Endosomal Receptor Trafficking and Signal Transduction in Schwann cells : Regulation of the Nrg1-induced PI3-kinase pathway

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ABSTRACT OF THE DISSERTATION

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Neuregulin1-ErbB signaling is important for various functions during Schwann cell development and myelination. Activation of the ErbB receptors also triggers myelin breakdown in mature myelinating Schwann cells. The mechanism by which the activated ErbB receptor complex elicits multiple biological functions in Schwann cells is unclear. In Charcot-Marie-Tooth (CMT) disease - the most common demyelinating neuropathy in the peripheral nervous system - many proteins involved in regulating intracellular vesicular trafficking and sorting through the endocytic pathway are found mutated. Endocytic pathways are also strongly implicated in the regulation of signal transduction by cell surface receptors. It is possible that aberrant regulation of the ErbB receptors and downstream signal activation by the impaired endocytic components contribute to the disease manifestation. The function of ErbB receptor trafficking and signal modulation in Schwann cells is largely unknown. We hypothesized that Nrg1-induced ErbB2 and ErbB3 receptor trafficking can differentially regulate signaling by spatially and temporally localizing receptors in different endocytic compartments. In this study, we show that following treatment with soluble Nrg1, internalized ErbB receptors are sorted

into the late endosome/lysosome for degradation or transported to the recycling endosome and reappear on the cell surface. ErbB receptor recycling is also regulated by Nrg1 dose. Inhibition of receptor endocytosis by impairing dynamin activity blocked the Nrg1-induced Akt activation and abrogated the pro-myelinating effect in co-cultures. Interestingly, allowing receptor endocytosis but inhibiting the subsequent recycling from the early endosome enhanced Akt activation, indicating the importance of the early endosomal signaling for the Nrg1-induced Akt activity. Supporting this, sub-cellular fractionation showed that active Akt was enriched in the endosomal fraction in Schwann cells. We also investigated the mechanism by which membrane-bound Nrg1 Type III regulates ErbB receptor trafficking in Schwann cells. Binding of the axonal Nrg1 induced both ErbB2 and ErbB3 downregulation indicating receptor internalization. The membrane-bound Nrg1 Type III was also internalized into the Schwann cells, appearing in Rab5-positive early endosomes. The Nrg1-induced Akt activation, which is necessary for myelination was abrogated when receptor endocytosis in Schwann cells was blocked. Our results show that endocytic trafficking is important for the pro-myelinating function of Nrg1. The results also suggest that impaired endocytic pathways may contribute to the development of demyelinating neuropathy by resulting in aberrant regulation of the Nrg1-ErbB signaling in Schwann cells.

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3 Chapter One: Introduction

Many signals regulate the process of Schwann cell development and function in the peripheral nervous system (PNS). Dynamic bi-directional interactions between the growing neuron and its associated Schwann cell are required for proper myelination and the maintenance of a healthy myelin sheath. Aberrant communication between these two cells, during injury or disease conditions, causes severe forms of demyelinating neuropathies.

Neuregulins expressed by neurons bind to ErbB2 and ErbB3 receptors on Schwann cells to elicit a diversity of functions. Signals initiated by this interaction are crucial for the proper myelination of the axons. It has recently been discovered that various proteins involved in trafficking are mutated in some types of peripheral neuropathies. Therefore elucidating this mechanism will provide insights into understanding the disease. The molecular mechanism by which the Nrg1-ErbB ligand-receptor system plays diverse roles in Schwann cells is unknown.

The goal of the study presented in this thesis was to elucidate the signaling function of Nrg1 that regulates Schwann cell myelination. Specifically I focus on investigating the role of intracellular receptor trafficking in modulating the promyelinating function of Nrg1. There is increasing evidence that suggest that intracellular signaling events associated with receptor trafficking play a crucial role in modulating signaling and downstream pathway activation.

3.1 The Neuregulins

The neuregulin1 gene produces various splice variants, whose translational products have numerous other specific functions in the nervous system, heart and breast (Falls, 2003a, Mei and Xiong, 2008). Neuregulin1 is composed of 33 isoforms that are further classified into 6 different types, based on the identity of their N-terminus, (Mei and Xiong, 2008). Type I and II share an Ig (immunoglobulin) domain, while Type III has a cysteine-rich domain. All three neuregulins share the EGF domain on their N-terminus, which is sufficient to activate the receptor. Nrg1 Type I and II are inserted in the membrane as single pass transmembrane proteins with a Nout/Cin topology. Nrg1 Type III, because of the hydrophobic nature of its CRD region, remains in an Nin/Cin conformation as a two-pass transmembrane protein (Falls, 2003b). Neuregulins are synthesized as proproteins. Type I and II are released on proteolysis - at the stalk region - as soluble factors, while type III remains tethered to the axonal membrane by virtue of its hydrophobic N-terminus (Wang et al., 2001, Falls, 2003b, ffrench-Constant et al., 2004) (Figure 1A)

3.1.1 TypeIII Neuregulin processing:

Ligand proteolytic processing and release from the membrane can serve as an important regulatory mechanism for membrane tethered neuregulins. Key metalloproteases like the BACE, ADAMs (a disintegrin and metalloprotease), and gamma secretases, are known to cleave neuregulins and regulate their release and activity. While gamma secretases are known to cleave the cytoplasmic tail of neuregulin1 and release it into the cell, the beta and alpha secretases act as extracellular metalloproteases.

BACE1 (beta-site amyloid precursor protein-cleaving enzyme 1) is an aspartyl protease known to play a role in Alzheimers disease pathology by cleaving APP to Ab1 plaques whose accumulation is the initiating factor of the disease (Hussain et al., 1999, Vassar et al., 1999, Kandalepas and Vassar, 2012). BACE I is localized to the golgi, endoplasmic reticulum and endosomes (Hussain et al., 1999, Vassar et al., 1999). BACE1 is co-expressed with Nrg1 typeIII in DRG (dorsal root ganglion neurons) and the spinal cord and is especially high during early stages of myelination. Phosphorylation in its c-terminus and low pH regulate BACE1 activity (Walter et al., 2001). BACE1 null mice show PNS hypomyelination and disrupted segregation of small diameter axons, similar to that shown by Nrg1 TypeIII mutant mice. This phenotype is caused by the reduced processing of Nrg1 TypeIII and activation of Akt, a known positive regulator of myelination (Hu et al., 2006, Willem et al., 2006).

ADAM10 and ADAM17(TACE) were also shown to cleave Neuregulin1 typeIII at a site 8 amino acids upstream of the BACE1 cleavage site (La Marca et al., 2011, Luo et al., 2011a). Luo et al., 2011, claim that ADAM17 cleavage is much weaker than that of ADAM10 and also occurs at multiple sites, and that ADAM10 cleaved Nrg1 is capable of activating Akt and Erk1/2 pathways downstream of Nrg1 in Schwann cells. La Marca et al., 2001, however claim that TACE (ADAM17) cuts within the EGF domain of Nrg1 typeIII and inactivates it, thus inhibiting Nrg1 typeIII mediated Akt activation in Schwann cells and inhibiting myelination in the PNS. On the same lines, TACE knock-out animals showed hypermyelination that was a correlative to the increase in the amount of Nrg1 TypeIII expressed on the axonal surface. Both agree however, that BACE1 cleavage is activating and is required for proper myelination to occur.

Neuregulin1 TypeIII B1a (henceforth referred to as Nrg1 Type III aka, CRD-Nrg) is thus, tethered in an upside-down conformation (after processing). The full-length proprotein being around 110kD or 140kD, because of differential glycosylation , and the cleavage at the stalk region results in two membrane tethered fragments – the n-terminal fragment (NTF) which is around 75kD and the c-terminal fragment (CTF) which is around 60kD. It was shown that it is this 75kD form that is predominantly accumulated at the cell surface. There is a potential 'second cleavage' site that exists c-terminal to the CRD that enables the release of a soluble form of this Nrg1 TypeIII (Wang et al., 2001) Although Wang et al., show traces of this soluble Nrg1 TypeIII in the extracellular medium release by cells, it is proposed to be just a small percentage of the total cleaved (1st) Nrg1 typeIII. This potential 2nd cleavage site remains to be studied, and its relevance in Schwann cells has not been determined.

The intracellular cytoplasmic domain of TypeIII Neuregulin-1 has been shown to back signal in neurons, and increase the surface expression of Alpha 7 nicotinic aetylcholine receptors on neurons in sensory neurons, via a PI3K dependent pathway, thereby acting as a bi-directional molecule (Bao et al., 2003, Hancock et al., 2008).

3.2 The ErbB Receptor family

The receptors in this family include the EGFR/ErbB1, ErbB2, ErbB3 and ErbB4 (Figure 1B.). ErbB receptors consist of an extracellular region that contains two-ligand binding domains, an extracellular juxtamembrane region, a transmembrane region, a cytoplasmic kinase domain and cytosplasmic tyrosine residues that serve as receptor

phosphorylation and docking sites for adaptor proteins (Riese et al., 2007). The extracellular region of EGFR, ErbB3 and ErbB4 exist in two distinct conformations, the open and closed, with 95% of the receptors existing in the closed conformation in the absence of ligand. The extracellular domain of EGFR is composed of four sub-regions – I, II, III and IV. Regions I and III form a ligand-binding pocket in the open conformation. In the closed conformation interactions between regions II and IV, do not allow regions I and III to form this binding pocket. Regions II and IV contain the dimerization arms, thus the closed conformation inhibits receptor dimerization (Garrett et al., 2002, Ogiso et al., 2002, Riese et al., 2007). Each ligand interacts with a single receptor molecule, causing conformational changes after binding. Exposing of the dimerization arm on region II then causes two receptors to dimerize. ErbB2 has an inactive ligand-binding domain while, ErbB3 receptor lacks intrinsic kinase activity (Kraus et al., 1989). For efficient receptor activation and intracellular signal propagation, these two receptors need to heterodimerize. Dimerization on ligand binding, activates the kinase domain of ErbB2 which goes on to phosphorylate its tyrosine residues in cis and also those of ErbB3 in trans, thereby recruiting receptor specific signal adaptors (Wang et al., 2006). In Schwann cells, only the ErbB2 and the ErbB3 are expressed (Vartanian et al., 1997).

3.3 Diversity of Neuregulin1ErbB signaling in the Peripheral Nervous system

Evidence from previous studies on neuregulin1-ErbB signaling have shown the role played by various downstream molecules on Schwann cell biological function. Neuregulin1, ErbB2 and ErbB3 knock-out mice die before birth due to developmental abnormalities, however knock-out mice have revealed the importance of neuregulins and

the ErbB receptors for the generation of Schwann cell precursors (Garratt et al., 2000, Falls, 2003b). Neuregulins promote survival, proliferation, differentiation and myelination of the Schwann cells via activation of the PI3kinase pathway (Maurel and Salzer, 2000, Ogata et al., 2004), whereas activation of the Ras/Raf/Erk1/2 inhibits myelination and triggers Schwann cell migration, de-differentiation and demyelination (Mahanthappa et al., 1996, Zanazzi et al., 2001, Ogata et al., 2004, Guertin et al., 2005). It was recently shown however, that specific deletion of Erk1/2 in Schwann cell precursors is required for Schwann cell myelination(Newbern et al., 2011). C-jun N-terminal kinase (JNK), another downstream effector of ErbB2, mediates Schwann cell migration.(Yamauchi et al., 2008) Axonal Nrg1 TypeIII also activates NFkappa B in Schwann cells to positively regulate myelination independent of proliferation (Limpert and Carter, 2010). This diversity in signaling patterns and functional outcomes, implicates that there may be a fine balance between the positive and negative regulators of these processes during development (Parkinson et al., 2008).

3.4 Neuregulin isoforms in signaling

Nrg1 Type III heterozygous mice develop scarce myelination. Overexpression of the membrane-bound Nrg1 type III, results in hypermyelination, through activation of the PI3kinase pathway (Garratt et al., 2000, Michailov et al., 2004, Taveggia et al., 2005). On the other hand, soluble Type II neuregulin induces demyelination in Schwann cell-neuron cultures (Zanazzi et al., 2001) and induces hyper-proliferation and demyelination in vivo. The question of how isoform-specific differences between TypeII and TypeIII, and also in the mere presentation of the ligand (soluble vs membrane-bound) mediates such

diverse functions is intriguing. One explanation could be due to the differences in the n-terminal region of these two ligands. TypeII Nrg1 n-terminus has an immunoglobulin-rich domain, that is lacking in TypIII Nrg1. Immunoglobulin-domains bind to heparan sulfate proteoglycans (HSPGs) expressed on cell surfaces, and potentiate soluble growth factor signaling by keeping ligands at high concentrations and accessible to receptors at the membrane, increasing signaling (Sudhalter et al., 1996, Li and Loeb, 2001, Pankonin et al., 2005). A second reason could be the ability of receptors of the EGFR family to discriminate types of ligands, thus allowing for differential signaling by differential phosphorylation site usages (Muthuswamy et al., 1999, Sweeney and Carraway, 2000).

A recent study in the Kim lab (Syed and Kim, 2010) has described the importance of neuregulin dose on having a bi-functional biological outcome in Schwann cells. The study concluded that the two neuregulin1 isoforms (solube TypeII and TypeIII) have the same functional consequence on myelination depending on the dosage. Soluble Nrg1 TypeIII treated cultures promoted myelination as assessed by the number of myelin segments, while Nrg1 TypeII treated cultures inhibited it. When used at lower doses, however, TypeII promoted myelination, indicating that the biological outcome of the Nrg1 is determined by the dose presented to the Schwann cells. Furthermore, we have shown that the inhibitory function of Nrg1 at high doses is associated with the appearance of Erk1/2 activation. At low concentration, PI3-kinase is the predominant pathway activated by the Nrg1 treatment. These results indicate that activation of the ErbB-induced down-stream signaling pathways are differentially regulated in a dose-dependent manner.

3.5 Differential signaling at the ligand-receptor level

Multiple theories exist on how a ligand-receptor complex can elicit different biological outcomes. One, through ligand-mediated homo and heterodimerisation of the receptors (mentioned above), leading to differential receptor site-usages (Muthuswamy et al., 1999, Sweeney and Carraway, 2000). Two, by the action of co-receptors or adaptor proteins aiding in stabilizing or actively taking part in key signaling events. For example, ASGP2 a component of the MUC4 sialomucin complex is a transmembrane glycoprotein known to contain an EGF-like domain that binds to ErbB2 and regulate proliferation by increasing Nrg1-mediated ErbB2/3 phosphorylation (Carraway et al., 1999). Erbin an adaptor protein expressed in Schwann cells, binds to ErbB2 and stabilizes Nrg1 induced signaling, thereby positively regulating myelination. In Erbin null mice, there is aberrant ensheathment of nerves in the Remak bundle and gross hypomyelination in the PNS (Tao et al., 2009). Three – by receptor trafficking – by regulating receptor activity upon ligand binding spatially and temporally, cells can elicit a differential signaling paradigm.

3.6 The Endocytic Machinery

Mayor and Pagano, 2007, have proposed a system of classification for endocytic mechanisms, in which they have broadly divided all endocytic mechanisms into clathrin dependent and clathrin independent classes. These are then further classified on the basis of dynamin dependence (Mayor and Pagano, 2007). All routes of endocytic trafficking eventually merge on the Rab5/EEA1 early endosome compartment.

Clathrin – made up of a triskelion of 3 heavy and 3 light chains. These assemble to form hexagonal and pentagonal lattices once binding AP2 and getting recruited to the plasma membrane (Brodin et al., 2000). Lattice formation is required to form the curvature of the forming vesicle.

Dynamin – a 100kD, GTPase that is capable of severing clathrin-coated vesicles and has a role in the regulation of receptor trafficking in cells. Three dynamin isoforms have been identified. Dynamin1 and 3 are tissue specific - being found in neuronal cells and testes, lung and heart, respectively – while Dynamin 2 is ubiquitously expressed. Dynamins have five domains, namely a PH domain, a GTPase domain, a GTPase effector domain, a gamma-tubulin binding domain and a proline-rich domain (McNiven et al., 2000, Praefcke and McMahon, 2004).

Rabs – are a Ras family of monomeric GTPases that regulate membrane trafficking and are specific for each subcellular compartment in Eukaryotic cells. Like other GTPases, Rabs exist in two different forms, an ‘active’ or GTP bound form and an ‘inactive’ or GDP bound form. However, the regulatory function of the Rabs is determined by the rates of this nucleotide exchange and hydrolysis (Zerial and McBride, 2001). Rab5 regulates early endosome formation by aiding in transport of the clathrin coated vesicles from the plasma membrane and fusion to the endosomes. Early Endosomal Antigen (EEA1) has two Rab5-binding sites and is required for tethering and docking of the early endosomes, via Rab5 binding (Christoforidis et al., 1999, Rubino et al., 2000). Rab5 function is important for Insulin induced activation of Akt (Hunker et al., 2006, Su et al., 2006). Rab11- recycling endosomes are involved in targeted trafficking of membrane components from the endocytic and biosynthetic pathways to regions in the

cell that are dynamically involved in reorganization (van Ijzendoorn, 2006). Rab7 is associated with regulating late endosome-lysosomal trafficking (Zhang et al., 2009).

Ubiquitination – is a process in which a 76 amino acid ubiquitin moiety is added onto a substrate by sequential processes culminating in the action of an E3 ligase, namely Cbl. A protein can be monoubiquitinylated or multi-monoubiquitinylated at lysine residues. These ubiquitins can be further extended at lysine 48 or lysine 63 residues, forming ubiquitin-linked chains, that are destined for proteasomal degradation or are important for endocytic trafficking and signaling, respectively (Pickart, 2000, Polo et al., 2002, von Zastrow and Sorkin, 2007). Sigismund et al., 2005 along with others have shown that Cbl recruitment is important for clathrin-mediated internalization of cargo, by efficient ubiquitination of endocytic machinery components and not necessarily that of cargo. Ubiquitinylation of cargo, that happens at high doses of EGF is necessary, however for the caveolin-dependent endocytosis of cargo.

3.7 Endocytosis and signaling

Endocytosis of receptors is commonly viewed as a mechanism to attenuate signal once triggered. However, endocytosis of a receptor has two fates: recycling/turnover of receptors and downregulation of receptor levels (Sorkin and Goh, 2009). Recycling maintains active receptor pools at the surface in close proximity to the ligand, while endosomal sorting involves trafficking and sustained signaling in the endosomes and/or ubiquitin-mediated downregulation, thus attenuating signals (Sorkin and Von Zastrow, 2002). There is no ‘unifying theory’ for trafficking (Gonzalez-Gaitan, 2008), depending

on the cell type and ligand many different pathways exist. Ligand binding, receptor dimerization and activation are processes that occur before receptor internalization. Once ligand-receptor complexes are internalized they move through the early endosome compartment and then to multivesicular bodies, from which they are either recycled back to the surface via recycling endosomes or degraded in lysosomes. In each of these endosomal compartments, the ligand-receptor complexes face different environments in terms of pH and a variety of signaling platforms rich in lipids, adaptor proteins etc (Sadowski et al., 2009). Many modifications like ubiquitination, phosphorylation, dephosphorylation, ligand-dissociation etc occur in these compartments. All these events lead to changes in receptor level and activation and determine the overall signaling outcome.

Sorting of receptors that are ubiquitinated (mono and lys-63 poly) occurs in the Multivesicular bodies (MVBs) - that are large membranes that are formed from the invaginations of the early endosomes, forming intraluminal vesicles (ILVs) – by components of the ESCRT (endosomal sorting complex required for transport) (Gruenberg and Stenmark, 2004, Hurley, 2008). Endosomal sorting into MVBs is important to downregulate receptor signaling, as the cytoplasmic active site of receptors is not exposed to the cytosol, where it could have interacted with signaling molecules. However, this sorting step is crucial as not all endocytosed cargo are eventually degraded, thus it is very tightly regulated by the ESCRT proteins (Katzmann et al., 2002, Williams and Urbe, 2007).

Receptor recycling pathways consist of two arms – the fast recycling pathway from the peripheral endosomal compartment or the slow recycling pathway via the perinuclear Rab11-rich recycling endosomes (Sheff et al., 1999).

3.8. Receptor endocytosis and trafficking as a regulator of signaling in other cell-types

In PC12 cells, NGF-activated TrkA (tropomyosin-receptor-kinase) receptors mediate differentiation via endocytosis and Erk1/2 signaling; while mediating survival via membrane associated sustained activation of Akt (Zhang et al., 2000). In cortical neurons however, BDNF-dependent TrkB endocytosis is required for Akt and not Erk1/2 mediated neuronal protection and dendritic growth (Zheng et al., 2008). It has been proposed that differences in the requirement for endocytosis for Akt activation among the Trk family could be dependent on cell-type specific differences in doswnstream regulators of neurotrophin signaling. PDGF receptor signaling in NIH3T3 cells, has a dose-dependent bidirectional response. With smaller doses (1ng/ml) triggering a migratory response while larger doses (>5ng/ml) triggering a proliferative response. This mechanism being modulated by PDGF receptor endocytosis, wherein low doses stimulates clathrin mediated endocytosis, and a recycling fate of receptor and high doses a clathrin-independent, degradative fate (De Donatis et al., 2008). Furthermore, IGF Type-I receptor internalization and recycling mediate the sustained phosphorylation of Akt, by recycling receptor pools to the surface in oligodendrocyte progenitor cells (Romanelli et al., 2007). Antigen-induced BCR (B cell receptor) internalization and signaling is initaitated at the plasma membrane but continous in the endosomes.

Dynamin-mediated inhibition of this receptor leads to hyperphosphorylation of Erk1/2 and hypophosphorylation of Akt (Chaturvedi et al., 2011)

3.9 Receptor endocytosis and trafficking as a regulator of signaling in the EGFR family

The EGFR family contains the ErbB1/EGFR, ErbB2, ErbB3 and ErbB4 receptors. It has long been believed that only the EGF receptor is capable of getting endocytosed while the other ErbB receptors are either endocytosis-deficient or have slower kinetics of internalization (Baulida et al., 1996, Hendriks et al., 2003, Haslekas et al., 2005, Shen et al., 2008). Although some reports claim that the ErbB receptor defect in endocytosis is because of poor recruitment of Cbl-the E3 ubiquitin ligase-it was shown recently that the Cbl binding domain in ErbB2/4 has no defect in recruiting Cbl when replaced with the Cbl binding domain of the EGF receptor (Jansen et al., 2009) ErbB2 defect in endocytosis was also speculated to be because of inability to form clathrin coated pits (Haslekas et al., 2005). The internalization rate of EGF by EGFR is three folds higher than that by EGFR-ErbB2/3/4 chimeras, ke ~ 0.13 and ke ~ 0.04, respectively (Baulida et al., 1996). Also the half-life of the EGFR receptors decrease 3-4 fold upon EGF stimulation, whilst that of the chimeras does not change over time. ErbB3 chimera has a shorter half-life than the other chimeras. The ErbB2 receptor, which is over expressed in cancers is known to be endocytosis resistant (Hommelgaard et al., 2004). However, receptor endocytosis and trafficking is cell-type specific, as many cell types, like the hippocampal neurons and myoblasts do in fact exhibit endocytosis of the ErbB2, ErbB3

and ErbB4 receptors (Burke et al., 2001, Waterman and Yarden, 2001, Sorkin and Goh, 2008).

EGFR signaling is controlled by clathrin-mediated endocytosis in Hela cells, which is important for cell proliferation via Erk1/2 (Vieira et al., 1996). ErbB4 receptor internalization in hippocampal neurons is required for sustained Akt and Erk1/2 activity (Liu et al., 2007). In myoblast cells, neuregulin-induced acetylcholine receptor expression requires ErbB4 receptor endocytosis and Erk1/2 signaling. A recent study has shown that the concentration of EGF ligand determines the fate of the receptor (Sigismund et al., 2005, Sigismund et al., 2008). Low EGF, causing internalization and recycling via the clathrin-dependent pathway, while, high EGF leading to the degradation of the receptors via a clathrin-independent, caveolin-dependent mechanism. Receptors were also (Gruenberg, 2001) significantly more ubiquitinated with higher doses of EGF than with low doses. Physiological levels of EGF in serum are 1-2ng/ml and in many biological fluids is 10-100ng/ml ((Sigismund et al., 2005) Thus the type of endocytic machinery used also determines kinetics and trafficking patterns (Mayor and Pagano, 2007, Miaczynska and Stenmark, 2008) and becomes relevant for downstream signaling events. However, endocytosis of the ErbB2 and ErbB3 receptors in Schwann cells and the effect of their trafficking on ligand mediated signaling have not been determined.

Mutants of EGFR that are only defective in internalization but are still capable of a complete signaling capacity have not been generated. This makes it challenging to look for the specific effect of internalization on receptor signaling. Goh et al.,2010 recently generated the first internalization-defective EGFR mutant - that had functional kinase activity and normal tyrosine phosphorylation – by mutating sites on the receptor that are

responsible for various redundant internalization mechanisms of EGFR (Goh et al., 2010). Using these mutant EGFRs they show that inhibiting activated EGFR endocytosis inhibits sustained Akt and not Erk1/2 activation.

3.10. Plasma membrane versus endosomal signaling

Endosomal signaling may involve events that are biochemically different from those at the plasma membrane but also similar to those occurring at the plasma membrane but differing in spatial, temporal and quantitative strength of the signal (von Zastrow and Sorkin, 2007). Endocytosis and signaling are closely linked processes that are often bi-directionally linked in cells. Endocytosis can regulate cell signaling. Conversely signaling events are known to regulate endocytosis as well. Grb2 for example is known to regulate EGFR endocytosis by the recruitment of Cbl ubiquitin ligase to the receptor, and allowing proper localization in clathrin coated pits (Jiang et al., 2003)

Endosomal signaling can be divided into two kinds. One, signaling that occurs at the endosomal level and that can also take place at the plasma membrane; and two, signaling that requires receptor endocytosis and occurs solely in the endosomes (Sorkin and von Zastrow, 2009)

Experiments using dominant negative mutants of the endocytic pathway have underscored the need for endocytosis to achieve full Erk1/2 activation. This can be explained by research that indicates that MAPK scaffold complexes like MP1-p14 along with p18 exist on late endosomes. By bringing together MAPK and Erk1/2, they are believed to potentiate Erk1/2 signaling here. However this depends on how fast

internalized RTKs are sorted into the late endosome pathway (Teis et al., 2002). GPCRs that remain bound to ß Arestins also potentiate MAPK signaling in endosomes where the ß arrestin are known to bind MAPK signaling components (DeFea et al., 2000). In PC12 cells, endocytosis is required for Erk1/2 activation. Also by the use of compartmentalised neuron cultures, it has been shown elegantly that neurotrophin receptors signal as retrograde signals in endosomes that are required for Erk5 and CREB phosphorylation and survival signals, however activation in the soma alone induced Erk1/2 phosphorylation and not CREB and not sufficient for survival signaling (Watson et al., 2001)

3.10.1 PI3K/Akt trafficking and signaling in endosomes – Class1 PI3Ks are divided into ClassA and ClassB. They are both composed of a catalytic and a regulatory subunit. Class1A has three catalytic isoforms – p110 alpha, beta and delta forms and five p85 subunits, while Class1B has the p110 gamma form bound to either the p101 or p84 reuglatory subunit (Cain et al., 2010). Class1A isoforms associated with phosphorylated tyorsine residues, while Class1B isoforms associated with GPCRs. Upon mitogenic activation and phosphorylation of receptor tyrosine kinases, the p85 subunit binds phosphorylated tyrosine residues of the receptor, while the catalytic domain p110 activates and enables PI3K to convert phosphotidylinositol-4,5-bisphosphate (PI(4,5)P2) to phosphotidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3). Signaling proteins with PH domains like Arf6, BTK, PDK1 and Akt (also known as Protein kinase B) get recruited to this activated PIP3 site. Once Akt is recruited here, it is phosphorylated in its activation loop at Threonine 308, by PDK1 (phosphoinositide-dependent kinase), followed by its activation in its hydrophobic domain at its Serine 473 site by mTORC2 complex

(mammalian target of rapamycin) to attain full activity (Alessi et al., 1996, Guertin et al., 2006). Akt gains catalytic activity and is known to phosphorylate and inhibit the activity of many of its downstream effectors like GSK3 (and isoforms) (Brazil and Hemmings, 2001, Lawlor and Alessi, 2001, Cantley, 2002).

Akt signal termination occurs via two mechanisms. One, the absence of its activating phospholipid (PIP3), by the phospatase PTEN which dephosphorylates PIP3 to PIP2 (Maehama and Dixon, 1998) or by the dephosphorylation of Akt itself, by two known molecules – PP2A-type phosphatases (Andjelkovic et al., 1996) and PHLPP (Brognard et al., 2007, Brognard and Newton, 2008).

Angiotensisn II activation in vascular smooth muscle cells, via its GPCR AT1R, causes the activation of Akt in EEA1 postive compartments (Nazarewicz et al., 2011). Downregulation of EEA1 protein in these cells prevented Akt activation. In Zebrafish, APPL1 (adaptor protein containing pH domain, PTB domain, and Leucine zipper motif) which is a Rab5 effector is present on endosomal compartments and positively regulates Akt activation upon growth factor treatment (Schenck et al., 2008).

3.10.2 Endosomal signaling/Relevance: Wang et al., 2002, showed very elegantly that EGFR receptors can form signaling platforms at the endosomal level that can elicit biological outcomes. By using AG-1478, which is an EGFR tyrosine kinase inhibitor, to inhibit EGF mediated activation and using Monensin, that would inihibt receptor recycling, they have shown that EGFR can get activated in the endosomes, and that this endosomal signaling is sufficient to drive P13K/Akt (and also Erk1/2 and Shc) pathway which is necessary for cell survival in serum-withdrawal conditions.

Since endosome associated EGFR signaling (as described by (Wang et al., 2002)) remained active only for 2hrs, which does not stimulate DNA synthesis and proliferation of MDCK cells, these cells were stimulated with two short pulses of endosomal signaling EGFR signaling 8hrs apart. This technique was able elicit MDCK (Pennock and Wang, 2003) and primary hepatocyte (Luo et al., 2011b) cell proliferation, showing that EGFR endosomal signaling alone is sufficient to trigger cell division.

3.11. Defective Trafficking in Schwann cell myelination during disease

Charcot-Marie-Tooth (CMT) or hereditary motor and sensory neuropathies (HMSN) are an umbrella of diseases divided into two categories, based on their cell of origin. CMT type 1 are those that include demyelinating forms of the disease, whose gene mutations arise in Schwann cells and in which nerve conduction velocities (NCV) are reduced; CMT type 2 are the axonal forms of the disease in which the NCVs largely remain unaffected (Suter and Scherer, 2003). Both types affect the myelinated axons of the PNS, with the commonly occurring CMT1 manifesting earlier in childhood and causing severe demyelination, remyelination and axonal degeneration. CMTs are further subdivided into more than a dozen sub-types based on gene mutations, duplication and deletion events.

The main genes mutated in CMT1 diseases fall into three groups. First, mutations that arise in myelin components that are integral to the maintenance of the structure and correct composition of a dynamic myelin sheath, for example PMP22 and P0. Second, proteins that are important for myelin gene transcription like EGR2/Krox20 and Sox10.

Third, proteins that are involved in endocytic sorting, trafficking, and degradation of myelin proteins, for example, MTMR2, Dynamin 2 and SIMPLE (Berger et al., 2006).

Schwann cells require large amounts of plasma membrane to enwrap axons – leading to important trafficking events that need to occur in a regulated manner (Cowling et al., 2012). Trafficking proteins mutated in CMTs that lead to disease pathology are discussed below.

3.11.1.Dynamin 2 – Dynamins belong to a large family of GTPases that are involved in the pinching of newly formed vesicles from the plasma membrane and Golgi. The Dynamin family is comprised of DNM1, present ubiquitously; DNM2, present in the brain and; DNM3, present in the brain and testes (McNiven et al., 2000, Praefcke and McMahon, 2004). DNM2 mutations are associated with CMTDIB (a dominant intermediate neuropathy) (Zuchner et al., 2005). Mutations associated with this disease are found in the pleckstrin homology (PH) domain of DNM2. DNM2 PH domain interacts with phosphoinositides present on membranes. Mutant proteins show reduced receptor mediated endocytosis, reduced vesicle binding and cytoskeletal reorganizations (Zuchner et al., 2005). DNM2 trafficking defects in Schwann cells could lead to defects in polarity and organization of the myelin sheath (Niemann et al., 2006). Recently DNM2-CMTD1B specific mutations in Schwann cells reduced surface protein levels and impaired myelination in the PNS (Sidiropoulos et al., 2012).

3.11.2.Myotubularin-Related Proteins – Myotubularin (MTM) and myotubularin-related-proteins (MTMRs) belong to a family of active and inactive phosphoinositide

lipid-3-phosphatases (Wishart and Dixon, 2002). They specifically remove the phosphate group at position D-3 of PI-3-P and PI-3,5-P2, thus playing a role in trafficking by affecting these lipid endosomal regulators and their effector proteins (Michell et al., 2006). MTMR2 mutations are responsible for CMT4B1, a form of CMT (Bolino et al., 2000). MTMR13/SBF2 is an example of an inactive MTMR family member and mutations in this protein cause a form of CMT called CMT4B2 (Azzedine et al., 2003). Both these diseases show similar features of gross myelin outfoldings, possibly because they are known to interact with each other (Bolino et al., 2004, Berger et al., 2006). MTMR2 also binds to Disc large 1(Dlg1), a scaffold protein involved in Schwann cell polarization and membrane addition through exocytosis. MTMR2 acts as a negative regulator by binding Dlg1 and regulating membrane addition during myelination, thereby providing a break to the process. In MTM2 null mice this break is lost, and myelin outfoldings are a result of the overactive Dlg1 function (Bolino et al., 2004, Bolis et al., 2009).

3.11.3 Small Integral Membrane Protein of the Late Endosome (SIMPLE) – Mutations in SIMPLE cause defects in ubiquitination, sorting and degradation in myelinating Schwann cells, leading to a from of deymyelinating CMT known as CMT1C (Saifi et al., 2005). SIMPLE interacts with NEDD4 an E3 ubiquitin ligase that is expressed in Schwann cells and plays a role in ubiquitination and lysosomal degradation (Rotin et al., 2000, Shirk et al., 2005). It is proposed that SIMPLE mutations could lead to dysregulated degradation of some critical myelin proteins like PMP22, leading to CMT neuropathies (Ryan et al., 2002, Saifi et al., 2005, Fortun et al., 2006).

3.11.4. SH3TC2 (Src homology 3 domain and tetratricopeptide repeats 2) – Is a novel protein that has not yet been functionally characterized. It is mutated in CMT4C-type neuropathy (Arnaud et al., 2009). CMT4C neuropathy is characterized by disorganized nodes of ranvier and abnormal Schwann cell protrusions (Gabreels-Festen et al., 1999, Arnaud et al., 2009). SH3TC2 was recently shown localized to the plasma membrane and endosomal compartments, but most predominantly to the recycling endosomes (Lupo et al., 2009) (Arnaud et al., 2009). It was also found to be a novel effector of Rab11, found in recycling endosomes. Mutations in SH3TC2, cause mistargeting of this protein away from the recycling endosomes (Roberts et al., 2010, Stendel et al., 2010). Rab11, that interacts with SH3TC2 positively regulates myelination. A dominant negative form (RAB11a_S25N) inhibited SC-DRG myelination while a constitutively active form (Rab11a_Q70L) moderately promoted myelination (Stendel et al., 2010).

4. Research Aims:

Specific Aim1 (Chapter Two): To characterize Nrg1-mediated ErbB2 and ErbB3 receptor trafficking in Schwann cells

Endosomal trafficking has the ability to localize active receptor to various sub-cellular compartments of the cell, thereby having a profound effect on signaling. Our aim was to characterize ErbB receptor trafficking in Schwann cells. We hypothesized that activated ErbB receptors in Schwann cells get endocytosed upon Nrg1-stimulation and are routed to either a recycling or a degradative fate. Members of the ErbB receptor family are differentially trafficked, based on their dimerization partners and ligands. We also hypothesized thus, that ErbB2 and ErbB3 may get differentially trafficked upon Nrg1 stimulation.

Specific Aim2 (Chapter Three): To determine how receptor trafficking regulates ErbB signaling and Schwann cell function

Nrg1-ErbB signaling regulates multiple functions during Schwann cell development and myelination. In Schwann cells PI3-kinase/Akt signaling is required for Nrg1-induced survival, proliferation and myelination. On the other hand Nrg1-mediated Erk1/2 causes demyelination and de-differentiation. A key question is how a single ligand-receptor complex can elicit multiple, sometimes even opposing biological outcomes. In this Aim, we hypothesized that receptor trafficking has the unique ability to segregate active receptors and their downstream signaling molecules in different

endocytic compartments thereby modulating signaling and Schwann cell function. We hypothesize that Nrg1-mediated ErbB receptor trafficking differentially regulates PI3-kinase/Akt and Ras/Raf/Erk1/2 pathways.

Specific Aim3 (Chapter Four): To determine membrane-bound Nrg1 Type III mechanism of ErbB receptor trafficking

Nrg1 Type III is the sole Nrg1 isoform required for Schwann cell myelination in the PNS. Since Nrg1 Type III is membrane bound, it acts as a source of juxtacrine signaling. Our previous studies have been done on the soluble Nrg1 Type II isoform. In this aim we hypothesized that membrane-tethered Nrg1 Type III by maintaining ErbB receptors at the membrane elicits differential signaling of Nrg1-induced downstream pathways.

5. Chapter Two: Characterization of ErbB2 and ErbB3 receptor trafficking in Schwann cells

5.1 Introduction

Although endocytosis is commonly viewed as a mechanism to downregulate receptors and attenuate signaling, receptor trafficking upon ligand-activation has the ability to localize active ligand-receptor complexes to different sub-cellular compartments of the cell. Endosomal sorting of these complexes re-routes endocytosed cargo to various compartments, thereby either potentiating signals via recycling/endosomal trafficking, or attenuating signals via lysosomal degradation (Gonzalez-Gaitan, 2003, Sorkin and von Zastrow, 2009). This spatial segregation of receptors allows the recruitment of a variety of adaptor proteins and signaling molecules onto these platforms, thereby having a profound affect on signaling (Sadowski et al., 2009).

Out of the four ErbB family members, the Epidermal growth factor receptor (EGFR)/ErbB1 receptor internalization and trafficking has been most extensively studied and best characterized (Baulida et al., 1996, Sorkin et al., 1996, Burke et al., 2001). EGFR undergoes ligand-induced activation and endocytosis followed by either recycling or lysosomal degradation, depending on the pathway of endocytosis – clathrin versus caveolin, respectively (Sigismund et al., 2008). Internalized EGFR is known to traffick back to the cell surface by either a fast recycling (Rab11-negative endosomes) or a slow recycling pathway (Rab11-positive endosomes) (Sorkin et al., 1991, Sheff et al., 1999, Sorkin and Von Zastrow, 2002, van Ijzendoorn, 2006). All other ErbB receptors are known to have slower kinetics of internalization, with ErbB2 reported to be endocytosis-deficient in many cell types and in various cancers (Baulida et al., 1996, Waterman and Yarden, 2001). However, ErbB2/3/4 in myoblast cells and ErbB4 in hippocampal

neurons undergo ligand-dependent internalization, which is necessary for receptor signaling (Yang et al., 2005, Liu et al., 2007, Sorkin and Goh, 2008). Schwann cells express both the ErbB2 and ErbB3 receptors. The fate of the ErbB2-ErbB3 heterodimer following Nrg1 binding at the cell surface is currently unknown.

Guertin et al., showed evidence of activated ErbB2 receptor diffusion away from the nodes and into the Schwann cell body, in the myelinated nerves of the adult rats. This preliminary evidence suggested that ErbB receptors could be undergoing ligand-dependent endocytosis in Schwann cells.

Using both biochemical and confocal imaging analysis, in this study we characterized ErbB2 and ErbB3 receptor trafficking in Schwann cells. We show that ErbB2 and ErbB3 receptors internalize in a Nrg1-dependent manner and are localized to $Rab5^{+}$ early endosomes. Following internalization, both receptors are either targeted for degradation or are recycled back to the membrane. Recycling receptors co-localized with fluorescently labeled transferrin in perinuclear compartments, indicating trafficking via the recycling endosomes.

5.2 Materials and Methods

Antibodies and growth factors - For immunofluorescence staining, monoclonal antibody to green fluorescent protein was used at 1:1000 (Upstate), ErbB2 at 1:500 (Santa Cruz, Biotech), antibody to haemagglutinin (HA) at 1:1000(Covance). Dylight 488, 594 and 649 fluorophore conjugated secondary antibodies were all obtained from Jackson Immuno Research Laboratories and used at 1:400 dilution. EEA1 1:1000(BD Biosciences) ,Caveolin1 1:500 (BD Biosciences). For Western blot analysis, monoclonal antibody to phospho-Akt (Cell signaling) and polyclonal antibody to phospho- Erk1/2 (Promega) were used at 1:1000 and 1:5000, respectively. Polyclonal antibodies to Akt (Cell Signaling) and Erk1/2 (Promega) were used at 1:1000 and 1:5000, respectively. Polyclonal antibodies to ErbB, phospho-ErbB2, ErbB3 and phospho-ErbB3 were used at 1:500 (Santa Cruz). Monoclonal antibody to α-actin (Sigma-Aldrich) was used at 1:5000 dilution. HRP conjugated secondary antibody and ECL substrate were purchased from PIERCE. Recombinant human sensory and motor neurons derived factor (rhSMDF, type III Nrg1) and recombinant human EGF domain fragment from R&D systems (Minneapolis, MN). Recombinant human glial growth factor-II (rhGGF-II, type II Nrg1) from Acorda.

Recombinant Nrg1 Type II and Type III

Recombinant human sensory and motor neurons derived factor (rhSMDF, type III Nrg1) was purchased from R&D systems (Minneapolis, MN). Recombinant human glial growth factor-II (rhGGF-II, type II Nrg1) was obtained from Acorda (Hawthorne, NY) under a material transfer agreement. In this study, rhSMDF and rhGGF-II are referred simply as soluble Nrg1 Type III and Nrg1 Type II, respectively. The soluble Nrg1-type III was the peptide with N-terminus 296 amino acid residues containing both the EGF domain and CRD domain . The GGF was the peptide with N-terminus 519 amino acid residues containing the EGF like domain and the Ig-like domain. Both Nrg1 peptides lacked the transmembrane and cytoplasmic domains.

Schwann cell preparation – Schwann cells were isolated from neonatal rat pups between

day one and day three as described previously The sciatic nerve fragments were harvested and pooled after incision at the thigh area. Nerves were then dissociated in 0.25% trypsin (Mediatech) with 0.1% collagenase (Worthington Biochemical). Nerves were dissociated using a narrow-bored glass pipette. After dissociation, Schwann cells were plated on 60mm culture dishes in Schwann cell medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 100µg/ml penicillin/streptomycin (P/S, Mediatech). The next day, the culture media was changed to Schwann cell media containing 0.24µg/ml cytosine arabinoside (AraC) and maintained in this medium for three to four days to eliminate fibroblasts. After this period, cells were trypsinised off plates using 0.25% tryspin, resuspended in anti-thy1 antibody (0.25µg/ml in DMEM), and exposed to rabbit complement to remove any remaining fibroblasts. The Schwann cells were then plated on poly-L-lysine coated culture dishes in Schwann cell media. Next day, the Schwann cell media was changed to Schwann cell growth medium (SC media with 10ng/ml neuregulin (R&D systems) and 5µM forskolin) and grown till confluency. The cells were passaged two to three times before use for experiments/infections/plating on DRG neurons.

DRG - Schwann cell co - cultures - Dissociated DRGs were prepared from embryonic day (E) 15.5 rat embryos as described previously (Eldridge et al., 1987) and plated in droplets on matrigel(150-200ng/ml) coated coverslips in neurobasal media supplemented with NGF (25ng/ml). The next day, they were flooded in NBM (neurobasal medium) + NGF + 15µM FUDR (that kills all the dividing cells). Cultures were cycled out of FUDR. After a week, Schwann cells were plated (100,000/ well) in MEM, containing 0.4% glucose,10%FBS and NGF (50ng/ml). After another week, the cultures were moved into c-VitC media (Schwann cell media+NGF+VitC (50µg/ml)). Cultures were removed and stained or lysed as mentioned.

Immunofluorescence staining – Cultures were rinsed in PBS twice and fixed with 4% paraformaldehyde made up in PBS, for 20min. They were then rinsed in PBS and

permeablised with methanol for 20′ at –20 degrees. Cultures were then blocked in 5% normal goat serum, 0.4% Triton x 100 in PBS for 45′. Primary antibody (as mentioned in the antibodies section) dilutions were made up in blocking and incubated overnight at +4 degrees. Cultures were rinsed in PBS thoroughly with 2′ intervals and placed in secondary antibody (Jackson laboratories) made up in PBS for 45′. Then stained for DAPI, mounted with fluoromount on coverslips and viewed under the fluorescence microscope. Confocal images were obtained using a Nikon eclipse TE2000-U microscope.

SDS PAGE and Western blotting – Cell lysates were prepared with lysis buffer (10% glycerol, 1% NP40, 20mM Tris (pH – 7.4), 2.5 mM EDTA (pH-8), 2.5mM EGTA (pH – 8), 150mM NaCl, PMSF(1mM), leupopeptin (2ug/ml), aprotinin (2µg/ml), sodium orthovanadate (1mM)). The lysates were spun down at 14000rpm for 15´ at +4 degrees. The cleared supernatant was now collected for the protein assay using BCA protein assay kit and run on a 10% SDS-PAGE gels. For immunoprecipitation, 200-400µg of protein was incubated with 5µl of antibody for 3hr. Then incubated with proteinA-sepharose beads overnight. These were then spun down at 14000rpm for 5´ and the supernatant discarded. This step was performed 5 times. The beads were now boiled in sample buffer and dye for 10´ and loaded onto SDS gels and run till good separation was got. The protein was then transferred onto PVDF (Immobilon) membranes overnight. The membrane was blocked in 5% NGS, 0.3% TritonX 100 in TBS for 45´. After incubation with primary (overnight) and secondary (45´) antibodies, the membranes were washed, and ECL western blotting substrate (Pierce) was used to develop the blots on X-ray films.

Schwann cell proliferation - Purified Schwann cells were plated on Poly-L-Lysine/Matrigel coated glass coverslips in 10%FBS/DMEM media at a density of 60,000 per well, serum starved for two days, and then treated with varying amounts of neuregulins and/or drugs. Sixteen hours following NRG treatment, the cells were pulsed with 10uM BrdU for 20 hours, fixed in 4% PFA then processed for BrdU

immunolabeling . BrdU incorporation was assessed by immunolabeling with mouse anti-BrdU antibodies, after 4%PFA fixation, coverslips were washed in PBS and then incubated in 2N Hcl for 15 minutes at 37 C to partially denature DNA, the HCl was then washed off and the coverslips were incubated in 0.1M boric buffer 3 times for 5 minutes each. Samples were then blocked in 5% NGS, 0.2% Tx-100 in PBS for 1 hour. Anti-BrdU antibodies (Sigma) were then applied at dilution of 1:1000 in blocking solution for 2 hours overnight at 4 degrees. Next day coverslips were washed 3 times with PBS, then secondary antibody were used at 1:1000. BrdU incorporation was assessed under fluorescence microscope as number of BrdU positive per DAPI (percentage).

Live-cell imaging on Schwann cells transfected with ErbB2-EYFP

Schwann cells (100,000/chamber) from early passages (3-5) were plated in an 8 chambered Lab-Tek borosilicate cover glass system. Cells were plated in Schwann cell media (DMEM + 10% FBS + 100ug/ml Penicillin/Streptomycin) one day before transfection. Lipofectamine (Invitrogen) was used to transfect the cells the next day. The concentration of DNA-Lipofectamine was 1.6ug DNA : 1ul of Lipofectamine. 1.6ug of DNA were dissolved in 25ul of DMEM. Similarly 1ul of Lipofectamine was dissolved in 25ul of DMEM. They were let to sit for 5′ and both the volumes were mixed and incubated for 30′for the DNA-Lipofectamine complexes to form. Meanwhile the Schwann cells were taken off Schwann cell media and incubated in DMEM. After the 30′ incubation 200ul of DMEM was added to the DNA-Lipofectamine mixture. This transfection mixture (250ul) was now placed on the Schwann cells and let sit for 4-6 hours. 200ul of Schwann cell growth media was added to the transfected cells. The next day the media was changed to Schwann cell growth media only, thus removing the transfection mix. On day 3, the Schwann cells were replaced in Schwann cell media only and left in the absence of growth factors for 48 hours. Post 48 hours the cells were imaged using the Zeiss LSM 510 microscope at 60x oil-immersion magnification. 10ng/ml of GGF (Glial Growth factor) was used to stimulate the Schwann cells.

Receptor biotinylation protocols:

Internalized - Schwann cells were growth factor starved for 48hrs. Surface proteins were then biotinylated with Sulfo-NHS-SS-Biotin (Pierce) for 1hr at 4°C. Schwann cells were then washed free of biotin, quenched with 0.1M glycine and left either untreated (NT) or treated with 0.3nM of GGF and CRD-Nrg for 20,50 and 90minutes.After incubation, cells were washed free of growth factor, and cell surface biotinylated proteins were cleaved off their biotin label by the glutathione cleavage buffer. Schwann cells were lysed and harvested in modified RIPA buffer and immunoblotted for ErbB2 after a pull down for biotin using the streptavidin agarose beads(Thermoscientific).

Surface Biotinylation to measure receptor downregulation: Schwann cells were growth factor starved for 48hrs, surface proteins were biotinylated with Sulfo-NHS-LC biotin for 1hr at 4°C. They were then washed free of biotin and stimulated with Nrg1 Type II or Nrg1 Type III (10nM) for 120min and 240min. After each time point cell were lysed, biotin labeled protein pulled down with streptavidin agarose beads and immunoblotted for ErbB2/ErbB3.

Receptor recycling: For the recycling assay, after biotinylation with sulfo-NHS-S-S-biotin at 4°C, Schwann cells were incubated with Nrg1 Type II or Type III as indicated, for 45 min to drive internalization of biotinylated receptors. The cells were cooled on ice, and the surface-exposed biotinylated proteins were stripped of their biotin tag by treatment with the reducing agent glutathione. Internalized biotinylated receptors were protected from biotin-stripping. Subsequently, pre-warmed medium was added, and the cells were incubated for 30 min and 60 min at 37°C, to allow recycling of internalized receptors. This incubation was followed by glutathione treatment on ice to remove biotin from the surface-exposed biotinylated receptors that had recycled back to the cell surface during the 37°C. Internalized biotinylated receptors were detected by streptavidin pulldown followed by Western blot analysis for ErbB2 and ErbB3.

Crude Endosomal fractionation protocol: Was conducted as described by (de Araujo et al., 2008). Briefly, three 15cm plates were used for each condition. Schwann cells were scraped in homogenization buffer after Nrg1 treatments. Cells were then homogenized using 25-30strokes of the Dounce Homogenizer, to break the cells and release the nuclear fractions. The cells were then spun at 14000xg for 20min and the post-nuclear superantant (TL) loaded at the bottom of a sucrose step-gradient. The concentrations of the sucrose were 40.1%, 34%, 25% and 8%. The gradients were centrifuged at 35,000xg for 2hrs and the 1ml fractions were collected from the top. The protein in each fraction was precipitated using ethanol and total precipitated protein from each fraction was run on an SDS-PAGE. Western blot analysis for the presence of pAkt, pErk1/2, Akt, Erk1/2 and Rab5 were then conducted.

Preparation of neurite membrane fraction from neuron cultures

Neurite membrane fractions were prepared as described previously (Maurel and Salzer, 2000)Briefly, dissociated DRG neurons were prepared as described above in 35mm culture dishes until the axons extend out to the periphery. Neurons were scraped off the plate, homogenized in PBS using a Dounce homogenizer (Wheaton, USA) and centrifuged at 80xg for 20 minutes at 4˚C to remove any cell debris and collagen. The supernatant was collected and the membrane fractions were collected by ultracentrifugation at 35,000xg for 1 hour at 4˚C. After determining the protein concentrations, an equal amount of adult and embryonic neurite membrane fractions were centrifuged (200 X g, 10 minutes, 4˚C) onto serum starved rat primary Schwann cells. After incubating at 37˚C for 20 minutes, cell lysates were prepared, size fractionated on SDS-PAGE and then subjected to Western blot analysis.

Generation of Adenoviruses – Adenoviruses encoding Wild-type and Temperature-sensitive Dynamin1 constructs and ErbB2-EYFP were cloned into the PadTrack adenoviral expression shuttle vector from Stratagene. Next, the shuttle vectors containing

the transgenes were linearized with Pme I and electroporated into the BJ5183 E coli along with the adenovirus vector backbone. The bacteria then carried out homologous recombination, which resulted in the insertion of the transgene into the adenovirus vector backbone. Positive clones were identified via restriction digest, and suitable clones were selected by their ability to induce high levels of the transgene when transfected into the helper cell line AD293. Adenoviruses encoding the appropriate transgenes were then amplified in AD293 cells in 10cm dishes. When ~50% of the cells were exhibiting cytopathic effects, they were rinsed off the plate, spun down, and resuspended in 2ml of PBS. The adenovirus was then released from the cells via 3 rounds of freeze-thaw in ethanol-dry ice bath and RT water. After centrifugation, the virus is the aliquoted and stored in -80 degrees. For in vitro infections, virus from the previous step was used to infect three 15cm dishes of AD293 cells for amplification, and collected as above. Schwann cells were infected with varying titers of virus to select for best possible expression. The cells were then either used for biochemistry analysis for endocytosis using dynamin isoforms or the ErbB2-EYFP expressing Schwann cells were used for imaging and immunofluorescence analysis.

Statistical analysis – One way ANOVA followed by Tukey's post hoc analysis was performed using Instat Prism Graph Pad.

5.3 Results

Nrg1 induces internalization of the ErbB2 and ErbB3 receptors in Schwann cells

To determine whether ErbB2 and ErbB3 receptors are internalized into Schwann cells after activation at the cell surface, we used cell surface biotinylation assays to track the fate of the receptors following Nrg1 stimulation. For this, Schwann cell surface proteins were first biotinylated at 4°C and then either left in ligand-free media or stimulated with soluble Nrg1 at 37°C to initiate receptor activation. After various time points (15′, 45′ and 90′) any remaining surface biotin was stripped off. The internalized biotinylated protein was purified by streptavidin beads and the amount of ErbB2 and ErbB3 receptors were determined by Western blot analysis. As shown in Figure 4A, Lane1 represents total biotinylated surface ErbB2 at 4°C. After shifting to 37°C, control cultures in the absence of Nrg1 show low levels of internalized ErbB2 over time, suggesting constitutive ligand-independent endocytosis. Following Nrg1 stimulation, ErbB2 internalization was increased compared to the controls, indicating ligand-dependent endocytosis. Maximum ErbB2 internalization was detected at 45′ upon Nrg1 treatment. Nrg1 stimulation showed an activation of the ErbB2 receptors (pErbB2) that accompanied the increase in receptor internalization. ErbB3 receptors followed a similar kinetics of Nrg1-dependent internalization as seen with ErbB2. Quantification of the results represented as the percentage of surface biotinylated ErbB2 and ErbB3 internalized over time are shown in Figure 4B.

Next, we used live-cell imaging to visualize ErbB receptor internalization in Schwann cells. For this, we transfected Schwann cells with fluorescently labeled ErbB2

and monitored receptor localization following Nrg1 stimulation. In control cells without Nrg1 treatment, ErbB2-EYFP was localized mainly on the Schwann cell membrane, with no clear indication of constitutive internalization over time, as detected using the biochemical analysis earlier (Figure. 4C). In Nrg1 stimulated cells, several puntca of ErbB2-EYFP were detected inside the cells as early as 25′ and increasing at 40′. This was accompanied by a decrease in fluorescent intensity at the plasma membrane. ErbB2-EYFP appeared localized to the perinuclear region at 40′ and 75′, indicating that upon Nrg1 stimulation ErbB2 is internalized over time. Next we asked if this internalization could be blocked by dynasore, which is a quick acting, reversible, specific inhibitor of dynamin GTPase activity(Macia et al., 2006). Dynamins belong to a family of GTPases that function in the pinching of newly formed vesicles at the plasma membrane and Golgi (Praefcke and McMahon, 2004). ErbB2-EYFP expressing Schwann cells were treated with dynasore in the presence or absence of Nrg1. Schwann cells treated with Nrg1 showed clear intracellular localization of ErbB2-EYFP puncta at 90′(Figure. 4D) whereas, cells co-treated with dynasore and Nrg1 showed ErbB2-EYFP localization mainly at the plasma membrane, indicating that dynamin activity is required for ErbB2 internalization upon Nrg1 stimulation.

Altogether these results indicate that Nrg1 induces ErbB2 and ErbB3 internalization in Schwann cells in a dynamin-dependent manner.

ErbB2 and Nrg1 are seen localized to Rab5-positive endosomes upon internalization

To further determine the endocytic property of ErbB2, we assessed whether the internalized receptors appear in Rab5-positive early endosomes. Schwann cells transfected with ErbB2-EYFP were either left untreated or stimulated with Nrg1 for 15′, 45′ and 90′ and then immunostained for ErbB2-EYFP and Rab5. In the absence of Nrg1, ErbB2-EYFP remained localized mainly at the membrane (Figure. 5A). Upon Nrg1 stimulation ErbB2-EYFP was localized to Rab5 endosomes as early as 15′ and more prominently at 90′ upon Nrg1 treatment, indicating that ErbB receptors traffic through the Rab5 endosomal compartments upon internalization.

Next we monitored internalization of the endogenous ErbB receptors following Nrg1 stimulation. To track ligand-bound receptor complexes in Schwann cells, recombinant Nrg1 was conjugated to Alexa Fluor 555 and purified using resin packed spin filters (as described in Material and Methods). Nrg1-Alexa 555 was successful in activating the ErbB receptors, as tested by Western blot analysis (data not shown). Schwann cells were stimulated with the labeled Nrg1 for 20′ at 4°C, to allow surface receptor labeling without internalization. Cultures were then moved to 37°C for various time points (15′ and 45′) to allow for endocytosis. As shown in Figure 5B, in cultures left at 4°C most of the labeled Nrg1 was found on the Schwann cell surface outlining the plasma membrane (Figure. 5B). At 15′ and more predominantly at 45′, Nrg1-Alexa 555 was seen inside the Schwann cell colocalizaing with the Rab5-positive compartments. This result indicates that the endogenous ErbB receptors along with their bound ligands are internalized into an early endosome.

Internalized ErbB receptors are either degraded or recycled back to the cell surface

Once internalized into the early endosome, ligand-bound receptors either traffic to the lysosome via the late endosome for degradation, or recycle back to the cell surface (Gonzalez-Gaitan, 2008). To determine whether internalized ErbB receptors are targeted for degradation, Schwann cells were treated with increasing doses of Nrg1 (0.3nM, 1nM and 10nM) for various time points. The ErbB2 and ErbB3 receptor activation and total amounts were assessed over time by Western blots analysis. ErbB receptors were activated upon 15′ of Nrg1 treatment with all concentrations of Nrg1 (Figure. 6A). Higher doses of Nrg1 increased ErbB2 and ErbB3 activation, as expected. ErbB2 activation was decreased as early as 90′ and more at 150′ with all doses of Nrg1. ErbB3 receptor followed a similar pattern of activation as ErbB2. Western blots analysis for total ErbB2 and ErbB3 receptors indicated overall receptor downregulation over time with significant downregulation at 150′ with 1nM Nrg1 and at 90′ and 150′ with 10nM Nrg1 for ErbB (Figure. 6B). In addition to showing a similar pattern of receptor downregulation as ErbB2, ErbB3 showed enhanced downregulation, seen as early as 15′ with 10nM Nrg1.

Next we determine if ErbB2 and ErbB3 receptors are capable of trafficking back to the plasma membrane. One of the markers for the receptor recycling pathway is transferrin. Transferrin undergoes both a fast ($t_{1/2}$ – 5 min) and a slow recycling ($t_{1/2}$ – 15-30 min) pathway upon internalization (Mayor et al., 1993, Sheff et al., 1999). We stimulated ErbB2-EYFP transfected Schwann cells with Nrg1 along with transferrin-555 (Tfn-555) for various time points (15′ and 45′). Transferrin-555 labeling was seen on

Schwann cell membrane and intracellularly at 15′ and in perinuclear compartments at 45′ (Figure. 7A). Most ErbB2-EYFP remained predominantly at the cell surface at 15′, however ErbB2 receptor was co-localised with Tfn555 at 45′, suggesting that the ErbB2 receptors undergo recycling via the slow recycling pathway.

To verify biochemically whether endogenous ErbB2 and ErbB3 reappear on the cell surface after being internalized we used the biotinylation assay to track the receptors following their activation with Nrg1. Briefly, Schwann cells were surface biotinylated at 4°C and stimulated with Nrg1 at 37°C for 45′ to allow for receptor internalization. Cell surface biotin was then stripped off and the cells were incubated in Nrg1-free media at 37°C to allow for internalized biotinylated-receptors to recycle back to the surface for another 30′ and 60′. Biotinylated proteins that recycled back were again stripped of biotin or left intact. If biotinylated internalized receptors reappeared on the cell surface, they would become sensitive to biotin stripping. Total biotinylated protein was purified with streptavidin beads and the amount of ErbB2 and ErbB3 in each sample was determined by Western blot analysis. Lane 1 represents total biotinylated receptor at 4°C. Lane 2 represents internalized receptors at 45′ in the absence of Nrg1. ErbB2 and ErbB3 receptors internalized after 45′ of Nrg1-treatment, as seen earlier (Figure. 7B – Lane 3). After a 30′ chase in Nrg1-free media, non-stripped conditions (Lane 6) showed higher amounts of ErbB2 and ErbB3 receptors compared to the biotin-stripped conditions (Lane 4), indicating that internalized ErbB2 and ErbB3 receptors were recycled to the membrane. After the 60′ chase however, non-stripped conditions (Lane 7) showed lower amounts of ErbB2 and ErbB3 receptors compared to the biotin-stripped conditions (Lane

5), indicating that most internalized ErbB2 and ErbB3 receptors did not reappear on the surface and might have been routed for degradation. It was interesting to note that ErbB3 receptor had higher rates of constitutive endocytosis, seen in control non-stimulated Schwann cells (Figure. 7B). Quantitative representation of 3 independent experiments show that upon Nrg1 stimulation, almost 51% of ErbB2, while only 32% of internalized ErbB3 recycled back to the surface after the 30′ chase (Figure. 7C).

To determine if ligand dose has a differential effect on receptor recycling, we compared 0.3nM and 10nM Nrg1 treatment on ErbB receptor recycling. The recycling assay was conducted as described above. As seen in Figure. 8A, Lane 1 represents total biotinylated receptor at 4°C. Lane 2 represents internalized receptor at 45′ in the absence of Nrg1. Lane 3 and 6 represents internalized receptor at 0.3nM and 10nM, respectively. Lane 4 and 5 correspond to biotinylated receptor that remained after the second biotin cleavage with the different Nrg1 doses. Lane 5 and 8 correspond to total biotinylated receptor in cultures that were not subject to the second biotin removal. Our preliminary data shows that a dose of 10nM Nrg1 increased overall internalization of ErbB2 and ErbB3 receptor (Figure. 8A, compare Lane 3 and 6). ErbB2 receptor recycles at 42.5% and 41.65% at 0.3nM and 10nM respectively (Figure. 8B), whereas ErbB3 recycles at 34.9% and 24.4% at 0.3nM and 10nM, respectively. This suggests that higher Nrg1 dose decreases ErbB3 receptor recycling. The percentage of internalized receptor that is not recycled is indicated as percentage remaining (Figure. 8B, % Remaining), while the unaccounted receptor population is assumed to be that which has degraded (Figure. 8B, % Degraded). ErbB3 receptor decrease in recycling (34.9% to 24.4%) corresponds to its increase in degradation (18% to 43.4%) from 0.3nM to 10nM. Our preliminary data

suggest that ErbB2 and ErbB3 receptors might have differential recycling rates dependent on Nrg1 dose.

5.4 Discussion

ErbB2 and ErbB3 receptors are endocytosed upon Nrg1-stimulation and localize to Rab5-positive compartments in Schwann cells

The results shown in this chapter collectively provide evidence that ErbB2 and ErbB3 receptors in Schwann cells undergo ligand-dependent endocytosis in a dynamin-dependent manner. Although many studies indicate that ErbB2 receptor is endocytosis-deficient because of regions in its cytoplasmic domain that confer this inhibitory affect, in Schwann cells we observed that ErbB2 is capable of internalization (Baulida et al., 1996, Haslekas et al., 2005). Pedersen et al, have shown that co-expression of EGFR or ErbB3 along with ErbB2 in PAE cells, induces downregulation of ErbB2 (Pedersen et al., 2009). Since both ErbB2 and ErbB3 receptors are expressed in Schwann cells and must dimerize to propagate Nrg1-mediated signals, ErbB3 receptor internalization upon ligand binding could be a driving force for the internalization of ErbB2 and could explain why the ErbB2 receptor is not deficient in its endocytic property in Schwann cells.

We also observed that the kinetics of internalization of both ErbB2 and ErbB3 in Schwann cells were slower in comparison to that proposed of EGFR in other cell types. EGFR becomes internalized within 5′ of EGF stimulation (Vieira et al., 1996, Wiley, 2003). The mechanism of EGFR internalization upon EGF stimulation has been

extensively characterized the internalization and trafficking of all other ErbB receptors upon Nrg stimulation remain not fully characterized and controversial. Liu et al, show that in hippocampal neurons 50% of ErbB2 and ErbB4 internalize after 30′ to 60′ of stimulation with Nrg1 (Liu et al., 2007). In contrast ErbB2/3/4 like the EGFR become rapidly endocytosed upon Nrg1 stimulation in HEK293 (Yang et al., 2005). In Schwann cells, although endocytosis of ErbB2 and ErbB3 is observed in negligible amounts as early as 15′(Figure. 4A), detection of maximal internalized ErbB2 and ErbB3 is observed only at 45. The constitutive internalization of the ErbB receptors in the absence of Nrg1 likely represents the normal membrane flow and dynamics in the cell(Wiley, 2003).

Whether ErbB receptor kinase activity is required for its endocytic function also remains controversial. Multiple studies using receptor chimeras and deletion mutants of the EGFR, report that kinase activation is a pre-requisite for internalization (Jiang et al., 2003, Schmidt et al., 2003). However, inhibition of EGFR receptor kinase activity using pharmacological inhibitors show that ligand binding and dimerization but not kinase activity is sufficient for EGFR endocytosis (Wang et al., 2002, Wang et al., 2005). For the other ErbB receptors, it has been shown that inhibiting the kinase activity of the ErbB2/4 receptors decreased Nrg1-mediated internalization (Yang et al., 2005, Liu et al., 2007), indicating the requirement of receptor activity. Our data in Schwann cells shows that ErbB2 and ErbB3 internalization is accompanied by receptor activation. However whether receptor activation is required for internalization in Schwann cells is yet to be investigated.

Using confocal imaging analysis we also monitored ErbB2 receptor trafficking in live cells. ErbB2-EYFP-positive puncta were seen accumulating in the cell body of

Schwann cells within 40′ of Nrg1 treatment (Figure. 4C), which corresponds to the timing of maximum internalization we observed earlier using the biotinylation assay. However we did not observe the evidence of constitutive internalization in the absence of Nrg1, which was detected as early as 15′ in Figure. 4A. This could be due to the overexpression of ErbB2-EYFP which has been shown to increase and maintain receptors on membrane protrusions (Hommelgaard et al., 2004). Using both pharmacological and genetic analysis we demonstrate that ErbB2 internalization is dynamin-dependent (Figure. 4D). This result is analogous to that obtained when EGFR endocytosis is inhibited with mutant dynamin proteins in HELA cells (Vieira et al., 1996).

Our data also demonstrates that ErbB2 is trafficked to Rab5-positive endosomes upon Nrg1-stimulation, indicating that ErbB receptors in Schwann cells follow a canonical endosomal route (Figuer. 5A and 5B). Sorkin et al showed that EGF ligand remains bound to the receptors in the endosomes (Sorkin et al., 1988). Using fluorescently labled Nrg1 to follow endogenous ErbB3 receptors, we show that Nrg1-555 is also localized to Rab5-positive compartments in Schwann cells. This suggests that Nrg1 and ErbB receptors remain complexed in the early endosome.

Upon internalization ErbB2 and ErbB3 receptors are either degraded or recycled back to the cell surface

Once in the early endosomal compartment, internalized receptors are sorted into various routes. The pH sensitivity of the ligand binding, the dose of the ligand and the route of the receptor endocytosis are a few factors that determine the sorting process

(French et al., 1995, Wiley, 2003, Sigismund et al., 2008). Our data shows that with increasing Nrg1 concentrations more ErbB2 and ErbB3 are targeted for degradation (Figure. 6A). This is somewhat consistent with the mechanism of EGFR trafficking. Sigismund et al showed that significant EGFR ubiquitination was detected only at high doses of EGF, which is not only required for increased internalization of EGFR but also for targeted trafficking and degradation (Sigismund et al., 2005). They also showed that at low doses EGFR was targeted to the regulatory pathway rather than being degraded (Sigismund et al., 2008). Thus whereas low doses target receptors to the recycling pathway high doses target receptors for degradation. The dose-dependent differential trafficking of EGFR was associated with the use of the clathrin and non-clathrin-mediated endocytic pathways at low and high doses, respectively. It would be interesting to investigate if differential usage of internalization mechanisms (clathrin versus caveolin) may have a role in dose-dependent ErbB receptor downregulation in Schwann cells.

As mentioned above we further show that both ErbB2 and ErbB3 travel back to the plasma membrane following Nrg1-induced internalization (Figure. 7B). We also demonstrated that internalized ErbB2 receptors co-localize with transferrin in perinuclear compartments 45′ following Nrg1 stimulation (Figure. 7A), which corresponds to this phase of transferrin trafficking through the Rab11-positive recycling endosomes. This indicates that recycling ErbB2 receptors travel via the slow-recycling pathway (Hopkins and Trowbridge, 1983, Sheff et al., 1999).

It was interesting to note here that 51% of ErbB2 while only 32% of ErbB3 receptors re-appeared on the cell surface with the same dose of Nrg1 (0.3nM), one

possible explanation for this could be that ErbB3 receptors are preferentially sorted for degradation over recycling, as they remain bound to the ligand Nrg1. ErbB2 receptors however, do not bind ligand and might be sorted into recycling endosomes as empty receptors. However, for this to occur ErbB2 and ErbB3 must dissociate from one another. The mechanism of this dissociation and differential sorting is currently unknown. Our preliminary data on dose-dependent recycling (Figure. 8A) shows that while ErbB2 recycling rates do not vary with at low (0.3nM - 42.5%) and high (10nM – 41.65%) Nrg1 doses ErbB3, recycling decreases with increasing Nrg1 dose (0.3nM – 34.9% versus 10nM – 24.4%). This result provides further evidence that ErbB2 and ErbB3 receptors may be differentially sorted upon internalization.

6. Chapter Three: To determine how receptor trafficking regulates ErbB signaling and Schwann cell function

6.1 Introduction:

Many different signals lie downstream of the Nrg1-ErbB complex in the PNS. Nrg1-ErbB receptor signaling is required for various Schwann cell functions such as survival, proliferation, differentiation and myelination (Dong et al., 1995, Morrissey et al., 1995, Syroid et al., 1996, Dong et al., 1999, Garratt et al., 2000, Maurel and Salzer, 2000, Zanazzi et al., 2001, Michailov et al., 2004, Ogata et al., 2004, Kao et al., 2009). Nrg1- mediated signaling also plays a role in the inhibition of myelination and the onset of demyelination (Zanazzi et al., 2001, Ogata et al., 2004).

In Schwann cells PI3-kinase/Akt signaling is required for Nrg1-induced survival, as well as proliferation (Dong et al., 1999, Maurel and Salzer, 2000). PI3-kinase/Akt pathway is also crucial for promoting Nrg1-mediated Schwann cell myelination (Ogata et al., 2004, Taveggia et al., 2005, Syed et al., 2010). On the other hand, Erk1/2 is a key de-differentiation signal in Schwann cells independent of Akt signaling (Harrisingh et al., 2004, Ogata et al., 2004). Erk1/2 and c-jun N terminal kinase (JNK) pathways are also implicated in Nrg1-mediated Schwann cell migration (Yamauchi et al., 2008)

The mechanism by which the Nrg1-ErbB receptor ligand system activates different downstream effectors and elicits multiple roles in Schwann cells is unknown. One possible mechanism that might underlie this functional diversity is endosomal signaling of the receptor modulated by the intracellular endocytic pathways.

In many cell types intracellular receptor trafficking has been shown to regulate receptor signaling by modulating downstream effectors (De Donatis et al., 2008, Sigismund et al., 2008). In PC12 cells, NGF-induced differentiation is regulated by TrkA receptor internalization that activates Erk1/2 within endosomes, whereas cell survival is mediated by the PI3-kinase activation, which occurs at the plasma membrane (Zhang et al., 2000). On the other hand, TrkB receptors internalization upon BDNF stimulation is required for PI3-kinase/Akt activation (Zheng et al., 2008). Of the ErbB receptors, ErbB4 receptor internalization in hippocampal neurons is required for sustained Akt and Erk1/2 activation (Liu et al., 2007). Whereas in primary rat myoblasts, ErbB2/3/4 internalization promotes Erk1/2 activation that induces acetylcholine receptor synthesis (Yang et al., 2005).

In chapter two we show that both ErbB2 and ErbB3 receptors become internalized into Schwann cells following Nrg1 stimulation. The results also show that the receptors follow two distinct pathways, one that targets the receptors for degradation and the other that recycles them back to the cell surface. Therefore in this study, we investigated the roles of ErbB receptor internalization and trafficking in modulating the downstream signal activation. We hypothesized that Nrg1-induced receptor endocytosis by affecting the kinetics of downstream molecules such as Akt and Erk1/2 may have a quantifiable effect on cellular functions such as proliferation and myelination. To test this, we investigated the effect of endocytic block on Nrg1-mediated ErbB receptor signaling in Schwann cells.

Our data shows that ErbB receptor internalization is important for Nrg1-induced PI3-kinase/Akt activation but not the Ras/Raf/Erk pathway. Nrg1-induced Schwann cell

proliferation and myelination also required receptor internalization. We also provide evidence that the Nrg1-induced PI3-kinase pathway activation is modulated within the receptor recycling pathway associated with Rab11 function.

6.2 Results

Nrg1-mediated ErbB receptor internalization is required for maximal Akt activation

To determine the effect of Nrg1-mediated ErbB receptor endocytosis on its downstream signaling, we first used pharmacological endocytic inhibitors to block receptor internalization. Monodansylcadaverine (MDC), is an inhibitor of membrane transglutaminases that actively take part in receptor-mediated endocytosis (Schlegel et al., 1982). To determine whether MDC inhibits Nrg1-induced ErbB receptor endocytosis in Schwann cells, Schwann cells transfected with ErbB2-EYFP were stimulated with Nrg1 in the presence of MDC. In control cultures in the absence of MDC, Nrg1-induced ErbB2-EYFP internalization was seen inside the Schwann cells (Figure. 9A). In the presence of MDC, ErbB2-EYFP remained on the cell surface, indicating that MDC was successful in inhibiting Nrg1-mediated ErbB receptor endocytosis.

Next we determined the effect of endocytic block on Nrg1-induced signaling. Schwann cells were pretreated with MDC (100μM) for 45′, then stimulated with Nrg1 for varying time-points in the continuing presence of MDC (15′, 45′ and 90′). MDC treatment did not have a significant effect on Nrg1-induced ErbB receptor activation, however, drastically decreased the downstream Akt activation (Figure. 9B). Nrg1-induced Erk1/2 activation was not affected by the inhibition, indicating that Erbb receptor endocytosis is required for the PI3-kinase/Akt pathway. To confirm the endocytic block

on Nrg1-mediated signaling function we also used the pharmacological drug – dynasore. Dynasore is a reversible, specific inhibitor of dynamin GTPase activity (Macia et al., 2006). In the presence of dynasore, Nrg1-induced Akt activation is attenuated in Schwann cells (Figure. 10A). As shown with MDC, dynasore did not affect Nrg1-induced Erk1/2 activation.

We also employed adenoviruses to deliver wild-type dynamin-1 (WT) and temperature-sensitive dynamin-1 mutants (TS, G273D), into Schwann cells, to further confirm the role of Nrg1-mediated ErbB receptor internalization on the PI3-kinase/Akt pathway (Zhang et al., 2000). The TS-dynamin mutant protein becomes inactive when moved from a permissive temperature of 32°C to a non-permissive temperature of 39°C, thereby inhibiting dynamin driven internalization. After infections with adenoviruses, expression of the HA-tagged WT-dynamin or the TS-dynamin was confirmed by immunostaining. As shown in Figure 11A, all infected Schwann cells expressed the dynamin proteins. Next, Schwann cells expressing either WT or TS-dynamin were grown to confluency and were placed in either the permissive (32°C) temperature or the non-permissive (39°C) temperature to inactivate TS-dynamin, for 1hour. Schwann cells were then treated with Nrg1 for 45′ to stimulate receptor activation and internalization. Under permissive temperatures, Nrg1 stimulation induced Akt and Erk1/2 activation in both the WT and TS-dynamin cells (Figure. 11B). When switched to the non-permissive temperature (39°C) there was an overall decrease in the activation level of Akt and Erk1/2 in WT-dynamin cells, likely reflecting the effect of high temperature on Nrg1 signaling. However in the TS-dynamin cells, there was a complete inhibtion of Akt activation while Erk1/2 activation was still observed.

Taken together, these results indicate that Nrg1-induced ErbB receptor internalization is important for the activation of the downstream PI3-kinase pathway.

ErbB receptor endocytosis is required for Nrg1-induced Schwann cell proliferation and myelination

So far, our data suggests that ErbB receptor internalization is required for Akt activation. Previous studies have shown that the activation of PI3-kinase/Akt but not the Ras/Raf/Erk pathway induces Nrg1-mediated Schwann cell proliferation (Dong et al., 1999, Maurel and Salzer, 2000). To determine whether the Akt activity associated with ErbB receptor internalization is involved in Nrg1 function, we investigated the effect of endocytic block on Nrg1-induced Schwann cell proliferation. Schwann cells were treated with Nrg1 in the presence of absence of dynasore (0.1μM, 1μM and 10μM) and the proliferation was assessed by BrdU incorporation. Control cultures were co-treated with Nrg1 along with LY294002 (2.5μM and 5μM) or U0126 (2.5μM and 5μM), specific inhibitors of the PI3-kinase and the Ras/Raf/Erk pathway, respectively. As shown in Figure 12, dynasore inhibited Nrg1-induced Schwann cell proliferation in a dose-dependent manner. A similar inhibitory effect was seen in cultures treated with LY294002. As reported previously, inhibition of the Ras/Raf/Erk pathway did not affect Nrg1-induced proliferation. This result suggests that ErbB receptor internalization, which is likely associated with activation of downstream Akt is required for Nrg1-induced Schwann cell proliferation.

Previously we have shown that soluble Nrg1 at low doses elicits a promyelinating effect on Schwann cells by its selective activation of the PI3-kinase pathway (Syed et al.,

2010). Therefore, we employed a similar approach as above to determine the effect of endocytic block on the Akt-dependent promyelinating function of Nrg1. In Schwann cell-DRG co-cultures, treatment with dynasore for an extended period of time, would block other non-Nrg1 dependent endocytic traffic, which may be essential for myelination. In our earlier study, we observed a transient (7 hour) exposure to Nrg1 was sufficient to elicit a promyelinating response at the end of a 10-day culture period. Therefore, we designed the following experiment to determine the role of ErbB receptor internalization in mediating the promyelinating function of soluble Nrg1, which minimizes the non-specific effect of the endocytic block. Co-cultures were treated with varying doses of dynasore (0.1μM, 1μM and 10μM) in the presence of Nrg1 for 7 hours. Nrg1 and dynasore were then washed out and co-cultures were maintained in normal myelinating media for 10 more days. Control cultures were treated with dynasore for 7 hours before being placed in normal media. Myelination was assessed by immunostaining for MBP.

Our data shows that the myelin-promoting effect of soluble Nrg1 was blocked in the presence of dynasore in a dose-dependent manner (Figure. 13A). The 7 hours dynasore treatment alone (0.1μM and 1μM) had no effect on myelination, indicating that we were able to localize the endocytic block on the soluble Nrg1 function. Dynasore treatment alone at 10μM however, had an inhibitory effect on myelination, suggesting that this concentration may have a deleterious effect of the co-cultures. Representative images for the statistically significant results are shown in Figure. 13B. Taken together these results suggest that ErbB receptor internalization is important for the pro-myelinating function of Nrg1.

We next asked if the inhibitory effect of dynasore on the Nrg1-function was associated with an inhibition of the Nrg1 induced Akt activation. Co-cultures were treated with Nrg1 in the presence of dynasore (0.1μM, 1μM and 10μM) for 15′, 45′ and 90′. As seen in Figure 13C (column 3), Nrg1 treatment increased Akt activation overtime in co-cultures, as we have shown previously (Syed et al., 2010). Co-treatment with dynasore resulted in a dose-dependent decrease in Akt activation (column 4, at 1μM), which had no effect on the Erk1/2 activity. In control cultures without Nrg1 stimulation, the dynasore treatment had no effect on Akt and Erk1/2 activity (compare column 1 and 2). These results suggest that Nrg1 mediated Akt activation in co-cultures requires endocytosis.

Inhibition of ErbB receptor recycling enhances Nrg1-induced Akt activation

We have shown that ErbB receptors internalize to elicit Nrg1-mediated Akt activation. Our studies in chapter two show that internalized ErbB receptors are also capable of recycling to the cell surface. In many cell types, PI3-kinase/Akt activation occurs at the plasma membrane (Sadowski et al., 2009, Sorkin and von Zastrow, 2009). Furthermore, it has been shown that sustained Akt activation requires continuous recycling of growth factor receptors back to the plasma membrane (Maxfield and McGraw, 2004, Romanelli et al., 2007). Therefore we asked whether ErbB receptor recycling is required for Nrg1-induced Akt activation in Schwann cells. Schwann cells were pretreated with monensin, an ionophore that inhibits receptor recycling, for 45′ (Maxfield, 1982, Stein et al., 1984, Yamashiro and Maxfield, 1984, Wang et al., 2002).

Cells were then stimulated with Nrg1 for various time points (15′, 45′, 90). Monensin treatment increased the overall levels of active Akt in Nrg1 treated cultures compared to the cultures in the absence of monensin (Figure. 14A). In contrast monensin inhibited the levels of Nrg1-induced Erk1/2 activation. The activation levels of ErbB3 (pErbB3) which provide docking sites for the PI3-kinase were not affected by monensin, indicating that the effect of monensin on the Akt activation was not due to altered receptor activity. This result indicates that inhibiting ErbB receptor from travelling back to the cell surface or retaining the receptors inside the cells promotes Akt activation.

Together with the data above that showed the presence of active Akt within endosomes, this result also suggests that localization within endosomal compartments may be crucial for sustaining Nrg1-induced Akt activation in Schwann cells.

Next, we asked whether inhibition of receptor recycling enhances Akt activation in co-cultures treated with soluble Nrg1, a condition that promotes myelination, as we have shown previously (Syed et al., 2010). We prepared lystaes from co-cultures treated with monensin in the presence or absence of Nrg1. As expected, Nrg1-induced Akt and Erk1/2 activation in co-cultures. Monensin treatment enhances Nrg1-induced Akt activation (Figure. 14B, compare column 2 and 1) but attenuated that of Erk1/2, as seen in Schwann cell monocultures above. Monensin also did not affect the endogenous Akt Erk1/2 activation in co-cultures. These results indicate that inhibition of recycling promotes Nrg1-induced Akt activation in Schwann cell-DRG co-cultures.

Monensin does not affect initial ErbB receptor endocytosis

To determine if monensin affected receptor endocytosis, ErbB3-citrine expressing Schwann cells were first treated with monensin and stimulated with Nrg1. Control cultures, in the absence of Nrg1 stimulation had a clear plasma membrane localization of ErbB3 (Figure. 14C). Nrg1 stimulation for 45′ showed ErbB3 co-localised on the plasma membrane and intracellularly with early endosomal marker (EEA1). Nrg1 treatment in the presence of monensin showed more ErbB3 present in the early endosomes, suggesting that monensins did not affect endocytic function of the ErbB3 receptor, but may have increased the accumulation of these receptors in endosomal compartments. Taken together, these results indicate that Nrg1-induced PI3-kinase/Akt activation is regulated by an intracellular endosomal event.

Active Akt is enriched in endosomal fractions upon Nrg1 stimulation

Endosomal Akt activation and signaling has been reported in other cell types (Hunker et al., 2006, Schenck et al., 2008, Nazarewicz et al., 2011). In Schwann cells, whether Nrg1-induced Akt activation associates with endosomal compartments is unknown. To investigate this, we purified crude endosomal fractions from Schwann cells stimulated with Nrg1 for 15′ and determined the levels of active Akt and Erk1/2. Briefly Schwann cells were treated with or without Nrg1 and homogenized. The post-nuclear supernatant (TL) was loaded on a sucrose step floatation gradient, optimized to separate endosomal fractions from other membrane organelles (Gorvel et al., 1995, de Araujo et al., 2008). The fractions were concentrated and analysed for the presence of active Akt

and Erk1/2 by Western blot analysis. Rab5 was used as a marker for the early endosomes. In control cultures without Nrg1 stimulation, Rab5 was predominantly present in fraction 6 (Figure. 15A), indicating its enrichment in the early endosome. No detectable levels of phospho-Akt (pAkt) or phospho-Erk1/2 (pErk1/2) were present across the fractions. Upon Nrg1 stimulation both active Akt and Erk1/2 were detected in the Rab5-enriched fraction 6. Quantification of the relative amounts of the active Akt and Erk1/2 showed an approximate nine-fold increase in the enrichment of the active Akt in fraction 6 compared to the level detected in the total post nuclear supernatant (Figure. 15B). In comparison, the proportion of active Erk1/2 was not significantly increased in this fraction. This result, although preliminary suggests the presence of endosomal Akt signaling in Schwann cells.

Rab11 enhances association of ErbB receptors with recycling endosomes and may promote PI3-kinase/Akt signaling

Our experiments so far indicate a role of endosomes in promoting Nrg1-induced PI3-kinase/Akt signaling. However, it is not clear which endosomal compartment is associated with PI3-kinase/Akt activation and possibly the promyelinating function of Nrg1.

A recent study by Stendel et al, indicate a role of recycling endosomes and the associated Rab11a in regulating Schwann cell myelination. Their study showed that Schwann cell-specific overexpression of a constitutively active Rab11a mutant (GTP-bound, Rab11a_Q70L) that lacks GTPase activity, enhanced myelination in co-cultures

whereas expression of a dominant negative Rab11a mutant (GDP-bound, Rab11a_S25N), inhibited the myeliantion process.

To determine the role of Rab11 on ErbB receptor trafficking and the PI3-kinase activation we co-transfected WT Rab11a-DsRed or DN Rab11a-DsRed along with ErbB3-citrine into Schwann cells. Cells were stimulated with Nrg1 for 45′ then immunostained for the presence of ErbB3 and WT or DN Rab11a. As shown in Figure 16A, WT Rab11a-DsRed appears in the perinuclear region as condensed punctate compartments. In the absence of Nrg1 stimulation ErbB3 was predominantly localized to the plasma membrane in WT Rab11a-DsRed co-transfected cells (Figure. 16A). Upon Nrg1 stimulation, ErbB3 (also ErbB2, data not shown) localized strongly with WT Rab11a-DsRed in perinuclear compartments, indicating a likely arrest in the recycling endosomes. In some cells ErbB2 and ErbB3 receptors were also constitutively recruited into recycling endosomes in the absence of Nrg1 in WT Rab11a-DsRed Schwann cells (data not shown). In cells co-transfected with ErbB3 and DN Rab11a-DsRed, Rab11a showed a diffused pattern of presence all throughout the Schwann cell body. ErbB3 appears as punctate structures intracellularly with no strong colocalization to any perinuclear compartment. These results indicate that Rab11 plays a role in regulating ErbB receptor trafficking by promoting its association with recycling endosomes.

We next asked whether intracellular PI3-kinase/Akt activity is modulated by Rab11 in Schwann cells. To monitor intracellular PI3-kinase activity we used a fluorescent probe PH-Akt-GFP that binds to PIP3, an inositol lipid produced by PI3-kinase (Fields et al., 2010). Schwann cells were co-transfected with PH-Akt-GFP along with WT Rab11a-DsRed. As shown in Figure 15B, control Schwann cells in the absence

of Nrg1 stimulation, show diffused appearance of PH-Akt-GFP, suggesting no specific localization to membrane compartments. Rab11a in these Schwann cells appeared condensed in the perinuclear compartment, as seen above. Upon Nrg1 stimulation PH-Akt-GFP localized to the Schwann cell plasma membrane and was also accumulated in the Rab11a positive compartments, indicating PI3-kinase activation occurring at the membrane and also localized to the recycling endosomes upon Nrg1 stimulation. This result also indicates a possible role of Rab11a in generating the endosomal PI3-kinase activity.

6.3 Discussion

Nrg1-mediated Akt activation and function requires ErbB receptor endocytosis

In this chapter we have confirmed, by using three approaches, that ErbB receptor endocytosis plays an important role in eliciting Nrg1-mediated PI3-kinase/Akt activation. Once believed to be solely occurring at the plasma membrane where phosphatidylinositol-(3,4,5)-phosphate (PIP_3) is generated, it is becoming increasingly clear that PI3-kinase/Akt activation, requires receptor endocytosis to sustain signaling (Zheng et al., 2008, Goh et al., 2010, Chaturvedi et al., 2011, Nazarewicz et al., 2011). An early study by Vieira et al, showed that EGFR endocytosis is required not only to attenuate EGF signaling but to control specific signaling pathways (Vieira et al., 1996). By using mutant dynamin to inhibit active EGFR internalization in HELA cells, they showed that some pathways such as the PI3-kinase/Akt and Ras/Raf/Erk1/2 are

downregulated upon endocytic block while others such as PLCγ are enhanced. Liu et al, similarly show that blocking Nrg1-induced ErbB2 and ErbB4 receptor endocytosis in hippocampal neurons also inhibits PI3-kinase/Akt and Erk1/2 activation (Liu et al., 2007). Taken together we provide here strong evidence that ErbB2 and ErbB3 endocytosis modulates Nrg1-mediated PI3-kinase/Akt activation in Schwann cells (Figures. 9B, 10A and 11B).

Interestingly, in our study we did not observe a drastic change on Erk1/2 activation upon inhibiting Nrg1-mediated ErbB receptor endocytosis (Figures. 9B, 10A and 11B). Erk1/2 activation downstream of NGF-TrkA, EGF-EGFR and other ligand-receptor interactions however, has been shown to be dependent on endocytosis (Vieira et al., 1996, Zhang et al., 2000, Sigismund et al., 2008). Recently, Goh et al, generated the first EGFR mutant that is endocytosis-deficient and showed that Akt and not Erk1/2 activation is dependent on EGFR endocytosis in PAE cells (Goh et al., 2010). Also BDNF-dependent Akt but not Erk1/2 activation has been shown to require TrkB endocytosis (Zheng et al., 2008). Therefore the notion that receptor endocytosis is required for Erk1/2 activation is also controversial and may vary within ligand-receptor families and/or between cell types (Zheng et al., 2008, Sorkin and Goh, 2009, Chaturvedi et al., 2011). We thus suggest that Nrg1-induced ErbB2 and ErbB3 receptor endocytosis may serve to downregulate Erk1/2 activation while enhancing that of Akt in Schwann cells.

Our results show that ErbB receptor endocytosis is required for Nrg1-induced Schwann cell proliferation. Inhibition of proliferation mimics that of the LY294002 (PI3-kinase inhibitor) but not the U0126 (Mek1/2-inhibitor) in a dose-dependent and

significant manner (Figure. 12A). It has been shown that inhibition of the PI3-kinase but not the Ras/Raf/Erk1/2 pathway reversibly inhibits Nrg1-mediated Schwann cell proliferation (Dong et al., 1999, Maurel and Salzer, 2000). Luo et al, have shown that the early phase of EGF-stimulated cell cycle progression in hepatocytes is mediated by endosomal Akt activity (Luo et al., 2011b). Taken together, we implicate Akt activation downstream of the ErbB receptor endocytic event as being required for Schwann cell proliferation mediated by Nrg1.

We next showed that inhibiting ErbB receptor endocytosis suppresses the promyelination function of soluble Nrg1 (Figure. 13B). We observe that endocytic block attenuates Nrg1-mediated Akt activation in Schwann cell-DRG co-cultures. We showed previously that the promyelinating function of Nrg1 corresponds to a selective increase in Akt activation in Schwann cell-DRG co-cultures (Syed et al., 2010). This supports the notion that ErbB receptor endocytosis plays an important role in Nrg1-induced Akt activation that is crucial for myelination.

Nrg1-induced Akt activity is regulated in the endosomes

A key finding of our study was that inhibiting receptor recycling enhances Nrg1-induced Akt activation in both Schwann cell monocultures and Schwann cell-DRG co-cultures (Figure. 14A and 14C). Monensin treatment did not inhibit ErbB3 receptor endocytosis or its activity, establishing that the increased Akt activation we observed was not due to an increase in the active Akt at the plasma membrane or altered receptor activity, respectively (Figure. 14C and 14A). This provided us with evidence suggesting

that inhibition of receptor recycling increases Akt activation by sustaining the receptor signaling within endosomes.

Our results although preliminary, suggest a role for Nrg1-mediated endosomal Akt signaling in Schwann cells. Upon Nrg1 stimulation, we observed a nine-fold enrichment of phospho-Akt (pAkt) in Rab5-positive fractions in comparison to that of phospho-Erk1/2(pErk1/2) (Figure. 15A and 15B), indicating an endosomal localization of Akt activity. The functional significance of endosomal Akt activity in EGF stimulated cells has been investigated. By localizing EGFR activity to endosomes, Wang et al have shown that endosomes not only serve as active platforms for the recruitment of EGFR-associated signaling molecules such as Akt and Erk1/2 but also are sufficient in eliciting a functional outcome (Wang et al., 2002). More recently, Luo et al, by using a similar approach show that endosome-associated Akt and Erk1/2 activation is sufficient to induce EGF-stimulated cell cycle progression in primary hepatocytes (Luo et al., 2011b). These studies support our results, suggesting that endosomal Akt activity may play a significant role in mediating Nrg1 function in Schwann cells.

PI3-kinase converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidyl inositol 3,4,5-triphosphate (PIP_3) that provide docking sites for Akt and its subsequent activation by PDK1. Akt has also been shown to be recruited by APPL1 to endosomes where its activation and substrate specificity is modulated (Mitsuuchi et al., 1999, Yang et al., 2003, Schenck et al., 2008). Kelly et al, provided early evidence of active PI3-kinase enrichment in low density membranes upon insulin stimulation in adipocytes, suggesting their presence in endosomes (Kelly et al., 1992). Using crude endosomal preparations we observed that active Akt is enriched in Rab5 containing fractions,

however, we cannot rule out the possibility that this fraction also contains other endosomal components such as the recycling endosomes (Rab11). Therefore, endosomal Akt activation downstream of PI3-kinase could be associated with either the early/sorting endosomes (Rab5/EEA1) or the recycling endosomes (Rab11).

Infact our data indicate the involvement of Rab11 and the recycling endosomes in regulating Nrg1-signaling in Schwann cells. Rab11 is a regulatory GTPase that is recruited to recycling endosomes (Lock and Stow, 2005). In CHO cells, expression of wild-type or constitutively active Rab11a (GTP-bound, Rab11a_Q70L) that lacks GTPase activity accumulates transferrin receptor in the recycling endosomes, while dominant negative Rab11a (GDP-bound, Rab11a_S25N) accumulates receptor in the early endosome (Ren et al., 1998). Interestingly both mutants inhibit receptor recycling back to the cell surface suggesting the importance of Rab11 in regulating receptor traffic.

We showed that overexpression of WT Rab11a in Schwann cells accumulated ErbB3 (and ErbB2, data not shown) receptors in Rab11-positive perinuclear compartments (Figure 16A). Expression with DN Rab11a on the other hand, showed a punctate appearance of ErbB3 upon Nrg1-stimulation throughout the cytosol without specific localization.

Furthermore using PH-Akt-GFP to localize Nrg1-induced PI3-kinase function in Schwann cells, we observed that expression of WT Rab11a localizes PH-Akt-GFP to the plasma membrane as well as the Rab11a-positive compartments, following Nrg1 stimulation (Figure. 16B). This finding demonstrates that Nrg1-induced PI3-kinase activity accumulates in the recycling endosomes in a Rab11a-dependent manner. This

data is interesting in light of recent evidence by Stendel et al, that showed that Schwann cell-specific expression of constitutively active Rab11a enhanced myelination while dominant negative Rab11a impaired it (Stendel et al., 2010). Although this study did not investigate the levels of active PI3-kinase/Akt in each of the mutant phenotypes, it is interesting to speculate that misregulation of recycling could also alter Nrg1-mediated endosomal PI3-kinase signaling during Schwann cell myelination. Since PI3-kinase/Akt is an important positive regulator of myelination, their study supports the hypothesis that PI3-kinase activation in the recycling endosomes could be functionally important for myelination.

Many proteins found mutated in inherited peripheral neuropathies such as CMT (Charcot-Marie-Tooth) disease, are regulators of the endocytic pathway. Therefore it is possible that misregulation of receptor trafficking may result in myelin phenotype by abnormal regulation of the endosomal PI3-kinase activity.

7 Chapter Four: To determine membrane-bound Neuregulin1 Type III mechanism of ErbB receptor trafficking

7.1 Introduction:

In our earlier studies (Chapter Two and Three) we used recombinant soluble Nrg1 Type II to characterize the role of ErbB receptor trafficking on Nrg1 signaling in Schwann cells. In the PNS, however, the predominant Nrg1 isoform important for Schwann cell development and myelination is the Nrg1 Type III (Michailov et al., 2004, Taveggia et al., 2005). Nrg1 Type II and Type III differ in their N-terminal region, with Type II containing an Ig (immunoglobulin) domain, while Type III contains a CRD (cysteine-rich-domain) (Garratt et al., 2000). On proteolytic cleavage of these transmembrane ligands, Nrg1 Type II becomes released and available as a paracrine signal, while Nrg1 Type III remains membrane-bound because of the hydrophobic property of its CRD, making it available as a juxtacrine signal. The first cleavage of Nrg1 is mediated by two proteases (ADAM17/TACE and BACE1) that cleave in close proximity to each other (Sahin et al., 2004, Willem et al., 2006, Hu et al., 2008). A second cleavage by an unknown mechanism has been proposed by Wang et al to release the membrane-bound Nrg1 Type III, making it also available as a paracrine factor (Wang et al., 2001).

Nrg1 Type III is the sole axonal protein that activates the PI3-kinase/Akt pathway - in associated Schwann cells - required for myelination. It has been proposed that upon binding to ErbB receptors on the Schwann cells Nrg1 Type III activates the PI3-kinase/Akt pathway and sustains signaling by maintaining the active receptors at the membrane.

Our data so far, shows that ErbB receptor internalization is necessary for Nrg1 Type II - mediated PI3-kinase/Akt activation. However, it is not clear whether activation of the PI3-kinase/Akt pathway induced by membrane bound Nrg1 Type III requires ErbB receptor internalization. The endocytic property of the ErbB receptors activated by Nrg1 Type III also remains to be elucidated.

Therefore in this study we determined if Nrg1 Type II and Type III, both provided in a soluble form differ in their properties to induce ErbB receptor internalization in Schwann cells. We also asked if binding and activation by membrane-tethered Nrg1 Type III initiates ErbB endocytosis into Schwann cells. Lastly, we determined whether upon binding the ErbB receptors, the membrane bound Nrg1 Type III is cleaved to generate the N-terminal fragment (NTF) that can be released from the plasma membrane.

Our data shows that both Nrg1 Type II and Type III, in a soluble form elicit a similar effect on ErbB receptor endocytosis. When tethered to the plasma membrane Nrg1 Type III also induced ErbB receptor downregulation in Schwann cells upon cell-cell contact. We also provide evidence that a direct contact with Schwann cells facilitates the processing of membrane-tethered Nrg1 Type III expressed on the opposing cells.

7.2 Results:

ErbB receptor endocytosis is not Neuregulin1 isoform-specific

To investigate whether Nrg1 isoform-type determines ErbB receptor internalization, we compared the endocytic property of ErbB receptors upon stimulation with Nrg1 Type II or Type III provided in a soluble form. To test this, we conducted a surface biotinylation assay to determine Nrg1-induced downregulation of the biotinylated receptors over time. Schwann cell surface proteins were first biotinylated at 4°C. Excess biotin was washed off and cells were stimulated at 37°C with Nrg1 Type II or Type III for 120′ and 240′, to allow for activated ErbB receptors to internalize. Total biotinylated proteins, were purified by streptavidin beads and the biotinylated ErbB2 and ErbB3 receptors were assessed by Western blot analysis. Control cultures at 4°C in the absence of Nrg1 represent total surface biotinylated ErbB2 (Figure. 17). Over time, in control cultures ErbB2 levels show a gradual decrease, suggesting ligand-independent ErbB2 receptor downregulation corresponding to receptor turn over in Schwann cells. Upon Nrg1 Type III stimulation, total biotinylated ErbB2 levels were similar to that of controls at 120′. At 240′, almost no ErbB2 was detected, indicating ligand-dependent receptor degradation. Nrg1 Type II stimulated cultures showed a similar pattern of ErbB2 receptor downregulation. In comparison, ErbB3 receptor levels in control cultures were abundant at 0′ and 120′. At 240′, ErbB3 protein was drastically decreased, suggesting that ErbB3 half-life in Schwann cells is shorter than that of ErbB2. Upon Nrg1 Type III or Type II stimulation, ErbB3 levels decreased predominantly at 240′ compared to controls, also

indicating ligand-dependent downregulation of ErbB3. Taken together, these results indicate that ErbB receptor internalization and downregulation is not isoform-specific.

Membrane-bound Neuregulin1 Type III induces the activation and downregulation of the ErbB receptors

Our previous data (Chapter Three) shows that ErbB receptor internalization is required for Akt activation. Since Nrg1 Type III is the predominant neuregulin isoform that is necessary for PI3-kinase/Akt activation and myelination, we first asked if membrane-tethered Nrg1 Type III mediates ErbB receptor endocytosis in Schwann cells. We used neurite membranes prepared from DRG neurons as a source of membrane-tethered Nrg1 Type III. Neurite membranes of increasing amounts (indicated as 0.5x, 1x and 2x) were briefly centrifuged onto Schwann cells. These cultures were then incubated for varying lengths of time (15′, 60′, 120′ and 240′) to activate the ErbB receptors (Maurel and Salzer, 2000). Western blot analysis on these cultures was conducted to determine the active (pErbB2 and pErbB3) and total levels (ErbB2 and ErbB3) of these receptors. As seen in Figure 18A, activation of both ErbB2 and ErbB3 receptors was detected upon 15′, which decreased over time. The overall activation levels were also increased with increasing amounts of the neurite membrane. There was also a corresponding decrease in the total levels of ErbB2 and ErbB3 over time upon neurite membrane stimulation. These results suggest that that membrane-bound Nrg1 Type III association is capable of inducing ErbB receptor downregulation and degradation in Schwann cells.

Membrane-bound Nrg1 Type III activation of Akt and not Erk1/2 in Schwann cells is dynamin-dependent

Our previous data showed that dynasore attenuated soluble Nrg1-induced Akt activation in Schwann cells. Next we asked if membrane-tethered Nrg1 Type III activation of the PI-3kinase/Akt pathway is also dependent on endocytosis. For this neurite membrane fragments were generated and briefly centrifuged onto Schwann cells in the presence or absence of dynasore. Cultures were stimulated with neurite membranes for 15′ and 120′. The activation levels of Akt and Erk1/2 were analysed by Western blot analysis. Figure 18B, shows the activation of Akt and Erk1/2 pathways in the presence of neurite fractions plus or minus dynasore. Upon 15′ of stimulation by membrane-tethered Nrg1 TypeIII, both Akt and Erk1/2 pathways are activated, and this activation decreases over time (seen at 120′). In the presence of dynasore, neurite membrane stimulation of active Akt in Schwann cells was much reduced. In contrast, no effect on active Erk1/2 was seen. This suggests that endocytic block in Schwann cells selectively inhibits Akt activation, induced by neurite membranes, the source of the membrane-bound Nrg1 Type III.

Nrg1 Type III is processed in a contact-dependent manner

Our data so far suggests that ErbB receptor endocytic function is required for Akt activation mediated by membrane-tethered Nrg1 Type III in Schwann cells. We next

asked whether Nrg1 Type III undergoes processing after activating the ErbB receptors allowing the cleavage and release of its N-terminal fragment from the membrane. To better test this we ectopically expressed HA (Haemaglutinin)-epitope tagged Nrg1 Type III in Cos-7 cells, and used them as a source of membrane-tethered Nrg1 Type III. Cos-7 cells were transfected with Nrg1 Type III and immunostained with antibodies against Nrg1 (C-terminus) and the HA tag (present on the N-terminus). Transfected cells show a clear membrane localization of Nrg1 Type III, with both antibodies, indicating that the ligand was properly expressed and inserted in the membrane (Figure. 19A).

To further test if the membrane-tethered Nrg1 Type III was functional in eliciting ErbB receptor activation in Schwann cells, we transfected a confluent layer of Cos-7 cells with Nrg1 Type III. After 48 hours, Schwann cells were briefly centrifuged onto the Nrg1 Type III expressing Cos-7 cells and allowed to associate for varying lengths of time (0′, 45′, 90′, 150′). Schwann cells were also centrifuged onto untransfected Cos-7 cells as controls. All conditions were analysed by Western blots analysis. Schwann cells in contact with untransfected Cos-7 cells show no activation of ErbB3 receptor, while Schwann cells in contact with membrane-tethered Nrg1 Type III expressing Cos-7 cells showed ErbB3 activation initiated upon contact (Figure. 19B). Although not robust, there was a small increase in the activation of downstream Akt and Erk1/2 that corresponded with the increased ErbB3 activation. Cos-7 cell alone cultures did not express ErbB3 and showed background levels of activated Akt and Erk1/2 activation. These results suggest that membrane-tethered Nrg1 Type III was expressed in a manner that allowed it to engage and activate the ErbB3 receptors and its downstream signaling molecules.

We next asked whether Nrg1 Type III undergoes processing after activating the ErbB receptors, allowing its release from the membrane. Figure. 20A, is a model of Nrg1 Type III membrane presentation and cleavage sites (Wang et al., 2001). Full-length Nrg1 Type III pro-protein is 110kD, with a glycosylated mature form measuring 140kD. Upon cleavage at the first cleavage site, by TACE or BACE1, two isoforms of 76kD (N-terminal fragment – NTF) and 60kD (C-terminal fragment – CTF) are generated. A second cleavage - by an unknown protease - of the bound 75kD fragment has been proposed to release a 63kD soluble fragment (Wang et al., 2001). The α-HA antibody recognizes the 140kD, 110kD, 75kD and the 63kD fragments, while the α-Nrg1 c-terminus antibody recognizes the 140kD, 110kD and the 60kD fragments.

To determine whether Schwann cell contact alters Nrg1 Type III processing and release of the the NTF in its neighboring cell, Schwann cells were centrifuged onto Cos-7 cells expressing Nrg1 Type III and allowed to associate with them for varying lengths of time (0′, 15′, 45′, 90′ and 150′). Cultures were analysed by Western blot analysis for the presence of Nrg1 Type III cleavage products. Untransfected Cos-7 cells did not show any background bands for the α-HA and α-Nrg1 C-terminus antibodies, suggesting that these antibodies were specific to the ectopically expressed Nrg1. At time 0′, upon Schwann cell contact, the 75kD NTF fragment was the predominantly expressed form, while the 140kD fragment is expressed at low amounts. This confirms previous studies which show that the predominant Nrg1 Type III product expressed in Cos-7 cells is the processed 75kD membrane-tethered form. Nrg1 Type III 75kD fragment increased in intensity over time while the 140kD full-length protein decreased. The detection of increased amounts of a 63kD band was also visible over time, suggesting a cleavage at the second site on Nrg1

Type III. Various CTFs (between 37kD and 65kD, recognized by the Nrg1 C-terminus antibody) increased in intensity over time (Figure. 20B). This suggests that Schwann cell-contact mediates Nrg1 Type III processing of the pro-protein at the first cleavage site in the Cos-7 cells and could also mediate the cleavage at the second site to release to receptor bound NTF fragment from the Cos-7 cell membrane.

To better detect and confirm the presence of the released NTF, we asked if increasing the Schwann cell number would enable increased Nrg1 Type III processing and better detection of the NTF. For this we centrifuged 0.75 million or 3 million Schwann cells onto Nrg1 Type III transfected Cos 7 cells, and assessed Nrg1 Type III processing over time by Western blot analysis. Schwann cells centrifuged onto untransfected Cos-7 cells were used as controls. Schwann cell contact activated the ErbB3 receptors (Figure. 20C). Total ErbB3 amounts were more in conditions with four times the amount of Schwann cells, as expected. Increased Schwann cell contact increased the presence of the 75kD band (HA), while decreasing that of the 140kD. The 63kD NTF was not detectable. Therefore Schwann cell contact increased the processing of the full-length Nrg1 Type III from its 140kD to the 75kD form indicating that increased Schwann cell contact mediated Nrg1 Type III processing at its 1st cleavage site.

Schwann cells actively endocytose membrane-bound Neuregulin1 Type III

We have shown so far that Schwann cell contact mediates Nrg1 Type III processing in its neighbouring cell. We also know that ErbB receptors are downregulated upon stimulation by membrane-bound Nrg1 Type III. We hypothesized that the NTF,

although not detected in large amounts by our previous western blot analysis, must be getting cleaved and released after binding the ErbB receptors on the Schwann cells. To investigate this we expressed ErbB2-EYFP in Schwann cells and co-cultured them with Nrg1 Type III expressing Cos-7 cells for two hours. The cultures were immunostained with the α-HA antibody. Z-stack images were taken of positively transfected Schwann cells and Cos-7 cells making contact with each another. We noticed punctate structures appearing within the Schwann cell cytoplasm at the site of contact with the Nrg1 Type III expressing Cos-7 cells in the ErbB2 expressing Schwann cells (Figure. 21A). These puncta were HA-positive and some localization with the ErbB2-EYFP was seen in the respective cells. This suggested a mechanism of uptake of the NTF of membrane-tethered Nrg1 Type III from the Cos-7 cell into Schwann cells.

To determine whether these punctate structures represent endocytosed ligand in a cellular compartment, Schwann cells expressing Rab5-RFP were cocultured with Nrg1 Type III expressing Cos-7 cells for 2 hours. They were then immunostained for α-HA antibodies. As seen in Figure 21B, punctate structures that were positive for Rab5-RFP and the α-HA N-terminus antibody appeared co-localised in the Schwann cells. A 3-D reconstitution of a Z-stack of these two cells is shown in Figure 21C (along the y-z axis). This image shows that the HA-positive puncta were present within the Schwann cell body, in close proximity to the Rab5-RFP, indicating that these puncta were localized intracellularly. To further confirm the above results in adherent cells having a flat morphology, Cos-7 cells expressing Nrg1 Type III were first plated on coverslips and allowed to adhere and spread for 4hours. Schwann cells expressing Rab5 RFP were then plated onto the Cos-7 cells and allowed to associate for 2 hours. Cultures were

immunostained with the same antibodies as mentioned above. Small puncta arising from Cos-7 cells that were positive for the α-HA N-terminus antibody were localized to Rab5 RFP-positive compartments in Schwann cells (Figure. 21D). This result indicates that upon contact with Nrg1 Type III expressing neighboring cells, Schwann cells actively endocytose this ligand into Rab5-positive compartments. It was also interesting to note here that upon co-staining with α-Nrg1 (C-terminus) antibody, this fragment was also seen localized to the Rab5 RFP compartments in Schwann cells, suggesting a mechanism of uptake of the full-length form of the ligand (Figure. 20E).

7.3 Discussion

Nrg1 Type III presented in a paracrine or a juxtacrine manner induces ErbB receptor downregulation in Schwann cells

The endocytic property of the ErbB receptors activated by Nrg1 Type III has not been investigated. Since Nrg1 Type II and Type III differ in their N-terminus region, we first asked if Nrg1 isoform-type determines ErbB receptor endocytosis. To investigate this we compared soluble Nrg1 Type III and Type II and showed that Nrg1 Type III, like Type II induced ErbB receptor downregulation in Schwann cells (Figure. 16A). Therefore, indicating that ErbB receptor endocytosis is not Nrg1 isoform-specific.

We next investigated whether presenting Nrg1 Type III in a juxtacrine manner differs in its ability to induce ErbB receptor endocytosis. Neurite fractions used as a source of membrane-tethered Nrg1 Type III elicited ErbB receptor downregulation in

Schwann cells (Figure. 17A). This result demonstrates that despite being membrane-bound Nrg1 Type III can also induce ErbB receptor downregulation in Schwann cells.

Since both soluble and membrane-tethered Nrg1 Type III stimulates ErbB receptor endocytosis, we next asked if ErbB receptor endocytosis triggered by Nrg1 Type III is required for PI3-kinase/Akt activity in Schwann cells. Our data shows that dynasore, a dynamin inhibitor attenuated neurite membrane induced Akt activation and not Erk1/2 (Figure. 17B). These results suggest that receptor internalization is important for membrane-tethered Nrg1 Type III induced PI3-kinase/Akt pathway in Schwann cells.

Nrg1 Type III is processed in a Schwann cell contact-dependent manner

Our results so far collectively provide evidence for a membrane-tethered Nrg1 Type III mediated ErbB receptor endocytic event in Schwann cells. We also demonstrate that this dynamin-dependent endocytic event is required for Nrg1-mediated Akt activation. However, the key question is whether Nrg1 Type III by being structurally tethered to the membrane and immobile gets cleaved to release a soluble fragment upon ErbB receptor binding. Wang et al, observed a soluble released NTF (N-terminal fragment) in Cos-7 cells ectopically expressing Nrg1 Type III and proposed an additional 2^{nd} cleavage of the processed membrane tethered isoform, illustrated in Figure 19A (Wang et al., 2001). To test if Schwann cell contact mediates this 2^{nd} cleavage, we ectopically expressed Nrg1 Type III in Cos-7 cells and cultured them with Schwann cells. Small amounts of a ~63kD NTF that matched the size predicted by Wang et al., increased in amounts upon contact, over time (Figure. 19B). However, the most significant change

was the increase in the 75kD NTF upon Schwann cell contact, over time. There was also a corresponding decrease in the 140kD full-length Nrg1 form. This suggests that Schwann cell contact increased Nrg1 Type III processing at the 1st cleavage site. This processing of Nrg1 Type III is determined by two proteases TACE/ADAM17 and BACE1 that cleave 8 amino acids apart from each other (Hu et al., 2006, La Marca et al., 2011, Luo et al., 2011a). La Marca et al, showed that TACE cleavage inactivates Nrg1 Type III while BACE1 cleavage produces an active Nrg1 cleaved product (La Marca et al., 2011). Though our data does not answer the question of which protease cleaves Nrg1 Type III at the 1st cleavage site, we observe that there is an increased processing of this cleaved 75kD NTF upon Schwann cell contact over time (Figure. 19B). Esper et al, demonstrated in CHO cells that neurotrophins increased the release of soluble neuregulins in a PKCδ-dependent manner (Esper and Loeb, 2009). It is interesting to speculate whether this increase in Nrg1 Type III processing is dependent on the increased PKC-dependent protease activity in Cos-7 cells, upon Schwann cell contact. We next increased Schwann cell number to determine processing at the 2nd cleavage site more clearly. Although we did not observe a clear band at 63kD upon increasing Schwann cell contact on Cos-7 cells, we observed that increased Schwann cell contact increased Nrg1 Type III processing at the 1st cleavage site (Figure. 19C). This determines that a Schwann cell contact –dependent factor increased the Nrg1 Type III processing in Cos-7 cells.

Our results also provide evidence that upon contact with Cos-7 cells expressing Nrg1 Type III, punctate structures positive for the NTF of Nrg1 Type III appeared in the Schwann cells (Figure 20A and 20B). This indicated internalization of the NTF into the Schwann cells. To confirm this intracellular localization and internalization into Schwann

cells we expressed Rab5 RFP in Schwann cells and see that the NTF from the Cos-7 cells also colocalised with Rab5 in Schwann cells (Figure. 20C). This suggests that Schwann cells must cleave this membrane-tethered Nrg1 to internalize it. Interestingly, we also saw co-localization of the C-terminal fragment in the Rab5 RFP endosomes of the Schwann cells (Figure. 20D). This fragment could either correspond to the full-length fragment or the CTF fragment alone of Nrg1 Type III (Figure. 19A). Although preliminary, this result demonstrates that upon contact Schwann cells actively endocytose membrane-tethered Nrg1 Type III. Lauterbach et al, showed that astrocytes co-cultured with hippocampal neurons pinched off EphB2-containing vesicles at neuron-glia contact sites indicating a mechanism of trans-endocytosis (Lauterbach and Klein, 2006). Also binding of Notch to Delta, which are both membrane proteins, causes ADAM protease-mediated cleavage of the extracellular domain of Notch that is then endocytosed into the neighbouring Delta expressing cell (Nichols et al., 2007, Coumailleau et al., 2009). Thus a similar mechanism could be occurring between the membrane-tethered Nrg1 Type III and the ErbB receptors in Schwann cells. Taken together, the relevance of a Nrg1 Type III cleavage and trans-endocytic mechanism on Schwann cell function would be interesting to investigate further.

8 Chapter Five Conclusions and Future Directions

8.1 Conclusions and Future Directions

One of the most intricate and intimate cell-cell interactions in the nervous system is the one that exists between a Schwann cell and its associated neuron. Dynamic bi-directional signaling between these two cells is not only required for the efficient generation and development of the Schwann cell lineage but also the myelination of the PNS neuron(Nave, 2010). Schwann cells are important not only for myelination but for the long term survival and maintenance of the axons. Although multiple factors regulate Schwann cell myelination, the overexpression of a single growth factor, Nrg1 Type III was shown to change the fate of a non-myelinating Schwann cell to a myelinating one, underscoring the importance of Nrg1 signaling in the peripheral nervous system (Taveggia et al., 2005).

Neuregulins on the axon bind to and activate the ErbB2-ErbB3 receptor heterodimer on the Schwann cells (Garratt et al., 2000). This interaction leads to activation and signaling by multiple downstream second-messenger cascades such as PI3-kinase/Akt, Ras/Raf/Erk1/2, NF-kB, Calcineurin/NFAT, that determine the functional outcome in Schwann cells (Maurel and Salzer, 2000, Ogata et al., 2004, Taveggia et al., 2005, Kao et al., 2009, Limpert and Carter, 2010). Since Nrg1-ErbB signaling triggers multiple, sometimes even opposing biological outcomes in Schwann cells, we hypothesized that receptor trafficking may provide a mechanism that regulates differential signaling downstream of the Nrg1-ErbB ligand-receptor axis. Receptor

trafficking has the ability to modify active receptor-associated signaling as it travels through endosomal compartments within the endocytic pathway (Sorkin and Von Zastrow, 2002). Therefore endosomal signaling enables both the temporal and spatial regulation of receptor signaling, contributing to functional diversity. EGFR endocytosis for example is required for EGF-mediated Akt and Erk1/2 activity but not for PLCγ (Vieira et al., 1996).

Our study investigated the role of Nrg1-mediated ErbB2 and ErbB3 receptor endocytosis and recycling on the receptor signaling function and the physiological outcome in Schwann cells. So far there has been no other study characterizing the ErbB receptor trafficking in Schwann cells. Understanding Nrg1-ErbB mechanism of trafficking and signal regulation will not only elucidate important molecular mechanisms during the process of myelination, but will also throw light on how this signaling axis is modified and regulated during development and more important, during injury and disease.

The results from this study are summarized as follows: In Chapter two, we have shown that Nrg1 induces ErbB2 and ErbB3 receptor internalization in a dynamin-dependent manner in Schwann cells. ErbB receptors and Nrg1 co-localize to Rab5-positive endosomes upon internalization. Internalized receptors either follow a degradative route or recycle back to the surface. We demonstrate that a larger percentage of ErbB2 gets recycled compared to ErbB3, suggesting that recycling is differentially regulated. We also observe that ErbB3 recycling but not that of ErbB2 is decreased with increasing dose of Nrg1, further confirming differential recycling between these receptors. Taken together the results in this chapter collectively provide evidence for

Nrg1-mediated ErbB receptor internalization and differential trafficking in Schwann cells.

In Chapter three, we investigated the role of Nrg1-mediated ErbB2 and ErbB3 receptor endocytosis and recycling on the receptor downstream signaling in Schwann cells. Here we demonstrate that internalization of ErbB2/3 attenuates PI3-kinase/Akt signaling while not having a significant affect on the Ras/Raf/Erk1/2 pathway. Inhibition of receptor internalization also inhibits Nrg1-induced proliferation and the promotion of myelination in Schwann cells. Inhibition of receptor recycling potentiates PI3-kinase/Akt and not Erk1/2 signaling. Active Akt is enriched in Rab5 compartments upon Nrg1 stimulation. Expression of WT Rab11a accumulates ErbB receptors and PH-Akt-GFP in Rab11a positive recycling compartments upon Nrg1 stimulation. Taken together the data presented here underscores the importance of Nrg1-mediated PI3-kinase/Akt signaling from an endosomal compartment.

In Chapter four, we investigated the role of membrane-tethered Nrg1 Type III in eliciting ErbB receptor endocytosis and asked whether this process requires Nrg1 cleavage and subsequent release from the membrane. We show here that membrane-bound Nrg1 Type III can elicit ErbB receptor downregulation. We also observe that Schwann cell contact promotes Nrg1 Type III processing in the opposing Nrg1 presenting cell. Also preliminary evidence indicates a possible cleavage of the full length Nrg1 Type III and uptake into the Schwann cells.

Our study has led to the following key questions: How might differential ErbB receptor trafficking affect signaling? Is endosomal PI3-kinase activity significant and

required for Schwann cell function? How do Schwann cells mediate contact dependent processing of Nrg1 Type III? What is the mechanism of cellular uptake and/or cleavage of Nrg1 Type III upon ErbB receptor binding? What is the role of Nrg1-ErbB endocytic transport in health and disease? We discuss some of these questions below.

Differential routing of internalized ligand-receptors and its role in signaling

As mentioned previously, ErbB3 binds Nrg1 but lacks kinase activity while ErbB2 has a kinase domain but does not bind ligand. Upon ligand binding to ErbB3 and dimerization with ErbB2, this ligand-receptor complex recruits various adaptor proteins to its cytoplasmic domain. ErbB3 has docking sites for the p85 regulatory subunit of PI3-kinase, while ErbB2 has sites for Shc/Grb2 proteins. Therefore activation of PI3-kinase/Akt and Ras/Raf/Erk1/2 pathways is likely to originate from activation of different receptors. Thus the trafficking and activation kinetics of these receptors becomes crucial for modulating differential signaling (Schulze et al., 2005).

Our work suggests a differential mechanism of internalized ErbB receptor recycling upon Nrg1-stimulation. Receptors traveling within the recycling pathway not only escape degradation but are also, able to sustain signaling within these compartments. The recycling pathway also transports receptors to different regions within the cell or to the plasma membrane, thus providing spatial regulation of receptor signaling resulting in different biological outcomes (Sigismund et al., 2008).

Our data shows differential regulation of ErbB2 versus ErbB3 receptor recycling in Schwann cells in response to increasing doses of Nrg1. While ErbB2 receptors

continue to recycle independent of Nrg1 dose, ErbB3 receptors become targeted to the degradation pathway with increasing doses of Nrg1. Since ErbB2 and ErbB3 provide unique docking sites for different adaptor proteins, differential regulation of the receptor recycling and degradation is likely to contribute to dose-dependent differential Nrg1 signaling in Schwann cells.

French et al, showed that the pH sensitivity of a ligand determines the trafficking route of the receptor (French et al., 1995). They showed that EGF and TGFα ligands both bind to EGFR with the same affinities. However, because EGF is less sensitive than TGFα to the acidic pH of the early endosomes, it remains bound to the EGFR while TGFα dissociates from it upon internalization into early endosomes. This results in the EGF-EGFR complexes to be routed to the late endosomes and lysosomes, for degradation while the TGFα-EGFR complexes dissociate and recycle back to the membrane. The EGFR associated with TGFα is able to get activated at the cell surface again. As mentioned previously, since the ErbB3 receptor remains bound to the Nrg1 ligand - which is also internalized and localized to the Rab5-positive compartments - it would indicate that ErbB3 receptor and not ErbB2 is preferentially routed for degradation. ErbB2 is not ligand bound, but dimerized with ErbB3. The key question here again is whether this receptor-dimer dissociates in the early endosomes, allowing ErbB2 to be sorted back to the surface via the recycling pathway.

Implication of defective ErbB trafficking and signaling in Charcot-Marie-Tooth Disease (CMT)

As described briefly in Chapter One, recent studies have identified SH3TC2 as one of the proteins mutated in CMT4 type-C, a peripheral neuropathy with Schwann cell pathology (Arnaud et al., 2009). SH3TC2$^{-/-}$ mice exhibit decreased motor and sensory nerve conduction velocities and also show marked hypomyelination. In the PNS, a common feature occurring in patients of CMT4C is disorganized nodes of Ranvier, abnormal myelin outfoldings and hypomyelination (Gabreels-Festen et al., 1999). SH3TC2 is normally found localized to the plasma membrane and endocytic compartments including the perinuclear recycling endosomes (Lupo et al., 2009) (Arnaud et al., 2009). Recently Stendel et al, showed that SH3TC2 interacts with and is an effector of Rab11 in the recycling endosomes (Stendel et al., 2010). They also showed that SH3TC2 mutations that lead to CMT4C disrupt SH3CT2 and Rab11 interaction. Furthermore Schwann cells expressing dominant-negative Rab11a mutants fail to form myelin in co-cultures (Stendel et al., 2010). Expression of wild-type or constitutively active Rab11 promoted myelination in co-cultures. These observations suggest that the Rab11a associated receptor recycling pathways are important for the regulation of Schwann cell myelination. Our data shown in this study demonstrates that ErbB receptors travel via the recycling pathway after internalization. We show that expression of wild-type Rab11a mutants in Schwann cells accumulates ErbB receptors and PI3-kinase activity in the recycling endosomes upon Nrg1 stimulation. Garcia-Regalado et al, demonstrated that in yeast the G protein-coupled receptor subunit GβϒInteracts with Rab11a in the recycling endosomes leading to the recruitment of PI3-kinase and the phosphorylation of Akt in these compartments (Garcia-Regalado et al., 2008).Taken together our data suggests that ErbB receptors normally traveling through the recycling pathway upon Nrg1 stimulation

generate PI3-kinase specific endosomal signaling that could regulate Schwann cell myelination.

Regulation of Nrg1 Type III processing by secretases

Nrg1 Type III processing is an important regulatory step of myelination. Willem et al, showed that mice lacking $BACE^{-/-}$ (a β secretase) mice show hypomyelination and abberant segregation of small diameter axons in the PNS (Willem et al., 2006). These defects are similar to those seen in mice with Schwann cell specific knockouts of ErbB2 or Nrg1 Type III (Michailov et al., 2004). Hu et al, showed decreased processing of Nrg1 Type III from its full length to its N-terminal fragment form in $BACE^{-/-}$ mice cortices (Hu et al., 2006). This reduced Nrg1 processing also correlated with decreased activation of Akt. These results indicate that BACE1 is a positive regulator of myelination. These results also indicate that Nrg1 Type III processing determines Akt activation which is a positive regulator of myelination. La Marca et al, recently showed that another α-secretase (TACE) known to shed neuregulins cleaves 8 amino acids away from the Bace1 cleavage site, cleaving within the EGF domain of Nrg1 Type III, required for receptor activation (La Marca et al., 2011). Thus TACE-mediated processing inactivates Nrg1 Type III. These two studies demonstrate the opposing function of both BACE1 and TACE on Schwann cell myelination.

Our data in chapter four, indicates contact-dependent processing of Nrg1 Type III in Cos-7 cells. Schwann cell-contact generates the 75kD N-terminal Nrg1 Type III fragment (75kD-NTF). However, we do not know if Schwann cell-contact upregulates

specific proteases to enable a feed forward regulatory loop. To specifically determine if Schwann cell contact upregulates BACE1 activity, a point mutation could be generated at the Bace1 cleavage site of Nrg1 Type III and expressed in Cos-7 cells. A similar contact experiment that we conducted could be done to investigate this process.

It was shown by Esper et al, that neurotrophins increase the release of processed Nrg1s from CHO cells (Esper and Loeb, 2009). They showed that this Nrg1 processing is sensitive to inhibition of the PKC pathway. In the PNS, Schwann cells and neurons signal in a bi-directional manner with Schwann cells secreting neurotrophins upon axonal contact and neurons in turn producing Nrg1s (Esper and Loeb, 2004). It is interesting to speculate that upon Schwann cell contact, Bace1 activity in neurons is upregulated, to increase the processing and activation of Nrg1 Type III in a PKC-dependent manner.

Intercellular protein transfer as a mechanism of cell-cell communication

Our preliminary data shows membrane-bound Nrg1 Type III from the Cos-7 cells co-localized to Rab5 endosomal compartments in Schwann cells, upon cell-cell contact. We also detect the cytoplasmic tail fragment of Nrg1 Type III (60kD-CTF) being internalized into the cell.

Transendocytosis is a process of cell-cell communication during which proteins from one cell are taken up by a neighboring cell. Notch-Delta, Eph-Ephrins, MHC molecules CD86 interactions are examples of the proteins that exhibit this process (Rechavi et al., 2009). Although predominantly a phenotype of immune cells at what is termed the ‘IS’ or the immune synapse, which is the interface that exists between

lymphocytes and antigen presenting cells, this process can be paralleled to other biological synapses like that of the neuron. Court et al., 2008, reported that Schwann cells are capable of transferring ribosomes to desomatized axons, possibly aiding in protein translation (Court et al., 2008). Although evidence suggested by Court et al, is unprecedented, their work implies a trans-endocytic mechanism present between Schwann cells and neurons.

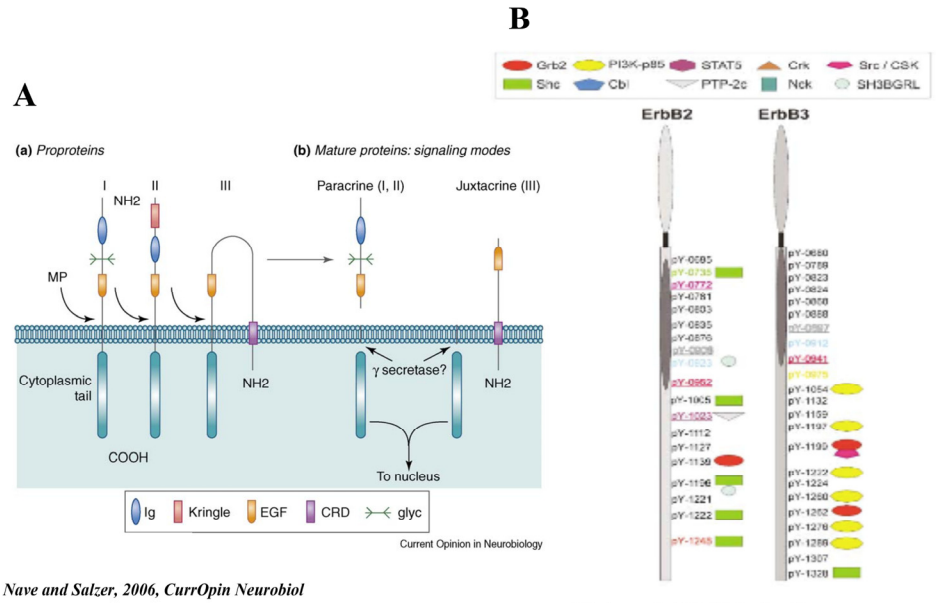
Other cells types, exhibit contact-dependent cleavage or endocytosis of membrane-tethered proteins expressed in the opposing cells. Endothelial cells expressing EphB4 receptor tyrosine kinase receptor are known to interact with their ligand, ephrinB2 on another endothelial cell and undergo contact-dependent repulsion by transendocytosis. Upon cell-cell contact EphrinB2 is transferred into the EphB4 expressing cell, by being pinched off along with the adjacent membrane. This process is important for maintaining the cellular environment and boundary between these two cell types (Marston et al., 2003). Astrocytes expressing ephrin ligands have also been shown to endocytose membrane vesicles containing EphB2 clusters when co-cultured with hippocampal neurons (Lauterbach and Klein, 2006). This process is thought to regulate neuronal structures during growth and development. A direct contact between an osteoblast and a haematopoietic stem-progenitor cell (HSPC) also results in uptake of membrane embedded CD63 as a whole into the osteoblast by transendoytosis, which then travels into SARA-positive endosomes. This process is required for the production of stromal-derived factor-1, which is a chemokine required for the migration of HSPCs into the bone marrow (Gillette et al., 2009). Furthermore, numerous reports have indicated a similar mechanism of transendocytosis in Notch and Delta signaling. In this process the

heterodimer-Notch receptor gets cleaved and its extracellular domain gets endocytosed into the ligand- expressing cell, thereby mediating forward signaling with its extracellular domain and backward signaling after the cleavage of its intracellular domain in the receptor expressing cell (Parks et al., 2000). This process of receptor cleavage and endocytosis has only recently been described to be the result of mechanical forces of stress at this contact interface, that pulls the receptor into the ligand expressing cell without requiring enzymatic cleavage to occur (Nichols et al., 2007). Delta-Notch complexes after internalization have been shown to signal from endosomal platforms (Furthauer and Gonzalez-Gaitan, 2009).

In light of these transendocytic events in other cells, it would be interesting to determine the mechanism of Nrg1 Type III uptake by Schwann cells. However, further experiments with plasma membrane labeling fluorescent probes need to be done to visualize and confirm this process.

9. Figures

Figure 1



Nave and Salzer, 2006, CurrOpin Neurobiol

Schulze et al., 2005, Molec Syst Bio

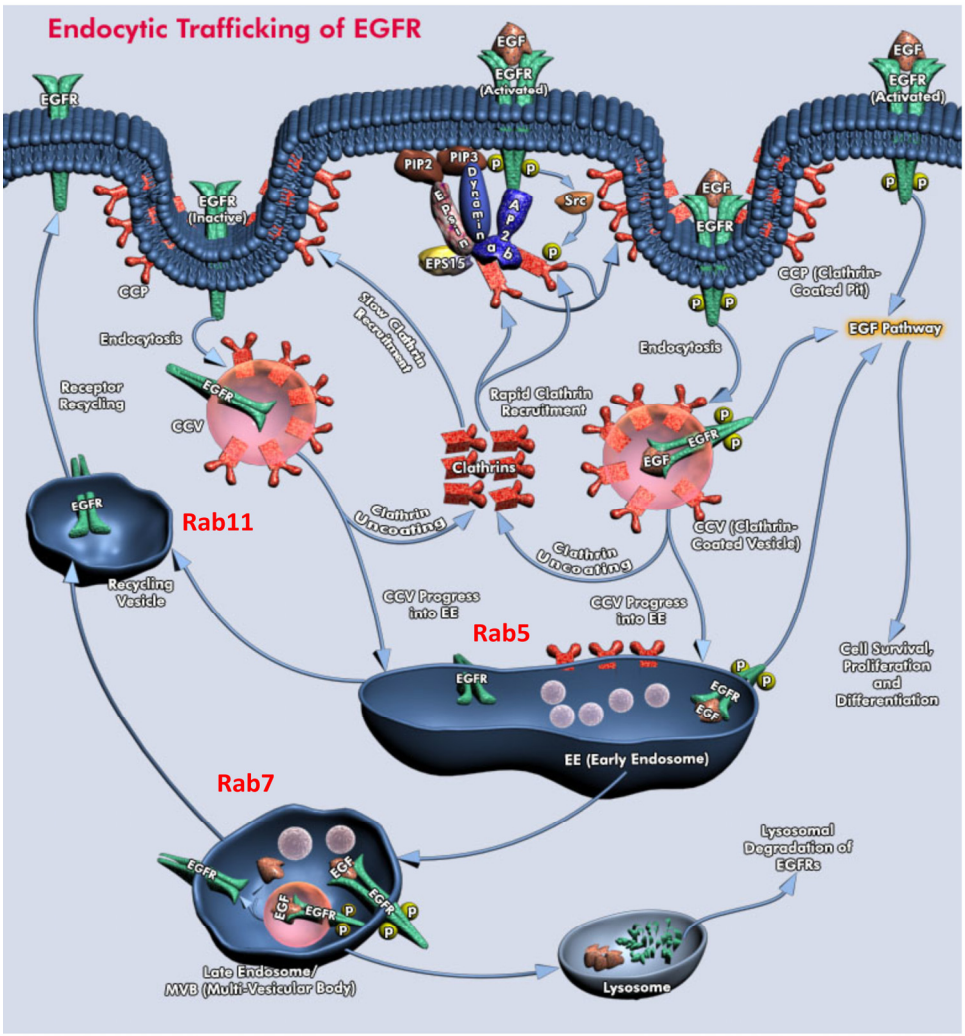
Figure 1. Neuregulins and the ErbB receptors

(A) Neuregulin1 Isoforms: Type I and Type II are presented as single pass transmembrane proteins, while Type III has two transmembrane domains. Type I and Type II are released as soluble factors upon cleavage by metalloproteinases. Type III remains membrane-tethered via its cystein rich domain (CRD). Type I and Type II signal as paracrine factors while Type III signals in a juxtacrine manner.

(B) Systematic interaction profiling of ErbB2 and ErbB3 receptor tyrosine kinases:

ErbB2 has few interaction partners and primarily interacts with Shc. ErbB3 interacts mainly with the p85 subunit of PI3-kinase. ErbB2 *has* an active kinase domain while ErbB3 lacks a kinase domain. ErbB3 and not ErbB2 binds to ligand.

Figure 2

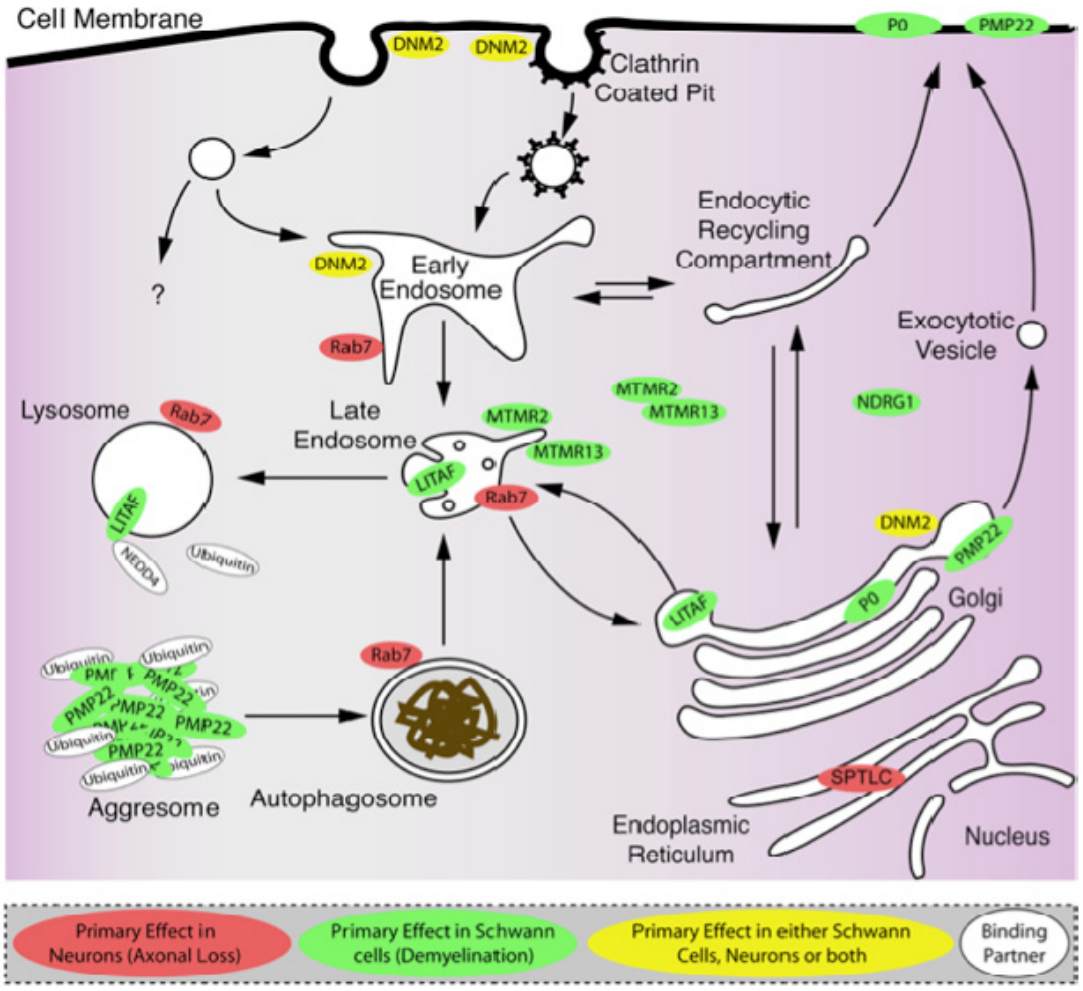


Modified from gene globe pathways:Qiagen technologies

Figure 2. Endocytic Trafficking of EGFR

Ligand (EGF) bound EGFR gets endocytosed via a clathrin-dependent pathway to reach the Rab5 positive early endosomes. From here the receptors are either recycled via the Rab11 positive recycling endosomes, or targeted for lysosomal degradation via the Rab7 positive late endosomes/multivesicular bodies. Empty receptors are seen constitutively internalized and trafficked back to the membrane.

Figure 3



Niemann et al., 2006, NeuroMolecular Medicine

Figure 3. Defective trafficking during Charcot-Marie-Tooth disease

Schematic overview of a myelinating Schwann cell highlighting the subcellular localization of proteins mutated in demyelinating peripheral neuropathies and their binding partners (Nieman et al., 2006). Of these, mutations in dynamin2 (DNM2) and Src homology 3 domain and tetratricopeptide repeats 2 (SH3TC2) proteins that lead to disruption in endocytosis and recycling, respectively cause hypomyelination in Schwann cells (Stendel et al., 2010, Sidiropoulos et al., 2012).

Figure 4

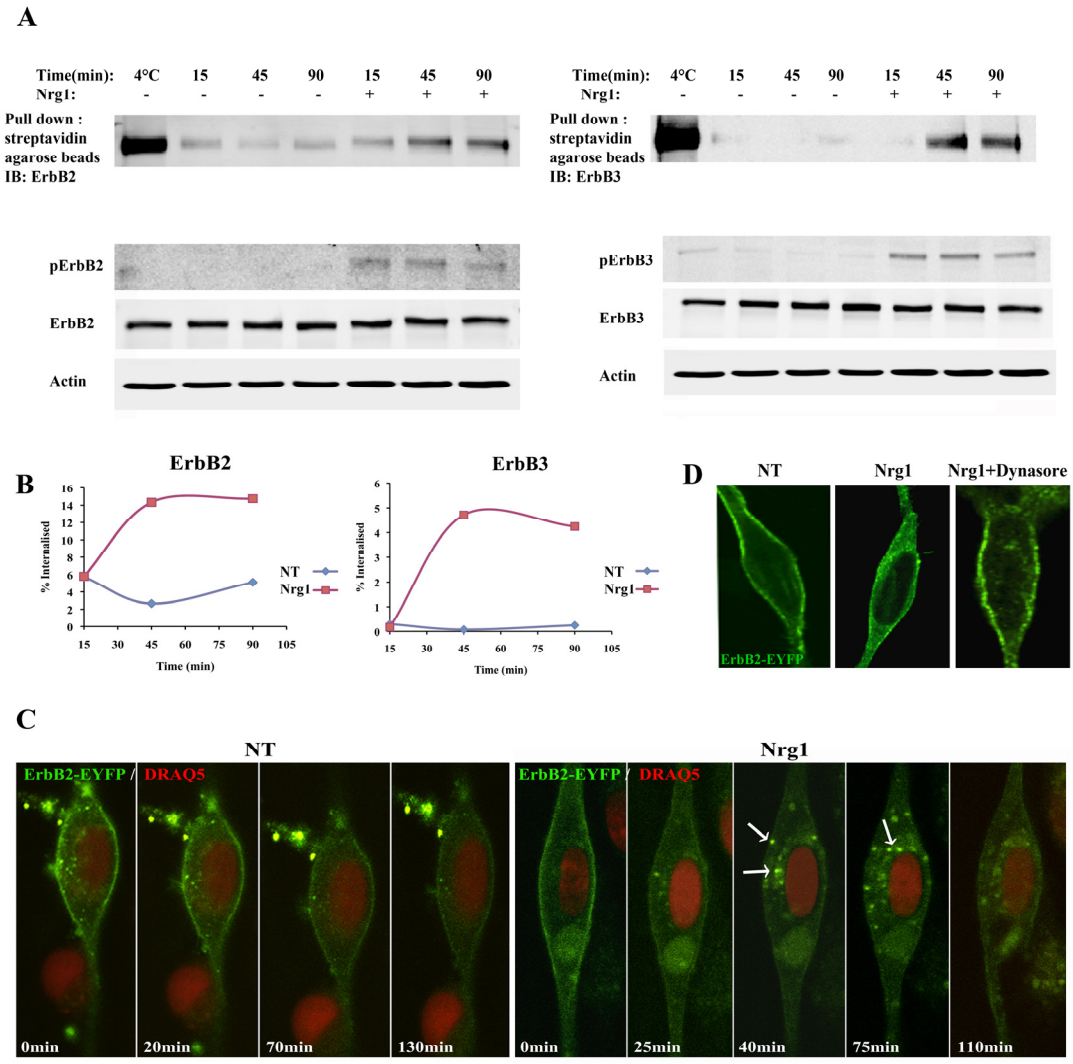

A
Time(min): 4°C 15 45 90 15 45 90
Nrg1: - - - - + + +
Pull down : streptavidin agarose beads
IB: ErbB2
IB: ErbB3
pErbB2
ErbB2
Actin
pErbB3
ErbB3
Actin
B
ErbB2
ErbB3
% Internalised
Time (min)
NT
Nrg1
D
NT
Nrg1
Nrg1+Dynasore
ErbB2-EYFP
C
NT
Nrg1
ErbB2-EYFP / DRAQ5
0min
20min
70min
130min
25min
40min
75min
110min

Figure 4. Nrg1 induces Erb2 and Erb3 receptor internalization in Schwann cells

(A) Schwann cells were growth factor starved for 48 hours. Surface proteins were biotinylated with Sulpho-NHS-SS-Biotin at 4°C. Cells were washed free of Biotin and left either untreated or treated with Nrg1 at 37°C for the indicated time points (15′, 45′, 90′). After incubation cells were washed free of Nrg1, cell surface Biotin was cleaved with glutathione. A Western blot analysis for internalized ErbB2 and ErbB3 after a streptavidin-agarose pulldown was conducted. Total activated ErbB2 (pErbB2) and ErbB3 (pErbB3) were also determined. ErbB2 and ErbB3 receptors showed maximum internalization at 45′ (B) Quantification of the percentage of the surface biotinylated ErbB2 and ErbB3 that was internalized over time. (C) ErbB2-EYFP transfected Schwann cells were monitored by live cell imaging with or without Nrg1 stimulation over time. ErbB2-EYFP puncta appeared at 40min after Nrg1 stimulation (D) ErbB2-EYFP transfected Schwann cells were treated with dynasore in the presence or absence of Nrg1 for 90 minutes. ErbB2-EYFP internalization was inhibited in a dynamin-dependent manner.

Figure 5

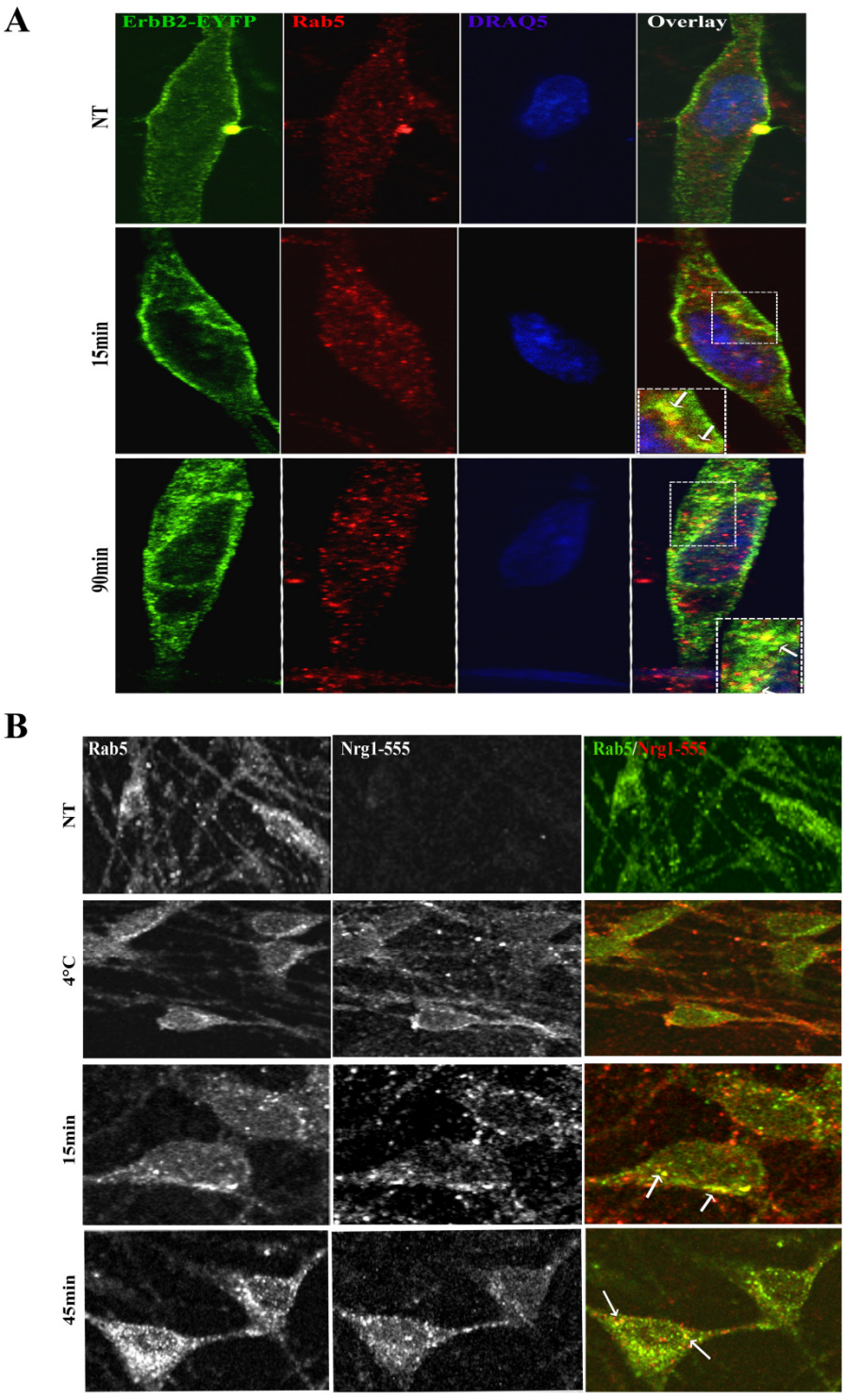

A
ErbB2-EYFP
Rab5
DRAQ5
Overlay
NT
15min
90min
B
Rab5
Nrg1-555
Rab5/Nrg1-555
NT
4°C
15min
45min

Figure 5. ErbB2 and Nrg1 are co-localized to Rab5 positive endosomes upon internalization

(A) ErbB2-EYFP transfected Schwann cells were treated with Nrg1 for 15′ and 90′ and immunostained for Rab5, an early endosomal marker. ErbB2-EYFP was seen co-localized to Rab5 positive compartments upon Nrg1 stimulation. (B) Schwann cells were stimulated with recombinant Nrg1 conjugated Alexa Fluor 555 (Nrg1-555) for 20′ at 4°C to allow surface labeling. Cultures were then moved to 37°C for 15′ and 45′ minutes to allow for endocytosis. Schwann cells were immunostained for Rab5. Nrg1-555 was seen co-localized to Rab5 positive endosomes predominantly at 45′.

Figure 6

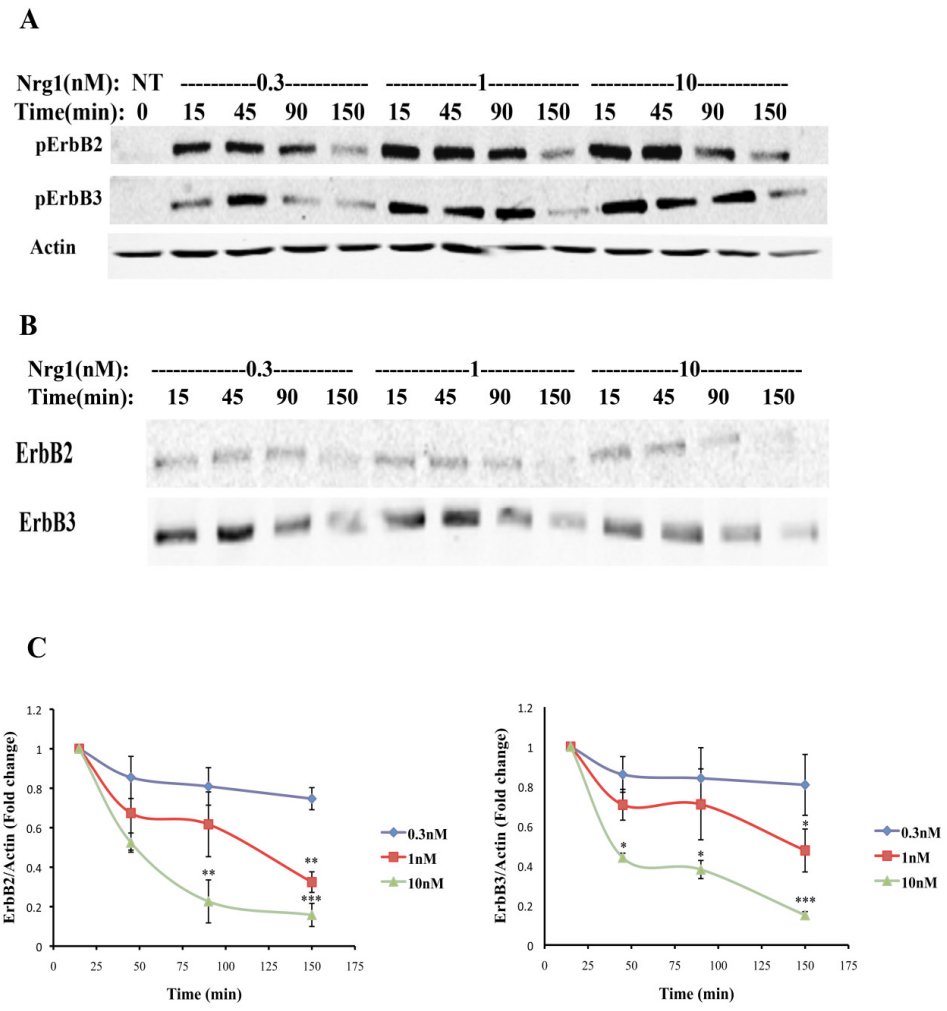

A
Nrg1(nM): NT ----------0.3---------- ----------1---------- ----------10----------
Time(min): 0 15 45 90 150 15 45 90 150 15 45 90 150
pErbB2
pErbB3
Actin
B
Nrg1(nM): ----------0.3---------- ----------1---------- ----------10----------
Time(min): 15 45 90 150 15 45 90 150 15 45 90 150
ErbB2
ErbB3
C
ErbB2/Actin (Fold change)
Time (min)
0.3nM
1nM
10nM
ErbB3/Actin (Fold change)
Time (min)
0.3nM
1nM
10nM

Figure 6. Downregulation of ErbB receptors upon Nrg1 stimulation

(A) Schwann cells were growth factor starved for 48 hours. They were then treated with 0.3nM, 1nm and 10nM Nrg1 for the indicated time points. Total activate ErbB2 and ErbB3 receptor was analysed by Western blot. ErbB2 and ErbB3 receptors showed robust activation upon Nrg1 stimulation that decreased over time. (B) Representative Western blots for ErbB2 and ErbB3 levels over time from A. (C) Quantification of blots from three separate experiments as in (B). Quantification was conducted with LICOR imaging software and statistical significance determined by ANOVA with Tukey's post-hoc analysis using INSTAT Prism Software. Asterisk indicates difference from 15′,

*$p<0.05$, **$p<0.01$ and ***$p<0.001$. Results indicate that ErbB2 and ErbB3 undergo ligand-mediated downregulation over time.

Figure7

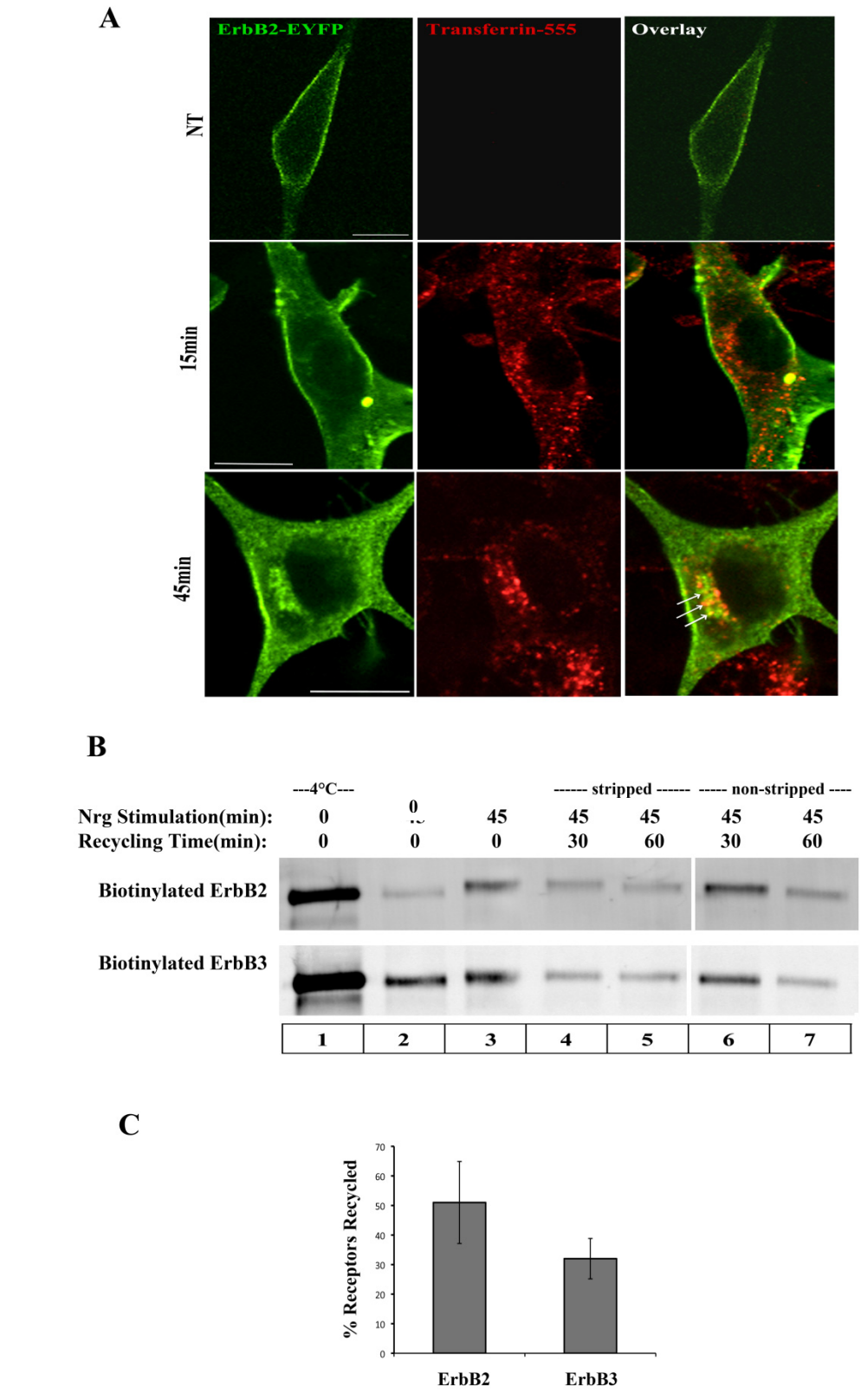

A
ErbB2-EYFP
Transferrin-555
Overlay
NT
15min
45min
B
---4°C---
------ stripped ------
----- non-stripped ----
Nrg Stimulation(min):
0
0
45
45
45
45
45
Recycling Time(min):
0
0
0
30
60
30
60
Biotinylated ErbB2
Biotinylated ErbB3
1
2
3
4
5
6
7
C
% Receptors Recycled
70
60
50
40
30
20
10
0
ErbB2
ErbB3

Figure 7. Internalized ErbB2 and ErbB3 receptors recycle back to the surface

(A) ErbB2-EYFP Schwann cells were stimulated with Nrg1 along with Transferrin-555. Cells were immunostained after co-stimulation for 15′ and 45′. ErbB2-EYFP localizes along with Transferrin-555 in perinuclear compartments, indicating a common trafficking mechanism via the slow recycling pathway. (B) Briefly Schwann cells were surface biotinylated and receptors were allowed to internalize upon Nrg1 stimulation for 45′. The remaining surface biotin was stripped and internalized receptors were allowed to recycle back to the surface for 30′ and 60′, upon which time a second round of biotin stripping was conducted (Lane 4 and Lane 5). A parallel set of cultures were left unstripped (Lane 6 and Lane 7). Lane1 represents total surface biotinylated receptors at 4°C. Lane 2 and Lane 3 represent internalized receptors minus or plus Nrg1 stimulation. An increase in receptor levels in the non-stripped versus the stripped conditions indicates recycling. Both ErbB2 and ErbB3 receptors show recycling at 30′ after a 45′-Nrg1 stimulation. (C) Average results from three separate blots shows that of the internalized receptors, 51% of ErbB2 while only 32% of ErbB3 is recycled, indicating a differential recycling mechanism between the two receptors.

Figure 8

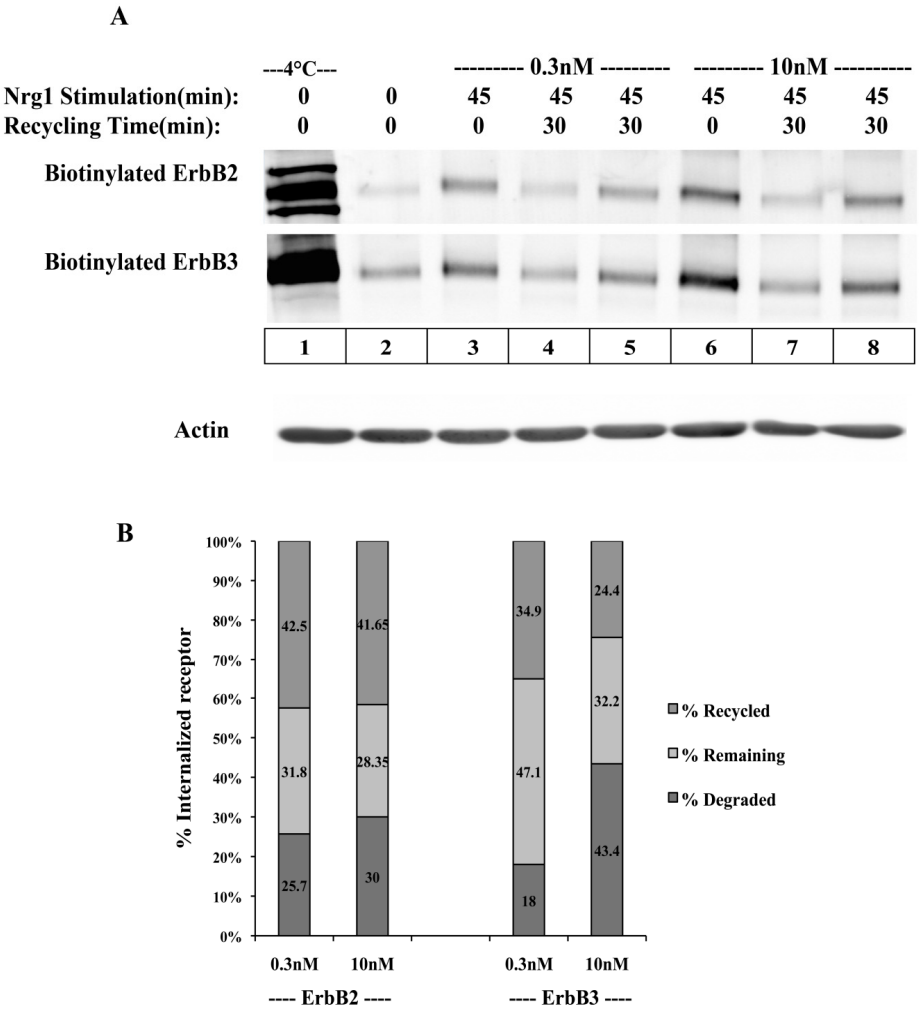

A
---4°C---
--------- 0.3nM ---------
--------- 10nM ----------
Nrg1 Stimulation(min): 0 0 45 45 45 45 45 45
Recycling Time(min): 0 0 0 30 30 0 30 30
Biotinylated ErbB2
Biotinylated ErbB3
1 2 3 4 5 6 7 8
Actin
B
% Internalized receptor
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
42.5
31.8
25.7
41.65
28.35
30
34.9
47.1
18
24.4
32.2
43.4
% Recycled
% Remaining
% Degraded
0.3nM 10nM 0.3nM 10nM
---- ErbB2 ---- ---- ErbB3 ----

Figure 8. Dose-dependent differential recycling between ErbB2 and ErbB3 receptors

(A) Receptor recycling assay as described previously (Figure 7.) was conducted here upon different doses of Nrg1 stimulation – 0.3nM versus 10nM. ErbB2 and ErbB3 receptors showed recycling at both doses. (B) Quantification from one experiment shows the populations of biotinylated, internalized receptor for ErbB2 and ErbB3. The % receptor recycled was calculated as that amount of internalized receptor that was sensitive to the second biotin strip. The % remaining is that percentage of internalized receptor that remains after the second strip. The % degraded is that percentage of internalized receptor that is unaccounted for (100% internalized - % recycled + % degraded). The data here suggests that ErbB3 and not ErbB2 recycling decreases with higher Nrg1 dose. Also ErbB3 and not ErbB2, is preferentially degraded with higher Nrg1 dose.

Figure 9

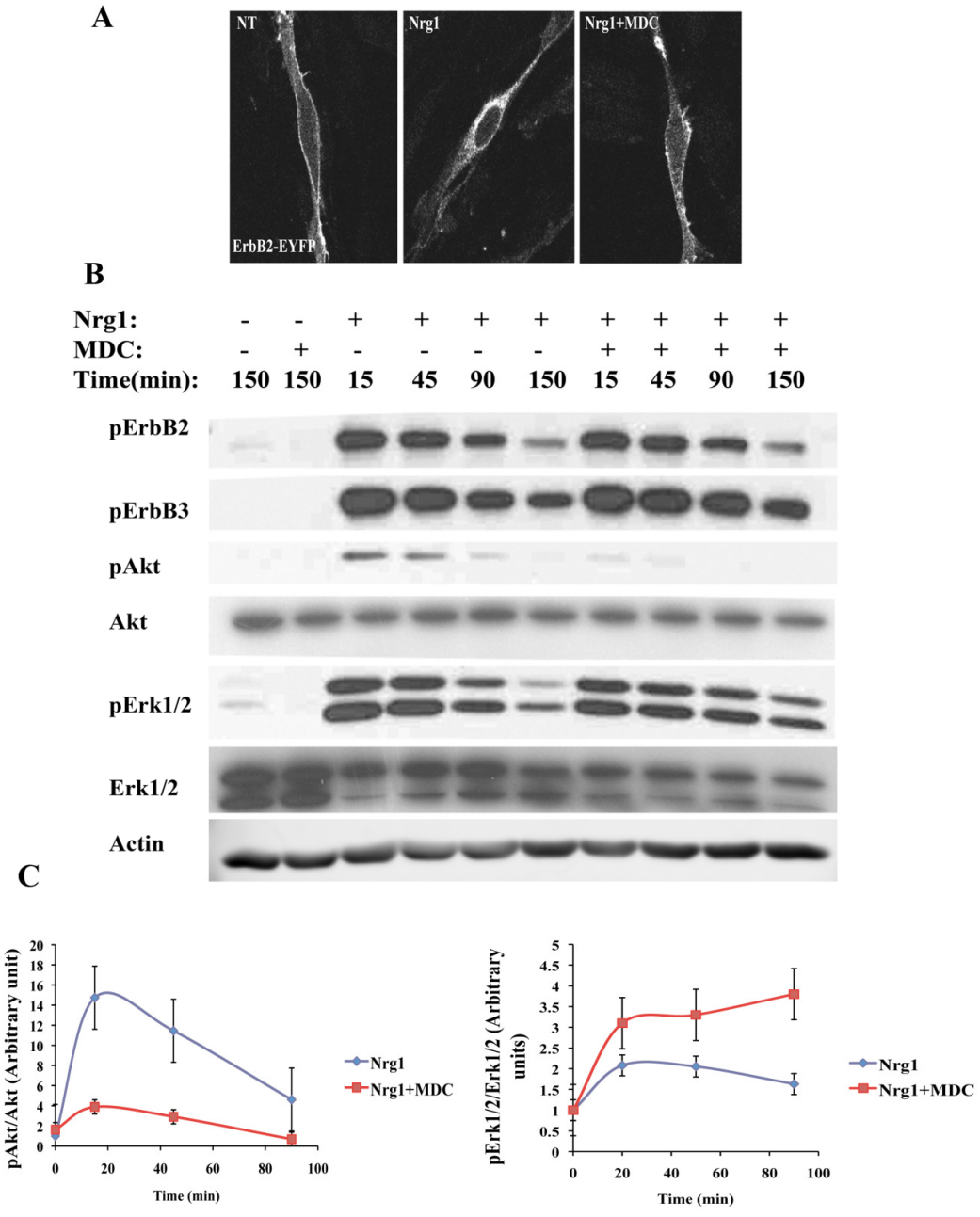

A
NT
Nrg1
Nrg1+MDC
ErbB2-EYFP
B
Nrg1: - - + + + + + + + +
MDC: - + - - - - + + + +
Time(min): 150 150 15 45 90 150 15 45 90 150
pErbB2
pErbB3
pAkt
Akt
pErk1/2
Erk1/2
Actin
C
pAkt/Akt (Arbitrary unit)
Time (min)
Nrg1
Nrg1+MDC
pErk1/2/Erk1/2 (Arbitrary units)
Time (min)
Nrg1
Nrg1+MDC

Figure 9. Inhibition of Nrg1-induced ErbB receptor internalization by monodansylcadaverine (MDC) decreases Akt and not Erk1/2 activity

(A) ErbB2-EYFP Schwann cells were treated with Nrg1 in the presence or absence of MDC. MDC blocked Nrg1-mediated ErbB2-EYFP internalization in Schwann cells.

(B) Growth factor starved Schwann cells were stimulated with Nrg1 with or without MDC for 15′, 45′, 90′ and 150′, respectively. Cells were then lysed and analyzed by Western blot for pErbB2, pErbB3 and downstream pAkt and pErk1/2. MDC treatment inhibited Nrg1-induced Akt activation without causing a decrease in Erk1/2 activity. (C)

Quantification of pAkt/Akt and pErk1/2/Erk1/2 from three separate experiments using LICOR imaging software, shows that MDC inhibits Nrg1-induced Akt activation and might even cause Erk1/2 activity to remain sustained upon Nrg1 stimulation.

Figure 10

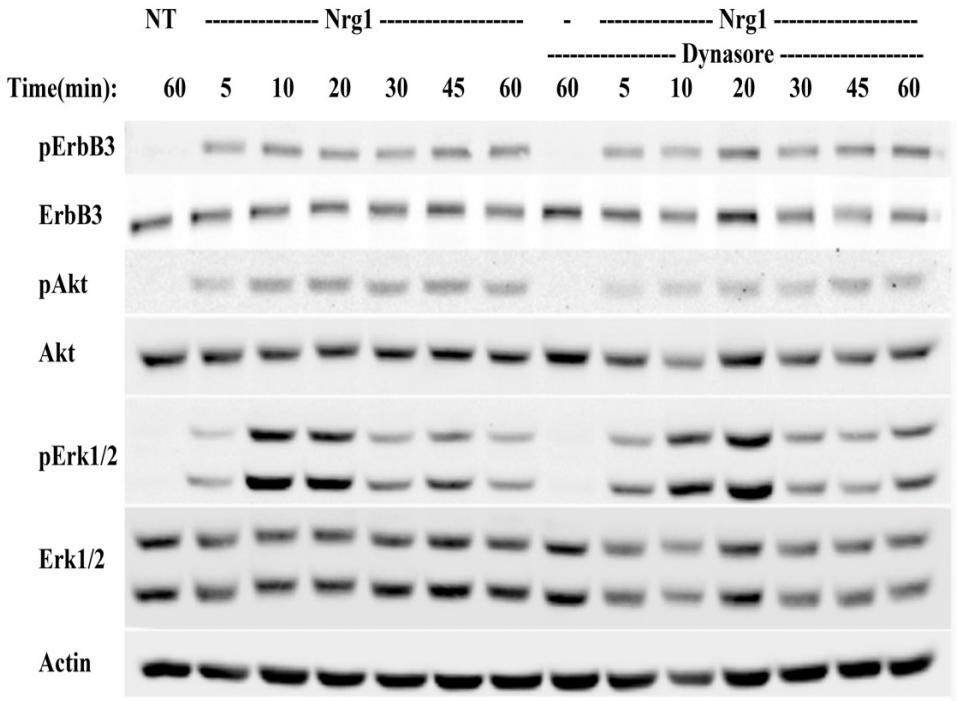

NT
Nrg1
-
Nrg1
Dynasore
Time(min):
60 5 10 20 30 45 60 60 5 10 20 30 45 60
pErbB3
ErbB3
pAkt
Akt
pErk1/2
Erk1/2
Actin

Figure 10. Inhibition of dynamin-mediated endocytosis by dynasore inhibits maximal Akt and not Erk1/2 activation

Growth factor starved Schwann cells were treated with Nrg1 in the presence or absence of dynasore for the indicated time points (5′,10′,20′,30′,45′ and 60′). Dynasore treatment decreased Nrg1-induced maximal Akt activation, without affecting Erk1/2 activity.

Figure 11

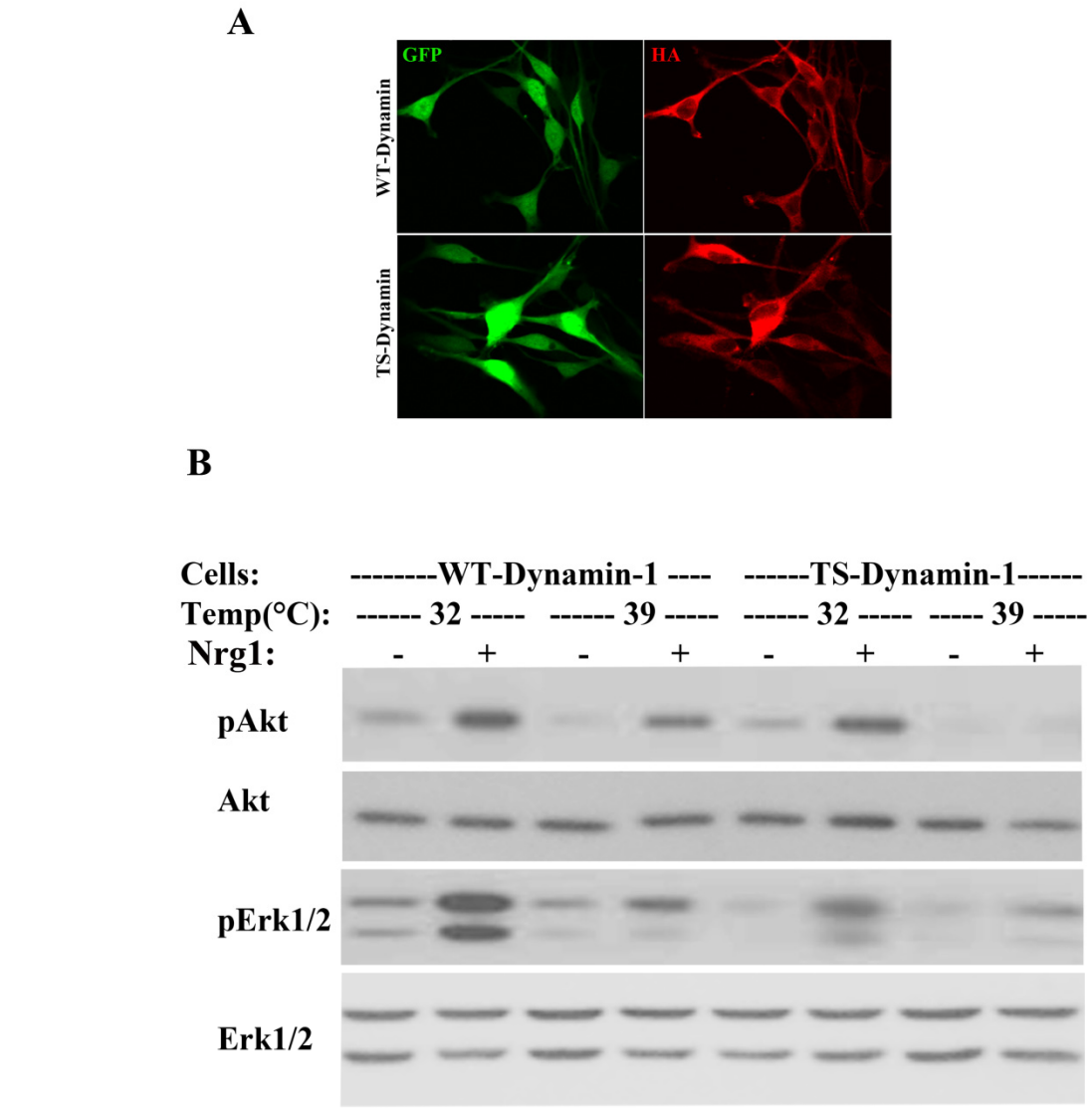

A
GFP
HA
WT-Dynamin
TS-Dynamin
B
Cells: --------WT-Dynamin-1 ---- ------TS-Dynamin-1------
Temp(°C): ------ 32 ----- ------ 39 ----- ------ 32 ----- ----- 39 -----
Nrg1: - + - + - + - +
pAkt
Akt
pErk1/2
Erk1/2

Figure 11. Inhibition of dynamin-induced endocytosis by temperature-sensitive dynamin-1 mutants inhibits Akt activation mediated by Nrg1 in Schwann cells

(A) Schwann cells were adenovirally transduced to express WT or TS-dynamin mutant proteins. Cells were immunostained for the HA-tag, expressed on the dynamin mutant proteins and GFP, present on the adenoviral reporter plasmid. Most infected cells also expressed dynamin protein abundantly in the cells. (B) Schwann cells expressing either WT or TS-dynamin proteins were briefly switched to a temperature of 39°C (the non-permissive temperature) to inactivate TS-dynamin. Parallel cultures were maintained at 32°C (permissive temperature) for 1hour. Cells were then stimulated with Nrg1 for 45′ at the these temperatures. Cultures were lysed and analysed by Western blot for pAkt and pErk1/2. Schwann cells expressing TS-dynamin1 show a decreased ability in achieving Nrg1-mediated maximal Akt activation at the non-permissive temperature. Although Erk1/2 activation is also slightly inhibited in the TS-dynamin cells at the non-permissive temperature, the fold difference compared to the WT-dynamin1 cells at the non-permissive temperatures is smaller for activated Erk1/2 than Akt. This data confirms the importance of dynamin activity in regulating Nrg1-induced Akt activation in Schwann cells.

Figure 12

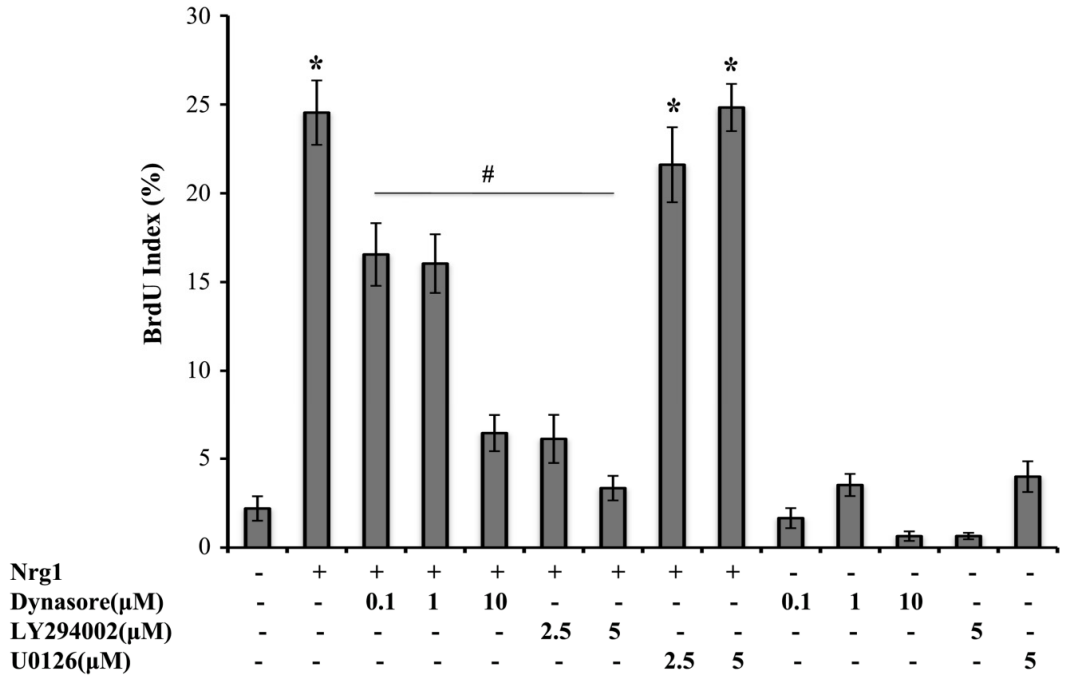

BrdU Index (%)
30
25
20
15
10
5
0
*
*
*
#
Nrg1 - + + + + + + + + - - - - -
Dynasore(μM) - - 0.1 1 10 - - - - 0.1 1 10 - -
LY294002(μM) - - - - - 2.5 5 - - - - - 5 -
U0126(μM) - - - - - - - 2.5 5 - - - - 5

Figure 12. Inhibition of endocytosis inhibits Schwann cell proliferation induced by Nrg1

Schwann cells were growth factor starved for 48hrs and then stimulated with Nrg1 in the presence or absence of dynasore (0.1μ, 1μ and 10μM) or LY294002, an inhibitor of PI3-kinase or U0126, an inhibitor of Mek1/2. Cultures were fixed after 24hrs and stained to assess for BrdU incorporation. Total BrdU positive cells as a percentage of DAPI are represented here. Dynasore treatment inhibited Nrg1-induced Schwann cell proliferation in a dose-dependent manner. Inhibition of the PI3-kinase and not the Ras/Raf/Erk1/2 pathway inhibited Schwann cell proliferation, as also shown previously. Data from 2 independent experiments (2-3 coverslips/experiment) were collected. ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments. Asterisk indicates significant difference from NT *$p<0.05$; '#', indicates values significantly different from Nrg1 treatment, #$p<0.05$. This data suggests that Nrg1-mediated ErbB receptor internalization is likely to be associated with PI3-kinase/Akt activation required for Schwann cell proliferation.

Figure 13

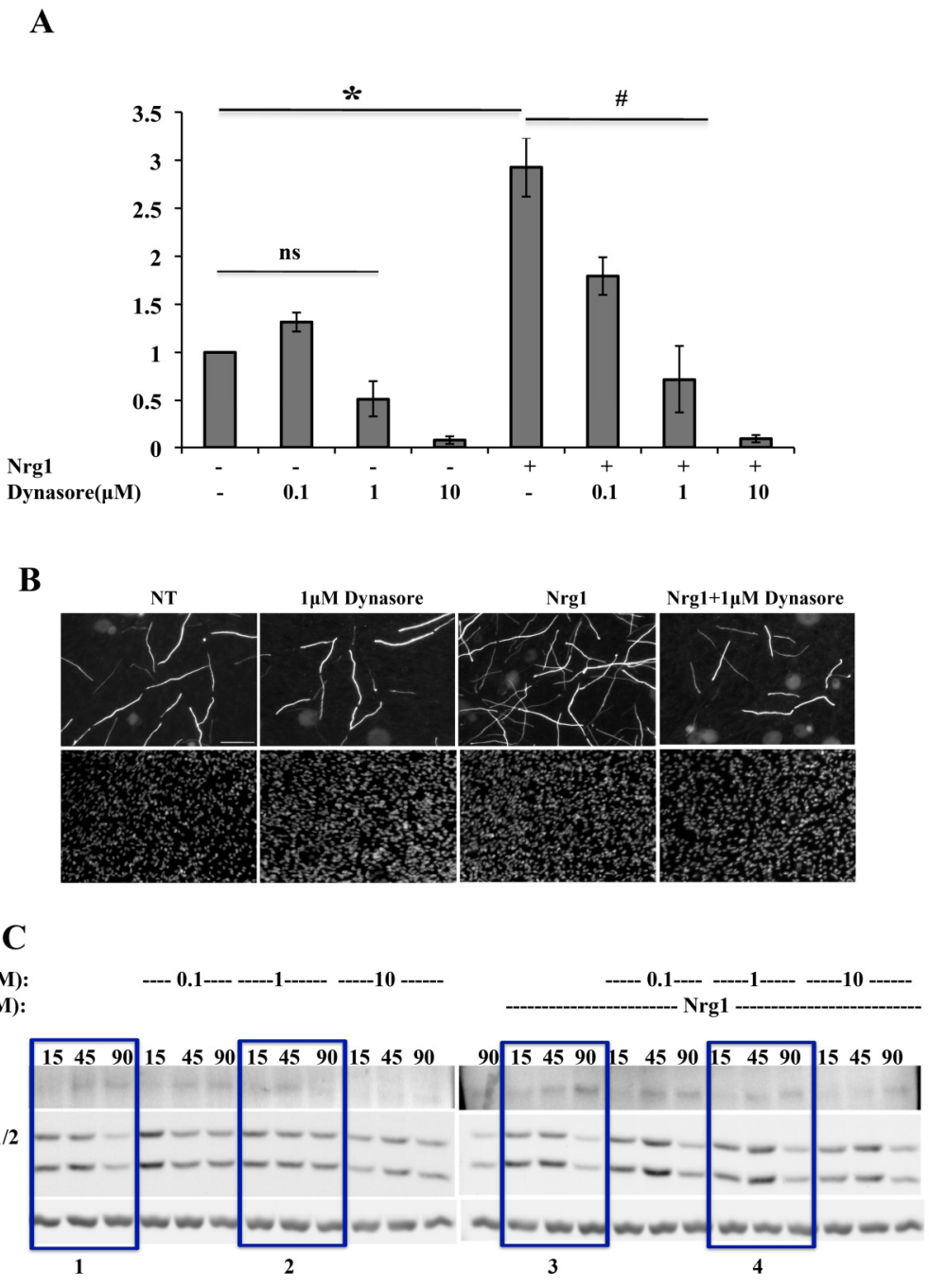
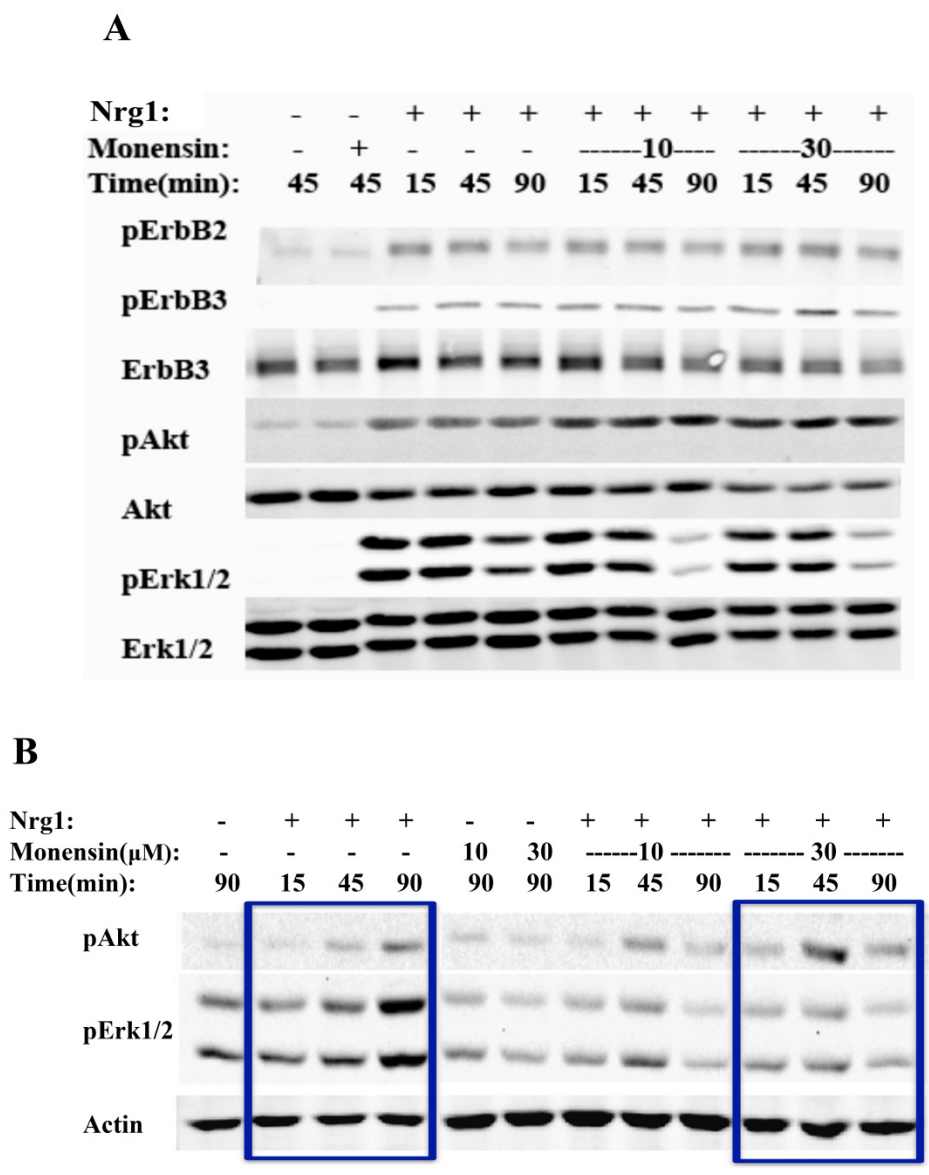

A
*
#
ns
3.5
3
2.5
2
1.5
1
0.5
0
Nrg1 - - - - + + + +
Dynasore(μM) - 0.1 1 10 - 0.1 1 10
B
NT
1μM Dynasore
Nrg1
Nrg1+1μM Dynasore
C
Dynasore(μM): ---- 0.1---- -----1------ -----10 ------ ----- 0.1---- -----1----- -----10 ------
Nrg1(0.01μM): ---------- Nrg1 ----------
Time(min): 15 45 90 15 45 90 15 45 90 15 45 90 90 15 45 90 15 45 90 15 45 90 15 45 90
pAkt
pErk1/2
Actin
1
2
3
4

Figure 13. ErbB receptor endocytosis is required for Nrg1-induced Schwann cell promotion of myelination

(A) Schwann cells were seeded onto DRG neuron cultures, as described in the Materials and Methods section. After proper alignment and Schwann cell proliferation along the axon, dynasore was added at varying doses for 1hr. Nrg1 at 0.01nM was added in the presence or absence of dynasore. The co-cultures were maintained in this co-treatment for a period of 7hours. Cultures were then thoroughly rinsed and placed back in myelinating media for 10 days. After 10 days, cultures were fixed and stained for the presence of MBP-positive segments. The data represented here is from three independent experiments (2-3 coverslips/ experiment). One-way ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments.

Asterisk indicates significant difference from NT *$p<0.05$; '#" indicates significant difference between Nrg1 treatment and the dynasore treated culures. This result indicates that dynamin function regulates Nrg1-induced promyelination function in Schwann cells, likely via the PI3kinase/Akt pathway. (B) Representative figures of conditions in A, are shown here. Although dynasore at 1μm did not significantly affect myelination in normal cultures, dynasore treatment inhibited the promyelination effect of Nrg1. (C) Schwann cell-DRG co-cultures were stimulated with Nrg1 for 15′, 45′ and 90′ with or without varying doses of dynasore (0.1μM, 1μM and 10μM). Western blot analysis for the pAkt and pErk1/2 were conducted. Dynasore treatment inhibited the activation of Akt observed in co-cultures in a dose-dependent manner.

Figure 14


A
Nrg1: - - + + + + + + + + +
Monensin: - + - - - ------10---- -------30------
Time(min): 45 45 15 45 90 15 45 90 15 45 90
pErbB2
pErbB3
ErbB3
pAkt
Akt
pErk1/2
Erk1/2
B
Nrg1: - + + + - - + + + + + +
Monensin(μM): - - - - 10 30 ------10 ------- ------- 30 -------
Time(min): 90 15 45 90 90 90 15 45 90 15 45 90
pAkt
pErk1/2
Actin

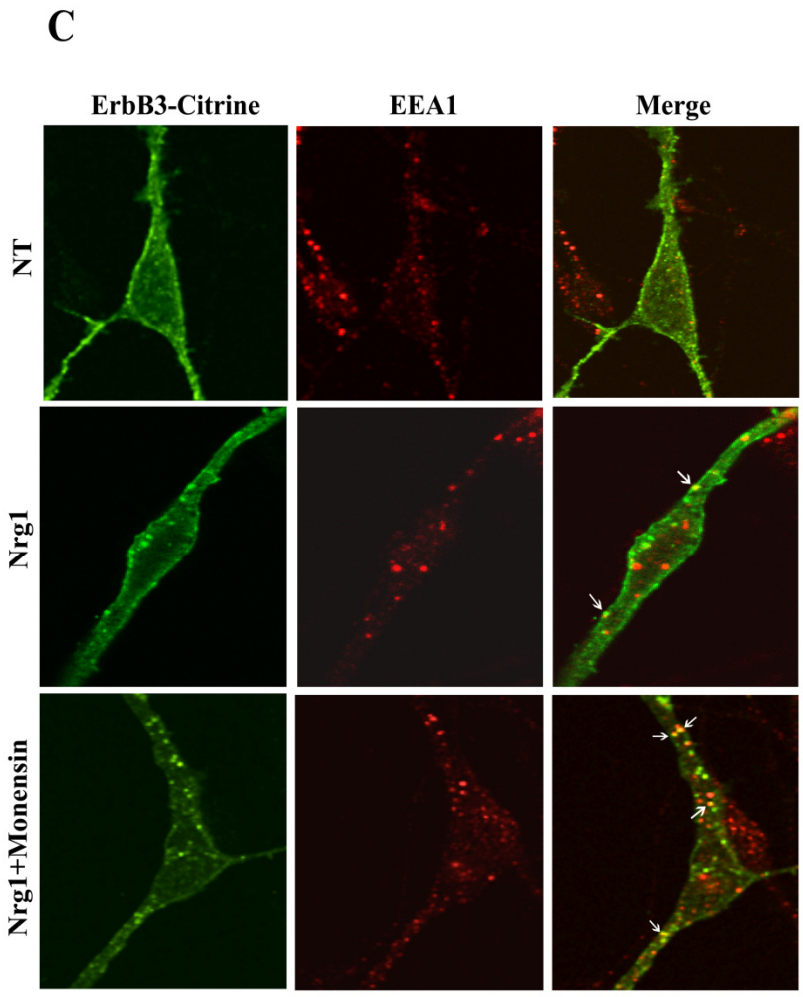

C
ErbB3-Citrine
EEA1
Merge
NT
Nrg1
Nrg1+Monensin

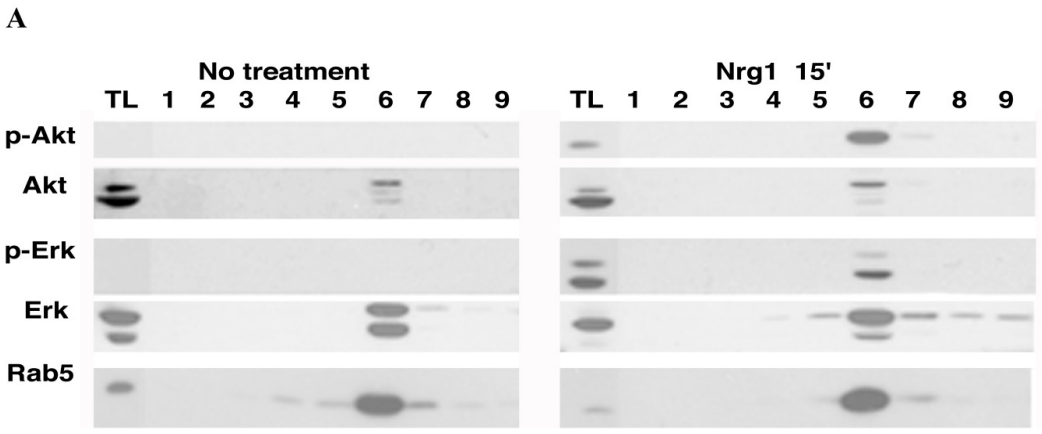
Figure 14. Inhibition of receptor recycling enhances Nrg1-induced Akt activation in Schwann cells

(A) Schwann cells were growth factor starved for 48hrs. They were then pretreated with monensin at 10μM and 30μ for 1hour and stimulated with Nrg1 for 15′, 45′ and 90′. Western blot analysis for pErbB2, pErbB3, pAkt and pErk1/2 was conducted. Monensin treatment increased Nrg1-induced Akt activation in Schwann cell monocultures. Inhibition of recycling attenuated Erk1/2 activity.

(B) Schwann cell-DRG co-cultures were treated as above. Nrg1 stimulation of co-cultures sitmulates Akt and Erk1/2 activation, however inhibition of recycling increases Nrg1-induced Akt activation, seen clearly at 45′, while having an opposing effect on Erk1/2 activity.

(C) ErbB3-Citrine Schwann cells were stimulated with Nrg1 in the presence or absence of monensin. Cultures were then immunostained for early endosomal marker, EEA1. Treatment with Nrg1 increased ErbB3-citrine endocytosis in Schwann cells. Monensin treatment did not affect the endocytic function of ErbB3 upon Nrg1 stimulation. Monensin treated cells even showed an increased accumulation of ErbB3 in EEA1 positive cells.

Figure 15


A
No treatment
Nrg1 15'
TL 1 2 3 4 5 6 7 8 9
TL 1 2 3 4 5 6 7 8 9
p-Akt
Akt
p-Erk
Erk
Rab5

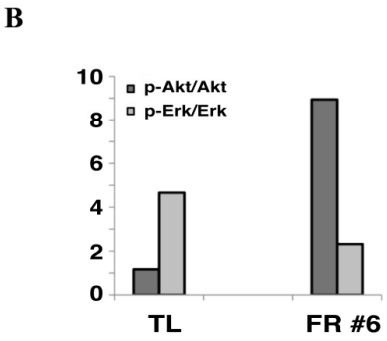

B
10
8
6
4
2
0
p-Akt/Akt
p-Erk/Erk
TL
FR #6

Figure 15. Active Akt is recruited to endosomal fractions upon Nrg1 stimulation

(A) Schwann cells were stimulated with Nrg for 15′. Briefly, cells were homogenized and the post-nuclear supernatant (TL) loaded onto a sucrose step floatation gradient optimized to separate endosomal fractions from other membrane organelles. The fractions were collected and concentrated and analysed by Western blot for pAkt and pErk1/2 and Rab5 as a marker for early endosomal compartments. Upon Nrg1 stimulation both active Akt and Erk1/2 were detected in endosomal compartments.

(B) Quantification of the relative amounts of active Akt and Erk1/2 shows an approximate nine-fold increase in enrichment of the active Akt in fraction 6 compared to the level detected in the TL. In comparison the proportion of active Erk1/2 was not significantly increased in these fractions.

Figure 16

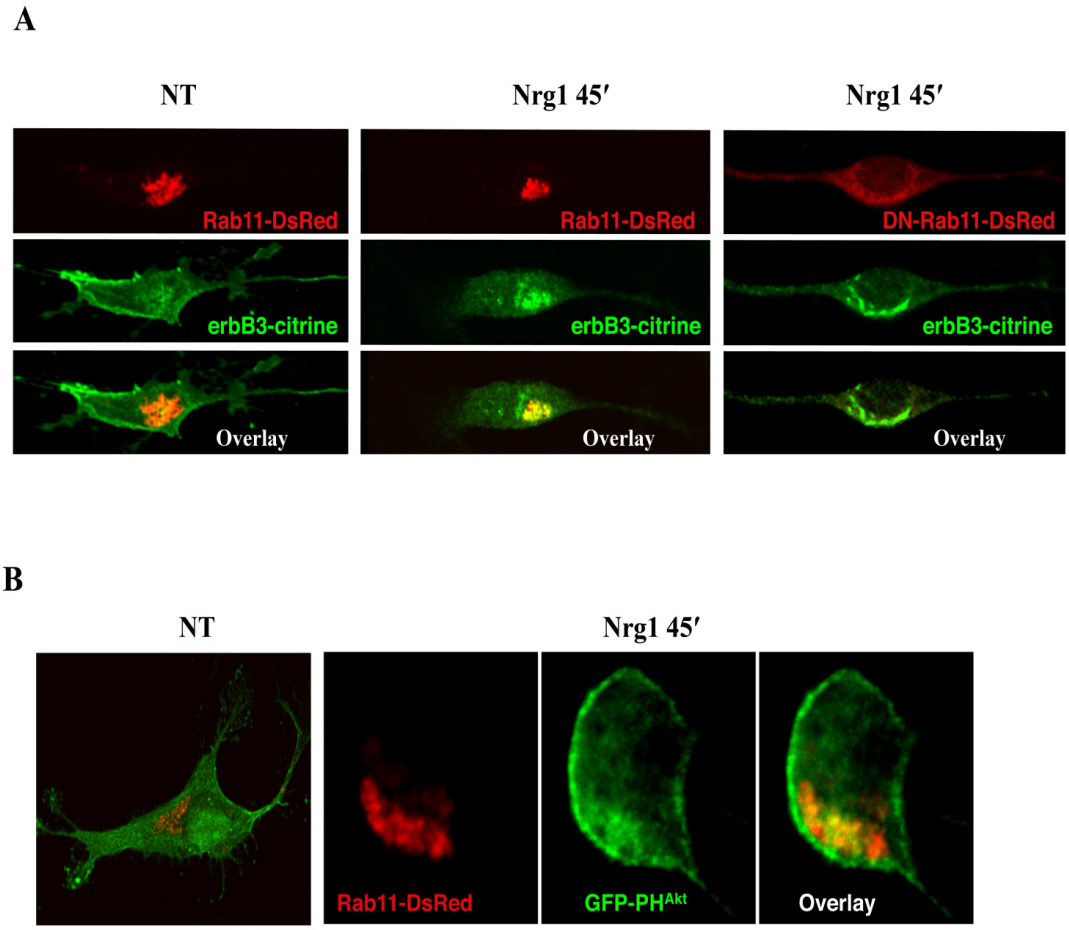

A
NT
Nrg1 45′
Nrg1 45′
Rab11-DsRed
Rab11-DsRed
DN-Rab11-DsRed
erbB3-citrine
erbB3-citrine
erbB3-citrine
Overlay
Overlay
Overlay
B
NT
Nrg1 45′
Rab11-DsRed
GFP-PHAkt
Overlay

Figure 16. Rab11 increases accumulation of ErbB receptors within recycling endosomes and may promote PI3-kinase/Akt signaling

(A) Schwann cells co-transfected with WT Rab11a-DsRed or DN Rab11a-DsRed and ErbB3-citrine were stimulated with Nrg1 for 45′. Cells were then fixed and immunostained for the presence of ErbB3 and WT or DN Rab11a. WT Rab11a-DsRed had a strong punctate perinuclear localization that was diffused and disrupted in cells expressing DN Rab11a-DsRed. Upon Nrg1 stimulation ErbB3-citrine which is normally present mostly at the plasma membrane is seen co-localised to the WT Rab11a compartments, indicating an arrest in the recycling endosomes. No clear ErbB3 perinuclear localization is seen in DN Rab11a expressing cells. ErbB3 in these cells was still internalized. (B) Schwann cells were transfected, with WT Rab11a-DsRed along with PH-Akt-GFP to visualize the localization of PI3-kinase activity. Cells were stimulated with Nrg1 for 45′ and then immunostained. Upon Nrg1 stimulation PH-Akt-GFP was seen localized to the plasma membrane as well as WT Rab11a-DsRed positive compartments in the cells, indicating an endosomal origin for Nrg1-induced PI3-kinase signaling.

Figure 17

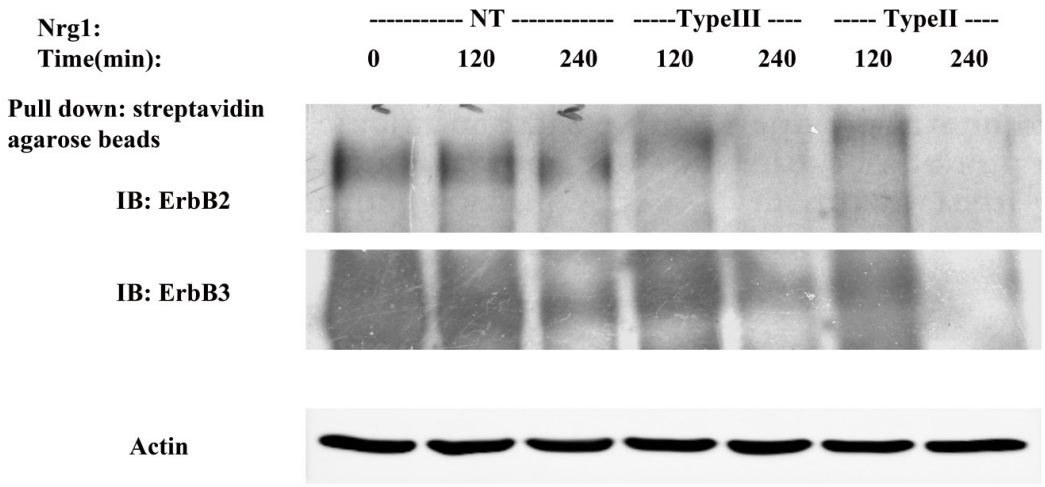

Nrg1:
---------- NT ----------
-----TypeIII ----
----- TypeII ----
Time(min):
0
120
240
120
240
120
240
Pull down: streptavidin agarose beads
IB: ErbB2
IB: ErbB3
Actin

Figure 17. ErbB receptor endocytosis is not Nrg1 isoform-specific

Schwann cells were surface labeled with biotin and stimulated with Nrg1 Type II or Type III for 120′ and 240′. After the indicated times, cells were lysed and the remaining biotin labeled protein was purified and analysed by Western blot for the presence of ErbB2 and ErbB3. Time 0′ indicates total surface biotinylated receptors at the start at 4°C. Both Nrg1 Type II and Type III caused increased downregulation of the biotinylated surface receptors over time, indicating receptor internalization and degradation.

Figure 18

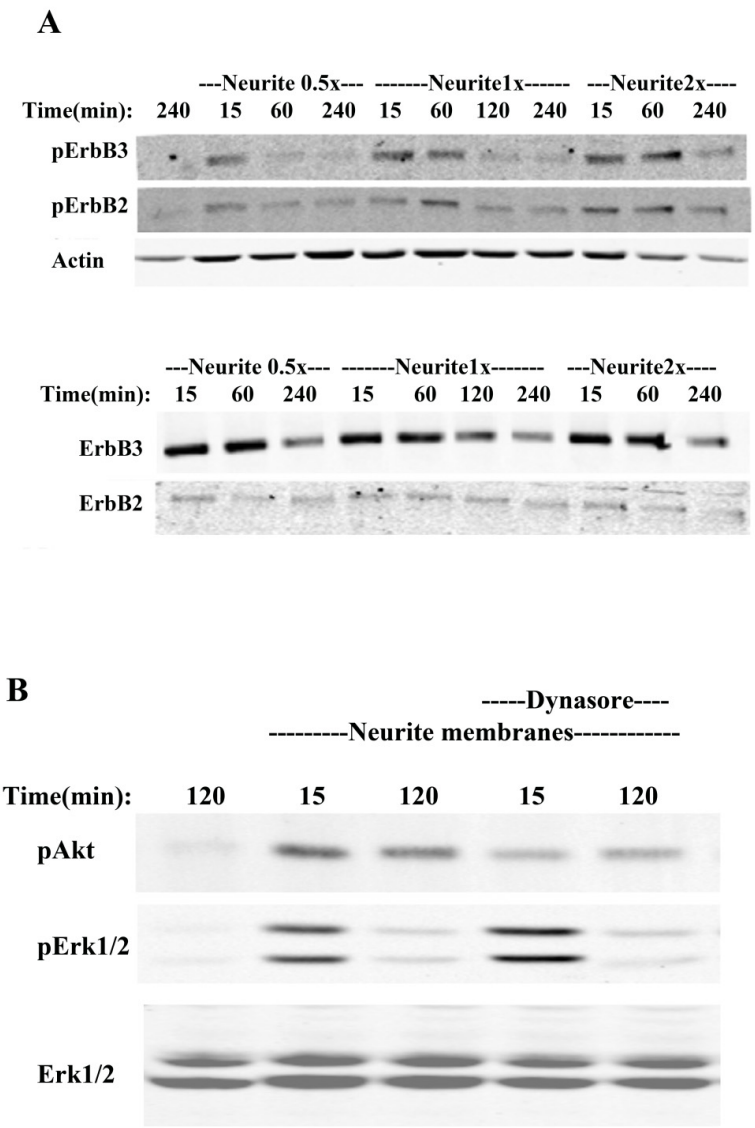

A
---Neurite 0.5x--- -------Neurite1x------ ---Neurite2x----
Time(min): 240 15 60 240 15 60 120 240 15 60 240
pErbB3
pErbB2
Actin
---Neurite 0.5x--- -------Neurite1x------- ---Neurite2x----
Time(min): 15 60 240 15 60 120 240 15 60 240
ErbB3
ErbB2
B
-----Dynasore----
---------Neurite membranes----------
Time(min): 120 15 120 15 120
pAkt
pErk1/2
Erk1/2

Figure 18. Membrane-tethered Nrg1 Type III induces the activation and downregulation of ErbB receptors in Schwann cells

(A) Different amounts of increasing neurite membrane fractions were centrifuged onto growth factor starved Schwann cells and allowed to stimulate them for 15′, 60′, 120′ and 240′. Cells were then lysed and analysed by Western blot for the presence of pErbB2, pErbB3, ErbB2 and ErbB3. Neurite fractions stimulated ErbB receptor activation in a dose-dependent manner. Total ErbB2 and ErbB3 receptors were seen downregulated upon Nrg1 stimulation over time, indicating degradation.

(B) Schwann cells were stimulated with neurite membranes in the presence or absence of dynasore for 15′ and 120 ′. Cultures were lysed and analysed by Western blot for the presence of pAkt and pErk1/2. Inhibition of endocytosis selectively decreased neurite membrane-induced Akt and not Erk1/2 activation in Schwann cells.

Figure 19

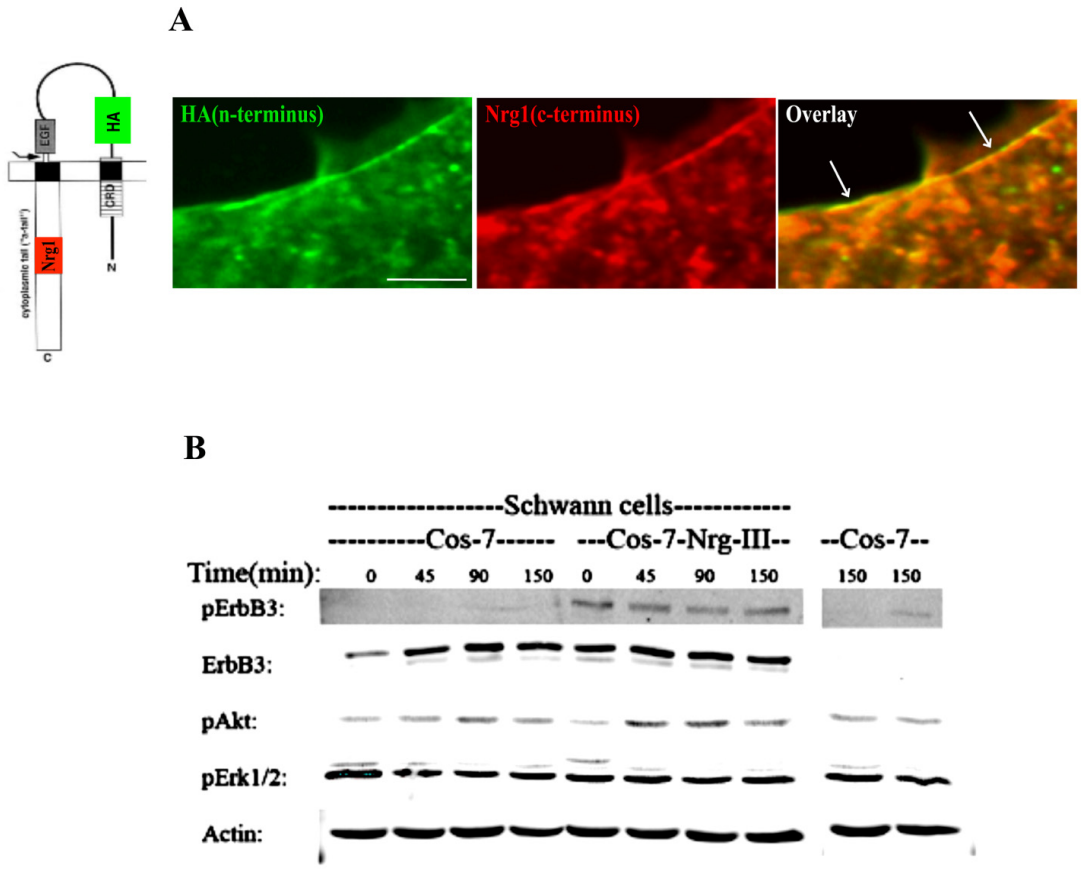

A
HA
EGF
CRD
Nrg1
N
C
HA(n-terminus)
Nrg1(c-terminus)
Overlay
B
----------Schwann cells----------
---------Cos-7------ ---Cos-7-Nrg-III-- --Cos-7--
Time(min): 0 45 90 150 0 45 90 150 150 150
pErbB3:
ErbB3:
pAkt:
pErk1/2:
Actin:

Figure 19. Ectopic expression of Nrg1 Type III in Cos-7 cells enables its membrane localization and activity

(A) Cos-7 cells were transfected with HA Nrg1 Type III and immunostained for α HA-present on the N-terminus and α-Nrg1 recognizing the C-terminus of Nrg1 Type III. Transfected cells show clear membrane localization of Nrg1 Type III with both antibodies. (B) Cos-7 cells were either left untreated or transfected with Nrg1 Type III. Growth factor starved Schwann cells were centrifuged onto a bed of Cos-7 cells and allowed to associate with them for the indicated time points. Cells were lysed and analysed by Western blot for the presence of pErbB3, pAkt and pErk1/2. Membrane-tethered Nrg1 Type III on the Cos-7 cells was successful in activating the ErbB3 receptors and to a lesser extent also Akt and Erk1/2.

Figure 20

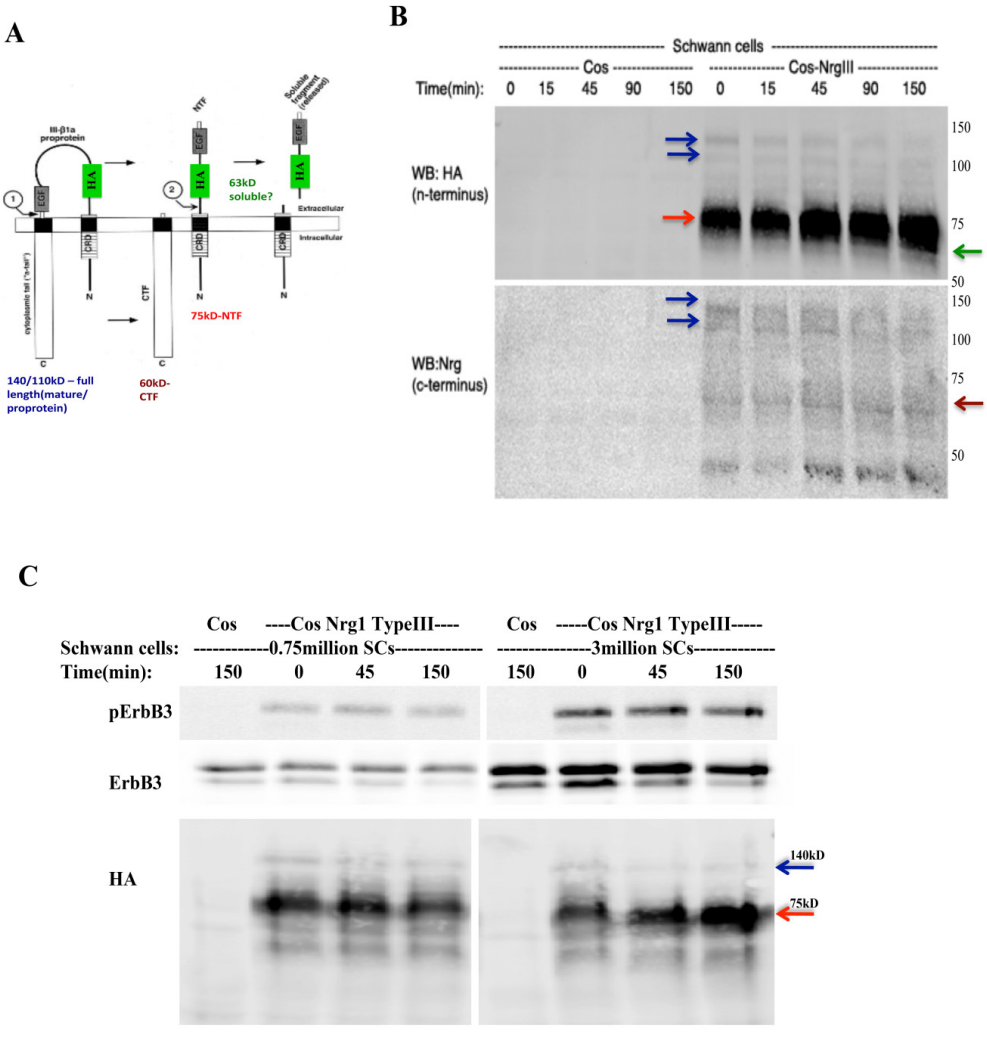

A
III-β1a proprotein
EGF
HA
CHD
NTF
63kD soluble?
Soluble fragment (ectodomain)
Extracellular
Intracellular
CTF
75kD-NTF
140/110kD – full length(mature/ proprotein)
60kD-CTF
B
Schwann cells
Cos
Cos-NrgIII
Time(min): 0 15 45 90 150 0 15 45 90 150
WB: HA (n-terminus)
WB:Nrg (c-terminus)
150
100
75
50
C
Cos
----Cos Nrg1 TypeIII----
Cos
-----Cos Nrg1 TypeIII-----
Schwann cells: ----------0.75million SCs---------- ----------3million SCs----------
Time(min): 150 0 45 150 150 0 45 150
pErbB3
ErbB3
HA
140kD
75kD

Figure 20. Schwann cell contact increases the processing of Membrane-tethered Nrg1 Type III

(A) Model of Nrg1 Type III processing – Full length isoforms, 140kD/110KD; cleavage at site 1 (BACE1/TACE) releases a membrane-bound C-terminal fragment of 60kD (60kD CTF) and a membrane-bound N-terminal fragment of 75kD (75kD NTF); cleavage at site 2 (unknown protease) releases a soluble N-terminal fragment of 63kD (63kD NTF).

(B) Schwann cells were centrifuged either onto untransfected or Nrg1 Type III transfected Cos-7 cells and allowed to associate for varying lengths of time as indicates. Cells were lysed and analysed by Western blot using antibodies α-HA and α-Nrg1 c-terminus, to detect Nrg1 Type III processed fragments. The colors of the arrows are coded to those on the model (A) and indicate the specific cleavage products. Upon Schwann cell contact, Nrg1 Type III is processed largely at its 1st cleavage site. A faint band ~63kD NTF is visible at 90′ and 150′.

(C) Schwann cells were plated at 0.75million and 3million onto Cos-7 cells to increase contact-mediated Nrg1 Type III processing and better detection of the 63kD NTF. Cultures were analysed by Western blot as described above. Robust activation of ErbB3 receptors increasing with increased Schwann cell number was seen. Although a 63kD NTF form is not clearly detected, increased Schwann cell contact increased Nrg1 Type III processing at its 1st cleavage site.

Figure 21

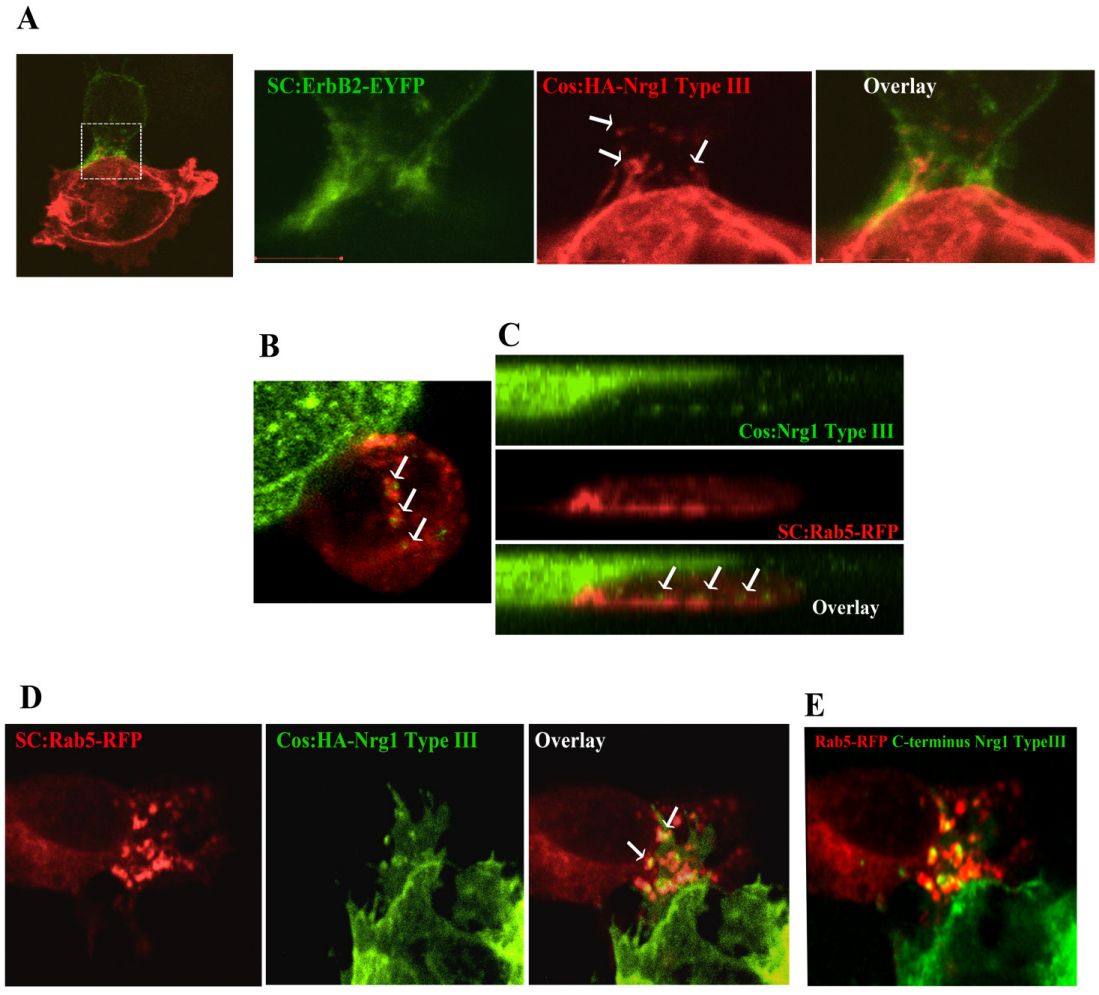

A
SC:ErbB2-EYFP
Cos:HA-Nrg1 Type III
Overlay
B
C
Cos:Nrg1 Type III
SC:Rab5-RFP
Overlay
D
SC:Rab5-RFP
Cos:HA-Nrg1 Type III
Overlay
E
Rab5-RFP C-terminus Nrg1 TypeIII

Figure 21. Schwann cells endocytose membrane-bound Nrg1 Type III

(A) ErbB3-citrine expressing Schwann cells were cultured with HA Nrg1 Type III Cos-7 cells for 2hours. Cells were fixed and immunostained and Z-stack images at the cell-cell contact were taken (0.5μM). Two positively transfected cells making contact are seen here. Small puncta labeled with α-HA were seen travelling (arrows) into the Schwann cells at the contact point. Some co-localization of ErbB3 with these puncta was observed, indicating endocytic uptake of the ligand.

(B) Schwann cells expressing Rab5-RFP were cultured with HA Nrg1 Type III Cos-7 cells for 2hours. Cells were fixed and stained and imaged as above. Small Rab5-RFP compartments were seen adjacent to punctate Nrg1 Type III (arrows), indicating an endosomal uptake of ligand

(C) A 3-D reconstitution of a z-stack of the cells shown in (B) was done along the y-z axis. These images show that the Nrg1 Type III positive puncta are present within the Schwann cell body.

(D) Cos-7 cells transfected with HA Nrg1 Type III were first plated on coverslips and allowed to adhere and spread for 4hours. Schwann cells were then plated onto the Cos-7 cells and allowed to associate for 2 hours. Clear localization between the Rab5-RFP in Schwann cells and the HA Nrg Type III was detected confirming the endocytic uptake of the ligand into Rab5 compartments in the Schwann cells.

(E) Visualizing Nrg1 Type III using its α-C-terminal antibody also shows co-localization with Rab5-RFP in Schwann cells. This suggests either the uptake of the full length Nrg1 Type III form into the Schwann cells or that upon the 1st cleavage the CTF was being endocytosed by default along with the NTF, by its proximity to it.

Figure 22

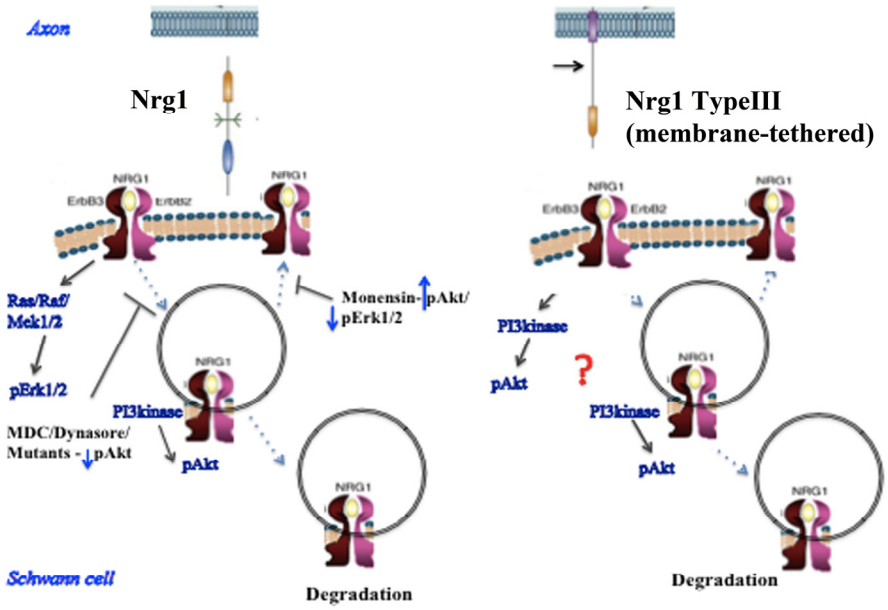


Figure 22. Proposed model of Nrg1 - ErbB trafficking in Schwann cells

Our work is summarized in this proposed model of Nrg1-mediated ErbB receptor trafficking and signaling in Schwann cells. ErbB2 and ErbB3 receptor internalization is required for soluble Nrg1-mediated PI3-kinase/Akt and not Ras/Raf/Erk1/2 signaling in Schwann cells. Inhibition of receptor recycling enhances PI3-kinase/Akt signaling while attenuating Erk1/2 activity. Upon Nrg1 stimulation PI3-kinase/Akt signaling is also generated in an endosomal compartment, possibly Rab11. Membrane-tethered Nrg1 Type III also down regulates ErbB receptors and stimulates PI3-kinase/Akt signaling that is sensitive to dynamin inhibition, indicating a mechanism of ligand cleavage and uptake into the Schwann cells for efficient endosomal signaling.

10. Bibliography

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