

**The role of the NG2 proteoglycan in migration and
characterization of the interaction with the intracellular
binding partner, Syntenin.**

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1. INTRODUCTION

NG2 or 'nerve-glia antigen 2' is a surface protein expressed by immature cell types in developing and adult brain (Nishiyama et al., 1991) and certain undifferentiated cells elsewhere (Stallcup, 2002; Stegmuller et al., 2002). The literature on NG2 suggests that like many proteoglycans (PGs) it is involved in adhesion-migration (Grumet et al., 1996; Hartmann and Maurer, 2001). The current work investigates binding partners of NG2 and elucidates the protein's role in migration to help expound the role(s) of NG2-positive cells in developing and adult brain.

1.1 Glia

Glial cells were traditionally thought to provide a supporting matrix to neurons in the brain. In some regions of human brain, glia out-number neurons by as much as 10 to 1 (Newman, 2003a). Recent work suggests that they play a much more active role including coordination of synaptic signalling (Araque and Perea, 2004; Ullian et al., 2004). Glia are broadly divided into three categories; astrocytes, microglia, oligodendrocytes and their peripheral nervous system (PNS) counterpart, Schwann cells. NG2-expressing cells in the brain have been suggested as a fourth class of glia (Butt et al., 2002).

Glial classification was originally based on cell morphology. Astrocytes derived their name from their elaborate local processes that give a sub-population of these cells a star-like appearance. Astrocytes maintain, in a variety of ways an appropriate chemical environment for neuronal signalling (Newman, 2003a). Oligodendrocyte morphology is dependant on the stage of cellular differentiation, varying from bipolar to multipolar in appearance (Chap. 2, pp 29-56, Myelin Biol., Elsevier, 2004). They are the myelinating cells in the Central Nervous System (CNS). Microglial cells are small in size and are of mesodermal origin (Chap. 2, pp 18-33, Principl. Neural Sci., Elsevier, 1991). They share many properties with tissue macrophages, and are the primary scavenger cells that remove cellular debris from sites of injury or normal cell turnover.

NG2-positive cells comprise a highly complex and distinct adult glia population within the CNS (Lin and Bergles, 2002; Schools et al., 2003). Under the electron microscope these cells are clearly distinct from any other type of cell in the adult CNS (Butt et al., 2002) and resemble astrocytes rather than oligodendrocytes. The roles of NG2-positive cells will be enumerated later.

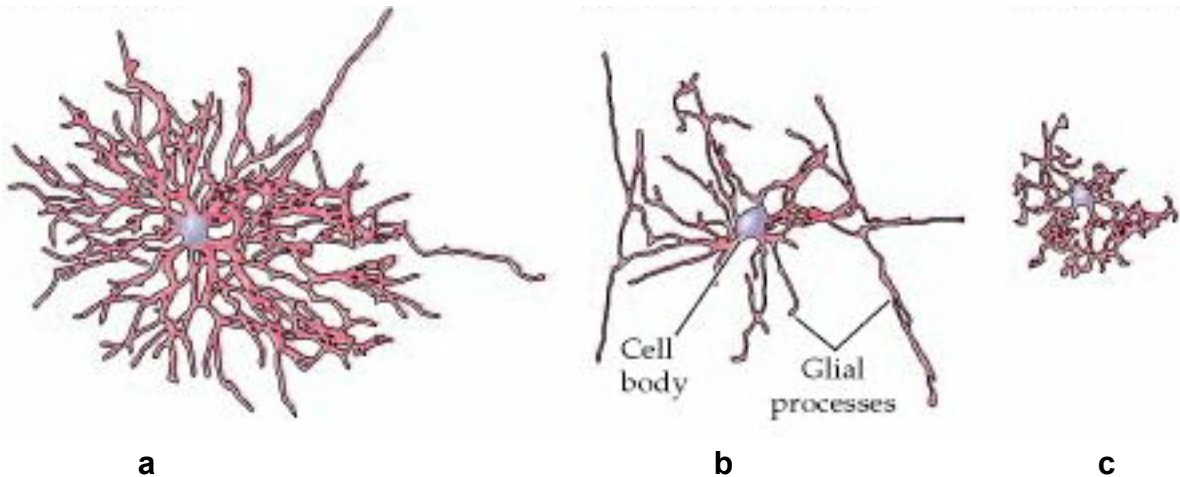


Figure 1.1.1

Neuroglial cells

Tracings of an astrocyte (a), an oligodendrocyte (b), and a microglial cell (c) visualized by impregnation with silver salts. Adapted from Introductory Chapter, Neuroscience, 2nd Edition, Sinauer Associates, 2001.

1.1.1 Functions of Glia

The earliest function attributed to glia was providing nutritional sustenance to neurons (Chap. 2, pp 18-33, Principl. Neural Sci., Elsevier, 1991) as well as modulating synaptic action by controlling the uptake of neurotransmitters (Marcaggi and Attwell, 2004). In this respect, astrocytes were first implicated in neurotransmitter uptake from synaptic clefts thereby limiting the diffusion of neurotransmitters to neighbouring cells (Newman, 2003a). Some glia release neurotransmitters in response to neural activity, which can locally modulate neuronal excitability and synaptic transmission (Auld and Robitaille, 2003). NG2+ glia can respond to neurotransmitter release from neurons and might be

involved in bidirectional signaling (Bergles et al., 2000; Newman, 2003a). Recent work showed that glia can increase the number of functional boutons at synaptic junctions by release of soluble factors: release of thrombospondin (Christopherson et al., 2005) from astrocytes leads to increased efficiency of synapses, while Apo E regulates neurotransmission, growth factor release, and immune responses (Pfrieger, 2003; Gee and Keller, 2005).

Additionally, glia provide scaffolding for neural development. During the development of the cerebral cortex, radial glia serve as a scaffold to support and direct neurons during their migration (Rakic, 2003). Guidance cues for developing axons are also provided by glia releasing chemotactic factors (Bunge, 1994).

Microglial cells are the first to respond to injury (Nimmerjahn et al., 2005). They phagocytose injured cells as well as apoptotic cells in the developing nervous system (Chap. 2, pp 18-33, *Principi. Neural Sci.*, Elsevier, 1991). They are also known to be involved in neuropathic pain (Watkins et al., 2001; Watkins and Maier, 2002).

Oligodendrocytes and Schwann cells are responsible for generating the myelin sheath in the CNS and PNS respectively (Bunge, 1968). The myelin sheath, a lipid-rich insulating envelope prevents the transfer of ions between the axonal cytosol and the extracellular fluids. All electrical activity in axons is confined to the nodes of Ranvier, where the ion channels are located allowing flow of ions across the axonal membrane (Peles and Salzer, 2000) leading to saltatory conduction and increased rate of conduction along axons. Myelinating glia are also essential for axonal health (Dubois-Dalcq et al., 2005).

Glial cells have also been implicated in preventing neurite outgrowths after injuries (Sandvig et al., 2004). Scars in injured CNS have hypertrophic astrocytes, activated microglia and oligodendrocyte precursor cells. Scarred tissue express high levels of chondroitin sulphate proteoglycans (CSPG) like NG2 (Levine, 1994). These scars act as a physical and biochemical barrier to axon regeneration and neurite outgrowth in which CSPGs have been demonstrated to play a role (Fawcett and Asher, 1999; Levine et al., 2001). Glia can therefore either express factors in aiding the repair of injured segments, or prevent in some instances recovery from neural injury by expression of

molecules which deter process outgrowth (Levine et al., 2001; Sandvig et al., 2004). Glial cells expressing NG2 have been implicated in deterring repair of damaged neuronal tracts, but this point is still open to debate (Schneider et al., 2001).

1.1.2 Oligodendrocytes

Oligodendrocytes are the cells that are responsible for producing myelin, the stack of specialized plasma membrane sheets that ensheath an axon. Postnatally, precursors of oligodendrocytes migrate from the subventricular zone (SVZ) into the striatum and white matter as well as medial, dorsal, and lateral regions of the cerebral cortex (Marshall et al., 2003). They develop into myelinating oligodendrocytes through a series of intermediate steps. Each step of this lineage is defined by morphology, proliferative ability and antigenic profile. NG2 is one of the proteins expressed by OPCs.

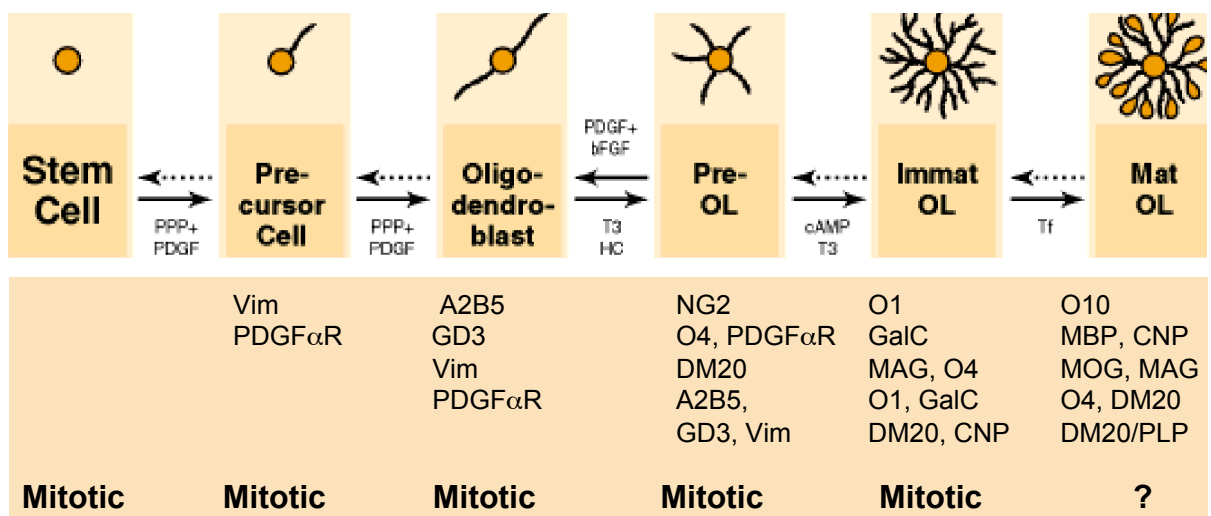


Figure 1.1.2

The oligodendrocyte lineage

Generation of myelinating oligodendrocytes is through several steps. At each stage the cells have a specific antigenic profile. NG2 is expressed by Pre-oligodendrocytes (Pre-OL). Immat = Immature, Mat = Mature. Adapted from Chap. 2, pp 29-56, Myelin Biol., Elsevier, 2004.

1.1.3 Role of precursor cells

Precursor cell populations have the ability to give rise to certain differentiated cell types while persisting throughout development into adulthood as a pool retaining self-renewal properties. They are generated from stem cells which, have the potential to develop into many different cell types in the body and can theoretically divide without limit to replenish other cells. Precursor cells come into action to replace disseminated mature cells and often multiply during injury and in lesions. The density of precursors is highest at embryonic and postnatal stages and decreases over time. They retain the capacity to proliferate even in the adult.

Lineage studies on NG2+ glia

Lineage studies and clues from transcription factor patterns showed that NG2+ precursor cells situated in the SVZ express the transcription factor Olig2 *in vivo* (Aguirre et al., 2004). Basic helix-loop-helix (bHLH) transcription factors Olig2 and Olig1 are important in the specification of both oligodendrocyte and motorneurons (Lu et al., 2002) and in myelinogenesis and axonal recognition (Xin et al., 2005). Olig1-null animals show a delayed oligodendrocyte maturation (Arnett et al., 2004). QRT-PCR (Quantitative Reverse Transcriptase Polymerase Chain Reaction) for *Mbp*, *Plp1*, and *Mag* in these mice showed a 200-fold reduction compared to heterozygous mice (Xin et al., 2005) suggesting the importance of *Olig1* in generating mature oligodendrocytes. Olig2-knockout mice have a more severe effect and die at birth due to oligodendrocyte and motorneuron deficits (Lu et al., 2002). However, there is uncertainty about the specificity of NG2+ precursors (Aguirre et al., 2004). Identified as an oligodendrocyte precursor cell (OPC) marker, NG2 expressing cells appear to be a more heterogenous population displaying multipotency with possible ability to generate electrically excitable neurons, as well as astrocytes and oligodendrocytes (Berry et al., 2002; Belachew et al., 2003; Aguirre et al., 2004). NG2 immunoreactivity in some new neocortical neurons suggested that

they may be generated from the NG2+ precursors that reside within the cortex itself (Dayer et al., 2005).

Antigenic Profile of NG2-positive cells

It has been demonstrated that *in vitro*, NG2 cells behave like O2A precursors. Antibodies against NG2 label O-2A cells, (Raff et al., 1983a) which give rise to oligodendrocytes and type 2 astrocytes *in vitro* (Diers-Fenger et al., 2001). In the CNS, Western blots show NG2 expression beginning at embryonic day (E) 13 (Niehaus et al., 1999) overlapping with PDGF α R (Platelet-derived-growth-factor alpha receptor) expression, which starts around E16-17. In cultured immature oligodendrocytes, NG2 expression partially overlaps with O4 and CNP (2', 3' Cyclic nucleotide phosphodiesterase) and decreases in mature cells expressing antigens such as MAG (Myelin Associated Glycoprotein), MOG (Myelin Oligodendrocyte Glycoprotein) and PLP (Proteolipid Protein). NG2-positive glia does not show immunoreactivity for Glial Fibrillary Acidic Protein (GFAP), a marker for astrocytes (Zhou et al., 2000). Some NG2+ cells bear a complex morphology. These are positive for the glutamate/aspartate transporter (GLAST), glutamate transporter-1 (GLT-1) and S100 β , the calcium-binding protein expressed in astrocytes (Lin and Bergles, 2002).

Expression of NG2 in the nervous system

Western blots of murine brain show NG2 expression peaking between postnatal day (P) 6 to 12 and gradually decreasing thereafter (Niehaus et al., 1999). NG2+ cells are present in adult brain as pericytes along the vasculature (Stallcup 2002), synantocytes (Butt et al., 2002) around nodes of Ranvier and as a distinct population lacking in markers characteristic of differentiated cells but with a phenotype different from NG2+ OPCs (Lin and Bergles, 2002). In the PNS, NG2 is expressed in immature Schwann cells. NG2 expression also increases in Schwann cells when there is direct axon-Schwann cell contact, in comparison to cultures where dorsal root ganglion (DRG) neurons are absent

(Schneider et al., 2001). Identification of the ligands and signalling pathways of NG2 will thus be instrumental in ascribing functions to NG2+ cells.

Roles of NG2-positive glia in developing and adult brain

The finding that PLP and MBP (Myelin Basic Protein) mRNA (Ye et al., 2003) are expressed in a subpopulation of NG2+ cells emphasise their status as precursors of myelinating oligodendrocytes in the developing and adult brain. Depletion of NG2+ cells in myelinating cultures by lysis with AN2 monoclonal antibody plus complement prevented the development of MAG and MOG-expressing cells (Niehaus et al., 2000). The ability to replenish the pool of myelinating oligodendrocytes is important in demyelinating diseases like Multiple Sclerosis (MS) where NG2-positive cells have been shown to be present at lesions and suggested as an important component of the repair machinery (Watanabe et al., 2002; Arnett et al., 2004). However, a subpopulation of NG2+ cells in the brain, defined by absence of the PLP gene expression, may not function as oligodendrocyte precursors (Mallon et al., 2002).

The roles of NG2+ cells in the adult brain are still not completely defined. The fact that a subpopulation retains PDGF α R expression, suggests a mitotically active pool with progenitor potential. They however exhibit rate of migration, cell-cycle length and lineage restriction distinctly different from embryonic OPCs (Raff et al., 1983a, b, c). Adult NG2+ OPCs express PDGF-alpha receptor and O4, are stellate in shape, possess a long cell cycle and are slow to differentiate into oligodendrocytes without mitogen (Chekenya and Pilkington, 2002).

NG2+ cells are often found contacting neurons. This includes perisynaptic wrappings of NG2+ cell processes around the nodes of Ranvier and between neuronal synaptic contacts in the cortex and hippocampus (Ong and Levine, 1999; Butt et al., 2002). Electronmicrographs show that these cells termed synantocytes have the potential to link with a host of surface proteins along axons (Butt et al., 1999; Butt et al., 2002). These cells can be seen to have dense areas that look like synapses, when making contact to axons (Peters,

2004). They thus possess the capacity to respond to bidirectional signalling. Their exact role remains unclear.

It has been recently shown that NG2+ cells in the developing and adult hippocampus expressing α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) receptors form glutamatergic and (γ -amino-butyric acid) GABAergic synapses with neurons (Bergles et al., 2000; Lin and Bergles, 2002, 2004a, b). Stimulation of the neurons resulted in glutamate and GABA release and activation of the AMPA and GABA A receptors respectively in the NG2+ cells. Synaptic structures such as synaptic density and synaptic vesicles could be seen in the neurons contacting the NG2+ cells. NG2 could thus be involved in the alignment and formation of glia-neuron synapses (Stegmuller et al., 2003) and play a role in the modulation of synaptic activity.

An increase in the number of NG2+ cells in response to demyelination around lesions has been seen in several studies (Keirstead et al., 1998; Watanabe et al., 2002). Retroviral lineage experiments in induced lesions demonstrated that immature cycling cells give rise to remyelinating oligodendrocytes (Gensert and Goldman, 1997). Studies carried out have also identified NG2+ cells in Multiple Sclerosis lesions (Chang et al., 2002), which may be involved in regenerating oligodendrocytes. However, as is evident from the above discussion, it remains open as to whether these cells are OPCs.

Importance of NG2-positive cells in Multiple Sclerosis

The importance of myelination is dramatically demonstrated in demyelinating diseases like Multiple Sclerosis (MS), in which myelin sheaths in some regions of the central nervous system are segmentally destroyed. Where this happens, the propagation of nerve impulses is greatly slowed, often with devastating neurological consequences (Lassmann, 2005).

Among demyelinating diseases Multiple Sclerosis (MS) is the commonest with a prevalence of 100 out of 100000 adults in the higher latitudes of Western Hemisphere (Chap. 29, pp 691-700, Myelin Biol., Elsevier, 2004) and a higher susceptibility in females (2 Female: 1 Male). Its incidence varies geographically, with higher frequency at more extreme latitudes. The cause of MS is still under

debate. Classically it has been considered an autoimmune disease with development of T-cell reactivity and antibodies against myelin components and subsequent inflammation. Systematic screening for candidate infectious agents failed to identify a microbial or viral cause for Multiple Sclerosis (Chap. 30, pp 701-734, *Myelin Biol.*, Elsevier, 2004). This however does not rule out possibilities of infections pre-disposing the immune system towards MS. A strong association of MS and the major histocompatibility complex HLA-DR/DQ loci has been found. Other candidate genes which might increase susceptibility include more than fifty adhesion molecules like ICAM, immune receptors, accessory molecules, cytokines, chemokines and their receptors and antagonists (Chap. 30, pp 701-734, *Myelin Biol.*, 2004).

MS is a chronic inflammatory demyelinating disease with loss of axons, oligodendrocytes and gliosis (Raine, 1997). It features demyelinated plaques, partial axonal preservation and reactive glial scar formation. Demyelination by activated macrophages or microglia lead to the formation of pits and vesicles on the axon (Epstein et al., 1983). This process of attack involves antibodies directed against myelin proteins expressed on the sheath (Trotter et al., 1986; Linington et al., 1992) and OPC antigens (Niehaus et al., 2000). Repeated demyelination and failure of remyelination in some patients may be because of compromised axonal integrity, loss of neurons, (Trapp et al., 1998) as well as changes in oligodendrocyte and OPC population (Niehaus et al., 2000; Trotter, 2005).

Analysis of serial sections of tissue from MS patients showed NG2+ OPCs containing nuclear Olig1 at the edge of MS lesions. This distribution suggests that Olig1 nuclear translocation is important in the repair process in MS patients (Arnett et al., 2004). Lysolecithin-induced injuries in Olig1^{-/-} rodents on analysis confirmed an impaired ability to generate mature myelinating cells from precursors and consequent lack in remyelination (Arnett et al., 2004). Additionally, antibodies against NG2 have been shown to be generated in patients with relapsing-remitting MS (Niehaus et al., 2000; Trotter, 2005). The exact effect of the antibodies against the NG2 protein in MS is unclear.

Some symptoms of MS can be reproduced in Experimental Allergic Encephalomyelitis (EAE), an animal model of Multiple Sclerosis. Studies on

NG2+ cells and their role in remyelination have been therefore explored in EAE. EAE is an acute, inflammatory and in some instances demyelinating autoimmune disease. The animals are injected with myelin proteins, which induce an autoimmune response in the animals (Zamvil et al., 1985; Zamvil and Steinman, 1990; Wekerle et al., 1994; Steinman and Zamvil, 2005).

1.1.4 Expression of NG2 in non-neuronal cells

NG2 is expressed in myoblasts (Petrini et al., 2003), endothelial cells (Grako et al., 1999), a wide variety of immature cell types like cartilage, bone and pericytes in developing vasculature (Levine and Nishiyama, 1996; Stallcup, 2002) as well as in some tumour types. Although NG2 was initially identified as an OPC marker, it has also been shown to be in other glial types (Stallcup, 1981; Lin and Bergles, 2002). It is also upregulated in almost all human melanomas (Harper et al., 1984) and gliomas (Chekenya et al., 1999). The common feature of these NG2-positive cells is their ability to migrate under pathological conditions and invade by modulating cell-extracellular matrix and cell-cell adhesion components (Garrigues et al., 1986; Chekenya et al., 1999; Reynolds et al., 2002)

1.2 The NG2 Protein

1.2.1 Homology

NG2 also called CSPG4, is a high molecular weight membrane proteoglycan, with a single transmembrane domain (Stallcup, 1981; Nishiyama et al., 1991; Levine and Nishiyama, 1996). It was subsequently identified in humans as Melanoma Chondroitin Sulphate Proteoglycan (accession number NP001888) or MCSP (Pluschke et al., 1996), in mouse as AN2 (Schneider et al., 2001) with accession number NP620570, in *Drosophila* as CG10275 and in *C.elegans* as C48E7.6.p (Celniker et al., 2002).

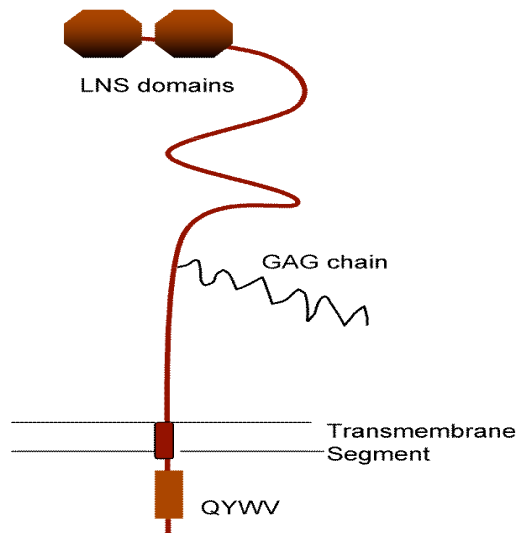


Figure 1.2.1

Schematic diagram of the NG2 proteoglycan

The NG2 proteoglycan is a surface protein with a large extracellular segment. It possesses two LNS-domains (Laminin-Neurexin-Sex Hormone binding globulin) at its extreme N-terminal, one confirmed GAG chain (Glycosaminoglycan), a short transmembrane domain and a C-terminal PDZ-recognition (PSD-95, Disc-large protein, Zona occludens) motif.

1.2.2 Structure of NG2

Primary and secondary structure of NG2

The mouse homologue of NG2 termed AN2 is composed of 2327 amino acids (aa). The N-terminal extracellular region can be divided into two cysteine-rich domains separated by a serine-glycine rich domain in the middle. The N-terminal cysteine rich part possesses disulphide bonds whilst the central region of the extracellular domain conforms to the consensus sequence required for chondroitin sulphate attachment (Nishiyama et al., 1991). Though fourteen potential N-linked glycosylation sites are known, only Serine 999 on the rat NG2 has been confirmed to carry a GAG chain. The attachment of GAG chains is developmental and cell-type specific (Schneider et al., 2001).

The type I transmembrane helix in NG2 stretches from amino acids 2225 to 2249.

The cytoplasmic domain of NG2 contains three threonine residues. Among these, Threonine 2256 has been shown to be phosphorylated by PKC- α (Makagiansar et al., 2004). At the extreme C-terminal of the 76 aa cytoplasmic tail there is a PDZ-binding (Postsynaptic density protein-95, Discs-large, Zona occludens-1) motif. The presence of this 4-amino acid recognition sequence suggested that some of NG2's intracellular partners could be PDZ-proteins.

The NG2 molecule can be categorised within two important groups of proteins; it is a proteoglycan by virtue of its GAG chains and it is a LNS (Laminin, Neurexin, Sex-hormone-binding globulin) - domain protein.

NG2 as an LNS domain protein

An important secondary structure of NG2 is the two LNS domains present at the N-terminal end (47-179 and 224-364; PROSITE). LNS domains are ovoid shaped, approximately 190aa 35-40 Angstrom β -sandwiches formed by two anti-parallel seven-stranded β -sheets. In the case of neurexin, laminin and Sex Hormone Binding Globulin where the crystal structures have been solved, ligand binding occurs at the rim of the β -sandwich opposite the N- and C-termini and involves β 10- β 11 and several loops that run along the rim of the β -sandwich. A loop-forming region of highly conserved amino acids is also involved in chelating metal ions such as Ca^{2+} in laminin and Zn^{2+} in SHBG (Rudenko et al., 2001). The presence of two high affinity LNS domains in NG2 suggests that ligands may exist interacting with these domains in *trans*.

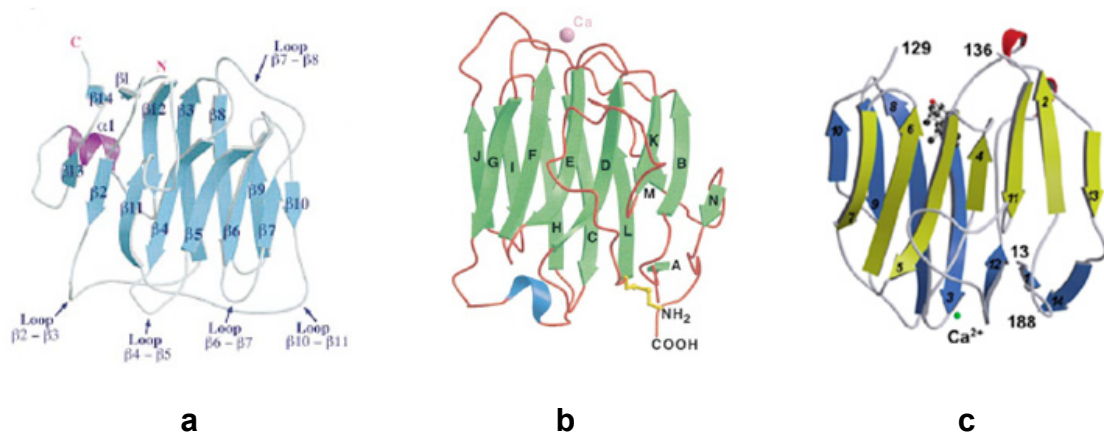


Figure 1.2.2

Crystal structure of LNS domains from three proteins

Orthogonal view of LNS domain with chelated metal ion in Neurexin (a) LamininG (b) and Sex-hormone binding globulin (c). Adapted from (Hohenester et al., 1999; Rudenko et al., 1999; Grishkovskaya et al., 2000).

LNS-domain proteins bind diverse ligands with a wide range of biological functions. Several of these proteins are involved in cell migration, adhesion and recognition as shown in the table below.

Table 1

LNS Domain possessing proteins and their functions

Laminin alpha chains	Heparin, sulfatides, α -dystroglycan, integrins, fibulin-1 and 2, nidogen-2, syndecan-1	Cell adhesion and migration
Agrin	Heparin, α -dystroglycan, agrin receptor	Cell adhesion and migration
Perlecan	Heparin, α -dystroglycan, nidogen-1, fibulin-2	Cell adhesion and migration
Neurexin	Neurologin, α -latrotoxin, neurexophilin and likely others to be yet identified	Neuron–neuron recognition and adhesion
SHBG	Sex steroids (testosterones, estradiols and derivatives)	Steroid transport
Protein S	C4BP, tyro-3 receptor family	Blood coagulation, Mammalian spermatogenesis
	Tyro-3 receptor family	Mammalian spermatogenesis
Slit, crumbs, Fat		Development

NG2 as a proteoglycan

A large number of proteoglycans are expressed in a spatio-temporal manner in both neurons and glia. Several of these have been implicated in proliferation, migration, adhesion, elongation and path-finding of processes and even in stabilizing synapses (Kjellen and Lindahl, 1991). The NG2 proteoglycan has been implicated in cell-cell adhesion (Legg et al., 2003), invasion (Pluschke et al., 1996) as well as in signal transduction complexes mediating migration (Pluschke et al., 1996). For some of these interactions at the extracellular segment the GAG chains are necessary. The invasive potential (Iida et al., 2001) of WM1341D melanoma cell line is dependant on the presence of the GAG chains on NG2. CS-affinity columns alone could bind tagged matrix-metalloprotease (MT3) and enzymatic digestion or treatment of melanoma cells with Chondroitinase ABC, β -D-xylopyransoside and α DX (its inactive analogue) effectively inhibited the process. MT3-MMP was also precipitated out from a

CS-affinity column to which biotinylated melanoma cell surface proteins were applied (Iida et al., 2001).

Below is a table listing some common proteoglycans and their functions.

Table 2

Examples of Proteoglycans and their functions

Proteoglycan	Appro MW	Type of GAG chain	Number of GAG chains	Location	Function
Aggrecan	210kD	Chondroitin Sulphate+keratan sulphate	130	cartilage	Mechanical support
Betaglycan	36kD	Chondroitin Sulphate/ Dermatan sulphate	1	Cell surface and matrix	Binds TGF-beta
Decorin	40kD	Chondroitin Sulphate/Dermatan sulphate	1	In connective tissue	Binds TGF-beta, binds collagen
Perlecan	600kD	Heparan sulphate	2-15	Basal lamina	Structural and filtering function
Syndecan	32kD	Chondroitin Sulphate+heparan Sulphate	1-3	Epithelial cell surface	cell adhesion; binds FGF and other growth factors
Dally (in <i>Drosophila</i>)	60kD	Heparan sulphate	1-3	Cell surface	Co-receptor for <i>Wingless</i>

PDZ-binding motifs

NG2 possesses a PDZ-recognition motif, QYWV (Glutamine-Tyrosine-Tryptophan-Valine) at its extreme C-terminus (Stegmuller et al., 2003). The QYWV sequence is classified as Class II recognition site. Specificity for the

domain is conferred by the –2 residue of the recognition site and forms the basis for classification (Sheng and Sala, 2001).

The second and fourth amino acid residues in a PDZ-binding motif are widely conserved and hydrophobic in nature. Though four or more C-terminal residues function as the commonest form of PDZ-binding motif, internal peptide sequences with beta-hairpin structure can also recognize PDZ-proteins (Hillier et al., 1999). The amino acids at –2 and –3 of the recognition site make specific contact with the PDZ domain.

Three PDZ-proteins were identified as intracellular ligands of NG2 in a yeast-two-hybrid screen, using the complete cytoplasmic domain as bait (doctoral dissertation, J. Stegmüller).

1.2.3 PDZ Domain Proteins

The first PDZ domain was identified from Membrane associated guanylate kinases or MAGUKs (Ponting et al., 1997). Subsequently, they were found to be one of the commonest domains and have been identified in a variety of proteins.

They are classified into 3 classes according to the specificity for the binding motif (X = any amino acid, ϕ = hydrophobic amino acids).

Class I PDZ domains recognise X-S/T-X-V/L, as in NMDA-R2A by the PDZ protein PSD-95.

Class II PDZ domains recognise X- ϕ -X- ϕ , as in NG2 with the PDZ proteins GRIP and Syntenin.

Class III PDZ domains recognise X-D-X-V, as in the interaction of Melatonin with the PDZ protein nNOS.

PDZ domains are modular in nature and the proteins possessing them are involved in sorting, targetting and assembly of supramolecular complexes.

Self-folding domains can be appended together to form multi-PDZ proteins that can then interact with more than one protein simultaneously. Combined PDZ domains have significantly different binding affinities compared to the individual domains. Amongst themselves, PDZ proteins can also form homo- or hetero-multimers (Srivastava et al., 1998; Xu et al., 1998; Dong et al., 1999). Since

PDZ domains have different binding specificities, they can bind a wide variety of partners and are thus capable of assembling multiple ligands into a supramolecular complex, exemplified at the synapse.

The presence of a PDZ-binding motif at the C-terminal of NG2 supported the contention of PDZ-domain proteins as scaffolding candidates linking NG2 to cytoplasmic components.

Structure of PDZ domains

PDZ domains consist of six anti-parallel beta-strand sandwiches flanked by two alpha helices. The N and C-termini of the PDZ domain lie close to each other. The peptide binding occurs in a groove between β B and α B helix, an area designated as the carboxylate-binding loop. The PDZ domain is designed to accommodate a free carboxylate group at the end of the peptide. Crystal structures indicate that the main chain amides of the β A- β B loop donate hydrogen bonds to the carboxylate of the terminal residue of the interacting peptide and that a hydrophobic cavity formed by conserved amino acids in the primary structure interact with the C-terminal recognition sequence (Doyle et al., 1996). The amino acids of this cavity determine the nature of the C-terminal residues selected. However, since hydrophobic interactions are not stringent, the C-terminal residues specified by individual PDZ domains are quite varied.

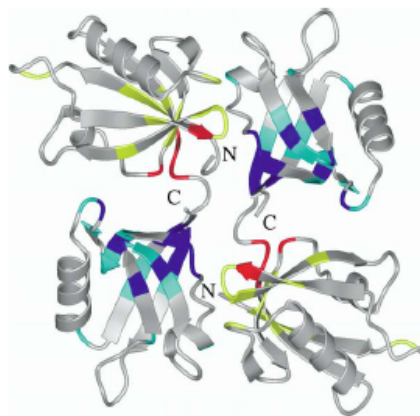


Figure 1.2.3

PDZ domain structure.

Crystal structure of Syntenin PDZ domains in tandem. Adapted (Cierpicki et al., 2005). Red indicates PDZ1 and blue PDZ2.

Phosphorylation and PDZ interactions

Interaction between a PDZ protein and the recognition motif is frequently influenced by the phosphorylation status of the latter. The –2 position of PDZ-recognition sites are frequently phosphorylatable amino acids such as threonine, serine or tyrosine. However, the phosphorylatable residue need not necessarily be part of the recognition motif affecting binding (Sheng and Sala, 2001). Phosphorylation at the motif leads to disruption of the PDZ interaction (Sheng and Sala, 2001) in some cases.

NG2 has been shown to be part of signal transduction pathways implicating tyrosine kinases (Iida et al., 1995), ERK/FAK (Iida et al., 1995; Yang et al., 2004) and Ack-1 or Activated Cdc-42 kinase (Eisenmann et al., 1999; Majumdar et al., 2003; Makagiansar et al., 2004). More recent work showed that isoforms $\epsilon 1$ and $\epsilon 3$ of PKC α can phosphorylate Threonine 2256 on NG2 and increases the migratory ability of astrocytoma cells. Phosphorylation of NG2 also localises it into different cell surface microdomains in astrocytomas (Makagiansar et al. 2004). Though the phosphorylated threonine in NG2 is not within the PDZ-motif, interaction of NG2 with its PDZ domain ligands could conceivably be affected by phosphorylation of this amino acid.

NG2 – repulsive or attractive in nature for neurite outgrowth?

The effect of the NG2 molecule on neurite outgrowth has been under considerable debate. Data from the Levine Lab would suggest that NG2 has a negative effect with NG2 upregulation occurring in injured areas and associated neurite outgrowth inhibition (Dou and Levine, 1994; Levine and Nishiyama, 1996; Ughrin et al., 2003). However, experiments from the Trotter group suggest NG2 to be more equivocal in nature; immunoaffinity purified NG2 (AN2 in mice) from early post-natal mouse brain neither showed inhibition or enhancement of neurite outgrowth (Schneider et al., 2001). Cerebellar granule cells extend neurites on NG2-coated as well as NG2-free areas of the coverslip (Niehaus et al., 1999). Furthermore, NG2 upregulation in regenerating PNS (Rezajooi et al., 2004) as well as neurite outgrowth *in vivo* in the NG2 knockout

versus wild type mice (de Castro et al., 2005) are evidences against a purely inhibitory role of NG2. In view of the ability of proteins such as MAG to act as inhibitors or promoters of neurite outgrowth (Mukhopadhyay et al., 1994) depending on age, type of neuron and status of intracellular cyclic nucleotide levels (Henley et al., 2004), NG2 can probably be either inhibitory or repulsive depending on the circumstances.

1.2.4 Binding partners of NG2

Several extracellular partners of NG2 that have been identified include the extracellular matrix components Collagen Type VI and V through a putative collagen-binding site (Stallcup et al., 1990; Burg et al., 1996; Tillet et al., 1997), growth factors like PDGF AA and bFGF, the galectin-3 protein, kringle domain proteins like plasminogen (Goretzki et al., 1999) and PDGF-alpha receptor (Nishiyama et al., 1996b). Extracellular ligands binding to the two LNS domains have yet to be identified.

Identified intracellular binding partners of NG2 all possess PDZ domains. MUPP1 a protein with 13 PDZ modules was shown to interact with NG2 (Barritt et al., 2000) as well as GRIP (Glutamate receptor interaction protein), which contains 7 PDZ domains (Stegmuller et al., 2003). The intracellular ligands of NG2 thus reflect its structure. Syntenin, whose interaction with NG2 has been characterised in the current work, possesses 2 PDZ domains.

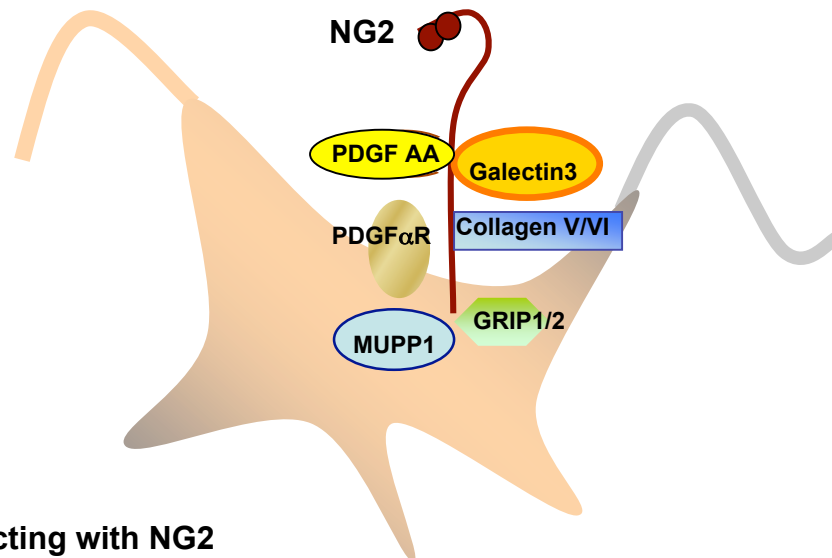


Figure 1.2.4

Ligands interacting with NG2

Confirmed intra- and extracellular partners of NG2. The precise regions, to which the extracellular partners bind, are yet to be defined. All known cytoplasmic partners interact through the PDZ-recognition motif.

1.3 Cell Migration

NG2 and its homologues have already been shown to play a role in migration and invasion (Eisenmann et al., 1999; Iida et al., 2001). The signal transduction pathways involved have also been tentatively explored in some cell types (Iida et al., 1995; Miyamoto et al., 1996; Eisenmann et al., 1999; Makagiansar et al., 2004). The current work focuses on the role of NG2 in migration.

Migration is an integral part of embryonic development, homeostasis and during pathogenic conditions such as cancer. It is a dynamic, cyclical process in which a cell extends a protrusion at its front, which in turn attaches to the substratum on which the cell is migrating, with simultaneous retraction of the other. The cell thus becomes polarized in the direction of movement. The cycle is initiated by chemotactic molecules, which are sensed and communicated to the cell's interior by specialized receptive proteins on the cell membrane. In response to these signals cells extend protrusions by polymerizing actin. Polarisation provides the foci where the machinery for enabling movement assembles. Adhesive complexes collect at the front of the protrusion. Actomyosin filaments contract at the front of the cell and pull the cell body towards the protrusion.

Release of adhesive connections in the rear of the cell and retraction of the tail completes the cycle. The orchestration of this complex process resides in many molecules that serve to distinguish the front from the rear of the cell and whose actions are carefully timed.

1.3.1 Motility in OPCs

Oligodendrocyte precursor cells generated in the ventral neural tube migrate out throughout the CNS (Pringle and Richardson, 1993) where they proliferate and differentiate into myelinating oligodendrocytes which in turn begins the process of enveloping the sheath around the axon. Radial migration of OPCs follows the direction of radial glia fibres and is primarily confined to a coronal plane while ventral to dorsal migration possibly uses axons (Marshall et al., 2003). OPCs therefore are highly motile (Niehaus et al., 1999). Migration is also necessary when cells have to re-populate areas depleted of undifferentiated cells such as during injury.

The stimulation for migration can be soluble chemotactic factors like netrins and semaphorins (Newman, 2003b) as well as cell adhesion molecules attached to the ECM or a neighbouring cell. NG2-positive glia respond to netrin-1 and class 3 semaphorins, sema3F (Spassky et al., 2002) and sema3A (Sugimoto et al., 2001) for which the cells possess receptors. PSA-NCAM (Zhang et al., 2004) and N-cadherin (Schnadelbach et al., 2000), integrin $\alpha v \beta 1$ (Milner et al., 1996) and growth factors such as PDGF-AA released by neurons (Armstrong et al., 1990) have been shown to have an effect *in vitro* on migration. Proteoglycans in *in vitro* experiments have also been implicated in adhesion, migration and playing a role in directing processes along appropriate pathways (Hartmann and Maurer, 2001).

1.3.2 Role of NG2 in migration and adhesion

The *Oli-neu* cell line possessing glial precursor properties (Jung et al., 1995) expresses NG2 endogenously and is remarkably motile (Niehaus et al., 1999). Human melanomas over-expressing the protein MCSP show invasive

properties (Eisenmann et al., 1999) and endothelial cell motility is promoted by the NG2 proteoglycan (Fukushi et al., 2004). Similarly, U251 human astrocytoma transfected with the wild type form are considerably more motile in comparison to those expressing 'chimeric proteins' where either the cytoplasmic or the transmembrane portion of NG2 had been replaced. It was concluded that the proximal-membrane segment of NG2 is necessary for actin cytoskeleton reorganisation and cell motility (Fang et al., 1999).

The rate of migration also differed with the substrate engaging the NG2 extracellular domain (Fang et al., 1999); 'chimeric substrates' with fibronectin synthetic peptide and anti-NG2 mAb, 9.2.27, are preferred over single substrates (Iida et al., 1995). Subsequent work was aimed at identifying the cytoskeletal reorganization occurring with simulation of engagement of the molecule.

The cytoplasmic domain of NG2 is highly conserved and mediates several functions, including integrin-mediated adhesion. Experiments in cells expressing a chimeric protein where the cytoplasmic portion has been replaced with CD8 shows decreased keratinocyte cohesiveness without affecting adhesiveness to the extracellular matrix or motility. The adherens junctions in such cells are disorganized and it is suggested that a disruption of E-cadherin-based adherens junction occurs (Legg et al., 2003).

1.3.3 NG2 and reorganisation of cytoskeleton during migration

Since migration involves polarization and change in morphology with alternate steps of adhesion and retraction of processes, extensive reorganisation of the cytoskeleton is essential. Studies from the Stallcup lab have tried to define the nature of the rearrangement with respect to NG2 in B28 glioma, U251MG astrocytoma and U373 glioblastoma cell lines.

The cytoplasmic part and the chondroitin sulphate chain(s) may dictate the localisation of NG2 in subcellular microdomains thus playing an additional role in deciding polarity (Fang et al., 1999). Lin et al (Lin et al., 1996b) suggest that NG2 can associate with two distinct types of actin-containing structures depending on the stimulus, and this may define cell motility and morphogenesis.

Localisation of NG2 in filopodial extensions lacking in myosin and focal adhesion plaques occurs only in motile cells (Lin et al., 1996a). In more static cells, NG2 associates with myosin-containing stress fibres.

It was also observed that monoclonal antibodies against NG2 engaging different epitopes of the proteoglycan result in distinct forms of actin reorganisation (Fang et al., 1999).

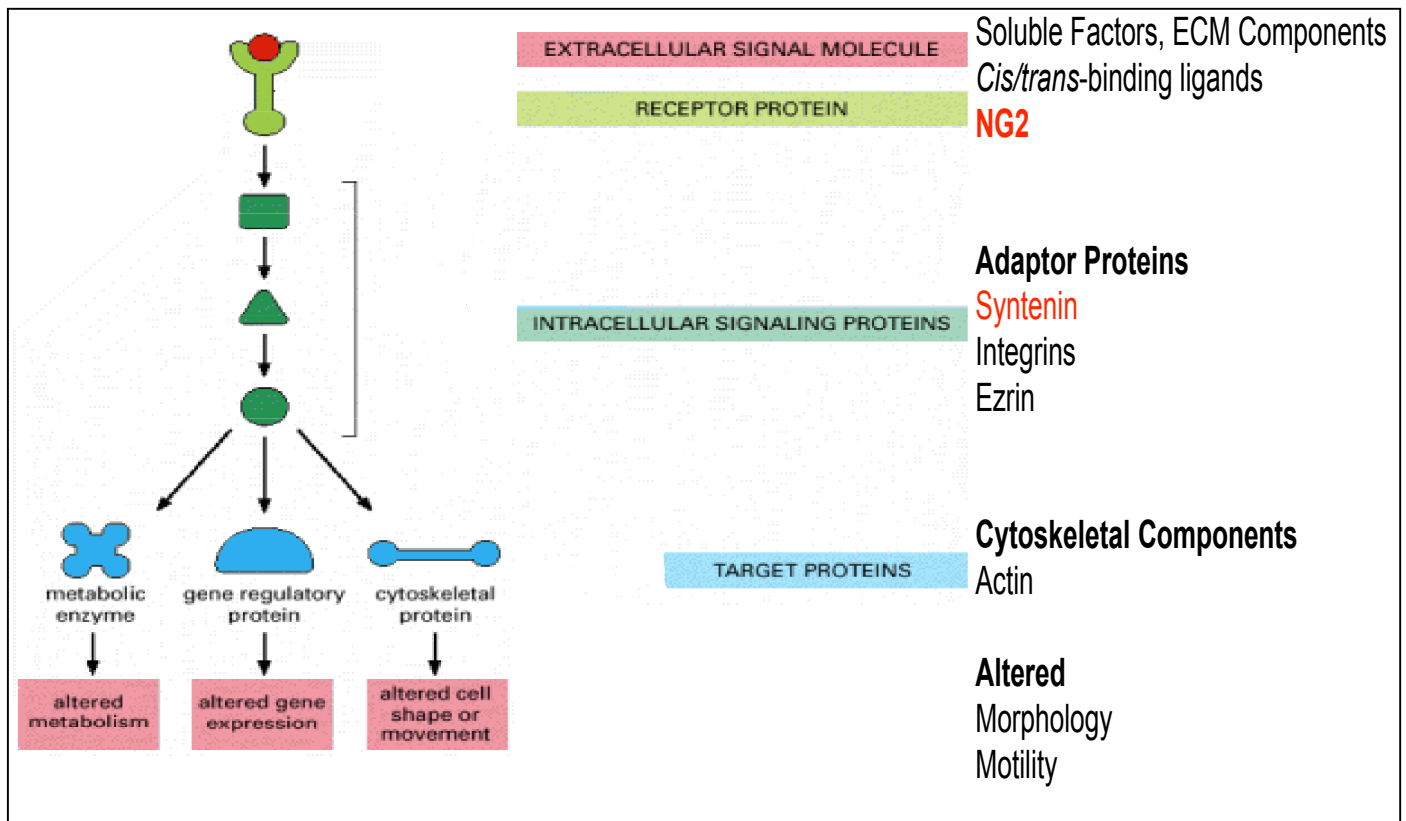


Figure1.3.1

Schematic diagram of sequence of steps involved in signal transduction.

Signalling between cytoplasm and the extracellular milieu involving the cortical machinery is mediated by sequential interaction and assembly of multiple proteins leading to change in cell physiology. Surface receptors like NG2 binding to extracellular signal proteins initiate a cascade of reaction. Membrane proteins are usually part of multi-protein complexes involving adaptor proteins such as PDZ-domain proteins and membrane cytoskeletal markers, which trigger the signal transduction pathways. The target genes or proteins of these pathways lead to altered behaviour of the cell. Adapted from Chap. 15, pp 832-907, Mol. Biol. of Cell, 4th Edition, Garland Publishing, 2002.

1.3.4 Adaptor proteins and membrane cytoskeletal linkers

The cortex of cells consists of the plasma membrane and an underlying network of cytoskeletal components. It is heavily involved in determination of cell morphology, attachment to other cells and ECM, membrane trafficking, cell migration and division during both normal cellular physiology and pathology. Establishment and maintenance of cell polarity involve signalling between cytoplasm and the extracellular milieu involving the cortical machinery. Signal transduction from the external milieu to internal target organelles provides the connection between the environment and the cell. Regulation of this complex communication system involves cell surface receptors, enzymes, and the cytoskeleton, culminating in a cascade of conformational and functional changes in cytosolic and nuclear proteins leading to specific intracellular events. The integration between the initial signalling molecules and the downstream second messengers are facilitated by a group of proteins that mediates interaction with other proteins through their specific domains. These adapter molecules possess no enzymatic or transcriptional activity but function as scaffolds and linkers. Since NG2-actin interaction has not been directly shown, it is probable that adaptor proteins help to link and direct the cytoskeletal reorganisation on engagement of the molecule by extracellular ligands.

Some proteins like the PDZ protein Syntenin and the membrane cytoskeleton linkers (MCL) Ezrin-Radixin-Moesin (ERM) proteins are completely intracellular (Bretscher, 1999), others like integrins can themselves be transmembrane proteins. Frequently, these adaptor proteins compartmentalize with the surface proteins they associate with.

Syntenin has been implicated in migration and invasion of tumorigenic cells (Koo et al., 2002) and shown to be responsible for morphogenesis through its association with the plasma membrane (Zimmermann et al., 2001). It can also interact through its PDZ domains with cell surface signalling molecules like syndecans (Grootjans et al., 1997) as part of a large complex.

The ERM proteins have a domain organization in which the 300 most amino-terminal residues are suggested to directly or indirectly bind to the plasma membrane (Pietromonaco et al., 1998) and the 30 most carboxyl-terminal

residues bind to F-actin (Gary and Bretscher, 1995). The cytoplasmic tails of several surface receptors have been shown to bind to one or the other of the ERM proteins but the interactions are likely to be cell-specific (Bretscher, 1999; Jannatipour et al., 2001; Ng et al., 2001).

Integrins are a family of cell surface receptors that bind extracellular matrix molecules such as laminins, fibronectin, and collagens. A wide array of downstream molecules interacts with integrins and integrin-associated proteins. These factors cooperate to transduce extracellular messages, and ultimately influence processes such as cell division, polarity, movement and gene expression. Oligodendrocytes and *Oli-neu*, which have a similar pattern, have restricted integrin expression. $\alpha 3\beta 1$ is expressed throughout oligodendrocyte differentiation while αv integrins showed developmental regulation with sequential expression of $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta v$, $\alpha v\beta 8$ and $\alpha 8\beta 1$ (Ffrench-Constant et al., 1994; Milner et al., 1996).

1.4 Functions of NG2 and NG2+ Glia

Given that NG2 is present on multiple cell types at differing stages of development, it would not be surprising if NG2 and the cells expressing it have multiple roles. We briefly recapitulate the functions in order to emphasise the protein's importance.

Most evidence suggests that a subpopulation of NG2-positive OPCs can generate oligodendrocytes in developing brain as well as having potential for regenerating myelinating oligodendrocytes in demyelinated plaques (Berry et al., 2002)

In vitro assays with primary oligodendrocyte cell cultures, *Oli-neu* (Niehaus et al., 1999) and an immortalised Schwann cell clone (Schneider et al., 2001) showed that antibodies generated against immunopurified NG2 (AN2) from P6 mouse brain inhibit migration. Overexpression of NG2 in melanoma and glioma as well as enhanced motility in pericytes and cells transfected with the NG2 molecule confirm its role in invasion (Makagiansar et al., 2004). Interference

with NG2's extracellular and cytoplasmic segments leads to cytoskeletal reorganization (Lin et al., 1996a): details of adaptor proteins involved in this interaction are unclear.

NG2, GRIP (glutamate receptor interaction protein) and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subunit GluRB form a complex at the cell surface of glial progenitor cells (Stegmuller et al., 2003). AMPA receptors mediate fast synaptic transmission in the CNS and are composed of subunits GluR1-4. It is possible that GRIP helps to cluster and orient NG2 and GluRB towards neurons thus facilitating signalling and synaptic efficacy in the CNS (Bergles et al., 2000). MUPP1 has been similarly suggested to help incorporate NG2 into supramolecular complexes (Barritt et al., 2000).

NG2 can bind growth factors like PDGF-AA and bFGF (Goretzki et al., 1999). This might be of importance in view of observations that in the NG2-knockout mice smooth muscle cells respond poorly to PDGF (Grako et al., 1999). It has been suggested that NG2 in association with PDGF α R sequester (Nishiyama et al., 1996a, b) growth factor(s).

1.5 AIMS OF THE STUDY

NG2 possesses a PDZ-binding motif, GAG chain(s) and LNS domains suggesting its ability to bind multiple partners. Defining the interaction partners of the NG2 proteoglycan in *cis* and *trans* will help clarify the functions of NG2-expressing cells in the brain. NG2-positive cells in primary cultures and NG2+ cell lines are motile and exhibit local process motility. Elucidation of connections between NG2 and the cell cytoskeleton would clearly be important. This work set to characterise NG2's interaction with the PDZ protein Syntenin that had been previously identified by yeast-two-hybrid screen as a putative partner of NG2, and explores its possible role as an adaptor protein.

Antibodies against NG2 inhibit the migration of NG2+ OPC and NG2+ immature Schwann cells in *in vitro* assays. In order to identify which parts of the molecule are involved, we generated polyclonal antibodies against extracellular segments of the molecule and observed the rate of migration in each case, to pin-point the region(s) which is most likely to be involved in motility.

Several biochemical approaches were undertaken in this study to identify probable ligands in the brain that might be interacting with the extracellular portion of the molecule.

2. MATERIALS AND METHODS

The Materials Section (2.1) lists the reagents, kits and organisms used. The Methods Section (2.2) details the techniques used in the course of this work. It is divided into Cell Culture, Immunofluorescence, Biochemistry and Molecular Biology.

2.1 MATERIALS

2.1.1 Reagents

Acros Organic: beta-Mercaptoethanol, Acetone

AGS (Heidelberg) : 30% Acrylamide / bis-acrylamide solution

Amersham-Pharmacia: [³⁵S]- in vitro Cysteine-Methionine labelling mix, Protein A Sepharose (pre-packed column, unswelled beads), Protein G Sepharose

Applichem (Bensheim): Bovine serum albumin (BSA), IPTG, Reduced Glutathione, Ampicillin, Ethidium bromide, n-Octyl-beta-glucoside

Biochrom: Fetal Calf Serum, Horse Serum

Collaborative Research, Bedford, USA: PDGF, BFGF

Difco: Agar-agar, Peptone

Fluka: NP-40

Hoechst (Frankfurt): Moviol

GIBCO: DMEM, BME, L-Glutamine, Agarose, DMEM,

Invitrogen: L-Glutamine, Trypsin

MBI Fermentas: Restriction Enzymes and Buffers, DNA Markers

New England Biolab: Restriction enzymes and buffers

Roth: APS, DMSO, EDTA, EGTA, Glucose, Sucrose, Glycerol, Para-formaldehyde, Tris, Ethanol, Methanol

Serva (Heidelberg): Coomassie Blue G250, Bromophenol Blue, Gentamycin, Hygromycin, Glutaraldehyde

Sigma: Insulin, poly-L-lysine, progesterone, PMSF, putrescine, TEMED, transferrin, Tween20, Soyabean Trypsin Inhibitor, bis-benzamide, PonceauS,

Methionine, Cysteine, Pepstatin, Leupeptin, Aprotinin, Antipain, DTT, PLL,

Triton X 100

Chemicals for general purposes were obtained from Merck, Roth, Riedel de Haen and Sigma. They were all of p.a grade.

2.1.2 Animals

NMRI mice of both sexes were obtained from the Central Animal Facility of the University of Mainz and the Central Animal facility of the University of Heidelberg.

2.1.3 Antibodies

Polyclonal AN2-domain specific antibodies: pcGST-LamG1, pcGST-LamG2, pcGST-extra, pcGST-cyto; produced in Trotter lab against specific segments.

Polyclonal Ezrin: Upstate; generated against a synthetic peptide corresponding to 479-498aa

Polyclonal Syntenin: Synaptic Systems; generated against synthetic peptide corresponding to 286-298aa in mouse, Group of Fritz Rathjen, MDC Berlin generated against GST-syntenin-1 (Koroll et al., 2001).

Polyclonal NCAM: (Trotter et al., 1989)

Polyclonal PLP: P6; generated against the carboxy-terminal, Group of Chris Linington

Polyclonal Phospho-Threonine: Upstate Biotechnologies

Polyclonal EGFP: Clontech; generated against full-length rGFP

Polyclonal L1: Schachner Lab

Phalloidin-FITC/TRITC: Sigma

Polyclonal PDGF α R: Santa Cruz Biotechnology; generated against a peptide mapping at the carboxy-terminus.

Rat Monoclonal and Polyclonal AN2 (mcAN2 and pcAN2, Niehaus et al, 1999); polyclonal AN2 antibody produced against NG2 purified from postnatal day six mouse brains.

Rat Monoclonal NCAM: BSP2, glycoprotein fraction of neonatal mouse brain used as immunogen.

Mouse Monoclonal GRIP: BD Biosciences; recognises within 877-1067aa in mouse.

Mouse Monoclonal Tubulin: Sigma, clone DM1A

Mouse Monoclonal MOG: Clone 8-18C5 (recognises an extracellular epitope),
Group of Chris Linington

Mouse Monoclonal CNPase: Sigma, clone 11-5B

Mouse Monoclonal L1: Clone 555, Group of Fritz Rathjen, MDC Berlin

Mouse Monoclonal beta-Dystroglycan: Group of Stephan Kröger, Uni Mainz

Mouse Monoclonal alpha-Dystroglycan: Upstate, clone IIH6C4

Monoclonal Alexa543-conjugated anti-BrdU: Sigma

Fluorescently coupled secondary antibodies, Horse radish peroxidase coupled antibodies for ECL and goat-anti-rat and rabbit-anti-rat bridge antibodies were obtained from Dianova, Hamburg, Germany.

2.1.4 Bacterial cells

E.coli TOP10F', E.coli TOP10, Competent bacteria (Invitrogen)

2.1.5 Kits and Markers

Amersham-Pharmacia: Hi-Trap Column, SilverQuest Staining Kit

Invitrogen: NuPage Pre-cast gels and accessories

ProMega: Cell Titer Non-radioactive Cell Proliferation Assay Kit

Qiagen and ProMega: Maxi and Mini-Prep Kits

Stratagene: Mycosensor PCR Assay Kit

Biorad: Precision Plus Protein Standards

Invitrogen: HMW Standard, Magic Mark, Prestained marker

Sigma: High and Low Molecular weight Markers, Silver Stain SDS MW Mix

2.1.6 Membranes and Films

Amersham Biosciences: Hybond-P {polyvinylidene difluoride (PVDF) membrane},
HyperFilm MP for visualising proteins analysed by Western blots.

2.1.7 Plasticware

Sarstedt (Cell culture disposables), Nunc (Cell culture disposables), Falcon (15ml, 50ml tubes), Greiner (syringes), Millipore (filters), Eppendorf (tips and gridded coverslips).

2.2 METHODOLOGY

2.2.1 CELL CULTURE

2.2.1.1 Solutions and Media

Solutions and Media are listed alphabetically.

BME (Basal Medium Earles's) /10%HS: 9.34g/l BME, 2.2g/l NaHCO₃, 2.24g/l alpha-D-Glucose, 10000 i.u/l penicillin, 10000 i.u/l streptomycin, 2mM L-Glutamine (0.2923 g/l), 10% heat-deactivated at 57°C Horse Serum.

Collecting Medium for FACS: 20mM HEPES, 10%HS, 2mM Penicillin-Streptomycin

COS Medium: 10% Fetal Calf Serum, 2mM Penicillin-Streptomycin, DMEM, steri-filtered.

DMEM: Dulbecco's Modified Eagle's Medium (Vicario-Abejon et al., 1995): 1mM Sodium pyruvate, 4mM L-Glutamine, 5 µg/ml insulin, 100µM putrescine, 100 µg/ml transferrin, 20nM progesterone and 30nM Sodium-selenite in DMEM.

HBSS (Hanks Balanced Salt Solution): For 1000ml, 0.4g KCl, 0.06g KH₂PO₄, 8g NaCl, 1.13g Na₂HPO₄xH₂O, 1g alpha-D-Glucose, 10mg Phenol Red, sterile.

10 X HBSS+: For 1000ml, 80g NaCl, 4g KCl, 0.6g Na₂HPO₄xH₂O, 0.6g CaCl₂xH₂O, 10g Glucose, 0.1g Phenol Red, 23.8g HEPES.

HEK and 293THEK Medium: 10% heat-deactivated at 57°C Fetal Calf Serum (FCS), 2mM L-Glutamine, 2mM Penicillin-Streptomycin, DMEM.

L-Glutamine: 200mM stock solution. Used at final concentration of 2µM

MEM:10ml 10x MEM, 90 ml H₂O, 0.22g NaHCO₃

Methods

Penicillin-Streptomycin: For stock solution 3,16g Benzyl Penicillin (Penicillin G), 6,75g Streptomycin-sulphate dissolved in 500ml distilled water and aliquoted as stock solution. 10ml /litre for final usage.

Poly-L-Lysine: 12,5ml 10xPLL, 97.5ml H₂O, steri-filtered, 10xPLL prepared as stock solution 1mg/ml

Sato (Bottenstein and Sato, 1979); modified as in Trotter et al., 1989): 13.4g/l DMEM, 0.2% NaHCO₃, 0.01mg/ml insulin, 0.01mg/ml transferrin, 200nM Progesterone, 100µM Putrescine, 220nM Sodium selenite, 500nM 3,3', 5-Triiodo-L-thyronine (T3), 500nM L-Thyroxine (T4), 25µg/ml gentamycin, steri-filtered.

Sodium butyrate: 1M Sodium butyrate in H₂O.

Sorting Medium for FACS: Sorting Medium with 5% Dissociation Buffer (GIBCO), 10mM HEPES, 10µg/ml DNase

Trypsin-Low: 1% trypsin (6ml 10xHBSS+, 54ml H₂O, 40ml 2.5% trypsin solution, 50mg DNase, adjust pH to 7.8), 0.2% EDTA in HBSS-, sterile-filtered.

2.2.1.2 Culture of Cells

Culture of COS cells

COS cells were grown in COS medium on uncoated plastic flasks or petridishes at 37°C in 5% CO₂. The cells were passaged by releasing them from plate or dish with trypsin-low. Trypsinization was stopped with BME/10% HS, the cells collected by centrifugation (10 min at 800rpm or 130g) and seeded at required density.

Culture of HEK293 cells

HEK cells were grown in HEK medium on uncoated plastic flasks or petridishes at 37°C in 5% CO₂. The cells were passaged by releasing them from plate or dish with trypsin-low. Trypsinization was stopped with BME/10% HS, the cells collected by centrifugation (10 min at 800rpm or 130g) and seeded at a density decided by the assay in use.

Culture of *Oli-neu*

Oli-neu was grown in Sato-Medium in PLL- coated plastic flasks or petridishes at 37°C in 5% CO₂. PLL coating was carried out by incubation in PLL for a minimum of 15 minutes followed by washing thrice with PBS. The cells were released from plates with trypsin-low. Trypsinization was stopped with BME/10% HS, the cells collected by centrifugation (10 min at 800rpm or 130g) and seeded at the required density.

2.2.1.3 Fluorescence Activated Cell Sorting (FACS)

The cells, in this case NG2delHEK cells expressing the NG2del construct were grown in flasks for 3 days or until 95% confluent. The cells were released by incubating them with 0.01% Trypsin/0.02% EDTA in HBSS- until they floated free in the medium. All steps henceforth were carried out on ice. Soya-bean Trypsin Inhibitor was immediately added and the cells transferred to a tube containing 10x excess of chilled culture medium with 10% HS. The cells were recovered by centrifugation for 10 minutes at 130g and resuspended in a minimal amount of medium and divided to prepare the sorting fraction and the unstained control. Polyclonal AN2 antibody was added at the dilution used for immunofluorescence (1:800). The suspension was gently mixed by flicking and left on ice for 20 min with periodic mixing. The cells were then overlaid on an equal volume of cushion of Horse Serum with the help of a plugged Pasteur pipette and centrifuged at 300g, 10 min. The supernatant was removed and 1 ml of medium was added for titration through progressively finer (three different bores were used) bores of Pasteur pipettes to give a single cell

Methods

suspension. The cells were then counted and suspended to a final cell count of 1 million/ml. The secondary antibody was added for 15 min and mixed. The secondary antibody was added also to the 'control' to gate out autofluorescence. The cell suspension was under-laid again with Horse Serum and centrifuged at 800rpm, 5 min. The pelleted cells were titrated, this time in Sorting Medium with 1µg/ml Propidium Iodide. The suspension was filtered to eliminate cell clumps before applying to the sample tube of the cell sorter.

The first cells are allowed to run through the machine so that the sort parameters could be set, and dead and weakly fluorescing cells can be gated out. Samples of the positive and negative sort fractions are checked under a fluorescence microscope to check setting of the sorter gates. Deflected fractions are collected in Collecting Medium, centrifuged and plated in the growing medium at regular intervals.

2.2.1.4 Migration Assay

The Migration Assay is based on the Wound Assay

Previous work from this lab had shown an involvement of NG2 in migration. Migration assays were performed with clusters of cells forming spheres. A single cell suspension was cultured under rotation at 70 rpm. Aggregates formed were plated and the distance/radius the cells migrated out was measured.

However this assay suffered from certain disadvantages. While primary cultures usually do form spheres in rotating cultures, several cell lines have difficulty in forming cell aggregates. Moreover, it takes considerable time to make detectable observations. This introduces the chances of errors arising from proliferating cells. Lastly, the volume of medium and therefore antibody needed is relatively large.

To overcome these shortcomings, an assay based on the typical Wound Assay used frequently in immunological studies was developed (Kodama et al., 2003; Li et al., 2003). It is based on the principle that damage to a cell layer would be repaired by the movement of uninjured cells into the injured area from which cells have been removed, thus restoring the continuity of the layer (Fig

Methods

2.2.1.4.1). The assay consists of monolayer of cells seeded on gridded coverslips. It can be adapted to any cell type that can attach on coated coverslips. In our experiments, single cell suspension of *Oli neu*, released by trypsin were seeded at a density of 30000 cells per coverslip. Coverslips were coated with PLL. The cells were grown overnight in Sato / 1%HS. The following day, scratches were drawn on every coverslip with 200 microlitre sterile, plugged tips, washed with PBS and carefully transferred to Nunc four-well plates. Total volume of 300 microlitre of medium was added in each well. Except for the 'Control' well (without any antibody), antibody(s) were added to all wells. To simulate untreated conditions, 1X sterile PBS was added to the Control well. A particular frame (the area where the scratches form a cross or an arm of the cross) in the wounded area was photographed every two hours for a total period of 14 hours (Fig 2.2.1.4.2, A). During each experiment, positive and negative controls were included. Treatment with 80µg/ml polyclonal AN2 antibody (generated against the whole AN2 molecule purified from mice brains) was used as a positive control. The percentage area covered by the cells in the frame of interest with respect to time was measured at each time point. Calculation (Fig 2.2.1.4.2, B) of percentage area covered by cells was performed with the IPLAb Software (Scanalytics Inc.). The values thus obtained were then normalised with respect to the area covered at time zero for that particular frame. Since the percentage area covered by cells at time point zero varied with each 'wound', normalisation allowed the elimination of this variance. For each time point, the normalised percentage area covered was plotted with respect to time.

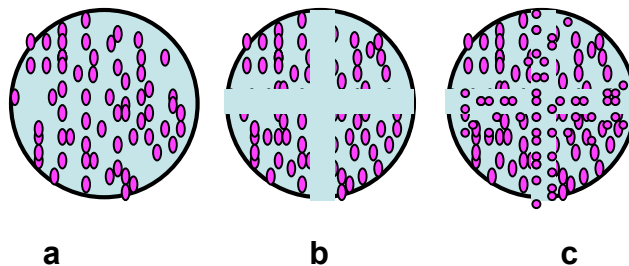


Figure 2.2.1.4.1
Schematic diagram of the assay.

The assay is based on the typical Wound Assay. (a) Cells were seeded at optimum density (b) 'Wound' scratched on cell monolayer at time 0hr (c) Rate of movement of cells into cell-free 'wounded' area measured as percentage area covered by cells with time.

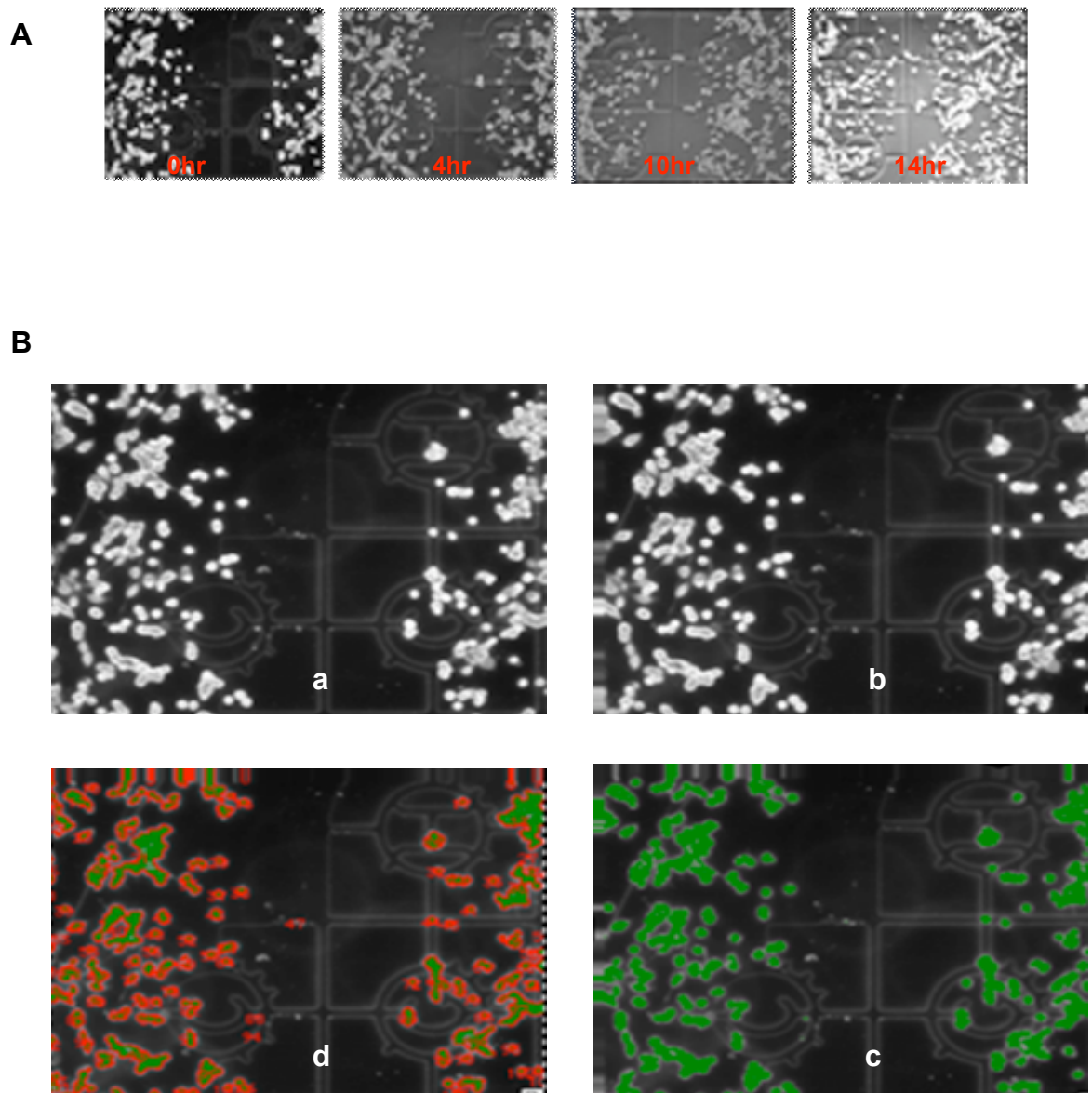


Figure 2.2.1.4.2
Steps of analysis of the Migration Assay

A: Example of frame-by-frame movement of cells into 'wounded' area. Four time points shown; 0hr, 4hr, 10hr, 14hr

B: Using the IPLab software to measure percentage area covered by cells.

(a) Original File **(b)** Conversion to grayscale **(c)** Segmentation **(d)** Quantitation of percentage area

The normalized percentage area covered by cells with time indicated rate of movement. The data were analysed with Microsoft Excel.

2.2.1.5 Transfection

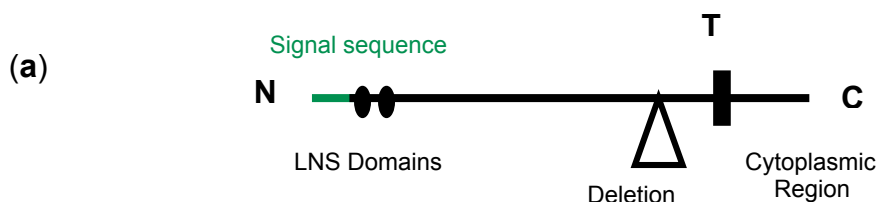
By Electroporation

Cells were grown to 70% confluency. Thereafter the cells were harvested by trypsinisation and washed in BME/10%HS before being centrifuged at 800rpm for 10 min to pellet them down. The cells were re-suspended in media at a density of 3-5 million cells/ml. 300µl of cell suspension was electroporated with 10-20µg of plasmid DNA after the cuvette had been incubated on ice for 5 min. Transfection parameters differed according to cell type and was dependant on the instrument used. As an example, transfection parameters used for 300-600µl *Oli neu* cell suspension with the Biorad GenePulser Xcell in a 4mm cuvette requires 220V and 950µF. The cuvette was returned to 4°C and the transfected cells plated immediately on PLL-coated dishes.

For transient transfections 2mM Sodium-butyrate was added when using constructs with pCMV promoters.

When multiple plasmids were transfected, the total DNA amount was kept constant at 20µg and the amount of DNA for each plasmid is decided according to its transfection efficiency. For example co-transfection of EGFP-Syntenin and NG2del requires 10µg DNA of each construct.

For making stable lines an antibiotic-resistance plasmid is electroporated along with the plasmid containing the protein of interest. Thereafter, the cells are selected for incorporation of both plasmids by growing them in the presence of the antibiotic for 4 weeks. For establishment of the NG2delHEK cell line the pBabe and NG2del (Stegmuller et al., 2003) construct were co-transfected. Four different concentrations of hygromycin were used to select for HEK293 cells expressing both plasmids.



(b)

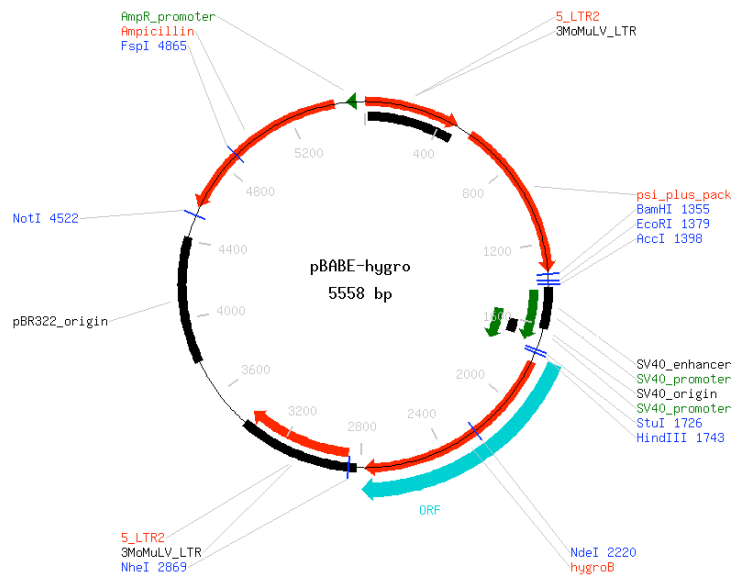


Figure 2.2.1.5.1

Schematic diagrams of the constructs used for cotransfection.

(a) The NG2del construct possesses the full cytoplasmic and transmembrane segments and one-fourth of the extracellular segment towards the N-terminal including the two LNS domains. (b) The pBabe possesses a hygromycin resistance gene.

By FuGENE

Cells were grown overnight at a cell density of 1 million/ml in the minimum amount of medium necessary. DMEM:Reagent ratio of 3:1 was used according to manufacturer’s instructions. To 97µl of DMEM, 3µl Reagent was added and mixed, in that order and incubated for 5 min. 1µg of DNA was added to the pre-mixed FuGene. This was gently mixed again and allowed to incubate for 30 min. The mixture was added dropwise to the petridish containing the cells and swirled for even distribution. The cells were allowed to grow overnight. The cells can be re-transfected if the efficiency is low.

2.2.2 IMMUNOFLUORESCENCE

2.2.2.1 Solutions

Solutions are listed alphabetically.

Blocking Solution: 10ml Horse serum in 100ml BME

DAPI (4',6-Diamidino-2-phenylindole) : Stock 5mg/ml dissolved in PBS; Final dilution 1:10000

Moviol: Dissolve 2.4g moviol in 6ml glycerine and 6ml water overnight. Add 12ml 0.2M Tris/HCl pH 8.5, heat for 1hr at 56°C, centrifuge 15 min at 4000g and aliquot the supernatant. Store at -80°C.

Neutralisation Solution for BrdU staining: 9,5g Borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in 250ml H_2O . pH adjusted to 8,5 with 12N HCl.

4% Para-formaldehyde (PFA): Dissolve 20g 37% PFA in 350ml 60°C water by adding a few drops of NaOH. Add 50ml 10X PBS , cool, adjust p to 7.3 and fill up to 500ml with water.

Permeabilisation Buffer: Dissolve 10 μl TX100 in 10ml PBS

Permeabilisation Solution for BrdU staining: 12ml H_2O , 6ml 37% HCl, 90 μl Tween 20.

2.2.2.2 Immunocytochemical stainings of cells

For labelling with antibodies, cells on coverslips were first washed in PBS. Cells were fixed (except for live staining) with 4% PFA for 10 min, washed, and incubated with BME / 10% HS for 15 min. Cells were permeabilised for intracellular proteins with 0.1% T X 100_in PBS, followed by PBS wash and blocking for 10 min. All antibodies were diluted in Blocking Solution. Primary antibodies, except when live staining was necessary, were added together for

30 min. For live staining, the primary antibody was added after blocking the cells for 15 min with Blocking Solution (BME / 10% HS). The cells were then washed and incubated with fluorescently labelled secondary antibodies for 15 min. To visualise nuclei the cells were incubated for 10 mins with DAPI (1:10000) or VectaShield Hardset, followed by washing thrice with PBS, rinsing in water and mounting in moviol.

2.2.2.3 Bromo-deoxy-Uridine (BrdU) Staining

Cells on coverslips were washed in PBS before being fixed in 4% PFA for 10min. They were again washed with PBS before adding the Permeabilisation Solution for 20min. The Neutralising Solution was used afterwards to wash the Permeabilisation Solution. This step was repeated thrice. At each washing step, the wash solution was allowed to rest for 2 min. The cells were washed again in PBS before adding the BrdU antibody (1:100) diluted in 0.5% Tween in DMEM or PBS. BrdU marks only mitotically active cells; to visualise all nuclei, the cells were further incubated with DAPI for 5min. The cells were then again subjected to PBS wash, dipped in water before being mounted. BrdU staining can also be coupled with other primary antibody. The VectaShield Hardset does not work with the BrdU protocol.

2.2.2.4 Capping of Surface molecules

Primary oligodendrocytes (*2div*) on coverslips were incubated with monoclonal AN2 antibody (mcAN2) at a dilution of 1:10 for 10 mins at 4°C. The primary antibody (mcAN2) was then washed away with PBS and incubated with goat-anti-rat secondary antibody coupled to Cy2 for 20 mins at 37°C, or 4°C: the latter for control cells. The secondary antibody was washed away and the cells fixed with 4% PFA for 5 mins. The cells were then permeabilised with 0,1% TX100 for 5 mins, blocked with BME/10%HS and incubated overnight with pcSyntenin. The goat-anti-rabbit secondary coupled to Cy3 against pcSyntenin antibody was added the next day, before mounting the cells in moviol.

2.2.3 BIOCHEMISTRY

2.2.3.1 Solutions

Solutions are listed alphabetically.

Electrophoresis and Western Blot

Blocking Solution: 4% milk powder in PBS-Tween

Butanol over water: 50% Butanol, 50% H₂O. Shake vigorously and then allowed to settle.

Coomassie Stain G250: 34% Methanol, 2% phosphoric acid, 17%(w/v) ammonium sulphate, 0.066%(w/v) Colloidal Coomassie Blue, 47% H₂O.

De-staining Solution: 10% Isopropanol, 10% Acetic acid, 80% H₂O.

Developing Solution for silver staining: 2.5% Na₂CO₃ in H₂O, 25µl formaldehyde

Fixation solution

(1) For Coomassie: 50% methanol, 2% phosphoric acid, 48% H₂O

(2) For Silver staining: 30% ethanol, 10% acetic acid, 60% H₂O

Gel Solutions:

30% Acrylamide/Bis-acrylamide solution

Lower Buffer: 1.5M Tris pH 8.8, 0.4% SDS

H₂O

2,25g Sucrose in higher than ten percent gels

Upper Buffer: For Stacking gels. 0.5M Tris, pH 6.8, 0.4% SDS

10% Ammonium peroxy-disulphate

TEMED

Methods

Gradient Gels

For gradient gels, differing percentages of Separating gel solutions were freshly prepared. Polymerisation was started by adding 60µl 10% APS and 6µl TEMED (Sambrook et al., Mol. Clon. Vol.3, Appendix A8.9-8.10). These mixtures were poured into a gradient mixer and a peristaltic pump used for an even layering. 50% Butanol was overlaid.

Incubation Solution for Coomassie staining: 35% methanol, 2% phosphoric acid, and 17% ammonium sulphate

10X Phosphate buffered saline (PBS): For 1l, 80g NaCl, 2g KCl, 2g KH₂PO₄, 14.4g Na₂HPO₄·xH₂O.

PBS-T: 0.1% Tween-20 in 1 X PBS

PonceauS solution: 0.2% w/v PonceauS, 3% w/v Trichloroacetic acid, H₂O.

Reducer Solution for silver staining: 30% ethanol, 2.5% 4M Na-acetate, pH 6 in 100ml H₂O, 0.1% Sodium thiosulphate in 100ml H₂O

10X Running Buffer: 1.9M Glycine, 0.25M Tris, 1% SDS, pH 8.8, Used at 1X.

4X Sample Buffer: 62.5mM Tris-HCl, 10% Glycerine, 3% beta-Mercaptoethanol, Bromophenol Blue, pH 6.8.

Strip Buffer: 0.2M HCl, adjusted to pH 2.00 with 1M Glycine

Transfer Buffer 1: 0.3M Tris, 20% Methanol, pH 10.4

Transfer Buffer 2: 0.025M Tris, 20% Methanol, pH 10.4

Transfer Buffer 3: 0.04M amino-n-caproic acid, 0.025M Tris, 20% Methanol, pH 9.4

Methods

Immunoprecipitation and Pulldown Assays

Ampicillin: Stock solution of 175mg ampicillin in H₂O

DMEM minus Cys-Met: DMEM (see section 2.2.1.1), 5mM L-Cysteine, 5mM L-Methionine, steri-filtered.

Glutathione reduced: 10mM reduced Glutathione, 50mM Tris, pH 8.0

Glutathione Sepharose: 1ml of ethanol suspended Glutathione Sepharose beads were washed thrice before use in 10ml PBS.

Homogenisation Buffer:

(1) Immunoprecipitation from whole brain: 1mM NaHCO₃, 3mM CaCl₂, 2.5mM MgCl₂, 1mM Spermidine, protease inhibitor complex

(2) For GST-pulldown experiment: 20mM HEPES-NaOH pH 7.4, 3mM CaCl₂, 2.5mM MgCl₂, protease inhibitor complex

(3) For Synaptosomes: 0.32M Sucrose, 5mM HEPES pH 7.4, protease inhibitor complex, phosphatase inhibitor complex

IPTG (isopropyl-beta-D-thiogalactopyranoside): Stock solution of 1M stock in H₂O

Luria-Bertani (LB) Medium: For 1l, 10g tryptone, 5g yeast extract, 10g NaCl, distilled H₂O, pH was adjusted to 7.2. Solid media were supplemented with 2% agar.

Lysis Buffer: 150mM NaCl, 20mM Tris pH 7.4, 0.5% TX100, 3mM CaCl₂, 2.5mM MgCl₂, fresh cocktail of protease inhibitors to be added.

Phosphatase Inhibitor complex: 100µM Na₃VO₄, 10mM Sodium Fluoride

Protease inhibitor complex: Iodoacetamide (18mg/ml H₂O), PMSF (100mM in Isopropanol), Pepstatin (5mg/ml in DMSO), Antipain (1mg/ml in DMSO),

Methods

Aprotinin (1mg/ml in water), Benzamidine-HCl (26mg/ml in H₂O, Leupeptin (5mg/ml in DMSO)

Protein A Sepharose: 300mg of lyophilised Protein A Sepharose (PAS) with 10 ml Solubilisation Buffer with 1% TX100. Left on rocker overnight at 4°C. The swelled beads were then resuspended in 10ml of Homogenisation Buffer. 0.02% Sodium azide was added to preserve the beads.

Protein G Sepharose (PGS): 1ml of Protein G Sepharose beads washed in 10 ml of PBS, thrice. Pelleted and stored with 0.02% Na-azide.

Coupling bridge antibody (rabbit anti-rat, goat anti-rat) to PAS or PGS: 50µl antibody added to 1.6ml bead solution, rotated overnight at 4°C, centrifuged at 3min at 3000rpm, followed by removal of supernatant and resuspended in equal volume of triton-solubilisation buffer.

Re-IP Buffer: 50mM Tris pH 7.4, 0.5% SDS, 1% β-mercaptoethanol

Resuspension Buffer for Synaptosomes: 0.32M Sucrose, 5mM Tris-HCl, pH 8.1

RIPA Buffer: 50mM Tris HCl pH 7.2 or 7.5, 150mM NaCl, 1% TritonX-100, 1%Deoxycholate, 0.1% SDS, 1mM DTT, Protease inhibitors

Sato-Met-Cys: Sato Medium (Section 2.2.1.1), 5µM L-Cysteine, 5µM L-Methionine.

Sucrose Solution for synaptosomes: 0.85M, 1.0M, 1.2M sucrose solutions in 5mM Tris/HCl pH 8.1

Solubilisation Buffer (S.B.) I and II: 20mM Tris/HCl, 150mM NaCl, 3mM CaCl₂, 2.5mM MgCl₂. S.B. I and II contain 1% Triton X 100 and 1% NP-40, respectively.

Methods

Sulphate-free medium (Starvation Medium): 1ml MEM, 2mM Glutamine, 10mM HEPES (0,2383g N-2-Hydroxyethylpiperazine N`2-ethansulfonic acid), 50µl Gentamycin

Protein concentration and purification

Acetone/TCA: 12.5% TCA, Acetone, 3mg/ml DTT. Chilled at -20-°C

Binding Buffer for antibody purification: 20mM sodium phosphate, pH 7.0

Elution Buffer for antibody purification: 0.1M Sodium citrate, pH 3.5

2.2.3.2 Western Blot

Before analysis by Western blot, all samples were denatured at 95°C, 10min in 4X Sample Buffer and analysed on gels in 1x running buffer. The gels were then blotted in a semi-dry blot chamber. The time and current used for protein transfer onto the PVDF-membrane was dependant on the size of the gel, the molecular weights of the proteins to be analysed and the blotting chambers used. The blot chamber was packed from plus pole onwards as follows: 3 layers Whatman paper soaked in transfer buffer 1, 3 layers Whatman paper soaked in transfer buffer 2 followed by PVDF-membrane activated for 3 min in methanol, the gel, and then 4 layers of Whatman in transfer buffer 3. After blotting, the membrane was stained with PonceauS solution for 10 min and washed in H₂O to ensure proper transfer and define the markers and lanes. The membrane was blocked for 1 hour in Blocking Solution, incubated 1 hour or overnight in primary antibody diluted in blocking solution, washed thrice for 10min in PBS-T, incubated for 30 min in HRP-coupled secondary antibody and washed again in PBS-T followed by PBS. The signal was developed with chemiluminescence using ECL followed by exposure of the membrane to X-ray sensitive films.

2.2.3.3 Gel Staining

Silver Staining of gels

All steps were at room temperature. The solutions were discarded in a special canister. Gels were fixed by shaking gently in Fixation solution (2) for silver staining in two steps of 15 min or overnight. It was then shaken in the Reducer solution for 30 min and washed thrice in water for a total period of 30 min. At each step the solution from the previous step was discarded. The Silver solution was then added for another 30 min. 25µl formaldehyde may be added at this stage if the gel is not to be used for mass spectrometry. The silver solution was removed and the gel briefly washed with water. The Development solution was then added to the washed gel along with 25µl of formaldehyde and the gel shaken to develop the bands. To stop the reaction the gel was washed in water with 1% acetic acid.

Coomassie Staining of gels

The gel was fixed overnight in Fixation solution (1) for Coomassie staining. All steps were carried out at room temperature. It was then washed thrice in water, each lasting 30 min. This was followed by an incubation step in Incubation solution for 1 hour. For staining in the Coomassie Staining Solution, the gel was left to shake gently for up to 5 days. Water was used for destaining, when necessary.

2.2.3.4 Immunoprecipitation

All steps were carried out at 4°C and with pre-cooled reagents.

Immunoprecipitation (IP) from both radioactively labelled and unlabelled cell lysates were performed. For a radioactive immunoprecipitation, the cells (either transfected or non-transfected) were grown in 6cm petridishes at an approximate density of 1 million cells per plate. The cells were starved for 1 hour in Sulphate-free medium, followed by radioactive labelling by addition of 100µCi/ml [³⁵S]-*in vitro* Cysteine-Methionine labelling mix for 4 hours or overnight

Methods

at 37°C, 5% CO₂. The radioactive medium was then removed and the cells washed with HBSS- with cold Cysteine and Methionine, before adding the Lysis buffer to the cells for 30 min on ice to prepare the lysates. To the collected lysate or homogenate was added a cocktail of fresh protease and phosphatase inhibitors. Smaller organelles, membrane fragments and nuclei were removed by a 10 min 13000rpm centrifugation. Proteins binding non-specifically were removed with a 'pre-clear' step by incubating supernatants with PAS and PGS (with or without the bridge antibody complex) or both sequentially for 1 hour on a wheel. After a brief centrifugation step of 3min at 3000rpm, the supernatant was transferred to new tubes and incubated with primary antibody(s), overnight. The following day the samples were incubated with the beads (with or without the bridge antibody complex) for 1 hour, centrifuged (1 min, 3000rpm) and washed at least four times.

The wash buffer was RIPA buffer. The washed beads were resuspended in 4X Sample Buffer for denaturation at 95°C and subsequent analysis by electrophoresis, or re-eluted with Re-IP Buffer for concentrating the precipitated protein(s).

Immunoprecipitation from whole mouse brain

All steps were carried out at 4°C and with pre-cooled reagents.

Mice brains were homogenized in Homogenisation Buffer (1), with Potter Homogeniser. Cell debris and nuclei were removed by a 10min, 200g run, followed by a 100000g run for 1 hour to pellet only membrane fractions. The membrane fraction was extracted with Solubilisation Buffer I and II for 1 hour at 4°C. After each extraction, the solution was centrifuged for 1 hour at 100000g and the supernatant in each case saved to be ultimately pooled. The pooled brain extract was divided into a 'control' (no primary antibody added) and 'mcAN2' fraction. Monoclonal AN2 antibody was added at a dilution of 1:10. The immunoprecipitation method has been described in Section 2.2.3.4.

2.2.3.5 GST-Pulldown Assays

All steps were carried out at 4°C and with pre-cooled reagents.

150µg of GST-Fusion Proteins (GST-LNS1, GST-LNS2 and GST-LNS(1+2)) were attached to 100µl Glutathione-Sepharose beads by incubating them together for 4 hours.

Mice brains were homogenized in Homogenisation Buffer (2), with Potter Homogeniser. Cell debris and nuclei were removed by a 10min, 200g run. To the supernatant was added the Extraction Buffer for 1 hour. The extracted solution was centrifuged for 30 min at 100000g and the supernatant supplemented with 3mM CaCl₂ and 2.5mM MgCl₂. To the above brain homogenate, GST-fusion proteins coupled to Glutathione-Sepharose beads were added and left to incubate overnight. The beads were centrifuged down, next day and washed thrice with PBS or PBS-T. They were then incubated with 10mM reduced Glutathione to release the pulled-down protein(s).

2.2.3.6 Preparation of Brain Membrane Fractions

All steps were carried out at 4°C and with pre-cooled reagents.

Preparation of Synaptosomes

Mouse brains were homogenized in Homogenisation Buffer (3) for synaptosomal preparation with a Teflon Douncer and centrifuged for 10 min at 1000g. This procedure was repeated twice and the combined homogenate was centrifuged at 12000g for 15 min. The pellet was resuspended in Homogenisation Buffer (3) and subjected to another step of homogenisation before centrifuging at 12000g for 20 min. The pellet thus formed was re-suspended in Resuspension Buffer and loaded on a sucrose step gradient. The step gradient is prepared by layering the highest density of sucrose at the bottom of the tube followed by lower percentage sucrose solutions (0.85; 1.0; 1.2M). The sample was loaded on top of the gradient. This was centrifuged at 85000g for 2 hours. On top of the sucrose gradient lies the 'Myelin' fraction. At the next interface of (0.85/1.0M) the 'light membrane' fraction is formed.

Methods

Synaptosomes are harvested at the (1.0/1.2M). At the bottom is found the 'mitochondrial' fraction.

Preparation of Axolemmal Fraction

The same protocol as above was carried out to obtain the 'Myelin' fraction. To the myelin, chilled H₂O was added and mixed before briefly homogenizing it. The osmotic shock step loosens the membrane. The shocked myelin is loaded on another sucrose step gradient (10%-40%) and centrifuged overnight at 81000g. The axolemmal fraction is collected from the 28%-32% fraction.

2.2.3.7 Concentrating proteins

All steps were carried out at 4°C and with pre-cooled reagents.

The TCA/Acetone solution was prepared earlier and left to chill at -20°C for at least 1 hour. DTT was added freshly. Directions stated are for 100µl protein solution. 400µl TCA/Acetone/DTT was added to the protein and left to be incubated for 2 hours at -20°C after vortexing. All steps were carried on ice and centrifugation steps were at 12000g and for 5 min. After incubation, the protein solution was centrifuged and the supernatant thoroughly removed before adding 1ml of chilled acetone. This procedure of washing with acetone was repeated thrice. Finally, the supernatant was removed and the pellet left to air-dry.

2.2.3.8 Antibody purification by Immunoaffinity

All steps were carried out at 4°C and with pre-cooled reagents.

The serum pH was adjusted to 8.0 by adding 1M Tris; 1/10th volume of serum to be purified, followed by 10000g spin for 2 min to remove any debris in the serum. Since the polyclonal antibodies were generated against GST-FP proteins, the adjusted serum was first passed through an equilibrated GST-Glutathione-Sepharose column to remove any antibody generated against the GST-fraction of the fusion protein. The eluate was then run through the Protein A Sepharose column taking care not to overload it. Manufacturer's instructions

Methods

were followed. The serum was re-run three times to extract maximal amount of IgG. The column was then washed with 6 volumes of Binding Buffer and eluted with 4 volumes of Elution Buffer. The purified IgG fractions were adjusted to a neutral pH with 1M Tris HCl of pH 9.0 and aliquoted in sterile tubes.

2.2.3.9 Protein Quantification

Bovine serum albumin stocks were diluted to give a standard curve ranging from 0 to 25µg/ml. The sample(s) were prepared in at least two dilutions in PBS. All dilutions were referred to 1000µl total volume; 200µl BioRad Reagent was added to all standards and samples. Absorbance was measured at 592nm.

2.2.3.10 Generating polyclonal Domain specific antibodies

The DNA sequence of LNS1, LNS2 and LNS(1+2) were inserted into the pGEX2T Glutathione-S-Transferase containing vector and cloned to be expressed as bacterial Glutathione-S-transferase tagged fusion proteins (GST-FP); GST-LNS1, GST-LNS2, GST-LNSG(1+2). For maximum yield the induction time and temperature were checked for every construct. The FPs produced were verified by electrophoresis and Coomassie staining for molecular weight and purity. Bacterial cultures were grown in LB medium with ampicillin to an O.D (optical density) of 0.6 at either 37°C or 30°C. IPTG was added for a final concentration of 1mM/l the next day and the cells induced for a few hours. All steps henceforth were done at 4°C. The bacterial culture was then centrifuged at 4000rpm, 10 min and the pelleted cells resuspended in PBS-T. The cells were sonicated to lyse them. The resulting homogenate was centrifuged at 10000rpm for 20 min. The supernatant is collected and loaded on an equilibrated Glutathione-Sepharose column. The GST-FPs were eluted off the column with 10mM reduced Glutathione. After each purification step, the eluted protein was quantified and checked for purity and then concentrated by lyophilisation.

A dose of 2mg at the primary immunisation in rabbit was used, followed by half that value during secondary injections and later boosts. The secondary and tertiary injections were given at a gap of 4-6 weeks and blood from the injected

Methods

animals collected two weeks after each injection. The collected blood was prepared in 50ml Falcon tubes by allowing the blood to clot for 45 min at 37°C and then using a clean glass rod to separate the clot from the collecting vessel. The clot was left overnight at 4°C for further contraction. The serum was centrifuged at 10000g for 10 min to remove insoluble material. pcGST-LNS2 was generated for the migration assays as part of the current thesis.

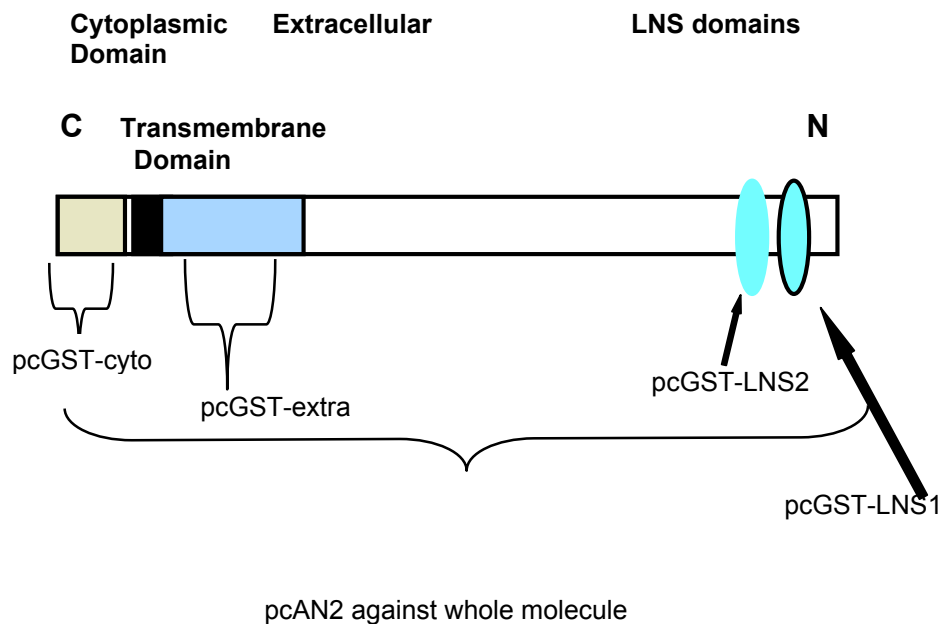


Figure 2.2.3.10.1

Polyclonal antibodies generated against NG2 (AN2)

GST-fusion proteins were injected into rabbits to generate polyclonal antibodies against specific domains of the molecule. pcAN2 was generated against the complete 2437 amino acids that encode the mouse NG2. For pcGST-LNS1 and pcGST-LNS2 amino acids 47-179 and 223-364, respectively were incorporated into the pGEX-2T vector for GST-tagged proteins. pcGST-extra was generated against amino acids 1891 to 2226. pcGST-cyto was raised against the amino acids 2252-2357 (Doctoral dissertation J.Stegmüller, Diploma dissertation B. Neubar).

2.2.4 MOLECULAR BIOLOGY

2.2.4.1 Solutions

Ampicillin: Stock solution of 175mg ampicillin in H₂O

Kanamycin: Stock solution 30mg/ml ampicillin in H₂O

Luria-Bertani (LB) Medium: For 1l, 10g tryptone, 5g yeast extract, 10g NaCl, distilled H₂O, pH was adjusted to 7.2. For solid media this was supplemented with 2% agar.

50x TAE Buffer: 242g Tris, 37.2g EDTA, 57.1ml acetic acid

2.2.4.2 Agarose gel Electrophoresis

1% Agarose gels were prepared fresh in 1x TAE Buffer by dissolving the agarose in a microwave. To the cooled gel 0.01% Ethidium Bromide was added and the gel poured into a chamber to solidify. The samples were mixed with 6x Loading Buffer and loaded on the gels and the gel ran in 1x TAE Buffer. Generally, for a minigel 45V for 45 minutes was sufficient to resolve out bands.

2.2.4.3 Maxi and Mini-Prep

DNA/plasmid were purified either with Mini or Maxi-Prep kits according to manufacturer's instructions after growing the bacteria in LB Medium.

2.2.4.4 Restriction Enzyme Digestion

Restriction enzyme digests were usually done at 37°C for an hour. 1 Unit of enzyme digests 1µg DNA. Volume and amounts were varied depending on whether a single or double digest was being performed. The total volume was either 30 or 50µl. Amount of buffers and enzymes added were according to

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manufacturer's instructions and the total volume was made up with double-distilled water.

2.2.4.5 Transformation

TOP10 competent cells were thawed on ice. 100µl of cells were taken in a sterile Eppendorf. 5µl Ligation Mix/DNA was added. The mixture was incubated at 4°C for 30 min. The cells were then immediately heat-shocked for 1 min at 37°C. 1ml of warmed (at 37°C) LB Medium was added to the heat-shocked cells and allowed to grow resistance at 37°C on a bacterial shaker. The cells were then plated, in at least two dilutions on LB-agar plates with the appropriate antibiotic and incubated at 37°C overnight on a shaker.

3. RESULTS

This section has been divided into four segments. The establishment of a stable cell line carrying the NG2del construct (Stegmuller et al. 2003) has been explained in Section 3.1. In section 3.2 the segment(s) of the extracellular portion of NG2 involved in migration has been mapped. Characterisation of the NG2-Syntenin interaction is demonstrated in Section 3.3. Finally, in Section 3.4 several approaches to identify extracellular partner(s) of NG2 have been explored.

3.1 Usefulness of a stable line possessing NG2

A mammalian cell line without other antigens common to oligodendrocytes allows elucidation of NG2's role without interference from other glial surface molecules. We established a line of HEK293 cells stably expressing the NG2del construct encoding for 741 of 2437 aa of the full length NG2/AN2 molecule. It consists of one fourth part of the extracellular region including the two LNS domains at the N-terminal and the complete transmembrane and cytoplasmic regions (Stegmuller et al. 2003).

The NG2delHEK cell line was used to confirm the NG2-Syntenin interaction; EGFP-tagged Syntenin was transfected into cells, radioactively labeled and subjected to immunoprecipitation for Syntenin and NG2 (Sect 3.3).

Additionally, the cell line was used to test for the generation of antibodies against the LNS domains of NG2 in the serum of rats with MOG-induced EAE (Experimental Allergic Encephalomyelitis) after DNA vaccination with NG2del to induce an antibody response against NG2.

3.1 Establishment of the NG2delHEK cell line

The NG2del construct was transfected into HEK293 cells along with the pBabe vector conferring hygromycin resistance. HEK293 cells were selected for four weeks with hygromycin for incorporation of both plasmids. The population surviving hygromycin selection was sorted by a Fluorescence Activated Cell Sorter to select cells expressing the highest levels of NG2del (Fig 3.1; A). This fraction (R4) represented twenty percent of the original population. Cells from R4 were further cultured and after 48 hours *in vitro* restained for NG2del expression (Fig 3.1; B). These cells were radioactively labeled and NG2del from the stable line lysate immunoprecipitated by pcAN2 (Fig 3.1; C).

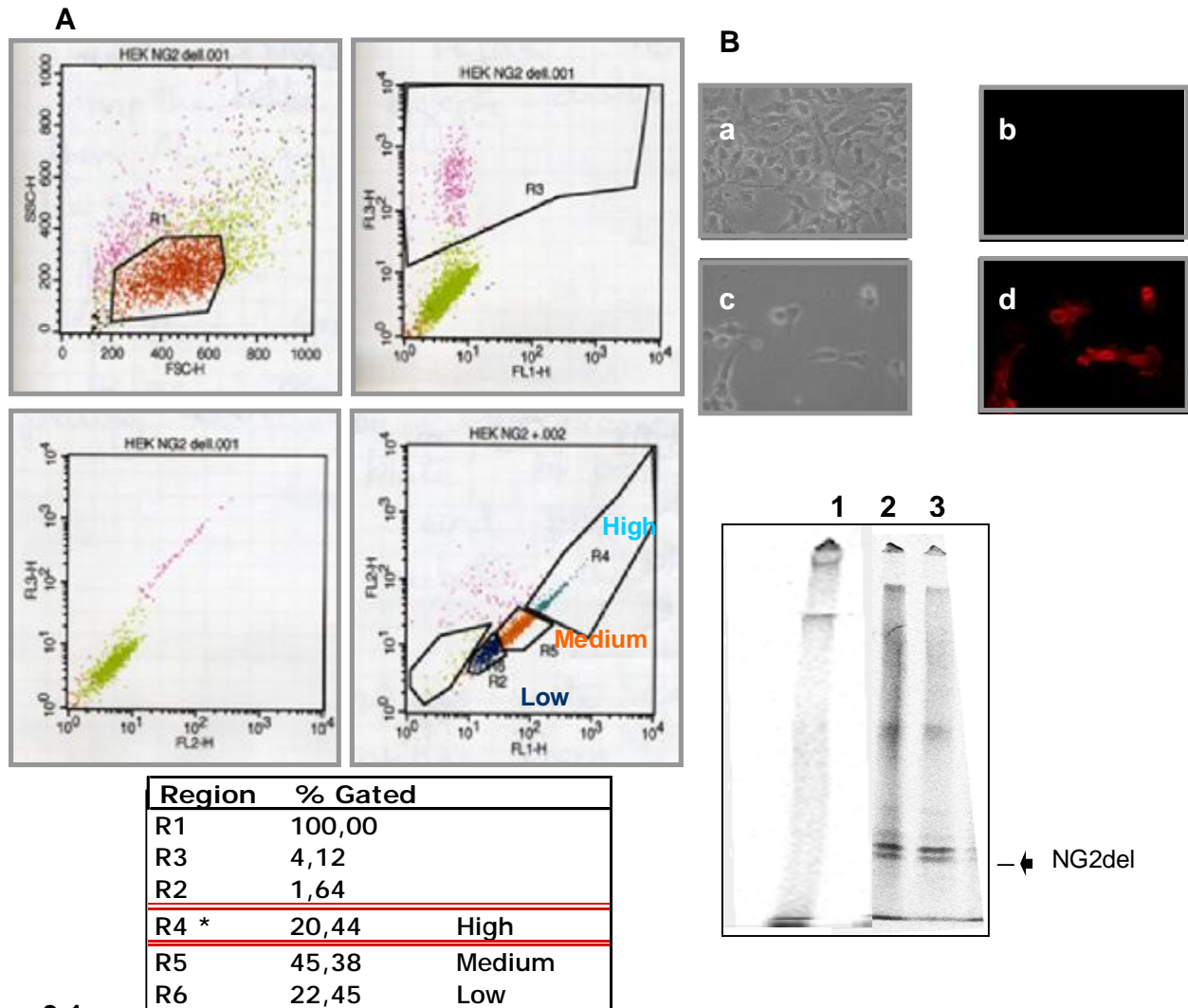


Figure 3.1
Establishment of NG2delHEK stable line

A: Fluorescence activated cell sorting of NG2del-positive cells. The four panels are dot plot representations of successive gatings to sort out negative and dead cells. The cells were sorted into three groups; low, medium and high (R6, R5, R4 respectively) expression of the stably transfected NG2del plasmid (lower right panel).

B: R4 (red-bordered fraction in the table) was further cultivated for 48 hours and stained for NG2del expression. (a and b) are phase contrast and fluorescence image respectively, of HEK293 cells without the NG2del construct, used as negative control.

(c and d) are phase contrast and fluorescence image respectively, of NG2delHEK (R4 fraction, marked with an asterisk in table).

C: Radioactive immunoprecipitation of the NG2del construct from the stable line with polyclonal AN2 against the whole molecule. Lane 1; Preclear, Lane 2 and 3; Precipitates from the stable line lysate washed with Tx100 and RIPA respectively. Arrow marks the 77 kD band corresponding to the NG2del protein.

Results

3.2 Dissecting the extracellular regions of NG2 involved in migration

Addition of polyclonal AN2 antibody against the whole molecule to oligosphere assays showed the involvement of the extracellular part of NG2 in migration (Niehaus et al. 1999).

In order to dissect the regions of the extracellular portion of NG2 which are involved in migration, we generated polyclonal antibodies against fragments of the extracellular portion and used them in migration assays.

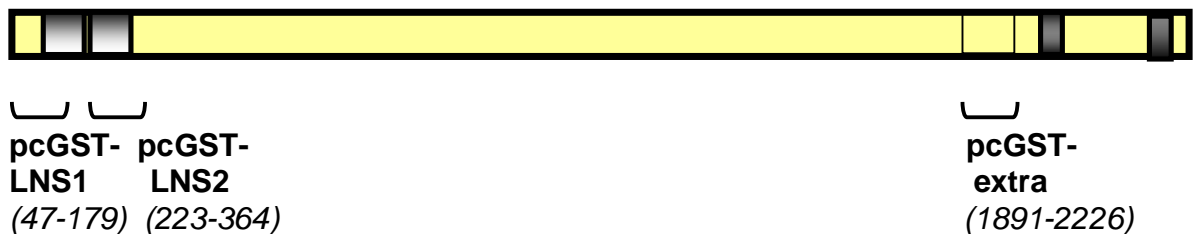


Fig 3.2 Regions of NG2 against which the antibodies pcGST-LNS1, pcGST-LNS2 and pcGST-extra were targeted.

Schematic diagram of the regions of NG2 against which polyclonal antibodies raised for usage in the subsequent migration assays. Amino acids of NG2 against which antibodies were generated are mentioned within brackets.

3.2.1 Polyclonal antibody against AN2 has no effect on proliferation

The cell migration assay measures the increase in area covered by cells. Increase in area covered by cells could result from migrating cells as well as cell proliferation. Two assays were used to measure cell proliferation, in order to exclude that our interpretations from the migration assay were influenced by cell proliferation during the time period of the assay.

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Cell Proliferation Assay (Promega) was performed on *Oli neu*. The assay measures the number of cells metabolising formazan crystals as a function of optical density at 570nm. Effect of polyclonal AN2 antibody (pcAN2) on proliferation was measured with respect to untreated cells after incubating the cells for 14 hours. pcAN2 in two differing concentrations were used. Addition of pcAN2 did not make any striking difference in the rate of proliferation of the cells (Fig 3.2.1; A).

Cells were incubated with BrdU (Bromo-deoxy-uridine) under conditions identical to those used in the migration assay. BrdU is incorporated only in mitotically active cells. The cells were then fixed and stained with an anti-BrdU antibody as well as DAPI to stain all nuclei (Fig 3.2.1; B). Twelve percent of all cells were shown to have gone through a cell cycle (Fig 3.2.1; C). Data were collected from three experiments.

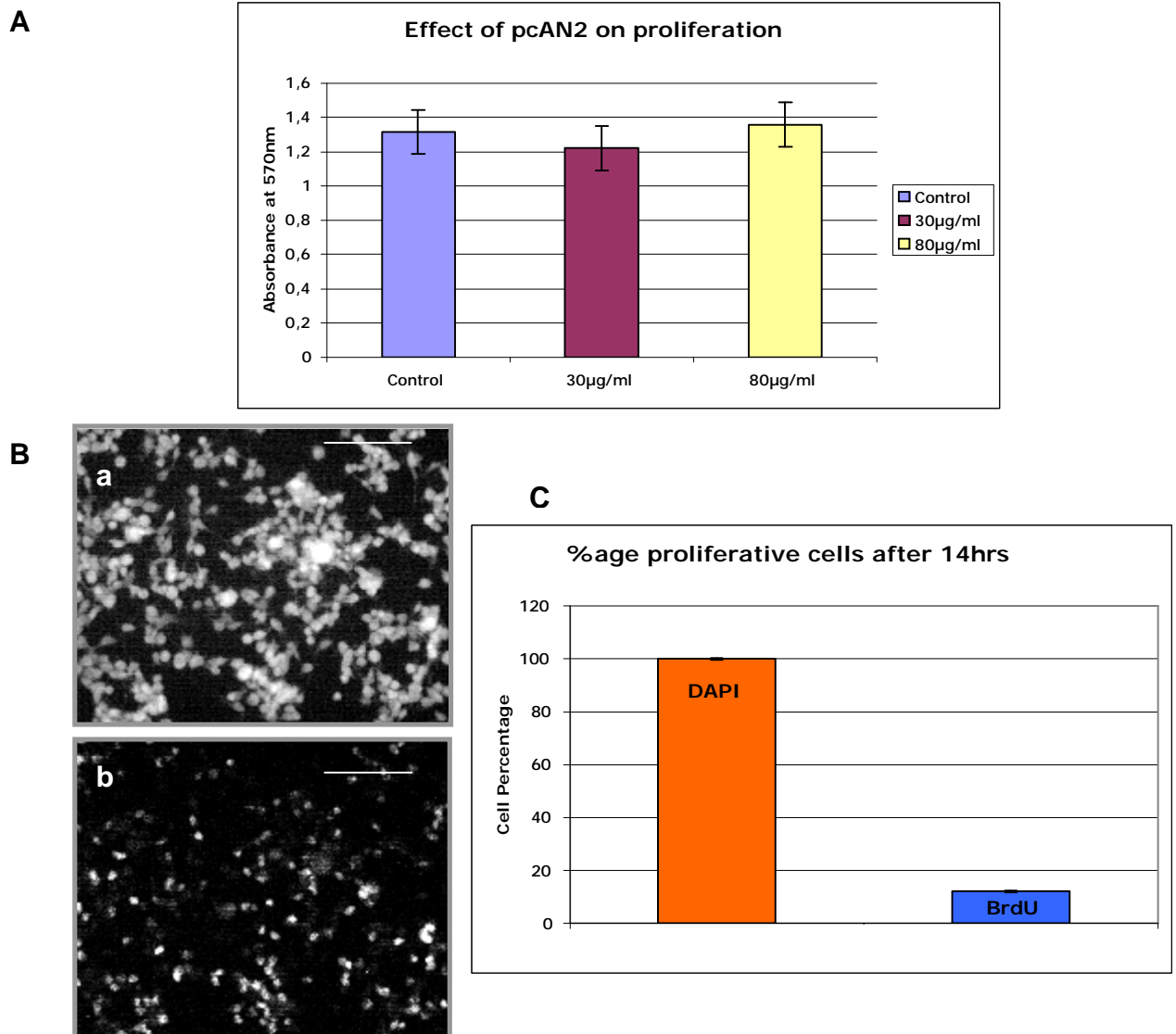


Figure 3.2.1
Polyclonal antibody against AN2 has no effect on proliferation within the 14-hour period of the migration assay

A: 30000 cells per well (*Oli neu*) were seeded and incubated for 14 hours in presence or absence of pcAN2. The rate of proliferation was then measured with the MTT Cell Proliferation assay as an index of incorporation of MTT into cells. No significant difference was observed.

B: Cell proliferation as measured by BrdU incorporation. Scale bar equals 100µm. (a) DAPI stains all nuclei. (b) BrdU is incorporated only in the nuclei of mitotically active cells (brightly stained).

C: The percentage of cells with BrdU incorporation within the 14-hour period of the migration assay was measured and plotted as percentage of total cell number. Within the 14hr duration of the assay, 12% of all cells were mitotically active.

3.2.2 High concentrations of polyclonal antibody against AN2 lead to process retraction

In previous experiments using IgG fractions of polyclonal AN2 antibody to inhibit migration in sphere assays with duration of 72 hours, a maximum concentration of 800µg/ml was used. On applying the same concentrations to the monolayer of cells in the 'Wound Assay' the cells were found to be lifting up.

Concentrations above 150µg/ml caused cells to lose their shape with retraction of processes in a dose-dependant manner (Fig 3.2.2; A). High concentrations (400µg/ml) lead to total loss of attachment to substrates. Any filopodia-like structure at X40 magnification was considered as a process.

The percentage of cells without processes was quantified and plotted with respect to concentration of antibody (Fig 3.2.2; B). A total number of 1000 cells were counted for each concentration. Data collected from three experiments.

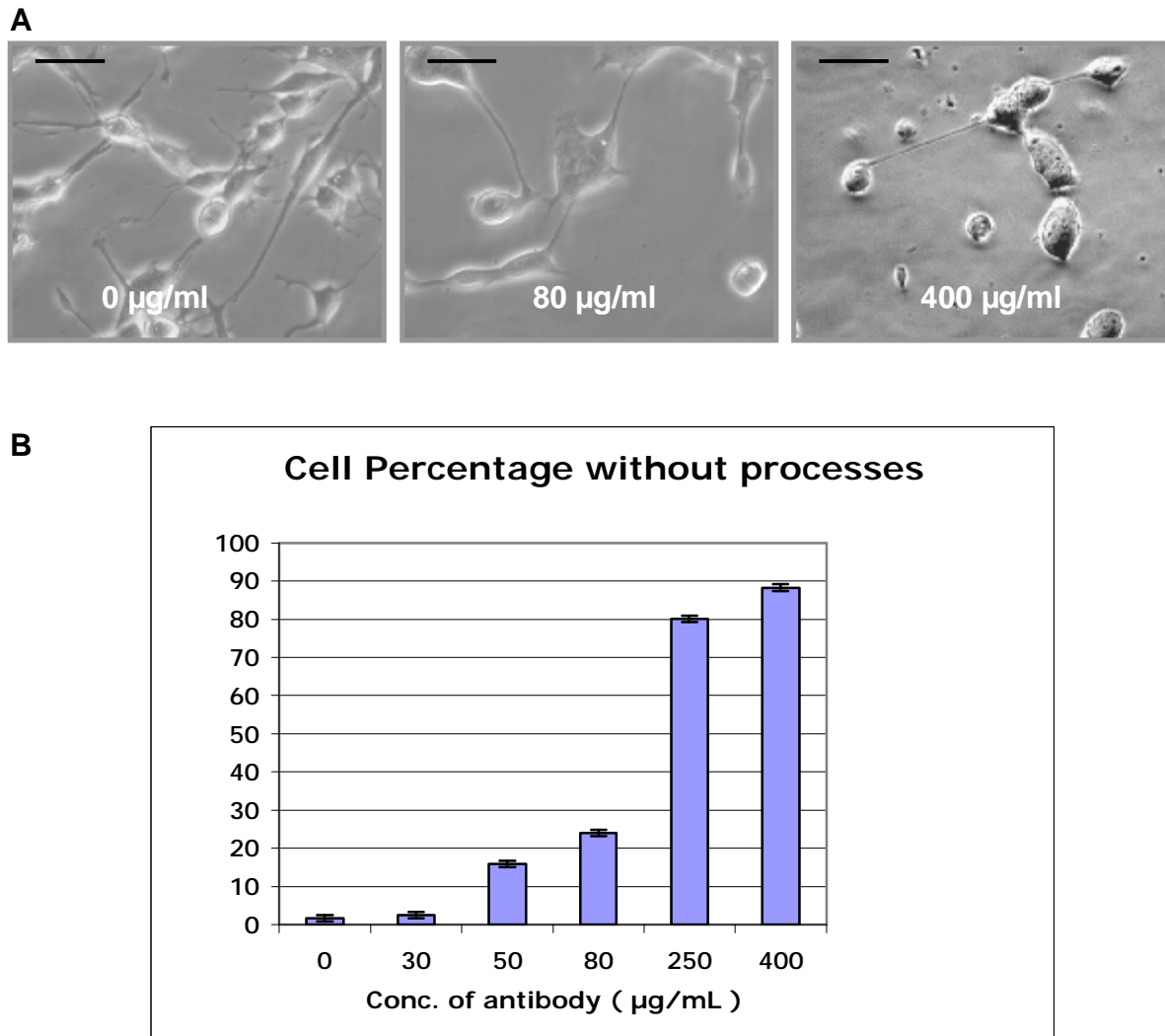


Figure 3.2.2
Polyclonal AN2 antibody in high concentrations lead to process retraction in *Oli-neu*

A: *Oli-neu* growing in a monolayer on PLL-coated coverslips retract their processes on addition of high concentrations IgG fractions of pcAN2 antibody. At the highest concentration used, 400µg/ml, 88% of cells were rounded up. Images were taken at X40 magnification after four hours of incubation with pcAN2 antibody. Scale bar equals 50µm.

B: Percentage of cells without processes plotted against concentrations of antibody. High concentration of pcAN2 antibody (higher than 150µg/ml) leads to retraction of processes and detachment of cells from the substrate-coated coverslips.

3.2.3 Polyclonal antibodies generated against specific parts of the extracellular region of NG2 have differential effects on migration

To accurately map the extracellular region(s) of NG2 involved in migration, polyclonal (pc) GST-LNS1, pcGST-LNS2 and pcGST-extra antibodies (Ab) were generated and used as functional tools in migration assays. For each serum, the experiments were repeated three times. The IgG fractions were used in the assays.

Rate of migration with respect to time was measured as a function of area covered by cells within the square of observation between two time points (Sect. 2.2.1.4). To ascertain the degree of significance for each serum, paired 1-tail test of significance were performed with respect to untreated control cells for each time point to obtain a p value. The p value indicates whether the results observed under untreated and treated conditions are significantly different.

Inhibition relative to the untreated cells occurred on addition of all three antibodies. The rate of migration was most severely inhibited in the presence of polyclonal antibody against GST-extra (Fig 3.2.3.1); p values were below 0,05. Furthermore, the effect was dose-dependant. Addition of pcGST-LNS1 (Fig 3.2.3.2) does not have such a significant effect; p values were less than or equal to 0,25, but the effect was dose-dependant. A reduction in migration was also seen with pcGST-LNS2. p value was less than 0,05 (Fig 3.2.3.3), but the effect was not dose-dependant.

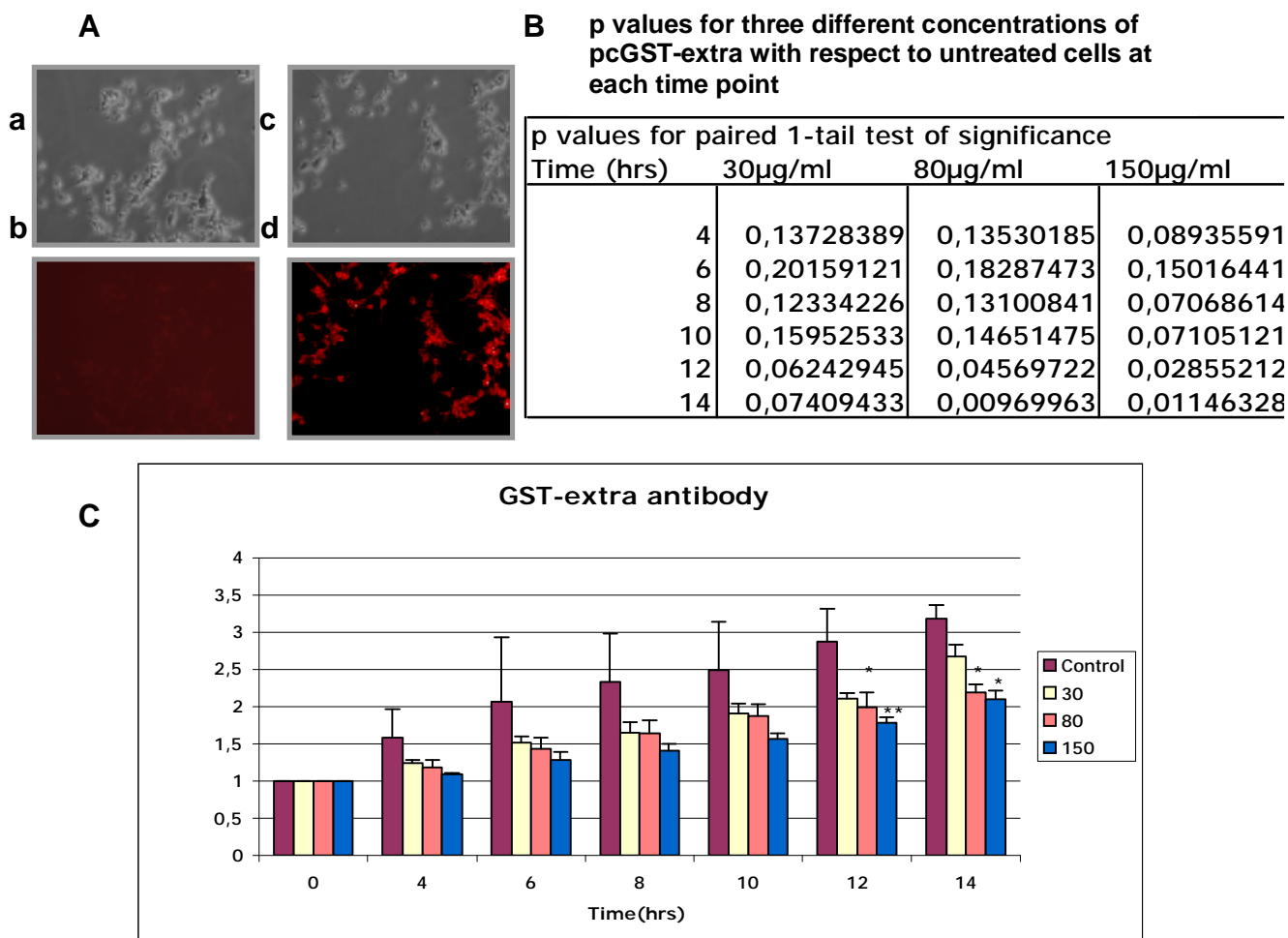


Figure 3.2.3.1
Polyclonal GST-extra antibody has a pronounced inhibitory effect on rate of migration in a dose-dependant manner

A: IgG fraction of pcGST-extra stains living *Oli neu* cells.

a and **b:** Phase contrast and fluorescence images; negative control (secondary antibody). **c** and **d:** Phase contrast and fluorescence image; purified fraction, respectively. Images taken at identical exposure times

B: Test of significance at each time point with respect to Control (without antibody). Three antibody (Ab) concentrations; 30µg/ml, 80µg/ml, 150µg/ml were used. * indicate degree of statistical significance. Paired 1-tail t-tests were performed.

* $p < 0,05$ is, 95% probability of observed effects being significant

** $p < 0,01$ is, 99% probability of observed effects being significant

*** $p < 0,001$ is, 99,9% probability of observed effects being significant.

C: Graph shows effect of addition of IgG fraction of polyclonal GST-extra antibody. Rate of migration decreases with increased concentration of antibody. Standard deviation from three experiments.

Pronounced effect on migration was seen only after some hours. On addition of 150µg/ml Ab migration decreased strikingly after 8 hrs of incubation with Ab, while with 30µg/ml the effect was more pronounced only after 12 hrs.

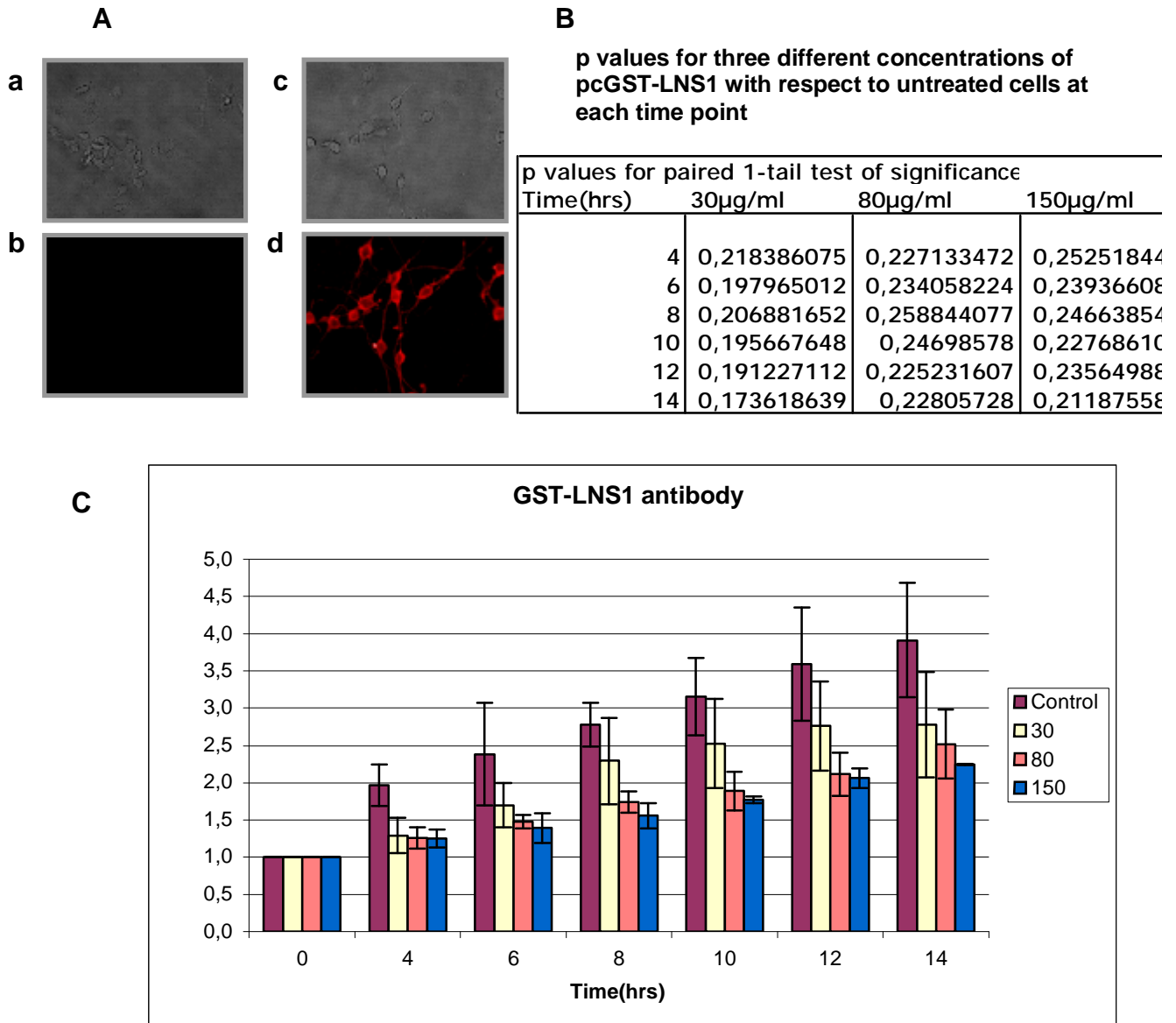


Figure 3.2.3.2
Polyclonal GST-LNS1 antibody has a dose-dependant inhibitory effect on rate of migration

A: IgG fraction of pcGST-LNS1 stains living *Oli neu* cells.
a and b: Phase contrast and fluorescence images; negative control (secondary antibody). **c and d:** Phase contrast and fluorescence images; purified fraction. Images taken at identical exposure times
B: Test of significance at each time point with respect to Control (without antibody). Three antibody (Ab) concentrations; 30µg/ml, 80µg/ml, 150µg/ml were used. Paired 1-tail t-tests were performed with respect to untreated cells. p is less than or equal to 0,25.
C: Graph shows effect of addition of polyclonal GST-LNS1 antibody. Dose-dependant effect of antibody was seen.

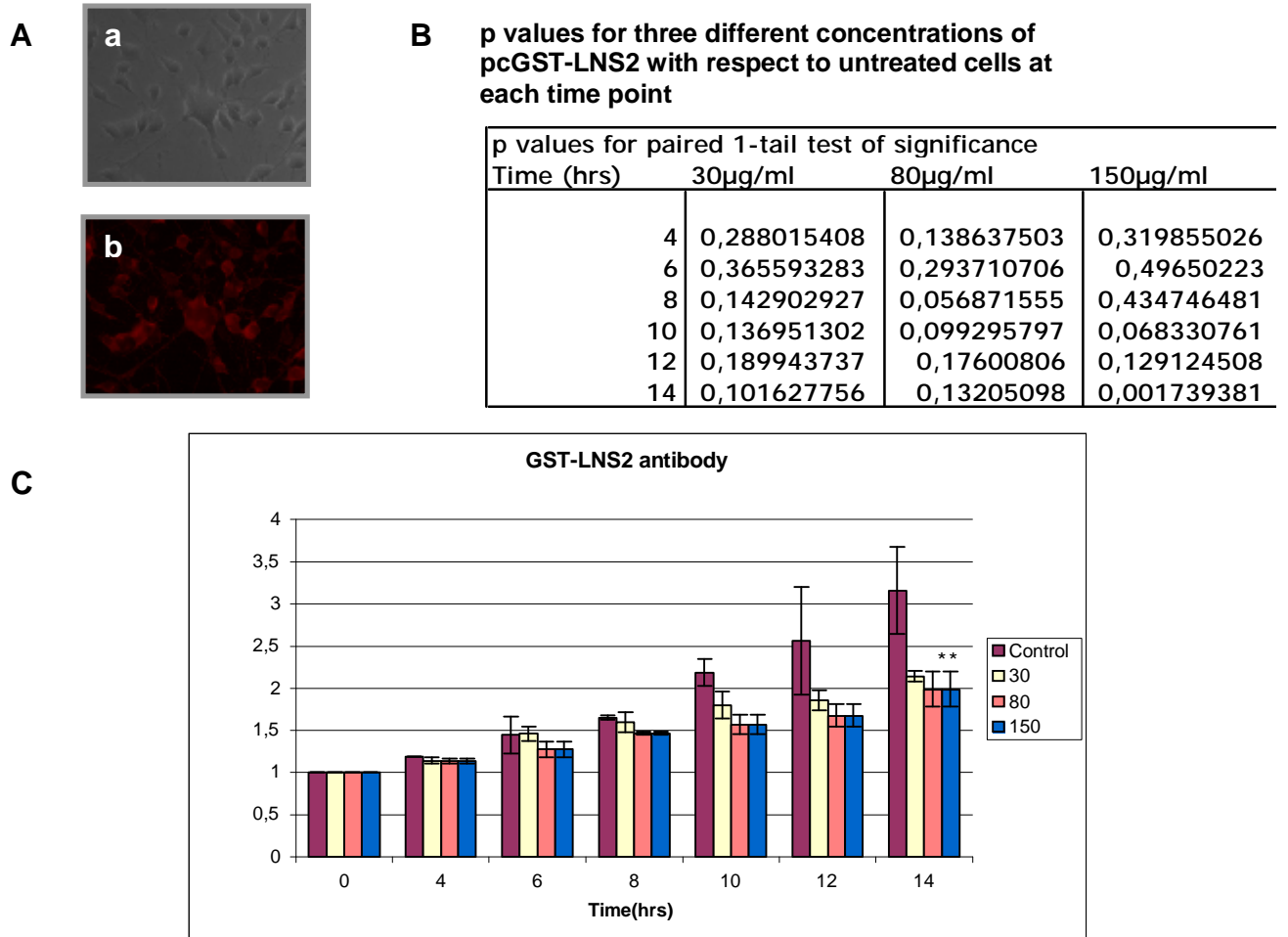


Figure 3.2.3.3
Polyclonal GST-LNS2 antibody also inhibits migration but is less dose-dependant

A: IgG fraction of pcGST-LNS2 stains *Oli neu* cells.

(a) Phase contrast and (b) fluorescence images; purified fraction.

B: Test of significance at each time point with respect to Control (without antibody). Three antibody concentrations; 30µg/ml, 80µg/ml, 150µg/ml were used. Paired 1-tail t-tests were performed.

* $p < 0,05$

** $p < 0,01$

*** $p < 0,001$

C: Graph shows effect of addition of polyclonal GST-LNS2 antibody. pc-GST-LNS2 shows a less dose dependant effect. There is however a reduction in rate of migration.

3.2.4 Addition of Phorbol 12-myristate 13-acetate (PMA) promotes migration in *Oli neu* cells

Migration Assays with *Oli neu* were undertaken to investigate whether migration is promoted by PMA in these cells as reported for astrocytomas. PMA, a diacylglycerol analogue stimulates PKC-alpha. PMA-induced activation of PKC-alpha increases the rate of migration in astrocytoma cells by phosphorylating NG2 (Makagiansar et al. 2004).

A significant increase in rate of migration on incubation of *Oli neu* cells with 200nM of PMA was observed (Fig 3.2.4); p values were less than 0,05. Such an effect of PMA may imply that migration in *Oli neu* uses a similar mechanism involving PKC-alpha as in astrocytomas.

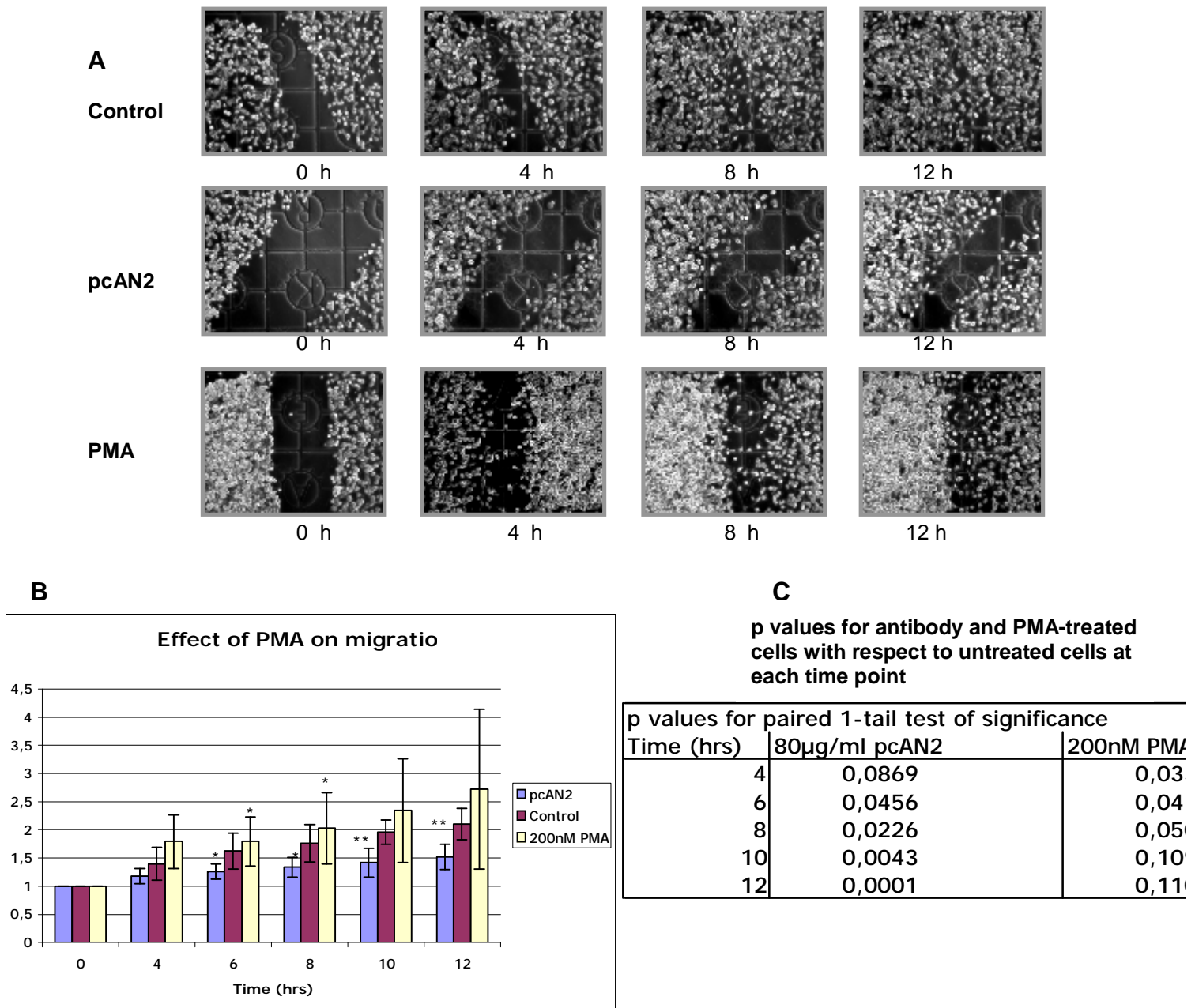


Figure 3.2.4
Addition of PMA leads to increase in rate of migration in *Oli neu*

A: Images of migrating cells; Control (without any antibody), with polyclonal antibody against AN2 and 200nM PMA-treated cells.

B: Graph showing effect of addition of PMA. There is an increase in rate of migration.

C: 1-tail test of significance at each time point with respect to Control (without antibody). Paired 1-tail t-tests were performed.

* is $p < 0,05$

** is $p < 0,01$

*** is $p < 0,001$

Significant increase in migration was seen on addition of PMA. Data obtained from three separate experiments.

3.2.5 PMA leads to phosphorylation of NG2 but fails to cause a redistribution of NG2 and ezrin in *Oli neu*

In astrocytomas, PMA-induced phosphorylation of NG2 leads to redistribution of NG2 along with the ERM family member ezrin. Ezrin and NG2 compartmentalise together on addition of PMA (Makagiansar et al. 2004). The effect of PMA on NG2 phosphorylation and distribution of NG2 in *Oli neu* was investigated.

To ascertain whether PMA stimulated phosphorylation of NG2 in *Oli neu*, cells were treated with PMA and lysates probed for enhanced levels of phosphorylation. Localisation of endogenous ezrin and NG2 was investigated by staining.

Oli neu indeed show phosphorylation of NG2 on treatment with 100nM PMA for 4 hours (Fig 3.2.5; A). We found no evidence that expression of ezrin is upregulated on treatment with PMA under the prevailing conditions (Fig 3.2.5; B). In addition, no re-distribution in endogenous NG2 and ezrin is observed upon PMA incubation (Fig 3.2.5; C and D) in contrast to astrocytomas (Makagiansar et al. 2004).

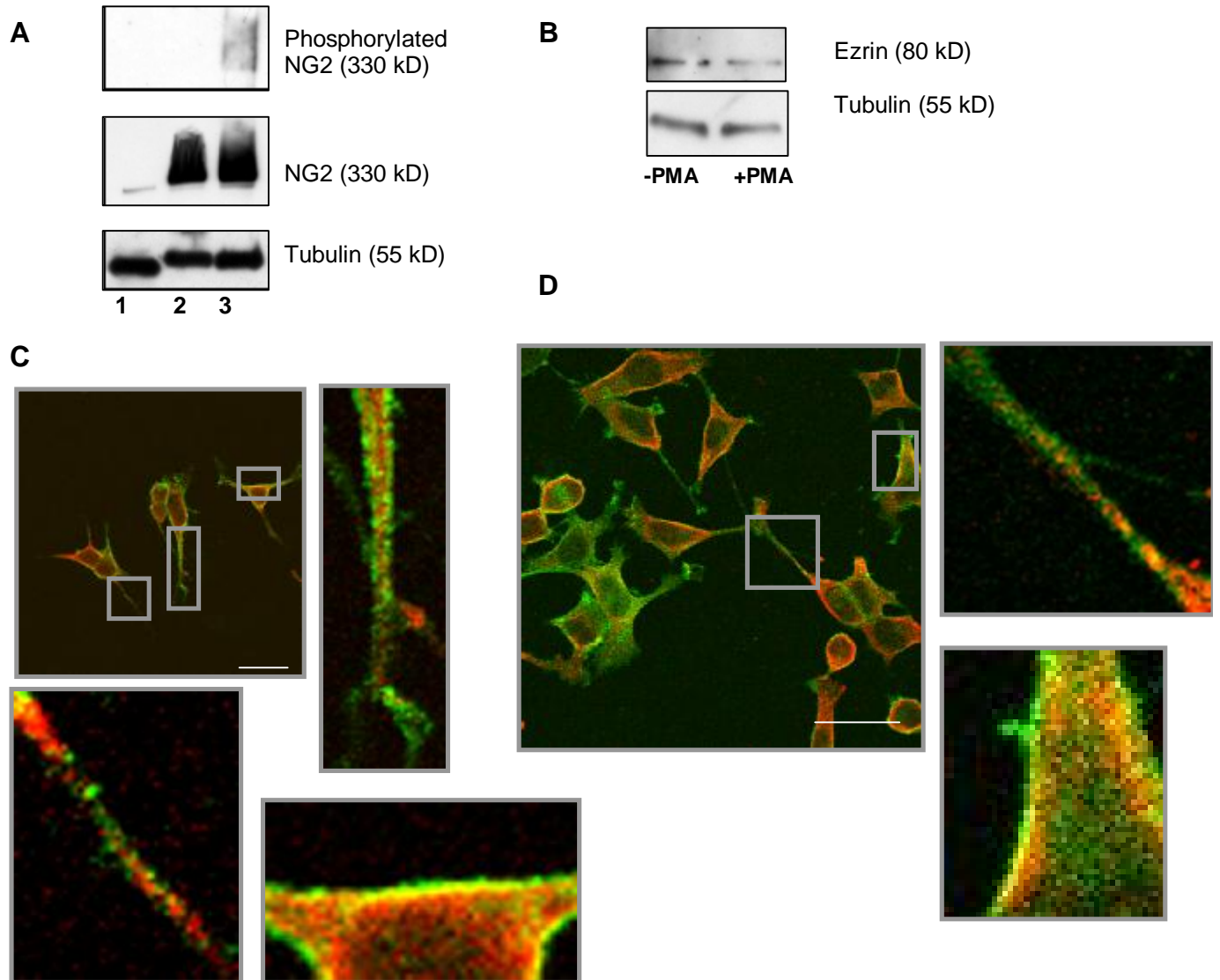


Figure 3.2.5
Incubation with PMA leads to phosphorylation of NG2 but does not lead to a re-distribution of ezrin and NG2 in *Oli neu* cells

A: Stimulation of PKC-alpha by PMA leads to phosphorylation of NG2 in *Oli neu*. Lysate from treated and untreated cells were probed with monoclonal antibody against AN2 and a polyclonal antibody against phospho-threonine.

Lane 1: whole brain lysate.

Lane 2: lysate from untreated cells.

Lane 3: lysate from PMA-treated cells shows a signal at the position of NG2 when probed with antibody against phospho-threonine.

B: Ezrin is not upregulated on treatment of *Oli neu* with 100nM PMA for 4 hours.

C, D: Single section confocal images of *Oli neu* stained with polyclonal antibody against ezrin (red). Live staining with monoclonal antibody against NG2 (green). Scale bar equals 30 μm.

Incubation of *Oli neu* cells with 100nM PMA for 4 hours (C) does not lead to any change in distribution of either ezrin or NG2 in comparison to control cells (D). Both ezrin and NG2 are endogenously expressed in these cells and found at the cell surface.

3.3 Characterisation of the interaction between NG2 and Syntenin

NG2 has a cytoplasmic tail with a QYWV Class II PDZ-recognition motif, implying PDZ proteins as intracellular binding partners. Syntenin is one of three known intracellular proteins binding to NG2, the other two being MUPP1 and GRIP (Barritt et al. 2000; Stegmuller et al. 2003).

Syntenin as an intracellular partner of NG2.

To identify intracellular partners, the C-terminal cytoplasmic region of NG2 was used as bait in a yeast-two-hybrid screen with embryonic mouse brain cDNA library (Doctoral dissertation, J.Stegmüller). Syntenin was identified as one of three binding partners in the yeast two-hybrid system. Point mutations showed that the first three amino acid residues in the C-terminal PDZ-recognition site of NG2 (QYWV) are important for binding to Syntenin. Both the PDZ domains in Syntenin are required for binding to NG2 (unpublished work, J.Stegmüller).

Syntenin and NG2 have similar developmental profiles; both are down-regulated in primary oligodendrocytes *in vitro* as the cells mature. Biochemical confirmation of the interaction was shown by co-immunoprecipitating both NG2 and Syntenin from transfected cells. It was subsequently shown that Syntenin colocalises with NG2 in cultured oligodendrocyte precursor cells. Lastly, the possible functional significance of the Syntenin-NG2 interaction was explored.

3.3.1 Syntenin-1 is expressed in oligodendrocyte lineage cells and downregulated with maturation

Syntenin is present in cultured primary oligodendrocytes and the glial progenitor cell line *Oli neu* (Fig. 3.3.1, A; Lane 2 and 3). Whole brain extract was taken as a positive control (Fig 3.3.1, A; Lane 1). Primary oligodendrocytes *in vitro* were tested for NG2 and Syntenin to define the development profile at three stages at different *days in vitro (div)*; *div2*, *div8*, *div14* (Fig 3.3.1, B and C). Primary oligodendrocytes at the early stage express PDGF α R and NG2. CNP appears at an intermediate stage while MOG is expressed only in more mature cells (Fig 1.1.2). As the oligodendrocytes mature, Syntenin is down-regulated. To check for equal loading of protein, the lanes were probed with antibodies against tubulin.

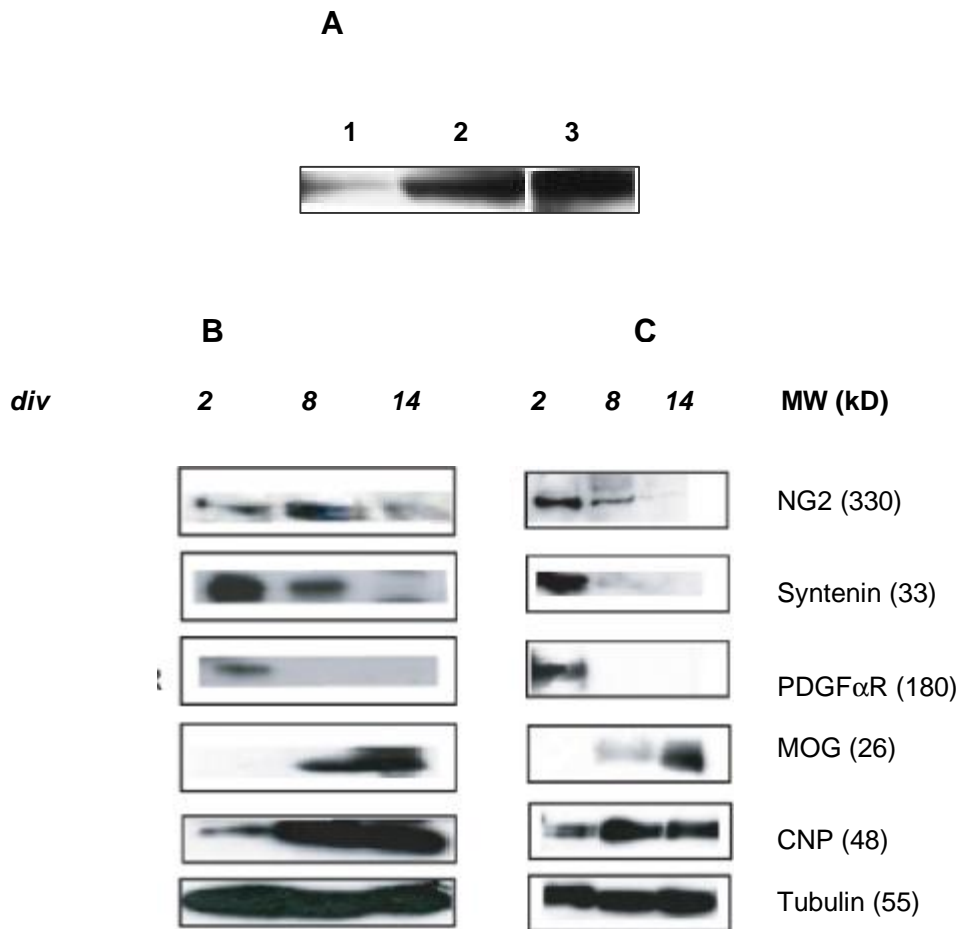


Figure 3.3.1
Syntenin expression is downregulated as oligodendrocytes mature in culture

A: Primary oligodendrocyte and *Oli neu* were tested for expression of Syntenin. Lane 1: Whole brain homogenate. Lane 2 and 3 were loaded with lysates from primary oligodendrocytes and *Oli neu*, respectively.

B and C: Primary oligodendrocytes at different stages of maturity were probed by Western blotting for expression of Syntenin along with other oligodendroglial markers. Lysates were prepared from cultures at different *days in vitro (div)* and then blotted for stage-specific markers.

3.3.2 Co-immunoprecipitation of NG2 and Syntenin from transfected cells

To corroborate the apparent binding shown by the yeast-two-hybrid screen, the interaction between NG2 and Syntenin was demonstrated by biochemical techniques. HEK293 cells were co-transfected with the NG2del construct (Stegmuller et al. 2003) and Enhanced Green Fluorescence tagged Syntenin-1 (EGFP-ST1). NG2del has one-fourth of the extracellular domain and the complete transmembrane and cytoplasmic tail of NG2, and EGFP-ST1 possesses the full length Syntenin (Koroll et al. 2001). The cells were then radioactively labelled with [³⁵S] cysteine and methionine. The proteins were precipitated with polyclonal AN2 antibody (pcAN2) that recognises NG2del (Fig 3.3.2.1 C; Lane 4) and polyclonal antibody against EGFP (pcEGFP) targeting the tagged Syntenin (Lane 6). Polyclonal GST-LNS1 (pcGST-LNS1) also precipitated NG2del and EGFP-ST1 (Fig 3.3.2.1, C; Lane 5). EGFP-ST1 is absent as expected in untransfected cell lysates: lane 2 (from HEK293 cells) and 3 (from NG2delHEK stable line) of Fig 3.3.2.1, C.

NG2, which is endogenously expressed in *Oli-neu*, was co-immunoprecipitated along with the EGFP-ST1 and endogenous Syntenin (Fig 3.3.2.2, B) from EGFP-ST1 transfected *Oli neu* lysate. pcAN2 precipitated NG2 as well as the 61kD EGFP-ST1 (Fig 3.3.2.2, B; Lane 2). pcEGFP precipitated EGFP-ST1, endogenous Syntenin and NG2 (Fig 3.3.2.2, B; Lane 3). Polyclonal antibody against Syntenin immunoprecipitated (IP) EGFP-ST1, endogenous NG2 and endogenous syntenin (Fig 3.3.2.2, B; Lane 4). All experiments were performed thrice.

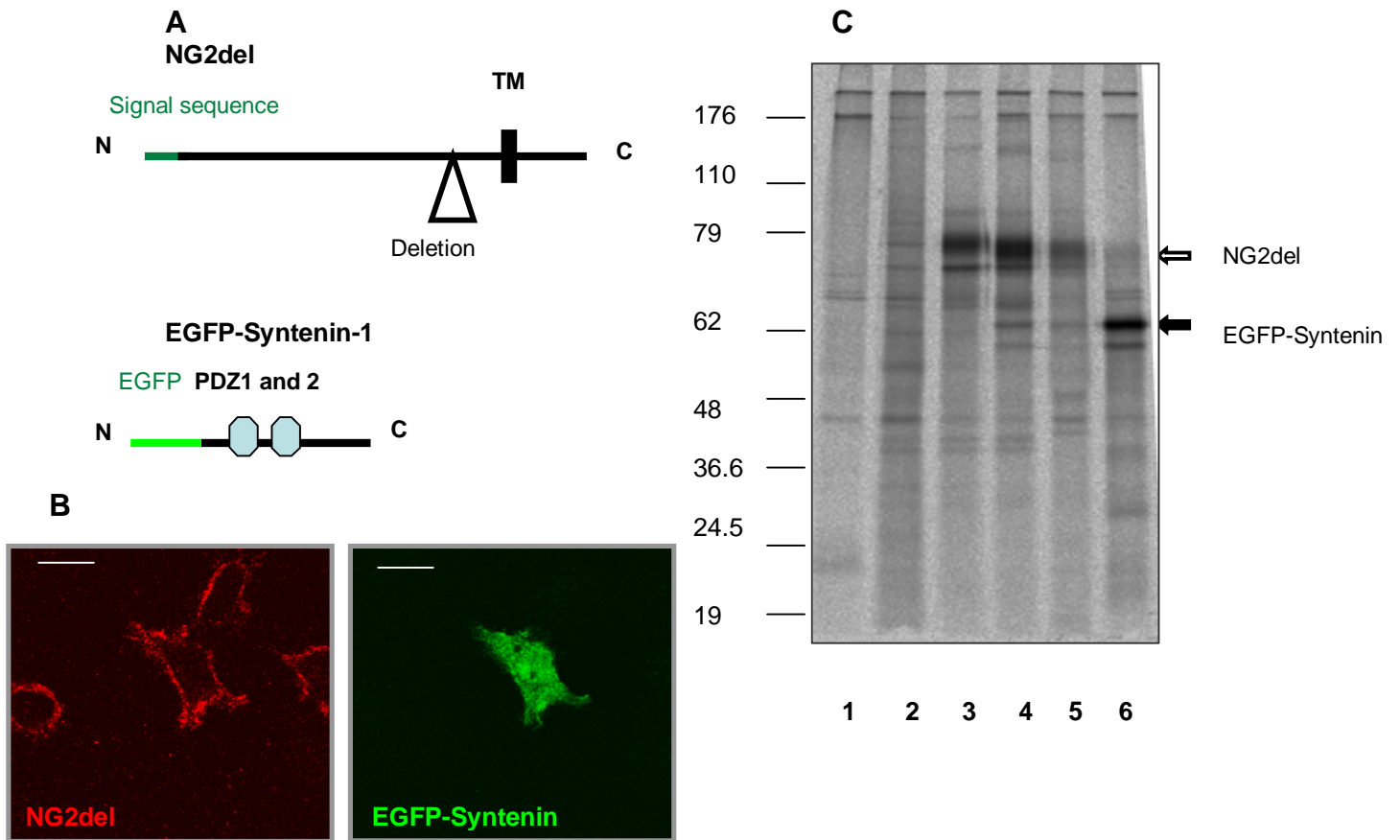


Figure 3.3.2.1
EGFP-Syntenin-1 and NG2del form a complex in transfected cells

A: Diagram of the constructs used.

NG2del construct consists of one-fourth part of the extracellular segment including the LNS domains and the transmembrane and cytoplasmic regions. EGFP-Syntenin-1 construct. Full length Syntenin-1 cDNA was cloned into pEGFP-C1 vector.

B: Confocal image of transfected HEK293 cells. Scale bar equals 10µm.

C: Radioactive IP (with labelled [³⁵S] containing Cys/Met for 4 hours) from EGFP-ST1 and NG2del transfected HEK293 cells. Arrows indicate NG2del at 77kD and EGFP-ST1 at 61kD.

Lane 1: Pre-clear; beads without primary antibody.

Lane 2: Non-transfected cells, immunoprecipitated with pcAN2 functions as a negative control.

Lane 3: Lysate from the stable line expressing NG2del, immunoprecipitated with pcAN2
 Lane 4: HEK293 cells transfected with NG2del and EGFP-Syntenin, immunoprecipitated with pcAN2

Lane 5: HEK293 cells transfected with NG2del and EGFP-Syntenin, immunoprecipitated with pcGST-LamG1 raised against the LamG1 domain of NG2

Lane 6: HEK293 cells transfected with NG2del and EGFP-Syntenin, immunoprecipitated with pcEGFP.

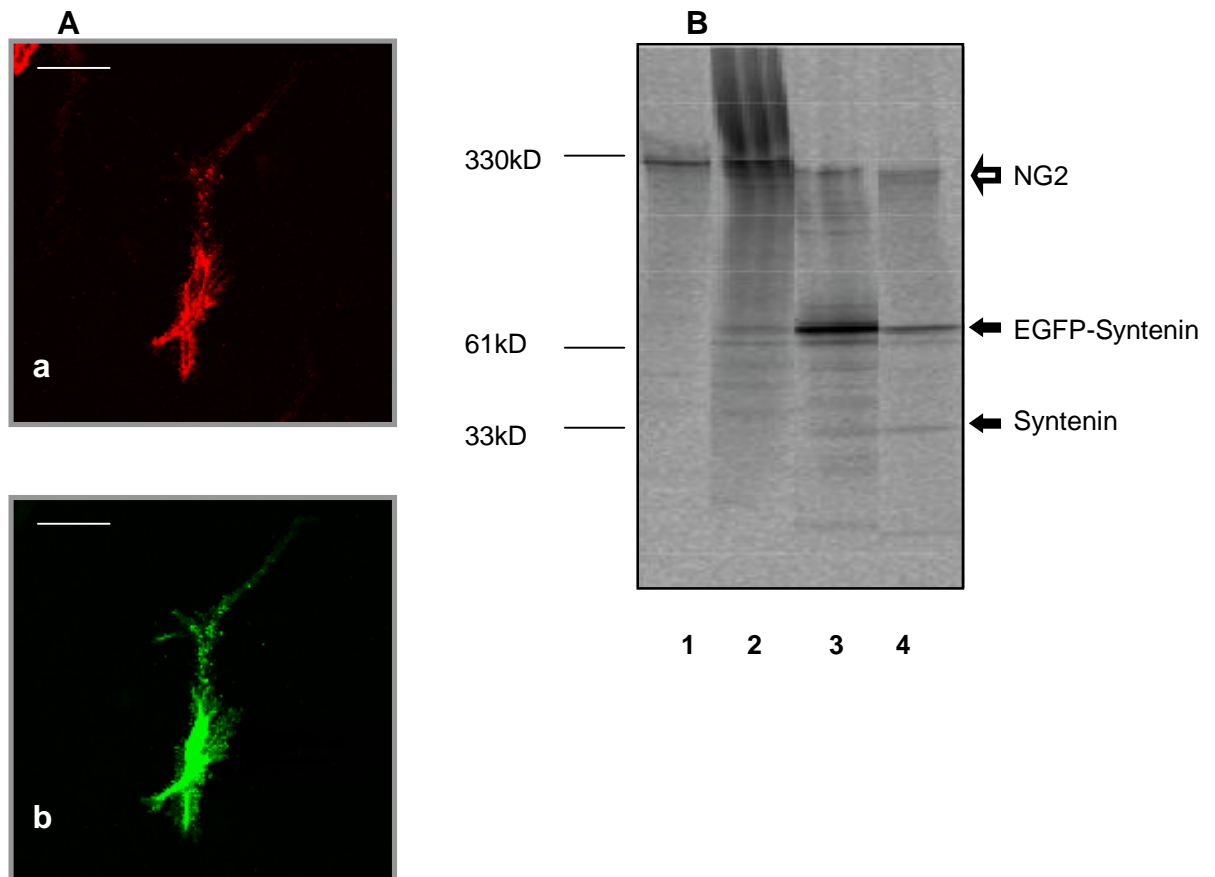


Figure 3.3.2.2

Endogenous NG2 in *Oli-neu* interacts with Syntenin on transfection with EGFP-ST1

Oli-neu expressing endogenous NG2 were transfected with EGFP-ST1 and subjected to IP from radiolabelled cells for NG2 and Syntenin.

A: Images of EGFP-ST1 transfected *Oli neu*. Scale bar equals 10 μ m.

(a) mcAN2 stains NG2. (b) Fluorescent EGFP-ST1.

B: Phosphorimager picture of radiolabelled proteins coimmunoprecipitated

Lane 1: Immunoprecipitation with polyclonal AN2 antibody; lysate from untransfected *Oli neu*

Lane 2: Immunoprecipitation with polyclonal AN2 antibody; transfected cells

Lane 3: Immunoprecipitation with polyclonal EGFP antibody; transfected cells

Lane 4: Immunoprecipitation with polyclonal Syntenin antibody; transfected cells
Note the endogenous syntenin coimmunoprecipitated in lane 3 and 4 at 33 kD.

Syntenin is known to form homodimers. The EGFP-tagged syntenin immunoprecipitated could be the result of dimerisation with endogenous syntenin and vice versa.

Arrows mark the respective positions of NG2, EGFP-ST1 and endogenous Syntenin.

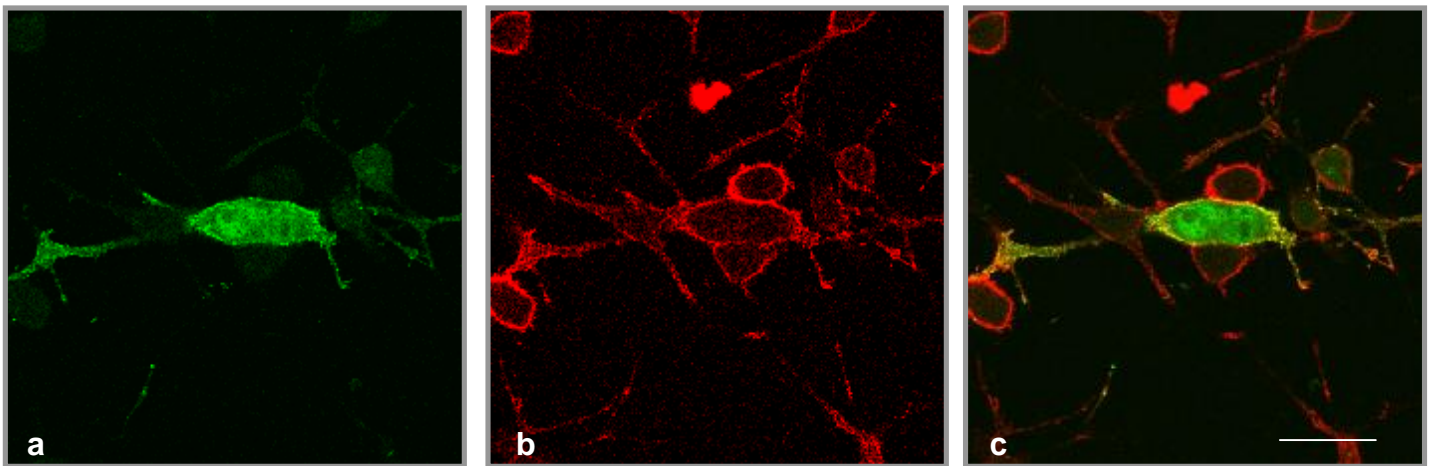
3.3.3 NG2 colocalises with Syntenin-1 at the plasma membrane in primary oligodendrocytes and transfected cells

The distribution of NG2 and Syntenin was shown by staining for both proteins in cultured oligodendrocytes and transfected cells.

Fig 3.3.3.1; A shows HEK293 cells transfected with NG2del and EGFP-ST1, as well as *Oli-neu* (B) transfected with EGFP-ST1 both showed a high degree of surface colocalisation (c) of Syntenin and NG2. Overexpression of Syntenin as EGFP-ST1 leads to a nuclear localization of Syntenin.

Fig 3.3.3.2; A shows primary oligodendrocyte cultures at different *days in vitro (div)* were double-labelled with monoclonal antibody against NG2/AN2 and polyclonal antibody against Syntenin-1 to colocalise the endogenous proteins. In Fig 3.3.3.2; B, the rat AN2 monoclonal antibody stains the surface of the NG2-positive cells. The rabbit polyclonal antibody against Syntenin-1 stains the whole cell in a punctate manner. The yellow punctate fluorescence in the merge indicates a colocalisation of the two proteins (Fig 3.3.3.2; B). The colocalisation is particularly intense at varicosities in the processes.

A HEK293 cells transfected with NG2del and EGFP-ST1



B *Oli neu* transfected with EGFP-ST1

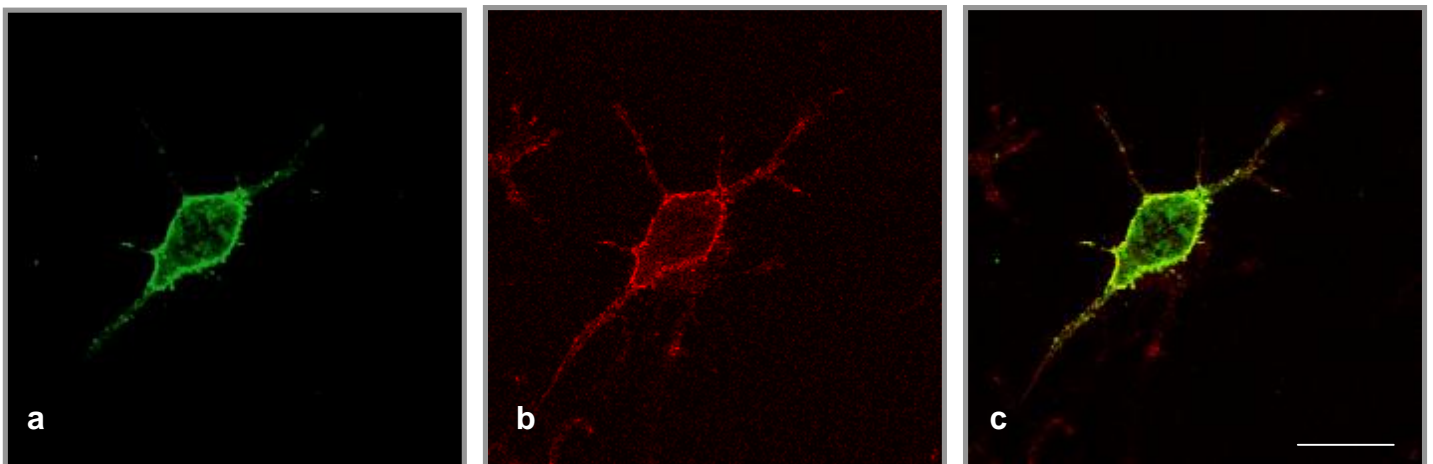


Figure 3.3.3.1

NG2/NG2del and EGFP-Syntenin colocalise at the plasma membrane in transfected cells

Single section confocal microscopy images of cells transfected with **EGFP-tagged syntenin** (green). Live staining with polyclonal AN2 antibody, which recognises NG2del and stains for **NG2** (red). Scale bar equivalent to 20µm.

A (a, b): Staining of HEK293 cells transfected with NG2del and EGFP-Syntenin.

B (a, b): Staining of *Oli-neu* transfected with EGFP-Syntenin.

(c) Overlay.

Primary Oligodendrocytes

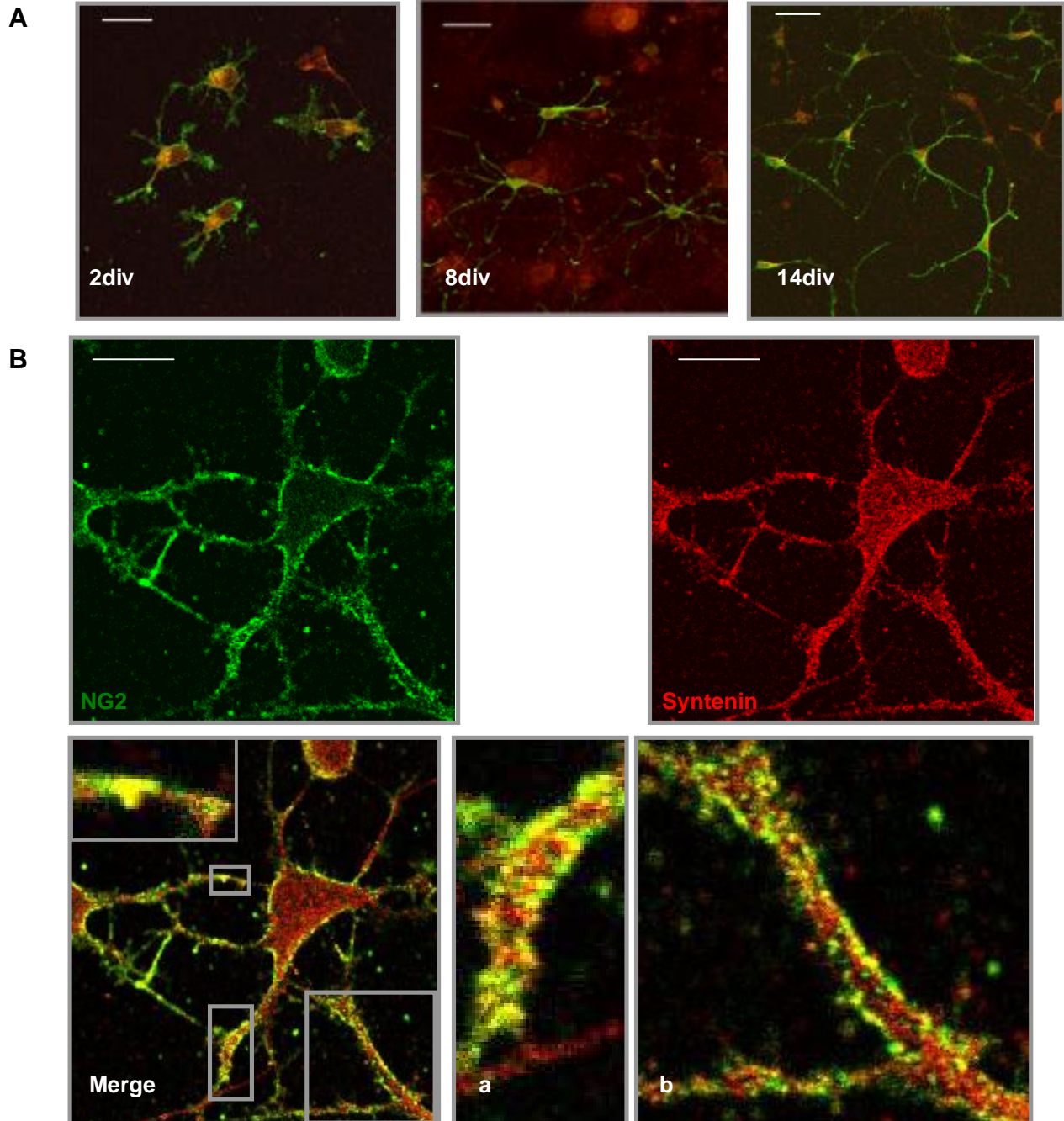


Figure 3.3.3.2
NG2 and syntenin colocalise in immature primary oligodendrocytes

Single section confocal microscopy images of cultured primary oligodendrocytes. NG2 identified by **monoclonal AN2** antibody (green) and Syntenin by a rabbit polyclonal antibody against **Syntenin-1** (red). Scale bar in both (A) and (B) equals 20 μ m.

A: Syntenin and NG2 staining at different times *in vitro* in primary oligodendrocytes.

B: *div2* primary oligodendrocyte stained for NG2 (green) and Syntenin (red). Overlay showing colocalisation (Merge). **(a)**, **(b)** and Inset show zoomed-in areas on the processes. Inset focuses on a varicosity with intense colocalisation.

3.3.4 Investigating the effect of addition of pc and mcAN2 antibodies on distribution of NG2, Syntenin and actin

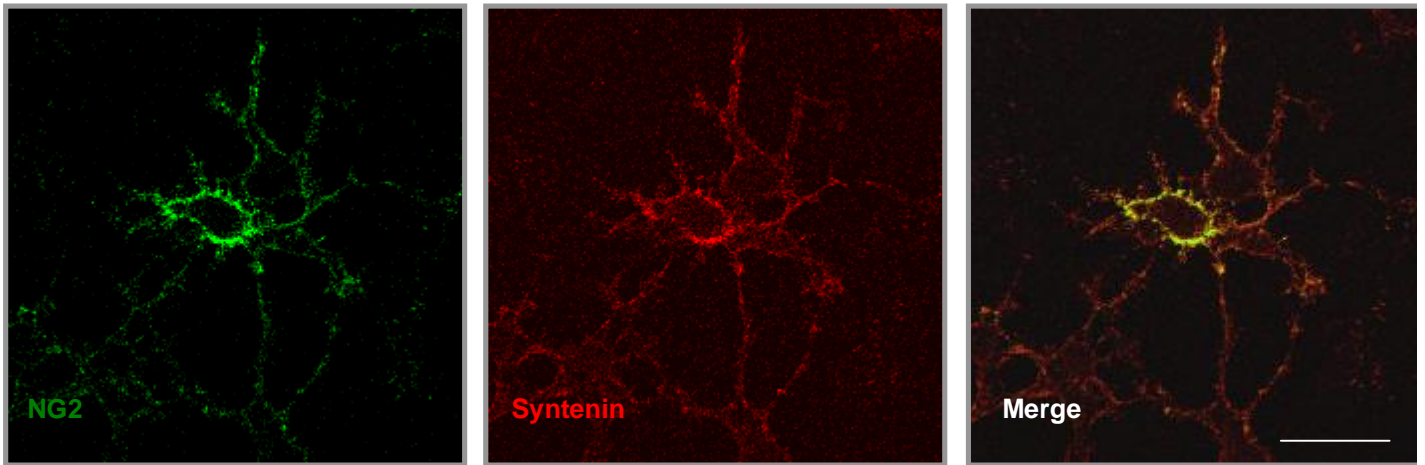
To test whether NG2 and Syntenin cluster together, co-capping was analysed (Fig 3.3.4.1). NG2 on primary oligodendrocytes were clustered with monoclonal antibody against AN2. NG2 and Syntenin distribute together on clustering with mcAN2 antibody.

In order to investigate Syntenin's possible role in cytoskeletal reorganisation in association with NG2, EGFP-Syntenin (Koroll et al. 2001) transfected *Oli-neu* were incubated with polyclonal antibody against AN2 (pcAN2) and stained for F-actin localized by binding of TRITC-coupled Phalloidin.

Addition of pcAN2 for 1 hour at 37°C leads to more intense staining with Phalloidin-TRITC resembling 'contractile actin bundles' along the surface in processes. In untreated cells (Fig 3.3.4.2; B) the surface of processes show presence of both actin, stained by Phalloidin and EGFP-ST1. In cells incubated with pcAN2, actin forms contractile bundles at the inner surface of the plasma membrane. EGFP-Syntenin presence diminishes, apparently redistributing away from the surface in processes (Fig 3.3.4.2; C).

Oli-neu does not show stress fibres when stained with Phalloidin-TRITC. Actin staining in *Oli-neu* resembles the 'loose-network' staining common to actin in the cell cortex (Fig 3.3.4.2, A). It might be that actin stress fibres are absent in highly motile cells like *Oli-neu*. Addition of pcAN2 inhibits cell motility. The appearance, at least at the surface of processes, of contractile bundles of actin could be an outcome of the cell's more stationary state.

A



B

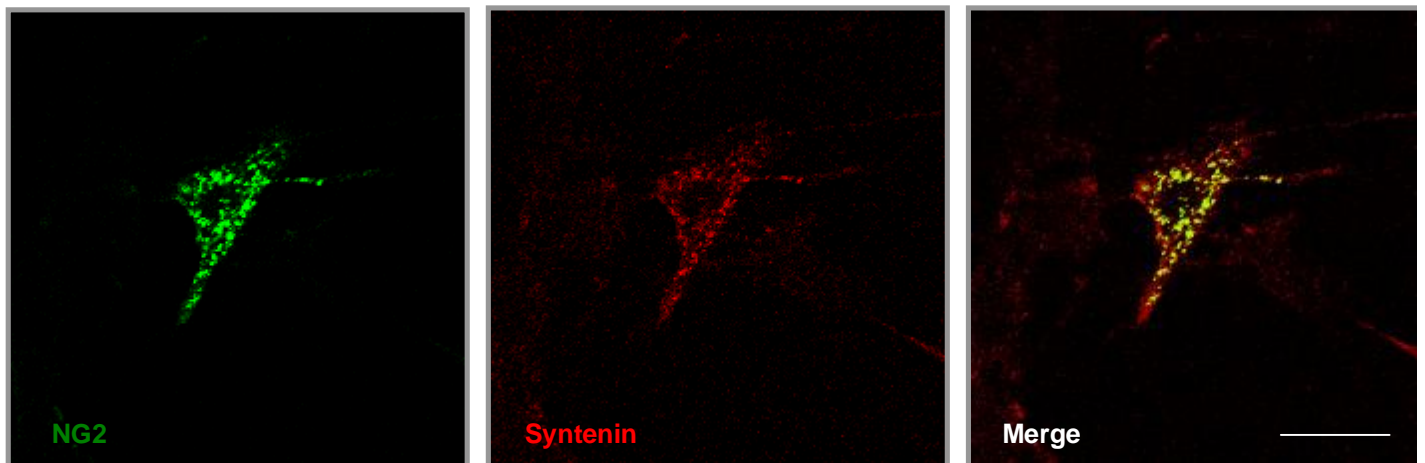
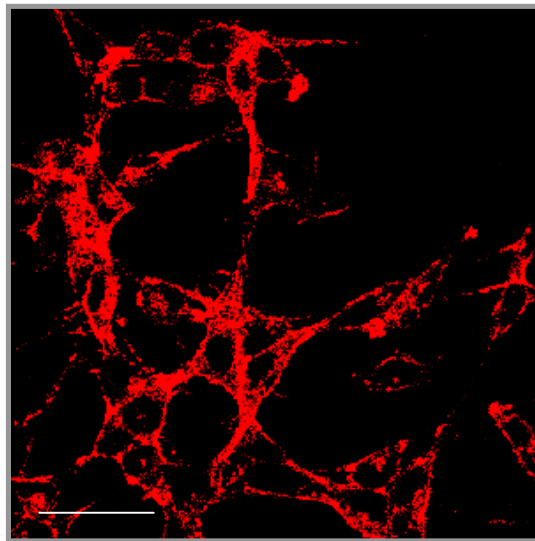


Figure 3.3.4.1
NG2 and Syntenin co-cap in immature primary oligodendrocytes

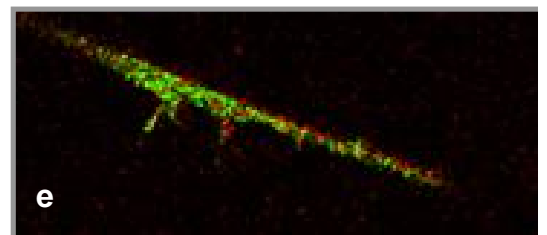
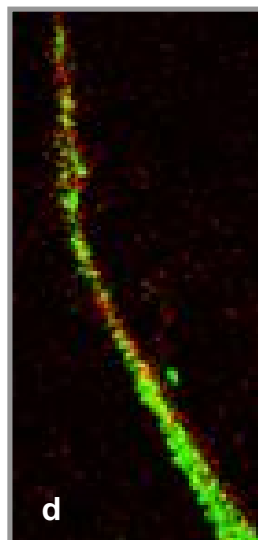
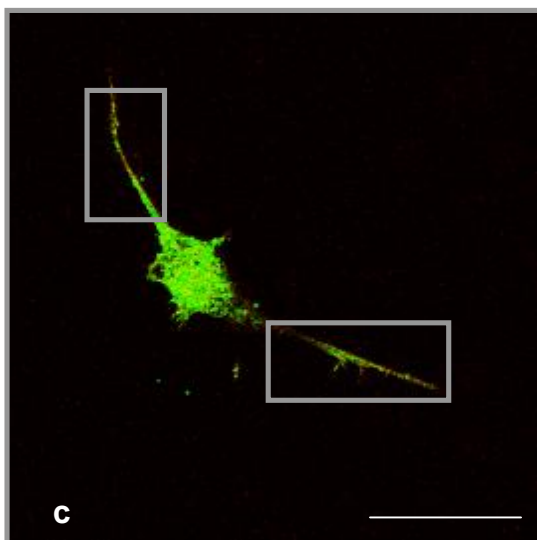
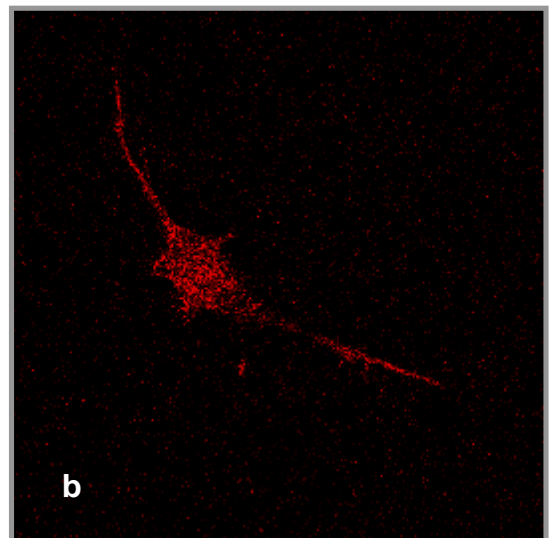
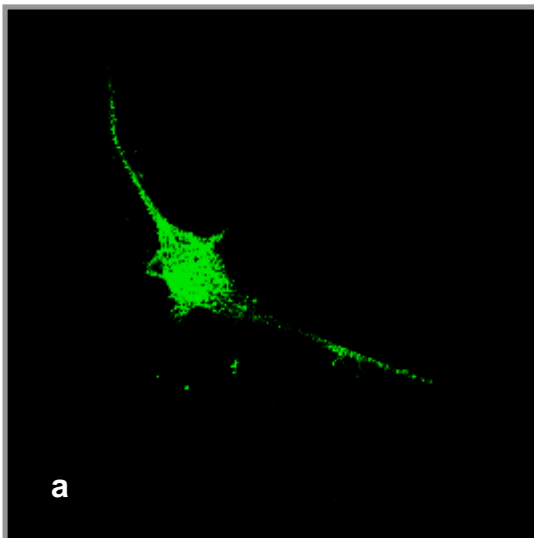
Single section confocal images of primary oligodendrocytes. Scale bar equals 20 μ m. Primary oligodendrocytes (*div2*) were incubated with mcAN2 antibody at 4°C, followed by incubation with fluorescent secondary antibody against mcAN2 and then stained for Syntenin. Staining for Syntenin was performed at room temperature after fixation and permeabilisation of the cells.

A: Control cells were incubated at 4°C with secondary antibody against mcAN2.
B: Capped cells were incubated at 37°C with secondary antibody against mcAN2. Cells showed co-capping of NG2 and Syntenin.

A



B



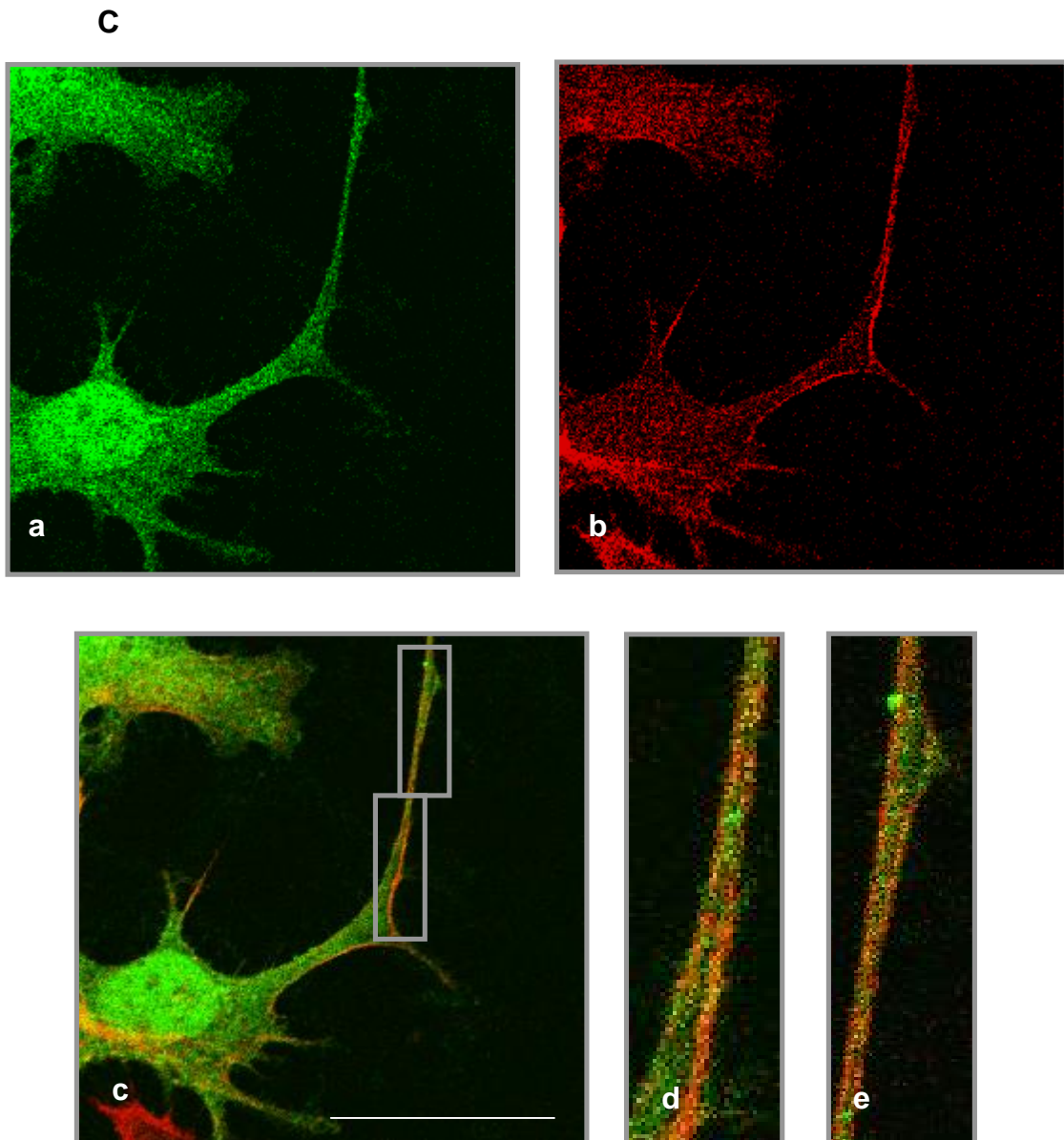


Figure 3.3.4.2
Actin forms contractile bundles at inside surface of plasma membrane of processes on incubation with polyclonal AN2 antibody

Single section confocal microscopy images of EGFP-ST1 transfected *Oli-neu*. Scale bar equals 30µm.

A: F-actin staining in *Oli-neu* localized by Phalloidin conjugated to TRITC
B: EGFP-Syntenin (a) and Actin staining with Phalloidin-TRITC (b) in untreated cells. Overlay (c). (d) and (e) zoomed-in areas. Both EGFP-ST1 and actin are present in the surface of the process.

C: *Oli-neu* transfected with EGFP-ST1 after 1 hour incubation with pcAN2. EGFP-Syntenin (a) and Phalloidin-TRITC staining of F-actin in (b). Overlay (c). Strong expression of EGFP-ST1 in the nucleus. (d) and (e): Zoomed in area. Intense staining of actin at the surface of the process.

3.3.5 Subpopulations of more differentiated oligodendrocytes express Syntenin

The expression profile of Syntenin was tested along with oligodendrocyte markers identifying different stages of differentiation.

Primary oligodendrocyte cultures were stained for Syntenin, CNP and MOG. CNP is an intermediate stage marker; while MOG appears later in development in oligodendrocytes capable of myelination (Fig 1.1.2).

In vitro cultures show double-labelling of these markers with Syntenin (Fig 3.3.5; B and C) in a sub-population of cells. Images of cells stained for NG2 and Syntenin included in Fig 3.3.5; A.

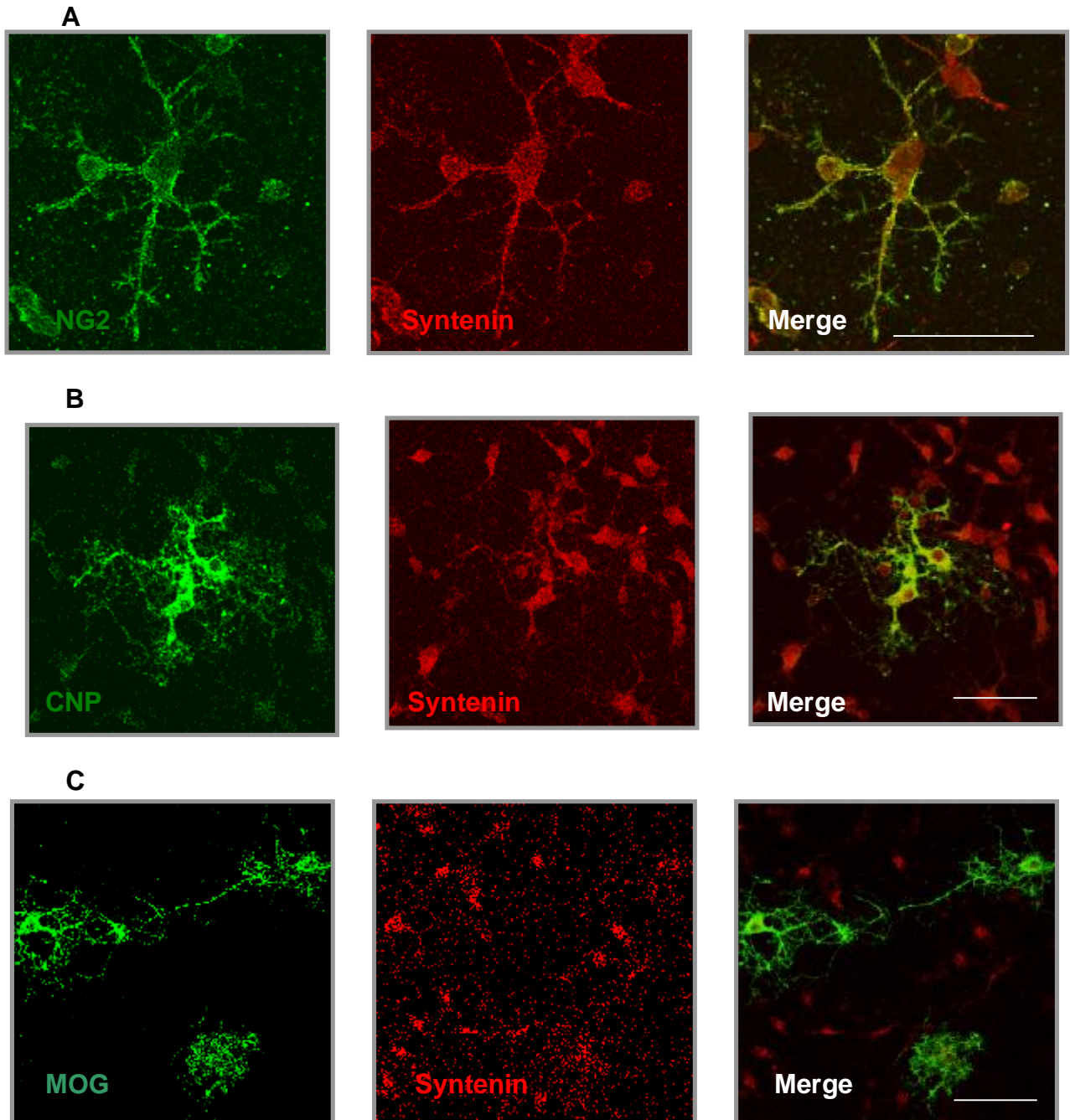


Figure 3.3.5
Subpopulation of CNP- and MOG-expressing oligodendrocytes *in vitro* (*div 2*) express Syntenin

Single section confocal microscopy images of cultured oligodendrocytes. Scale bar equals 40 μm . Polyclonal antibody stains **Syntenin** (red), monoclonal antibodies stain for NG2, **CNP** and **MOG** (green).

A: *div2* oligodendrocytes stained for NG2 and Syntenin and merged image.

B: *div2* oligodendrocytes stained for CNP and Syntenin and merged image.

C: *div2* oligodendrocytes stained for MOG and Syntenin and merged image.

3.3.6 Phorbol 12-Myristate 13-Acetate treated *Oli neu* cells do not show a re-distribution of NG2 and Syntenin

Interaction between a PDZ domain and its recognition motif frequently depends on phosphorylation (Cao et al. 1999; Matsuda et al. 1999; Zimmermann et al. 2002). Phosphorylation usually abolishes binding (Sheng and Sala 2001). NG2's phosphorylation status on Threonine 2256 in the cytoplasmic part of the molecule (Makagiansar et al. 2004) determines its distribution and has been shown to enhance migration.

To investigate whether phosphorylation of NG2 regulates NG2-Syntenin interaction and localization *Oli neu* transfected with the fluorescent-tagged Syntenin, EGFP-Syntenin were treated with Phorbol 12-Myristate 13-Acetate (PMA), an analogue of diacylglycerol which stimulates PKC-alpha.

Transfected *Oli neu* showed no perceptible difference in EGFP-Syntenin and NG2 distribution on treatment with 100nM PMA for four hours (Fig 3.3.6; B) with respect to untreated cells (Fig 3.3.6; A). The processes however appeared to be thicker in comparison to untreated cells.

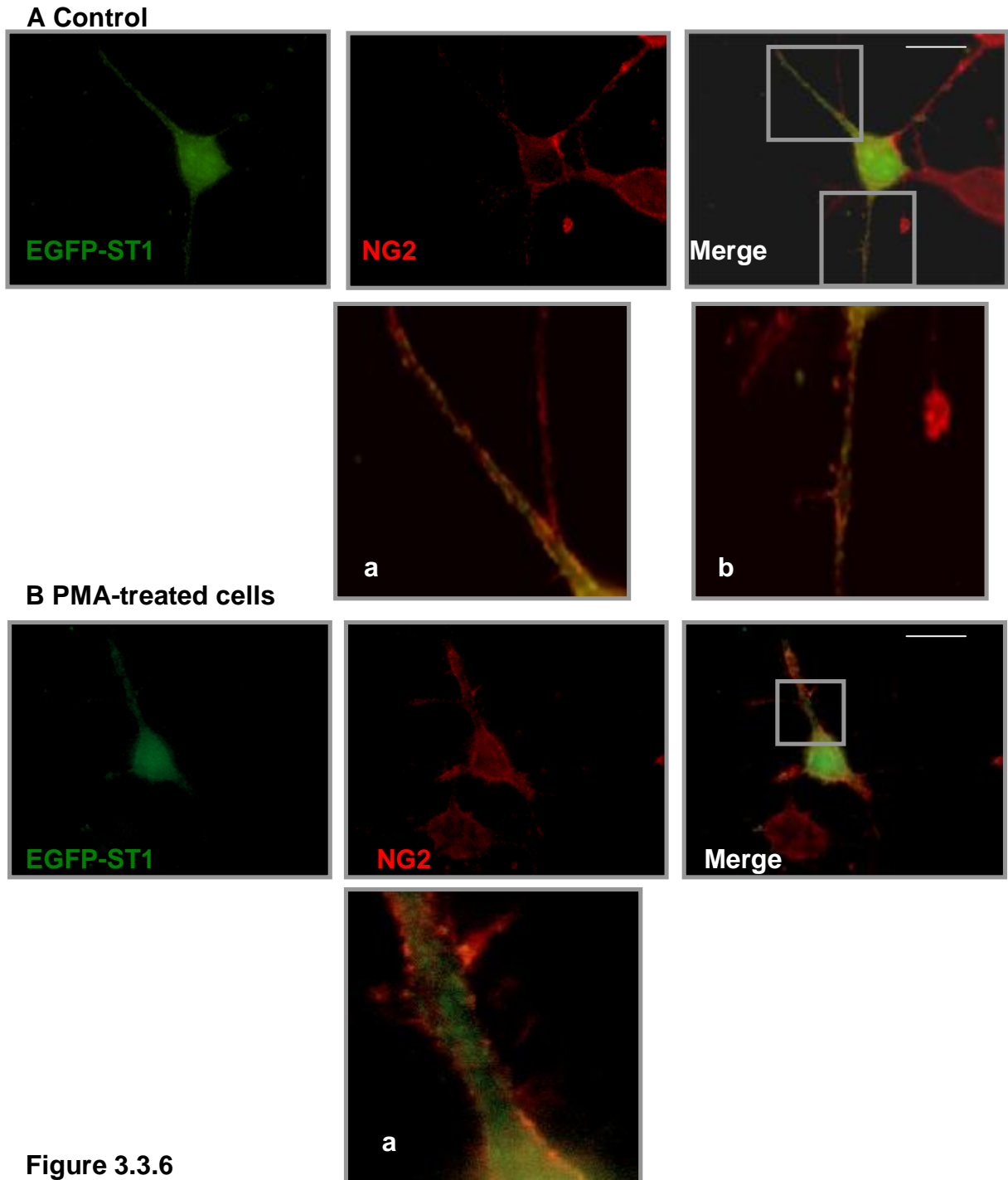


Figure 3.3.6
PMA-induced phosphorylation does not cause re-distribution of either EGFP-Syntenin or NG2 in transfected *Oli neu* cells

EGFP-ST1 transfected *Oli neu*. Live staining for NG2 with monoclonal AN2 antibody. Scale bar equals 30µm.

A: Control cells; no PMA added. Zoomed area (a and b)

B: Transfected *Oli neu* treated with 100nM PMA for 4 hours. Zoomed area (a). There is no striking re-distribution on treating EGFP-Syntenin transfected cells with PMA, which is known to change distribution of NG2 in astrocytoma cells. The processes are however thicker in comparison to control cells (zoomed in areas).

3.4 Investigating extracellular partners of NG2

Brief overview of structure of NG2

AN2 is the mouse homologue of NG2 (Schneider et al. 2001; Stegmuller et al. 2002). The extreme C-terminal consists of a PDZ-binding (Postsynaptic density protein-95, Discs-large, Zona occludens-1) motif, which specifies binding to a class II PDZ domain (Sheng and Sala 2001) followed by a 25 aa transmembrane helix and a large extracellular domain. At the N-terminal there are two LNS domains (29-193 and 203-281; PROSITE). NG2 has two predicted disulfide bridges at 170-193 and 355-381. The molecule has several potential GAG (Glycosaminoglycan) -sites but only one has been confirmed to be attached at Serine 999 (Stallcup and Dahlin-Huppe 2001).

Approaches

Experiments were designed bearing in mind that the LNS domains (Butt et al. 1999; Rudenko et al. 2001) are probably involved in high affinity interactions involving the extracellular portion of the molecule. In order to investigate the presence of NG2 in brain membrane fractions, we fractionated brain into myelin, synaptosomes and the bottom fraction consisting of pelleted membranes (Fig 3.4.1). Two biochemical approaches to identify interacting molecules were explored, by GST-Pulldown assays (Fig 3.4.2) and non-radioactive co-immunoprecipitation (Fig 3.4.3).

3.4.1 Investigating the presence of NG2 in brain membrane fractions.

NG2-positive cells are often close to neurons, enwrapping them, and indeed making synaptic contacts (Bergles et al. 2000; Butt et al. 2002). We fractionated developing mouse brain into membrane fractions; synaptosomes (Fig 3.4.1; A), myelin and axolemma (B). The goal was to investigate the presence of NG2 in these fractions by Western Blotting and Electron Micrographs.

NG2 was found on Western blotting in myelin, synaptosomes and to a lesser extent also in the mitochondrial fraction (Fig 3.4.1; C). Electron micrographs showed that the bottom (mitochondrial) fraction was sometimes contaminated with small synaptosomes with mitochondria inside; the NG2 signal in the mitochondrial fraction could be from these contaminating synaptosomes. The specificity of the fractionation was checked by blotting the fractions with antibodies against proteins which are known to be enriched in these fractions. PLP/DM20, the main myelin protein appeared as two isoforms at 20 kD and 17 kD only in the myelin fraction. Synaptophysin which functions as a synaptic vesicular marker was mostly limited to the synaptosomal and mitochondrial fractions (Fig 3.4.1; C). Immunolabelling for gold-tagged pcAN2 was attempted in the enriched fractions but could not locate NG2 in any recognisable structure like post-synaptic densities (data not shown).

This source of material for finding prospective partners of NG2 was abandoned since the yield was inadequate and NG2 could not be observed on any recognisable structures in electron micrographs.

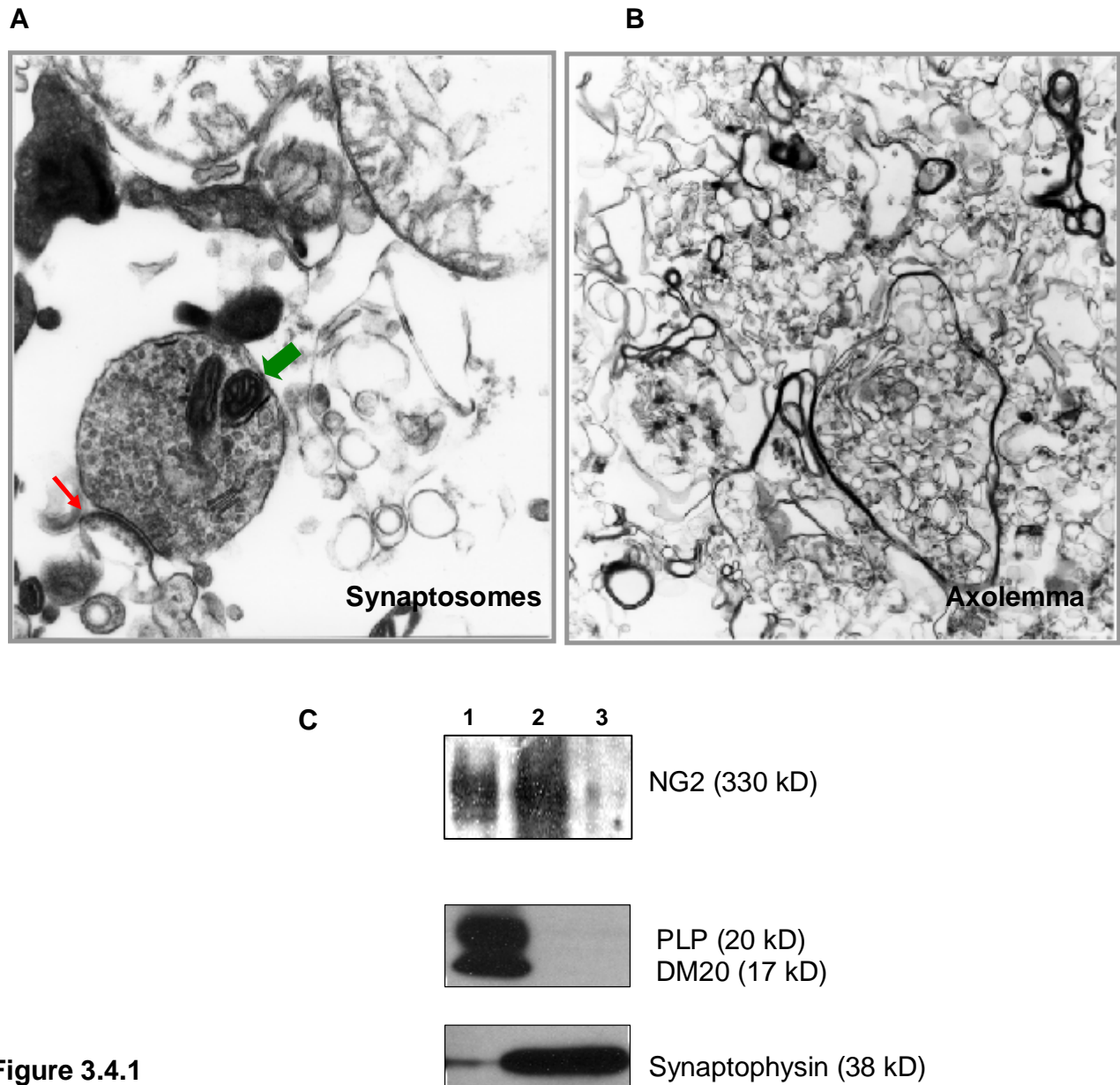


Figure 3.4.1
NG2 is enriched in synaptosomal fractions

A: Synaptosomal preparation from four week old mouse. Some of the synaptosomes contain mitochondria within them (Green arrow) and have an electron-dense post-synaptic density attached to it (Red arrow).

B: Axolemmal fraction. From two-month-old mouse.

C: Western Blot of the fractions

Lane 1: Myelin,

Lane 2: Synaptosomes,

Lane 3: Bottom fraction with mitochondria.

3.4.2 GST-Fusion Protein Pulldown Experiments from mouse brain

GST Pulldowns are a popular method to immunoprecipitate associated proteins. The whole NG2 molecule was fragmented into overlapping sequences and tagged with GST (Fig 3.4.2.1;A). To investigate possible extracellular binding partners of NG2 three of these constructs, GST-LNS1 (Fig 3.4.2.1,B; Lane 1), GST-LNS2 (Lane 2) and GST-LNS (1+2) (Lane 3) were used to probe brain homogenates prepared according to Sugita et al (Sugita et al. 2001).

A band at approximate molecular weight of 130 kD (7-15% gradient gel) was immunoprecipitated with GST-LNS1 (Fig 3.4.2.2; A, Lane 2). A table lists the bands pulled down by GST alone and GST-LNS1, from five experiments (Fig 3.4.2.2; B).

The assay had certain disadvantages. Yields of the fusion proteins were low; only GST-LNS1 gave a reasonable yield. Moreover, bound proteins did not dissociate from the column completely; repeated elution with reduced Glutathione could not remove all of the attached bait and immunoprecipitated proteins from the Glutathione-Sepharose beads. Higher concentrations of reduced Glutathione did not increase the yield of immunoprecipitated proteins. The band pattern after pulldown when analysed on Silver stained gels, was also variable between experiments. However, a protein band at 130 kD was reproducibly immunoprecipitated.

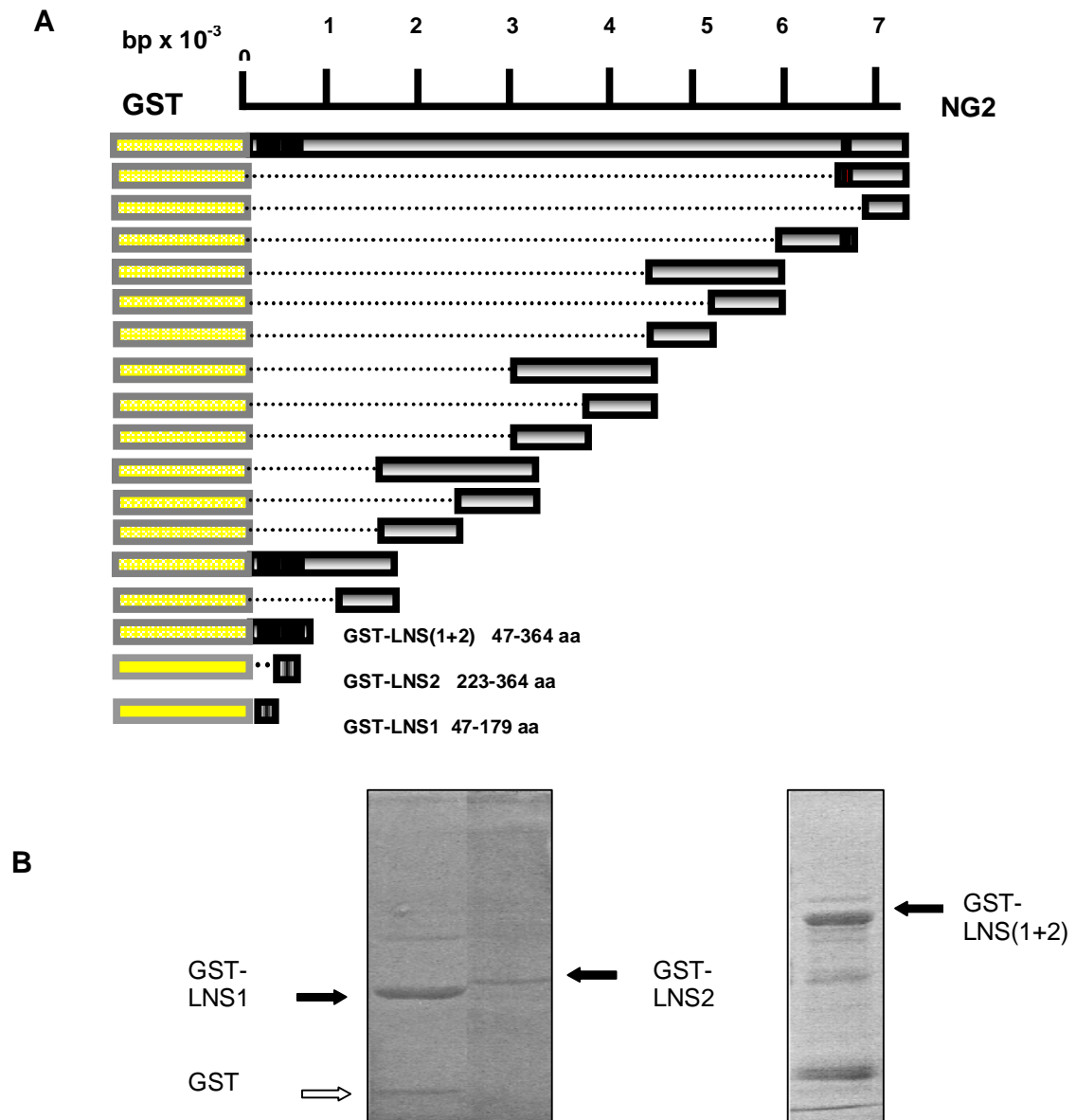


Figure 3.4.2.1
LNS domains tagged with GST

Fragments of NG2 inserted in pGEX-2T vector to form GST-Fusion proteins were purified over a Glutathione-Sepharose column and analysed by SDS-PAGE and Coomassie staining.

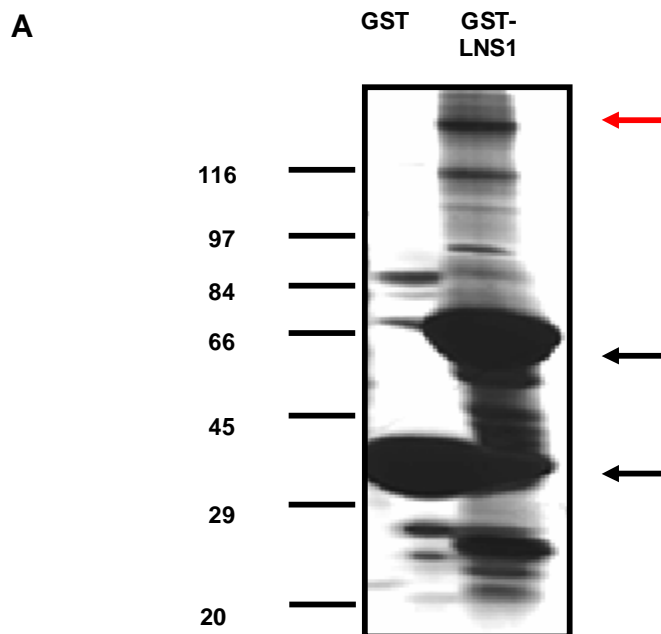
A: Schematic diagram of NG2 fragments inserted into pGEX2T vector to generate the GST-FP constructs. Numerals on scale indicate every 1000 base pairs.

B: Constructs used in Pulldown Assay analysed on SDS-PAGE.

GST-LNS1 at 40,6 kD

GST-LNS2 at 41,6 kD

GST-LNS (1+2) at 63,9 kD and GST at 26 kD



B Molecular weights of proteins precipitated with GST and GST-LNS1

MW	kD	kD	kD	kD	kD	kD	kD
GST	48	50	64		94		205
GST-LNS1	48	50	64	81	94	130	205

Figure 3.4.2.2
GST Pulldown Assay with GST-LNS1 immunoprecipitates a 130 kD protein

GST and GST-LNS1 and associated proteins were analysed on 7-15% gradient gels. **A:** Representative silver stained gel showing typical band pattern of precipitated proteins with GST alone (control) and GST-LNS1. Black arrows indicate relative position of GST and GST-LNS1. The protein at 130 kD, marked by a red arrow was consistently immunoprecipitated with GST-LNS1.

Lane1: GST alone

Lane2: GST-LNS1

B: Table listing molecular weights of GST- and GST-LNS1 pulldown associated proteins; summary of five experiments.

3.4.3 Co-immunoprecipitation of NG2 and potential partners from brain homogenates using the monoclonal AN2 antibody

Immunoprecipitation of NG2 with associated partners using the mcAN2 antibody was another technique used to identify partner proteins interacting with NG2. The binding site of the monoclonal antibody against NG2 (mcAN2) has been mapped to within 1237 and 1532 aa. This leaves both the LNS domains and the cytoplasmic part of NG2 free to interact with other proteins.

Briefly, NG2 was extracted from pelleted brain (P6) membrane fractions with mild detergents (Sect. 2.2.3.4) and the brain extract subjected to immuno-affinity chromatography using the mcAN2 antibody attached to beads.

A band at approximately 140 kD (in 4-10% gradient gel) was immunoprecipitated reproducibly (Fig 3.4.3.1 A, B and C; Lane 2). The control, bridge antibody attached to beads did not precipitate this protein (Lane 1). When analysed on a 10% gel (B), the 140 kD protein corresponded to the marker at 180 kD. On a 10% gel, Analysis of NG2 on an SDS-PAGE gel requires a gradient gel of 4-10%: on a 10% gel therefore the NG2 band could not be observed.

The band pattern survives more vigorous washes with RIPA buffer.

An attempt to identify the band at approximate molecular weight (MW) of 140 kD was made by probing with antibodies against candidate proteins of similar MW. The coimmunoprecipitated proteins were analysed on SDS-PAGE in duplicates. Half of the gel was silver stained, the other half blotted and probed with distinct antibodies. Thus, the relative position of the stained bands and Western blot signals could be compared. GRIP is present in lanes loaded with whole brain lysate (Fig 3.4.3.2, B; Lane 2) and IP with mcAN2 (Lane 3). Comparison with the silver stained gel showed that the band at 140 kD does not correspond to the band recognized by the antibody against GRIP. The band does not coincide with signals on the blots with antibodies against L1, dystroglycans or PDGF-alpha receptor (Fig 3.4.3.2, B; Lane 3).

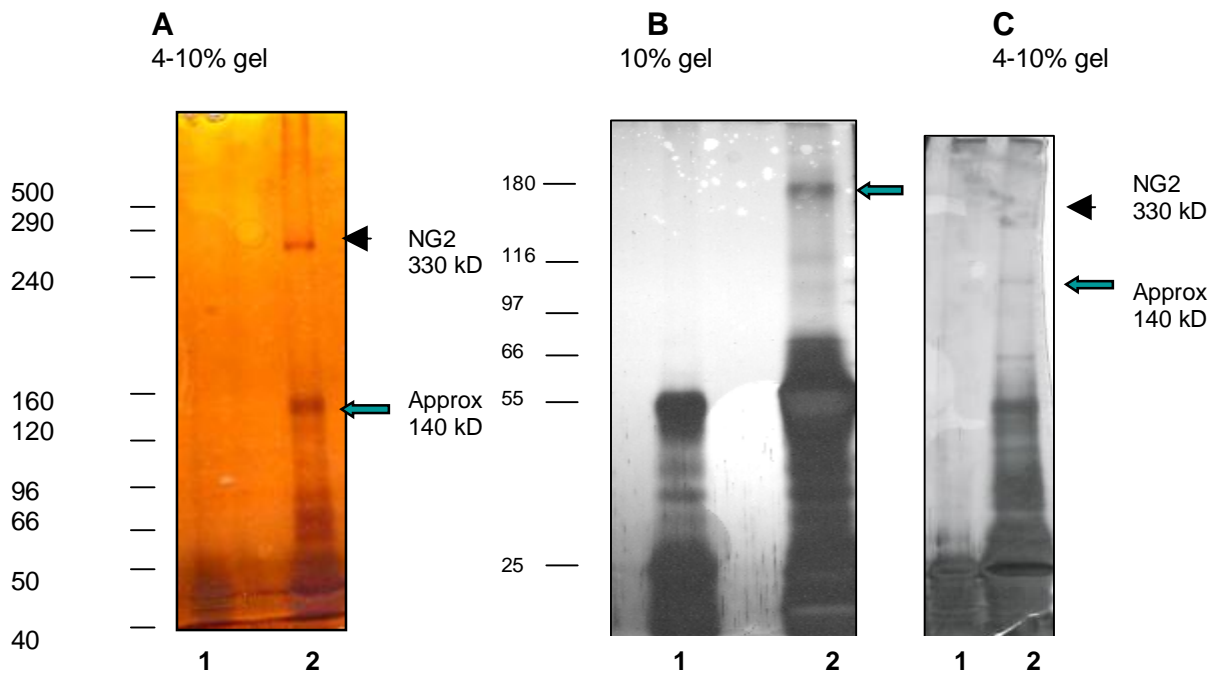


Figure 3.4.3.1

A 140kD band was identified in non-radioactive co-immunoprecipitation with monoclonal antibody against AN2

NG2 was isolated by immunoprecipitation from brain homogenates extracted as in Section 2.2.3.4. The complex precipitated was then analysed by SDS-PAGE. A consistent band pattern with a protein of approximate molecular weight of 140 kD was observed repeatedly.

Lane 1 in each case was loaded with a negative control; IP with bridge antibody but without mcAN2.

Lane 2 IP with mcAN2. Arrowhead and arrow indicate NG2 and the band of interest.

A: Silver staining of Coimmunoprecipitates with control IP and IP with mcAN2 respectively (Lane 1 and 2), analysed on 4-10% gradient gel.

B and C show silver stained gels of differing percentages from a single experiment.

To resolve the molecular weight of the band at 140 kD it was analysed on a 10% SDS-PAGE (B). The band runs around 180kD (B) and at 140kD on a 4-10% gradient (C). **Green arrows** indicate the protein band of interest. Black arrowhead indicates relative position of NG2.

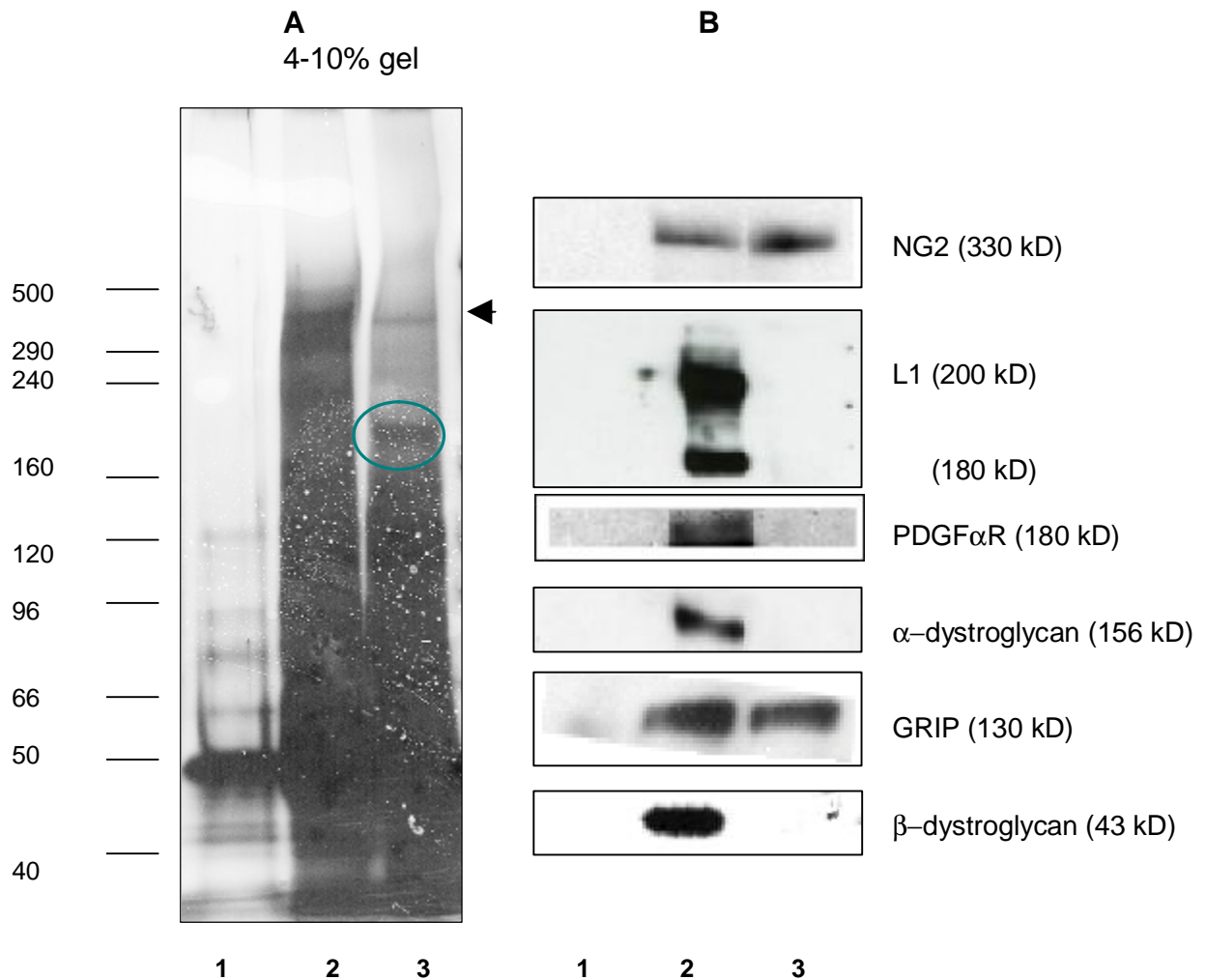


Figure 3.4.3.2
Western Blot of co-immunoprecipitated bands probed with various antibodies

The immunoprecipitated proteins were analysed on SDS-PAGE in duplicates and then stained with silver (**A**) and blotted on PVDF membrane (**B**). Blots were tested for NG2 with pcAN2 and GRIP with monoclonal GRIP antibody as positive controls. The lanes were sequentially probed with antibodies against alpha/beta-dystroglycan, PDGF-alpha receptor and L1. Arrowhead indicates position of NG2. Green ring marks band of interest.

Lane1 Control IP; without addition of mcAN2 antibody

Lane2: Whole brain extract

Lane3: IP with mcAN2

Results

4. DISCUSSION

NG2-positive cells are present both in white and gray matter of developing and adult brain, as well as in the PNS where immature Schwann cells express the protein (Stegmuller et al., 2002). NG2 is overexpressed in highly migratory melanomas and gliomas where it enhances disease progression (Chekenya et al., 2002). In the current thesis, the regions of the molecule involved in migration were dissected (Sect 4.1). The binding of Syntenin to NG2 has been characterised and the functional significance of the interaction explored in Section 4.2. Preliminary results regarding the identification of a possible extracellular partner of NG2 in mouse brain is discussed in section 4.3.

4.1 The role of NG2 in migration

In vitro assays with primary oligodendrocyte cultures, *Oli-neu* and an immortalised Schwann cell clone showed that antibodies generated against immunopurified NG2 from early mouse brain inhibit migration (Niehaus et al., 1999; Schneider et al., 2001). Similarly, human melanomas over-expressing the protein MCSP show increased invasive properties (Eisenmann et al., 1999) and endothelial cell motility is promoted by the NG2 proteoglycan (Fukushi et al., 2004). Though the NG2-knockout mouse does not show any dramatic phenotype in the brain, smooth muscle cells are affected (Grako et al., 1999). However, a detailed analysis is yet to be performed. Whether the migration of cells is affected remains an open question.

In this study the segments of the extracellular part of NG2 that are involved in migration have been defined. The observation that polyclonal NG2 antibody in high concentrations lead to process retraction was noted while standardising the migration assay. Defining the regions of NG2 involved in migration is also clinically important in view of literature describing the presence of antibodies against NG2 in the CSF (cerebrospinal fluid) of relapsing-remitting MS patients (Niehaus et al., 2000). It is unclear how these antibodies affect the function of NG2+ glia in MS lesions. It could be that some of these polyclonal antibodies against NG2 generated in MS patients inhibit the mobility of the NG2+ OPCs and contribute to failure of remyelination (Franklin, 2002).

Antibodies against NG2 may also affect local process motility of NG2⁺ cells. These processes might be particularly important in the NG2⁺ cells enwrapping synapses where they may modulate synaptic signalling by altering the size of the synaptic cleft.

4.1.1 Effect of polyclonal AN2 antibody on cell adhesion

Cell surface proteoglycans have been implicated before in cell-cell adhesion and adhesion to the ECM (Gallagher, 1989). It is therefore not surprising that antibodies against NG2 can perturb adhesion of cells to the substratum, causing cells to round up. Publications from the group of W. Stallcup showed that chondroitin sulfate and cytoplasmic domain-dependent membrane targeting of NG2 lead to promotion of cell polarization and retraction fiber formation (Stallcup and Dahlin-Huppe, 2001).

Polyclonal antibody concentrations (200µg/ml, 400µg/ml and 800µg/ml) at which process retraction was observed in this study were previously used in the oligosphere migration assays (Niehaus et al., 1999). In the oligosphere assay, aggregates of cells were allowed to grow over 72 hours. The distance of the migrating cell front from the foci was considered as the measure of migratory ability of the cells. Major differences between the two assay systems consisted of the cell monolayer in the present system (Sect 3.2) in comparison to aggregates, and the shorter duration of the current assay (14 hours).

It may be that this retraction of processes and subsequent loss in attachment involves integrins, which are widely known to mediate cell adhesion. $\alpha 4\beta 1$ is known to bind to the GAG chain(s) of MCSP (Eisenmann et al., 1999; Iida et al., 2001). NG2 may regulate cell-ECM interactions by serving as co-receptor for $\alpha 4\beta 1$ integrin during adhesion (Iida et al., 1995). The NG2 core protein communicates with $\alpha 4\beta 1$ (Midwood and Salter, 2001) via the LDV (leucine-aspartate-valine) motif (Nishiyama et al., 1991). It follows that blocking of these sites by polyclonal antibodies against the whole molecule would perturb adhesion to the substratum. NG2 also interacts and compartmentalises with the $\beta 1$ subunit in human dermal microvascular epithelial and astrocytoma cell lines,

where it modulates adhesion to type II and type VI collagen (Fukushi et al., 2004; Makagiansar et al., 2004). Interference with this interaction will also lead to loss of adhesion.

While generating the NG2delHEK line (Sect 3.1), it was observed that the transfected cells were remarkably adherent when compared to untransfected HEK293 cells. Transfected cells expressed high levels of the NG2del construct (Stegmüller et al., 2003) consisting of one-fourth part of the extracellular segment of NG2 including the two LNS domains at the N-terminal and the complete transmembrane and cytoplasmic terminal. LNS domains are commonly found in cell adhesion molecules where they are involved in protein-protein interactions (Gallagher, 1989). Loss of attachment to the substratum at high antibody concentrations could also be due to interference with the LNS domains. Furthermore, 200µg/ml of pcGST-extra did not show a similar retraction of processes (experiment performed once, data not shown).

4.1.2 Effect of polyclonal domain-specific antibodies against NG2 on migration

12% of the cells were mitotically active within the 14 hour period of the assay (Fig 3.2.1; C). The increase in percentage area covered by migrating cells at the end time point was typically three times with respect to time zero (Fig 3.2.3.1, 3.2.3.2 and 3.2.3.3; C). We can therefore conclude that the increased coverage of injured area observed in the migration assays is not due to proliferation. Measuring cell number after 14 hours by the MTT Assay showed no noticeable influence of pcAN2 antibodies on proliferation.

Migration assays (Section 2.2.1.4) were performed in the presence of three polyclonal antibodies directed against 'LNS1', 'LNS2' and the sequence encoded by 1891-2226 referred to as 'extra'. Addition of antibodies directed against specific segments would block interaction with ligands binding to these regions as well as simulate engagement. LNS domains and the extracellular segment immediately before the transmembrane segment (1891 to 2226 aa) seemed likely to be involved in migration in view of literature on other LNS

domain proteins (Rudenko et al., 2001) and NG2- α 3 β 1 interaction (Fukushi et al., 2004).

The most striking effects were seen on addition of polyclonal antibody against GST-extra (pcGST-extra). On addition of this antibody a pronounced dose-dependant inhibition was observed (Fig 3.2.3.1). Integrins, which are known to be associated and compartmentalised with NG2 may bind here. Integrins transmit signals following ligand binding by the assembly of a signal complex often in association with cytoplasmic domains of other proteins. Downregulation of α v β 1 correlates with loss of migratory potential in OPC (Milner et al., 1996). Similarly, blocking with anti-integrin antibodies lead to inhibition in migration (Iida et al., 2001) of the WM1341D melanoma line. β 1 integrin, a major player in migration in OPC, (Milner et al., 1996) is therefore an ideal candidate for amplifying a signal cascade downstream and galvanising the cytoskeletal machinery. NG2 and its homologues have been shown to be compartmentalized and associated in *cis* with a number of integrins. Interactions between MCSP- α 2 β 1, MCSP- α 3 β 1 and MCSP- α 4 β 1 have all been suggested (Iida et al., 1998; Fukushi et al., 2004). Integrins can also bind adaptor proteins (Chen et al., 2000; Fernandez-Valle et al., 2002); for example β 1 integrin associates with the ERM protein Schwannomin which is expressed in Schwann cells (Chen et al., 2000; Jannatipour et al., 2001; Fernandez-Valle et al., 2002). Our work showed that both *Oli-neu* and primary oligodendrocytes express Syntenin and Ezrin, another ERM protein. Both Syntenin and Ezrin show plasma membrane localisation along with NG2 (Sect 3.2.5, Sect 3.3.2). It is reasonable to suppose that there are several multi-protein complexes linking the NG2 proteoglycan to the cytoplasm. Whether the integrin(s), Ezrin or Syntenin separately or collectively form such a complex to regulate Actin dynamics, motility and adhesion remains open. Experiments involving coimmunoprecipitation of Syntenin and NG2 did not show any proteins within the molecular range of 120 kD which could have corresponded with some of the integrin subunits (Sect 3.3.2).

Addition of polyclonal antibody against GST-LNS1 (pcGST-LNS1) showed a dose-dependant inhibition in rate of migration (Fig 3.2.3.2). Published work from

the group of W. Stallcup has shown that engaging the extracellular portion at the N-terminal of NG2 by an antibody (named D120) localised NG2 in close apposition to radial Actin spikes characteristic of filopodia, while addition of an antibody (named N143) which binds nearer to the transmembrane segment leads to appearance of cortical Actin bundles (Lin et al., 1996a). It might be that addition of pcGST-LNS1 mimics the cytoskeletal effect seen on addition of D120 and stabilises the processes and hinders movement.

Polyclonal antibody against GST-LNS2 (pcGST-LNS2) does not have a significant effect on rate of migration except at high concentrations (Fig 3.2.3.3). Blockage of this domain, might interfere with binding of *cis* interacting proteins functioning as co-receptors which are necessary in migration as well as *trans* interactions with the LNS2 domain itself.

4.1.3 PMA leads to increase in rate of migration of *Oli neu* but does not cause redistribution of Ezrin or NG2

NG2-mediated migration had been shown to be affected by phosphorylation of NG2 at Threonine 2256 by PKC α leading to cytoskeletal reorganisation in astrocytomas (Makagiansar et al., 2004). To investigate whether *Oli neu* migrate using a similar signal transduction pathway, they were treated with PMA, a diacylglycerol analogue and stimulator of PKC-alpha. The distribution of the adaptor proteins Syntenin and Ezrin that may connect the extracellular milieu with the intracellular machinery was also explored.

Oli neu cells did not show a noticeable re-distribution of Ezrin, NG2 or Syntenin when cells were treated with 100nM PMA for 4 hours (Fig 3.3.6 and Fig 3.2.5; C and D) or rearrangement of Actin (data not shown). Furthermore, radioactive immunoprecipitation of NG2 and Syntenin with antibodies against the respective proteins did not immunoprecipitate any 81 kD proteins, possibly corresponding to Ezrin (Sect 3.3.2).

Oli neu lysate from PMA-treated cells showed an increased phosphorylation of NG2 (Fig 3.2.5; A). It would seem that NG2 in *Oli neu* can be phosphorylated on Threonine residues (as identified by anti-phospho-threonine antibody) by PKC-

alpha in a similar fashion to NG2 in the astrocytoma cell lines (Makagiansar et al., 2004). *Oli neu* cells treated with 200nM PMA also showed enhanced rate of migration (Fig 3.2.4).

PMA being an analogue of diacylglycerol may however perturb more than one signal transduction pathway. Therefore it is not certain whether the enhanced rate of migration is induced only by PKC-alpha activation in *Oli neu*. NG2 and its homologues have been implicated as interacting with a host of kinases including Cdc42 through the Ack-1 pathway (Eisenmann et al., 1999), Rac/Rho GTPases (Stallcup and Dahlin-Huppe, 2001), extracellular signal regulated kinase (ERK) and focal adhesion kinases (Yang et al., 2004); some or any of these might be involved in regulating migration of *Oli neu* cells.

4.2 PDZ proteins as partners of NG2

PDZ domain proteins act as intracellular scaffolds around which signalling complexes are formed to mediate protein-protein interactions through a variety of functional domains. These proteins thus coordinate and compartmentalise interactions between molecules involved in signal transduction. The majority of the PDZ-domain containing proteins are associated with plasma membrane proteins and they are generally restricted to specific subcellular domains such as synapses, cell-cell contact points or at apical, basal, or lateral cell surfaces (Sheng and Sala, 2001). The NG2 proteoglycan is known to bind to several PDZ-domain proteins with its C-terminal QYWV motif (Barritt et al., 2000; Stegmuller et al., 2003). The current work characterises Syntenin as an additional intracellular ligand of NG2, with the potential to link NG2 to the cytoskeletal machinery.

4.2.1 Expression of Syntenin

First identified binding syndecan as an adaptor protein in focal adhesion complexes (Grootjans et al., 1997), Syntenin later was observed to be associated with a number of membrane proteins as part of multi-protein assemblies, notably at the pre- and post-synaptic terminal (Torres et al., 1998;

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Lin et al., 1999; Hirbec et al., 2002). As a component of early secretory pathways (Fernandez-Larrea et al., 1999), it has been found to be present in early apical recycling endosomes (Fialka et al., 1999). Overexpression of Syntenin has implicated it in morphogenesis and metastasis suggesting a role in cytoskeletal reorganisation (Zimmermann et al., 2001). Syntenin shows a widespread tissue expression at both fetal and adult stages (Grootjans et al., 1997). The expression is particularly high in fetal kidney, liver, lung and brain, of both the protein and mRNA (Zimmermann et al., 2001). Cloning of mouse and rat syntenins (also known as melanoma differentiation associated gene-9 or (*mda*) revealed that the molecule is highly homologous (Sarkar et al., 2004); the rodent and human *mda-9*/syntenin is nearly identical at the level of PDZ-1 and the COOH-terminal domains. The N-terminal portion is the most divergent; in humans this domain is 81% and 77% identical to the mouse and rat domains, respectively. Humans show a second isoform, Syntenin-2 α and -2 β (Koroll et al., 2001).

In vivo cell bodies and dendrites of pyramidal cells show a diffuse staining of Syntenin while granule cell cytoplasm show no staining with antibody (Ohno et al., 2004). Other cell types like lymphocytes show both membrane and cytosolic distribution (Gimferrer et al., 2005).

Cell lysates from cultured primary oligodendrocytes, the cell line *Oli neu* and whole mouse brain homogenate all show expression of Syntenin by Western blotting (Fig 3.3.1; A). Probing for Syntenin and NG2 in primary oligodendrocyte lysates at different stages of differentiation illustrated that both NG2 and Syntenin have similar developmental profiles; both are downregulated with age of the cultures, within the same time frame (Fig 3.3.1; B). In lysates from cultures comprised of more differentiated cells, which are identified by the expression of MOG and CNP, Syntenin expression is downregulated (Fig 3.3.1; B). There are some cells in primary cultures that however double-label for both Syntenin and the above-mentioned markers (Fig 3.3.5; B and C).

4.2.2 Colocalisation of NG2 and Syntenin

Staining for endogenous Syntenin in primary oligodendrocytes and *Oli-neu* showed a diffuse staining within the cells (Fig 3.3.3.2; B), which often appeared punctate and may represent vesicles. Colocalisation of Syntenin and NG2 occur at the plasma membrane. Co-transfection of HEK293 with NG2del and EGFP-Syntenin, and *Oli neu* expressing EGFP-Syntenin-1 also show an overlap at the plasma membrane (Fig 3.3.3.1). Overexpression of Syntenin leads to localisation of Syntenin in the nucleus, where it is known to interact with Sox4 (Geijsen et al., 2001).

The expression profile of NG2 and Syntenin in primary oligodendrocytes and colocalisation pattern suggest a temporal correlation of expression of the two proteins. The high levels of expression of both proteins in immature oligodendrocytes coincide with the motility of these cells and may reflect the greater requirement for changes in membrane architecture and remodelling in terms of process arrangement in a developing nervous system. The intense colocalisation of NG2 and Syntenin at varicosities in OPC processes (Fig 3.3.3; B) is also significant in view of recent work in neurons where overexpression of Syntenin led to marked changes in neuronal morphology by increasing the number of dendritic protrusions (Hirbec et al., 2005). It has been suggested that Syntenin might have a role in CNS maturation by stabilising dendritic outgrowths (Hirbec et al., 2005). PDZ proteins are known to assemble large complexes and in this instance it is possible that Syntenin and NG2 interact in a similar fashion with Syntenin stabilising NG2 at branching points where filopodia are most dynamic.

Functional protein interactions require physical colocalisation of the interacting proteins. NG2 is almost exclusively localised along the plasma membrane, while Syntenin shows both a membrane and a prominent cytosolic distribution. This cell distribution pattern is compatible with the partial colocalisation of the two proteins at the plasma membrane. The ability of the two molecules to co-cap was analysed. Clustering and parallel movement of both NG2 and Syntenin was explored by incubating primary oligodendrocytes (*div2*) with monoclonal

antibody against AN2 for 10 minutes at 4°C. This was followed by clustering of the primary antibody with a fluorescent secondary antibody at 37°C. Cocapping occurs as evidenced by clustering of NG2 and Syntenin in primary oligodendrocytes (Fig 3.3.4.1). NG2 and Syntenin are not just co-associated at the plasma membrane but also seen inside the cell after capping. It would appear that some NG2 associated with Syntenin has endocytosed inside the cell. NG2 and Syntenin interact under both basal (control) and capping conditions. However, only a sub-population of Syntenin colocalises with NG2. This further reiterates that Syntenin binds multiple partners and only a subpopulation of Syntenin interacts with NG2 at any given time point. Our results are similar to observations that show Syntenin systematically accumulating with capped CD6 to which Syntenin is known to bind in T and B lymphocytes (Gimferrer et al., 2005). CD6 like NG2 is a type I membrane glycoprotein and is expressed on thymocytes, mature T and B lymphocytes and CNS cells.

4.2.3 Binding of NG2 to Syntenin

Some of Syntenin's ten putative and confirmed partners show non-cooperative binding (Kang et al., 2003), while others require a cooperative mechanism (Grootjans et al., 2000) as in EphB, ProTGF η , neurofascin, protein phosphatase h , IL-5R α , and Schwannomin. The second PDZ domain in Syntenin has binding capacity for both class I and class II peptides (Grootjans et al., 2000; Koroll et al., 2001), which might explain the degenerate nature (Kang et al., 2003) of Syntenin's binding partners (Xu et al., 1998). Wild type Syntenin can therefore recognise type I (neurofascin, ProTGF η), type II (ephrinB2, EphA7, rPTP η), and an unconserved PDZ-binding motif as in Schwannomin (Kang et al., 2003).

NG2 possesses a PDZ-binding motif with the sequence QYWV. Mutation studies showed that for Syntenin-NG2 interaction the -1 (Tryptophan) and -2 (Tyrosine) position of the motif are important (unpublished work, J.Stegmüller). These results are in concurrence with other PDZ domain – motif interactions (Sheng and Sala, 2001). However there is a level of tolerance involved; the

Glutamine residue at -3 position (Q) is necessary only when a fraction of the molecule with the 2 PDZ domains is used but not in the presence of full-length Syntenin. Syntenin's interaction with NG2 requires both its PDZ domains. It has not been ascertained whether the binding is cooperative (unpublished work, J.Stegmüller). Immunoprecipitation of NG2 with anti-AN2 antibodies and of Syntenin with polyclonal Syntenin antibody, also show that only a sub-population of NG2 and Syntenin interact with each other (Fig 3.3.2.1 and Fig 3.3.2.2). The ability to form multimers increases the possibility of Syntenin interacting with multiple ligands simultaneously.

It is important to recall that Syntenin has the capacity to multimerize (Koroll et al., 2001). PDZ domains themselves have the propensity to multimerize as homo-multimers or hetero-multimers (Srivastava et al., 1998). Additionally, PDZ proteins can multimerize via PDZ-independent mechanisms (Hsueh et al., 1997); a PDZ domain may simultaneously be involved in multimerization and peptide binding without interference in either interaction. Moreover, not all interactions with Syntenin require both domains (Xu et al., 1998). It is therefore reasonable to conclude that a Syntenin-NG2 interaction does not exclude simultaneous binding of other ligands at the same time.

Below are suggested models for NG2-Syntenin complex formation.

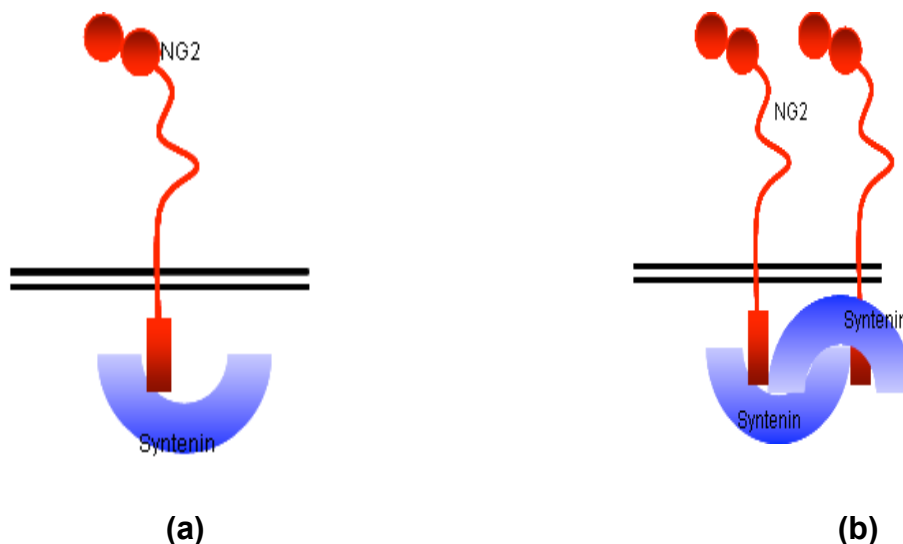


Figure 4.2.1

Possible models for NG2-Syntenin interaction

Syntenin-NG2 interaction requires both PDZ domains of Syntenin. Syntenin can therefore bind only a single NG2 (a). Syntenin can however cluster NG2 as well

as other PDZ-recognition motif containing proteins by virtue of its ability to multimerize (b).

Regulation of NG2 and Syntenin interaction by phosphorylation of NG2

NG2 and Syntenin probably interact under distinct conditions. It has been suggested (Zimmermann et al., 2001) that Syntenin possesses partially cryptic domains which bind only upon activation, reminiscent of the model that is proposed for ERM proteins where a conformational dormancy masks the binding site for membrane and cytoskeletal association (Bretscher, 1999).

Phosphatidylinositol 4,5 bisphosphate (PIP₂) has been demonstrated to interact with and affect binding of Syntenin. PIP₂ reduces receptor peptide binding in the hydrophobic PDZ pocket. Local concentration of phosphoinositide can control association of Syntenin with its target receptors at the plasma membrane (Zimmermann et al., 2002). Recent work showed that Syndecan recycling is controlled by Syntenin–PIP₂ interaction (Zimmermann et al., 2005). Phosphorylation of the PDZ-binding motif also regulates affinity for PDZ domains (Cohen et al., 1996). Interaction of the β₂-adrenergic receptor with the PDZ domain of NHERF (Na⁺/H⁺ regulatory exchanger factor) is regulated by the G-protein coupled receptor kinase (GRK5) activity (Hall et al., 1998). Phosphorylatable residues need not be part of the recognition site to regulate binding (Matsuda et al., 1999). Phosphorylation of residues near the C-terminus, catalyzed by broad-spectrum kinases is likely to be a common mechanism for regulating distribution of interacting proteins and their interactions. NG2 has several Threonine and Tyrosine residues at its cytoplasmic segment and phosphorylation of Threonine 2256 redistributes the molecule to a different compartment in astrocytomas (Makagiansar et al., 2004). In order to investigate whether phosphorylation of NG2 influences Syntenin-NG2 localisation, *Oli neu* transfected with EGFP-Syntenin were treated with Phorbol Myristate Acetate (Sect. 3.3.6). Treatment of *Oli neu* with PMA noticeably enhances phosphorylation of NG2 (Fig 3.2.5), but did not result in any redistribution of NG2 or change in colocalisation of NG2 and EGFP-Syntenin (Fig 3.3.6). Further, co-immunoprecipitation of radiolabelled NG2 and Syntenin in presence of PMA did not show any noticeable difference in

association between the two molecules compared to untreated cells (data not shown). This however, does not exclude PIP₂ having an influence on the NG2-Syntenin interaction.

4.2.4 Association of Syntenin with the Actin Cytoskeleton

Syntenin partitions in microsomes from cell fractionation experiments. Extraction of Syntenin from membrane fractions by detergents, high salt, carbonate and freeze-thawing failed (Zimmermann et al., 2001). This intractability suggests that Syntenin is partly associated with the cytoskeleton. Various experiments have confirmed that Syntenin can mediate cytoskeletal dynamics at the plasma membrane (Grootjans et al. 1997). The N-terminal domain of Syntenin containing both PDZ domains plus the ten following amino acids localises it to stress fibres, and induces the formation of long, branching plasma membrane extensions (Grootjans et al., 1997). Change in cell morphology (Zimmermann et al., 2001; Hirbec et al., 2005) with Syntenin overexpression has also been reported. It is conceivable, considering the literature on NG2's role in migration and invasion (Eisenmann et al., 1999) that Syntenin might be involved in mediating cytoskeletal changes necessary for movement.

The distribution of Actin in cell processes changes on addition of polyclonal AN2 antibody. This inhibits migration of NG2+ cells (Sect 3.3.4). Immunofluorescence of untreated cells transfected with EGFP-Syntenin shows a diffuse Phalloidin staining pattern for F-Actin in processes (Fig 3.3.4.2; B). In contrast, pcAN2 antibody-treated cells showed intense Actin staining along processes, resembling contractile bundles. Moreover, distribution of EGFP-tagged Syntenin, at the surface of cell processes is reduced when cells were incubated with pcAN2 (Fig 3.3.4.2; C). It is likely that NG2, Actin and Syntenin interact during migration. Simulation of a more stationary state on addition of pcAN2 antibody could lead to a redistribution of Syntenin and interacting Actin. The Actin distribution in *Oli neu* does not show the fibrillary structure similar to cortical bundles familiar in most cells. *Oli-neu* does not show any stress fibres when stained with Phalloidin (Fig 3.3.4.2; A). Stress fibres are usually seen in

stationary and differentiated cells. The discrepancy of our observations with respect to other publications (Makagiansar et al., 2004) and absence of stress fibres in *Oli neu* could be due to the resemblance of this cell line to progenitors which are highly motile. Over-expression of Syntenin in *Oli neu* did not lead to any dramatic changes in morphology in our experiments; no increased process arborisation was observed.

4.2.5 Functional significance of the Syntenin-NG2 interaction

The association of NG2 with defined subsets of Actin-containing structures has been extensively studied by the group of W. Stallcup (Lin et al., 1996a). NG2 and the Actin-binding protein fascin define separate populations of Actin-containing structures (Lin et al., 1996b). NG2 is restricted to filopodia and fascin to lamellipodia. Actin-binding domains have however never been identified in the cytoplasmic tail of NG2. Yet, NG2 has been implicated in migration, metastasis and adhesion complexes. The association of NG2 with downstream cytoskeletal machinery must therefore be mediated by linker proteins. NG2 has been compartmentalised with membrane cytoskeletal linkers such as ERM proteins which interact with Actin (Makagiansar et al., 2004). This role might similarly be undertaken by PDZ domain proteins. Syntenin has been found to be part of supramolecular complexes with ability to bind a wide variety of proteins including syndecan (Zimmermann et al., 2001) and Schwannomin (Jannatipour et al., 2001). Syntenin (mda-9) is also upregulated in melanoma (Sarkar et al., 2004)). The structure and localization of Syntenin is ideal for an intermediate linker. NG2 participates in metastasis through a variety of pathways including the Rho/rac kinases and FAK/ERK (Iida et al., 1995). Engagement of NG2 has been shown to activate the GTPase rac and p130cas leading to cell spreading (Eisenmann et al., 1999; Majumdar et al., 2003). We propose Syntenin as an adaptor molecule, which connects NG2 to downstream components.

The possible significance of the interaction of NG2 with Syntenin homodimers in influencing clustering or redistributing metabotropic receptors also cannot be overlooked. The interaction of Syntenin with GluR5-GluR7 subunits of kainic acid receptors (KAR) has been suggested to be important during CNS

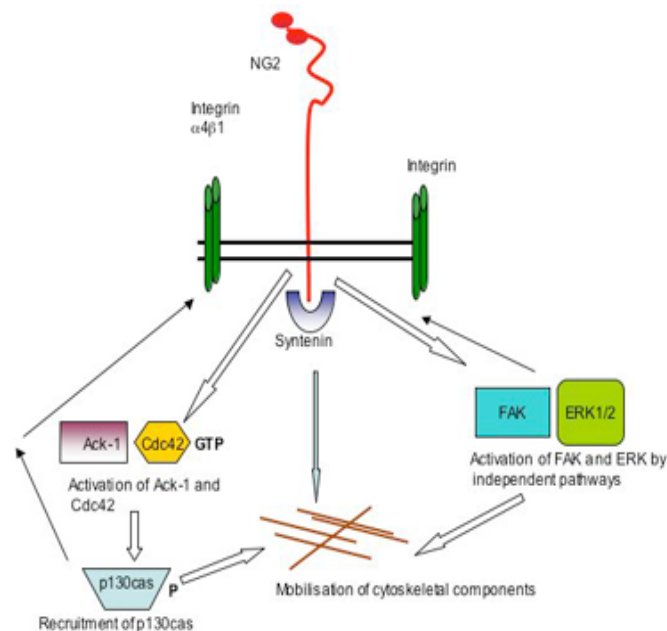
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maturation (Hirbec et al., 2003; Hirbec et al., 2005). KAR are ligand-gated ion channels comprised of tetrameric assemblies of GluR5-7 and KA1-2 subunits. They are present at both pre and post-synaptic sites and involved in developmental and activity-dependant plasticity of excitatory synapses (Hirbec et al., 2005). Identification of a similar complex with NG2 and the GluRB subunit of AMPA receptors through its interaction with the PDZ-protein GRIP has been shown and proposed to have significance in the developing and adult brain for glial-neuronal recognition and signalling (Stegmuller et al., 2003). NG2 could play a role in specifically clustering either KAR or AMPA receptors depending on whether it is compartmentalised and interacting with Syntenin or GRIP.

Syntenin-1 has been shown to bind neurofascin (Koroll et al., 2001). The neurofascin isoform, NF155 expressed by glia binds to the Paranodin/Caspr-contactin complex on neurons at the paranodal axoglial junction in both CNS and PNS (Peles and Salzer, 2000). OPCs and other types of NG2+ cells issue processes at these locations in the CNS (Butt et al., 1999; Martin et al., 2001). It is plausible that NG2 and Syntenin function together at this junction. Adaptor proteins like Syntenin could mediate in downstream signalling through their cytosolic partners to recruit specific regulatory components or help selectively anchor receptors at specialised sites.

Below are schemes of possible signal transduction pathways involving NG2 and Syntenin.

(a)



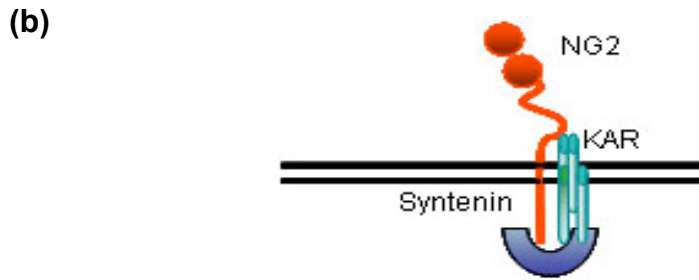


Figure 4.2.2

Signalling pathways involving NG2 and Syntenin in glia

Syntenin can cluster NG2 at the plasma membrane by virtue of its ability to homodimerise. (a): Syntenin and NG2 interaction may modulate the cytoskeleton through pathways involving multiple kinases like ERK, FAK and CdC42. Clustering of NG2 is known to facilitate activation of adhesion-related signalling pathways leading to cell spreading and migration. Based on (Eisenmann et al., 1999, Yang et al., 2004, Zimmermann et al., 2001). (b) Syntenin and NG2 could be involved in clustering metabotropic receptors (Hirbec et al., 2005).

4.3 Extracellular ligands of NG2

Does NG2 have neuronal partners?

In their role as myelinating cells in the CNS and PNS respectively, oligodendrocytes and Schwann cells signal bidirectionally with neurons (Charles et al., 2002; Chan et al., 2004). They can respond to electrical activity to stimulate myelination in the CNS (Demerens et al., 1996). OPCs express receptors for GABA (Kettenmann et al., 1991; Kirchhoff and Kettenmann, 1992) and glutamate (Borges et al., 1994; Steinhauser and Gallo, 1996). NG2⁺ cells at synapses react to these excitatory and inhibitory neurotransmitters released by neurons (Bergles et al., 2000; Lin and Bergles, 2002). NG2⁺ cells in PNS and CNS are often found contacting neurons. This includes perisynaptic wrappings of NG2⁺ cell processes around and between neuronal synaptic

contacts in the cortex and hippocampus (Ong and Levine, 1999; Martin et al., 2001; Butt et al., 2002). The molecule has also been implicated as a negative modulator in deterring neurite outgrowth during development and after CNS injury (Dou and Levine, 1994).

In the adult mouse brain, 5-8% of all glia have been cited to be NG2-positive (Butt et al., 2002). Isolating fractions (Sect. 2.2.3.6) enriched in NG2 therefore seemed a plausible approach to investigate potential interactions. The synaptosomal fraction was positive for NG2. Recognisable NG2-positive structures could not however be visualised by EM. The infrequent occurrence of these cells reduces the likelihood of isolating and observing NG2-labelled structures.

We therefore decided to use whole brain as source material and immunoprecipitate NG2-interacting proteins.

4.3.1 LNS-domains as bait for extracellular ligands

Preliminary experiments using biochemical approaches to identify neuronal partner(s) of NG2 were performed bearing in mind the nature of LNS domains. LNS domains possess both high affinity and versatility in their capacity to bind ligands (Rudenko et al., 2001). It was thus assumed that *trans* interactions between NG2 and its partners are likely to involve the LNS domains. These domains have also been found on proteins involved in adhesion, migration and recognition. To date no extracellular binding partners for the LNS domains of NG2 have been identified. However, the frequent and close association of NG2+ cells with neurons during myelination and at the nodes of Ranvier suggest the existence of putative neuronal receptor(s) for NG2 (Butt et al., 1999, Sugita et al., 2001).

GST-LNS fusion proteins were harvested from bacteria expressing the constructs. We assumed that since LNS domains are self-folding, the fusion proteins produced by the bacteria were functional. The GST-LNS1 fusion protein was used to probe for putative partners from whole brain extracts (Sect 2.2.3.5). Brain homogenate was prepared according to Sugita et al (Sugita et al., 2001). A protein at approximate molecular weight of 130 kD was

consistently precipitated by GST-LNS1 (Fig 3.4.2.2). To identify the protein Western blotting with antibodies against candidate partner proteins was performed. The results proved that it is not L1 or alpha-dystroglycan.

4.3.2 A 140 kD protein as a possible NG2 ligand

As an alternative approach, co-immunoprecipitation with mcAN2 antibody was performed. The monoclonal AN2 antibody is ideal for such a purpose; it recognises an epitope on the ectodomain (within 1237-1531 aa) away from the LNS domains and identified GAG site at Serine 999 and leaves the cytoplasmic region free to interact. Immunoprecipitating NG2 from P6 mouse brain (Sect 2.2.3.4) along with associated proteins was therefore performed.

Immunoprecipitation using the AN2 monoclonal antibody reproducibly co-precipitated a protein at approximate molecular weight of 140,000 dalton (Sect 3.4.3). In order to resolve out the molecular weight more accurately it was analysed on SDS-PAGE of different percentages. On a ten percent gel the protein was detected as running at 180,000 dalton (Sect 3.4.3, Fig 3.4.3.1, B). The immunoprecipitated proteins were analysed on SDS-PAGE, blotted and probed with several antibodies (Sect 3.4.3, Fig 3.4.3.2). NG2 is proteolytically released (Nishiyama et al., 1995) as a soluble form and is part of a complex containing the MT3-MMP metalloprotease (Iida et al., 2001). In the injured cerebral cortex, much of the overall increase in NG2 is due to the saline-soluble, cleaved ectodomain. NG2 is sensitive to two types of metalloproteases: the ones inhibited by hydroxamic acid and TIMP (tissue inhibitors of metalloproteases) 2 and 3 (Asher et al., 2005). It is therefore susceptible to degradation. The 140 kD protein did not react with pcAN2 antibody, ascertaining that it was not a degraded fragment of NG2.

The primary antibody-coated beads employed as a control did not bind non-specifically: blotting for NCAM did not show any signals in both control or mcAN2-coated beads.

Blotting with anti-GRIP antibody (identifies a protein at 130 kD) did not give a signal coinciding with the protein identified at approximate molecular weight of 140 kD. However, GRIP was present among the immunoprecipitated proteins,

proving that the protocol is successful in identifying interaction partners. α -dystroglycan (156 kD) and β -dystroglycan (43 kD) both failed to give any signals in the lanes loaded with the immunoprecipitates with mcAN2. Alpha - and beta dystroglycans have been shown to be part of a supramolecular structure at synapses, where they bind neurexin regulating cell adhesion (Sugita et al., 2001). The 140 kD protein did not react with antibodies against PDGF α R (Goretzki et al., 1999) which had been shown to bind NG2 in *cis* or against L1. Bearing in mind that several integrins associate or compartmentalize with NG2 (Iida et al., 1998; Chen et al., 2000; Jannatipour et al., 2001; Fernandez-Valle et al., 2002; Fukushi et al., 2004; Makagiansar et al., 2004), we cannot exclude that the protein at 140 kD (Sect 3.4.3) or the one immunoprecipitated with GST-LNS1 at 130 kD (Sect 3.4.2) is an integrin subunit. However we could not identify it as such. It is conceivable that the 140 kD protein seen in 4-10% gradient gels is the same one identified at 130 kD in 7-15% gradient gels which was immunoprecipitated by GST-LNS1 (Sect 3.4.2).

The protein corresponding to the protein at 140 kD may be a constituent of the plasma membrane since the extraction protocol favoured interaction within the membrane fractions. In spite of a strong signal with silver staining, the protein sequence could not be obtained from the MALDI (Matrix Assisted Laser Desorption/Ionization) spectra. It was suggested that the immuno-precipitated protein is heavily glycosylated.

4.4 Outlook

To expand the studies concerning the NG2 proteoglycan and its interaction with Syntenin, the exact conditions under which the two proteins interact need to be identified. NG2 and Syntenin have both been implicated in migration and adhesion. The complete signal transduction pathway involving both molecules needs to be elucidated. The role NG2 plays in clustering different receptor types at the cell surface should be examined; AMPA subunits in complex with GRIP and Syntenin with KAR. The role of NG2 in plasticity would be consolidated if indeed such specificity holds true.

Discussion

The components, linkers and kinases, functioning in conjunction with NG2 in migration of *Oli neu* cells need to be explored. It would also be important to investigate whether antibodies generated against NG2 in MS have an inhibitory effect on migration. This would in part answer the question whether the presence of such antibodies in relapsing-remitting patients hamper myelinating efforts of OPCs in MS lesions.

Identification of the protein at 140 kD and exploring other approaches to identify a *trans*-acting extracellular partner is important. This might contribute to explaining the role of NG2+ cells near nodes of Ranvier and around neuronal synapses as well as NG2+ OPC-axon interaction.

5. SUMMARY

NG2 was initially identified as a specific marker of immature oligodendrocyte precursors but subsequently NG2⁺ cells have been shown to be a heterogeneous population in the brain. The role(s) of cells expressing this antigen has remains open. NG2's involvement in migration has been extensively studied and the regions of the extracellular part of the molecule involved in this function have been mapped as part of this work. The current work focuses on characterisation of NG2 – Syntenin interaction and its possible functional significance. Finally, several approaches to identify extracellular ligands interacting with the LNS domains of NG2 have also been explored.

NG2 carries a PDZ-binding motif at its intracellular cytoplasmic end. Syntenin, a syndecan-binding protein was identified as an intracellular ligand of NG2. Syntenin, among its other roles has been implicated in migration. It functions as an adaptor protein in supra-molecular complexes. Syntenin and NG2 have a similar expression profile, with highest expression in the developing brain and extensive surface colocalisation in immature cultured oligodendrocytes. The interaction of these two molecules coincides with the period of maximum motility in these pre-oligodendrocytes. Additionally, treatment with antibodies against the NG2 molecule lead to clustering of NG2 and Syntenin as well as redistribution of Actin and EGFP-tagged Syntenin in primary oligodendrocytes and transfected *Oli neu*, respectively. Syntenin may function as a link between NG2 and the cytoskeletal machinery through which extracellular signals could be translated to change in morphology and locomotion of the cell.

In light of the role NG2 plays in migration of cells and enhanced invasive potential, the regions of the extracellular segment of NG2 that are involved in this function were defined. Polyclonal antibodies against GST-tagged proteins representing portions of the extracellular domain of the molecule were generated. The region immediately next to the transmembrane component (encoded within GST-extra) had a major role in mediating migration. It might be that *cis*-binding partners of NG2 like integrins are interfered with on addition of polyclonal antibodies against this specific section. The LNS domains with their

high binding capacity were also implicated but appear to play a lesser role. Treatment with PMA, a stimulator of PKC-alpha increases phosphorylation on NG2 as well as rate of migration. It is yet to be determined whether the signal transduction pathway modulating migration in *Oli neu* involves PKC-alpha. PMA treatment however does not redistribute possible membrane cytoskeletal linkers like Ezrin or Syntenin in *Oli neu*.

Furthermore, preliminary experiments were performed to address the issue of extracellular partners. Multiple biochemical approaches were explored for *trans* partners of NG2, possibly through its high affinity LNS domains on its extracellular arm. We have repeatedly observed a 140 kD protein associating with NG2. Its identity remains to be determined.

6. ABBREVIATIONS

AMPA α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
APS Ammonium-persulphate
BME Basal metabolic Eagle's medium
BrdU Bromo deoxy Uridine
CNP 2', 3' Cyclic nucleotide phosphodiesterase
CNS Central nervous system
COS Simian fibroblasts transformed by SV40 viral genome
CSPG Chondroitin sulphate proteoglycan
DAPI 4', 6-Diamidino-2-phenylindole
DMEM Dulbecco's modified Eagle's medium
DTT Dithiothreitol
EAE Experimental Allergic Encephalomyelitis
ECM Extra cellular matrix
EGFP Enhanced green fluorescent protein
ERK Extracellular signal regulated kinase
ERM Ezrin Radixin Moesin
FACS Fluorescence activated cell sorter
FAK Focal adhesion kinase
FGF Fibroblast growth factor
FP Fusion proteins
GABA γ -amino-butyric acid
GAG Glycosaminoglycan
GFAP Glial Fibrillary Acidic Protein
GLAST glutamate/ aspartate transporter
GLT-1 glutamate transporter-1
GRIP Glutamate receptor interaction protein
GST Glutathione S transferase
HBSS Hanks balanced salt solution
HEK Human embryonic kidney
IP Immunoprecipitation
KAR Kainic acid receptor
LNS Laminin-Neurexin-Sex Hormone binding globulin
MAG Myelin associated glycoprotein
MAGUK Membrane associated guanylate kinases
MBP Myelin basic protein
MCSP Melanoma Chondroitin Sulphate Proteoglycan
MOG Myelin oligodendrocyte glycoprotein
MS Multiple Sclerosis
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUPP1 Multi-PDZ domain protein 1
NMDA *N*-methyl-D-aspartic acid
OPC Oligodendrocyte precursor cell
PBS Phosphate buffered saline
PDGF- α -R Platelet-derived-growth-factor α receptor
PDZ PSD-95, Disc-large protein, Zona occludens
PFA para-formaldehyde
PIP2 Phosphatidylinositol 4,5 bisphosphate
PKC Protein kinase C

PLL Poly L-lysine
PLP Proteolipid protein
PMA Phorbol 12-Myristate 13-Acetate
PNS Peripheral nervous system
SDS Sodium dodecyl sulphate
SVZ Subventricular zone
TEMED N, N, N', N'-Tetramethylethylenediamine
TIMP Tissue inhibitors of metalloproteases

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Publications during the course of the current thesis

Karram K., Chatterjee N., Trotter J. NG2-expressing cells in the nervous system: role of the proteoglycan in migration and glial-neuron interaction. *J. Anatomy* in press.

Chatterjee N., Stegmüller J., Koroll M., Werner H, Nave KA., Trotter J.

Identification of syntenin as an intracellular binding partner of the NG2 proteoglycan in glial cells. *Manuscript under preparation.*

Conference Presentations

Poster presentation at EUROGLIA 2005, 7th European Meeting on Glial Cell Function in Health and Disease, May 17-21 2005, Amsterdam.

Poster presentation at Gordon Research Conference, Glial Biology: Functional Interactions Among Glia & Neurons March 13-18 2005, California, "The PDZ protein Syntenin is an intracellular partner of NG2 in glial cells"