

**From Lgi4 to Adam22:
Novel Players in Peripheral Nervous System Development
and Myelination**

Ekim Özkaynak

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Designed by Ekim.

Front cover: "Big Hug", A Schwann cell is about to hug a neuron.

Back cover: "SCG", An axon myelinated by two Schwann cells.

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*Van Lgi4 tot Adam22: nieuwe spelers in perifeer zenuwstelsel ontwikkeling
en myeline vorming*

Thesis

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Ekim Özkaynak

born in Ankara, Turkey



Doctoral Committee

Promoters:

Prof.dr. F.G. Grosveld
Prof.dr.ir. D.N. Meijer

Other Members:

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"If you try and take a cat apart to see
how it works, the first thing you have
on your hands is a non-working cat."
Douglas Noel Adams

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List of Abbreviations

- Adam22: A Disintegrin And Metalloprotease 22
 ADPEAF/ADLTE: autosomal-dominant partial epilepsy with auditory features/autosomal dominant lateral temporal epilepsy
 AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
 APP: Amyloid beta A4 protein
 BDNF: Brain Derived Neurotrophic Factor
 BMP: Bone Morphogenic Protein
 CAM: Cell Adhesion Molecule
 cAMP: cyclic Adenosine Monophosphate
 cDNA: complementary DNA
 Clp: Claw paw
 CMT: Charcot-Marie-Tooth
 CNP: 2',3'-cyclic-nucleotide 3'-phosphodiesterase
 CNS: Central Nervous System
 Dhh: Desert hedgehog
 DLG: Disks Large homolog
 DNA: Deoxyribonucleic Acid
 DRG: Dorsal Root Ganglia
 DRP2: Dystrophin Related Protein 2
 EAR/EPTP: Epilepsy Associated Repeat/Epitempin
 ECM: Extracellular Matrix
 EGF: Epidermal Growth Factor
 EGR2: Early Growth Response protein 2
 ERK: Extracellular signal Regulated Kinase
 ERM: Ezrin-Radixin-Moesin
 FAK: Focal Adhesion Kinase
 Fc: Fragment crystallizable region
 FGF: Fibroblast Growth Factor
 Fxyd3: FXYD domain-containing ion transport regulator 3
 GDNF: Glial cell-line Derived Neurotrophic Factor
 GGF: Glial Growth Factor
 GTP: Guanosine triphosphate
 HLH: Helix Loop Helix
 HNPP: Hereditary neuropathy with liability to pressure palsies
 Ig: Immunoglobulin
 IGF: Insulin-like Growth Factor
 ILK: Integrin Linked Kinase
 JNK: C-jun-amino-terminal kinase
 Kv: Voltage gated potassium channel
 Lgi4: Leucine-rich Glioma Inactivated 4
 LIF: Leukemia Inhibitory Factor
 LIM: Lin-Isi-Mec
 LPA: Lysophosphatidic acid
 LRR: Leucine-rich repeat
 MADM: Mammalian Disintegrin Metalloprotease
 MAG: Myelin Associated Glycoprotein
 MAGUK: Membrane Associated Guanylate kinases
 MAPK: Mitogen Activated Protein Kinase
 MBP: Myelin Basic Protein
 MPZ: Myelin Protein Zero
 mRNA: messenger RNA
 NaV: Voltage gated sodium channel
 NC: Neural Crest
 Necl: Nectin-like Protein
 NF155/NF186: Neurofascin
 Caspr: Contactin Associated Protein
 NFATc4: Nuclear factor of activated T-cells, cytoplasmic 4
 NFkB: Nuclear Factor kappa B
 NGF: Nerve growth factor
 Ngn: Neurogenin
 NMSC: Non-Myelinating Schwann Cell
 NrCAM: Neuronal cell adhesion molecule
 Nrg: Neuregulin
 NSCL: Nescient helix loop helix
 NT: Neurotrophin
 P2: Myelin P2 protein
 p75NTR: p75 Neurotrophin Receptor
 PC12: Pheochromocytoma cell line 12
 PDGF: Platelet Derived Growth Factor
 PDZ: PSD-Dlg-ZO1
 PI3: Phosphoinositide 3
 PKA: Protein Kinase A
 PKB: Protein Kinase B
 PLP/DM-20: Myelin Proteolipid Protein
 PMP22: Peripheral Myelin Protein 22
 PNS: Peripheral Nervous System
 POU: Pit-Oct-Unc
 PSD: Post Synaptic Density
 RNA: Ribonucleic Acid
 Robo: Roundabout
 ROCK: Rho Associated Protein Kinase
 RT-PCR: Reverse Transcription Polymerase Chain Reaction
 SCE: Schwann Cell-specific Enhancer
 SCP: Schwann cell Precursor
 SH3: Src homology 3
 Shh: Sonic hedgehog
 TACE: TNF-alpha Converting Enzyme
 TAG1: Transient Axonal Glycoprotein 1
 TGF: Transforming Growth Factor
 TNF: Tumor Necrosis Factor
 Trk: Neurotrophic Tyrosin Kinase receptor
 UTR: Untranslated Region
 Wg: Wingless

Aim and scope of this thesis

The evolution of complex nervous systems from simple sensory apparatuses in animals provides great advantages to these organisms. Not only does this facilitate their interactions with the outside world, but it also helps them to regulate homeostasis. Like all information transfer protocols, speed is a very important aspect of nerve impulse propagation. Neurons themselves are capable of very fast nerve conduction velocities, but myelination by glial cells gives a new meaning to the term conduction velocity as it cranks up the speed by two orders of magnitude. In the peripheral nerves of higher vertebrates, large calibre axons are myelinated by Schwann cells, whereas small calibre axons are supported by non-myelinating Schwann cells. Development and myelination of peripheral nerves involves the intimate association of neurons and glia, and the molecular cross-talk between axons and Schwann cells. Whereas several mechanisms both in the extra- and intracellular environments have been studied extensively, many unknowns governing the development and myelination of the peripheral nervous system remain. The main aim of this thesis is to identify and characterize such mechanisms, and the molecules involved. Throughout this project, we have worked with Claw paw, Lgi4, and Adam22 mutant mice. These animals display similar limb abnormalities, and hypomyelinated peripheral nerves. Combined efforts from our lab and our collaborators enabled us to characterize several aspects of these phenotypes, and to study the role of Lgi4 and Adam22 proteins.

Chapter 1 of this thesis presents a general introduction on the cells of the nervous system, and their function. It continues with aspects of peripheral nervous system development related to myelination by Schwann cells, including the role of several proteins and the outcome of their dysfunction. Chapter 2 describes the identification of Lgi4 as a molecule involved in myelination. Data presented in this chapter shows that a mutation in the Lgi4 gene causes the Claw paw phenotype. This finding is followed by the initial characterization of Lgi4 protein, and its participation in myelination. Chapter 3 identifies the interaction between Lgi4 and Adam22, and the cellular compartments of their action. Chapter 4 deals with the functional implications of the Claw paw mutant form of Lgi4, and the differences observed in Claw paw and Lgi4 knock out mice. Finally, Chapter 5 gives a comprehensive discussion of the results obtained, together with some future perspectives and concluding remarks.

Chapter 1

Introduction

1 - INTRODUCTION

1.1 - A brief introduction to cells and nervous system

Life resides in a cell, and the cell defines life. Cells are considered to be the smallest functional and structural unit of life. Life in turn, is defined by the properties -or abilities- of cells [1]. Many living organisms are single cells, and are often not visible to the naked eye. They survive in many different environments by adapting their lifestyle to often extremely unfriendly habitats.

A cell is basically a chemical factory that can reproduce [2]. Cells need a blueprint so that they know how to function and can pass this information on to their progeny. This blueprint is coded in the form of DNA. The DNA code is transcribed into RNA as an intermediary step, which provides a degree of control over expression. Then, the RNA is translated into proteins, which perform most cellular functions. The inside and outside of a cell are separated by a lipid membrane, which enables exchange of certain materials. Proteins, smaller peptides, and carbohydrates embedded or bound to this structure provide further support and add functionality. Furthermore, cells contain specialised regions that perform specific functions. For example, tail-like extensions such as flagella facilitate movement, or organelles like chloroplasts provide energy. Thus, a cell gathers supplies from the outside, and turns them into energy or uses them as raw material to survive and procreate.

While some cells cope with the world on their own, others form groups and work together. In multicellular organisms, cells adapt to perform specific functions, such as secretion of enzymes to digest food. Cells with similar function form tissues and organs, and tissues and organs that carry out a certain aspect of life, such as digestion or reproduction, form systems [1, 2].

The nervous system gathers information from an animal's environment and body, and processes this information to control the animal's general behaviour [3]. Animals have different ways of gathering data, ranging from magnetic acuity to the pressure of their bladders. These data are then carried to tissues and organs which decipher and interpret them, deciding where to go or when to go. Depending on the complexity of the organism, nervous systems with different levels of organisation have evolved: From simple, disorganized nerve nets of hydra without any form of control centre, to a concentration of nerve cells in worms forming tissues (ganglia) to add a layer of centralization; from dispersed control centres of octopuses, with many cells lying in ganglia outside their brain, to the more densely packed brains of primates with billions of cells, which provide a more centralized control[1, 4]. Finally, the response to sensory stimuli is relayed to other parts

of the body to perform an action; a bird flaps its wings and migrates to the south, or a dog relieves itself and in the meantime marks its territory.

In higher invertebrates and all vertebrates, the nervous system can be divided into two parts: central and peripheral [1, 3, 4]. In vertebrates, the brain and the spinal cord are contained within an endoskeleton, and make up the central nervous system (CNS). The brain controls general behaviour, and contains many substructures responsible for different functions. The spinal cord carries information to and from the brain. It also controls some reflexes, and certain aspects of coordinated and rhythmic outputs. The peripheral nervous system (PNS) consists of the rest of the nervous system. It gathers information that will be interpreted by the CNS, and relays the instructions of the CNS.

1.2 - Cells of the nervous system and their functions

1.2.1 - Neurons

Neurons perform the basic function of the nervous system by transferring and processing information. All neurons contain four essential parts: a cell body, dendrites, an axon, and axon terminals (Figure 1) [1, 4]. The cell body contains the nucleus of the neuron, and many metabolic activities take place here. Dendrites (dendron = tree) are branch-like extensions of neurons, and most of the stimuli are received here. Sensory input such as mechanical, thermal, or chemical stimuli can be directly recognized by neurons [2, 3]. These induce nerve impulses (action potentials), which are carried along the neuron (See Box 1). The axon is a thin and long cellular process that is responsible for the propagation of action potentials. Axon terminals transmit these signals to other neurons, as well as other cell types such as muscle cells. Neurons communicate with each other either chemically by secreting molecules, or directly by the propagation of action potentials through gap junctions [5, 6]. Chemical synapses that

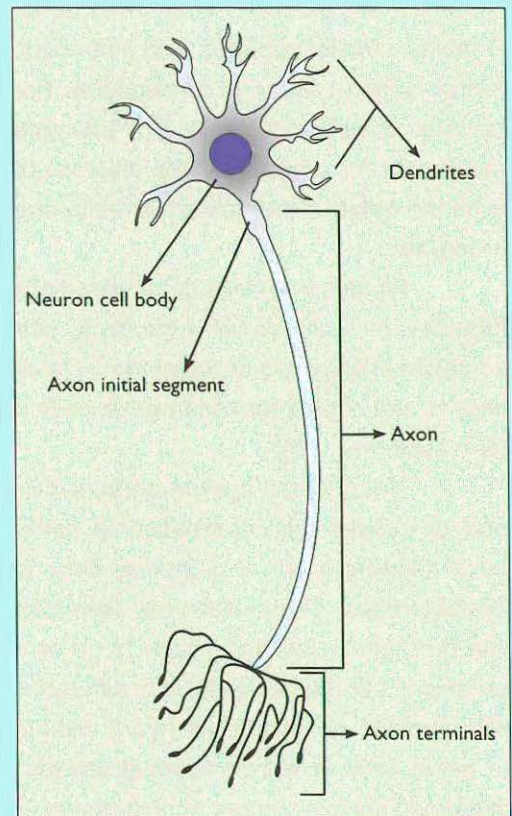


Figure 1. **Neuron**
Schematic representation of a neuron and its parts.

transmit information between neurons are usually formed by axon-dendrite interactions, although other types such as axon-cell body, dendrite-dendrite, are also observed [7]. Neurons form a large network of connections through which they communicate with each other. The connections neurons make, and the possibility to direct and modify the signal, enable them to process different stimuli and act accordingly.

BOX 1

Nerve impulse propagation

Normally, the plasma membrane of neurons is polarized, with the inside being more negatively charged (Figure 2). This resting potential is achieved by selective separation of ions across the membrane. Ion channels are activated upon binding of neurotransmitters to their receptors, or by other signals such as light or pressure. This results in a difference in the resting potential. The axon initial segment (AIS) contains a high density of voltage-gated ion channels. Excitatory and inhibitory signals converge here, and when a certain threshold is reached, the influx of Na^+ amplified by a positive feedback loop fires the action potential. This impulse propagates along the length of the axon, consecutively activating Na-channels on the next section. While the active Na-channels slowly inactivate, outflow of K^+ repolarises the membrane. The inactivated Na-channels enter a refractory period and cannot be reactivated during this time. Thus, the action potential flows unidirectionally. When the action potential reaches the axon terminal, it opens up voltage-dependent calcium channels. The influx of Ca^+ causes synaptic vesicles to fuse with the presynaptic membrane, and neurotransmitters are released. They cross the synaptic cleft, and are recognized by the receptors on the post-synaptic sites. Afterwards, neurotransmitters are readily removed from the synaptic cleft by endocytosis or enzymatic degradation [1-4].

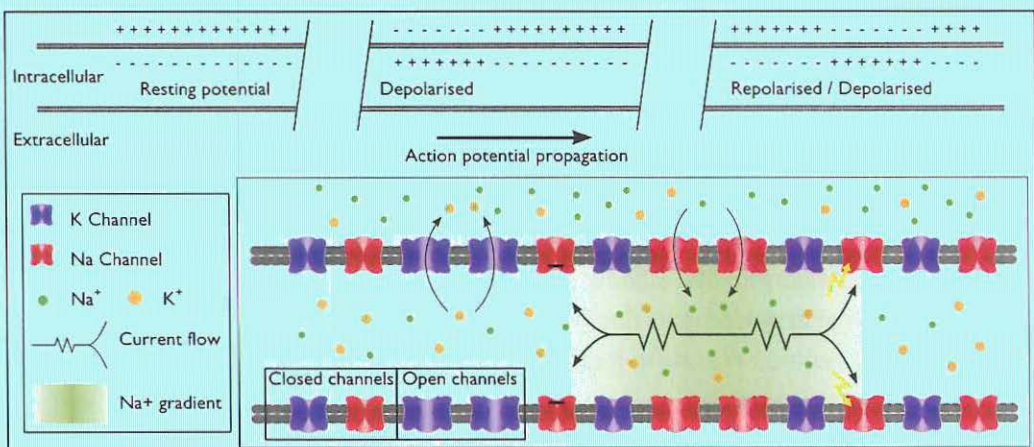


Figure 2. Action potential propagation

Activation of Na^+ channels results in the depolarization of the axonal membrane and the propagation of action potentials.

1.2.2 - Glia

Glia is the term given to the non-neuronal cells of the nervous system. In 1856, Rudolph Virchow published a paper in which he described a mesodermal connective tissue in the brain, and named it accordingly: neuroglia -nerve glue [8]. It is now known that most glial cell types are derived from the ectoderm [9]. And although their function is much more than just “glue”, the name is still used to describe the cells that support and accompany neurons throughout their lives.

The presence of glia in the lowest groups of invertebrates can be debated, but glial cells are thought to have evolved after bilaterian branching [10, 11]. Studies in invertebrates focused on certain species of worms, arthropods and molluscs. Here, glial cells have been found to facilitate several aspects of neuronal life, such as growth, survival, protection, synaptic function, and metabolic support. Their function is highly similar in the vertebrate nervous system. In vertebrates they are more abundant than their invertebrate counterparts and they have been characterised more thoroughly.

Vertebrate glia are categorized according to their morphology, cellular contacts and/or function (see reference [9] for further details). In the CNS, microglia function as the immune system. They are the only glia derived from the mesoderm. They are responsible for the phagocytosis of cellular debris and foreign materials. Their function is similar to that of macrophages throughout the rest of the body. Macroglia of the CNS include astrocytes, ependymal glia, and oligodendrocytes. Astrocytes form cellular processes thereby linking the neurons to each other, to the blood vessels, or the pia mater (the innermost layer of the meninges). They provide physical support to the neurons, structure the nervous tissue, regulate neuronal growth, metabolism, extracellular ion concentration, transmitter release or uptake, and synaptic modulation. Furthermore, they can influence and support the blood-brain barrier to maintain homeostasis. Ependymal cells are found surrounding the ventricular system. They produce the cerebrospinal fluid and provide exchange of materials with it. Cerebrospinal fluid protects the nervous tissue, and plays a role in maintaining the homeostasis. Oligodendrocytes affect neuronal growth, and produce the myelin sheath in the CNS. In addition to the mature forms of glia mentioned above, their precursors also affect neuronal development. For example, radial glia not only guide the migration of neurons from the ventricular zone in the developing brain, but can also give rise to different neuronal and glial cell types [9].

In the PNS, Schwann cells provide protection and support for the axons. They can influence their growth by providing trophic factors, and assist regeneration upon injury. They also form the myelin sheath, regulate extracellular ion concentration and synaptic transmission to facilitate axonal function. The enteric glia influence gastrointestinal function

by regulating and complementing neuronal and synaptic activity. Satellite cells surround neuronal cell bodies in the peripheral ganglia to regulate their homeostasis and provide trophic support. Apart from these major types, more specialized glia, such as Müller cells of the retina or the olfactory ensheathing cells, can be found in both CNS and PNS assisting neurons.

1.3 - Myelin sheath and rapid nerve impulse propagation

It is evident that the nervous system provides a useful set up for an animal to evaluate and control both its internal clockwork and external interactions. As such, the uninterrupted and efficient operation of this organization is paramount for the survival of the organism. Furthermore, advancements in this system in a particular organism can provide an advantage over its competitors. The myelin sheath is an example of such progress.

Speed, is one of the most important aspects of information transfer and processing. This is evident in our everyday lives where the computer and internet speeds double every year or so, enabling us to do the same work in shorter amounts of time. As the sensory inputs increase, the environment becomes more challenging, and the behaviour of animals becomes more complex, rapid information processing can mean life or death. What myelin does is to provide a framework where neurons can transfer action potentials faster, and more efficiently. This is provided by glial ensheathment of nerve fibres with several layers of cytoplasmic wraps [12]. While some invertebrates such as shrimps and earthworms contain insulating glial ensheathments to provide a myelin-like structure with closely apposed extracellular spaces, composition of the membranes and the distance of cytoplasmic spaces differ from vertebrate myelin [13-15]. Vertebrate species, except for agnatha, all contain myelin sheaths with spiral glial wrappings that surround larger calibre axons to provide faster nerve impulse propagation. But how does this cellular sheath increase the speed of propagation?

Two factors important in electrical current propagation by axons are resistance and capacitance [16]. Both the resistance of axonal fibres to the electrical current, and the capacitance of axonal surface (the amount of charge that is necessary to initiate ion exchange over the membrane) hinder nerve impulse propagation. A simple way to increase conductance is achieved by increasing axonal diameter, which decreases resistance. Giant axons that enable rapid response to external stimuli are found in many animals such as squids and insects. Although indispensable in its own right, this system has disadvantages such as the amount of space and energy needed. The myelin sheath on the other hand, increases conductance by way of decreasing axolemmal capacitance, and to a lesser extent by providing insulation. This way, it can provide at least ten-fold faster axonal propagation

compared to unmyelinated axons of same diameter. Myelinated axons of mammals can conduct up to 120m/sec, while unmyelinated axons conduct less than 1m/sec [5, 12].

Glial cells myelinate axons along their length except for short gaps, termed Nodes of Ranvier. This is where Na-channels are clustered, and the ion exchange occurs across the axonal membrane. Nerve impulses thus propagate in a saltatory manner. The myelin sheath insulates axons over the internodes by increased resistance to ion exchange. More importantly, the capacitance along the internodes is decreased, reducing the current flow over the axolemma. These properties are achieved both by the nonconductive lipid sheath, and by restraining ion channel localization. This way, nerve impulses from the active node can be propagated along the internodes without loss of their potential. Furthermore, the restriction of Na-channels at the nodes also decreases their capacitance. This increases their charging rates by enabling them to reach threshold rapidly and activate with less charge. Therefore in comparison, the myelin sheath enables more rapid action propagation while reducing energy costs [12, 16]. The optimal conduction velocity is determined by several factors including axon diameter, myelin thickness, and internodal distance. These factors are also correlated with each other, for example the axon diameter has a linear relationship with myelin thickness (Figure 3) [17, 18].

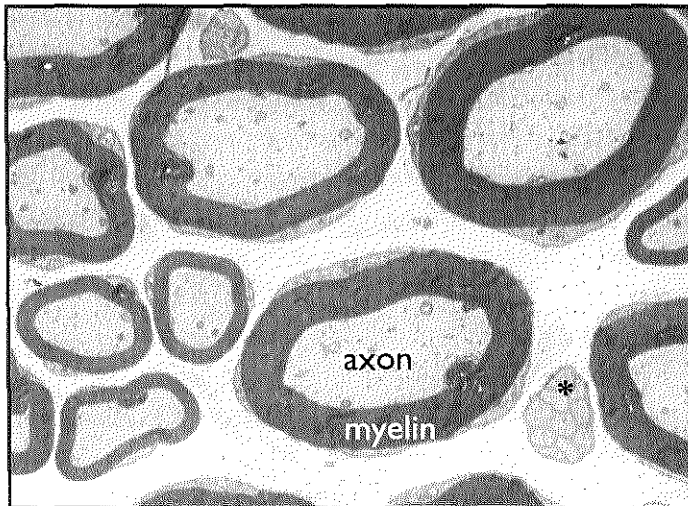


Figure 3. Myelinated axons in the sciatic nerve

An electron micrograph of a transverse sciatic nerve section. Myelin is visible as dark rings around the axons. The myelin sheath thickness is correlated to the axonal diameter; thicker axons have thicker myelin sheaths. A Remak bundle is marked by an asterisk.

In vertebrates, larger calibre axons of both CNS and PNS are myelinated. Although functionally similar, there are structural differences between the two systems. The myelinating cells of the CNS are oligodendrocytes, whereas peripheral axons are myelinated by Schwann cells (Figure 4). Although their origins and structure are different,

myelination by both oligodendrocytes and Schwann cells depends on neurons and other environmental factors. Axons play more a regulatory role for oligodendrocyte myelination. Oligodendrocyte differentiation is controlled by extracellular cues such as Sonic hedgehog (Shh), Notch signalling, and Platelet-derived growth factor (PDGF). Premyelinating oligodendrocytes express many myelin proteins such as DM-20, MAG, CNP, and MBP without axonal input. Axons modulate oligodendrocyte numbers, and may provide signals for protein redistribution, or cytoskeletal rearrangements [9, 12]. On the other hand, Schwann cell survival, differentiation, and myelination depend highly on axonal signals, as well as autocrine factors [19]. For example, axonally derived neuregulin-1 (Nrg1) controls both the development of Schwann cells and myelination [20, 21]. Some of the structural and organisational differences between CNS and PNS myelin are listed below:

Oligodendrocytes can myelinate multiple axons by extending several processes, while one Schwann cell surrounds and myelinates a single axon. Schwann cells contain numerous incisures with junctional complexes, which facilitate communication between inner and outer segments of myelin. PNS myelin periodicity as well as extracellular leaflet separation is slightly larger. Schwann cells extend microvilli over the Nodes of Ranvier. Furthermore, the protein constituents of myelin differ between the two systems. In PNS, MPZ, MBP, PMP22 and P2; in CNS, PLP and MBP are the major compact myelin components.

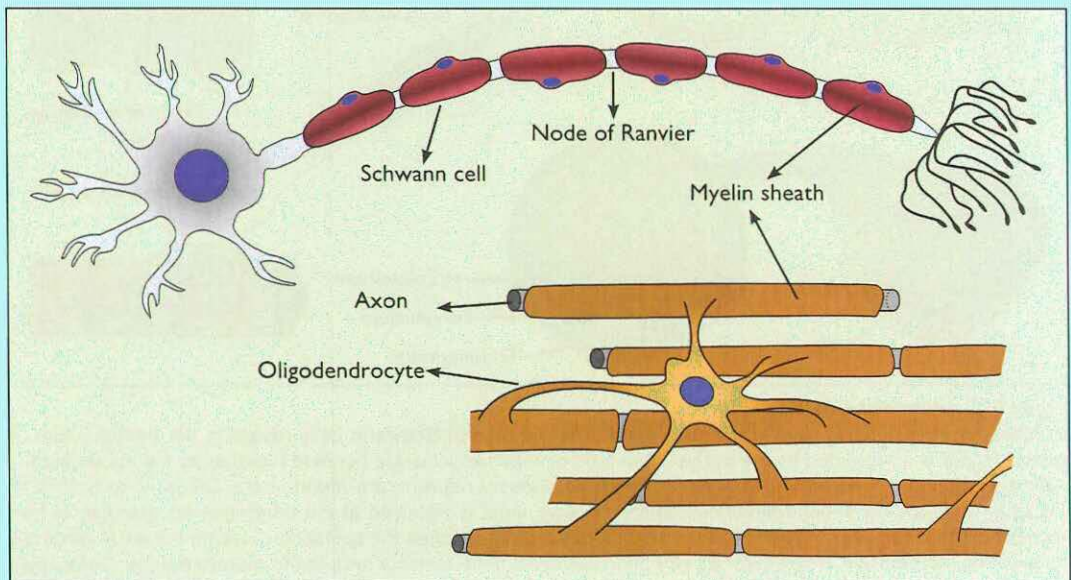


Figure 4. **Myelinated axons**
Schematic representation of myelinated axons.

1.4 - Morphology of peripheral nerves

Two types of neurons are found in the PNS: afferent and efferent nerves, or sensory and motor neurons. While the cell bodies of sensory neurons reside in ganglia outside the CNS, cell bodies of motor neurons can reside inside or outside. Sensory neurons gather information and relay it to the CNS. Motor neurons relay information from the CNS to tissues such as muscles and glands. The axons of peripheral neurons are closely associated with Schwann cells, either myelinating or non-myelinating. Other types of Schwann cells, such as satellite cells or teloglia, can be found in ganglia or at axon terminals where neurons interact with muscle tissues.

Nerve fibres are surrounded with layers of connective tissue that protect them from environmental factors (Figure 5) [22]. This tissue also contains blood vessels that branch towards the inner layers through capillaries, to supply the cells. Major cellular components of this tissue are fibroblasts, which produce an extracellular matrix (ECM) that is rich

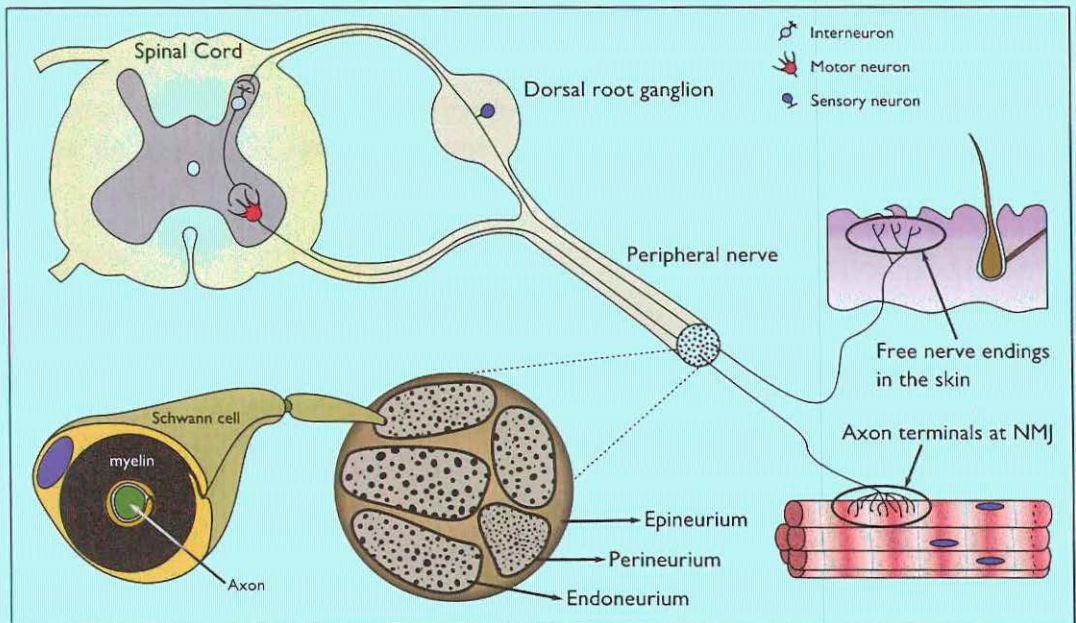


Figure 5. Overview of peripheral nerves

Morphology of peripheral nerves is represented. The axons and Schwann cells reside in the endoneurium. A nerve fascicle is surrounded by the perineurium, and several fascicles are bundled together by the epineurium. A simple reflex arc is shown. Cell bodies of somatic sensory neurons are found in the DRGs. One branch of the axon reaches the peripheral tissues where sensory input is received at the dendrites; an example of free nerve endings in the skin is given. Another branch of the axon reaches the spinal cord, where the axon terminals contact the interneurons. Interneurons carry information from the sensory neurons to motor neurons. Cell bodies of somatic motor neurons reside in the spinal cord. The axon terminals of motor neurons innervate the muscle at the neuromuscular junctions (NMJ).

in collagen. The outermost layer, called epineurium, groups the fascicles together. The collagen and fat in this layer protects the fibres from mechanical stress. In addition to the blood vessels, it also connects with the lymphatic system. The middle layer, perineurium, is made up of cells that surround the individual fascicles. Specialized fibroblasts in the perineurium produce basal lamina, and are connected by tight junctions, forming concentric and flat cellular layers. This provides a selective barrier against a large array of molecules such as proteins, ions, pathogens, etcetera, much like the blood-brain barrier of the CNS. The innermost layer, endoneurium, embeds the nerves in collagen fibres. Along with the fibroblasts, Schwann cells also contribute to the production of ECM.

1.5 - Development of the PNS

1.5.1 - Origins: the neural crest

All cells of the PNS arise from the neural crest (NC), with the exception of a number of cranial ganglia that contain contributions from ectodermal placodes. During the development of an embryo, the neural plate which is located dorsally, folds inward to form the neural groove (Figure 6) [19]. The edges of the neural fold move closer and eventually fuse to form the neural tube that pinches off from the remaining ectoderm. The neural tube forms the brain and spinal cord, and the remaining ectoderm on the dorsal surface of the embryo forms the epidermis. The NC arises during the fusion of the neural folds as a group of cells that delaminate from the neuroectoderm, and moves out laterally. NC cells differentiate into a multitude of cell types. These include the neurons and glia of the peripheral and enteric nervous systems, cartilage and bones of the head and neck, pigment cells of the skin, fibroblasts, vascular smooth muscle cells, and connective tissues in muscles and glands [23].

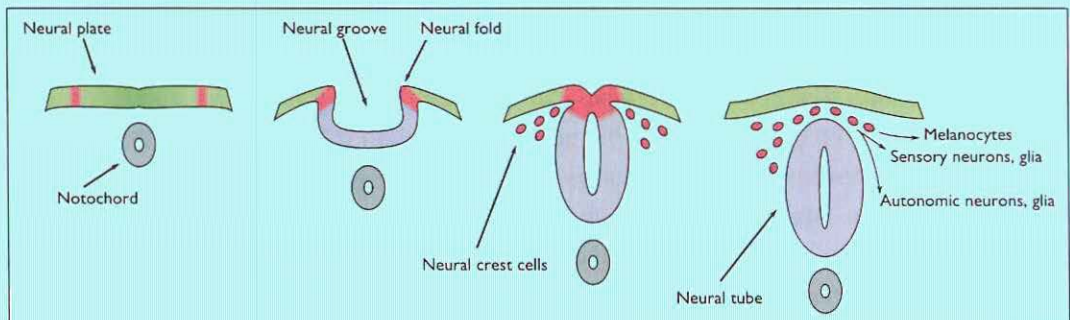


Figure 6. Formation of the neural tube and neural crest cells

The neural plate folds inward to form the neural groove. The neural folds fuse forming the neural tube which pinches off from the ectoderm. The neural crest cells arise from the cells that delaminate from the neural folds. These cells migrate along different routes, for example laterally migrating cells give rise to melanocytes in the skin, and ventrally migrating cells give rise to glia, sensory neurons of the DRGs, and autonomic neurons. (Figure based on [19])

Neural crest cell specification is initiated by signals from the underlying mesoderm and the adjacent non-neural ectoderm [24-26]. Several studies indicate that BMP, Wnt, and FGF signalling events emanating from the ectoderm or the mesoderm activate NC formation [27-33]. The interplay between these signals and their relative importance in NC specification seems to differ upon context [34]. Furthermore, these signals need to be modulated for proper specification [35, 36]. This ensures that a specific group of cells will give rise to NC, whereas the adjacent cells differentiate to their prospective tissues. In a similar fashion, a Shh gradient along the dorsoventral axis of the neural tube induces the formation of specific motor neuron and interneuron populations [37]. The extracellular signals controlling NC cell formation are relayed to the nucleus. Here, activation of transcription factors regulate NC cell specification (Msx and Pax), and ensure the survival of the precursors (Sox) [38].

The NC cells delaminate from their initial site at the neural folds, and migrate along specific routes to their targets [39]. As with NC formation, competing activities of BMP and Noggin are thought to influence rostral to caudal delamination of NC cells [40]. It has been suggested that Snail family transcription factors such as Slug [41, 42], and members of Cadherin cell adhesion molecules [43] are involved in the delamination process.

The NC progenitors show stem-cell like properties in that they can give rise to different cell types [44, 45], along with self renewal properties observed in some subtypes [23, 46]. They undergo several steps of differentiation, giving rise to precursors with different levels of potency. These cells represent a heterogeneous population in the migrating NC [47], some of which persist into adulthood [48, 49]. Differentiation of NC cells depends on the environmental factors of their migratory pathways and the timing of migration. For example, glial differentiation can occur from populations along the whole anteroposterior axis, while facial cartilage and bone cells emerge from the cranial region [50]. Similarly, an initial ventrally migrating group of cells give rise to neurons, whereas a later group moving in the lateral direction generates melanocytes [51]. It was shown that GGF (glial growth factor/Nrg1) signalling allows for glial differentiation [52], whereas BMP and Wnt signalling induce neuronal differentiation [53-55]. BMP and Wnt result in instructive signals on NC progenitors, rather than selective signals. They induce or repress expression of downstream molecules, which control differentiation along different cell lineages. An interesting aspect of these multiple signals converging on the same pool of NC cells is how the instructions are read. It was found that neuregulin signalling reaches a threshold later than BMP or Wnt signalling, therefore an initial wave of neuronal cells emerge [56, 57]. On the other hand, Notch signalling can invert neuronal fate, and induce glial differentiation in peripheral ganglia [58, 59]. While the exact sequence of events determining NC cell fates

have not been fully elucidated, it is evident that combined effects of several factors with different levels of expression, and cells with different sensitivities to these factors give rise to different precursors [60].

1.5.2 - Neuronal differentiation

Upon activation, transcription factors Mash1 [53], Phox2a and 2b induce the formation of autonomic neurons [61-63]. Together with receptor tyrosine kinase Ret (a receptor for GDNF), these factors are involved in the enteric nervous system differentiation [64, 65]. On the other hand, expression of neurogenins (Ngn-1 and -2) induce the formation of sensory neurons [66, 67]. Neurogenins activate a cascade of several transcription factors to generate different sensory neuron subtypes [67, 68]. Along with these, POU and LIM homeodomain proteins (Brn3a [69] and Isl-1 [70]) have been identified as important players in neuronal differentiation.

The next stage in neuronal differentiation is characterized by the expression of neurotrophin receptors. Neurotrophins form a family of structurally related, secreted proteins that control neuron growth and survival. Four genes have been identified encoding neurotrophins; NGF, BDNF, NT3 and NT4/5. The proteins form homodimers, and have different affinities for different receptors. The receptors for NTs are Trk receptors (A, B, and C) from the receptor tyrosine kinase family, and p75NTR from the TNF receptor superfamily. Upon binding to their ligands, Trk receptors dimerize and are phosphorylated, which leads to their activation. Via different adaptor molecules, they activate signalling pathways, such as MAPK or PKB/AKT, leading to differentiation or survival mechanisms. NT binding to p75 induces signalling pathways through p75 interaction partners. Therefore, the outcome of these signals depends highly on the cellular context. p75 can also interact with Trk receptors to modulate their function and the affinity to their ligands. In this way, the same ligand can induce survival and differentiation in one cell, while another cell will undergo cell death. Furthermore, the different variants of NT receptors are thought to facilitate or compete with signalling events. Through these diverse set of interactions, NTs and their receptors control the formation of different types and numbers of neurons [71, 72].

For proper neuronal development, a constant supply of NTs and other neurotrophic factors is needed. These factors can be expressed from neurons themselves, or from the surrounding tissues. The role of Schwann cells on neuronal development comes from mouse mutant studies, such as the ErbB3 knock-out mice. In these mice, Schwann cells and their precursors are missing. Along with this, both sensory and motor neurons are lost during embryonic development. It is thought that the expression of GDNF and possibly other neurotrophic factors (BDNF, NT3, NGF among others) from Schwann cells are necessary for the continued survival and proper development of neurons. Therefore, while

initial differentiation of neurons occurs normally in these mice, in later stages, neurons gradually die off [73]. Similar problems are observed in other mutants where Schwann cells or their precursors do not develop properly. Target tissues of neurons also produce trophic factors. During development, neurons are produced in excess, and only those that reach their target will survive. This ensures that the axons growing out from neuron cell bodies will reach to their intended termination points.

1.5.3 - Schwann cell differentiation

Schwann cells develop from NC cells through distinct cellular stages characterised by expression of specific genes. The first stage is the Schwann cell precursor (SCP) stage. SCPs can be found in the peripheral nerves of mice around embryonic day (E) 11.5. Between E12.5 and E15.5, these precursors give rise to immature Schwann cells [74]. Postnatal fate decision of Schwann cells depend on the axons they associate with. Only those that envelope a large calibre axon initiate myelination, while the smaller calibre axons will be ensheathed with non-myelinating Schwann cells. Schwann cell lineage develops in contact with axons, starting from the initial association of NC precursors with axons (Figure 7) [75].

It is not clear how the gliogenic fate is induced by transcription factors, and whether extracellular signals actively instruct NC cells, or it is a default pathway for those that do not receive an input. Sox10 is expressed in glia of the peripheral nerves and is absent in neurons, but it is also present in migrating NC cells [76]. Sox10 is thought to be important in NC cell survival as well as gliogenesis [77]. Mice carrying Sox10 mutations fail to develop peripheral glia [78], and haploinsufficiency of Sox10 in humans causes Waardenburg/Hirschsprung disease, which is characterized by neural crest defects. Sox10 activity is therefore a prerequisite for Schwann cell differentiation, but because it is involved in other pathways as well, there should be other factors leading the cells through this lineage.

It is also not clear how NC cells become associated with the axons. Axons may secrete molecules to attract NC subpopulations, or they present cell adhesion molecules that will induce NC cell attachment. Regardless of the mechanisms, upon their association, axons provide a signal in the form of Nrg1 that will induce the transformation of NC cells to SCPs. One aspect of Sox10 activity in Schwann cell differentiation may involve this pathway. One of the receptors for Nrg1, ErbB3, was found to be downregulated in Sox10 mutants [78]. Nrg1 inhibits neurogenesis from NC cells, although it is not known whether it also provides an active signal for gliogenesis [52]. Nonetheless, it is clear from mouse mutant studies that Nrg1 and its receptors on the Schwann cells, ErbB2 and ErbB3, are necessary for SCP survival [79-81]. Furthermore, this interaction was shown to be necessary for proper migration of Schwann cells along the lateral line nerve in zebrafish

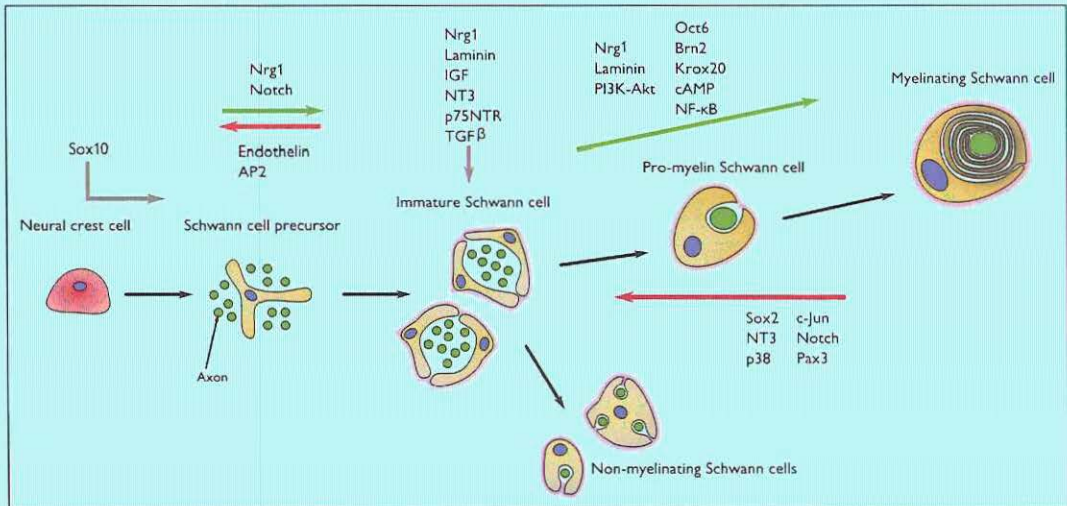


Figure 7. Development of the Schwann cells from the neural crest

Schematic representation of Schwann cell development. Developmental stages, and the molecules involved in these stages are shown. Green and red arrows indicate molecules that regulate differentiation. Sox10 is necessary for the development of peripheral glia from the neural crest. Nrg1, laminin and autocrine factors such as IGF and NT3 influence immature Schwann cell survival. Together with p75NTR and TGFβ, they control Schwann cell numbers. Intracellularly, transcription factors such as Sox2 and c-Jun are necessary to control both Schwann cell numbers and delay differentiation. See text for further details. (Figure based on [19])

[82]. Therefore, Nrg1 derived from the axons not only provides signals for SCP survival, but also guides SCP along the nerve trunks ensuring the formation of immature Schwann cells.

Schwann cells populating the dorsal and ventral roots of the spinal cord develop from the boundary cap cells [83]. Boundary cap cells also originate from the neural crest, but they reside in the entry and exit points of the roots and give rise to satellite cells and a small subset of nociceptive neurons. Another role for boundary cap cells, or their derivatives, appears to be to maintain a border between CNS and PNS. Studies done in mice where boundary cap cells are not present, demonstrated that the motor neuron cell bodies shift towards the ventral roots rather than staying in the spinal cord [84].

Differentiation of SCPs to immature Schwann cells involves a shift in the expression of factors involved in survival, proliferation, cell adhesion and cellular structure. Upon differentiation, immature Schwann cells downregulate cell adhesion molecules such as N-cadherin and Cadherin-19 [85, 86], and sort the axons into smaller groups. These events also coincide with the formation of endoneurial space. The endoneurial fibroblasts are thought to have arisen alongside SCPs, from NC cells that express Dhh (possibly from SCPs) [87]. Both Schwann cells and their precursors express Dhh during embryonic development as well as during the initial weeks of postnatal development. The Dhh receptor Patched, on the other hand, can be found on the surrounding mesenchymal cells. Loss

of Dhh results in epineurial and perineurial abnormalities, suggesting that Schwann cells influence the formation of surrounding connective tissue [88, 89].

Although the factors involved in the transition from SCPs to immature Schwann cells are not entirely clear, *in vitro* and *in vivo* experiments point to the involvement of Nrg1, Notch, and endothelin. Notch promotes immature Schwann cell formation [90], whereas endothelin inhibits differentiation [91]. Nrg1 is important for Schwann cell survival [92-94]. It is also possible that Nrg1 plays a role during the transition from SCPs [95]. An important difference between SCPs and Schwann cells is the ability of the latter to support their own survival. The autocrine survival is mediated by factors such as IGF2, NT3, PDGFB, LIF, and LPA [96-98].

The final steps before myelination involve radial sorting of axons by immature Schwann cells. Schwann cells segregate large calibre axons from the bundles by extending cytoplasmic processes. This continues along the length of the axons with a single Schwann cell associating with a portion of a single axon. Acquisition of this 1:1 ratio is necessary for subsequent myelination. Schwann cells acquire this stage through cytoskeletal rearrangements that require interaction between cells and their ECM. The importance of these interactions has been demonstrated in mouse through genetic studies. Mouse mutants carrying inactivating deletions for laminin [99-102], and $\beta 1$ integrin [103, 104] show a lack of Schwann cell processes and improper axonal segregation. Also, sporadic myelination of multiple axons by a single Schwann cell suggests inhibition of radial sorting. These ECM interactions signal through RhoGTPases (Rac1, Rho, Cdc42) intracellularly, which mediate diverse cellular responses such as cytoskeletal rearrangements or cell cycle control [105].

Inactivation of Rac1 in Schwann cells results in a delay in radial sorting, and a subsequent arrest in myelination [106, 107]. This indicates that Rac1 is involved in Schwann cell radial sorting and ensheathment. In addition, Rac1 mutants show defects in cellular process extension and stability. In mice lacking $\beta 1$ integrin, Rac1 activity is impaired, suggesting that it is activated by $\beta 1$ integrins. The defect in radial sorting in these mice can be overcome by the expression of constitutively active Rac1. Rac1 activity can be modulated by Merlin, so that cells can balance axonal elongation with radial sorting [108]. Merlin is encoded by the Nf2 tumour suppressor gene. It belongs to the ERM family of proteins (named after Ezrin, Radixin, Moesin proteins), which act as cytoskeleton-membrane linkers.

RhoA/Rho kinase (ROCK) signalling is also suggested to have roles in ensheathment or early myelination events. It is thought that they regulate timing of radial sorting and myelin sheath length, by controlling Schwann cell morphology and adhesion

[109]. A recent study identified Integrin-linked kinase (ILK) as a possible regulator of radial sorting and myelination by Schwann cells. ILK associates with integrins and links them to cytoskeletal molecules and several signalling pathways. For example, ILK inactivates Rho/ROCK signalling via focal adhesion kinase (FAK), which facilitates the progression of radial sorting. Furthermore, it influences Akt signalling pathway, thereby promoting myelin formation [110].

An immediate implication of proper radial sorting is the necessity of Schwann cell migration and control of Schwann cell numbers. It was shown that growth factors such as Nrg1, IGF, NT3 and BDNF affect and regulate Schwann cell migration. These signals, along with laminin dependent activity, modulate Schwann cell motility and alignment with axons via kinases or phosphatases controlling cytoskeletal rearrangements [111-114]. One aspect of Schwann cell proliferation depends on axonally derived signals. *In vitro* studies show that neurite membrane preparations, or co-culturing with neurites induce Schwann cell proliferation [115]. At least one constituent of this signal is Nrg1 [116]. It is possible that ErbB-FAK interactions facilitate this. FAK is linked to ErbB signalling as well as $\alpha 6 \beta 1$ integrin, and was found to be required for Schwann cell proliferation [117]. It was recently shown that Notch signalling also regulates Schwann cell proliferation. This could result from upregulation of ErbB2 by notch signalling, which would increase Nrg1 responsiveness [90]. In addition to their roles in motility, laminins can also influence Schwann cell proliferation and survival [118]. *In vivo* studies confirm the importance of axonal contact on Schwann cell proliferation. Transection of nerves results in axonal degeneration distal to the site of injury by a process called Wallerian degeneration. In the peripheral nerves of newborn rats, Schwann cells proliferate actively. But the loss of axonal contact in transected sciatic nerves, results in a decrease in Schwann cell proliferation [119].

An intracellular regulator of Schwann cell numbers is Cdc42. Cdc42 is strongly activated by Nrg1 in culture, linking these two factors in control of Schwann cell proliferation. Loss of Cdc42 in Schwann cells results in reduced proliferation, and subsequent defects in sorting and myelination [106]. The Cdc42 mutant phenotype illustrates the importance of Schwann cell numbers in both radial sorting and myelination. Although Rac1 mutant nerves do recuperate to a certain extent in time, Cdc42 mice show a more severe phenotype.

While extracellular growth signals, together with autocrine loops induce proliferation, a negative regulation by cell death is also necessary to obtain correct ratio to axons. It was shown that p75^{NTR} induces Schwann cell death upon injury, possibly in an NGF dependent manner [120]. TGF β signalling also induces cell death in Schwann cells, both *in vivo* and *in vitro* [121]. In an odd twist of fate, TGF β was also shown to induce Schwann cell proliferation [122-124]. It was suggested that TGF β signalling exerts a dual effect which

(Box 2 continued)

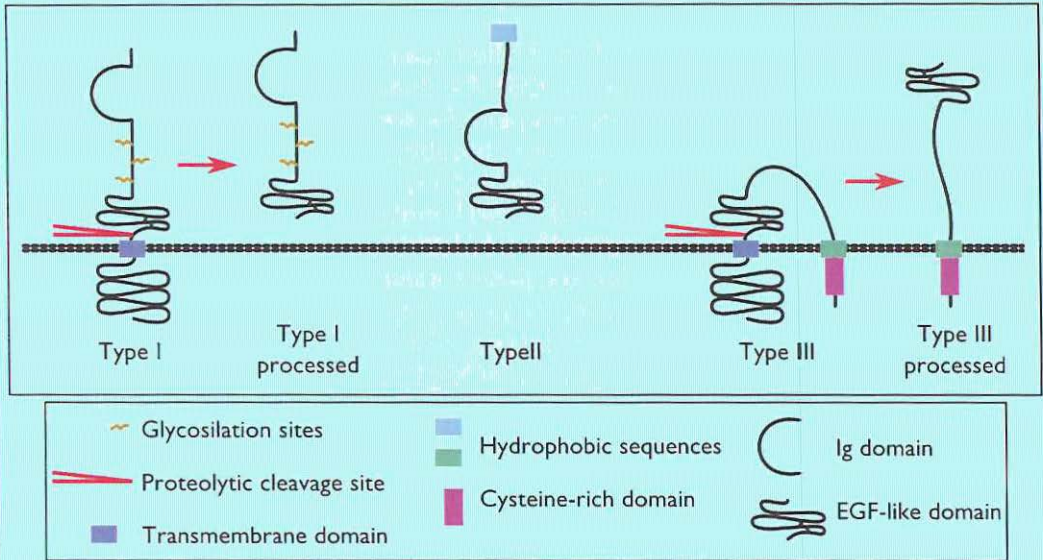


Figure 8. Neuregulin1 isoforms

The three major isoforms of Nrg1 involved in PNS development is depicted. (Figure based on [20, 197])

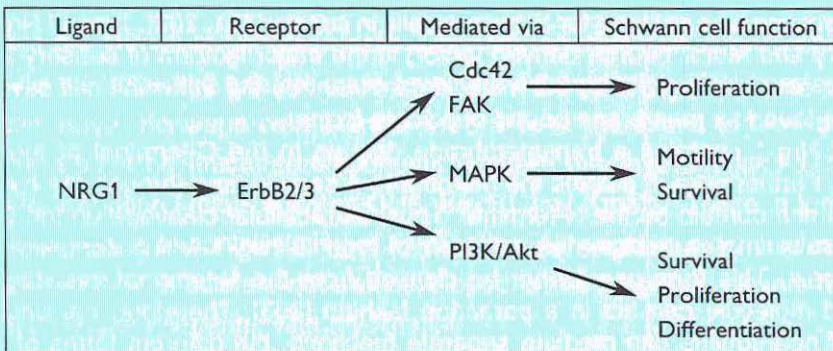


Figure 9. Nrg1-ErbB2/3 signalling in Schwann cells

Nrg1-ErbB2/3 interaction is mediated via intracellular signalling cascades to control several aspects of Schwann cell function. (Figure based on [20, 118])

and acts in a juxtacrine fashion. Addition of Nrg1 type II, a secreted form, perturbs myelin formation and causes demyelination in culture [131]. Similarly, overexpression of Nrg1 type I does not cause hypermyelination, and both type I and type II cannot compensate the loss of type III (see Box 2 for further information) [129]. Studies in mice using ErbB receptor mutants corroborate the effect of Nrg1 in myelination. Both ErbB2 knock-out mice [80] and dominant-negative ErbB4 transgenic mice [132] display hypomyelination in peripheral nerves. While these data show that Nrg1 signalling is necessary for the initiation

of myelination, it was found to be dispensable for the maintenance of myelin [133]. This study makes use of a conditional mutant allele of ErbB2, induced for recombination after the completion of myelination in mice. Nrg1 signalling cascade is also activated during Wallerian degeneration. On the contrary, Schwann cell proliferation and survival are not affected in the conditional ErbB2 mutant mice after nerve injury. It is important to note that these mice still express low levels of ErbB2, which could contribute to this observation.

Neurotrophins are also implicated in differentiation of Schwann cells. It was found that GDNF can induce proliferation and myelination of non-myelinating Schwann cells in rats [134]. NGF and GDNF expression by Schwann cells induce Nrg1 secretion, and Nrg1 in turn can influence GDNF expression by Schwann cells. It is possible that this crosstalk influences myelination of Schwann cells [21]. Both *in vivo* and *in vitro* experiments suggest competing roles for neurotrophins in myelination. Addition of BDNF in culture or injection into developing sciatic nerves induces myelin formation. In contrast NT3 exerts an inhibitory function [135]. Addition of NGF to DRG-Schwann cell cultures induces myelination, but it inhibits DRG-oligodendrocyte myelination [136]. Although, this study also shows that it is the neurons responding to NGF rather than the glial cells.

Although the direct links between these extracellular signals have not been established as yet, it is clear that myelination is initiated by the activities of several transcription factors that are the targets of these signalling routes. The cAMP-PKA cascade is one of the intracellular signals that initiate the myelin machinery along with the associated transcription factors. *In vitro*, proliferation of rat Schwann cells isolated from embryonic and neonatal stages becomes dependent on the levels of cAMP activation [137]. Furthermore, elevation of cAMP results in differentiation of Schwann cells and activation of myelin proteins such as MPZ and MBP [138, 139]. Activation of cAMP-dependent protein kinase (PKA) is found to increase transcriptional activity of NFκB [140]. And the activation of NFκB has been identified as one of the events necessary for myelination by Schwann cells [141].

The role of NFκB in regulating downstream transcription factors has been exemplified by Oct6 activation. Inhibition of NFκB drastically reduces the upregulation of Oct6 protein in DRG co-cultures, and its activation correlates with Oct6 expression [140, 141]. Oct6 is a POU domain transcription factor, and its expression is tightly controlled during Schwann cell development via a gene regulatory element termed Schwann cell-specific enhancer (SCE) [142, 143]. Its expression peaks at the promyelinating stage and gradually decreases as myelination progresses [144-146]. This suggests that it regulates downstream targets, switching on the myelination machinery [147, 148]. Oct6 knock-out mice display a delay in myelination. The Schwann cells in these mice are temporarily arrested at the promyelin stage, indicating the presence of a compensation mechanism.

Overexpression of Brn2 (another POU domain transcription factor) has been shown to ameliorate the Oct6 knock-out phenotype. Furthermore, double knock-outs of Oct6 and Brn2 display a more severe hypomyelination phenotype, suggesting that the two proteins have an overlapping function in peripheral nerve myelination [149]. Upregulation of Oct6 is thought to be controlled by binding of Sox10 on the SCE, possibly accompanied by other factors ([150] and unpublished observations Noorjahan Jagalur). Timely downregulation of Oct6 is also necessary for proper myelination. In mice that constitutively express Oct6, a persistent hypomyelination phenotype is observed, accompanied by axonal loss. This study also shows that although Krox20 is present, myelin proteins MPZ, MBP, and PMP22 are downregulated [151]. Downregulation of Oct6 is thought to stem from a negative feedback loop facilitated by Oct6 as well as Krox20 [152, 153].

Oct6, together with Brn2 and Sox10 was found to upregulate Krox20 expression that is necessary for myelination [154, 155]. A recent study also shows that addition of Nrg1 on Schwann cells results in a calcineurin mediated activation of NFATc4, which then forms a complex with Sox10 and activates Krox20 expression [156]. In addition to its role in Schwann cell development and upregulation of transcription factors involved in myelination, Sox10 is thought to regulate the expression of myelin proteins as well. Sox10 binding sites are found on several myelin genes such as Mbp, Mpz, Connexin32, and Mag. Binding of Sox10 and Krox20 to their respective sites in these regulatory elements seems to control the expression of such proteins [157-159].

Krox20 mutations are found in human hereditary neuropathies, such as Charcot-Marie-Tooth and congenital hypomyelinating neuropathy. The role of Krox20 in myelination was already suspected from previous mouse mutant studies. Knock-out mice, as well as mice with a hypomorphic allele of Krox20 ($Egr2^{Lo/Lo}$) display severe hypomyelination in the peripheral nerves [160, 161]. Close examination of gene expression profiles in $Egr2^{Lo/Lo}$ mice showed that many of the genes involved in myelination are decreased, whereas genes related to immature and promyelinating stages are increased. Krox20 activity was also shown to be important for myelin maintenance and remyelination following nerve trauma. Inactivation of Krox20 in adult Schwann cells results in demyelination, and although Schwann cells try to reactivate the myelination machinery, they fail to do so [162]. Finally, Krox20 not only activates the expression of myelin proteins, but also regulates the activation of cholesterol/lipid biosynthesis pathways [163]. Myelin production by Schwann cells therefore results from the combined effects of several extracellular signals that converge on the Schwann cell nucleus to activate transcription factors that initiate a myelin-related transcriptional program.

1.5.5 - Non-myelinating Schwann cells

Before going into the structure and organization of the myelin sheath, I will briefly discuss the non-myelinating Schwann cells (NMSC) of the nerve fibres. These cells differentiate from the same pool of immature Schwann cells and are associated with small calibre (usually $<1\mu\text{m}$) axons. An immature Schwann cell ensheaths several axons engulfing them in its cellular processes during normal development. If these axons are of smaller diameter, then the Schwann cells differentiate into NMSCs rather than myelinating Schwann cells. These axon-Schwann cell units are called Remak bundles. The NMSCs surround and insulate axons in individual pockets of cytoplasmic processes. The axons are surrounded along their entire length by neighbouring, interdigitating Schwann cells. Usually several axons are engulfed by a single Schwann cell. It is however not uncommon to see a 1:1 ratio with axons by NMSCs as well. In contrast to myelinating Schwann cells, NMSCs express molecular markers very similar to those expressed in immature Schwann cells [164, 165]. It is possible that they gradually change into NMSCs during the postnatal development, by regulating gene expression and exiting cell cycle.

1.6 - Structure and organization of the myelin sheath in the PNS

In the myelin sheath, the close appositions of membrane surfaces are held in place by protein interactions. The cytoplasm is excluded from between the myelin membranes, except for regions such as the paranodal loops. Myelin membranes are highly enriched in certain lipids such as cholesterol and galactocerebroside, and contain high amounts of myelin specific proteins such as MPZ. This composition provides a more packed and organized, stable structure.

The myelin sheath along the internodes (in between the Nodes of Ranvier) is made up of two main domains, compact and non-compact myelin. The protein composition of non-compact myelin allows for more space, and 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 (lateral ends of the internode), the outer- and innermost layers, and Schmidt-Lanterman incisures - canals that run along the compact myelin [166]. The abundance of the incisures in PNS is thought to facilitate radial transport. It is possible that they also play a role in myelin organization and stability.

During myelination, Schwann cells extend longitudinally, and surround the axon radially. The inner cytoplasmic lip turns around the axon while the outer lip is anchored to the basal lamina. Thus, the spiral wraps are formed followed by compaction [167]. Two alternating stripes can be observed in compact myelin when viewed at high magnification under the electron microscope (Figure 10). These are called the major dense line and the

intraproduct line. The major dense lines are formed by the intracellular layers of the plasma membrane with highly reduced intracellular space. The intraproduct lines (a doublet with extracellular space in between) are formed by the two extracellular layers closely apposing each other. MPZ is the major component of peripheral myelin. The extracellular interactions of MPZ tetramers in cis and trans are needed for the compaction of myelin and to maintain its ultrastructure. Mutations in MPZ can lead to diseases resulting from both myelin abnormalities and axonal loss [168]. PMP22, a protein of unknown function, is thought to be important for the stability of myelin. Mutations that result in changes of protein amounts and protein trafficking, cause myelin abnormalities including some of the most common peripheral neuropathies such as CMT1A and HNPP (Hereditary neuropathy with liability to pressure palsies). Along with the transmembrane proteins MPZ and PMP22, MBP is found at the cytoplasmic surface of compact myelin. It does not seem to be important in PNS compaction as MPZ can mimic its function to fuse the intracellular leaflets. Nonetheless, MBP could function to regulate protein interactions and lipid organization, to control myelin sheath thickness [169].

Non-compact myelin contains many junctional complexes (Figure 10 and 11) [166]. Adherens junctions are particularly enriched in the outer mesaxon and the outer layers of paranodes and incisures. These are formed by cadherins such as E-cadherin, and are possibly linked to the actin cytoskeleton. Gap junctions, which contain Connexin32 and possibly other connexins, form radial channels that allow for transport of small molecules.

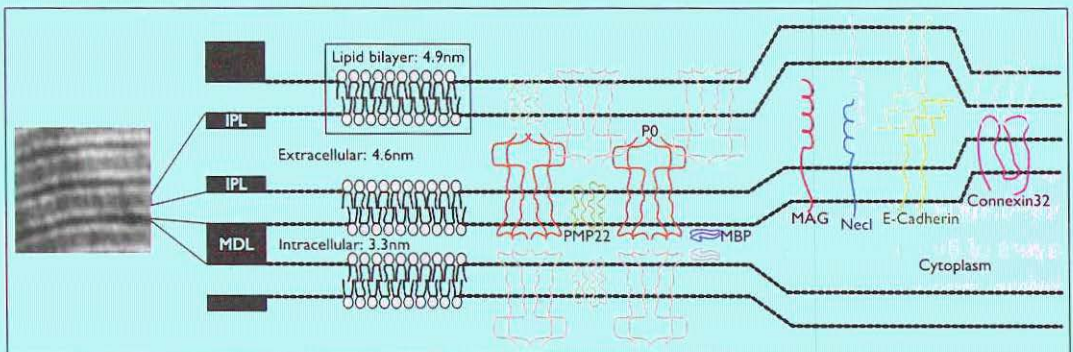


Figure 10. Schwann cell myelin

An electron micrograph (on the left) of Schwann cell compact myelin in cross section, and the schematic representation of compact and non-compact myelin proteins. The compact myelin is seen as alternating layers of major dense lines and intraproduct lines under the EM. The apposition of the myelin membranes is held in place by P0 (MPZ) tetramer interactions. The approximate thicknesses of the lipid bilayer, as well as the intracellular and extracellular spaces are given. Non-compact myelin is represented on the right side of the figure. These contain junctional complexes, formed by connexins and cadherins, as well as an increased cytoplasmic space that facilitate transportation of molecules between the layers of myelin. MAG and Necls are thought to mediate the separation of extracellular leaflets of Schwann cell membrane in the Schmidt-Lanterman incisures. (Figure based on [166])

Mutations in Connexin32 cause an X-linked form of CMT characterised by demyelination and axonal degeneration. Tight junctions are found surrounding the other junctions.

In addition to junctional complexes, other adhesion molecules are located along the internodes, at the axo-glial surface, and the Schmidt-Lanterman incisures. MAG expression on the Schwann cell surface is thought to regulate the axonal cytoskeleton through neurofilament phosphorylation which results in increased neurofilament spacing and axonal diameter. Therefore, MAG affects axonal structure and stability via its receptors, sialoglycans and Nogo receptor on the axonal surface. MAG is also thought to be important in myelin maintenance and regeneration [170]. Due to its location, it is possible that MAG maintains the extracellular space between opposing membranes. Myelinating Schwann cells also cause paranodal-nodal constriction of the axons [171]. Recently, a new set of proteins, the Necls (Nectin-like protein/SynCAM-synaptic cell adhesion molecule), were identified that are thought to regulate Schwann cell-axon interactions, and induction of myelination thereof. Along the internodes, axons express Necl1 and Necl2, whereas Schwann cells express Necl4 and possibly Necl2 on the periaxonal membranes. The interaction between these molecules, especially between Necl1-Necl4, is thought to enable

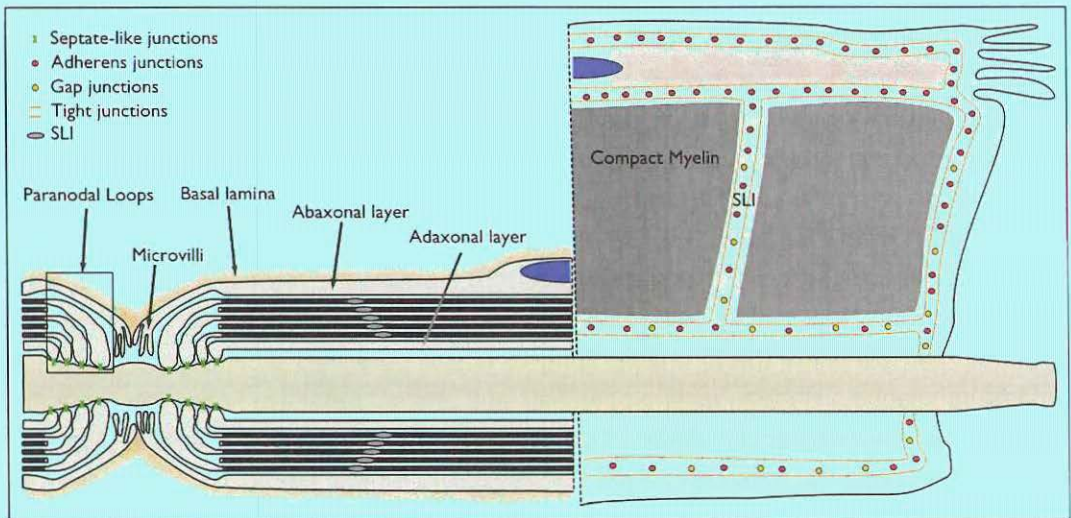


Figure 11. **Overview of myelinating Schwann cell**

Schematic representation of a myelinated axon. The left part of the figure shows a longitudinal section through the myelin spirals, and the right part shows unwrapped myelin membrane. Compact myelin is indicated with dark colour. Non-compact myelin is indicated with light colour. Non-compact myelin is found in Schmidt-Lanterman incisures, paranodal loops, microvilli, as well as adaxonal and abaxonal layers. Junctional complexes of non-compact myelin are also indicated. The paranodal loops contact the axons and form septate-like junctions. Schwann cell microvilli extend over the nodes of peripheral nerves. Schwann cells produce basal lamina on the abaxonal surface, and interact with the axon on the adaxonal surface. (Figure based on [166])

Schwann cell adhesion to axons. Studies done to identify the role of this interaction in myelination suggested that it is important for the wrapping of axons and the initiation of myelination, possibly by potentiating other signals. They are also thought to facilitate the polarisation of Schwann cells [172, 173].

Schwann cells are considered to be polarised cells. The adaxonal surface of the Schwann cell, the surface apposing the axolemma, could be compared to the apical surface of an epithelial cell. Whereas the abaxonal surface, the surface contacting the basal lamina, could be compared with the basal membrane (Figure 11). The basal lamina of Schwann cells mainly composed of laminin and collagen, acts as an anchor for the intracellular molecules (see Box 3 for further information). Myelinating Schwann cells express $\alpha 6\beta 4$ integrin and dystroglycan as laminin-2 receptors [174, 175]. These receptors cooperate to provide stability to myelin. Integrins interact with intermediate filaments and PMP22. Dystroglycan interacts with utrophin, dystrophin (Dp116), and DRP2. DRP2 is localized in clusters directly opposing the myelin sheath. Its interaction with L-periaxin, a PDZ domain protein, is thought to facilitate this localization [176]. Through these interactions, dystroglycan possibly links the basal lamina to the actin cytoskeleton, and regulates microvilli formation, as well as Na-channel clustering at the nodes [177, 178].

The nodal regions of myelin and axons show specialized domains that enable action potential propagation by saltatory conduction. These domains are Nodes of Ranvier, paranodes, and juxtaparanodes (Figure 13). Interactions between cell surface molecules at these sites not only organize ion channels to distinct domains, but also regulate axonal properties such as cytoskeletal rearrangements [171, 179].

Schwann cells extend microvilli over the nodes. The microvilli are enriched in ERM proteins, and also provide a perinodal matrix. It is thought that these cytoskeletal molecules help cluster cell surface molecules that facilitate the formation and function of the nodes [180]. Nodes of Ranvier are enriched in voltage-gated ion channels. Voltage-gated sodium channels (NaV) enable firing of action potentials at each node, and the potassium channels stabilize resting potentials and prevent repetitive firing. The assembly of nodes starts with the accumulation of cell adhesion molecules NrCAM and NF186 [181]. Schwann cells express gliomedin, a cell surface ligand for the axonal NrCAM and NF186 [182]. At the nodal regions, gliomedin is secreted by proteolytic cleavage, and is integrated into the perinodal matrix. Thereby, gliomedin clusters NrCAM and NF186, which brings about the formation of nodal structures at the axolemma [183]. In contrast to the situation in axonal initial segments (AIS), where cytoskeletal protein Ankyrin G recruits NF186 and NaV channels [184], in the nodes NF186 recruits Ankyrin G. Here, Ankyrin G stabilizes the CAMs. Furthermore, it interacts with β IV spectrin. Together they recruit and stabilize NaV

BOX 3**ECM molecules**

Laminins are formed as heterotrimeric complexes of α -, β -, and γ - subunits. Each subunit is encoded by different genes, and their expression is regulated dependent on the cell type. Laminin-2 ($\alpha 2, \beta 1, \gamma 1$) and Laminin-8 ($\alpha 4, \beta 1, \gamma 1$) are the major types produced by Schwann cells. Laminin-8 is expressed in higher levels during adulthood, whereas both are found during development. Furthermore, Laminin-10 ($\alpha 5, \beta 1, \gamma 1$) is found over the nodes and paranodes. Other subunits can be upregulated in case a certain form is missing, or in diseases and injury. Laminins interact with other ECM molecules such as nidogen, and can form polymers contributing to basal lamina formation.

Integrins are type I transmembrane proteins. They are formed through dimerization of α - and β - subunits. Schwann cells predominantly express $\alpha 6 \beta 1$ and $\alpha 6 \beta 4$. Expression of different integrins is developmentally regulated. They are found on NC cells, neurons, and Schwann cells. Integrins can also act as receptors for fibronectin, vitronectin, and collagens. Dystroglycan is composed of an extracellular α subunit, and a transmembrane β subunit. It also acts as a receptor for the proteoglycan agrin, which is important for neuromuscular junction function. Laminin-receptor interactions are relayed intracellularly via kinases or adaptor molecules to induce signalling pathways, or link ECM and cytoskeleton (Figure 12) [118, 207-209].

Collagen forms another major component of Schwann cell ECM. Although their function has not been studied to the same level of detail as laminins, collagens are also important for Schwann cell function, such as adhesion and migration. Collagens form heterotrimers, which are guided by their C-terminal non-collagen domains. Posttranslational modifications on N- and C-terminal domains of collagens contribute to the formation of extended structures such as fibrils (types I, III and V) and networks (type IV). Cell surface heparan-sulfate proteoglycans, such as syndecan-3 and glypican-1, act as type V collagen receptors on the Schwann cells. Glypican-1 has been found to mediate adhesion, cell spreading, and cytoskeleton assembly of Schwann cells. Type IV collagens can mediate Schwann cell attachment and spreading, as well as axonal growth via integrins. Collagens are also thought to facilitate myelination [118].

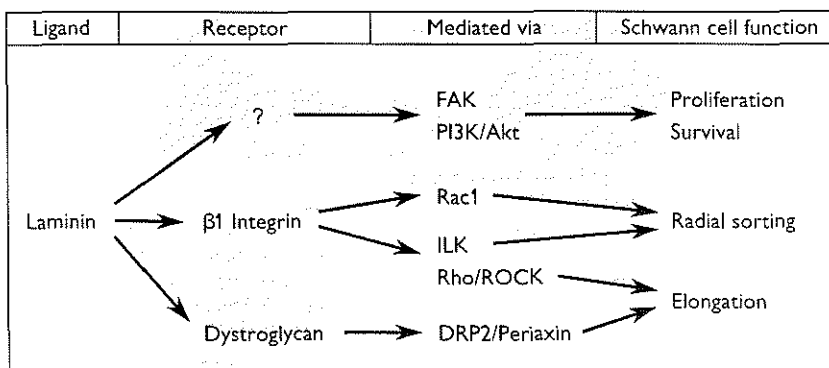


Figure 12. **Laminin signalling in Schwann cells**

Laminin signals through integrins and dystroglycan to control different aspects of Schwann cell function. (Figure based on [118])

and potassium channels. The organization of the nodes is further supported by targeting of molecules to these sites, or by removal of mistargeted proteins.

Paranodes present a physical barrier to the nodes by forming axoglial junctions. This not only prevents diffusion of cell surface molecules, but also provides a partial barrier to the diffusion of ions from both sides. Consecutive layers of Schwann cell membrane loops contact the axolemma, forming the paranodes. Contactin and Caspr complexes form the axonal side of the paranodal loops [185]. On the glial surface, NF155 probably acts as the ligand for this complex [186]. Although the direct mechanism of this interaction seems to be more complicated, as Caspr can also interfere with NF155–contactin interactions [187]. Furthermore, clustering of Caspr by Schwann cells requires the initiation of myelination, unlike by oligodendrocytes [188]. Although the expression of NF155 coincides with myelination [189], which is necessary for paranodal junction formation [190], it is

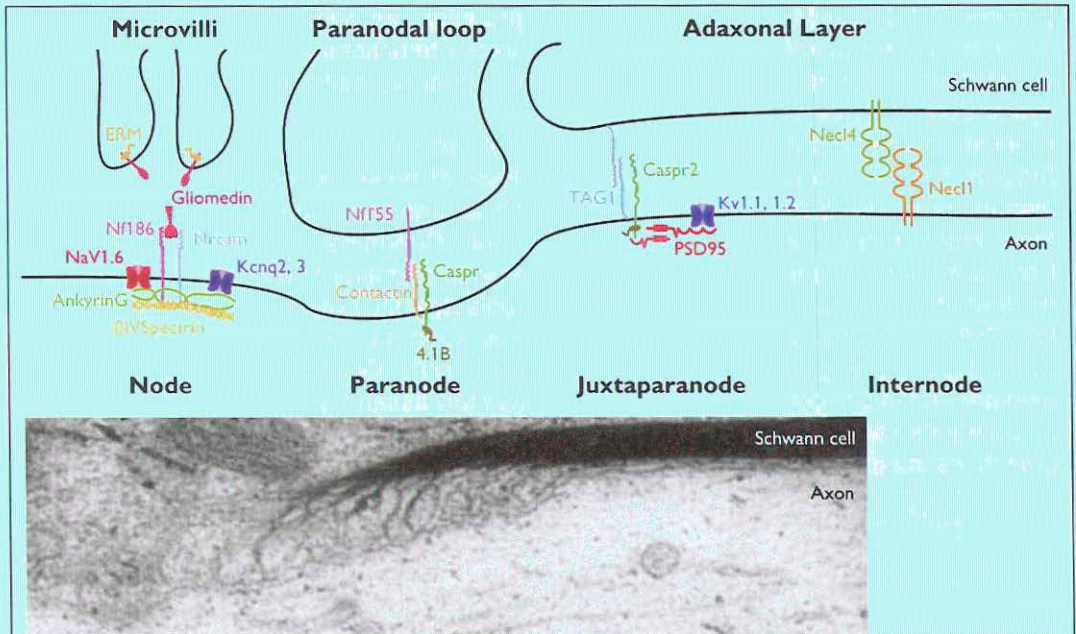


Figure 13. Molecular domains of myelinated axons in the PNS

The molecular domains are characterised by the expression of cell adhesion molecules and ion channels. Nodal axolemma contains voltage gated sodium channels (Nav1.6), and potassium channels (Kcnq2 and 3). Nf186 and NRCAM are clustered by Gliomedin that is incorporated in the nodal basal lamina. Gliomedin is localised at the Schwann cell microvilli by ERM proteins, and it is secreted by proteolytic cleavage from here. Ankyrin G and β IV Spectrin act as cytoskeletal linkers, and facilitate ion channel localisation via CAMs. The septate-like junctions at the paranodal loops are formed by Nf155, Contactin, and Caspr. Contactin and Caspr are linked to the axonal cytoskeleton via protein 4.1B. The juxtaparanodes are characterised by the accumulation of voltage gated potassium channels (Kv1.1 and 1.2). Localisation of ion channels is facilitated by TAG1–Caspr2 interactions extracellularly, and cytoskeletal linkers that link the CAMs to the ion channels. Internodal regions contain Necl1 and 4 that are necessary for Schwann cell–axon adhesion. (Figure based on [171, 179])

possible that other glial molecules are also needed. Caspr/Contactin bind to cytoskeletal protein 4.1B via Caspr, and this interaction stabilizes these complexes [191]. Defects in the septate-like paranodal junctions result in juxtaparanodal molecules to relocate towards the nodes, widening of nodal regions, and microvillar intrusions towards paranodes.

Juxtaparanodes, as the name implies, are found near the paranodes. They are characterized by a high density of Shaker-type potassium channel (Kv). Studies on nerve conduction during development and disease suggest that these channels regulate and stabilize the excitability of the nodes [192]. On the axonal surface of juxtaparanodes, Caspr2 and TAG1 adhesion molecules interact with each other. Trans interactions of TAG1 on the axonal and glial surfaces are thought to mediate the organization of juxtaparanodes [193, 194]. Caspr2 also binds to protein 4.1B cytoplasmically, which is suggested to facilitate the recruitment of Kv1.1 and 1.2 to the juxtaparanodes [195]. Similar to NaV recruitment by Ankyrin G, clustering of Kv channels at the AIS requires PDZ domain protein PSD-93. Although Caspr2 also contains a PDZ domain, and thus interacts with PDZ domain scaffolding proteins, neither PSD-93 nor PSD-95 is required for the accumulation of Kv channels at the juxtaparanodes [195, 196]. The significance of these molecules at the juxtaparanodes is not yet clear.

1.7 - Novel players

Evidently, several proteins in the extracellular environment play important roles in Schwann cell development and myelination. Some of these molecules are secreted, and some are transmembrane proteins. Some are presented by Schwann cells and others by axons. In this last section, I will introduce two proteins: Lgi4 and Adam22. These molecules are involved in PNS development and myelination, and are the main focus of research in this thesis project.

1.7.1 - Lgi4

Lgi4 belongs to a subfamily of leucine-rich repeat (LRR) proteins [197]. The first member identified, Lgi1, was found to be inactivated in the T98G glioblastoma cell line, due to a rearrangement of the gene from the t(10;19)(q24;q13) balanced translocation. Hence, it was named Leucine-rich gene - Glioma [n]activated [198]. This small family consists of four proteins (Lgi1-4) characterized by an N-terminal signal peptide, the LRR repeats which are flanked by cysteine-rich residues, a putative transmembrane region; and on the C-terminal portion, putative phosphorylation sites, and the EAR (Epilepsy Associated Repeat) or EPTP (Epitempin) domain characterized by tandem repeat elements [197, 199, 200]. Later studies showed that Lgi proteins were secreted rather than being transmembrane [201].

Human Lgi proteins show an overall similarity of more than 60% between different members, and more than 90% similarity across human and mouse. LRR repeats flanked by cysteine-rich residues are found in many extracellular LRR proteins. Lgi LRRs belong to the F-20 family characterized by a conserved phenylalanine at position 20 [197, 202]. It is known that LRR domains facilitate many protein interactions, and the EAR/EPTP domain is also thought to have a similar role [199, 200, 202, 203].

Several studies were directed towards the role of Lgi1 in tumour formation and progression. Although some studies show that Lgi1 expression correlates with proliferation/apoptosis, and invasion through the ERK1/2 pathway [204, 205], others argue that the role of Lgi1 in cell-ECM interactions does not correlate with a tumour suppressor function in gliomas [206]. On the other hand, involvement of Lgi1 in epilepsy is well established. Mutations of Lgi1 have been linked to the autosomal-dominant partial epilepsy with auditory features (ADPEAF; or ADLTE - autosomal dominant lateral temporal epilepsy) by a study in 2002, and since then several others have found different mutations of Lgi1 in familial or sporadic cases [207, 208]. It was shown that mutations in Lgi1 result in the retention of the protein inside the cell [201]. It is possible that the protein structure and folding is tightly controlled intracellularly, because not only large deletions or truncations but also point mutations or insertions result in protein retention. Two isoforms of Lgi1 have been identified: a long secreted isoform, and a short, truncated isoform that is retained inside the cell. Furthermore, the long isoform was found to bind the cell surface of differentiated PC12 cells [209]. A study based on mass spectrometry of proteins interacting with Kv1.1 shows that this protein forms a complex with other α - and β - Kv subunits, MAGUKs (Membrane Associated Guanylate Kinases, including PSD93 and PSD95), cell adhesion molecules (including Caspr2 and contactin1), Lgi1 and ADAM22. It is thought that Lgi1 inhibits the inactivation of these channels through Kv β 1, and ADLTE mutations result in a loss of function [210]. Although this study suggested that the secretion of Lgi1 is not necessary for this function, another study shows that the extracellular interactions of Lgi1 and ADAM22 enhance AMPA-receptor mediated synaptic transmission [211]. It is possible that Adam22 forms a link between extracellular Lgi1 and the cytoplasmic events. So far, mutations of Kv1 channel genes or ADAM22 have not been linked to ADLTE [212, 213]. On a more colourful note, Lgi1 loss of function has been speculated to be the cause of the voices (as epileptic auras) Jeanne D'arc heard [214].

Other Lgi molecules are not as well characterized as Lgi1. Although expression of Lgis has been found in many different tissues, they are predominantly expressed in the nervous system. No studies show a functional role for Lgi2. Lgi3 has been suggested to play a role in amyloid beta uptake by astrocytes, endocytosis and exocytosis [215-218].

Functional analysis of *Lgi4* has also been limited, and the studies on *Lgi4* are fragmented. Polymorphisms of *Lgi4* are associated with childhood absence epilepsy, but their relevance remains unknown [219]. One study alludes to the transcriptional regulation of *Lgi4* and *Fxyd3*. These genes are located on mouse chromosome 7, and are transcribed in opposite orientation. Together with this, the 3'UTR of *Lgi4* overlaps with the last exon of *Fxyd3*. It was therefore suggested that the transcripts could form double stranded RNA molecules *in vivo*, which might affect their expression [220]. Another study shows that *Lgi4* is absent in zebrafish and pufferfish. It is thought that *Lgi4* was either lost in ray-finned fish, or originated in the lineage leading to lobe-finned fish (sarcopterygii) [221]. Finally, our studies on Claw paw mice led to the identification of *Lgi4* as a candidate molecule controlling PNS development and myelination (Chapter 2 - [222]). The Claw paw mice were first observed in the 1970s, in an inbred strain of C57Bl/6J-ob mice. This autosomal recessive phenotype was characterized by limbs flexed at joints and extended at the elbows, hence the name Claw paw [223]. Claw paw mice also show impaired movement, and the pups are smaller than their littermates, possibly because of malnutrition. Furthermore, these mice display delayed myelination in the PNS, with implications of a role in axon-Schwann cell signalling. If they survive until after weaning, their condition improves. Furthermore, although the males breed very rarely, the females breed normally and can nurture their young. It was also shown that the formation of nodes is delayed, and the adult internodal space is reduced in Claw paw mice [224]. Consistent with the delay in myelination, these mice display similar expression profiles of certain genes downregulated in Oct6 knock out mice, although Oct6 itself is not downregulated properly in Claw paw mice [225]. Experiments performed previously in our laboratory showed that Claw paw mutation affects Schwann cell function, but a neuronal contribution is also likely. The Schwann cells display an overall delay in the myelination program, starting at least from axonal sorting. Although Oct6 is expressed in Claw paw nerves, *Krox20* is not; suggesting a role for Claw paw signalling in *Krox20* activation. MPZ expression is delayed, and periaxin expression is abnormal [226]. Furthermore, basal lamina formation, and laminin deposition appears to be delayed in Claw paw nerves [227]. These observations suggest that Claw paw signalling is involved in Schwann cell-axon interactions, and myelination in the PNS.

1.7.2 - Adam22

Adam22 belongs to the protein family termed A Disintegrin And Metalloprotease. Adam proteins contain a propeptide domain with a signalling sequence, a metalloprotease domain, a disintegrin domain, a cysteine-rich region, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail on the C-terminus (Figure 14). These proteins are involved

in protein interactions, cellular adhesion, intracellular signalling, and proteolytic cleavage.

Fertilin α and β (ADAM1 and 2) were the first described ADAMs. They were identified in sperm cells as type I integral membrane glycoproteins that are involved in sperm-egg fusion [228]. It was suggested that they bind to egg integrins to facilitate fertilization. Further characterization revealed the presence of metalloprotease and disintegrin domains, similar to those found in snake venom disintegrins [229]. The snake venom proteins inhibit platelet aggregation by competing with fibrinogen binding to integrin α IIb β 3. Although it is now known that fertilins bind integrins (such as α 9 β 1), their function during fertilization is not yet clear [230]. Since their initial discovery, about 40 ADAMs have been identified in different tissues, among different species from *C. elegans* to human. Many ADAM genes encode multiple protein products that result from alternative splicing. These events usually give rise to proteins with different cytoplasmic tails, or secreted forms.

The prodomain of ADAMs are cleaved off by for example furin protease, which enables the activation of the catalytic domain. Catalytically active ADAMs have a wide array of substrates ranging from growth factors to matrix proteins. Some are also termed sheddases, because of their role in the juxtamembrane cleavage and secretion of other transmembrane proteins, such as APP, TNF- α , and EGF. Although several ADAMs contain a metalloprotease activity, others are inactive and are thought to be involved in protein interactions, especially with integrins. The disintegrin loops of many ADAMs contain sequences similar to the classical integrin binding motif RGD, which facilitates these interactions. The cysteine-rich domain also facilitates protein interactions. These interactions could therefore lead to cell adhesion and cell fusion. Furthermore, some ADAMs, such as the fertilins are found as dimers. The cytoplasmic tail of ADAMs can interact with intracellular proteins through binding motifs they present such as SH3, or PDZ. In turn, these events could facilitate signalling, or cytoskeletal rearrangements, giving ADAMs a bidirectional function [231, 232].

Several ADAMs are expressed in the nervous system, and function both during development and in adult life. Of special interest are the ADAMs 10 (Kuzbanian, MADM), 17 (TACE), and 19 (Meltrin β). These molecules are possibly involved in the development of the NC and the PNS. Knock out mice of Adams 10, 17, and 19 die during embryonic development or at birth, due to defects in the cardiovascular system. ADAM10 knock outs also display defects in the CNS and somites [233]. This is at least partially caused by a defect in Notch signalling. ADAM10 was shown to affect Notch signalling by cleavage of Notch as well as its ligand delta [234, 235]. It could function in a similar way during NC development and Schwann cell differentiation. ADAM10 also mediates EGF signalling, functions in axon growth via Slit/Robo or ephrins and cell adhesion via L1 CAM or N-cadherin. ADAM17

protease activity has been well characterized, and several substrates were identified, such as TNF α , TGF α , EGF, ErbB4, and collagen XVII. Furthermore, it cleaves Notch, and its ligand jagged [236, 237]. Therefore it is possible that ADAM10 and 17 cooperate to mediate Notch signalling. As mentioned before, both ADAM17 and ADAM19 are involved in processing neuregulin. This effect is also reflected in the heart defects observed in the mutants. Unfortunately, the lethality of the phenotype precludes the characterization of their role in PNS development and myelination. Nonetheless, ADAM19 has been shown to function in the shedding of Nrg1 in cultured neurons [238, 239]. Recently, a study

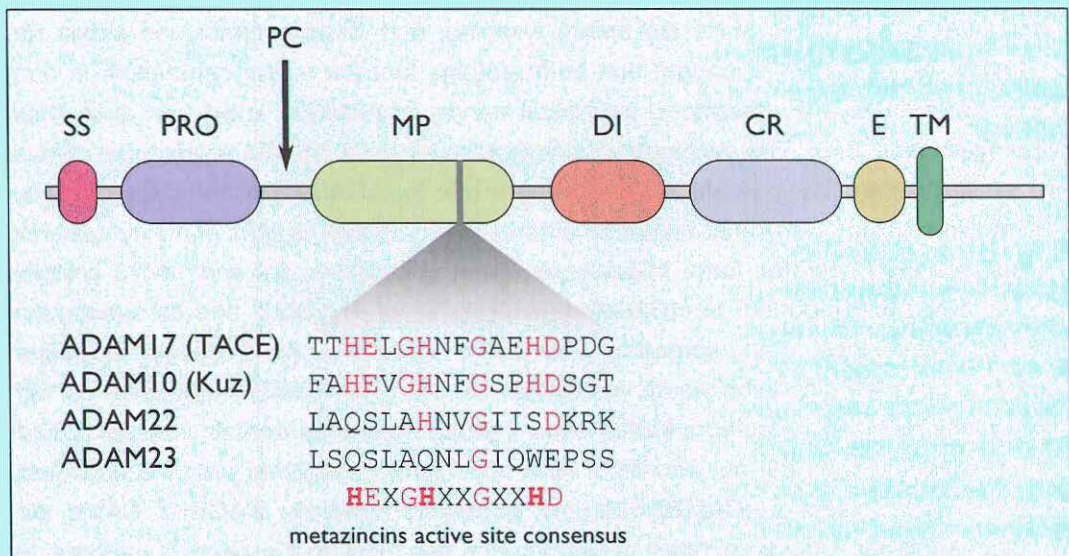


Figure 14. The Adams Family

The domain structure of Adam proteins is represented. The signalling sequence (SS) and the pro-domain (PRO) is cleaved off during the maturation of the proteins. The amino acid sequence of the zinc-binding site, which is necessary for the metalloprotease activity, is given. This is compared with the consensus sequence of Metazincin superfamily. The Metazincins are composed of Adam, Adamts (Adam with thrombospondin motifs), and MMP (matrix metalloprotease) proteins. Note the differences from the consensus sequence in the proteolytically inactive Adam22 and 23. The histidine (H) residues are necessary for zinc ion binding. PC: proteolytic cleavage site; MP: metalloprotease domain; DI: disintegrin domain; CR: cysteine-rich domain; E: EGF-like domain; TM: transmembrane domain.

performed on the ADAM19 knock out mice that survive until adulthood shed some light on this process. It was found that these mice display delayed remyelination upon injury, and the neuron membrane preparations failed to activate Akt signalling on Schwann cell cultures that is necessary to initiate myelination [240]. Unfortunately, the authors do not refer to a role during normal development. It is possible that other molecules substitute for such a function. Cell type and time point specific knock outs of these ADAMs could help identify their roles in PNS development and myelination.

ADAM22 together with ADAM11 and ADAM23 make up a small group in the Adams family. These three proteins are highly expressed in brain, show sequence similarity with each other, and all lack the metalloprotease activity due to the absence of a zinc binding motif in the metalloprotease domain [241]. ADAM11 knock out mice appear normal [242]. Although other studies indicate impaired learning and motor skills, and altered nociception, suggesting a role in synaptic transmission [243, 244]. ADAM23 mice in contrast die during early postnatal development. Furthermore, these mice show tremor and ataxia, possibly related to the role of ADAM23 in cerebellum [245, 246]. ADAM23 was also shown to mediate cell-cell interactions via $\alpha v \beta 3$ integrin, and is involved in tumour progression [247, 248]. ADAM22 knock out mice die before weaning, and display tremor and ataxia like ADAM23 knock outs. It is thought that both proteins function in the cerebellum in non-redundant pathways. Furthermore, peripheral nerves of ADAM22 knock out mice show hypomyelination [249]. The accompanying limb abnormalities and the myelination defects are similar to the Claw paw mice, suggesting a role for ADAM22 in the same pathway. Alternative splicing of ADAM22 results in isoforms with different lengths of the cytoplasmic tail, as well as a secreted form. Interactions of the cytoplasmic tail and 14-3-3 proteins was shown to be important in cell surface expression of ADAM22, and its subsequent function in cell adhesion and spreading [250-252]. Furthermore, the disintegrin domain of ADAM22 inhibits proliferation via its interaction with integrins, possibly by interfering with ILK activation [253]. The authors further show that the disintegrin domain induces cellular adhesion via integrins $\alpha 9$, $\alpha 6$, and $\beta 3$. These observations, together with the previously mentioned interactions of ADAM22 with Lgi1, potassium channels, MAGUKs, CAMs, etc. puts ADAM22 in the middle of many developmental and functional aspects of neurons, as well as Schwann cells.

The following chapters describe our efforts in the characterization of Lgi4 and Adam22 molecules in PNS myelination.

References:

1. Solomon, E.P., L.R. Berg, and D.W. Martin, *Biology*. 6th ed. 2002, [Pacific Grove, CA]: Brooks/Cole Thomson Learning. 1 v. (various pagings).
2. Alberts, B., *Molecular biology of the cell*. 4th ed. 2002, New York: Garland Science. xxxiv, [1548] p.
3. Sanes, D.H., T.A. Reh, and W.A. Harris, *Development of the nervous system*. 2000, San Diego, Calif.: Academic Press. xiv, 500 p.
4. Liem, K.F. and W.F. Walker, *Functional anatomy of the vertebrates : an evolutionary perspective*. 3rd ed. 2001, Fort Worth: Harcourt College Publishers. xvii, 703 p.
5. Kandel, E.R., J.H. Schwartz, and T.M. Jessell, *Principles of neural science*. 4th ed. 2000, New York: McGraw-Hill, Health Professions Division. xli, 1414 p.
6. Hormuzdi, S.G., et al., *Electrical synapses: a dynamic signaling system that shapes the activity of neuronal networks*. *Biochim Biophys Acta*, 2004. **1662**(1-2): p. 113-37.
7. Ribak, C.E. and R.C. Roberts, *GABAergic synapses in the brain identified with antisera to GABA and its synthesizing enzyme, glutamate decarboxylase*. *J Electron Microscop Tech*, 1990. **15**(1): p. 34-48.
8. Jacobson, M., *Developmental neurobiology*. 3rd ed. 1991, New York: Plenum Press. ix, 776 p.
9. Kettenmann, H. and B.R. Ransom, *Neuroglia*. 2nd ed. 2005, New York: Oxford University Press. xix, 601 p.
10. Vernadakis, A. and B.I. Roots, *Neuron-glia interrelations during phylogeny*. *Contemporary neuroscience*. 1995, Totowa, N.J.: Humana Press.
11. Kettenmann, H. and B.R. Ransom, *Neuroglia*. 1995, New York: Oxford University Press. xxii, 1079 p.
12. Lazzarini, R.A., *Myelin biology and disorders*. 2004, Sand Diego, Calif.: Elsevier Academic Press.
13. Waehneltd, T.V., *Phylogeny of myelin proteins*. *Ann N Y Acad Sci*, 1990. **605**: p. 15-28.
14. Davis, A.D., et al., *Myelin-like sheaths in copepod axons*. *Nature*, 1999. **398**(6728): p. 571.
15. Roots, B.I., *The phylogeny of invertebrates and the evolution of myelin*. *Neuron Glia Biol*, 2009: p. 1-9.
16. Hartline, D.K. and D.R. Colman, *Rapid conduction and the evolution of giant axons and myelinated fibers*. *Curr Biol*, 2007. **17**(1): p. R29-35.
17. Friede, R.L. and T. Samorajski, *Relation between the number of myelin lamellae and axon circumference in fibers of vagus and sciatic nerves of mice*. *J Comp Neurol*, 1967. **130**(3): p. 223-31.
18. Friede, R.L. and R. Bischhausen, *How are sheath dimensions affected by axon caliber and internode length?* *Brain Res*, 1982. **235**(2): p. 335-50.
19. Jessen, K.R. and R. Mirsky, *The origin and development of glial cells in peripheral nerves*. *Nat Rev Neurosci*, 2005. **6**(9): p. 671-82.
20. Birchmeier, C. and K.A. Nave, *Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation*. *Glia*, 2008. **56**(14): p. 1491-7.

21. Nave, K.A. and J.L. Salzer, *Axonal regulation of myelination by neuregulin 1*. *Curr Opin Neurobiol*, 2006. **16**(5): p. 492-500.
22. Dyck, P.J., *Peripheral neuropathy*. 3rd ed ed. 1993, Philadelphia ; London: Saunders. 2 v. (xx, 1720, xlii p., 4. p. of col. plates).
23. Dupin, E., et al., *Neural crest progenitors and stem cells*. *C R Biol*, 2007. **330**(6-7): p. 521-9.
24. Meulemans, D. and M. Bronner-Fraser, *Gene-regulatory interactions in neural crest evolution and development*. *Dev Cell*, 2004. **7**(3): p. 291-9.
25. Basch, M.L., M.I. Garcia-Castro, and M. Bronner-Fraser, *Molecular mechanisms of neural crest induction*. *Birth Defects Res C Embryo Today*, 2004. **72**(2): p. 109-23.
26. Sasai, Y. and E.M. De Robertis, *Ectodermal patterning in vertebrate embryos*. *Dev Biol*, 1997. **182**(1): p. 5-20.
27. Liem, K.F., Jr., et al., *Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm*. *Cell*, 1995. **82**(6): p. 969-79.
28. Ikeya, M., et al., *Wnt signalling required for expansion of neural crest and CNS progenitors*. *Nature*, 1997. **389**(6654): p. 966-70.
29. LaBonne, C. and M. Bronner-Fraser, *Neural crest induction in Xenopus: evidence for a two-signal model*. *Development*, 1998. **125**(13): p. 2403-14.
30. Bonstein, L., S. Elias, and D. Frank, *Paraxial-fated mesoderm is required for neural crest induction in Xenopus embryos*. *Dev Biol*, 1998. **193**(2): p. 156-68.
31. Marchant, L., et al., *The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient*. *Dev Biol*, 1998. **198**(2): p. 319-29.
32. Bang, A.G., et al., *Expression of Pax-3 in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm*. *Dev Biol*, 1999. **212**(2): p. 366-80.
33. Monsoro-Burq, A.H., R.B. Fletcher, and R.M. Harland, *Neural crest induction by paraxial mesoderm in Xenopus embryos requires FGF signals*. *Development*, 2003. **130**(14): p. 3111-24.
34. Garcia-Castro, M.I., C. Marcelle, and M. Bronner-Fraser, *Ectodermal Wnt function as a neural crest inducer*. *Science*, 2002. **297**(5582): p. 848-51.
35. Anderson, R.M., et al., *Endogenous bone morphogenetic protein antagonists regulate mammalian neural crest generation and survival*. *Dev Dyn*, 2006. **235**(9): p. 2507-20.
36. Endo, Y., N. Osumi, and Y. Wakamatsu, *Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development*. *Development*, 2002. **129**(4): p. 863-73.
37. Briscoe, J. and J. Ericson, *The specification of neuronal identity by graded Sonic Hedgehog signalling*. *Semin Cell Dev Biol*, 1999. **10**(3): p. 353-62.
38. Steventon, B., C. Carmona-Fontaine, and R. Mayor, *Genetic network during neural crest induction: from cell specification to cell survival*. *Semin Cell Dev Biol*, 2005. **16**(6): p. 647-54.
39. Christiansen, J.H., E.G. Coles, and D.G. Wilkinson, *Molecular control of neural crest formation, migration and differentiation*. *Curr Opin Cell Biol*, 2000. **12**(6): p. 719-24.
40. Sela-Donenfeld, D. and C. Kalcheim, *Regulation of the onset of neural crest*

- migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development*, 1999. **126**(21): p. 4749-62.
41. Nieto, M.A., et al., *Control of cell behavior during vertebrate development by Slug, a zinc finger gene*. *Science*, 1994. **264**(5160): p. 835-9.
 42. Mayor, R., R. Morgan, and M.G. Sargent, *Induction of the prospective neural crest of Xenopus*. *Development*, 1995. **121**(3): p. 767-77.
 43. Taneyhill, L.A., *To adhere or not to adhere: the role of Cadherins in neural crest development*. *Cell Adh Migr*, 2008. **2**(4): p. 223-30.
 44. Baroffio, A., E. Dupin, and N.M. Le Douarin, *Common precursors for neural and mesectodermal derivatives in the cephalic neural crest*. *Development*, 1991. **112**(1): p. 301-5.
 45. Le Douarin, N.M. and E. Dupin, *Multipotentiality of the neural crest*. *Curr Opin Genet Dev*, 2003. **13**(5): p. 529-36.
 46. Trentin, A., et al., *Self-renewal capacity is a widespread property of various types of neural crest precursor cells*. *Proc Natl Acad Sci U S A*, 2004. **101**(13): p. 4495-500.
 47. Dupin, E., C. Ziller, and N.M. Le Douarin, *The avian embryo as a model in developmental studies: chimeras and in vitro clonal analysis*. *Curr Top Dev Biol*, 1998. **36**: p. 1-35.
 48. Li, H.Y., E.H. Say, and X.F. Zhou, *Isolation and characterization of neural crest progenitors from adult dorsal root ganglia*. *Stem Cells*, 2007. **25**(8): p. 2053-65.
 49. Lagares, A., et al., *Primary sensory neuron addition in the adult rat trigeminal ganglion: evidence for neural crest glio-neuronal precursor maturation*. *J Neurosci*, 2007. **27**(30): p. 7939-53.
 50. Anderson, D.J., *Cellular and molecular biology of neural crest cell lineage determination*. *Trends Genet*, 1997. **13**(7): p. 276-80.
 51. Henion, P.D. and J.A. Weston, *Timing and pattern of cell fate restrictions in the neural crest lineage*. *Development*, 1997. **124**(21): p. 4351-9.
 52. Shah, N.M., et al., *Glial growth factor restricts mammalian neural crest stem cells to a glial fate*. *Cell*, 1994. **77**(3): p. 349-60.
 53. Shah, N.M., A.K. Groves, and D.J. Anderson, *Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members*. *Cell*, 1996. **85**(3): p. 331-43.
 54. Hari, L., et al., *Lineage-specific requirements of beta-catenin in neural crest development*. *J Cell Biol*, 2002. **159**(5): p. 867-80.
 55. Lee, H.Y., et al., *Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells*. *Science*, 2004. **303**(5660): p. 1020-3.
 56. Shah, N.M. and D.J. Anderson, *Integration of multiple instructive cues by neural crest stem cells reveals cell-intrinsic biases in relative growth factor responsiveness*. *Proc Natl Acad Sci U S A*, 1997. **94**(21): p. 11369-74.
 57. Greenwood, A.L., E.E. Turner, and D.J. Anderson, *Identification of dividing, determined sensory neuron precursors in the mammalian neural crest*. *Development*, 1999. **126**(16): p. 3545-59.
 58. Morrison, S.J., et al., *Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells*. *Cell*, 2000. **101**(5): p. 499-510.

59. Taylor, M.K., K. Yeager, and S.J. Morrison, *Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems*. *Development*, 2007. **134**(13): p. 2435-47.
60. Kubu, C.J., et al., *Developmental changes in Notch1 and numb expression mediated by local cell-cell interactions underlie progressively increasing delta sensitivity in neural crest stem cells*. *Dev Biol*, 2002. **244**(1): p. 199-214.
61. Schneider, C., et al., *Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons*. *Neuron*, 1999. **24**(4): p. 861-70.
62. Stanke, M., et al., *The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons*. *Development*, 1999. **126**(18): p. 4087-94.
63. Pattyn, A., et al., *The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives*. *Nature*, 1999. **399**(6734): p. 366-70.
64. Blaugrund, E., et al., *Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence*. *Development*, 1996. **122**(1): p. 309-20.
65. Durbec, P.L., et al., *Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts*. *Development*, 1996. **122**(1): p. 349-58.
66. Perez, S.E., S. Rebelo, and D.J. Anderson, *Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo*. *Development*, 1999. **126**(8): p. 1715-28.
67. Ma, Q., et al., *Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia*. *Genes Dev*, 1999. **13**(13): p. 1717-28.
68. Anderson, D.J., *Lineages and transcription factors in the specification of vertebrate primary sensory neurons*. *Curr Opin Neurobiol*, 1999. **9**(5): p. 517-24.
69. McEvelly, R.J., et al., *Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons*. *Nature*, 1996. **384**(6609): p. 574-7.
70. Sun, Y., et al., *A central role for Islet1 in sensory neuron development linking sensory and spinal gene regulatory programs*. *Nat Neurosci*, 2008. **11**(11): p. 1283-93.
71. Bibel, M. and Y.A. Barde, *Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system*. *Genes Dev*, 2000. **14**(23): p. 2919-37.
72. Roux, P.P. and P.A. Barker, *Neurotrophin signaling through the p75 neurotrophin receptor*. *Prog Neurobiol*, 2002. **67**(3): p. 203-33.
73. Jessen, K.R. and R. Mirsky, *Schwann cells and their precursors emerge as major regulators of nerve development*. *Trends Neurosci*, 1999. **22**(9): p. 402-10.
74. Dong, Z., et al., *Schwann cell development in embryonic mouse nerves*. *J Neurosci Res*, 1999. **56**(4): p. 334-48.
75. Mirsky, R. and K.R. Jessen, *Schwann cell development, differentiation and myelination*. *Curr Opin Neurobiol*, 1996. **6**(1): p. 89-96.
76. Kuhlbrodt, K., et al., *Sox10, a novel transcriptional modulator in glial cells*. *J Neurosci*, 1998. **18**(1): p. 237-50.
77. Paratore, C., et al., *Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic*

- combinatorial signaling*. *Development*, 2001. **128**(20): p. 3949-61.
78. Britsch, S., et al., *The transcription factor Sox10 is a key regulator of peripheral glial development*. *Genes Dev*, 2001. **15**(1): p. 66-78.
79. Riethmacher, D., et al., *Severe neuropathies in mice with targeted mutations in the ErbB3 receptor*. *Nature*, 1997. **389**(6652): p. 725-30.
80. Garratt, A.N., et al., *A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool*. *J Cell Biol*, 2000. **148**(5): p. 1035-46.
81. Garratt, A.N., S. Britsch, and C. Birchmeier, *Neuregulin, a factor with many functions in the life of a schwann cell*. *Bioessays*, 2000. **22**(11): p. 987-96.
82. Lyons, D.A., et al., *erbb3 and erbb2 are essential for schwann cell migration and myelination in zebrafish*. *Curr Biol*, 2005. **15**(6): p. 513-24.
83. Maro, G.S., et al., *Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS*. *Nat Neurosci*, 2004. **7**(9): p. 930-8.
84. Vermeren, M., et al., *Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points*. *Neuron*, 2003. **37**(3): p. 403-15.
85. Wanner, I.B., et al., *Role of N-cadherin in Schwann cell precursors of growing nerves*. *Glia*, 2006. **54**(5): p. 439-59.
86. Takahashi, M. and N. Osumi, *Identification of a novel type II classical cadherin: rat cadherin19 is expressed in the cranial ganglia and Schwann cell precursors during development*. *Dev Dyn*, 2005. **232**(1): p. 200-8.
87. Joseph, N.M., et al., *Neural crest stem cells undergo multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells*. *Development*, 2004. **131**(22): p. 5599-612.
88. Parmantier, E., et al., *Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths*. *Neuron*, 1999. **23**(4): p. 713-24.
89. Mirsky, R., et al., *Schwann cell-derived desert hedgehog signals nerve sheath formation*. *Ann N Y Acad Sci*, 1999. **883**: p. 196-202.
90. Woodhoo, A., et al., *Notch controls embryonic Schwann cell differentiation, postnatal myelination and adult plasticity*. *Nat Neurosci*, 2009. **12**(7): p. 839-47.
91. Brennan, A., et al., *Endothelins control the timing of Schwann cell generation in vitro and in vivo*. *Dev Biol*, 2000. **227**(2): p. 545-57.
92. Grinspan, J.B., et al., *Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins*. *J Neurosci*, 1996. **16**(19): p. 6107-18.
93. Syroid, D.E., et al., *Cell death in the Schwann cell lineage and its regulation by neuregulin*. *Proc Natl Acad Sci U S A*, 1996. **93**(17): p. 9229-34.
94. Dong, Z., et al., *Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors*. *Neuron*, 1995. **15**(3): p. 585-96.
95. Leimeroth, R., et al., *Membrane-bound neuregulin1 type III actively promotes Schwann cell differentiation of multipotent Progenitor cells*. *Dev Biol*, 2002. **246**(2): p. 245-58.
96. Meier, C., et al., *Developing Schwann cells acquire the ability to survive without axons by establishing an autocrine circuit involving insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB*. *J Neurosci*, 1999. **19**(10): p.

- 3847-59.
97. Weiner, J.A. and J. Chun, *Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid*. Proc Natl Acad Sci USA, 1999. **96**(9): p. 5233-8.
 98. Dowsing, B.J., et al., *Leukemia inhibitory factor is an autocrine survival factor for Schwann cells*. J Neurochem, 1999. **73**(1): p. 96-104.
 99. Xu, H., et al., *Murine muscular dystrophy caused by a mutation in the laminin alpha 2 (Lama2) gene*. Nat Genet, 1994. **8**(3): p. 297-302.
 100. Chen, Z.L. and S. Strickland, *Laminin gamma1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve*. J Cell Biol, 2003. **163**(4): p. 889-99.
 101. Yu, W.M., et al., *Schwann cell-specific ablation of laminin gamma1 causes apoptosis and prevents proliferation*. J Neurosci, 2005. **25**(18): p. 4463-72.
 102. Wallquist, W., et al., *Impeded interaction between Schwann cells and axons in the absence of laminin alpha4*. J Neurosci, 2005. **25**(14): p. 3692-700.
 103. Feltri, M.L., et al., *Conditional disruption of beta 1 integrin in Schwann cells impedes interactions with axons*. J Cell Biol, 2002. **156**(1): p. 199-209.
 104. Pietri, T., et al., *Conditional beta1-integrin gene deletion in neural crest cells causes severe developmental alterations of the peripheral nervous system*. Development, 2004. **131**(16): p. 3871-83.
 105. Feltri, M.L., U. Suter, and J.B. Relvas, *The function of RhoGTPases in axon ensheathment and myelination*. Glia, 2008. **56**(14): p. 1508-17.
 106. Benninger, Y., et al., *Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development*. J Cell Biol, 2007. **177**(6): p. 1051-61.
 107. Nodari, A., et al., *Beta1 integrin activates Rac1 in Schwann cells to generate radial lamellae during axonal sorting and myelination*. J Cell Biol, 2007. **177**(6): p. 1063-75.
 108. Shaw, R.J., et al., *The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling*. Dev Cell, 2001. **1**(1): p. 63-72.
 109. Melendez-Vasquez, C.V., S. Einheber, and J.L. Salzer, *Rho kinase regulates schwann cell myelination and formation of associated axonal domains*. J Neurosci, 2004. **24**(16): p. 3953-63.
 110. Pereira, J.A., et al., *Integrin-linked kinase is required for radial sorting of axons and Schwann cell remyelination in the peripheral nervous system*. J Cell Biol, 2009. **185**(1): p. 147-61.
 111. Cheng, H.L., et al., *IGF-I promotes Schwann cell motility and survival via activation of Akt*. Mol Cell Endocrinol, 2000. **170**(1-2): p. 211-5.
 112. Meintanis, S., et al., *The neuron-glia signal beta-neuregulin promotes Schwann cell motility via the MAPK pathway*. Glia, 2001. **34**(1): p. 39-51.
 113. Yamauchi, J., J.R. Chan, and E.M. Shooter, *Neurotrophins regulate Schwann cell migration by activating divergent signaling pathways dependent on Rho GTPases*. Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8774-9.
 114. Frago, G., et al., *Inhibition of p38 mitogen-activated protein kinase interferes with cell shape changes and gene expression associated with Schwann cell myelination*.

- Exp Neurol, 2003. **183**(1): p. 34-46.
115. Salzer, J.L., et al., *Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction.* J Cell Biol, 1980. **84**(3): p. 753-66.
 116. Morrissey, T.K., et al., *Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2.* Proc Natl Acad Sci U S A, 1995. **92**(5): p. 1431-5.
 117. Grove, M., et al., *FAK is required for axonal sorting by Schwann cells.* J Cell Biol, 2007. **176**(3): p. 277-82.
 118. Chernousov, M.A., et al., *Regulation of Schwann cell function by the extracellular matrix.* Glia, 2008. **56**(14): p. 1498-507.
 119. Komiyama, A. and K. Suzuki, *Age-related differences in proliferative responses of Schwann cells during Wallerian degeneration.* Brain Res, 1992. **573**(2): p. 267-75.
 120. Syroid, D.E., et al., *Induction of postnatal schwann cell death by the low-affinity neurotrophin receptor in vitro and after axotomy.* J Neurosci, 2000. **20**(15): p. 5741-7.
 121. Parkinson, D.B., et al., *Transforming growth factor beta (TGFbeta) mediates Schwann cell death in vitro and in vivo: examination of c-Jun activation, interactions with survival signals, and the relationship of TGFbeta-mediated death to Schwann cell differentiation.* J Neurosci, 2001. **21**(21): p. 8572-85.
 122. Ridley, A.J., et al., *Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells.* J Cell Biol, 1989. **109**(6 Pt 2): p. 3419-24.
 123. Einheber, S., et al., *Transforming growth factor-beta 1 regulates axon/Schwann cell interactions.* J Cell Biol, 1995. **129**(2): p. 443-58.
 124. Guenard, V., et al., *Effect of transforming growth factor-beta 1 and -beta 2 on Schwann cell proliferation on neurites.* Glia, 1995. **13**(4): p. 309-18.
 125. D'Antonio, M., et al., *TGFbeta type II receptor signaling controls Schwann cell death and proliferation in developing nerves.* J Neurosci, 2006. **26**(33): p. 8417-27.
 126. Parkinson, D.B., et al., *c-Jun is a negative regulator of myelination.* J Cell Biol, 2008. **181**(4): p. 625-37.
 127. Parkinson, D.B., et al., *Krox-20 inhibits Jun-NH2-terminal kinase/c-Jun to control Schwann cell proliferation and death.* J Cell Biol, 2004. **164**(3): p. 385-94.
 128. Jessen, K.R. and R. Mirsky, *Negative regulation of myelination: relevance for development, injury, and demyelinating disease.* Glia, 2008. **56**(14): p. 1552-65.
 129. Michailov, G.V., et al., *Axonal neuregulin-1 regulates myelin sheath thickness.* Science, 2004. **304**(5671): p. 700-3.
 130. Taveggia, C., et al., *Neuregulin-1 type III determines the ensheathment fate of axons.* Neuron, 2005. **47**(5): p. 681-94.
 131. Zanazzi, G., et al., *Glial growth factor/neuregulin inhibits Schwann cell myelination and induces demyelination.* J Cell Biol, 2001. **152**(6): p. 1289-99.
 132. Chen, S., et al., *Neuregulin 1-erbB signaling is necessary for normal myelination and sensory function.* J Neurosci, 2006. **26**(12): p. 3079-86.
 133. Atanasoski, S., et al., *ErbB2 signaling in Schwann cells is mostly dispensable for maintenance of myelinated peripheral nerves and proliferation of adult Schwann cells after injury.* J Neurosci, 2006. **26**(7): p. 2124-31.
 134. Hoke, A., et al., *Glial cell line-derived neurotrophic factor alters axon schwann cell units and promotes myelination in unmyelinated nerve fibers.* J Neurosci, 2003.

- 23(2): p. 561-7.
135. Chan, J.R., et al., *Neurotrophins are key mediators of the myelination program in the peripheral nervous system*. Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14661-8.
 136. Chan, J.R., et al., *NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes*. Neuron, 2004. **43**(2): p. 183-91.
 137. Dong, Z., et al., *Response of Schwann cells to mitogens in vitro is determined by pre-exposure to serum, time in vitro, and developmental age*. Glia, 1997. **20**(3): p. 219-30.
 138. Morgan, L., K.R. Jessen, and R. Mirsky, *The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition*. J Cell Biol, 1991. **112**(3): p. 457-67.
 139. Lemke, G. and M. Chao, *Axons regulate Schwann cell expression of the major myelin and NGF receptor genes*. Development, 1988. **102**(3): p. 499-504.
 140. Yoon, C., Z. Korade, and B.D. Carter, *Protein kinase A-induced phosphorylation of the p65 subunit of nuclear factor-kappaB promotes Schwann cell differentiation into a myelinating phenotype*. J Neurosci, 2008. **28**(14): p. 3738-46.
 141. Nickols, J.C., et al., *Activation of the transcription factor NF-kappaB in Schwann cells is required for peripheral myelin formation*. Nat Neurosci, 2003. **6**(2): p. 161-7.
 142. Mandemakers, W., et al., *A distal Schwann cell-specific enhancer mediates axonal regulation of the Oct-6 transcription factor during peripheral nerve development and regeneration*. EMBO J, 2000. **19**(12): p. 2992-3003.
 143. Ghazvini, M., et al., *A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration*. EMBO J, 2002. **21**(17): p. 4612-20.
 144. Scherer, S.S., et al., *Axons regulate Schwann cell expression of the POU transcription factor SCIP*. J Neurosci, 1994. **14**(4): p. 1930-42.
 145. Blanchard, A.D., et al., *Oct-6 (SCIP/Tst-1) is expressed in Schwann cell precursors, embryonic Schwann cells, and postnatal myelinating Schwann cells: comparison with Oct-1, Krox-20, and Pax-3*. J Neurosci Res, 1996. **46**(5): p. 630-40.
 146. Arroyo, E.J., et al., *Promyelinating Schwann cells express Tst-1/SCIP/Oct-6*. J Neurosci, 1998. **18**(19): p. 7891-902.
 147. Bermingham, J.R., Jr., et al., *Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration*. Genes Dev, 1996. **10**(14): p. 1751-62.
 148. Jaegle, M., et al., *The POU factor Oct-6 and Schwann cell differentiation*. Science, 1996. **273**(5274): p. 507-10.
 149. Jaegle, M., et al., *The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development*. Genes Dev, 2003. **17**(11): p. 1380-91.
 150. Schreiner, S., et al., *Hypomorphic Sox10 alleles reveal novel protein functions and unravel developmental differences in glial lineages*. Development, 2007. **134**(18): p. 3271-81.
 151. Ryu, E.J., et al., *Misexpression of Pou3f1 results in peripheral nerve hypomyelination and axonal loss*. J Neurosci, 2007. **27**(43): p. 11552-9.
 152. Jaegle, M. and D. Meijer, *Role of Oct-6 in Schwann cell differentiation*. Microsc Res

- Tech, 1998. **41**(5): p. 372-8.
153. Zorick, T.S., et al., *Krox-20 controls SCIP expression, cell cycle exit and susceptibility to apoptosis in developing myelinating Schwann cells*. *Development*, 1999. **126**(7): p. 1397-406.
154. Kuhlbrodt, K., et al., *Cooperative function of POU proteins and SOX proteins in glial cells*. *J Biol Chem*, 1998. **273**(26): p. 16050-7.
155. Ghislain, J. and P. Charnay, *Control of myelination in Schwann cells: a Krox20 cis-regulatory element integrates Oct6, Brn2 and Sox10 activities*. *EMBO Rep*, 2006. **7**(1): p. 52-8.
156. Kao, S.C., et al., *Calcineurin/NFAT signaling is required for neuregulin-regulated Schwann cell differentiation*. *Science*, 2009. **323**(5914): p. 651-4.
157. LeBlanc, S.E., R.M. Ward, and J. Svaren, *Neuropathy-associated Egr2 mutants disrupt cooperative activation of myelin protein zero by Egr2 and Sox10*. *Mol Cell Biol*, 2007. **27**(9): p. 3521-9.
158. Jones, E.A., et al., *Interactions of Sox10 and Egr2 in myelin gene regulation*. *Neuron Glia Biol*, 2007. **3**(4): p. 377-87.
159. Svaren, J. and D. Meijer, *The molecular machinery of myelin gene transcription in Schwann cells*. *Glia*, 2008. **56**(14): p. 1541-51.
160. Topilko, P., et al., *Krox-20 controls myelination in the peripheral nervous system*. *Nature*, 1994. **371**(6500): p. 796-9.
161. Le, N., et al., *Analysis of congenital hypomyelinating Egr2Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell differentiation and myelination*. *Proc Natl Acad Sci U S A*, 2005. **102**(7): p. 2596-601.
162. Decker, L., et al., *Peripheral myelin maintenance is a dynamic process requiring constant Krox20 expression*. *J Neurosci*, 2006. **26**(38): p. 9771-9.
163. Leblanc, S.E., et al., *Regulation of cholesterol/lipid biosynthetic genes by Egr2/Krox20 during peripheral nerve myelination*. *J Neurochem*, 2005. **93**(3): p. 737-48.
164. Griffin, J.W. and W.J. Thompson, *Biology and pathology of nonmyelinating Schwann cells*. *Glia*, 2008. **56**(14): p. 1518-31.
165. Woodhoo, A. and L. Sommer, *Development of the Schwann cell lineage: from the neural crest to the myelinated nerve*. *Glia*, 2008. **56**(14): p. 1481-90.
166. Scherer, S.S. and E.J. Arroyo, *Recent progress on the molecular organization of myelinated axons*. *J Peripher Nerv Syst*, 2002. **7**(1): p. 1-12.
167. Bunge, R.P., M.B. Bunge, and M. Bates, *Movements of the Schwann cell nucleus implicate progression of the inner (axon-related) Schwann cell process during myelination*. *J Cell Biol*, 1989. **109**(1): p. 273-84.
168. Scherer, S.S. and L. Wrabetz, *Molecular mechanisms of inherited demyelinating neuropathies*. *Glia*, 2008. **56**(14): p. 1578-89.
169. Martini, R., et al., *Mice doubly deficient in the genes for P0 and myelin basic protein show that both proteins contribute to the formation of the major dense line in peripheral nerve myelin*. *J Neurosci*, 1995. **15**(6): p. 4488-95.
170. Schnaar, R.L. and P.H. Lopez, *Myelin-associated glycoprotein and its axonal receptors*. *J Neurosci Res*, 2009.
171. Salzer, J.L., *Polarized domains of myelinated axons*. *Neuron*, 2003. **40**(2): p. 297-318.

172. Spiegel, I., et al., *A central role for Necl4 (SynCAM4) in Schwann cell-axon interaction and myelination*. *Nat Neurosci*, 2007. **10**(7): p. 861-9.
173. Maurel, P., et al., *Nectin-like proteins mediate axon Schwann cell interactions along the internode and are essential for myelination*. *J Cell Biol*, 2007. **178**(5): p. 861-74.
174. Feltri, M.L., et al., *Beta 4 integrin expression in myelinating Schwann cells is polarized, developmentally regulated and axonally dependent*. *Development*, 1994. **120**(5): p. 1287-301.
175. Yamada, H., et al., *Dystroglycan is a dual receptor for agrin and laminin-2 in Schwann cell membrane*. *J Biol Chem*, 1996. **271**(38): p. 23418-23.
176. Sherman, D.L., et al., *Specific disruption of a schwann cell dystrophin-related protein complex in a demyelinating neuropathy*. *Neuron*, 2001. **30**(3): p. 677-87.
177. Saito, F., et al., *Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization*. *Neuron*, 2003. **38**(5): p. 747-58.
178. Occhi, S., et al., *Both laminin and Schwann cell dystroglycan are necessary for proper clustering of sodium channels at nodes of Ranvier*. *J Neurosci*, 2005. **25**(41): p. 9418-27.
179. Salzer, J.L., P.J. Brophy, and E. Peles, *Molecular domains of myelinated axons in the peripheral nervous system*. *Glia*, 2008. **56**(14): p. 1532-40.
180. Melendez-Vasquez, C.V., et al., *Nodes of Ranvier form in association with ezrin-radixin-moesin (ERM)-positive Schwann cell processes*. *Proc Natl Acad Sci U S A*, 2001. **98**(3): p. 1235-40.
181. Lambert, S., J.Q. Davis, and V. Bennett, *Morphogenesis of the node of Ranvier: co-clusters of ankyrin and ankyrin-binding integral proteins define early developmental intermediates*. *J Neurosci*, 1997. **17**(18): p. 7025-36.
182. Eshed, Y., et al., *Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier*. *Neuron*, 2005. **47**(2): p. 215-29.
183. Eshed, Y., et al., *Secreted gliomedin is a perinodal matrix component of peripheral nerves*. *J Cell Biol*, 2007. **177**(3): p. 551-62.
184. Dzhashiashvili, Y., et al., *Nodes of Ranvier and axon initial segments are ankyrin G-dependent domains that assemble by distinct mechanisms*. *J Cell Biol*, 2007. **177**(5): p. 857-70.
185. Rios, J.C., et al., *Contactin-associated protein (Caspr) and contactin form a complex that is targeted to the paranodal junctions during myelination*. *J Neurosci*, 2000. **20**(22): p. 8354-64.
186. Charles, P., et al., *Neurofascin is a glial receptor for the paranodin/Caspr-contactin axonal complex at the axoglial junction*. *Curr Biol*, 2002. **12**(3): p. 217-20.
187. Gollan, L., et al., *Caspr regulates the processing of contactin and inhibits its binding to neurofascin*. *J Cell Biol*, 2003. **163**(6): p. 1213-8.
188. Eisenbach, M., et al., *Differential clustering of Caspr by oligodendrocytes and Schwann cells*. *J Neurosci Res*, 2009.
189. Basak, S., et al., *Differential expression and functions of neuronal and glial neurofascin isoforms and splice variants during PNS development*. *Dev Biol*, 2007. **311**(2): p. 408-22.
190. Pillai, A.M., et al., *Spatiotemporal ablation of myelinating glia-specific neurofascin (Nfasc NF155) in mice reveals gradual loss of paranodal axoglial junctions and*

- concomitant disorganization of axonal domains.* J Neurosci Res, 2009. **87**(8): p. 1773-93.
191. Gollan, L., et al., *Retention of a cell adhesion complex at the paranodal junction requires the cytoplasmic region of Caspr.* J Cell Biol, 2002. **157**(7): p. 1247-56.
192. Rasband, M.N., *It's "juxta" potassium channel!* J Neurosci Res, 2004. **76**(6): p. 749-57.
193. Traka, M., et al., *Association of TAG-1 with Caspr2 is essential for the molecular organization of juxtaparanodal regions of myelinated fibers.* J Cell Biol, 2003. **162**(6): p. 1161-72.
194. Poliak, S., et al., *Juxtaparanodal clustering of Shaker-like K⁺ channels in myelinated axons depends on Caspr2 and TAG-1.* J Cell Biol, 2003. **162**(6): p. 1149-60.
195. Horresh, I., et al., *Multiple molecular interactions determine the clustering of Caspr2 and Kv1 channels in myelinated axons.* J Neurosci, 2008. **28**(52): p. 14213-22.
196. Rasband, M.N., et al., *Clustering of neuronal potassium channels is independent of their interaction with PSD-95.* J Cell Biol, 2002. **159**(4): p. 663-72.
197. Birchmeier, C., *ErbB receptors and the development of the nervous system.* Exp Cell Res, 2009. **315**(4): p. 611-8.
198. Falls, D.L., *Neuregulins: functions, forms, and signaling strategies.* Exp Cell Res, 2003. **284**(1): p. 14-30.
199. Mei, L. and W.C. Xiong, *Neuregulin 1 in neural development, synaptic plasticity and schizophrenia.* Nat Rev Neurosci, 2008. **9**(6): p. 437-52.
200. Hayes, N.V. and W.J. Gullick, *The neuregulin family of genes and their multiple splice variants in breast cancer.* J Mammary Gland Biol Neoplasia, 2008. **13**(2): p. 205-14.
201. Brockes, J.P., G.E. Lemke, and D.R. Balzer, Jr., *Purification and preliminary characterization of a glial growth factor from the bovine pituitary.* J Biol Chem, 1980. **255**(18): p. 8374-7.
202. Lemke, G.E. and J.P. Brockes, *Identification and purification of glial growth factor.* J Neurosci, 1984. **4**(1): p. 75-83.
203. Wang, J.Y., S.J. Miller, and D.L. Falls, *The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain.* J Biol Chem, 2001. **276**(4): p. 2841-51.
204. Montero, J.C., et al., *Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor-alpha-converting enzyme.* Mol Cell Neurosci, 2000. **16**(5): p. 631-48.
205. Shirakabe, K., et al., *Roles of Meltrin beta /ADAM19 in the processing of neuregulin.* J Biol Chem, 2001. **276**(12): p. 9352-8.
206. Willem, M., et al., *Control of peripheral nerve myelination by the beta-secretase BACE1.* Science, 2006. **314**(5799): p. 664-6.
207. Berti, C., et al., *Role of integrins in peripheral nerves and hereditary neuropathies.* Neuromolecular Med, 2006. **8**(1-2): p. 191-204.
208. Court, F.A., L. Wrabetz, and M.L. Feltri, *Basal lamina: Schwann cells wrap to the rhythm of space-time.* Curr Opin Neurobiol, 2006. **16**(5): p. 501-7.
209. Feltri, M.L. and L. Wrabetz, *Laminins and their receptors in Schwann cells and hereditary neuropathies.* J Peripher Nerv Syst, 2005. **10**(2): p. 128-43.

210. Gu, W., et al., *The LGI1 gene involved in lateral temporal lobe epilepsy belongs to a new subfamily of leucine-rich repeat proteins*. FEBS Lett, 2002. **519**(1-3): p. 71-6.
211. Chernova, O.B., R.P. Somerville, and J.K. Cowell, *A novel gene, LGI1, from 10q24 is rearranged and downregulated in malignant brain tumors*. Oncogene, 1998. **17**(22): p. 2873-81.
212. Scheel, H., S. Tomiuk, and K. Hofmann, *A common protein interaction domain links two recently identified epilepsy genes*. Hum Mol Genet, 2002. **11**(15): p. 1757-62.
213. Staub, E., et al., *The novel EPTP repeat defines a superfamily of proteins implicated in epileptic disorders*. Trends Biochem Sci, 2002. **27**(9): p. 441-4.
214. Senechal, K.R., C. Thaller, and J.L. Noebels, *ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy*. Hum Mol Genet, 2005. **14**(12): p. 1613-20.
215. Buchanan, S.G. and N.J. Gay, *Structural and functional diversity in the leucine-rich repeat family of proteins*. Prog Biophys Mol Biol, 1996. **65**(1-2): p. 1-44.
216. Kajava, A.V., *Structural diversity of leucine-rich repeat proteins*. J Mol Biol, 1998. **277**(3): p. 519-27.
217. Kunapuli, P., et al., *LGI1, a putative tumor metastasis suppressor gene, controls in vitro invasiveness and expression of matrix metalloproteinases in glioma cells through the ERK1/2 pathway*. J Biol Chem, 2004. **279**(22): p. 23151-7.
218. Gabellini, N., et al., *Increased expression of LGI1 gene triggers growth inhibition and apoptosis of neuroblastoma cells*. J Cell Physiol, 2006. **207**(3): p. 711-21.
219. Piepoli, T., et al., *Expression studies in gliomas and glial cells do not support a tumor suppressor role for LGI1*. Neuro Oncol, 2006. **8**(2): p. 96-108.
220. Kalachikov, S., et al., *Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features*. Nat Genet, 2002. **30**(3): p. 335-41.
221. Michelucci, R., E. Pasini, and C. Nobile, *Lateral temporal lobe epilepsies: clinical and genetic features*. Epilepsia, 2009. **50 Suppl 5**: p. 52-4.
222. Sirerol-Piquer, M.S., et al., *The epilepsy gene LGI1 encodes a secreted glycoprotein that binds to the cell surface*. Hum Mol Genet, 2006. **15**(23): p. 3436-45.
223. Schulte, U., et al., *The epilepsy-linked Lgi1 protein assembles into presynaptic Kv1 channels and inhibits inactivation by Kvbeta1*. Neuron, 2006. **49**(5): p. 697-706.
224. Fukata, Y., et al., *Epilepsy-related ligand/receptor complex LGI1 and ADAM22 regulate synaptic transmission*. Science, 2006. **313**(5794): p. 1792-5.
225. Chabrol, E., et al., *Absence of mutations in the LGI1 receptor ADAM22 gene in autosomal dominant lateral temporal epilepsy*. Epilepsy Res, 2007. **76**(1): p. 41-8.
226. Diani, E., et al., *Autosomal dominant lateral temporal epilepsy: absence of mutations in ADAM22 and Kv1 channel genes encoding LGI1-associated proteins*. Epilepsy Res, 2008. **80**(1): p. 1-8.
227. d'Orsi, G. and P. Tinuper, *"I heard voices...": from semiology, a historical review, and a new hypothesis on the presumed epilepsy of Joan of Arc*. Epilepsy Behav, 2006. **9**(1): p. 152-7.
228. Kimura, N., et al., *Abeta upregulates and colocalizes with LGI3 in cultured rat astrocytes*. Cell Mol Neurobiol, 2007. **27**(3): p. 335-50.
229. Okabayashi, S. and N. Kimura, *Immunohistochemical and biochemical analyses of LGI3 in monkey brain: LGI3 accumulates in aged monkey brains*. Cell Mol

- Neurobiol, 2007. **27**(6): p. 819-30.
230. Okabayashi, S. and N. Kimura, *Leucine-rich glioma inactivated 3 is involved in amyloid beta peptide uptake by astrocytes and endocytosis itself*. Neuroreport, 2008. **19**(12): p. 1175-9.
231. Park, W.J., et al., *Leucine-rich glioma inactivated 3 associates with syntaxin 1*. Neurosci Lett, 2008. **444**(3): p. 240-4.
232. Gu, W., et al., *Genotypic association of exonic LGI4 polymorphisms and childhood absence epilepsy*. Neurogenetics, 2004. **5**(1): p. 41-4.
233. Runkel, F., M. Michels, and T. Franz, *Fxyd3 and Lgi4 expression in the adult mouse: a case of endogenous antisense expression*. Mamm Genome, 2003. **14**(10): p. 665-72.
234. Gu, W., et al., *Using gene-history and expression analyses to assess the involvement of LGI genes in human disorders*. Mol Biol Evol, 2005. **22**(11): p. 2209-16.
235. Bermingham, J.R., Jr., et al., *The Claw paw mutation reveals a role for Lgi4 in peripheral nerve development*. Nat Neurosci, 2006. **9**(1): p. 76-84.
236. Henry, E.W., E.M. Eicher, and R.L. Sidman, *The mouse mutation Claw paw: forelimb deformity and delayed myelination throughout the peripheral nervous system*. J Hered, 1991. **82**(4): p. 287-94.
237. Koszowski, A.G., G.C. Owens, and S.R. Levinson, *The effect of the mouse mutation Claw paw on myelination and nodal frequency in sciatic nerves*. J Neurosci, 1998. **18**(15): p. 5859-68.
238. Bermingham, J.R., Jr., et al., *Identification of genes that are downregulated in the absence of the POU domain transcription factor pou3f1 (Oct-6, Tst-1, SCIP) in sciatic nerve*. J Neurosci, 2002. **22**(23): p. 10217-31.
239. Darbas, A., et al., *Cell autonomy of the mouse Claw paw mutation*. Dev Biol, 2004. **272**(2): p. 470-82.
240. Darbas, A., *The Mouse Mutant Claw paw: Cellular and genetic aspects*. Phd Thesis, 2005, Erasmus University.
241. Blobel, C.P., et al., *A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion*. Nature, 1992. **356**(6366): p. 248-52.
242. Wolfsberg, T.G., et al., *The precursor region of a protein active in sperm-egg fusion contains a metalloprotease and a disintegrin domain: structural, functional, and evolutionary implications*. Proc Natl Acad Sci U S A, 1993. **90**(22): p. 10783-7.
243. Eto, K., et al., *Functional classification of ADAMs based on a conserved motif for binding to integrin alpha 9beta 1: implications for sperm-egg binding and other cell interactions*. J Biol Chem, 2002. **277**(20): p. 17804-10.
244. Yang, P., K.A. Baker, and T. Hagg, *The ADAMs family: coordinators of nervous system development, plasticity and repair*. Prog Neurobiol, 2006. **79**(2): p. 73-94.
245. Novak, U., *ADAM proteins in the brain*. J Clin Neurosci, 2004. **11**(3): p. 227-35.
246. Hartmann, D., et al., *The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts*. Hum Mol Genet, 2002. **11**(21): p. 2615-24.
247. Lieber, T., S. Kidd, and M.W. Young, *kuzbanian-mediated cleavage of Drosophila Notch*. Genes Dev, 2002. **16**(2): p. 209-21.
248. Qi, H., et al., *Processing of the notch ligand delta by the metalloprotease Kuzbanian*. Science, 1999. **283**(5398): p. 91-4.

249. Brou, C., et al., *A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE*. *Mol Cell*, 2000. **5**(2): p. 207-16.
250. LaVoie, M.J. and D.J. Selkoe, *The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments*. *J Biol Chem*, 2003. **278**(36): p. 34427-37.
251. Wakatsuki, S., T. Kurisaki, and A. Sehara-Fujisawa, *Lipid rafts identified as locations of ectodomain shedding mediated by Meltrin beta/ADAM19*. *J Neurochem*, 2004. **89**(1): p. 119-23.
252. Yokozeki, T., et al., *Meltrin beta (ADAM19) mediates ectodomain shedding of Neuregulin beta1 in the Golgi apparatus: fluorescence correlation spectroscopic observation of the dynamics of ectodomain shedding in living cells*. *Genes Cells*, 2007. **12**(3): p. 329-43.
253. Wakatsuki, S., et al., *Roles of meltrin-beta/ADAM19 in progression of Schwann cell differentiation and myelination during sciatic nerve regeneration*. *J Biol Chem*, 2009. **284**(5): p. 2957-66.
254. Sagane, K., et al., *Metalloproteinase-like, disintegrin-like, cysteine-rich proteins MDC2 and MDC3: novel human cellular disintegrins highly expressed in the brain*. *Biochem J*, 1998. **334** (Pt 1): p. 93-8.
255. Sagane, K., et al., *Cloning and chromosomal mapping of mouse ADAM11, ADAM22 and ADAM23*. *Gene*, 1999. **236**(1): p. 79-86.
256. Takahashi, E., et al., *Deficits in spatial learning and motor coordination in ADAM11-deficient mice*. *BMC Neurosci*, 2006. **7**: p. 19.
257. Takahashi, E., et al., *Altered nociceptive response in ADAM11-deficient mice*. *Brain Res*, 2006. **1097**(1): p. 39-42.
258. Leighton, P.A., et al., *Defining brain wiring patterns and mechanisms through gene trapping in mice*. *Nature*, 2001. **410**(6825): p. 174-9.
259. Mitchell, K.J., et al., *Functional analysis of secreted and transmembrane proteins critical to mouse development*. *Nat Genet*, 2001. **28**(3): p. 241-9.
260. Cal, S., et al., *ADAM 23/MDC3, a human disintegrin that promotes cell adhesion via interaction with the alphavbeta3 integrin through an RGD-independent mechanism*. *Mol Biol Cell*, 2000. **11**(4): p. 1457-69.
261. Verbisck, N.V., et al., *ADAM23 negatively modulates alpha(v)beta(3) integrin activation during metastasis*. *Cancer Res*, 2009. **69**(13): p. 5546-52.
262. Sagane, K., et al., *Ataxia and peripheral nerve hypomyelination in ADAM22-deficient mice*. *BMC Neurosci*, 2005. **6**: p. 33.
263. Godde, N.J., et al., *Efficient ADAM22 surface expression is mediated by phosphorylation-dependent interaction with 14-3-3 protein family members*. *J Cell Sci*, 2006. **119**(Pt 16): p. 3296-305.
264. Zhu, P., et al., *The interaction between ADAM 22 and 14-3-3zeta: regulation of cell adhesion and spreading*. *Biochem Biophys Res Commun*, 2003. **301**(4): p. 991-9.
265. Zhu, P., et al., *ADAM22 plays an important role in cell adhesion and spreading with the assistance of 14-3-3*. *Biochem Biophys Res Commun*, 2005. **331**(4): p. 938-46.
266. D'Abaco, G.M., et al., *ADAM22, expressed in normal brain but not in high-grade gliomas, inhibits cellular proliferation via the disintegrin domain*. *Neurosurgery*, 2006. **58**(1): p. 179-86; discussion 179-86.

Chapter 2

The *claw paw* mutation reveals a role for *Lgi4* in peripheral nerve development

John R Bermingham Jr, Harold Shearin, Jamie Pennington, Jill O'Moore,
Martine Jaegle, Siska Driegen, Arend van Zon, Aysel Darbas, Ekim Özkaynak,
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and Dies Meijer

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The *claw paw* mutation reveals a role for *Lgi4* in peripheral nerve development

John R Bermingham Jr¹, Harold Shearin¹, Jamie Pennington¹, Jill O'Moore¹, Martine Jaegel², Siska Driegen², Arend van Zon², Aysel Darbas², Ekim Özkaynak², Elizabeth J Ryu^{3,4}, Jeffrey Milbrandt³ & Dies Meijer²

Peripheral nerve development results from multiple cellular interactions between axons, Schwann cells and the surrounding mesenchymal tissue. The delayed axonal sorting and hypomyelination throughout the peripheral nervous system of *claw paw* (*clp*) mutant mice suggest that the *clp* gene product is critical for these interactions. Here we identify the *clp* mutation as a 225-bp insertion in the *Lgi4* gene. *Lgi4* encodes a secreted and glycosylated leucine-rich repeat protein and is expressed in Schwann cells. The *clp* mutation affects *Lgi4* mRNA splicing, resulting in a mutant protein that is retained in the cell. Additionally, siRNA-mediated downregulation of *Lgi4* in wild-type neuron–Schwann cell cocultures inhibits myelination, whereas exogenous *Lgi4* restores myelination in *clp/clp* cultures. Thus, the abnormalities observed in *clp* mice are attributable to the loss of *Lgi4* function, and they identify *Lgi4* as a new component of Schwann cell signaling pathway(s) that controls axon segregation and myelin formation.

In the peripheral nervous system (PNS), Schwann cells synthesize myelin sheaths, which permit the rapid, saltatory conduction of nerve impulses. The debilitating effects of diseases that disrupt myelin emphasize the importance of understanding the mechanisms that control its synthesis. The development of peripheral nerve tissue results from a series of coordinated interactions between neurons and Schwann cells (reviewed in refs. 1 and 2). Schwann cell precursors originate from the neural crest and migrate along embryonic nerve trunks, proliferating in response to axonal cues and subdividing the nerve into smaller groups of fibers; ultimately, they differentiate into myelinating or non-myelinating Schwann cells. Several signaling proteins that regulate Schwann cell development and myelin formation are known. Axonal membrane-bound neuregulin-1 (NRG1) isoform III, signaling through the ErbB2–ErbB3 receptor on Schwann cells, is required for Schwann cell survival, proliferation and myelination^{3–6}. The competing effects of neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF) modulate Schwann cell myelination⁷, and nerve growth factor (NGF) seems to stimulate Schwann cell myelination indirectly through binding to the TrkA receptor on dorsal root ganglion (DRG) axons⁸. In parallel, neuronal activity triggers axonal release of ATP that inhibits Schwann cell proliferation and myelination (reviewed in ref. 9). Nevertheless, many important aspects of axon–Schwann cell signaling are not yet understood. In addition to axonal contact, the sorting of nerve fibers and Schwann cell myelination also require the synthesis of a basal lamina (reviewed in ref. 1).

Axon–Schwann cell and Schwann cell–basal lamina interactions activate multiple signaling pathways that converge onto the nucleus

to regulate the transcriptional programs for myelinating or non-myelinating fates^{10,11}. Critical roles in Schwann cell proliferation, differentiation and myelination have been established for the transcription factors Sox10, Sox2, Jun, NFκB, Oct6 (the product of the *Pou3f1* gene) and Krox-20 (the product of the *Egr2* gene) (reviewed in refs. 12 and 13). In particular, the cessation of Schwann cell proliferation and the initiation of myelin formation require the downregulation of Sox2 and the upregulation of *Pou3f1*/Oct6 to induce *Egr2*/Krox-20, which in turn—together with Sox10—activates the myelination program^{14–17}. Sox10 is also required in Schwann cell precursors for their survival, where its actions are mediated at least in part through the regulation of the neuregulin receptor ErbB3 gene¹⁸.

The *clp* mouse is a particularly interesting model for the study of Schwann cell development *in vivo*. These mice are characterized by limb posture abnormalities and peripheral hypomyelination, with no sign of dysmyelination in the CNS^{19–21}. The *clp* mutation, which arose spontaneously in C57BL/6-*ob* mice at the Jackson Laboratory, is recessive and has been mapped to proximal mouse chromosome 7 (ref. 19). The similarities in delayed myelination observed in *Pou3f1*/Oct6 mutant mice and in *clp* mice suggest an interaction between the two genes. We have shown previously that perinatal *Pou3f1*/Oct6 upregulation is not affected by the *clp* mutation, but that downstream effectors, such as *Egr2*/Krox-20, are activated with delayed kinetics^{21,22}. Additionally, the radial sorting of nerve fibers is delayed in *clp* animals, and nerve-grafting experiments demonstrate that *clp* gene function is required in Schwann cells and possibly in neurons²¹. Taken together, these data demonstrate that *clp* function

¹McLaughlin Research Institute, 1520 23rd Street South, Great Falls, Montana 59405, USA. ²Department of Cell Biology & Genetics, Erasmus MC University Medical Center, PO Box 1738, 3000 DR Rotterdam, The Netherlands. ³Department of Pathology and Immunology and ⁴Department of Psychiatry, Washington University, Box 8118, 660 South Euclid Avenue, St. Louis, Missouri 63110, USA. Correspondence should be addressed to J.R.B. Jr. (jrjb@p.mri.montana.edu) or D.M. (d.meijer@erasmusmc.nl).

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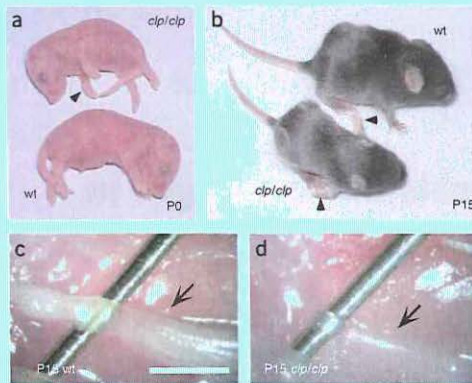


Figure 1 The claw paw phenotype. (a,b) P0 (a) and P15 (b) wild-type and *clp/clp* mutant mice on a C57BL/6 genetic background. Arrowheads highlight the forelimb defect in these mice. (c,d) Sciatic nerves from the mice shown in b. For clarity, a 30-gauge needle has been inserted behind the nerves. Note hypotrophy of the *clp/clp* sciatic nerve (arrows).

is required for peripheral nerve development and that, in Schwann cells, *clp* functions in a myelination pathway that is distinct from *Pou3f1/Oct6* activation.

To understand the molecular basis of the claw paw phenotype, we identified the genetic defect by positional cloning. Here we show that the *clp* mutation is caused by a repetitive element insertion that precludes splicing of exon 4 of *Lgi4*, one of four members of the leucine-rich glioma-inactivated (*Lgi*) family of genes that encode putative secreted proteins²³. *Lgi4* is dynamically expressed in Schwann cells and restricted populations of neurons. The *clp* mutation results in the production of *Lgi4* protein with an interstitial deletion that fuses two of its leucine-rich repeats (LRRs). We demonstrate that, although *Lgi4* is glycosylated and secreted, *Lgi4*^{clp} is retained within the cell. Moreover, we show that siRNA-mediated downregulation of *Lgi4* in sensory neuron–Schwann cell cocultures inhibits myelination, whereas the addition of *Lgi4* to *clp/clp* neuron–Schwann cell cocultures significantly restores myelination. Thus, our data indicate that the claw paw phenotype results from a loss of *Lgi4* and identify *Lgi4* as an important signaling molecule that controls axon sorting and gene expression in peripheral nerve myelination.

RESULTS

The *clp* mutation produces an arthrogryposis-like phenotype that affects the forelimbs (Fig. 1a,b) and, in severe cases, also the hind limbs. The growth of *clp/clp* pups is often delayed, and many of the most severely affected individuals die before weaning. Notwithstanding the variability of the limb posture abnormality, all peripheral nerves in *clp* mice are hypotrophic (Fig. 1c,d; refs. 19 and 21).

Positional cloning of the *clp* gene

The initial backcross involving the *clp* locus revealed that it resides on proximal chromosome 7 (ref. 19). As a first step toward the identification of the *clp* gene, we localized it more precisely using *clp/+* C57BL/6J × BALB/c intercrosses and *clp/+* C57BL/6J × CAST backcrosses. These genetic mapping studies (Supplementary Figs. 1 and 2 online) resulted in the definition of a *clp* candidate region that spanned approximately

150 kilobase (kb) from microsatellite marker D7MRI6 to D7MIT155 and contained at least eight genes (Fig. 2).

To identify the *clp* mutation, we analyzed the *clp* candidate region for exons and putative regulatory sequences. Each of these sequences was amplified using the polymerase chain reaction (PCR) from *clp/+* and *clp/clp* DNA, and sequenced. PCR products from primers that flanked exon 4 of the *Lgi4* gene consistently produced a larger than expected product from *clp/clp* mice. The sequencing of this product revealed that 4 base pairs (bp), CTCT, located six nucleotides 5' to exon 4 were replaced with a 225-bp repetitive sequence. This repetitive sequence was 100% homologous to a repeat element located 797 bp upstream, but in the reverse orientation (Fig. 3a,b). To confirm the presence of the insertion in *clp/clp* mice, we performed exon 4 PCR on *clp/clp* mice that were congenic with BALB/c mice (data not shown) and, using Southern hybridization, we examined DNA from *clp/clp* mice and wild-type mice for the *clp* restriction fragment length polymorphism (RFLP, Fig. 3c). In each case, a larger fragment segregated with the claw paw phenotype, and therefore it is likely that this insertion allele is the *clp* mutation. Formally, *clp* is an allele of *Lgi4* (*Lgi4*^{clp}), but for simplicity we will continue to call it *clp*.

The identification of an insertion associated with the *clp* mutation facilitated genetic complementation studies with wild-type DNA from the *clp* candidate region. We generated a transgenic mouse line with a bacterial artificial chromosome (BAC), 75-D7, containing wild-type mouse genomic DNA that spanned the *clp* candidate region (Fig. 2b). To confirm that the *clp* locus resides within the candidate region, we crossed mice that were heterozygous for *clp* to mice that were heterozygous for *clp* and carried the BAC 75-D7 transgene (Supplementary Fig. 3 online summarizes the genotyping of these mice and Table 1 details the offspring from such a complementation cross). We observed no *clp/clp* transgene-positive mice with the claw paw phenotype, whereas all *clp/clp* homozygous mice without the transgene were phenotypically claw pawed. To confirm the rescue of the claw paw phenotype, we performed direct microscopic examination of the sciatic nerves from *clp/+* and *clp/clp* offspring carrying the 75-D7 BAC transgene (Fig. 3c and 3d, respectively). These sciatic nerves appeared healthy and myelinated, and in marked contrast to nerves from *clp/clp* mice (Fig. 1c), were indistinguishable from the nerves of wild-type mice (Fig. 1d). Thus, we concluded that the 75-D7 BAC transgene complements the *clp* mutation. Because the *Lgi4* insertion was the only polymorphism identified in this region that segregated with the *clp* mutation, the *Lgi4* insertion is the most probable cause of the claw paw phenotype.

Expression of the *Lgi4* gene in the PNS

Previously we showed that *clp* gene function is required by Schwann cells and possibly by neurons²¹. Therefore, we examined the expression

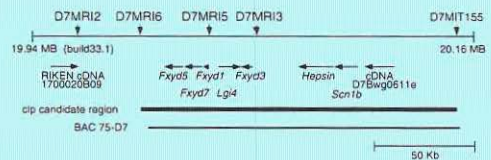


Figure 2 The *clp* candidate region (NCBI build 33.1) consists of eight genes: *Fxyd5*, *Fxyd7*, *Fxyd1*, *Lgi4*, *Fxyd3*, *Hepsin*, *Scn1b* and *D7Bwg0611e*. The *clp* candidate region as defined by 27R and 187R backcross mice (Supplementary Fig. 2) is shown as a thick bar; below it, CAST BAC 75-D7 is shown as a thinner bar.

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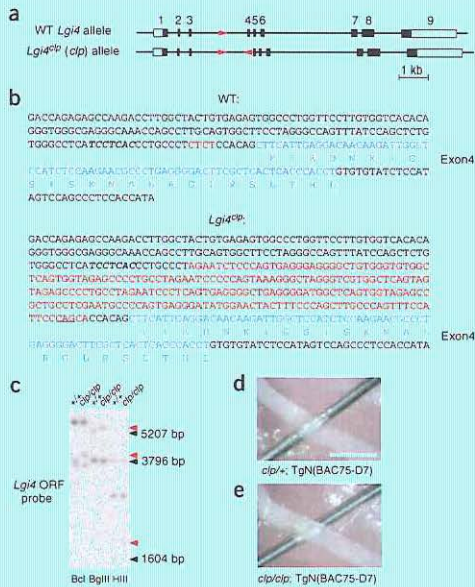


Figure 3 The *clp* mutation is an insertion in *Lgi4*. (a) The structure of the wild-type *Lgi4* gene is shown, with a repetitive element in intron 3 shown in red. This repetitive element has been duplicated and inserted in the opposite orientation adjacent to exon 4 in the *clp* allele of the *Lgi4* gene (*Lgi4*^{clp}). (b) Partial sequence of wild-type *Lgi4* and *Lgi4*^{clp}. Exon 4 is shown in blue, the four bases that are replaced by the *clp* insertion are shown in red and a sequence with strong homology to the branchpoint consensus⁴⁹ is shown in bold italics. The CAG sequence for a potential 3' splice site in the *clp/clp* sequence is underlined. (c) Southern blot of genomic DNA from wild-type and *Lgi4*^{clp} mice depicting the RFLP that results from the *clp* insertion. (d, e) BAC transgene complementation of the *clp* mutation. Two F2 FVB-Tg75-D7 × C57BL/6J-*clp* mice, (d) no. 145 and (e) no. 148 (not shown) that presence of the 75-D7 transgene permits development of normal sciatic nerves in *clp/clp* mice (compare with Fig. 1d). Genotyping of these mice is depicted in Supplementary Figure 3.

Egr2/Krox-20 transcription. To determine whether the reduction in wild-type *Lgi4* activity was compensated for by the upregulation of other members of the Lgi family, we examined the expression of *Lgi1*, *Lgi2* and *Lgi3* in sciatic nerves from P4 wild-type and *clp/clp* mice. In nerves from wild-type mice, we observed modest expression of *Lgi1* (Fig. 4f) and no expression of *Lgi2* or *Lgi3* (data not shown). In nerves from P4 *clp/clp* mice, we observed no expression of *Lgi1* (Fig. 4f), *Lgi2* or *Lgi3* (data not shown). These observations revealed a potential role for *Lgi1* in mature Schwann cells and indicated that compensation for the *Lgi4*^{clp} mutation by *Lgi1*, *Lgi2* and *Lgi3* was unlikely.

To determine if *Lgi4* and the other *Lgi* genes were expressed in sensory neurons during postnatal development, we examined their expression in P4 DRGs by *in situ* hybridization. *Lgi1* was expressed at very low levels throughout the DRG (Fig. 4g), presumably reflecting its low expression in Schwann cells (Fig. 4g). *Lgi2* and *Lgi3* were expressed abundantly in a pattern that corresponded to the position of sensory neurons in the DRG. In contrast, *Lgi4* was expressed at moderate levels uniformly throughout the DRG, probably by intraganglionic Schwann cells. A comparison of these patterns of expression with the embryonic data suggests that, during development, early *Lgi4* expression in DRG is supplanted postnatally by *Lgi2* and *Lgi3* in sensory neurons. To determine whether *Lgi4* is expressed by peripheral motor neurons, we hybridized adjacent transverse sections of P4 spinal cord with probes for *Lgi4* and, as a control, the proteolipid protein gene (*Plp1*; Fig. 4h). *Plp1* expression was observed in white matter tracts, as

pattern of *Lgi4* in the embryonic and early postnatal PNS of wild-type mice and compared it to the expression of the closely related *Lgi2* and *Lgi3* genes. On embryonic day 14 (E14), *Lgi4* was expressed in developing peripheral nerves. *Lgi4* was strongly expressed in the trigeminal nerve and ganglion, and was particularly abundant in the boundary cap cells (Fig. 4a,b)—a transient population of cells that contributes to the Schwann cell population of the dorsal root nerve²⁴. The pattern of *Lgi4* expression at E14 was distinct from those of *Lgi1*, *Lgi2* and *Lgi3* (data not shown). Moderate *Lgi4* expression was observed in the DRG (Fig. 4c), and in the intestine in a pattern consistent with its expression in the developing enteric nervous system (Fig. 4d). Together, these observations demonstrate that *Lgi4* is expressed in the entire PNS at E14. Although it was clear that *Lgi4* is expressed in the Schwann cells of the embryonic nerve, it was not clear, from *in situ* hybridization to embryonic DRG, whether *Lgi4* expression is in sensory neurons, satellite glia or both.

To determine whether *Lgi4* is also expressed in postnatal Schwann cells and whether its expression is altered by the absence of Oct6, we performed *in situ* hybridization on sciatic nerves from wild-type and *Pou3f1*^{-/-} (Oct6-null) mice. Previous data suggest that in postnatal nerve development, *Lgi4* acts downstream of Oct6 or in parallel with it^{21,22}. We found that *Lgi4* was expressed independently of Oct6 (Fig. 4e). Thus, *Lgi4* and Oct6 function in independent pathways in myelinating Schwann cells.

To further define the effects of the *clp* mutation, we examined the expression of *Lgi4* and additional genes in *clp/clp* mice (Fig. 4f). In *clp/clp* sciatic nerves on postnatal day 4 (P4), *Lgi4* expression was upregulated, most probably reflecting the temporal delay in Schwann cell differentiation. *Egr2*/Krox-20 mRNA expression was strongly downregulated in *clp/clp* mice, demonstrating that the reduction of Krox-20 protein in these mice²¹ occurs as a result of reduced

Table 1 Rescue of the claw paw phenotype by BAC 75-D7

<i>Lgi4</i> alleles	BAC 75-D7 transgene	Number of mice	Phenotype
FVB/FVB	no	4	normal
<i>clp</i> /FVB	no	4	normal
<i>clp</i> / <i>clp</i>	no	17	claw paw
FVB/FVB	yes	10	normal
<i>clp</i> /FVB	yes	18	normal
<i>clp</i> / <i>clp</i>	yes	17	normal

To determine whether homozygous *clp/clp* mice that carry the BAC 75-D7 transgene are phenotypically claw paw, *clp*⁺/C57BL/6J × FVB Tg(BAC75-D7) mice were mated to generate F1 *clp*⁺ Tg(BAC75-D7) and *clp*^{-/-} mice. These F1 mice possess a wild-type *Lgi4* allele on an FVB chromosome and a *clp* allele on a C57BL/6 chromosome. This makes it possible to unambiguously genotype alleles at the endogenous *Lgi4* locus in the presence of the wild-type *Lgi4* locus on the BAC75-D7 transgene, by using C57BL/6 and FVB-specific markers that are linked to *Lgi4* but are not present on BAC 75-D7. Genotype analysis of the F1 mice *clp*⁺ no. 108 and *clp*^{-/-} Tg(BAC75-D7) no. 93 are shown in Supplementary Figure 3. These mice were mated to obtain the F2 progeny that are listed in the table. No *clp/clp* transgene-positive mice that were phenotypically claw paw were observed, eliminating the hypothesis that the transgene has no effect on the claw paw phenotype. This result indicates that the sequences within BAC 75-D7 complement the *clp* mutation. A statistically significant excess of transgene carrying mice was observed, likely as a result of segregation of two independent insertion sites of the transgene in mouse no. 93. Southern blot and quantitative RT-PCR analysis (data not shown) indicate that transgene-positive mice possess two or three copies of BAC 75-D7.

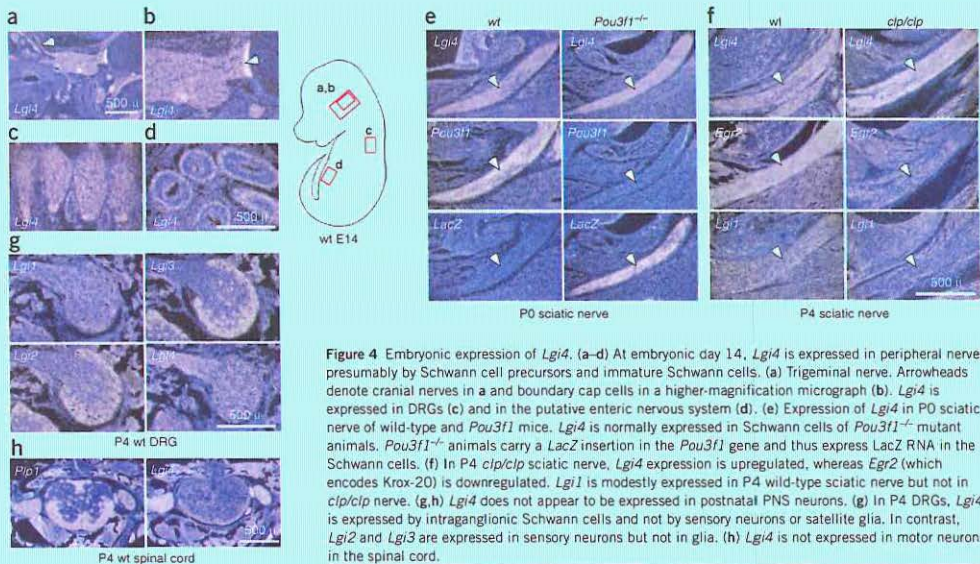


Figure 4 Embryonic expression of *Lgi4*. (a–d) At embryonic day 14, *Lgi4* is expressed in peripheral nerves, presumably by Schwann cell precursors and immature Schwann cells. (a) Trigeminal nerve. Arrowheads denote cranial nerves in a and boundary cap cells in a higher-magnification micrograph (b). *Lgi4* is expressed in DRGs (c) and in the putative enteric nervous system (d). (e) Expression of *Lgi4* in P0 sciatic nerve of wild-type and *Pou3f1* mice. *Lgi4* is normally expressed in Schwann cells of *Pou3f1*^{-/-} mutant animals. *Pou3f1*^{-/-} animals carry a *LacZ* insertion in the *Pou3f1* gene and thus express LacZ RNA in the Schwann cells. (f) In P4 *clp/clp* sciatic nerve, *Lgi4* expression is upregulated, whereas *Egr2* (which encodes Krox-20) is downregulated. *Lgi1* is modestly expressed in P4 wild-type sciatic nerve but not in *clp/clp* nerve. (g, h) *Lgi4* does not appear to be expressed in postnatal PNS neurons. (g) In P4 DRGs, *Lgi4* is expressed by intraganglionic Schwann cells and not by sensory neurons or satellite glia. In contrast, *Lgi2* and *Lgi3* are expressed in sensory neurons but not in glia. (h) *Lgi4* is not expressed in motor neurons in the spinal cord.

expected. Although *Lgi4* expression was observed in dorsal and ventral roots, it was not observed in motor columns. Thus, *Lgi4* does not seem to be expressed in postnatal motor neurons. Together, these data demonstrate that in the PNS, it is primarily Schwann cells that express *Lgi4*.

The *clp* mutation alters splicing of *Lgi4*^{*}

The proximity of the *clp* insertion to *Lgi4* exon 4 suggested that it could alter *Lgi4* splicing in three possible ways: (i) the insert could disrupt exon 4 splicing by interrupting the polypyrimidine tract of the adjacent 3' splice site and shifting a putative branchpoint 221 bp away; (ii) it could introduce a new 3' splice site that would enlarge exon 4 and prematurely terminate *Lgi4* translation; or (iii) the *clp* insertion and the oppositely oriented repeat (located 797 bp away in intron 3; Fig. 5a) could form a pre-mRNA stem-loop structure that occludes the third intron 3' splice site. To distinguish between these possibilities, we performed reverse transcriptase PCR (RT-PCR) on RNA from the brain and sciatic nerves of *clp/+* and *clp/clp* animals, using primers mapping to exons 2 and 5. A shorter DNA fragment was amplified from *clp/clp* brain and nerve samples than from *clp/+* cDNA samples (Fig. 5b). We cloned and sequenced these DNA fragments and found that the *clp*-derived cDNAs lacked exon 4. To assess whether stem-loop formation blocked the use of the 3' splice site on exon 4 in *Lgi4*^{clp} pre-mRNAs, we generated minigenes that spanned exons 2 through 6 and contained the *clp* insertion, but with the nearby oppositely oriented repeat sequence either removed or inverted (Supplementary Fig. 4 online). The expression of these minigenes in COS1 cells and the sequencing of the resulting cDNAs demonstrated that *Lgi4* exon 3 was spliced to exon 5, regardless of the presence or orientation of the additional cognate repeat. Thus, we concluded that the insertion of the repeat element in the *clp* allele disrupted the third intron 3' splice site,

which resulted in the skipping of exon 4. As exon 4 is 72 nucleotides long, we predict that the *Lgi4* protein encoded by the *clp* allele has a 24-amino acid interstitial deletion.

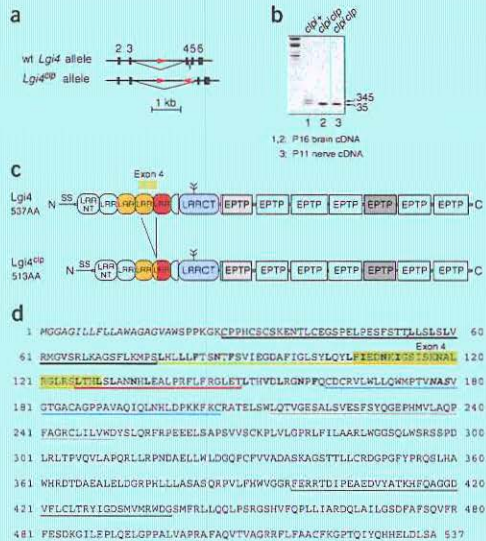
Cellular processing of *Lgi4* and *Lgi4*^{clp}

Lgi4 shares common protein structural domains with the other three members of the *Lgi* family: an N-terminal signal sequence, LRRs and a C-terminal epilepsy-associated repeat (EAR) or epitempin domain^{25,26}. The predicted domain organization of *Lgi4* and *Lgi4*^{clp} (Fig. 5c), based on the wild-type amino acid sequence (Fig. 5d), revealed a putative signal sequence; the presence of this sequence suggested that *Lgi4* enters the secretory pathway and may be secreted. In support of this, the LRR repeats of *Lgi4* and the *Lgi1*, *Lgi2* and *Lgi3* proteins were flanked by cysteine-rich sequences (LRRNT and LRRCT in Fig. 5d) that typified extracellular LRR proteins^{27,28}. In addition, one potential glycosylation site (Asn177) was present in the LRRCT of *Lgi4*.

To determine whether *Lgi4* and *Lgi4*^{clp} are indeed secreted proteins, we stably expressed a Myc-epitope- and 6×Histidine-tagged *Lgi4* or *Lgi4*^{clp} in CHO cells. Using an antibody to Myc, we observed *Lgi4* protein expression in a reticular pattern that largely overlapped with the ER of the cell, suggesting that it enters the secretory pathway (Fig. 6a). To check whether *Lgi4* and *Lgi4*^{clp} were secreted into the medium, we purified the recombinant proteins from cell extracts and medium on NiNTA-agarose beads. In this analysis, we included CHO cells expressing V5- and 6×Histidine-tagged *Lgi1* as a positive control and Oct6-expressing cells as a negative control. *Lgi4* and *Lgi1* could be purified from the culture medium, whereas *Lgi4*^{clp} and Oct6 could not (Fig. 6b). Thus, in contrast to the readily secreted wild-type *Lgi4* and *Lgi1* proteins, *Lgi4*^{clp} was retained within the cell.

We next checked whether *Lgi4* and *Lgi4*^{clp} proteins are glycosylated by treating them with endoglycosidase H (EndoH) or peptide

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N-glycosidase F (PNGaseF). EndoH cleaves the high-mannose structures that are found in ER resident proteins, whereas PNGaseF cleaves both the high-mannose structures and the complex oligosaccharides present on mature glycosylated proteins. We observed that secreted Lgi4 was not cleaved by EndoH but was cleaved by PNGaseF—as was apparent from the downward shift in molecular weight (Fig. 6c). Thus, secreted Lgi4 carries complex oligosaccharides. Lgi4 purified from cell extracts was largely EndoH sensitive, indicating that the proteins were rapidly secreted after maturation in the Golgi compartment. Lgi4^{cp} also was largely EndoH sensitive, demonstrating that the interstitial deletion in Lgi4^{cp} did not inhibit glycosylation *per se*. The observation that little mature glycosylated Lgi4^{cp} accumulated in the cell suggested that the protein was retained within the ER. These data suggested that Lgi4 functions extracellularly and that the claw paw phenotype results from loss of Lgi4 function. An alternative possibility, that Lgi4^{cp} retention exerts a dominant-negative effect on Schwann cells, is unlikely because the claw paw phenotype is largely recessive and is complemented by wild-type Lgi4 genes.

Figure 6 The *clp* mutation affects processing and sorting of Lgi4. (a) Myc-tagged Lgi4 protein expressed in CHO cells is detected with an antibody to Myc (α -Myc) in a pattern that largely overlaps (overlay) with that of the GRP-94 antigen, present on the ER resident protein GRP-94. (b) Lgi4 and Lgi1 protein can both be purified from cell lysates (cell) and culture medium (medium) of stably transfected CHO cells. Lgi4^{cp} is present in cell extracts but not in medium. The nuclear protein Oct6 can be purified only from the cell extract, demonstrating that there is very little contamination of the medium fraction with the cellular fraction. Lgi4 is detected with a Myc antibody, Lgi1 with a V5 antibody and Oct6 with an Oct6 antibody. (c) Lgi4 purified from medium is resistant to cleavage by EndoH but sensitive to PNGaseF as judged by the shift in electrophoretic mobility (indicated with arrows labeled 'a' and 'b'). Lgi4 protein isolated from cell lysates (cell) is largely EndoH sensitive (below).

Figure 5 Skipping of *Lgi4*^{cp} exon 4 generates a protein with an internal deletion. (a) Diagram of *Lgi4* splicing patterns in wild-type and *clp/clp* mice, showing skipping of exon 4 in *Lgi4*^{cp} transcripts. (b) PCR of cDNA from *clp*⁺ brain and nerve and *clp/clp* nerve, showing absence of exon 4 in *clp/clp* transcripts. (c) Structure of mouse wild-type Lgi4 and Lgi4^{cp} proteins. SS, signal sequence that is predicted to be cleaved between amino acids 19 and 20 (SignalP3.0; ref. 46); LRRNT, leucine-rich N-terminal domain; LRR, leucine-rich repeat; LRRCT, leucine-rich C-terminal repeat; EPTP, epitope repeat. Colored or shaded domains are recognized by the SMART database. The red LRR has a lower *E* value (probability with which the sequence would appear at random) than the orange LRR domains, and the dark gray epitope domain has a lower *E* value than the light gray domain. White domains are not recognized by the SMART or CDART databases. A branched structure depicts a putative glycosylation site within the LRRCT (blue). The part of the protein that is encoded by exon 4 is overshadowed in green. 513AA Lgi4^{cp} is shown below wild-type Lgi4, with the third and fourth putative leucine-rich repeats fused. (d) Mouse Lgi4 protein sequence. Signal sequence is depicted in italics. A putative N-linked glycosylation site (Asn177 or NXST1⁵⁰) in the LRRCT domain is shown in bold italics, and conserved amino acids in leucine-rich repeats are shown in bold. Amino acids encoded by exon 4 are highlighted in green. Leucine-rich domains and conserved epitope domains depicted in a are underlined. The sequence is derived from mouse Lgi4 cDNA NM_144556.1.

Lgi4 controls myelination in neuron-Schwann cell cocultures
 If the loss of Lgi4 function caused the claw paw phenotype, then the downregulation of Lgi4 by means of siRNA should phenocopy the *clp* defect. We established myelinating neuron-Schwann cell cocultures from dissociated DRGs derived from *clp*⁺ and *clp/clp* E13 mouse embryos. *clp/clp* cultures did not myelinate, whereas *clp*⁺ cultures exhibited robust myelination (Fig. 7a). A lentivirus that expressed Lgi4-specific siRNA was constructed and shown to suppress Lgi4 mRNA expression in cultured rat Schwann cells by approximately 80% (Fig. 7b). The transduction of neuron-Schwann cell cocultures with the Lgi4 siRNA lentivirus resulted in reduced numbers of myelin internodes as compared to cultures transduced with a control lentivirus (Fig. 7a,c). Quantification of these results showed that the expression of Lgi4 siRNA resulted in a decrease of myelin internodes by approximately 70% (Fig. 7c). These experiments strongly suggested that the myelination defect in *clp* mice and in *clp* DRG explant cultures results from a loss of Lgi4 function.

The data presented above suggested that Lgi4 acts extracellularly. Therefore, we anticipated that the addition of Lgi4 to *clp/clp* neuron-Schwann cell cocultures would restore their myelination defect (Fig. 7a). We treated *clp/clp* DRG cultures with medium conditioned by either CHO cells expressing Lgi4 or, as a control, non-recombinant CHO cells. We analyzed the progression of Schwann cell differentiation

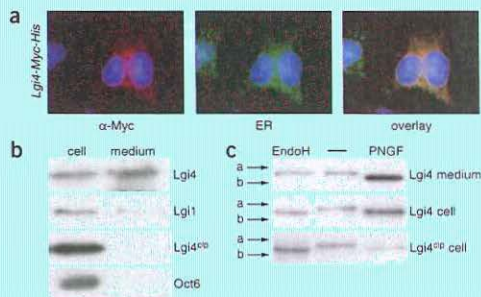
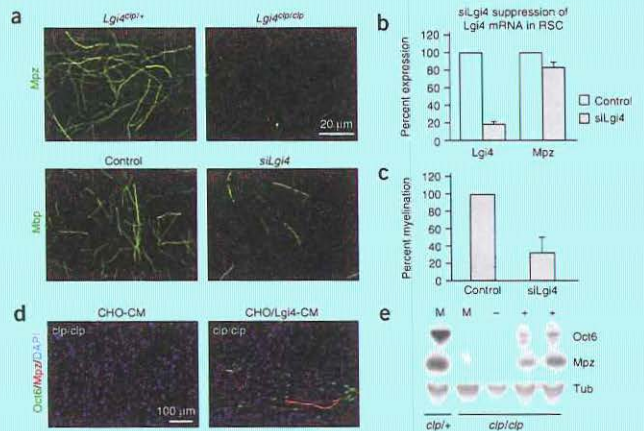


Figure 7 Inhibition of Lgi4 results in reduced myelination in DRG cultures and exogenous Lgi4 restores myelination in *clp/clp* DRG cultures. (a) Anti-Mpz immunostaining of dissociated *clp/+* or *clp/clp* DRG cultures 12 d after addition of ascorbic acid to induce myelination (top panels). Immunostaining by antibodies to Mbp of sensory neuron–Schwann cell cocultures infected with lentivirus expressing Lgi4 siRNA (siLgi4) or non-recombinant lentivirus (control) after ascorbic acid treatment. (b) Quantitative RT-PCR of Lgi4 and Mpz RNA expression levels in Schwann cells infected with siLgi4 or control lentivirus. (c) Quantification of myelin profiles in control and siLgi4 panels. Percent myelination value is calculated from three independent experiments. Error bars represent s.e.m. (d) Mpz (red) and Oct6 (green) immunostaining of dissociated *clp/clp* DRG cultures 12 d after addition of ascorbic acid to induce myelination. Nuclei are blue (DAPI). Cultures were grown in the presence of 50% non-recombinant CHO cell–conditioned medium (CHO-CM) or Lgi4-expressing CHO cell–conditioned medium (CHO/Lgi4-CM). Myelin internodes are recognized by their strong Mpz staining. (e) Expression of Oct6 and Mpz in dissociated DRG cultures 12 d after addition of ascorbic acid was assessed on immunoblots. Acetylated α -tubulin (Tub), which is highly enriched in neurites, was used as a protein loading control. DRG cultures derived from heterozygous (*clp/+*) or *clp*-homozygous (*clp/clp*) embryos were grown in standard medium (M) or standard medium supplemented (1:1) with CHO-CM (–) or CHO/Lgi4-CM (+).



and myelination in these cultures by immunocytochemistry and western blotting (Fig. 7d,e). No myelin internodes were observed in *clp/clp* Schwann cell cocultures grown in the presence of medium conditioned by non-recombinant CHO cells (CHO-CM; Fig. 7d), and very little of the myelin-associated protein Mpz was expressed in these *clp/clp* cultures (Fig. 7e). In contrast, *clp/clp* DRG cultures grown in the presence of medium conditioned by Lgi4-expressing CHO cells (CHO/Lgi4-CM) showed myelination, as demonstrated by the strong Mpz staining of several internodes (Fig. 7d) and elevated Mpz protein expression (Fig. 7e). High Oct6 expression marks immature and premyelinating Schwann cells. We observed many Oct6-positive Schwann cells in the CHO-Lgi4-CM cultures, but not in the CHO-CM cultures (Fig. 7d). This was corroborated by increased Oct6 protein expression in the CHO/Lgi4-CM *clp/clp* cultures as compared to CHO-CM *clp/clp* cultures (Fig. 7e).

Taken together, these experiments demonstrated that exogenous Lgi4 can restore the myelination defect in *clp/clp* neuron–Schwann cell cocultures, suggesting that Lgi4 can indeed function extracellularly.

DISCUSSION

Through genetic and cell biological studies of *clp* mutant mice, we have identified the secreted protein Lgi4 as an essential molecule in PNS development. The *clp* mutation results in intracellular retention of mutant Lgi4. Downregulation of Lgi4 in wild-type neuron–Schwann cell cocultures inhibits myelination, whereas exogenous Lgi4 restores myelination in *clp/clp* cocultures. Thus, Lgi4 is involved in Schwann cell signaling pathways that control axonal ensheathment and myelination.

Lgi4 protein

The Lgi4 protein belongs to a small family of proteins that are characterized by the presence of a signal peptide and two putative protein interaction domains: an LRR domain and an epitimpin or EAR domain. The function of these proteins is unknown, although their structural characteristics suggest that they function extracellularly and are involved in multiple molecular interactions.

LRR domains provide a versatile protein interaction interface that is found in many proteins from phylogenetically diverse organisms^{27,28}. The LRR domain of Lgi4 has highest homology to the *Drosophila* Slit D3 LRR. Slit proteins are extracellular guidance molecules for both neurons and some non-neuronal cells, and function by binding to the transmembrane Roundabout (Robo) receptors (reviewed in ref. 29)—an interaction that is mediated through the LRR domain of Slit³⁰. Also, the C-terminal half of the Lgi proteins is potentially involved in direct molecular interactions. This domain is predicted to adopt a β -sheath structure and may represent a version of the seven-bladed β -propeller fold, a structural element involved in protein–protein interactions. For example, this structural motif is also found in the semaphorins, a family of proteins involved in axon guidance (reviewed in ref. 31), and in the integrins, a major family of basal lamina receptors. The epitimpin domain is shared by the four members of the Lgi family, by C21orf29 (TSPEAR) and by the extracellular domain of the G protein–coupled receptor MASS1 (also known as VLGR). Two of these proteins are altered in epilepsy: *LG11* mutations have been found in patients with autosomal-dominant partial epilepsy with auditory features (ADPEAF), whereas the *MASS1* (or *VLGR*) gene is mutated in Frings audiogenic seizure mice³² and in humans with audiogenic seizures³³. In humans with ADPEAF, point mutations in *LG11* have been found in both the LRR and epitimpin domains^{34–37}. The human *LG14* gene resides in 19q13, a region linked to benign familial infantile convulsions (BFIC; OMIM 601764), and *LG14* polymorphisms are associated with childhood absence epilepsy³⁸. Thus far, however, no mutations in *LG14* have been detected in patients with either type of epilepsy.

The interstitial deletion of 24 amino acids within the LRR domain of Lgi4 results in intracellular retention of the mutant protein, most probably within the ER. The unique peptide junction in Lgi4^{clp} does not resemble any known ER retention signal and thus refutes a simple gain-of-function mechanism. A stringent quality control regime within the ER eliminates improperly folded or assembled proteins³⁹. In the peripheral nerve, the association of contactin-associated protein (Caspr) and contactin within the ER is required for maturation and

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surface expression of the Caspr-contactin complex⁴⁰. Perhaps the altered protein-interaction characteristics of the mutant LRR domain of Lgi4 inhibit its incorporation into a complex destined for transport to the cell surface. Alternatively, possibly Lgi4^{clp} is recognized as a misfolded protein, retained within the ER and eliminated through proteasome-mediated decay. Notably, a variety of ADPEAF-associated mutations in Lgi1 all result in intracellular retention of the protein when overexpressed in 293T cells, whereas the wild-type Lgi1 protein is normally secreted by these cells⁴¹.

Possible roles for Lgi4 in peripheral nerve development

The transplantation of wild-type sciatic nerve segments from wild-type mice into nerves of *clp/clp* mice and vice versa suggested both Schwann cell-autonomous and Schwann cell-non-autonomous functions for the *clp* gene product²¹. We found that Lgi4 is expressed primarily by Schwann cells in the PNS, implicating it in Schwann cell-axon interactions. The embryonic and postnatal secretion of Lgi4 by Schwann cells suggests several potential roles for this protein in nerve development. First, it is possible that Schwann cells signal axons through Lgi4 to stimulate axon growth, reduce axon-axon adhesiveness or trigger axonal signaling back to Schwann cells. Lgi4 could bind directly to an axonal receptor or it could bind to an extracellular ligand. Second, Lgi4 could modulate an axonal signal to Schwann cells. Third, the axonal sorting defect in *clp* nerves resembles the defects observed in several different mice that possess laminin or integrin mutations²¹. Similarly, in neuron-Schwann cell cocultures treated with a low concentration of cyochalasin D that only subtly affects the actin cytoskeleton, Schwann cells project processes into fascicles of axons but fail to segregate them completely⁴². These similarities raise the possibility that Lgi4 modulates basal lamina signaling through integrins, thereby triggering the cytoskeletal rearrangements that are required for elaboration of Schwann cell processes and subsequent axon segregation.

clp/clp mice have a forelimb phenotype (Fig. 1) that is uncommon among peripheral dysmyelinating mutants. Abnormal forelimb posture at birth is also observed in Sox10 and cysteine-rich domain-NRG1 knockout mice; however, in these mice, it is associated with paralysis resulting from significant motor and sensory neuron cell death as a consequence of the loss of Schwann cells^{18,43}. *clp* mice are not paralyzed and do not show excessive Schwann cell death or loss of sensory and motor neurons^{19,21}. In fact, it is possible that this particular aspect of the phenotype is not associated with loss of Lgi4 expression in Schwann cells. Lgi4 is expressed in restricted regions of the CNS (refs. 41,44 and data not shown), and therefore the limb defect could be related to the CNS. Purkinje cells in the adult cerebellum express Lgi4, but the appearance of the limb defect precedes Purkinje cell maturation⁴⁵, suggesting that their involvement is unlikely. Identification of the etiology of the limb defect in *clp* mutant mice will be facilitated by the generation of tissue-specific deletions of *Lgi4*.

In summary, the identification and characterization of the *clp* mutation presented here demonstrates that the loss of Lgi4 expression underlies the claw paw phenotype and implicates the Lgi4 protein in Schwann cell differentiation and peripheral nerve development. Identification of Lgi4-interacting proteins will be instrumental in uncovering the signaling pathways in which Lgi4 participates and, additionally, may elucidate the functions of other Lgi proteins in oncogenesis and epileptogenesis. As a secreted protein that is required for Schwann cell development, Lgi4 is a promising target for therapies to stimulate myelination.

METHODS

Mice. We obtained C57BL/6J *clp/+* mice from the Jackson Laboratory, and FVB and CAST mice from the McLaughlin Research Institute breeding colony.

Animal experiments were performed according to protocols that had been approved by the Institutional Animal Care and Use Committee at the McLaughlin Research Institute and the Institutional Animal Experimentation Committee at Erasmus University. Procedures for mapping of the *clp* mutation are presented in Supplementary Methods online.

Genotyping of *Lgi4*^{clp} mutant mice. Mice were genotyped by PCR using two primers that flank the *clp* insertion (5'-CTTGACAATGACCAGAGAGC CAAGAC-3' and 5'-TGGCACCCTCGGATGACAGC-3') and a third primer that covers the 3' junction of the repeat insert (5'-CCATTCAGCAGC CACAGCTTCA-3').

cDNA amplification. We isolated RNA from adult C57BL/6 *Lgi4*^{+/+} and *clp/+* mouse sciatic nerves using Trizol (Invitrogen) and then carried out cDNA synthesis using Superscript II (Invitrogen) and a Clontech Advantage kit (BD Biosciences and Clontech). The Lgi4 open reading frame was amplified from this cDNA using the primers 5'-AAACTCGAGGAGCAACATGGGAGG GCGGGCATCTTG-3' and 5'-GGGCTCTTCCCGGTACCCGGCAGCTAG ATCCAATTCATGGTG-3' to improve the Kozak consensus around the ATG start site and to remove the stop codon. We cloned the 1,645-bp PCR product into pCRII+ (Invitrogen), sequenced it and subsequently cloned it into pcDNA3.1/Myc-His(-)C (Invitrogen) as an EcoRI-XbaI fragment.

In situ hybridization. We anesthetized and perfused mice with formalin. Hindquarters were dissected to expose the sciatic nerve and stored at 4 °C in formalin. Tissues were frozen in a 1:1 mixture of OCT and Aquamount, and sectioned at 20 µm. We performed *in situ* hybridization as described²² and prepared single-stranded ³⁵S-labeled antisense transcripts from the clones described above. Emulsion-dipped sections were exposed for 7–21 d, counterstained in 0.001% bisbenzidine (Sigma) and photographed using dark-field optics and Kodak Ektachrome 160T film.

Transgenic complementation of the *clp* mutation. BACs were identified from the CAST/Ei BAC library at the Children's Hospital of Oakland Research Institute, by hybridizing filters with radiolabeled *Foxp3* cDNA according to the manufacturer's protocol. BAC transgenic mice were made by pronuclear injection of 75-D7 BAC DNA that was purified using the Qiagen large construct kit (Qiagen), purified by density centrifugation in a CsCl gradient and dialyzed against 10 mM Tris (pH 8.0), 0.1 mM EDTA using Slide-A-Lyzer 10K dialysis cassettes (Pierce). We identified BAC transgenic mice by PCR amplification of a 350-bp sequence from the pTARBAC 2.1 plasmid backbone using the primers 5'-CGCAGGTAATGTGTGCAAAATCTG-3' and 5'-CCCGTTCGGTAACTCAG CATC-3'. We analyzed FVB and C57BL/6 chromosome-7 sequences using MIT microsatellite markers that differ in size between FVB and C57BL/6. These microsatellites were identified using the Center for Inherited Disease Research website (http://www.cidr.jhmi.edu/mouse/mouse_dif.html). M13-tagged primers for the D7MIT267 microsatellite marker were used for PCR amplification and analysis by electrophoresis on LI-COR apparatus (LI-COR Biosciences) as described above.

Bioinformatics. We analyzed sequences using the Celera Discovery System and the public mouse and human genome databases. We performed signal sequence prediction using Signal P3.0 (ref. 46) and TMHMM (version 2.0). Intron splice sites prediction using NetGene2. We identified conserved sequences between mouse and human using VISTA⁴⁷.

Cell transfection, immunohistochemistry and western blotting. CHO1 cells were grown in DMEM/F10 (Whitaker) medium supplemented with 5% fetal calf serum, penicillin and streptomycin. We obtained polyclonal cell lines stably expressing Lgi4, Lgi4^{clp} or Lgi1 after transfection of CHO1 cells with pcDNA3.1 Myc-His- or pcDNA3.1 V5-His-based expression cassettes and selection in G418 (1 mg ml⁻¹). For immunohistochemistry, the cells were grown in two-well microchamber slides, washed with phosphate-buffered saline (PBS) and fixed for 5 min in 4% paraformaldehyde (PFA) followed by 5 min in ice-cold methanol. Cells were preincubated with blocking solution (0.5% normal goat serum (DAKO), 0.5% bovine serum albumin (BSA), 0.01% Triton X-100, 0.2 mM glycine in PBS) for 5 min. First antibodies were diluted in blocking solution (1:200 for the anti-Myc 9E1 (Roche) mouse monoclonal antibody and

1:100 for the GRP-94 (DAKO) ER marker rabbit serum, applied to the cells and incubated for 2 h. Cells were washed extensively with PBS-0.001% Triton X-100 before addition of dilutions of secondary antibodies in blocking buffer (1:100 of Alexa 594-conjugated goat anti-mouse antibody and 1:100 Alexa 488-conjugated goat anti-rabbit antibody (Molecular Probes)). After 1 h, cells were extensively washed with PBS-0.01% Triton X-100, PBS and demineralized water; and mounted. We examined the cells using a Leica Aristoplan epifluorescence microscope and collected images using a Sony video camera.

We purified proteins from cell extracts and medium using Ni²⁺-NTA beads (Qiagen) and separated them on a 12% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) and detected with primary antibodies to Myc (Lg14), V5 (Lg11) or Oct6. We visualized proteins using an ECL western blot detection kit (Pierce).

Neuron-Schwann cell cocultures and lentiviral infection. Dissociated mouse DRG cultures: DRGs were dissected from E13 *clp/clp*, *clp/+* and wild-type embryos, and collected in Leibovitz's L-15 medium (Invitrogen). DRGs from individual embryos were dispersed using trypsin and plated in a small drop of MEM (Invitrogen) supplemented with 10% FBS (HyClone) and 100 ng ml⁻¹ 7S NGF (Promega) on collagen-coated coverslips. We cultured and induced myelination by the addition of 50 µg ml⁻¹ ascorbic acid (Sigma) as described in ref. 8. We visualized myelin figures by staining PFA-fixed cultures with antibodies to the major myelin proteins Mbp (ref. 48) or Mbp. Lg14-Myc-His-expressing CHO cells (polyclonal) or non-recombinant CHO cells were grown in MEM supplemented with 10% FBS for 5 d. The conditioned medium was harvested, filtered and used to supplement medium for dissociated *clp/clp* DRG cultures. The conditioned medium was added the day after plating and was present during the whole time period of the experiment. For rat DRG-Schwann cell cocultures, primary DRG neuron cultures were established using DRGs harvested from E16 rat pups. DRGs were dissociated, and approximately two DRG were plated per well in four-well Nunc plates coated with Matrigel (BD Biosciences). Neurons were grown in supplemented neural basal medium (Invitrogen) (B27 supplement, Invitrogen; 4 g l⁻¹ D-glucose, 2 mM glutamine and 50 ng ml⁻¹ NGF, Harlan) with or without a mixture of 10 µM 5-fluoro-2'-deoxyuridine and uridine (Sigma) for two or three 48-h cycles to eliminate fibroblast growth. Primary rat Schwann cells were harvested from sciatic nerves of P1 and P2 rat pups according to Brookes' method and cultured as described¹⁷. For myelination, 100,000 lentiviral-infected Schwann cells were seeded onto DRG cultures in DMEM/F12 medium (Invitrogen) supplemented with 15% FBS (Sigma), 2 mM glutamine and 50 ng ml⁻¹ NGE. After 48–96 h, the medium was switched to myelin-promoting medium consisting of DMEM/F12, 15% FBS, 2 mM glutamine, 50 ng ml⁻¹ NGF and 50 µg ml⁻¹ ascorbic acid (Sigma). Myelination medium was changed three times every week, and the cultures were allowed to myelinate for up to 2 weeks.

The Lg14 siRNA vector was produced by cloning annealed oligos, which encoded a hairpin loop and the targeting sequence 5'-CTGTCACTCTCTCTCGTCA-3' into a lentiviral vector containing the U6 promoter and a puromycin resistance cassette. Non-recombinant lentivirus without a hairpin oligo insertion was used as the control. Lentivirus production and infection was performed as described¹⁶. Infected Schwann cells were selected for by treating with 1 µg/ml puromycin for 48 h.

Immunohistochemistry of rat DRG-Schwann cell cocultures. Cultures were fixed for 10 min in 4% paraformaldehyde and then permeabilized for 20 min in cold methanol at -20°C. Incubation with primary antibody was performed overnight at 4°C with monoclonal antibodies to Mbp (1:200; Sternberger Monoclonals) in blocking buffer (5% BSA (Sigma), 1% goat serum (Zymed), 0.2% Triton in PBS). Goat anti-mouse IgG-Cy3 (Jackson ImmunoResearch) was used at 1:200 dilution for 1 h at room temperature. Percent myelination was calculated by counting the total number of Mbp-positive internodes in five random fields per culture viewed at 10× magnification and setting the control to 100%. Each condition was performed in triplicate or quadruplicate, and the data presented is the average ± s.e.m. of three independent experiments.

Quantitative RT-PCR. For cDNA synthesis, 1 µg of total RNA isolated from lentiviral-infected cultured Schwann cells (Trizol reagent, Invitrogen) was used. Gene expression was quantified as previously described¹⁷ and normalized to

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Each sample was quantified in triplicate, and the results shown are the averages ± s.e.m. of a representative experiment.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Jessen, K.R. & Mirsky, R. Schwann Cell Development. in *Myelin Biology and Disorders* Vol. 1 (ed. Lazzarini, R.A.) 329–370 (Elsevier Academic, San Diego, 2004).
- Lobsiger, C.S., Taylor, V. & Suter, U. The early life of a Schwann cell. *Biol. Chem.* **383**, 245–253 (2002).
- Britsch, S. et al. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev.* **12**, 1825–1836 (1998).
- Garrett, A.N., Britsch, S. & Birchmeier, C. Neuregulin, a factor with many functions in the life of a Schwann cell. *Bioessays* **22**, 987–996 (2000).
- Michailov, G.V. et al. Axonal neuregulin-1 regulates myelin sheath thickness. *Science* **304**, 700–703 (2004).
- Taveggia, C. et al. Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* **47**, 681–694 (2005).
- Cosgaya, J.M., Chan, J.R. & Shooter, E.M. The neurotrophin receptor p75NTR as a positive modulator of myelination. *Science* **298**, 1245–1248 (2002).
- Chan, J.R. et al. NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. *Neuron* **43**, 183–191 (2004).
- Fields, R.D. & Stevens-Graham, B. New insights into neuron-glia communication. *Science* **298**, 556–562 (2002).
- Atanasiou, S. et al. The protooncogene Ski controls Schwann cell proliferation and myelination. *Neuron* **43**, 499–511 (2004).
- Ogata, T. et al. Opposing extracellular signal-regulated kinase and Akt pathways control Schwann cell myelination. *J. Neurosci.* **24**, 6724–6732 (2004).
- Wegner, M. Transcriptional control in myelinating glia: the basic recipe. *Glia* **29**, 118–123 (2000).
- Topilko, P. & Meijer, D. Transcription factors that control Schwann cell development and myelination. in *Glia Cell Development* (eds. Jessen, K.R. & Richardson, W.D.) 223–244 (Oxford Univ. Press, Oxford, 2001).
- Ghislaian, J. et al. Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development. *Development* **129**, 155–166 (2002).
- Jaegle, M. et al. The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes Dev.* **17**, 1380–1391 (2003).
- Le, N. et al. Analysis of congenital hypomyelinating Egr2/Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell differentiation and myelination. *Proc. Natl. Acad. Sci. USA* **102**, 2596–2601 (2005).
- Nagarajan, R. et al. EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. *Neuron* **30**, 355–368 (2001).
- Britsch, S. et al. The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66–78 (2001).
- Henry, E.W., Eicher, E.M. & Sidman, R.L. The mouse mutation claw paw: forelimb deformity and delayed myelination throughout the peripheral nervous system. *J. Hered.* **82**, 287–294 (1991).
- Kozowski, A.G., Owens, G.C. & Levinson, S.R. The effect of the mouse mutation claw paw on myelination and nodal frequency in sciatic nerves. *J. Neurosci.* **18**, 5859–5868 (1998).
- Darbas, A. et al. Cell autonomy of the mouse claw paw mutation. *Dev. Biol.* **272**, 470–482 (2004).
- Birmingham, J.R., Jr. et al. Identification of genes that are downregulated in the absence of the POU domain transcription factor pou3f1 (Oct-6, Tst-1, SCIP) in sciatic nerve. *J. Neurosci.* **22**, 10217–10231 (2002).
- Gu, W. et al. The Lg11 gene involved in lateral temporal lobe epilepsy belongs to a new subfamily of leucine-rich repeat proteins. *FEBS Lett.* **519**, 71–76 (2002).

ARTICLES

24. Maro, G.S. *et al.* Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. *Nat. Neurosci.* **7**, 930–938 (2004).
25. Staub, E. *et al.* The novel EPTP repeat defines a superfamily of proteins implicated in epileptic disorders. *Trends Biochem. Sci.* **27**, 441–444 (2002).
26. Scheel, H., Tomiuk, S. & Hofmann, K. A common protein interaction domain links two recently identified epilepsy genes. *Hum. Mol. Genet.* **11**, 1757–1762 (2002).
27. Buchanan, S.G. & Gay, N.J. Structural and functional diversity in the leucine-rich repeat family of proteins. *Prog. Biophys. Mol. Biol.* **65**, 1–44 (1996).
28. Kajava, A.V. Structural diversity of leucine-rich repeat proteins. *J. Mol. Biol.* **277**, 519–527 (1998).
29. Wong, K., Park, H.T., Wu, J.Y. & Rao, Y. Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr. Opin. Genet. Dev.* **12**, 583–591 (2002).
30. Howitt, J.A., Clout, N.J. & Hohenester, E. Binding site for Robo receptors revealed by dissection of the leucine-rich repeat region of Slit. *EMBO J.* **23**, 4406–4412 (2004).
31. Gherardi, E., Love, C.A., Esnouf, R.M. & Jones, E.Y. The sema domain. *Curr. Opin. Struct. Biol.* **14**, 669–678 (2004).
32. Skradski, S.L. *et al.* A novel gene causing a mendelian audiogenic mouse epilepsy. *Neuron* **31**, 537–544 (2001).
33. Nakayama, J. *et al.* A nonsense mutation of the MASS1 gene in a family with febrile and afebrile seizures. *Ann. Neurol.* **52**, 654–657 (2002).
34. Kalachikov, S. *et al.* Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nat. Genet.* **30**, 335–341 (2002).
35. Berković, S.F. *et al.* LGI1 mutations in temporal lobe epilepsies. *Neurology* **62**, 1115–1119 (2004).
36. Ottman, R. *et al.* LGI1 mutations in autosomal dominant partial epilepsy with auditory features. *Neurology* **62**, 1120–1126 (2004).
37. Pisano, T. *et al.* Abnormal phonologic processing in familial lateral temporal lobe epilepsy due to a new LGI1 mutation. *Epilepsia* **46**, 118–123 (2005).
38. Gu, W., Sander, T., Becker, T. & Steinlein, O.K. Genotypic association of exonic LGI4 polymorphisms and childhood absence epilepsy. *Neurogenetics* **5**, 41–44 (2004).
39. Ellgaard, L. & Helenius, A. Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.* **4**, 181–191 (2003).
40. Bonnon, C., Goutetbroze, L., Denisenko-Nehrbass, N., Girault, J.A. & Faivre-Sarrailh, C. The paranodal complex of F3/contactin and caspr/paranodin traffics to the cell surface via a non-conventional pathway. *J. Biol. Chem.* **278**, 48339–48347 (2003).
41. Senechal, K.R., Thaller, C. & Noebels, J.L. ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy. *Hum. Mol. Genet.* **14**, 1613–1620 (2005).
42. Fernandez-Valle, C., Gorman, D., Gomez, A.M. & Bunge, M.B. Actin plays a role in both changes in cell shape and gene-expression associated with Schwann cell myelination. *J. Neurosci.* **17**, 241–250 (1997).
43. Wolpowitz, D. *et al.* Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. *Neuron* **25**, 79–91 (2000).
44. Runkel, F., Michels, M. & Franz, T. Fxyd3 and Lgi4 expression in the adult mouse: a case of endogenous antisense expression. *Mamm. Genome* **14**, 665–672 (2003).
45. McKay, B.E. & Turner, R.W. Physiological and morphological development of the rat cerebellar Purkinje cell. *J. Physiol. (Lond.)* **567**, 829–850 (2005).
46. Bendtsen, J.D., Nielsen, H., von Heijne, G. & Brunak, S. Improved prediction of signal peptides: signalP 3.0. *J. Mol. Biol.* **340**, 783–795 (2004).
47. Mayor, C. *et al.* VISTA: visualizing global DNA sequence alignments of arbitrary length. *Bioinformatics* **15**, 1045–1047 (2000).
48. Archelos, J.J. *et al.* Production and characterization of monoclonal antibodies to the extracellular domain of PO. *J. Neurosci. Res.* **35**, 46–53 (1993).
49. Zhang, Y.A., Goldstein, A.M. & Weiner, A.M. UACUAAC is the preferred branch site for mammalian mRNA splicing. *Proc. Natl. Acad. Sci. USA* **86**, 2752–2756 (1989).
50. Hart, G.W., Brew, K., Grant, G.A., Bradshaw, R.A. & Lennarz, W.J. Primary structural requirements for the enzymatic formation of the N-glycosidic bond in glycoproteins. Studies with natural and synthetic peptides. *J. Biol. Chem.* **254**, 9747–9753 (1979).



Chapter 3

Adam22 is a neuronal receptor for Lgi4 mediated Schwann cell signalling

Ekim Özkaynak, Gina Abello, Martine Jaegle,
Laura van Berge, Diana Hamer, Linde Kegel, Siska Driegen,
Koji Sagane, John R. Bermingham Jr and Dies Meijer

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Brief communication

Adam22 is a neuronal receptor for Lgi4 mediated Schwann cell signalling

Ekim Özkaynak¹, Gina Abello¹, Martine Jaegle¹, Laura van Berge¹, Diana Hamer¹, Linde Kegel¹, Siska Driegen¹, Koji Sagane², John R. Bermingham Jr³ and Dies Meijer^{1,4}

1. Department of Cell Biology & Genetics, ErasmusMC, Rotterdam, Netherlands
2. Tsukuba Research Laboratories, Eisai Co Ltd, Tsukuba, Japan
3. McLaughlin Research Institute, Great Falls, Montana, USA
4. Corresponding author: Dies Meijer, PhD

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The *Lgi4* gene (Leucine-rich-glioma inactivated4) is an important regulator of myelination in the peripheral nervous system and is found mutated in *claw paw* mice. Its mechanism of action is unknown. Here we demonstrate that Schwann cell secreted *Lgi4* functions through binding of axonal Adam22 (A disintegrin and metalloprotease22) to drive the differentiation of Schwann cells, thus revealing a novel signalling axis in peripheral nerve myelination.

The structural and functional maturation of myelinated nerve fibers in the peripheral nervous system (PNS) is governed by molecular interactions between the axon and the Schwann cell¹. For example, engagement of axonally derived Neuregulin1 (NRG1) with ErbB2/3 receptors on Schwann cells (SCs) mediates SC proliferation and survival, and myelination of axons²⁻³. Neurotrophins and their receptors also modulate myelination⁴. Recently, a mutant form of *Lgi4* has been shown to underlie the congenital hypomyelinating phenotype of *Claw paw* mice, implicating this protein as a positive regulator of myelin formation in the PNS^{5,6}. However, its precise mechanism of action has not been determined: The principal cell source of *Lgi4* in the PNS is unknown (both Schwann cells and neurons express the gene (ref 6 and **Supplementary Fig.1**), the *Lgi4* receptor has not as yet been identified, and it is unknown whether *Lgi4* is involved in paracrine or autocrine events. To address the cell-autonomous function of *Lgi4*, we generated mice that carry a conditional null allele of *Lgi4* (**Supplementary Fig. 2**). *Lgi4* was specifically deleted in SCs during embryonic development using a *DhhCre* transgene⁷. At postnatal day 12 (P12), SC-specific deletion of *Lgi4* results in hypomyelination that is comparable to that observed in *claw paw* (*Lgi4^{clp/clp}*) nerves (**Fig. 1**). Similar deletion of *Lgi4* in both SCs and sensory neurons using a *Wnt1Cre* transgene⁸ also results in peripheral hypomyelination at P12. These results demonstrate that SCs are the principal source of *Lgi4* in the developing nerve.

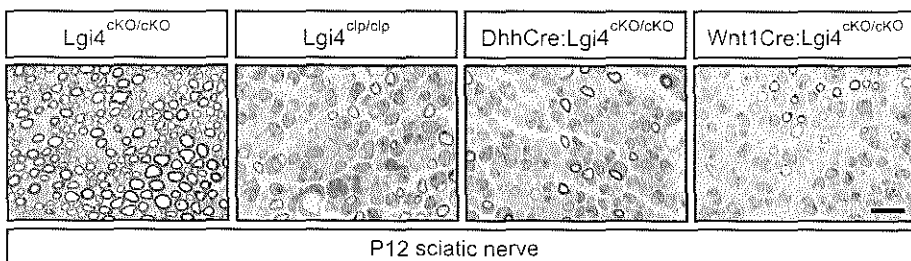


Figure 1 - Schwann cell specific deletion of *Lgi4* results in a congenital hypomyelinating phenotype in the peripheral nervous system.

Cross sections of plastic embedded nerves derived from animals homozygous for the conditional *Lgi4* allele (*Lgi4^{cKO/cKO}*) at P12, show a pattern of myelin figures that is comparable to wildtype (see Fig. 2a). Deletion of the *Lgi4* gene in Schwann cells (*DhhCre; Lgi4^{cKO/cKO}*) or in Schwann cells and sensory neurons (*Wnt1Cre; Lgi4^{cKO/cKO}*) 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 .

Several lines of evidence suggested that members of the Adams family are receptors for Lgi proteins: Lgi1 and Adam22 co-immunoprecipitated in hippocampal neuron membrane preparations⁹. Lgi1 and Lgi4 bind to cells that ectopically express Adam22 or its relative Adam23^{9,10}. Deletion of *Adam22* results in a peripheral nerve phenotype similar to that observed in *claw paw* animals¹¹. Our comparison of cross-sections from P12 sciatic nerves of *claw paw* and *Adam22* mutant animals shows that the peripheral nerves in both animals are hypomyelinated, although the *claw paw* nerve phenotype appears more severe (**Fig. 2a**). Indeed, immunohistochemical and Western blot analyses show that more SCs 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) protein and the larger number of cells expressing Mpz and Krox20 (**Fig. 2 b,c**). This less severe *Adam22* mutant nerve phenotype suggests that additional receptors can partially compensate for the loss of *Adam22*. Both *Adam11* and *Adam23* are expressed in neurons and SCs of the developing nerve (**Supplementary Fig.1**) and Lgi4 binds to these proteins when expressed on the cell membrane (**Fig. 2d**). Moreover, Lgi2 and 3, which are both expressed in the sensory neurons of the dorsal root ganglia (DRGs) and SCs⁶, also bind to these Adam proteins, albeit with different specificity (**Fig. 2d**). To test whether binding of Lgi4 to Adam22 is direct or requires the presence of additional molecules at the cell membrane, we utilised fusion proteins that consist of the ectodomain of Adam22 and the human IgG Fc moiety. Adam22-Fc efficiently precipitates Lgi4 from conditioned medium (**Fig2. e**). In contrast, Adam22-Fc with the D509N mutation in the disintegrin-like domain mutant does not. This mutation is analogous to the *ju160* allele of *C.elegans Adm1/Unc-71*, which causes axon guidance and sex myoblast migration defects¹² 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.

Adam22 is expressed by neurons as well as SCs (**Supplementary Fig.1** and ref. 11) raising the question whether Lgi4 functions in an autocrine or/and paracrine fashion. To answer this question, we generated primary sensory neuron cultures from *Adam22^{+/neo}* and *Adam22^{neo/neo}* embryos and seeded these cultures with rat SCs. Rat SCs readily ensheath and myelinate *Adam22^{+/neo}* neurons (myelin basic protein (MBP) positive myelin sheaths surrounding neurofilament positive (green) neurites), whereas *Adam22^{neo/neo}* neurons are not myelinated (**Fig. 3a & 3b**). These results indicate that Adam22 function is required in the neuron, but do not exclude an additional function in SCs. To further explore the SC-autonomous function of Adam22 in nerve development, we generated a conditional Adam22 allele (**Supplementary Fig. 3**) and bred it to homozygosity in the presence of the Wnt1Cre transgene (*Adam22* deletion occurs in all neural crest derivatives, including

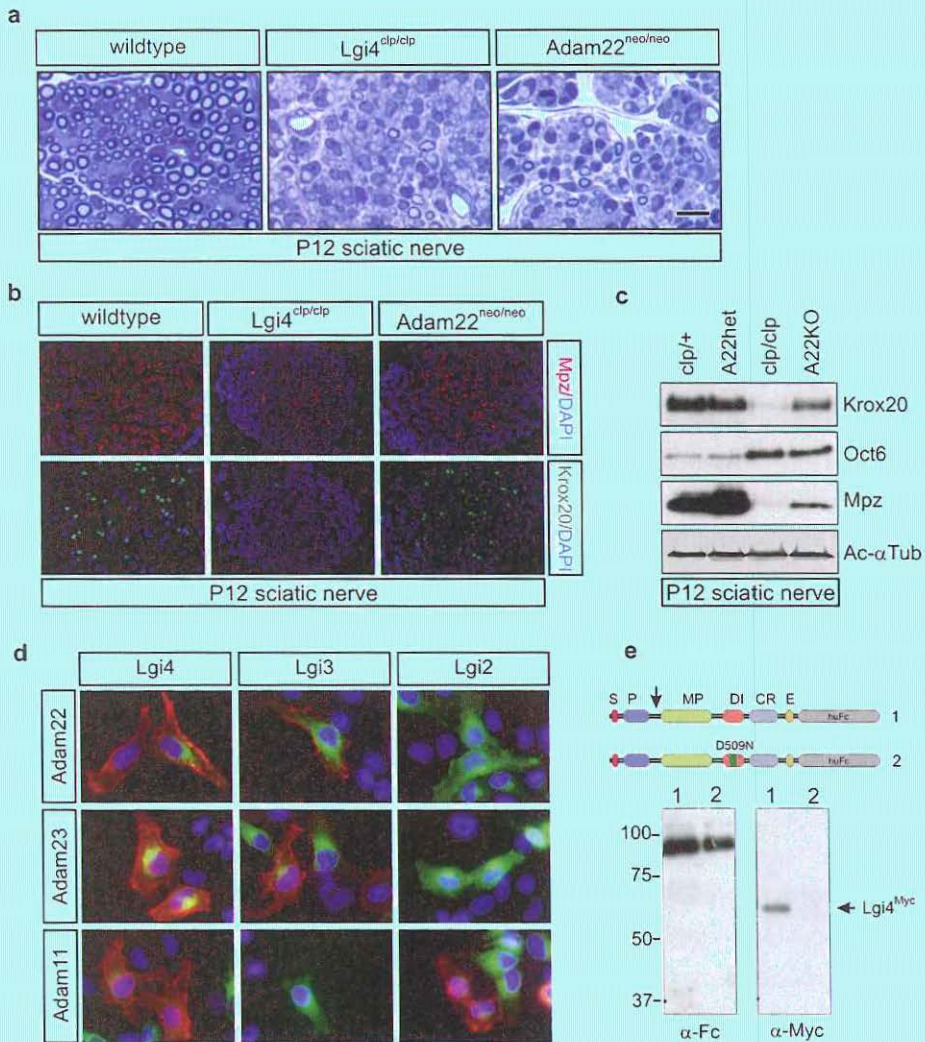


Figure 2 - Similarities between *Lgi4* and *Adam22* deficient mice and direct interaction between *Lgi4* and *Adam22*. (a) Toluidine blue stained cross sections of sciatic nerve of Claw paw (*Lgi4*^{clp/clp}) and *Adam22* (*Adam22*^{neo/neo}) mutant show similarities in extent of myelin formation at P12. Representative pictures of 3 individuals per genotype examined. Scale bar = 10 μm (b) Myelin protein zero (Mpz) and Krox20 staining of cross sections of sciatic nerves derived from Claw paw, *Adam22* mutant and wildtype 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 a pro-myelinating marker, the transcription factor 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 (d) *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 (**Supplementary Fig. 1**), 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

sensory neurons and SCs, but not in spinal motor neurons; see also **Supplementary Fig. 4**). Analysis of myelin formation in the sciatic nerve of these mice revealed a mosaic pattern of normally myelinated fibers and promyelinated arrested fibers (**Fig. 3c & 3d**). Most of the myelinated axons expressed Choline acetyltransferase (CHAT), indicating they are motor fibers. As in claw paw mice at P12, non-myelinated fibers are associated with predominantly Oct6 positive promyelinating cells, whereas Krox20 positive SCs are invariably myelinating cells (**Fig. 3d and Fig. 2b**). Examination of the ventral and dorsal roots revealed normal myelinated motor fibers and severely hypomyelinated sensory fibers respectively (**Fig. 3c and e**). These data demonstrate that Adam22 is required in neurons, whereas deleting Adam22 in SCs has no effect on myelin formation. Thus, our data suggest that axonal Adam22 is the major receptor for SC-derived Lgi4 and that the myelin promoting activity of Lgi4 is mediated largely through Adam22.

But how does Lgi4 binding to axonal Adam22 drive SC myelination? At least two possible scenarios can be envisioned. Adam22 interacts through its carboxy-terminal PDZ binding motif with scaffolding proteins such as PSD95. It is conceivable that Lgi4 binding to Adam22 modulates these interactions with scaffolding proteins, resulting in clustering or presentation of important axonal signalling molecules such as NRG1 or Trk receptors. Such a mechanism is similar to that proposed by Fukata and colleagues⁹ for Lgi1-Adam22 mediated AMPA receptor clustering in hippocampal synapses. Alternatively, 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^{13,14}. However, the actions of these integrins have been mainly interpreted in terms of their interaction with extracellular matrix proteins and not with the axon.

In conclusion, we have identified Adam22 as the major axonal receptor for the SC derived factor Lgi4, thus revealing a new signalling axis in SC-neuron interactions that govern myelin formation in the PNS.

tagged Lgi2 (red) or Lgi3 (red). (e) Soluble fusion proteins between the ectodomain of Adam22, either wildtype 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. Lgi4 was efficiently precipitated by Adam22-Fc (1) bound to ProteinA beads while the disintegrin mutant (2) protein did not precipitate Lgi4.

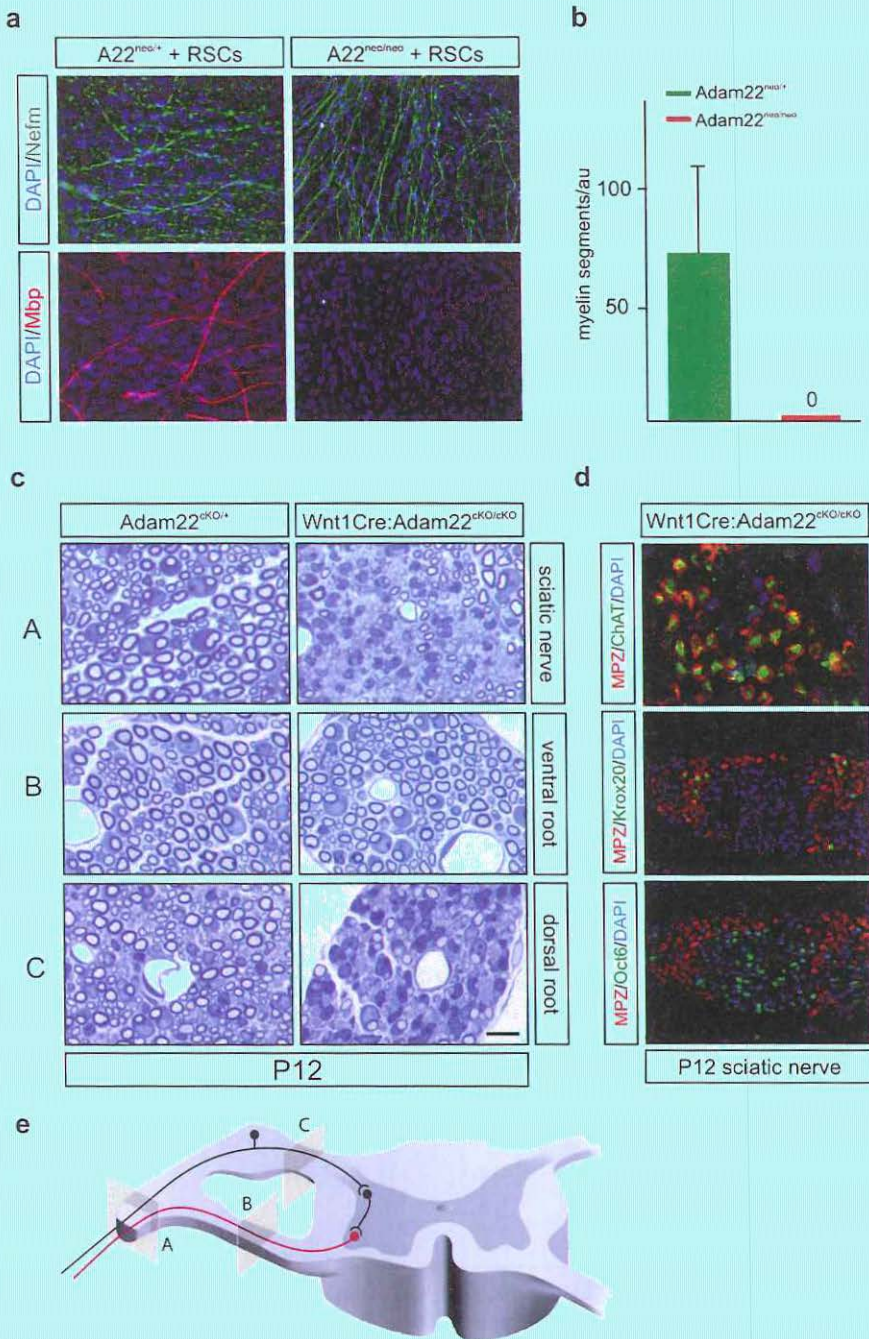


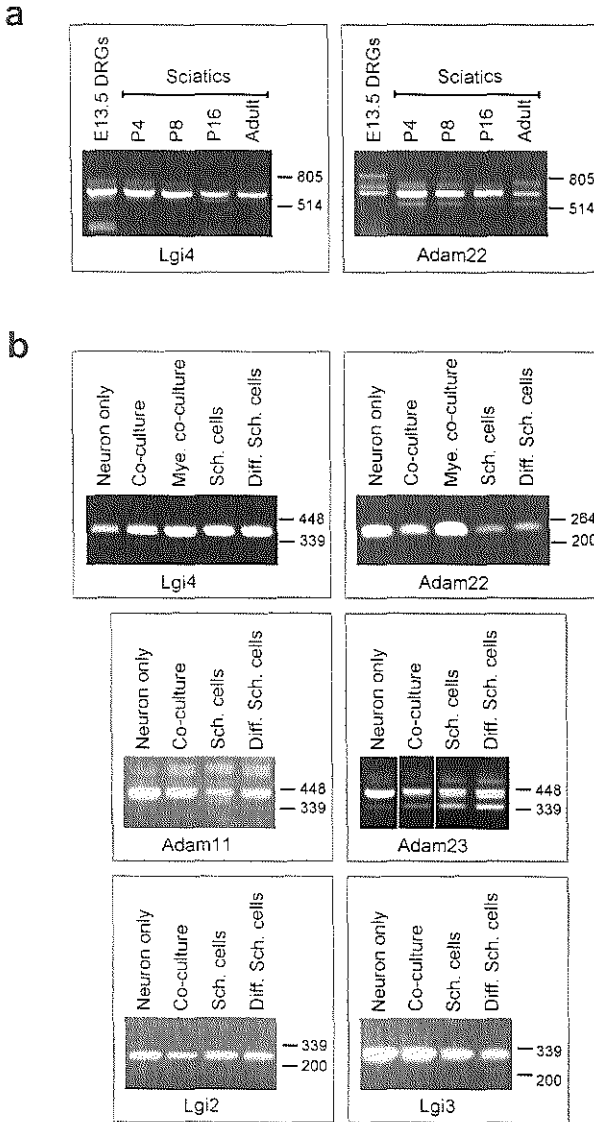
Figure 3 - Adam22 is a neuronal receptor required for myelin formation by Schwann cells.
 (a) Wildtype rat Schwann cells ensheath and myelinate the neurites of embryonic sensory neurons derived from Adam22^{neo/+} but not Adam22^{neo/neo} mutant embryos *in vitro*. The neurites in these cultures are 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. (b) Quantification of myelin formation in the *in vitro* cultures shown in (a). The bar shows the mean of three independent experiments. (c) Conditional deletion of Adam22 in neural crest derivatives using the Wnt1Cre driver. Toluidine blue staining of sections of nerves from Adam22^{CKO/+} and Adam22^{CKO/CKO} Wnt1Cre mice at three different anatomical levels A, B and C. These levels are schematically depicted in (e). Motor fibers are indicated in red and sensory fibers in black. Scale bar represents 10 μ m. (d) Immunostaining for choline-acetyl transferase (Chat; green) and Mpz (myelin; red) shows that most myelinated axons are positive for Chat. Schwann cells in the non-myelinated areas of the nerve are Krox20 negative and Oct6 positive.

References:

1. Jessen, K.R. & Mirsky, R. *Nat Rev Neurosci* **6**, 671-82 (2005).
2. Nave, K.A. & Salzer, J.L. *Curr Opin Neurobiol* **16**, 492-500 (2006).
3. Birchmeier, C. & Nave, K.A. *Glia* **56**, 1491-7 (2008).
4. Chan, J.R., Cosgaya, J.M., Wu, Y.J. & Shooter, E.M. *Proc Natl Acad Sci U S A* **98**, 14661-8 (2001).
5. Henry, E.W., Eicher, E.M. & Sidman, R.L. *J Hered* **82**, 287-94 (1991).
6. Bermingham, J.R., Jr. et al. *Nat Neurosci* **9**, 76-84 (2006).
7. Jaegle, M. et al. *Genes Dev* **17**, 1380-91. (2003).
8. Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K. & McMahon, A.P. *Curr Biol* **8**, 1323-6 (1998).
9. Fukata, Y. et al. *Science* **313**, 1792-5 (2006).
10. Sagane, K., Ishihama, Y. & Sugimoto, H. *Int J Biol Sci* **4**, 387-96 (2008).
11. Sagane, K. et al. *BMC Neurosci* **6**, 33 (2005).
12. Huang, X., Huang, P., Robinson, M.K., Stern, M.J. & Jin, Y. *Development* **130**, 3147-61 (2003).
13. D'Abaco, G.M. et al. *Neurosurgery* **58**, 179-86; discussion 179-86 (2006).
14. Berti, C., Nodari, A., Wrabetz, L. & Feltri, M.L. *Neuromolecular Med* **8**, 191-204 (2006).

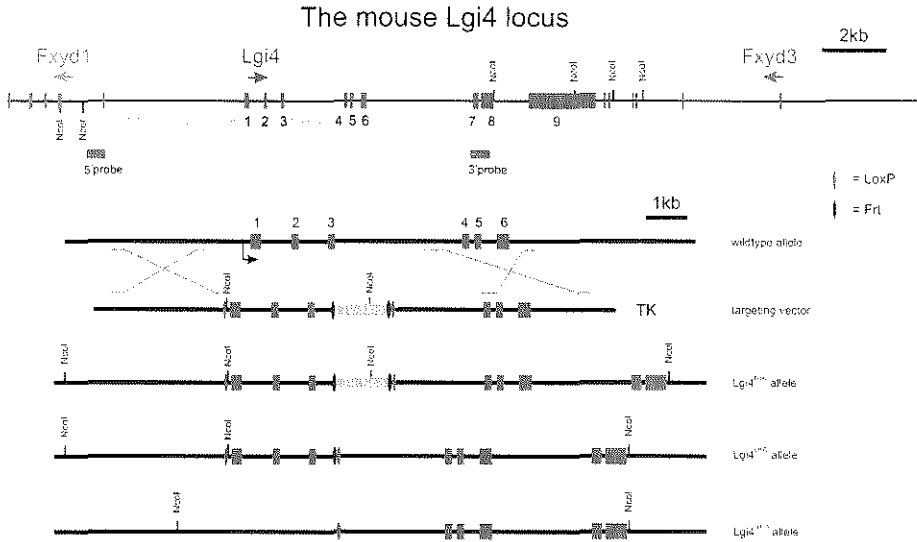
Supplementary information



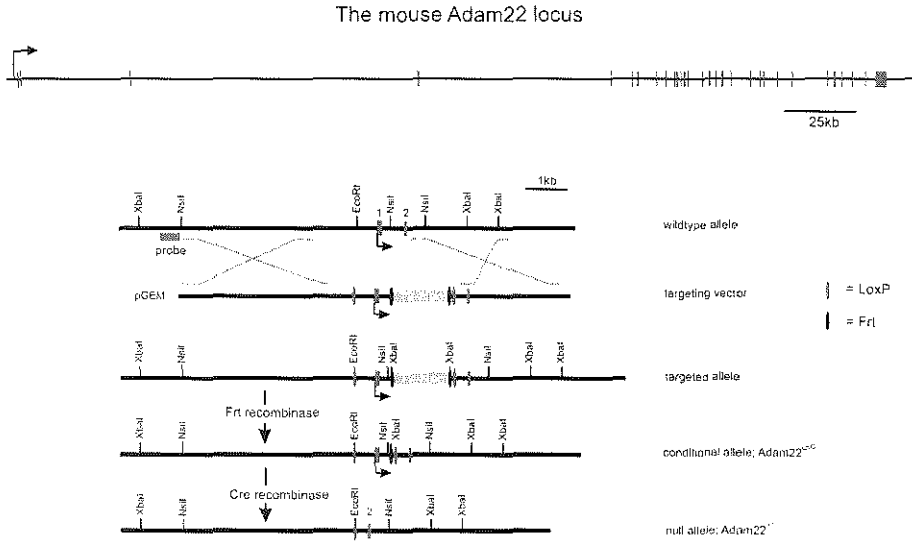
Supplementary Figure 1 - RT-PCR analysis of Lgi2, 3, 4 and Adam11, 22, and 23 expression in different tissues and cell types.

(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^{1,2}. The differences in length of the amplified Adam22 cDNAs represent different mRNA species expressed in neurons and Schwann cells².

(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 results³. 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 rats⁴. Note that the Adam22 RT-PCR here does not differentiate for different isoforms.

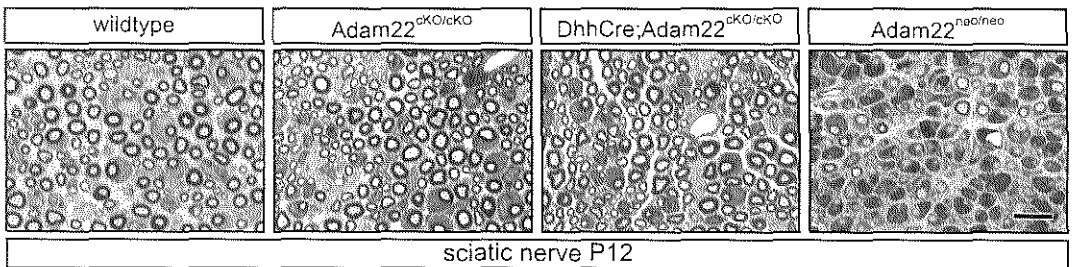


Supplementary Figure 2 - Targeted mutagenesis at the mouse Lgi4 locus to create a conditional Lgi4 null allele. The mouse Lgi4 gene consists of 9 exons spanning approximately 12kb of genomic sequences on mouse chromosome 7. To create a conditional null allele we introduced LoxP sites in front of exon1 and downstream of exon3 through homologous recombination in ES cells. After establishing a mouse line from correctly targeted ES cells, we removed the TK-neo cassette by crossing the mice with the ROSA26-Frt recombinase deleter⁵ mouse. Offspring of this cross was used to establish the Lgi4 conditional knock out (Lgi4^{CKO}) 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 to 3.



Supplementary Figure 3 - Targeted mutagenesis at the mouse Adam22 locus to create a conditional Adam22 null allele.

The mouse Adam22 gene consists of more than 30 exons that span around 300kb of genomic sequences on chromosome 5. To create a conditional null allele for Adam22 (Adam22cKO), we introduced LoxP sequences around exon1 through homologous recombination in ES cells. Exon1 contains the start codon and encodes the signal peptide. Deletion of exon1 will therefore result in a null allele. Correctly targeted ES cells were used to generate a mouse carrying the targeted allele containing the TK-neo selection cassette. Deletion of exon1 by Cre recombinase results in the generation of the Adam22 null (Adam22^{Δ1}) allele.



Supplementary Figure 4 - Schwann cell specific deletion of Adam22 does not affect myelination in the PNS. Myelination of nerve fibers in the sciatic nerve of mice homozygous for the Adam22 conditional null allele (Adam22^{cKO/cKO}) appears normal and is comparable to that observed in sciatic nerve of wildtype and DhhCre;Adam22^{cKO/cKO} 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

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 ². Conditional alleles for Adam22 and Lgi4 were generated through homologous recombination in ES cells using standard techniques. The strategy to create these alleles and a schematic representation of these alleles is depicted in Figures 2 and 3. Mice were genotyped using the following primers;

For Lgi4^{CKO} allele; forward 5'-TCTGACCTCCTCCTTCCCTACCC-3' and reverse 5'-CCCAAATCACTACATCTCCGTGG-3', annealing T=65°C.

For Lgi4^{cb} allele; forward 5'-AGAGAGCCAAGACCTTGGCTAC-3' and reverse 5'-GTCATCTGAAGGTCGAGGATGGC-3', annealing T=60°C.

For Lgi4^{Δ1-3} knock out allele; forward 5'-ACTCTCGGAGGAATGGGAAGGAGGG-3' and reverse 5'-CCATCATCCACACCAGCGAC-3', annealing T=60°C.

For Adam22^{neo} allele; forward 5'-CATTGCTTACATGACTACTGACC-3' and reverse 5'-AAGCAGAGCTGTACAATCC-3', annealing T=58°C.

For Adam22^{CKO} allele; forward 5'-TGCACTCAAGGCATGGTCTC-3' and reverse 5'-GTAGCTAAGTGGCTTCTAGC-3', annealing T=55°C.

For Adam22KO null allele; forward 5'-TGCACTCAAGGCATGGTCTC-3' and reverse 5'-CTGCACTCCTCATCACCTTA-3', annealing T=55°C.

Animal experiments were performed according to protocols that had been approved by an independent animal experimentation committee.

Light microscopy:

Mice were deeply anaesthetized with sodium pentobarbital and transcardially perfused with PBS followed by 4% paraformaldehyde/1% glutaraldehyde in 0,1M phosphate (Millonig's) buffer pH7,2. Sciatic nerves, spinal cords and DRGs were dissected and placed in the same fixative at 4C for at least 16 hours. Tissues were washed with 0,1M Millonig's buffer and osmicated overnight at 4 C in 1% osmium tetroxide/ferricyanide in Millonig's buffer. Tissues were subsequently dehydrated through an ascending acetone series and embedded in plastic resin. One μm sections were cut on a glassknife, mounted on microscope slides and stained with either para-phenylene-diamine (PPD) or Toluidine Blue, as described ^{6,7}. Sections were examined using an Olympus BX40 microscope and pictures were collected using a ColorviewIIIu camera.

Immunofluorescence:

P12 mice were sacrificed and sciatic nerves were dissected and fixed in 4% PFA in PBS overnight at 4°C. After dehydration through an ascending series of ethanol, tissues were

washed in xylene, infiltrated with, and embedded in paraffin. Six μm sections were cut on a Microm microtome, and were mounted on SuperFrost Plus slides (Menzel-Gläser). Sections were de-waxed in xylene, and rehydrated through a descending series of ethanol dilutions and distilled water. After washing 5 minutes each in PBS, and PBS/0.1% Tween-20, antigen retrieval was performed with 15mM $\text{Na}_3\text{-Citrate}$ Buffer containing 0.05% Tween-20 at 80-90°C for 20-30 minutes in a waterbath. After cooling down to room temperature, slides were washed with PBS, then PBS/0.1% Tween-20. Sections were blocked for at least 1 hour at room temperature with filtered blocking solution containing PBS, 1% BSA, 0.2mM 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. Slides were washed 5 times with PBS/0.1% Tween-20 at room temperature. After an additional blocking step of 10 minutes, fluorescent conjugated secondary antibody incubations were done in block solution for 2 hours at room temperature. Slides were washed 5 times with PBS/0.1% Tween-20, once with PBS, and for 2 minutes with running tap water. After rinsing in distilled water, sections were dried, and mounted with polyvinyl alcohol 4-88 containing DABCO anti-fading agent and DAPI for DNA staining.

Binding assays: Lgi2, Lgi3 or Lgi4 conditioned media (CM) were incubated with antibodies against V5 (Lgi2 and Lgi3) or Myc-tag (Lgi4) for 45 minutes at room temperature. CM were then added to transfected cells and incubated at 37°C/5% CO_2 for 45 minutes. Cells were washed 2 times with PBS, fixed with 4% PFA in PBS for at least 15 minutes, then washed 2 times with PBS, once with PBS/0.05% Triton X-100, and blocked for at least 20 minutes at room temperature. Block contained 1% BSA, 0.2mM Glycine, 0.01% Triton X-100, and 0.5% Normal Goat Serum (Dako). Primary antibody incubation was done at room temperature for at least 2 hours, and secondary antibody incubation was done at room temperature for at least 45 minutes.

For DRG cultures; the protocol was the same as above, except for an additional fixation step: After PFA fixation, cells were washed several times with PBS, then fixed and permeabilized with ice-cold methanol for 20 minutes, to facilitate myelin staining.

Samples were examined under a Zeiss AxioImager.Z1 microscope using 40x or 63x objectives. In some cases, optical sections were obtained using the apotome system (Zeiss). Images were captured with an AxioCam MRm digital camera, and analyzed with AxioVision software. Brightness and contrast levels were adjusted in photoshop.

Sciatic nerve tissue extraction and Western blotting:

Nerves were dissected from euthanized 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

minute. Samples were denatured at 95°C for 10 minutes, and proteins were separated by SDS-PAGE (12.5% gel), and blotted onto Immobilon-P PVDF membranes (Millipore). The blots were blocked with 3% BSA or blotting grade non-fat dry milk (Bio-Rad), and 0.05% Tween-20 in PBS for 1 hour at room temperature. Primary antibody incubation in block solution was done overnight at room temperature. The blots were washed 5 times for 10 minutes with PBS/0.1% Tween-20, and incubated with HRP- or AP-conjugated secondary antibodies for at least 1 hour at room temperature (For Fc staining, blots were blocked overnight at 4°C, then incubated for 1 hour at room temperature with HRP-conjugated anti-human antibodies). After washing with PBS/Tween-20, the HRP-bound protein bands were detected by chemiluminescence with ECL kit (GE Healthcare) on Kodak BioMax MR films, and the AP-bound protein bands were detected by chromogenic assay with NBT/BCIP (Roche).

Antibodies:

Primary antibodies: (Immunofluorescence; IF, Western Blot; WB) Mouse-anti-P0⁸ (IF, 1:500; WB, 1:1000), rabbit-anti-Oct6⁷ (IF, 1:200; WB, 1:500), rabbit-anti-Krox20⁹ (IF, 1:200; WB, 1:500), mouse-anti-Neurofilament M (supernatant clone 2H3, Developmental Studies Hybridoma Bank; IF, 1:300), rat-anti-MBP (Millipore; IF, 1:300), goat-anti-ChAT (Millipore; IF, 1:200), mouse-anti-myc 9E10 (clone 9E10, 0.4mg/ml, Roche; IF, 1:300), mouse-anti-V5 (custom made monoclonal antibody; IF, 1:50), rat-anti-Lgi4 (clone 11H12, Absea Biotechnology; IF, 1:300; WB, 1:1000), mouse-anti-acetylated tubulin (Sigma; WB, 1:1000).

Secondary antibodies: The following secondary antibodies were used. The commercial source and dilution are indicated in brackets. Alexa594 conjugated Goat-anti-mouse and Alexa488 conjugated goat-anti-rabbit (Molecular Probes, Invitrogen, and 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 (Amersham Biosciences; WB, 1:5000).

Cloning:

Lgi2, 3, 4 and Adam22 cDNAs were amplified from E13.5 mouse DRG cDNA. cDNAs were generated using the SuperScript II (Invitrogen) reverse transcription system, on total RNA isolated by RNA-Bee reagent (Tel-Test, Inc). After cDNA amplification with Phusion polymerase (Finnzymes), the fragments were ligated to Zero-Blunt TOPO vector (Invitrogen) and sequenced. Adam11 and Adam23 were amplified from IMAGE cDNA clones. V5-His (Lgi2, 3) or myc-His (Lgi4) tagged Lgi proteins were obtained by re-cloning into pcDNA3.1

based expression cassettes. Full length Adam-GFP-His fusion proteins and Ectodomain Adam-Fc fusion proteins were obtained by re-cloning into CMV-based expression cassettes (G20 isoform for Adam22 and alpha isoform for Adam23 were used).

Primers: Restriction site used for cloning are underlined. Start codon is in bold.

Lgi2; forward 5'-CAAGCTTGCCACC**ATGG**CGCTATGGAGAGG-3',
reverse 5'-CCTCGAGACAACTTAAATCAACAATGATATGTTTC-3'.

Lgi3; forward 5'-CAAGCTTGCCACC**ATGG**CCGGGCTACGAGC-3',
reverse 5'-CCTCGAGAGGCACTAAGGTCCACCACAACATG-3'.

Lgi4; forward 5'-CATCGATGCCACC**ATGG**GAGGGGCGGGCATC-3',
reverse 5'-CTCTAGAGGCACTGAGATCCAATTCATGGTG-3'

Adam22; forward 5'-CATCGATGCCGCCACC**ATGC**AGGCAG-3',
reverse 5'-AGAATTCAATGGATGTCTCCCATAGCC-3'

The 3' ends of different variants were amplified by;

forward 5'-GTTTGCAGCAATGAGCTCAAGTG-3',

reverse 5'-AGAATTCAATGGATGTCTCCCATAGCC-3', making use of the internal SacI site for subsequent cloning steps.

Cell culture, binding and immunoprecipitation assays:

All cell culture reagents were from Lonza, and all cultures dishes were from Greiner Bio-one unless otherwise mentioned. CHO and HeLa cells were grown in DMEM, 10% FCS, Penicillin and Streptomycin (PS), and HEK 293T cells were grown in DMEM/F10, 10% FCS, PS. HeLa, or 293 cells were transiently transfected by Lipofectamine reagent (Invitrogen), or Calcium phosphate transfection method (ref). Stable CHO cell lines expressing Lgi4 were obtained by linearization of plasmid with SspI, transfection with lipofectamine, and G418 selection.

For immunoprecipitation assays, 6 days CM were collected and filtered. CM were buffered with 0.1M Tris pH 8. Adam22-ED-Fc proteins were bound to ProteinA 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 CM and incubated overnight at 4°C. After washing, beads were resuspended in reducing sample buffer, and SDS-PAGE (10% resolving gel) and western was performed as above.

RT-PCRs:

For RT-PCR experiments, RNA was isolated from various tissues with RNA-Bee, first strand cDNA synthesis was performed with SuperScript III (Invitrogen), 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 (T_m ; 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 (sec)	Expected product length (bp)
Mouse Lgi4 forward	CAGCTTCTTAAAGATGCCGTCATTG	69	15	620
Mouse Lgi4 reverse	CCAGCACCCAGAGGCTTGCAGG			
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	CTCAGGCACTGAGATCCAATC			
Rat Adam22 forward	GGTAAATCACGTCCACACTG	67.5	10	240
Rat Adam22 reverse	GAGCTCATTGCTACAACTCCG			
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	GGTCCCATCATCTTGCAGT	66	10	275
Rat Lgi2 reverse	ACGTGTAGTCACTCCCCAGG			
Rat Lgi3 forward	CCTTGTAATCGCCTAGCTGC	66	10	328
Rat Lgi3 reverse	TGGGATGAGAAAGGAGATGG			

Rat Schwann cell and DRG cultures:

Primary rat SC cultures were set up essentially as described before¹⁰. Briefly, sciatic nerves were isolated aseptically from P4 rats and disrupted mechanically by teasing with needles. Subsequently, teased nerves were treated with 0,1% collagenase (Roche) in Leibovitz15 (L15) medium for 45 minutes at 37°C. The cells were plated on Primaria

Falcon Plates (BD Biosciences) in DMEM, 10% FCS, 10ng/ml 7S NGF (Harlan), PS. The next day medium was changed to medium containing Ara-C (Sigma) at 10mM to kill dividing fibroblasts. Cells were treated with Ara-c containing medium for three days and medium was changed twice during this period. After this treatment, pure SCs were expanded and maintained in DMEM, 3% FCS, 5% NDF- β CM¹¹, 2 μ M Forskolin (Sigma), PS; (Schwann Cell Proliferation Medium - SCPM). The cells were maintained in SCPM and were differentiated using 20 μ M Forskolin in DMEM/F12 (Invitrogen), N2 supplements (Invitrogen), 5% NDF- β CM/PS for 48 hours.

DRGs were isolated aseptically from E13.5 mouse embryos. After dissociation by trypsinization and trituration, the cells were plated onto 18mm PLL (Sigma)/collagen (home-made) coated coverslips (Thermo Scientific), and maintained in MEM (GIBCO), 3% FCS, 100ng/ml NGF, PS (M1 Medium). The medium was refreshed every other day. To obtain neuron-only cultures, the cells were treated with 10 μ M FUDR (Sigma) in M1 medium, one cycle with and one cycle without FUDR. Two to three cycles of FUDR treatment was enough to obtain neuron only cultures. Prior to seeding, the RSCs were starved in DMEM, 10% FCS, PS for 3 days, and after trypsinization 2x10⁵ cells in M1 medium were seeded onto neuron-only cultures. The Schwann cells were allowed to associate with the neurons for an additional 5 days, and then myelination was induced by addition of ascorbic acid to 50 mg/ml (Sigma). Cells were grown in M1 medium containing 50 μ g/ml ascorbic acid, and 10% FCS for 2 weeks for myelination, and immunofluorescence was performed as above.

References:

1. Godde, N.J., D'Abaco, G.M., Paradiso, L. & Novak, U. Differential coding potential of ADAM22 mRNAs. *Gene* **403**, 80-8 (2007).
2. Sagane, K. et al. Ataxia and peripheral nerve hypomyelination in ADAM22-deficient mice. *BMC Neurosci* **6**, 33 (2005).
3. Bermingham, J.R., Jr. et al. The claw paw mutation reveals a role for Lgi4 in peripheral nerve development. *Nat Neurosci* **9**, 76-84 (2006).
4. Sun, Y.P. et al. Two novel isoforms of Adam23 expressed in the developmental process of mouse and human brains. *Gene* **325**, 171-8 (2004).
5. Farley, F.W., Soriano, P., Steffen, L.S. & Dymecki, S.M. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* **28**, 106-10 (2000).
6. Estable-Puig, J.F., Bauer, W.C. & Blumberg, J.M. Technical note; paraphenylenediamine staining of osmium-fixed plastic embedded tissue for light and phase microscopy. *J Neuropathol Exp Neurol* **24**, 531-536 (1965).
7. Jaegle, M. et al. The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes Dev* **17**, 1380-91. (2003).
8. Archelos, J.J. et al. Production and characterization of monoclonal antibodies to

- the extracellular domain of P0. *J Neurosci Res* **35**, 46-53 (1993).
9. Ghazvini, M. et al. A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration. *Embo J* **21**, 4612-20. (2002).
 10. Brockes, J.P., Fields, K.L. & Raff, M.C. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res* **165**, 105-18 (1979).
 11. Spiegel, I. & Peles, E. A novel method for isolating Schwann cells using the extracellular domain of Nectin1. *J Neurosci Res* (2009).

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Chapter 4

Claw paw revisited: A study on the function of wild type and mutated forms of Lgi4

Ekim Özkaynak, Martine Jaegle, Siska Driegen,
Koji Sagane, John R. Bermingham Jr and Dies Meijer

Work in progress

Claw paw revisited: A study on the function of wild type and mutated forms of Lgi4

Ekim Özkaynak¹, Martine Jaegle¹, Siska Driegen¹,
Koji Sagane², John R. Bermingham Jr³ and Dies Meijer¹

1. Department of Cell Biology & Genetics, ErasmusMC, Rotterdam, Netherlands
2. Tsukuba Research Laboratories, Eisai Co Ltd, Tsukuba, Japan
3. McLaughlin Research Institute, Great Falls, Montana, USA

Abstract

Myelination in the peripheral nervous system depends on the intercellular communication of axons and Schwann cells. In previous chapters, we have identified Lgi4-Adam22 interaction as an essential part of this crosstalk. The claw paw mutated form of the normally secreted Lgi4 protein is retained intracellularly, although minute amounts of the mutant protein are secreted. This led to the hypothesis that Lgi4^{clp} represents a hypomorphic allele that produces reduced amounts of mutant, but functional protein. Indeed, we show that peripheral nerves of Lgi4 null mice are completely devoid of myelination, whereas nerves of Claw paw animals are hypomyelinated. Furthermore, Lgi4^{clp/clp} Adam22^{neo/neo} compound mutants exhibit a more severe hypomyelinating phenotype than the single homozygous mutants, indicating that Lgi4^{clp} activity, like Lgi4, is mediated through Adam22. We conclude that the claw paw phenotype results from decreased Lgi4 function. Additionally, expression of Lgi4 during earlier stages of nervous system development, as well as expression in non-neuroectodermal cell types could contribute to myelination.

Introduction

Previously we have shown that the hypomyelination phenotype of Claw Paw mice results from a mutation in the *Lgi4* gene [1]. *Lgi4* encodes a secreted protein that contains two repeat domains, leucine-rich repeat (LRR) and epilepsy associated repeat (EAR or EPTP), both of which are thought to mediate protein interactions. The claw paw mutation causes skipping of exon four during mRNA splicing, resulting in a mature mRNA that encodes a mutant *Lgi4* protein. The *Lgi4*^{clp} protein misses one LRR unit in its LRR domain. Consequently, it contains three LRR domains instead of four. The *Lgi4*^{clp} protein is retained intracellularly possibly by the effects of quality control mechanisms in the ER. Peripheral nerves of claw paw mice are hypomyelinated and delayed in the initiation of myelination. If the mice survive the initial postnatal weeks, they recuperate to a certain extent over time, and behave normally. This suggests that the *Lgi4* protein functions in the extracellular environment to control the timing, and possibly, the extent of myelination.

We and others have shown that *Lgi4* binds to Adam22 on the cell surface of transfected cells, suggesting that Adam22 is a receptor for *Lgi4* ([2] and Chapter 3). In line with this, Adam22 knock out mice also display a hypomyelination phenotype, and limb abnormalities similar to that of claw paw mice [3]. Furthermore, we suggested that the expression of *Lgi4* from the Schwann cells and its recognition by Adam22 on the axons is necessary for proper myelination of the peripheral nerves (Chapter 3).

In spite of these observations, not all aspects of this interaction are completely understood. The fact that *Lgi4*^{clp/clp} neuron-Schwann cell co-cultures myelinate in the presence of exogenous *Lgi4* in the culture medium suggests that the *Lgi4*^{clp} mutation results from a loss of function. Although it is clear that retention of *Lgi4* inside the cell in the claw paw animals results in the myelination defect, it is not clear whether *Lgi4*^{clp} would still be functional if it were secreted. Interestingly, in transfected cells, small amounts of *Lgi4*^{clp} can be found in the culture medium, suggesting that some of the protein escapes the quality control mechanisms of the cell. Therefore, if functional, the minute amounts of *Lgi4*^{clp} in the extracellular environment *in vivo* could contribute to the delayed kinetics in myelination of the claw paw mice. In line with this, the severity of the claw paw phenotype also depends on the genetic background of the mice, with mice containing a mixed background showing less severe defects.

To address the possibility that *Lgi4*^{clp} is in fact a hypomorphic allele, we analyzed full null and conditional null alleles of *Lgi4*. Compared to claw paw animals, *Lgi4* null mice display a more severe phenotype, with peripheral nerves that are completely amyelinated. In addition, tissue specific deletions of *Lgi4* result in intermediary phenotypes, suggesting that both the exact place and the timing of *Lgi4* expression is of great importance. Furthermore,

we analyzed the myelination status of peripheral nerves in Adam22/Lgi4 double mutants and found them to be more severely hypomyelinated than the nerves of single mutants, thus corroborating our observations on Lgi4-Adam22 interaction. Finally, we show that co-expression of Adam22 with Lgi4^{clp} does not overcome the intracellular retention of Lgi4^{clp}, refuting a possible increased secretion scenario in vivo.

Results

Secretion of Lgi4^{clp}

It has been shown that not only large deletions, but also point mutations in the epileptic forms of Lgi1 result in the intracellular retention of the mutant protein [4]. This suggests that the protein structure and folding is tightly controlled. It is no surprise then to see that the loss of 24 amino acids in Lgi4^{clp} results in the retention of the protein. Despite this, small amounts of Lgi4^{clp} can be purified from the culture medium of stably transfected CHO cell lines. (Figure 1) Therefore, the low amounts of extracellular Lgi4^{clp}, if still functional, could drive myelination albeit in a delayed fashion.

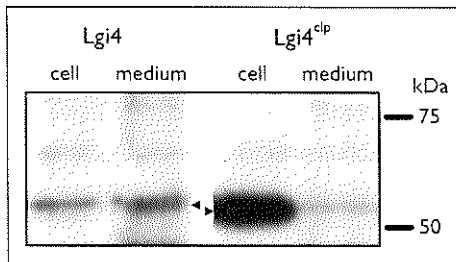


Figure 1 - Assessment of the secretion of Lgi4^{clp}.

Myc and 6xHis tagged wild type or mutant Lgi4 proteins were purified from cell extracts and conditioned media of stably transfected CHO cells. The proteins were detected with α -Myc antibody by Western blot. Lgi4 is secreted to the medium (left panel). Compared to Lgi4 protein, very small amounts of Lgi4^{clp} can be purified from the culture medium (right panel).

Lgi4^{clp} is a hypomorphic allele

The observations above suggest two straightforward possibilities. One is that Lgi4^{clp} is functional, and the hypomyelination phenotype of the claw paw (Lgi4^{clp/clp}) mice results from decreased amounts of mutant but functional Lgi4 in the extracellular environment. Alternatively, Lgi4^{clp} is not functional, and the phenotype observed in claw paw mice is simply the result of the absence of Lgi4 activity in the peripheral nerves. The fact that inbred claw paw mice show a more severe phenotype than those with mixed genetic backgrounds might suggest that Lgi4^{clp} is indeed functional, and that inbreeding affects the secretion of Lgi4^{clp}. Deletion of the Lgi4 receptor Adam22 results in a peripheral nerve phenotype that is less severe than that of claw paw animals, which could be interpreted to suggest that alternative receptors for Lgi4 exist in the developing nerve.

To distinguish between the above-mentioned possibilities, we generated conditional null alleles of the Lgi4 gene in mice, and crossed these with mice expressing

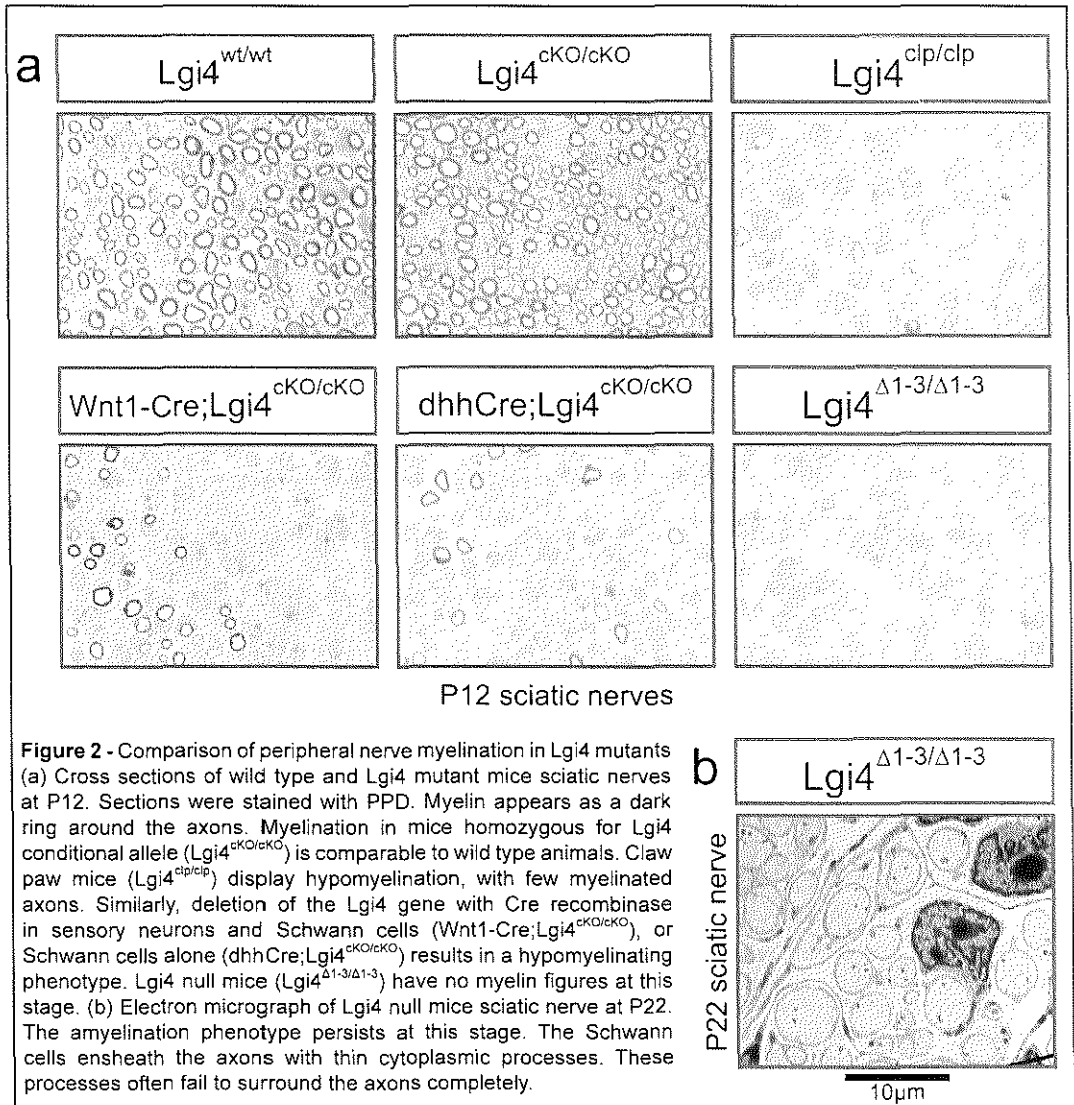
Cre recombinase under the control of different regulatory sequences. This enables us to delete the *Lgi4* gene at different time points and in different tissues. We compared both the overall phenotype, and the peripheral nerve phenotype of these mice with claw paw mice. We used CAG-Cre to delete *Lgi4* in the germline and obtain a complete knock out [5]. *Wnt1*-Cre enabled recombination in all neural crest derivatives, including sensory neurons and Schwann cells [6]. *Dhh*-Cre was used for Schwann cell specific recombination [7].

We microscopically analyzed myelination in the sciatic nerves of the different animals at postnatal day 12 (P12) (Figure 2a). Compared to claw paw mice, full *Lgi4* knock out nerves completely lack myelination, whereas Schwann cell specific and neural crest specific deletion of *Lgi4* results in a hypomyelination phenotype similar to claw paw. These results demonstrate that complete loss of *Lgi4* results in a more severe phenotype, supporting the hypothesis that *Lgi4*^{clp} is a hypomorphic allele. In addition, the characteristic limb phenotype of claw paw mice is observed in both full *Lgi4* null mice and neural crest specific (*Wnt1*-Cre) *Lgi4* null mice, but not in mice in which *Lgi4* is specifically deleted in Schwann cells only (*Dhh*-Cre). Full and neural crest specific knock out mice rarely survived after the second postnatal week. In contrast, claw paw mice, as well as Schwann cell specific *Lgi4* mutants, can survive well into adulthood. For the full *Lgi4* null mutant, we were able to analyze one mouse at P22, which showed no myelin figures in the sciatic nerves, with all cells arrested at what appears to be the promyelin stage (Figure 2b). This suggests that myelination does not progress in these mice beyond the one to one sorting stage.

Cooperation of *Lgi4* and *Adam22* in myelination

The interaction between *Lgi4* and *Adam22*, and the comparable phenotypes of the mice mutated for these genes, suggest that these proteins act together to control PNS myelination. The *Lgi4* or *Adam22* mutations analyzed so far do not show a phenotype in heterozygous mice, indicating that these mutations represent recessive alleles and a single copy of the wild type gene is sufficient for proper myelination. However, *Adam22* and *Lgi4* homozygous mutants show subtle differences in their phenotypes. For example, claw paw nerves are translucent, and have less myelin figures compared to *Adam22* mutants ([1] and Chapter 3). On the other hand, the hind limbs of *Adam22* null mice are invariably affected. In claw paw mice, this is highly variable, and severity appears to correlate with the degree of inbreeding (Figure 3).

To confirm the hypothesis that *Lgi4* and *Adam22* cooperate to control myelination, and to investigate the dosage relationship of the genes, we crossed claw paw mice with *Adam22* mutant mice. If indeed *Lgi4* and *Adam22* act in concert to drive myelination, double homozygous mutants (*Adam22*^{neo/neo};*Lgi4*^{clp/clp}) are expected to show a more severe



phenotype then single gene homozygous mutants (Adam22 null or claw paw animals).

We generated mice that are either double heterozygous or homozygous for the Adam22^{neo} and *Lgi4*^{clp} alleles. In addition, we generated mice that carry an *Lgi4*^{clp} allele on an Adam22^{neo/neo} background, and mice that carry an Adam22^{neo} allele on a claw paw background. The sciatic nerves of these mice were microscopically analysed. At P12, the nerves of double heterozygous animals (Adam22^{neo/+}; *Lgi4*^{clp/+}) are myelinated similar to wild type or single heterozygous animals (Figure 4a). Thus, reducing the gene dose to one

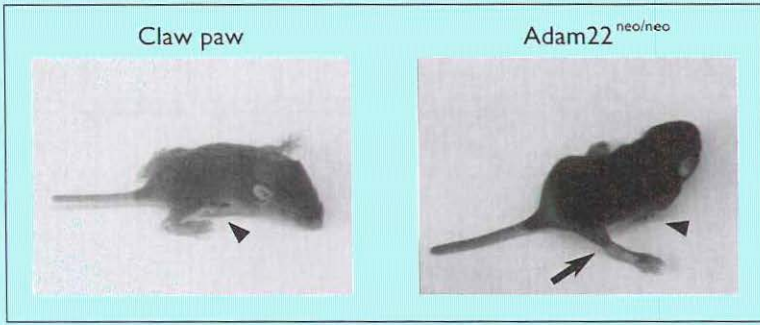


Figure 3 - Limb posture abnormalities in Claw paw and Adam22 knock out mice ($Adam22^{neo/neo}$). Arrowheads indicate the forelimb defects in these mice. In severe cases, hindlimbs of Claw paw mice can be affected as well (data not shown). Hindlimbs of Adam22 knock out mice are invariably affected (arrow).

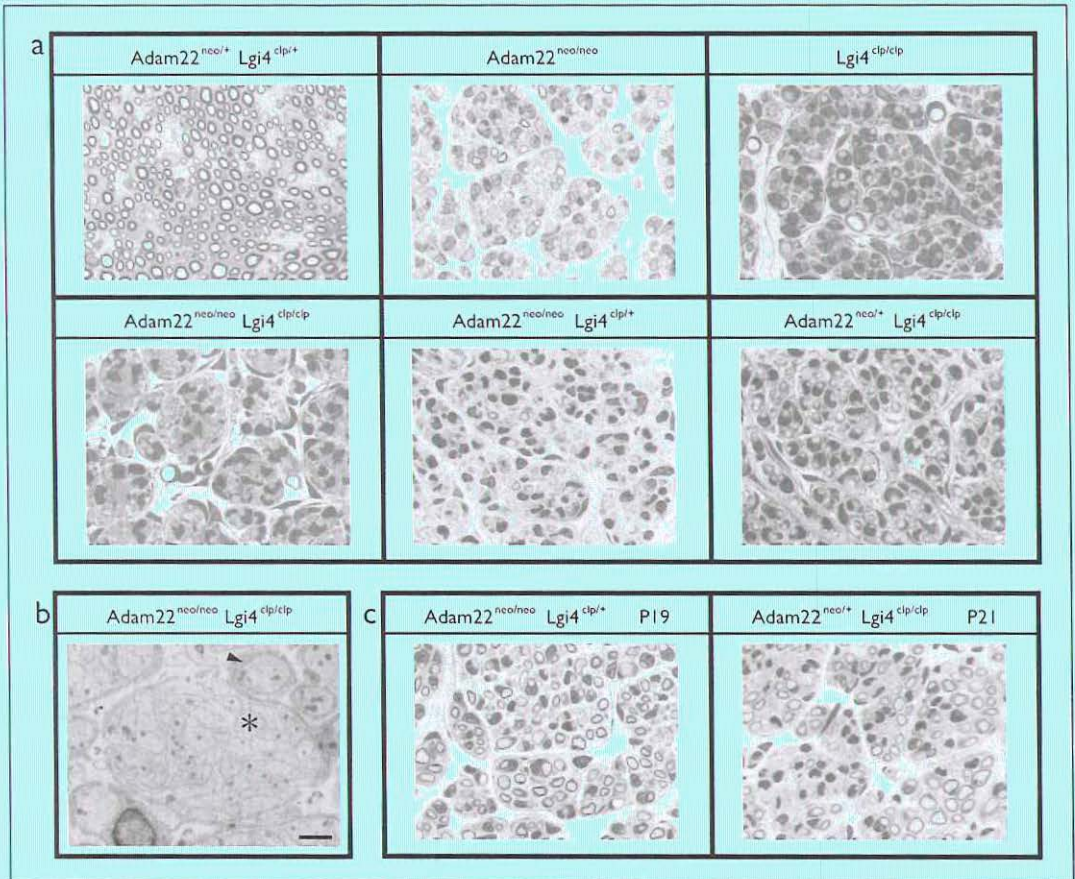


Figure 4 - Myelination in the sciatic nerves of *Lgi4* and *Adam22* compound mutants. (a) Toluidine blue stained cross sections of mutant mice at P12. Myelination in the double heterozygote mice ($Adam22^{neo/+} Lgi4^{clp/+}$) appears unaffected. *Adam22* knock out ($Adam22^{neo/neo}$) and Claw paw ($Lgi4^{clp/clp}$) mice display hypomyelination. Mice homozygous mutant for one gene and heterozygous for the other ($Adam22^{neo/neo}$

for both Adam22 and Lgi4 does not affect the myelination process. Mice homozygous for one gene and heterozygous for the other mimic the homozygous mutant phenotype. That is; Adam22^{neo/neo};Lgi4^{clp/+} nerves are similarly hypomyelinated at P12 as Adam22 mutant nerves, and Adam22^{neo/+};Lgi4^{clp/clp} nerves are similarly hypomyelinated as nerves in claw paw animals. Myelination progresses at later stages of postnatal development in these mice. Several axons are thinly myelinated at P19 or P22 (Figure 4b). On the other hand, Adam22^{neo/neo};Lgi4^{clp/clp} nerves are completely devoid of myelin figures (Figure 4a), and several axons remain in large bundles, suggesting that radial sorting is incomplete at P12 (Figure 4b). Taken together these results demonstrate the physiological relevance of the Lgi4-Adam22 interaction, and its importance for proper development and myelination of the PNS.

Adam22 does not affect the secretion of Lgi4^{clp}

Adam22, like Lgi4, is expressed by both Schwann cells and neurons ([1, 3] and chapter 3). If Adam22-Lgi4 interaction also takes place intracellularly, in the vesicular transport system, it is possible that Adam22 facilitates the secretion of Lgi4. A similar scenario could be envisioned for the possible interaction of Lgi4^{clp} and Adam22. Therefore, while transfection studies show that Lgi4^{clp} is largely retained intracellularly, the co-expression of these two proteins in the same cell type could result in its secretion.

To test this possibility, we co-expressed wild type or mutant Lgi4, with Adam-Fc fusion proteins in HEK293T cells. We transfected ectodomain-Fc fusion constructs of Adam22 and Adam23, together with Lgi4 constructs. We then assessed the secretion of the proteins into the medium. These experiments showed that the secretion of Lgi4^{clp} is not affected, even though the Adams are also present in these cells (Figure 5). Furthermore Lgi4^{clp} is not immunoprecipitated by the Adam-Fcs from the conditioned medium (CM). These data suggest that, Adam22 does not facilitate the secretion of Lgi4, and it is unlikely that such mechanisms contribute to the claw paw phenotype.

Discussion

Myelination in the PNS of claw paw mice represents an intermediate state compared to myelination in wild type and Lgi4 null animals. Homozygosity for Lgi4^{clp} results in hypomyelinated nerves, while complete loss of Lgi4 gives rise to amyelinated nerves.

Lgi4^{clp/+}, or Adam22^{neo/+} Lgi4^{clp/clp}) display hypomyelinating phenotypes. P19 or P21 nerves of these mice contain several, thinly myelinated axons (c). Nerves of double homozygous mutant mice (Adam22^{neo/neo} Lgi4^{clp/clp}) are unmyelinated. (b) Electron micrograph of double homozygous mutant mice at P12. Radial sorting of axons is delayed. An axon ensheathed with Schwann cell processes are indicated with an arrowhead. A large bundle of axons without Schwann cell protrusions is highlighted with an asterisk (Scale bar: 1µm).

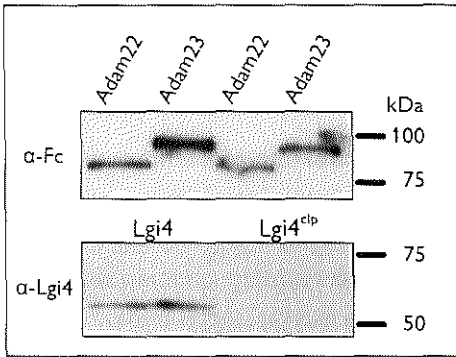


Figure 5 - The secretion of Lgi4^{clp} is not affected by Adam proteins.

Lgi4, but not Lgi4^{clp}, is co-immunoprecipitated with Adam-ED-Fc proteins from the conditioned media. Lgi constructs were co-transfected with Adam22 or 23 ectodomain-Fc fusion constructs into HEK293T cells. Proteins were immunoprecipitated with protein A beads. Western blotting was performed to detect secreted and immunoprecipitated proteins. α-Fc antibody is used to detect Adam-ED-Fc fusion proteins, α-Lgi4 antibody was used to detect Lgi4 proteins.

Lgi4 functions via Adam22 interactions, and the small amount of secreted Lgi4^{clp} is relevant for the myelination observed in claw paw mice. Furthermore, while the expression of Lgi4 from Schwann cells is a determining factor in its role in myelination, expression from other cell types could contribute to myelination as well.

The claw paw mice contain a hypomorphic allele of Lgi4. It is at present unclear whether the hypomorphic nature of Lgi4^{clp} results from reduced secretion of the mutant protein that is fully functional, or from both reduced secretion and impaired function due to the deletion of one LRR motif. The LRR domain presents a motif that is involved in protein interactions [8]. It is therefore possible that Lgi4^{clp} does not interact with other proteins necessary for Lgi4 function during peripheral nerve myelination. Lgi4 binds to Adam22 through its EPTP domain, and binding is independent of the LRR domain (Linde Kegel unpublished observations). Experiments with Lgi1 have suggested that the LRR domain is involved in dimerization of the Lgi1 protein, and that dimerization is important for function [9]. However, we have found no evidence for homo- or heterodimerization of Lgi4 and other Lgi proteins (E.O. unpublished observations). Thus, it remains to be established which proteins are bound to the Lgi4 LRR domain, and how this contributes to Lgi4 function.

The results of our studies on conditional and full Lgi4 mutant animals have several implications for our understanding of Lgi4 function in PNS development and myelination. First, full ablation of Lgi4 results in a complete block of myelin formation, demonstrating that Lgi4 is essential for this process. Second, comparison of Schwann cell and neural crest specific ablation of Lgi4 demonstrated the Schwann cell autonomous role of Lgi4 in myelination. However, in these cell type specific mutants, we observed significant numbers of myelinated axons, in an otherwise severely hypomyelinated nerve (Figure 2a). This strongly suggests that neurons or other cells associated with the developing nerve could be an alternative source for Lgi4. Indeed, we have demonstrated that neurons express low levels of Lgi4 [1], but it is unclear at present whether this expression contributes significantly

to myelin formation. Moreover, nerve transplantation experiments between claw paw and wild type animals has suggested that a non-Schwann cell source of Lgi4 contributes to nerve repair [10]. In the conditional Lgi4 null animals, it is not possible to determine the contribution of neurons by sciatic nerve analysis alone. Similar to the experiments described in Chapter 3, the analysis of dorsal and ventral roots for myelination in Wnt1-Cre/Lgi4cko mice would be useful to assess the neuronal contribution by sensory and motor neurons respectively.

The fact that Wnt1-Cre Lgi4 mutant mice display limb posture abnormalities and die postnatally, but Dhh-Cre Lgi4 mutants do not, also suggests a function for Lgi4 not related to myelinating Schwann cells. In addition to the cell specificity of Lgi4 ablation, it is also possible that the time of Lgi4 deletion during embryonic development, as well as the protein's stability could contribute to the phenotype observed in these conditional Lgi4 mutant animals. Effects of Dhh-Cre recombination in the DRGs could be observed as early as E12.5, and that of Wnt1-Cre as early as E9.5 [7, 11]. Lgi4 in DRGs is detected at E10.5 as an early wave of high expression, and subsequently at E13.5 and later (J. B. unpublished observations and [1]). These observations and the fact that both the initiation of myelination and axonal sorting are delayed in claw paw animals, suggest that Lgi4 is also important for earlier development of the peripheral nerves. Therefore, in the conditional null mice, the early events could take place normally (more so in dhhCre than in Wnt1Cre), and the remaining Lgi4 protein in the nerves contribute significantly to the further differentiation of immature Schwann cells toward the myelinating lineage.

Clearly, future studies focusing on Lgi4/Adam22 protein expression patterns in peripheral nerve development and other tissues will contribute to our understanding of Lgi4-Adam22 interactions, and how these interactions affect nerve development and function. Furthermore, identification of Lgi4 interaction partners, particularly through the LRR domain, will be essential to gain further insight into Lgi4's role in myelination.

Materials and Methods

Mice:

The generation of Adam22 knock out and Lgi4 conditional knock out mice have been previously described ([3] and Chapter 3). Claw paw heterozygous mice were obtained from the Jackson laboratory. Mice were genotyped by PCR as previously described ([1], and Chapter 3). Animal experiments were performed according to protocols that had been approved by an independent animal experimentation committee.

Light/electron microscopy:

Mice were deeply anaesthetized with sodium pentobarbital and transcardially perfused with PBS followed by 4% paraformaldehyde/1% glutaraldehyde in 0,1M phosphate (Millonig's) buffer pH7,2. Sciatic nerves were dissected and placed in the same fixative at 4°C for at least 16 hours. Tissues were washed with 0,1M Millonig's buffer and osmicated overnight at 4 C in 1% osmium tetroxide/ferricyanide in Millonig's buffer. Tissues were subsequently dehydrated through an ascending acetone series and embedded in plastic resin. One μm sections were cut on a glassknife, mounted on microscope slides and stained with either para-phenylene-diamine (PPD) or Toluidine Blue for light microscopy, as described [12, 13] Sections were examined using an Olympus BX40 microscope and pictures were collected using a ColorviewIIIu camera. Sections were stained with uranyl acetate and lead citrate for electron microscopic analysis (Philips CM100).

Cell culture:

Cells were grown in DMEM (for CHO cells) or DMEM/F10 (for HEK293T cells) containing 10%FCS and PS. Stable CHO cell lines expressing myc and 6xHis tagged Lgi4 and Lgi4^{cp} proteins were obtained by Lipofectamine (Invitrogen) transfection, followed by G418 (1mg/ml) selection. Co-transfection of Lgi and Adam constructs were done by calcium phosphate transfection method. Confluent dishes of HEK293T cells were split 1:5 the day before transfection. Next morning, culture medium was refreshed with DMEM/F10 containing 10%FCS. 20 μg of DNA (Lgi:Adam 1:1) was diluted in tissue culture grade water and 50 μl of 2.5M CaCl_2 was added (end volume 500 μl). 500 μl 2xHBS (pH 7.2) was added while vortexing to precipitate the DNA. After 5 minutes, the reaction mix was added onto the cells. 6 hours later the medium was refreshed with normal growth medium. The cell extracts and conditioned media were collected 4 to 6 days later. Lgi4 and Lgi4^{cp} DNA constructs were obtained by cloning cDNAs into pcDNA3.1 based expression cassettes containing myc-His tag sequences. Ectodomain Adam-Fc fusion constructs were obtained by re-cloning the ectodomains into CMV based expression cassettes containing the human Ig Fc sequence.

Protein purification and detection:

Lgi4 and Lgi4^{cp} were purified from cell extracts or medium by Ni-NTA beads (Qiagen) according to the manufacturer's protocol. Immunoprecipitation and Western blotting experiments were described previously (Chapter 3). Proteins were separated by 10% SDS-PAGE. The PVDF membranes were blocked with 3% blocking grade non-fat

dried milk (BioRad). Mouse- α -myc (Roche, clone 9E10, 0.4 mg/ml), and rat- α -Lgi4 (Absea Biotechnology, clone 11H12) antibodies were diluted 1:500 in block. HRP conjugated antibodies were used for protein detection by ECL kit (GE Healthcare). Goat- α -human (Amersham Biosciences), goat-anti-mouse and goat-anti-rat (Dako) antibodies were diluted 1:5000 in block. Chemiluminescence was detected on Kodak BioMax MR films.

References:

1. Bermingham, J.R., Jr., et al., *The claw paw mutation reveals a role for Lgi4 in peripheral nerve development*. Nat Neurosci, 2006. **9**(1): p. 76-84.
2. Sagane, K., Y. Ishihama, and H. Sugimoto, *LGI1 and LGI4 bind to ADAM22, ADAM23 and ADAM11*. Int J Biol Sci, 2008. **4**(6): p. 387-96.
3. Sagane, K., et al., *Ataxia and peripheral nerve hypomyelination in ADAM22-deficient mice*. BMC Neurosci, 2005. **6**: p. 33.
4. Senechal, K.R., C. Thaller, and J.L. Noebels, *ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy*. Hum Mol Genet, 2005. **14**(12): p. 1613-20.
5. Sakai, K. and J. Miyazaki, *A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission*. Biochem Biophys Res Commun, 1997. **237**(2): p. 318-24.
6. Danielian, P.S., et al., *Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase*. Curr Biol, 1998. **8**(24): p. 1323-6.
7. Jaegle, M., et al., *The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development*. Genes Dev, 2003. **17**(11): p. 1380-91.
8. Bella, J., et al., *The leucine-rich repeat structure*. Cell Mol Life Sci, 2008. **65**(15): p. 2307-33.
9. Fukata, Y., et al., *Epilepsy-related ligand/receptor complex LGI1 and ADAM22 regulate synaptic transmission*. Science, 2006. **313**(5794): p. 1792-5.
10. Darbas, A., et al., *Cell autonomy of the mouse claw paw mutation*. Dev Biol, 2004. **272**(2): p. 470-82.
11. Chai, Y., et al., *Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis*. Development, 2000. **127**(8): p. 1671-9.
12. Estable-Puig, J.F., W.C. Bauer, and J.M. Blumberg, *Technical note; paraphenylenediamine staining of osmium-fixed plastic embedded tissue for light and phase microscopy*. J Neuropathol Exp Neurol, 1965. **24**: p. 531-536.
13. Jaegle, M., et al., *The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development*. Genes Dev, 2003. **17**(11): p. 1380-91.

Chapter 5

Discussion

5 - DISCUSSION

Several lines of research have demonstrated the importance of Schwann cell-axon and Schwann cell-ECM interactions for myelination of nerve fibers. Secreted and transmembrane proteins regulate the development of Schwann cells. These extracellular signals are directed towards the Schwann cells to control their proliferation, morphology, and protein expression profile. Notwithstanding the previous research in the field, our understanding of peripheral nerve development is incomplete. Studies on patients and mutant animals with peripheral neuropathies have proven useful for the identification of molecules involved in myelination. In this project, we used classical and modern molecular genetic approaches to identify a novel set of molecules that play an essential role in neuron-Schwann cell communications that govern axonal ensheathment and myelin formation in the peripheral nervous system (PNS).

5.1 - Analysis of Claw paw mice and characterization of Lgi4

Claw paw mutant mice display hypomyelination in the PNS and limb posture abnormalities [1, 2]. Other hypomyelinating phenotypes, such as those observed in mice carrying mutations in laminin, integrin, ErbB, or Adam22, also show limb posture abnormalities due to ataxia and muscle paralysis, often accompanied by tremors [3-6]. These findings suggest that myelination and proper function of peripheral nerves could be an important determinant for normal posture development and gait. The Claw paw phenotype shows some variability in both the severity of limb abnormalities and the survival of the pups during postnatal development. However, the hypomyelination phenotype is fully penetrant, and myelination occurs with delayed kinetics.

The mutation in the Claw paw mice was linked to the Lgi4 gene by genetic mapping studies [7]. Lgi genes encode glycosylated and secreted proteins. Their secretion is tightly controlled, and mutations ranging from large deletions to single amino acid changes result in intracellular retention [8]. The Lgi proteins contain LRR and EAR (or Epitempin-EPTP) domains [9-11]. The LRRs have been identified in several proteins with diverse functions, ranging from cell signalling to RNA processing. They fold into a solenoid structure, which is thought to facilitate protein-protein interactions [12]. The LRR domains of Lgis are functionally related to those found in Trk receptors and Slit [13]. In Trk receptors, these repeats are thought to modulate NT binding [14]. In Slit, they are necessary for binding to the Ig domains of Slit receptor Robo. Slit-Robo signalling has been identified as an important regulator of axon guidance, and neuronal migration [15]. The role of LRRs in Lgi molecules remains elusive. It is possible that they are involved in oligomerization of

Lgis [16]. It is thought that the EPTP domain also plays a role in protein interactions [10, 11]. It has been demonstrated that Lgi1-Adam22 interactions are mediated by the EPTP domain [16]. Other than the Lgis, the EPTP domain has been identified in VLGR1 and TSPEAR proteins. VLGR1 (Very large G-protein coupled receptor 1) mutations have been linked to seizures and Usher syndrome (USH2C), which is characterized by hearing loss and retinitis pigmentosa [17, 18]. TSPEAR (TNEP1) is a protein of unknown function. It is named after the thrombospondin-N terminal domain and the EAR.

The Claw paw mutation is an insertion of a repeat element in the 3rd intron of the Lgi4 gene. As a consequence, exon 4 is skipped during mRNA processing. This mRNA encodes a protein with an internal deletion. Lgi4^{cbp} protein misses an LRR domain, and is retained intracellularly. Secretion of Lgi4 is necessary for proper myelination. This is demonstrated in Claw paw DRG cultures. These cultures fail to myelinate, but the addition of wild type Lgi4 protein in the medium initiates myelination. *In situ* hybridization experiments demonstrate that Lgi4 is expressed throughout the PNS, primarily by Schwann cells [7]. As a secreted molecule, Lgi4 could play a role in Schwann cell-axon interactions. We proposed possible mechanisms by which Lgi4 could mediate PNS development and myelination (Figure 1). These mechanisms involve paracrine (or juxta-paracrine) signalling from Schwann cells to axons, or autocrine signalling in Schwann cells. Lgi4 could bind to an axonal receptor to initiate signalling, or it could modulate an already present signalling event between Schwann cells and axons. Alternatively, Lgi4 could initiate or modulate signalling events by binding to receptors on Schwann cells.

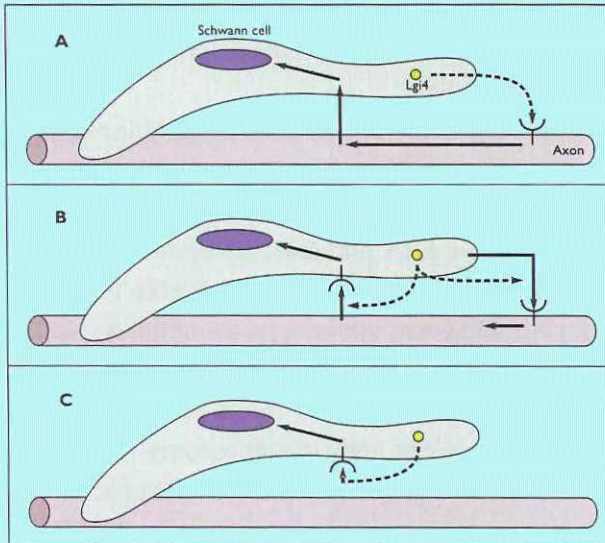


Figure 1. Lgi4 modes of action

Three possible mechanisms by which Lgi4 could function in Schwann cell-axon interactions.

A) Lgi4 could bind an axonal receptor in a juxta-paracrine fashion.

B) Lgi4 could modulate an already present signalling event between Schwann cells and axons.

C) Lgi4 could bind a receptor on the Schwann cell surface in an autocrine fashion.

5.2 - Lgi4 and Adam22, a novel protein complex involved in myelination

The identification of Lgi1-Adam22 interaction suggested new possibilities for the function of these proteins and their relatives in synaptic transmission and cytoskeletal re-arrangements [16, 19]. Lgi1-Adam22 further interacts with PSD95 and voltage gated potassium channels. It is thought that these interactions stabilize ion channel localisation at the synapses, and modulate ion channel function. The tremor observed in Adam22 knock out mice could be related to this function. Similar to Lgi4 mutant mice, Adam22 knock-out mice also display peripheral nerve hypomyelination and limb abnormalities [6]. Taken together, these observations suggest that Lgi4 and Adam22 interact with each other.

In chapter 3, we showed that Lgi proteins bind to Adams on the cell surface with different specificities: Lgi2 binds to Adam11, Lgi3 binds to Adam22 and Adam23, and Lgi4 binds to all three Adams. These interactions could regulate different developmental or functional aspects of the nervous system depending on the context. We also showed by immunoprecipitation experiments that Lgi4-Adam22 interaction does not depend on other transmembrane proteins. Although, it is possible that other soluble proteins mediate or modulate Lgi4-Adam22 interaction.

We crossed Claw paw and Adam22 knock out mice to generate double mutant animals (Chapter 4). Double heterozygous mice do not show a hypomyelination phenotype. Mice homozygous mutant for one gene and heterozygous for the other, are similar to the homozygous mutant phenotype. Therefore, the expression of these proteins from a single allele is sufficient for proper myelination. Finally, the peripheral nerves of double mutant mice are completely amyelinated. Taken together with our previous observations, these data strongly indicate that Lgi4 functions through Adam22 to control myelination in the PNS.

RT-PCR analysis revealed that both Lgi4 and Adam22 are expressed in Schwann cells and neurons. Unfortunately, technical difficulties or the relatively low amounts of these proteins in sciatic nerves and cultured DRGs, precluded the examination of their localisation by immunofluorescence microscopy. To identify the cellular source of Lgi4 and Adam22 function we generated cell type specific knock out mice. Analysis of the myelination phenotypes of these mice revealed that Schwann cell derived Lgi4 and neuronally expressed Adam22 are required for proper myelination. Also, non-Schwann cell derived Lgi4 (possibly from neurons) could still be relevant (further discussed in the next section). To answer this possibility, the DRG culture system could be used. For example, wild type neuron only cultures could be seeded with Schwann cells isolated from Lgi4 null mutant animals. This would enable us to identify whether neuronal Lgi4 is able to initiate myelination. A similar experiment was performed, using Adam22 knock-out neurons and

wild type Schwann cells, to demonstrate that Adam22 on Schwann cells is not sufficient for myelination (Chapter 3).

In line with a paracrine mode of action for Lgi4, we suggest different mechanisms by which Lgi4-Adam22 interaction could control myelination. The first possibility involves Adam-integrin interactions (Figure 2). The disintegrin domain of Adam22 has been implicated in cellular adhesion and control of cell proliferation through integrins [20]. It is therefore possible that Lgi4-Adam22 interaction plays a role in ECM-Schwann cell or axon-Schwann cell communication via integrins. Integrins play important roles in radial sorting of axons and progression of the Schwann cell mesaxon [21]. Also, in Claw paw mice radial sorting of axons is delayed, suggesting an interaction between Lgi4-Adam22 and the integrin pathway [2]. A similar defect is possibly present in Adam22 knock out mice, and deserves further examination. Adam22 has been implicated in cellular adhesion and proliferation [20, 22, 23]. We investigated the role of Adam22 in Schwann cell adhesion and migration in cell culture experiments, where Adam22 coated coverslips were used as a substrate. These experiments did not show a difference compared with controls, in the presence or absence of Lgi4 (unpublished observations). Although, it is possible that either the protein amounts used in these experiments were insufficient, or other proteins in the ECM or on the axons are necessary for such a role.

A second possible role involves extracellular protein interactions to influence membrane localisation of other proteins. Adam10, 17 and 19 are involved in Notch and Nrg1 signalling by proteolytic cleavage of these molecules, or Notch ligands [24, 25]. Both signalling pathways are involved in Schwann cell development and differentiation [26]. The domains responsible for substrate recognition by Adams have not been fully elucidated. If Adam22 contains similar motifs, it may play a role in mediating or regulating the processing of these proteins. It is important to stress here that Adam22 has no proteolytic activity by itself. Alternatively, Adam22 could organise membrane localisation of integrins and/or Nrg1 to potentiate signalling events (Figure 2). Identification of such interactions and the role of Lgi4 in mediating these events would help clarify the mechanism by which Lgi4 and Adam22 control PNS myelination.

Finally, Lgi4 binding to Adam22 could induce intracellular signalling, or regulate protein localisation via the cytoplasmic domain of Adam22. Several isoforms of Adam22 are produced by alternative splicing of the cytoplasmic domain sequences [6], but the significance of these isoforms is unknown. We detected two major isoforms in DRGs: G20 isoform is predominantly expressed in neurons, and G3 isoform is predominantly expressed in Schwann cells. G3 is a shorter isoform that lacks internal sequences in the C-terminal. To identify their significance in myelination, we generated lentiviral constructs with different

isoforms. These will be used to infect DRG cultures isolated from Adam22 knock out mice, and myelination assays will be performed. This will show whether the differences in the cytoplasmic domain are functionally relevant. Like Adam22, Adam11 and 23 are also expressed in Schwann cells and neurons (Chapter 3). The cytoplasmic domains of Adam11 and 23 are very short compared to Adam22. Therefore, it is likely that they lack functional domains contained within Adam22. We will generate constructs with swapped cytoplasmic domains of these three Adams. They will be used together with normal constructs in similar experiments, to identify: a) whether the cytoplasmic domain is necessary, and b) whether a compensation mechanism exists between different Adams.

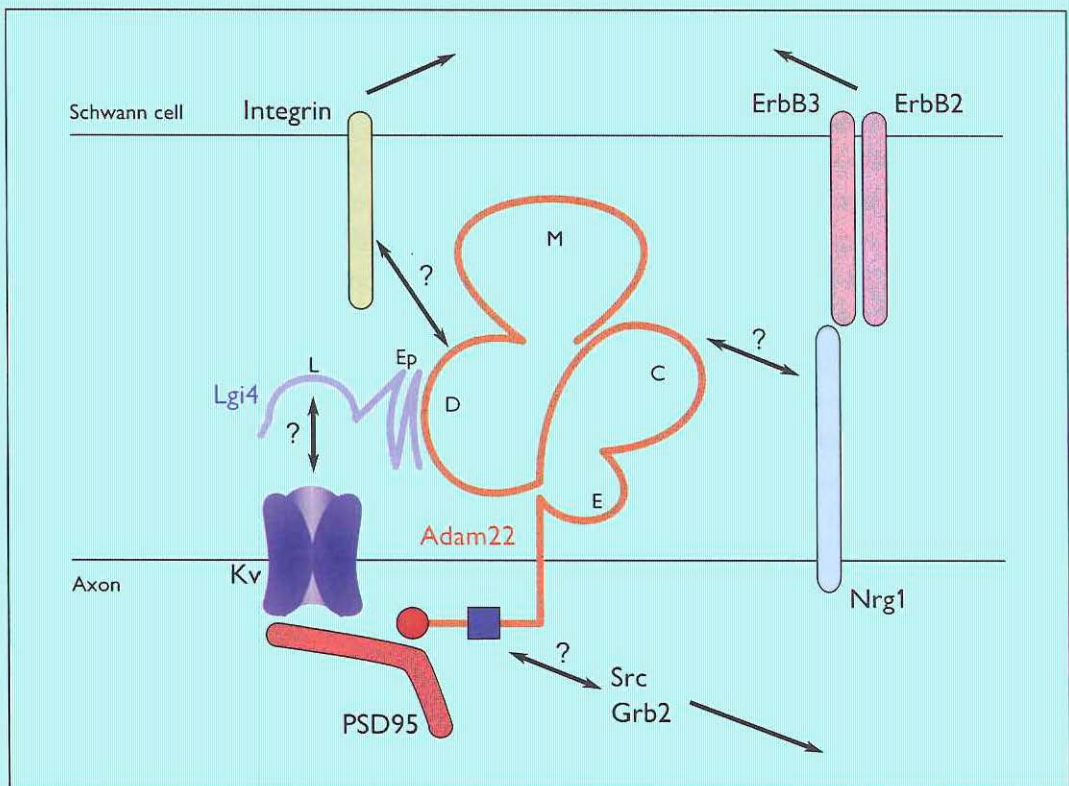


Figure 2. Possible mechanisms by which Lgi4-Adam22 interaction controls PNS development

The Eptempin domain (Ep) of Lgi4 binds to the disintegrin domain (D) of Adam22. Adam22 could interact with integrins (via the disintegrin domain), or Nrg1. Lgi4 could modulate these interactions, as well as potassium channel function. In the cytoplasmic domain, Adam22 could interact with SH3, or PDZ domain containing molecules to initiate signalling cascades (via Src or Grb2), or organise protein localisation at the axonal membrane (via MAGUKs). M: metalloprotease domain; C: cysteine-rich domain; E: EGF-like domain; square: proline-rich sequence (SH3 domain binding); circle: ETS1 sequence (PDZ domain binding). L: LRR domain.

The proline-rich sequences in the cytoplasmic domain of Adams bind SH3 domain containing proteins. These interactions could affect cytoskeletal rearrangements via adaptor molecules, or initiate intracellular signalling. For example, Adam12 C-terminal domain interacts with Src (Proto-oncogene tyrosine-protein kinase) and Grb2 (Growth factor receptor-bound protein 2) [27]. It is possible that these interactions are involved in intracellular signal transduction through Ras. Furthermore, the ETS1 sequence in the C-terminal end of Adam22 is required for PDZ domain interactions [16]. The MAGUK family proteins contain PDZ, SH3 and Guanylate kinase-like domains that mediate protein interactions. These proteins are similar to the *Drosophila* disks large protein (Dlg1), which is an important regulator of neuronal development [28]. MAGUKs function as scaffolding molecules at the cytoplasmic surface of the cell membrane, to regulate protein targeting and signalling. They are especially important in synaptic structure and function. This is due to their interactions with neurotransmitter receptors, ion channels, cell adhesion molecules, and signalling molecules such as GAPs (GTPase activating proteins) and GEFs (Guanine nucleotide exchange factors) [29, 30]. So far, DLG1 (SAP97), DLG2 (PSD93) and DLG4 (PSD95) have been identified to interact with Adam22 in mass spectrometry experiments [16, 31]. These molecules could be an important part of Adam22 function. For example, Adam22 and Lgi4 could play a role in the juxta-paranodal localisation of potassium channels via PSD95 (Figure 2). Although this is probably not necessary for myelination per se, it could be important for proper function of myelinated axons. To identify if Lgi4-Adam22 interaction mediates membrane localisation of potassium channels via PSD95, we performed co-transfection and immunofluorescence experiments on cells. We did not observe a differential expression pattern of potassium channels in the presence or absence of Lgi4 and Adam22. Although plausible, Lgi4-Adam22 interaction may not be necessary for potassium channel clustering. Alternatively, the presence of other molecules such as Caspr2 and TAG1 could be necessary. Nonetheless, analysis of potassium channel localisation in Lgi4 and Adam22 mutant mice will provide insights for such function.

5.3 - Functional implications of Lgi4 and Lgi4^{clp}

While myelination in Claw paw mice is delayed, peripheral nerves of Lgi4 null mice are completely amyelinated. Schwann cell specific or neural crest specific deletion of Lgi4 results in a hypomyelinating phenotype. These observations have several implications for Lgi4 function.

One of these implications is that, Lgi4^{clp} is a hypomorphic allele. We showed that small amounts of Lgi4^{clp} can be detected in the culture medium of transfected cells (Chapter 4). Together with the myelination phenotypes, this suggests that Lgi4^{clp} could be functional

and could interact with Adam22 on the axonal surface. We did not observe an interaction between Adam22 and Lgi4^{clp} in the culture medium. We expect such interactions to occur. It is possible that either the relative protein amounts in the medium or the protein interaction dynamics present experimental difficulties. For example, Lgi4-Adam22 binding could result in a stable complex, whereas Lgi4^{clp}-Adam22 interaction is transient. This could result in a build-up of the signal and delayed myelination *in vivo*, but prevents the detection of an interaction by immunoprecipitation. Experiments are under way to use cell extracts of Lgi4^{clp} transfected cells for immunoprecipitation and binding assays. The use of antibodies to precluster the protein of interest in binding assays increases the avidity of the interactions. Furthermore, the crosslinking step prevents the dissociation of the interactions during washing steps. These could be beneficial for the detection of Lgi4^{clp}-Adam22 interactions on the cell surface.

Other functional implications of our observations involve the cellular source and the properties of Lgi4 protein. Expression of Lgi4 from neurons could still be relevant for myelination. In Dhh-Cre knock outs both sensory and motor neurons, and in Wnt1-Cre knock outs motor neurons still express Lgi4. Although the hypomyelination phenotype is comparable, Wnt1-Cre knock out mice display limb abnormalities and die postnatally, but Dhh-Cre knock outs do not. This could be due to the amounts of protein, or another function of Lgi4 outside myelination. To identify the contribution of neuronal Lgi4, additional experiments on Wnt1-Cre mice are planned to compare myelination of motor versus sensory axons. We identified the role of neuronal Adam22 in such a manner, by analysing the myelination of the dorsal and ventral roots of Wnt1-Cre/Adam22 knock out mice (Chapter 3).

The timing of Lgi4 deletion in the conditional knock out mice also differs. Wnt1-Cre is active before Dhh-Cre. If Lgi4 is already secreted before recombination occurs, depending on the stability of the protein and its interactions, myelination could be initiated albeit in a delayed fashion. This would involve a suboptimal signal, like the clp mice. It is possible to determine protein stability *in vitro*, by adding known amounts of Lgi4 protein onto DRG cultures and analysing its degradation over time by western blot analysis. This experimental setup has the advantage of being similar to an *in vivo* situation. It is also important to add that the Cre recombination events in the conditional knock out mice could be incomplete, or some cells may recombine later than others depending on the expression of the recombinase.

5.4 - Final remarks

As discussed previously, Nrg1 signalling pathway is an important regulator of PNS myelination. It is possible that Lgi4 and Adam22 interaction influences this pathway. Experiments are being performed in our laboratory to determine if Nrg1 signalling is affected in Lgi4 and Adam22 mutant mice. For example, we are looking into the phosphorylation status of ErbB receptors (activation of Nrg1 signalling), MAPK (Schwann cell motility) and Akt (initiation of myelination) in Schwann cells.

The DRG co-culture system is an invaluable tool for the analysis of Schwann cell-neuron interactions and myelination *in vitro*. This system provides a more controlled environment. For example, it enables exogenous addition of proteins in culture media or knocking down their expression, to follow the effects on myelination. We are currently setting up experiments that will utilize viral transduction of Lgi and Adam genes. As previously mentioned, we will use this system to determine the significance of different Adam22 isoforms. It is also possible to test for example, whether overexpression of Lgi4^{clp}, or other Lgi molecules will result in myelination in Lgi4 mutant nerves. This would reveal whether Lgi molecules are functionally equivalent. Furthermore, we can analyze the expression pattern of proteins, such as Nrg1, integrins, potassium channels, or PSD95, by immunofluorescence to further characterize the role of Lgi4 and Adam22.

A major drawback in this research has been the difficulty to detect Lgi4 and Adam22 protein in tissues and cells. The characterisation of Lgi4 and Adam22 expression patterns will be informative for their function. So far, we only detected Lgi4 in the Purkinje cells, and Adam22 in the Pinceaux formations of the basket cells (the contact point of Purkinje cell AIS and basket cell axons). These cells are located in the cerebellum. Purkinje cells are responsible for motor coordination and provide the sole output from cerebellar cortex. They receive excitatory and inhibitory inputs from neurons such as granule cells or stellate cells. Basket cells provide inhibitory inputs [32]. Neurologic disorders such as Ataxia-Telangiectasia and Spinocerebellar ataxia are caused by impaired cerebellar function. These are characterised by degeneration of Purkinje cells and malfunction of other neurons [33, 34]. Because of the ataxia and tremor observed in Lgi4 and Adam22 mutant mice, it is not surprising to see the expression of these proteins in the cerebellum. It is possible that Lgi4-Adam22 interactions modulate synaptic function at the Pinceaux formations. However, Purkinje cell specific deletion of Lgi4 by L7-Cre recombinase does not result in a visible phenotype. This could be due to the timing of recombination, or redundancy between Lgi proteins. It would be interesting to further study the role of Lgis and Adams in the cerebellum.

Identification of protein interaction partners is an important step for the

characterisation of a protein's function. The studies of Schulte et al. and Fukata et al. not only suggested that an interaction between Lgi4 and Adam22 exists, but also provided clues about the function of these proteins. Therefore, the discovery of other proteins that interact with Lgi4 and Adam22 will be an important part of future research. Mass spectrometry analysis could be used to this end. These experiments will be performed by overexpressing tagged proteins to pull down interactors from tissues such as DRGs or sciatic nerves. The amount of tissue material and the specificity of the pull down methods will be a determinant in the results obtained. This method could be used to identify Adam22 isoform specific interactors, by using G3 and G20 cytoplasmic domain constructs for protein expression. In a similar fashion specific domain or intact protein constructs could be used to identify partners of Lgi4 and Adam22. Another possible method to identify partners is to utilize cDNA libraries for protein expression and binding assays on transfected cells.

The future of this project is indeed very exciting, and full with possibilities. It will be interesting to see how the Lgi-Adam story develops, and which developmental or functional mechanisms are affected by their action in the nervous system and elsewhere.

References:

1. Henry, E.W., E.M. Eicher, and R.L. Sidman, *The mouse mutation Claw paw: forelimb deformity and delayed myelination throughout the peripheral nervous system*. J Hered, 1991. **82**(4): p. 287-94.
2. Darbas, A., et al., *Cell autonomy of the mouse Claw paw mutation*. Dev Biol, 2004. **272**(2): p. 470-82.
3. Chen, Z.L. and S. Strickland, *Laminin gamma1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve*. J Cell Biol, 2003. **163**(4): p. 889-99.
4. Feltri, M.L., et al., *Conditional disruption of beta 1 integrin in Schwann cells impedes interactions with axons*. J Cell Biol, 2002. **156**(1): p. 199-209.
5. Garratt, A.N., et al., *A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool*. J Cell Biol, 2000. **148**(5): p. 1035-46.
6. Sagane, K., et al., *Ataxia and peripheral nerve hypomyelination in ADAM22-deficient mice*. BMC Neurosci, 2005. **6**: p. 33.
7. Bermingham, J.R., Jr., et al., *The Claw paw mutation reveals a role for Lgi4 in peripheral nerve development*. Nat Neurosci, 2006. **9**(1): p. 76-84.
8. Senechal, K.R., C. Thaller, and J.L. Noebels, *ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy*. Hum Mol Genet, 2005. **14**(12): p. 1613-20.
9. Gu, W., et al., *The LGI1 gene involved in lateral temporal lobe epilepsy belongs to a new subfamily of leucine-rich repeat proteins*. FEBS Lett, 2002. **519**(1-3): p. 71-6.
10. Scheel, H., S. Tomiuk, and K. Hofmann, *A common protein interaction domain links two recently identified epilepsy genes*. Hum Mol Genet, 2002. **11**(15): p. 1757-62.
11. Staub, E., et al., *The novel EPTP repeat defines a superfamily of proteins implicated in epileptic disorders*. Trends Biochem Sci, 2002. **27**(9): p. 441-4.
12. Bella, J., et al., *The leucine-rich repeat structure*. Cell Mol Life Sci, 2008. **65**(15): p. 2307-33.
13. Krex, D., et al., *Physical and functional characterization of the human LGI1 gene and its possible role in glioma development*. Acta Neuropathol, 2002. **103**(3): p. 255-66.
14. Roux, P.P. and P.A. Barker, *Neurotrophin signaling through the p75 neurotrophin receptor*. Prog Neurobiol, 2002. **67**(3): p. 203-33.
15. Wong, K., et al., *Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes*. Curr Opin Genet Dev, 2002. **12**(5): p. 583-91.
16. Fukata, Y., et al., *Epilepsy-related ligand/receptor complex LGI1 and ADAM22 regulate synaptic transmission*. Science, 2006. **313**(5794): p. 1792-5.
17. Nakayama, J., et al., *A nonsense mutation of the MASS1 gene in a family with febrile and afebrile seizures*. Ann Neurol, 2002. **52**(5): p. 654-7.
18. Weston, M.D., et al., *Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II*. Am J Hum Genet, 2004. **74**(2): p. 357-66.
19. Schulte, U., et al., *The epilepsy-linked Lgi1 protein assembles into presynaptic Kv1 channels and inhibits inactivation by Kvbeta1*. Neuron, 2006. **49**(5): p. 697-706.
20. D'Abaco, G.M., et al., *ADAM22, expressed in normal brain but not in high-grade gliomas, inhibits cellular proliferation via the disintegrin domain*. Neurosurgery, 2006.

- 58**(1): p. 179-86; discussion 179-86.
21. Nodari, A., et al., *Beta1 integrin activates Rac1 in Schwann cells to generate radial lamellae during axonal sorting and myelination*. *J Cell Biol*, 2007. **177**(6): p. 1063-75.
 22. Zhu, P., et al., *The interaction between ADAM 22 and 14-3-3zeta: regulation of cell adhesion and spreading*. *Biochem Biophys Res Commun*, 2003. **301**(4): p. 991-9.
 23. Zhu, P., et al., *ADAM22 plays an important role in cell adhesion and spreading with the assistance of 14-3-3*. *Biochem Biophys Res Commun*, 2005. **331**(4): p. 938-46.
 24. Novak, U., *ADAM proteins in the brain*. *J Clin Neurosci*, 2004. **11**(3): p. 227-35.
 25. Yang, P., K.A. Baker, and T. Hagg, *The ADAMs family: coordinators of nervous system development, plasticity and repair*. *Prog Neurobiol*, 2006. **79**(2): p. 73-94.
 26. Jessen, K.R. and R. Mirsky, *The origin and development of glial cells in peripheral nerves*. *Nat Rev Neurosci*, 2005. **6**(9): p. 671-82.
 27. Suzuki, A., et al., *Meltrin alpha cytoplasmic domain interacts with SH3 domains of Src and Grb2 and is phosphorylated by v-Src*. *Oncogene*, 2000. **19**(51): p. 5842-50.
 28. Mendoza, C., et al., *Novel isoforms of Dlg are fundamental for neuronal development in Drosophila*. *J Neurosci*, 2003. **23**(6): p. 2093-101.
 29. Fujita, A. and Y. Kurachi, *SAP family proteins*. *Biochem Biophys Res Commun*, 2000. **269**(1): p. 1-6.
 30. Garner, C.C., J. Nash, and R.L. Haganir, *PDZ domains in synapse assembly and signalling*. *Trends Cell Biol*, 2000. **10**(7): p. 274-80.
 31. Sagane, K., Y. Ishihama, and H. Sugimoto, *LGI1 and LGI4 bind to ADAM22, ADAM23 and ADAM11*. *Int J Biol Sci*, 2008. **4**(6): p. 387-96.
 32. Sotelo, C., *Development of "Pinceaux" formations and dendritic translocation of climbing fibers during the acquisition of the balance between glutamatergic and gamma-aminobutyric acidergic inputs in developing Purkinje cells*. *J Comp Neurol*, 2008. **506**(2): p. 240-62.
 33. Lavin, M.F., et al., *Current and potential therapeutic strategies for the treatment of ataxia-telangiectasia*. *Br Med Bull*, 2007. **81-82**: p. 129-47.
 34. Koeppen, A.H., *The pathogenesis of spinocerebellar ataxia*. *Cerebellum*, 2005. **4**(1): p. 62-73.

Summary

Glial cells support neuronal function to gather and process information to control an animal's general behaviour. One important function of glial cells is to produce and maintain myelin, a fatty insulating sheath around axons. Myelinated axons exhibit nerve conduction velocities that are one to two orders of magnitude larger than unmyelinated axons of the same diameter. This enables faster responses to stimuli, and is advantageous for the survival of the organism. Debilitating effects of diseases such as multiple sclerosis or Charcot-Marie-Tooth, which result from problems in the myelin sheath, demonstrate the importance of myelination for proper function of the nervous system.

In the peripheral nervous system, the myelin sheath is produced by glial cells called Schwann cells named after Theodor Schwann (1810-1882), one of the founders of the cell theory in the 19th century. Schwann cells develop in close association with the outgrowing axons of neurons, and the continuous molecular crosstalk between these two cell types regulates Schwann cell development, differentiation and myelination. Schwann cells produce a basal lamina, and the interaction between Schwann cells and their basal lamina is of prime importance for axonal sorting and myelin formation. Furthermore, Schwann cells dictate the formation of specialisations in the axonal membrane that result in enrichment of voltage gated sodium and potassium channels at the nodal and juxtaparanodal regions. Thus, the development of the peripheral nerve tissue results from reciprocal interactions between Schwann cells and their associated axons.

To gain further insight into the development and myelination of peripheral nerves, we studied the natural mouse mutant claw paw. In claw paw mice, myelination of the peripheral nerves is delayed, suggesting that the normal function of the mutated gene is necessary for proper development and function of the peripheral nervous system. This thesis describes the identification of a mutation in the *Lgi4* (Leucine-rich glioma inactivated 4) gene, which causes the claw paw phenotype, and the initial characterisation of the *Lgi4* protein. The mutation in claw paw mice severely reduces the secretion of the *Lgi4* protein. By *in vitro* Schwann cell-neuron co-culture studies, we showed that secretion of *Lgi4* is necessary for peripheral nerve myelination and that Schwann cells are a major source of *Lgi4*. Ablation of *Lgi4* in all tissues results in a peripheral nerve phenotype in which Schwann cells are arrested at the premyelinating stage with no evidence of myelin formation. This phenotype is much more severe than that observed in the nerves of claw paw mice. We conclude that the claw paw form of the *Lgi4* gene represents a hypomorphic allele of *Lgi4*, and that the reduced secretion of claw paw *Lgi4* protein is responsible for the milder hypomyelination phenotype.

Secreted Lgi4 interacts with the transmembrane protein Adam22. Adam22 belongs to the large family of transmembrane proteins known as 'A Disintegrin And Metalloprotease' proteins. Both Lgi4 and Adam22 are expressed in Schwann cells and neurons during embryonic and postnatal development. Using tissue specific genetic ablation studies, we showed that expression of Lgi4 from Schwann cells and Adam22 from neurons is necessary for myelination. Therefore, we suggest that Lgi4-Adam22 interaction represents a novel signalling axis from Schwann cells to axons during peripheral nerve development.

It is not yet clear how Lgi4-Adam22 interaction controls myelination, and how this signal is relayed back to Schwann cells to control their differentiation. It is possible that they function directly to regulate Schwann cell proliferation, migration or adhesion. It is also possible that they function in a more indirect manner, and are required for proper localisation and function of other proteins, or for signalling events controlling axonal function. Further studies will focus on the downstream events of Lgi4-Adam22 interaction to identify their role in peripheral nervous system development and myelination. This will greatly enhance our understanding of peripheral nerve development and function, and might open up new possibilities for therapeutic approaches towards peripheral nerve injuries and diseases of the myelin sheath.

Nederlandse samenvatting

Glia cellen ondersteunen neuronen structureel en functioneel en spelen een essentiële rol in de informatie verwerking die ten grondslag ligt aan het gedrag van dieren, inclusief de mens. Een belangrijke functie van glia cellen is de aanleg en onderhoud van een isolerende laag rondom een groot aantal axonen in het zenuwstelsel. Deze zogenaamde myeline laag bestaat uit een glia celmembraan dat vele malen om het axon gewonden is en gespecialiseerde myeline eiwitten bevat. Gemyelineerde axonen geleiden actiepotentialen 10-100 maal sneller dan niet gemyelineerde axonen van gelijke diameter. De snellere zenuwgeleiding en compacte structuur van gemyelineerde axonen hebben ongetwijfeld een belangrijke rol gespeeld bij de evolutie van grotere organismen. Met de evolutionaire voordelen van myeline kwamen ook de nadelen aan het licht. Destabilisatie of beschadiging van de myelineschede lijden tot een breed scala aan neurologische ziekten zoals multiple sclerose en de erfelijke sensorische en motorische neuropathieën. Deze ziekten onderstrepen het belang van de myelineschede voor het normaal functioneren van het zenuwstelsel.

In het perifeer zenuwstelsel wordt de myeline laag gemaakt door glia cellen die Schwann cellen worden genoemd naar Theodor Schwann, een van de grondleggers van de celtheorie in de 19^{de} eeuw. Schwann cellen ontwikkelen zich gedurende de embryogenese in nauw contact met de uitgroeiende axonen. De voortdurende moleculaire communicatie over en weer tussen deze cellen ligt ten grondslag aan de ontwikkeling, differentiatie en myelinisatie van perifere zenuwen. Schwann cellen produceren een basaal membraan en de communicatie over en weer tussen de Schwann cel en zijn basaal membraan is van groot belang voor het uit sorteren van individuele axonen en myeline vorming. Verder dicteren de Schwann cellen een aantal structurele specialisaties in het axonale membraan die resulteren in een enorme verrijking van natrium en kalium kanalen in en rond de knoop van Ranvier. Hoewel een aantal van de moleculen die betrokken zijn bij bovengenoemde processen bekend zijn, is er nog veel onduidelijkheid over de moleculaire mechanismen die deze processen beheersen.

Om meer inzicht te verkrijgen in de ontwikkeling en myelinisatie van het perifeer zenuwweefsel en de moleculaire mechanismen die hieraan ten grondslag liggen, hebben wij de natuurlijke muis mutant *claw paw* bestudeerd. In *claw paw* muizen is de myelinisatie van de perifere zenuwen ernstig vertraagd, wat er op duidt dat het in *claw paw* muizen gemuteerde gen een belangrijke rol speelt in het myelinisatie proces en de functionele ontwikkeling van de zenuw. In dit proefschrift worden experimenten beschreven die geleid hebben tot de identificatie van het Lgi4 (Leucine-rich glioma inactivated 4) gen dat

gemuteerd is in claw paw muizen, gevolgd door een initiële karakterisering van het Lgi4 eiwit en de identificatie van een receptor voor het Lgi4 eiwit. De claw paw mutatie leidt tot een zeer sterke reductie van de secretie van het Lgi4 eiwit. Met behulp van Schwann cel-sensorische neuronen co-cultures hebben we laten zien dat secretie van Lgi4 belangrijk is voor myelinisatie en dat Schwann cellen een belangrijke bron van Lgi4 zijn. Volledige uitschakeling van het Lgi4 in het genoom van de muis leidt tot een volledige blokkering van het myelinisatie proces, een fenotype dat ernstiger is dan wordt waargenomen in claw paw muizen. We concluderen dat de claw paw vorm van het Lgi4 gen een hypomorf allel vertegenwoordigt en dat de zeer geringe secretie van het gemuteerde eiwit verantwoordelijk is voor het mildere myelinisatie fenotype.

Het uitgescheiden Lgi4 eiwit bindt aan het extracellulaire domein van het transmembraan eiwit Adam22. Adam22 behoort tot de grote familie van Adam receptoren wat staat voor 'A Disintegrin And Metalloprotease'. Lgi4 en Adam22 genen komen in zowel neuronen als Schwann cellen tot expressie gedurende de embryonale en postnatale ontwikkeling. Door middel van celtype specifieke uitschakeling van het Adam22 of het Lgi4 gen hebben we laten zien dat Lgi4 voornamelijk geproduceerd wordt door Schwann cellen, wat de conclusie uit eerdere celweek experimenten bevestigt, en dat Adam22 expressie in neuronen noodzakelijk is voor myelinisatie van die neuronen. We stellen voor dat de binding van Lgi4 aan het extracellulaire domein van axonaal Adam22 een moleculaire schakelaar is die de normale associatie en myelinisatie van Schwann cellen beheerst.

Het is op dit moment nog onduidelijk hoe Lgi4 binding aan Adam22 leidt tot myelinisatie en hoe deze gebeurtenis de differentiatie van de Schwann cel stuurt. Het is mogelijk dat Lgi4 binding aan Adam22 direct de adhesie, migratie of proliferatie van Schwann cellen reguleert. Ook is het mogelijk dat Lgi4 door Adam22 indirect andere signaalmoleculen die myelinisatie sturen in stelling brengt. Toekomstig onderzoek zal zich richten op deze vragen. Een beter inzicht in de moleculaire mechanismen van Schwann cel differentiatie en myelinisatie, en de rol die Lgi4 en Adam22 hierin spelen, zal hopelijk in de toekomst leiden tot nieuwe mogelijkheden voor therapieën die er op gericht zijn de remyelinisatie na axonale schade te bevorderen en de schadelijke effecten van demyeliniserende neuropathieën te verminderen.

Curriculum Vitae

Personal Details

Name: Ekim Özkaynak
Birth date: 19 January 1983
Birthplace: Ankara, Turkey

Education

2004-2009: PhD student
Department of Cell Biology and Genetics, Erasmus University Medical Centre, Rotterdam, The Netherlands

2000-2004: Bachelor of Science
Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

1997-2000: High School
Antalya Anadolu Lisesi, Maths and Sciences Division, Antalya, Turkey

Research experience

2004-2009: PhD project
ErasmusMC, Department of Cell Biology and Genetics
Prof. Dr. Frank G. Grosveld (promoter), Prof. Dr. Ir. Dies N. Meijer (promoter, supervisor); "Study of Claw Paw Mice to Identify Novel Mechanisms Controlling Peripheral Nerve Development"

2003-2004: Undergraduate senior year project
Bilkent University, Molecular Biology and Genetics
Prof. Dr. Tayfun Özçelik, MD (principal investigator), Akin Sevinç MSc. (supervisor); "Analysis of X Chromosome Inactivation Status in Breast Cancer Patients"

2003: Internship
ErasmusMC, Department of Cell Biology and Genetics
Prof. Dr. Frank G. Grosveld (principal investigator), Dr. Arnd Hostert (supervisor); "Putative Interactions of LDB1 Protein on Mouse beta-Globin Locus"

List of Publications

Bermingham J.R. Jr, Shearin H., Pennington J., O'Moore J., Jaegle M., Driegen S., van Zon A., Darbas A., Ozkaynak E., Ryu E.J., Milbrandt J., Meijer D. The claw paw mutation reveals a role for Lgi4 in peripheral nerve development. *Nat Neurosci.* 2006; 9(1):76-84.

Ozkaynak E., Abello G., Jaegle M., van Berge L., Hamer D., Kegel L., Driegen S., Sagane K., Bermingham J., Meijer D. Adam22 is a neuronal receptor for Lgi4 mediated Schwann cell signaling. Submitted to *Nat Neurosci.*

PhD Portfolio Summary

Name PhD Student: Ekim Özkaynak ErasmusMC department: Cell Biology and Genetics Research School: Medisch-Genetisch Centrum Zuid-West Nederland	PhD period: November 2004-November 2009 Promotors: Prof.dr. F.G. Grosveld, Prof.dr.ir. D.N. Meijer Supervisor: Prof.dr.ir. D.N. Meijer
1. PhD Training	Year
General academic skills Biomedical English Writing and Communication Contemporary Research Topics	2006 2005
Research skills Working with test animals Radiation safety course Safe laboratory techniques	2005 2005 2007
In-depth courses (e.g. Research school, Medical Training) Experimental Approach to Molecular and Cell Biology Transgenesis and Gene Targeting In vivo Imaging: From Molecule to Organism From Development to Disease (Embryos, Genes and Developmental Defects) Basic and Translational Oncology Applied Bioinformatics "Finding Your Way in Biological Information" Signal Transduction and Control of Cellular Proliferation Epigenetic Regulation (Epigenetic Mechanisms in Health and Disease) Genome Maintenance and Cancer	2005 2005 2005 2005 2006 2006 2007 2008 2009
Presentations Winterschool Kleinwalsertal 5 th Dutch Endo-Neuro-Psycho Meeting, Doorwerth 13 th MGC-CRUK PhD Workshop, Oxford 1 st European Life Science for Young Scientists Conference, Twente	2005-2008 2006 2006 2007
International conferences 8th European Meeting on Glial Cells in Health and Disease, London, UK (poster contribution)	2007
Seminars and workshops MGC PhD workshops (poster contribution) European Career Event in Molecular Cell Biology and Medicine, Porto, Portugal 1 st and 2 nd SCDD symposium BSIK, Amsterdam	2005, 2006 2008 2008 2007, 2008
Teaching Activities Supervision of MSc students Developmental Biology elective course MSc program (oral presentation on PhD project)	2006-2008 2007

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"Bana ayrılan yaşam alanını protesto ediyorum." Derin Esmer

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