Role and Specificity of LGI4-ADAM22 Interactions in Peripheral Nerve Myelination

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ISBN: 978-94-6203-332-0

The research presented in this thesis was performed at the department of Genetics and Cell Biology of the Erasmus MC in Rotterdam, The Netherlands.

The studies described in this thesis were supported by grants from the BSIK Innovation programme “Stem Cells in Development and Disease” (SCDD, BSIK 03038),the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant No. FES0908), VICI grant 918.66.616 and the European Union (NGIDD FP7 HEALTH-F2-2008-201535).

Financial support by Erasmus MC for the publication of this thesis is gratefully acknowledged.

This thesis was printed by CPI-Wöhrmann Print Service, Zutphen.

Cover designed by Linde: G key transformed into a neuron and a Schwann cell.

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Rol en specificiteit van LGI4-ADAM22 interacties in myelinisatie van de perifere zenuw

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H.G. Schmidt

and in accordance with the decision of the Doctorate Board

The public defense shall be held on
Wednesday 10 April 2013 at 11.30 o'clock

by

Linde Kegel

born in Vlaardingen

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Voor mijn ouders en Arno

Contents

List of abbreviations

ADAM : A Disintegrin And Metalloprotease
ADLTE : Autosomal Dominant Lateral Temporal lobe Epilepsy
ADPEAF : Autosomal-Dominant Partial Epilepsy with Auditory Features
ANS : Autonomic nervous system
AP2α : Activator Protein 2α
APP : Amyloid Precursor Protein
BACE : Beta-site APP cleaving enzyme 1
BDNF : Brain Derived Neurotrophic Factor
BFABP : Brain Fatty Acid-Binding Protein
BFJE : Benign Familial Juvenile Epilepsy
BMP : Bone Morphogenic Protein
Cad19 : Cadherin19
CAM : Cell Adhesion Molecule
cAMP : cyclic Adenosine Monophosphate
Caspr : Contactin Associated Protein
Cdc42 : Cell division control protein 42 homolog
Clp : Claw paw
CMT : Charcot-Marie-Tooth
CN : Calcineurin
CNP : 2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNS : Central Nervous System
CRD : Cysteine Rich Domain
Cx : Connexin
DGC : Dystrophin-Glycoprotein Complex
DHH : Desert Hedgehog
DRG : Dorsal Root Ganglion
DRP2 : Dystrophin Related Protein 2
EBP50 : ERM-Binding Phosphoprotein 50 kDa
ECM : Extracellular Matrix
EGF : Epidermal Growth Factor
EGR2 : Early Growth Resonse protein 2
EPTP : Epitempin
ERK : Extracellular signal Regulated Kinase
ERM : Ezrin-Radixin-Moesin
FAK : Focal Adhesion Kinase
Fc : Fragment crystallizable region
FGF : Fibroblast Growth Factor
GDNF : Glial cell-line Derived Neurotrophic Factor
GAP-43 : Growth-Associated Protein-43
GFAP : Glial Fibrillary Acidic Protein
GGF : Glial Growth Factor
Gpr : G protein-coupled receptor
HMSN : Hereditary Motor and Sensory Neuropathy
Ig : Immunoglobulin
IGF : Insulin-like Growth Factor
ILK : Integrin-Linked Kinase
K_v : Voltage gated potassium channel
LE : Limbic Encephalitis
LGI : Leucine-rich Glioma Inactivated
LRR : Leucine-rich Repeat
MAG : Myelin Associated Glycoprotein
MAPK : Mitogen Activated Protein Kinase
MBP : Myelin Basic Protein
MPZ : Myelin Protein Zero
mRNA : messenger RNA
Na_v : Voltage gated sodium channel
NCad : Cadherin-2/N-Cadherin
NCAM : Neural cell-adhesion molecule
NCC : Neural Crest Cell
NECL : Nectin-like Protein
NF155 : Neurofascin 155
NF186 : Neurofascin 186
NFATc4 : Nuclear Factor of Activated T-cells, cytoplasmic 4
NFκB : Nuclear Factor kappa B
NGF : Nerve growth factor
NICD : Notch intracellular domain
NMSC : Non-Myelinating Schwann Cell
NrCAM : Neuronal cell adhesion molecule
NRG : Neuregulin
NT : Neurotrophin
P75NTR : p75 Neurotrophin Receptor
PDG : Periaxin-DRP2-Dystroglycan
PDGF : Platelet Derived Growth Factor
PDZ : PSD-Dlg-ZO1
PI3K : Phosphatidylinositol 3 Kinase
PKA : Protein Kinase A
PLCγ : Phospholipase Cγ
PMP22 : Peripheral Myelin Protein 22
PNS : Peripheral Nervous System
POU : Pit-Oct-Unc
PSD : Post Synaptic Density
RNA : Ribonucleic Acid
ROCK : Rho Associated Protein Kinase
RT-PCR : Reverse Transcription Polymerase Chain Reaction
SCE : Schwann Cell-specific Enhancer
SCP : Schwann cell precursor
SNS : Somatic nervous system
SLI : Schmidt-Lanterman incisures
SREBP : Sterol Regulatory Element Binding Protein
TACE : TNF-alpha Converting Enzyme
TAG1 : Transient Axonal Glycoprotein 1
TGF : Transforming Growth Factor
TNF : Tumor Necrosis Factor

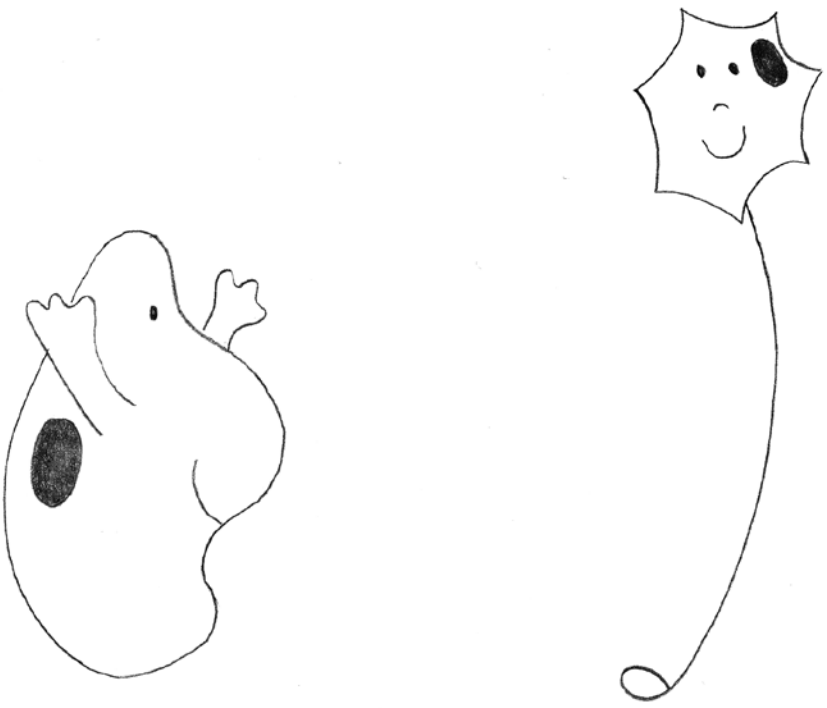
Scope of the thesis

In the peripheral nervous system, large caliber axons are ensheathed and myelinated by Schwann cells. Myelin is crucial for a faster signal transduction along the nerve. Hence it is not surprising that defects in this myelination process cause serious neurological disease. Despite the medical importance of these cells, our understanding of the cellular and molecular mechanisms that control Schwann cell development and myelination is still incomplete. Continuous communication between Schwann cells and neurons is essential for the development, differentiation and myelination of peripheral nerves. Previous studies showed that LGI4 is a secreted protein that is crucial for myelination and might be a key player in this communication process. The main aim of this thesis is to elucidate the mechanism of LGI4 function in peripheral nerve myelination and to identify its interaction partners.

Chapter 1 of this thesis presents an overview of the different types of cells in the peripheral nervous system with an emphasis on Schwann cell development and myelination. Chapter 2 describes the LGI protein family of which the function of most members largely remains a mystery. Chapter 3 identifies the interaction between LGI4 and ADAM22, and the cellular compartments of their action. In chapter 4 we show that three amino acids in LGI4 explain its specific function in myelination. Finally, chapter 5 presents an overall discussion about our findings together with future perspectives and concluding remarks.

Chapter 1

Introduction



1 – Introduction

1.1 The vertebrate nervous system: a brief introduction

The nervous system is the control center of our body. It enables animals to successfully monitor, control and respond to changes in their environment and it manages our thoughts and all the vital body processes such as blood pressure and breathing. At the most basic level, the function of the nervous system is to send signals from one site of the body to the other (1). The *central nervous system* (CNS) consists of the brain and spinal cord and the rest of the nerves make up the *peripheral nervous system* (PNS)(1, 2)(Figure 1). The latter carries information back and forth between the control center (the brain) and the periphery (our senses and other parts of the body). Without information provided by the PNS, the CNS could not function. The PNS is traditionally divided into two systems. The *somatic nervous system* carries information about conscious sensations and innervates the voluntary (striated) muscles of the body. The *autonomic nervous system* on the other hand is strictly motoric and controls most of the involuntary, visceral activities. The autonomic nervous system again consists of three subdivisions: the *parasympathetic system*, which in general promotes the visceral activities characteristic of periods of peace and relaxation, the *sympathetic system*, which controls involuntary activities that occur under stress, and the *enteric system*, which is also sometimes considered an independent system; it comprises a largely self-contained system and consists of both sensory and motor neurons in the gastrointestinal tract that mediate digestive reflexes (1, 2)(Figure 1).

Like all tissues of the body, the nervous system is composed of cells, the majority being *neurons* and *glia* (1, 2). A neuron is extraordinary for its enormously extended shape. It consists of a cell body with short branching dendrites and a longer axon (Figure 2). Neurons transmit information throughout the entire body in the form of electrical signals along their axons and neurotransmitter release at the synapses. In this way motor neurons send information away from the brain via the spinal cord to muscles and peripheral organs. Sensory neurons in turn transmit signals in the opposite direction from the periphery via the spinal cord to the brain, which then decides how to respond based on this peripheral information.

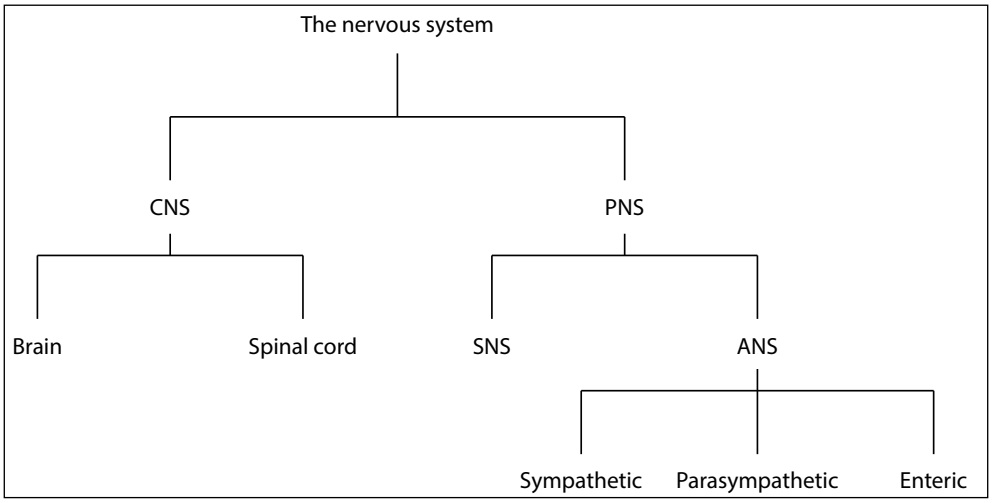


Figure 1. The overall organization of the nervous system
The central nervous system (CNS) consists of the brain and spinal cord. The peripheral nervous system (PNS) consists of the somatic (SNS) and autonomic (ANS) nervous system. The latter in turn consists of the sympathetic, parasympathetic and enteric nervous system.

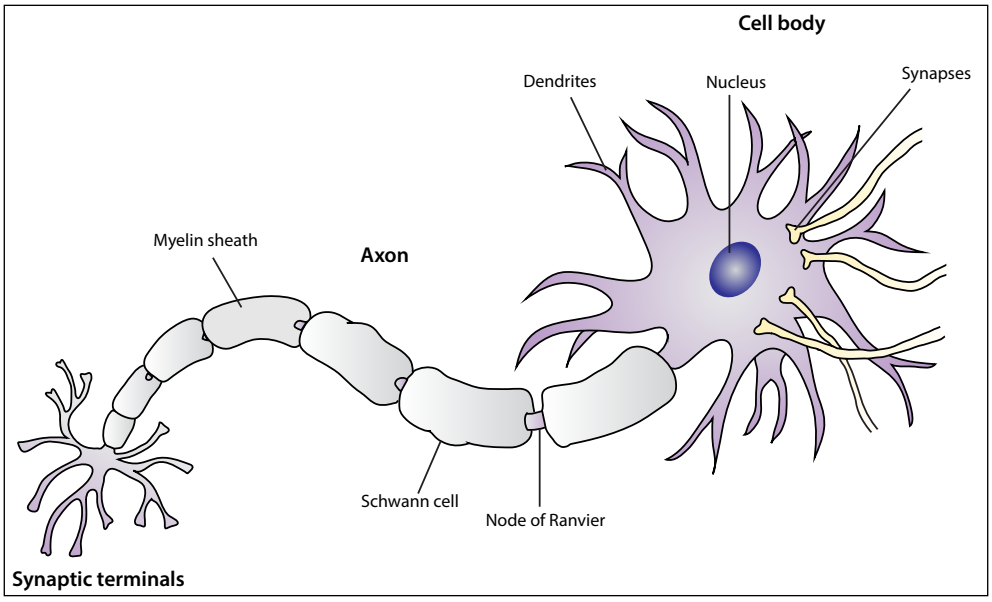


Figure 2. Neuron
Schematic representation of a neuron and its cellular domains. In this example the axon is myelinated by Schwann cells. Along the length of the axon there are gaps up to a millimeter apart where the axon is not covered by myelin. These gaps are called '*Nodes of Ranvier*'.

Glia nurture and protect the neurons. These cells come in many types and have multiple functions in the developing and mature nervous system (reviewed in (3-5)). In the CNS, the main glia cell types are astrocytes and oligodendrocytes and in the PNS, the Schwann cells, enteric glial cells and satellite cells. *Astrocytes* contribute to homeostasis by providing neurons with energy and substrates for neurotransmission. Besides that astrocytes act as physical barriers at synapses between neighboring neurons, remove excess neurotransmitters from the extracellular space and are involved in the formation of synapses and in modulating synaptic function (6, 7). *Enteric glia* regulate neuronal maintenance, survival and function and synaptic activity of the enteric nervous system (reviewed in (8)). *Satellite cells* in turn surround neuronal cell bodies in the peripheral ganglia (dense clusters of neuronal soma) to regulate their homeostasis and to provide trophic support (6, 7).

Oligodendrocytes and *Schwann cells* have very similar functions (3). During evolution animals became bigger, developed more sophisticated behaviors and needed to survive in riskier environments. Thus, nerve fibers that conducted more rapidly became an advantage for fast computation and action (9). Action potentials in invertebrates are generally conducted at a speed of about 1 meter per second or less, which is still fast enough for small animals to survive. However, larger body sizes require larger distances to cross for these action potentials, and thus an increase in action potential velocity. One way to achieve this is to increase the diameter of the axons, which lowers the internal resistance of the axon (2). However, this would require more space within the body. Vertebrates have developed a much more efficient system, in which both oligodendrocytes and Schwann cells produce a lipid-rich membrane called *myelin*, which enwraps axons providing electrical insulation (2). Myelin decreases capacitance across the membrane, and increases electrical resistance (2). This reduces the ability of current to leak out of the axon and thus increases the distance along the axon that a given local current can flow. By acting as an electrical insulator, myelin greatly speeds up action potential conduction (1, 2, 11, 12). Oligodendrocytes can myelinate multiple axons by extending several processes, while a Schwann cell only myelinates a single axon (1-3, 13)(Figure 3). Along the length of the axon there are gaps up to a millimeter apart where the axon is not covered by myelin. These gaps are called '*Nodes of Ranvier*'. At these sites, ion channels are clustered. This allows the signal to jump from one node to the next increasing the signal transduction 100 times compared to non-myelinated axons. This process is called '*saltatory conduction*' (2)(Box 1). In contrast, the flow is continuous in non-myelinated nerves, consistent with the

diffuse distribution of ion channels in these fibers. Axonal insulation by myelin not only facilitates rapid nerve conduction but also regulates axonal transport and protects against axonal degeneration (14-16).

It is understandable that loss of myelin leads to various serious diseases, including multiple sclerosis and hereditary sensorimotor neuropathy. Communication between oligodendrocytes or Schwann cells and the axon is crucial for proper myelination. Consequently, better knowledge about the precise molecular mechanisms in these interactions is of the highest scientific and clinical importance and will lead to improved therapies for myelin-associated disorders.

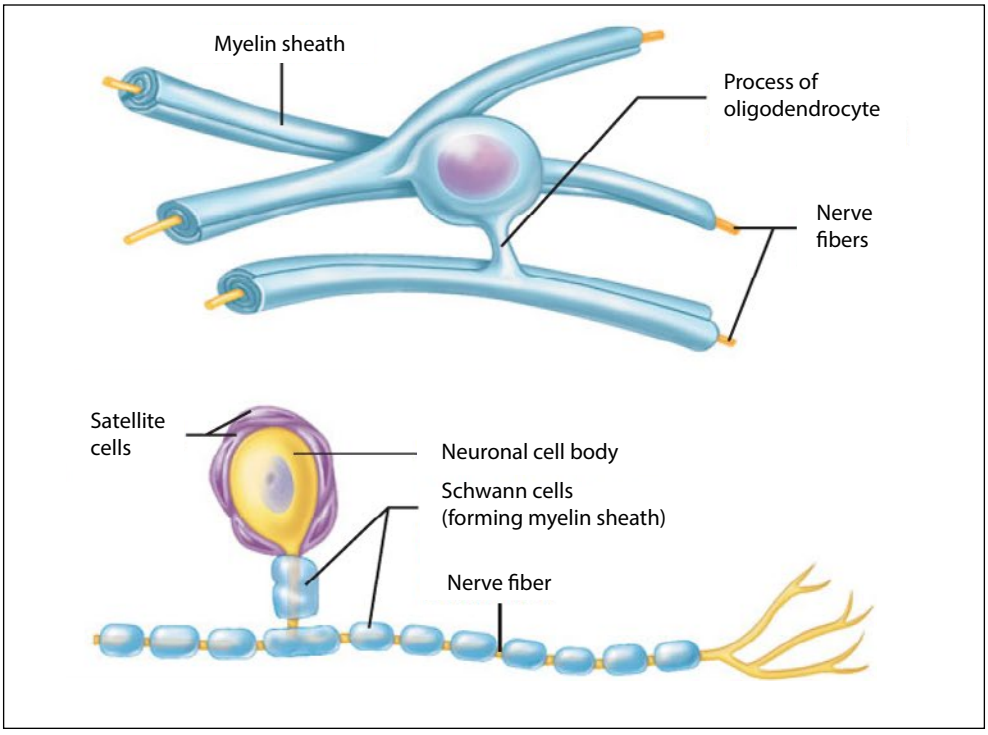


Figure 3. Myelinated axons

Oligodendrocytes in the CNS (upper) and Schwann cells in the PNS (lower) myelinate axons. Satellite cells surround neuronal cell bodies in the peripheral ganglia (5).

Box 1 Nerve impulse propagation

Neurons have a more negatively charged cytoplasmic side compared to the extracellular side. This *resting potential* results from a difference in ion-concentration at both sides of the membrane: the extracellular side has more positively charged Na^+ ions compared to the cytoplasmic side, which has more K^+ ions. The incoming signals to dendrites and cell bodies consist of small, graded changes in the resting potential caused by the actions of neurotransmitters or by other signals such as light and pressure. These are summated in the axon hillock close to the cellbody (2). When the strength of the summated synaptic potentials is sufficient to locally reduce the resting potential at the axon hillock below threshold voltage gated Na^+-channels (Na_V) open, resulting in an influx of Na^+ over the axonal membrane and the generation of an action potential at the axon hillock (Figure 4A, upper axon). The resulting inward current flows passively along the axon, depolarizing the adjacent region of the axon. Depolarization of the adjacent membrane opens Na_V-channels, resulting in the initiation of the action potential at this site and additional inward current that again spreads passively to an adjacent point farther along the axon (Figure 4B, upper axon). This cycle continues along the full length of the axon (2). As the action potential spreads, the active Na_V-channels slowly inactivate and outflow of K^+ repolarizes the membrane. The inactivated Na_V-channels cannot be reactivated at this point, resulting in a unidirectional flow of the action potential down the axon. In non-myelinated axons the action potential moves continuously down the axon like a wave. However, in myelinated axons the myelin prevents ions from entering or leaving the axon. Local current in response to action potential initiation at the axon hillock flows locally as described for non-myelinated axons. However, the presence of myelin prevents the local current from leaking across the membrane; it therefore flows farther along the axon than it would in the absence of myelin (Figure 4, lower axons). Moreover, Na_V-channels are only present at the nodes of Ranvier. As a result, the time consuming process of action potential generation occurs only at these non-myelinated regions, greatly enhancing velocity of action potential conduction. Thus, in myelinated axons the ionic current from an action potential at one node provokes another action potential at the subsequent node; this “jumping” of the action potential from node to node is termed ‘*saltatory conduction*’(2).

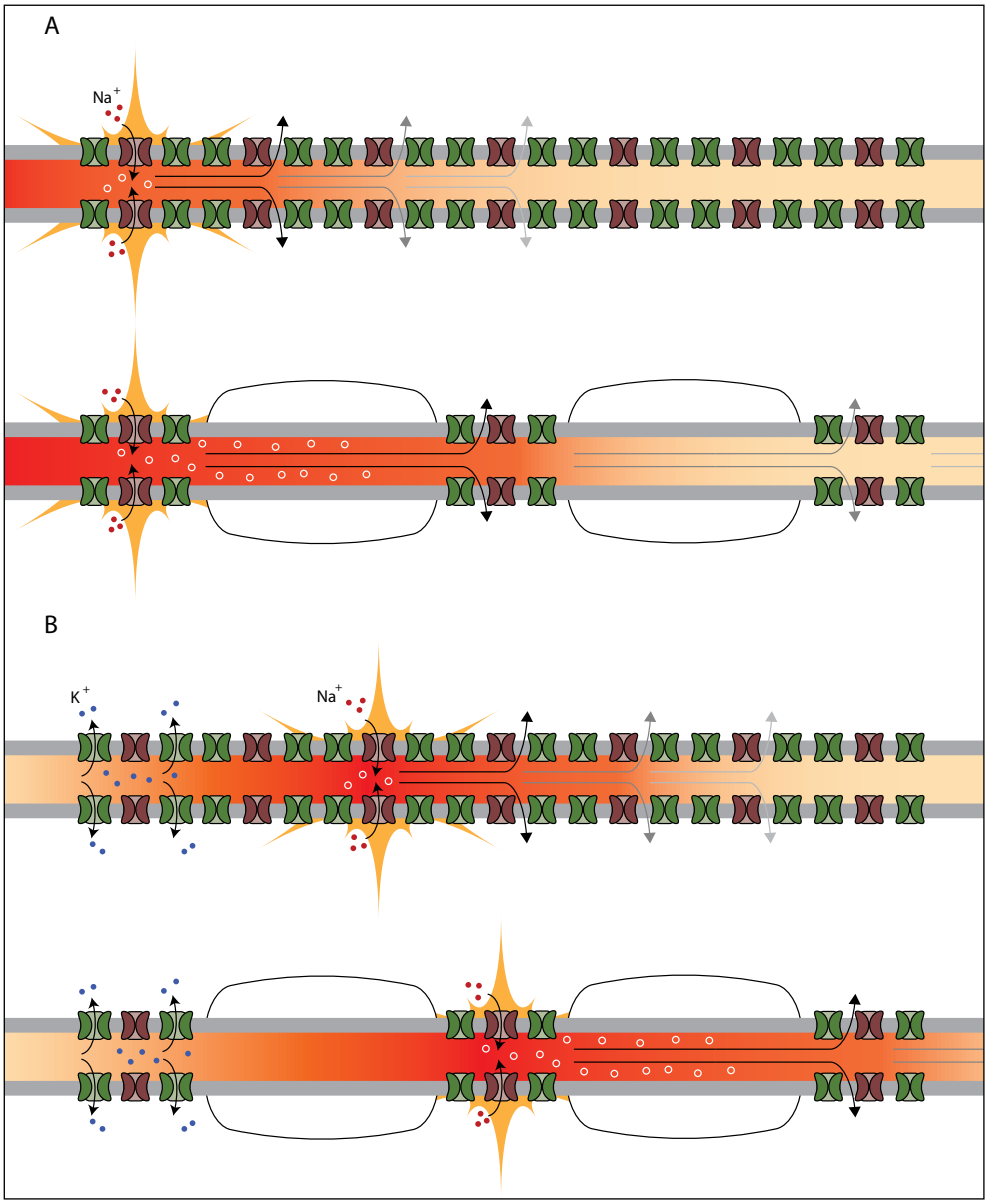


Figure 4. Action potential conduction

Comparison of the speed of action potential conduction in umyelinated (upper) and myelinated (lower) axons. The presence of myelin prevents the current from leaking (outward arrows) across the membrane; it therefore flows further along the axon than it would in the absence of myelin. Moreover Na_V-channels are present only at the nodes of Ranvier. Therefore the time consuming process of action potential generation occurs only at the non-myelinated regions, resulting in a greatly enhanced velocity of action potential conduction (B). Outflow of K^+ repolarizes the membrane (Figure based on (2)).

1.2 The structure of peripheral nerves

As described above, peripheral neurons are crucial for proper functioning of the body, and can be divided in sensory and motor neurons. Sensory neurons gather information, which is then transmitted to the CNS. Motor neurons transmit information from the CNS to tissues such as muscles and glands. Cell bodies of motor neurons can reside inside or outside the CNS, while cell bodies of sensory neurons are organized in ganglia (cranial and dorsal root ganglia) that lay outside the anatomical limits of the CNS (Figure 5). The axon of a DRG neuron has two branches, one projecting to the periphery and one projecting to the CNS (1, 2, 5). The peripheral terminal of the DRG neuron is sensitive to natural stimuli. The properties of the nerve terminal determine the sensory function of each DRG neuron, which can be *discriminative touch* (required to recognize the size, shape and texture of objects and their movements across the skin), *proprioception* (the sense of static position and movement of the limbs

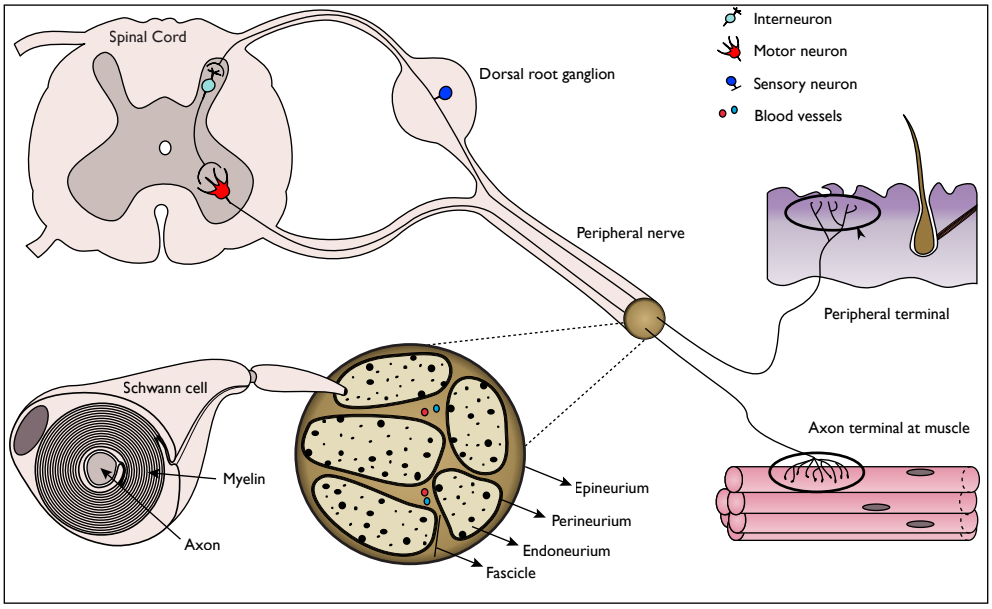


Figure 5. Peripheral nerve morphology

Cell bodies of sensory neurons reside in ganglia outside the CNS. Depicted here is a dorsal root ganglion (DRG). The axon of a DRG neuron has two branches, one projecting to the periphery and one projecting to the CNS. Covering the Schwann cells is a delicate layer of loose connective tissue called endoneurium. Groups of axons are bound into bundles called nerve fascicles by a wrapping of connective tissue called the perineurium. A tough fibrous sheath, the epineurium, surrounds the whole nerve.

and body), *nociception* (the signaling of tissue damage or chemical irritation, typically perceived as pain or itch), and *temperature sense* (warmth and cold) (2). Both sensory and motor peripheral axons are closely associated with Schwann cells.

Inside the nerves, groups of neurons are organized into bundles called fascicles (Figure 5) (5, 13). Each individual fascicle is surrounded by the multilayered *perineurium*. This layer is composed of flattened cells, basement membrane, and collagen fibers and provides a selective barrier against a large array of different molecules such as proteins, ions and pathogens. Inside the fascicles, neurons and blood vessels are held in place by loose connective tissue referred to as the *endoneurium*. The *epineurium* in turn is a layer of dense connective tissue that loosely surrounds the fascicles, grouping them together, and protects the nerves from environmental factors such as mechanical stress associated with movement of the body. The epineurium mainly consists of fibroblasts, fat and collagen. Arteries and veins are located between the fascicles, of which branches enter the fascicles and supply the neurons with nutrients and gasses (13).

1.3 Schwann cell development

Schwann cells are the support cells of peripheral neurons and are crucial for the proper development and functioning of the nervous system. Hence, accurate knowledge about Schwann cell development is highly important. Schwann cell development involves three main transitional stages (4, 11, 17-19); when *neural crest cells* (NCCs) have migrated out to contact developing axons, these cells are specified to form *Schwann cell precursors* (SCPs), typically found at mouse embryonic day (E) 12-13. SCPs then proliferate and give rise to *immature Schwann cells*, present from E15 to the time of birth. Around the time of birth, these cells adopt either a myelinating or non-myelinating Schwann cell fate, depending on the size of the axon these cells associate with. The fate of each of these transitional stages is controlled by different signals from the axon and extracellular matrix. Different stages are recognized by a distinct set of molecular markers (Figure 6). Besides that, a number of molecules is universally expressed at all developmental stages of the Schwann cells, including the Neuregulin1 (NRG1) receptor ErbB3, the transcription factor SOX10, L1 cell adhesion molecule and p75 neurotrophin receptor ($p75^{NTR}$) (11, 20-23).

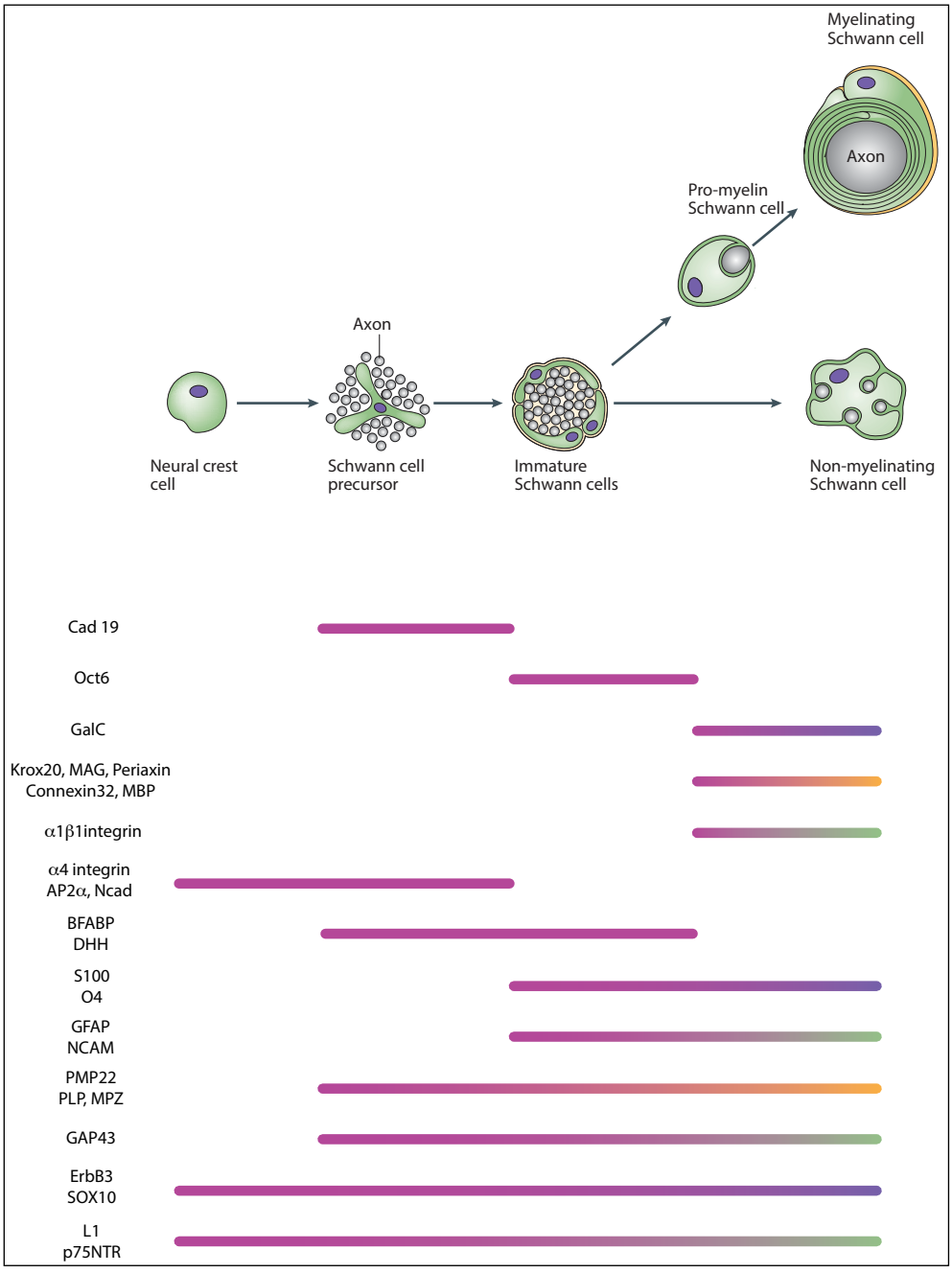


Figure 6. The developmental stages of the Schwann cell lineage

The expression profile is indicated by pink lines for neural crest cells, Schwann cell precursors and immature Schwann cells. Markers expressed by mature Schwann cells are yellow (myelinating Schwann cells), green (non-myelinating Schwann cells) or purple (both myelinating and non-myelinating Schwann cells) (Figure based on (4, 11, 23)).

During development of the peripheral nerves, interactions between Schwann cells and neurons are crucial. Schwann cells provide survival signals to neurons, define domains of the axolemma and determine the diameter of axons (24-26). In turn, axons provide signals that regulate the proliferation, survival and differentiation of the Schwann cells, as well as myelin formation (27). In adulthood, myelinating Schwann cells maintain axolemma organization, axonal diameter and neuronal health, while axons maintain glial differentiation and myelin integrity (25). In the next sections, this will be discussed for every stage in Schwann cell development.

1.3.1 The Schwann cell lineage originates in the neural crest

With the exception of a number of cranial ganglia derived from the ectoderm, all cells of the PNS arise from the neural crest. This cell population is derived from the dorsal-most surface of the neural tube. During *neurulation* the dorsally localized *neural plate* folds inward to form the *neural groove* (Figure 7)(11, 28). As the neural folds fuse to form the neural tube it is pinched off from the remaining ectoderm. The neural tube will form the brain and spinal cord while the remaining ectoderm on the dorsal surface of the embryo will form the epidemis.

During the fusion of the neural folds, neural crest cells (NCCs) delaminate and migrate extensively throughout the embryo (29). Their migration starts as a continuous wave, moving away from the neural tube, but quickly splits into discrete streams. To arrive at their target region, NCCs must interpret multiple environmental signals that directly influence their direction. As most NCCs migrate collectively, cell-cell interactions play a crucial role in polarizing the cells and interpreting these external cues. As a result of migration along these specific pathways that exposes the NCCs to additional inductive signals, NCCs give rise to a variety of progeny, including most of the neurons and all of the glial cells of the PNS, melanocytes, a major part of the cartilage and bone of the craniofacial structures, endocrine cells and smooth muscle cells (30-36).

When NCCs have migrated out to contact developing axons, these cells are specified to the Schwann cell lineage and differentiate into Schwann cell precursors (SCPs)(22, 37). It is unclear which specific transcription factors are needed for a NCC to adopt a glial cell fate. The transcription factor SOX10 regulates glial cell differentiation and is expressed throughout Schwann cell development, including NCCs (38)(Figure 6). However, SOX10 is also expressed

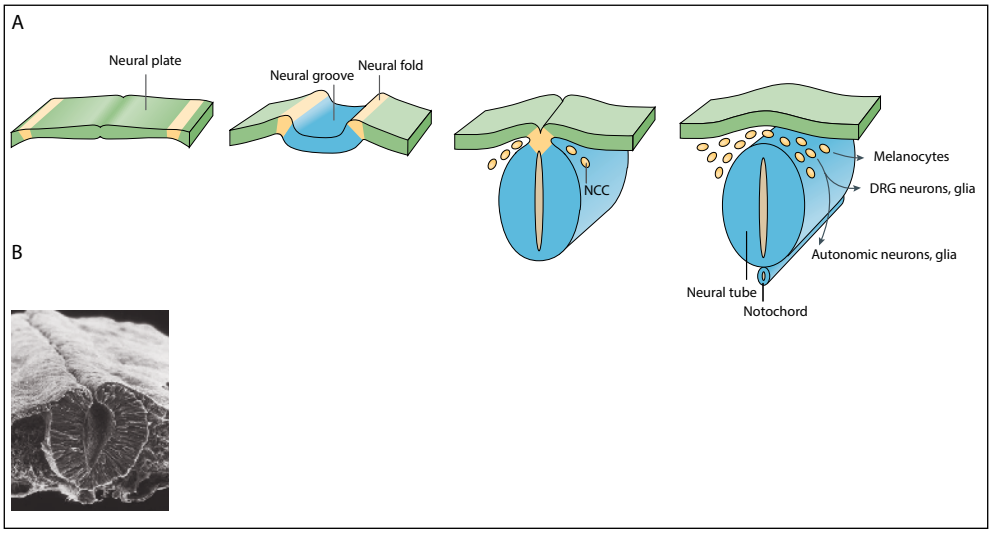


Figure 7. Neurulation and formation of neural crest cells
A) The neural plate folds inward to form the neural groove. The neural folds fuse forming the neural tube, which pinches off from the ectoderm. Neural crest cells (NCCs) delaminate and migrate away from the neural tube, giving rise to melanocytes, glia, DRG and autonomic neurons (Figure based on 11). B) Scanning electron micrograph showing a cross section through the trunk of a 2-day chick embryo. The neural tube is about to close and pinch off from the ectoderm (28).

by other NCCs that do not differentiate into SCPs, suggesting that SOX10 alone is not sufficient to activate glial cell acquisition (39, 40). In mice in which SOX10 has been inactivated, SCPs, Schwann cells and satellite cells are absent and the number of melanocytes is reduced. NCCs need to maintain contact with the extracellular matrix (ECM) for NRG1-dependent survival. Thus, impaired NRG1 signaling probably causes some of the effects on the glial lineage in these *Sox10* mutant mice, since SOX10 regulates expression of the NRG1 receptor ErbB3 on the Schwann cell (39, 41). The fundamental role of SOX10 in NCC development is underscored by the observation that dominant *Sox10* mutations are associated with complex neurocristopathies, including Waardenburg syndrome and Hirschsprung disease, and peripheral neurophaties (42). This is partly attributed to a requirement for SOX10 in early neural crest for survival, maintenance of pluripotency and specification to several cell lineages, including peripheral glia. It should be noted that SOX10 is required at all stages of Schwann cell development including myelin maintenance (43).

1.3.2 Schwann cell precursors

Transition from NCCs to Schwann cell precursors (SCPs) can be identified by the appearance of stage specific markers such as Brain Fatty Acid-Binding Protein (BFABP), Desert Hedgehog (DHH), Proteolipid Protein (PLP), Growth-Associated Protein-43 (GAP-43), Cadherin19 (Cad19) and low levels of the myelin associated Myelin Protein Zero (MPZ) and Peripheral Myelin Protein 22 kDa (PMP22) (11, 23). Except for Cadherin19, which is only expressed in SCPs (44), these markers persist in later stages of Schwann cell development (11). Besides these markers, the SCPs express Integrin-α4, the transcription factor activator protein 2α (AP2α) and Cadherin-2/N-Cadherin (NCad), which are also expressed by NCCs (Figure 6).

Unlike NCCs, survival of SCPs is ECM independent. However, SCPs do depend on interactions with the axon. The axonal signal regulating SCP survival is NRG1. Studies on NRG1 and its receptors ErbB2 and ErbB3 have revealed crucial roles for these molecules throughout Schwann cell development. One of the most striking observations is that SCPs are basically absent in the nerve trunks of mice lacking NRG1, ErbB2 or ErbB3. However, the presence of either NRG1 isoform I and II, or isoform III is sufficient to establish a significant number of SCPs in early embryonic nerves (45, 46) (Box 2).

Box 2 Neuregulins

The neuregulin (NRG) family has emerged as important regulators of most aspects of Schwann cell development (53-55). Of the four distinct neuregulin genes (*Nrg1-4*), *Nrg1* is best characterized and ErbB2/ErbB3 heterodimers are the major NRG1 receptors expressed by Schwann cells (55, 56). The *Nrg1* gene is transcribed from different promoters, which, together with alternative splicing of the mRNAs give rise to different isoforms (54). These isoforms are classified into different subtypes based on their distinct N-terminal domains. The three major isoforms of NRG1 that are involved in Schwann cell development are NRG1 Type I (NDF-Neu differentiation factor/ heregulin/GGF), Type II (GGF2-Glial Growth Factor 2) and Type III (SMDF-

sensory and motor neuron derived factor). NRGs share an extracellular growth factor (EGF)-like signaling domain (Figure 8), which is the domain that activates the ErbB tyrosine kinase receptors (53, 57, 58). Besides that, some isoforms contain glycosylation sites, an Ig like domain, or a cysteine-rich domain. Both NRG1 Type I and III are cleaved at the C-terminal site of the EGF-like domain. For NRG1 Type I this results in release from the cell and binding to the ErbB receptors on the Schwann cell. NRG1 Type III passes the membrane a second time through the hydrophobic cysteine-rich domain on the N-terminus. After cleavage this results in a membrane bound form, which can further be cleaved from the N-terminus, giving rise to a secreted molecule that can act in a paracrine fashion (53, 54, 59). NRG1 Type II is a secreted isoform. Other less well studied isoforms include NRG1 Type IV-VI, which have shorter amino termini (55).

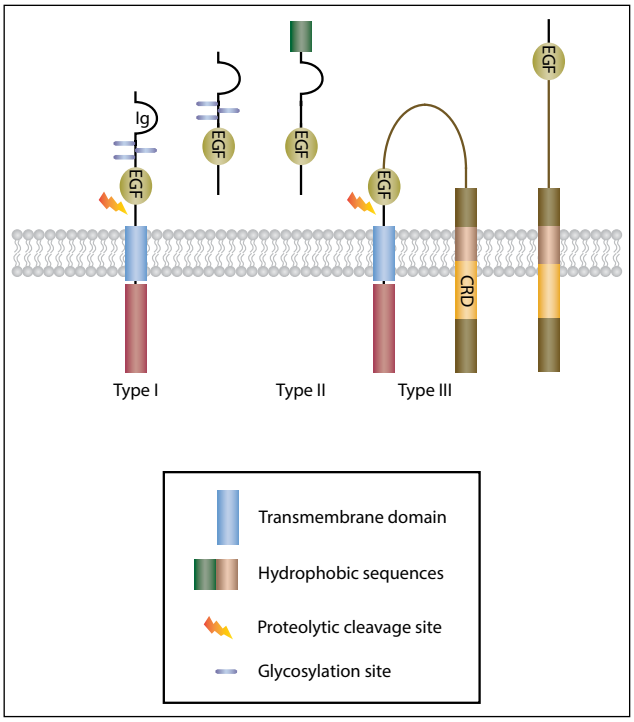


Figure 8. Major NRG1 isoforms involved in myelination
Common to all isoforms is an EGF-like domain (EGF). Other sequences present in NRG1 are an Ig domain (Ig), domains rich in potential glycosylation sites (purple bands), and a cysteine rich domain (CRD)(Figure based on (54)).

Box 2 continued

Metalloproteases A Disintegrin And Metalloprotease 10, ADAM17 (TACE) and ADAM19 (meltrin-β) function as α-secretases for NRG1 processing (60-63). However, altered ADAM10 expression does not affect myelination (62, 64, 65) and ADAM19 deficient mice also myelinate normally, but exhibit a delay in remyelination due to delayed activation of KROX20 and myelin-related proteins (66). NRG1 cleavage by ADAM17 probably inactivates it, thereby negatively regulating PNS myelination, since motor neurons of neuron-specific ADAM17 knock out mice are significantly hypermyelinated and even small-caliber fiber myelination is observed (61). Another NRG1 regulator is BACE1, a Type I transmembrane aspartyl protease, which is enriched in neurons. Mice lacking BACE1 display hypomyelination in the PNS (62, 67, 68).

ErbBs are composed of two cysteine-rich portions in the extracellular domain and a kinase domain located intracellularly. ErbB3 binds NRG1 with high affinity, but lacks catalytic activity. This interaction induces dimerization with ErbB2, which then by its tyrosine kinase activity initiates signaling cascades involving RAS, PI3-kinase and FAK. This way, NRG1-ErbB2/3 signaling modulates Schwann cell proliferation, differentiation, motility and survival (23, 69, 70).

NRG1 is also important for SCP differentiation into immature Schwann cells. When precursors are cultured and exposed to NRG1, these cells differentiate into Schwann cells with a similar time course as observed for embryonic nerves *in vivo* (47). Endothelins in turn block maturation effects of neuregulins and regulate the timing of SCP differentiation (48). This suggests that SCP differentiation is controlled by positive and negative signals.

SCPs provide trophic support for survival of sensory and motor neurons in developing nerves and are important for nerve fasciculation. The absence of SCPs in peripheral nerves of *ErbB2*, *ErbB3* and *Sox10* mutants thus leads to nerve fasciculation defects and aberrant axonal projections to target tissues and eventually neuronal degeneration (39, 49-51). Altogether, it is clear that NRG1 signaling is crucial for SCP survival (50, 52).

1.3.3 Immature Schwann cells

The transition from SCPs to immature Schwann cells involves again a shift in expression markers. Integrin-α4, the transcription factor AP2α and the adhesion molecules N-cadherin and Cadherin-19 are strongly downregulated in immature Schwann cells (23, 44, 71), while immature Schwann cells start expressing Glial Fibrillary Acidic Protein (GFAP), Neural cell-adhesion molecule (NCAM), S100, the transcription factor OCT6 and glycolipid antigen O4 (23) (Figure 6). Unlike SCPs, immature Schwann cells have a basal lamina. Another important difference between SCPs and immature Schwann cells is that the latter can survive without axonal signals. Molecules like Insulin-like Growth Factor 2 (IGF2), Neurotrophin 3 (NT3) and Platelet Derived Growth Factor-BB (PDGF-BB) mediate this autocrine survival (72).

Immature Schwann cells send cytoplasmic processes into groups of axons, to form axon-Schwann cell bundles. At this stage also a distinct layer of developing perineurium appears at the nerve surface, and the nerve becomes vascularized. Only large caliber axons (diameter larger than 1 μm) become ensheathed in a one-to-one relationship with the Schwann cells that will differentiate into mature myelinating Schwann cells in a process called *radial sorting* (11). This process involves Schwann cell proliferation, polarization of the cell and process extension (73). Extensive Schwann cell proliferation guarantees a precise match of Schwann cell number with axon number for individual axon ensheathment and requires NRG1, transforming growth factor-β (TGF-β) and Notch signaling (56, 74, 75). Importantly, ablation of genes involved in Schwann cell proliferation such as *Cdc42* (76) and *Fak* (77) results in radial sorting impairment. Furthermore, polarization of the cell and process extension are needed for interdigitation and ensheathment of axons (73).

Radial sorting can be divided into the following steps (Figure 9): 1) Formation of Schwann cell families with a common basal lamina, 2) Insertion of Schwann cell processes into axon bundles, 3) Contact and recognition of large axons, 4) Segregation of large axons at the periphery, 5) Matching of axon and Schwann cell number by proliferation (not shown in figure 9), 6) Defasciculation of the axon in a one-to-one relationship with the Schwann cell, with formation of an independent basal lamina (11, 78). During this process immature Schwann cells undergo cytoskeletal rearrangements that require interaction between the Schwann cells and the ECM (79-81). ECM components typically include collagens, heparin sulfate proteoglycan and the non-collagenous glycoproteins laminin, fibronectin and entactin (24). Laminins are heterotrimeric proteins

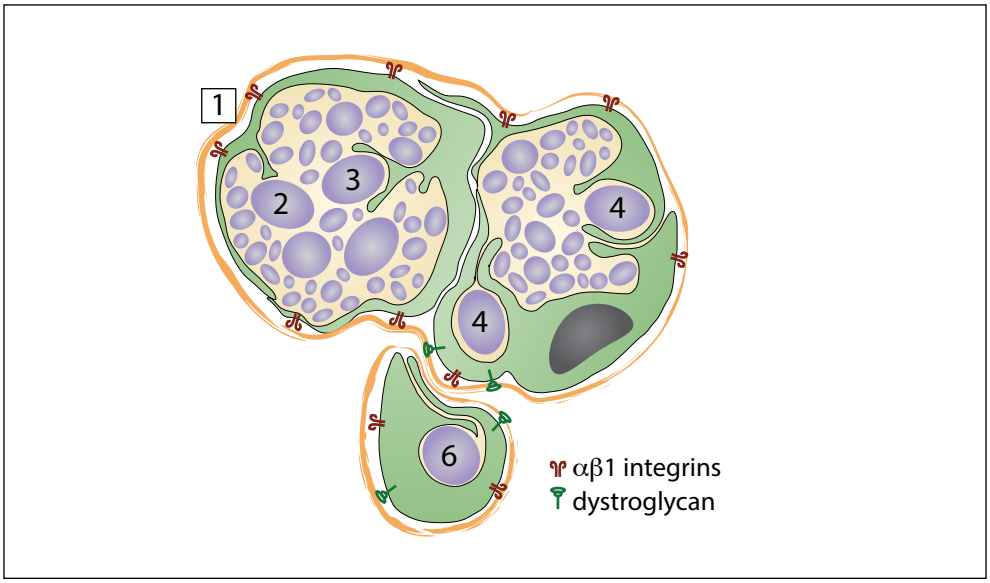


Figure 9. The multiple steps in radial sorting
Schematic representation of the radial sorting process. The orange line represents the basal lamina. Schwann cells are shown in green, with a grey nucleus, and axons are indicated in purple. Numbering indicates the different stages as described in the text. Step 5 (matching of axon and Schwann cell number by proliferation) is not shown in this figure (78).

formed by α-, β-, and γ-subunits and bind integrins, which are composed of two noncovalently associated transmembrane glycoprotein subunits, α and β (82). Laminin binding to β1-integrin recruits cytoskeleton and signaling molecules to cell matrix contact sites, linking the actin cytoskeleton of the Schwann cell to the ECM. Above all, integrins activate the Rho GTPase RAC1, which leads to generation of radial lamellae, thereby promoting the formation of Schwann cell processes (76, 79-81, 83-85). However, the precise signaling pathways controlling this process are largely unknown.

Integrin-linked kinase (ILK) interacts with the β1 and β3-integrin cytoplasmic domains (86, 87) and is linked to Rho GTPases (88) including CDC42 and RAC1 (76, 85). Interestingly, ILK was shown to negatively regulate Rho/Rock activity to promote Schwann cell process extension (89). Since ILK also binds directly to β3-integrin and indirectly to other receptors this might explain why ILK regulation of RAC1 does not mirror β1-integrin signaling (89). The laminin receptor dystroglycan was also shown to be involved in radial sorting (78). Dystroglycan is a key component of the dystrophin-glycoprotein

complex (DGC) that links the ECM with the cytoskeleton in various tissues (90). It is expressed in the abaxonal Schwann cell membrane, which is surrounded by the basal lamina, and consists of an extracellular α-subunit and a transmembrane β-subunit (91). Dystroglycan functions as a receptor for laminin-2 and this interaction is important for axon sorting into a one-to-one relationship (78, 92-95)(Figure 9). Dystroglycan and β1-integrins activate different pathways (78). In addition, ErbB signaling was found to be important in process extension in radial sorting in zebrafish (96). In conclusion, a combination of several extracellular signals and intracellular mechanisms regulate this radial sorting and preparation for myelination.

1.3.4 Mature Schwann cells

Schwann cells differentiate into either myelinating or non-myelinating Schwann cells, depending on the caliber of the axon the Schwann cells associate with. Upon association with large caliber axons (>1μm), Schwann cells elongate and exit the cell cycle to initiate myelination, referred to as the promyelin stage. Schwann cells will then wrap around the axons forming the myelin sheath. Non-myelinated Schwann cells (NMSCs) remain associated with multiple small diameter axons (< 1 μm) forming Remak-bundles (11, 97). These NMSCs surround and insulate axons in individual pockets of thin cytoplasmic processes. NMSCs express molecular markers very similar to those expressed in immature Schwann cells (11, 23, 27, 97). Additionally, NMSCs express α1β1 integrin (Figure 6). Both myelinating and NMSCs express O4, GalC and S100 and are enveloped by a basal lamina, which is in close associating with the abaxonal Schwann cell membrane.

Expression of structural components of the myelin sheath is regulated by combined activities of transcription factors (98). The POU homeo-domain transcription factors OCT6 and, to a lesser extent, BRN2 are important regulators for the transition from promyelin to myelinating stage (99-101) and Schwann cells express both transcription factors in a similar developmental profile. OCT6 expression peaks at the promyelinating stage and gradually decreases as myelination progresses (102-104). Upregulation of OCT6 is tightly controlled by binding of SOX10 to a gene regulatory element termed Schwann cell-specific enhancer (SCE) (105, 106), possibly accompanied by other factors (107, 108). NFκB is also required for OCT6 activation and myelination (109) (Figure 10).

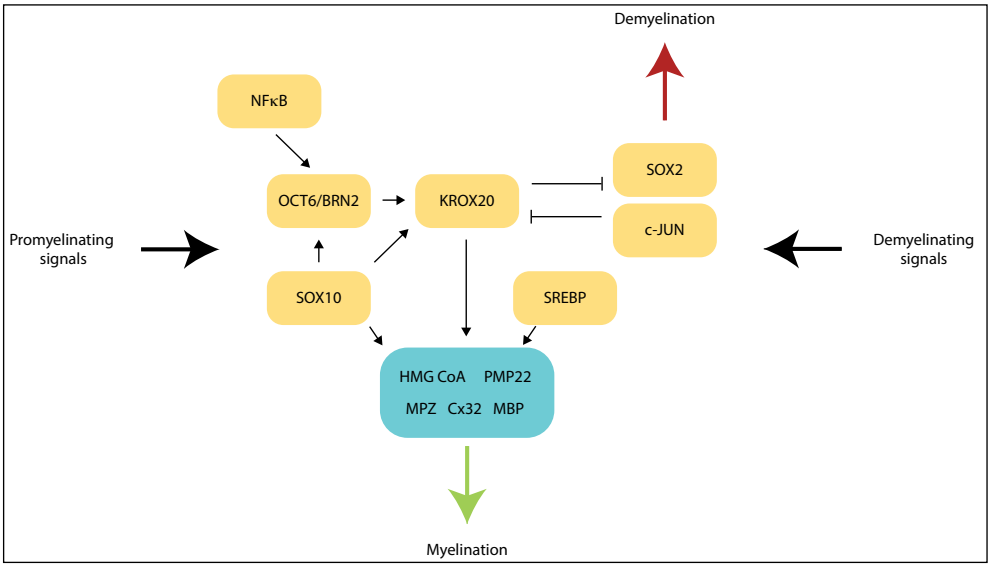


Figure 10. Outline of the gene regulatory network of myelin-associated Schwann cell differentiation

The major transcription factors involved in the transition from promyelinating to myelinating stage are depicted. Arrows indicate activation while blunt lines indicate repression. This regulatory pathway results in upregulation of several genes, including *Myelin protein zero* (*MPZ*), *Peripheral myelin protein 22* (*PMP22*), *Myelin basic protein* (*MBP*), *Connexin 32* (*Cx32*) and *HMG CoA reductase* (*HMG CoA*) (Figure based on (98)).

Not surprisingly, *Oct6* knock out mice show a transient arrest of Schwann cells at the promyelinating stage and a delay in nerve myelination (99, 101). Overexpression of BRN2 in OCT6-deficient Schwann cells results in partial rescue of this delay. BRN2 activation does not depend on OCT6 and the absence of both BRN2 and OCT6 results in a more severe phenotype (100). OCT6 and BRN2, together with SOX10, accelerate myelination by upregulating KROX20 and inducing myelin genes (38, 98, 110) such as *Myelin protein zero* (*Mpz*), *Peripheral myelin protein 22* (*Pmp22*), *Myelin basic protein* (*Mbp*), *Myelin-associated glycoprotein* (*Mag*), *Connexin32* (*Cx32*) and *Periaxin* (111-113) (Figure 6 and 10). In addition, KROX20, in synergy with SREBP (Sterol regulatory element binding protein), transactivates *HMG CoA reductase*, which is involved in lipid synthesis during myelination (114-116). These lipids are required for the creation of the multiple layers of cholesterol-rich myelin membrane.

Sustained expression of KROX20 in adult nerves is required for myelin

sheath maintenance (117). Above all, mutations in *Krox20/Erg2* in humans cause peripheral neuropathies including Charcot Marie Tooth disease (CMT) and Dejerine-Sottas Syndrome (reviewed in 118). Although OCT6 activates *Krox20* expression, *Oct6* knock out mice only show a transient arrest of the Schwann cells at the promyelin stage and Schwann cells do progress to the myelinating stage, indicating the presence of a compensatory mechanism. Besides that, in claw paw (*clp/clp*) mice, which are further discussed in later chapters, Schwann cells express normal levels of OCT6 but reduced levels of KROX20 (119). These findings indicate the presence of pathway(s) that do not include OCT6 but do play a role in *Krox20* regulation. Additionally, SOX10 is thought to, next to *Krox20* expression, regulate the expression of myelin proteins directly. SOX10 binding sites are found on several myelin genes such as *Mbp*, *Mpz*, *Connexin32* and *Mag*. Chromatin immunoprecipitation sequencing has confirmed that SOX10 binds to the regulatory sequences of these genes (120). Binding of SOX10 and KROX20 to their respective sites in these regulatory elements seems to control the expression of such proteins (98, 111, 121).

The transcription programs and expression of the major myelin genes in Schwann cells in turn depend on extracellular signals (122, 123). Some molecular players have been identified as driving or interfering with myelination. It is known that the amount of axonally expressed NRG1 type III determines the thickness of the Schwann cell myelin sheath (53, 55, 69, 70). The optimal myelin sheath thickness is determined by the ratio of axon diameter to fiber diameter (g-ratio), which is approximately 0.68 in vertebrate peripheral nerves. Accordingly, large diameter axons have thicker myelin sheaths than axons with a smaller diameter. Besides regulating myelin sheath thickness, NRG1 signaling is necessary for the initiation of myelination and is activated during Wallerian degeneration. However, NRG1 was found to be dispensable for myelin maintenance (124, 125). As mentioned earlier, NRG1 acts by binding ErbB2/3 receptors on the Schwann cell. The NRG1-ErbB2/3 receptor interaction activates multiple pathways, including PI3/AKT, RAS/ERK1/2, FAK and Calcineurin/NFAT signaling (126, 127).

Another important receptor residing on the Schwann cell is the G protein-coupled receptor GPR126. A ligand for this receptor has not been identified. It was proposed that GPR126 elevates cyclic AMP (cAMP) levels, thereby triggering *Oct6* expression to drive myelination (128, 129) (Figure 11). In addition, heterophilic interactions between Nectin-like protein1 (NECL1) on the axolemma and NECL4 on Schwann cells have a key role in myelination (130, 131)(Figure 11). Schwann cells must polarize with axonal and basal lamina

surfaces to form myelin, and NECLs are suggested to have a role in this process (132). PAR (partitioning defective) polarity complexes are implicated in PNS myelination as well. PAR-3, a component of this complex, accumulates at the adaxonal Schwann cell membrane contacting the axon (Figure 11), and plays a crucial role in initiation of myelination (133). Myelination is impaired when PAR-3 localization is disrupted. Whether NECL4 interacts with components of the PAR polarity complex, as suggested by NECL4 localization in the Schwann cell and its PDZ-binding sequences, is so far unknown. Transgenic mice lacking *Necl1* expression display no defects in PNS myelination (134). Functional compensation by other NECL proteins or unrelated adhesion molecules might account for this lack of phenotype. Myelination is also regulated by Notch signaling cascades (75). After binding of Notch to its ligands that belong to the Jagged protein family, cleavage of Notch releases the active Notch intracellular domain (NICD) from the plasma membrane, which translocates to the nucleus and complexes with other proteins to activate gene transcription of target genes (127, 135)(Figure 11). Notch signaling regulates multiple stages of Schwann cell development including SCP differentiation to immature Schwann cells and proliferation, but also inhibits myelination by opposing KROX20 (75).

Besides axon-Schwann cell signaling, signaling from the Schwann cell to the axon is also crucial for myelination. Leucine rich gene glioma inactivated 4 (LGI4) is secreted by Schwann cells (136) and is suggested to bind to axonal ADAM22, a member of the A Disintegrin And Metalloprotease family that lacks metalloprotease activity (137)(Figure 11). LGI4 is not secreted in *clp/clp* mice, causing congenital hypomyelination and defects in radial sorting in the PNS (136, 137). Additionally *Adam22* KO mice show severe PNS hypomyelination (138), underscoring the importance of this pathway in myelination. However, the exact mechanism through which LGI4-ADAM22 interaction drives myelination is unknown.

AKT1 is an integrator of various signals that increase myelination and is phosphorylated by phosphatidylinositol 3-kinase (PI3K) in response to in particular NRG1 type III and insulin growth factor 1 (IGF-1) (70). Another separate pathway involved in activating myelination in Schwann cells is the CN (calcineurin)–NFAT (nuclear factor of activated T cells) pathway (139). This signaling pathway is downstream of NRG1 and independent of PI3K (139). A model is suggested in which activation of ErbB2/ErbB3 heterodimers increases intracellular Ca^{2+} levels via phospholipase Cγ (PLCγ), activating CN, which promotes nuclear translocation of NFATc4 where it complexes with SOX10 to upregulate KROX20 (139) (Figure 11).

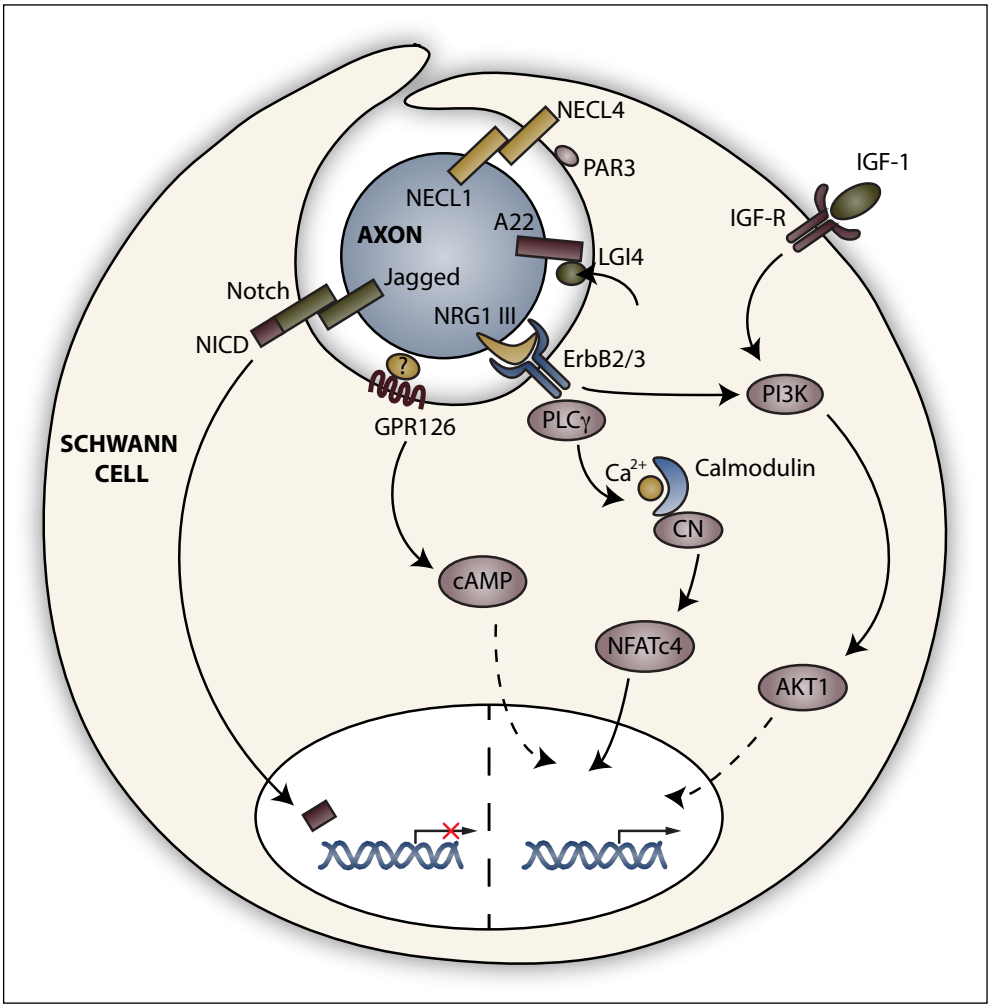


Figure 11. Axon-Schwann cell signaling in myelination: A simplified schematic overview showing the main signaling pathways.

The figure depicts a promyelinating Schwann cell. The pathways focus on signaling cascades that converge to the nucleus. Dashed lines indicate signals of uncertain targets. NRG1 III: Neuregulin 1 type III; IGF-1: Insulin-like Growth Factor 1; IGF-R: IGF Receptor; NECL: Nectin-like protein; PAR3: Partition defective 3; GPR: G protein coupled receptor; NICD: Notch-1 intracellular domain: PI3K: phosphatidylinositol-3 kinase; PLC γ : phospholipase γ ; NFATc4: Nuclear factor of activated T-cells, cytoplasmic 4; A22: A Disintegrin And Metalloprotease 22; LGI4: Leucine-rich gene Glioma Inactivated 4. (Figure based on (127)).

In conclusion, myelin production by Schwann cells results from the combined effects of several extracellular signals that converge on the Schwann cell nucleus to activate transcription factors that initiate a myelination-transcription program.

1.3.5 Schwann cell dedifferentiation and remyelination

Myelinating Schwann cells show striking plasticity and are able to switch off the myelin program, re-enter the cell cycle and adopt again a phenotype that is superficially similar to that of immature Schwann cells (reviewed in (11, 140)). Typically, Schwann cell dedifferentiation is caused by nerve injury and loss of contact with the axon, promoting axon regrowth and nerve repair.

When Schwann cells dedifferentiate, mechanisms are activated to break down the myelin sheaths and to cause transformation into cells with long, parallel processes. This way the Schwann cells form uninterrupted regeneration tracks (Bands of Bungner) that guide axons back to their targets (141). Collectively, these events are called Wallerian degeneration, which changes the normally growth-hostile environment of intact nerves to a growth supportive environment. When repair is finished, Schwann cells envelop the regenerated axons again either to transform to myelinating or non-myelinating Schwann cells, driven by both axonal and Schwann cell signals.

An important regulator in this Schwann cell plasticity is the basic leucine zipper transcription factor c-JUN, a key component of the AP-1 transcription factor complex. Most importantly, c-JUN activates a repair program in Schwann cells, which creates a cell specialized in supporting regeneration. Interestingly, c-JUN acts in conjunction with SOX2 (Figure 10), which inhibits myelination, is suppressed during myelination and re-expressed during injury (142, 143). Both c-JUN and SOX2 are in a cross-antagonistic circuit with KROX20, governing active demyelination following nerve injury (143, 144).

1.4 Domains and structure of the myelin sheath in the PNS

Myelin forming Schwann cells are highly polarized and develop a complex geometric organization with distinct subcellular domains on the radial and longitudinal axes (reviewed in (145)). Radial polarity in a myelinating Schwann cell includes the adaxonal membrane (Figure 12), which harbors adhesion molecules that mediate interactions between the axon and the Schwann cell, and the abaxonal surface, which is surrounded by a basal lamina and harbors ECM receptors (146, 147). The longitudinal polarization results in distinct domains each containing a specific subset of junctional proteins. These domains include the node of Ranvier, contacted by cytoplasmic extensions formed by the Schwann cell called microvilli, and immediately adjacent the paranodal loops, juxtaparanodes and internodes (26, 145, 148).

1.4.1 The radial polarity of the myelin sheath

During myelination the inner cytoplasmic lip of the Schwann cell turns around the axon while the outer lip is anchored to the basal lamina. In this process Schwann cells also extend longitudinally (149). As a result, when myelination is completed, a myelin sheath is comprised of multiple wraps of Schwann cell membrane that surround the axon. This sheath is essentially comprised of two domains—compact and non-compact myelin—each of which contains a non-overlapping set of proteins. The nucleus is located on the outside of the sheath. A small collar of cytoplasm persists adjacent to the axon (the adaxonal compartment) and on the outside of the sheath (abaxonal compartment) (150). The periaxonal space separates the adaxonal Schwann cell membrane from the axon (148, 150)(Figure 12).

The non-compact myelin provides cytoplasmic continuity along the layers of the myelin sheath. While the majority of the myelin sheath is made up of compact myelin, non-compact myelin is found along the paranodes and the outer- and innermost layers of myelin sheath (150). An important non-compact myelin protein is L-Periaxin (95, 151). L-Periaxin homodimerizes at its PDZ domain and associates with Dystroglycan Related Protein 2 (DRP2), which interacts with β-dystroglycan, forming a transmembrane complex, also termed the Periaxin-DRP2-Dystroglycan (PDG) complex (95). Periaxin (PRX) is crucial for clustering and stabilization of this complex and mutations in *PRX*

cause CMT4F (reviewed in (152)). The PDG complex is concentrated at zones of adhesion between the abaxonal surface of compact peripheral myelin and the Schwann cell plasma membrane (95, 151, 153, 154), named Cajal bands after the initial description by Ramon y Cajal. These cytoplasmic channels create a network of cytoplasm that radiates outward from the nucleus (153)(Figure 12). Besides that, gap junctions containing Connexin32 form radial channels. Mutations in Connexin32 cause an X-linked form of CMT characterized by demyelination and axonal degeneration (155). Junctional proteins that form adherens and tight junctions are found in non-compact myelin as well. Schmidt-Lanterman incisures (SLIs) are cytoplasmic channels formed by tight junctions that spiral through the myelin sheath and provide a conduit between the inner and outer cytoplasmic compartments (145, 148, 150, 156). All these different channels are thought to facilitate radial transport.

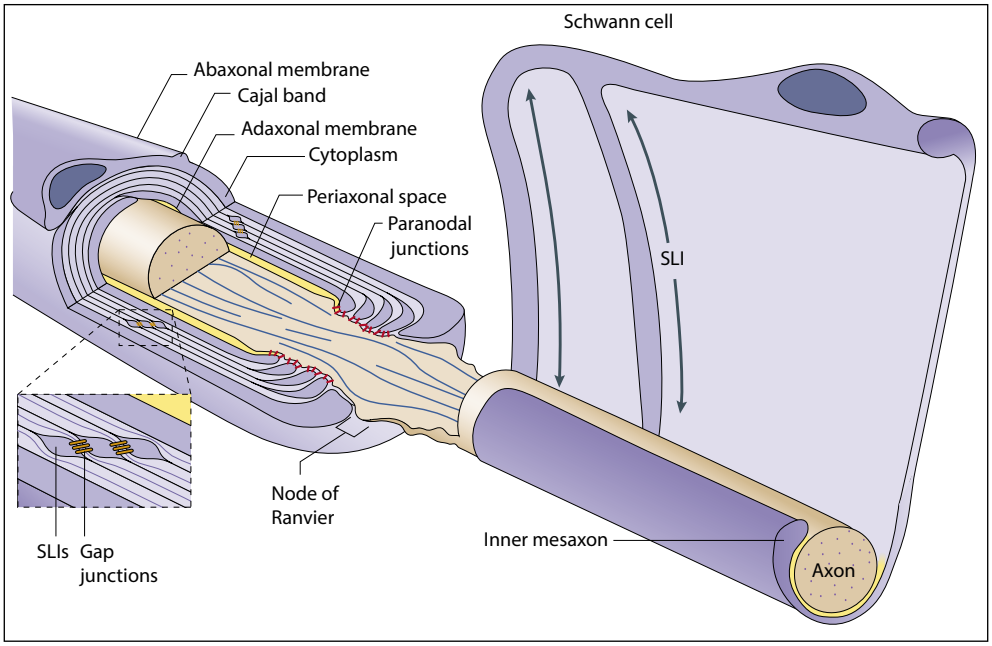


Figure 12. Morphology of domains of myelinated axons in the PNS

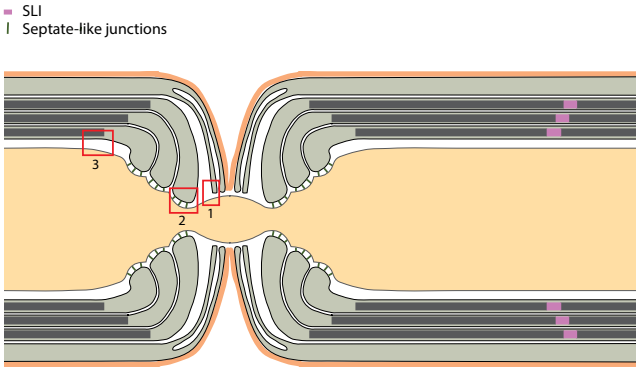
Schematic representation of a myelinated peripheral axon with the unrolled sheath of a Schwann cell on the right. Compacted myelin (pale purple) serves as an insulator that inevitably deprives the axoplasm of access to the extracellular milieu, except at the Nodes of Ranvier. The channel system of the non-compacted myelin is continuous with the Schwann cell cytoplasm and brings cellular metabolites close to the periaxonal space (yellow). The Schmidt-Lanterman incisures (SLIs) – local stacks of non-compacted myelin that spiral around the axon – are linked by gap junctions when stacked (Figure based on (148)).

Compact myelin forms the bulk of the myelin sheath, and is composed of closely packed membranes. Myelin membranes are highly enriched in lipids such as cholesterol and galactocerebroside, and contain high amounts of myelin specific proteins. These proteins are involved in interactions crucial for holding in place the close appositions of membrane surfaces. Myelin Protein Zero (MPZ) is the main protein of compact PNS myelin (157), which is an immuno-globulin (Ig)-like cell adhesion molecule with an extracellular domain forming tetramers that interact with each other both in *cis* and in *trans*, making MPZ the molecular glue of PNS myelin (158). *Mpz* mutations lead to disease resulting from both myelin abnormalities and axonal loss (152). Peripheral Myelin Protein 22 kDa (PMP22) is another transmembrane protein in compact myelin, which is important for myelin stability and its overexpression causes CMT1A (159). Myelin Basic Protein (MBP) on the other hand is found at the cytoplasmic surface of compact myelin and does not seem to be important in PNS compaction, as MPZ can mimic its function. However, MBP could function to regulate protein interactions and lipid organization, in order to control myelin sheath thickness (160).

1.4.2 The longitudinal polarization of the myelin sheath

The entire length of myelinated axons is organized into a series of polarized domains that center around the Nodes of Ranvier (Figure 13). These sites contain highly organized multimolecular complexes of ion channels and cell adhesion molecules, closely connected with the cytoskeleton, which appear essential for the stability of the nodal regions (reviewed in 161, 162). At the nodes of Ranvier, voltage-dependent Na^+-channels are clustered in the axonal membrane, flanked by paranodal junctions that provide a strong diffusion barrier (163, 164). In the adjacent juxtaparanodal region, fast K^+ channels are concentrated. The internodal Schwann cell membranes are fused to form compact myelin. Together, these domains are critical for the propagation of action potentials (reviewed in (145)). Thus, deciphering the molecular organization of the various cell contacts in these regions and elucidating how this organization is realized during development is of great importance.

A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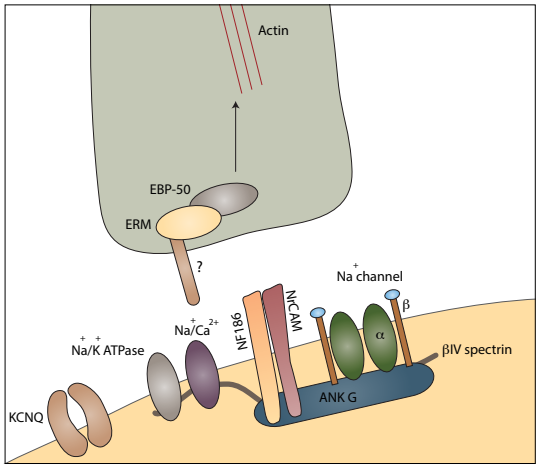
Actin
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βIV spectrin
ANK G
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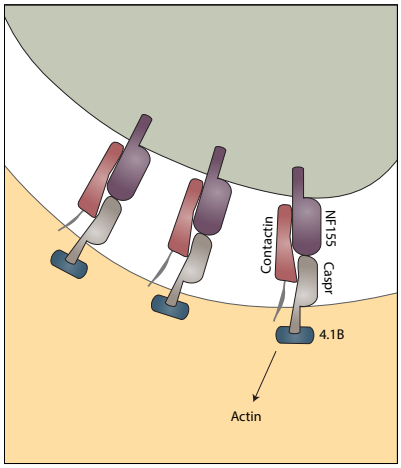
Contactin
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Actin

3

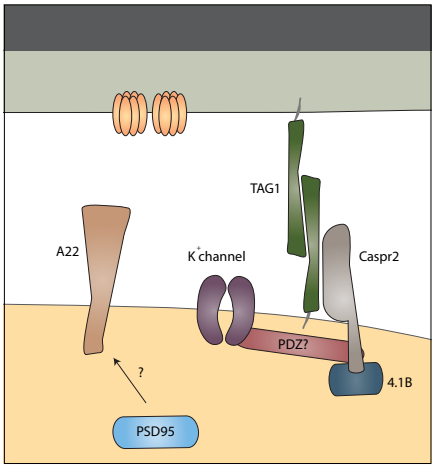
TAG1
A22
K+ channel
Caspr2
PDZ?
4.1B
?
PSD95

Figure 13. Schematic representation of longitudinal specialization in the PNS
A) Schematic depiction of the node (red box 1), paranode (red box 2) and juxtaparanode (red box 3). Compact myelin is indicated in dark grey. Non-compact myelin is indicated with light colour. The basal lamina is shown in orange. B) Enlargements of the red boxes in A. Schematic depiction of possible *cis* and *trans* interactions between the molecular components of the longitudinal specializations. B1) Schematic organization of nodal regions in peripheral nerves. Microvilli contain ERMs and EBP50. These ERMs may provide a connection to actin filaments. Transmembrane proteins thought to interact with axonal proteins are unknown (?). In the axon, NF186 and NrCAM are anchored to Ankyrin G (ANK G), as are the Na^+ channels. βIV spectrin is also associated with Ankyrin G. Additional proteins enriched in the nodal axolemma include the Na^+/K^+ ATPase, voltage-gated potassium channels (KCNQ) and the Na^+/Ca^+ exchanger (Na^+/Ca^+). B2) Schematic organization of paranodal regions. The main components of paranodal axolemma are Caspr associated with Contactin, a GPI-anchored protein. This complex interacts with NF155 on the paranodal loops. The intracellular region of Caspr interacts with protein 4.1B, which provides a potential link with actin filaments. B3) Schematic organization of juxtaparanodal regions. The juxtaparanodal axolemma contains K^+ channels and Caspr2, which are associated through an as yet unidentified PDZ domain containing protein (PDZ?). PSD95 is enriched at the juxtaparanode and requires ADAM22 for its localization by unknown mechanisms (?). The Caspr2 intracellular region can associate with protein 4.1B, which provides a link to the actin cytoskeleton. TAG1, a GPI-anchored glycoprotein, is enriched in Schwann cell membranes and possibly on neuronal membranes and could be involved in cis or trans assoiations with Caspr2, or in trans hemophilic interactions with itself. Cx29 is located in the Schwann cell membrane where it may form functional hemichannels (150, 161).

1.4.2.1 The Nodes of Ranvier

Nodes of Ranvier are the sites of myelinated axons where insulating layers of myelin are interrupted and where the ion fluxes occur which are required for propagation of action potentials. This is realized by the presence of Na^+/K^+ ATPases, Na^+/Ca^{2+}exchangers, voltage-gated potassium channels (KCNQ) and a high density of voltage-gated Na^+ channels (Na_V) (161, 162, 165). These Na_V-channels are heterotrimeric complexes of one large, pore-forming α-subunit and two transmembrane β-subunits, which anchor the channel to extracellular, as well as intracellular components (166, 167). Na_V-channels at the node of Ranvier are part of a multiprotein complex. Within the axoplasm, Na_V-channels directly bind Ankyrin G (168, 169), which belongs to a family of intracellular adaptor proteins involved in targeting membrane proteins to specialized domains (170). Ankyrin G in turn binds to βIV Spectrin (171, 172)

and also interacts with NrCAM and the 186 kDa isoform of Neurofascin (NF186) (173, 174). The latter two proteins also associate with each other (175). Thus, nodal proteins appear to form a meshwork of interacting components, in which transmembrane proteins are associated directly and through intracellular adaptor proteins (Figure12B).

The nodal axon segment is in close contact with hundreds of specialized microvilli of the Schwann cells. Schwann cell microvilli contain Ezrin, Radixin, Moesin (ERM) proteins and an associated PDZ domain-containing protein, ERM-binding phosphoprotein 50 kDa (EBP50) (176, 177). ERM proteins provide a membrane anchoring mechanism for actin microfilaments.

1.4.2.2 The molecular organization of the paranodes

At the paranodes, the glial loops of the Schwann cell are tightly attached to the axolemma through a septate-like junction. This region is the site of closest apposition between the membranes of the axon and myelinating glia (2.5–3 nm) and provides a barrier between the extracellular space at the nodes and the periaxonal space (reviewed in (150, 178, 179)).

The proteins Caspr (Contactin-associated protein)(180-182) and Contactin (180) interact with each other in the paranodal axolemma (183). Knockout mice lacking Caspr or Contactin display ataxia, motor deficits and a dramatically reduced nerve conduction velocity. In these mutants, the ultrastructure of the paranodes is severely altered; the glial paranodal loops are disorganized, the gap between glial and axonal membranes is increased and the electron-dense material forming the septa is absent (184, 185). Although the nodal clustering of Na^+ channels is minimally affected in the Caspr and Contactin mutant mice, K^+ channels from the juxtaparanodes are mislocalized at the paranodes. This underscores the role of the paranodal junction in providing a barrier that restricts the movement of juxtaparanodal proteins (164, 184-187). The paranodal loops also contain the 155 kDa splice isoform of Neurofascin (NF155), which binds to the Caspr–Contactin complex (188)(Figure 13B).

Paranodal junctions are tightly associated with the cytoskeleton in both glial loops and the axoplasm. On the axonal side, the short intracellular domain of Caspr binds protein 4.1B (181, 189), which is concentrated at paranodes and juxtaparanodes (190, 191). Because protein 4.1B has a conserved actin–spectrin-binding domain, it may directly connect the transmembrane protein complexes with the axonal cortical cytoskeleton (Figure 13B).

1.4.2.3 The molecular organization of the juxtaparanodal region

Just under the compact myelin sheath immediately adjacent to the paranodes lies the juxtaparanodal domain. This region is enriched in the shaker-type K^+ channels, Kv1.1, Kv1.2 and their Kvβ2 subunit, which are thought to play a role in stabilizing the action potential discharge (192). Caspr2 is associated with K^+ channels, presumably through a PDZ domain-containing protein (193). Post Synaptic Density-95 (PSD-95), another PDZ domain-containing protein, is enriched at juxtaparanodes but its binding-partners are not known. Similarly to Caspr, Caspr2 directly interacts with protein 4.1B (194). Transient Axonal Glycoprotein 1 (TAG1), a GPI-anchored cell adhesion molecule, is highly enriched in the juxtaparanodal region in the Schwann cell membrane and possibly the axonal membrane, and could be involved in *cis* or *trans* hemophilic interactions with itself and Caspr2. The entire juxtaparanodal complex is dispersed along axons in mice deficient of TAG1 or Caspr2, underscoring the importance of this complex in juxtaparanodal formation. However, the significance of the localization of the juxtaparanodal domain itself is not yet known, as both knock out mice are phenotypically normal with normal conduction velocities (195, 196). Finally, the juxtaparanodal adaxonal Schwann cell membrane contains Connexin 29 (Cx29), a gap junction protein that may be capable of forming functional hemichannels and is possibly involved in K^+ clearance (Figure 13).

ADAM22 is localized at the juxtaparanode as well. This catalytically inactive ADAM consists of an extracellular domain containing a metalloprotease-like domain, a disintegrin domain (which binds LGI4 and integrins), a cysteine rich domain and an EGF like domain (137, 138, 197). Besides that, ADAM22 has a cytoplasmic domain containing an ETSI (-Glu-Thr-Ser-Ile-) domain, which is a PDZ binding motif through which it potentially interacts with PDZ domain containing proteins (198). Indeed ADAM22 is capable of binding PSD-93 and PSD-95 (198, 199) and ADAM22 is required to recruit both proteins to the juxtaparanode (199). However, ADAM22 clustering at the juxtaparanodes itself requires neither PSD-93 or PSD-95, suggesting that ADAM22 also interacts with other proteins.

1.5 Myelin and disease

Multiple diseases target myelin in the PNS, including Charcot-Marie-Tooth disease (CMT), also termed Hereditary Motor and Sensory Neuropathy (HMSN), and Guillain-Barré syndrome.

CMT includes a clinically and genetically diverse group of disorders affecting the PNS and is the most frequently inherited peripheral neuropathy. This disorder is classified into two major groups, namely a demyelinating form (CMT1, CMT3 and CMT4) when myelinating Schwann cells are primarily affected, and an axonal form (CMT2) when axons are the principal disease target (152, 155, 200-202). The major disease characteristics include slowly progressive muscular atrophy and weakness, with sensory loss in the distal extremities of the limbs and absence of deep tendon reflexes. Deformed feet and claw toes are common features of the disease, leading to gait impairment. Loss of myelin from single internodes explains the reduced nerve conduction velocity (NCv) that precedes the clinical onset of the demyelinating CMT. However, the secondary axonal pathology and degeneration typically begin later, leading to functional denervation, progressive muscle weakness and sensory deficits, which are more clinically important than reductions in NCv (203). As mentioned earlier, mutations in genes of myelin components including Pmp22, Mpz, Connexin32 and Periaxin are responsible for various subtypes of CMT and some subtypes of CMT are caused by mutations in genes encoding transcription factors such as KROX20 and SOX10 (reviewed in (98, 118, 152, 204-207)).

In general, the degree of disability observed in myelin disorders correlates best with the level of associated axonal damage (152, 208). In most disorders, axonal damage is recognized after myelin damage, suggesting that signals from Schwann cells to axons are altered. The cause of axonal loss in demyelinating diseases has remained elusive; energetically inefficient conduction, loss of trophic support, disturbed axonal transport, and even superimposed inflammatory changes could be involved (209). A remarkable body of work in patients, as well as animal and cellular models, has defined the clinical and molecular genetics of these illnesses and shed light on how mutations in associated genes produce the demyelinating phenotypes (118, 152, 209).

In summary, continuous communication between axons and Schwann cells is highly important during development and maintenance of the myelin sheath. Various types of molecules, acting at diverse regulatory levels, mediate myelination. Some of these molecules normally promote myelination, and might be perturbed in myelin diseases, whereas others normally inhibit myelination and can be inappropriately active in such disorders. The following chapters of this thesis discuss one of the key players in myelination, LGI4, which is crucial in Schwann cell-neuron communication. We identify the interaction between LGI4 and ADAM22, and the cellular compartments of their action, and we show that three amino acids in LGI4 explain its specific function in myelination.

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Chapter 2

LGI proteins in the nervous system

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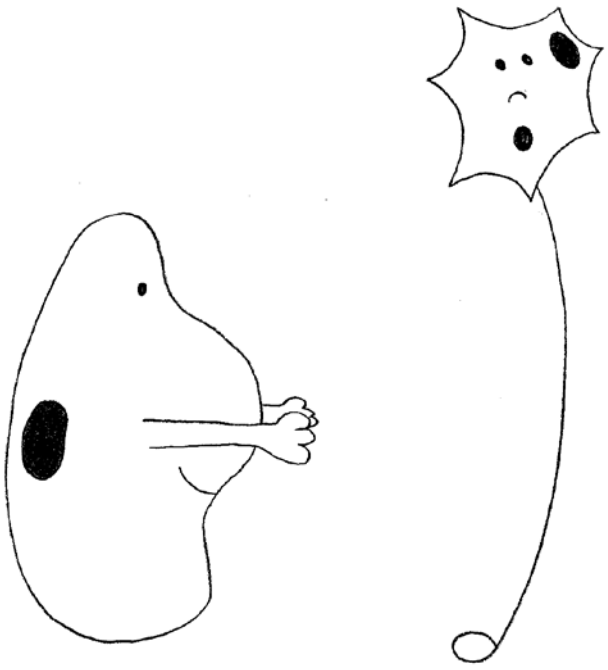
Accepted pending revisions ASN Neuro



LGI proteins in the nervous system

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Acknowledgements
Research in the lab of D.M. is supported by grants from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant No. FES0908), VICI grant 918.66.616 and the European Union (NGIDD FP7 HEALTH-F2-2008-201535)

Abstract

The development and function of the vertebrate nervous system depends on specific interactions between different cell types. Two examples of such interactions are synaptic transmission and myelination. Leucine-rich Glioma Inactivated proteins (LGI1-4) play important roles in these processes. They are secreted proteins consisting of a Leucine-rich repeat (LRR) domain and a so-called epilepsy associated or epitempin (EPTP) domain. Both domains are thought to function in protein-protein interactions. The first *LGI* gene to be identified, *LGI1*, was found at a chromosomal translocation breakpoint in a glioma cell line. It was subsequently found mutated in Autosomal Dominant Lateral Temporal lobe Epilepsy (ADLTE) also referred to as Autosomal Dominant Partial Epilepsy with Auditory Features (ADPEAF). LGI1 protein appears to act at synapses and antibodies against LGI1 may cause the autoimmune disorder limbic encephalitis. A similar function in synaptic remodelling has been suggested for LGI2, which is mutated in canine Benign Familial Juvenile Epilepsy (BFJE). LGI4 is required for proliferation of glia in the peripheral nervous system and binds to a neuronal receptor, ADAM22, to foster ensheathment and myelination of axons by Schwann cells. Thus, LGI proteins play crucial roles in nervous system development and function and their study is highly important, both to understand their biological functions and for their therapeutic potential. Here we review our current knowledge about this important family of proteins, and the progress made toward understanding their functions.

Cell-cell interactions in nervous system development

Synapse formation and maturation require multiple interactions between presynaptic and postsynaptic neurons that are mediated by a diverse set of synaptic proteins (1-3). Initial synapse formation needs both the binding of secreted proteins to presynaptic and postsynaptic receptors, and the direct binding between presynaptic and postsynaptic transmembrane proteins. Many synaptogenic proteins have been described, some of which, like the secreted LGI proteins discussed here, are specific to vertebrates.

Myelination also requires cell-cell interactions; myelin is required for rapid axonal transmission of electrical signals in the vertebrate nervous system. It consists of multiple wraps of membrane that permit saltatory conduction between specialized structures, the nodes of Ranvier. Myelination requires reciprocal interactions between the axon and oligodendrocytes in the central nervous system (CNS), or Schwann cells in the peripheral nervous system (PNS) (Reviewed in (4-6)). As will be described below, LGI4 participates in axon-Schwann cell communication; it is crucial for PNS (but not CNS) myelination. In addition to synaptogenesis and myelination, LGI proteins are likely to participate in other cell-cell interactions as well. The study of this interesting family of proteins is critical for understanding proper development and function of the vertebrate nervous system, and for gaining insights into therapies for diseases that affect them.

Identification of the LGI family

As suggested by its name, Leucine-rich Glioma Inactivated, the LGI protein family was discovered in gliomas. Loss of one copy of chromosome 10 is a common event in high-grade gliomas. In a search for genes that are mutated in these gliomas, Cowell and colleagues identified a gene in the 10q24 region which was rearranged as a result of a t(10;19)(q24;q13) balanced translocation in a T98G glioblastoma multiforme cell line (7). It was suggested that the complete loss of this gene, which contains 4 Leucine-rich repeats, contributes to the malignant progression of glial tumors. *LGI1* was proposed to function as a tumour suppressor gene, but the involvement of *LGI1* deletion in tumour formation or progression is still a controversial issue (8-13).

In a completely independent line of inquiry, *LGI1* was found implicated in epilepsy. Epilepsy is a heterogeneous disease, and families with multiple epileptic individuals are important resources for identifying causal genes. In one such family multiple individuals presented with partial seizures with auditory features, thereby permitting a causal gene to be mapped to a 10-centimorgan region of chromosome 10 (14). This type of epilepsy was named Autosomal Dominant Partial Epilepsy with Auditory Features (ADPEAF; (15), and additional families with similar symptoms confirmed linkage to the same region of chromosome 10 (16). Independently, a large five-generation Basque family with similar dominant partial epilepsy also demonstrated linkage to chromosome 10q (17). This inherited epilepsy was named Autosomal Dominant Lateral Temporal lobe Epilepsy (ADLTE). Making the assumption that the same gene was mutated in these two families reduced the chromosomal region containing the epilepsy gene to a 4,2 Mb sequence. All genes within this region were sequenced in three affected individuals of 3 different families revealing distinct mutations in the *LGI1* gene. Extending the analysis to two additional families revealed two more mutations (18). Independently, Morante-Redolat and colleagues identified mutations in the *LGI1* gene in two families (19). Since these initial publications many more mutations in the *LGI1* gene have been found in ADLTE/ADPEAF patients and the total number is now in excess of 33 (see (20, 21)). The terms ADLTE and ADPEAF refer to the same clinical entity (OMIM 600512). In this review we will follow the nomenclature of Winawer and colleagues (15) and refer to the disease as ADPEAF.

As we will discuss here, LGI1 is found associated with synapses in the nervous system and most recent research has focused on the role of LGI1 in nervous system function and disease. The other *LGI* family members, *LGI2*, *-3* and *-4*, were cloned following *in silico* identification of LGI1 homologous genes in vertebrate genomes (22).

Lgi2 is associated with canine epilepsy. In the Italian water dog *Lagotto Romagnolo*, the disease is transient, generally disappearing by 10 weeks of age, and could serve as a model for human remitting epilepsies. Analysis of a Finnish pedigree of these dogs indicated a primarily recessive inheritance (23). Using an expanded pedigree of these dogs, Seppala and colleagues identified 11 pairs of discordant (one affected, one not) siblings. Their DNA was subjected to genome wide association analysis, revealing a region of homozygosity on canine chromosome 3. Subsequent sequencing revealed a truncating mutation in *Lgi2* that segregated with the juvenile epilepsy (24), indicating that *Lgi2*, like *Lgi1*, is an epilepsy gene.

LGI3 has not been linked yet to a disease state in humans or in animals. However, *in vitro* experiments using cell lines have associated LGI3 with a wide range of biological activities ranging from endocytosis to promotion of survival of keratinocytes following UV irradiation. It is at present unclear what common LGI3 mediated mechanism might underlie these diverse biological functions.

Study of the *claw paw* mutation in mice has revealed a novel LGI4-mediated signalling pathway that controls peripheral myelination. In 1977, Nelda Blaisdell, an animal technician handling C57BL/6-*obese* mice at The Jackson Laboratory in Bar Harbor, Maine, noticed a litter in which two pups had limb abnormalities. Rather than holding their forelimbs up towards the head as most mouse pups do, they were held out, away from the body, or lowered towards the thorax. Mating of the littermates of these affected pups demonstrated that the phenotype was heritable. The new spontaneous mutation, called *claw paw* (*clp*), caused PNS hypomyelination without affecting central myelin (25). Because of the similarity in its myelination phenotype with that of mice lacking the POU domain transcription factor POU3f1/OCT6 (26, 27), the *clp* mutation was investigated further. However, the *clp* mutation mapped to a separate locus on chromosome 7, and initial *Oct6* expression was not affected (26, 28). The *clp* mutation was positionally cloned using a series of backcrosses, and found to result from an insertion in the *Lgi4* gene (26), demonstrating a critical role for LGI4 in peripheral myelination.

Evolution of LGI Genes

Lgi genes made their appearance in vertebrates. They have not been found in any invertebrates studied to date. Early vertebrate genomes underwent two rounds of whole genome duplication (29, 30) and presumably a single primordial *Lgi* gene gave rise to four *Lgi* genes. A third whole genome duplication occurred in the teleost fish lineage after the tetrapod-teleost split (31-33). The zebrafish, *Danio rerio*, a teleost, possesses two copies of *lgi1* (*lgi1a* and *lgi1b*), two copies of *lgi2* (*lgi2a* and *lgi2b*), a single copy of *lgi3* and no copy of *lgi4* (34). A similar picture - with duplicated *lgi1* and *lgi2* genes, a single *lgi3* gene and no *lgi4* gene - emerges from the genomes of other teleost fishes such as cod, stickleback and platyfish (see Ensembl.org). Following whole genome duplication events, gene families often contract, as genes with redundant functions are lost (32). In the case of fishes, *lgi4* may have been lost as one or more other *lgi* genes assumed its

functions in peripheral nerve development. Alternatively, *Lgi4* diverged from an ancestral *Lgi* gene specifically in the tetrapod lineage. Coelacanths (*Latimeria sp*) are an order of fish that diverged from teleost fishes prior to their third genome duplication and are thus more closely related to the ancestors of the tetrapods. The coelacanth genome (see Ensembl.org) contains single copies of *lgi1*, *lgi2*, and *lgi3* genes, but not *lgi4*. Surprisingly, the recently sequenced *Xenopus tropicalis* genome (35) has been found to contain *lgi1*, *lgi2*, and *lgi4*, but not *lgi3*. Although it remains to be seen if the absence of individual *Lgi* genes in coelacanth and frog genomes is real the current genome sequence data support the appearance of *lgi4* in tetrapods. As more genomes are sequenced, the pattern of *Lgi* gene evolution will become clearer.

Structure of LGI proteins

All LGI family members have a calculated molecular mass of ~60 kDa (7) and contain a signal peptide that is cleaved off, a leucine-rich repeat (LRR) domain containing 4 LRR repeats flanked by cysteine-rich sequences (36) and an epitempin domain (EPTP) consisting of seven epitempin (EPTP) repeats (Figure 1) (37, 38). The EPTP repeats most likely fold into a so-called 7 bladed beta-propeller, a structure that resembles a slightly conical doughnut. Both the LRR domain and the beta-propeller structure provide a scaffold for specific protein interactions and are found in a wide range of proteins with diverse physiological function. The LRR and EPTP domains are discussed below.

The LGI proteins' LRR domain is most homologous to Slit proteins (39). Slit proteins are large secreted proteins involved in axonal guidance and neuronal migration through interaction with their receptor Roundabout (Robo). The Slit proteins contain four LRR domains, D1-D4, and each domain is approximately 31-33% homologous to the LGI LRR domain. A model of the LGI1 LRR domain (21), based on the structure of Slit LRRs, suggests that it consists of four 24 amino acid long LRRs. The intron-exon structure of LGI genes further corroborates this suggestion as the repeats are encoded by four 72nt long exons (exon 2 to 5). The LRRs with the amino- and carboxyl-terminal flanking sequences form a slightly curved structure with parallel beta strands forming the concave face of the domain (Figure 1B). Because Slit proteins dimerize (40), one hypothesized function of the LRR domain is dimerization. Indeed, LGI1

is secreted as an oligomer (41) raising the possibility that the LGI proteins homo- or heterodimerize with other LGI proteins, or possibly with other LRR-containing proteins.

At its carboxyl-terminal end, the EPTP domain directly abuts the LRR domain. The name epitempin derives from the observation that this domain is found in two epilepsy associated proteins namely LGI1 and Gpr98/VLGR/MASS, a transmembrane protein mutated in human and mouse auditory epilepsy (42-44). This name is a bit misleading as LGI3 and -4, and the deafness-associated protein TSPEAR (45), also contain EPTP repeats but have not yet been associated with epilepsy. As already mentioned above, the EPTP repeats of LGI proteins likely form a seven bladed beta-propeller domain (37, 38). Beta-propeller domains consist of 4-10 modules (the blades), and are found in a wide variety of proteins in both prokaryotes and eukaryotes (46-49). Each beta-propeller module consists of four antiparallel beta strands that form a sheet warped like a propeller blade (Figure 1B). In the case of the EPTP domain, seven of these modules associate to form a disc with a conical central pore, and stabilized by hydrophobic interactions between the blades (Figure 1B). Sites for interactions with other proteins reside on the outer surfaces, and for some beta-propellers, the pore contains a substrate-binding site, but whether or not anything associates with the central pore of LGI proteins is unknown.

Recently, *in silico* model(s) of the LGI1 EPTP domain have been published, based on the structure of the beta-propeller protein WDR5 (21, 50). A notable feature of the WDR5 structure is that the seven clusters of four beta strands, seen in the primary sequence, are out of register with the seven blades in the structure (reviewed in (47, 51)). Accordingly, the register of beta strands in the *in silico* model of LGI1 structure proposed by Leonardi and colleagues (21) is shifted by one beta strand relative to the boundaries of each EPTP repeat that were described initially (37, 38). A characteristic of beta-propeller domains with six or more repeats is the presence of a "Velcro" or "molecular clasp", sequence that holds the circular structure closed (reviewed in (52)). These Velcro sequences consist of one or more beta strands contributed by one terminal repeat module that are integrated into the beta sheet formed by the repeat at the other end, permitting inter-beta-strand interactions to pull the entire beta-propeller closed, thereby stabilizing its circular structure. Leonardi and colleagues propose that for LGI1, the N-terminal EPTP beta strand resides at the outside of the C-terminal beta-propeller, with the C-terminal beta strand nested inside it (Figure 1B). The Leonardi model provides a plausible explanation for the deleterious phenotype of the 1639insA mutation in one family of ADPEAF

patients (18), and the LGI2 truncation seen in Benign Familial Juvenile Epilepsy (BFJE) dogs (24). These mutations replace or remove the 11 C-terminal amino acids of LGI1 or LGI2, respectively. Their absence may preclude formation of the first EPTP domain, and preclude folding of the entire beta-propeller structure.

All LGI proteins possess consensus N-linked glycosylation sites (Figure 1A). Asn192, located within the carboxyl-terminal portion of the LRR domain (LRR-CT), resides in a glycosylation site that is conserved among all LGI members (Figure 1). Asn277 is part of a glycosylation site in some LGI1 and LGI2 orthologs, and a glycosylation site that includes Asn422 is found only in mammalian LGI1 proteins. These sites have been demonstrated to be glycosylated in LGI1 (53). A triple glycosylation mutant of LGI1 is not secreted and secretion of the N192Q mutant is severely diminished, underscoring the importance of these glycosylation sites for normal maturation and secretion of LGI1. LGI4 is glycosylated at the LRR-CT site (26), but the functional significance of this is unknown. Based on the observations with LGI1, it is reasonable to postulate that LGI proteins require glycosylation for one or more steps in their secretion.

How the LRR and EPTP domains of LGI proteins are oriented relative to one another is not known. Currently, this important question cannot be answered by modelling alone. The crystal structure of an intact LGI protein is needed to provide insight into the specific functions of the four LGI proteins, and to provide better understanding of the effects of the different human *LGI1* mutations.

Both LRR and EPTP domains are known to be capable of protein-protein interactions (54, 55), but only a handful of LGI binding partners have been identified (Table1). Some of these proteins interact with all LGI proteins whereas others bind more selectively (41, 56-58) and (E.A. unpublished). These proteins are discussed below in the context of the individual LGI proteins to which they bind.

The role of LGI1 in central nervous system development and function

All LGI's seem to interact with select members of the A Disintegrin And Metalloprotease (59) transmembrane protein family. More than 40 ADAMs have been identified in species from C. elegans to human. Some members are

catalytically active metalloproteases and control cell signalling by activating membrane-bound growth factors or by shedding the ectodomain of cell-surface receptors (60, 61). Other members are inactive and are thought to be involved in protein interactions, especially with integrins (62). LGI1 binds to the extracellular domain of ADAM22, which binds to the third PDZ domain of PSD-95 through its cytoplasmic C-terminal ETSI-motif (41). The first two PDZ domains of PSD-95 in turn bind to the C-terminal tail of stargazin, a transmembrane regulatory subunit of AMPA-receptors that is critical for AMPA-receptor trafficking and gating (63-66). Fukata's group found that, in addition to the stargazin/AMPA receptor complex, PSD-95 strongly associated with LGI1 and ADAM22 in rat brain (41). Thus, PSD-95 may tether two protein complexes, stargazin/AMPAR and ADAM22/LGI1 (Figure 3). Since PSD-95 together with stargazin controls synaptic AMPA receptor number, this association could explain the increase of synaptic AMPA/NMDA ratio in hippocampal slices after incubation with LGI1; it suggests that LGI1 is an extracellular factor controlling synaptic strength at the excitatory synapses (41). Additionally, an interaction between ADAM23 and LGI1 in the brain has been identified (58, 67). Both *Adam*22 KO and *Adam23* KO mice show strong overlap in phenotype with *Lgi1* KO mice, which is characterized by severe spontaneous epilepsy and premature death (58, 68-71). Additionally, a point mutation in *Adam23* has been found in epileptic Belgian Shepherd dogs (72). Importantly, ADAM22 and ADAM23 co-assemble in the brain dependent on LGI1 (58). Thus, it was hypothesized that LGI1 forms a bridge between presynaptic ADAM23 and postsynaptic ADAM22 (58) (Figure 3) effectively regulating trans-synaptic interactions contributing to synaptic strength.

In addition to ADAM22 and ADAM23, LGI1 also binds to ADAM11 (73), which is an ADAM protein essential for spatial learning, motor coordination and nociceptive responses (74, 75). However, whether ADAM11 resides within the same complex with LGI1, ADAM22 and ADAM23 is not known, and electrophysiological studies will be needed to analyse if ADAM11 has a role in synaptic transmission and plasticity. Furthermore, the interaction between ADAM23 and LGI1 affects neurite outgrowth. This conclusion is based on the observation that addition of LGI1 to DRGs or hippocampal neurons causes a dose dependent increase in neurite outgrowth, and this effect is reduced for neurons cultured from *Adam23* KO mice (69).

LGI1 protein appears to affect NMDA-receptor subunit expression. The NMDA receptor (NMDA-R) forms a hetero-tetramer between two NR1 and two NR2 subunits. A hallmark of glutamatergic synapse maturation is the change

in postsynaptic NMDA-R NR2 subunit composition (76, 77). The NR2B subunit is mainly expressed in immature neurons of the early postnatal brain. During development, the number of NR2A subunits grows, and eventually NR2A subunits outnumber NR2B subunits. LGI1 shows an increase *in vivo* when NR2B/NR2A decreases, and when mutant ADTLE LGI1 is overexpressed in mice NR2B/NR2A increases (78), suggesting that LGI1 regulates postsynaptic function during development. The same study showed that overexpressed mutant ADTLE LGI1 blocks the normal developmental pruning of excess neuronal dendrites, resulting in an increase of excitatory synaptic transmission and seizure susceptibility. Both LGI1 and integrins (79) seem to be crucial to the synchronous maturation of pre- and postsynaptic membrane functions of glutamatergic synapses during postnatal development and both bind the same disintegrin domain of ADAM proteins (41, 80). Furthermore, both integrin and LGI1 regulate NR2 subunit composition via tyrosine kinase signalling (78, 79). These observations suggest that LGI1, integrins and ADAM proteins may cooperate to promote glutamatergic synapse maturation.

LGI1 also indirectly binds Kv1.1 voltage gated potassium channels, which is a major constituent of presynaptic A-type channels that modulate synaptic transmission in CNS neurons (81). LGI1 protein expression was reported to reduce Kv1.1 inactivation via the intracellular β-subunit of the channel (81). However, since LGI1 has a signalling sequence and is secreted, it is unlikely that LGI1 competes with Kvβ1 for binding to Kv1.1. In contrast to wild-type LGI1 protein, certain mutant LGI1 proteins that cause ADTLE in patients could not reduce Kv1.1 inactivation (81). Interestingly, these same mutant LGI1 proteins are not secreted (53, 82, 83) suggesting that LGI1 first needs to be secreted before having an effect on channel inactivation.

Since the LRR domains of LGI proteins are very homologous to the Slit proteins, they might have similar functions. Indeed, LGI1 counteracts myelin-induced growth cone collapse and neurite outgrowth inhibition (57). LGI1 binds to Nogo receptor 1 (NgR1), raising the possibility that LGI1 is an antagonist of myelin based growth inhibitors (57). ADAM22 was present in a complex with NgR1 and facilitates LGI1 binding to this receptor, suggesting that NgR1 and ADAM22 collaborate to create an LGI1 binding complex that is important for synapse formation (57). It has not been tested whether other LGI proteins bind any of the Nogo receptors. Together these observations underscore an important role for LGI1 in brain excitation and development, explaining why LGI1 mutations result in epilepsy.

Role of LGI1 in ADPEAF

As mentioned earlier, LGI1 mutations result in 'Autosomal Dominant Partial Epilepsy with Auditory Features' (ADPEAF; 600512) (18). ADPEAF patients presents with complex partial and secondarily generalized seizures that are often associated with auditory auras and have a predominant involvement of the left hemisphere (82). Subtle abnormalities have been observed in MRI from ADPEAF patients (84, 85) in which the average age of onset is in early adulthood (15). This suggests that ADPEAF results from defects in brain development, which is in line with the hypothesized functions for LGI1. To date, more than 30 mutations have been identified in LGI1 with a penetrance of ~67% (82, 86, 87) (Figure 2). Mice and rats that lack LGI1 die of seizures during the third postnatal week, showing that normal development or function of the CNS requires LGI1 (58, 68, 71, 88). Two lines of *Lgi1* knock out mice were created in two different laboratories. Despite the similar phenotype of spontaneous seizures, both studies contradict each other, suggesting that loss of *Lgi1* either reduces (58) or increases (71) AMPA-miniature excitatory postsynaptic currents (mEPSCs) in CA1 hippocampal neurons. Other electrophysiological studies on BAC transgenic mice overexpressing either wild-type or mutant LGI1 that is found in ADPEAF, suggest that LGI1 acts both pre- and postsynaptically (78). Mice expressing mutant LGI1 also display reduced developmental pruning of dendritic arbors of hippocampal granule cells and increased spine density, thereby increasing neural excitability (78). Similarly, it was demonstrated recently that LGI1 also regulates the pruning of retinogeniculate fibers (89). These observations are in line with increased neurite outgrowth of wild-type mice neurons after addition of LGI1 (69). Thus, LGI1-ADAM complexes add to a growing list of trans-synaptic complexes whose precise role in synaptic maturation and preservation are yet to be elucidated (reviewed in (2, 3)).

Recently, rats that carry an ENU-generated Leucine to Arginine mutation (L385R) in the fourth epitempin domain of LGI1 have been described (88). These rats recapitulate many aspects of ADPEAF, and provide a useful model system for developing new therapies for this and perhaps other epilepsies as well.

As mentioned above, LGI1 was initially identified from a chromosomal breakpoint in a high-grade glioma cell line (7), but its role in oncogenesis remains controversial. LGI1 expression is reduced or absent in many glioma cell lines (7, 39), and is downregulated in gliomas (90) and in Barrett's-related adenocarcinoma of the esophagus (91). However, ADPEAF patients do not show

increased frequencies of gliomas (8, 9). An important use of *Lgi1* KO mice will be to assess the susceptibility to tumorigenesis when these mice lack both copies.

LGI1 and limbic encephalitis

Limbic encephalitis (LE) is a neurological autoimmune disease associated with antibodies against a variety of antigens (reviewed in (92-94)). Symptoms include memory loss, confusion, brain MRI abnormalities, and seizures. Recently, in such patients auto-immune antibodies against LGI1 have been found (95, 96). As some of the symptoms of LE patients are also observed in ADPEAF patients and LGI1 mutant rats and mice, it is reasonable to assume that at least part of these symptoms result from a reduction in LGI1 levels.

Initially, LE was considered rare and tumour-associated. Currently it is recognized also in patients free of tumours, to present with a variety of symptoms, and to involve tissues beyond the limbic system. Voltage gated potassium channels (VGKC) are a common autoimmune antigen in these patients. Anti-VGKC antibodies typically are detected by their ability to immunoprecipitate radio-labelled α-dendrotoxin, a potassium channel binding protein, after it is added to lysates of brain tissue. However, upon further study, it was found that most of these antibodies reacted with LGI1 and not with potassium channel subunits (95, 96). In fact, almost 90% of these LE cases possess LGI1-reactive sera. Some cases of LE are preceded (prodrome) or overlap with faciobrachial dystonic seizures, which are characterized by frequent brief seizures that typically affect an arm and ipsilateral face (97). Recognition of these clinical symptoms as a prelude to full LE provides a time window for early therapeutic intervention to limit the severity of symptoms and maybe prevent permanent disability. These clinical observations confirm that LGI1 is essential for proper functioning of vertebrate synapses, not only for their maturation. About 1% of epilepsy cases worldwide are idiopathic and many of these cases may have an autoimmune etiology. Therefore the low prevalence of ADPEAF may understate the importance of *LGI1* as an epilepsy gene. Should pathogenic anti-LGI1 antibodies bind to specific epitopes, these epitopes could be druggable and lead to effective new epilepsy treatments.

What can anti-LGI1-mediated LE tell us about LGI1 function? First, the onset of pathology in LE patients suggests that LGI1 is required for functioning of fully developed synapses, in addition to its role in synaptic maturation (69).

The differences between the symptoms of ADPEAF and LE patients may reflect disruption in both developing and mature synapses in the former, versus only mature synapses in the latter. Second, faciobrachial dystonia likely involves the basal ganglia (95), suggesting that LGI1 expression there (98) is functionally significant. Third, patient anti-LGI1 antibodies trigger epileptiform activity in hippocampal slices (99). This experimental model system will help identify mechanisms by which LGI1 controls synaptic activity. Fourth, LE patient LGI1 antibodies may disrupt specific LGI1 interactions with ADAM proteins or other accessory proteins. Characterization of these interactions will provide additional insights into synaptic function. Curiously, one patient's serum was positive for LGI1 but negative in the immunoprecipitation assay for VGKCs, suggesting VGKC-independent functions for LGI1 (95). Thus, the identification of a connection between LGI1 and LE will advance both our understanding of synaptic biology, and our approach toward diagnosis and treatment of epilepsy.

The role of LGI2 in central nervous system development

Roughly 36% of *Lagatto Romagnolo* dogs carry a point mutation in *Lgi2* that results in truncation of 11 amino acids from the C-terminus of the mutant LGI2 protein and causes Benign Familial Juvenile Epilepsy (24). Structurally, this mutation is adjacent to the frameshift mutation in LGI1 in ADPEAF patients described by Kalachikov and colleagues (18), providing additional evidence for the requirement of the C-terminal amino acids of LGI proteins to zip up the structure, as predicted by the Velcro model of beta-propeller folding.

The protein interactions of LGI2 are less well studied than are those of LGI1. In culture, LGI2 was found to bind to the cell surface of ADAM11 expressing cells, but not to cells that expressed either ADAM22 or ADAM23 (56). However, a later study in rat brain showed that LGI2 was, like LGI1, co-immunoprecipitated with both ADAM22 and ADAM23 antibodies, suggesting that LGI2 interacts - at least indirectly - with these ADAM proteins (24). As mentioned above, a truncating mutation in *Lgi2* causes benign juvenile epilepsy in dogs. Just like most mutations in *LGI1* causes the mutated protein to be retained in the cell and degraded, this mutation of *Lgi2* completely abolishes its secretion (24). These observations suggest that LGI1 and LGI2 function through a similar mechanism that affects synaptic maturation at different time points of postnatal nervous

system development. Importantly, *Lgi2* expression in the brain is highest preceding axonal pruning and before the onset of epilepsy, suggesting that LGI2 acts during the network construction phase (24). LGI1 might then act during the pruning phase to ensure an electrically stable network to serve the rest of the animal's life, explaining why in *Lgi2* mutant dogs epileptic episodes are only seen in young animals.

Multiple potential functions of LGI3

Unlike *Lgi1*, *Lgi2*, and *Lgi4*, mutations in *Lgi3* have yet to be associated with a pathological phenotype in humans or experimental animals. What is known about LGI3 suggests that it may perform several distinct functions. Putative LGI3-specific antibodies co-immunoprecipitate Syntaxin1, but not SNAP-25 or other components of the secretory apparatus (100). Curiously, the C-terminus of Syntaxin1 is buried within the plasma membrane, and is inaccessible (101). Therefore its interaction with LGI3 a) occurs in the cytoplasm, b) is indirect, perhaps mediated by ADAM proteins, or c) occurs as a result of tissue homogenization. The first two possibilities are reminiscent of the interaction between LGI1 and Kvβ1 (81). Amyloid Aβ40 and Aβ42 peptides transiently upregulate *Lgi3* expression in astrocytes, and LGI3 appears to promote Aβ endocytosis through an interaction with Flotillin1 (102-104). Thus LGI3 may mediate endocytosis for both Syntaxin1 and amyloid peptides.

LGI3 may also function in cell types other than astrocytes. *Lgi3* expression in brain increases postnatally and it is expressed in neurons but not in oligodendrocytes (103, 105). It is enriched in homogenates of CNS myelin relative to homogenates of whole brain, and it appears to be localized to nodes of Ranvier (106). These observations suggest that it functions in myelinated nerve fibers. Additionally, LGI3 induces neurite outgrowth and increases phosphorylation of the signal transduction proteins AKT and FAK (107). *Lgi3* is expressed in keratinocytes, and may promote their survival following UV irradiation (108). *Lgi3* is expressed in multiple neural crest-derived cell types, including melanoma cell lines (109), DRG neurons (26) and adipocytes (110). It attenuates pre-adipocyte differentiation through binding to ADAM23 (110). Together these observations suggest multiple functions for LGI3 that await further elucidation by the analysis of *Lgi3* mutant mice.

Involvement of LGI4 in cell-cell interactions in the peripheral nervous system

Cell-cell interactions are required during PNS development for Schwann cell proliferation, migration, survival and myelination. Yet the molecular mechanisms mediating these interactions are poorly understood. Claw paw (*clp/clp*) mice, in which LGI4 is not secreted (26), have limb posture abnormalities and peripheral hypomyelination, with no sign of dysmyelination in the CNS (25). They display a delay in radial sorting of axon fibers, and nerve-grafting experiments demonstrate that LGI4 function is required in Schwann cells and possibly in neurons (28). *Adam22* KO mice show a similar phenotype to claw paw mice (56, 111) and LGI4 was found to bind ADAM22. Thus, Schwann cell-secreted LGI4 might interact with ADAM22 on peripheral axons and in this way enable Schwann cell-neuron communication. The interaction could trigger a reciprocal ensheathment and/or myelination signal to Schwann cells (Figure 3). The nature of these putative signalling components is unknown. One possibility is that LGI4-ADAM22 modulates Neuregulin 1 (NRG1) signalling, but if so, it does not modulate the surface expression of NRG1, a major regulator of Schwann cell migration and myelination (56). Another possibility is that LGI4 modulates an already existing interaction between ADAM22 and another ADAM22-binding protein on the Schwann cell membrane, for example integrins (Figure 3). In addition to ADAM22, LGI4 can also bind to ADAM23 and ADAM11 (56, 73). Whether these ADAM proteins play a role in PNS myelination is at present unknown. It is possible that - in analogy with the proposed role of LGI1 in regulating trans-synaptic adhesion (Figure 3)- LGI4 links axons and Schwann cells through interactions with ADAM22 in the axonal membrane and ADAM23 or ADAM11 in the Schwann cell membrane.

Lgi4 is initially expressed by neural crest stem cells but its expression becomes restricted to the glial cells that derive from them. These neural crest derived cells include, in addition to Schwann cells, enteric glial cells with properties resembling those of astrocytes in CNS, and satellite cells of (para-) sympathetic and dorsal root ganglia (reviewed in (112)). Analysis of cultures derived from embryonic enteric and (para-) sympathetic ganglia revealed that LGI4 is required for proper levels of proliferation of glial precursors, but not for glial fate determination of neural crest stem cells (113). Interestingly, embryonic sciatic nerve Schwann cells proliferated normally in the absence of LGI4 (113), demonstrating that different types of PNS glial cells have different requirements for LGI4. Whether the proliferative effect of LGI4 on these glial

precursors is mediated through an ADAM receptor is an important unanswered question. Thus, LGI4 has multiple functions including proliferation of enteric glia and satellite cells in PNS, and later, myelin formation in Schwann cells. The requirement for LGI4 for embryonic enteric glial cell and satellite cell proliferation contrasts with that of LGI1 for glioma cells as LGI1 inhibits proliferation of glioma cells (7, 39).

Concluding remarks

Over the last decade, LGI proteins have emerged as important regulators of cellular interactions in the nervous system and their mutation has been associated with diverse pathologies such as epilepsy, psychiatric disorders and hypomyelination. However, their mechanism of action is still not understood. LGI proteins are secreted proteins and the observation that most mutations in LGI proteins affect their secretion suggests that the diverse pathologies all result from loss of function. These mutations do not inform us about the mechanism of action of LGI proteins. Therefore, of interest is the recent description of a disease-associated mutation in *LGI1* that does not affects its secretion but might instead affect the stability of functionally relevant interactions with other accessory proteins possibly including the ADAM22 and Nogo receptors (114). Identification of additional disease-associated LGI mutant proteins that are normally secreted, map the functionally relevant interfaces of LGI proteins and provides a first step towards a fuller understanding of the interactions these proteins engage in.

A further understanding of LGI function might come from studies that map the epitopes within LGI1 recognized by sera of different LE patients. It is not known at present whether the sera from different LE patients recognize the same or different epitopes. Mapping of these epitopes might tell us how these antibodies interfere with normal LGI1 function and cause disease.

All LGI proteins appear to interact with ADAM22/23/11 receptors albeit probably with very different affinities. The high degree of identity among LGI proteins and their affinity for these ADAM receptors suggest that they act through a similar mechanism. However, it is not known whether the LGI proteins are functionally equivalent or serve distinct functions in different parts of the nervous system at different developmental stages. The one published experiment that speaks to this issue demonstrated that LGI3 couldn't replace

LGI1 in CNS synaptic development, possibly due to a much lower affinity of LGI3 for the ADAM22 receptor (58). An understanding of the functional relationship of LGI proteins will require the determination of the relative affinities of these proteins for the ADAM receptors.

Another open question is how LGI-ADAM interactions influence ADAM-integrin interactions: Are they mutually exclusive or does LGI binding potentiate ADAM-integrin interactions? Answering these questions, in addition to identifying the full repertoire of LGI receptors in different parts of the nervous system will shed light on mechanistic aspects that are common to the LGI protein family as well as to those that are member specific.

Current and future clinical, genetic and biochemical studies directed towards an understanding of the biology of LGI proteins and their role in human neurological disease will ultimately lead to novel approaches in combatting the neurological diseases associated with mutations in the LGI gene family.

Binding partner:	LGI1	LGI2	LGI3	LGI4
ADAM11	Weak (Sa8) Binds (W)	Binds (Oz)	Does not bind (Oz)	Moderate (Sa8) Binds (Oz, N)
ADAM22	Moderate (Sa8) Binds (T, W)	Does not bind (Oz) Binds (Sp)	Binds (Oz)	Strong (Sa8, Sa10) Binds (Oz, N)
ADAM23	Strong (Sa8) Binds (T, W)	Does not bind (Oz) Binds (Sp)	Binds (O) Binds (K)	Strong (Sa8) Binds (Oz, N)
Syntaxin1	ND	ND	Binds (Ok)	ND
Flotillin	ND	ND	Binds (P)	ND
NgR1	Binds (T)	ND	ND	ND

Table 1. Potential binding partners of LGI proteins

Proteins whose association with LGI proteins have been tested directly by co-immunoprecipitation or by co-localization of tagged proteins in cultured cells, are shown. References are abbreviated as follows: K: Kim (2012); Ok Okabayashi; Oz: Ozkaynak (2010); P: Park (2008); Sa8: Sagane (2008); Sa10: Sagane (2010); Sp: Seppala (2012); T: Thomas; W: Owuor; F: Fukata, 2010, N: Nishino. In addition, LGI1 has been shown not to bind to ADAM12 (W), NgR2(T) and NgR3(T). ADAM9 does not bind to LGI1(F), or to LGI4 (N).

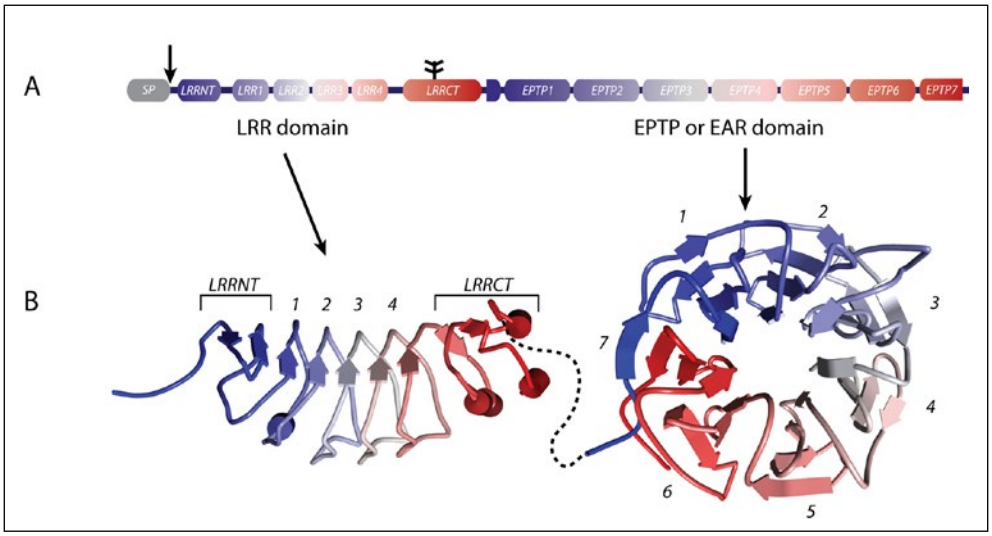

Figure 1. Structural characteristics of the LGI protein family

In A, the general domain structure of the LGI protein family is depicted. All LGI proteins have a signal peptide (SP) that is cleaved of (arrow in A) and is not included in the putative protein structures shown in B. The glycosylation site present in all LGI members is indicated in A with a branched line structure. The putative structures of the LRR domain and EPTP domain were predicted separately using the HHpred tool (http://toolkit.tuebingen.mpg.de/hhpred) and the WDR5 protein structure (PDB 2GNQA) as template. Structures were visualized using the Accelrys Discovery Studio visualizer. The structure is colour-coded from amino-terminus (purple) to carboxyl-terminus (red) and corresponds with the colour code in A. The colour-code graphically reveals the Velcro beta strand interacting (purple) with the last beta strand (red) of the EPTP domain to zip up the structure. How the LRR domain and the EPTP domain are oriented towards each other is unknown. The stippled black line does not represent any structural feature but is only intended to show the linkage between the two domains.

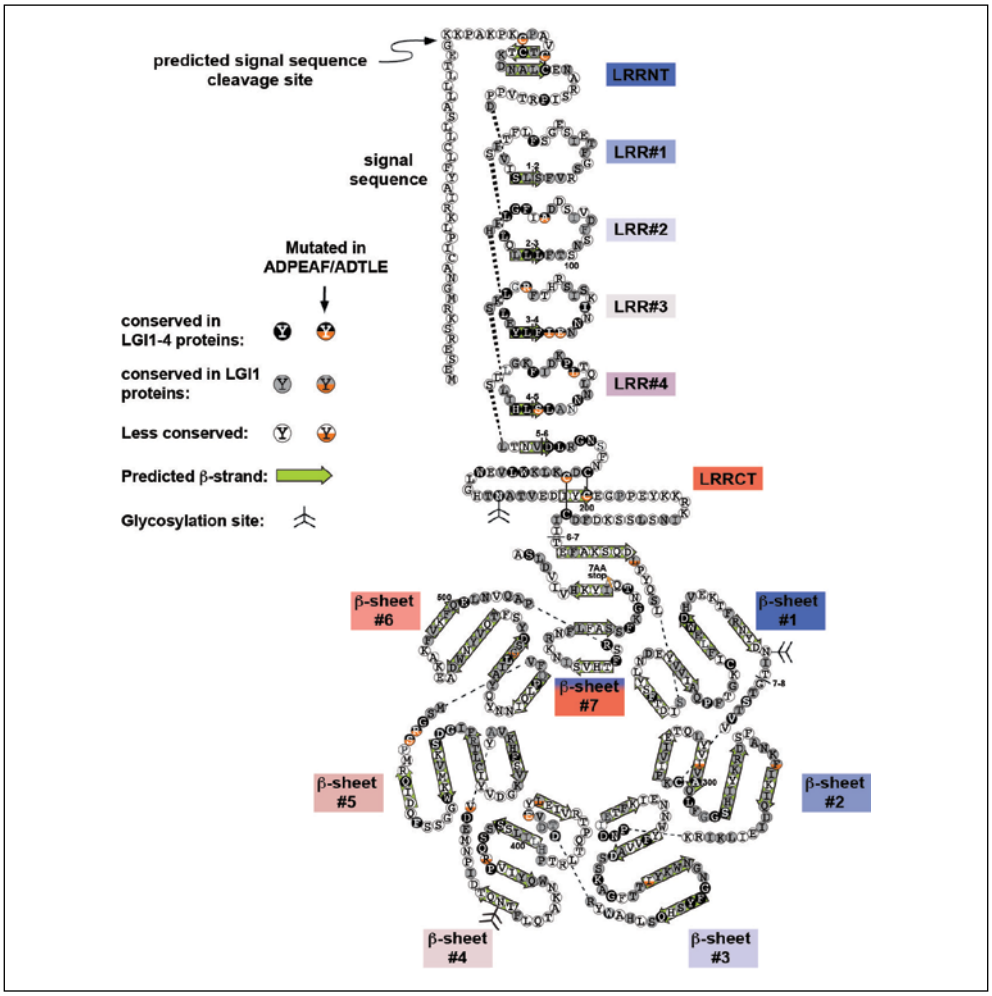

Figure 2. A schematic diagram of human LGI1 protein.

In this "exploded" view of LGI1, the LRR and EPTP modules are separated from one another; dashed lines connect amino acids that are linked in the intact protein. Amino acids are represented as filled, shaded, or open circles, depending on their level of conservation. The bottom half of circles for amino acids that are changed by point mutations are colored orange; all of these mutations are from human except for the mutation at L385, which is from rat. For simplicity, frameshift mutations are omitted, with the exception of one at the C-terminus that provides evidence that the "Velcro" model of beta-propeller closure, in which the N-terminal beta strand is included in the seventh EPTP propeller fold. Human *LGI1* mutations were obtained from (20, 82, 115) and the rat mutation from (88). For the EPTP domain, the beta-propeller blades, which in large part would intersect the plane of the figure in the intact protein, have been laid flat. Beta strands are outlined by green arrows. Disulfide bonds are depicted as solid lines, and glycosylation sites have a branched structure. Boundaries of the eight *LGI1* exons are presented as lines through the sequence, with the relevant exon numbers juxtaposed. Every 100th amino acid is labelled.

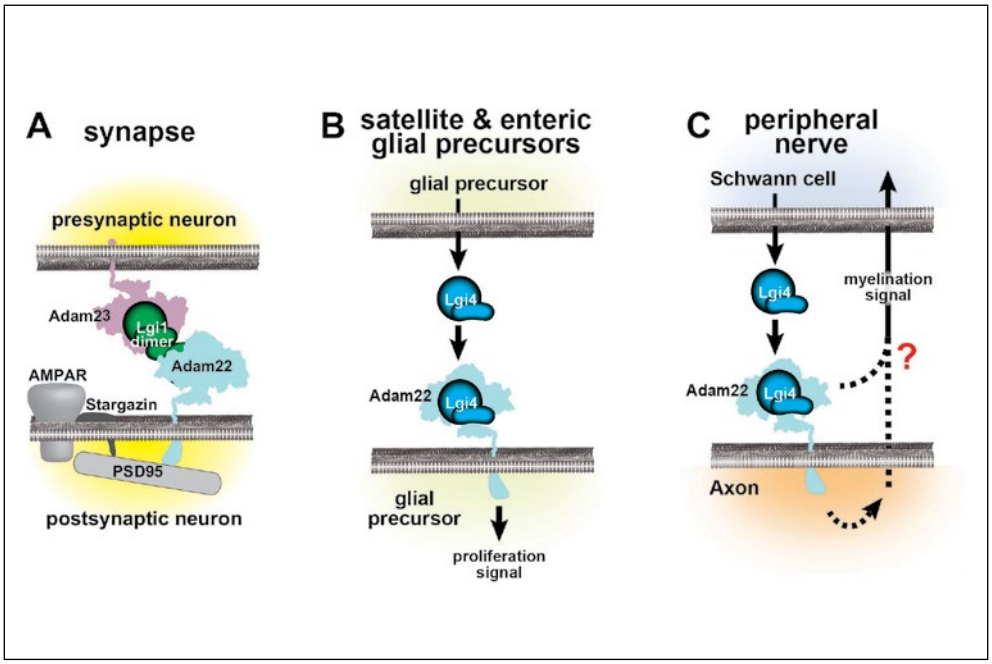

Figure 3. Possible mechanisms by which LGI proteins participate in cell-cell interactions.
Panels A-C depict several possible mechanisms by which LGI1 controls synapse development and function, and LGI4 controls PNS development. Note that these mechanisms are not mutually exclusive; the figure simply summarizes what is currently known for each interaction.

A. At synapses, LGI1 dimers may form a bridge between postsynaptic ADAM22, which is linked to the Stargazin/AMPAR complex through PSD-95 and Stargazin, and presynaptic ADAM23 (58). LGI1 has an effect on Kv1.1 inactivation through mechanisms that are unclear (81).

B. In peripheral glial precursors, LGI4 appears to act as a paracrine or autocrine factor through binding to ADAM22 (113).

C. During Schwann cell ensheathment and myelination of axons, LGI4 binds to the disintegrin domain of ADAM22. This interaction could trigger signalling or protein localization within the axon, thereby activating signal(s) to the Schwann cell. An LGI4-ADAM22-PSD-95 interaction could cluster proteins at the axonal membrane. Alternatively, LGI4 binding to ADAM22 could induce the formation of protein complex(es) extracellularly. LGI4 might modulate ADAM22-integrin interactions, which also utilize the ADAM22 disintegrin domain (62).

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Chapter 3

ADAM22 is a major neuronal receptor for LGI4 mediated Schwann cell signaling

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Journal of Neuroscience, 2010, 30(10): 3857-64

The Journal of Neuroscience, March 10, 2010 • 30(10):3857–3864 • 3857

Cellular/Molecular

Adam22 Is a Major Neuronal Receptor for Lgi4-Mediated Schwann Cell Signaling

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The segregation and myelination of axons in the developing PNS, results from a complex series of cellular and molecular interactions between Schwann cells and axons. Previously we identified the *Lgi4* gene (leucine-rich glioma-inactivated4) as an important regulator of myelination in the PNS, and its dysfunction results in arthrogryposis as observed in claw paw mice. Lgi4 is a secreted protein and a member of a small family of proteins that are predominantly expressed in the nervous system. Their mechanism of action is unknown but may involve binding to members of the Adam (A disintegrin and metalloprotease) family of transmembrane proteins, in particular Adam22. We found that Lgi4 and Adam22 are both expressed in Schwann cells as well as in sensory neurons and that Lgi4 binds directly to Adam22 without a requirement for additional membrane associated factors. To determine whether Lgi4-Adam22 function involves a paracrine and/or an autocrine mechanism of action we performed heterotypic Schwann cell sensory neuron cultures and cell type-specific ablation of Lgi4 and Adam22 in mice. We show that Schwann cells are the principal cellular source of Lgi4 in the developing nerve and that Adam22 is required on axons. Our results thus reveal a novel paracrine signaling axis in peripheral nerve myelination in which Schwann cell secreted Lgi4 functions through binding of axonal Adam22 to drive the differentiation of Schwann cells.

Introduction

The myelin sheath is a highly ordered lamellar structure that surrounds a large fraction of nerve fibers in the vertebrate nervous system. Myelinated nerve fibers conduct nerve impulses with a speed that is approximately two orders of magnitude faster than unmyelinated fibers of similar diameter. Dedicated neuroglial cells, oligodendrocytes in the CNS and Schwann cells in the PNS, produce myelin. The importance of the myelin sheath for the normal function of the nervous system is underscored by the large range of neurological diseases associated with its dysfunction.

The structural and functional maturation of myelinated nerve fibers in the PNS is governed by a temporally and spatially controlled series of molecular interactions between the axon and the Schwann cell (Jessen and Mirsky, 2005). For example, cell adhesion molecules of the nectin-like family are important for proper Schwann cell–axon interactions during myelination and along the internode of the structurally mature myelin sheath (Maurel et al., 2007; Spiegel et al., 2007). Furthermore, engagement of axonally derived neuregulin-1 (Nrg1) with ErbB2/3 tyrosine-kinase receptors on Schwann cells mediates Schwann cell proliferation and survival, and myelination of axons (Nave and Salzer, 2006; Birchmeier and Nave, 2008). Other ligands for tyrosine kinase receptors also modulate myelination, including platelet-derived growth factor and insulin-like growth factor-1 (Ogata et al., 2006) and the neurotrophins (Chan et al., 2001). Additionally, the G-protein coupled receptor gpr126 is an orphan receptor that is Schwann cell-autonomously required to regulate expression of the transcription factor Oct6 and induce myelination (Monk et al., 2009).

Recently, a mutant form of Lgi4 has been shown to underlie the congenital hypomyelinating phenotype of claw paw mice (Henry et al., 1991; Bermingham et al., 2006). Lgi4 is a secreted molecule that belongs to a small family of proteins predominantly expressed in the nervous system. In claw paw mice the Lgi4 mutant protein is not secreted (Bermingham et al., 2006). Thus, the Lgi4 protein is implicated as a positive regulator of myelin formation in the PNS.

Previous studies suggest that proteins of the ADAM (A disintegrin and metalloproteinase) family (Seals and Courtneidge, 2003; Yang et al., 2006) are receptors for Lgi proteins. Lgi1 and Adam22 coprecipitate from brain membrane preparations (Fukata et al., 2006; Schulte et al., 2006) and Lgi1 and Lgi4 both bind to cells that ectopically express Adam22 or Adam23 (Fukata et al., 2006; Sagane et al., 2008). Strikingly, deletion of *Adam22* presents a peripheral nerve phenotype similar to that of claw paw

Received Dec. 20, 2009; revised Jan. 25, 2010; accepted Feb. 8, 2010.

This work was supported by grants from the Dutch government; Netherlands Organization for Scientific Research VICI Grant 918.66.616 (D.M.), BSIK programme 03038, 'Stem cells in development and disease' (D.M.), the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement No. HEALTH-F2-2008-201535 (D.M.), National Institute of Neurological Disorders and Stroke Grant R01NS40751 (J.R.B.), and Muscular Dystrophy Association Grant 4147 (J.R.B.). G.A. is a recipient of a European Union FP7 Marie Curie Fellowship (PIEF-GA-2009-236638). We thank Tom de Vries Lentsch for help in preparing the figures, Ivo Spiegel and Elior Peles (Weizmann Institute, Rehovot, Israel) for Fc-fusion expression cassettes, Carla Taveggia (San Raffaele, Milan, Italy) for ErbB2-Fc and ErbB3-Fc vectors and expert advice, and Elaine Dzierzak for critically reading the manuscript.

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DOI:10.1523/JNEUROSCI.6287-09.2010

 0270-6474/10/303857-08$15.00/0

animals (Sagane et al., 2005) suggesting that Adam22 is a receptor for Lgi4 in the developing nerve. However, it is unknown whether Lgi4 interacts directly with Adam22 and whether the Lgi4-Adam22 ligand receptor pair functions through an autocrine or (juxta)paracrine mechanism of action. Here we show that Lgi4 binds directly to the ectodomain of Adam22. Lgi4 binding depends on an intact Adam22 disintegrin domain but is independent of additional membrane associated factors. Using cell type-specific deletion of Lgi4 or Adam22 and heterotypic sensory neuron–Schwann cell cultures we show that Schwann cells are the principal source of Lgi4 and require binding to axonal Adam22 to drive myelin formation.

Materials and Methods

Mice

The neo allele of *Adam22* (*Adam22^{neo}*), in which a TK-neo cassette is inserted into exon 8, has been described before (Sagane et al., 2005). Conditional alleles for *Adam22* and *Lgi4* were generated through homologous recombination in ES cells using standard techniques (Jaegle et al., 2003). To create a conditional *Lgi4*-null allele we introduced LoxP sites into a nonconserved CA repeat 5′ to the *Lgi4* translation start site, and a second LoxP site, along with a PGK-neomycin cassette that was flanked with frt sites, into intron 3 through homologous recombination in ES cells. We established mouse lines from two independently targeted ES cell lines; these mice were phenotypically indistinguishable. The PGK-neo cassette was removed by crossing the mice with the ROSA26-Frt recombinase deleter mouse (Farley et al., 2000). Offspring of this cross was used to establish the *Lgi4* conditional knock out (*Lgi4^{Lox}*) mouse line used here. Crossing these mice with Cre recombinase drivers such as *DhhCre* and *Wnt1Cre*, results in the tissue-specific deletion of exon 1–3, including the Lgi4 translation start site, signal sequence and N-terminal leucine-rich repeat, thereby generating a null allele (see Fig. 1*A*). To create a conditional null allele for *Adam22* (*Adam22^{Lox}*), we introduced LoxP sequences around exon1, along with a TK-neo cassette that was flanked by Frt sites, through homologous recombination in ES cells. Correctly targeted ES cells were used to generate a mouse carrying the targeted allele containing the TK-neo selection cassette. The TK-neo cassette was removed by crossing the mice with the ROSA26-Frt recombinase deleter mouse (Farley et al., 2000). Offspring of this cross was used to establish the *Adam22* conditional knock out (*Adam22^{Lox}*) mouse line. Deletion of exon1 by Cre recombinase results in the generation of the *Adam22*-null (*Adam22$^{\Delta 1}$*) allele (see Fig. 5*A*). All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by an independent committee (DEC) on the ethical use of experimental animals.

Light microscopy

Mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with PBS followed by 4% paraformaldehyde/1% gluteraldehyde in 0.1 M phosphate buffer pH 7.2. Sciatic nerves, spinal cords and DRGs were dissected and placed in the same fixative at 4°C for at least 16 h. Tissues were washed with 0.1 M phosphate buffer and osmicated overnight at 4°C in 1% osmium tetroxide/ferricyanide in phosphate buffer. Tissues were embedded in plastic resin and 1 μm sections were cut, mounted and stained with either paraphenylenediamine (PPD) or toluidine blue, as described previously (Estable-Puig et al., 1965; Jaegle et al., 2003). Sections were examined using an Olympus BX40 microscope and pictures were collected using a ColorviewIIIu camera.

Immunofluorescence

Mice were killed at postnatal day 12 (P12) and sciatic nerves were dissected and fixed in 4% PFA in PBS overnight at 4°C and embedded in paraffin. Six micron sections were rehydrated and washed in PBS and PBS/0.1% Tween 20. Antigen retrieval was performed with 15 mM Na_3-Citrate Buffer containing 0.05% Tween 20 at 80−90°C for 20–30 min in a water bath. Sections were blocked for at least 1 h at room temperature with PBS, 1% BSA, 0.2 mM glycine, 0.05% Tween 20, and 0.5% Normal Goat Serum (Dako) or Normal Donkey Serum (Sigma) depending on the secondary antibody used. Primary antibodies were diluted in blocking solution, and sections were incubated overnight at 4°C. Secondary antibodies were incubated in block solution for 2 h at room temperature. After washing and rinsing in distilled water, sections were dried, and mounted with polyvinyl alcohol 4–88 containing DABCO anti-fading agent and DAPI for DNA staining. Samples were examined under a Zeiss AxioImager.Z1 microscope and images were captured with an AxioCam MRm digital camera, and analyzed with AxioVision software. Brightness and contrast levels were adjusted in Adobe PhotoShop.

Binding assays

Lgi2, Lgi3 or Lgi4 conditioned media (CM) were incubated with antibodies against V5-tag (Lgi2 and Lgi3) or Myc-tag (Lgi4) for 45 min at room temperature. CM were then added to transfected cells and incubated at 37°C/5%CO_2 for 45 min. Cells were washed twice with PBS, fixed with 4% PFA in PBS for at least 15 min, then washed twice with PBS, once with PBS/0.05% Triton X-100, and blocked for at least 20 min at room temperature. Block contained 1% BSA, 0.2 mM glycine, 0.01% Triton X-100, and 0.5% Normal Goat Serum (Dako). Secondary antibody incubation was done at room temperature for at least 45 min.

Sciatic nerve tissue extraction and Western blotting

Nerves were dissected from killed animals at P12 and frozen on dry ice. Tissues were extracted in reducing sample buffer by sonicating on ice, with 4 on/off cycles of 1 min. Proteins were separated by SDS-PAGE (12.5% gel) and blotted onto Immobilon-P PVDF membranes (Millipore). Blots were probed with different antibodies exactly as described before (Jaegle et al., 2003).

Antibodies

Primary antibodies. Primary antibodies were from the following sources: Mouse-anti-P0 (Archelos et al., 1993) [immunofluorescence (IF), 1:500; Western blot (WB), 1:1000], rabbit-anti-Oct6 (Jaegle et al., 2003) (IF, 1:200; WB, 1:500), rabbit-anti-Krox20 (Ghazvini et al., 2002) (IF, 1:200; WB, 1:500), mouse-anti-neurofilament M (IF, 1:300) (The 2H3 monoclonal antibody developed by Jessell and colleagues was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa, Iowa City, IA), rat-anti-myelin basic protein (Millipore; IF, 1:300), goat-anti-ChAT (Millipore; IF, 1:200), mouse-anti-myc 9E10 (clone 9E10, 0.4 mg/ml, Roche; IF, 1:300), mouse-anti-V5 (custom-made monoclonal antibody; IF, 1:50), rabbit-anti-phospho AKT (Cell Signaling Technology; WB, 1:1000), rabbit-anti-Erk (Cell Signaling Technology; WB, 1:1000), mouse-anti-acetylated tubulin (Sigma; WB, 1:1000).

Secondary antibodies. The following secondary antibodies were used. Alexa594-conjugated goat-anti-mouse and Alexa488-conjugated oat-anti-rabbit (Invitrogen; used at 1:300 for IF), Cy2-conjugated donkey-anti-mouse (IF, 1:100), AffiniPure DyLight594-conjugated donkey-anti-rat and AffiniPure DyLight594-conjugated donkey-anti-goat (Jackson ImmunoResearch; IF: 1:500), HRP-goat-anti-mouse, AP-goat-anti-mouse, and HRP-goat-anti-rabbit (Dako; WB, 1:5000), goat-anti-human-HRP (GE Healthcare; WB, 1:5000).

Cloning

Lgi2, 3, 4 and Adam22 cDNAs were amplified from E13.5 mouse DRG cDNA using Phusion polymerase (Finnzymes), cloned into Zero-Blunt TOPO vector (Invitrogen) and sequenced. cDNAs were generated using the SuperScript II (Invitrogen) reverse transcription system, on total RNA isolated by RNA-Bee reagent (Tel-Test, Inc). Adam11 and Adam23 were amplified from IMAGE cDNA clones. V5-His (Lgi2, 3) or myc-His (Lgi4) tagged Lgi proteins were obtained by recloning into pcDNA3.1-based expression cassettes. Full-length Adam-GFP-His fusion proteins and Ectodomain Adam-Fc fusion proteins were obtained by recloning into CMV-based expression cassettes [G20 isoform for Adam22 and α isoform for Adam23 were used (Sun et al., 2004; Sagane et al., 2005)].

Cell culture and immunoprecipitation assays

All cell culture reagents were from Lonza, and all cultures dishes were from Greiner Bio-One. CHO and HeLa cells were grown in DMEM, 10%

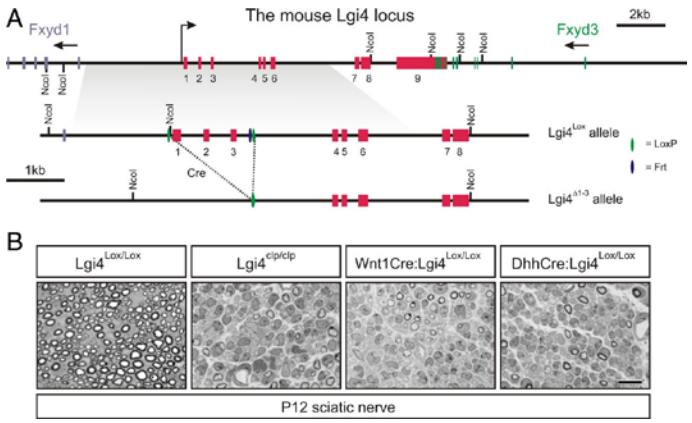

Figure 1. Schwann cell-specific deletion of Lgi4 results in a congenital hypomyelinating phenotype in the peripheral nervous system. ***A***, Schematic depiction of the *Lgi4* gene, the conditional allele ($Lgi4^{Lox}$), and the null allele ($Lgi4^{\Delta 1-3}$) that results from the action of Cre recombinase. The *Lgi4* gene consists of 9 exons spanning ~12 kb of genomic sequences on mouse chromosome 7. The gene is located between the *Fxyd7* and *Fxyd3* genes. The *Lgi4* and *Fxyd3* 3′ ends overlap. The arrows indicate the direction of transcription. ***B***, Cross sections of plastic embedded nerves derived from animals homozygous for the conditional *Lgi4* allele ($Lgi4^{Lox/Lox}$) at P12, show a pattern of myelin figures that is comparable to wild type (see Fig. 2*A*). Deletion of the *Lgi4* gene in Schwann cells ($DhhCre:Lgi4^{Lox/Lox}$) or in Schwann cells and sensory neurons ($Wnt1Cre:Lgi4^{Lox/Lox}$) results in a hypomyelinating phenotype with few myelinated axons, similar to that observed in claw paw ($Lgi4^{clp/clp}$) animals. Sections are stained with PPD to accentuate the myelin sheath, which appears as a dark ring. Scale bar represents 10 μm.

FCS, penicillin and streptomycin (PS), and HEK 293T cells were grown in DMEM/F10, 10% FCS, PS. HeLa, or HEK 293T cells were transiently transfected using Lipofectamine reagent (Invitrogen) or the calcium phosphate transfection method. For immunoprecipitation assays, media conditioned for 6 d were collected and filtered. The conditioned medium was buffered with 0.1 M Tris pH 8 and Adam22-ED-Fc proteins were bound to protein A beads (RepliGen) overnight at 4°C. Adam22-ED-Fc beads were washed three times with PBS/0.1% Triton X-100 and then added to Lgi4 conditioned medium and incubated overnight at 4°C. After washing, beads were resuspended in reducing sample buffer. Bound proteins were separated by SDS-PAGE (10% resolving gel) and analyzed by Western blotting.

Rat Schwann cell and DRG cultures

Primary rat Schwann cell cultures were set up essentially as described before (Brockes et al., 1979) with slight modifications.

DRGs were isolated aseptically from E13.5 mouse embryos. After dissociation by trypsinization and trituration, the cells were plated onto 18 mm poly-L-lysine (Sigma)/collagen-coated coverslips (Thermo Scientific), and maintained in MEM (Invitrogen), 3% FCS, 100 ng/ml NGF, PS (M1 Medium). The medium was refreshed every other day. To obtain neuron-only cultures, the cells were treated with 10 μM FUDR (Sigma) in M1 medium for 2 d. Two cycles of FUDR treatment (2 d with FUDR, 2 d without FUDR) was usually sufficient to obtain neuron-only cultures. Rat Schwann cells were starved of neuregulin in DMEM, 10% FCS, PS for 3 d before seeding 2×10^5 cells in M1 medium onto neuron-only cultures. The Schwann cells were allowed to associate with the neurons for an additional 5 d, and then myelination was induced by addition of ascorbic acid to 50 μg/ml (Sigma). Cultures were maintained in M1 medium containing 50 μg/ml ascorbic acid and 10% FCS for 2 weeks and subsequently analyzed for the presence of myelin.

Results

Cellular source of Lgi4 in the developing nerve

Schwann cells are a likely source of Lgi4 as siRNA inhibition of Lgi4 expression in Schwann cells reduces myelin formation in neuron–Schwann cell coculture experiments (Bermingham et al., 2006). However, both Schwann cells and sensory neurons express *Lgi4* mRNA (Bermingham et al., 2006) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), and nerve transplantation experiments have suggested both Schwann cell autonomous and possibly nonautonomous functions of Lgi4 (Darbas et al., 2004). Whether Lgi4 is expressed in mesenchymal derivatives such as the perineural cells or endothelial cells and pericytes, is unknown. To address the cell-autonomous function of Lgi4 in the PNS, we generated mice that carry a conditional null allele of *Lgi4* (Fig. 1*A*). *Lgi4* was specifically deleted during embryonic development in Schwann cells, sensory neurons (but not motor neurons) and endoneurial fibroblasts, using a *Wnt1Cre* transgene (Danielian et al., 1998; Joseph et al., 2004). Microscopic examination of cross sections of sciatic nerves of $Wnt1Cre:Lgi4^{Lox/Lox}$ animals at postnatal day 12 (P12) reveals a hypomyelination phenotype that is indistinguishable from that observed in claw paw ($Lgi4^{clp/clp}$) nerves (Fig. 1*B*). To further define the cellular source of Lgi4, we examined nerves of animals in which *Lgi4* is deleted during embryonic development in Schwann cells and endoneurial fibroblasts only, using a *DhhCre* transgene (Jaegle et al., 2003). At P12, $DhhCre:Lgi4^{Lox/Lox}$ animals present with a hypomyelination phenotype that is identical to that of claw paw and $Wnt1Cre:Lgi4^{Lox/Lox}$ mice (Fig. 1*B*). Taken together, these results demonstrate that Schwann cells are the principal source of Lgi4 in the developing nerve.

Lgi4 receptors

The strikingly similar peripheral nerve phenotype of *Adam22* mutant animals and claw paw animals (Henry et al., 1991; Sagane et al., 2005; Bermingham et al., 2006), together with the observation that Lgi1 and Lgi4 bind to cells ectopically expressing Adam22 (Fukata et al., 2006; Sagane et al., 2008), suggest that Adam22 functions as a Lgi4 receptor in nerve development. To test whether binding of Lgi4 to Adam22 is direct and/or requires the presence of additional molecules at the cell membrane, we used human IgG-Fc fusion proteins that contain the ectodomain of Adam22 (Fig. 2*A*). The fusion proteins contained either normal Adam22 or Adam22 with a D509N (Asp to Asn substitution at position 509) mutation in the disintegrin-like domain that is analogous to the *ju160* allele of *C. elegans Adm1/Unc-71*, which causes axon guidance and sex myoblast migration defects (Huang et al., 2003). Fusion proteins were incubated with conditioned medium of CHO cells expressing and secreting myc-tagged Lgi4, and precipitated with protein A-Sepharose beads. The precipitated proteins were analyzed by Western blot (Fig. 2*B*). Adam22-Fc protein was found to efficiently precipitate Lgi4 from conditioned medium. In contrast, no Lgi4 protein was found precipitated with the Adam22-Fc protein containing the D509N mutation. These results indicate that binding of Lgi4 to Adam22 does not require additional membrane associated receptors and that binding depends on the integrin-binding domain of Adam22.

Since several other LGI and ADAM family molecules, in addition to Lgi4 and Adam22, are expressed in the PNS (sup-

plemental Fig. 1, available at www.jneurosci.org as supplemental material), we examined binding of Lgi4, Lgi2 and Lgi3 to cell surface expressed Adam proteins Adam22, Adam23 and Adam11. Conditioned medium of CHO cells overexpressing tagged versions of Lg4, Lgi2 and Lgi3 were incubated with HeLa cells expressing C-terminal GFP fusions of Adam22, Adam23 or Adam11 and binding was detected by immunocytochemistry. Lgi4 binds, in addition to Adam22, also to Adam11 and Adam23, both of which are expressed in Schwann cells and sensory neurons (Fig. 2*C*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Lgi2 and Lgi3 are also expressed in the peripheral nerve and bind to Adam11 and Adam22/Adam23, respectively (Fig. 2*C*).

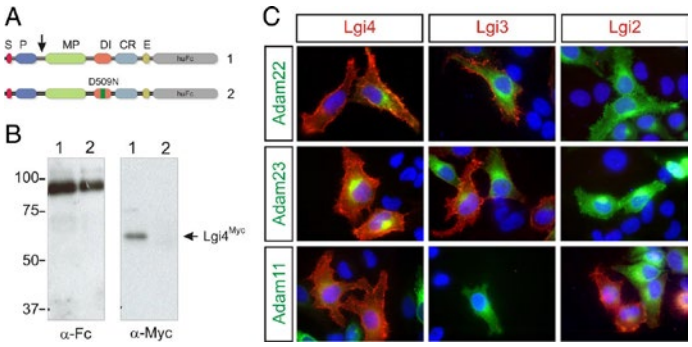

Figure 2. Lgi4 binds to Adam receptors. ***A***, Soluble fusion proteins between the ectodomain of Adam22, either wild type or carrying a missense mutation in the disintegrin domain (D509N) and the human IgG1 Fc domain were generated and their domain structure is schematically depicted here. S, Signal peptide; P, pro-domain; MP, metalloprotease-like domain; DI, disintegrin-like domain; CR, cysteine-rich domain; E, Egf-like domain. The arrow indicates the site at which the precursor protein is cleaved by furin-like proteases during processing. ***B***, Lgi4 is efficiently precipitated by Adam22-Fc from conditioned medium of CHO cells transfected with a myc-tagged Lgi4 expression cassette (1). The disintegrin mutant Adam22 Fc-fusion protein (2) does not precipitate Lgi4 from conditioned medium. The precipitated Adam22-Fc fusion proteins were detected using an anti-human Fc antibody (left panel) while myc-tagged Lgi4 was detected using a myc antibody (right panel). ***C***, Lgi4 (red) binds to the surface of HeLa cells expressing Adam22-GFP fusion proteins. Lgi4 also binds to the Adam22 related proteins Adam23 (-GFP fusion; green) and Adam11 (-GFP fusion; green). Lgi2 (red) and Lgi3 (red), both of which are expressed in sensory neurons and in Schwann cells (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), bind with different specificity to Adam22, Adam23 and Adam11. Lgi4, Lgi2 and Lgi3 are added to transfected HeLa cells in the form of conditioned media derived from CHO cells overexpressing either myc-tagged Lgi4 (red), or V5 tagged Lgi2 (red) or Lgi3 (red).

Whether Adam11 and/or Adam23 contribute to Lgi4 function as alternative Lgi4 receptors in the peripheral nerve is unclear. If Adam11 or Adam23 function as ancillary receptors for Lgi4, the peripheral nerves in Lgi4-deficient mice should be more severely affected than the nerves in Adam22 mutant mice. When we compared the developmental stage of sciatic nerves derived from wild-type, claw paw ($Lgi4^{clp/clp}$) and Adam22-deficient ($Adam22^{neo/neo}$) animals at P12, the peripheral nerves in both mutant animals were found to be hypomyelinated. However, the claw paw nerve phenotype appears more severe with fewer myelin figures (Fig. 3*A*). Immunohistochemical and Western blot analyses confirm the histological analyses and show that more Schwann cells have progressed to the myelinating stage in *Adam22* mutant animals compared with claw paw animals, as evidenced by the higher levels of myelin protein zero (Mpz) and Krox20 protein (Fig. 3*C*) and the larger number of cells expressing Mpz and Krox20 (Fig. 3*B*). This less severe *Adam22* mutant nerve phenotype suggests that additional Lgi4 receptors exist and can partially compensate for the loss of Adam22.

Cell autonomous function of Adam22

Adam22 is expressed by neurons as well as Schwann cells (Sagane et al., 2005) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) suggesting that Lgi4 could function in an autocrine or/and paracrine manner. To determine whether Adam22 expression is required on Schwann cells, on neurons, or both, we generated primary sensory neuron cultures from $Adam22^{+/neo}$ and $Adam22^{neo/neo}$ embryos and seeded these cultures with wild-type rat Schwann cells. Rat Schwann cells readily ensheath and myelinate $Adam22^{+/neo}$ neurons [myelin basic protein-positive (red) myelin sheaths surrounding neurofilament-positive (green) neurites], whereas $Adam22^{neo/neo}$ neurons are not myelinated (Fig. 4). These results indicate that Adam22 function is required in the neuron, but do not exclude an additional function in Schwann cells.

To further explore a possible Schwann cell-autonomous function of Adam22 and to corroborate its neuronal function in nerve development, we generated a conditional *Adam22*-null allele (Fig. 5*A*) and bred it to homozygosity in the presence of the *Wnt1Cre* transgene, thereby deleting *Adam22* in all neural crest derivatives, including sensory neurons, endoneurial fibroblasts and Schwann cells, but not in spinal motor neurons. The sciatic nerve is a mixed nerve containing both primary sensory and motor fibers (see diagram in Fig. 5*C*). Histological and immunostaining analysis of myelin formation in the sciatic nerve of these mice revealed a mosaic pattern of normally myelinated fibers and promyelin arrested fibers (Fig. 5*B*,*C*). Most of the myelinated axons express choline acetyltransferase (ChAT) (Fig. 5*C*, top), indicating they are motor fibers. Therefore, motor neurons are myelinated normally by Adam22-deficient Schwann cells. In P12 $Wnt1{:}Adam22^{Lox/Lox}$ sciatic nerves, as in Lgi4-deficient claw paw mice of similar age, nonmyelinated fibers are associated with predominantly Oct6-positive promyelinating cells (Fig. 5*C*, bottom), whereas Krox20-positive Schwann cells are invariably myelinating cells as evidenced by Mpz protein expression (Fig. 5*C*, middle; see also Fig. 3*B*). Examination of the ventral motor and dorsal sensory roots revealed normal myelinated motor fibers and severely hypomyelinated sensory fibers, respectively (Fig. 5*B*,*D*). These data demonstrate that Adam22 is required in neurons, whereas deleting Adam22 in Schwann cells has no effect on myelin formation. The latter conclusion is further illustrated by the normal microscopic appearance of cross sections of sciatic nerves derived from mice in which *Adam22* is deleted, through the action of the *DhhCre* transgene, in Schwann cells only (Fig. 5*E*). Thus, axonally expressed Adam22 serves as the major receptor for Schwann cell-derived Lgi4 in PNS myelination.

Does Lgi4-Adam22 interaction modulate Nrg1 signaling?

How does Lgi4 binding to axonal Adam22 drive Schwann cell myelination? The multitude of axon-Schwann cell signaling and interacting molecules provide numerous possible mechanisms by which Lgi4-Adam22 may promote myelin formation. One possibility is that Lgi4-Adam22 promotes myelination through mod-

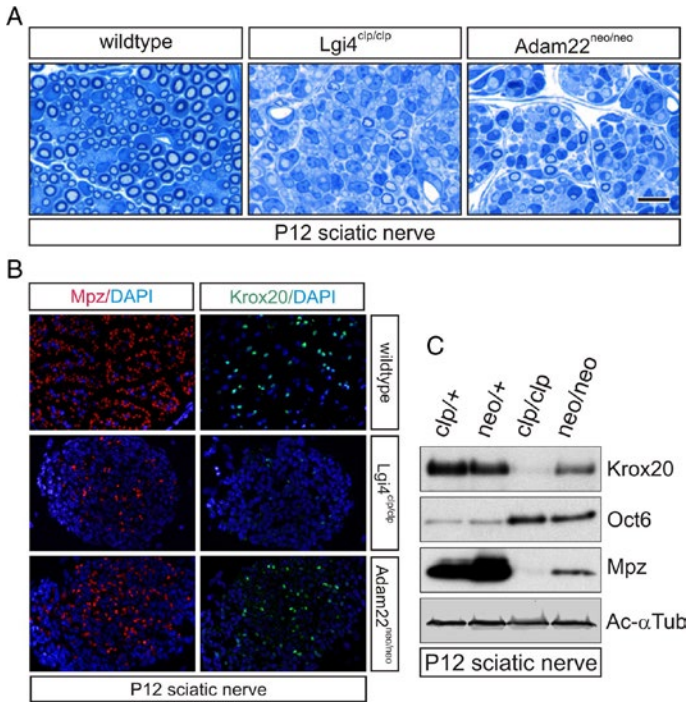

Figure 3. Comparison of sciatic nerves of Lgi4 and Adam22 deficient animals. ***A***, Toluidine blue-stained cross sections of sciatic nerve of claw paw (*Lgi4*$^{clp/clp}$) and Adam22 (*Adam22*$^{neo/neo}$) mutant show similarities in extent of myelin formation at P12. Representative pictures of 3 individuals per genotype examined. Scale bar, 10 μm. ***B***, Mpz and Krox20 staining of cross sections of sciatic nerves derived from Claw paw, *Adam22* mutant and wild-type animals demonstrate a larger fraction of myelinating Schwann cells (Krox20 and Mpz positive) in *Adam22* mutants versus claw paw animals. ***C***, Western blotting corroborates this observation as elevated levels of the promyelinating marker Oct6 and reduced Mpz expression are observed in both mutant animals. The more advanced stage of Schwann cell differentiation in *Adam22*$^{neo/neo}$ versus claw paw animals is again evident from the higher Krox20 and Mpz levels in *Adam22*$^{neo/neo}$ nerves.

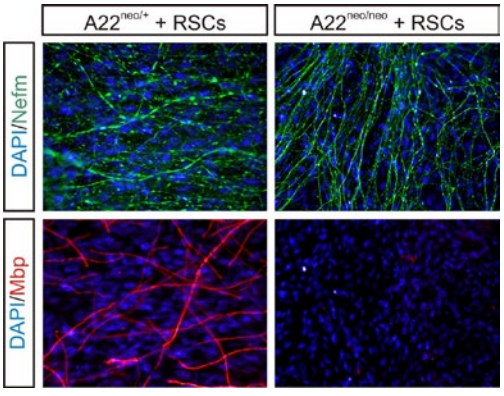

Figure 4. Adam22 is a neuronal receptor required for myelin formation by Schwann cells. Wild-type rat Schwann cells ensheath and myelinate the neurites of embryonic sensory neurons derived from *Adam22*$^{neo/+}$ but not *Adam22*$^{neo/neo}$ mutant embryos *in vitro*. The neurites in these cultures are visualized by neurofilament staining (Nefm; green) and myelin is visualized by myelin basic protein (Mbp; red) staining. The nuclei of Schwann cells and neurons are revealed with DAPI (blue) stain.

ulating the axonal membrane expression or processing of type III Nrg1. Type III Nrg1 is required for myelination and the extent of myelination is proportional to the level of type III Nrg1 expression on the axonal membrane (Michailov et al., 2004; Taveggia et al., 2005). To test whether deficient clustering or processing of Nrg1 on Adam22 deficient axons could account for the hypomyelination phenotype in these mutant mice, we examined the surface expression and signaling potential of type III Nrg1. Heterodimeric fusion proteins of the ErbB2 and ErbB3 extracellular domains with the human Ig Fc domain have been used previously to demonstrate type III Nrg1 expression on axons (Taveggia et al., 2005). Using these fusion proteins, membrane bound Nrg1 could be readily detected on the axons of wild-type as well as Adam22 mutant primary sensory neurons (Fig. 6*A*).

We next tested the ability of Nrg1 on wild-type and Adam22 deficient axons to activate signaling in Schwann cells. Axonal membrane of sensory neurons robustly activate the phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase (Erk) pathways in Schwann cells and it has been demonstrated that phosphorylation of AKT, a major target of PI3K, depends on the activity of type III Nrg1 (Ogata et al., 2004; Taveggia et al., 2005). We prepared membranes from sensory neuron cultures derived from wild-type or *Adam22* mutant embryos and seeded these on Schwann cells. Both preparations activate the PI3K and Erk pathways to the same extent (Fig. 6*B*) in Schwann cells. Thus, these experiments eliminate the simple hypothesis that Lgi4-Adam22 is required for membrane localization or activity of Nrg1 type III.

Discussion

Here we show, through selective deletion of the *Lgi4* gene in the Schwann cell lineage, that Schwann cells are the principal source of Lgi4 in the developing peripheral nerve tissues. This result corroborates our earlier conclusions from nerve transplantation and siRNA-mediated inhibition of Lgi4 expression in Schwann cells (Darbas et al., 2004; Bermingham et al., 2006). Interestingly, we found that sensory neurons also express *Lgi4* at low levels. Either this neuronal Lgi4 is not transported and secreted along the axon or its expression is too low, as deletion of *Lgi4* in both Schwann cells, endoneurial fibroblasts and sensory neurons (as in *Wnt1-Cre:Lgi4*$^{Lox/Lox}$ animals) results in a similar hypomyelinating nerve phenotype as observed in *DhhCre:Lgi4*$^{Lox/Lox}$ animals in which *Lgi4* is only deleted in Schwann cells and endoneurial fibroblasts. Also, we found that *Lgi2* and *Lgi3* are expressed at low level in Schwann cells and at higher levels in sensory neurons (Bermingham et al., 2006). Apparently, Lgi2 and Lgi3 proteins do not substitute for the loss of Lgi4 in claw paw animals. In line with this we found that Lgi2 does not bind to Adam22 at all. However, Lgi3 does bind to Adam22, suggesting that Lgi3 is not secreted at sufficient levels at the right developmental time, or that Lgi4 and

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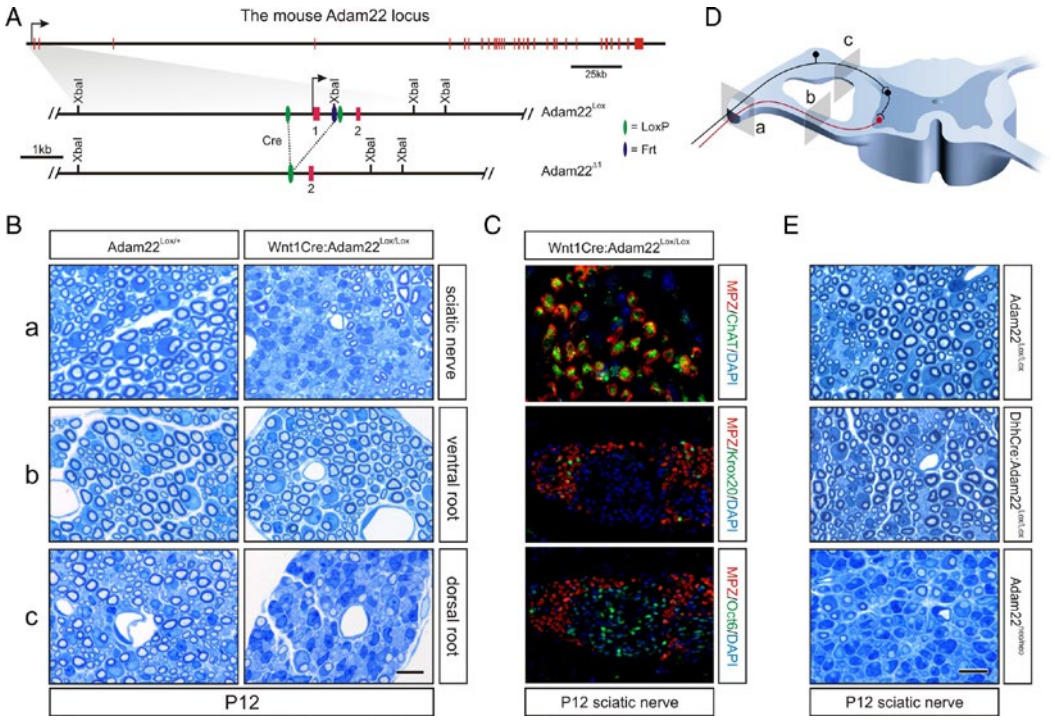

Figure 5. Neuron cell-autonomous function of Adam22. ***A***, Schematic depiction of the mouse *Adam22* locus, the conditional allele (*Adam22*^{Lox}), and the null allele (*Adam22*$^{\Delta 1}$) that results from the action of the Cre recombinase. The *Adam22* gene consists of >30 exons (red vertical bars on top line) that span ~300 kb of genomic sequences on mouse chromosome 5. The promoter is indicated with an arrow. ***B***, Conditional deletion of *Adam22* in neural crest derivatives using the *Wnt1Cre* driver. Toluidine blue staining of sections of nerves from *Adam22*$^{Lox/+}$ and *Wnt1Cre:Adam22*$^{Lox/Lox}$ mice at three different anatomical levels ***a–c*** (scale bar, 10 μm). These levels are schematically depicted in ***D***. Motor fibers are indicated in red and sensory fibers in black. ***C***, Immunostaining for ChAT (green) and Mpz (myelin; red) shows that most myelinated axons are positive for ChAT. Schwann cells in the nonmyelinated areas of the nerve are Krox20 negative and Oct6 positive. ***E***, Myelination of nerve fibers in the sciatic nerve of mice homozygous for the *Adam22* conditional null allele (*Adam22*$^{Lox/Lox}$) appears normal and is comparable to that observed in sciatic nerve of *DhhCre:Adam22*$^{Lox/Lox}$ mice. In the latter, *Adam22* is deleted selectively in Schwann cells. Complete deletion of *Adam22* (*Adam22*$^{neo/neo}$) results in a hypomyelinating phenotype. The scale bar represents 10 μm.

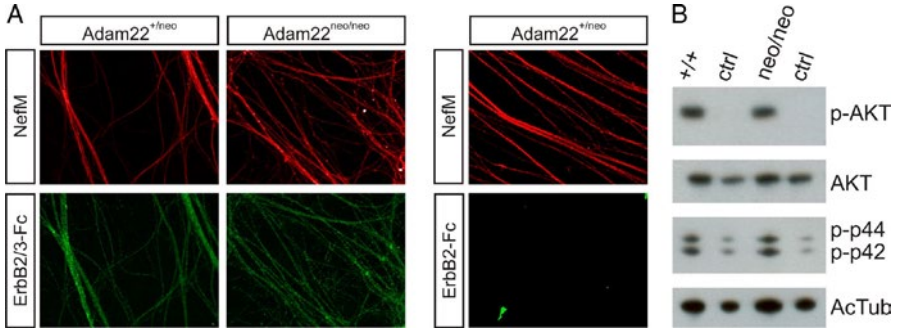

Figure 6. Adam22 and contact-dependent Nrg1 signaling. ***A***, Embryonic sensory neuron cultures derived from *Adam22*$^{neo/+}$ (9 cultures derived from 5 embryos; 9/9 positive for ErbB2/3 binding) and *Adam22*$^{neo/neo}$ (6 cultures from 3 embryos; 6/6 positive for ErbB2/3 binding) mutant embryos express typeIII Nrg1 on their neurites as evidenced by binding of the ErbB2/3-Fc fusion proteins. Neurites are visualized with neurofilament (NefM) antibodies (red) and the Fc moiety of the fusion proteins is visualized with an anti-human Fc antibody (green). ErbB2-Fc does not bind Nrg1 and serves here as a control. Magnification 20×. ***B***, Axonal membranes were prepared from *Adam22*$^{+/+}$ (+/+) and *Adam22*$^{neo/neo}$ (neo/neo) primary sensory neuron cultures and centrifuged onto Schwann cells. After 30 min lysates were probed with antibodies for phosphorylated-Akt (p-AKT), total Akt (AKT), and for phosphorylated Erks (p-p42 and p-p44). A lysate of Schwann cells exposed to fresh serum-free medium served as a control (ctrl). The experiment was performed twice, in duplicate.

Lgi3 are qualitatively different, perhaps reflecting differential interactions through their LRR domains. Thus, we find that Lgi4 is the major LGI protein required for Schwann cell differentiation and myelination.

Lgi4 appears to function in PNS myelination principally through interaction with the transmembrane protein Adam22 as Lgi4 binds directly to the extracellular domain of Adam22 and deletion of Adam22 in mice results in a peripheral nerve phenotype that is very similar to that observed in claw paw animals (Sagane et al., 2005) (Fig. 3). Adam22 belongs to a small subfamily of ADAM proteins, including Adam23 and Adam11, which are predominantly expressed in the nervous system (Yang et al., 2006). Adam22, like Adam23 and Adam11, lacks metalloproteinase activity, and therefore these ADAM proteins have been suggested to mainly function as integrin receptors through their disintegrin domain (Yang et al., 2006). Soluble forms of the Adam22 extracellular domain inhibit cell proliferation in an integrin-dependent manner (D'Abaco et al., 2006). Interestingly, it was found that reexpression of Lgi1 in the glioma cell line T98G (the glioma cell line in which Lgi1 originally was found inactivated; hence the name Leucine-rich Glioma Inactivated) reduces proliferation and cell migration (Chernova et al., 1998; Kunapuli et al., 2003). It is unclear whether these observations are relevant for the role of Adam22 in the peripheral nervous system. It is possible that Lgi4 binding to Adam22 modulates (either positively or negatively) Adam22-integrin interactions, as Lgi4 binds to the disintegrin-like domain of Adam22. Indeed, Adam22 interacts with a number of integrins that are expressed on Schwann cells (in particular $\alpha 6\beta 1$ and $\alpha 6\beta 4$) and are involved in axonal sorting and myelin stabilization (Berti et al., 2006; D'Abaco et al., 2006). However, the actions of these integrins have been interpreted in terms of their interaction with the Schwann cell extracellular matrix and not with the axon. It is tempting to speculate that Lgi4 regulates interactions between axonal Adam22 and Schwann cell integrins to allow progression of the Schwann cell's inner mesaxon over the axonal membrane and initiate myelin formation, a process that is inhibited in the absence of Lgi4 or Adam22.

An alternative mechanism of Lgi4-Adam22 function is suggested by work of Fukata and colleagues (Fukata et al., 2006). Adam22 was found associated with Lgi1 when both Adam22 and Lgi1 coprecipitated with Stargazin and PSD95 from rat brain membrane preparations. Lgi1, which is found frequently mutated in autosomal dominant partial epilepsy with auditory features (ADPEAF) (for review, see Nobile et al., 2009), enhances AMPA-receptor-mediated synaptic transmission. It was suggested that Lgi1 binding to Adam22 stabilizes the Stargazin/AMPAR/PSD95/Adam22 complex in the postsynaptic membrane. Similarly, it is possible that Lgi4 binding to axonal Adam22 stabilizes complexes at the axonal surface that are involved in Schwann cell mesaxon progression and myelination. We have begun to explore this possibility here by examining the surface expression and signaling potential of membrane bound type III NRG1, a known regulator of myelination. We found that NRG1 is expressed on the axonal membrane of Adam22$^{neo/neo}$ neurons and that these membranes activate PI3K and Erk as effectively as membranes derived from wild-type neurons, suggesting that Lgi4-Adam22 does not function by modulating Nrg1-ErbB2/3 interactions.

To further explore the mechanism of Adam22 function in myelination it will be important to identify proteins that bind to the cytoplasmic tail of Adam22, which includes a PDZ binding motif. Interestingly, it was recently demonstrated that accumulation of the MAGUKs PSD93/PSD95 at the juxtaparanodal membrane of myelinated axons depends on Adam22 (Ogawa et al., 2010). It is unlikely that these interactions are important for the earlier function of Adam22 as PSD93/PSD95 double knock out animals, though severely affected, form myelin (Horresh et al., 2008).

In conclusion, by identifying Adam22 as the major axonal receptor for the Schwann cell-derived factor Lgi4, we have revealed a new molecular component in Schwann cell–neuron interactions that govern myelin formation in the PNS. In contrast to previously identified ligand-receptor pairs that function in PNS development, Lgi4 is secreted from Schwann cells and binds to axonally expressed Adam22. How Lgi4 and Adam22 interact with other axon-Schwann cell signaling molecules is an important unanswered question, the answer to which may provide novel approaches for treating myelin disease.

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Supplementary information

RT-PCRs:

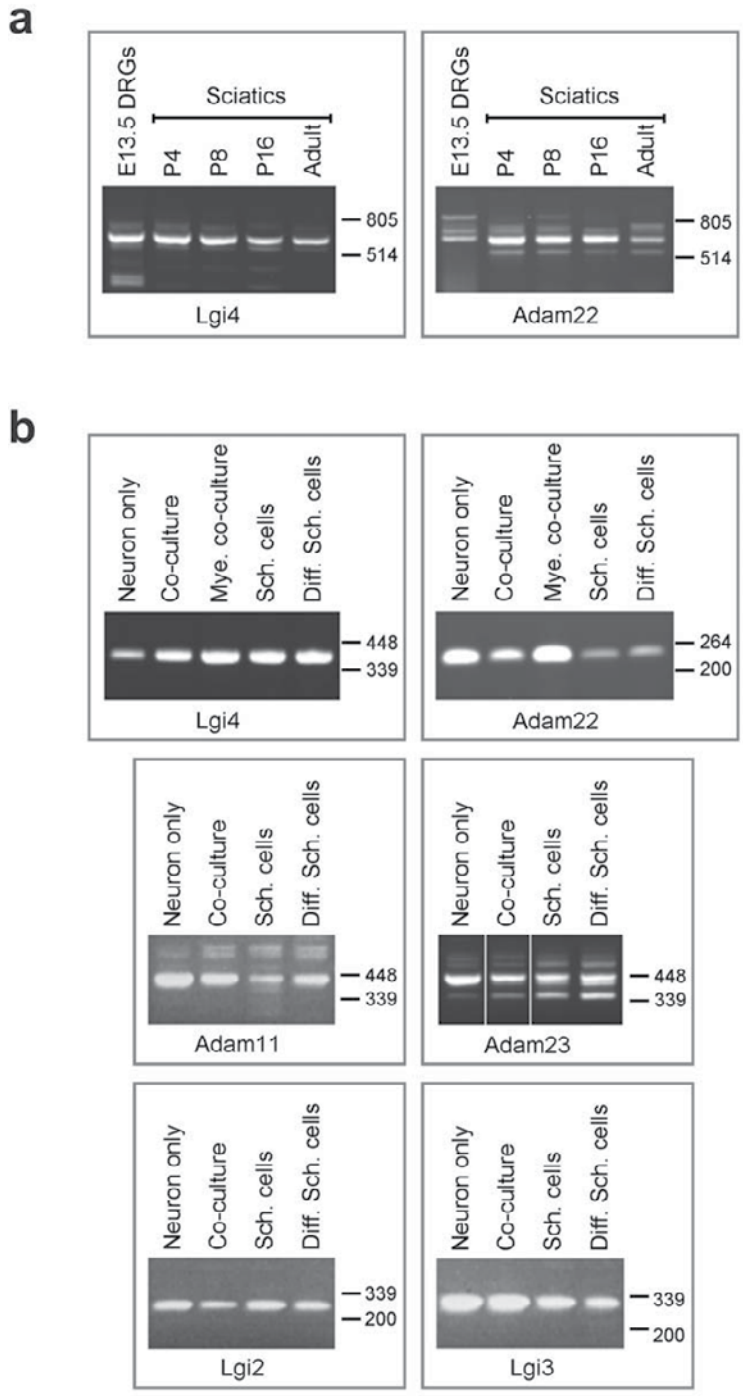
For RT-PCR experiments, RNA was isolated from various tissues with RNA-Bee, first strand cDNA synthesis was performed with SuperScript III (Invirtogen), and PCRs were done with Phusion. The PCR products were run on an agarose gel, and pictures were made with a Typhoon Imager (GE, Healthcare). The PCR steps were 98°C, 5 minutes, initial denaturing; (98°C, 20 seconds, denaturing; annealing 20 seconds (Tm; see table); extension 30 seconds at 72°C) for 30 cycles; 72°C, 10 minutes, final extension.

The primers used and related information are listed below:

Primer	Sequence	Annealing Temp. (°C)	Extension time (s)	Expected product length (bp)
Mouse Lgi4 forward	CAGCTTCTTAAAGATGCCGTCATTG	69	15	620
Mouse Lgi4 reverse	CCAGCACCAGAGGCTTGCAGG			
Mouse Adam22 forward	GTTTGCAGCAATGAGCTCAAGTG	68	20	560-1000 (different isoforms)
Mouse Adam22 reverse	CCTCTGGTATCTCAGAGGGTCTTG			
Rat Lgi4 forward	CAGAGGCAGAGGACGTCTATG	66.5	10	405
Rat Lgi4 reverse	CTCAGGCACTGAGATCCAACTC			
Rat Adam22 forward	GGTGAAATCACGTCCACACTG	67.5	10	240
Rat Adam22 reverse	GAGCTCATTGCTACAAACTCCG			
Rat Adam11 forward	GAGGGGACAAGGCAACACAG	69	10	441
Rat Adam11 reverse	TGCAAAGTTGCTGGTGAGGA			
Rat Adam23 forward	GACTGCAGTGGTGCTCACGTAG	69.5	10	445 (possible different isoforms)
Rat Adam23 reverse	CTCCAGTGCAGCTGATTCAGATG			
Rat Lgi2 forward	GGTTCCCATCATCTTGCAGT	66	10	275
Rat Lgi2 reverse	ACGTGTAGTCACTCCCCAGG			
Rat Lgi3 forward	CCTTGTAATCGCCTAGCTGC	66	10	328
Rat Lgi3 reverse	TGGGATGAGAAAGGAGATGG			

Figure S1

RT-PCR analysis of Lgi2, 3, 4 and Adam11, 22, and 23 expression in different tissues and cell types. (a) Lgi4 and Adam22 are expressed in embryonic DRGs and sciatic nerves of mice at different stages of postnatal development. Embryonic DRGs at this stage contain both sensory neurons and Schwann cell precursors; therefore both cell types could express Lgi4 and Adam22. Expression in sciatic nerve most likely represents expression in Schwann cells, as these cells are the most numerous. However, as some neuronal RNAs are transported down the axon, we cannot rule out significant neuronal expression from these data alone. Different isoforms of Adam22 are expressed as a result of differential splicing of a number of exons encoding the C-terminal intracellular portion of the protein (Sagane et al. 2005; Godde et al. 2007). The differences in length of the amplified Adam22 cDNAs represent different mRNA species expressed in neurons and Schwann cells (Sagane et al. 2005). (b) To establish the cell types within DRGs and nerves that express Lgi4 and Adam22 and their close relatives Lgi2, Lgi3 and Adam11, Adam23 respectively, we examined purified cultures of rat neurons and rat Schwann cells and co-cultures of the two cell types. Whereas neuronal cultures always contain small numbers of fibroblasts and Schwann cells, Schwann cell cultures are absolutely devoid of neurons and are virtually free of fibroblasts. RT-PCR analysis shows that Lgi4 is expressed in sensory neurons and in Schwann cells, in accordance with our previous results (Bermingham et al. 2006). Also Adam22 is expressed in neurons and in Schwann cells. This is also true for Lgi2 and 3 and for Adam11 and 23. In addition, possible different isoforms of Adam23 may be present, mainly in the Schwann cells. Differential splicing of 3’ exons have been described in mouse and human but not in rats (Sun et al. 2004). Note that the Adam22 RT-PCR here does not differentiate for different isoforms.


a
E13.5 DRGs
Sciatics
P4
P8
P16
Adult
805
514
Lgi4
Adam22
b
Neuron only
Co-culture
Mye. co-culture
Sch. cells
Diff. Sch. cells
448
339
264
200
Adam11
Adam23
Lgi2
Lgi3

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Chapter 4

Myelination depends on a critical interaction surface of LGI4

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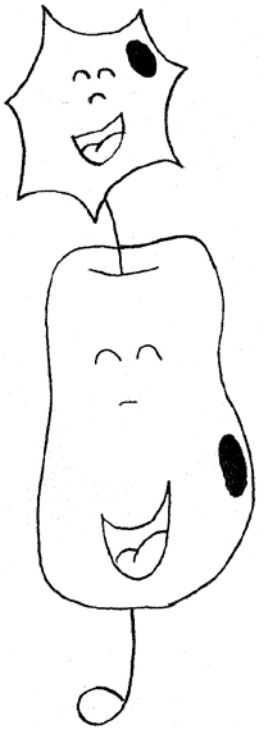
Submitted for publication

Chapter 5

Discussion



5 – Discussion

Demyelination or errors in peripheral myelin development cause serious neurological disease. Continuous communication between axons and Schwann cells is crucial for the proper development and maintenance of the myelin sheath. As a consequence, a deeper understanding of the precise molecular mechanisms that govern these interactions is highly important and will eventually lead to improved therapies for myelin-associated disorders. Various types of molecules, including secreted and transmembrane proteins, mediate this communication. In this project, we have shown that the interaction between Schwann cell-derived LGI4 and axonal ADAM22 is essential for proper axon ensheathment and myelin formation in the peripheral nervous system (PNS). While also other LGI family members are expressed in the PNS and interact with ADAM22, the role of LGI4 in myelination is specific. This specificity critically depends on a three amino acid motif that forms a cluster on the outer surface of the LGI4 epitempin domain and provides a specific interaction interface with ADAM22 and/or accessory proteins.

5.1 The cellular source of LGI4 in the developing peripheral nerve

Claw paw mice display severe hypomyelination in the PNS due to a mutation in *Lgi4* (1-3). *In situ* hybridization experiments in P4 mouse sciatic nerves showed that *Lgi4* is expressed by Schwann cells, and not by neurons (1). However, the RT-PCR experiments in chapter 3 suggest that *Lgi4* is also expressed in neuron only cultures. It should be taken into account that *Lgi4* expression in these RT-PCR experiments is not quantified and does not give any information about the actual protein levels. Schwann cell specific knock down of *Lgi4* mRNA resulted in a decrease of myelin internodes in wild-type Schwann cell-neuron cultures, suggesting that Schwann cells are the principal source of LGI4 (1). To address the cell-autonomous function of LGI4 in the PNS, mice were generated that carry a conditional null allele of *Lgi4*. In *Wnt1Cre: Lgi4*$^{Lox/Lox}$ mice *Lgi4* was specifically deleted during embryonic development in Schwann cells, sensory neurons (but not motor neurons), and endoneurial fibroblasts. In *DhhCre: Lgi4*$^{Lox/Lox}$ mice *Lgi4* was specifically deleted in Schwann cells and endoneurial fibroblasts only. At P12, both mutant mice presented an identical hypomyelination phenotype,

demonstrating that Schwann cells are the principal source of LGI4 in the developing peripheral nerve. Accordingly we could restore myelination in claw paw Schwann cell-neuron cultures that normally fail to myelinate by introducing *Lgi4* selectively in Schwann cells using retroviral transduction.

5.2 LGI4-ADAM22 interactions in peripheral nerve myelination

The claw paw mutation is an insertion of a repeat element in the 3rd intron of the *Lgi4* gene. Consequently, exon 4 is skipped during mRNA processing resulting in a protein with an internal deletion in the Leucine-rich repeat (LRR) domain. As a result, $LGI4^{clp}$ is retained in the cell causing severe hypomyelination (1). Additionally, LGI4 conditioned medium initiates myelination in claw paw Schwann cell-neuron cultures (1). This shows that LGI4 secretion is crucial for proper myelination and the functional development of the nerve. Also, LGI4 might be involved in communication between Schwann cells and neurons by interacting with proteins outside the Schwann cell. Identification of the LGI4 receptor is critical in regard to LGI4 function during myelination. A good candidate receptor was shown to be ADAM22; LGI1 is highly similar in structure to LGI4 and binds synaptic ADAM22 in the CNS (4). ADAM22 is also expressed on axons and possibly Schwann cells in the peripheral nerve. Additionally, both claw paw mice and *Adam22* knock out mice show severe peripheral nerve hypomyelination and limb abnormalities, suggesting that both proteins are involved in the same signaling pathway. Indeed, we could show that LGI4 directly binds ADAM22. This binding is dependent on an intact ADAM22 disintegrin-like domain, since ADAM22-Fc with the D509N (Asp to Asn substitution at position 509) disintegrin-like domain mutation does not precipitate LGI4 (5). This mutation is analogous to the *ju160* mutation of *C.elegans Adm1/Unc-71*, which causes defects in axon guidance and sex myoblast migration (6). Accordingly, the ADAM22 disintegrin domain binds specific integrins expressed on cultured cells (7). Thus, LGI4 could have an effect on ADAM22-integrin binding by binding the disintegrin domain of ADAM22.

RT-PCR detected *Adam22* mRNA expression in both neurons and Schwann cells. Thus, LGI4 might be involved in paracrine signaling from Schwann cells to axons, or autocrine signaling in Schwann cells. We have shown that wild-type rat Schwann cells do not myelinate ADAM22 deficient neurons in culture. Additionally, myelination is not affected when *Adam22* is specifically deleted in Schwann cells. Together, these data clearly demonstrate that

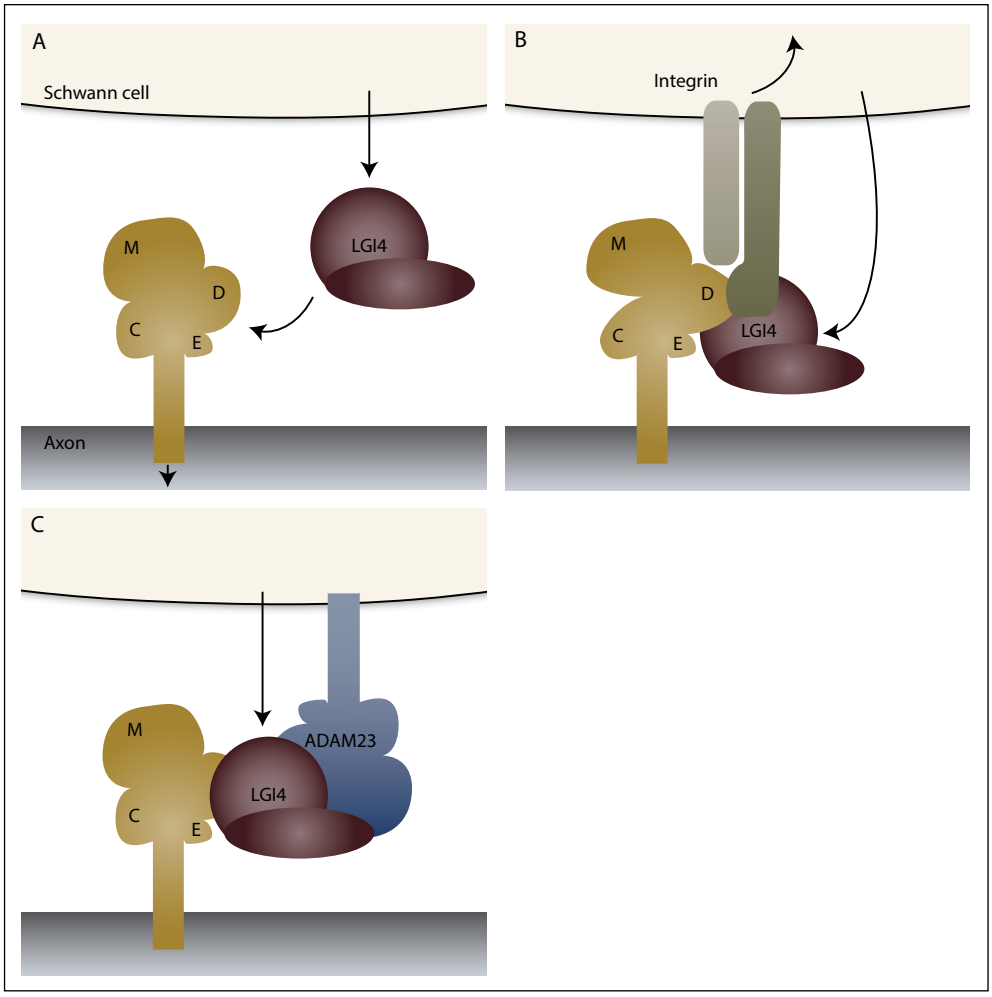


Figure 1. Possible mechanisms by which LGI4-ADAM22 interactions mediate PNS myelination

A) LGI4 is secreted by Schwann cells and binds the disintegrin domain of ADAM22. This interaction could trigger signaling or protein localization within the axon and/or cause a conformational change in ADAM22 (8). B) Binding of LGI4 to the ADAM22 disintegrin domain might cause a conformational change in ADAM22, which has an effect on interactions between ADAM22 and other ADAM22 binding partners such as integrins. C) LGI4 might form a bridge between ADAM23 (or ADAM11 not depicted here) on the Schwann cell and ADAM22 on the axon. M, metalloprotease-like domain; D, disintegrin-like domain; C, cysteine-rich domain; E, EGF-like domain.

ADAM22 is required on the axonal membrane for Schwann cells to myelinate. This demonstrates that LGI4 is secreted by Schwann cells and acts by binding to axonal ADAM22 (Figure 1A).

A major obstacle to further understanding the nature of LGI4 function has been the difficulty in detecting LGI4 and ADAM22 in tissues and cells. To date, we could not visualize LGI4 binding to endogenous axonal ADAM22 in neuron only cultures, which is most probably due to the diffuse distribution of ADAM22 on the axon. However, when membrane fractions of neuron only cultures, either derived from wild-type or *Adam22* knock out mice, are analysed by Western blot, LGI4 is only detected in the membrane fractions of wild-type-derived samples (G. Abello, unpublished results). This suggests that LGI4 needs ADAM22 for docking to the membrane.

LGI proteins contain a leucine rich repeat (LRR) domain and an epitempin (EPTP) domain, which are both known to be capable of protein-protein interactions. We have shown that the LGI4-EPTP domain is responsible for binding to ADAM22. However, LGI1-3 also bind to ADAM22. Using a Schwann cell-neuron culture system derived from claw paw embryos we demonstrated that LGI4 function is specifically required in Schwann cells to stimulate myelination as its function cannot be replaced by any of the other LGI proteins. We demonstrated that this specificity depends on a protein sequence within the carboxyl-terminal half of LGI4-EPTP. To further identify functionally relevant sequences within LGI4 we took advantage of more than 400 million years of independent evolutionary history between modern day teleost fishes and mammals. To date, comparative genomics has failed to identify an *Lgi4* homologue among the five *lgi* genes in the zebrafish genome (a teleost fish). However, we found that zebrafish Lgi1a and Lgi1b can functionally replace mouse LGI4 and stimulate myelin formation in our claw paw Schwann cell-neuron culture system. Multi-species alignment is a powerful tool for identifying functionally relevant amino acids. Close examination of the sequence alignments between fish Lgi1a-1b and mammalian LGI4 proteins identified three conserved amino acids that closely map to the outer surface of LGI4-EPTP. Introducing these amino acids in LGI1 transformed LGI1 into a myelination-stimulating protein, proving that these amino acids determine LGI4 specificity in myelination. Mapping the position of these three amino acids shows that their side chains are solvent exposed and form a potential interaction interphase at the side of the donut shaped EPTP domain. In these rescue experiments one of these amino acids replaced an LGI1 glycosylation site (N422) (9). As a consequence we could not rule out that changing LGI1

into a myelination-stimulating molecule was due to removal of the glycosylation site. However, none of the LGI1 proteins encoded in non-mammalian tetrapod genomes are glycosylated at this site. It is therefore unlikely that sugar side chains obstructing a potential interaction surface cause the lack of myelination-stimulating properties in mammalian LGI1. As expected, transducing the glycosylation mutant LGI1^{N422Q} in claw paw Schwann cell-neuron cultures did not restore myelination. Thus, removing the sugar modifications on this side of the LGI1 protein does not unmask a latent myelin-promoting activity. It is more likely that the three amino acids we identified here are part of an interaction interphase that is functionally significant. Another LGI1 glycosylation site, N192, is located within the carboxyl-terminal portion of the LRR domain and is conserved among all LGI members. N277 is part of a glycosylation site in some LGI1 and LGI2 orthologs. The N422 is specific for LGI1 (9). A triple glycosylation mutant of LGI1 is not secreted and secretion of the LGI1^{N192Q} mutant is severely diminished, underscoring the importance of these glycosylation sites for normal maturation and secretion of LGI1. As a consequence, secretion of the LGI1^{N422Q} mutant might also be severely diminished. We showed that the secretion of LGI1^{N422Q} was not affected and that the lack of myelination-stimulating activity is not due to reduced amounts of secreted protein. LGI4 is glycosylated at the LRR-CT site (N177) (1). However, introducing the LGI4^{N177Q} mutant in claw paw Schwann cell-neuron cultures restores myelination while secretion is severely affected but not absent (unpublished data). This suggests that the glycosylation site in LGI4 is not crucial for its function in myelination.

Despite the fact that both LGI1 and LGI4 biological functions are mediated through ADAM22, the functional outcome of these interactions is distinct. It is suggested by the crystal structure of ADAM22 that ligand binding to the ectodomain causes a conformational change (8). Therefore, LGI4 binding might influence ADAM22 interactions with other molecules, for example with integrins (7, 10) on the adaxonal side of the Schwann cell (Figure 1B). However, so far it is not known which integrins reside here. Another possibility is that the LGI4 specific amino acids cause an interaction with as yet unknown proteins that reside either on the axon or the Schwann cell. These interactions might lead to recruitment of these proteins to the ADAM22-LGI4 complex. Interestingly, ADAM22 and ADAM23 co-assemble in the brain and this is dependent on LGI1 (11). Thus, it was suggested that LGI1 forms a bridge between presynaptic ADAM23 and postsynaptic ADAM22. Using RT-PCR we detected *Adam23* and *Adam11* expression in both neuron only and rat Schwann cell cultures. If ADAM23 and ADAM11 are actually localized in the Schwann cell membrane, a

similar model can be hypothesized for LGI4, in which LGI4 could form a bridge between ADAM23 and/or ADAM11 on the Schwann cell and ADAM22 on the neuron (Figure 1C).

Since all LGI family members bind ADAM22 we expected a dominant negative effect on myelination in *Lgi1-3* infected wild-type cultures. However, quantification of the amount of myelin sheaths and MPZ protein showed that this was not the case. A possible explanation is that LGI4 binds ADAM22 more efficiently as compared to other LGI family members. This might be due to a difference in binding efficiency or by LGI4 specific binding partners that strengthen the interaction with ADAM22. Determination of the relative affinities of LGI's for the ADAM receptors would improve our understanding of the functional relationship between these proteins.

Also, LRR domains are known to be capable of protein-protein interactions. We showed that the LGI4-LRR domain is interchangeable with the LRR domain of the other LGI family members. However, the LGI4-EPTP domain alone cannot restore myelination in claw paw Schwann cell-neuron cultures, showing that the LRR domain is crucial for the function of LGI4. One possibility is that the LGI4-LRR domain interacts with a binding partner that is shared by other LGI family members, but is crucial for its function. Interestingly, LGI1 is secreted as an oligomer (4) and we have shown recently that LGI4 is capable of forming a dimer (unpublished data). The LGI-LRR domain is most homologous to Slit proteins (12), which also form dimers (13), suggesting that LGI protein dimerization is caused by the LRR domain.

How LGI4-ADAM22 interactions lead to myelination of the Schwann cell is so far unknown. Changes in protein expression and/or localization on the Schwann cell membrane and changes in cytoskeleton dynamics are all possibilities. We found that NRG1 is expressed on the axonal membrane of *Adam22* knock out neurons and that these membranes activate PI3K and ERK as effectively as membranes derived from wild-type neurons, suggesting that LGI4-ADAM22 does not function by modulating NRG1-ErbB2/3 interactions. Since ADAM22 is able to interact with integrins it would be interesting to test whether integrin signaling is affected in *Lgi4* and *Adam22* mutant mice. Introducing constitutively active ILK or RAC1 (two proteins involved in integrin signaling) into the Schwann cells of DRG co-cultures derived from these mice could test this hypothesis.

5.3 The role of LGI4 in myelination of the peripheral nervous system

Lgi4 is initially expressed by neural crest stem cells but its expression becomes restricted to Schwann cells, enteric glia and satellite cells. Importantly, both LGI4 and ADAM22 are not required for glial fate determination, but are essential for proliferation of enteric glia and satellite cells and not for Schwann cell proliferation (14). This demonstrates that different types of PNS glial cells have different requirements for LGI4 and ADAM22. Thus, LGI4-ADAM22 interactions have multiple functions including proliferation of enteric glia and satellite cells in PNS, and later myelin formation in Schwann cells. LGI4 binding to specific accessory proteins thereby activating distinct signalling pathways in different cells within the glial lineage may cause these distinctive effects.

To date, the exact stage in Schwann cell development during which LGI4 action takes place has not been defined. Schwann cell development involves multiple transitional stages; when neural crest cells (NCCs) have migrated out to contact developing axons, these cells are specified to form Schwann cell precursors (SCPs), which then proliferate and give rise to immature Schwann cells. When these cells associate with large caliber axons they will sort and myelinate this axon. To get a better idea about in which stage of PNS development LGI4-ADAM22 interactions play a crucial role we need to know when the proteins are actually expressed. In the future we will test membrane fractions derived from sciatic nerves at different time points in development to determine the relative amount of LGI4 and ADAM22 proteins.

Claw paw and *Adam22* knock out mice show a delay in axonal sorting, suggesting that LGI4-ADAM22 interactions at least have a role in this stage of development. β1-integrin mutants show a delay in axonal sorting and have impaired myelination also (15, 16). Schwann cells devoid of β1-integrin migrate to and elongate on axons but cannot extend radial lamellae of cytoplasm. Activation of RAC1 by β1-integrins allows Schwann cells to switch from migration/elongation to the extension of radial membranes required for axonal sorting and myelination. ADAM22 binds integrin αvβ3 and α9β1 though its disintegrin-like domain (7). Since LGI4 binds the ADAM22 disintegrin domain as well it is tempting to suggest an interaction between LGI4-ADAM22 and the integrin pathway (Figure 1B). Measuring RAC1 activity in *Lgi4* and *Adam22* knock out mice will be required to test this hypothesis.

Since myelin is not properly formed in *Lgi4* and *Adam22* knock out mice, we cannot rule out that LGI4-ADAM22 interactions are also required for myelin maintenance and/or repair. Selectively deleting *Lgi4* in Schwann cells of adult

mice in a tamoxifen-dependent manner will show whether LGI4 functions in myelin maintenance. Similarly, deletion can be induced after nerve injury to test if LGI4 is required for repair.

In summary, future experiments will increase our knowledge about new binding partners of both LGI4 and ADAM22 and the result of their interactions. Hopefully, once we understand the mechanisms of these interactions, this fundamental knowledge will lead to improved therapies for peripheral myelination disorders.

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Summary

Our actions are coordinated by the nervous system, which passes signals from one site of the body to the other in the form of electrical currents. The isolating layer of myelin that surrounds the axons of neurons makes it possible that these signals are processed faster. Myelin is a specialized membrane containing specific proteins and lipids and is produced by glia, which wrap around the neuronal axon multiple times. Along these axons we find gaps that are not covered by myelin, called "Nodes of Ranvier". The electrical signal jumps from one node to the next, increasing the signal transduction 100 times compared to non-myelinated axons. Hence, it is not surprising that defects in myelination cause serious neurological disease like Multiple Sclerosis and Charcot-Marie-Tooth disease.

The glia cells that make myelin in the peripheral nervous system (PNS) are called Schwann cells. The continuous communication between Schwann cells and neurons is essential for the development, differentiation and myelination of peripheral nerves. Although some molecules involved in this are known, details about the mechanisms involved are still largely unclear.

Lgi4 (Leucine-rich glioma inactivated 4) is mutated in claw paw mice, of which myelination of the peripheral nerves is severely delayed. The claw paw mutation results in a very strong reduction of the secretion of the LGI4 protein. This shows that LGI4 secretion is highly important for proper myelination and the functional development of the nerve and that LGI4 most probably is involved in communication between Schwann cells and neurons.

In this thesis we show that LGI4 binds the extracellular domain of ADAM22, a member of the ADAMs (A Disintegrin And Metalloprotease) receptor family. LGI4 and ADAM22 are both expressed in Schwann cells and neurons. Using tissue specific gene ablation approaches, we showed that *Lgi4* expression from Schwann cells and *Adam22* expression from neurons are necessary for myelination. We propose that axonal ADAM22 functions as a receptor for Schwann cell secreted LGI4. This interaction may induce activation of signaling pathways that start myelination in Schwann cells.

Since *Lgi1-3* are also expressed in the PNS and bind ADAM22, we asked whether these proteins are functionally equivalent to LGI4 in the PNS and, additionally, what protein domains within the LGI proteins are relevant for stimulating myelin formation. Using a Schwann cell-neuron culture system derived from claw paw embryos we demonstrated that myelination

specifically requires LGI4 as its function cannot be replaced by other LGI proteins. Furthermore, we show that this specificity critically depends on protein sequence within the carboxyl-terminal half of the epitempin domain of LGI4. Zebrafish Lgi1a and Lgi1b could functionally replace LGI4 and stimulate myelin formation in our claw paw Schwann cell-neuron culture system. Close examination of sequence alignments of fish Lgi1a-1b and mammalian LGI4 proteins identified three amino acids that closely map to the outer surface of the epitempin domain, suggesting that this patch represents an interaction domain that uniquely distinguishes LGI4 function from that of LGI1. Introducing these amino acids into LGI1 confers myelination-stimulating activity and thus provides proof of the specific functional interaction interface with ADAM22 and/or accessory proteins.

Identifying such accessory proteins is essential to fully understand the function of LGI4. For example, these three LGI4 specific amino acids might cause a conformational change in ADAM22, which can influence ADAM22-integrin interactions. Alternatively, LGI4 binding to ADAM22 results in recruitment of unknown proteins to the LGI4-ADAM22 complex. It is hoped that once we understand the mechanism of LGI4-ADAM22 interactions in developmental myelination and in repair following nerve trauma, this fundamental knowledge will lead to improved therapies for peripheral myelination disorders.

Nederlandse samenvatting

Het zenuwstelsel coördineert onze acties waarbij elektrische signalen door worden gegeven tussen verschillende lichaamsdelen. Dankzij de isolerende myeline laag die om de axonen van neuronen ligt kan een signaal sneller doorgegeven worden. Myeline bestaat uit eiwitten en lipiden die geproduceerd worden door glia cellen die zich meerdere malen om het axon van een neuron winden. Langs het axon bevinden zich uitsparingen waar het axon niet bedekt is met myeline, ook wel de "knopen van Ranvier" genoemd. Het elektrische signaal springt van knoop naar knoop waardoor het signaal 100 maal sneller wordt doorgegeven vergeleken met niet-gemyeliniseerde axonen. Het is dus niet verwonderlijk dat defecten in het myelinisatie proces ernstige neurologische ziekten veroorzaken zoals Multiple Sclerose en de ziekte van Charcot-Marie-Tooth.

De glia cellen die axonen in het perifere zenuwstelsel myeliniseren worden Schwann cellen genoemd. De constante communicatie tussen Schwann cellen en neuronen is essentieel voor de ontwikkeling, differentiatie en myelinisatie van perifere zenuwen. Hoewel een aantal van de moleculen die hierbij zijn betrokken bekend zijn, is er nog veel onduidelijkheid over de moleculaire mechanismen die bij deze processen betrokken zijn.

Lgi4 (Leucine-rich glioma inactivated 4) is gemuteerd in claw paw muizen, waarvan de myelinisatie van de perifere zenuwen ernstig is vertraagd. De claw paw mutatie leidt tot een zeer sterke reductie van de secretie van het LGI4 eiwit. Dit duidt er op dat de secretie van LGI4 een belangrijke rol speelt in myelinisatie en de functionele ontwikkeling van de zenuw en dat LGI4 hoogst waarschijnlijk betrokken is bij communicatie tussen Schwann cellen en neuronen.

In dit proefschrift wordt aangetoond dat het LGI4 eiwit bindt aan het extracellulaire domein van ADAM22, welke behoort tot de familie van ADAM (A Disintegrin And Metalloprotease) receptoren. *Lgi4* en *Adam22* genen komen in zowel de Schwann cellen als de neuronen tot expressie. Door gebruik te maken van weefsel specifieke uitschakeling laten we zien dat *Lgi4* voornamelijk in Schwann cellen geëxpresseerd wordt en dat *Adam22* expressie cruciaal is in neuronen. We stellen voor dat axonaal ADAM22 een receptor is voor het door Schwann cellen uitgescheiden LGI4 eiwit en dat deze interactie kan leiden tot activatie van signalen die de Schwann cel aanzetten tot myelinisatie.

Aangezien *Lgi1-3* ook in het perifere zenuwstelsel tot expressie komen en binden aan ADAM22 hebben we getest of deze eiwitten functioneel equivalent zijn aan LGI4 in het perifere zenuwstelsel en daarnaast, welke LGI eiwit domeinen relevant zijn voor het stimuleren van myelinisatie. Door gebruik te maken van Schwann cel-neuron cultures verkregen van claw paw embryos hebben we laten zien dat LGI4 een specifieke functie heeft in myelinisatie, aangezien LGI1-3 niet kunnen compenseren voor het verlies van LGI4 secretie in deze cultures. Verder laten we zien dat deze specificiteit afhankelijk is van een eiwitsequentie in de carboxyl-terminale helft van het epitempin domein van LGI4. Zebravis Lgi1a en Lgi1b kunnen LGI4 functioneel vervangen en myelinisatie stimuleren in ons claw paw Schwann cell-neuron culture systeem. Zorgvuldige bestudering van de sequenties van vis Lgi1a-1b en zoogdier LGI4 eiwitten identificeerde drie aminozuren die samen een uniek interactiedomein zouden kunnen vormen en LGI4 functie zouden kunnen onderscheiden van LGI1. Het introduceren van deze aminozuren in LGI1 veranderde de myelinisatie-stimulerende activiteit en bewijst dat deze aminozuren een specifieke functionele interactie interface vormen met ADAM22 en/of andere eiwitten.

Identificatie van deze andere eiwitten is noodzakelijk voor het begrijpen van LGI4 functie. Deze drie aminozuren zouden bijvoorbeeld een conformatie verandering in ADAM22 kunnen veroorzaken, wat de ADAM22-integrin interactie zou kunnen beïnvloeden. Een andere mogelijkheid is dat LGI4 binding aan ADAM22 resulteert in het rekruteren van onbekende eiwitten naar het LGI4-ADAM22 complex. Hopelijk zal het begrijpen van de rol van deze interacties in myelinisatie en herstel na zenuw schade leiden tot het verbeteren van therapieën voor perifere neurologische ziekten.

Curriculum Vitae

Personal details

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Education:

2009-2013	PhD student Department of Cell Biology and Genetics, Erasmus University Medical Centre, Rotterdam, The Netherlands
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2003–2006	Bachelor Biomedical Sciences, Leiden University, Leiden, The Netherlands Part of second year at Karolinska Institutet, Stockholm, Sweden
1997-2003	High School S.G. Spieringshoek, Schiedam, The Netherlands

Research experience

2009-2013	PhD project Department of Cell Biology and Genetics, Erasmus University Medical Centre Prof. Dr. Ir. Dies N. Meijer (promotor, supervisor) “LGI4-ADAM22 interactions in peripheral nerve development”
2008-2009	Masterinternship research project Department of Neuroscience, Erasmus University Medical Centre Prof. dr. C. Hoogenraad (principal investigator), dr. S.A. Spangler (supervisor) “Characterization of the Liprin-binding proteins KIF21B and p140cap”
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Department of Cellular and Molecular Neurobiology, Vrije Universiteit Amsterdam
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"The hippocampal distribution of doublecortin and calretinin in CARP overexpressing mice"

List of Publications:

MacGillavry, H. D., Stam, F. J., Sassen, M. M., **Kegel, L.**, Hendriks W.T., Verhaagen, J., Smit, A. B., van Kesteren, R. E. NFIL3 and cAMP response element-binding protein form a transcriptional feedforward loop that controls neuronal regeneration-associated gene expression. J. Neurosci. 2009 Dec 9; 29(49):15542-50.

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Kegel, L., Driegen, S., Leslie, K., Jaegle, M., Meijer, D. Myelination depends on a critical interaction surface of LGI4. Submitted to Nature

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PhD Portfolio

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PhD Training	**Year**
General courses	
Working with test animals	2009
Safe laboratory techniques	2009
Literature course	2010
Adobe Photoshop and Illustrator Course	2011
Specific courses	
Molecular Cell Biology	2009
Signal Transduction	2009
From Development to Disease	2011
Ensembl	2011
(Inter)national conferences	
Winter School Kleinwalsertal, Austria (oral presentation)	2010
Neuron-Glia Interactions in Development and Disease meeting 2010, Rome (oral presentation)	2010
Federation of European Neuroscience forum 2010, Amsterdam (poster)	2010
19th MGC Symposium, Rotterdam	2010
Cancer Genomics Center meeting, Utrecht	2010
Intercity Young Scientist Meeting, Apeldoorn (oral presentation)	2010
Neuron-Glia Interactions in Development and Disease meeting 2011, Mallorca (oral presentation)	2011
ISN Myelin Meeting 2011, Crete (poster)	2011
Dutch Developmental Biology meeting 2011	2011
20th MGC Symposium, Leiden	2011
Gordon Conference (Myelin) 2012, Italy (poster)	2012
Dutch Developmental Biology meeting 2012	2012
21st MGC Symposium, Leiden (oral presentation)	2012
Dutch Developmental Biology meeting 2013 (oral presentation)	2013

Seminars and workshops	
Cell Biology and Genetics Department guest lectures	2009-2013
Department of Neuroscience seminars	2009-2013
16th MGC PhD student workshop, Brugge	2009
17th MGC PhD student workshop, Cologne (poster)	2010
18th MGC PhD student workshop, Maastricht (oral presentation)	2011
PhD Career Day, Rotterdam	2012
Presentations	
Monday Morning Meeting, Cell Biology	2009-2013
Department Journal Club	2009-2013
Supervising practicals and excursions, Tutoring	
Junior Science Program	2011
Master students thesis projects	2009-2013
HLO student supervision	2009-2013
Other	
Organizer 18th MGC PhD student workshop, Maastricht	2011

Acknowledgements

Doing a PhD project and writing a thesis was only possible with the help of many great colleagues, friends and family who supported me. Thanks to them, these past four years have been an incredible experience and I have enjoyed every second of it. This, of course, means I want to thank a lot of people:

First of all I would like to show my deepest gratitude to my promotor and supervisor Prof. Dies Meijer. Thank you Dies, for giving me an opportunity to explore this fascinating project. Thank you for your immeasurable patience and always being there to answer my endless questions, which has broadened my knowledge and thinking. Your door was always open (although you sometimes locked the office with me still in it!!!). Thanks to you, I could go to great meetings and congresses to meet new people and talk about my results and future career. You are a great mentor and also very generous. Thank you for all your delicious cooking and taking us out for dinner so many times! It is great to know that we will be working in the same department again by the end of this year, in Edinburgh. This means I don't have to miss your statements like "It clones like a tiger", and we can still update each other about nice music.

Prof. Frank Grosveld. Thank you for giving me the opportunity to work in this fantastic department, with so many things to learn and many fabulous colleagues. Thank you for being in my PhD committee.

The small committee who critically read this thesis: Niels, Willy and David. Thank you for all your critical comments, valuable suggestions and corrections. Niels, thank you for coordinating the PhD program, and for all your Apple-updates. Willy, thank you for the nice discussions we had. David, I am really excited that I will soon be working in your lab. I am looking forward to your visit to Rotterdam to discuss my thesis. Ype and Sjaak, thank you for taking the time to be in the PhD committee.

The Schwann cell group: Martine, The Sweet and Charming Lady. Thank you for all your patience, help and answering my endless list of questions. I was so lucky to have you as a mouse/ams-database mentor! Thank you for calming me down during the last few months when I was over the top stressed with the thesis work. I will miss all the French-speaking people surrounding me during pipetting, when they came to chat with you or have a coffee… Merci beaucoup!

I am looking forward to see you in Edinburgh again when you come to visit us! Siska, 1000x thank you for teaching me different techniques and for everything you do for the lab. Your assistance in my project was of a great value. Thank you for your inputs in making this thesis a reality.

I would also like to thank all the former members of the lab, who helped me a lot during their stay here: Sweet Noorie, my paranimph. Thanks for all your great help. You are a super kind and enthusiastic girl. I learned so much from you; not only about techniques but also about life in general…. You are never afraid to try new things. I will never forget the Death march we did in Belgium (and that you finished it two times!!!). I was honored to be present when you got your swimming diploma A (I still think you were the best of the whole group, especially watertrappelen!). I hope one day you will go for diploma B! Gina, I also learned a lot from you. I really enjoyed long discussions about our partly overlapping projects. You were always an excellent example in the lab and I appreciate your dedication and confidence. I am very glad you found a nice project in Barcelona. Eerik, thank you for all our delightful conversations about music. I am honored to have shared a bench with an Estonian artist! I wish you all the best in your future career. Ekim, I really enjoyed working with you on the LGI4 project during my first year in the lab. I hope you will like the outcome of this project! All the former students: Ali, Slawek, Cheryl, Gijs, Elif, Shane, Jim, Aschwin, Mike, Sayanne and Annelies, thanks for all your efforts (and teaching me how to supervise students).

Elaine, an unofficial Schwann cell family member, thank you for all the expert advices, checking drafts of the papers, and all the encouragements.

My paranimph Erik. Thank you Erik for all your generous help. I really enjoyed organizing the PhD workshop with you. You have a talent in organizing but also in creating nice and funny videos with the rest of your lab. I wish you all the best with the rest of your PhD research.

Marike, Jasperina, Bep, Marielle, computer guys (Pim, Leo, Sjozef and Nils), Melle, Arthur, Leo, Rini, Sonja, Benno, Marjoleine, Marja and the kitchenladies thanks for helping with all those things that no scientist wants to deal with. Alex, and all the people from EDC, thanks for all your help with the mice. I also want to thank Gert, Kerstin and Bob for taking care of the ML labs. Jeroen, thank you for your help with the mass spectrometry.

Big thanks to the rest of the department: Your input was indispensable. I have

met so many great people that have contributed to an unforgettable four years. There was time for work and time for fun ("networking"). With joy I will think about the meetings, workgroups, congresses, dinners, music festivals and parties. Kris, thank you for helping me out with modeling of the LGI proteins and checking some chapters of my thesis. I hope all the Scottish are as friendly and as funny as you are! Luna, thanks for being one of my lunch buddies and for the nice chit-chats and help with planning my defence. Mehrnaz, although you were not officially working in the Schwann cell lab during my PhD project it feels like you did. Thanks for all the nice discussions and encouragement. Wormlab, it was fun to have you as our new neighbors! Gert, thank you for organizing the journal club. It is a great way to discuss interesting papers and techniques that can be applied (or not). Suzanne, you are so friendly and always willing to help. Aniek, you are a sweet and intelligent girl and I am glad I was your colleague. Weng Yee, I was very honored to be at your wedding in KL. You and your family and friends were so generous! Good luck with finishing the last bits of your thesis. Joost, thanks for all the nice discussions. Toroti, you were always friendly and interested in how other people are doing. Thank you for all your advice for writing my thesis. There is still a long list of other people that I want to thank for the discussions and all the fun times: Dorota, Jeffrey, Thomas, Nesrin, Michaela, Chris, Fanny, Polynikis, Lalini, Jessica, Dave, Widia, Liu, Michael, Umut, Jean-Charles, Catherine R., Parham, Parisa, Gerben, Emma, Alvin, Derk, Alex, Kerstin, Robbert, Marjon, Anne, Marta, Heleen, Kim, Joshua, Marialuisa, Romana, Rick, Gert, Xiao, Ralph, Nynke, Divine, Maikel, Petra, Loes, Frank S., Natasja, Kishan, Koos, Maarten, Ernie, Maaike, Ruud, Raymond, Johan, Eveline, Aristea, Catherine D., Friedman, Federica, Francesca (thanks for all the great parties and nice Italian food!), Maureen, Stefan, Tracy, Cristina, Joost, Anton…. If I have missed mentioning someone here, blame it on my memory lapse due to late night thesis writing... It was a pleasure working with you pals. I wish you all the luck with your projects and life in general.

Carol, although you are an ex-Erasmus MC fellow now, I am so glad that we still catch up once in a while and update each other. You are so sweet and I wish you all the luck with your last bit of experiments and writing your thesis.

I also want to thank the people who guided me through my bachelor and master studies. Without their help there was no PhD career for me: Geert, thank you for supervising me during my bachelor thesis project and for introducing me to the scientific world. Prof. Erno Vreugdenhil and Ronald van Kesteren, thank you

for letting me work in your lab. Harold, thank you for supervising me during my first master project and letting me do all those exciting experiments, making me even more enthusiastic about neurobiology. Prof. Guus Smit, thank you for all your great advice and for being my mentor during the master of Neuroscience. Samatha, thank you for supervising me during my second master thesis project and helping me to get ready for my PhD journey. Prof. Casper Hoogenraad, thank you for letting me work in your lab in the Neuroscience department and for helping me find a PhD project. I would also like to thank the rest of the Neuroscience department for the nice discussions and fun times. I have a great memory of those days. Dick, special thanks for your help, also during my PhD project when we started working on KIF1B.

Al mijn familie en vrienden: Dank jullie voor alle steun en motiverende woorden. Jullie waren altijd erg geïnteresseerd in mijn project, ook wanneer jullie misschien geen idee hadden wat ik nou precies aan het doen was ("iets met eiwitten…"). Zonder jullie was het nooit gelukt.

Lieve papa en mama, jullie hebben me altijd de vrijheid en mogelijkheden gegeven te doen wat ik wilde en hebben mij gebracht waar ik nu ben. Bedankt voor al jullie liefde en steun.

Broertjes Koen en Tommie, jullie ook bedankt voor jullie steun en motivatie. Koen, bedankt voor al je tips bij de vormgeving van mijn thesis en voor je interesse in mijn project. Tommie, bedankt voor het aanhoren van mijn gezeur als er iets niet goed ging.

Lieve Arno, jij bent er altijd voor me. Ik prijs mezelf gelukkig dat ik elke dag weer mag genieten van je humor, positiviteit en zorgzaamheid. In Edinburgh en daarna, voor altijd.

Thank you all! Dank jullie wel!

Linde