage (Liu et al., 1998b; Wang et al., 2001). In contrast to the isoforms type I and type II, NRG1 type III bears it its cysteine-rich domain an additional hydrophobic sequence that serves as an (uncleaved) insertion signal, thus exposing the membrane-bound ligand to the extracellular environment in a "reversed" orientation (Schroering and Carey, 1998; Wang et al., 2001).

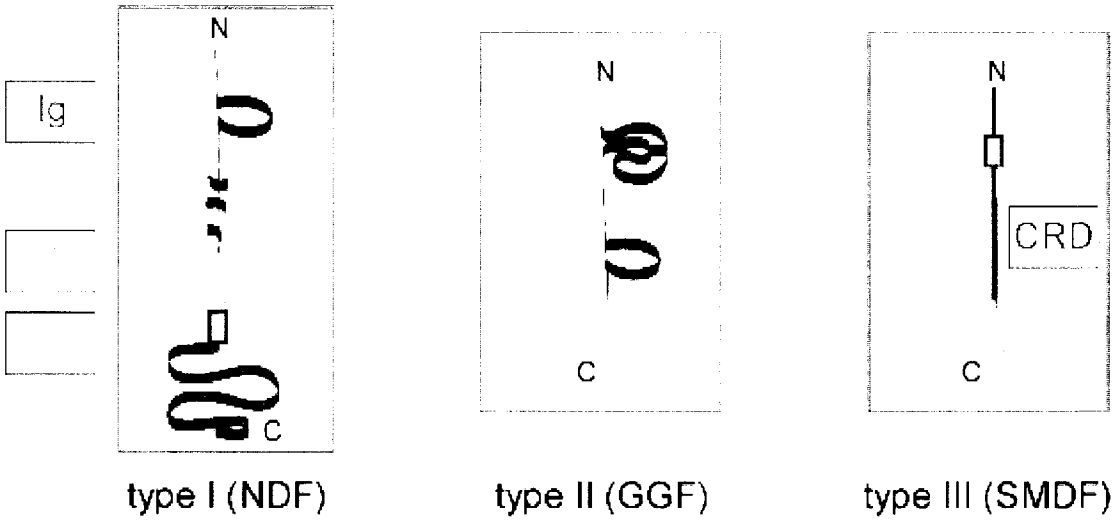



**Figure 3:** Schematic structure of the major Neuregulin 1 isoforms

Different major isoforms (type I-III) are produced from the single Neuregulin 1 gene by different promoter usage and alternative splicing. Common to all isoforms is an EGF-like domain (orange). Other domains present in Neuregulins are an Ig-like domain (green), a domain rich in potential glycosylation sites (type I: N-terminal to the EGF-like domain), a kringle-like domain (type II: N-terminus), and a cysteine-rich domain (red). Blue boxes indicate signal peptides or internal hydrophobic sequences that are thought to serve as transmembrane domains.

*2.2. Neuregulin 1 binds with high affinity to the receptors erbB3 and erbB4*

NRG1 isoforms exert their biological activity through a subfamily of cell-surface receptor protein-tyrosine kinases, the erbB receptors (reviewed in Buonanno and Fischbach, 2001). Two members, erbB3 and erbB4, display high affinity to NRG1. Binding of the ligand results in heterodimerization with erbB2 (or homodimerization with erbB4), a prerequisite for signal transmission (Plowman et al., 1993; Carraway and Cantley, 1994; Sliwkowski et al., 1994; Riese et al, 1995; Pinkas-Kramarski et al., 1996, 1998; Jones et al., 1999). Interestingly, NRG1 activity in varying developmental processes was found to be mediated by distinct receptor combinations. Signaling during heart development appears to be mediated by erbB2/erbB4 while the heterodimers erbB2/erbB3 and erbB2/erbB4 are involved in development of the neural crest cell lineage. Furthermore, it was observed that signaling through erbB receptors is context-dependent (Garcia et al., 2000; Paratore et al., 2001; Sherman et al., 2000; this review). Thus, the different NRG1 isoforms interacting with diverse receptor assemblies constitute a rich source of signaling combinations that - as will be discussed below with an emphasis on the peripheral nervous system - participate in regulation of development at various levels.

## *3. Emigration and pathfinding of neural crest cells*

After early specification, a process which takes place at the interface of neural and non-neural ectoderm, neural crest cells undergo an epithelial to mesenchymal transition, delaminate from the dorsal neural tube, and start to migrate to their target locations throughout the embryo (reviewed in Le Douarin, 1982; Garcia-Castro and Bronner-Fraser, 1999; Christiansen et al., 2000, LaBonne and Bronner-Fraser, 1999). Even though NRG1 expression at the site of neural crest cell origin in the dorsal part of the neural tube has been reported (Meyer and Birchmeier, 1995) no involvement of NRG1 in neural crest induction or delamination has been described to date.

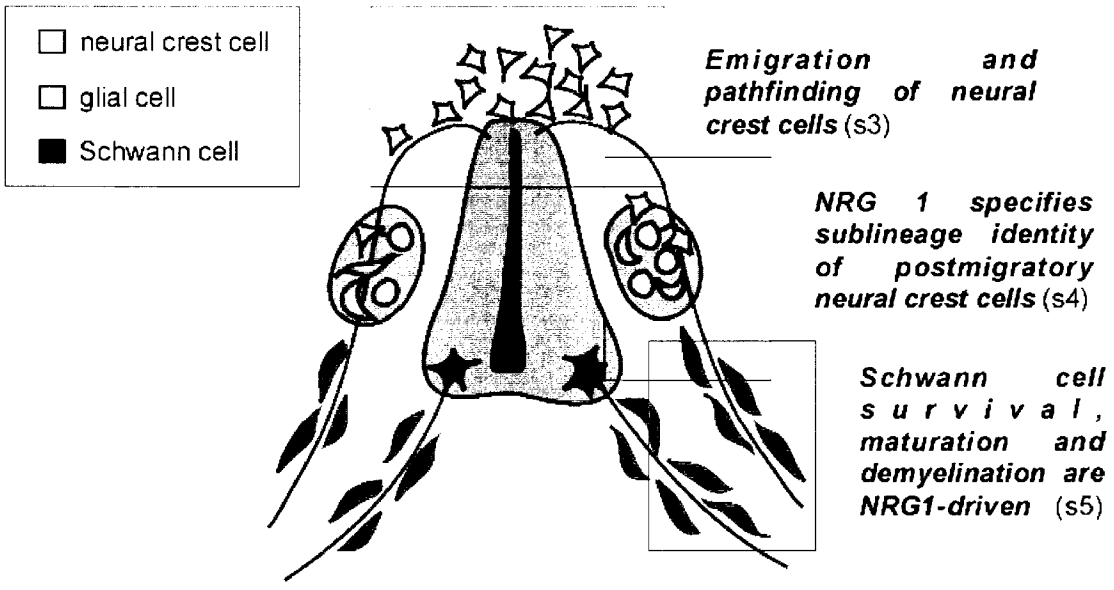



**Figure 4:** Neural crest cells and their derivatives in the developing peripheral nervous system

Schematic representation showing a crossection through the dorsal trunk of a developing vertebrate embryo. Depicted are the neural tube (grey, with motoneurons), the dorsal root ganglia (grey, with sensory neurons) and the glial neural crest cell derivatives. NRG1-related developmental events that are discussed in sections 3 to 5 of this review are enframed: **Section 3** (s3) discusses NRG1 activity in the context of neural crest cell migration. **Section 4** (s4) concerns NRG1-dependent glial lineage determination and differentiation of postmigratory neural crest cells. **Section 5** (s5) deals with NRG1 functions in Schwann cell survival and discusses recent findings that indicate a role for NRG1 in myelination and demyelination processes.

### *3.1. NRG1 type I is required for migration of sympathogenic crest cells*

By the analysis of NRG1, erbB2, and erbB3 mutant mice, however, it became clear that the Neuregulin signaling system is required for appropriate migration of at least a subpopulation of neural crest cells, those which give rise to the sympathetic nervous system (Britsch et al., 1998). Overall, delamination and emigration of the neural crest cells seemed unaffected in the mutants. However, the cells did not reach the anlage of the sympathetic

ganglion chain lateral to the dorsal aorta, but rather clustered in a dorsal position of the embryo. Consistent with this observation, NRG1 type I, the first NRG1 isoform to be expressed at detectable levels (Meyer and Birchmeier, 1995; Meyer et al., 1997; Britsch et al., 1998), is found in the dorsal neural tube, in the mesenchyme along the migration routes, and in the target of the sympathogenic crest cells. Strikingly, homing of sensorigenic and enteric neural crest cells seemed to be unaffected, indicating that migration of this lineages is independent of NRG1 activity. Furthermore, embryos expressing only the NRG1 isoform type III, show a similar phenotype (Britsch et al., 1998). Since NRG1 type II is not expressed in the region of neural crest cell migration, the population giving rise to the sympathetic nervous system must be dependent on type I activity for migration to the sites of terminal differentiation. Nevertheless, NRG1 type II could also be shown to promote neural crest cell migration and survival (Bannerman et al., 2000).

*3.2. Cranial neural crest cell migration potentially depends on NRG1 type I signaling*

The weight of evidence suggests that the sensorigenic neural crest cell lineage in the trunk is independent of NRG1 activity for proper migration to the forming dorsal root ganglion (DRG). In contrast, cranial sensory ganglia of NRG1, erbB2, and erbB3 mutant mice showed severe hypoplasia due to decreased contribution of neural crest cells (Lee et al., 1995; Meyer and Birchmeier, 1995; Erickson et al., 1997; Meyer et al., 1997; Riethmacher et al., 1997; Woldeyesus et al., 1999). Studies on NRG1 isoform-specific mutant mice indicated that expression of NRG1 type I at the sites of ganglia formation is responsible for correct formation of neural crest-derived cranial sensory neurons: Neural crest cells of mice devoid of the type I isoform emerged normally, but were absent in forming cranial ganglia (Kramer et al., 1996; Meyer et al., 1997). Furthermore, sensory neurons were generated normally in mice lacking the isoform type III only (Wolpowitz et al., 2000). Together, these findings are consistent with a role of NRG1 type I in migration of cranial neural crest cells, but could as well be interpreted in terms of promotion of postmigratory neural crest cell survival.

*3.3. NRG1 type I-regulated cell motility is mediated by the receptor erbB2*

The mechanisms by which NRG 1 type I might influence motility of neural crest cells are still unknown. Using breast cancer cells, Hijazi, *et al.*, (2000) presented data suggesting that NRG1 type I can regulate the actin cytoskeleton and induce motility and invasion. Similarly, it was previously shown that NRG1 type I stimulates conversion of globular to filamentous actin, formation of lamellipodia, and cell migration of non-invasive breast cancer MCF-7 cells (Adam et al., 1998). These effects were shown to be regulated by the p21-activated kinase1 (PAK1) through phosphatidylinositol-3 kinase (PI-3) activation. Inhibition of either PAK1, PI-3, or erbB2 receptor activity blocked NRG1 type I-induced cell migration (Adam et al., 1998). That NRG1 type I acts on cell migration was supported by the observation that Neuregulin promotes physical interaction between PAK1, actin and erbB2 receptor. Likewise, Neuregulin-mediated activation of ectopic heterodimeric receptors erbB2/erbB3 was reported to trigger PI-3 kinase-dependent cell motility of CHO and 32D myeloid cells (Chausovsky et al., 2000). In addition, constitutive activation of the Neuregulin receptor erbB2 in MDCK epithelial cells induces breakdown of cell-cell junctions, reorganization of the actin cytoskeleton, and leads to increased cell motility (Khoury et al., 2001). Although some of these studies were performed in the context of breast cancer progression, it is conceivable that at least in a subpopulation of forming neural crest cells Neuregulin activity might play a role in processes of epithelial to mesenchymal transition or in migration, events that share common features with invasive behavior of cancerogenous cells.

*3.4. Is Ets-1 a downstream target of NRG1 during emigration of neural crest cells?*

Detailed analysis of the developmental expression patterns of several members of the Ets family of transcription factors revealed striking co-localization with expression of NRG1 (Mercader et al., unpublished data). In addition, NRG1-regulated expression of members of the Pea-3 subfamily was observed *in vitro* (Hagedorn et al., 2000b). Ets-1, a member of a Pea-3-related Ets gene subfamily, was shown to be expressed during emigration of neural

crest cells (Maroulakou et al., 1994; Fafeur et al., 1997) and to be an actor in the process of epithelial cell dissociation (Fafeur et al., 1997). This finding, together with the observed co-localization of Ets-1 and Neuregulin in the dorsal neural tube and along the migrational pathways of crest cells, suggests that Ets-1 may be a target of NRG1 type I signaling during emigration of the neural crest. Alternatively, Ets-1 might modify Neuregulin activity by controlling erbB2 expression. This hypothesis would be consistent with the observation that Ets transcription factor binding sites were found in the enhancer region of the NRG1 receptor gene erbB2 (Scott et al., 2000). Investigating the responsiveness to Neuregulin of neural crest cells from Ets-1 knockout mice and investigating Ets-1 expression in Neuregulin knockout mice might help to resolve the relationship of Ets-1 and NRG1 activities during neural crest emigration.

*3.5. Neuregulin 1-induced cell migration might be controlled by Sox10 activity*

Investigations on sox10 deficient mice revealed that sox10 controls the expression of the high affinity NRG1 receptor erbB3 in cells where the genes are coexpressed, leading to similar phenotypes in erbB3 and sox10 mutant mice (Britsch et al., 2001). Downregulation of erbB3 due to loss of sox10 function leads to a loss of sensitivity to NRG1 activity (Paratore et al., 2001). It is conceivable that *in vivo*, responsiveness of at least a subpopulation of neural crest cells (cells of the cranial ganglia and sympathogenic neural crest which are absent in Sox10-deficient mice) to Neuregulin signaling is conferred by sox10, thus controlling aspects of crest cell migration. That erbB receptor activity is involved in cell motility was nicely demonstrated by Morris, *et al.*. Migration of Schwann cell precursors out of cultured erbB2-deficient DRGs was clearly impaired when compared to wildtype control (Morris et al., 1999; for detailed discussion of NRG1-induced Schwann cell migration, see below).

Taken together, the studies and observations discussed above suggest that early migration of sympathogenic (and cranial) neural crest cells is directly controlled by NRG1 type I signaling and mediated through erbB2/erbB3-induced PI3 activation. To further elucidate the function of NRG1 in neural

crest cell migration, and possibly in induction and delamination, it will be of interest to ectopically express distinct isoforms at various sites in the developing embryo.

*3.6. Patterning of cranial neural crest cells requires the NRG1 receptor erbB4*

Analysis of erbB4 deficient mice suggested that Neuregulin also plays a crucial role in the patterning of migrating cranial neural crest cells (Golding et al., 2000; Gassmann et al., 1995). ErbB4 receptor expressed in rhombomeres r3 and r5 of the developing hindbrain was proposed to bind and thereby sequester NRG1 type I diffusing from the neighboring rhombomeres r2, r4, and r6. In this model, erbB4-depleting activity would hinder emigrating crest cells of the adjacent rhombomeres from migrating in the mesenchyme laterally to the erbB4 expressing rhombomeres, thus segregating the r2 and r4-derived crest streams (Wehrle-Haller and Weston, 1997). However, this model can not explain all of the observations: Even if NRG1 type I is sequestered in rhombomeres 3 and 5 (Wang et al., 2001), one would expect to find migrating cells in the mesenchyme *adjacent* to these erbB4-positive rhombomeres (due to absence of neutralizing erbB4 activity), which is not the case. Previous transplantation studies (Farlie et al., 1999; Sechrist et al., 1994; Golding et al., 2000) are more consistent with the existence of an indirect patterning mechanism: Interaction of NRG1 type I and erbB4 at the borders of adjacent rhombomeres would elicit a putative repulsive patterning cue that accumulates in the mesenchyme lateral to r3, thus segregating streams of migrating neural crest cells (Golding et al., 2000). Further investigations will be needed to elucidate the exact mechanisms by which erbB4 affects pathfinding of neural crest cells.

*3.7. Conclusion*

In spite of the recognized role of NRG1 in migration, the exact mechanism by which Neuregulin affects migration is unknown. However, it appears that Neuregulin may control aspects of cytoskeleton reorganization, induction of motility, and maintenance of migration of at least a subpopulation of the neural crest cells by activation of PI-3 and PAK1 kinase pathways.

## *4. Neuregulin 1 specifies sublineage identity of postmigratory crest cells*

Much attention has been paid to the question of whether Neuregulin has an impact on cell fate or differentiation. Experiments with Schwann cell precursors indicated that NRG1 might be involved in differentiation (Dong et al., 1995). Previously, it was shown that neural crest stem cells commit to a glial fate when cultured in the presence of NRG1 (Shah et al., 1994). Furthermore, the cells were observed to lose their neurogenic capacity when treated with NRG1 (Shah et al., 1994; Shah and Anderson, 1997). However, glial cells can be found in peripheral sensory ganglia of animals that lack functional NRG1-signaling, indicating that for at least a subset of neural crest cells Neuregulin is not required to instruct gliogenesis *in vivo*. The activity more likely is needed for specification of the Schwann cell lineage, which is absent in mutant mice (Meyer and Birchmeier, 1995; Riethmacher et al., 1997; Morris et al., 1999). Firm evidence that NRG1 plays an instructive role in respect to cell lineage commitment *in vivo* is still elusive. Experimental findings that suggest a role for NRG1 in processes of fate acquisition are discussed below.

### *4.1. Fate acquisition is a context-dependent process*

Initial steps of neural fate segregation are likely to take place in postmigratory multipotent crest cells of forming peripheral ganglia under the influence of the Numb/Notch signaling system. It has been suggested that lineage commitment starts with an asymmetric cell division, leading to Numb activity over a threshold value that might be the key event in generating first neurons (Wakamatsu et al., 2000). Subsequent Delta1 expression in these prospective neurons mediates Notch activation in neighboring cells of the developing ganglion. Interestingly, activated Notch *in vitro* not only inhibits neuronal differentiation (Wakamatsu et al., 2000) but also irreversibly instructs multipotent crest cells to enter the glial lineage (Morrison et al., 2000). Further,

*in vitro* it deprives the cells of their responsiveness to BMP, a strong neurogenic signal that itself was shown to override the gliogenic effect of NRG1 (Shah and Anderson, 1997). Whether the reported gliogenic promotion by Notch reflects an instructive cue or a permissive cue rendering the cells susceptible to gliogenic factors and/or insensitive to neurogenic factors remains to be elucidated. In any event, together these observations raise the question of how coexistence of glial, neuronal, and progenitor cells in the same ganglion is possible and how acquisition of alternative fates can occur in parallel. Presumably, potent instructive signals such as those mediated by Delta, BMP or TGFβ have to be regulated and modulated by antagonistic and synergistic mechanisms that then might account for the complex constitution of a peripheral ganglion. In this context it was found that NRG1 attenuates neuronal induction of cultured neural crest cells by low concentrations of BMP (Shah and Anderson, 1997), indicating that instructive signals to a certain extent might modulate and/or counterbalance each other. According to this model, it was found that neurons, which express the Notch ligand Delta also express the neuron-inducing factor TGFβ (Hagedorn et al., 2000a). In addition, the local environment of a multipotent neural crest cell was shown to be crucial for the cell's fate choice, a phenomenon referred to as a community effect (Gurdon et al., 1993): single progenitor cells generate smooth muscle in response to TGFβ, whereas clusters of progenitors produce neurons. Higher doses of TGFβ induce apoptosis in communities while single cells remain non-neural (Hagedorn et al., 1999, 2000a). This context-dependent interpretation of instructive signals was also observed in conjunction with NRG1-driven glial commitment of neural crest cells. Single neural crest cells as well as communities of crest cells predominantly undergo gliogenesis, when cultured in the presence of NRG1 type II. In the absence of one Sox10 allele, however, single cells acquire a non-neural fate while clusters retain their gliogenic response to NRG1 type II signaling (Paratore et al., 2001). This indicates that the interpretation of NRG1 signaling is also context-dependent and confirms that communities provide the cells the opportunity to differentially interpret instructive signals.

In summary, lineage commitment of multipotent neural crest cells is to an increasing degree being recognized as the result of an interplay among multiple cell intrinsic and extrinsic factors. The understanding of these phenomena requires both *in vivo* and *in vitro* investigations.

*4.2. Satellite cell specification is regulated by NRG1 type II*

Cells of the peripheral ganglia were shown to express the Ets-domain transcription factor Erm (Hagedorn et al., 2000b), and Erm expression serves, both *in vivo* and *in vitro*, as a marker to distinguish satellite cells, which are Erm positive, from Schwann cells, which are Erm negative. It could be demonstrated that the glial cells which are generated from NRG1 type II-treated neural crest cell cultures express Erm and therefore display a satellite phenotype (Hagedorn et al., 2000b). Moreover, NRG1 type II was shown to regulate Erm expression *in vitro* (Hagedorn et al., 2000b). Following this observations, it is tempting to suggest that *in vivo* NRG1 type II might control the glial subtype identity by promoting terminal differentiation into satellite cells (Wakamatsu et al., 2000). So far, it is not clear whether the glial cells present in the peripheral ganglia of NRG1, erbB2, or erbB3-deficient mice display a disturbance in development of satellite glia, a question which could be addressed by testing these cells for Erm expression. Interestingly, dominant negative repression of Erm activity in NRG1 type II-treated neural crest cell cultures led to decreased mitosis, but glial fate acquisition was not affected. Surprisingly, decreased neurogenesis was observed, suggesting that Erm is a downstream target of additional factors, other than NRG1 (Paratore et al., submitted). Taken together, these results could indicate that NRG1 type II, though displaying the potential to induce satellite gliogenesis, *in vivo* rather controls proliferation of precursor and/or satellite cells in the developing DRG through Erm activity. In contrast, Shah, *et al.*, observed no increase in proliferation when postmigratory neural crest cells were cultured in medium containing a soluble form of NRG1 type II (1994). Thus, in the context of proliferation, Erm might fulfill NRG1-independent functions. Preliminary observations in gain of function experiments, overexpressing Erm in cultured neural crest cells, indicate that an excess of Erm is not sufficient to increase

the rate of mitosis (Paratore, personal communication). Thus, Erm seems to be a differentially regulated transcription factor, that depending on the intra- and extracellular context displays distinct activities in fate acquisition and proliferation of multipotent precursor cells. The data suggest that at least some of these activities are regulated by NRG1 type II.

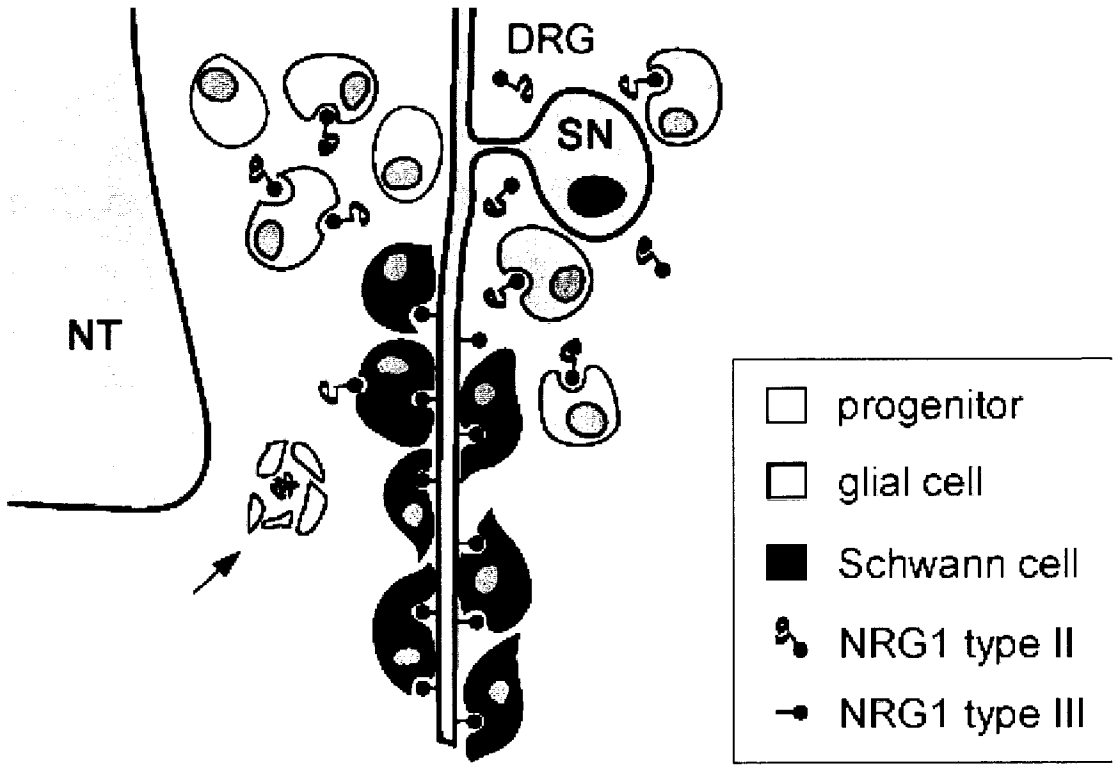
*4.3. The isoform NRG1 type III actively promotes Schwann cell differentiation*

After NRG1 had been described as a mitogen for postnatal Schwann cells (Marchionni et al., 1993), it was found that it might also play an important role in the development of Schwann cell precursors: Administration of soluble forms of NRG1 type I to cultures of freshly isolated Schwann cell precursors both rescued the cells from apoptosis and promoted their maturation (Dong et al., 1995). In accordance with this, Schwann cell precursors were absent in the developing nerve of mice deficient for NRG1 signaling (Meyer and Birchmeier, 1995; Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999). The presence of Schwann cell precursors in the developing nerve of mice lacking NRG1 type I and type II led to the suggestion that NRG1 type III might be the isoform involved in early development of the Schwann cell lineage (Meyer et al., 1997). Specific ablation of the type III isoform was associated with initial presence of Schwann cell precursors along peripheral projections followed by their loss at later stages (Wolpowitz et al., 2000). The rather unexpected existence of precursor cells proximal to dorsal and ventral roots could be due to the remaining NRG1 type II activity that is generated in the neurons of the DRG. Overall, the experiment confirmed the dependence on NRG1 type III for appropriate development of Schwann cell precursors in the peripheral nerve. Yet it left unanswered the question of whether NRG1 type III is solely required for survival of developing Schwann cell precursors or whether it promotes differentiation of the precursors as well. Cultures of endothelin-treated Schwann cell precursors survived for as long as 4 days, but did not differentiate, showing that long-term survival *per se* is not sufficient for Schwann cell maturation (Brennan et al., 2000). Seemingly, the cells require an instructive signal promoting their differentiation. Recent experiments in which soluble forms of NRG1 type III promoted differentiation of Schwann cell

precursors into S100-positive early Schwann cells, indicated that this isoform might be such an instructive factor (Leimeroth et al., submitted). Fate acquisition can be studied independently of survival in cultures of DRG-derived multipotent progenitor cells, thus obviating the difficulties of interpretation presented by experiments with Schwann cell precursors (Hagedorn et al., 1999, 2000b). Progenitor cells did not show any increase in Schwann cell maturation in response to long-term treatment with soluble type III isoform (Leimeroth et al., submitted; Part II of this thesis). Moreover, preliminary studies indicate that in contrast to NRG1 type II the soluble type III isoform is not able to induce the expression of the satellite cell markers Erm or S100 in cells of DRG-derived progenitor cultures (Leimeroth, unpublished data). Surprisingly, when the same cells were exposed to membrane-bound forms of NRG1 type III they developed the molecular features of committed Schwann cells (increased S100 and Oct6 expression) - even in the absence of neurons (for more details, see Part II of this thesis). Thus, *in vitro* NRG1 type II promotes satellite gliogenesis from multipotent progenitors while the membrane-bound type III isoform promotes Schwann cell differentiation. This points out that type II and type III isoforms might be intrinsically different in respect to their functions. When cells were exposed to a combination of membrane-bound type III isoform and soluble type II isoform differentiation into Schwann cells was unaffected, indicating that NRG1 type III overrules the activity of NRG1 type II to induce presumptive satellite fate (Leimeroth et al., submitted; Part II of this thesis). Based on this, we propose a model for Schwann cell precursor recruitment from DRG progenitors: It would predict that as soon as progenitor cells make contact with the axonal roots of sensory neurons in the DRG they undergo type III-driven differentiation needed for appropriate onset of migration into the nerve, despite the presence of type II isoform in the DRG itself (**Figure 5**). This hypothesis is supported by the observation that Schwann cell precursors in culture, unlike DRG precursors, do not respond to NRG1 type II by the upregulation of the satellite marker Erm (Hagedorn et al., 2000b). Possible regulatory mechanisms that might account for the differential response to type II and type III isoforms will be discussed subsequently (see *section 6*).

**Figure 5:** Schwann cell precursors are recruited by axonal NRG1 type III

Schematic diagram of the distinct activities of the NRG1 isoforms type II and type III in the dorsal root ganglion (DRG) and the ventral nerve root. The model suggests a role for NRG1 type III in recruiting Schwann cell precursors from multipotent progenitor cells (or glial cells) in the DRG by inducing their differentiation and subsequently their emigration along the sensory axon (grey, SN). Soluble forms of NRG1 type II might support survival of the precursors in proximal regions of the nerve. A potential *in vivo* role of the type II isoform in satellite specification remains to be elucidated. Cells that do not remain in the proximity to the axon are deprived from the axon-derived survival signals and undergo programmed cell death (cell carcass, arrow). NT, neural tube; DRG, dorsal root ganglion; SN, sensory neuron.



*4.4. Differentiation of Schwann cell precursors as a prerequisite for migration*

Explant cultures of DRG are currently used to study migrational behavior of Schwann cell precursors. Such an *in vitro* assay with mice chimaeric for functional erbB3 revealed a dependence on NRG1 signaling for appropriate population of the developing nerve by Schwann cell precursors (Riethmacher et al., 1997). In accordance with this observation, migration of precursors was severely reduced in explant cultures of mice mutant for erbB2 (Morris et al., 1999). Unexpectedly, NRG1 failed to induce increased precursor migration in wildtype DRG isolated from embryos. However, when DRG derived from neonatal mice were used for the assay, a clear induction of migration was found in response to NRG1 treatment. The increase in motility of Schwann cells derived from newborn animals was observed as well by other groups (Mahanthappa et al., 1996; Meintanis et al., 2001). These findings make it conceivable that Neuregulin in the context of Schwann cell development plays at least two distinct roles which supposedly are tightly linked: a prerequisite for NRG1-driven migration into peripheral nerves might be differentiation to a certain committed stage, a process which on itself is likely regulated by NRG1 activity (Leimeroth et al., submitted).

*4.5. Conclusion*

Overlapping *in vivo* expression of NRG1 type II and type III in sensory neurons of the DRG make it difficult to assign distinct activities to the two isoforms. Yet, as mentioned, *in vitro* studies suggest that type II and type III isoforms display different activities. Besides, each of the isoforms supposedly has a wide scope of functions, that might overlap with activities of the other isoform. This obviously complicates the attempt to take apart the diverse tasks of the NRG1 isoforms.

## 5. Adjusting glial cell numbers by Neuregulin 1 activity

Survival of postmigratory ganglionic and peripheral nerve glial cells seems to be regulated by NRG1. Moreover, Schwann cell survival and proliferation along peripheral nerves appears to be tightly defined by the axons of motor and sensory neurons. Axonally-derived NRG1 might thus control Schwann cell numbers in the nerve.

### *5.1. Axonal NRG1 regulates proliferation of Schwann cell precursors and Schwann cells*

Apart from basic fibroblast growth factor (Jessen et al., 1994), axon-associated NRG1 was suggested to display the signal that regulates survival and mitosis of Schwann cells: Schwann cell precursor cells that were cultured in the presence of soluble forms of NRG1 type I showed increased proliferation in a 20hr culturing assay (Dong et al., 1995). Furthermore, Levi et al. showed that addition of erbB2-specific blocking antibody to postnatal Schwann cell cultures in the presence of NRG1 type I resulted in decreased Schwann cell proliferation (Levi et al., 1995). Thereby, erbB2-specific activation through tyrosine kinase phosphorylation was inhibited, suggesting that the mitogenic activity is mediated by the NRG1 receptor erbB2. NRG1 immunoreactivity along the neurites of sensory neurons further suggests, that Schwann cells have to contact the axon in order to receive the mitogenic signal (Morrissey et al., 1995). *In vivo* expression studies, however, suggest that NRG1 type I is unlikely the isoform controling peripheral glial cell numbers. Rather this function is fulfilled by the isoforms type III and type II which are found to be expressed in sensory and motor neurons (Meyer et al., 1997).

*5.2. Neuregulin provides a survival cue for ganglionic postmigratory neural crest cells*

Besides its role in fate specification, Neuregulin was also recognized as a survival factor for postmigratory crest cells. As mentioned above, it was shown that expression of the NRG1 receptor erbB3 is controlled by Sox10 (Britsch et al., 2001), a key regulator in peripheral gliogenesis, which has two distinct effects: Sox10 is required for glial fate acquisition as well as for survival of at least a subpopulation of DRG-derived neural crest cells *in vitro*. Survival of these cells was shown to be mediated by NRG1 type II, presumably via Sox10-dependent expression of erbB3 (Paratore et al., 2001; Britsch et al., 2001). In agreement with this observation, mice deficient for erbB2 displayed increased apoptosis rates of nonneuronal cells within the DRG at E14.5, as compared to controls (Morris et al., 1999). These data suggest a role for NRG1 as a survival factor for a subpopulation of ganglionic postmigratory neural crest cells in dependence of Sox10 activity.

*5.3. NRG1 type II and III as survival factors for migrating Schwann cell precursors*

During population of the developing nerve, Schwann cell precursors are dependent on axonally derived survival and mitogenic cues, amongst them NRG1 (reviewed in Jessen and Mirsky, 1999a). Previous observations described a mitogenic activity of NRG1 that is confined to the axonal surface of sensory neurons and that could be abolished by NRG1 or erbB2-specific antibody (Salzer et al., 1980; Morrissey et al., 1995). Seemingly, *in vitro* proliferation and survival of Schwann cell precursors can be elicited by any of the three isoforms of NRG1 (Dong et al., 1995; Leimeroth et al., submitted; Lobsiger, personal communication). This could indicate a rather unspecific activity provided by the EGF-like domain which all isoforms have in common. Indeed, the domain was reported to be sufficient to induce enhanced proliferation of Schwann cells (Dong et al., 1995; Baek and Kim, 1998). Further, peptides that comprise the EGF-like region only are sufficient to provide specificity for binding and activation of the Neuregulin receptor (Holmes et al., 1992; Barbacci et al., 1995). Analysis of mice that lack the type

III isoform of NRG1 (Wolpowitz et al., 2000) suggests that survival of Schwann cell precursors along the nerve projections is mediated by NRG1 type III and that the isoform type II in the proximal regions is partially able to substitute for type III mediated survival of developing Schwann cells (**Figure 3**). Beside the membrane-associated activity, the cleaved extracellular domain of NRG1 type III may serve as paracrine survival factor for migrating Schwann cell precursors (Wang et al., 2001; see subsequent chapter).

*5.4. Neuregulin 1 is a survival cue for perinatal Schwann cells*

Prevention of Schwann cell death by NRG1 activity was not only seen in precursor cultures but was also observed in cultures of early postnatal Schwann cells (Syroid et al, 1996). The Schwann cells dependency on axonally derived survival factors extends to the first postnatal weeks. *In vivo*, this might provide a mechanism that allows to adjusting the number of Schwann cells that align the axons. Lack of survival factors could account for apoptosis of perinatal Schwann cells, which was observed in normal development and to an increased degree after axotomy (Grinspan et al., 1996). Naturally occuring apoptosis of Schwann cells during early postnatal development was lowered by injection of NRG1 type II. Surprisingly, even increased apoptosis as a result of axotomy during early postnatal development could be markedly inhibited by exogenous Neuregulin (Grinspan et al., 1996). Preceding experiments had shown that reinnervation and axonal sprouting normally occurring in axotomized adult animals were absent when neonatal muscles were denervated (Trachtenberg and Thompson, 1996). Here as well, increased apoptotic Schwann cell death was abolished by injection of NRG1 type II. These findings indicate that *in vivo* perinatal Schwann cell development depends on axon-Schwann cell interactions and that axonal NRG1 is at least required for survival.

*5.5. Axonal NRG1 adjusts Schwann cell numbers along the axon*

Together with the *in vitro* observation that axonally derived NRG1 has a mitogenic effect on Schwann cells, the role of NRG1 in survival suggests that NRG1 is involved in the process of adjusting the number of Schwann cells

along the sensory and motor axons: the availability of NRG1 near the axon might account for whether a Schwann cell survives and proliferates or whether it undergoes apoptosis. Interestingly, it was found that NRG1 type III, in contrast to the isoforms type I and II, does not bind heparin, a compound of the extracellular matrix (Burgess et al., 1995; Holmes et al., 1992; Loeb et al., 1995; Sudhalter et al., 1996; Schroering and Carey, 1998). In the context of neuromuscular junction (NJM) formation, it was proposed that once NRG1 is released from the cell surface it accumulates in the ECM by binding heparin leading to a local increase in NRG1 concentration at the NMJ. As a consequence, the increased levels of the factor were suggested to maintain a high density of AChRs on the receptor cell side (Adlkofer and Lai, 2000). Conceivably, the inability of NRG1 type III to bind heparin might prevent concentration of shedded factor aside the axon. Accordingly, developing Schwann cells would neither survive nor proliferate unless they have close axonal contact. This hypothesis is in accordance with the observation that *in vitro* the process of membrane-bound NRG1 type III release by proteolytic shedding is not highly active (Schroering and Carey, 1998). High levels of NRG1 type III solely available at the axonal surface would allow a tight regulation of Schwann cell numbers and development in the nerve.

*5.6. Schwann cell-derived NRG1 might be part of an autocrine survival loop*

Axonally derived survival signals seem to loose their importance during Schwann cell development. Autocrine survival activity was shown to be the mechanism by which Schwann cells are able to maintain their own survival even in the absence of axons (Meier et al., 1999; Cheng et al., 1998). Studies performed by Rosenbaum et al. (1997) suggest that NRG1 isoforms might play a role in mediating *in vitro* survival by an autocrine loop. This would be in contradiction to the observation that the expression of the NRG1 receptor erbB2 is drastically reduced in myelinating and adult Schwann cells (Grinspan et al., 1996). However, it was found that the levels of erbB2/3 expression increase within days, subsequent to nerve transection (Kwon et al., 1997). Furthermore, NRG1 was found to be upregulated by Schwann cells within 3 days after nerve axotomy suggesting that it acts in an autocrine and/or

paracrine manner (Carroll et al., 1997). Together these findings show that Schwann cells, as they progress during development, likely loose their dependency on erbB2/3-mediated NRG1 activity, a process which is reversed in response to nerve transection. The autocrine survival loop displayed by adult Schwann cells makes sense in the light of nerve regeneration: the autocrine and/or paracrine activity renders the Schwann cells independent of axonally derived survival factors and allows nerve regeneration in the absence of the axon. Possible mechanisms that might regulate axon-independent Schwann cell survival are considered in more detail in several reviews (Adlkofer and Lai, 2000; Jessen and Mirsky, 1999a; Jessen and Mirsky, 1999b).

## *6. Schwann cell maturation and demyelination - Neuregulin 1-driven processes*

Interestingly, NRG1 activity was not only shown to play an important role during Schwann cell migration and survival along the developing nerve but likewise has to an increasing degree been associated with aspects of nerve myelination and regeneration.

### *6.1. The degree of myelination is dependent on the NRG1 signaling*

The process of myelination and the factors involved therein have been extensively discussed (reviewed in Jessen and Mirsky, 1998, 1999a; Wegner, 2000a, 2000b; Mirsky et al., 2001). Recent findings have ascribed a role in myelination to NRG1. With the help of a *Krox20-cre* allele, ablation of *erbB2* was restricted to Schwann cells. While Schwann cells in the roots of mutant mice loose their functional erbB2 already at E11, cells of the peripheral nerve become erbB2 deficient perinatally (Garratt et al., 2000a). Mutant animals display a peripheral neuropathy that is marked by abnormally thin myelin sheaths, with a decreased number of myelin wraps. It was speculated whether the relative amount of Neuregulin and other trophic factors at the axonal

surface might provide the signal responsible for the degree of myelination (Garratt et al., 2000a; Garratt et al., 2000b). In any case, these findings demonstrate, that at least some of the developmental events that take place after the transition to a committed Schwann cell are dependent on NRG/erbB signaling. In further accordance with a role of NRG1 in myelination, it was found that Brn-5, a POU gene present in myelinating Schwann cells, is upregulated when Schwann cells are cultured in the presence of NRG1 type II. Increased levels of the factor, however, inhibit Brn-5 expression (Wu et al., 2001) indicating that Neuregulin might as well maintain cells in a unmyelinated state, dependent on its concentration.

*6.2. A role for NRG1 in nerve regeneration?*

NRG1 was proposed by different authors to be involved in nerve regeneration. Mahanthappa et al. for instance, reported that NRG1 type II in explant cultures led to increased neurite outgrowth. Subsequent experiments suggested that the growth promoting activity was mediated by the Schwann cells present along the neurites (Mahanthappa et al., 1996). Li et al. suggested that responsiveness of chronically denervated Schwann cells to signals that derive from regrowing axons might increase after exposure to NRG1 type II, thereby promoting nerve regeneration (Li et al., 1998). In accordance with this, decreased levels of NRG1 in axons of patients with chronic axonal neuropathies were observed suggesting that axonally derived NRG1 might support denervated Schwann cells and thus indirectly enhance nerve regeneration (Oka et al., 2000). Similarly, lesions in peripheral nerves of adult erbB2 mutant mice were – in contrast to wildtype counterparts - not repaired indicating that the Neuregulin/erbB signaling system provides the factors at least needed for Schwann cell regeneration (Garratt et al., 2000a). Evidence for a direct enhancing effect of NRG1 on remyelination comes from studies with central nervous system models showing that myelin regeneration by oligodendrocytes could be enhanced in the presence of NRG1 type II (Cannella et al., 1998; Marchionni et al., 1999). In contrast, NRG1 was found to inhibit myelination when added to neuron-Schwann cell cocultures. When myelinated cultures were treated with NRG1 they displayed demyelination

associated by dedifferentiation of Schwann cells to a promyelinating stage. Furthermore, a substantial number of dedifferentiated cells reentered the cell cycle (Zanazzi et al., 2001).

These somehow contradictory findings might be explained as follows: normal levels of NRG1 may promote aspects of myelination while increased levels of NRG1, present after nerve injury, might favor demyelination and proliferation of dedifferentiated Schwann cells thus supporting nerve regeneration.

## *7. Cellular functions of Neuregulin 1 in Schwann cell development; a concluding comment*

In summary, we propose a model for multiple Neuregulin functions in Schwann cell development. Axonal NRG1 provides the signal that promotes differentiation of multipotent progenitor cells into committed Schwann cells and supports emigration of the latter into the periphery of nerve projections (**Figure 5**). During early stages of Schwann cell development, axonally derived NRG1 contributes to survival and proliferation of precursors and committed Schwann cells, thus adjusting cell numbers along the axon. At later stages survival is mediated by Schwann cell derived para-/autocrine survival factors, possibly including NRG1. Eventually, NRG1 does regulate aspects of Schwann cell myelination, demyelination and regeneration in a concentration-dependent manner: increasing levels of Neuregulin activity seem to favor the unmyelinated Schwann cell state and promote proliferation of demyelinated cells in order to repair local deficits.

## *8. Differential signaling by Neuregulin 1 isoforms*

The expression of different NRG1 isoforms in the same cell type – such as sensory neurons – make it likely that these isoforms display distinct

activities. In agreement with this consideration, NRG1 isoform-specific mutant mice show different phenotypes (Meyer and Birchmeier, 1995; Meyer et al., 1997; Wolpowitz et al., 2000). Moreover, our own data demonstrate that NRG1 type II and type III are factors that elicit different responses (submitted; see also Part II of this thesis). These findings and the observation that the membrane-bound type III isoform, in contrast to soluble NRG1 isoforms, is able to induce the expression of Schwann cell-specific markers in crest-derived multipotent progenitors raise the question of how specificity of NRG1 signaling is realized.

*8.1. Neuregulins interact with different erbB receptors eliciting distinct responses*

Conceivably, different combinations of Neuregulin receptors of the erbB family could alter the readout after ligand binding. Interestingly, it was found that different Neuregulins are able to activate distinct signaling cascades differing in erbB receptor phosphorylation and signaling molecules (Pinkas-Kramarski et al., 1998; Sweeney et al., 2000; Sweeney et al., 2001). Even though differences in erbB receptor combinations were shown to influence the outcome of NRG1 signaling (reviewed in Burden and Yarden1997; Buonanno and Fischbach, 2001) this regulation mechanism is unlikely to be utilized in the developing peripheral glia in order to modulate NRG1 signaling since solely the receptor heterodimer erbB2/3 was found to mediate NRG1 signaling in glial crest derivatives (Shah et al., 1994; Levi et al., 1995; Grinspan et al., 1996; Vartanian et al., 1997).

*8.2. The way NRG1 is presented or processed might account for isoform-specific effects*

Isoform-specific domains or the orientation of the signaling domain might affect signal interpretation by the signal receiving cell, eliciting distinct signaling pathways. Although many NRG1 isoforms are synthesized as transmembrane proproteins, NRG1 type III is accumulated at the cell surface while the bioactive ectodomain of other NRG1 isoforms appears to be released from the expressing cell (Schroering and Carey, 1998; Wang et al.,

2001). Disruption of the cytoplasmic tail of membrane-anchored Neuregulin isoforms in mice led to similar phenotypes as observed when all Neuregulin isoforms were ablated (Meyer and Birchmeier, 1995; Liu et al., 1998a). Additional experiments had demonstrated that proper release of the bioactive Neuregulin ectodomain of the proprotein is regulated by its cytoplasmic tail (Liu et al., 1998b; Wang et al., 2001). Furthermore, this process of Neuregulin solubilization was suggested to be differentially regulated by metalloproteases (Montero et al., 2000). However, membrane association of NRG1 type III is mediated by an additional hydrophobic domain in its amino-terminal cystein rich domain, resulting in exposure of the C-terminus to the extracellular environment (Schroering and Carey, 1998). Thus, the reverse orientation might affect signal interpretation by the signal receiving cell. Alternatively, persistent or increasing signaling by locally accumulated NRG1 might influence its biological effects. Such mechanisms might account for the observation that, in contrast to soluble NRG1 type III, a membrane-bound form of NRG1 type III actively promoted Schwann cell differentiation from multipotent progenitors (Leimeroth et al., submitted). Together, these findings stress the importance of how NRG1 is presented to the receptors. Apparently, it makes a great difference whether the factors are membrane-bound or processed in order to be shedded.

*8.3. Neuregulin activity is modulated by cofactors*

Conceivably, regulating the expression levels of the erbB receptors might be a mechanism by which Neuregulin signaling can be modified (**Figure 6**). In the context of promoter studies, an Ets binding site was identified in the enhancer region of the Neuregulin 1 receptor gene erbB2 (Scott et al., 2000). Possibly, Ets transcription factors take part in regulating NRG1 signaling by adjusting the availability of the erbB receptor. Furthermore, it was shown that sensitivity to NRG1 activity is dependent on the levels of erbB3, which is regulated by Sox10 (Paratore et al., 2001; Britsch et al., 2001).

Cofactors such as coligands or coreceptors may as well modulate the interpretation of NRG1 signaling. PDZ-containing proteins or the transmembrane protein CD44, for instance, were shown to interact with erbB

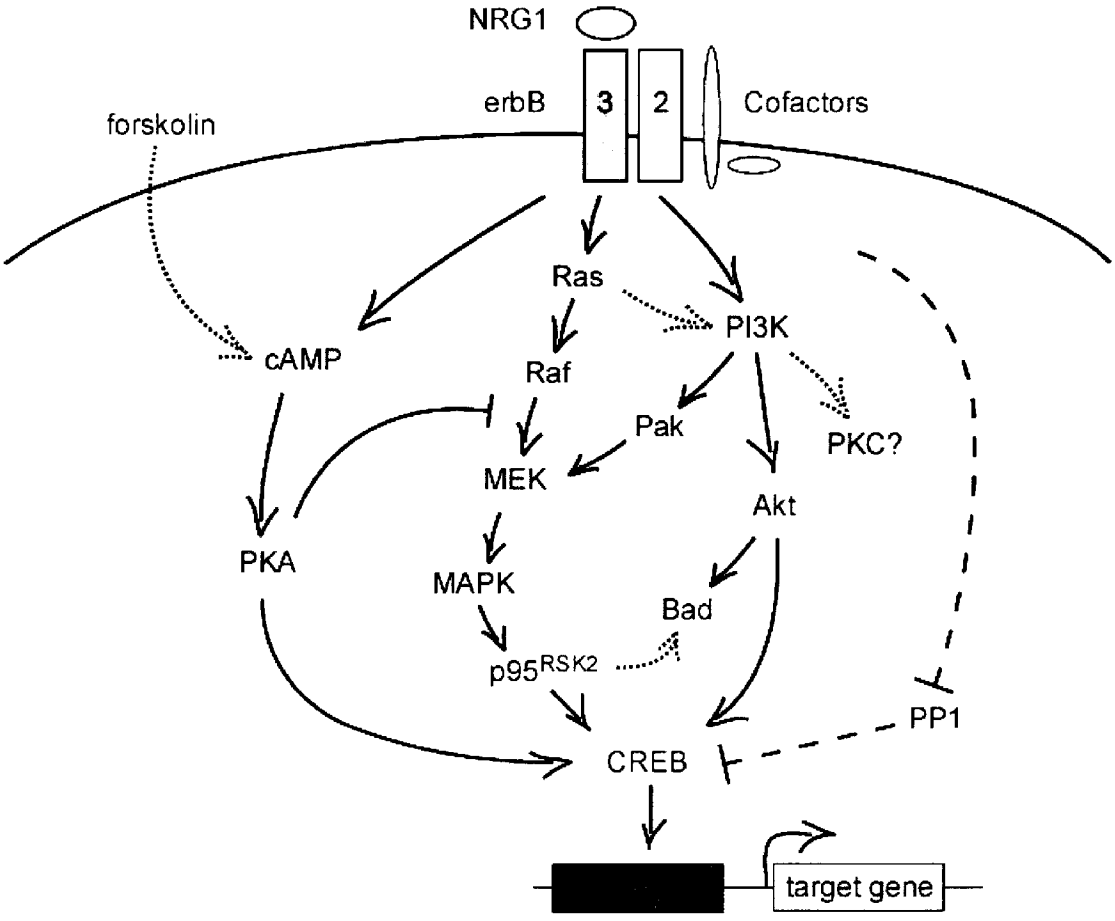
receptors and to alter NRG1 activity (Garcia et al., 2000; Sherman et al., 2000). The association of Neuregulin ligands and receptors with the extracellular matrix and integrins, respectively, might sequester Neuregulin signaling to specific subcellular localizations and influence signal transduction (Loeb and Fischbach, 1995; Gambaletta et al., 2000).

*8.4. Activation of different signaling pathways by distinct NRG1 isoforms*

Modulation of Neuregulin signaling might underlie the differential pathway usage observed in postnatal Schwann cell survival, proliferation, motility and myelination (Maurel and Salzer, 2000; Li et al., 2001; Meintanis et al., 2001). While NRG1 type II activates both the MAP kinase and PI3 kinase pathway to support survival (Maurel and Salzer, 2000; Li et al., 2001), NRG1 type I only signals through the MAP kinase pathway to promote Schwann cell motility (Meintanis et al., 2001). Furthermore, only PI3 kinase pathway is required for axon mediated survival, proliferation and initial myelination (Maurel and Salzer, 2001). Promyelinating Schwann cells express the transcription factor Oct-6 which is regulated by the adenylyl cyclase-protein kinase A (PKA) pathway (Lemke and Chao, 1988; Monuki et al., 1989). When neural crest-derived multipotent progenitors are cultured in the presence of forskolin, an activator of the adenylate cyclase, they upregulate Oct-6 (Hagedorn et al., 2000b). Oct-6 upregulation was as well observed, when these progenitors were exposed to membrane-bound NRG1 type III (Leimeroth et al., submitted) indicating that signaling of this isoform might be performed by the PKA pathway, activating similar target proteins of the signaling cascade. In accordance with this model, it was found in NRG1-treated Schwann cell cultures that the PKA and MAP kinase cascade show an intersection in their signal transduction pathways, activating the common downstream effector CREB (cAMP response element-binding protein) (Kim et al., 1997; Tabernero et al., 1998, **Figure 6**). Thus, it is conceivable that activation of the same receptor heterodimer by similar ligands can lead to distinct cellular responses. However, it remains to be determined by which mechanisms the various activities of the NRG1 isoforms may be translated into differentially acting signaling cascades.

**Figure 6:** Neuregulin elicits various signaling pathways with distinct effects

NRG1 binding to the erbB3 receptor leads to heterodimer formation with erbB2 and to activation of the complex by autophosphorylation. ErbB-mediated signaling is likely to be modulated by interaction with distinct cofactors such as the transmembrane glycoprotein CD44 or PDZ-containing proteins (Garcia et al., 2000; Sherman et al., 2000). The various signaling cascades that are triggered by NRG1 have been implicated in distinct developmental events of Schwann cells. Survival of Schwann cells is mediated through the PI3 kinase pathway leading to Bad and Bcl activation, and thus rescuing the cell from apoptosis (Li et al., 2001). Eventhough the PI3 kinase signals also via the MAPK kinase (MEK), the MAP kinase pathway might only partially provide a survival cue for the cell and rather controls cell motility (Maurel and Salzer, 2000; Meintanis et al., 2001). Schwann cell proliferation as well as initial events of myelination requires PI3 kinase (Maurel and Salzer, 2000). Specific inhibition of the cyclic AMP-dependent protein kinase A (PAK) blocks NRG1-induced Schwann cell proliferation. Parallely, activation of PAK pathway has an inhibitory effect on NRG1-triggered MAP kinase activity (Kim et al., 1997).



Eventhough PKC is in some cases a downstream effector of PI3 kinase (dotted line), its role in NRG1-mediated Schwann cell regulation is not clear. The diverse signaling pathways meet at a common downstream target, the cAMP response element (CRE) binding protein (CREB), which as a result of phosphorylation binds to regulatory elements in the promoter region of various genes and induces their transcription. Conceivably, prolonged phosphorylation of CREB might be maintained by inhibition of CREB-specific phosphatases such as the protein phosphatase 1 (PP1), thus promoting CREB-regulated expression of late response genes involved in differentiation (dashed lines; Tabernero et al., 1998).

Presumably, the cell-extrinsic and cell-intrinsic context specifies which parts of the signaling network are recruited in response to NRG1 receptor activation.

## *9. Conclusion*

A variety of developmental processes in the forming peripheral nervous system are regulated by Neuregulin 1. Even though many aspects of Neuregulin 1 activity remain to be determined it seems clear that the different NRG1 isoforms are intrinsically different, displaying distinct activities. Their differential processing at various sites and different time-points allows regulation of multiple steps in the generation and maintenance of the PNS. *In vivo* and *in vitro* approaches together revealed many facets of Neuregulin signaling: NRG1 activity was described to regulate migration of neural crest cells and Schwann cells. Glial fate acquisition was shown to be promoted by NRG1. Axonally-derived NRG1 might initiate Schwann cell development and regulates Schwann cell number along the nerve, supplying a survival cue for Schwann cell precursors. Furthermore, aspects of Schwann cell myelination are controlled by axonal NRG1. The way of how NRG1 is presented to the signal-receiving cell was shown to have an impact on the signaling outcome. As part of a signaling network, Neuregulin conceivably interacts with different cofactors that modulate its varying isoform activities. Thus, specification of the glial lineage is regulated by an interplay of different factors acting in a context-dependent manner. To approach the problem of how the different players of the Neuregulin signaling network interact and regulate development and

maintenance of the peripheral nervous system, further *in vivo* and *in vitro* experiments will be required. In this context, cell culture experiments performed with cells derived from specific mutants might allow insight into often complex phenotypes that result from genetic knockout approaches.

# REFERENCES PART I


Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J., Kumar, R. (1998). Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. *J. Biol. Chem.* **273**,28238-46.

Adlkofer, K., and Lai, C. (2000). Role of neuregulins in glial cell development. *Glia* **29**, 104-111.

Baek, S. Y., and Kim, S. U. (1998). Proliferation of human Schwann cells induced by neu differentiation factor isoforms. *Dev. Neurosci.* **20**, 512-517.

Bannerman, P. G., Puhalla, S., Sahai, A., Shieh, A., Berman, M., and Pleasure, D. (2000). Glial growth factor-2 promotes the survival, migration and bromodeoxyuridine incorporation of mammalian neural crest cells in caudal neural tube explant cultures. *Brain Res. Dev. Brain Res.* **124**, 93-99.

Barbacci, E. G., Guarino, B. C., Stroh, J. G., Singleton, D. H., Rosnack, K. J., Moyer, J. D., Andrews, G. C. (1995). The structural basis for the specificity of epidermal growth factor and heregulin binding. *J. Biol. Chem.* **270**, 9585-9589.

Bermingham-McDonogh, O., Xu, Y. T., Marchionni, M. A., and Scherer, S. S. (1997). Neuregulin Expression in PNS Neurons: Isoforms and Regulation by Target Interactions. *Mol. Cell. Neurosci.* **10**, 184-195.

Brennan, A., Dean, C. H., Zhang, A. L., Cass, D. T., Mirsky, R., and Jessen, K. R. (2000). Endothelins control the timing of Schwann cell generation in vitro and in vivo. *Dev. Biol.* **227**, 545-557.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C., and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66-78.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C., and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev.* **12**, 1825-1836.

Buonanno, A., and Fischbach, G. D. (2001). Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr. Opin. Neurobiol.* **11**, 287-296.

Burden, S., and Yarden, Y. (1997). Neuregulins and their receptors: a versatile signalling module in organogenesis and oncogenesis. *Neuron* **18**, 847-855.

Burgess, T. L., Ross, S. L., Qian, Y. X., Brankow, D., and Hu, S. (1995). Biosynthetic processing of neu differentiation factor. Glycosylation trafficking, and regulated cleavage from the cell surface. *J. Biol. Chem.* **270**, 19188-19196.

Cannella, B., Hoban, C. J., Gao, Y. L., Garcia-Arenas, R., Lawson, D., Marchionni, M., Gwynne, D., and Raine, C. S. (1998). The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis. *Proc. Natl. Acad. Sci. U S A* **95**, 10100-10105.

Carraway, K. L. III, and Cantley, L. .A. (1994). A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**, 5-8.

Carroll, S. L., Miller, M. L., Frohnert, P. W., Kim, S. S., and Corbett, J. A. (1997). Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. *J. Neurosci.* **17**, 1642-1659.

Chausovsky, A., Waterman, H., Elbaum, M., Yarden, Y., Geiger, B., and Bershadsky, A. D. (2000). Molecular requirements for the effect of neuregulin on cell spreading, motility and colony organization. *Oncogene* **19**, 878-888.

Cheng, L., Esch, F. S., Marchionni, M. A., and Mudge, A. W. (1998). Control of Schwann cell survival and proliferation: autocrine factors and neuregulins. *Mol. Cell Neurosci.* **12**, 141-56.

Christiansen, J. H., Coles, E. G., and Wilkinson, D. G. (2000). Molecular control of neural crest formation, migration and differentiation. *Curr. Opin. Cell Biol.* **12**, 719-724.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R., and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-596.

Erickson, S. L., O'Shea, K. S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L. H., and Moore, M. W. (1997). ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development* **124**, 4999-5011.

Fafeur, V., Tulasne, D., Queva, C., Vercamer, C., Dimster, V., Mattot, V., Stehelin, D., Desbiens, X., and Vandenbunder, B. (1997). The ETS1 transcription factor is expressed during epithelial-mesenchymal transitions in the chick embryo and is activated in scatter factor-stimulated MDCK epithelial cells. *Cell Growth Differ* **8**, 655-65.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the Neu ligand family. *Cell* **72**, 801-815.

Farlie, P. G., Kerr, R., Thomas, P., Symes, T., Minichiello, J., Hearn, C. J., and Newgreen, D. (1999). A paraxial exclusion zone creates patterned cranial neural crest cell outgrowth adjacent to rhombomeres 3 and 5. *Dev. Biol.* **213**, 70-84.

Gambaletta, D., Marchetti, A., Benedetti, L., Mercurio, A. M., Sacchi, A., and Falcioni, R. (2000). Cooperative signaling between alpha(6)beta(4) integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. *J. Biol. Chem.* **275**, 10604-10610.

Garratt, A. N., Britsch, S., and Birchmeier, C. (2000a). Neuregulin, a factor with many functions in the life of a Schwann cell. *Bioessays* **22**, 987-996.

Garratt, A. N., Voiculescu, O., Topilko, P., Charnay, P., and Birchmeier, C. (2000b). A dual role of erbB2 in myelination and in expansion of the Schwann cell precursor pool. *J. Cell. Biol.* **148**, 1035-1046.

Garcia, R. A., Vasudevan, K., and Buonanno, A. (2000). The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. *Proc. Natl. Acad. Sci. USA* **97**, 3596-3601.

Garcia-Castro, M., and Bronner-Fraser, M. (1999). Induction and differentiation of the neural crest. *Curr. Opin. Cell Biol.* **11**, 695-698.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.

Golding, J. P., Trainor, P., Krumlauf, R., and Gassmann, M. (2000). Defects in pathfinding by cranial neural crest cells in mice lacking the neuregulin receptor ErbB4. *Nat. Cell Biol.* **2**, 103-109.

Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M., and Scherer, S. S. (1996). Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *J. Neurosci.* **16**, 6107-6118.

Gurdon, J. B., Lemaire, P., and Kato, K. (1993). Community effects and related phenomena in development. *Cell* **75**, 831-834.

Hagedorn, L., Floris, J., Suter, U., and Sommer, L. (2000a). Autonomic neurogenesis and apoptosis are alternative fates of progenitor cell communities induced by TGFbeta. *Dev. Biol.* **228**, 57-72.

Hagedorn, L., Paratore, C., Brugnoli, G., Baert, J. L., Mercader, N., Suter, U., and Sommer, L. (2000b). The Ets domain transcription factor Erm distinguishes rat satellite glia from Schwann cells and is regulated in satellite cells by neuregulin signaling. *Dev. Biol.* **219**, 44-58.

Hagedorn, L., Suter, U., and Sommer, L. (1999). P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-ß family factors. *Development* **126**, 3781-3794.

Hijazi, M. M., Thompson, E. W., Tang, C., Coopman, P., Torri, J. A., Yang, D., Mueller, S. C., and Lupu, R. (2000). Heregulin regulates the actin cytoskeleton and promotes invasive properties in breast cancer cell lines. *Int. J. Oncol.* **17**, 629-641.

Ho, W.-H., Armanini, M. P., Nuijens, A., Phillips, H. S., and Osheroff, P. L. (1995). Sensory and motor neuron-derived factor. A novel heregulin variant highly expressed in sensory and motor neurons. *J. Biol. Chem.* **270**, 14523-14532.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W. J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205-1210.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. (1994). The Schwann cell precursor and its fate: a

study of cell death and differentiation during gliogenesis in rat embryonic nerves. *Neuron* **12**, 509-527.

Jessen, K. R., and Mirsky, R. (1998). Origin and early development of Schwann cells. *Microsc. Res. Tech.* **41**, 393-402.

Jessen, K. R., and Mirsky, R. (1999a). Schwann cells and their precursors emerge as major regulators of nerve development. *Trends Neurosci.* **22**, 402-410.

Jessen, K. R., and Mirsky, R. (1999b). Why do Schwann cells survive in the absence of axons? *Ann. N Y Acad. Sci.* **883**, 109-115.

Jones, J. T., Akita, R. W., and Sliwkowski, M. X. (1999). Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett.* **447**, 227-231.

Khoury, H., Dankort, D. L., Sadekova, S., Naujokas, M. A., Muller, W.J., and Park, M.(2001). Distinct tyrosine autophosphorylation sites mediate induction of epithelial mesenchymal like transition by an activated ErbB-2/Neu receptor. *Oncogene* **20**, 788-99.

Kim, H. A., DeClue, J. E., and Ratner, N. (1997). cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways. *J. Neurosci. Res.* **49**, 236-247.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E., and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc. Natl. Acad. Sci. USA* **93**, 4833-4838.

Kwon, Y.K., Bhattacharyya, A., Alberta, J. A., Giannobile, W. V., Cheon, K., Stiles, C. D., and Pomeroy, S. L. (1997). Activation of ErbB2 during wallerian degeneration of sciatic nerve. *J. Neurosci.* **17**, 8293-8299.

LaBonne, C., and Bronner-Fraser, M. (1999). Molecular mechanisms of neural crest formation. *Annu. Rev. Cell Dev. Biol.* **15**, 81-112.

Le Douarin, N. M. (1982). *The neural crest.* Cambridge University Press, Cambridge, UK.

Le Douarin, N. M., and Kalcheim, C. (1999). *The Neural Crest.* New York: Camebridge University Press.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398.

Leimeroth, R., Lobsiger, C., Lüssi, A., Taylor, V., Suter, U., and Sommer, L. (2002). Membrane-bound neuregulin 1 type III actively promotes Schwann cell differentiation of multipotent progenitor cells. *Submitted.*

Lemke, G., and Chao, M. (1988). Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development* **102**, 499-504.

Levi, A. D., Bunge, R. P., Lofgren, J. A., Meima, L., Hefti, F., Nikolics, K., and Sliwkowski, M. X. (1995). The influence of heregulins on human Schwann cell proliferation. *J. Neurosci.* **15**, 1329-1340.

Li, H., Wigley, C., and Hall, S. M. (1998). Chronically denervated rat Schwann cells respond to GGF in vitro. *Glia* **24**, 290-303.

Li, Y., Tennekoon, G. I., Birnbaum, M., Marchionni, M. A., and Rutkowski, J. L. (2001). Neuregulin signaling through a PI3K/Akt/Bad pathway in Schwann cell survival. *Mol. Cell. Neurosci.* **17**, 761-767.

Liu, X., Hwang, H., Cao, L., Buckland, M., Cunningham, A., Chen, J., Chien, K. R., Graham, R. M., and Zhou, M. (1998a). Domain-specific gene disruption reveals critical regulation of neuregulin signaling by its cytoplasmic tail. *Proc. Natl. Acad. Sci. U S A* **95**, 13024-13029.

Liu, X., Hwang, H., Cao, L., Wen, D., Liu, N., Graham, R. M., and Zhou, M. (1998b).

Release of the neuregulin functional polypeptide requires its cytoplasmic tail. *J Biol Chem.* **273**, 34335-34340.

Loeb, J. A., and Fischbach, G. D. (1995). ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. *J. Cell Biol.* **130**, 127-135.

Mahanthappa, N. K., Anton, E. S., and Matthew, W. D. (1996). Glial growth factor 2, a soluble neuregulin, directly increases Schwann cell motility and indirectly promotes neurite outgrowth. *J. Neurosci.* **16**, 4673-4683.

Marchionni, M. A., Cannella, B., Hoban, C., Gao, Y. L., Garcia-Arenas, R., Lawson, D., Happel, E., Noel, F., Tofilon, P., Gwynne, D., and Raine C. S. (1999). Neuregulin in neuron/glial interactions in the central nervous system. GGF2 diminishes autoimmune demyelination, promotes oligodendrocyte progenitor expansion, and enhances remyelination. *Adv. Exp. Med. Biol.* **468**, 283-295.

Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., and Kobayashi, K. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-318.

Marikovsky, M., Lavi, S., Pinkas, K. R., Karunagaran, D., Liu, N., Wen, D., and Yarden, Y. (1995). ErbB-3 mediates differential mitogenic effects of NDF/heregulin isoforms on mouse keratinocytes. *Oncogene* **10**, 1403-1411.

Maroulakou, I. G., Papas, T. S., and Green, J. E. (1994). Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. *Oncogene* **9**, 1551-1565.

Maurel, P., and Salzer, J. L. (2000). Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity. *J. Neurosci.* **20**, 4635-4645.

Meier, C., Parmantier, E., Brennan, A., Mirsky, R., and Jessen, K. R. (1999). Developing Schwann cells acquire the ability to survive without axons by establishing an autocrine circuit involving insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB. *J .Neurosci.* **19**, 3847-3859.

Meintanis, S., Thomaidou, D., Jessen, K. R., Mirsky, R., and Matsas, R. (2001). The neuron-glia signal beta-neuregulin promotes Schwann cell motility via the MAPK pathway. *Glia* **34**, 39-51.

Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L. E., and Birchmeier, C. (1997). Isoform-specific expression and function of neuregulin. *Development* **124**, 3575-3586.

Mirsky, R., Parkinson, D. B., Dong, Z., Meier, C., Calle, E., Brennan, A., Topilko, P., Harris, B. S., Stewart, H. J., and Jessen, K. R. (2001). Regulation of genes involved in Schwann cell development and differentiation. *Prog. Brain Res.* **132**, 3-11.

Monuki, E. S., Weinmaster, G., Kuhn, R., and Lemke, G. (1989). SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* **3**, 783-793.

Montero, J. C., Yuste, L., Diaz-Rodriguez, E., Esparis-Ogando, A., and Pandiella, A. (2000). Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor-alpha-converting enzyme. *Mol. Cell Neurosci.* **16**, 631-648.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D., and Lee, K. F. (1999). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273-283.

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G., and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.

Morrissey, T. K., Levi, A. D., Nuijens, A., Sliwkowski, M. X., and Bunge, R. P. (1995). Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. *Proc. Natl. Acad. Sci. U S A* **92**, 1431-1435.

Oka, N., Kawasaki, T., Matsui, M., Tachibana, H., Sugita, M., and Akiguchi, I.(2000). Neuregulin is associated with nerve regeneration in axonal neuropathies. *Neuroreport* **11**, 3673-3676.

Paratore, C., Brugnoli, G., Lee, H. Y., Suter, U., and Sommer, L. (2002). Differential roles of the Ets domain transcription factor Erm in neuronal fate decision and glial proliferation of neural crest stem cells. *Submitted.*

Paratore, C., Goerich, D. E., Suter, U., Wegner, M., and Sommer, L. (2001). Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* **128**, 3949-3961.

Pinkas-Kramarski, R., Shelly, M., Guarino, B. C., Wang, L. M., Lyass, L., Alroy, I., Alimandi, M., Kuo, A., Moyer, J. D., Lavi, S., Eisenstein, M., Ratzkin, B. J., Seger, R., Bacus, S. S., Pierce, J. H., Andrews, G. C., Yarden, Y., and Alamandi, M. (1998). ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. *Mol. Cell. Biol.* **18**, 6090-6101.

Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* **15**, 2452-2467.

Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton ,G. W., Rothwell, V.M., and Buckley, S. (1993). Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. *Nature* **366**, 473-475.

Riese, D. J. II, van Raaij, T. M., Plowman, G. D., Andrews, G. C., Stern, D. F. (1995). The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell Biol.* **15**, 5770-5776.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R., and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.

Rosenbaum, C., Karyala, S., Marchionni, M. A., Kim, H. A., Krasnoselsky, A. L., Happel, B., Isaacs, I., Brackenbury, R., and Ratner, N. (1997). Schwann cells express NDF and SMDF/n-ARIA mRNAs, secrete neuregulin, and show constitutive activation of erbB3 receptors: evidence for a neuregulin autocrine loop. *Exp. Neurol.* **148**, 604-615.

Salzer, J. L., Bunge, R. P., and Glaser, L. (1980). Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *J. Cell Biol.* **84**, 767-778.

Schroering, A., and Carey, D. J. (1998). Sensory and motor neuron-derived factor is a transmembrane heregulin that is expressed on the plasma membrane with the active domain exposed to the extracellular environment. *J. Biol. Chem.* **273**, 30643-30650.

Scott, G. K., Chang, C. H., Erny, K. M., Xu, F., Fredericks, W. J., Rauscher, F. III, Thor, A. D., Benz, C.C. (2000). Ets regulation of the erbB2 promoter. *Oncogene* **19**, 6490-502.

Sechrist, J., Scherson, T., and Bronner-Fraser, M. (1994). Rhombomere rotation reveals that multiple mechanisms contribute to the segmental pattern of hindbrain neural crest migration. *Development* **120**, 1777-1790.

Shah, N. M., and Anderson, D. J. (1997). Integration of multiple instructive cues by neural crest stem cells reveals cell-intrinsic biases in relative growth factor responsiveness. *Proc. Natl. Acad. Sci. U S A* **94**, 11369-11374.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* **77**, 349-360.

Sherman, L. S., Rizvi, T. A., Karyala, S., and Ratner, N. (2000). CD44 enhances neuregulin signaling by Schwann cells. *J. Cell. Biol.* **150**, 1071-1084.

Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R.L., and Carraway, K. L. III. (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J Biol Chem.* **269**, 14661-14665.

Sommer, L. (2001). Context-dependent regulation of fate decisions in multipotent progenitor cells of the peripheral nervous system. *Cell Tissue Res.* **305**, 211-216.

Sudhalter, J., Whitehouse, L., Rusche, J. R., Marchionni, M. A., and Mahanthappa, N. K. (1996). Schwann cell heparan sulfate proteoglycans play a critical role in glial growth factor/neuregulin signaling. *Glia* **17**, 28-38.

Sweeney, C., Fambrough, D., Huard, C., Diamonti, A. J., Lander, E. S., Cantley, L. C., and Carraway, K. L., 3rd. (2001). Growth factor-specific signaling pathway stimulation and gene expression mediated by ErbB receptors. *J. Biol. Chem.* **276**, 22685-22698.

Sweeney, C., Lai, C., Riese, D. J., 2nd, Diamonti, A. J., Cantley, L. C., and Carraway, K. L., 3rd. (2000). Ligand discrimination in signaling through an ErbB4 receptor homodimer. *J. Biol. Chem.* **275**, 19803-19807.

Syroid, D. E., Maycox, P. R., Burrola, P. G., Liu, N., Wen, D., Lee, K. F., Lemke, G., and Kilpatrick, T. J. (1996). Cell death in the Schwann cell lineage and its regulation by neuregulin. *Proc. Natl. Acad. Sci. U S A* **93**, 9229-9234.

Tabernero, A., Stewart, H. J., Jessen, K. R., and Mirsky, R. (1998). The neuron-glia signal beta neuregulin induces sustained CREB phosphorylation on Ser-133 in cultured rat Schwann cells. *Mol. Cell. Neurosci.* **10**, 309-322.

Trachtenberg, J. T., Thompson, W. J. (1996). Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature* **379**, 174-177.

Vartanian, T., Goodearl, A., Viehover, A., and Fischbach, G. (1997). Axonal neuregulin signals cells of the oligodendrocyte lineage through activation of HER4 and Schwann cells through HER2 and HER3. *J. Cell Biol.* **137**, 211-220.

Wakamatsu, Y., Maynard, T. M., and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-2821.

Wang, J. Y., Miller, S. J., and Falls, D. L. (2001). The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. *J. Biol. Chem.* **276**, 2841-2851.

Wehrle-Haller, B., and Weston, J. A. (1997). Receptor tyrosine kinase-dependent neural crest migration in response to differentially localized growth factors. *Bioessays* **19**, 337-345.

Wegner, M. (2000a), Transcriptional control in myelinating glia: flavors and spices. *Glia* **31**, 1-14.

Wegner, M. (2000b), Transcriptional control in myelinating glia: the basic recipe. *Glia* **29**, 118-123.

Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Luo, Y., and Yarden, Y. (1992). Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* **69**, 559-72.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P., and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev.* **13**, 2538-2548.

Wolpowitz, D., Mason, T. B., Dietrich, P., Mendelsohn, M., Talmage, D. A., and Role, L. W. (2000). Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. *Neuron* **25**, 79-91.

Wu, R., Jurek, M., Sundarababu, S., and Weinstein, D. E. (2001). The POU gene Brn-5 is induced by neuregulin and is restricted to myelinating Schwann cells. *Mol. Cell Neurosci.* **17**, 683-695.

Yang, X., Kuo, Y., Devay, P., Yu, C., and Role, L. (1998). A cysteine-rich isoform of neuregulin controls the level of expression of neuronal nicotinic receptor channels during synaptogenesis. *Neuron* **20**, 255-270.

Zanazzi, G., Einheber, S., Westreich, R., Hannocks, M. J., Bedell-Hogan, D., Marchionni, M. A., and Salzer, J. L. (2001). Glial growth factor/neuregulin inhibits Schwann cell myelination and induces demyelination. *J. Cell Biol.* **152**, 1289-1299.

Zhao, J. J., and Lemke, G. (1998). Selective disruption of neuregulin-1 function in vertebrate embryos using ribozyme-tRNA transgenes. *Development* **125**, 1899-1907.

# PART II: *MEMBRANE-BOUND NEUREGULIN 1 TYPE III ACTIVELY PROMOTES SCHWANN CELL DIFFERENTIATION OF MULTIPOTENT PROGENITOR CELLS*

## *1. Introduction*

In the first part of this thesis the diverging activities of Neuregulin 1 (NRG1) isoforms are reviewed. As described, the implication of the type III isoform in Schwann cell development was demonstrated by various studies. However, it could not be shown to date, whether NRG1 type III acts as a differentiation cue or whether it is solely a survival cue. Part II of this thesis is focussing on our data, demonstrating that NRG1 type III actively promotes Schwann cell generation of multipotent progenitor cells *in vitro*.

As mentioned, roles for NRG1 signaling in neural development have been analyzed in mice carrying selective mutations of NRG1 ectodomains. Mice homozygous for NRG1 lacking the EGF-like domain common to all isoforms display cardiac malformation and die around embryonic day 10.5 (E10.5) (Meyer and Birchmeier, 1995). Besides considerable loss of neuronal cell populations, they exhibit severely reduced numbers of presumptive glial

cells in cranial ganglia and of Schwann cell precursors along peripheral nerves. Homozygous deletion of the exon encoding the immunoglobulin domain results in the specific loss of NRG1 type I and type II isoforms without affecting expression of NRG1 type III. Mice carrying this mutation also die around E10.5 with similar heart defects to those described above, however, early development of the peripheral glial lineage is not affected (Kramer et al., 1996; Meyer et al., 1997). Furthermore, the nerves of NRG1 type III-deficient mice are initially populated by Schwann cell precursors, but are devoid of Schwann cells later in development (Wolpowitz et al., 2000). The culmination of these genetic data indicates that NRG1 type III is required for Schwann cell development. However, it is unclear from these findings whether NRG1 type III is solely required to support survival of developing Schwann cells along the axons or whether NRG1 type III can also act as a differentiation cue that promotes maturation of Schwann cell precursors into early Schwann cells.

In vitro studies might allow discrimination between the specific roles of NRG1 in migration, survival, and differentiation of neural crest derivatives. With respect to early Schwann cell development, variants of NRG1 type I were shown to promote survival and maturation of cultured Schwann cell precursors isolated from peripheral nerves (Dong et al., 1995; Dong et al., 1999; Brennan et al., 2000). A role for NRG1 in glial differentiation has also been shown in cultures of multipotent neural crest stem cells and neural crest derivatives that are instructed by NRG1 type II to choose a glial fate (Hagedorn et al., 1999; Shah et al., 1994). However, NRG1 type II-treated cells differentiate into satellite glia expressing the transcription factor Erm and not into Schwann cells of the peripheral nerve that express the transcription factor Oct-6 (Hagedorn et al., 2000). Based on the expression and genetic data, Schwann cell development in vivo might rather be regulated by NRG1 type III. Therefore, we here elucidated the potential functions of NRG1 type III in the Schwann cell lineage, using culture systems in which survival and differentiation can be investigated separately and independently of migration.

## 2. Results

### *2.1. NRG1 type III rescues Schwann cell precursors in a 24 hr survival assay*

In order to address the cellular role of the NRG1 type III isoform in early stages of Schwann cell development, we exposed Schwann cell precursor cells to a soluble form of NRG1 type III. Schwann cell precursors were freshly isolated from embryonic rat sciatic nerves at E14.5 and cultured in defined medium, as described previously (Dong et al., 1995; Jessen et al., 1994; Lobsiger et al., 2000). In a first experiment, a standard 24 hr survival assay was performed (Jessen et al., 1994). Approximately 200 cells were plated per 15-mm coverslip and the medium was supplemented with different concentrations of NRG1 type III. In parallel, cells on sister coverslips were exposed to a soluble NRG1 type II isoform (GGF2) (Marchionni et al., 1993) that has been shown to have a gliogenic effect on neural crest cells (Shah et al., 1994).

The survival of Schwann cell precursors was assessed by comparing the number of viable p75-positive cells after 24 hours to the number of p75-positive cells plated (Jessen et al., 1994) (**Figures 7** and **8**). Virtually no cells expressing the precursor marker p75 were found 24 hours after plating when cultured in defined medium alone (**Figure 7C** and **7D**). In contrast, when the medium was supplemented with NRG1 type II or type III, a significant number of p75-positive cells survived (**Figure 7E-7H**). Survival in the presence of 1nM NRG1 type II was close to 100%, while in the presence of 1nM or more NRG1 type III maximal survival was 60% (**Figure 8**). Thus, similar to NRG1 type I isoforms (Dong et al., 1995), both type II and type III isoforms of NRG1 prevented death of Schwann cell precursors, albeit at different efficiencies.

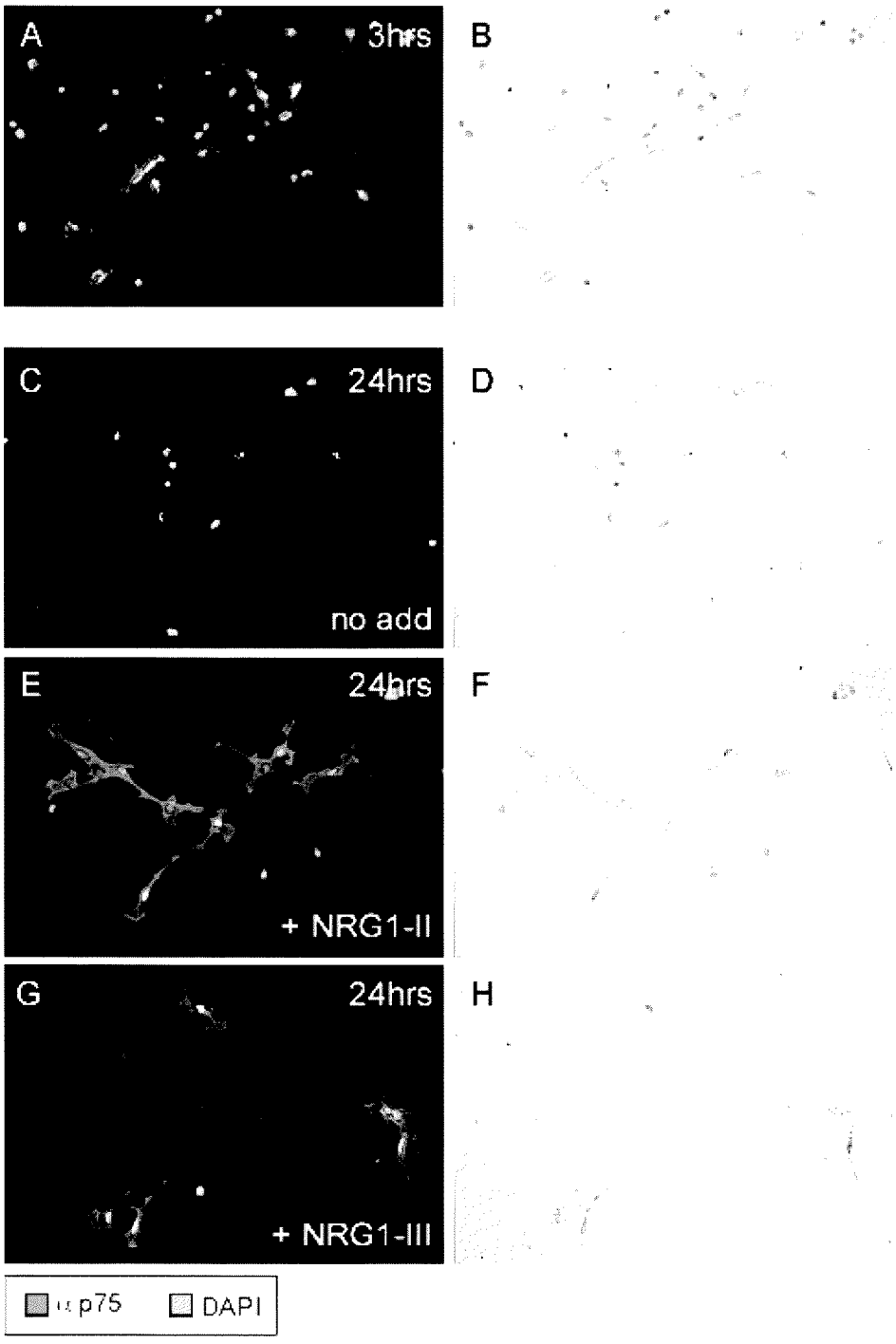



**Figure 7:** 24 hours survival assay with Schwann cell precursors

Sciatic nerves isolated from rat E14.5 embryos were dissociated and the cells were fixed at 3 hours or at 24 hours after culturing in the presence of soluble NRG1 type II (NRG1-II) or type III (NRG1-III). Immunostaining for p75 using a fluorescent Cy3-coupled secondary antibody revealed that the culture mainly consisted of Schwann cell precursor cells. Shown are

representative fields of Schwann cell precursor cultures 3 hours (A, B) and 24 hours (C-H) after plating. Cells that were immunoreactive to p75 antibody and that showed an intact nucleus (defined by means of DAPI staining) were considered for counting in order to determine relative survival rate. Phase-contrast (B, D, F and H). Magnification, X20.

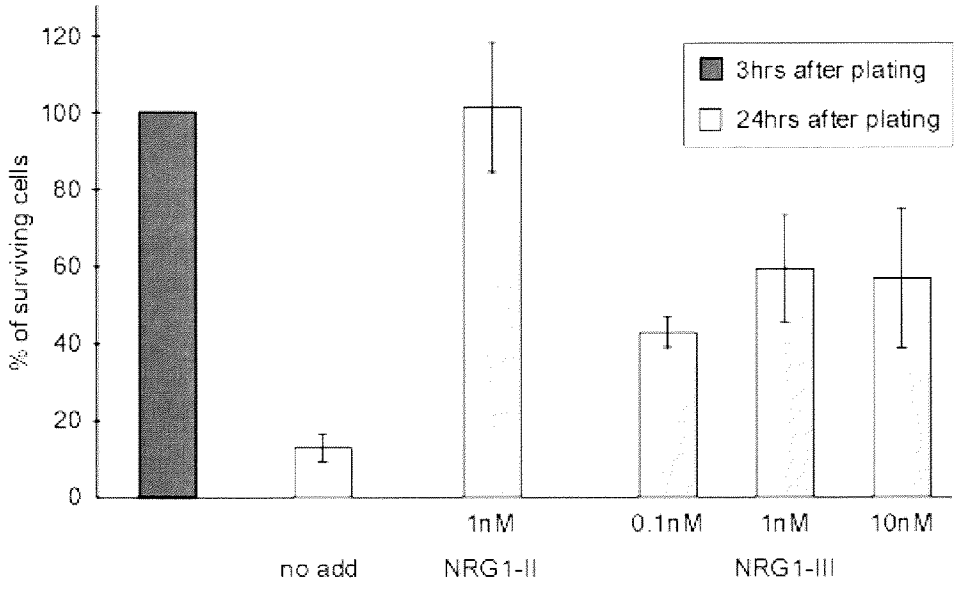



**Figure 8:** NRG1 type II and III prevent death of Schwann cell precursor cells

Schwann cell precursors from sciatic nerves were isolated and cultured as described in the figure legend of Figure 7. Survival capacity was measured by counting all p75-positive cells that displayed an intact nucleus present per coverslip. Total number of cells per coverslip of 24 hours cultures at different medium conditions were compared to the total number of p75-positive cells plated (3 hours cultures, approx. 200 cells / coverslip). Each of the three independent experiments was performed in triple. Relative survival rate of Schwann cell precursors is given in percentage ± s.d.

### *2.2. Soluble NRG1 type III supports maturation of Schwann cell precursors in a long-term survival assay*

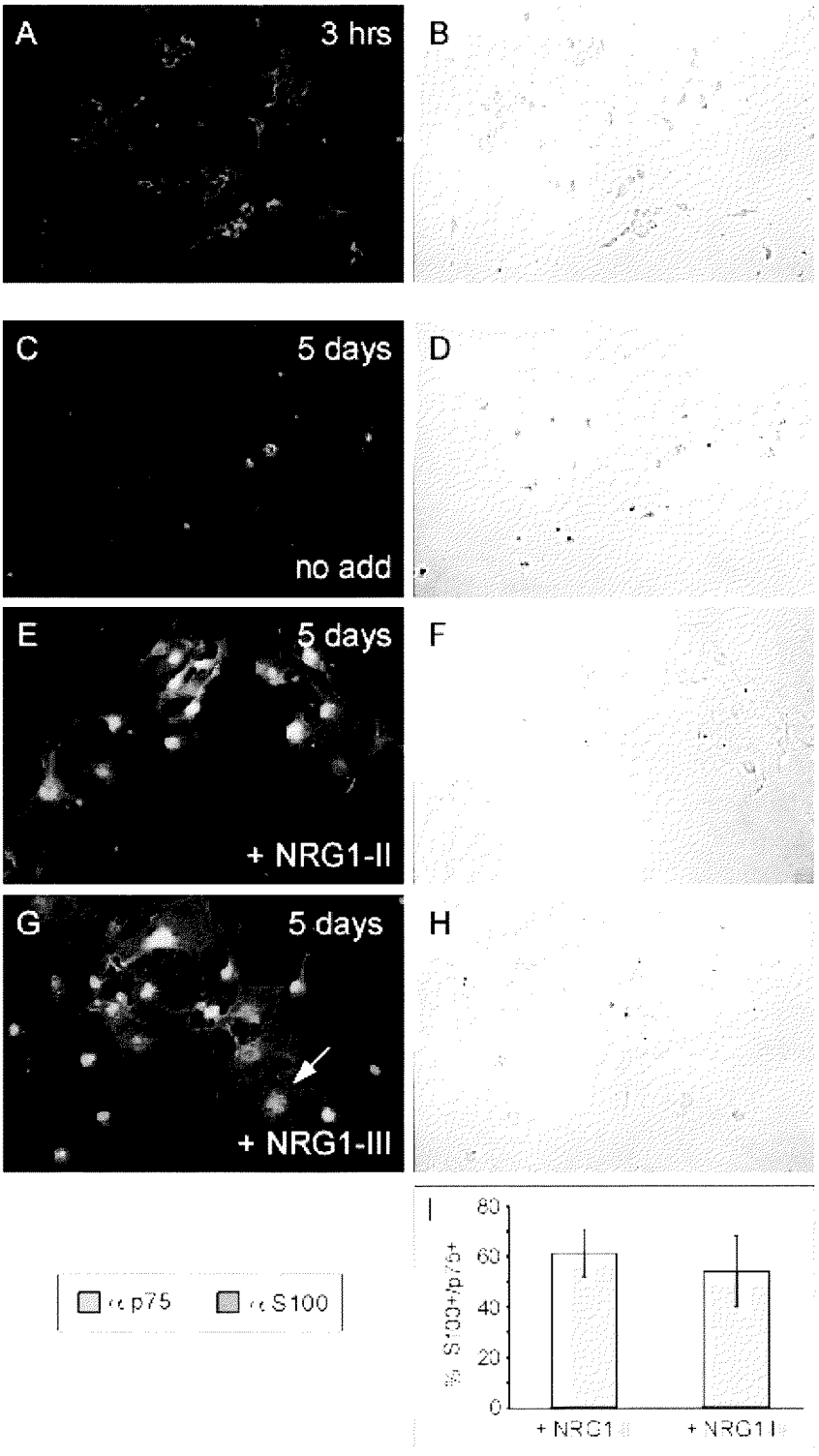
Previously it has been shown that long-term survival of Schwann cell precursors promoted by NRG1 type I isoforms was accompanied by differentiation of the precursors to early Schwann cells (Dong et al., 1995;

Dong et al., 1999). Therefore, we addressed whether other NRG1 isoforms also support long-term survival and maturation of Schwann cell precursors. Precursor cells were plated at high density in defined medium and treated with soluble forms of either NRG1 type II or type III over a period of five days. Differentiation into early Schwann cells was monitored by the upregulation of S100. In the presence of NRG1 type II, little cell death was observed as monitored by DAPI-staining of fragmented nuclei (data not shown). Moreover, 61±9% of the NRG1 type II-treated p75-positive cells displayed increased S100 immunoreactivity when compared to precursors on sister plates that were fixed 3 hours after plating (**Figure 9A-F**, **I**). In medium supplied with NRG1 type III, many cells showed fragmented nuclei (data not shown), but a substantial proportion of the cells survived in these conditions (**Figure 9G**, **H**). While virtually all of the surviving cells were p75 immunoreactive, 54±14% of these cells also expressed S100 (**Figure 9G**, **I**). Thus, many NRG1 type II and type III-treated cells survived without differentiation, suggesting that the transition from Schwann cell precursors to early Schwann cells is not a cell-autonomous process that unfolds in cells prevented from undergoing apoptotic death (Jessen and Mirsky, 1998; Brennan et al., 2000). Rather, it is likely that both survival and differentiation have to be actively promoted by extracellular cues.

**Figure 9:** Longterm survival of Schwann cell precursors in the presence of NRG1 type II and III is accompanied by their differentiation into early Schwann cells

Dissociated rat E14.5 nerves were plated and cultured over a period of 5 days in medium supplemented with soluble forms of NRG1 type II or type III. Fixed cells were immunolabeled using anti-p75 and anti-S100 antibody visualized by FITC-conjugated and Cy-3 conjugated secondary antibody respectively (A, C, E and G). Differentiation of Schwann cell precursor cells (A, B, 3 hours cultures) into early Schwann cells (E-H, 5 days cultures) is marked by the upregulation of S100 expression. In the absence of any survival cue virtually all cells died within the 5 days of culturing (C, D). (I) Percentage of cells (± s.d.) that were found to be double-positive for the expression of p75 and S100 was determined in four independent experiments. Between 100 and 150 p75-positive cells were scored per experiment. Note that

many of the p75-positive cells do not express S100 (arrow). Phase-contrast (B, D, F and H). Magnification, X20.



*2.3. Membrane-bound but not soluble NRG1 type III promotes Schwann cell differentiation from multipotent progenitor cells*

To identify signals that promote Schwann cell differentiation independent of survival, we employed a culture system of neural crest-derived cells in which cell death is minimal. Non-neuronal neural crest-derived cells isolated from early dorsal root ganglia (DRG) are multipotent, giving rise to neurons, satellite glia and Schwann cells as well as to non-neural cells, depending on the environmental conditions (Hagedorn et al., 2000; Hagedorn et al., 1999). Moreover, although the survival of at least a subpopulation of these cells is supported by NRG1 signaling (Paratore et al., 2001), we have established conditions in which more than 95% of progenitor cells isolated from embryonic rat DRG survive in the absence of exogenously added NRG1 (Hagedorn et al., 1999). In agreement with previous studies (Hagedorn et al., 2000; Zorick and Lemke, 1996), such progenitor cells derived from rat E15 DRG generate Schwann cells upon treatment for 4 days with forskolin, which elevates intracellular cAMP. Schwann cell differentiation was monitored by increased expression of the transcription factor Oct-6 (also called SCIP or Tst-1; He et al., 1989; Meijer et al., 1990; Monuki et al., 1989) (**Figure 10A-D**) and S100 (not shown). The NRG1 type II isoform GGF2 has been shown to regulate satellite gliogenesis in DRG-derived progenitor cells but does not induce expression of the Schwann cell marker Oct-6 (**Figure 10E**, **F**; Hagedorn et al., 2000). Likewise, addition of soluble NRG1 type III (1nM) had no effect on Oct-6 levels and did not induce S100 expression (**Figure 10G**, **H**; and data not shown). Moreover, Schwann cell differentiation of DRG-derived progenitor cells was not observed even when using concentrations of NRG1 type III (10nM) 10-fold above saturation for survival and differentiation of sciatic nerve-derived Schwann cell precursors (data not shown).

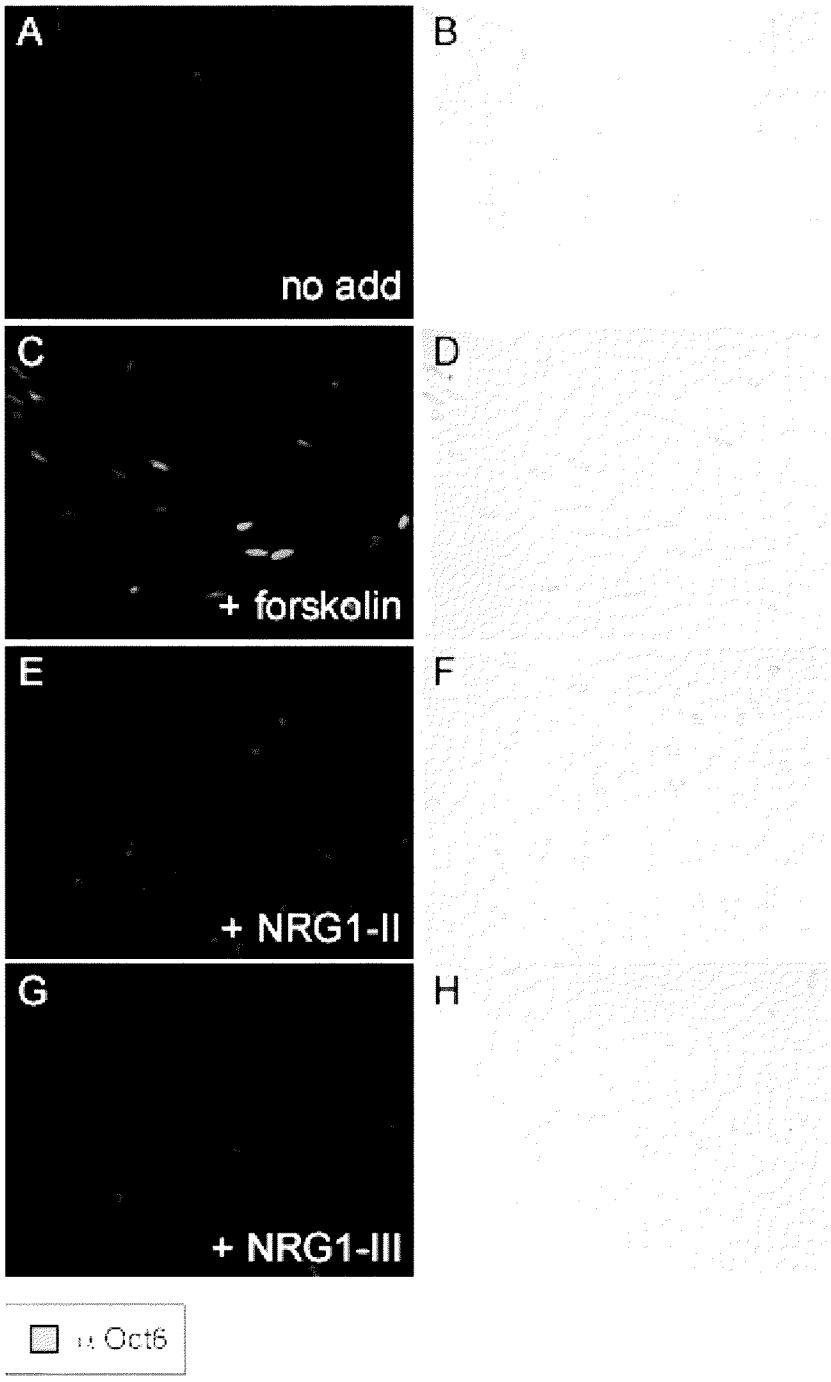



**Figure 10:** Soluble NRG1 type II and type III do not induce expression of the Schwann cell-specific marker Oct-6 in neural crest-derived multipotent progenitor cells

Dorsal root ganglia isolated from rat E15 embryos were dissociated and the cells were plated at high density. Cultures were challenged with soluble forms of NRG1 type II or type III over a period of 4 days. Fixed cells were labeled immunocytochemically for Oct-6. Staining was

visualized with FITC-coupled secondary antibody. Schwann cell differentiation, as monitored by the expression of Oct-6, is induced by supplying the culture medium with forskolin (C, D). Addition of NRG1 type II or type III to the medium had no effect on Oct-6 expression (E-F) when compared to untreated sister plates (A, B). Phase-contrast (B, D, F and H). Magnification, X20.

Hydrophobic amino acids in the N-terminal domain of NRG1 type III confer membrane association (Schroering and Carey, 1998; Wang et al., 2001). Thus, the activity of NRG1 type III might depend on whether the factor is presented to cells as soluble or membrane-bound form. To address this issue, we sought to overexpress the factor in cultured cells to allow local NRG1 signaling to neighboring cells. Retroviral expression vectors were generated encoding a) the NRG1 type III isoform carrying a human myc-epitope tag; b) NRG1 type III without a myc-epitope tag; and c) an inert protein (nuclear green fluorescence protein, GFP) fused to a myc-epitope tag, serving as control (**Figure 11**). As a further marker of infected cells, all three vectors encoded alkaline phosphatase (AP) driven from an internal ribosomal entry site (IRES). Immunostaining using an antibody to the myc-epitope tag was consistent with cell surface localization of NRG1 type III in infected postmigratory neural crest cells (**Figure 11**), in agreement with previous reports that biochemically demonstrated membrane association of NRG1 type III upon cellular expression (Schroering and Carey, 1998; Wang et al., 2001).

To address the role of cell surface-associated NRG1 type III in Schwann cell differentiation of multipotent progenitor cells, non-neuronal cells were isolated from DRG (Experimental Methods) and plated at high density to allow short range cell-cell interactions. The undifferentiated cells were infected 3 hours after plating either with retroviral vector encoding NRG1 type III fused to a myc-epitope tag (mycNRG1-III) or with control retroviral vector (**Figure 11**). Four days post-infection the cells were fixed and immunostained with antibodies against the myc-epitope tag and S100. Many NRG1 type III-expressing cells were associated with cells expressing high levels of S100 (**Figure 12C**, **D**). Intriguingly, processes of NRG1-expressing cells were often found in close contact to S100-positive cells (arrow in **Figure 12C**).

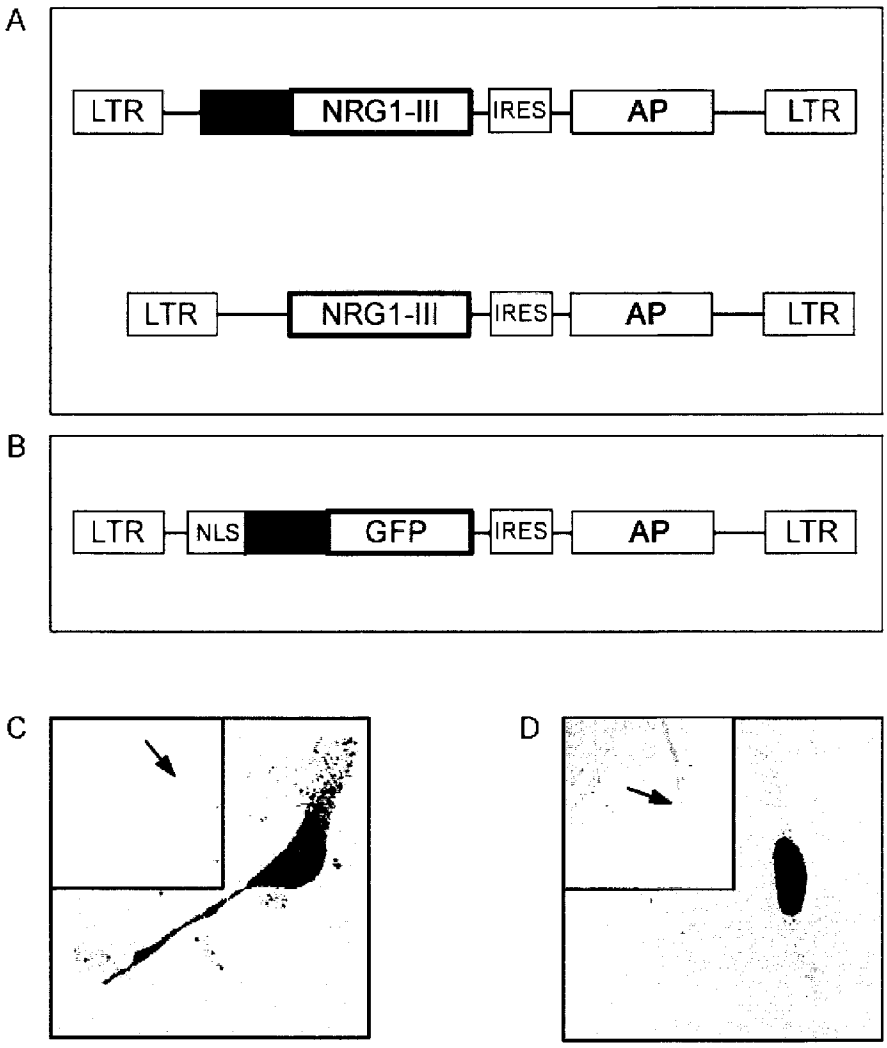



**Figure 11:** Retroviral expression constructs encoding membrane-bound NRG1 type III

cDNA's encoding myc-tagged NRG1 type III (mycNRG1-III; A) or NRG1 type III without epitope tag (NRG1-III; A) were inserted into retroviral expression constructs. Control infections were performed using a virus encoding a GFP fusion protein with a myc-epitope tag and a nuclear localization signal (NLS; B). Infected cells can be revealed by immunocytochemical detection of the myc-epitope tag (C, mycNRG1-III-infected; D, mycGFP-infected) or alkaline phosphatase (AP). (C, D) Three-dimensional representations (calculated from confocal data stacks) of Myc-immunostained infected DRG cells (green fluorescence; arrows indicate the infected cell in the phase contrast view). (C) The subcellular distribution of the dye is consistent with a membrane association of mycNRG1-III. Note that the mycNRG1-III

containing vesicles are distributed throughout the whole cell to the distal tips of the process. IRES: internal ribosomal entry side, LTR: long terminal repeats.

In addition, cells in close proximity to NRG1 type III-expressing cells also expressed Oct-6 (**Figure 12G, H**). As with S100-expressing cells, a striking correlation between Oct-6 labeling and cellular association with processes of NRG1 type III-positive cells was seen (arrow in **Figure 12G**). By contrast, control-infected cells were mostly not associated with S100- or Oct-6-positive cells (**Figure 12A, B, E, F**). Expression of Oct-6 in some cells of control cultures might have been due to cellular association with neurites of residual neurons possibly present in the cultures. However, staining for neurofilament only revealed very few neurons both in control and in mycNRG1-III-infected cultures, so that neuronal contact could not account for induction of Oct-6-expression in adjacent cells (data not shown). While the presence of few Oct-6-positive cells in control cultures might thus reflect a possible heterogeneity in the cultures, the strong association of Oct-6-expressing cells with mycNRG1-III-infected cells indicates that direct cell-cell contact resulted in NRG1 type III-mediated induction of the Schwann cell markers.

To exclude an involvement of membrane-associated myc-epitope tag in Schwann cell differentiation, neural crest-derived progenitor cells were infected with a retrovirus encoding NRG1 type III without epitope tag (**Figure 11**). Expression from the retroviral construct was monitored by staining for AP. Similar to epitope-tagged NRG1, the untagged form was also able to induce expression of both S100 and Oct-6 in neighboring cells, confirming the specificity of NRG type III in inducing a Schwann cell phenotype (**Figure 13**).

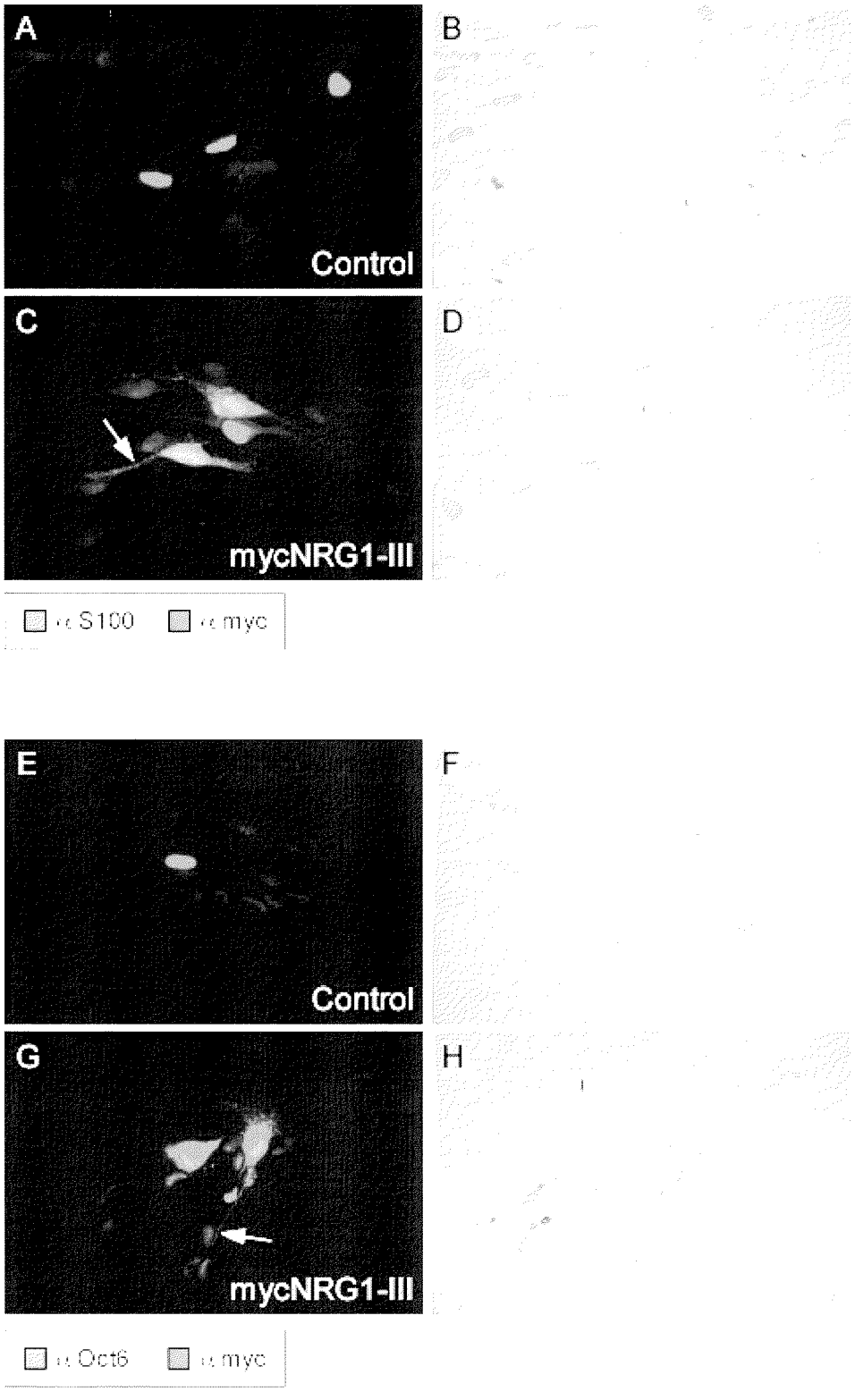



**Figure 12:** Oct-6- and S100-positive Schwann cells associate with cells expressing membrane-bound myc-tagged NRG1 type III

Cells dissociated from E15 rat DRG were plated at high density onto fibronectin-coated plates and cultured over a period of 3 to 4 hours. In order to remove neurons from the plate, the cultures were rinsed with cold PBS. Subsequently cells were replated onto pDL/fibronectin-coated plates at high density and were infected with retrovirus expressing mycNRG1-III or with control retrovirus. Cultures were fixed after 4 days and stained for the tagged fusion protein using anti-Myc antibody visualized by Cy3-conjugated secondary antibody (A, C, E and G). Anti-Oct-6 or anti-S100 antibody followed by a secondary antibody conjugated to FITC were used to reveal the presence of Schwann cells in the cultures. Non-neuronal cells in close vicinity to mycNRG1-III expressing clusters (as monitored by anti-Myc immunoreactivity, arrows) were found to express the Schwann cell markers S100 (C, D) or Oct-6 (G, H). Association of control-infected cells with Schwann cells was found with a significantly lower frequency (see Figure 14). Phase-contrast (B, D, F and H). Magnification, X40.

To quantify NRG1 type III-induced upregulation of Schwann cell markers, we counted cohorts of infected cells that displayed Schwann cell marker expression in their vicinity (**Figure 14**). Thereby, association of Schwann cells with infected cells was scored when at least one Oct-6- or S100-positive cell was found in direct contact with an infected cell. Using these criteria, 30±10% of the control-infected cells were associated with S100-positive cells. In contrast, 71±5% of the NRG1 type III-expressing cells were found in close proximity to S100-positive Schwann cells. Likewise, Oct-6 staining was detectable adjacent to 71±7% of the NRG1 type III-expressing cells, while this number amounted to only 26±5% in the control-infected cultures. Similar numbers were obtained when Schwann cell differentiation was monitored in the vicinity of cohorts of untagged NRG1-expressing cells (data not shown). Thus, the highly significant relationship (**Figure 14**) between cells expressing NRG1 and cells marked by S100 or Oct-6 suggests a prominent role for membrane-bound NRG1 type III in promoting Schwann cell differentiation.

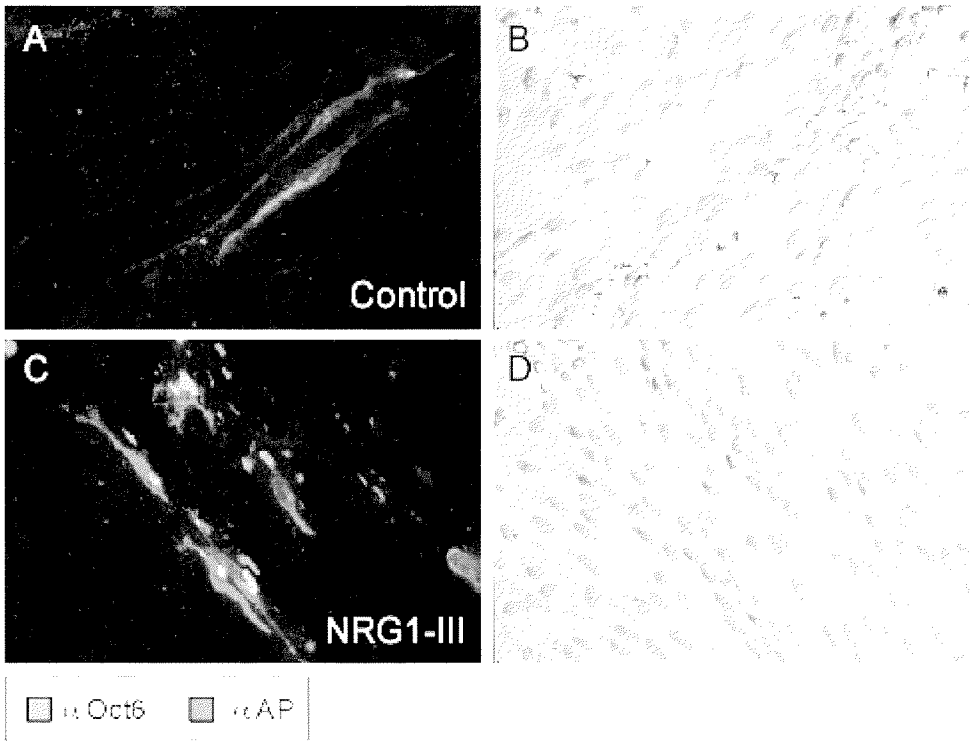


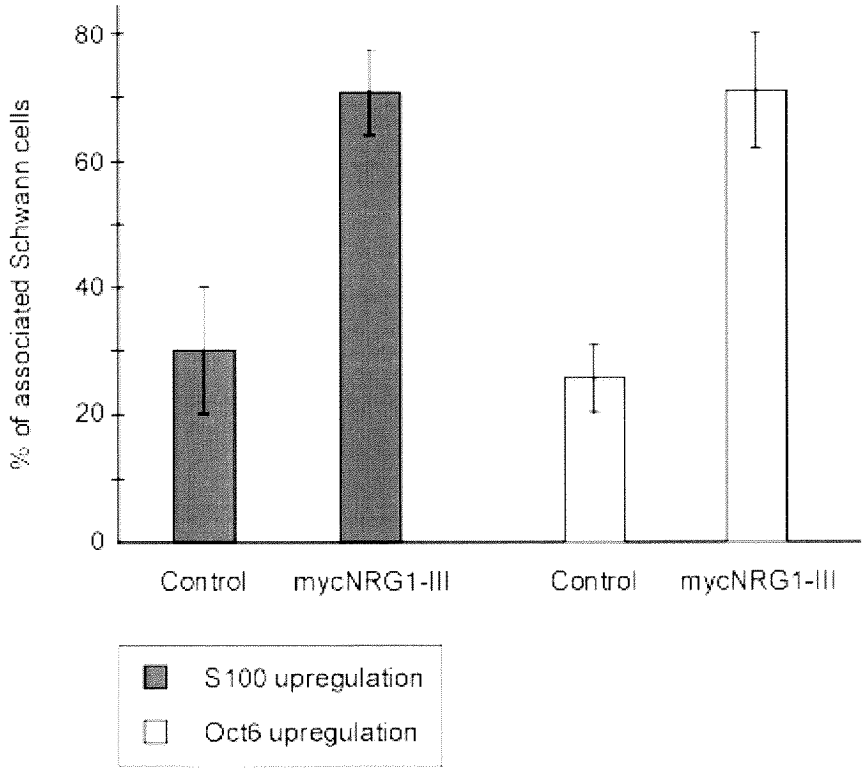

**Figure 13:** NRG1 type III-expressing cells, monitored by alkaline phosphatase expression, are associated with Oct-6-positive cells

Dissociated embryonic rat DRG were infected and cultured for 4 days. Infection with NRG1-III virus (C, D) or with control virus (A, B) was monitored by the presence of alkaline phosphatase (AP). Note that in the few cases in which control-infected cells were associated with Schwann cells, usually only one cell expressed the Schwann cell marker Oct-6. In contrast, NRG1 type III-expressing cells were usually associated with more than three Schwann cells. Phase-contrast (B, D). Magnification, X20. (Courtesy by A. Lüssi).

**Figure 14:** Membrane-bound NRG1 type III promotes Schwann cell differentiation

NRG1 type III-induced upregulation of Schwann cell markers was quantified. Cells that were found to be in the proximity of infected cells expressing mycNRG1-III and that showed increased expression of the Schwann cell markers S100 or Oct-6 were scored. Association of S100- or Oct-6-positive cells with infected cells was scored when at least one Schwann cell was found in direct cell contact (determined by means of phase-contrast microscopy) with an

infected cell. Relative numbers of neighboring Schwann cells are given in percentage (± s.d.). Between 100 and 120 infected cohorts were scored per experiment and condition. Scores were significantly different between NRG1 type III-expressing cells and control-infected cells for both S100 (Student's t-test, $p<0.01$) and Oct-6 expression (Student's t-test, $p<0.03$).




## *3. Discussion*

The loss of Schwann cell precursors in mice deficient in components of the NRG1 signal transduction pathway precludes the analysis of a direct involvement of NRG1 in Schwann cell differentiation (Meyer et al., 1997; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999; Wolpowitz et al., 2000). Appropriate culture systems turn out to be valuable tools to address this issue. Although NRG1 promotes survival and differentiation of Schwann cell precursors isolated from peripheral nerves (Dong et al., 1995; Dong et al., 1999; Brennan et al., 2000), soluble NRG1 induces the generation of satellite glia rather than Schwann cells in multipotent

neural crest cells (Hagedorn et al., 2000). In this study, we demonstrate that membrane-associated NRG1 type III, in contrast to soluble NRG1 isoforms, has the potential to induce the Schwann cell markers Oct-6 and S100 in cultures of multipotent neural crest-derived progenitors. Since cell death is minimal in the conditions chosen (Hagedorn et al., 1999), our data identify locally-presented NRG1 type III as an active Schwann cell differentiation signal.

*3.1. Role of NRG1 isoforms in survival of peripheral progenitor cells*

In previous reports, variants of the type I isoform have been used to demonstrate the survival-promoting activity of NRG1 on Schwann cell precursors (Dong et al., 1995; Dong et al., 1999). However, the late and restricted expression of NRG1 type I in the peripheral nervous system (PNS) makes this isoform unlikely to be involved in early Schwann cell development *in vivo* (Meyer et al., 1997). Given their strong expression in sensory and motor neurons and based on *in vivo* functional analysis, NRG1 type II and, in particular, type III might be crucial for the early development of the Schwann cell lineage (Ho et al., 1995; Meyer et al., 1997; Wolpowitz et al., 2000). Schwann cell precursors are absent in mutants in which signaling by both of these NRG1 forms is affected, whereas Schwann cell precursors are found along peripheral nerves in mutants lacking either type II or type III isoform (Meyer et al., 1997; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999; Wolpowitz et al., 2000). Thus, type II and type III isoforms might compensate each other to regulate the earliest steps in Schwann cell precursor generation. Depletion of Schwann cell precursors in NRG1 mutants might be due to cell death of neural crest-derived progenitors. The survival of at least some multipotent progenitors is supported by NRG1 signaling (Paratore et al., 2001), and, as we show here, both NRG1 type II and type III are able to promote survival of Schwann cell precursors *in vitro*. In addition, a role of NRG1 isoforms in regulating progenitor migration into peripheral nerves is conceivable (Britsch et al., 1998; Morris et al., 1999).

Survival at a somewhat later stage in development, during the transition from a Schwann cell precursor to an early Schwann cell, is likely controlled by NRG1 type III. In mice carrying a type III-specific mutation, Schwann cell precursors initially form along peripheral nerves but are then progressively lost (Wolpowitz et al., 2000). Our results indicate that soluble NRG1 type III is able to support survival of Schwann cell precursors, although less efficiently than other NRG1 isoforms (Dong et al., 1995; Dong et al., 1999; Brennan et al., 2000). Thus, it is likely that NRG1 type III acts in combination with factors such as FGF2, PDGF-BB and NT-3 to promote survival of Schwann cell precursors (Dong et al., 1995; Dong et al., 1999; Lobsiger et al., 2000). Moreover, the retention of NRG1 type III at the cell surface (Schroering and Carey, 1998; Wang et al., 2001) might increase its anti-apoptotic activity. According to this model, neuron-derived NRG1 signaling would support only those Schwann cell precursors that directly contact the axon. Such a mechanism, in concert with mitotic stimuli, would ensure a tight control of the Schwann cell number to be produced along the nerve.

*3.2. Isoform-specific activities of NRG1 in Schwann cell differentiation*

In cultures of Schwann cell precursors isolated from sciatic nerve, only about 54 to 61% of the surviving cells displayed upregulated expression of Schwann cell differentiation markers after treatment with soluble NRG1 type II or type III. This number is lower than the differentiation rate observed by other authors, who used various soluble NRG1 type I forms or an isolated EGF domain of NRG1 (Dong et al., 1995; Dong et al., 1999; Brennan et al., 2000). The discrepancy might be due to different NRG1 variants used or to subtle differences in culture conditions. In any case, our data suggest that survival *per se* is not sufficient for full differentiation of Schwann cell precursors. Rather, differentiation might have to be actively promoted independently of survival. This is also supported by the recent report that endothelins act as survival factors of Schwann cell precursors while delaying their differentiation (Brennan et al., 2000). Apparently, secreted NRG1 type II and III isoforms do not represent efficient Schwann cell differentiation factors. This is particularly evident in multipotent progenitors isolated from early DRG. These cells did not

adopt features of Schwann cells in the presence of soluble NRG1 isoforms, even when added well above saturation. In contrast, forced cellular expression of NRG1 type III induced Schwann cell traits in adjacent cells, demonstrating that local presentation of NRG1 is required to reveal its direct involvement in Schwann cell differentiation. In agreement with a role of membrane-bound, axon-derived NRG1 type III in regulating Schwann cell features, axon-Schwann cell interactions have been shown to upregulate the expression of Oct-6 in Schwann cells (Scherer et al., 1994). As mentioned above, soluble NRG1 type II regulates a satellite glial fate rather than Schwann cell development in DRG-derived multipotent progenitors (Hagedorn et al., 2000). Intriguingly, simultaneous exposure of multipotent progenitors to NRG1 type II and membrane-bound NRG1 type III did not block the Schwann cell promoting activity of the type III isoform (data not shown), suggesting that axon-mediated NRG1 signaling is dominant over signaling by secreted NRG1 (**Figure 5**).

Analysis of mutant mice revealed an important function of the transcription factor Oct-6 in Schwann cell myelination (Bermingham et al., 1996; Jaegle et al., 1996). Previously, it has been shown that expression of Oct-6 in Schwann cells is regulated by the adenylyl cyclase-protein kinase A (PKA) pathway (Monuki et al., 1989), and neural crest-derived multipotent progenitors upregulate Oct-6 expression upon treatment with the adenylate cyclase activator forskolin (Hagedorn et al., 2000). Moreover, it has been proposed that the PKA pathway synergizes with NRG1 signaling to stimulate Oct-6 expression (Mandemakers et al., 2000). It is noteworthy, therefore, that in multipotent progenitor cells profound upregulation of Oct-6 expression by locally-presented NRG1 type III occurred in the absence of exogenously added forskolin. Membrane-bound NRG1 may mimic the PKA pathway by activating similar target proteins. In support of this hypothesis, crosstalk between NRG1- and PKA-signal transduction pathways occurs and likely activates CREB (cAMP response element-binding protein) as a common downstream effector in cultures of postnatal Schwann cells (Kim et al., 1997; Tabernero et al., 1998).

Our findings that membrane-bound NRG1 type III as opposed to soluble NRG1 isoforms is able to induce Oct-6 and S100 in multipotent neural crest-derived progenitors raise the question of how the specific activities of NRG1 signaling are brought about. NRG1 signaling can be affected by varying ErbB receptor combinations (reviewed in Buonanno and Fischbach, 2001; Burden and Yarden, 1997). In the developing PNS, this is an unlikely mechanism to control differential NRG1 signaling since the receptor heterodimer of ErbB2 and ErbB3 appears to be responsible for NRG1 signaling in all neural crest-derived glia at different stages (Grinspan et al., 1996; Levi et al., 1995; Shah et al., 1994; Vartanian et al., 1997). Moreover, similar to soluble NRG1 isoforms, membrane-bound NRG1 type III stimulates phosphorylation of ErbB2 and ErbB3 receptors (Ho et al., 1995; Schroering and Carey, 1998). It is conceivable, however, that a given ErbB receptor combination can discriminate among signaling by different forms of NRG1. Different NRG gene products can elicit differential usage of ErbB phosphorylation sites or recruit different signaling proteins to activated receptors, thereby stimulating distinct signaling pathways (Pinkas-Kramarski et al., 1998; Sweeney et al., 2001; Sweeney et al., 2000). Membrane association of NRG1 type III is mediated by a hydrophobic domain in its N-terminus, resulting in exposure of the C-terminal end of the ectodomain to the extracellular environment (Schroering and Carey, 1998). Thus, the specific orientation of the NRG1 type III signaling domain might affect signal interpretation by the signal-receiving cell. In addition, cofactors such as PDZ-containing proteins or the transmembrane protein CD44 that interact with ErbB receptors might modulate NRG1 signaling (Garcia et al., 2000; Sherman et al., 2000). Such mechanisms might also underlie the differential pathway usage observed in postnatal Schwann cell motility, survival, proliferation and myelination (**Figure 6**, Li et al., 2001; Maurel and Salzer, 2000; Meintanis et al., 2001).

*3.3. Stage-specific responses of the peripheral glial lineage to NRG1 signaling*

The multiple biological responses of neural crest derivatives to NRG signaling are not only triggered by the differential activities of NRG forms. Rather, the competence of a neural crest cell to interpret a given NRG signal

apparently changes with time. NRG1 type II regulates expression of the satellite glia marker Erm in multipotent neural crest stem cells and DRG-derived progenitors, but once such cells have populated peripheral nerves as Schwann cell precursors, NRG1 type II is able to support survival rather than Erm expression (Hagedorn et al., 2000; this study). As mentioned above, signaling by membrane-bound NRG1 type III appears to be dominant over NRG1 type II signaling, consistent with the idea that the exposure of a cell to axon-mediated NRG1 type III alters its intrinsic program and its capacity to interpret signaling by soluble NRG1. Thereby, priming by NRG1 type III signaling might be irreversible since a transition of an Erm-positive multipotent progenitor to a Schwann cell precursor but never of a Schwann cell precursor to an Erm-expressing cell has been observed (Hagedorn et al., 2000). Based on our data we conclude that continuous exposure to axon-derived, membrane-bound NRG1 type III would then allow Schwann cell precursors to survive and to differentiate into Schwann cells.

# OUTLOOK

## *1. NRG1 type III-supported maturation of Schwann cell precursors in a long-term survival assay*

The differentiation of cultured p75-positive Schwann cell precursors into S100-positive early Schwann cells by means of soluble NRG1 isoforms has been reported by independent studies (Dong et al., 1995; Dong et al., 1999; Leimeroth et al., this study; Lobsiger, personal communication). However, the question of how the distinct isoforms influence later differentiation steps in Schwann cell generation remain unanswered. It would be of interest to expose Schwann cell precursors to the various NRG1 isoforms for a longer culture period testing them for late differentiation markers like Oct-6 or Krox-20. Conceivably, the different soluble isoforms have distinct effects on differentiation processes beyond the early Schwann cell state. Exposure of Schwann cell precursors to membrane-bound NRG1 type III might reveal a role for the membrane-associated isoform in late Schwann cell maturation. Performing this experiment under the conditions described in this study would require a survival cue for the Schwann cell precursors during the initial phase of infection with a transgenic retrovirus. Apoptosis of the precursors in defined culture medium could be prevented by addition of soluble NRG1 type III.

## *2. Differential receptor activation in response to diverse NRG1 isoforms?*

DRG-derived multipotent progenitor cells that are cultured in the presence of NRG1 type II display the molecular features of satellite glia (Hagedorn et al., 2000). The absence of satellite cell markers in cultures of such progenitors treated with soluble NRG1 type III (this study; and data not shown) indicates that the activities of NRG1 isoforms type II and type III are intrinsically different. This difference might be reflected on the level of receptor activation. This possibility could be approached by investigating the level of erbB receptor phosphorylation of cells exposed to either NRG1 type II or type III, similar to studies performed by Sweeney *et al.* (2000) in which distinct NRGs induced differential receptor phosphorylation. A number of experiments were performed analysing erbB2 phosphorylation by Western, using crude protein extracts of NRG1-treated DRG-derived primary cell cultures (Leimeroth, data not shown). However, interpretation of the results proved to be intricate. Residual erbB2 phosphorylation was found even in untreated cultures indicating that endogenous NRG1, derived from the sensory neurons, may activate erbB2 receptors. Thus, it might be worth performing these experiments with cultures of non-neural erbB2/3 expressing cell lines such as the non-invasive breast cancer MCF-7 cells (Adam et al., 1998).

## *3. Activation of distinct signaling cascades by NRG1 type II and membrane-bound NRG1 type III?*

In contrast to the effect of NRG1 type II, it was found that the membrane-bound type III isoform promotes Schwann cell differentiation. The unequal effects of these factors in respect to glial differentiation are likely to be reflected by activities of distinct signaling cascades in the receptor cells. For

instance, NRG1 type I was described to signal through the MAP kinase pathway to regulate Schwann cell motility, while NRG1 type II activates both the MAP kinase and PI3 kinase pathway to support survival of Schwann cells (see part I; Maurel and Salzer, 2000; Li et al., 2001; Meintanis et al., 2001). Thus, specific blockage of either the PI3 or MAPK signaling pathway (by LY294002 and U0126, respectively) during culturing of progenitor cells in the presence of either soluble NRG1 type II or membrane-bound NRG1 type III might allow to determine differential usage of these signaling cascades for the specification of distinct glial sublineages (Maurel and Salzer, 2000).

## *4. Are Schwann cells DRG-derived?*

In Figure 5 I propose that NRG1 type III recruits presumptive Schwann cells in the DRG by promoting their initial differentiation and subsequently their emigration along the axonal projections. Whether this model reflects the *in vivo* situation could be addressed by taking advantage of a Cre-recombinase-based method for *in vivo* lineage-tracing (Zinyk et al., 1998). DRG cells, in contrast to Schwann cells, specifically express the transcription factor Erm (see part I; Hagedorn et al., 2000). Thus, crossing of mice expressing Cre-recombinase under the control of Erm regulatory elements to reporter mice strains (ROSA26 or cACT) in which persistent expression of LacZ is initiated by Cre-mediated recombination should allow to identify Schwann cells derived from Erm-positive progenitor cells (Soriano, 1999; Zinyk et al., 1998).

## *5. Is NRG1 required for Schwann cell differentiation?*

The findings presented in part II of this work show, that *in vitro* NRG1 can function as a Schwann cell differentiation factor. Mice lacking the NRG1 type III indicate that this isoform is required for appropriate development of the Schwann cell lineage (Meyer and Birchmeier, 1995; Riethmacher et al., 1997;

Wolpowitz et al., 2000). These observations, however, let the question unanswered, whether *in vivo* the factor is needed for Schwann cell survival alone or whether it is required as an active differentiation cue, as well. Neural crest cell cultures derived from mice that are mutated in the NRG1 signaling system could shed light on this issue. NRG1 or erbB2/3 mutant neural crest cells that fail to form mature Schwann cells in the presence of forskolin would indicate that the NRG1 signaling system is required for Schwann cell differentiation.

# MATERIAL AND METHODS

## *1. Cell culture*

### *1.1. Animals*

Time-mated OFA rats were obtained from RCC Ltd (Füllinsdorf, Switzerland).

### *1.2. Schwann cell precursor cultures*

Cultures of rat Schwann cell precursors were prepared essentially as described by Lobsiger et al. (2000). Briefly, sciatic nerves were dissected from rat E14.5 embryos and dissociated by incubation for 60 min at 37°C in 0.2% collagenase typeIV (Worthington Biochemical Corp.), 0.12% hyaluronidase typeIV-S (SIGMA) and 0.03% trypsin inhibitor (SIGMA). Cells were plated onto poly-L-lysine- (SIGMA), laminin- (SIGMA) coated 15mm glass coverslips (Arnold Bott AG) and cultured in defined medium with or without additional growth factors that were given to the medium 3 hours after plating. Defined culture medium was used as previously described by Dong et al. (1995). For the Schwann cell precursor survival assay cells were plated at low density (approx. 200 cells / coverslip) and cultured in defined medium supplemented with soluble forms of either recombinant human NRG1 type III (also referred to as SMDF, R&D Systems) or recombinant human NRG1 type II (also referred to as rhGGF2, a gift from M. A. Marchionni, Cambridge NeuroScience), for 24

hours prior to fixation with 3.7% paraformaldehyde in PBS. Long-term survival and differentiation was tested by plating the cells at high density and by extending the survival assay described above to 5 days. All assays were performed in triplicate.

*1.3. DRG cell cultures*

Cultures of rat DRG cells were prepared exactly as described by Hagedorn et al. (2000). DRG were dissected from rat E15 embryos and dissociated by incubation in 0.25% trypsin (GIBCO BRL), 0.03% collagenase type I (Worthington Biochemical) for 20 min at 37°C. Cells were plated at approximately 10.000 to 20.000 cells per 35-mm culture dish (Corning) coated with poly-D-lysine (Roche Diagnostics) and fibronectin (Roche Diagnostics). DRG cultures were maintained in standard culture medium (Hagedorn et al., 1999; Stemple and Anderson, 1992). 4 hours after plating, the medium of some culture dishes was supplemented with either soluble NRG1 type III (SMDF, R&D Systems), soluble NRG1 type II (rhGGF2, Cambridge NeuroScience), or forskolin (SIGMA). Cells were cultured for 4 days prior to fixation with 3.7% formaldehyde in PBS.

## *2. Construction of retroviral vectors*

Vector construction was performed using standard molecular cloning techniques.

*2.1. mycNRG1-III retrovirus*

A NRG1 type III cDNA (SMDF, a gift from D. J. Carey, Penn State College of Medicine) was cloned in-frame into a retroviral vector called pBabeMT.APPuro. The vector has a myc-epitope tag upstream of the cloning site and is described in detail by Lo et al. (1997). The plasmid contains an internal ribosomal entry site (IRES)-human placental alkaline phosphatase cassette downstream of the cloning site.

*2.2. NRG1-III retrovirus*

The NRG1 type III cDNA described above was cloned in-frame into a retroviral vector called pBabe.APPuro. The vector corresponds to pBabeMT.APpuro lacking the myc-epitope tag.

*2.3. Control retrovirus*

A GFP cDNA (pGreen Lantern, GIBCO/ Life Technologies) was cloned into a re-engineered form of the retroviral vector pBabe.APPuro. The vector was modified by introducing GFP cDNA downstream of a tag that consisted of a nuclear localization signal (NLS) fused in-frame to the myc-epitope.

Viral supernatants for infection of DRG cell cultures were produced by transient transfection in Phoenix$^{TM}$ cells as described (http://www.uib.no/mbi/nolan/NL-phoenix.html).

## *3. Retroviral infection of DRG cells*

Approximately 250'000 freshly isolated rat E15 DRG cells were plated on a fibronectin (Roche Diagnostics) substrate in a 35mm dish (Corning). Cell cultures were maintained in standard culture medium (SM) for 3.5 hours and then flushed four to five times with ice-cold PBS in order to remove most of the sensory neurons present in the culture. Cells were removed from culture dishes with 0.25% trypsin (Gibco BRL) for 2 min and replated at a density of 10.000 to 20.000 cells per 35-mm culture dish coated with poly-D-lysine (Roche Diagnostics) and fibronectin (Roche Diagnostics). NRG1-III- and mycNRG1-III-expressing as well as control retroviral supernatants were diluted 1:4 with SM and added to the cells. After a 3 hours incubation at 37°C, the viral solution was removed and the culture was allowed to recover in fresh SM for 1 hour. The infection procedure was repeated once. Following infection, incubation continued in fresh SM. After 4 days of culture, cells were fixed in 3.7% formaldehyde in PBS and stained for the desired epitopes.

## 4. Immunocytochemistry

Cells were fixed in PBS containing 3.7% formaldehyde for 10 min at RT. Labeling of the cell surface antigen p75/LNGFR was performed in 10% goat serum, 0.1% BSA in PBS using a polyclonal rabbit anti-mouse p75 antibody (1:300 dilution; Chemicon) visualized by either FITC-conjugated donkey anti-rabbit IgG (1:200 dilution; Jackson Immuno Research Laboratories) or Cy3-coupled goat anti-rabbit IgG (1:500 dilution; Jackson Immuno Research Laboratories). To label intracellular antigens, cells were permeabilized for 20 min at RT using 10% goat serum, 0.3% Triton X-100, 0.1% BSA in PBS. To reveal expression of S100, labeling (rabbit anti-cow S100; DAKO; 1:400 dilution) was performed for 1 hour at RT and visualized by either Cy3 or FITC fluorescence. Rabbit anti-Oct-6/SCIP antibody (Zwart et al., 1996) (a gift from D. Meijer, Erasmus University, Rotterdam, The Netherlands) was used at a 1:200 dilution and visualized with FITC fluorescence. To visualize myc-epitope tagged proteins, mouse monoclonal anti-human c-Myc antibody (9E10; 1:3 dilution; Developmental Studies Hybridoma Bank) was applied for 1 hour at RT, followed by staining with Cy3-conjugated (1:300 dilution; Jackson ImmunoResearch Laboratories) goat anti-mouse IgG. AP labeling was performed with monoclonal anti-human alkaline phosphatase antibody (placental, clone 8B6; 1:300 dilution; SIGMA) followed by incubation with Cy3-coupled IgG. Nuclei of the cells were visualized with DAPI (1µg/ml), a chromosome-specific fluorescent dye. Stained fixed cultures were embedded in AF1/Citifluor™ (Chemical Laboratory).

## 5. Confocal microscopy

Confocal data sets were recorded woth a step size in the Z-axis of 284 nm using a Leica inbverted microscope DM IRB/E equipped with a true

confocal scanner SP1, argon-helium-neon lasers and a PL APO 63x/1.32 oil immersion objective.

Three-dimensional representations were calculated from the data stacks using Imaris (Bitplane AG, Zürich, Switzerland); a software specialized for confocal data sets.

# REFERENCES PART II


Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J., Kumar, R. (1998). Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. *J. Biol. Chem.* **273**,28238-46.

Bermingham, J. R., Jr., Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L., and Rosenfeld, M. G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev* **10**, 1751-1762.

Brennan, A., Dean, C. H., Zhang, A. L., Cass, D. T., Mirsky, R., and Jessen, K. R. (2000). Endothelins control the timing of Schwann cell generation in vitro and in vivo. *Dev. Biol.* **227**, 545-557.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C., and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev.* **12**, 1825-1836.

Buonanno, A., and Fischbach, G. D. (2001). Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr. Opin. Neurobiol.* **11**, 287-296.

Burden, S., and Yarden, Y. (1997). Neuregulins and their receptors: a versatile signalling module in organogenesis and oncogenesis. *Neuron* **18**, 847-855.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R., and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal

and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-596.

Dong, Z., Sinanan, A., Parkinson, D., Parmantier, E., Mirsky, R., and Jessen, K. R. (1999). Schwann cell development in embryonic mouse nerves. *J. Neurosci. Res.* **56**, 334-348.

Garcia, R. A., Vasudevan, K., and Buonanno, A. (2000). The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. *Proc. Natl. Acad. Sci. USA* **97**, 3596-3601.

Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M., and Scheres, S. S. (1996). Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *J. Neurosci.* **16**, 6107-6118.

Hagedorn, L., Paratore, C., Brugnoli, G., Baert, J. L., Mercader, N., Suter, U., and Sommer, L. (2000). The Ets domain transcription factor Erm distinguishes rat satellite glia from Schwann cells and is regulated in satellite cells by neuregulin signaling. *Dev. Biol.* **219**, 44-58.

Hagedorn, L., Suter, U., and Sommer, L. (1999). P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-ß family factors. *Development* **126**, 3781-3794.

He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W., and Rosenfeld, M. G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* **340**, 35-42.

Ho, W.-H., Armanini, M. P., Nuijens, A., Phillips, H. S., and Osheroff, P. L. (1995). Sensory and motor neuron-derived factor. A novel heregulin variant highly expressed in sensory and motor neurons. *J. Biol. Chem.* **270**, 14523-14532.

Jaegle, M., Mandemakers, W., Broos, L., Zwart, R., Karis, A., Visser, P., Grosveld, F., and Meijer, D. (1996). The POU factor Oct-6 and Schwann cell differentiation. *Science* **273**, 507-510.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. (1994). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. *Neuron* **12**, 509-527.

Jessen, K. R., and Mirsky, R. (1998). Origin and early development of Schwann cells. *Microsc. Res. Tech.* **41**, 393-402.

Kim, H. A., DeClue, J. E., and Ratner, N. (1997). cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the

cAMP and neuregulin/tyrosine kinase pathways. *J. Neurosci. Res.* **49**, 236-247.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E., and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc. Natl. Acad. Sci. USA* **93**, 4833-4838.

Levi, A. D., Bunge, R. P., Lofgren, J. A., Meima, L., Hefti, F., Nikolics, K., and Sliwkowski, M. X. (1995). The influence of heregulins on human Schwann cell proliferation. *J. Neurosci.* **15**, 1329-1340.

Li, Y., Tennekoon, G. I., Birnbaum, M., Marchionni, M. A., and Rutkowski, J. L. (2001). Neuregulin signaling through a PI3K/Akt/Bad pathway in Schwann cell survival. *Mol. Cell. Neurosci.* **17**, 761-767.

Lo, L., Sommer, L., and Anderson, D. J. (1997). MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. *Curr. Biol.* **7**, 440-450.

Lobsiger, C. S., Schweitzer, B., Taylor, V., and Suter, U. (2000). Platelet-derived growth factor-BB supports the survival of cultured rat Schwann cell precursors in synergy with neurotrophin-3. *Glia* **30**, 290-300.

Mandemakers, W., Zwart, R., Jaegle, M., Walbeehm, E., Visser, P., Grosveld, F., and Meijer, D. (2000). A distal Schwann cell-specific enhancer mediates axonal regulation of the Oct-6 transcription factor during peripheral nerve development and regeneration. *Embo J.* **19**, 2992-3003.

Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., and Kobayashi, K. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-318.

Maurel, P., and Salzer, J. L. (2000). Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity. *J. Neurosci.* **20**, 4635-4645.

Meijer, D., Graus, A., Kraay, R., Langefeld, A., Mulder, M. P., and Grosveld, G. (1990). The octamer binding factor Oct6: cDNA cloning and expression in early embryonic cells. *Nucl. Acids Res.* **18**, 7357-7365.

Meintanis, S., Thomaidou, D., Jessen, K. R., Mirsky, R., and Matsas, R. (2001). The neuron-glia signal beta-neuregulin promotes Schwann cell motility via the MAPK pathway. *Glia* **34**, 39-51.

Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L. E., and Birchmeier, C. (1997). Isoform-specific expression and function of neuregulin. *Development* **124**, 3575-3586.

Monuki, E. S., Weinmaster, G., Kuhn, R., and Lemke, G. (1989). SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* **3**, 783-793.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D., and Lee, K. F. (1999). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273-283.

Paratore, C., Goerich, D. E., Suter, U., Wegner, M., and Sommer, L. (2001). Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* **128**, 3949-3961.

Pinkas-Kramarski, R., Shelly, M., Guarino, B. C., Wang, L. M., Lyass, L., Alroy, I., Alimandi, M., Kuo, A., Moyer, J. D., Lavi, S., Eisenstein, M., Ratzkin, B. J., Seger, R., Bacus, S. S., Pierce, J. H., Andrews, G. C., Yarden, Y., and Alamandi, M. (1998). ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. *Mol. Cell. Biol.* **18**, 6090-6101.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R., and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.

Scherer, S. S., Wang, D. Y., Kuhn, R., Lemke, G., Wrabetz, L., and Kamholz, J. (1994). Axons regulate Schwann cell expression of the POU transcription factor SCIP. *J. Neurosci.* **14**, 1930-1942.

Schroering, A., and Carey, D. J. (1998). Sensory and motor neuron-derived factor is a transmembrane heregulin that is expressed on the plasma membrane with the active domain exposed to the extracellular environment. *J. Biol. Chem.* **273**, 30643-30650.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* **77**, 349-360.

Sherman, L. S., Rizvi, T. A., Karyala, S., and Ratner, N. (2000). CD44 enhances neuregulin signaling by Schwann cells. *J. Cell. Biol.* **150**, 1071-1084.

Soriano, P., (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Stemple, D. L., and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973-985.

Sweeney, C., Fambrough, D., Huard, C., Diamonti, A. J., Lander, E. S., Cantley, L. C., and Carraway, K. L., 3rd. (2001). Growth factor-specific signaling pathway stimulation and gene expression mediated by ErbB receptors. *J. Biol. Chem.* **276**, 22685-22698.

Sweeney, C., Lai, C., Riese, D. J., 2nd, Diamonti, A. J., Cantley, L. C., and Carraway, K. L., 3rd. (2000). Ligand discrimination in signaling through an ErbB4 receptor homodimer. *J. Biol. Chem.* **275**, 19803-19807.

Tabernero, A., Stewart, H. J., Jessen, K. R., and Mirsky, R. (1998). The neuron-glia signal beta neuregulin induces sustained CREB phosphorylation on Ser-133 in cultured rat Schwann cells. *Mol. Cell. Neurosci.* **10**, 309-322.

Vartanian, T., Goodearl, A., Viehover, A., and Fischbach, G. (1997). Axonal neuregulin signals cells of the oligodendrocyte lineage through activation of HER4 and Schwann cells through HER2 and HER3. *J. Cell Biol.* **137**, 211-220.

Wang, J. Y., Miller, S. J., and Falls, D. L. (2001). The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. *J. Biol. Chem.* **276**, 2841-2851.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P., and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev.* **13**, 2538-2548.

Wolpowitz, D., Mason, T. B., Dietrich, P., Mendelsohn, M., Talmage, D. A., and Role, L. W. (2000). Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. *Neuron* **25**, 79-91.

Zinyk, D. L., Mercer, E. H., Harris, E., Anderson, D. J., and Joyner, A. L. (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. *Curr. Biol.* **8**, 665-668.

Zorick, T. S., and Lemke, G. (1996). Schwann cell differentiation. *Curr. Opin. Cell Biol.* **8**, 870-876.

Zwart, R., Broos, L., Grosveld, G., and Meijer, D. (1996). The restricted expression pattern of the POU factor Oct-6 during early development of the mouse nervous system. *Mech. Dev.* **54**, 185-194.

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Neuregulin 1 isoforms as players in signaling networks in neural crest cell migration, lineage determination and differentiation (2002), *review*, in preparation.