Executive Summary:

Myelin disorders present a significant health burden and are an important cause of disability. Myelin is laid down in segments around a majority of axons and contributes to the normal physiology of the nervous system. Schwann cells and oligodendroglia cells are the myelin-forming cells in the peripheral and central nervous system respectively and the formation and maintenance of myelin results from a continuous molecular dialogue with the neuronal axon. Despite the importance of myelin for the normal function of the nervous system, relatively little is known about the molecular mechanisms that underlie axon-glia communication in health and disease. Consequently, the therapeutic options for these disorders are very limited. The NGIDD project aimed to bridge the gap in our knowledge and identify new drug targets to stimulate the regenerative capacity of the nervous system.

To realize this overall aim, the members of the NGIDD consortium addressed the following questions related to; 1 the initial engagement of glial cells with axons, 2 the control of myelin formation and maintenance, 3 the functional organization of the axo-glial unit and 4, the pathology of de- and dismyelination.

Over the last four years, the NGIDD consortium has made major steps towards answering these questions. NGIDD has identified a completely novel molecular interaction consisting of the Schwann cell derived protein Lgi4 and its axonal receptor Adam22. NGIDD has elucidated the role of integrins, focal adhesion kinase and N-WASP in myelin formation and axonal sorting. Furthermore NGIDD has generated a range of mouse models of peripheral neuropathies and has provided evidence that the drug rapamycin is a beneficial modulator of myelin abnormalities in these mouse models. Additionally, NGIDD has generated and validated a cell-based assay to screen drugs that ameliorate the pathogenic effects of maladaptive unfolding protein responses that plays a role in a number of inherited neuropathies.

Thus, scientific and technological progress made by the NGIDD consortium has opened new avenues of inquiry to further our understanding of the molecular and cellular interactions that shape our myelinated nerves and govern their physiology. Importantly, this research has already yielded new lead compounds and preclinical models to explore new therapeutic approaches to combat the devastating neurological effects in de- and dismyelinating diseases in humans.

The NGIDD consortium united scientists from five different countries with different scientific expertise into a common cause. The consortium has contributed to the exchange of ideas, reagents and personnel, has trained a large number of scientist and has participated in scientific and civic conferences to disseminate the novel findings of the consortium, thus successfully contributing to the larger European Research Area.

Project Context and Objectives:

Although for many years it has been appreciated that myelinating glial cells and their underlying axons are engaged in a complex dialogue which is crucial for the normal function of both cell types, the identity of the molecules that mediate this interaction remained almost completely unknown. Studies by members of the consortium contributed significantly to our understanding how myelinating glial cells communicate with the axons they ensheath. The studies carried out in this collaborative effort provide important new insights on the molecular mechanisms that control myelination and the functional organization of the axon. Uncovering the mechanisms by which myelinating glial cells recognize and ensheath their associated axons is highly significant given the high prevalence of human diseases resulting from demyelination or alteration in myelin, such as multiple sclerosis and a number of inherited neuropathies.

Many developmental mechanisms are recapitulated after injury and pathology in these diseases, and can be exploited to promote regeneration, remyelination and re-establishment of functional axo-glial units. Furthermore, understanding the molecular basis for axo-glial contact is of particular interest as it is becoming increasingly evident that although in many of these disease the first pathological event is the disruption of myelin, secondary changes in the axon, which eventually lead to axonal loss is the main cause of clinical impairment. In summary, the present collaboration aimed to uncover the molecular mechanisms that govern the Schwann cell-axon engagement, myelination, and the establishment of distinct structural domains, all of which are processes that are crucial for the generation of a fully functional myelinated nerve. Increased knowledge about these mechanisms will prove useful in the design of therapeutic strategies for many neuropathies and demyelinating disorders.

The collaborative project NGIDD consisted of seven European research groups and one biotech company Axxam. The specific expertise of the individual members was mobilized to address four key objectives. These key objectives aligned with the four workpackages in this collaborative effort under one management (workpackage 5) to realize the main objective of this project; to push our understanding of myelination and myelination-related diseases beyond the state of the art and to identify new targets for pharmaceutical intervention to aid the regenerative capacity of the nervous system

Initial events leading up to myelination

The glial cells and neurons of the peripheral nervous system are in close apposition and interdependent from the moment they are generated from the neural crest during embryonic development. Schwann cell precursors proliferate and migrate along the outgrowing axonal bundles to give rise to the mature axon-glia unit that characterize the fully developed and functional nervous system.

One crucial step in achieving correct axo-glia relationships in peripheral nerve development is 'radial axonal sorting'. This developmental step allows Schwann cells to achieve a 1:1 relationship with large axons destined to be myelinated. Radial sorting is a pre-requisite for myelination, and is impaired in a common form of hereditary human neuropathy that accompanies congenital muscular dystrophy 1A. Sorting processes are also recapitulated during nerve repair. Thus, to clarify the mechanisms

governing axonal sorting is of utmost importance not only to understand normal nerve development, but also to promote therapeutic intervention in the context of hereditary diseases and nerve repair. Therefore, an important question addressed in this context is how molecules such as extracellular matrix laminins and their integrin receptors, together with Focal Adhesion Kinase and the small GTPase Rac1 enable radial sorting of axons.

Many other players in radial sorting remain to be identified. Work on the natural mouse mutant Claw paw (clp) has identified one such player. Delayed axonal sorting and abnormal myelination of peripheral nerve fibers characterize Claw paw mice. It was found that a mutation in the Lgi4 gene causes the defects in nerve development in these mice. Lgi4 is a member of a small family of genes that encode secreted Leucine-rich proteins of unknown function. Lgi1, the founding member of this family, is frequently mutated in autosomal dominant partial epilepsy with auditory features (ADPEAF). How Lgi4 contributes to sorting and myelination is not known. An important insight into the possible mechanism of action of Lgi4 was obtained during this project through the identification of the receptor for Lgi4.

Control of myelin formation

It has been known for some time that, in postnatal development, axons themselves induce the associated Schwann cells to make myelin. However, the nature of this axonal signal was unclear until recently when a member lab of the consortium showed that neuregulin-1 (NRG1) type III is a critical growth factor of axons regulating myelination by Schwann cells in a quantitative fashion, thereby optimizing myelin sheath thickness. Binding of NRG1 to glial ErbB2 and ErbB3 receptors (via the EGF-like signaling domain common to all NRG1 isoforms) is likely to activate multiple signaling pathways, but it is unknown how these are tied into the myelination program. One attractive second messenger for myelination, which is activated by NRG1-ErbB signaling, is the PI3K pathway with the generation of phosphatidyl-inositol (3,4,5)-triphosphate (PIP3) as a signaling lipid. Activation of PI3K has been shown to modulate Schwann cell growth and myelination in vitro, but the role of PIP3 in myelination in vivo was still unclear. PIP3 is an activator of Akt1 and the mTOR pathway in RNA transcription and translation, but is also a subcellular activator of rac/cdc42-dependent process formation. To address the function of glial PI3K and mTOR signalling in vivo conditional mouse mutants of the Pten and mTOR gene will be generated.

The requirement for membrane-bound NRG1 in the PNS indicates that Schwann cell-axon contact is a prerequisite for myelination. A participant of this consortium has identified potential candidate cell adhesion molecules that mediate such axon-Schwann cell contact. These Nectin-like (Necl)/SynCAM cell adhesion molecules are located at the axon-glia interface along the internodes where they mediate Schwann cell-nerve interaction necessary for myelination. The role of these molecules and their interaction with NRG1 signals will be investigated.

The functional organization of myelinated axons

Myelinating glial cells and their underlying axons are engaged in a complex dialogue that is crucial for the normal physiological function of both cell types. An important consequence of this interaction is the accumulation of voltage-gated Na+ channels at short, regularly spaced interruptions in the myelin sheath known as the nodes of Ranvier. At the nodes, these channels are found in a multiprotein

complex that includes cell adhesion molecules and cytoplasmic adaptor proteins. In peripheral myelinated axons Na+ channel clustering is controlled by a direct contact with Schwann cell microvilli that emanate from the outer collar of the cell. As demonstrated by participants 3 and 4, Schwann cell-specific ablation of dystroglycan and to a lesser extent of laminin 1 cause disruption of microvillar organization and reduction in nodal Na+ channel clustering, suggesting that the microvilli play a direct role in node assembly. As shown by participants 2 and 4, Na+ channels are recruited to clusters of two cell adhesion molecules Nfasc186 and NrCAM that were first positioned by binding to gliomedin present on the glial. These observations led us to propose that the focal presentation of gliomedin to the axon during myelination causes the initial clustering of the axonodal CAMs into higher-ordered oligomers, which facilitates the recruitment of ankyrin G and Na+ channels. The participation of the groups of participant 2, 3 and 4 in this framework allows a unique opportunity to examine and advance our current knowledge of how Na+ channels are clustered in PNS nodes.

The nodes of Ranvier are bordered at both sides by specialised junctions that are formed between the axon and the paranodal loops of myelinating glia. These paranodal junctions (PNJ) provide attachment of the myelin sheath to the axon, separate the electrical activity at the node of Ranvier from the internodal region under the compact myelin sheath, and serve as a boundary that limits the lateral diffusion of membrane components. It was shown previously by participants 2 and 4 that the generation of the PNJ depends on the presence of three cell adhesion molecules the axonal Caspr and contactin, as well as the glial isoform of neurofascin Nfasc155. These studies led to the notion that the organization of myelinated axons requires the presence of barriers that are present at the PNJ, and restrict the diffusion of axonal proteins. Remarkably, the PNJ shares morphological, functional and molecular similarities with invertebrate septate junction (SpJ), which is crucial for the formation of the blood-brain barrier that is formed by the subperineural glial cells. Similarly to the PNJ, SpJ require the fly homologues of Caspr (i.e., NrxIV), contactin (i.e., Dcon) and Nfasc155 (i.e., neuroglian). More recent data from participant 7 identified NrxIV as a target for the splicing factor crooked neck, which controls glial differentiation in Drosophila, and led to the identification of additional genes required for SpJ formation. The molecular and functional similarity between the PNJ and SpJ, coupled with the sophisticated genetic and histological tools in the fly, provide a unique opportunity to search for additional structural and regulatory components of the paranodal junction in vertebrates.

Disturbed interactions between glial cells and neurons result in neuropathy

CMT hereditary demyelinating neuropathies are prevalent and cause significant disability, but there is no treatment.

Multiple animal models of CMT neuropathies have been produced and validated as good functional and pathological models of the corresponding human diseases Pathogenetic mechanisms are beginning to be identified in these models, and preliminary studies suggest treatment strategies for CMT1A, due to peripheral myelin protein 22kD (PMP22) overexpression.

Whereas CMT1A accounts for approximately one half of hereditary neuropathies, other demyelinating neuropathies have been defined by mutations in 23 distinct genes. Many are dominantly inherited, and produce mutant proteins, suggesting toxic mechanisms. There are two fundamental questions: What range of toxic mechanisms produce such widely variable phenotypes

and how do they interfere with myelination? Second, presuming a broad array of mutation-specific mechanisms, are there common downstream mechanisms amenable to therapeutic intervention?

We hypothesize that in neuropathy, 'toxic' pathogenetic mechanisms in Schwann cells may target the axonal signals necessary for formation or maintenance of myelin. More specifically, activated protein quality control in Schwann cells, or inefficient proteasome function, may perturb the cell intrinsic pathways normally activated by axonal signals during development, or inappropriately reactivate them in adult myelin forming Schwann cells. We will test this hypothesis by:

1) providing further expression analysis of activation of these signals in our collective animal models;

2) Genetically or pharmacologically reversing superactivation of these signals in CMT1A and CMT1B animals to provide direct evidence for their pathogenetic role;

3) Cooperating with our SME partner 8 to develop a high content cell-based assay to identify small molecules that can reverse the inappropriate trafficking and activated protein quality control associated with CMT mutant proteins.

These aims represent an emerging direction in pathogenesis of hereditary neuropathy that exploits our collective animal models and complementary expertise. We expect a major advance beyond the current knowledge of pathogenesis in neuropathy and we expect to produce new animal models, information and small molecule tools that will inform pharmaceutical strategies to intervene where toxicity perturbs axoglial signals in neuropathy.

The S&T results of the NGIDD project has been described in period reports, scientific publications, conference contributions, contributions in civic debate, through our website and through a brochure.

What follows here is a description of the major achievements per workpackage.

WP1: Engagement: Glial cells and neurons

i. To identify the role of the newly identified Lgi4 in early glia-axonal contact.

ii. To determine the molecular mechanisms underlying radial axonal sorting.

WP2: Control of myelin formation and maintenance

i. Dissecting axon-glia interactions essential for myelination.

ii. Dissecting regulatory mechanisms downstream of Neuregulin-1/ErbB signalling.

iii. Understanding myelin outfoldings in mouse models of neurological disease.

WP3: Functional organization of the axo-glial unit

i. To determine how myelinating glial cells organize the axonal membrane they ensheath.

ii. To determine the molecular mechanisms underlying Na+ channel clustering at nodes.

iii. To identify novel paranodal axoglial junction proteins.

WP4: Disturbed axo-glial signaling and nerve pathology

i. Study axo-glia signals in our collective animal models of neuropathy.

ii. Establish a pathogenic role for these signals in our neuropathy mouse models.

iii. Develop a cell-based assay to identify small molecules that reverse or ameliorate the pathogenic mechanism of CMT mutant proteins.

Project Results:

WP1: Engagement: Glial cells and neurons

Activity 1.1

Previous studies suggest that proteins of the Adams family are receptors for Lgi proteins: Lgi1 and Adam22 co-precipitate from brain membrane preparations using PSD95 or Kv1.1 antibodies. Lgi1/Lgi4 bind to cells that ectopically express Adam22/23 and deletion of Adam22 presents a similar peripheral nerve phenotype to that of claw paw animals. We analysed expression of Lgi and Adam family members in the developing nerve and found that all members of the Lgi family are expressed in both neurons and Schwann cells. Out of the wide range of Adam family members that are expressed in peripheral nerve, we focused on the Adams closely related to Adam22: Adam23 and Adam11. All three genes are expressed in Schwann cells and neurons. To test whether binding of Lgi4 to Adam22 is direct and/or requires the presence of additional molecules at the cell membrane, we utilized fusion proteins that consist of the ectodomain of Adam22 (either normal Adam22 or Adam22 with a D509N mutation in the disintegrin-like domain that is analogous to the ju160 allele of C.elegans Adm1/Unc-71, which causes axon guidance and sex myoblast migration defects) and the human IgG Fc moiety. Adam22-Fc protein was found to efficiently precipitate Lgi4 from conditioned medium. In contrast, no Lgi4 protein was found precipitated with the Adam22-Fc protein containing the D509N mutation. These results indicate that binding of Lgi4 to Adam22 does not require additional membrane associated receptors and that binding depends on the integrin-binding domain of Adam22.

Additionally, we found that Lgi4 binds to Adam11, Adam22 and Adam23 when expressed on the cell membrane of HeLa cells. Lgi2 and Lgi3 bind to Adam11 and Adam22/23 respectively. Whether Adam11 and/or Adam23 contribute to Lgi4 function as alternative Lgi4 receptors in the peripheral nerve is unclear although a close comparison between the claw paw and Adam22 mutant nerve phenotypes suggest that this might be the case.

Lgi4 binds to Adam22 through its EPTP domain. There is reason to believe that the LRR domain of Lgi4 is involved in interactions with other proteins. See below

- -Role of Lgi4 Adam interactions in nerve development

Adam22 is expressed by neurons as well as SCs 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 Adam22neo/neo embryos and seeded these cultures with rat SCs. Rat SCs readily ensheath and myelinate Adam22+/neo neurons, whereas Adam22neo/neo neurons are not myelinated. These results indicate that Adam22 function is required in the neuron, but do not exclude an additional function in SCs.

To further explore a possible SC-autonomous function of Adam22 and to corroborate its neuronal function in nerve development, we generated a conditional Adam22 allele and bred it to homozygosity in the presence of the Wnt1Cre transgene, thereby deleting Adam22 in all neural crest derivatives, including sensory neurons, endoneurial fibroblasts and SCs, but not in spinal motor neurons. The sciatic nerve is a mixed nerve containing both primary sensory and motor fibers.

Analysis of myelin formation in the sciatic nerve of these mice revealed a mosaic pattern of normally myelinated fibers and promyelin arrested fibers. Most of the myelinated axons express Choline acetyltransferase (ChAT), indicating they are motor fibers. Therefore motor neurons are myelinated normally by Adam22-deficient SCs. As in Lgi4-deficient claw paw mice at P12, non-myelinated fibers in Wnt1:Adam22cKO/cKO sciatic nerves are associated with predominantly Oct6 positive promyelinating cells, whereas Krox20 positive SCs are invariably myelinating cells. Examination of the ventral motor and dorsal sensory roots revealed normal myelinated motor fibers and severely hypomyelinated sensory fibers respectively. These data demonstrate that Adam22 is required in neurons, whereas deleting Adam22 in SCs has no effect on myelin formation. Thus, axonally expressed Adam22 serves as the major receptor for SC-derived Lgi4 in PNS myelination.

Adam22 proteins have been shown to interact with integrins. As Lgi4 binds to the disintegrin-like domain of Adam22 it is possible that Lgi4 binding modulates (either positively or negatively) Adam22 integrin interactions. Indeed, Adam22 interacts with a number of integrins that are expressed on Schwann cells (in particular 6.1 and 6.4) and are involved in axonal sorting and myelin stabilization. However, the actions of these integrins have been interpreted in terms of their interaction with the Schwann cell extracellular matrix and not with the axon.

In future experiments we will study the role of the diverse domains of Adam22 in mediating Lgi4 function using a lentiviral system to transduce different isoforms of Adam22 into Adam22 null sensory neurons. These neurons will then be seeded with wildtype rat Schwann cells and myelin formation in these co-cultures will be monitored using antibodies against myelin proteins and by electronmicroscopy.

In a collaborative effort between partners 2 and 1 and Dr. Rasband from Baylor College of Medicine, we found that ADAM22 is a component of the Kv1 channel complex and is found together with Caspr2 at the juxtaparanodal region in myelinated axons. Furthermore, we found that among all the known Kv1 channel interacting proteins, only ADAM22 is found at every site where Kv1 channels are clustered. Analysis of Caspr2, PSD-93, PSD-95, and double PSD-93/PSD-95 null mice showed ADAM22 clustering at BCTs requires PSD-95, but ADAM22 clustering at juxtaparanodes requires neither PSD-93 nor PSD-95. In direct contrast, analysis of ADAM22 null mice demonstrated juxtaparanodal clustering of PSD-93 and PSD-95 requires ADAM22, whereas Kv1.2 and Caspr2 clustering is normal in ADAM22 null mice. Thus, ADAM22 is an axonal component of the Kv1 K+ channel complex that recruits MAGUKs (i.e., PSD-93 and PSD-95) to juxtaparanodes. These findings are of particular importance as the excitable properties of neurons depend not only on the kinds of ion channels expressed in the plasma membrane, but also on the location of these channels. Among the many different ion channels expressed in the nervous system, the Kv1 channels are an excellent example of channels that are restricted to distinct subcellular locations. Mutations or diseases that disrupt clustering, localization, or composition of Kv1 channels severely compromise nervous system function and lead to conduction block, episodic ataxia, and/or epilepsies. A major question is whether the clustering of Kv1 and Adam receptors is dependent on binding of an Lgi ligand.

Lgi proteins are characterized by a typical extra-cellular Leucine-Rich Repeat (LRR) and a WDR5 related seven-bladed propeller structure called the epitempin (EPTP) domain. Both these structural motifs provide a platform for specific protein-protein interactions. As described above, all members of the Lgi family bind to a subfamily of Adam proteins eg Adam11, Adam22 and Adam23. These Adam proteins lack metalloproteinase activity and are predominantly expressed in the nervous system. How binding of Lgi4 to Adam22 results in proper axonal ensheathment and myelination is a major unresolved question in this field of research. We began to address this question by asking whether

binding of an Lgi ligand to Adam22 is sufficient to promote myelination by Schwann cells. Using a well-defined embryonic dorsal root ganglion neuron Schwann cell co-culture system we demonstrated that Schwann cells will readily myelinate associated axons in wildtype or clp heterozygous cultures but that no myelin is formed in cultures derived from claw paw embryos. Viral transduction of Schwann cells with vectors expressing Lgi4, but not Lgi1, Lgi2 or Lgi3, restores myelin formation in claw paw cultures. Thus, despite the fact that all these proteins bind to Adam22 - although probably with different affinities - only Lgi4 elicits a myelination response. We next performed a number of experiments in which the LRR domain and the EPTP domain were exchanged between Lgi4 and Lgi3 or Lgi1. Again the chimaeric molecules were tested in the co-culture system and it was found that functional specificity resides in the EPTP domain of Lgi4 in particular in the last four blades of the beta propeller structure. Within this domain there is a short amino acid sequence that connects blade 5 and 6 and that is fully conserved in all Lgi proteins suggesting it might be important for Lgi function in general. Indeed, mutations in this region, especially the S440L mutation, in Lgi1 causes epilepsie and psychotic disorders in a Japanese family. To test whether this Serine residue is also essential for Lgi4 function we introduced this mutation in Lgi4 and tested the mutant protein in the co-culture system. We found that this mutation did not affect Lgi4 function. Thus, a crucial amino acid residue in Lgi1 function is not relevant for Lgi4 function suggesting they engage in different interactions. To further identify functionally relevant sequences within the Lgi4 protein we took advantage of the more than 900 million years of independent evolutionary history between modern day fishes and mammals. Curiously, comparative genomics had failed to identify an Lgi4 homologue among the five Lgi genes in the genomes of fishes (species examined; sea bass, salmon, pufferfish, medaka and zebrafish). We cloned the zebrafish Lgi cDNAs and expressed them in the claw paw co-culture system and found that only drLgi1A, and to a lesser extent drLgi1B, but not drLgi2A, drLgi2B and drLgi3, promotes myelination. Examination of a sequence alignment of the primary amino acid sequences of drLgi1A, drLgiB and Lgi4 revealed two short patches that are uniquely conserved between Lgi4 and drLgi1A and drLgi1B but not Lgi1. These patches map next to each other on the upper rim of the EPTP domain of Lgi4. We hypothesize that these short sequence patches represent unique interaction interfaces that distinguish Lgi4 from Lgi1. Whether this patch is involved in Adam22 interactions or perhaps with an accessory molecule is at present unclear and provides an important challenge for the future.

Thus, we have firmly established Lgi4-Adam22 interactions as absolutely required for proper ensheathment and myelination. Future studies should reveal the detailed mechanism of action and the possible interaction with adaxonal integrins in the Schwann cell membrane.

Activity 1.2

During peripheral nerve development Schwann cells (SCs) surround large bundles of mixed-calibre axons send cytoplasmic processes into the bundles, thereby pulling large calibre axons to the edge of the bundles (radial sorting). The sorted large axons are then surrounded in a 1:1 relationship with SCs, and are subsequently myelinated.The mechanisms by which molecules promote sorting are incompletely understood. Axonal sorting requires multiple events: the formation of 'families' of SC with multiple axons contained in a common basal lamina, the matching of the number of axons and SC, the insertion of Schwann cell processes around axons to recognize and segregate large ones, and the defasciculation of single axons with their own daughter Schwann cell and basal lamina. It is unknown which laminin-receptor pairs contribute to each step. For example laminins promote the formation of Schwann cell processes and the interaction with axons, via activation of Rac1 by a beta1

integrin receptor, but laminins also promote Schwann cell proliferation and survival, via activation of PI3K, FAK and Cdc42 by an unknown laminin receptor. Thus which laminin receptors control matching of axons and SC number is unclear. 1 integrin receptors, but not dystroglycan, transmit survival and proliferation laminin signals. Laminins promote SC proliferation and survival and dystroglycan is required in vitro for laminin-mediated protection of SC from apoptosis. To determine which laminin receptors promote proliferation and survival in vivo, we measured the rate of apoptotic and proliferating cells in laminin receptors mutant nerves by TUNEL and anti-histone H3 staining. Proliferation was significantly decreased in eta1 integrins and double mutants at P3 and P5. Dystroglycan mutants showed increased proliferation at P15. Decreased proliferation rates for 1 integrin and double mutants at P3 were confirmed using BrdU incorporation. Apoptosis was increased in double mutants (beta1-integrin/dystroglycan) at P3 and P5. Staining with the SC marker periaxin showed that 80% of the TUNEL positive cells were SC. Single 1 integrin, but not single dystroglycan mutants also showed increased apoptosis. Thus only 1 integrin receptor(s) mediate the survival and proliferation signal from laminins during axonal sorting.

Radial sorting is also impaired in models of human diseases, congenital muscular dystrophy (MDC) 1A, MDC1D and Fukuyama, owing to loss-of-function mutations in the genes coding for laminin 2, Large or fukutin glycosyltransferases, respectively. It is not clear which receptor(s) are activated by laminin 211, or glycosylated by Large and fukutin during sorting. We identified the relevant receptor as dystroglycan and reported these findings in a paper in Development. We showed that the absence of dystroglycan in a specific genetic background causes sorting defects with topography identical to that of laminin 211 mutants, and recapitulating the MDC1A, MDC1D and Fukuyama phenotypes. By epistasis studies in mice lacking both integrin beta1 and dystroglycan in SCs, we show that absence of eta1 integrins impairs proliferation and survival, and arrests radial sorting at early stages, that eta1 integrins and dystroglycan activate different pathways, and that the absence of both molecules is synergistic. Thus, the function of dystroglycan and eta1 integrins is not redundant but is sequential. These data identify dystroglycan as a functional laminin 211-receptor during axonal sorting and the key substrate relevant to the pathogenesis of glycosyltransferase congenital muscular dystrophies.

How are these integrin signals transduced in sorting Schwann cells? FAK associates with beta1 integrin in Schwann cells and the presence of basal lamina activates FAK. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase central to several signal transduction pathways, including those that stimulate proliferation. Here we have investigated the role of Schwann cell FAK by targeted deletion using the Cre-loxP system.

During the sorting process, SCs undergo a burst of proliferation, ensuring that an adequate number of SCs are available to sort all the required axons, and we have shown that Focal Adhesion Kinase (FAK) is partially required for this proliferation. The onset of sorting coincides with the formation by SCs of a shared basal lamina, a major constituent of which is the extracellular matrix (ECM) protein laminin. Indeed, in the absence of laminin, the basal lamina is not formed, SC proliferation is reduced, and process extension and thereby radial sorting does not occur (see above). Interestingly, at the onset of sorting, the SC basal lamina forms in a fragmentary manner; thus, by EM, at E18.5 in mice, in some areas of peripheral nerve the basal lamina appears to be fully-formed, whereas in others it is virtually absent. As ECM, via FAK signaling, can regulate cell migration and proliferation, and can also regulate differentiation, we have been trying to determine whether the SC phenotype is affected by large differences in ECM concentration, and whether FAK plays a role.

In summary, we have found that wildtype SC proliferation and differentiation is virtually unaffected over a relevant range of Laminin1 concentrations. In contrast, though FAK mutant SCs proliferated

and differentiated normally at the highest Laminin1 concentration, at lower concentrations FAK mutant SCs failed to spread, did not proliferate and were hyper-sensitive to and differentiated prematurely in response to dbcAMP. These experiments suggest that the FAK mutant SCs are sensitive to cAMP because they do not spread on low Laminin1. Interestingly, in vivo around the time of radial sorting, FAK KO SCs prematurely upregulate the differentiation markers Oct6 and Krox20. This suggests that FAK-mediated SC spreading in vivo may regulate both proliferation and differentiation of SCs.

WP2: Control of myelin formation and maintenance

Activity 2.1

Nectin-like (Necl)/SynCAM cell adhesion molecules are located at the axon-glia interface along the internodes where they mediate Schwann cell-nerve interaction necessary for myelination. The role of these molecules and their interaction with NRG1 signals will be investigated. We took two approaches to study the role of Necl4 in myelination in vivo. In the first, we have generated and begun analyzing transgenic mice expressing a normal and a deletion mutant of Necl4 in Schwann cells and oligodendrocytes using an MBP promoter. Analysis of the transgenes showed that expressing the deletion mutant of Necl4 resulted in profound neurological defects and abnormal myelin formation. These are encouraging results, which support a role for Necl proteins in myelination. In the second approach, we used gene targeting to eliminate the expression of Necl4 in myelinating glial cells. Necl4 was completely absent from homozygous animals as was evident by Western-blot analysis of spinal cord and sciatic nerve lysates, as well as by immunofluorescence analysis of teased sciatic nerves and brain sections. Homozygous Necl4-/- mice were viable and fertile, but displayed mild motor abnormalities, enhanced limb-clasping reflexes, and reduced conduction velocity of peripheral nerves. Histological examination of sciatic nerve cross-sections prepared from homozygous animals at different ages revealed a marked delay in myelination and formation of abnormal myelin membrane detachments and outgrowth. Furthermore, we show that deletion of Necl4 resulted in a profound effect on the organization of myelinated axons - generation of split paranodal junction that was reflected by abnormal distribution of Caspr, aberrant clustering of Kv1.2 channels away from the juxtaparanodal region, and absence of defined juxtamessaxonal lines along the internodes. Our data suggest that axoglial contact, mediated by Necl4, is required for proper myelination and the molecular organization of myelinated axons.

How does Necl4 affect Schwann cell behavior? We have identified protein 4.1G as a cytoplasmic protein that interact with Necl4 in Schwann cells. Extensive immunolabeling experiments showed that these two molecules co-localized along the myelin internode. Furthermore, clustering of Necl4 on the surface of Schwann cells recruits protein 4.1G into the clusters, indicating that these proteins are associated at the plasma membrane interface. Analysis of mice lacking protein 4.1G revealed that these mice exhibit abnormal distribution of Necl4 and several other non-compact myelin proteins, further supporting a functional connection between these two proteins. Similar to ablating Necl4, deletion of 4.1G in mice resulted in aberrant distribution of both glial adhesion molecules and axonal proteins that were present along the internodes. In wild-type nerves, juxtaparanodal proteins (i.e., Kv1 channels, Caspr2, and TAG-1) were concentrated throughout the internodes in a double strand that flanked paranodal junction components (i.e., Caspr, contactin and NF155), and apposes the inner mesaxon of the myelin sheath. In contrast, in 4.1G-/- mice, these proteins 'piled up' at the juxtaparanodal region or aggregated along the internodes. These findings suggest that protein 4.1G

contributes to the organization of the internodal axolemma by targeting and/or maintaining glial transmembrane proteins along the axoglial interface.

In our search for proteins that control myelin formation and maintenance, we concentrated on proteins that would translate extracellular signals to the movement of the actin cytoskeleton that, as we suspected, will be required for myelination. We have shown that N-WASP is required for membrane wrapping and myelination by Schwann cells. During peripheral nerve myelination, Schwann cells sort larger axons, ensheath them and eventually wrap their membrane to form the myelin sheath. These processes involve extensive changes in cell shape, but the exact mechanisms involved are still unknown. Neural Wiskott-Aldrich syndrome protein (N-WASP) integrates various extracellular signals to control actin dynamics and cytoskeletal reorganization through activation of the Arp2/3 complex. By generating mice lacking N-WASP in myelinating Schwann cells, we show that N-WASP is crucial for myelination. In N-WASP deficient nerves, Schwann cells sort and ensheath axons, but most of them fail to myelinate and arrest at the promyelinating stage. Yet, a limited number of Schwann cells form unusually short internodes, containing thin myelin sheaths, with the occasional appearance of myelin misfoldings. These data suggest that regulation of actin filament nucleation in Schwann cells by N-WASP is crucial for membrane wrapping, longitudinal extension and myelination.

Cajal bands are cytoplasmic channels flanked by appositions where the abaxonal surface of Schwann cell myelin apposes and adheres to the overlying plasma membrane. These appositions contain a dystroglycan complex that includes periaxin and dystrophin-related protein 2 (Drp2). Loss of periaxin disrupts appositions and Cajal bands in Schwann cells and causes a severe demyelinating neuropathy in mouse and man. We have investigated the role of mouse Drp2 in apposition assembly and Cajal band function and compared it to periaxin. We have shown that Periaxin and Drp2 are not only both required to form appositions, but they must also interact. Periaxin-Drp2 interaction is also required for Drp2 phosphorylation but phosphorylation is not required for the assembly of appositions. Drp2 loss causes corresponding increases in Dystrophin family members, utrophin and dystrophin Dp116 though dystroglycan remains unchanged. Drp2-null Schwann cells have disrupted appositions and Cajal bands, and they undergo focal hypermyelination and concomitant demyelination. Nevertheless, they do not have the short internodal lengths and associated reduced nerve conduction velocity seen in the absence of periaxin, showing that periaxin regulates Schwann cell elongation independent of its role in the dystroglycan complex. We conclude that the primary role of the dystroglycan complex in appositions is to stabilize and limit the radial growth of myelin.

Activity 2.2 and 2.3

Binding of NRG1 typeIII to glial ErbB2 and ErbB3 receptors is likely to activate multiple signaling pathways, but it is unknown how they are tied into the myelination program. One attractive second messenger is the PI3K pathway with the generation of phosphatidyl-inositol (3,4,5)-triphosphate ('PIP3') as a signaling lipid. To determine the role of PI3Kinase signaling in PNS myelination in vivo we analyzed a mouse line were the Pten gene (encoding a D3-lipid phosphatase that antagonizes PI3K signaling) was specifically ablated from the Schwann cell lineage by using a CnpCre driver line. We could demonstrate by PCR amplification of sciatic nerve genomic DNA that the Cre-mediated inactivation of Pten in Schwann cells resulted in efficient deletion of Pten exon 5 in mutant sciatic nerves. Western blot analysis confirmed a significant downregulation of PTEN protein and enhanced phosphorylation of known PI3 Kinase effector proteins, i.e. AKT and S6 in mutant sciatic nerves.

When the levels of PtdIns(3,4,5)P3 and PtdIns(4,5)P2 were analyzed in acidic lipid extracts of sciatic nerves by gas-chromatographic quantification of their associated fatty acids we found, as predicted, an increase in PtdIns(3,4,5)P3 levels and a decrease in PtdIns(4,5)P2.

When small caliber axons (below 2 µm in diameter) of sciatic nerves were quantified by g-ratio analysis using ultra-thin cross sections, loss of PTEN caused a significant increase of myelin sheath thickness. However, when the degree of myelination was quantified for axons larger than 2µm (in the same cohort of mice), g-ratios of Pten mutants were unaltered compared to controls. Instead, we noticed that one very prominent feature was focal hypermyelination (Tomacula) and myelin outfoldings reminiscent of the neuropathological features in a diverse group of demyelinating neuropathies, including hereditary neuropathy with liability to pressure palsies (HNPP). Teased fiber preparations and the electron microscopic analysis of longitudinal sciatic nerve sections at postnatal day 30 (P30) revealed that these myelin abnormalities predominantly originated from paranodal regions and Schmidt-Lantermann incisures. These two regions of non-compacted myelin might represent zones of controlled membrane incorporation and myelin sheath growth. We therefore investigated whether the lack of Pten would mimic abnormal axon-glia signaling and PI3K activation in these regions. Indeed, when p-AKT was used as a sensor of PtdIns(3,4,5)P3, and co-immunostained with MPZ (a marker of compact myelin) and myelin-associated glycoprotein (MAG; a marker of non-compacted myelin), we found a striking colocalization of p-AKT in MAG-positive Schmidt-Lantermann incisures and paranodes. This indicates that in those regions PTEN could be involved in regulating PtdIns(3,4,5)P3 levels. Levels of the two cell polarity proteins Par3 and Dlg1, normally located at regions of non-compact myelin, and suggested to play a role in the control of myelination, were unchanged when tested by immunohistochemistry on teased fibres.

Loss of Pten from Schwann cell lineage cells increased both, the overall size of sciatic nerves and the numbers of myelinating Schwann cells (controls: 168±9; mutants: 278±25; p=0.015) and non-myelinating Schwann cells (controls: 56±8; mutants 128±16; p=0.017). Also the absolute number of myelinated axons in the sciatic nerve, when counted at the same level and at 3 months of age, was significantly higher in Pten mutants (5234 ± 98) than in controls (4152 ± 278) (p=0.0214). Here the axon size distribution indicated that the increase was primarily caused by a recruitment of small caliber axons with diameters between 1 and 3 µm. Indeed, when sciatic nerve cross sections were immunostained for both myelin protein zero (MPZ) and peripherin, a marker of the non-myelinated C-fibers, we frequently observed peripherin-positive axons that were surrounded by myelin in the mutants. Also electron microscopic analysis revealed the presence of myelinated axons of unusually small caliber in the mutants (often in close proximity to Remak bundles). This strongly suggests that enhanced PtdIns(3,4,5)P3 levels stimulate Schwann cells to sort and wrap axons below the normal size threshold.

Cre recombination in "non-myelinating" Remak Schwann cells was expected and demonstrated indirectly by strong immunostaining of phosphorylated AKT. When analyzed in adult Pten mutant nerves, Remak Schwann cells were increased in size, containing small calibre axons in a reduced density. Surprisingly, when visualized by electron microscopy, C-fiber axons often revealed a spiral ensheathment by the Remak cell. Those axons were closely apposed by multiple layers of glial cell membrane, reminiscent of a cytoplasm-filled space in immature myelin, but developmentally stable. Occasionally, "Remak myelin" had incorporated the adhesive protein MPZ and appeared compacted. Unexpectedly, mutant Remak Schwann cells that wrapped C-fiber axons also ensheathed bundles of collagen fibrils with multiple membrane wraps. The discovery that Schwann cells can ectopically "myelinate" a non-cellular target, lacking a membrane or complex signalling platform, reveals that wrapping does not require continued exchange of signals between axon and Schwann cell, or axon-

specific attractants. Once PI3K activity stimulates Schwann cells above threshold, wrapping emerges as autonomous cell growth. Collagen fibrils may not be completely inert, however, as their interaction with integrins could have established the initial cell polarity required for membrane outgrowth.

PI3K signaling is also mediated trhough the mTOR complex. The mammalian target of rapamycin (mTOR) is a core kinase in two major complexes, mTORC1 and mTORC2, that regulate cell growth and differentiation in a variety of mammalian cells. We have shown that elimination of mTOR from murine Schwann cells prevented neither radial sorting nor the initiation of myelination. However, normal post-natal growth of myelinating Schwann cells, both radially and longitudinally, was highly retarded. The myelin sheath in the mutant was much thinner than normal; nevertheless, sheath thickness relative to axon diameter (g-ratio) remained constant in both wild-type and mutant nerves from P14 to P90. Although axon diameters were normal in the mutant at the initiation of myelination, further growth as myelination proceeded was retarded, and this was associated with reduced phosphorylation of neurofilaments. Consistent with thinner axonal diameters and internodal lengths, conduction velocities in mutant quadriceps nerves were also reduced. These data establish a critical role for mTOR signalling in both the longitudinal and radial growth of the myelinating Schwann cell.

Halfway the project we have added an extra goal 'gene expression profiling of Schwann cells stimulated by neuronal NRG1 in transgenic mice' because the initially planned analysis of DRG cultures derived from Pten mutant mice was no longer timely in its original form. During months 37-48 we continued to work on the alternative approach with a specific focus on the fatty acid binding protein PMP2 (FABP8) as a target of axonal NRG1 type III signaling in Schwann cells. We first confirmed a threefold increase in PMP2 protein expression in postnatal sciatic nerves from NRG1 type III transgenic mice by western blotting. Together with previous findings this establishes a continuous and permanent sciatic nerve overexpression of PMP2 in response to elevated axonal NRG1 type III. Next, we tested the dependence of PMP2 expression on the myelination status and NRG1 signaling in mouse mutants of BACE1 (B-site amyloid precursor protein-cleaving enzyme 1). BACE1 deficiency results in the accumulation of unprocessed NRG1 in the brain and sciatic nerve hypomyelination suggesting that proteolytic processing may regulate NRG1 function during myelination. In contrast to the major myelin protein P0, PMP2 protein was strongly downregulated in sciatic nerves of adult BACE1 mutants. However, peripheral myelination and PMP2 expression were fully restored, when we bred BACE1 mutants to transgenic mice that neuronally overexpress N-terminally HA epitope-tagged full-length NRG1 type III. We conclude from these findings that BACE1 is not essential for NRG1 function during myelination and that multiple neuronal proteases collectively regulate NRG1 processing.

WP3: Functional organization of the axo-glial unit

Activity 3.1

A major consequence of myelination is the establishment of axonal domains that restrict sodium and potassium channels distribution. We investigated the mechanism through which these domains are established and what molecules play a role in this process.

Heminodal clustering of Na+ channels requires Gliomedin - To examine the role of gliomedin in the assembly of the nodes of Ranvier, we generated gliomedin null mice. Homozygous gldn-/- mice exhibited no overt neurological abnormalities, formed compact PNS myelin that was indistinguishable from their wild type littermates, but displayed disorganization and impaired attachment of Schwann

cell microvilli to the nodal axolemma. As Schwann cell microvilli were implicated in node formation, we examined the distribution of Na+ channels in the developing sciatic nerves of wild type and gldn-/- at P6, when both nodes and heminodes can be detected. We found that while in wild type nerves Na+ channels were clustered in mature nodes (i.e., nodes that are flanked by two adjacent Caspr-labeled PNJs), as well as in heminodes, in gldn-/- nerves these channels were absent from heminodes and only detected in mature nodes. In adult sciatic nerves of gldn-/- mice, Na+ channels were colocalized at the nodes of Ranvier with NF186, NrCAM, ankyrin G and IV spectrin, Caspr was present at the PNJ and Kv1 channels were localized at the juxtaparanodal region similar to wild type nerves. As an additional experimental approach, we used myelinating Schwann-dorsal root ganglion (DRG) neurons cultures, which enable monitoring the detailed molecular assembly of the nodes in PNS axons. In contrast to wild type cultures, Na+ channels, NF186, as well as ankyrin G and IV spectrin were not clustered at heminodes in gldn-/- myelinating cultures. Notably, while both in vivo and in myelinating cultures of wild type nerves, heminodes appeared adjacent to Caspr-labeled PNJs, in gldn-/- nerves, heminodes were absent near Caspr-positive sites, indicating that heminodal clustering is not the result of PNJ formation. We then counted the number of sites in which Na+ channels were clustered near the edge of a myelin segment. In gldn-/- the majority of Na+ channels were either absent or diffused (gldn-/- culture 92%, nerve 81%), compared to wild type nerves where they were clustered in more than 90% of the sites. These results demonstrate that gliomedin is required for clustering of the nodal complex at heminodes during myelination.

NF186 and NrCAM are required for clustering of Na+ channels at heminodes but not for their accumulation in mature nodes - The observation that clustering of the nodal complex requires gliomedin prompted us to examine whether this activity is mediated by its two axonodal receptors, NrCAM and NF186. We first examined sciatic nerves of nrcam-/- mice, in which Na+ channel clustering in PNS nodes is delayed. Immunolabeling of teased sciatic nerves isolated from 6 day-old (P6) mice using antibodies to Na+ channels and Caspr (to label the PNJs) showed that Na+ channels were not clustered at heminodes that were already bordered by Caspr-labeled PNJs. In addition, Na+ channels, NF186 and gliomedin were all detected at heminodes in myelinating DRG cultures derived from wild type, but not from nrcam-/- mice. In both sciatic nerves and myelinated cultures, Na+ channels, NF186, gliomedin, ankyrin G and IV spectrin were accumulated at mature nodes. Thus, similar to gliomedin, NrCAM is required for heminodal clustering but not for the accumulation of Na+ channels at mature nodes of Ranvier. Previous observations by UEDIN showed that genetic ablation of the neuronal (NF186) and glial (NF155) isoforms of neurofascin leads to the disruption of both PNJs and nodes. In line with these findings, nodes fail to form in myelinating co-cultures of DRG neurons and Schwann cells isolated from nfasc-/- mice. To examine directly the role of NF186 in the assembly of PNS nodes, we determined whether Na+ channels accumulate at nodes in the absence of NF186 by co-culturing DRG neurons isolated from nfasc-/- mice with wild type Schwann cells. The expression of NF155 in myelinating Schwann cells promoted the full recovery of the PNJs as demonstrated by immunolabeling for Caspr. PNJ formation in these axons was accompanied by the accumulation of Na+ channels at nodes together with all components of the nodal complex (i.e., ankyrin G, IV spectrin and NrCAM) except NF186. However, as observed in gliomedin and NrCAM-deficient DRG cultures, in the absence of axonal NF186, Na+ channels were not clustered at heminodes. To further determine the contribution of the axonodal CAMs to the formation of mature nodes, we established myelinating cultures using DRG neurons isolated from double nrcam-/-/nfasc-/- mutant mice and wild type Schwann cells (to recover the PNJs). Analysis of these cultures showed that Na+ channels, as well as ankyrin G and IV spectrin, accumulated at nodes that were flanked by PNJs, but not at heminodes. Taken together, these results demonstrate that gliomedin and its axonodal receptors NrCAM and NF186, are all required for developmental clustering of Na+ channel at

heminodes. They further reveal the existence of an additional mechanism that enables the accumulation of Na+ channels in mature nodes independently of heminodal clustering and the axonodal CAMs.

Involvement of the PNJ in node formation - One possible mechanism that could compensate for the lack of heminodal clustering might be provided by the PNJ, which serves as a membrane barrier separating nodal Na+ channels and juxtaparanodal K+ channels. However, the role of the PNJ in node formation remains unclear since several studies using paranodal mutant mice have indicated that these structures are dispensable for PNS node formation. Myelinating DRG cultures lacking gliomedin, NrCAM or NF186, in which heminodes fail to form, allowed us to directly examine the role of the PNJ in the assembly of PNS nodes. We found that in wild type cultures Na+ channels were clustered at heminodes, which eventually merged, forming a mature focal node of Ranvier. In contrast, in gldn-/- and nrcam-/- cultures, as well as in cultures containing nfasc-/- neurons and wild type Schwann cells (i.e., thus considered as nf186-/-) Na+ channels were spread between the two growing myelin segments. A similar distribution of Na+ channels was detected in developing sciatic nerves of gldn-/- and nrcam-/- nerves. Interestingly, although Na+ channels co-clustered with NF186 during all developmental stages in wt cultures, NF186 was not detected in gldn-/- and nrcam-/- cultures until these channels were already accumulated at focal nodes, further indicating that accumulation of Na+ channels in mature nodes occurs independently of the axonodal CAMs. Immunolabeling of gldn-/-, nrcam-/- and nf186-/- cultures using antibodies to Caspr, Na+ channels and MBP, showed that Na+ channels were eliminated from the area occupied by the PNJ, but invaded the axolemma underneath the myelin sheath in its absence. These observations suggest that in addition to being actively clustered at heminodes, Na+ channels are constrained between two neighboring myelin segments by the PNJ. Furthermore, in the absence of the PNJ, as found in mice lacking Caspr for example, the presence of gliomedin and the axonodal CAMs enable the clustering of Na+ channels at heminodes and consequently in mature nodes.

Heminodal clustering and the PNJ provide reciprocal backup systems for node formation - To test whether axoglial contact at both heminodes and PNJ contribute to the formation of the nodes of Ranvier, we generated gldn-/-/caspr-/- and nrcam-/-/caspr-/- mice. In agreement with previous reports, caspr null mice exhibit progressive neurological defects that were already apparent at P11, but nevertheless have a long life span. Both homozygous gldn-/-/caspr-/- and nrcam-/-/caspr-/- mice exhibit dramatic proprioceptive and motor deficits that appeared earlier (gldn-/-/caspr-/- at P5-6, and nrcam-/-/caspr-/- at P3-4) and were more severe than single caspr-/- mutant mice. Double nrcam-/-/caspr-/- and gldn-/-/caspr-/- mice died at P8 and P14, respectively. Sciatic nerves isolated from gldn-/-/caspr-/- and nrcam-/-/caspr-/- pups had myelin that was indistinguishable from the single mutant or their wt littermates. However, in both homozygous mutants, Schwann cell microvilli were disorganized, did not attach the nodal axolemma and often penetrated the space between the paranodal loops and the axolemma. Immunofluorescence analysis of the sciatic nerves revealed that the total amount of Na+ channel clusters formed in gldn-/-/caspr-/- and nrcam-/-/caspr-/- nerves was considerably reduced, reaching only 20% and 13%, respectively, of their wild type age-matched littermates. In agreement, electrophysiological analysis of sciatic nerves dissected from P14 gldn-/-/caspr-/- and P7 nrcam-/-/caspr-/- animals showed a striking reduction in conduction velocity, and especially in the latter case, conduction is likely to be continuous (non-saltatory). Taken together, these results indicate that node formation in the PNS can be achieved by either clustering of Na+ channels at heminodes, or by restricting the distribution of these channels to the nodes by the PNJ. In conclusion, we found that initial channel clustering in heminodes that border each myelin segment requires gliomedin, NrCAM and neurofascin 186 (NF186), three cell adhesion molecules (CAMs)

that mediate the interaction between Schwann cells and the axon. We further show that heminodal clustering coincides with a second, paranodal junction (PNJ)-dependent mechanism that allows Na+ channels to accumulate in mature nodes by restricting their distribution between two growing myelin segments. Based on these findings, we propose that Schwann cells govern the assembly of nodes in the PNS by two independent adhesion systems that provide reciprocal backup capacities.

As described above, gliomedin induces the clustering of sodium channels at the edges of each growing myelin segment (i.e., heminodes) by binding to the axonal cell adhesion molecule neurofascin 186 (NF186). Gliomedin is a type II transmembrane protein that is shed from the Schwann cell surface and incorporates into the extracellular matrix via interactions with heparan sulfate proteoglycans. We found that proteolytic processing controls both activation and inhibition of gliomedin. We show that membrane shedding of gliomedin by a furin protease is necessary for its activity during node formation. In contrast to the wild type protein, a furin-resistant gliomedin mutant failed to induce heminodal sodium channel clustering in myelinating DRG neurons/Schwann cells cultures derived from gldn null mice. Furthermore, we demonstrate that clustering of sodium channels by gliomedin is negatively regulated through a second proteolytic cleavage by a BMP1/TLD metalloproteinase, an enzyme that is enriched at the paranodal loops that flank the forming nodes of Ranvier. BMP1/TLD cleavage releases the olfactomedin domain of gliomedin that mediates its binding to NF186, thereby restricting the sodium channel clustering activity of gliomedin to areas of developing nodes. Eliminating the BMP1/TLD cleavage site in gliomedin, or treatment of wild type myelinating cultures with a BMP1/TLD inhibitor, results in the formation of numerous ectopic nodal-like clusters along axons that are devoid of myelin segments. Our results indicate that two distinct, and functionally opposing proteolytic events tightly control the clustering activity of gliomedin. A paper entitled Proteolytic processing of gliomedin regulates sodium channel clustering at the developing nodes of Ranvier, is in preparation.

Like the node of Ranvier, the axon initial segment (AIS) is a critical sub-cellular domain for the initiation and propagation of action potentials. While assembly of the AIS relies on interactions of scaffolding molecules with voltage-gated sodium channels, the molecular mechanisms that maintain the AIS in a stable configuration suitable for neuronal communication are poorly understood. The neuronal isoform of the cell adhesion molecule Neurofascin, Nfasc186, clusters voltage-gated sodium channels at nodes of Ranvier in myelinated nerves. We have now compared the assembly and stabilization of the node of Ranvier with the AIS. By inactivating the Nfasc gene in adult mice we have shown show that the absence of Nfasc186 causes disintegration of the AIS in cerebellar Purkinje cells. Selective breakdown of the AIS complex abolishes the spontaneous tonic discharge typical of Purkinje cells and modifies the waveform of evoked action potentials. We have thus proposed that Nfasc186 maintains accurate communication between mature neurons by anchoring the key elements of the AIS complex.

Activity 3.3

The functional organization of the axo-glial unit has been remarkably well conserved during evolution. The main molecules involved and the underlying molecular mechanisms are even conserved in simple model animals such as Drosophila. Drosophila offers a large number of experimental advantages that make this system very attractive to identify additional gene functions mediating or controlling neuron-glia interaction in the nervous system. One of the key approaches towards this aim resides in cell-type specific gene silencing. We have generated a number of Gal4

driver lines, which now allow activating gene expression in almost every glial cell type of Drosophila. In combination with large collections of transgenic lines established in Vienna, Austria, Harvard, USA, or Kyoto, Japan, that contain constructs which allow a Gal4 dependent expression of double stranded RNA, cell-type specific RNA interference (RNAi) can be induced. Such genome wide RNAi screens will eventually unravel most of the genes required for glial differentiation.

One of the main aims of the proposal was to identify novel genes acting in Drosophila glial cells. In flies - just as in primitive vertebrate fish - the blood brain barrier is established by glial cells, which form extensive cellular junctions characterized by septate junctions. In the mammalian nervous system, very similar junctions are established at the paranodal junctions. In both systems, the core components of these junctions are NeurexinIV, (the mammalian homolog is the Caspr protein) and Neuroglian (the mammalian homolog is the Neurofascin155 protein). This and other cases of functional homology suggest that Drosophila can serve as a model system to identify novel components underlying neuron-glial interaction.

In the funding period we have followed two different experimental avenues to reach these goals. On the one hand we continued our efforts with direct genetic screens for mutants affecting the integrity of the blood brain barrier. On the other hand we silenced individual genes specifically in glial cells in a RNA-interference based approach and thus determined genes required for glial cell development. Whereas the first approach directly pointed towards a gene function, the second approach just gave an indication whether or not a particular gene has any role in glial cell development and thus requires additional genetic work.

In the later phase of the funding period we have extended the RNAi based approach by silencing gene functions not only in all glial cells but also in different subsets (subperineurial glial cells, wrapping glial cells and astrocytic glial cells).

The genetic approach was based on injection of Texas-red conjugated dextran into mutant embryos. We have established genetic stocks that allow identifying homozygous mutant embryos and first characterized the gene coiled (which was initially called leaky, but we had to switch the name due to an earlier publication of a competitor). Our further work now revealed three additional genes that are all required for the establishment of the septate junctions in Drosophila. This makes this structure very special, since we already know 20 proteins that need to assemble to form this intricate junction. Interestingly, three of these septate junction proteins (Coiled, Crooked and Wullar) encode GPI-anchored, Ly-6 related proteins. A possible role of similar proteins in the mammalian paranodal junction remains to be shown. This work has also disclosed a role of a protease during septate junction assembly and proteases are also known to function in the mammalian paranodal junction.

The glial cell specific RNAi approach turned out to be very fruitful, we have now silenced about 6.000 gene functions in glial cells. For most of these genes we have silenced the genes in all glial cell as wellas in subsets of glial cells. To date we have identified more than 800 genes required for glial cell development or function in Drosophila.

WP4: Disturbed axo-glial signaling and nerve pathology

Activity 4.1

Tomacula and myelin outfoldings are a striking neuropathological feature of a diverse group of demyelinating neuropathies, including Charcot-Marie-Tooth disease 4B (CMT4B) and hereditary neuropathy with liability to pressure palsies (HNPP). Despite major progress in defining the genetic basis of these diseases, the molecular mechanisms that cause focal myelin growth and disrupt Schwann cell-axon interaction in some forms of CMT, but not in others, have remained obscure. Partner 5 has hypothesized that myelin outfoldings and "tomacula" result from uncontrolled myelin membrane growth, a developmental program that is at least in part controlled by neuregulin-1/ErbB2 and PI3K signaling. Partner 5 found in mice that lack Pten expression in Schwann cells, that glia cell-specific hyperactivation of the endogenous PI3K pathway causes focal hypermyelination, myelin outfoldings, and tomacula, associated with a progressive peripheral neuropathy. In previous experiments (see WP 2) Partner 5 found activated AKT kinase associated with phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) at paranodal loops and Schmidt-Lantermann incisures of Pten mutants. This and the focal hypermyelination pathology indicate that regions of non-compact myelin permit membrane incorporation and myelin growth. We suggest a model that elevated PtdIns(3,4,5)P3 levels contribute to the pathology of tomaculous neuropathies at least partially by activation of the AKT/mTOR pathway. Indeed, a pharmacological reduction of aberrant mTOR activation by rapamycin, a specific mTOR inhibitor, prevented aberrant myelin growth in Pten conditional mutant nerves to a significant extent.

To analyze whether inhibition of mTOR signaling could prevent the formation of myelin abnormalities Partner 5 treated Pten mutants and littermate control mice with rapamycin. We selected 3 groups of abnormal myelin formations by histological criteria (tomacula, comma-shaped outfoldings, recurrent loops and quantified them in sciatic nerve cross sections of controls and conditional Pten mutants that were treated with vehicle (-Rap) or rapamycin (+Rap) from postnatal day (P) 12 to P43 (Del 4.8). In the vehicle treated groups, as expected, all myelin abnormalities were higher in Pten mutants than in controls. After 31 days of rapamycin treatment tomacula were 75% less frequent in the drug treated mutants (52 ± 12.87, n=8) when compared to vehicle treated mutants (209 ± 20.76, n=6) ($p<0.0001$). Similarly, recurrent loops were reduced by 75% in the rapamycin treated mutants (17.88 ± 3.393 n=8) when compared to vehicle treated mutants (72.17 ± 7.897 in Pten mutants, n=6) ($p<0.0001$). Comma-like outfoldings were reduced by 49% (60.13 ± 13.37 n=8 in treated Pten mutants vs. 117.2 ± 6.882, n=6 in Pten mutants) ($p=0.0051$). Rapamycin treatment had no visible effect on control mice. Thus, although the growth abnormalities of the myelin sheaths were not completely corrected, the results indicate that enhanced mTOR signaling plays a role in the formation of redundant myelin in the Pten mutants.

Guided by the analysis of aberrant PtdIns(3,4,5)P3/Akt/mTOR activation in a HNPP mouse model (PMP22 +/- mice) the rapamycin treatment paradigm was extended to analyze whether rapamycin has a beneficial effect also on PMP22 mutant mice. However, our preclinical trial with rapamycin in PMP22 heterozygous mice (an animal model of HNPP) yielded inconclusive results so far.

Activity 4.2

The proper cellular responses following nerve trauma, in particular demyelination, require the activity of the transcription factor c-jun. We asked whether the removal of c-jun in a neuropathy model could ameliorate the clinical symptoms in this model. To this end P0CreXcJunflox/C3 mice were created together with suitable P0CreXcJunflox and C3 control mice. This was done to investigate whether the elevated c-Jun expression detected in C3 mice (which over express PMP22 and serve as a model of

CMT1A neuropathy) was deleterious for C3 pathology (as predicted by the ability of c-Jun to suppress myelin differentiation) or helpful (as predicted by the ability of c-Jun to activate a neuron-supportive Schwann cell program during mechanical nerve injury).

The P0CreXcJunflox/C3 mice (that over express PMP22 but lack c-Jun) were subjected to morphometric nerve analysis and an extensive behavioral testing of sensory and motor performance. The results were compared to the same tests carried out on P0CreXcJunflox and C3 control mice.

Morphometric ultrastructural analysis of the tibial and femoral nerves, and dorsal and ventral roots in these mice revealed a selective loss of sensory axons.

The behavioral tests included, beam walking, grid walking, hanging wire grip strength test, Rotarod performance, von Frey hair sensory test, hotplate test and footprint analysis. It was found that deletion of c-Jun in PMP22 over expressing mice resulted in a clear worsening of motor and sensory motor performance. The outcome of pure sensory tests (von Frey hair sensory test, hotplate test) did not change. It should be noted that most/all motor tasks involve the function of sensory neurons, in particular sensory afferents from muscle spindles.

Electrophysiological measurements showed a strong reduction in F-wave persistence in P0CreXcJunflox/C3 mice compared to C3 controls. This indicates reduced excitability of spinal motoneurons, which in turn could be caused by reduced input from sensory neurons innervating muscle spindles.

In conclusion, these experiments indicate that c-Jun activation of a neuron-supportive Schwann cell phenotype that is particularly relevant for the DRG sensory neurons, is the dominant effect of the c-Jun activation detected in Schwann cells in the nerves of C3 mice. Previously we have identified a comparable role for Schwann cell c-Jun during nerve regeneration. This provides a unifying picture of the adaptive role for Schwann cell c-Jun in damaged nerves irrespective of whether the pathology is mechanical injury/regeneration or genetic distress. In principle this suggests that promotion of c-Jun activity in Schwann cells should improve neuronal health and neuromuscular performance in peripheral neuropathies.

Finally, a correlated study had provided similar evidence that dedifferentiation may be beneficial, not detrimental, to myelinating S63del (CMT1B) Schwann cells, in which the unfolded protein response is pathogenic. Two independent approaches had suggested that persistent expression of genes that limit differentiation, in this case Sox2 and Id2, in Schwann cells improves both plasticity after injury and limits proteo-toxic stress in neuropathy. To eliminate the possibility that compensation by either factor in the absence of the other produced this paradoxical conclusion, we examined S63del nerves in which both Sox2 and Id2 were ablated in Schwann cells. Again, the ablated nerves were more pathological than S63del with Id2 and Sox2 intact, demonstrating that the two factors are beneficial in S63del neuropathy. It is likely that these factors promote plasticity of Schwann cells in injured nerves, and this plasticity is advantageous in neuropathy. A similar conclusion was drawn based on studies of the role of the dedifferentiation factors cJun and Notch after nerve injury and in neuropathy.

Activity 4.3

Develop a cell-based assay to identify small molecules that reverse or ameliorate the pathogenic mechanism of CMT mutant proteins.

NGIDD has constructed the Xbp-1unspliced-EGFP reporter construct and performed titration assays with G418 in transfected Cos-7 cells. We have established that there is not obvious cell death in Cos-7 cells due to transient transfection of the P0S63del-dsRED or P0wt-dsRED. Based on these collective data, we performed a permanent transfection in Cos-7 cells with the Xbp-1unspliced-EGFP reporter construct, in order to generate the UPR reporter cell line (Del 4.11). After two weeks of selection in 700µg/ml G418, a limiting dilution, at 1, 5 or 10 cells/well, in ten 96 MTPs, was performed. The pool of G418 resistant cells was analyzed at fluorescence microscope, upon 18 hrs. stimulation with 10µM Tunicamycin (to induce a UPR and Xbp1 splicing, and hence GFP expression).

When almost all of the wells were confluent, plates were replicated into 384 MTPs, in order to obtain a quadruplicate for each single clone. The first so called clone pool analysis was carried out on these plates, on uncounted cells. Cells were incubated in complete medium with or without 10µM Tunicamycin for 18 hrs. Plates were analysed at FLIPRTETRA (MDS) and emitted fluorescence recorded. Positive clones were selected on the basis of the highest EGFP dependent fluorescence signal detected. A total of 23 clones, deriving from limiting dilution at 10 cells/well, were selected, picked up and seeded in 6 wells plate for cell amplification. After clone selection, Cos-7 reporter cells were maintained in complete medium supplemented with 500 µg/ml G418. Unfortunately, as these clones were expanded and carried longer in culture, most of them died. We suspect toxicity in the cells permanently transfected with the Xbp-1unspliced-GFP reporter construct. They die perhaps due to chronic, low-level, leaky expression of GFP in the permanently transfected Cos-7 cells (a basal level of Xbp-1 splicing in Cos-7 cells is observed and may activate GFP expression even in the absence of ER stress). Currently 4 remaining reporter clones are in culture, to be tested in further experiments.

To avoid the problem of Xbp1-EGFP fusion protein toxicity, we have engineered two alternative plasmids based on firefly luciferase reporter genes. First, using the single cistron with IRES strategy, we have completed construction of the alternative Xbp-1unspliced-firefly luciferase-IRES-renilla luciferase plasmid and will perform permanent transfections in Cos-7 cells as before (Del 4.11). Performing the same analysis as outlined above for Xbp-1EGFP cells, we found only very low level activation of luciferase after tunicamycin treatment to induce UPR in Xbp1-luciferaseIRESrenilla cells.

In parallel, we also have undertaken the cloning of a luminescence based UPR reporter construct with a bicistronic design and humanized luciferase, that may be less toxic. In particular, the 979 bps HindIII-XhoI fragment of pGL4.74hRluc that contained a humanized version of the Renilla luciferase was unidirectionally cloned into the MCS (multiple cloning site) of the bicistronic vector pBudCE4.1, digested with the corresponding restriction enzymes, under the control of the CMV promoter. The construct pBud_Rluc _K16 was generated and its sequence confirmed.

Subsequently, the inactive / unspliced version of firefly luciferase (mXbp-1µC /unLuc) was cloned into the MCS under the control of pEF-1 promoter. Therefore, the 1.8 kb NcoI-NotI fragment of the construct pRL-IXFL (Back et al., JBC 2006, Vol. 281, 27: 18691-18706) was blunted by KLENOW fill-in reaction (DNA Polymerase I, Large fragment) and bidirectionally cloned into the XhoI site of MCS pEF-1 action after treatment with first, KLENOW and second, alkaline Phosphatase, calf intestinal (CIP) for generating blunt and dephosphorylated the ends, respectively. The sequence of this final construct was verified by direct sequencing of the plasmid.

The construct pBud_hRluc_mXBP1µC/unLuc, which expresses luciferase as reporter gene and renilla as internal reference control, was permanently transfected into Cos-7 cells, using

Lipofectamine, to generate the Cos-7/Xbp1-Luci reporter cell line. Since this vector contains a zeocin resistant gene, an antibiotic titration curve was previously performed in order to identify the optimal antibiotic concentration to be used for the selection stably transfected cells: 500 µg/ml. After transfection, cells were kept in culture in the presence of zeocin. After about 3 weeks a pool of permanently transfected cells was collected and a preliminary experiment was carried out.

Cells were seeded at 3.000 c/w into a 384 MTP, in complete medium. 48 hours after seeding, medium was replaced with a new medium solution containing 1-10µg/ml Tunicamycin (to induce UPR and Xbp1 splicing, and hence luciferase expression). The incubation was carried on for 6 hours and O/N. Then medium was removed and cells lysis was performed upon addition of water. Two subsequent injections, respectively of luciferin and coelentrazine, were done directly by the reader (Bayer-CCD camera) and the luciferase and renilla luminescence signals were measured for 60. Since the luciferase induced signal is indicative for UPR, while the renilla dependent signal represents the internal reference control; the final results are always calculated by normalizing the luciferase-RLU values for the renilla-RLU values.

Cells pre-incubated in the presence of Tunicamycin showed about two times luciferase stimulation in comparison to cells incubated in medium without Tunicamycin. Based on these results, cells underwent a limiting dilution in five 96MTPs, at 3 cells/well.

When wells were almost confluent, after about 3 weeks, plates were replicated into 384 daughter MTPs. After 48 hours, cells were stimulated for 6 hours with 10µg/ml of Tunicamycin and luminescence signals recorded as indicated above.

12 clones, showing the highest ratio between the luciferase and renilla induced signals, were selected and picked up for retesting.

The clones were retested as counted cells: cells were seeded at 3000 c/w and after 24 hours were incubated with 10µg/ml Tunicamycin, for 6 hours. Luminescence signal was recorded as indicated above. Based on the highest ratio between the luciferase and renilla induced signals and on their growth rate, out of these 12 clones, 6 were selected for further analysis. These clones were further tested upon stimulation for 6 hours and O/N with increasing concentrations of Tunicamycin (0.3-10 µg/ml).

6 hours Tunicamycin incubation gave rise up to 6 fold induction while O/N Tunicamycin incubation gave rise up to 15 fold induction, this UPR is strongly reduced after treatment with chemical chaperones, indicating that the reporter system is functional and suitable to be used for the generation of the P0 and P0delS63 cell lines.

The two best clones (G3 and G6) were expanded in order to create a cell bank. P0S63del-DSred or P0wt-DSred are currently being stably introduced into these 'reporter' cells using the following expression constructs: pcDNA3.1Hygro-P0wt-red and pcDNA3.1Hygro-P0S63del-red. The two constructs are designed to express in a constitutive way either the P0wt or the P0delS63 protein fused the red fluorescent protein dsRED, which will be used as internal reference control and for translocation assays.

Both Cos-7/Xbp1-Luci G3 and G6 clones were permanently transfected with pcDNA3.1Hygro-P0-red or pcDNA3.1Hygro-P0delS63del-red expression constructs, respectively. In parallel Cos-7/Xbp1-Luci G3 and G6 clones were also transfected with an empty hygromicin resistant vector, to generate mock cells. After transfections, cells were kept in culture in the presence of 300 mg/ml hygromicin

(and 300 mg/ml zeocin). After about 2 weeks the stable pools of cells were collected and put under limiting dilutions, at 3 cells/well in ten 96 MTPs/each pool.

When confluent, plates will be replicated and basal RLU levels will be measured and compared among the different family. The goal will be the identification of P0delS63-red expressing clones showing a higher luciferase/renilla signal ratio in comparison to P0-red expressing clones and mock clones after normalization for dsRed levels by FACS analysis.

Thus, a cell-based assay suitable for the identification of compounds interfering with UPR was generated and proved to be suitable for running HTS campaigns. The LOPAC compound collection was indeed used as a pilot screening, applying a fully automated HTS protocol.

Based on the results obtained, two future directions can be envisaged:

Results obtained upon screening the LOPAC compound collection on the UPR cell lines allow the identification of some promising hits. Therefore next steps would be the further validation of these hits and the interpretation by a biological proof of concept, using proof-of-principle in vitro assays and in vivo models. Anyway it has to be underlined that these hits won't represent per se 'lead compounds' for med-chem programs, but the investigation on their activity on secondary assays might additionally confirm the validity of the overall system. Treatment of myelinating explant cultures from S63del mice with these hit compounds is underway to provide just this validation.

The development of a stable cell line expressing P0S63Del together with an appropriate reporter gene, suitable for the analysis of compounds interfering with UPR mechanisms and signaling represents a big success, since so far only transiently transfected cells have been produced. The screening of the LOPAC collection proved that the assay is very robust and suitable for running true screening campaigns. Therefore the next steps would imply the screening of larger compound collections, in order to identify hits that might indeed represent lead compounds for future med-chem programs.

Potential Impact:

NGIDD has contributed to a better understanding of nervous system function and dysfunction in disease. Furthermore, our work on patho-mechanism of neuropathy directly feeds into industrial development and new therapies and contributes to improved diagnosis and management of diseases of the nervous system.

Primary neurological diseases of peripheral nerves cause significant disability. In addition, the developmental and pathogenic processes in the PNS are of direct relevance for our understanding of CNS processes. Due to the simpler cell structure and accessibility of the PNS, many of the molecular mechanisms governing CNS development and pathology have been first elucidated in the PNS. Our work encompasses aspects of both normal development and pathology to find relevant pathogenetic mechanisms accessible to therapeutic intervention. On the one hand, several of the developmental mechanisms that we have elucidated are arrested or impaired in genetic diseases. Examples are lack of axonal sorting and Na+ channel clustering in laminin deficiencies, myelin protein overexpression in CMT1A, and myelin outfolding in CMT4B and HNPP. On the other hand, most mechanisms used during development to form functional myelinated fibers are recapitulated during repair after common multifactorial or acquired neurological diseases, such as multiple sclerosis, diabetes, spinal cord and nerve injury and inflammatory neuropathies. Finally, it is becoming clear that many dominant diseases in the nervous system act through toxic mechanisms, such as protein misfolding and dysregulation of quality control. Our studies of these processes in peripheral neuropathies has identified pathogenetic mechanisms and therapeutic targets relevant to other common diseases of the nervous system, such as neurodegenerative diseases.

An example of the latter is CMT hereditary neuropathies, which affect 3,000,000 people worldwide and account for significant lifelong disability and important economic loss. Small mutations in 23 genes account for approximately one third to one half of CMT hereditary neuropathies. Many of these mutations are associated with childhood-onset neuropathies that include severe Charcot Marie Tooth, Dejerine Sottas Disease or Congenital Hypomyelinating neuropathy. The pathomechanisms of many of these mutations is likely to be gain of abnormal function and mutation-specific. Although several studies have implicated protein quality control alterations in the pathogenesis of PMP22 point mutants, there is not yet direct genetic evidence as to whether or how the alterations cause demyelination. Our partnership has begun to unravel how these mutations may be toxic in various animal models of CMT related mutations. For example, we have shown, by ablating Chop, that one of these protein quality control mechanisms the unfolded protein stress response is necessary for neuromuscular impairment in the MpzS63del version of CMT1B neuropathy.

We have developed a high throughput cell-based assay that reports the early events of maladaptive stress response. We have validated this approach and we will use this cell based assay to identify small molecules useful as tools in proof of therapeutic principle studies in our animal models of neuropathy, and form the basis for medicinal chemistry to develop therapeutic agents that can reduce the toxicity of the integrated stress response. Our data indicate that modulation of such pathways in neuropathy (and other misfolded protein disease) patients may diminish both the severity of their disease, and the resulting disability. Since conformational or protein misfolding diseases include for example the more common Alzheimer and Parkinson diseases or Diabetes, the information produced by our experiments may find broader application in other diseases (Ron, 2002; Lindsten et al., 2003).

Finally, one of the central objectives of this project was to identify common downstream pathogenetic mechanisms and their targets in order to find a common therapeutic strategy for the wide array of neuropathies resulting from mutations in 23 genes. Altered trafficking of mutant proteins and activation of protein quality control mechanisms (unfolded protein response, proteasome degradation, lysosomal-based autophagy) are common features of hereditary neuropathies. We have focused our attention on how these features may perturb signal pathways in glia downstream of developmentally important axonal to glia interactions. Thus, what we have learned about this toxicity in our CMT animal models will likely be applicable to many neuropathies. Indeed, we have shown altered PIP3/AKT signaling and neuropathy in PTEN mutant mice resembling myelin outfoldings as in the rare CMT4B neuropathy. We demonstrated that rapamycin treatment meliorates myelin outfoldings in our animal model. Such myelin abnormalities are present in a much broader range of neuropathies, including the more common HNPP. Thus our results suggest that rapamycin treatment might be beneficial in a range of neuropathies.

List of Websites:

Website: http://www.ngidd.eu

List of all beneficiaries with the corresponding contact details is available on the website.

Contact details coordinator:

Dies Meijer, PhD
Professor in Developmental Neurobiology
Department of Genetics
Erasmus University Medical Center
Street address; Dr Molewaterplein 50
3015GE Rotterdam
Postal address: PO box 2040
3000CA Rotterdam
Tel +31-(0)10-7044250
d.meijer@erasmusmc.nl

Contact detail project manager:

Rini de Crom, PhD
Managing Director
FP7-CP NGIDD
Erasmus MC - Dept. Cell Biology & Genetics
s-Gravendijkwal 230
3015 CE Rotterdam
The Netherlands
phone: +31 10 7043459
fax: +31 10 7044743
ngidd@erasmusmc.nl