The central role of Fyn kinase in axon-glial signalling and translation of myelin proteins

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2 INTRODUCTION

2.1 The nervous system

Evolutional changes from lower to higher eukaryotes are demonstrated by their advancing complexity of the nervous system. Its increase in size and functional potential is a prerequisite for the astounding capabilities of higher organisms. The vertebrate nervous system can be divided into the central nervous system (CNS) consisting of the brain and the spinal cord and the peripheral nervous system (PNS) which comprises the remaining part of the nervous system and can be further distinguished in somatic and autonomic PNS.

2.1.1 The central nervous system

The spinal cord transmits sensory information from the PNS to the brain and conducts motor information from the brain to effectors such as skeletal and smooth muscles or glands (via the PNS). Furthermore, the spinal cord integrates sensory information directly by reacting with simple reflexes such as the withdrawal reflex. The majority of sensory information, however, is processed in the brain which receives sensory information not only from the spinal cord, but also from cranial nerves such as the optic or olfactory nerves. The brain can be anatomically separated into forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). Forebrain and hindbrain are further subdivided into Diencephalon/ Telencephalon and myelencephalon/ metencephalon, respectively. The roles of the forebrain include the control of cognitive, sensory and motor function, the regulation of temperature, reproductive functions, eating, sleeping and the display of emotions. The hindbrain plays a role in the coordination of motor activity and regulates unconscious activities like breathing and blood circulation. Functions of the midbrain include the regulation of sensory information (vision, hearing) as well as the “fine tuning” of motor functions.

2.2 Cell types of the central nervous system

The human brain comprises ~2% of the body mass, but captures ~20% of the body’s glucose utilisation (Sherwood et al., 2006), demonstrating the vast degree of activity of this organ’s cellular components. The main cell types of the

central nervous systems are neurons and glial cells. The latter outnumber neurons and the glia/ neuron ratio increases with phylogeny culminating at a factor of 10 in humans. The increasing number of glial cells in higher organisms has possibly evolved to fulfil the energy requiring tasks of their larger and more complex brains. Glial cells can further be classified into Macroglia (astrocytes and oligodendrocytes) and Microglia.

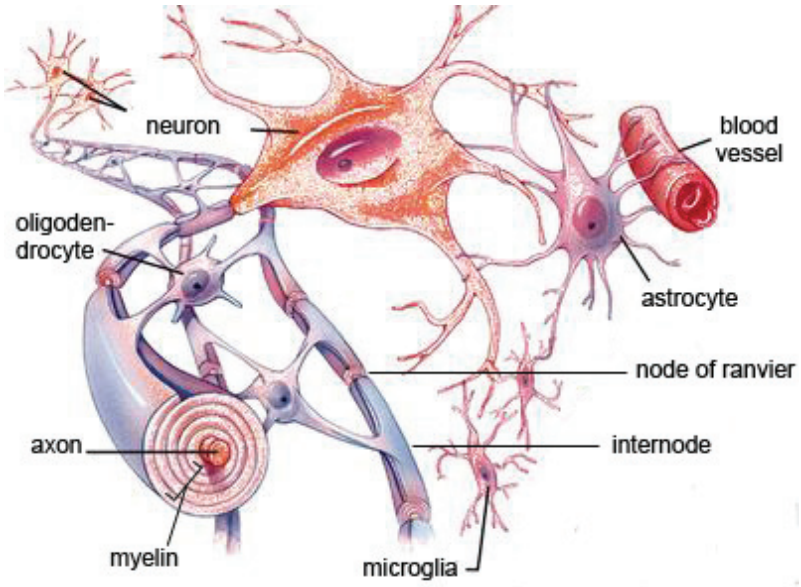

Figure 2.2-1: Cells types of the central nervous system

Neurons, oligodendrocytes, astrocytes and microglia are the main cell types of the CNS. They are illustrated here in agreement with their morphology and interplay. (Modified from Shier *et al*, 1999)

2.2.1 Neurons

Neurons are excitable cells that can transmit signals over long distances (see figure 2.2-1). Broadly, one distinguishes between neurons with sensory input (sensory neurons), neurons with muscle outputs (motoneurons) and neurons that connect with other neurons (interneurons) (Lackie & Dow, 1999). The number of neurons varies largely in different species. In invertebrates, the nematode worm *Caenorhabditis elegans* has 302, whereas the fruit fly *Drosophila melanogaster* possesses approximately 10^5 neurons. The human brain contains approximately 10^{11} neurons and one of these specialised cells can form up to 10^3 synaptic connections with other neurons forming a complex network allowing the processing of information in various ways. Although neurons can vary hugely in shape, most neurons are characterised by the presence of dendrites, somata and axons. Information is transferred by the

generation and conduction of action potentials, electrical impulses that depend on the presence of voltage gated channels in the soma and axon. The diameter of the axon determines the conductance velocity. Communication between different neurons is mediated by chemical or electrical synapses and can be inhibitory or stimulating.

2.2.2 Astrocytes

Astrocytes have been named according to their star-shaped morphology (see figure 2.2-1). Mature astrocytes characteristically express the intermediate filament protein GFAP (glial fibrillary acidic protein). They were originally associated with structural support for the CNS. It is now accepted that their relevance for the CNS is more versatile. Their functions include the regulation of extracellular ion and neurotransmitter concentration, blood-brain-barrier maintenance; they provide trophic factors for neurons and oligodendrocytes and serve as a scaffolding device for migrating neurons and glial cells (Lazzarini, 2004). They interact with blood vessels and neurons and form networks with other astrocytes which are connected by gap junctions that allow the intercellular transport of calcium and other small molecules (Abbott et al., 2006; Bennett et al., 2003). Recently, a role for astrocytes in myelination was proposed. Apparently, astrocytes embody a link between electrical activity in neuronal axons and myelination. They secrete LIF (leukaemia inhibitory factor) in response to ATP released from action potential firing neurons and LIF stimulates myelination by mature oligodendrocytes (Ishibashi et al., 2006).

2.2.3 Oligodendrocytes

Oligodendrocytes are the myelinating glial cells of the central nervous system (see figures 2.2-1, 2.3-1). Mature myelinating oligodendrocytes differentiate from oligodendrocyte precursor cells (OPC) that arise from neuroepithelium of the ventricular and subventricular zone in the brain. OPC migrate into the developing white matter areas until they reach their target axons. It has been controversially discussed from which area OPC emanate and start migrating to distinct target regions of the brain. Recently, it was proposed that the origin changes during time of development and that there are ventral and dorsal sources in the developing spinal cord and telencephalon (Richardson et al., 2006). Upon arrival at a target area precursor cells become post-mitotic, stop migrating and differentiate into myelin forming cells (Baumann and Pham-Dinh,

2001). Several factors affecting changes from a proliferation and migration competent precursor state to a differentiated myelination competent state have been postulated and originate from astrocytic as well as neuronal sources (Simons and Trajkovic, 2006). Moreover, OPC are generated in excess and only differentiated myelinating oligodendrocytes survive whereas surplus immature cells undergo apoptosis (Barres et al., 1993). Maturation and survival of oligodendrocytes are precisely regulated, but involved processes are only poorly understood (Simons and Trajkovic, 2006). Several expression markers (proteins and lipids) have been identified to distinguish different maturation stages of oligodendrocytes (see figure 2.2.3-1).

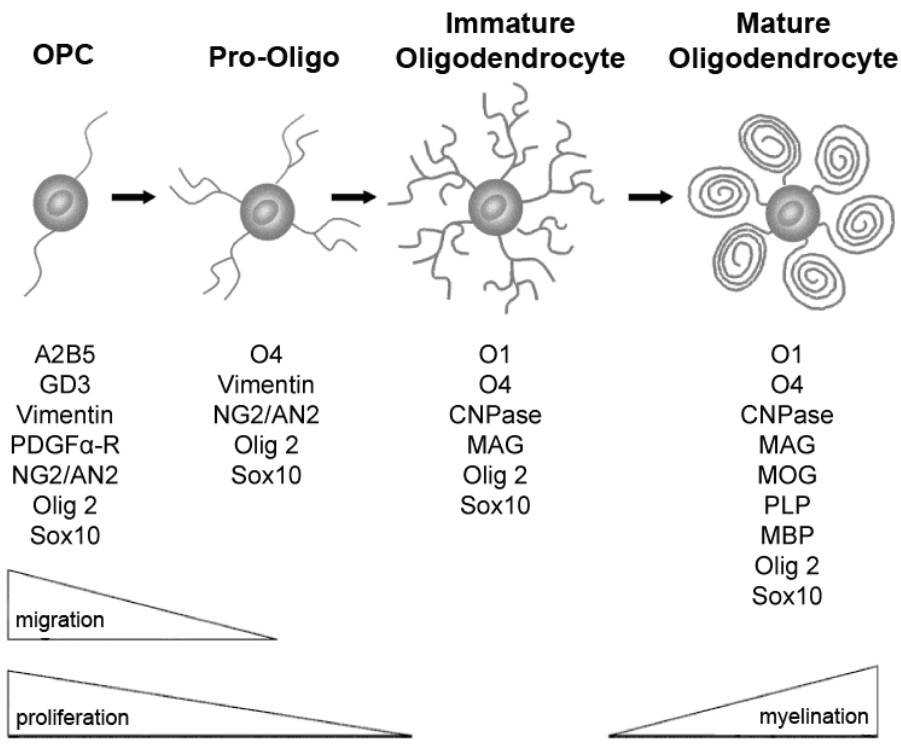

Figure 2.2.3-1: Oligodendroglial developmental stages and expression markers

Oligodendrocytes mature from proliferating oligodendrocyte precursor cells (OPC) that migrate and reach their destination where they become post mitotic and myelinate axons. Developmental stages can be identified by the presence of certain proteins or lipids as indicated below each oligodendrocyte illustration. Modified from (Pfeiffer et al., 1993).

2.2.4 Microglia

Microglial cells comprise approximately 10% of the entire glia population in the brain (Perry, 1998). They are smaller than astrocytes and oligodendrocytes (see figure 2.2-1) and their morphology changes drastically in response to their activation. They maintain brain homeostasis by clearing cellular debris from

apoptosed cells and microbes and support damaged neurons. Upon brain injury they extend processes to affected areas and thereby shield healthy tissue. They are regarded as the major cell type resident in the brain active in immune responses and are the first line of defence during brain damage (Kim and Joh, 2006). However, they have also been connected to diseases such as multiple sclerosis (MS) where antigen presenting microglia recruit T-cells which in turn enhance microglial activation resulting in the secretion of neurotoxic substances and neurological damage.

2.3 CNS Myelin

Myelin constitutes approximately 25-35% of the brain's dry weight (Lazzarini, 2004).

The myelin sheath is produced by the wrapping of the glial plasma membrane around neuronal axons (see figure 2.3-1). In the CNS single oligodendrocytes can myelinate up to 100 axons (Bjartmar et al., 1994) whereas Schwann cells, the myelinating cells of the PNS, always myelinate single axons. In both cases a myelinating cell and neuron form a structure also referred to as axon-glial apparatus containing distinct morphological and functional structures.

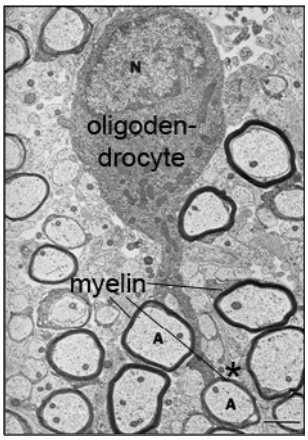

Figure 2.3-1: Myelination

EM picture showing a single oligodendrocyte myelinating 2 axons (A). Note that the thickness of the myelin sheath varies. The asterisk indicates an axon with a thinner sheath. N= nucleus. (Modified from Morell, 1984)

2.3.1 Structure of the myelinated axon

Myelin formation does not occur over the entire length of axon, but leaves unmyelinated areas termed nodes of Ranvier (see figure 2.2-1). Depolarisation

only takes place at these regions allowing rapid propagation of action potentials by saltatory conduction. Because the propagated impulse decays while travelling along the myelinated areas of the axon, nodes of ranvier are required to boost a new potential and expand the transmission over the entire length of an axon. The distances of these nodal regions vary from 100 µm for small diameter fibres to up to 1 mm for larger diameter fibres.

Generally, conduction velocity depends on the diameter of the axon, but myelination of axons increases the speed significantly (Waxman and Bennett, 1972). Therefore, vertebrates can transmit information at high speeds over long distances without requiring large diameter axons. To achieve conduction speeds comparable to myelinated vertebrate axons, unmyelinated axons would have to be 100 times larger in diameter (Hartline and Colman, 2007). This would further require similar increases in the soma size in order to accommodate the metabolic needs for such giant neurons. Consequently, this would result in an immense increase of the brain or spinal cord and affect the entire vertebrate anatomy and would alter evolutionary advantages of this anatomy dramatically. However, not all axons are myelinated. Axons with diameters larger than 0.2 µm in the CNS and 1 µm in the PNS are generally myelinated.

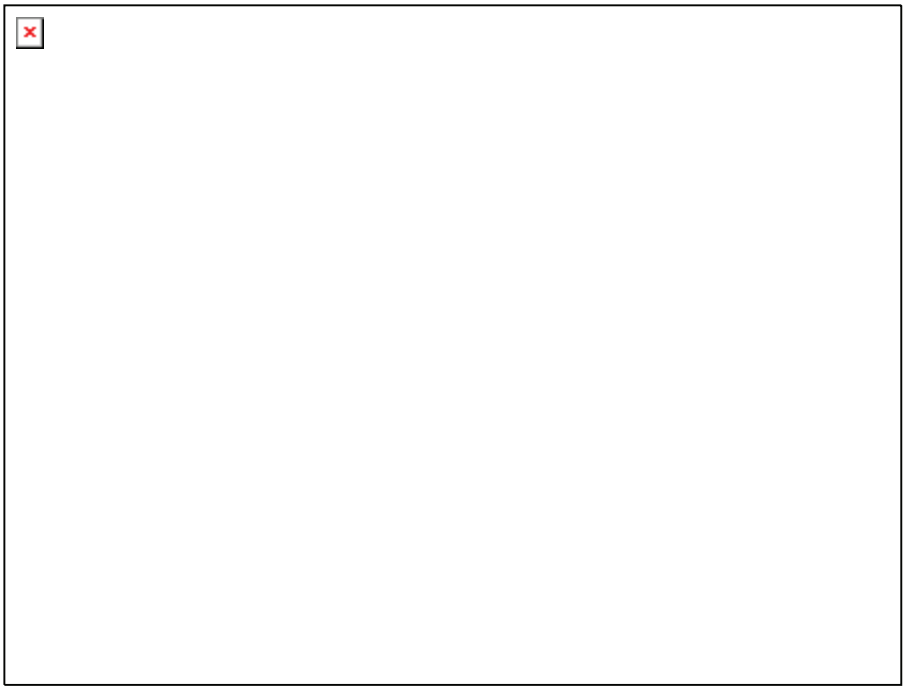

Figure 2.3.1-1 Structural organisation of the myelinated axon

The internodal, juxtaparanodal, paranodal and nodal regions are depicted including specific axonal and glial proteins localised in these areas (see text). Oligodendroglial proteins are underlined. CM, compact myelin; PNL, paranodal loops. Modified from (Peles and Salzer, 2000)

The number of layers around the axon determines the thickness of the myelin sheath and also depends on the diameter of the axon. Large axon fibres contain approximately 50 concentric spirals of myelin.

As mentioned earlier, there are myelinated (internodal) and unmyelinated areas (nodes) on myelinated axons. The internodes contain compacted myelin in which the cytoplasm has been excluded almost entirely. Moreover, one distinguishes two additional regions which differ in structure and molecular composition (see figure 2.3.1-1). Juxtaparanodes can be regarded as specialised regions of the internode. Potassium channels are located here which may play a role in repolarisation and maintaining the internodal resting potential. The paranodal region is located between the node and juxtaparanode. In this region the myelin membrane lamellae contain cytoplasm and are hence not as tightly packed as the compact myelin of the internodes. These paranodal loops forms septate-like junctions (paranodal junctions) with the axon and are thought to play a role in anchoring the myelin loops to the axon as well as serving as barriers inhibiting lateral diffusion of membrane components (Peles and Salzer, 2000). The neuronal proteins Caspr and contactin (F3) have been shown to form heterodimers and bind to glial neurofascin 155 (NF155) in these regions (Charles et al., 2002). Sodium and potassium channels are spatially separated by the formation of this axoglia junction (Bhat, 2003). These voltage gated sodium channels are concentrated in large amounts in the nodal region of the axolemma. It has been postulated that ankyrin G clusters these sodium channels as well as neurofascin 186 and the cell adhesion molecule NrCAM (Peles and Salzer, 2000). Interestingly, upon the first physical contact between axon and glial cell, sodium and potassium channels are redistributed in the axonal membrane to form clusters and can be seen as one of the initial myelination processes.

In the CNS, glial cells expressing the proteoglycan NG2 have been shown to form close contacts with the axolemma at the internodes, possibly affecting the distribution and maintenance of sodium channels, but their function is only poorly understood (Butt et al., 1999).

2.3.2 Myelin proteins and lipids

The lipid and protein composition of myelin differs from other cell membranes. In this specialised membrane, there is an enrichment of lipids (~70 - 85% of the dry mass) compared to proteins (15-30%) (Lazzarini, 2004). Cholesterol, phospholipids and glycosphingolipids (galactocerebroside and sulfatide) are especially enriched.

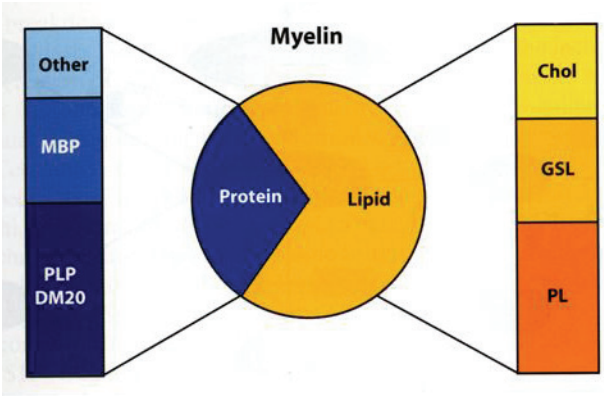

Figure 2.3.2-1: Protein and Lipid composition of myelin

In contrast to other membranes the lipid content is higher than the protein content in myelin. Myelin basic protein (MBP), proteolipid protein (PLP) and its splice variant DM20 are the major myelin proteins whereas the lipids are mainly represented by cholesterol (Chol), glycosphingolipids (GSL) and phospholipids (PL). (from Lazzarini, 2004)

Myelin Basic Protein (MBP) and Proteolipid protein (PLP) and its splice variant DM20 are the most abundant proteins in myelin. Together they account for ~ 80% of the total protein mass. Other myelin proteins include the third most abundant myelin-associated oligodendrocytic basic protein (MOBP) (Montague et al., 2006), and the characteristic myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and 2', 3'- cyclic nucleotide 3'-phosphodiesterase (CNP).

The generation of the myelin sheath is accompanied with a huge biosynthetic effort for an oligodendrocyte. It is estimated that a single cell synthesises up to 5-50 x 10^3 µm^2 myelin membrane surface area per day during the active phase of myelination (Pfeiffer et al., 1993). In order to fulfil this tremendous task, a highly specialised and organised protein and lipid delivery system must be required that needs to be regulated in a temporal and localised manner.

2.4 Proteins involved in this study

2.4.1 Fyn kinase

2.4.1.1 Domain organisation of Fyn and other Src family kinases

Fyn kinase is a 59kD (in *mus musculus*) protein of the Src family of non-receptor tyrosine kinases. In humans this includes 11 members (Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes). Only 2 members of this family have been found in *Drosophila* (Src42A and Src 64) and 3 in *C. elegans* (Manning et al., 2002). Initially, v-Src was identified as the cancer-causing protein encoded

by the avian rous sarcoma virus. C-Src (referred to as Src here) was found to be a physiological homologue of v-src and later characterised as the first proto-oncogene with transforming capabilities. Mutated forms of Src where found in certain types of cancer which motivated researchers to elucidate the basic functions and properties of this protein (Martin, 2001). Additional family members like Fyn were identified over the years and they all share the same protein organisation. They consist of an N-terminal region with acylation sites, a unique domain, an SH3 and an SH2 domain, an SH1 or kinase domain and a c-terminal regulatory tail (figure2.4.1-1).

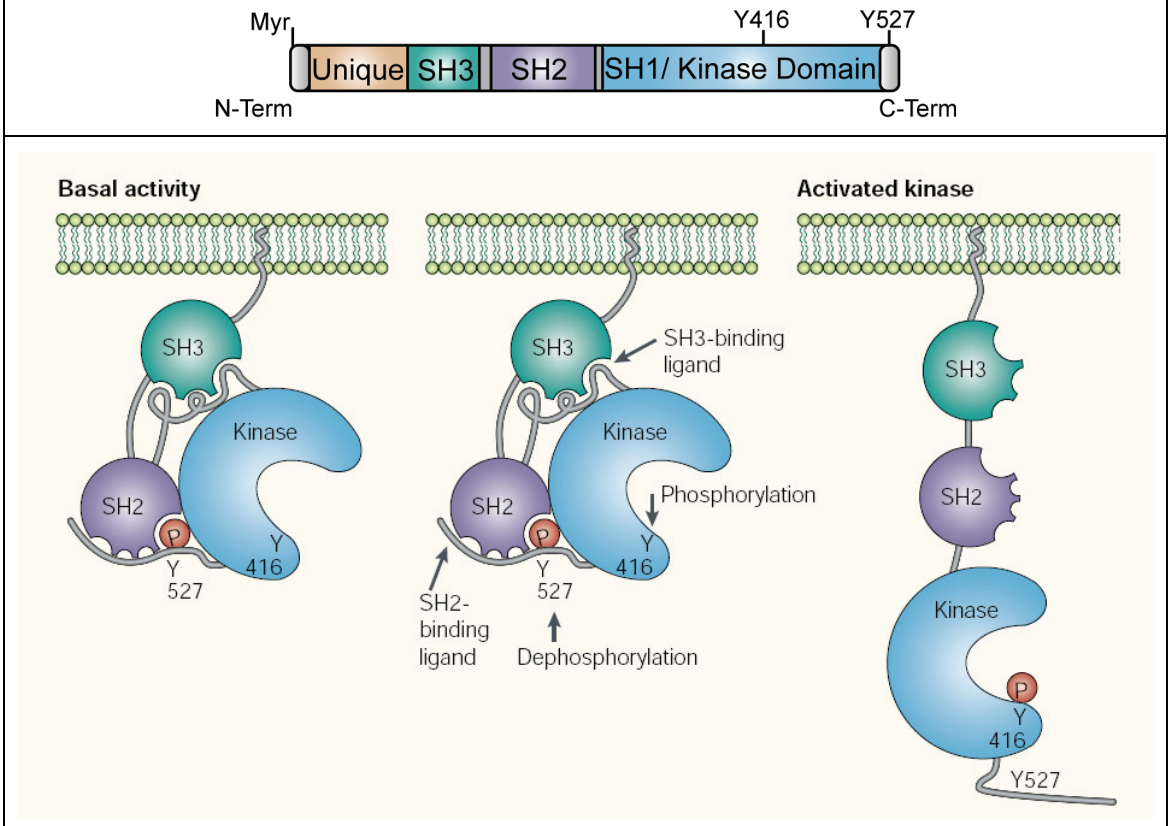

Figure2.4.1-1: Domain organisation and regulation of Src kinases

Top: Src kinases contain an N-terminal (SH4) domain anchoring the protein in the membrane by myristoylation (Myr), the SH3 and SH2 protein interaction domains, an SH1 or kinase domain and a C-terminus with a conserved regulatory tyrosine residue. **Bottom:** In the inactive or basal state the kinases is in a closed conformation due to intramolecular binding of the SH2 domain with the phosphorylated c-terminal tyrosine and the SH3 domain with a linker region between SH2 and kinase domain. Upon dephosphorylation of the c-terminal tyrosine or binding of external ligands to SH2 or SH3 domains, the conformation changes to an open form that is active. Intermolecular autophosphorylation of tyrosine 416 stabilises the active state of the kinase. (Martin, 2001)

The start methionine (which is cleaved off) at the N-Terminus is followed by a glycine and a cysteine residue which can be myristoylated and palmitoylated, respectively. Myristoylation occurs co-translationally on free polysomes and this acylation anchors the kinase to the plasma membrane. Here, palmitoylation can

occur which directs the kinase to lipid raft microdomains. The half-life of Fyn and the myristate modification is approximately 8 hours whereas palmitate has a half-life of only 1.5 - 2 hours. Palmitoylation could hence account for a distinct kind of regulation as the kinase could be deliberately brought in proximity with target proteins which are sorted into raft domains themselves (Resh, 1998). Interestingly, methylation of lysine residues 7 and 9 in the N-terminal region has been shown be important for Fyn function (Liang et al., 2004).

Downstream of this N-terminal region that is also referred to as the SH4 domain, a unique region is located which varies among the individual members of the Src family.

The following SH3 (Src-homology 3) domain of approximately 60 amino acids is a well defined protein-protein interaction site found not only in Src family kinases, but also in many other polypeptides (Mayer, 2001). SH3 domains bind to proline-rich ligands with the consensus sequences +xΦ**P**xΦ**P** (class I) or Φ**P**xΦ**P**x+ (class II) where + stands for a basic, Φ for a hydrophobic and x for any amino acid (Mayer, 2001). Moreover, further SH3 binding sequences have been identified which may alter slightly from the classic class I and class II motifs. The binding affinities of these non-canonical SH3 binding sequences are decreased (Roskoski, 2004).

Src kinases contain an SH2 (Src homology 2) domain of ~100 amino acids adjacent to the SH3 domain. SH2 domains are also rather common protein binding domains in mammals which have been shown to bind to phosphorylated tyrosine residues. There are different classes of SH2 domains which vary in their binding specificities. The variation in the sequences that are recognised lies within the first 5 amino acids carboxy-terminally of the phosphorylated tyrosine (Yaffe, 2002). The SH2 domains of Src and Fyn preferentially bind phosphotyrosine residues with negatively charged residues in the +1 and +2 position (e.g. pYEEI) (Roskoski, 2004; Yaffe, 2002).

The kinase or SH1 domain is structurally separated into a smaller N-terminal and larger C-terminal lobe. The smaller lobe consists predominantly of antiparallel β-sheets and is involved in ATP binding and orientation. The larger lobe is mostly α-helical and mainly responsible for protein substrate binding with additional impacts on ATP binding. The catalytic site is located in a cleft between the two lobes and the positioning of the two lobes determines the activity of the catalytic site of the kinase. An activation loop emerging from the larger lobe affects correct orientation of the catalytic site largely and phosphorylation of tyrosine residue 416 within this loop stabilises the active conformation (Roskoski, 2004).

The c-terminus of Src kinases contains a regulatory tyrosine residue which is phosphorylated in the majority of molecules in the cells and keeps the kinase in a basal (inactive) state.

2.4.1.2 Regulation of Fyn and other Src family kinases

As stated above, Src kinases are present either in an inactive or an active form. In the inactive state, the regulatory Tyrosine at the C-terminus (Y527) is phosphorylated and forms an intramolecular bond with the SH2 domain of the kinase (figure 2.4.1-1). This phosphorylation is regulated by tyrosine kinases such as Csk (C-terminal Src kinase) or Chk (Csk homologous kinase) (Chong et al., 2004; Okada and Nakagawa, 1989) and negatively regulates Src kinase activity. Furthermore, the SH3 domain binds intramolecularly to a linker region between the SH2 and the kinase domain. This keeps the kinase in a closed conformation in which both protein binding domains are occupied. Additional non catalytic means have been postulated to inhibit Src kinases. The binding of proteins such as Rack1, Caveolin-1 and WASP has been shown to inhibit Src activation. Caveolin-1 additionally recruits active Csk which phosphorylates the regulatory C-terminal Tyrosine and leads to further inhibition (Chong et al., 2005).

Activation of the kinase can occur by two independent means or perhaps a combination of both. Upon dephosphorylation of the c-terminal regulatory tyrosine residue, the intramolecular binding to the SH2 domain is abolished. This dephosphorylation is thought to be mediated by phosphatases such as transmembrane protein tyrosine phosphatases (PTP) alpha, epsilon and lambda and CD45 as well as cytoplasmic PTP1B, Shp1 and Shp2 (Roskoski, 2005). Upon loss of the SH2 interaction with the c-terminus, the kinase changes its conformation to a more open form which is accompanied with an intermolecular auto-phosphorylation of tyrosine 416 stabilising an active state of the catalytic site (Roskoski, 2004). Src kinases can also be activated by interfering with the inhibitory intramolecular binding of SH2 and SH3 domains. SH2 and SH3 domains can bind to external ligands leading to an activation of the kinase (Ostareck-Lederer et al., 2002; Sette et al., 2002).

Ubiquitination and proteasomal degradation of Src family kinases has been postulated as an additional means to regulate the activity of these enzymes (Hakak and Martin, 1999).

2.4.1.3 Fyn functions in oligodendrocytes and myelination

Two Fyn isoforms have been identified which differ by alternative splicing of exon 7. Exon 7a containing FynB is expressed in the brain and other tissues

whereas Exon 7b containing FynT is expressed in T cells. The differences between the functions of both isoforms are only poorly understood and their activity does not seem to differ largely (Resh, 1998).

Oligodendrocytes express the Src family members Fyn, Lyn and small amounts of Src. Fyn is the most prominent Src kinase in these cells. It has been shown to be crucial for the differentiation of oligodendrocytes as inhibition of Fyn kinase activity by chemical inhibitors or transfection with dominant negative constructs inhibited process outgrowth in cultured cells (Osterhout et al., 1999). The pivotal effect of Fyn kinase regarding myelination is demonstrated by the finding that Fyn knockout mice exhibit severe myelination defects. They are hypomyelinated in the forebrain and have shown to contain reduced levels of myelin basic protein, one of the main myelin proteins (Lu et al., 2005; Sperber et al., 2001; Umemori et al., 1994). Fyn is found in a complex with the GPI-anchored membrane protein F3/ contactin in lipid raft microdomains and antibody-mediated crosslinking of F3 leads to activation of Fyn kinase (Kramer et al., 1999). As active Fyn binds the cytoskeletal elements Tau and α-tubulin (Klein et al., 2002), is has been proposed that antibody-mediated crosslinking of F3 mimics the binding of an axonal ligand to oligodendroglial F3 leading to Fyn activation and recruitment of the cytoskeleton to the axon-glial contact site. The resulting polarisation of the cell towards this contact site can be regarded as one of the initial morphological changes required for the myelination process and may explain the striking consequences occurring in the absence of Fyn.

Intriguingly, Fyn has been implicated in the stimulation of MBP gene expression by acting (most likely indirectly) on a distinct region of the MBP promoter (Umemori et al., 1999).

2.4.2 L1-CAM and F3/ contactin

L1-CAM and F3/ contactin belong to the Immunoglobulin superfamily (IgSF) of cell adhesion molecules (CAM). Cell surface Proteins belonging to this superfamily mediate cell-cell interactions in a calcium independent way by homo- and/or heterophilic binding. Subfamilies within this superfamily differ by their number of characteristic immunoglobulin (IG) and fibronectin type III (FNIII) like domains (Grumet et al., 1991; Kenwrick et al., 2000).

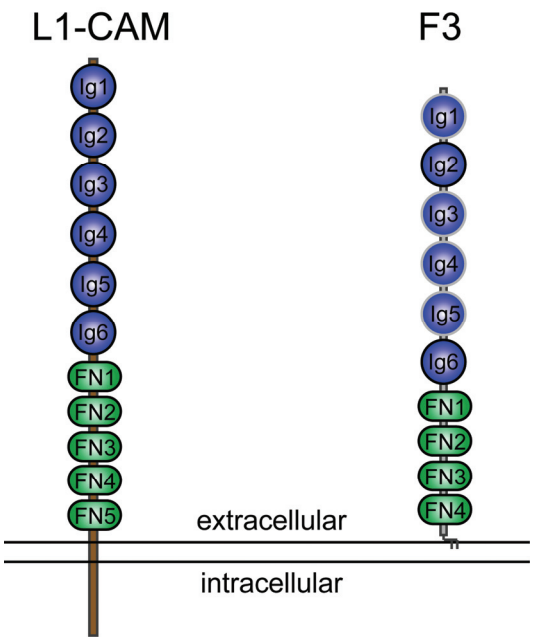

Figure 2.4.2-1: Domain organisation of L1 and F3

L1 and F3 belong to the Immunoglobulin superfamily of cell adhesion molecules: They both contain the characteristic immunoglobulin (Ig) and fibronectin type III (FN) domains. L1 is a single spanning transmembrane protein whereas F3 associated with the membrane by a GPI anchor. Note that the molecules differ in their number of FN domains.

2.4.2.1 L1-CAM

In vertebrates the L1 subfamily of Immunoglobulin superfamily CAMs contains the proteins L1 (NgCAM), CHL1, NrCAM and Neurofascin.

The primary structure of murine L1 contains 1260 amino acids and this glycoprotein has a molecular weight of 200-220 kD. L1 consists of a large extracellular part containing 6 Ig like domains and 5 fibronectin like (FNIII) domains, a single transmembrane region and a short but highly conserved cytoplasmic domain (see figure 2.4.2-1).

In the nervous system L1 is expressed on developing and mature neurons and as a splice variant on Schwann cells (Kamiguchi et al., 1998; Kenwrick et al., 2000). The presence of an alternative splice variant of L1 could also be detected in oligodendrocytes by RT-PCR in one study (Itoh et al., 2000). However, L1 expression by oligodendrocytes was excluded in other approaches (Barbin et al, 2004; Coman et al., 2005). L1 has been associated with several functions. Homophilic L1 binding in trans as well as heterophilic binding is probably responsible for L1s role in fasciculation, neurite outgrowth and axon guidance (Hortsch, 1996; Kamiguchi et al., 1998; Kenwrick et al., 2000). The heterophilic interaction of the cytoplasmic domain with the cytoskeleton via

ankyrin is regulated by MAP kinase signalling and is thought to be a crucial regulatory mechanism required for nerve growth (Whittard et al., 2006). Further binding partners include F3/ contactin, N-CAM, Tag-1 and integrins (Brummendorf et al., 1993; Malhotra et al., 1998; Oleszewski et al., 1999). L1 is downregulated on myelinated axons in culture (Barbin et al, 2004). In the adult mouse brain L1 is very strongly expressed on nerve fibres in the corpus callosum, but no immunoreactivity could be shown in the white matter tracts of the cerebellum (Munakata et al., 2003).

L1 deficient mice show a range of phenotypes including enlarged ventricles, learning and memory deficits and weakness of the hindlimbs (Kamiguchi et al., 1998).

2.4.2.2 F3/ contactin

F3/ contactin-1 belongs to the contactin family within the Immunoglobulin superfamily of cell adhesion molecules. The unprocessed molecular weight is approximately 113 kD and the domain organisation is similar to L1. It contains 6 Ig domains (4 of the C2 type), but only four fibronectin type III domains (see figure 2.4.2-1). F3 homologues have been identified in many species after the initial characterisation in chicken (F11) (Brummendorf et al., 1989). It is termed F3 in rodents and also found in human (contactin), *Drosophila* (Dcontactin) and *C. elegans* (C33F10.5/ RIG-4). F3 is associated with the membrane by a glycosyl-phosphatidyl-inositol (GPI)- anchor (Wolff et al., 1989) and is expressed by murine neurons (Gennarini et al., 1989) and oligodendrocytes (Einheber et al., 1997; Koch et al., 1997).

In myelinated axons, F3 is concentrated at the paranodes where it forms heterodimers with Caspr (contactin associated protein) and binds to glial neurofascin 155. This binding complex is thought to be instrumental for the formation of paranodal junctions (see section 2.3.1). Neuronal F3 also plays a role in axon-glial signalling. Binding of F3 to the glial transmembrane protein notch has been shown to lead to γ-secretase-mediated cleavage of notch. The cleaved intracellular domain seems to enter the nucleus. leading to an upregulation of myelin associated glycoprotein (MAG) and promoting oligodendrocyte differentiation (Hu et al., 2003).

Antibody-mediated crosslinking of F3 leads to the activation of Fyn in chick neurons and mouse oligodendrocytes (Kramer et al., 1999; Zisch et al., 1995). It was postulated for oligodendrocytes that a neuronal ligand could bind to F3 and trigger the activation of Fyn (Kramer et al., 1999). Interestingly, F3, Fyn and L1

could be isolated in a complex from mouse cerebellum (Olive et al., 1995). Moreover, F3 could be isolated in complexes with RPTPα, a receptor tyrosine phosphatase involved in the activation of Fyn kinase from murine and chicken brain and it was proposed that extracellular F3 could activate intracellular Fyn via transmembrane PTPα (Zeng et al., 1999; Zisch et al., 1995).

F3 deficient mice show aberrant paranodal organisation resulting in disruption of paranodal junctions and reduced conductance velocity of action potentials along the nerve. Furthermore, Potassium channels are mislocated to the paranodal region, but sodium channels are distributed normally at the node (Boyle et al., 2001).

2.4.3 Myelin basic protein (MBP)

Myelin basic protein is the second most abundant protein in CNS myelin and is also present in lower amounts in the PNS. It comprises 30% of the total amount of protein and 10% of the dry weight of CNS myelin (Boggs, 2006).

The complex organisation of the MBP gene leads to the generation of 3 pre-mRNAs by the use of 3 different transcription start sites. Alternative splicing of these mRNAs leads to the generation of a number of proteins which are separated into 2 families. Proteins originating from pre-mRNA transcribed from transcription site 1 in the MBP gene are expressed only at low levels in the nervous system and are mainly found in the immune system including thymus, spleen and lymph nodes. These are referred to as Golli (-MBP) proteins. The second family of proteins are denoted “classic” MBP proteins and are derived from pre-mRNAs transcribed from transcriptions sites 2 and 3. In mice alternative splicing of these mRNAs yields 6 different proteins ranging from 21.5 to 14 kD in size (21.5, 20.2, 18.5, 17.24, 17.22, and 14 kD) (Boggs, 2006). Only four classic MBP isoforms have been found in humans (21.5, 20.2, 18.5, and 17.2 kD).

All isoforms are found in compact myelin, but the isoforms seem to be differentially distributed. This could also be observed at mRNA and protein level in cells *in vitro*. Intriguingly, exon 2 containing isoforms (21.5, 17.22kD) could be detected in the nucleus in transiently transfected HeLa cells (Staugaitis et al., 1990). The distribution of different isoforms are not only localised differently spatially, but also temporally. Apparently, Exon 2 containing isoforms are expressed at higher levels during early stages of oligodendrocyte development and at lower levels during later stages whereas exon2 lacking isoforms show the opposite expression behaviour.

MBP is a very basic protein with an isoelectric point of ~10 and its main function in the CNS has been associated with the compaction of myelin membranes by interaction with negatively charged phospholipids (Boggs, 2006). MBP has been denoted the “executive molecule of myelin”, because so far it is the only structural protein essential for correct CNS myelin formation. In contrast to mice lacking the most abundant myelin protein PLP which elaborate relatively normal myelin, MBP ablation results in a drastic hypomyelinated phenotype. This could be shown by the analysis of MBP knock out mice as well as the naturally occurring *shiverer* mouse mutant. A large part of the MBP gene is deleted in the *shiverer* mouse mutant and the CNS lacks most compact myelin (Boggs, 2006). Furthermore, a naturally occurring rat mutant termed ‘*Long Evans shaker rat’ (les)* lacks compact myelin in the CNS due to abnormal MBP transcription caused by a mutation in the gene (Carre et al., 2002).

In the PNS proteins like P0 are thought to fulfil MBP’s function in membrane compaction. However, although MBP is expressed only in small amounts, the PNS of the *shiverer* mouse mutant develops abnormally. It shows reduced axonal diameter and myelin sheath thickness, aberrant axon-Schwann cell contacts and the number of Schmidt-Lantermann incisures is more than doubled (Boggs, 2006). Hence, MBP seems to be important for PNS development as well.

2.5 RNA transport granules and mRNA localisation

The differential distribution of molecules within the cell allows the formation of functional compartments and subdomains enabling the specialisation of eukaryotic cells. In higher eukaryotes this has lead to the establishment of distinct organs. Every organ serves a specialised function which depends on the presence of specialised cell types which is ultimately based on the differential expression and distribution of distinct molecules.

When a protein coding messenger RNA that has been synthesised and processed in the nucleus, enters the cytoplasm it faces different potential fates which depend on its sequence. Most mRNAs directly associate with free ribosomes and are translated on polysomes. Cytosolic proteins remain in the same compartment whereas proteins that are to be targeted to different compartments such as the mitochondrion or the nucleus contain specific targeting sequences that lead them to the desired organelle. The synthesis of membrane and secretory proteins is arrested during translation and the mRNA bound to the ribosome is targeted to the endoplasmatic reticulum into which the

protein it is then translocated co-translationally. These proteins are targeted and further processed by the so-called secretory pathway.

On the contrary, some mRNAs are not directly translated upon exiting the nucleus, but they are first transported to their destination where they are then translated locally. Generally, these mRNAs associate with a set of RNA binding proteins in the nucleus which mediate nuclear export and prevent the formation of polysomes upon entry into the cytoplasm. These trans acting factors bind to specific sequences in the mRNA termed localisation elements (LE), zipcodes or targeting elements (TE) which are often located in the 3' untranslated region (3'UTR) (Czaplinski and Singer, 2006). In the cytoplasm larger RNA- protein complexes are formed which have been denoted RNA (transport) granules or localising ribonucleoprotein particles (L-RNP) (Carson et al., 2001b; Czaplinski and Singer, 2006). These structures contain a number of mRNA molecules, RNA binding proteins, ribosomal subunits, factors required for translation initiation and in some cases adaptor proteins for the association with cytoskeletal motor proteins (figure 2.5.1).

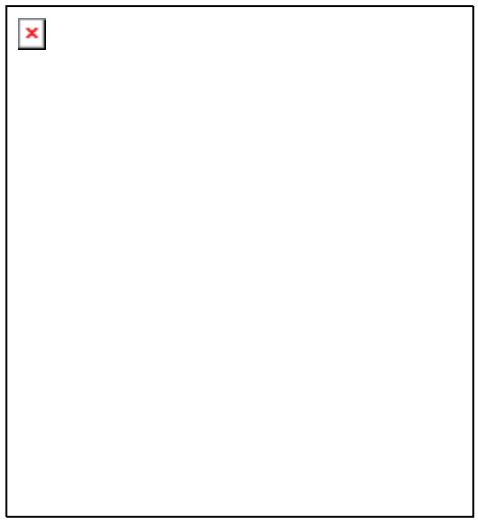

Figure 2.5.1: RNA transport granules

RNA granules consist of several mRNA molecules of the same type, a number of RNA binding proteins and sometimes components of the translation machinery. They often contain motor proteins that transport them along the cytoskeleton (modified from (Shav-Tal and Singer, 2005).

These granules are transported on cytoskeletal elements towards their destination where the encoded proteins are synthesised. Hence the mRNAs which are transported can be present in two states. They are either transport competent and translationally repressed or transport incompetent and translationally active. Translation inhibition is mediated by RNA binding proteins. The mechanisms and molecular components involved in triggering translation initiation are only poorly understood. It has been postulated that post

translational modifications of RNA binding proteins within the granules lead to a release of the transported mRNA allowing local translation. Recently, it could be shown that ZBP1, an RNA binding protein involved in the localisation of β-actin mRNA in fibroblasts and neurons, represses translation during transport within the granule. At the cell periphery ZBP1 is phosphorylated by Src kinase at a conserved tyrosine residue leading to a release of β-actin mRNA, thereby relieving inhibition and initiating translation (Huttelmaier et al., 2005).

Messenger mRNA transport in RNA granules has been extensively studied in oligodendrocytes. In these cells the RNA binding protein heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) has been identified as a trans acting factor mediating the transport of distinct mRNAs including MBP and MOBP (Ainger et al., 1997; Ainger et al., 1993). It could be shown that an 11 nucleotide (GCCAAGGAGCC) localisation element in the 3'UTR of MBP mRNA termed the A2 response element (A2RE) is required for hnRNP A2 binding (Munro et al., 1999). A2RE containing mRNA and hnRNP A2 associate in the nucleus before they are actively transported to the cytoplasm where RNA transport granules form. These complexes are transported on microtubules to the most distal processes of the oligodendrocyte (Carson and Barbarese, 2005). Each granule contains ~30 A2RE RNA molecules, motor proteins and components of the translation machinery including ribosomal subunits and initiation factors (Carson and Barbarese, 2005; Mouland et al., 2001). Interestingly, non A2RE containing localised mRNAs seem to be sorted into different types of granules showing that granule formation is sequence specific. Translation repression during granule transport seems to be mediated by hnRNP E1 which is recruited to the granule by hnRNP A2 (Kosturko et al., 2006). However, the factors and mechanisms required for localised translation initiation in oligodendrocytes are unknown.

2.6 Aim of the study

Oligodendroglial F3 had been shown to be involved in the activation of Fyn kinase. It was the aim of this study to identify a neuronal ligand capable of binding to glial F3 in order to activate Fyn. Furthermore, cellular consequences of Fyn activity in oligodendrocytes were to be assessed. Further elucidation of molecular interactions upstream and downstream of Fyn kinase was intended in order to contribute to the understanding of the central role of Fyn in oligodendrocytes and myelinogenesis.

3 MATERIALS AND METHODS

3.1 Equipment, Materials, Buffers and Media

3.1.1 Equipment & Software

Centrifuges	
Biofuge *fresco* (Tabletop centrifuge)	Heraeus
Megafuge 1.0 R (centrifuge)	Heraeus
Optima MAX-E (Ultracentrifuge)	Beckman
Transfection devices	
Gene Pulser	Biorad
Nucleofector II	AMAXA
Microscopes	
TCS SP5 (Laser scanning microscope)	Leica
DM LB	Leica
DFC 350F digital camera;	Leica
Other equipment	
Photospectrometer Ultrospect 2100 pro	Amersham/ GE Healthcare
T3 Thermocycler (PCR machine)	Biometra
Optimax X-Ray (Film processor)	MS Laborgeräte
Biotrak II Plate Reader (ELISA)	Amerhsam/ GE Healthcare
Fluoroskan Ascent FL (Luminometer)	Thermo Scientific
Image documentation and analysis (IDA) machine	Raytest
Software	
AIDA Image Analyzer 3.28	Raytest
CloneManager 7.01	Scientific & Educational Software
DNA Strider 1.3f9	CEA
ImageJ 1.38n	Wayne Rasband, NIH

3.1.2 Materials

Glassware was purchased from VWR, plastic ware from Nunc, Falcon and Sarstedt. Chemicals were obtained from Sigma and Roth. The origins of special materials, chemicals and kits are indicated in the text.

3.1.3 Buffers and Media

General Buffers	
PBS	150 mM NaCl; 8 mM Na_2HPO_4; 1.7 mM NaH_2PO_4; adjust pH to 7.2
TBS	150 mM NaCl ; 50 mM Tris , adjust pH to 7.2
TBST	TBS + 0.1% (v/v) Tween 20
Molecular Biology	
50 x TAE Buffer	242 g Tris (2M); 100 ml 0.5 M EDTA pH 8.0 (50mM); 57.1 ml acetic acid; fill to 1000ml with ddH_2O
LB medium	Bacto Trypton 10 g; Bacto Yeast extract 5 g; NaCl 10 g; Fill to 1000 ml with ddH_2O and adjust pH to 7.4 with NaOH.
LB agar	4.5 g agar-agar in 300ml LB medium; autoclave
Protein Biochemistry	
Lysis buffer	50mM Tris-HCl, pH 7.4; 1% (v/v) NP-40; 0.25% (w/v) sodium deoxycholate; 150mM NaCl; 1mM EDTA; Halt® Protease and Phosphatase Inhibitor Cocktails (Pierce) were added if appropriate
4x Sample Buffer	200 mM Tris-HCL (pH=6.8); 10% (w/v) SDS; 0.4% (w/v) bromphenol blue, 40% (v/v) glycerol, 400mM DTT (if reducing conditions are desired)
SDS Running Buffer for electro-phoresis (5x)	125 mM Tris;1.25 M glycine; 0.5 % (w/v) SDS; pH 8.3
Stacking/ Separation gel for SDS PAGE	See Tables A8-9 and A8-10 in „Molecular Cloning – A Laboratory Manual“, Sambrook und Russell, 2001, Cold Spring Harbour Press, New York, USA
Western Blot Transfer buffer	24 mM Tris, 192 mM Glycine, 20% Ethanol
Coomassie Protein staining solution	0.1% (w/v) Coomassie Brilliant Blue R250; 30% (v/v) ethanol; 10% (v/v) acetic acid
Destaining solution	30% (v/v) ethanol; 10% (v/v) acetic acid
10 x Ponceaus S	2% (w/v) Ponceau S; 30% (w/v) trichloric acid; 30% (w/v) Sulfosalicylic acid

Blocking solution	4% (w/v) dry milk powder in TBST or 3% (w/v) BSA in TBST (for Phospho-protein analysis)
Stripping solution	Add 16.7 ml 37% HCl to 700 ml ddH_2O,; adjust pH to 2.0 with approximately 230ml of 1M glycine
ECL solution	Solution A: 200 ml 0.1 M Tris-HCl, pH 8.6; 50 mg Luminol (store at 4°C); Solution B: 11 mg para-hydroxy coumaric acid Development: Combine 1ml solution A + 100 µl solution B + 0.3 µl H_2O_2
Immunodetection	
Fixation solution	4 % (w/v) paraformaldehyde in PBS
Blocking solution	PBS 10% horse serum
Mounting medium	2.4 g moviol 4-88; 6g glycerol; 6 ml ddH_2O; 12 ml 0.2 M Tris, pH 8.5
Cell culture	
10 x Poly-L-Lysine (PLL)	0.1 % PLL in ddH_2O
Sato 1 %HS (Oli-*neu*)	13.4 g/l DMEM; 2 g/l $NaHCO_3$;0.01 g/l Transferrin100 µg/l; Insulin (Stock 10 µg/ml); 100 µM Putrescine; 200 nM progesterone; 500 nM TIT; 220 nM Na-Selenite; 520 mM L-Thyroxine; 0.05% Gentamycin; 1% (v/v) horse serum
Differentiation medium (OLI-*neu*)	Sato 1% HS + daily addition of 1mM dbcAMP
Sato/ B27 supplement 1% HS (primary oligodendrocytes)	13.4 g/l DMEM; 2 g/l $NaHCO_3$; 20 ml/l B27 Zusatz; 0.011 % Pyruvate; 500 nM TIT; 520 mM L-Thyroxine; 0.05 % Gentamycin; 1% (v/v) horse serum
Trypsin/ EDTA „low“	0.01% Trypsin (Stock: 1 % in $HBSS^-$); 0.02% EDTA (Stock: 0.2 % in $HBSS^-$) in $HBSS^-$
Freezing medium	70% (v/v) RPMI 1640; 20% (v/v) FCS; 10% (v/v) DMSO
$HBSS^+$	500 ml HBSS + 7.5 ml $MgSO_4$ (Stock: 10% (w/v))
COS7 medium	DMEM + L-Glu containing 10% FCS
COS7 transfection medium	DMEM + L-Glu containing 1% FCS (5ml FCS were pretreated with 250µl Protein A Sepharose to remove IgGs from the serum)

3.2 Antibodies

3.2.1 Primary antibodies

Antigen	Name	Species	Application	Source
Actin	Actin	rabbit	1:200 (WB)	Sigma
F3/ contactin	11-111	mouse	1:1000 (WB); 1:10 (IXL)	Dr.Rathjen, Berlin
Fyn	Fyn3 (SC-16)	rabbit	1:1000 (WB); 1:100 (ICC/ IHC) 1:50-100 (IP)	Santa Cruz
Fyn	Clone 25	mouse	1:250 (WB)	BD Transduction laboratories
GAPDH	ab9485	rabbit	1:1000 (WB)	abcam
hnRNP-A2	EF67	mouse	1:500-1:1000 (WB) 1:400 (ICC) 1:200 (IHC) 1:100 (IP)	Dr. Rigby, Dartmouth Medical School, USA
hnRNP-B1	hnRNP-B1	rabbit	1:1000 (WB)	Dr. Sueko, Saga Medical School, Japan
hnRNP-E1	T-18	goat	1:100 (WB)	Santa Cruz
hnRNP-F	N-15	mouse	1:1000 (WB); 1:25 (ICC)	Santa Cruz
hnRNP-F	3H4	goat	1:100 (WB)	Santa Cruz
hnRNP-K	P20	goat	1:100 (WB)	Santa Cruz
L1	555	rat	1:100 (WB)	Dr.Rathjen, Berlin
MBP	MCA409S	rat	1:2500 (WB). 1:1000 (ICC)	Serotec
Olig2		rabbit	1:1000 (IHC)	Dr. Stiles, Harvard, USA
p130CAS	C-20	rabbit	1:100 (WB)	Santa Cruz
Phospho-tyrosine	4G10	mouse	1:750 (WB); 1:50 (ICC)	Upstate
Src-pY^{418}	Src-pY^{418}	rabbit	1:1000 (WB); 1:100 (ICC)	Biosource
Synatxin-6	Synatxin-6	rabbit	1:5000 (WB)	Synaptic Systems
α-tubulin	DM1a	mouse	1:5000 (WB); 1:500(ICC)	Sigma
WB: western blot; ICC: immunocytochemistry; ICH: immunohistochemistry; IP: immunoprecipitation; IXL: immunocrosslinking				

3.2.2 Secondary antibodies

Target species	Host species	Conjugation	application	Source
human	goat	HRP	1:2000 (CE)	Pierce
human	goat	Cy2	1:50 (ICC)	Dianova
human	goat	-	1:100 (IXL)	Dianova
mouse	goat	HRP	1:10000 (WB)	Dianova
mouse	goat	Cy3	1:1000 (ICC)	Dianova
mouse specific	goat	HRP	1:5000 (WB)	Dianova
mouse	Rabbit	-	1:100 (IXL)	Dianova
rabbit	goat	HRP	1:10000 (WB)	Dianova
rabbit	donkey	Cy2	1:100 (ICC)	Dianova
rat	goat	HRP	1:10000 (WB)	Dianova
rat specific	goat	Cy5	1:100 (ICC)	Dianova
WB: western blot; ICC: immunocytochemistry; HRP: horseradish peroxidase; IXL: immunocrosslinking				

3.3 DNA analysis and manipulation

3.3.1 Polymerase Chain Reaction (PCR)

Plasmid template DNA was amplified by polymerase chain reaction (PCR). Reaction conditions suitable for the used Polymerase and primers were chosen according to the manufacturer's instructions and the "Roche Molecular Biochemicals Lab FAQS" manual (Roche). The reactions mainly varied in annealing temperature (depending on the primers' melting temperature T_m) and extension time (depending on the efficiency of the used polymerase and the length of the desired product). Mainly, VentR (New England Biolabs)) and Pfu Turbo (Stratagene) polymerases were used.

Reaction conditions can be exemplified as follows:

	VentR	Pfu Turbo
Sense Primer (100 µM)	0.5 µl	0.5 µl
Antisense Primer (100 µM)	0.5 µl	0.5 µl
ThermoPol buffer (10x)	10 µl	-
Pfu DNA Polymerase	-	10 µl

reaction buffer (10x)		
$MgSO_4$	2 µl	-
dNTP (12.5 mM each)	2 µl	2 µl
Template plasmid DNA	~50 ng	~50 ng
Vent Polymerase (2 U /µl)	0.5 µl	-
Pfu Turbo Polymerase	-	1 µl
Nuclease free dH_2O	Fill to 100 µl	Fill to 100 µl

Thermo-cycling can be exemplified as follows (Pfu Turbo):

Step	Temperature	Time	
1) Initial Denaturation	95°C	2 min	
2) Denaturation	95°C	45 sec	**Steps 2 - 4 are repeated 25- 30 times.**
3) Primer Annealing	~60°C (~T_m-5°C)	45 sec	
4) Primer Extension	72°C	1 min/ kb	
5) Final Extension	72°C	10 min	

3.3.2 RT-PCR

As no template cDNA was available for the generation of hnRNP A2 expression vectors, reverse transcription and subsequent PCR (RT-PCR) with specific primers for the hnRNP A2 open reading frame were conducted.

Total RNA was isolated from Oli-*neu* cells using the RNeasy Plus® Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed with the OneStep RT-PCR Kit (Qiagen) following the user manual's suggestions:

Total (Oli-*neu*) RNA (1 µg/µl)	2 µl
RNAse free water	33.4 µl
Qiagen OneStep RT-PCR buffer (5x)	10 µl
dNTP Mix	2 µl
Sense Primer (100 µM)	0.3 µl
Antisense Primer (100 µM)	0.3 µl
Qiagen OneStep RT-PCR enzyme mix	2 µl

Temperature	Time	
50°C	30 min	
95°C	15 min	
94°C	45s	**35 cycles**
60°C	45s	
72°C	90s	
72°C	10 min	

The amplified products were analysed by agarose gel electrophoresis and processed further.

3.3.3 Plasmid Preparation from bacteria (Mini-/ Maxiprep)

Over night cultures of bacteria harbouring the plasmid of interest were grown in LB medium containing the selective antibiotic. Plasmid DNA was purified from 5 ml cultures for small scale (Miniprep) and 100 ml cultures for large scale (Maxiprep) preparation using the "Wizard® Plus SV Miniprep DNA Purification System" and the "Qiagen Plasmid Maxi Kit" according to the manufacturers' instructions.

3.3.4 DNA restriction digest

For analytical purposes approximately 1 µg of plasmid DNA was digested at 37°C for 60-90 minutes. Reactions were carried out as follows:

Plasmid DNA	~1 µg (~6µl MiniPrep DNA)
Enzyme buffer (10x)	2 µl
Enzyme (10U/ µl)	0.5 µl
Nuclease free dH_2O	Fill to 20 µl

For preparative purposes 10µg of plasmid DNA were digested at 37°C for 90 minutes as follows:

Plasmid DNA	10 µg
Enzyme buffer (10x)	10 µl
Enzyme (10 U/ µl)	1 µl
Nuclease free dH_2O	Fill to 100 µl

Restriction enzymes were purchased from Fermentas Life Sciences and Double digests were performed as suggested by the company's online software (http://www.fermentas.com/doubledigest/index.html). Digested DNA fragments were analysed by agarose gel electrophoresis.

3.3.5 Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis. Agarose (0.75-1.5% w/v) was dissolved by boiling in TAE buffer. Ethidium bromide (0.6 µg/ ml) was added to cooled agarose solution and poured into a horizontal gel chamber which was used for electrophoresis. DNA samples were combined with a 6x loading dye solution (Fermentas Life sciences) before loading the gel which was then run in TAE buffer at 5 V/ cm of distance between the electrodes.

3.3.6 DNA Extraction from agarose gels

DNA fragments were excised and extracted from agarose gels using the "QIAquick gel extraction kit" (Qiagen) according to the manufacturer's instructions.

3.3.7 DNA purification from PCR reactions

Amplified DNA fragments were isolated from PCR reactions by the "QIAquick PCR purification kit" (Qiagen) to remove primers, buffers and enzymes which may disturb downstream applications. This method was also applied to remove small DNA fragments, enzymes and buffers after restriction digestions of PCR fragments prior to ligation reactions.

3.3.8 DNA ligations

Vector backbone and insert DNA were digested with specific enzymes and purified as described in 3.3.4, 3.3.6 and 3.3.7. Ligations were carried out at molar ratios of 1 (vector) to 3-5 (insert) using T4 DNA ligase (Fermentas Life sciences):

Ligation buffer (10x)	2 µl
Vector backbone DNA	0,05 pmol
Insert DNA	0, 25 pmol
T4 DNA ligase	1 µl
Nuclease free dH_2O	Fill to 20 µl

Ligation reactions were incubated at 16°C over night.

3.3.9 Competent bacteria and transformation

For conventional cloning procedures, chemically competent Top10 F' *E. coli* were used (Invitrogen). 50 µl aliquots were thawed on ice and 5 – 15 µl of ligation reactions (see previous section) were added. The cells were incubated on ice for 30 minutes and heat-shocked at 37°C for 1 minute. Subsequently, 1ml of warm (37°C) LB medium (without antibiotics) was added and cells were incubated for 30 minutes at 37°C before plating on agar plates containing the selective antibiotics.

For propagation of hnRNP A2 plasmids, Stbl2 competent cells were used. The hnRNP A2 sequence contains repetitive elements that can apparently recombine with the *E. coli* genome which has made it impossible for several groups to generate expression vectors coding for hnRNP A2 fusion proteins (Dr. Nichols, Dartmouth School of Medicine, USA, personal communication). In order to overcome this problem Stbl2 *E. coli* were used which have been designed especially for the cloning of unstable inserts such as retroviral sequences or direct repeats (see Stbl2 user manual, Invitrogen). Transformation into Stbl2 cells was carried out mainly according to the manufacturer's instructions. However, 50 µl of cell suspension were sufficient for each transformation and LB medium was used instead of SOC medium. All over night cultures were grown at 30°C.

Genotype Top10F':

F´{*lac*Iq Tn*10* (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1*ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

Genotype Stbl2:

F$^-$ *mcr*A Δ(*mcr*BC-hsdRMS-*mrr*) *rec*A1 *end*A1*lon gyr*A96 *thi sup*E44 *rel*A1 λ$^-$ Δ(*lac-pro*AB)

3.3.10 Site directed mutagenesis

Mutations into the Fyn open reading frame were introduced using the Quikchange® II site directed mutagenesis kit (Stratagene) according to the manufacturer's suggestions. Mutagenic primers were designed using the "Quikchange® Primer design program" available online: http://www.stratagene.com/sdmdesigner/default.aspx. Primer sequences are listed in section 3.3.11.

3.3.11 Generated expression vectors and used Primers

The coding sequence of wildtype FynT was amplified by PCR from cDNA provided by Dr B. Schraven (Institute of Immunology, University of Magdeburg) and cloned into the NheI/ EcoRI site of pEGFP-C3 (BD Biosciences Clontech) after excising eGFP. Kinase inactive Fyn (K299M or Fyn-) and constitutively active Fyn (FynY528F or Fyn+) were obtained by site directed mutagenesis (Quikchange II, Stratagene) exchanging lysine 299 for methionine and tyrosine 528 for phenylalanine, respectively (figure 3.3-1). Lysine 299 is located in the ATP binding domain of the kinase close to lysine 296 which has been shown to be involved in ATP binding and its exchange leads to a kinase inactive mutant (Sperber et al., 2001). The exchange of lysine 299 seems to have a negative effect on the kinase's activity as well, but this mutant was not characterised in more detail (data are not shown). Tyrosine 528 (or 527, depending if the start methionine which is cleaved off is taken into account), however, regulates the activity of Fyn kinase in a phosphorylation-dependent manner (see introduction). The exchange of this tyrosine residue to phenylalanine results in a constitutively active Fyn mutant (Sette et al., 2002), because phenylalanine cannot be phosphorylated and hence mimics a dephosphorylated tyrosine.

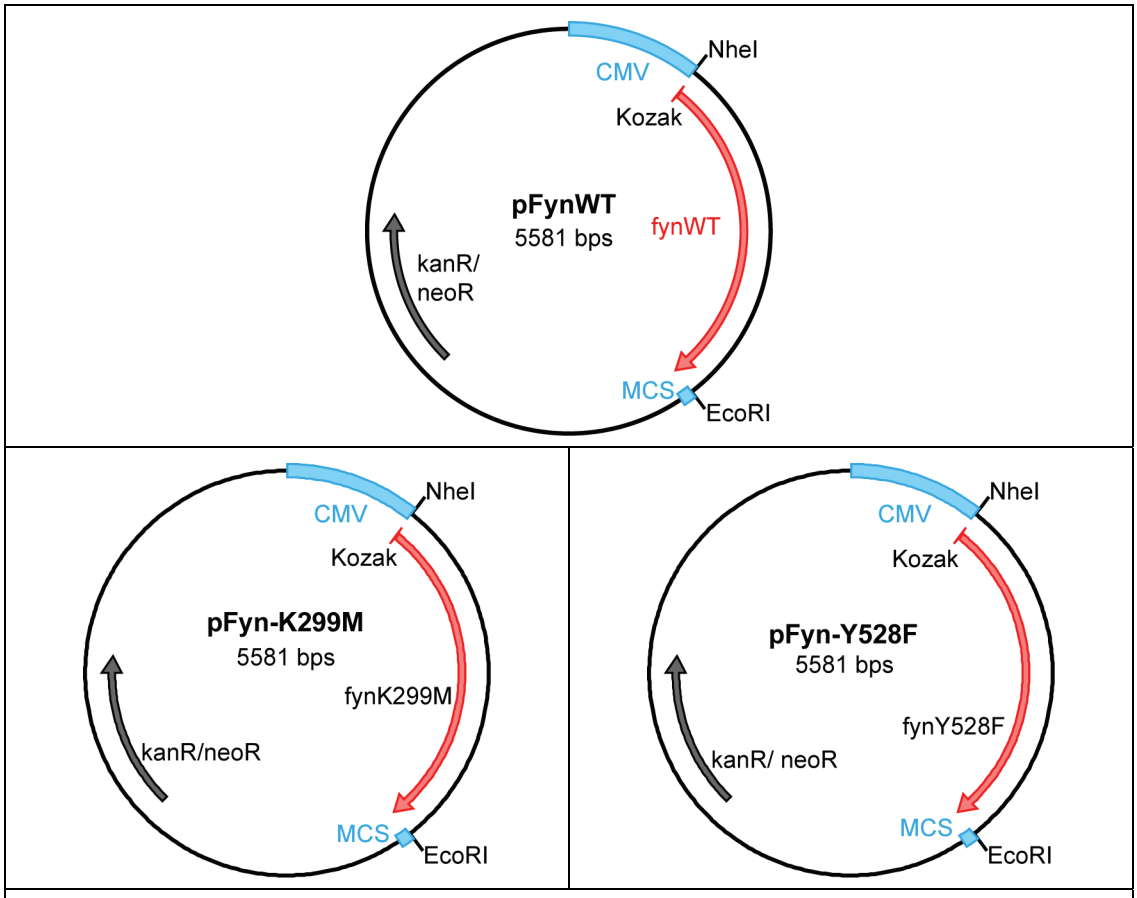

Figure 3.3-1: Fyn expression vectors

The wildtype FynT coding sequence (CDS) was amplified by PCR introducing NheI

and EcoRI restriction sites and cloned into these sites of pEGFP-C3 (BD Biosciences Clontech) vector after EGFP CDS had been excised (top). Site directed mutagenesis was performed on this plasmid to generate vectors expressing mutated Fyn proteins in which lysine 299 is changed to methionine (K299M, bottom left) or tyrosine 528 to phenylalanine (Y528F, bottom right).

Two luciferase reporter constructs were generated to analyse hnRNP A2-dependent translational activity. A 378 nucleotide long part of the 3' UTR of myelin basic protein mRNA containing the A2 response element (A2RE,(Munro et al., 1999)) was amplified by PCR from MBP14 3'UTR containing template cDNA (provided by Dr. M. Simons, University of Göttingen). Furthermore, a 357 long fragment was amplified that is identical to the former mentioned region apart from 21 nucleotides at the 3'end which contains the A2RE. The amplified fragments were fused 3' of the coding sequence of *firefly* luciferase into the EcoRI and XhoI sites of a pcDNA 3.1 vector (Huttelmaier et al., 2005) (figure 3.3-2).

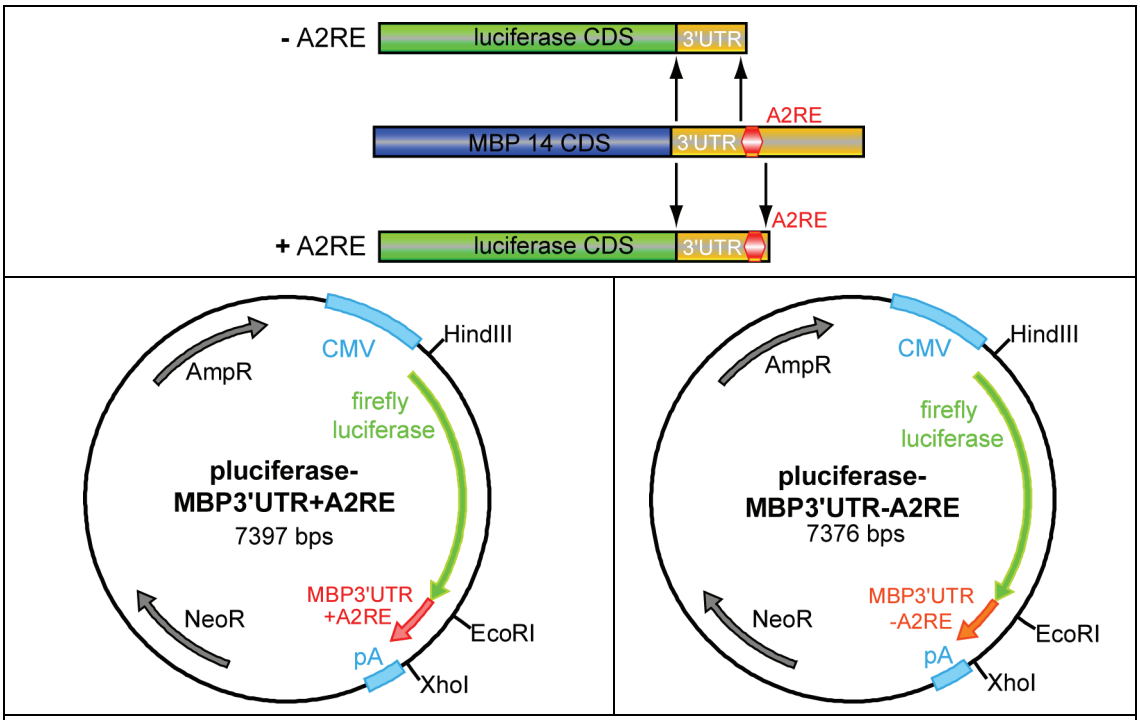

Figure 3.3-2: Luciferase reporter constructs

A region of MBP14 3'UTR lacking (-A2RE) or containing (+A2RE) the hnRNPA2 response element (A2RE) was amplified by PCR introducing EcoRI and XhoI restriction sites and cloned downstream of a *firefly* luciferase CDS (top) yielding luciferase reporter constructs (bottom left and right) used to analyse translational activity.

The coding sequence of hnRNP A2 was amplified by RT-PCR from Oli-*neu* cell RNA. Different primers were used to introduce either EcoRI and XhoI or XhoI and PstI restriction sites. The amplified products were cloned into a pcDNA3.1-FLAG vector (Huttelmaier et al., 2005) yielding an hnRNP A2 protein with a N-terminal Flag tag or into the pEGFP-C3 vector (BD Biosciences Clontech) giving rise to an eGFP-hnRNP A2 fusion protein (figure 3.3-3)

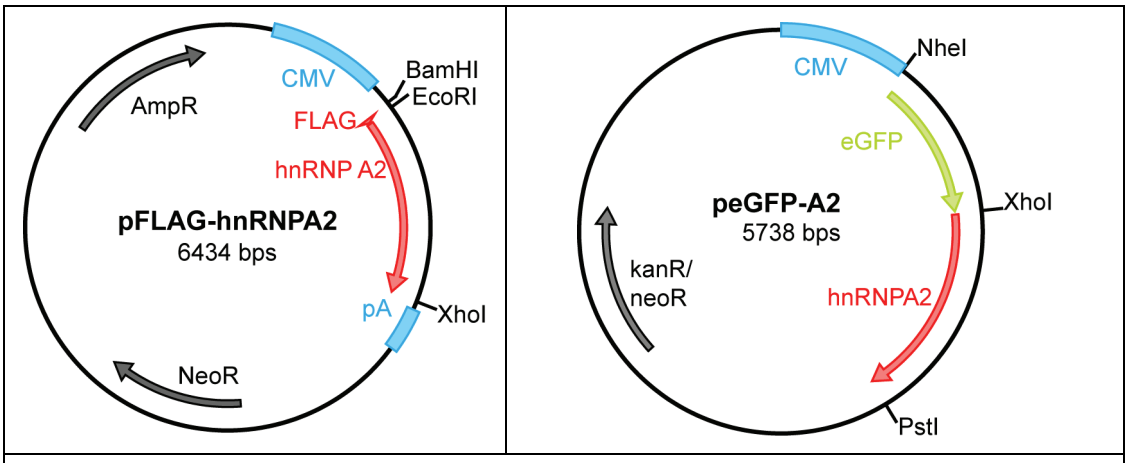

Figure 3.3-3: HnRNP A2 expression vectors

The hnRNP A2 coding sequence was obtained by RT-PCR of total Oli-*neu* RNA and EcoRI/ XhoI or XhoI/ PstI restriction sites were introduced. The amplified products were fused to a FLAG tag or to eGFP in a pcDNA3.1-FLAG (Huttelmaier et al., 2005) or pEGFP-C3 plasmid (BD Biosciences Clontech), respectively.

As stated in section 3.3.9 ligated hnRNP A2 constructs were transformed into Stbl2 *E.coli* (Invitrogen) due to the instability of the hnRNP A2 ORF in ordinary *E. coli* strains. Transformation into Top10 F' *E.coli* failed to produce full length hnRNP A2 constructs as apparently the RGG domains of hnRNP A2 are lost (Dr. Nichols, Dartmouth School of Medicine, USA, personal communication).

All generated plasmids were confirmed by DNA sequencing (Genterprise, Mainz) and the sequences are listed in the appendix.

The primers used for the mentioned PCR, RT-PCR and site directed mutagenesis reactions are listed in table 3.3-1.

Plasmid	Primer name	Primer Sequence 5'- 3'
pFynWT	Fyn-ATG_NheI	CCAGG**GCTAGC**CGCCACC**ATG**GGCTGTG
	Fyn-Stop_EcoRI	CGCTAC**GAATTC**T**CA**CAGGTTTTCACCGGGCTG
pFynY528F	FynY528F	CCACAGAGCCCCAGTTCCAGCCCGGTGAAAA
	FynY528Fantisense	TTTTCACCGGGCTGGAACTGGGGCTCTGTGG
pFynK299M	FynK299Msense	AGCCATAAAGACCCTTATGCCAGGCACCATGTC
	FynK299Mantisense	GACATGGTGCCTGGCATAAGGGTCTTTATGGCT

pluciferase-MBP3'UTR +A2RE	5'MBP_EcoRI	C**GAATTC**CTCAGCCTTCCCGAATCC
	3'MBP+A2RE_XhoI	GG**CTCGAG**ATGCTCTCT**GGCTCCTT**
pluciferase-MBP3'UTR +A2RE	5'MBP_EcoRI	C**GAATTC**CTCAGCCTTCCCGAATCC
	3'MBP-A2RE_XhoI	CA**CTCGAG**GTGTGCCTGTCTATCCGCAGTG
pFLAG-hnRNPA2	A2_ORF_ATG_EcoRI	C**GAATTCATG**GAGAGAGAAAAGG
	A2_ORF_Stop_XhoI	GG**CTCGAGTTA**ATATCTGCTCCTTCCACC
pEGFP-A2	A2_ORF_ATG_XhoI	CG**CTCGAGATG**GAGAGAGAAAAGG
	A2_ORF_Stop_PstI	CG**CTGCAGTTA**ATATCTGCTCCTTCCACC

Table 3.3-1: Primer sequences

The primers used to generate the indicated plasmids are listed. Restriction sites of used endonucleases are coloured red. Target sequences are underlined and Start and Stop codons are bold. A Kozak sequence was introduced in front of the Fyn coding sequence (green). In primers used for site directed mutagenesis orange colour indicates the desired mutation. Blue colour highlights a part of the A2 response element which is included in the 3'MBP+A2RE_XhoI primer.

3.4 RNA interference

Post transcriptional gene silencing (PTGS) by RNA interference (RNAi) has become a powerful tool in functional analysis in mammalian cells, because levels of a specific mRNA and its resulting protein can be strongly reduced. Briefly, a 21-25 nucleotide long double stranded RNA molecule (small interfering RNA or siRNA), is bound and processed by a multiprotein complex termed RISC (RNA induced silencing complex). The so called guide strand of the original RNA duplex, which is homologous to the target mRNA, remains bound to the RISC complex and this complex binds to the target mRNA. Depending on the origin of the RNA duplex, the mRNA is either degraded or translation is repressed (Hannon, 2002; Scherr and Eder, 2007; Valencia-Sanchez et al., 2006). The experimental introduction of a small double stranded RNA molecule is mainly achieved by two means. Firstly, a plasmid coding for a small hairpin RNA (shRNA) under the control of an RNA Polymerase III promoter can be transfected into the cell. The hairpin contains a stem of double stranded RNA and a loop region. The shRNA is synthesised in the nucleus and processed by an RNase III ribonuclease family enzyme such as dicer in the cytoplasm to yield a 21 - 25 nt long RNA duplex. Secondly, the dicer processing can be bypassed by transfecting a synthetically synthesised and annealed 21-25 nucleotide RNA duplex into the cytoplasm where it is recognised by the RISC complex. Synthetic siRNA can be efficiently introduced into cells, but the

suppression of gene expression only lasts for short periods of time, depending on factors such as cell proliferation, siRNA dilution and half life of the protein (Scherr and Eder, 2007). In contrast, shRNA coding plasmids need to be transfected into nuclei, which is less efficient and can be a limiting factor especially for postmitotic cells, but the expression of shRNA eventually leads to gene silencing for longer periods of time.

Here, both approaches were established for oligodendrocytes. The pSilencer vector system (ambion) for shRNA and synthetic siRNA from Qiagen were used (see appendix for shRNA results). However, synthetic siRNAs were used throughout the presented experiments. They seemed more effective compared to shRNA, because especially primary oligodendrocytes are generally difficult to transfect with plasmids at high efficiency, which restricts the use of the vector based system.

The sequences of synthetic siRNAs used in the shown experiments are listed in table 3.4-1:

Target	Target sequence	RNA sequence
F3/ contactin	CAGGTCTTTCATAGTACTCAA	r(GGUCUUUCAUAGUACUCAA)dTdT (sense)
		r(UUGAGUACUAUGAAAGACC)dTdG (antisense)
Fyn	CTCGTTGTTTCTGGAGAAGAA	r(CGUUGUUUCUGGAGAAGAA)dTdT (sense)
		r(UUCUUCUCCAGAAACAACG)dAdG (antisense)
Non-silencing control	AATTCTCCGAACGTGTCACGT	r(UUCUCCGAACGUGUCACGU)dTdT (sense)
		r(ACGUGACACGUUCGGAGAA)dTdT (antisense)

Table 3.4-1: siRNA sequences

The target cDNA sequence and the actual RNA sequence of sense and antisense strand of the synthetically synthesised siRNA duplexes are depicted for F3, Fyn and a non silencing control siRNA. siRNA was purchased from QIAGEN.

3.5 Cells

3.5.1 Primary Oligodendrocytes

Primary oligodendrocyte cultures were prepared from embryonic day 14 – 16 mice as described (Trotter et al., 1989). Principally, a single cell suspension was generated from total brains by trypsinisation and mechanically separating the cells by passing them through glass pipettes with decreasing diameters.

Neurons were removed from the cultures by an M5 antibody-mediated complement kill. Oligodendrocytes growing on top of astrocyte monolayers were manually shaken off ("Oligo shake") and plated in modified Sato medium containing 1% horse serum or Sato medium containing B27 supplement on poly-L-lysine-coated dishes. Immediately after the shake 10 ng/ml human recombinant platelet-derived growth factor (PDGF), and 5 ng/ml basic fibroblast growth factor (FGF) were added to increase the proportion of precursor cells as well as to promote survival. Cells were harvested after 4 - 6 days in vitro (DIV). The resulting population, which was used for all experiments with primary cultures, is enriched for differentiated oligodendrocytes but contains a fraction of progenitor cells.

3.5.2 Oli-*neu* cells

The cell line Oli-*neu* was generated by retroviral-mediated oncogene transfer of oligodendrocyte precursor cells (Jung et al., 1995). Oli-*neu* cells express the t-*neu* oncogene, a constitutively active form of the c-*neu* proto-oncogene under the control of a thymidine kinase promoter, rendering these progenitor cells in a proliferative state. These cells were cultured in Sato medium containing 1% horse serum. To induce differentiation of the precursor-like Oli-*neu* cells, cultures were treated with 1 mM dbcAMP for 3 – 4 days (daily additions to the culture medium).

3.5.3 Cos7 cells

Cos7 cells are derived from simian fibroblasts (CV-1 cells) and constitutively express the SV40 virus large t-antigen that leads to generation of multiple copies of transfected plasmids containing an SV40 origin of replication. This leads to high levels of expression of the gene encoded by the transfected plasmid. Cos7 cells were grown in DMEM 10% FCS in uncoated 15 cm cell culture dishes.

3.6 Transfection of primary oligodendrocytes and Oli-*neu* cells

3.6.1 Plasmids

Oli-*neu* cells were transfected with expression vectors either by conventional electroporation or by nucleofection using the "AMAXA Basic Nucleofection Protocol for Primary Mammalian Neural Cells" (VPI-1003).

For conventional electroporation 4-5 x 10^6 Oli-*neu* cells in 600 µl Sato medium were combined with 10-20 µg of plasmid DNA in an electroporation cuvette (4mm gap), incubated for 5 minutes at room temperature, pulsed (220 V, 960 µF) in a Biorad gene pulser device, incubated for 5 minutes at room temperature and plated into PLL coated cell culture dishes. For nucleofection 10^6 Oli-*neu* cells were pelleted and resuspended in 100µl of nucleofect solution. 2 µg of plasmid DNA were added and cells were carefully pipetted into an AMAXA cuvette. As quickly as possible, programme O-005 was executed in the nucleofector device, ~500 µl of 37°C Sato medium were carefully added with the AMAXA pipette and the entire solution (~600 µl) was taken up in the same pipette and carefully added dropwise to a PLL coated tissue culture dish containing 37°C Sato medium.

The demanding transfection of primary oligodendrocytes was established using the AMAXA Basic Nucleofection Protocol for Primary Mammalian Neural Cells. 4x10^6 primary oligodendrocytes were pelleted directly after the oligo shake, resuspended in 100 µl nucleofect solution and combined with 2 µg plasmid DNA. Nucleofection was executed using programme O-005 and cells were plated in 3 cm culture dishes in Sato 1% HS + 10 ng/ml PDGF and 5 ng/ml FGF. The complete medium (including growth factors) was changed 2 - 4 hours after nucleofection. Programme A-33 also yielded satisfactory nucleofection efficiencies, but programme O-005 resulted in better cell viability rates.

3.6.2 siRNA

160 pmol of siRNA were introduced into 10^6 Oli-*neu* cells or 4x10^6 primary oligodendrocytes by AMAXA nucleofection as described for plasmid DNA in the previous paragraph.

Alternatively, siRNA was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions:

The complete culture medium of primary oligodendrocytes cultured 5DIV in 6 cm cell culture dishes was replaced with 2 ml fresh medium (37°C). 100pmol siRNA and 5 µl Lipofectamine 2000 were diluted in 250µl OptiMEM I medium (Invitrogen) each and incubated for 5 minutes at room temperature. Both solutions were combined, mixed, incubated for 20 minutes at room temperature and added dropwise to the culture medium. Knock down of proteins was assessed by western blotting 2 days later and a 75% reduction could be observed.

3.7 Protein analysis

3.7.1 Cell lysis

Cells were scraped off cell culture dishes on ice in lysis buffer (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA and HALT Protease inhibitor cocktail from Pierce). A HALT Phosphatase Inhibitor cocktail (Pierce) was added for the analysis of phosphoproteins. Lysates were incubated for 45 minutes at 4°C on a rotating wheel. Lysates were then cleared of cellular debris and nuclei by a 300 x g centrifugation step for 10 minutes at 4°C.

3.7.2 Determination of protein concentration

Protein concentrations were analysed by the Bradford assay (Biorad) or BCA test (Pierce) according to the manufacturers' instructions. BSA was used to prepare standard curves.

3.7.3 SDS-PAGE

Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Stacking and separation gels with different polyacrylamide concentrations were prepared as described in *Molecular Cloning – A Laboratory Manual* (Sambrook und Russell, 2001) tables A8-9 and A8-10. To prepare and run the gels, either a Sigma Mini Gel system or a larger Hoefner gel system was used.

Alternatively, pre-cast 4-12% NuPAGE gels (Invitrogen) were used to separate proteins extremely reproducibly. According to the user manual either MOPS or MES buffer was used depending on the molecular weights of proteins intended for separation. These gels are especially suitable for subsequent mass spectrometric analysis, because the introduction of unwanted contaminating proteins during gel preparation is avoided.

3.7.4 Coomassie protein staining and trypsin digest for MS analysis

Proteins separated by SDS PAGE were visualised in the gel with the colloidal Coomassie stain Roti®-Blue (Roth) as suggested in the user manual.

The bands of interest were excised with a clean scalpel and transferred to a clean Eppendorf tube (0.5 ml). To remove the coomassie stain, 100 µl 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile were added and incubated at room temperature on a shaker for 35 minutes before removing the solution. After repeating this washing step twice, the destained gel piece was dehydrated by incubation in 100 µl acetonitrile until the colour of the piece turned opaque-white. The acetonitrile was removed and 100 µl of fresh acetonitrile were added. Then the acetonitrile was removed and the gel piece was air-dried for 10 minutes. For the purpose of reduction and alkylation of cysteine residues, the gel piece was incubated in 30 µl of 10 mM DTT in 25 mM ammonium bicarbonate for 35 minutes at room temperature followed by incubation for 45 minutes at 56°C in 30 µl of 55 mM iodoacetamide in 25 mM ammonium bicarbonate. The solution was replaced with 100 µl of 25 mM ammonium bicarbonate and incubated in a shaker for 10 minutes at room temperature. After this washing step the gel piece was dehydrated with 100 µl acetonitrile until it shrank and became an opaque-white colour. It was then dried in a vacuum centrifuge for 5 minutes. 25 µl of trypsin solution (25 µg/ ml trypsin in 25 mM NH_4HCO_3, pH 8,0) were added and the tube was incubated on ice for 15 minutes to swell the gel piece with the enzyme solution. Excess trypsin solution was removed and the gel piece was immersed with 30 µl of 25mM ammonium bicarbonate. The tube was incubated over night at 37°C for protease digestion. The peptides were extracted by the addition of 5 µl TFA (trifluoroacetic acid) and incubation at room temperature for 10 minutes (heavily shaking) followed by a quick centrifugation step at room temperature. The supernatant was transferred to a fresh 0.5 ml Eppendorf tube and the extraction was repeated twice with 20 µl 0.1% (v/v) TFA, 50% acetonitrile in 25 mM ammonium bicarbonate. All supernatants (containing the extracted peptides) were combined and concentrated in a vacuum centrifuge to a volume of approximately 5µl. These samples were stored at -20°C and analysed by MALDI-TOF by Dr. Kai Bruns, Zentrallabor, Klinikum, University of Mainz.

3.7.5 Western Blotting

Western-blots were performed with a semi-dry system as illustrated in *Molecular Cloning – A Laboratory Manual* (Sambrook und Russell, 2001). Gels were blotted onto PVDF membranes (HybondP, Amersham/ GE Healthcare)

which were treated as described in the user's manual. Blots were carried out for 1 - 2 hours at 180 - 250 mA depending on the size of the gel and the protein of interest.

NuPAGE gels were transferred to PVDF membranes using the XCell II blot module (Invitrogen) according to the manufacturer's instructions. Proteins were blotted at 30V/ gel for 90 minutes.

After transfer PVDF membranes were rinsed twice with dH_2O to remove residual SDS and proteins were visualised by staining with Ponceau S (see 3.1.3) and destaining with dH_2O.

Membranes were blocked for 20 - 30 minutes in blocking buffer (see 3.1.3) and incubated with the appropriate dilution of primary antibody in blocking buffer for 1 hour at RT or over night at 4°C. Subsequently, membranes were washed 3 x 10 minutes in TBST and secondary antibodies conjugated with horseradish peroxidase (HRP) were incubated for 30 - 60 minutes in blocking buffer at room temperature. Finally, the membranes were washed 2 x 10 minutes in TBST, 1 x 10 minutes in TBS and detected proteins were visualised on X-Ray films by enhanced chemiluminescence (ECL) reaction. Membranes were stored at -20°C and could be thawed and reblotted later.

If appropriate, primary and secondary antibodies were removed from membranes by incubation in stripping buffer (see 3.1.3) for 30 minutes at room temperature. To test if antibodies had been successfully removed, membranes were incubated with secondary antibodies only and washed and developed as described above. If necessary, membranes were stripped for longer periods of time until primary antibodies were completely removed.

3.8 Protein Purification

3.8.1 Purification of L1-Fc fusion protein

1.5 x 10^6 Cos7 cells were plated into several 15 cm dishes containing 18 ml transfection medium (3.1) The following day an expression vector coding for L1-Fc (Oleszewski et al., 1999) was transfected using Jetpei transfection reagent (Biomol):

12µg plasmid DNA or 24 µl Jetpei was added to 1 ml 150mM NaCl and vortexed. The two solutions were combined and incubated for 30 minutes at RT. The transfection mix (2 ml) was added dropwise to the 18ml transfection medium and the culture dish was swirled. Cells were grown to confluency (3-5

days) and the medium containing the secreted L1-Fc fusion protein was collected.

L1-Fc was purified from the medium with a HiTrap Protein A Column (1 ml, Amersham Biosciences) connected to a peristaltic pump. All steps were carried out at 4°C. The column was washed with ~50 ml of PBS before applying the entire collected medium at a flow rate of approximately 1.5 ml/ min. To remove unspecifically bound proteins, the column was washed with PBS (40-50 ml depending on the total volume of applied medium) until the flow through reached an OD_{280} of 0 (1 ml samples were analysed in a photometer). L1-Fc was eluted by connecting the HiTrap column to a 5 ml syringe and applying 2 ml of elution buffer (0.1 M glycine, pH2.7) the eluate was collected in 15 ml falcon tubes containing 120 µl 1M HEPES to neutralise the pH immediately. The elution step was repeated once or twice depending on the amount of starting material. The eluted L1-Fc solution was dialysed in 1l PBS using Slide-A-Lyzer® Dialysis Cassettes (0.5-3 ml, Pierce) for 2 x 2 hours, adding new PBS in between the incubation steps. Finally, the dialysed L1-Fc solution was concentrated using Centricon® centrifugal filter devices (YM-100, Millipore) to 250- 500 µl and the protein concentration was assessed (see 3.7.2). L1-Fc was stored at -20°C in 10 µg aliquots.

3.8.2 Purification of tyrosine phosphorylated proteins

Immunoaffinity chromatography was performed to purify tyrosine phosphorylated proteins from large amounts of starting material. 1 ml of Anti-Phosphotyrosine (clone 4G10), agarose conjugate slurry (Upstate) was applied to a Poly-Prep Chromatography column (0,8 + 4cm, Biorad) and washed with 10 ml of lysis buffer containing 0.2mM sodium-orthovanadate. Cell lysates were added to the column and allowed to pass through by gravity flow. The column was washed with ~20 ml lysis buffer containing 0.2 mM sodium-orthovanadate before eluting with 4 ml of lysis buffer containing 100 mM phenylphosphate. The eluates were concentrated with Centricon® centrifugal filter devices (YM-10, Millipore) to ~100 µl and 2 ml PBS were added. After a second concentration step to ~500 µl 1ml PBS was added and finally the solution was concentrated to ~150 µl. By this “buffer exchange” procedure most of the phenyl phosphate was lost.

For the purification of tyrosine phosphorylated proteins from smaller amounts of starting material, anti-phosphotyrosine agarose beads were used for immunoprecipitation as described in the user’s manual. 50 µl of slurry were used and eluted in 40 µl 2x sample buffer.

3.8.3 Immunoprecipitation (IP)

Protein A and Protein G sepharose beads (Amersham/ GE Healthcare) were used according to their optimal binding properties to the used antibody classes (see section 3.5 in “Roche Molecular Biochemicals Lab FAQS” manual). All steps were carried out at 4°C. Proteins were immunoprecipitated from cellular lysates by two approaches. In both cases the lysates were incubated in a rotating wheel for 1 - 2 hours with Protein A/G beads alone (with out a bound antibody) to remove cellular components that bind unspecifically to the Protein A/G sepharose matrix. In one approach the antibody was added directly to the lysate to form the antibody-antigen complex (incubation 3 hours - over night) and the Protein A/G sepharose was added subsequently for 1-2 hours to form the antigen-antibody-bead complex. For example, rabbit anti-Fyn antibodies (1:50 dilution) were incubated with the cleared lysates over night before 100µl of Protein A bead slurry were added and incubated for 2 hours. Alternatively, an antibody-bead complex was formed and this complex was incubated with the cleared lysate to form the antigen-antibody-bead complex. For instance 20µl mouse anti-hnRNP A2 antibody were incubated with 30µl Protein G slurry and 500µl PBS for 2 hours, washed twice with lysis buffer and added to the cleared lysate over night. In both cases the antigen-antibody-bead complexes were washed 4x with 1ml lysis buffer and 1 x with 1ml PBS before proteins were eluted by the addition of 40 µl 2x sample buffer and incubation at 90°C for 5 minutes.

3.9 Immunostaining

3.9.1 Immunocytochemistry

Oli-*neu* cells or primary oligodendrocytes were plated on PLL coated glass coverslips and analysed by indirect immunofluorescence:

All steps were carried out at room temperature. Cells were washed 2x with PBS to remove medium and fixed for 10 - 15 minutes in 4% paraformaldehyde. Subsequently, they were washed 2x with PBS and permeabilised with 0.1% (v/v) Triton X100 in PBS for 2 minutes if intracellular epitopes were to be detected. Cells were then incubated in blocking buffer (DMEM+ 10% HS or PBS+ 10%FCS) for 15 minutes before applying the primary antibody at the appropriated concentration in blocking buffer for 30 minutes (sometimes over night at 4°C). After 3x 2min washing steps with PBS, the secondary antibody was added diluted in blocking buffer for 20 minutes (sometimes up to 2 hours).

Finally, the cells were washed 3 x 2 minutes with PBS and briefly submerged in dH_2O water to remove residual salts before mounting in moviol.

3.9.2 Immunohistochemistry

Immunohistochemistry on mouse brains of different ages was performed in collaboration with Dr. Karram, Molecular Cell Biology, Mainz.

Paraformaldehyde fixed brains were permeabilised by incubation on a shaker in 0.4% Triton X-100 in PBS for 30 minutes The sections were then blocked with PBS 0.5% Triton X-100 containing 10% normal goat serum (NGS) for 30 minutes at room temperature (RT). Primary antibody incubation was performed either over night at RT or for longer periods of time at 4°C in PBS/ 0.5% Triton X-100/ 1% NGS using the appropriate antibody dilutions. The sections were then washed 3x 15 minutes with PBS before fluorophore coupled secondary antibodies were incubated for 2 hours at room temperature in PBS/ 1% NGS. The sections were then washed 3 x 15 minutes with PBS. Finally the sections were rinsed in dH_2O to remove residual PBS and mounted on PLL coated slides with moviol. Images were acquired by confocal microscopy and processed using ImageJ and Photoshop software.

3.10 L1-Fc live staining and Cell ELISA

3.10.1 L1-Fc live staining

Oli-*neu* cells adhering to cover slips were incubated with L1-Fc (150 µg/ ml in PBS containing 0,3 % (v/v) NGS) on ice for 60 minutes, washed 2x with PBS, fixed with 4% paraformaldehyde and blocked with 10% (v/v) NGS in PBS. L1-Fc was detected with a goat anti-human secondary antibody coupled to Cy2 as described in 3.9.1.

3.10.2 L1-Fc Cell ELISA

F3 Antibody competition

Untreated Oli-*neu* cells were plated in PLL coated 24 well plates (Nunc) at a density of 1.5 x 10^4 cells/ well in the presence of 1 mM dbcAMP to induce differentiation for four days. Cells in Sato medium containing 1% horse serum were treated with 0 or 25 nM L1-Fc and 25nM L1-Fc in the presence of mouse anti F3 antibodies (1:2 dilution). They were incubated for 1 hour at 4°C, washed

twice with PBS and fixed with 4% paraformaldehyde. After blocking for 30 minutes with PBS 10% FCS at room temperature, horseradish peroxidase (HRP) coupled goat anti human Fc antibody (Pierce, diluted 1:2000 in PBS) was incubated for 60 minutes to detect bound L1-Fc. After two washes with PBS, tetramethylbenzidine (Pierce) was used to detect bound HRP according to the manufacturer's instructions and colour development was quantified by transferring 100µl of each well to 96 well plates and measuring the absorbances in an ELISA reader.

F3 knock down

For RNA interference experiments, Oli-*neu* cells were transfected with control or F3 siRNA using the AMAXA system as described in 3.6.2. 30 µl of the final 500 µl cell suspension were plated into each 24 well plate and after two days, 20pmol siRNA were reapplied to the adherent cells with Lipofectamine 2000 as described in 3.6.2 (20 pmol siRNA in 50 µl OptiMEM I and 1 µl Lipofectamine 2000 in 50 µl OptiMEM I) Cells were grown in differentiation medium for a total of four days before 0 and 25 nM L1-Fc were added and binding was quantified as described for the F3 antibody competition assay in the previous paragraph. The ratio of L1-Fc treated to untreated control cells was determined for control siRNA and F3 siRNA treated cells.

3.11 Antibody-mediated crosslinking of F3 and L1-Fc binding

3.11.1 Antibody-mediated crosslinking of F3

Oligodendroglial F3 was crosslinked using mouse anti-F3 antibodies as described (Kramer et al., 1999). 10^5 Oli-*neu* cells were plated in PLL coated 10 cm cell culture dishes and cultured for 4 days in differentiation medium. Cells were washed 2x with PBS (cooled to 4°C) on ice and incubated with mouse anti-F3 antibodies (1:10 dilution of purified 11-111 hybridoma supernatant in PBS containing 3% (w/v) BSA) on a shaker for 1 hour at 4°C. Cells were washed 2x with cold PBS and incubated with a rabbit anti- mouse IgG antibody (1:100 in PBS 3% (w/v) BSA) for 2 minutes on ice and then for 5 minutes at 37°C. Cells were washed on ice with cold PBS as quickly as possible and lysed with lysis buffer containing phosphatase inhibitors. As a control, F3 antibodies were omitted and cells were only treated with rabbit anti mouse antibodies.

3.11.2 L1-Fc binding

Oli-*neu* cells were differentiated as described in the previous section and washed 2x with cold PBS on ice. L1-Fc was incubated in various concentrations (0-14nM) in PBS 3% (w/v) BSA for 1 hour at 4°C, washed 2x on ice with cold PBS and incubated with a goat anti-human IgG antibody (1:100 in 3% (w/v) BSA) for 2 minutes on ice and then for 5 minutes at 37°C. Alternatively, the incubation with goat anti human IgG was excluded and cells with bound L1-Fc were directly incubated at 37°C for 5 minutes. Cells were washed 2x with cold PBS and lysed in the presence of phosphatase inhibitors. Control cells were treated without L1-Fc and only with goat anti human control IgG (control Fc).

3.12 L1-Fc Bead binding

100 µl Protein G coated magnetic particles (4 µm diameter, Spherotec) were vortexed, placed in a water bath sonicator for 3 minutes and incubated in 0.3% (w/v) BSA with L1-Fc (2.4 µg) overnight at 4°C to bind L1-Fc efficiently. The beads (particles) were washed twice with PBS 0.3% (w/v) BSA to remove unbound L1-Fc and resuspended in 200 µl 0.3% (w/v) BSA. L1-Fc beads were placed in a water bath sonicator for 1 minute before adding 10 µl to Oli-*neu* cells which were grown on PLL coated glass cover slips placed in 24 well plates containing 200 µl Sato 1% HS. After 5 minutes incubation at 37°C cells were fixed and analysed by indirect immunofluorescence.

3.13 Luciferase assay

Oli-*neu* cells (10^6) were nucleofected with 250 ng *firefly* luciferase MBPA2RE reporter construct (pluciferaseMBP3'UTR+A2RE, figure3.3.-2), 100 ng *renilla* luciferase, 650 ng pEGFP C3 (BD Clontech) and 1 µg of either wildtype Fyn (FynWT, figure 3.3-1), constitutively active Fyn (pFynY528F/ Fyn+, figure 3.3-1) or additional pEGFP C3 (control) plasmids. Two days after transfection cells were scraped off in 700 µl PBS on ice and a luciferase assay was performed as instructed by the Dual-Glo manual (Promega). 50 µl (3 replicates) of cells in PBS were transferred to white 96 well plates and *firefly* luciferase activity was measured by adding 50 µl of Dual-Glo Luciferase Reagent, incubation at RT for 10 minutes and bioluminescence recording in a luminometer. To subsequently measure *renilla* luciferase activity, 50 µl Dual-Glo Stop & Glo® reagent were added to the wells, and bioluminescence was recorded in a luminometer after 10 minutes incubation. The ratio of *firefly* to *renilla* luciferase activity was

calculated for each well and the mean was determined from the 3 replicates. Each transfection (FynWT, Fyn+ or eGFP) was performed in triplicate and the normalised mean luciferase activities were compared.

3.14 *In vitro* Kinase assay

One confluent 15 cm dish of Oli-*neu* cells was lysed in 1ml lysis buffer (without phosphatase inhibitors), and a post nuclear supernatant was precleared with 30 µl of Protein G beads. The precleared lysate was divided equally and hnRNP A2 was immunoprecipitated in duplicate as described in 3.8.3. However, after incubation of the lysate with the hnRNP A2-antibody-bead complex, beads were washed (750 µl each) 2x with lysis buffer, 2x with kinase buffer and once with reaction buffer. The washed beads containing bound hnRNP A2 were then incubated moderately shaking for 20 minutes at 30°C in either 40µl reaction buffer or reaction buffer complemented with 40ng recombinant human Fyn kinase. (FynT purchased from Invitrogen). The reaction was stopped by the addition of 10 µl 4x sample buffer and incubation at 90°C for 10 minutes.

Kinase buffer	50 mM PIPES pH 7.02; 10 mM $MgCl_2$
Reaction buffer	Kinase buffer + 50 µM ATP + HALT® Phosphatase Inhibitor cocktail (Pierce)

4 RESULTS

Previous studies revealed a pivotal role for Fyn kinase in oligodendroglial maturation and CNS myelination (Kramer et al., 1999; Osterhout et al., 1999; Sperber et al., 2001). Interestingly, it was reported that oligodendroglial Fyn could be activated by antibody-mediated crosslinking of cell surface F3/ contactin (Kramer et al., 1999). It was proposed that this may mimic the binding of a neuronal ligand to oligodendroglial F3 representing the initiation of a neuron-glia signalling cascade involving Fyn as a key mediator. The L1 cell adhesion molecule was suggested as one of the potential neuronal ligands as it had been shown to bind to F3/ contactin *in vitro* and could be isolated from cerebellar tissue in a complex with F3 and Fyn. In order to investigate the potential interaction of L1 and F3 and the influence of this neuronal ligand on the proposed signalling cascade, a recombinant L1-Fc fusion protein was used (Oleszewski et al., 1999).

4.1 L1-Fc fusion protein

L1-Fc consists of almost the entire extra-cellular domain of murine L1 fused to the Fc region of human IgG (figure 4.1A). The Fc part of the molecule includes the disulfide bridge of the IgG hinge region and hence the generated fusion protein appears as a dimer in a non-reducing environment or as a monomer in

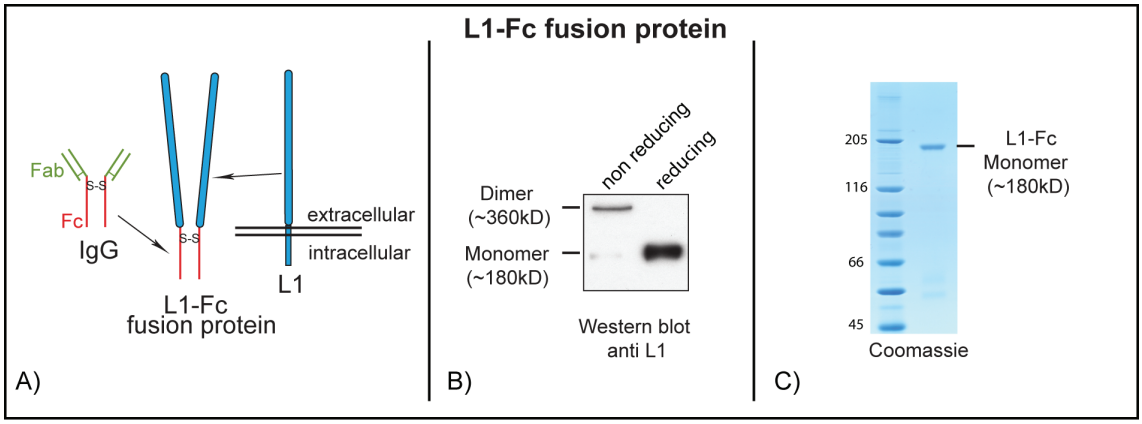

Figure 4.1: Purification of L1-Fc

A) Schematic diagram of L1-Fc fusion protein consisting of the extracellular domain of L1-CAM fused to the Fc part of human IgG. **B)** Western blot analysis of purified L1-Fc in reducing or non reducing conditions using an L1 specific antibody. **C)** SDS-PAGE and Coomassie protein staining of monomeric L1-Fc show no contaminating proteins after the purification.

reducing conditions. L1-Fc was expressed in Cos7 cells and the secreted

protein was purified from the medium by affinity chromatography using a Protein A column. Western blot analysis of the purified L1-Fc protein with an L1-specific antibody confirmed the identity of L1-Fc. Reducing and non reducing conditions were used to distinguish the monomeric and dimeric form of the molecule, respectively (figure 4.1A). To assess the purity of the protein, the reduced monomeric form of L1-Fc was analysed by SDS-PAGE and Coomassie protein staining. Figure 4.1C shows monomeric L1-Fc migrating at the expected size of approximately 180 kD and no major contaminating proteins. Hence, purified L1-Fc was generated as a tool to investigate a potential role for L1 in neuron-glial signalling.

4.2 L1-Fc binds to oligodendroglial F3/ contactin

In order to determine L1 as a neuronal binding partner of oligodendroglial F3/ contactin, L1-Fc binding to living oligodendrocytes in culture was analysed.

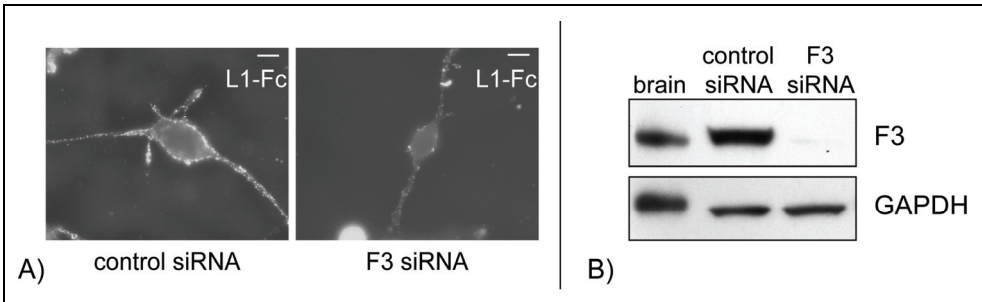

Figure 4.2-1: L1-Fc binds oligodendroglial F3/ contactin

A) Living Oli-*neu* cells were incubated with Oli-*neu* cells that had been treated either with control or F3 siRNA. Bound L1-Fc was detected with a Cy2 coupled anti-human Fc antibody. Binding is strongly reduced in F3 siRNA treated cells. **B)** The efficiency of the F3 knock down by siRNA was analysed by Western blotting using F3 antibodies. GAPDH served as a loading control. F3 protein levels are strongly reduced by F3 siRNA treatment. A P14 mouse brain lysate was used as blotting control.

The oligodendrocyte progenitor cell line Oli-*neu* (Jung et al., 1995) was treated with F3 directed small interfering RNA (siRNA) to silence F3 expression and reduce F3 protein levels or with non silencing control siRNA. The cells were cultured in cell culture dishes with glass cover slips which were removed 2 days after siRNA treatment. Living Oli-*neu* cells on coverslips were incubated with L1-Fc protein before they were fixed with paraformaldehyde. Bound L1-Fc was detected by incubation with an anti-human Fc secondary antibody coupled to Cy2. The remaining cells from the culture dishes were lysed and analysed for the presence of F3 protein by Western Blotting to assess the degree of siRNA-

mediated protein knock down. As shown in figure 4.2-1A, L1-Fc binds to the cell surface of control siRNA treated Oli-*neu* cells and the binding is strongly reduced in F3 siRNA treated cells. The efficiency of siRNA-mediated knock down is depicted in figure 4.2-1B. F3 protein levels are strongly reduced in F3 siRNA treated cells whereas a control protein (GAPDH) is not affected.

The experiment leads to the deduction that L1-Fc binds to oligodendrocytes in a F3-dependent manner.

To assess L1-Fc binding in a more quantitative manner, a Cell ELISA approach was used. Similarly to the experiment shown in figure 4.2-1, Oli-*neu* cells were incubated with L1-Fc and fixed. Bound L1-Fc was detected, however, by incubating the cells with a goat anti-human Fc antibody coupled with horseradish peroxidase (HRP) which catalyses a colour reaction in the presence of hydrogen peroxide and chromogenic tetramethylbenzidine (TMB). Colour development results in a change in absorbance at OD_{450} that can be measured in an ELISA reader and corresponds to the amount of bound L1-Fc. Figure 4.2-2A shows the summary of three experiments in which L1-Fc was incubated either alone or in the presence of F3 antibodies which may compete with binding to F3 protein. A significant increase in absorbance can be seen upon the addition of 25nM L1-Fc compared to a control in which only the secondary antibody was added. This implies that L1-Fc binds to Oli-*neu* cells. Binding of L1-Fc was largely reduced in the presence of competing F3 antibodies demonstrated by a significant decrease in absorbance suggesting that L1-Fc binds to oligodendroglial F3.

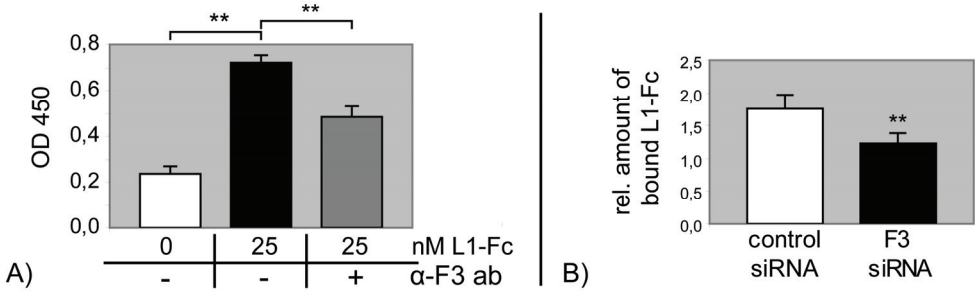

Figure 4.2-2: Cell ELISA quantification of F3-dependent L1-Fc binding

A) Oli-*neu* cells were grown and differentiated in 24 well plates and incubated with the indicated concentrations of L1-Fc protein in the presence (+) or absence (-) of F3 antibodies. L1-Fc binding was quantified by TMB ELISA. L1-Fc binds to Oli-*neu* cells and this binding can be reduced by the addition of competing F3 antibodies. **B)** Oli-*neu* cells were treated with control or F3 siRNA and grown as stated in A).Cells were then treated with 0 or 25nM L1-Fc and the ratio of mean absorbance at 25 and 0nM L1-fc was plotted to illustrate the relative amount of bound L1-Fc. Binding is significantly reduced in the presence of F3 siRNA. **A&B)** Error bars represent SEM. Student's t-tests were performed to assess significance. ** = $p < 0,01$; n = 3 (A) n=9 (B)

This Cell ELISA approach was also used to quantify the result visualised in figure 4.2-1A in which the reduction of F3 Protein levels leads to decreased L1-Fc binding to living cells. Figure 4.2-2B shows the summary of nine experiments in which L1-Fc was bound to Oli-*neu* cells in the presence of control or F3 siRNA. The relative amount of bound L1-Fc (absorbance ratio of 25nM/ 0nM L1Fc) is plotted for cells treated with control or F3 siRNA. Hence, the quantification is in agreement with the result shown in figure 4.2.-1. However, the degree of L1-Fc binding reduction is not as striking as the image in 4.2-1 suggests. This can be explained by the fact that the siRNA knock down of F3 was not as efficient in the Cell ELISA experimental set up (in which cells were cultured for longer periods of time), so that more F3 protein remained in the cells to which L1-Fc could bind.

The results depicted in figures 4.2-1and 4.2-2 lead to the deduction that L1-Fc binds to oligodendrocytes in an F3-dependent manner suggesting L1 as a neuronal ligand for F3/ contactin.

4.3 L1-Fc binding activates Fyn kinase

As antibody-mediated crosslinking of cell surface F3/ contactin leads to an activation of Fyn kinase mimicking binding of a neuronal ligand (Kramer et al., 1999) and L1-Fc was shown to bind oligodendrocytes in an F3-dependent manner, it was analysed if this L1-Fc binding could stimulate Fyn in a similar way. Differentiated Oli-*neu* cells and primary murine oligodendrocytes were treated with various concentrations of L1-Fc fusion protein and the activation state of Fyn was evaluated by Western Blot analysis of total cell lysates using antibodies that recognise active forms of Src family kinases (including Fyn) and total Fyn (independent of its activity). The results are shown in figure 4.3-1A. In Oli-*neu* cells, Fyn activity increases with raising concentrations of L1-Fc whereas total levels of Fyn are more or less unaffected. Similarly, in primary oligodendrocytes Fyn activity is higher upon treatment with L1-Fc. This indicates that L1-Fc binding leads to an activation of oligodendroglial Fyn kinase. In order to visualise the results obtained by the Western Blot approach, L1-Fc coupled Protein G coated magnetic particles were incubated with living Oli-*neu* cells. Cells were subsequently fixed and immunostained with the antibody recognising active Fyn. As shown in figure 4.3-1B, there seems to be a local concentration of active Fyn at the contact site of an L1-coated particle (see arrows). Most likely, this local activation of Fyn is triggered by L1-Fc binding. There also happen to be other areas within the cell where active Fyn is

concentrated (see arrowheads). This is not surprising because Fyn activity is likely to be required for different cellular actions independently of L1 binding

Figure 4.3-1: L1-Fc binding activates Fyn kinase

A) Living Oli *neu* cells (left) or primary murine oligodendrocytes (right, 6 DIV) were treated with the indicated concentrations of L1-Fc and total cell lysates were analysed with antibodies that recognise either active Fyn (top) or total Fyn (bottom). The level of active Fyn increases in response to L1-Fc binding whereas the total amount of Fyn is more or less unaffected. **B)** Oli-*neu* cells were incubated with L1-Fc coupled Protein G coated magnetic particles (~4 µm diameter). The left panel shows an immunostaining with an antibody recognising active Fyn with an enlargement of the boxed area. The arrows point to a concentration of active Fyn at the contact site of an L1-coupled particle whereas arrow heads indicate areas of concentrated activated Fyn in the absence of a particle. The right panel shows a Nomarski image of the same cell in which 3 spherical particles can be seen. Scale bars = 5 µm

It could be shown clearly by Western Blot analysis that L1 binding leads to an activation of Fyn kinase in oligodendrocytes and L1 coated beads seem to stimulate this effect locally in cellular processes.

4.4 L1 binding leads to tyrosine phosphorylation of a range of oligodendroglial proteins

As shown in section 4.2 and 4.3, L1-Fc binds to oligodendrocytes in an F3-dependent manner leading to an activation of Fyn kinase. As Fyn is a tyrosine kinase it was very likely that downstream targets of Fyn would be

phosphorylated in response to L1-Fc binding. This is shown in figure 4.4-1 which depicts an experiment in which differentiated Oli-*neu* cells were treated with a goat anti human IgG as a control Fc, L1-Fc fusion protein or F3 antibodies. The upper Phosphotyrosine western blot shows to which extent pro-

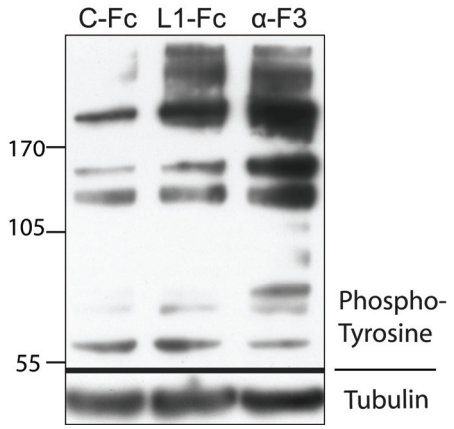

Figure 4.4-1: Tyrosine phosphorylation in response to L1-Fc binding

Differentiated Oli-*neu* cells were treated with L1-Fc fusion protein (L1-Fc) or F3 antibodies (α-F3) and compared with goat anti human IgG as a control Fc (C-Fc). Equal amounts of total cell lysates were analysed by Western Blotting using an antibody specific for phosphorylated tyrosine residues in Proteins and alpha-tubulin as a loading control.

teins were phosphorylated in response to L1-Fc or F3 antibody treatment. Compared to the control Fc it seems that a range of proteins are phosphorylated in response to L1-Fc binding (lane L1-Fc in 4.4-1). The same proteins seem to be phosphorylated after F3 antibody application (lane α-F3), only the level of tyrosine phosphorylation is higher in these cells which could be a concentration-dependent effect. One protein (see lowest band), however, is phosphorylated to a higher extent in L1-Fc than in F3 treated cells, alluding to an L1-Fc-specific effect.

This experiment shows that L1-Fc binding not only activates Fyn kinase as shown in section 4.3, but leads to the tyrosine phosphorylation of several oligodendroglial proteins. Furthermore, although the degree of phosphorylation of proteins is higher in F3 antibody compared to L1-Fc treated cells, the affected proteins are the same which suggests that L1-Fc binding to F3 leads to the phosphorylation of these downstream proteins. The phosphorylation is likely to be mediated either directly or indirectly by Fyn, as L1-Fc binding (section 4.3) as well as F3 antibody binding (Kramer et al., 1999) activate this kinase.

In order to investigate the impact of Fyn kinase on the tyrosine phosphorylation pattern of the entire oligodendroglial cell, siRNA was used to reduce Fyn levels. It could be shown clearly, that Fyn siRNA leads to a strong reduction of Fyn protein in Oli-*neu* cells (figure 4.4-2). Syntaxin 6 served as a loading control and

was not affected by the siRNA treatment. Reblotting with a Phospho-tyrosine specific antibody shows the effect of the absence of Fyn on the level of tyrosine phosphorylation of a range of proteins. Phosphorylation of most proteins is decreased whereas one protein appears to be phosphorylated to a higher extent (see asterisk in figure 4.4-2).

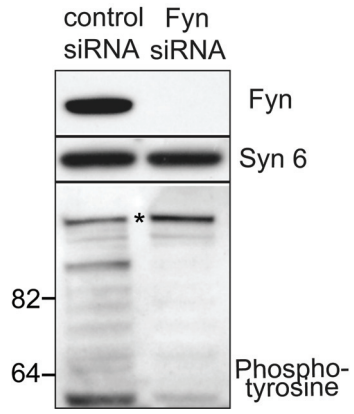

Figure 4.4-2:The reduction of Fyn kinase affects the phosphorylation of many proteins

Oli-*neu* cells were treated with non targeting control siRNA or Fyn directed siRNA. Total cell lysates were analysed by Western blotting using Fyn, Syntaxin 6 (Syn 6, as loading control) and phosphotyrosine specific antibodies (as indicated on the right).

4.5 Heterogeneous nuclear ribonucleoprotein A2/ B1 is a downstream target of Fyn signalling in oligodendrocytes initiated by F3 stimulation

In order to identify proteins that are tyrosine-phosphorylated in response to stimulation of F3 by antibodies or L1-Fc, the experiment introduced in section 4.4 was modified. Large (15 cm diameter) cell culture dishes with differentiated Oli-*neu* cells were treated with control or F3 antibodies, (see materials and methods) lysed and submitted to immunoaffinity chromatography using an antibody recognising tyrosine-phosphorylated proteins coupled to an agarose matrix. The purified proteins were separated by SDS-PAGE and visualised by Coomassie protein staining. Figure 4.5-1 shows an exemplary Coomassie gel in which purified tyrosine phosphorylated proteins can be seen. Some of these Phospho-proteins only appear in the Coomassie gel after F3 stimulation (see for example asterisks in fig. 4.5-1A).

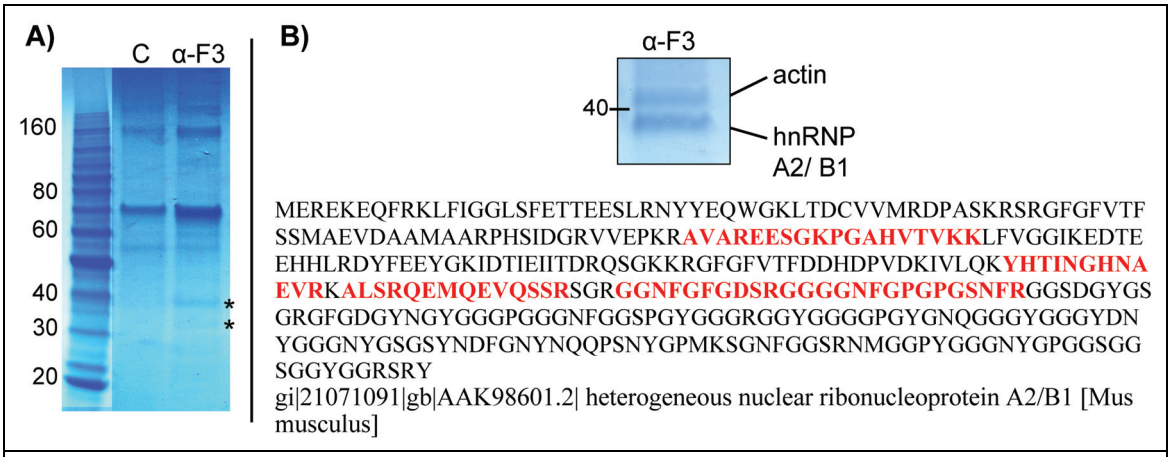

Figure 4.5-1: Purification of tyrosine phosphorylated proteins after antibody-mediated F3 stimulation

A) Differentiated Oli-*neu* cells were treated with control (c) or F3 antibodies (α-F3) as described previously. Subsequently, tyrosine phosphorylated proteins were purified by immunoaffinity chromatography and separated by SDS-PAGE (4-12%). Proteins were visualised by Coomassie protein staining. Certain proteins only appear in F3 antibody treated cells (see asterisks for example). A molecular weight marker was loaded on the left and several weights were indicated. **B)** An experiment as described in A was upscaled and tyrosine phosphorylated proteins were purified only from F3 treated cells. Proteins were separated by SDS-PAGE (4-12%), Coomassie stained and certain bands were excised. Proteins were subjected to in gel trypsin digestion (see materials and methods) and analysed by mass spectrometry. The top panel shows a part of the coomassie stained gel with two major protein bands before excision. As indicated on the right, those bands were identified as actin and heterogeneous nuclear ribonucleoprotein A2/B1. The primary structure of hnRNP A2 is shown at the bottom and peptides that were identified by mass spectrometry are coloured red. Sequence coverage: 20%; Protein score: 286 (scores higher than 53 are significant).

In order to obtain sufficient amounts of protein for mass spectrometric analysis, eight 15 cm dishes of differentiated Oli-*neu* cells were treated with F3 antibodies as described earlier, lysed and tyrosine phosphorylated proteins were purified from the pooled lysates by immunoaffinity chromatography. Purified proteins were separated by SDS PAGE, coomassie stained and subjected to “in gel” trypsin digestion and mass spectrometric analysis in collaboration with Dr. Bruns (Zentrallabor, Klinikum, University of Mainz). One of these proteins migrating between 30 and 40 kD in the gel (see figure 4.5-1B) was identified as heterogeneous nuclear ribonucleoprotein A2/ B1. Furthermore, the cytoskeletal protein actin was identified in this experiment. Tyrosine phosphorylation of actin has been described elsewhere (Kishi et al., 1998; Liu et al., 2006) and hence proved the functionality of the purification of Phosphoproteins with this approach. Tyrosine phosphorylation of oligodendroglial hnRNP A2 had not been reported before. Hence, further investigations concerning the expression and phosphorylation of this RNA binding protein were carried out.

4.6 Heterogeneous nuclear ribonucleoprotein A2/ B1 expression in oligodendrocytes

Heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) is a ubiquitously expressed RNA binding protein involved in various cellular processes (see section 5.3). In oligodendrocytes hnRNP A2 was shown to shuttle between the nucleus and the cytoplasm and to play a role in the intracellular localisation of distinct mRNAs.

Four transcripts of the hnRNP A2/B1/B0 gene have been identified which are generated by alternative splicing of exons 2 and 9, giving rise to the protein products hnRNP B1, A2, B0a and B0b (Hatfield et al., 2002). As seen in figure 4.6-1, the largest isoform hnRNP B1 (353 amino acids) contains exon 2 and 9 whereas hnRNP A2 (341 amino acids) lacks exon 2 but contains exon 9. Isoform B0b (313 amino acids) contains exon 2 (like hnRNP B1) but lacks exon 9. The smallest isoform hnRNP B0a (301aa) is deficient in exon 2 and exon 9.

A mouse monoclonal antibody (clone EF67 provided by Dr Rigby, Dartmouth Medical School, USA) was used to detect hnRNP A2 in Western Blots of primary murine oligodendrocyte lysates. Additionally, a rabbit polyclonal B1 specific antibody was used to distinguish the B1 from the A2 isoform. As seen in figure 4.6-1B (left panel), the mouse monoclonal antibody detects hnRNP A2 as a major band at 36kD in agreement with hnRNP A2's molecular weight. Furthermore, a weaker band at approximately 38 and 32 kD can be seen which correspond to hnRNP B1 and probably hnRNP B0a, respectively. It has been reported before that this antibody recognises hnRNP A2 and the B1 and B0 splice variants (Maggipinto et al., 2004). The hnRNP B1 specific antibody (Sueoka et al., 2004) detects a single band at ~38 kD (right panel). As this antibody must recognise a region coded for by exon 2 (which is missing in the A2 and B0a isoforms) it should also recognise the B0b isoform (which contains exons 2). However, only the B1 isoform at ~38 kD is detected. Hence, the lowest band in the hnRNP A2/B1 blot is most likely hnRNP B0a and no hnRNP B0b can be detected by the used reaction conditions. The two higher bands indicated with a question mark have been reported before (Hatfield et al., 2002) and could correspond to post-translationally modified forms of hnRNP A2/ B1.

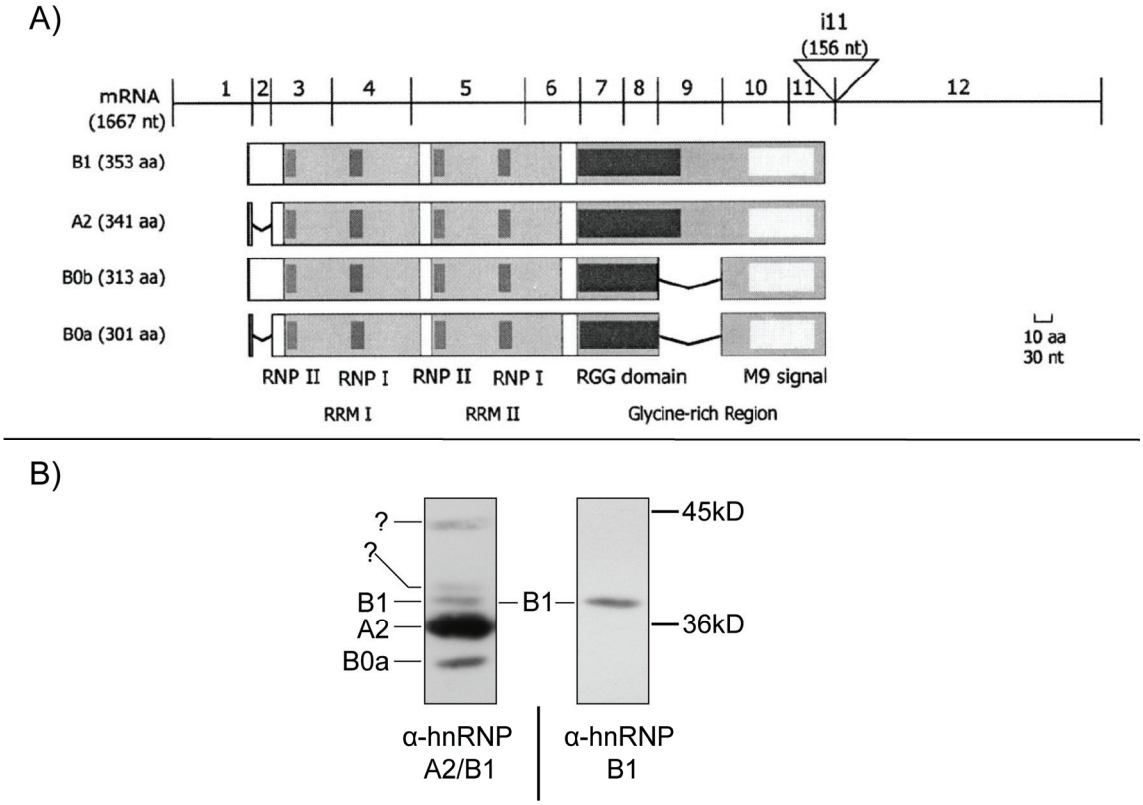

Figure 4.6-1: HnRNP A2/ B1/B0 in primary oligodendrocytes

A) Schematic diagram (Hatfield et al., 2002) depicting mRNA (top) and protein organisation (bottom 4) of hnRNP A2/ B1/ B0. Exons 2 and 9 are alternatively spliced yielding 4 protein isoforms with the indicated numbers of amino acids and domains. All four isoforms contain 2 RNA Recognition Motifs (RRM I & II) which include 2 highly conserved ribonucleoprotein motifs (RNP I & II). The C-terminal parts of the proteins contain a glycine-rich region including a RGG domain (RNA binding) and a M9 nuclear signal sequence. **B)** Western blot of a primary murine oligodendrocyte lysate using mouse anti hnRNP A2/B1/ B0 antibody recognising all isoforms (left) and the same blot stripped and reprobed with a rabbit anti hnRNP B1 specific antibody (right).

The expression of hnRNP A2 in Oli-*neu* cells had not been reported before. As shown by immunostaining using the mouse monoclonal EF67 antibody, hnRNP A2 is predominantly located in the nucleus, but a few cells also show a cytoplasmic distribution (figure 4.6-2). Since this antibody mainly recognises the A2 isoform (see 4.6-1) and only weakly detects other isoforms, a positive staining with this antibody is attributed to reactivity with hnRNP A2 in agreement with earlier publications (Brumwell et al., 2002; Shan et al., 2003).

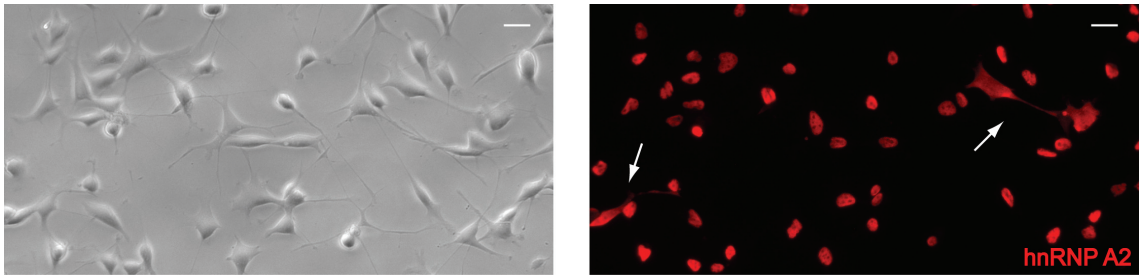

Figure 4.6-2: Endogenous hnRNP A2 Expression in Oli-*neu* cells

Oli-*neu* cells were fixed and immunostained with mouse anti hnRNP A2 (EF67) and Cy3 coupled goat anti mouse antibodies. Phase contrast (left) and fluorescence microscopic (right) images show that hnRNP A2 is predominantly located in the nuclei, but is also present in the cytoplasm of certain cells (arrows). Scale bar = 10µm

According to recent findings, hnRNP A2 levels are approximately 20 fold higher in the nucleus than in the cytoplasm of oligodendrocytes (Kosturko et al., 2006). The sensitivity of the immunostaining may not be adequate to visualise cytosolic localisation of hnRNP A2. To increase the total amount of hnRNP A2 in the cell, an expression vector was generated consisting of full length hnRNP A2 fused to an N-terminal FLAG tag (see materials and methods). Upon transfection of this vector into Oli-*neu* cells, hnRNP A2 could be detected in more detail in the cytoplasm (see figure 4.6-3). In this case hnRNP A2 seems to appear in a granular manner in the cell body and processes as described previously for neuronal and oligodendroglial RNA transport granules (Ainger et al., 1993; Brumwell et al., 2002; Thomas et al., 2005; Wickens and Goldstrohm, 2003)

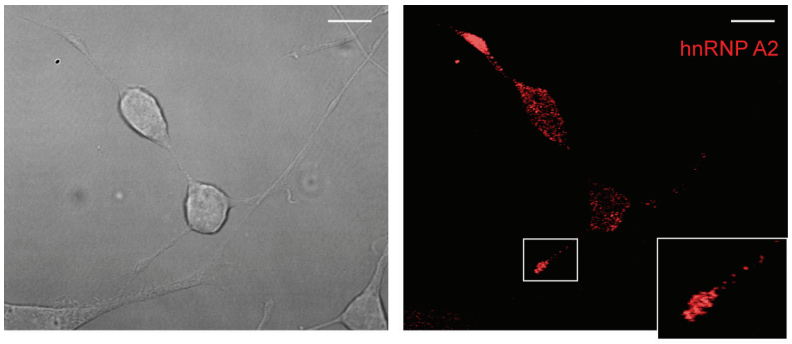

Figure 4.6-3: HnRNP A2 over-expression in Oli-*neu* cells

Oli-*neu* cells were transfected with a full length hnRNP A2 expression vector (FLAG-A2), immunostained with mouse anti hnRNP A2 (EF67) and Cy3 coupled goat anti mouse antibodies. Stainings were analysed by confocal microscopy. A single layer is depicted (left: DIC, right hnRNP A2/Cy3). An enlargement of the boxed area is shown on the bottom right. Scale bar = 10µm

Interestingly, these granular structures seem to accumulate at distal regions of the processes as seen in the enlargement of the boxed area in figure 4.6-3. The

single confocal slice depicted in this image does not reveal the predominant concentration of hnRNP A2 in the nucleus.

HnRNP A2 is ubiquitously expressed and has been detected in the brain (Hatfield et al., 2002; Matsui et al., 2000) and oligodendrocytes in culture (Brumwell et al., 2002; Kosturko et al., 2005) (and this manuscript) as mRNA and protein. To evaluate the expression of hnRNP A2 in murine brain, cortical tissue was analysed by immunohistochemistry. In collaboration with Dr. Karram (Molecular Cell Biology, University of Mainz) an adult (heterozygous) NG2-YFP mouse, a genetically modified “knock in” mouse expressing YFP under the control of the endogenous NG2 promoter (Karram, 2006), was fixed by perfusion and the brain was cut into 25µm thick sections using a vibratome. The sections were immunostained with hnRNP A2 and Olig 2 antibodies and analysed by confocal microscopy. Olig 2 (oligodendrocyte transcription factor 2) is a marker for immature and mature oligodendrocytes (Yokoo et al., 2004) whereas NG2 is expressed in a subpopulation of glial cells including immature oligodendrocytes (Karram et al., 2005; Trotter, 2005).

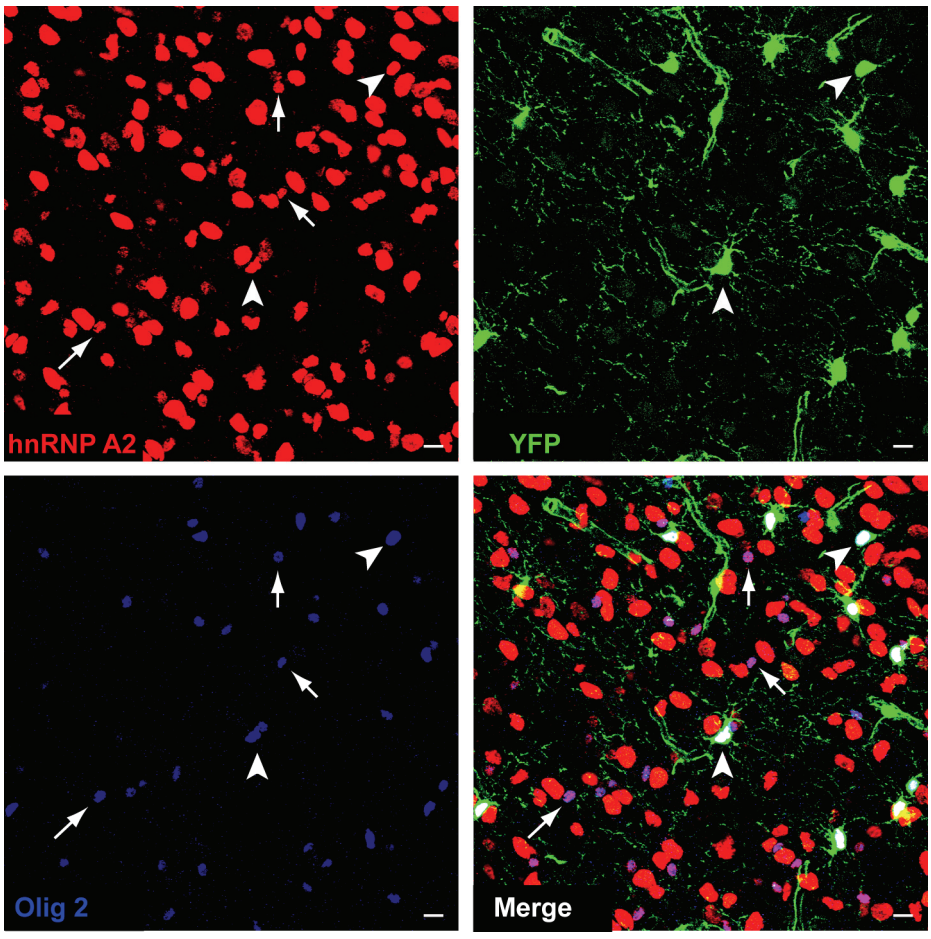

Figure 4.6-4: Endogenous hnRNPA2 expression in the adult mouse cortex

Confocal scan of a vibratome cut section (25 µm thick) of a P75 NG2-YFP mouse cortex. As indicated, the section was stained with antibodies against hnRNP A2 and

Olig 2, which is expressed by progenitor- and mature oligodendrocytes. The merged picture (bottom right) shows triple labelled (white) cells marked with arrowheads and hnRNP A2/ Olig 2 double labelled cells marked with an arrow (magenta). Scale bar = 10 µm.

Figure 4.6-4 shows that hnRNP A2 is ubiquitously expressed in the cortex and can be detected predominantly in the nuclei. There are cells that are triple labelled (marked with arrow heads) that appear white in the merged image. These cells are hnRNP A2, Olig 2 and YFP (indicating NG2 expression) positive and hence likely to be oligodendrocyte progenitors. The cells indicated with arrows are Olig 2 positive but YFP negative and appear in magenta in the merged image. Hence they are most likely more mature oligodendrocytes. The hnRNP A2 positive, but Olig 2 and YFP negative cells could be neurons due to their characteristically large nuclei. This immunohistochemical approach indicates that hnRNP A2 is expressed in immature and mature oligodendrocytes *in vivo*. The comparably low amounts of hnRNP A2 in the cytosol could not be clearly depicted by this approach.

To increase the amount of hnRNP A2 in the cytoplasm of primary murine oligodendrocytes, the full length hnRNP A2 expression vector mentioned above (figure 4.6-2) was nucleofected into primary murine oligodendrocytes using Amaxa® technology. Cells were fixed after 5 days in culture and stained with hnRNP A2 (EF67), Fyn and MBP antibodies. MBP (myelin basic protein) served as an oligodendrocyte marker to exclude the possibility of dealing with a contaminating non-oligodendroglial cell. As expected, hnRNP A2 is predominantly seen in the nucleus where it appears overexposed (white) in the MBP positive cell (figure 4.6-5). However, hnRNP A2 can also be detected extra-nuclearly in the cell body and processes, where it is located in the most distal regions as well. As observed in Oli-*neu* cells, hnRNP A2 appears in granular structures which are likely to be the previously described RNA transport granules (Ainger et al., 1993; Brumwell et al., 2002; Thomas et al., 2005; Wickens and Goldstrohm, 2003). Fyn seems to be relatively uniformly expressed which makes it difficult to demonstrate clearly that hnRNP A2 and Fyn co-localise in this cell. However, Fyn and hnRNP A2 are co-expressed by the same cell. Interestingly, in an MBP negative cell at the bottom right, hnRNP A2 seems to be present in the cell body and process as well. The identity of this cell remains uncertain, but it could possibly be an oligodendrocyte progenitor or neuronal cell.

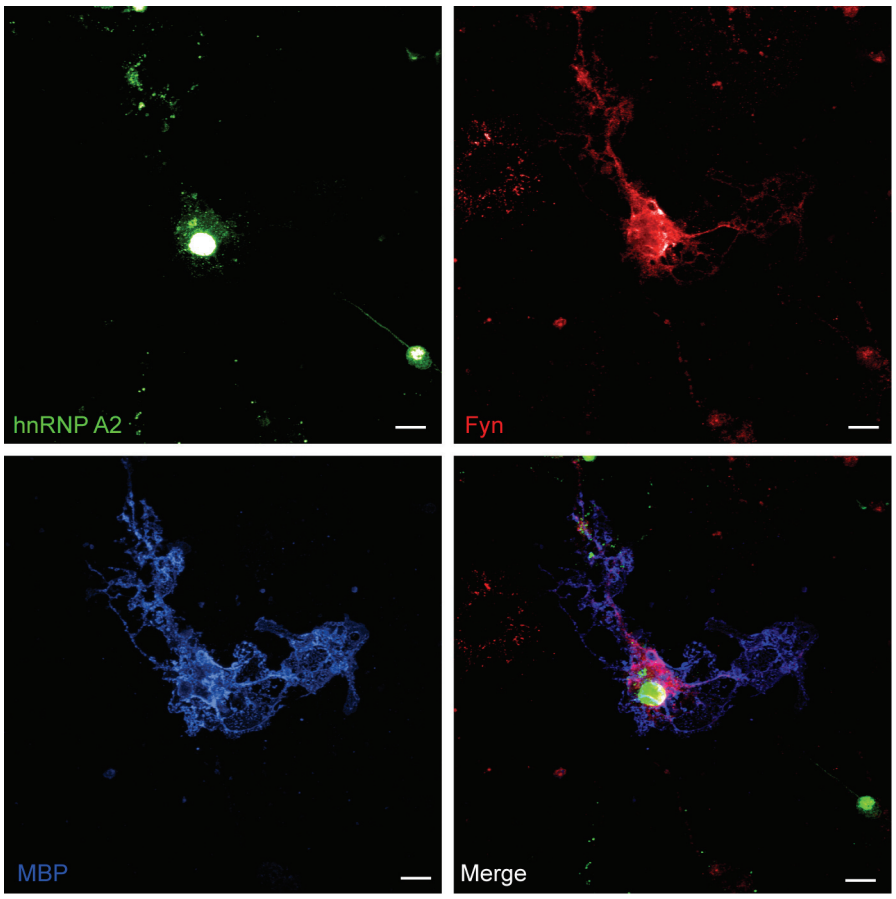

Figure 4.6-5: Over-expression of hnRNP A2 in primary oligodendrocytes

Primary murine oligodendrocytes were nucleofected with a full length hnRNP A2 construct using Amaxa® technology.. After 5 days in culture, cells were fixed and immunostained using the indicated antibodies. Myelin Basic Protein (MBP) served as an oligodendrocyte marker. A stacked confocal images scan is shown. Scale bar = 10 µm

4.7 Heterogeneous nuclear ribonucleoprotein A2 is phosphorylated by Fyn kinase

Heterogeneous nuclear ribonucleoprotein A2 was identified as one of the purified tyrosine phosphorylated proteins after antibody-mediated F3 stimulation (see section 4.5). Since it had been shown previously, that Fyn kinase is activated by this procedure (Kramer et al., 1999), it seems likely that the tyrosine kinase Fyn plays a role in phosphorylation of hnRNP A2.

In order to analyse the impact of Fyn on hnRNP A2 phosphorylation in more detail, Fyn expression vectors were generated. Besides a wild type Fyn vector, two additional vectors were constructed that carry a mutation in the ATP binding

site of the kinase domain (K299M) or a mutation in the activity regulating C-terminus of the protein (Y528F) (see materials and methods).

Oli-*neu* cells were transfected with a GFP-control plasmid or the Fyn Y528F active mutant (Fyn+) that remains constitutively active in the cells. Tyrosine phosphorylated proteins were immunoprecipitated from each set of transfected cells and analysed for the presence of hnRNP A2. By this approach, the tyrosine phosphorylated fraction of hnRNP A2 could be separated from total hnRNP A2 and a potential effect of Fyn kinase activity on this phosphorylation process could be assessed. Figure 4.7-1 shows that hnRNP A2 is present in both total cell lysates (before immunoprecipitation), but tyrosine phosphorylated hnRNP A2 (after immunoprecipitation) is predominantly present in cells transfected with the constitutively active Fyn construct (Fyn+). Heterogeneous nuclear ribonucleoproteins E1 and E2 served as a control and do not immunoprecipitate in this experiment.

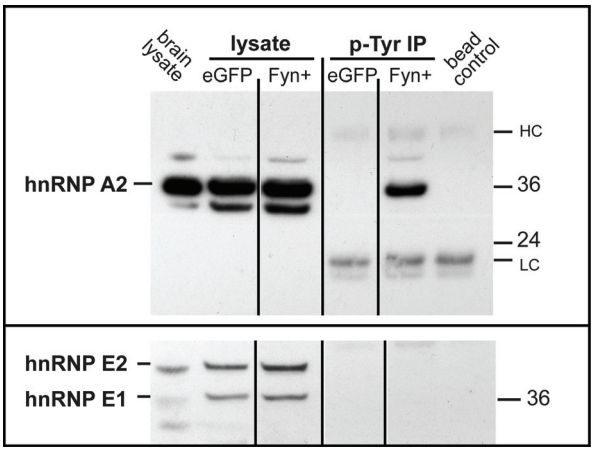

Figure 4.7-1:Fyn activity leads to hnRNP A2 phosphorylation

Tyrosine phosphorylated proteins were immunoprecipitated with a phospho-tyrosine specific antibody from Oli-*neu* cells that had previously been transfected with either eGFP (eGFP) or a constitutively active Fyn mutant (Fyn+).Total cell lysates before the Immunoprecipitation (lysate) and immunoprecipitated tyrosine phosphorylated proteins (p-Tyr IP) were analysed by Western blotting using hnRNP A2 (upper panel) or hnRNP E1/E2 antibodies (lower panel). A P14 murine brain lysate (brain lysate) served as a blotting control and antibody coupled beads used in the IP (bead control) were loaded to distinguish purified proteins from antibody heavy and light chains (HC, LC)

This result suggests that hnRNP A2 is tyrosine phosphorylated in response to Fyn activity. However, it cannot be entirely excluded that hnRNP A2 binds to a tyrosine phosphorylated protein and thus co-immunoprecipitates without being tyrosine phosphorylated itself. Furthermore, Fyn need not phosphorylate hnRNP A2 directly, but could lead to an activation of a second tyrosine kinase which in turn phosphorylates hnRNP A2. In order to investigate the ability of Fyn

kinase to phosphorylate hnRNP A2 directly, an *in vitro* kinase assay was performed with purified proteins. HnRNP A2 was isolated from Oli-*neu* cells by immunoprecipitation in duplicate and treated with or without purified recombinant Fyn kinase which was commercially available. As seen in figure 4.7-2, Western Blot analysis using a phospho-tyrosine specific antibody shows tyrosine phosphorylated hnRNP A2 protein migrating at 36 kD only in the presence of recombinant Fyn kinase. The subsequent analysis with an hnRNP A2 antibody confirmed this tyrosine phosphorylated protein to be hnRNP A2 and verified that hnRNP A2 was also present in the IP that had not been treated with recombinant Fyn and did not show a Phospho-band. Pure recombinant Fyn kinase, which is tyrosine phosphorylated itself and can hence be detected in the upper western blot, was loaded as a control, to exclude the possibility that the 36kD band could be a contamination introduced with the kinase. Interestingly, additional bands could be detected with the phospho-tyrosine antibody (figure 4.7-2, upper panel) in the A2 IP treated with recombinant Fyn kinase. These could possibly be proteins which co-immunoprecipitate with hnRNP A2.

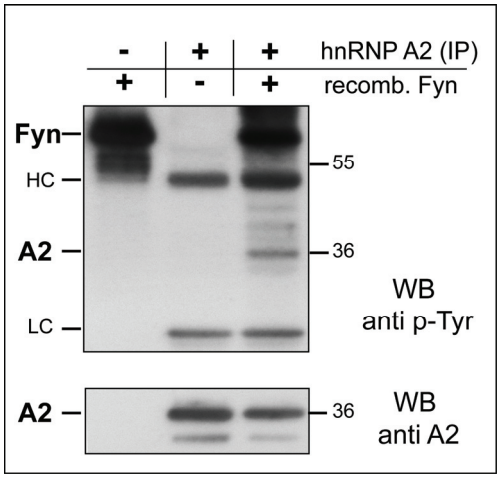

Figure 4.7-2: In-vitro Fyn kinase assay

hnRNP A2 was purified by immunoprecipitation in duplicate (A2 IP) and either treated with (+) or without (-) recombinant Fyn kinase (recomb. Fyn). 4-12 % SDS-PAGE and Western Blot analysis with a phospho tyrosine specific antibody (WB anti p-Tyr) revealed phosphorylated hnRNP A2 migrating at 36kD only in the presence of Fyn kinase Recombinant Fyn kinase alone was loaded as a control (C), to reassure that no contaminating proteins are introduced into the assay with Fyn kinase. The Western blot was stripped and re-blotted with hnRNP A2 antibodies (lower panel, WB anti-A2) to confirm that hnRNP A2 is present in both IPs and the 36kD phosphorylated protein is hnRNP A2. As indicated on the left of the upper blot, light and heavy chain of the antibody used in the IP are detected by the secondary antibody in the Western Blot analysis(LC/ HC)

Taking the former observations into consideration one can deduce that hnRNP A2 is a direct target of Fyn kinase.

The interaction between an enzyme like Fyn and its substrate like hnRNP A2 is probably rather transient within the cell. Hence, it can be rather demanding to show a direct interaction by biochemical or immunocytochemical methods. A wild-type Fyn construct was transiently transfected to increase Fyn levels within Oli-*neu* cells which maximises the number of possible interactions between Fyn and hnRNP A2. It was possible to co-immunoprecipitate endogenous hnRNP A2 with Fyn from these Oli-*neu* cells. Figure 4.7-3 depicts that Fyn was immunoprecipitated very efficiently and a small amount of hnRNP A2 was co-purified. The abundant protein α-tubulin that did not co-immunoprecipitate served as a control. The cytosolic protein GAPDH did not co-purify either (not shown).

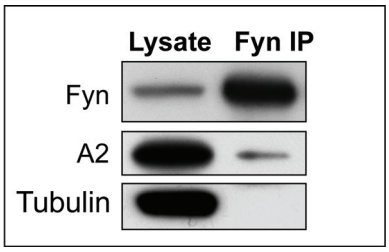

Figure 4.7-3: hnRNP A2 co-immunoprecipitates with Fyn

Oli-*neu* cells were transfected with a wild type Fyn construct and Fyn was immunoprecipitated from lysed cells. A fraction of the lysate before the IP (lysate) and immunoprecipitated Fyn (Fyn IP) were separated by SDS PAGE (4-12%) and analysed by Western blotting with rabbit anti Fyn (Fyn), mouse anti hnRNP A2 (A2) and mouse anti α-tubulin (Tubulin) antibodies as indicated on the left.

4.8 Fyn activity affects translation of an A2RE reporter

Heterogeneous nuclear ribonucleoprotein A2 has been implicated in the transport of distinct messenger RNAse within cells. These localised mRNAs contain a cis acting element in their 3' untranslated region (UTR) termed the A2 response element (A2RE). HnRNP A2 itself acts as a trans acting factor by binding to this region of the mRNA and seems to play a role in directing the mRNA to the periphery of the cell and recruiting other proteins that inhibit translation of the transported mRNA (Carson and Barbarese, 2005; Carson et al., 2001b; Kosturko et al., 2006). It has been shown for other RNA-binding proteins involved in mRNA localisation that they are tyrosine phosphorylated at the destination of their transported mRNA which results in localised translation (Huttelmaier et al., 2005) Therefore, it was analysed if Fyn activity, which had shown to lead to hnRNP A2 phosphorylation (section 4.7) affects translation of

mRNAs containing the A2RE. An A2RE containing region of the MBP14 3'UTR was cloned downstream of a *firefly* luciferase reporter (see materials and methods and figure 4.8-1).

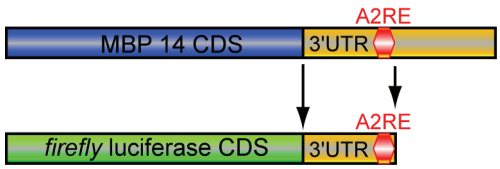

Figure 4.8-1: The luciferase A2RE reporter

A 378 nucleotide long part of the 3' UTR of myelin basic protein cDNA (isoform 14) containing the A2 response element (A2RE) was cloned downstream of a *firefly* luciferase coding sequence (CDS). The transcript of this reporter construct contains the A2RE binding site for hnRNP A2 in its 3'UTR and translational activity can be assessed by measuring *firefly* luciferase activity.

Transcription of this reporter construct yields an mRNA containing the regulatory A2 response element in the 3'UTR. Binding of hnRNP A2 to this element should repress translation (Kosturko et al., 2006). If Fyn activity and concurrent A2 phosphorylation affect translation of A2RE containing mRNAs, this should result in higher amounts of intracellular *firefly* luciferase enzyme which can be detected by measuring the enzyme activity as bioluminescence. In order to take transfection variations or general translational effects into account, *renilla* luciferase was co-transfected. Activities of *renilla* and *firefly* luciferases, which are derived from different organisms, are dependent on different substrates and result in bioluminescence of diverse wavelengths. Hence both luciferases can be incorporated into one assay and one of them, which is lacking additional cis regulatory elements such as an A2RE, can be used for normalisation (*renilla* luciferase in this case). In this assay *firefly* and *renilla* luciferases were co-transfected with either eGFP, wild-type Fyn (Fyn WT) or constitutively active Fyn (Fyn+).

Figure 4.8-2 shows that Fyn activity does indeed affect translation of an A2RE containing reporter. Compared to GFP transfected (control) cells, normalised *firefly* activity is increased by a factor of 2.1 in cells transfected with wild type Fyn and a factor of 4.6 in the presence of constitutively active Fyn (Fyn+).

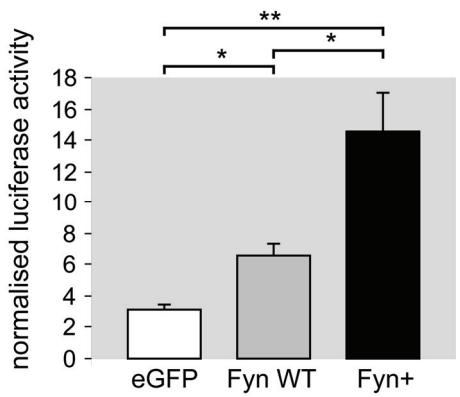

Figure 4.8-2: Fyn activity affects translation of A2RE containing mRNA

Oli-*neu* cells were co-transfected with the A2RE containing *firefly* luciferase reporter, *renilla* luciferase for normalisation and either a eGFP control plasmid (eGFP), a Fyn wild type vector (Fyn WT) or a vector expressing a constitutively active Fyn mutant (Fyn+). *Firefly* and *renilla* luciferase activity was assessed according to the DualGLO® manual (Promega) by measuring bioluminescence in a luminometer. The ratio of *firefly*/ *renilla* luminescence was taken to normalise luciferase activity and the mean of 3 individual experiments was plotted (n=3). Error bars represent SEM and paired student's t-tests assessed significance.(* = $p < 0,05$;** = $p < 0,01$)

To exclude an impact of Fyn over-expression on the stability of the luciferase reporter mRNA, which would also result in altered amounts of translated protein, total RNA was isolated from cells used in the assays and analysed by quantitative RT-PCR in collaboration with Dr. Hüttelmaier, University of Halle.

As illustrated in figure 4.8-3, mRNA levels do not seem to be affected by the over-expression of wild type or constitutively active Fyn kinase (FynWT and Fyn+, respectively). Variations introduced by different transfection efficiencies in the different experiments were overcome by the normalisation of *firefly* luciferase mRNA levels with *renilla* luciferase mRNA levels. The normalised *firefly* luciferase values of Fyn WT and Fyn+ transfected cells were then related to eGFP transfected control cells.

There is no significant difference in the means of normalised *firefly* luciferase in FynWT and Fyn+ transfected cells compared to eGFP transfected control cells as shown with a student's t-test.

Taken the results depicted in figures 4.8-2&3 into consideration one can deduce that the strong increase in *firefly* luciferase activity caused by Fyn activity is due to a translational effect and not due to increased mRNA stability. Hence, Fyn activity stimulates translation of an A2RE containing mRNA.

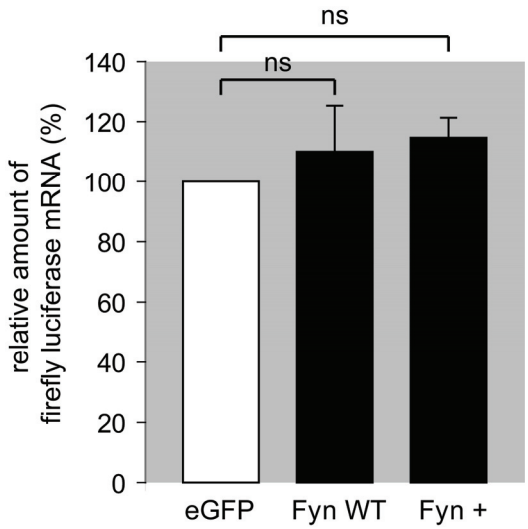

Figure 4.8-3: Fyn transfection does not significantly affect mRNA levels

Total RNA was isolated from cells used for the luciferase assay depicted in figure 4.8-2 and *firefly* and *renilla* luciferase mRNA levels were analysed by quantitative RT-PCR. *Firefly* luciferase mRNA levels were normalised with *renilla* luciferase mRNA and normalised levels in FynWT and Fyn+ transfected cells were related to eGFP transfected control cells. Error bars represent SEM and differences of the means were analysed by a student's t-test and shown to be not significant (ns). n=3.

4.9 Fyn activity and L1-Fc binding leads to an increase of hnRNP E1 protein levels in the cytoplasm

As mentioned earlier, hnRNP A2 binds to the A2 response element in certain mRNAs and forms granular ribonucleoprotein transport complexes termed RNA granules (Carson and Barbarese, 2005; Carson et al., 2001b). These granular structures can be clearly visualised after over-expression of hnRNP A2 in Oli-*neu* cells and primary oligodendrocytes (figures 4.6-3, 4.6-5). It was suggested recently, that hnRNP E1 is recruited to transport granules by hnRNP A2 and plays a role in the inhibition of A2RE containing mRNAs during transport (Kosturko et al., 2006). Hence, it seems plausible that the association of hnRNP A2 and hnRNP E1 with transported mRNA in the granules is altered to allow translation of the transported mRNA. Possibly, hnRNP A2 and E1 are released from the granule or the whole granule falls apart upon a stimulus that initiates

translation. In any case, the molecular composition of the granule is likely to change. It could be shown that L1-Fc binding activates Fyn kinase and active Fyn phosphorylates hnRNP A2 and leads to increased translation of an A2RE containing reporter (sections 4.3, 4.7, 4.8).

To analyse if hnRNP A2 or hnRNP E1 are released from granules in response to Fyn activity or L1-Fc binding, which may be required to initiate translation of the transported mRNA, a biochemical approach was attempted. Briefly, post nuclear supernatants of Oli-*neu* cell lysates were ultra-centrifuged at high speed to pellet all intracellular compartments and create a granule free supernatant which should contain only soluble cytosolic proteins (see figure 4.9-2A). By this method one should be able to distinguish between granule associated proteins, which should separate into the pellet and granule unassociated proteins, which should remain in the supernatant.

In order to test the functionality of this test, a post nuclear supernatant of an Oli-*neu* cell lysate was treated with RNAse A to degrade RNAs and dismantle RNA-Protein complexes such as the RNA granules. Subsequently, high speed ultracentrifugation was performed to obtain a granule free supernatant. Western blot analysis of the granule free supernatants shows that hnRNP A2 and E1 protein levels are increased in response to RNAse A treatment (figure 4.9-2B) from which one may deduce that this ultracentrifugation step distinguishes between granule associated and non-associated proteins.

To test if Fyn activity affects the association of hnRNP A2 and E1 with RNA granules, Oli-*neu* cells were transfected with either a control GFP plasmid or with a plasmid coding for constitutively active Fyn (Fyn+). A granule free supernatant was prepared as described above and subsequent Western blot analysis shows that the levels of hnRNP E1 are increased in Fyn+ transfected cells whereas hnRNP A2 levels seem slightly reduced (figure 4.9-2C).

Similarly, L1-Fc binding (as performed in section 4.3) leads to an increase of hnRNP E1 in the granule free supernatant, whereas hnRNP A2 levels are comparable to the control treatment (figure 4.9-2D).

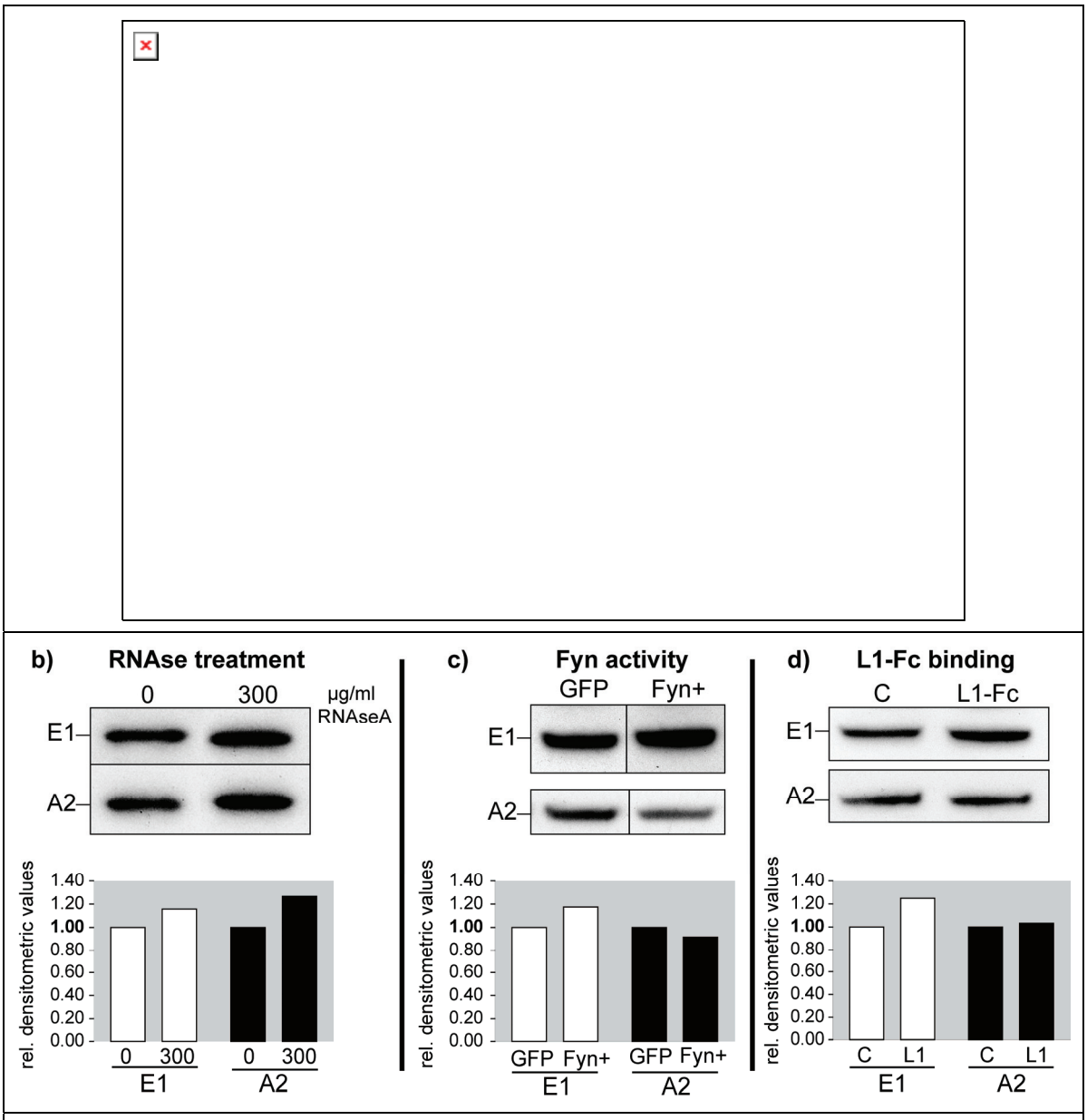

Figure 4.9-1: L1-Fc binding and Fyn activity lead to an increase of hnRNP E1 in the cytoplasm

A) Schematic diagram demonstrating the experimental setup to separate free cytoplasmic proteins from RNA transport granules. Briefly, cells are lysed with detergent containing buffer and nuclei and cellular debris are pelleted by low speed centrifugation (200 x g). A second high speed centrifugation step (136 000 x g) is carried out to clear the post nuclear supernatant from remaining intracellular organelles and structures such as RNA granules yielding a final supernatant consisting of free soluble cytosolic proteins **B) Top:** Equal amounts of a post nuclear supernatant were treated with 0 or 300 µg/ml RNAse A to disassemble Protein-RNA complexes such as RNA granules and centrifuged at high speed as described. The supernatant was analysed by SDS-PAGE (4-12%) and Western Blotting with hnRNP E1 and hnRNP A2 antibodies as indicated on the left (E1/A2). **C&D) Top:** Oli-*neu* cells were transfected with eGFP (GFP) or constitutively active Fyn (Fyn+) or treated with control Fc (C) or L1-Fc fusion protein (L1-Fc) before generation of a granule free supernatant as described in A). These supernatants were analysed by SDS-PAGE and Western blotting using the indicated antibodies. **B, C&D) Bottom:** Relative densitometric values of the band intensities seen in the Western blots (top). Control values were set to 1.00 and plotted in comparison to the ratio of treatment/ control to demonstrate relative quantities of hnRNP E1 and A2.

4.10 Identification of hnRNP A2 associated proteins

In order to identify proteins that are associated with hnRNP A2 in oligodendrocytes, hnRNP A2 was isolated from Oli *neu* cells by immunoprecipitation and visualised by SDS PAGE and Coomassie protein staining. Potentially associated proteins were co-purified and visible in the protein staining (see 4.10-1).

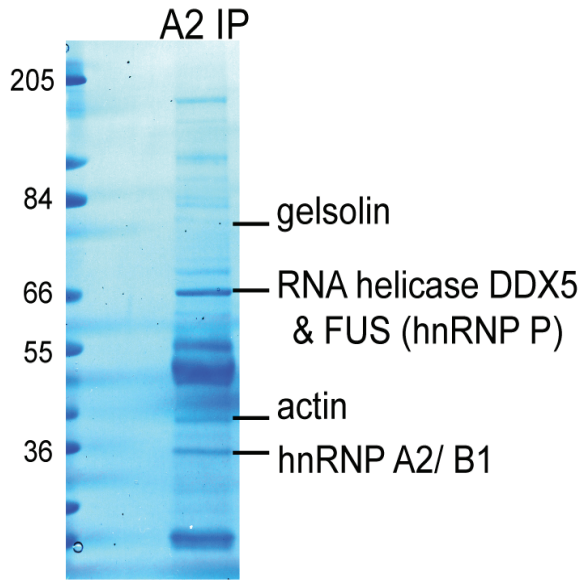

Figure 4.10-1:Identification of hnRNP A2 associated proteins

hnRNP A2 was immunoprecipitated from Oli-*neu* cells with mouse anti hnRNP A2 antibodies (EF67), separated by SDS-PAGE (4-12%) and visualised by Coomassie protein staining. The 3 indicated protein bands were excised and further processed for mass spectrometric analysis and identified as gelsolin, RNA helicase DDX5, FUS (hnRNP P) and hnRNP A2/ B1. Note that DDX5 and FUS co-migrated in the same band. A molecular weight marker is indicated on the left.

The indicated bands were excised, and identified by mass spectrometry in collaboration with Dr. Tenzer and Dr. Schild, Institute of Immunology, University of Mainz:

Gelsolin is an actin binding protein that has been implicated with Ca^{2+}-dependent capping, severing but also nucleation of actin filaments and has been connected to the transductions of signals into dynamic alterations of cytoskeletal architecture (McGough et al., 2003; Tellam and Frieden, 1982).

RNA helicase DDX5 is a member of the DEAD box family of proteins. These ATP-dependent helicases are required for various cellular processes involving RNA such as transcription, pre-mRNA processing, ribosome biogenesis, RNA transport, RNA decay and most interestingly translation initiation (Rocak and Linder, 2004):

Fus protein (also termed TLS or hnRNP P2) is an RNA binding protein shuttling between nucleus and cytoplasm associated with a chaperone-like function for transported RNAs (Calvio et al., 1995; Perrotti and Calabretta, 2002; Zinszner et al., 1997) .

Actin is an abundant cytoskeletal protein occurring either freely (g-actin) or in filamentous polymers (f-actin) in cells. It plays a major role in the cellular architecture as well as the polarisation of cellular processes leading to directed movement.

4.11 Identification of oligodendroglial Fyn targets

As depicted in section 4.4 (figure 4.4-2), the loss of Fyn leads to a decrease of tyrosine phosphorylation of several proteins in Oli-*neu* cells. This implies that Fyn activity affects many downstream targets either directly or indirectly. In order to identify additional Fyn targets, Oli-*neu* cells were transfected with an expression vector coding for a constitutively active Fyn mutant. Tyrosine phosphorylated proteins were immunoprecipitated from these cells using a phospho-tyrosine specific antibody coupled to an agarose bead matrix. Precipitated proteins were eluted from the antibody beads by boiling them in protein sample buffer. By this elution method, the coupled anti-phosphotyrosine antibodies are also removed from the beads and mix with the tyrosine phosphorylated proteins in the eluate. To distinguish antibody subunits (light and heavy chain) from precipitated proteins, as a control the antibody alone was incubated in sample buffer and was separated in addition to the immunoprecipitated proteins by SDS PAGE and visualised by Coomassie protein staining (see lane C in figure 4.11-1). Hence protein bands that are present in the control and the Phosphotyrosine IP are antibody subunits and not Fyn phosphorylated proteins derived from Oli-*neu* cells. Several potentially tyrosine phosphorylated proteins could be visualised by this method (see lane IP in 4.11-1) and some were subjected to mass spectrometric analysis.

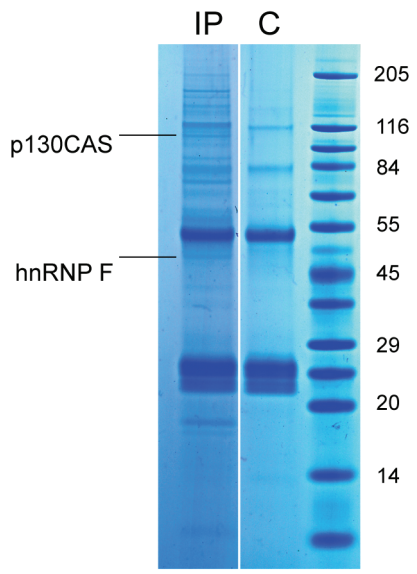

Figure 4.11-1: Purification of tyrosine phosphorylated proteins

Tyrosine phosphorylated proteins were immunoprecipitated from Oli-*neu* cells that had been transfected with constitutively active Fyn. Purified proteins (IP) as a well as control antibodies used in the IP (C) were separated by SDS-PAGE (4-12%) and Coomassie stained. The indicated bands were excised and identified by mass spectrometry.

The indicated bands in figure 4.11-1 were excised and identified by mass spectrometry in collaboration with Dr.Lechner, BZH Heidelberg:

P130Cas (Crk associated substrate) is a cytoplasmic docking protein phosphorylated by several kinases including Src or Fyn and is involved in the regulation of the actin cytoskeleton affecting morphological changes during migration or cell polarisation (Huang et al., 2006; Manie et al., 1997).

Heterogeneous nuclear ribonucleoprotein F (hnRNP F) is a ubiquitously expressed RNA binding protein with nuclear and cytoplasmic localisation (Honore et al., 2004; Matunis et al., 1994). HnRNP F has been associated with nuclear functions such as splicing and polyadenylation of pre-mRNAs or the binding to the nuclear cap binding complex (Min et al., 1995; Veraldi et al., 2001). However, recently it could be shown in *Drosophila* oocytes for the hnRNP F /H homologue Glorund, that this protein plays a role in translational repression of mRNAs (Kalifa et al., 2006).

Further protein bands were analysed but the amounts of protein in the gel were not sufficient to yield significant probability scores. Nevertheless, two proteins were identified as potential Fyn targets in oligodendrocytes by this approach. As discussed in section 5.6, hnRNP F was further analysed in collaboration with Constantin Gonsior (see Diploma thesis ‘Expression und Phosphorylierung des

heterogenen nukleären Ribonukleo-proteins F durch die Tyrosin-Kinase Fyn in Oligodendrozyten', AG Trotter, April 2007)

5 DISCUSSION

5.1 L1 binds to Oli-*neu* cells in an F3-dependent manner

The recognition of neuronal axons by oligodendrocytes represents one of the key incidents in CNS development leading to axonal ensheathment and formation of compact myelin culminating in the formation of an interdependent axon-glial apparatus required for highly efficient transmission of electrical impulses in higher vertebrates as well as maintenance of axonal integrity (Hartline and Colman, 2007). These recognition steps are likely to be mediated by proteins and lipids and the identification of these molecules will result in elucidating not only fundamental biological processes, but also the pathology of demyelinating and neurodegenerative diseases.

The immunoglobulin superfamily protein F3/ contactin was originally thought to be neuron-specific. However, it was later found to be additionally expressed by oligodendrocytes (De Benedictis et al., 2006; Einheber et al., 1997; Koch et al., 1997). It has been suggested that L1 is a potential neuronal binding partner of glial F3 (Kramer et al., 1999). The suggestion was in part based on the finding that F3 and L1 were isolated in a complex from murine cerebellar lysates (Olive et al., 1995). However, as L1 and F3 are both expressed in neurons, it cannot be distinguished if this association is *in cis* in neurons alone, or *in trans* between neuronal L1 and glial F3. Hence, one could not deduce from these results that neuronal L1 binds to oligodendroglial F3. Furthermore, F11, the F3 homologue in chicken, could be shown to bind to NgCAM (chicken L1) (Brummendorf et al., 1993). This binding could be shown by the binding of F11 coated microspheres to COS cells expressing NgCAM and thus demonstrates binding *in trans*. However, *in vivo* this binding *in trans* could be formed between neurons, as L1 of one axon could bind to F3 of a second axon.

Here, it could be demonstrated in section 4.2 that L1-Fc fusion protein binds to the murine oligodendroglial cell line Oli-*neu*. This binding was visualised (figure 4.2-1) and quantified by Cell ELISA (figure 4.2-2a). Binding occurs in an F3-dependent manner, as reduction of F3 protein levels by siRNA and the blocking of F3 protein with antibodies result in decreased binding of L1-Fc (figure 4.2-1&2) to the cells. This finding supports the above mentioned postulation of neuronal L1 as a neuronal binding partner for glial F3 (Kramer et al., 1999).

It cannot be excluded, however, that L1 binds to additional surface molecules on oligodendrocytes. Potential interaction partners of L1 on oligodendrocytes include integrins. Integrins are heterodimeric receptors that bind to the extracellular matrix or cell surface ligands (ffrench-Constant and Colognato, 2004). *In vitro*, L1 has been shown to bind to β1 integrins *in trans* (Oleszewski et al., 1999). However, in oligodendrocytes, α6β1 integrins have been associated with PDGF-mediated differentiation and survival in the context of binding to the α6β1 ligand laminin 2 (Colognato et al., 2004; ffrench-Constant and Colognato, 2004). Hence, the interaction of laminin 2 with α6β1 integrins seems to be important for this cell type, but no role of L1 and integrin binding has so far been postulated in neuron-glial interaction in the CNS. If L1- β1 binding occurred *in vivo*, perturbation of this binding should result in deficient axon-glial interaction. Interestingly, analysis of conditional transgenic mice lacking β1 expression in oligodendrocytes showed no myelination phenotype or deficits in remyelination. These mice myelinate normally, but apoptosis of premyelinating oligodendrocytes seems to be increased, suggesting that β1 integrins play a role in oligodendrocyte biology, but not in later stages of the myelination process *per se* (Benninger et al., 2006). Furthermore, no apparent CNS myelination phenotype was described in a knock in mouse model expressing mutant L1 protein lacking the 6th Ig domain required for L1 binding to β1 integrins (Itoh et al., 2004). It is unclear, however, in how much detail myelination defects were analysed. Taken these aspects into consideration it seems that L1-β1 integrin interaction occurs *in vitro*, but evidence for its role in axon-glial interaction and myelination is lacking.

Myelination in L1 and F3 knock out mice has not been analysed in detail. Nevertheless, the absence of either protein results in abnormal brain development. In addition to other abnormalities, L1 deficient mice show learning and memory deficits and delayed motor responses as well as weakness of the hind limbs (Dahme et al., 1997; Fransen et al., 1998). Especially the latter two phenotypes are likely to correlate with incorrect transmission of electrical impulses along nerve fibres which could be contributed to by aberrant myelination. F3 knock out mice show reduced transmission velocity and abnormally myelinated axons. The nodal organisation is perturbed and potassium channels are missorted (Boyle et al., 2001). The phenotypes observed in these mice cannot be interpreted solely due to a loss of oligodendroglial F3, because F3 is also strongly expressed by neurons and loss of neuronal F3 may explain the reported abnormalities.

Detailed analysis of CNS myelination in L1 and F3 knock out mice and generation of a conditional knock out mouse leading to F3 ablation in

oligodendrocytes alone would further elucidate the significance of F3-dependent binding of L1 to oligodendrocytes.

5.2 L1 binding leads to an activation of oligodendroglial Fyn kinase

Fyn activity plays an important role in oligodendrocyte maturation as demonstrated by the treatment of oligodendrocytes in culture with dominant negative constructs as well as small molecule inhibitors of Fyn kinase (Osterhout et al., 1999). Moreover, Fyn activity has been shown to be required for normal myelination *in vivo* as a knock in mouse expressing a kinase inactive Fyn mutant instead of wild type Fyn shows severe CNS hypomyelination similar to the Fyn knock out mouse (Sperber et al., 2001). It could be shown previously that antibody-mediated crosslinking of F3 molecules on Oli-*neu* cells leads to an activation of Fyn kinase (Kramer et al., 1999). Furthermore, F3 was co-immunoprecipitated with L1 and Fyn from brain tissue and with Fyn from cultured oligodendrocytes (Kramer et al., 1999; Olive et al., 1995). It was hence proposed that a neuronal ligand like L1 could bind to glial F3 and that this interaction could lead to an activation of Fyn kinase. Interestingly, L1 is downregulated on myelinated axons in culture, emphasising a potential function of L1 in early myelination processes that is no longer required once the myelin sheath is established. The activation of glial Fyn by axonal L1 could hence be part of an axon-glia communication mechanism to initiate myelination.

It could be shown here that L1 binding to Oli-*neu* cells as well as primary murine oligodendrocytes in culture leads to an activation of Fyn (figure 4.3-1a). Furthermore, localised Fyn activity could be visualised in response to local L1 binding (figure 4.3-1b). The latter could mimic binding of axonal L1 to oligodendrocytes in a simplified experimental setup. L1 binding as well as antibody-mediated crosslinking of F3 molecules leads to the phosphorylation of several downstream proteins and the phosphorylation pattern in response to both approaches appears very similar (figure 4.4-1). This reinforces the finding that L1 binds F3. Furthermore, the pivotal function of Fyn in oligodendrocytes could be shown by reduction of Fyn protein levels with siRNA. Tyrosine phosphorylation of several proteins seems decreased in the absence of normal Fyn levels (figure 4.4-2).

It remains unclear, how F3 stimulation by antibody crosslinking or L1 binding results in activation of intracellular Fyn. F3 is incorporated into the outer leaflet

of the plasma membrane by a GPI anchor (see introduction) and Fyn is inserted in the inner leaflet by acylation. It is hence likely that a transmembrane protein associated with F3 and Fyn transmits the signal from the outside of the cell to its intracellular part. As mentioned in the introduction, one possibility to activate Src-family kinases like Fyn is the dephosphorylation of a carboxy terminal regulatory tyrosine residue. Several transmembrane receptor protein tyrosine phosphatases (RPTP) have been shown to catalyse this reaction. Interestingly, RPTPα was coimmunoprecipitated with F3 and Fyn from total chicken and mouse brain (Zeng et al., 1999). The authors interpreted this interaction to be neuronal. However, from the experimental setup it cannot be excluded that coimmunoprecipitation is a result of interaction of these proteins in oligodendrocytes. Stimulation of F3 by L1 or antibody binding could hence co-stimulate associated RPTPα which could in turn dephosphorylate and thereby activate intracellular Fyn.

Furthermore, it has been proposed that F3 and Fyn are sorted into lipid raft microdomains where their interaction mainly takes part (Kramer et al., 1999; Kramer et al., 1997). GPI linked proteins and Src family kinases could often be detected in these specialised membrane regions and it has been postulated that the sorting of these molecules into lipid raft domains leads to a formation of signalling platforms in oligodendrocytes which are required for normal myelination (Debruin and Harauz, 2007). Possibly, the presence of longer and more saturated lipid acyl chains in these regions can not only form a more liquid ordered environment in the liquid disordered membrane, but can also transmit signals through the plasma membrane to proteins associated with the inner leaflet of the membrane. Alternatively, Fyn activating proteins could be directed into these lipid raft domains, stimulating Fyn in these regions of the cell. However, the mechanism by which extracellular binding of L1 leads to intracellular activation of Fyn kinase was not investigated in this study, but remains an important aspect of axon glial-communication.

5.3 HnRNP A2 is a novel downstream target of Fyn kinase

Myelinogenesis and the formation and maintenance of an axon-glial apparatus depend on bilateral communication between the neuron and the myelinating glial cell. The non receptor tyrosine kinase Fyn appears to play a fundamental role in these processes (see above). Therefore, the mechanisms of regulating Fyn activity as well as downstream implications of this activity need to be

elucidated to comprehend the function of this neuron-glial signalling cascade in the complex biological process of myelination.

Upon initial contact of a neuronal axon and the oligodendrocyte specific recognition must take place. An axon must not only be distinguished from processes of other cell types in the brain, but needs to be recognised as one of the axons that is to be insulated to guarantee rapid conduction of action potentials. Upon recognition of an axon as a myelination target, substantial morphological changes must occur in the oligodendrocyte. The cell must polarise towards the axon glial contact site which will be accomplished by major cytoskeletal rearrangements and must deliver large amounts of proteins and lipids in order to allow axonal ensheathment. It has been proposed that Fyn kinase embodies a crucial component of a signalling cascade involved in morphological changes in oligodendrocytes (Osterhout et al., 1999). As discussed in section 5.1 and 5.2, one of the upstream events triggering this signalling cascade appears to be the binding of neuronal L1 to glial F3. But what are the downstream consequences of this activation of Fyn kinase? How can Fyn activity lead to polarisation of the cell and an extension of cellular processes? A connection between Fyn activity and process outgrowth has been proposed by the finding that Fyn phosphorylates and thereby stimulates p190 RhoGAP. This stimulation leads to an inactivation of the small GTPase Rho resulting in the extension of glial processes probably mediated by changes in the actin cytoskeleton (Wolf et al., 2001). This regulation of Rho activity could also affect intracellular membrane transport and plasma membrane specialisation in oligodendrocytes as Rho was recently associated with these functions (Kippert et al., 2007). Furthermore, it was shown that activated Fyn recruits the microtubular cytoskeleton via the microtubule associated protein Tau and that this interaction is required for process outgrowth (Klein et al., 2002).

Here, an approach was used to combine the identification of downstream targets of activated Fyn with the stimulation of an extracellular component of the signalling cascade. Fyn was activated by F3 antibody-mediated crosslinking of F3 and tyrosine phosphorylated proteins were isolated. Heterogeneous nuclear ribonucleoprotein (hnRNP) A2 was identified as one of these proteins by mass spectrometry (section 4.5).

HnRNP A2 is localised in the nucleus and in the cytoplasm, but the concentration is approximately 20 fold higher in the nucleus (Kosturko et al., 2006). The protein is involved in nuclear processes such as splicing of pre mRNA and telomere regulation (Dreyfuss et al., 2002; Kamma et al., 2001;

McKay and Cooke, 1992) as well as cytoplasmic functions such as mRNA localisation and translation (Carson et al., 2001b; Kwon et al., 1999). In oligodendrocytes the role of hnRNP A2 in the localisation of MBP mRNA has been intensively investigated. HnRNP A2 binds to MBP mRNA in the nucleus. After export of this complex into the cytoplasm the formation of polysomes is prevented, probably by the formation of RNA transport granules mediated by the protein TOG (tumor overexpressed gene). It has been proposed that this large 215kD filamentous microtubule binding protein can bind 7 hnRNP A2 molecules. It colocalises with hnRNP A2 in A2RE RNA granules and is assumed to function as a scaffolding element for several hnRNP A2/ MBP RNA molecules within the transport granule. HnRNP A2 containing MBP mRNA granules are transported on microtubules to the most distal processes of oligodendrocytes and maintained translationally inactive during transport (Ainger et al., 1993; Carson and Barbarese, 2005; Carson et al., 2006; Carson et al., 2001b; Carson et al., 1997).

It could be confirmed in this study, that hnRNP A2 is expressed in oligodendrocytes and localises mainly to the nucleus, but is also present in granule-like structures in the cytoplasm (section 4.6). However, the presence of hnRNP A2-containing transport granules in Oli-*neu* cells as seen in figure 4.6-3 was depicted here for the first time. The phosphorylation of hnRNP A2 by Fyn kinase was confirmed by over-expressing constitutively active Fyn in Oli-*neu* cells and in an *in vitro* experiment in which immunoprecipitated hnRNP A2 was phosphorylated by purified recombinant Fyn kinase. The latter two approaches confirmed the direct interaction of Fyn and hnRNP A2 which was further substantiated by the coimmunoprecipitation of hnRNP A2 with Fyn (figure 4.7-3). The initial finding of hnRNP A2 as a tyrosine phosphorylated protein in response to F3 stimulation, as well as the more artificial assays used to confirm the direct action of Fyn in this phosphorylation process thus strongly suggests that F3, Fyn and hnRNP A2 function together within one signalling cascade.

5.4 Fyn activity regulates translation of myelin proteins

The identification of hnRNP A2 as a novel downstream target of the tyrosine kinase Fyn raised the question which cellular process is influenced by this interaction and posttranslational modification within this signalling cascade. Most likely the interaction of Fyn and hnRNP A2 is cytoplasmic. The coimmunoprecipitation and phosphorylation experiments in this study were performed with post nuclear cell lysates and Fyn is described to be located in

the cytoplasm and plasma membrane. Furthermore, L1-Fc- or F3 antibody-mediated Fyn activity would occur at the plasma membrane. Thus, phosphorylation of hnRNP A2 by Fyn is likely to affect a cytoplasmic function of hnRNP A2.

Intriguingly, Src kinase has been shown to phosphorylate RNA binding proteins leading to localised translation of transported mRNAs (Huttelmaier et al., 2005; Ostareck-Lederer et al., 2002). ZBP1 (zipcode binding proteins 1) binds to a region in β-actin mRNA termed the 'zipcode'. ZBP1 mediates the transport of β-actin mRNA in fibroblasts and neurons and represses translation until the actin rich protrusions of the cells are reached. At this destination, Src kinase phosphorylates a key tyrosine residue in ZBP1, leading to localised translation initiation of β-actin mRNA (Huttelmaier et al., 2005). In the light of these observations, it thus seems plausible that the phosphorylation of hnRNP A2 by the Src family member Fyn could affect the translation of hnRNP A2 associated mRNA. It could be shown clearly that Fyn activity positively affects translation of a reporter mRNA containing the A2RE of MBP in its 3'UTR (section 4.8). The presence of wildtype Fyn more than doubles the translation rate and constitutively active Fyn leads to an almost 5 fold increase. Variations in mRNA levels were excluded by qRT-PCR analysis and transcriptional effects can be outruled, because a common viral promoter was used in the experimental setup. It was important to use a system that is independent of transcriptional regulation, because it has been postulated that Fyn stimulates transcription of the MBP gene by transacting on a defined promoter region (Umemori et al., 1999). The reporter construct used in the translation assays contains a 378 nucleotide long region of the 3'UTR of MBP14 mRNA. This region includes the 11 nucleotide A2RE present in all classic MBP mRNAs to which the regulatory hnRNP A2 protein binds. The experiments show that this 378 nucleotide region is responsible for Fyn-mediated translational regulation of the luciferase reporter, because the control transfected *renilla* luciferase which is lacking the 3'UTR of MBP did not show a similar effect. It remains debatable, however, if the A2RE part of the region is governing translational control alone. It could also be possible that a different part of the 3' UTR used is responsible for controlling translation of the reporter gene in response to Fyn activity. Although there have been no reports in this respect it remains to be further investigated if there are additional cis regulatory elements in this region of the 3'UTR. Considering the current knowledge of regulatory cis-acting elements in MBP mRNA, and the finding of this study that hnRNP A2 is a direct target of Fyn kinase, it is very likely that translational regulation is mediated by Fyn, hnRNP A2 and the A2RE element.

Several other mRNAs in oligodendrocytes and other cell types contain the A2RE and are likely to be transported in a similar way as MBP mRNA (Ainger et al., 1997; Carson et al., 2001a). These include oligodendroglial MOBP (myelin associated/ oligodendrocyte basic protein), neuronal CaMKII (calcium calmodulin-dependent protein kinase II) and astrocyte GFAP (glial fibrillary acidic protein). It remains to be shown if Fyn stimulates the translation of these mRNAs as well and hence is a general activator of A2RE mRNA translation, or if Fyn specifically regulates translation of MBP.

5.5 L1 binding activates Fyn leading to changes in RNA granule protein composition

In addition to the function of hnRNP A2 in transporting A2RE containing mRNAs in oligodendrocytes, it was recently proposed to recruit hnRNP E1 which represses translation of the transported mRNA (Kosturko et al., 2006 8). It was suggested that hnRNP E1 must be released from RNA granules to de-repress the transported mRNA and to initiate translation. Post-translational modification of hnRNP A2 or hnRNP E1 was hypothesised as a means of removing hnRNP E1 from granules. The removal of hnRNP E1 from the granule is necessarily accompanied with an increase of free cytoplasmic hnRNP E1. It could be shown here that hnRNP E1 levels are increased in a cytoplasmic pool that has been separated from transport granules and other macromolecular cellular structures in response to Fyn activity (figure 4.9-1c). Furthermore, Fyn activity leads to phosphorylation of hnRNP A2 and enhanced translation of an mRNA containing a regulatory element (A2RE). Since it has been shown previously that hnRNP A2 binds to this A2RE region and recruits hnRNP E1, it is very likely that tyrosine phosphorylation of hnRNP A2 interferes with the binding of hnRNP E1 resulting in a release of hnRNP E1 from the granule.

HnRNP A2 alone enhances cap-dependent translation of A2RE containing mRNA (Kwon et al., 1999). It has been suggested that hnRNP A2 stimulates cap- dependent recruitment of the small ribosomal subunit and that hnRNP E1 prevents the association of the large ribosomal subunit. Release of hnRNP E1 would thus allow the formation of polysomes and subsequent protein synthesis. It remains unclear what happens to hnRNP A2 after the release of hnRNP E1. From the experiments presented in this study, it seems that hnRNP A2 levels are not increased in the cytoplasm in response to Fyn activity. Fyn activity even seems to reduce hnRNP A2 levels in the cytoplasm. One possible explanation could be that more free hnRNP A2 associates with A2RE mRNA (like MBP) into cytoplasmic granules and is hence pelleted in the ultracentrifugation step used

in the experimental setup (4.9-1a). This is possible, because Fyn activity additionally leads to increased transcription of the MBP gene (Umemori et al., 1999) resulting in higher amounts of MBP mRNA in the cytoplasm. Hence enhancement of Fyn activity would lead to a release of hnRNP E1 from the granules to initiate translation and would increase the amount of MBP mRNA in the cytoplasm to which free hnRNP A2 could bind to form new RNA granules. A subsequent transport of newly formed RNA granules to the site of translation would then increase the amount of MBP protein synthesised at the site where Fyn is activated.

Fyn-mediated translation initiation resulting from phosphorylation of hnRNP A2 and release of hnRNP E1 from RNA granules can thus be regarded as a novel mechanism used by oligodendrocytes to control myelin protein synthesis. Furthermore, neuronal L1 was identified as an extracellular ligand capable of activating Fyn. Most intriguingly, the finding that L1 binding leads to a release of hnRNP E1 from RNA granules (figure 4.9-1d) combines the intercellular communication between neuron and oligodendrocyte with an intracellular regulatory signalling mechanism. Binding of neuronal L1 thus leads to localised translation of MBP, the second most abundant myelin protein.

5.6 The identification of additional Fyn targets and hnRNP A2 associated proteins and their possible function in oligodendrocytes

In order to further elucidate the functional importance of Fyn kinase in oligodendrocytes, additional putative Fyn targets were identified by over-expressing constitutively active Fyn in Oli-*neu* cells and purifying tyrosine phosphorylated proteins by immunoaffinity chromatography. This approach is more artificial than the method used for identifying hnRNP A2, because the level of Fyn activity *in vivo* is unlikely to be as high. Furthermore, this approach can only identify downstream targets of Fyn whereas the upstream signalling mechanisms required to activate Fyn cannot be elucidated. Fyn can be activated by several pathways in oligodendrocytes and hence the downstream phosphorylated proteins found by this approach can result from different signalling cascades merging on Fyn. However, potential targets can be identified which need to be subsequently evaluated under more physiological conditions.

P130 CAS (Crk associated substrate) is ubiquitously expressed and is a known target of Fyn and other Src tyrosine kinases (Defilippi et al., 2006; Manie et al., 1997). It has not been described in detail in oligodendrocytes thus far, but has been associated in other cell types with various functions such as migration, process elongation, cell survival and apoptosis and cell transformation in cancer. It acts as a scaffolding protein to link ECM-mediated integrin signalling with Src kinase signalling (ffrench-Constant and Colognato, 2004). Phosphorylation of p130 CAS controls the association with other docking and effector proteins and affects the actin cytoskeleton (Defilippi et al., 2006). P130 CAS protein has been associated with the extension of cerebellar axons (Huang et al., 2006), a cellular mechanism that may resemble the polarisation of an oligodendrocyte wrapping its processes around axons. The phosphorylation of p130 CAS in oligodendrocytes by Fyn could hence play a role in rearrangements of the actin cytoskeleton required for the dynamic extension of cellular processes. Intriguingly, p130 CAS tyrosine phosphorylation increases in response to mechanical stretching of cells (Tamada et al., 2004). This phosphorylation changes the protein conformation and association with signalling proteins and transforms external force to intracellular signalling. The myelination process must involve stretching of cells and the degree of membrane curvature must be transmitted to the cellular cytoskeleton to evoke apparent changes. Perhaps p130 CAS plays a role during these events by connecting the sensation of the three-dimensional shape of the axon with cytoskeleton changes required for the ensheathing process.

Heterogeneous nuclear ribonucleoprotein F (hnRNP F) is an RNA binding protein for which nuclear functions have been primarily described. The drosophila homologue glorund has been shown, however, to play a role in translational silencing of nanos mRNA, and thus a cytoplasmic function could be hypothesised for hnRNP F (Kalifa et al., 2006). This protein was further analysed in collaboration with Constantin Gonsior (see Diploma thesis ‘Expression und Phosphorylierung des heterogenen nukleären Ribonukleo-proteins F durch die Tyrosin-Kinase Fyn in Oligodendrozyten’, April 2007). It could be shown by immunocytochemistry and an hnRNP F-GFP fusion protein, that hnRNP F localises mainly to the nucleus but can also be detected in smaller amounts in the cytoplasm. Furthermore, it seems to be upregulated during differentiation of oligodendrocytes in culture. HnRNP F was demonstrated to be tyrosine phosphorylated in response to Fyn activity in Oli-*neu* cells, but an *in vitro* phosphorylation assay with purified hnRNP F and Fyn kinase did not show a large degree of phosphorylation leading to the deduction that hnRNP F may not be a direct target of Fyn kinase. Possibly Fyn activity

stimulates a different tyrosine kinase which then phosphorylates hnRNP F. Initial experiments were performed to identify RNAs that bind to hnRNP F which will be pursued further in the near future and may elucidate functional roles of hnRNP F in oligodendrocyte biology. Due to the role of hnRNP F in translational repression, it is possible that this protein may embody a functional component of RNA granules.

In order to gain more insight into the function of hnRNP A2 in oligodendrocytes the identification of novel cytoplasmic interaction partners of hnRNP A2 was attempted by immunoprecipitation. Gelsolin, RNA helicase DDX5, FUS/ hnRNP P and actin were found to co-immunoprecipitate with hnRNP A2 from post nuclear Oli-*neu* cell lysates (section 4.10).

The best characterised cytoplasmic function of hnRNP A2 in oligodendrocytes is transporting MBP mRNA in RNA granules to the periphery of the cell where myelin protein is synthesised locally (Carson and Barbarese, 2005; Carson et al., 2006). The RNA binding protein FUS/ hnRNP P is possibly a yet unidentified component of the oligodendroglial A2RE RNA transport granule. Similar to hnRNP A2 it has been described to shuttle between the nucleus and the cytoplasm (Zinszner et al., 1997) and could thus perform a structural role in the granules.

Localised synthesis of MBP protein at the destination requires the termination of repression and the initiation of translation. HnRNP A2 containing granules contain components of the translation machinery including the small ribosomal subunit and the eukaryotic initiation factor eIF4a (Carson and Barbarese, 2005). Hence the transported mRNAs are primed for translation but repressed until they reach the destination where they can then be translated immediately. Here, the DEAD box family RNA helicase DDX5 was found to co-immunoprecipitate with hnRNP A2. DDX 5 and two other RNA helicases of this family (DDX1, DDX3) have been found associated with RNA granules isolated from total brain and in neuronal cell culture before (Elvira et al., 2006; Kanai et al., 2004). However, an interaction between hnRNP A2 and these proteins was not shown in oligodendrocytes. As DEAD box family RNA helicases function in translation initiation (Chuang et al., 1997), the function of DDX5 in respect to hnRNP A2 association could involve the initiation of MBP mRNA translation. DDX5 helicase activity could play a role in structural alterations of MBP RNA required to allow local translation of MBP mRNA that has reached its destination in the hnRNP A2-dependent transport pathway.

The elaboration of the myelin sheath at the axon glial contact site must include cytoskeletal rearrangements resulting in a polarisation of the oligodendrocyte

and extension of cellular processes. Hence the potential interaction of hnRNP A2 with actin and gelsolin seems meaningful. If hnRNP A2-mediated transport of MBP RNA and its localised translation correlates to the extension of the glial processes, actin fibres are likely to be involved. Interestingly, gelsolin is involved in depolymerisation and nucleation of actin fibres (McGough et al., 2003). Hence, it could function in this regard either in breaking down the cortical actin network to allow the formation and extension of the microtubule network found in larger oligodendrocyte processes, or it could lead to the formation of new actin fibres required to establish or elongate processes that subsequently ensheath the axon. Hence, hnRNP A2-mediated transport and the localised synthesis of myelin membrane proteins could coincide spatially and temporarily with the elaboration of cellular polarity required for early myelination events.

5.7 Neuron-glial signalling controls localised synthesis of myelin basic protein – a prerequisite for myelin deposition?

The findings of this study can be concluded to a novel signalling mechanism illustrated in figure 5.7-1.

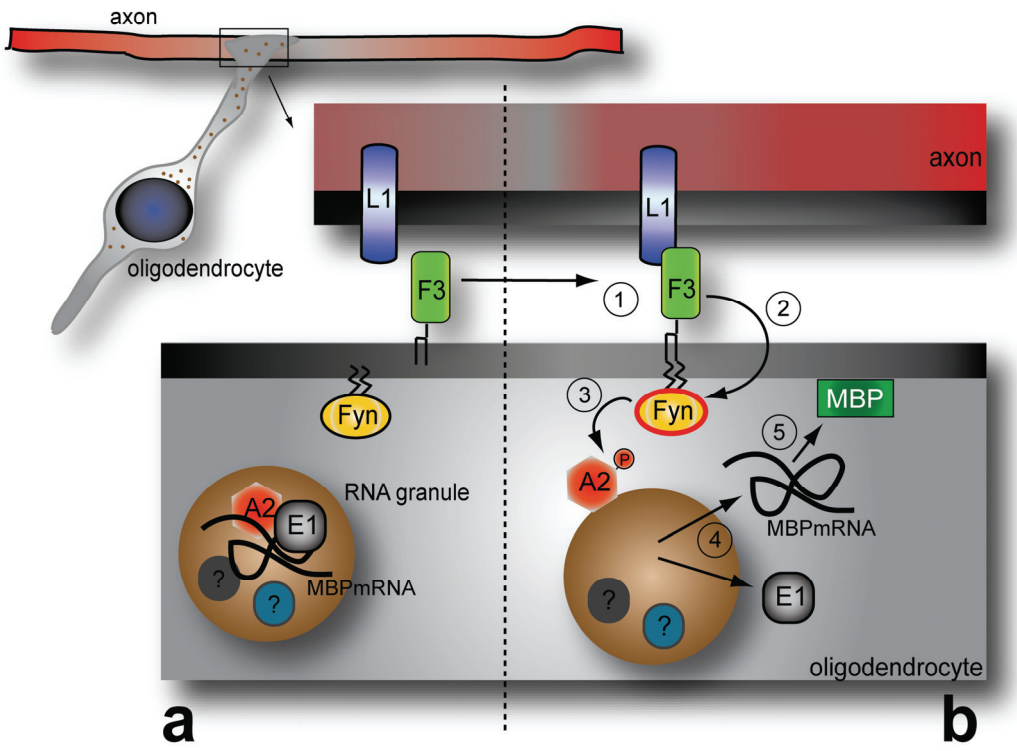

Figure 5.7-1: Model illustrating a novel axon glial signalling mechanism
See text for details

Before axon-glial contact (figure 5.7-1a), MBP mRNA is present in RNA transport granules in a translationally repressed state. Transport is mediated by hnRNP A2 and translational repression is achieved by hnRNP E1 which is recruited to hnRNP A2. Binding of neuronal L1 to oligodendroglial F3 (figure 5.7-1b 1) leads to an activation of glial Fyn kinase (2) resulting in phosphorylation of hnRNP A2 in granules (3) and subsequently a release of hnRNP E1 from the granules (4). Thereby MBP mRNA is released from the granule and can be translated at the site of axon-glial contact (5). Synthesised MBP proteins can associate with the membrane evoking a localised formation of the myelin sheath.

Furthermore, the cell regulates transport of the granules to the contact site. In oligodendrocytes, MBP RNA granules are transported to the periphery of the cell on microtubules by the action of the motor protein kinesin (Carson et al., 1997). It was proposed that pH microdomains regulate the velocity of granular transport because kinesin binding to microtubules is pH dependent (Carson and Barbarese, 2005; Verhey et al., 1998). Acidic and alkaline microdomains of approximately 1µm in diameter have been demonstrated along oligodendroglial processes (Ro and Carson, 2004). These microdomains are established by the conversion of H_2O and CO_2 into H^+ and HCO_3^- catalysed by the enzyme carbonic anhydrase II (CAII) combined with the association of H^+ or HCO_3^- membrane transporters which remove either H^+ or HCO_3^- from the cell. The removal of H^+ or HCO_3^- results in the formation of alkaline or acidic microdomains. As kinesin binding to microtubules is stronger at low pH, it was postulated, that RNA granules are slowed down in acidic microdomains (Carson and Barbarese, 2005). This hypothesis, however, does not explain how microtubules are specifically targeted to distinct regions of oligodendrocytes ensuring kinesin-mediated transport of RNA granules specifically to these areas. It has been shown that activated Fyn binds to the microtubule associated protein Tau and to tubulin directly (Klein et al., 2002). It has been hypothesised that active Fyn leads to a recruitment of the microtubule cytoskeleton. If these suggestions are taken into consideration with the findings of this study, it is plausible that neuronal L1 activates glial Fyn and that this activity recruits microtubules and thereby directs RNA granules towards this distinct region of the cell. L1 binding would hence not only lead to the initiation of local translation of MBP RNA from transport granules, it would additionally recruit MBP mRNA to the axon glial contact point. This would embody a neuronal signal for localised recruitment and translation of MBP mRNA. This could be an important mechanism for the generation of the myelin sheath, since the axon glial contact point must govern the synthesis of large quantities of myelin protein required for

the elaboration of copious amounts of oligodendroglial membrane. Neuronal expression of L1 would thus not only regulate the spatial organisation of myelinogenesis, but also control the timing of myelin formation.

As illustrated in figure 5.7-1, L1 binding to F3 and the resulting signalling steps may be one of the mechanisms controlling initial myelination events. If L1 plays a role in determining the axonal region that is to be myelinated by the oligodendrocyte, the question arises as to how L1 expression is regulated. Does L1 expression by neurons correlate with early myelination events? In the PNS, neuronal impulses control L1 expression and myelination (Stevens et al., 1998). It could be shown in co-cultures of DRG neurons and Schwann cells that application of low frequency stimulation results in downregulation of L1 and decreased myelination, whereas high frequency stimulation had no effect. *In vivo* DRG neurons develop spontaneous impulse activities even before synaptic connections have been established. The frequency of this impulse activity is slow in the beginning and increases with later stages of development. Furthermore, Schwann cells are closely associated with DRG axons when they are firing action potentials at low frequency, but do not start myelinating axons before later stages in development when the frequency of action potential is increased (Stevens et al., 1998). It has been proposed for the PNS that homophilic L1 binding in trans between axons and Schwann cells is required for myelination initiation (Seilheimer et al., 1989; Wood et al., 1990). Hence, there is a correlation between L1 expression mediated by electrical activity in the axon and myelination in the PNS. It may be that early myelination events in the CNS are similarly controlled. It is generally accepted, that L1 is not expressed by oligodendrocytes, and Schwann cells do not express F3 (Einheber et al., 1997). So perhaps the L1-F3 interaction in the CNS resembles an L1-L1 interaction in the PNS. Hence L1 expression may also be dependent on electrical activity in CNS neurons, but here myelination initiation is controlled by oligodendroglial F3.

The expression of L1 protein in the brain and potential consequences for myelination could also be regulated in alternative ways. The extracellular domain of L1 has been shown to be cleaved either in the third fibronectin type III domain by serine proteases like plasmin, or close to the membrane by a disintegrin and metalloproteinase (ADAM) 10 (Mechtersheimer et al., 2001). The cleavage products have been detected in the supernatants of carcinoma cell lines and the developing brain. Cleavage of the extracellular domain of L1 could inhibit binding of axonal L1 to binding partners such as oligodendrocyte F3. Alternatively, the binding effects in experiments with soluble L1-Fc presented in section 4 could also mimic the binding of a cleaved L1 product in

the developing brain. Hence, soluble rather than axon associated L1 could bind to F3 and lead to Fyn activation. However, ADAM 10 cleaved extracellular L1 fragments have been shown to become integrated in the extracellular matrix (Gutwein et al., 2000) and no association of soluble L1 with oligodendrocytes has been reported so far. L1 shedding has been associated with modulation of cellular migration (Mechtersheimer et al., 2001). Concerning the putative role in the initiation of myelination processes, it would be difficult to imagine how an unlocalised shedding of L1 could determine local specifications of axonal regions to be myelinated. It can be expected that many and redundant signalling pathways govern the generation of this complex structure. This is exemplified by the L1 knock out mouse which is lacking an obvious myelination defect phenotype (Dahme et al., 1997). Possibly compensatory factors rescue myelination in these mice or the revelation of myelination defects requires more detailed analysis.

As mentioned above, L1 is intimately regulating myelination in the PNS. Recently, however, the interaction of axonal neuregulin1 (NRG1) type III with Schwann cell ErbB2/ ErbB3 has been shown to control the formation of myelin *per se* and, furthermore, the regulation of myelin sheath thickness in response to axonal diameter (Michailov et al., 2004; Nave and Salzer, 2006). These findings in the PNS thus demonstrate that as to be expected myelination is regulated by a palette of interacting molecules.

It is very likely that CNS myelinogenesis also requires the involvement of multiple pathways that are involved in the activation of Fyn kinase: a central player in CNS myelination.

5.8 Outlook

The findings of this study are mainly based on *in vitro* data. Many of the results, however, can be applied to already existing *in vivo* data such as the analysis of fyn and MBP deficient mice. The particular difficulty in this respect, however, is the fact that many of the involved proteins are expressed not only by oligodendrocytes, but also by neurons and other cell types in the CNS. The generation of conditional knock out mice that lack Fyn, hnRNP A2 or F3 only in oligodendrocytes would be helpful to further substantiate the obtained results. As this will require a considerable amount of time and effort, more physiological *in vitro* conditions are required to analyse L1-mediated signalling in oligodendrocytes. This could be achieved by a neuron-oligodendrocyte co-culture system. Initial experiments were performed to establish such a system.

Neurons derived from total embryonic (E14-E16) murine spinal cords were combined with oligodendrocytes derived from embryonic mouse brains (figure 5.8-1).

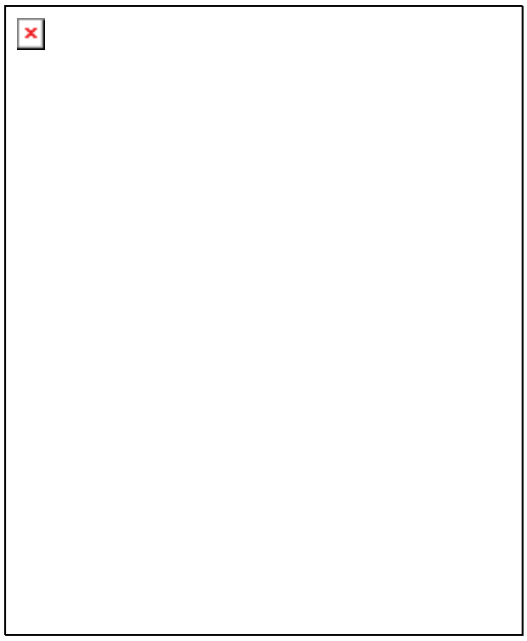

Figure 5.8-1: Co-culture of neurons and oligodendrocytes

Neurons derived from total embryonic spinal cords were co-cultured with oligodendrocytes originating from embryonic brains. Cell were fixed and immunostained with L1 (green) and O4 (sulfatide, red) antibodies to distinguish neuronal axons from oligodendrocyte processes, respectively. As indicated by arrows, oligodendrocyte processes intimately contact an axon.

Within this system, oligodendrocytes or neurons could be manipulated by over-expression of mutant fyn constructs or F3 or Fyn siRNA - tools that were established in the course of this study. It would be worthwhile to systematically knock down protein levels of L1, F3 and other components of the proposed signalling cascade to see if this resulted in differences in the number of contacts or the amount of activated Fyn (seen in immunostainings using the antibody recognising active fyn). Appropriate read out systems need to be established that may depict effects of the suggested manipulations. These could include the quantification of contact areas by staining methods or more subtle approaches like the analysis of redistribution of Na^+ channels or phosphorylation of axonal neurofilament proteins which are among the first incidences during myelination in response to oligodendrocyte contact. Moreover, local MBP protein synthesis could possibly be shown using this system and an effect of L1 or F3 reduction could be analysed.

Concerning the Fyn and hnRNP A2 interaction it would be interesting to identify the phosphorylated tyrosine residue by mutational analysis or mass spectrometry. This tyrosine residue could then be mutated to a phenylalanine or

aspartate, mimicking the unphosphorylated or phosphorylated form of hnRNP A2, respectively, and could be introduced into cells to study the effect of hnRNP A2 phosphorylation in detail.

The found Fyn targets and hnRNP A2 associated proteins need to be evaluated in more detail. Especially DDX5 and its potential role in translation initiation of granule transported mRNAs could possibly present a novel regulatory mechanism to control the synthesis of myelin proteins in oligodendrocytes. HnRNP F has already been analysed in more detail but its cytoplasmic function is yet to be characterised.

These and further experiments will contribute to the understanding of the complex molecular interactions during early phases of myelination in the central nervous system.

6 SUMMARY

During central nervous system myelination, oligodendrocytes extend membrane processes towards an axonal contact site which is followed by ensheathment resulting in a compacted multilamellar myelin sheath. The formation of this axon-glial unit facilitates rapid saltatory propagation of action potentials along the axon and requires the synthesis and transport of copious amounts of lipids and proteins to the axon-glial contact site. Fyn is a member of the Src family of non receptor tyrosine kinases and inserted into the inner leaflet of the oligodendrocyte membrane by acylation. Fyn activity plays a pivotal role in the maturation of oligodendrocytes and the myelination process. It was suggested previously that Fyn kinase can be stimulated by binding of a neuronal ligand to oligodendroglial F3/ contactin, a glycosyl-phosphatidyl-inositol anchored immunoglobulin superfamily (IgSF) member protein.

It could be shown here, that neuronal cell adhesion molecule L1 binds to oligodendrocytes in an F3-dependent manner and activates glial Fyn. In the search for downstream participants of this novel axon-glial signalling cascade, heterogeneous nuclear ribonucleoprotein (hnRNP) A2 was identified as a novel Fyn target in oligodendrocytes. HnRNP A2 was known to be involved in the localisation of translationally repressed myelin basic protein (MBP) mRNA by binding to a *cis* acting A2 response element (A2RE) present in the 3' untranslated region. Transport of MBP mRNAs occurs in RNA-protein complexes termed RNA granules and translational repression during transport is achieved by hnRNP A2-mediated recruitment of hnRNP E1 to the granules. It could be shown here, that Fyn activity leads to enhanced translation of reporter mRNA containing a part of the 3' UTR of MBP including the A2RE. Furthermore hnRNP E1 seems to dissociate from RNA granules in response to Fyn activity and L1 binding.

These findings suggest a novel form of neuron- glial communication: Axonal L1 binding to oligodendroglial F3 activates Fyn kinase. Activated Fyn phosphorylates hnRNP A2 leading to removal of hnRNP E1 from RNA granules initiating the translation of MBP mRNA. MBP is the second most abundant myelin protein and mice lacking this protein show a severe hypomyelination phenotype. Moreover, the brains of Fyn knock out mice contain reduced MBP levels and are hypomyelinated. Hence, L1-mediated MBP synthesis via Fyn as a central molecule could be part of a regulatory mechanism required for myelinogenesis in the central nervous system.

7 REFERENCES

Abbott, N.J., L. Ronnback, and E. Hansson. 2006. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci*. 7:41-53.

Ainger, K., D. Avossa, A.S. Diana, C. Barry, E. Barbarese, and J.H. Carson. 1997. Transport and localization elements in myelin basic protein mRNA. *J Cell Biol*. 138:1077-87.

Ainger, K., D. Avossa, F. Morgan, S.J. Hill, C. Barry, E. Barbarese, and J.H. Carson. 1993. Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. *J Cell Biol*. 123:431-41.

Barbin, G., M.S. Aigrot, P. Charles, A. Foucher, M. Grumet, M. Schachner, B. Zalc and C. Lubetzki. 2004. Axonal cell-adhesion molecule L1 in CNS myelination. *Neuron Glia Biology.* 1: 65-72

Barres, B.A., M.D. Jacobson, R. Schmid, M. Sendtner, and M.C. Raff. 1993. Does oligodendrocyte survival depend on axons? *Curr Biol*. 3:489-97.

Baumann, N., and D. Pham-Dinh. 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev*. 81:871-927.

Bennett, M.V., J.E. Contreras, F.F. Bukauskas, and J.C. Saez. 2003. New roles for astrocytes: gap junction hemichannels have something to communicate. *Trends Neurosci*. 26:610-7.

Benninger, Y., H. Colognato, T. Thurnherr, R.J. Franklin, D.P. Leone, S. Atanasoski, K.A. Nave, C. Ffrench-Constant, U. Suter, and J.B. Relvas. 2006. Beta1-integrin signaling mediates premyelinating oligodendrocyte survival but is not required for CNS myelination and remyelination. *J Neurosci*. 26:7665-73.

Bhat, M.A. 2003. Molecular organization of axo-glial junctions. *Curr Opin Neurobiol*. 13:552-9.

Bjartmar, C., C. Hildebrand, and K. Loinder. 1994. Morphological heterogeneity of rat oligodendrocytes: electron microscopic studies on serial sections. *Glia*. 11:235-44.

Boggs, J.M. 2006. Myelin basic protein: a multifunctional protein. *Cell Mol Life Sci*. 63:1945-61.

Boyle, M.E., E.O. Berglund, K.K. Murai, L. Weber, E. Peles, and B. Ranscht. 2001. Contactin orchestrates assembly of the septate-like junctions at the paranode in myelinated peripheral nerve. *Neuron*. 30:385-97.

Brummendorf, T., M. Hubert, U. Treubert, R. Leuschner, A. Tarnok, and F.G. Rathjen. 1993. The axonal recognition molecule F11 is a multifunctional protein: specific domains mediate interactions with Ng-CAM and restrictin. *Neuron*. 10:711-27.

Brummendorf, T., J.M. Wolff, R. Frank, and F.G. Rathjen. 1989. Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. *Neuron*. 2:1351-61.

Brumwell, C., C. Antolik, J.H. Carson, and E. Barbarese. 2002. Intracellular trafficking of hnRNP A2 in oligodendrocytes. *Exp Cell Res*. 279:310-20.

Butt, A.M., A. Duncan, M.F. Hornby, S.L. Kirvell, A. Hunter, J.M. Levine, and M. Berry. 1999. Cells expressing the NG2 antigen contact nodes of Ranvier in adult CNS white matter. *Glia*. 26:84-91.

Calvio, C., G. Neubauer, M. Mann, and A.I. Lamond. 1995. Identification of hnRNP P2 as TLS/FUS using electrospray mass spectrometry. *Rna*. 1:724-33.

Carre, J.L., B.D. Goetz, L.T. O'Connor, Q. Bremer, and I.D. Duncan. 2002. Mutations in the rat myelin basic protein gene are associated with specific alterations in other myelin gene expression. *Neurosci Lett*. 330:17-20.

Carson, J.H., and E. Barbarese. 2005. Systems analysis of RNA trafficking in neural cells. *Biol Cell*. 97:51-62.

Carson, J.H., N. Blondin, and G. Korza. 2006. Rules of engagement promote polarity in RNA trafficking. *BMC Neurosci*. 7 Suppl 1:S3.

Carson, J.H., H. Cui, and E. Barbarese. 2001a. The balance of power in RNA trafficking. *Curr Opin Neurobiol*. 11:558-63.

Carson, J.H., H. Cui, W. Krueger, B. Schlepchenko, C. Brumwell, and E. Barbarese. 2001b. RNA trafficking in oligodendrocytes. *Results Probl Cell Differ*. 34:69-81.

Carson, J.H., K. Worboys, K. Ainger, and E. Barbarese. 1997. Translocation of myelin basic protein mRNA in oligodendrocytes requires microtubules and kinesin. *Cell Motil Cytoskeleton*. 38:318-28.

Charles, P., S. Tait, C. Faivre-Sarrailh, G. Barbin, F. Gunn-Moore, N. Denisenko-Nehrbass, A.M. Guennoc, J.A. Girault, P.J. Brophy, and C. Lubetzki. 2002. Neurofascin is a glial receptor for the paranodin/Caspr-contactin axonal complex at the axoglial junction. *Curr Biol*. 12:217-20.

Chong, Y.P., K.K. Ia, T.D. Mulhern, and H.C. Cheng. 2005. Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. *Biochim Biophys Acta*. 1754:210-20.

Chong, Y.P., T.D. Mulhern, H.J. Zhu, D.J. Fujita, J.D. Bjorge, J.P. Tantiongco, N. Sotirellis, D.S. Lio, G. Scholz, and H.C. Cheng. 2004. A novel non-catalytic mechanism employed by the C-terminal Src-homologous kinase to inhibit Src-family kinase activity. *J Biol Chem*. 279:20752-66.

Chuang, R.Y., P.L. Weaver, Z. Liu, and T.H. Chang. 1997. Requirement of the DEAD-Box protein ded1p for messenger RNA translation. *Science*. 275:1468-71.

Colognato, H., S. Ramachandrappa, I.M. Olsen, and C. ffrench-Constant. 2004. Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. *J Cell Biol*. 167:365-75.

Coman, I., G. Barbin, P. Charles, B. Zalc, and C. Lubetzki. 2005. Axonal signals in central nervous system myelination, demyelination and remyelination. *J Neurol Sci*. 233:67-71.

Czaplinski, K., and R.H. Singer. 2006. Pathways for mRNA localization in the cytoplasm. *Trends Biochem Sci*. 31:687-93.

Dahme, M., U. Bartsch, R. Martini, B. Anliker, M. Schachner, and N. Mantei. 1997. Disruption of the mouse L1 gene leads to malformations of the nervous system. *Nat Genet*. 17:346-9.

De Benedictis, L., A. Bizzoca, P. Corsi, I. Albieri, G.G. Consalez, and G. Gennarini. 2006. Activation profile of the F3/Contactin gene in the developing mouse cerebellum. *Mol Cell Neurosci*. 32:403-18.

Debruin, L.S., and G. Harauz. 2007. White matter rafting--membrane microdomains in myelin. *Neurochem Res*. 32:213-28.

Defilippi, P., P. Di Stefano, and S. Cabodi. 2006. p130Cas: a versatile scaffold in signaling networks. *Trends Cell Biol*. 16:257-63.

Dreyfuss, G., V.N. Kim, and N. Kataoka. 2002. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol*. 3:195-205.

Einheber, S., G. Zanazzi, W. Ching, S. Scherer, T.A. Milner, E. Peles, and J.L. Salzer. 1997. The axonal membrane protein Caspr, a homologue of neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. *J Cell Biol*. 139:1495-506.

Elvira, G., S. Wasiak, V. Blandford, X.K. Tong, A. Serrano, X. Fan, M. del Rayo Sanchez-Carbente, F. Servant, A.W. Bell, D. Boismenu, J.C. Lacaille, P.S. McPherson, L. DesGroseillers, and W.S. Sossin. 2006. Characterization of an RNA granule from developing brain. *Mol Cell Proteomics*. 5:635-51.

ffrench-Constant, C., and H. Colognato. 2004. Integrins: versatile integrators of extracellular signals. *Trends Cell Biol*. 14:678-86.

Fransen, E., R. D'Hooge, G. Van Camp, M. Verhoye, J. Sijbers, E. Reyniers, P. Soriano, H. Kamiguchi, R. Willemsen, S.K. Koekkoek, C.I. De Zeeuw, P.P. De Deyn, A. Van der Linden, V. Lemmon, R.F. Kooy, and P.J. Willems. 1998. L1 knockout mice show dilated ventricles, vermis hypoplasia and impaired exploration patterns. *Hum Mol Genet*. 7:999-1009.

Gennarini, G., G. Cibelli, G. Rougon, M.G. Mattei, and C. Goridis. 1989. The mouse neuronal cell surface protein F3: a phosphatidylinositol-anchored member of the immunoglobulin superfamily related to chicken contactin. *J Cell Biol*. 109:775-88.

Grumet, M., V. Mauro, M.P. Burgoon, G.M. Edelman, and B.A. Cunningham. 1991. Structure of a new nervous system glycoprotein, Nr-CAM, and its relationship to subgroups of neural cell adhesion molecules. *J Cell Biol*. 113:1399-412.

Gutwein, P., M. Oleszewski, S. Mechtersheimer, N. Agmon-Levin, K. Krauss, and P. Altevogt. 2000. Role of Src kinases in the ADAM-mediated release of L1 adhesion molecule from human tumor cells. *J Biol Chem*. 275:15490-7.

Hakak, Y., and G.S. Martin. 1999. Ubiquitin-dependent degradation of active Src. *Curr Biol*. 9:1039-42.

Hannon, G.J. 2002. RNA interference. *Nature*. 418:244-51.

Hartline, D.K., and D.R. Colman. 2007. Rapid conduction and the evolution of giant axons and myelinated fibers. *Curr Biol*. 17:R29-35.

Hatfield, J.T., J.A. Rothnagel, and R. Smith. 2002. Characterization of the mouse hnRNP A2/B1/B0 gene and identification of processed pseudogenes. *Gene*. 295:33-42.

Honore, B., U. Baandrup, and H. Vorum. 2004. Heterogeneous nuclear ribonucleoproteins F and H/H' show differential expression in normal and selected cancer tissues. *Exp Cell Res*. 294:199-209.

Hortsch, M. 1996. The L1 family of neural cell adhesion molecules: old proteins performing new tricks. *Neuron*. 17:587-93.

Hu, Q.D., B.T. Ang, M. Karsak, W.P. Hu, X.Y. Cui, T. Duka, Y. Takeda, W. Chia, N. Sankar, Y.K. Ng, E.A. Ling, T. Maciag, D. Small, R. Trifonova, R. Kopan, H. Okano, M. Nakafuku, S. Chiba, H. Hirai, J.C. Aster, M. Schachner, C.J. Pallen, K. Watanabe, and Z.C. Xiao. 2003. F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. *Cell*. 115:163-75.

Huang, J., R. Sakai, and T. Furuichi. 2006. The docking protein Cas links tyrosine phosphorylation signaling to elongation of cerebellar granule cell axons. *Mol Biol Cell*. 17:3187-96.

Huttelmaier, S., D. Zenklusen, M. Lederer, J. Dictenberg, M. Lorenz, X. Meng, G.J. Bassell, J. Condeelis, and R.H. Singer. 2005. Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature*. 438:512-5.

Ishibashi, T., K.A. Dakin, B. Stevens, P.R. Lee, S.V. Kozlov, C.L. Stewart, and R.D. Fields. 2006. Astrocytes promote myelination in response to electrical impulses. *Neuron*. 49:823-32.

Itoh, K., L. Cheng, Y. Kamei, S. Fushiki, H. Kamiguchi, P. Gutwein, A. Stoeck, B. Arnold, P. Altevogt, and V. Lemmon. 2004. Brain development in mice lacking L1-L1 homophilic adhesion. *J Cell Biol*. 165:145-54.

Itoh, K., Y. Sakurai, H. Asou, and M. Umeda. 2000. Differential expression of alternatively spliced neural cell adhesion molecule L1 isoforms during oligodendrocyte maturation. *J Neurosci Res*. 60:579-86.

Jung, M., E. Kramer, M. Grzenkowski, K. Tang, W. Blakemore, A. Aguzzi, K. Khazaie, K. Chlichlia, G. von Blankenfeld, H. Kettenmann, and et al. 1995. Lines of murine oligodendroglial precursor cells immortalized by an activated neu tyrosine kinase show distinct degrees of interaction with axons in vitro and in vivo. *Eur J Neurosci*. 7:1245-65.

Jung, M., E.M. Kramer, T. Muller, H. Antonicek, and J. Trotter. 1998. Novel pluripotential neural progenitor lines exhibiting rapid controlled differentiation to neurotransmitter receptor-expressing neurons and glia. *Eur J Neurosci*. 10:3246-56.

Kalifa, Y., T. Huang, L.N. Rosen, S. Chatterjee, and E.R. Gavis. 2006. Glorund, a Drosophila hnRNP F/H homolog, is an ovarian repressor of nanos translation. *Dev Cell*. 10:291-301.

Kamiguchi, H., M.L. Hlavin, and V. Lemmon. 1998. Role of L1 in neural development: what the knockouts tell us. *Mol Cell Neurosci*. 12:48-55.

Kamma, H., M. Fujimoto, M. Fujiwara, M. Matsui, H. Horiguchi, M. Hamasaki, and H. Satoh. 2001. Interaction of hnRNP A2/B1 isoforms with telomeric ssDNA and the in vitro function. *Biochem Biophys Res Commun*. 280:625-30.

Kanai, Y., N. Dohmae, and N. Hirokawa. 2004. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. *Neuron*. 43:513-25.

Karram, K. 2006. Generation of a NG2-EYFP mouse for studying the properties of NG2-expressing cells**.** PhD Thesis, University of Mainz.

Karram, K., N. Chatterjee, and J. Trotter. 2005. NG2-expressing cells in the nervous system: role of the proteoglycan in migration and glial-neuron interaction. *J Anat*. 207:735-44.

Kenwrick, S., A. Watkins, and E. De Angelis. 2000. Neural cell recognition molecule L1: relating biological complexity to human disease mutations. *Hum Mol Genet*. 9:879-86.

Kim, Y.S., and T.H. Joh. 2006. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. *Exp Mol Med*. 38:333-47.

Kippert, A., K. Trajkovic, L. Rajendran, J. Ries, and M. Simons. 2007. Rho regulates membrane transport in the endocytic pathway to control plasma membrane specialization in oligodendroglial cells. *J Neurosci*. 27:3560-70.

Kishi, Y., C. Clements, D.C. Mahadeo, D.A. Cotter, and M. Sameshima. 1998. High levels of actin tyrosine phosphorylation: correlation with the dormant state of Dictyostelium spores. *J Cell Sci*. 111 (Pt 19):2923-32.

Klein, C., E.M. Kramer, A.M. Cardine, B. Schraven, R. Brandt, and J. Trotter. 2002. Process outgrowth of oligodendrocytes is promoted by interaction of fyn kinase with the cytoskeletal protein tau. *J Neurosci*. 22:698-707.

Koch, T., T. Brugger, A. Bach, G. Gennarini, and J. Trotter. 1997. Expression of the immunoglobulin superfamily cell adhesion molecule F3 by oligodendrocyte-lineage cells. *Glia*. 19:199-212.

Kosturko, L.D., M.J. Maggipinto, C. D'Sa, J.H. Carson, and E. Barbarese. 2005. The microtubule-associated protein tumor overexpressed gene binds to the RNA trafficking protein heterogeneous nuclear ribonucleoprotein A2. *Mol Biol Cell*. 16:1938-47.

Kosturko, L.D., M.J. Maggipinto, G. Korza, J.W. Lee, J.H. Carson, and E. Barbarese. 2006. Heterogeneous nuclear ribonucleoprotein (hnRNP) E1 binds to hnRNP A2 and inhibits translation of A2 response element mRNAs. *Mol Biol Cell*. 17:3521-33.

Kramer, E.M., C. Klein, T. Koch, M. Boytinck, and J. Trotter. 1999. Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. *J Biol Chem*. 274:29042-9.

Kramer, E.M., T. Koch, A. Niehaus, and J. Trotter. 1997. Oligodendrocytes direct glycosyl phosphatidylinositol-anchored proteins to the myelin sheath in glycosphingolipid-rich complexes. *J Biol Chem*. 272:8937-45.

Kwon, S., E. Barbarese, and J.H. Carson. 1999. The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation. *J Cell Biol*. 147:247-56.

Lackie, J.M. and J.A.T Dow. 1999. The dictionary of Cell & Molecular Biology. Academic Press, San Diego, London.

Lazzarini, R.A. 2004. Myelin Biology and Disorders. Elsevier Academic Press, San Diego, London.

Liang, X., Y. Lu, M. Wilkes, T.A. Neubert, and M.D. Resh. 2004. The N-terminal SH4 region of the Src family kinase Fyn is modified by methylation and heterogeneous fatty acylation: role in membrane targeting, cell adhesion, and spreading. *J Biol Chem*. 279:8133-9.

Liu, X., S. Shu, M.S. Hong, R.L. Levine, and E.D. Korn. 2006. Phosphorylation of actin Tyr-53 inhibits filament nucleation and elongation and destabilizes filaments. *Proc Natl Acad Sci U S A*. 103:13694-9.

Lu, Z., L. Ku, Y. Chen, and Y. Feng. 2005. Developmental abnormalities of myelin basic protein expression in fyn knock-out brain reveal a role of Fyn in posttranscriptional regulation. *J Biol Chem*. 280:389-95.

Maggipinto, M., C. Rabiner, G.J. Kidd, A.J. Hawkins, R. Smith, and E. Barbarese. 2004. Increased expression of the MBP mRNA binding protein HnRNP A2 during oligodendrocyte differentiation. *J Neurosci Res*. 75:614-23.

Malhotra, J.D., P. Tsiotra, D. Karagogeos, and M. Hortsch. 1998. Cis-activation of L1-mediated ankyrin recruitment by TAG-1 homophilic cell adhesion. *J Biol Chem*. 273:33354-9.

Manie, S.N., A. Astier, N. Haghayeghi, T. Canty, B.J. Druker, H. Hirai, and A.S. Freedman. 1997. Regulation of integrin-mediated p130(Cas) tyrosine phosphorylation in human B cells. A role for p59(Fyn) and SHP2. *J Biol Chem*. 272:15636-41.

Manning, G., D.B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam. 2002. The protein kinase complement of the human genome. *Science*. 298:1912-34.

Martin, G.S. 2001. The hunting of the Src. *Nat Rev Mol Cell Biol*. 2:467-75.

Matsui, M., H. Horiguchi, H. Kamma, M. Fujiwara, R. Ohtsubo, and T. Ogata. 2000. Testis- and developmental stage-specific expression of hnRNP A2/B1 splicing isoforms, B0a/b. *Biochim Biophys Acta*. 1493:33-40.

Matunis, M.J., J. Xing, and G. Dreyfuss. 1994. The hnRNP F protein: unique primary structure, nucleic acid-binding properties, and subcellular localization. *Nucleic Acids Res*. 22:1059-67.

Mayer, B.J. 2001. SH3 domains: complexity in moderation. *J Cell Sci*. 114:1253-63.

McGough, A.M., C.J. Staiger, J.K. Min, and K.D. Simonetti. 2003. The gelsolin family of actin regulatory proteins: modular structures, versatile functions. *FEBS Lett*. 552:75-81.

McKay, S.J., and H. Cooke. 1992. hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat TTAGGGn. *Nucleic Acids Res*. 20:6461-4.

Mechtersheimer, S., P. Gutwein, N. Agmon-Levin, A. Stoeck, M. Oleszewski, S. Riedle, R. Postina, F. Fahrenholz, M. Fogel, V. Lemmon, and P. Altevogt. 2001. Ectodomain shedding of L1 adhesion molecule promotes cell migration by autocrine binding to integrins. *J Cell Biol*. 155:661-73.

Michailov, G.V., M.W. Sereda, B.G. Brinkmann, T.M. Fischer, B. Haug, C. Birchmeier, L. Role, C. Lai, M.H. Schwab, and K.A. Nave. 2004. Axonal neuregulin-1 regulates myelin sheath thickness. *Science*. 304:700-3.

Min, H., R.C. Chan, and D.L. Black. 1995. The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event. *Genes Dev*. 9:2659-71.

Montague, P., A.S. McCallion, R.W. Davies, and I.R. Griffiths. 2006. Myelin-associated oligodendrocytic basic protein: a family of abundant CNS myelin proteins in search of a function. *Dev Neurosci*. 28:479-87.

Morell, P. 1984. Myelin, 2nd edition. Plenum Press, New York

Mouland, A.J., H. Xu, H. Cui, W. Krueger, T.P. Munro, M. Prasol, J. Mercier, D. Rekosh, R. Smith, E. Barbarese, E.A. Cohen, and J.H. Carson. 2001. RNA trafficking signals in human immunodeficiency virus type 1. *Mol Cell Biol*. 21:2133-43.

Munakata, H., Y. Nakamura, K. Matsumoto-Miyai, K. Itoh, H. Yamasaki, and S. Shiosaka. 2003. Distribution and densitometry mapping of L1-CAM immunoreactivity in the adult mouse brain--light microscopic observation. *BMC Neurosci*. 4:7.

Munro, T.P., R.J. Magee, G.J. Kidd, J.H. Carson, E. Barbarese, L.M. Smith, and R. Smith. 1999. Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking. *J Biol Chem*. 274:34389-95.

Nave, K.A., and J.L. Salzer. 2006. Axonal regulation of myelination by neuregulin 1. *Curr Opin Neurobiol*. 16:492-500.

Okada, M., and H. Nakagawa. 1989. A protein tyrosine kinase involved in regulation of pp60c-src function. *J Biol Chem*. 264:20886-93.

Oleszewski, M., S. Beer, S. Katich, C. Geiger, Y. Zeller, U. Rauch, and P. Altevogt. 1999. Integrin and neurocan binding to L1 involves distinct Ig domains. *J Biol Chem*. 274:24602-10.

Olive, S., C. Dubois, M. Schachner, and G. Rougon. 1995. The F3 neuronal glycosylphosphatidylinositol-linked molecule is localized to glycolipid-enriched membrane subdomains and interacts with L1 and fyn kinase in cerebellum. *J Neurochem*. 65:2307-17.

Ostareck-Lederer, A., D.H. Ostareck, C. Cans, G. Neubauer, K. Bomsztyk, G. Superti-Furga, and M.W. Hentze. 2002. c-Src-mediated phosphorylation of hnRNP K drives translational activation of specifically silenced mRNAs. *Mol Cell Biol*. 22:4535-43.

Osterhout, D.J., A. Wolven, R.M. Wolf, M.D. Resh, and M.V. Chao. 1999. Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. *J Cell Biol*. 145:1209-18.

Peles, E., and J.L. Salzer. 2000. Molecular domains of myelinated axons. *Curr Opin Neurobiol*. 10:558-65.

Perrotti, D., and B. Calabretta. 2002. Post-transcriptional mechanisms in BCR/ABL leukemogenesis: role of shuttling RNA-binding proteins. *Oncogene*. 21:8577-83.

Perry, V.H. 1998. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. *J Neuroimmunol*. 90:113-21.

Pfeiffer, S.E., A.E. Warrington, and R. Bansal. 1993. The oligodendrocyte and its many cellular processes. *Trends Cell Biol*. 3:191-7.

Resh, M.D. 1998. Fyn, a Src family tyrosine kinase. *Int J Biochem Cell Biol*. 30:1159-62.

Richardson, W.D., N. Kessaris, and N. Pringle. 2006. Oligodendrocyte wars. *Nat Rev Neurosci*. 7:11-8.

Ro, H.A., and J.H. Carson. 2004. pH microdomains in oligodendrocytes. *J Biol Chem*. 279:37115-23.

Rocak, S., and P. Linder. 2004. DEAD-box proteins: the driving forces behind RNA metabolism. *Nat Rev Mol Cell Biol*. 5:232-41.

Roskoski, R., Jr. 2004. Src protein-tyrosine kinase structure and regulation. *Biochem Biophys Res Commun*. 324:1155-64.

Roskoski, R., Jr. 2005. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun*. 331:1-14.

Scherr, M., and M. Eder. 2007. Gene silencing by small regulatory RNAs in mammalian cells. *Cell Cycle*. 6:444-9.

Seilheimer, B., E. Persohn, and M. Schachner. 1989. Neural cell adhesion molecule expression is regulated by Schwann cell-neuron interactions in culture. *J Cell Biol*. 108:1909-15.

Sette, C., M.P. Paronetto, M. Barchi, A. Bevilacqua, R. Geremia, and P. Rossi. 2002. Tr-kit-induced resumption of the cell cycle in mouse eggs requires activation of a Src-like kinase. *Embo J*. 21:5386-95.

Shan, J., T.P. Munro, E. Barbarese, J.H. Carson, and R. Smith. 2003. A molecular mechanism for mRNA trafficking in neuronal dendrites. *J Neurosci*. 23:8859-66.

Shav-Tal, Y., and R.H. Singer. 2005. RNA localization. *J Cell Sci*. 118:4077-81.

Sherwood, C.C., C.D. Stimpson, M.A. Raghanti, D.E. Wildman, M. Uddin, L.I. Grossman, M. Goodman, J.C. Redmond, C.J. Bonar, J.M. Erwin, and P.R. Hof.

2006. Evolution of increased glia-neuron ratios in the human frontal cortex. *Proc Natl Acad Sci U S A*. 103:13606-11.

Shier, D, J. Butler, R. Lewis. 1999. Human Anatomy and Physiology, 8th edition. McGraw-Hill.

Simons, M., and K. Trajkovic. 2006. Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. *J Cell Sci*. 119:4381-9.

Sperber, B.R., E.A. Boyle-Walsh, M.J. Engleka, P. Gadue, A.C. Peterson, P.L. Stein, S.S. Scherer, and F.A. McMorris. 2001. A unique role for Fyn in CNS myelination. *J Neurosci*. 21:2039-47.

Staugaitis, S.M., P.R. Smith, and D.R. Colman. 1990. Expression of myelin basic protein isoforms in nonglial cells. *J Cell Biol*. 110:1719-27.

Stevens, B., S. Tanner, and R.D. Fields. 1998. Control of myelination by specific patterns of neural impulses. *J Neurosci*. 18:9303-11.

Sueoka, E., M. Yukitake, K. Iwanaga, N. Sueoka, T. Aihara, and Y. Kuroda. 2004. Autoantibodies against heterogeneous nuclear ribonucleoprotein B1 in CSF of MS patients. *Ann Neurol*. 56:778-86.

Tamada, M., M.P. Sheetz, and Y. Sawada. 2004. Activation of a signaling cascade by cytoskeleton stretch. *Dev Cell*. 7:709-18.

Tellam, R., and C. Frieden. 1982. Cytochalasin D and platelet gelsolin accelerate actin polymer formation. A model for regulation of the extent of actin polymer formation in vivo. *Biochemistry*. 21:3207-14.

Thomas, M.G., L.J. Martinez Tosar, M. Loschi, J.M. Pasquini, J. Correale, S. Kindler, and G.L. Boccaccio. 2005. Staufen recruitment into stress granules does not affect early mRNA transport in oligodendrocytes. *Mol Biol Cell*. 16:405-20.

Trotter, J. 2005. NG2-positive cells in CNS function and the pathological role of antibodies against NG2 in demyelinating diseases. *J Neurol Sci*. 233:37-42.

Trotter, J., D. Bitter-Suermann, and M. Schachner. 1989. Differentiation-regulated loss of the polysialylated embryonic form and expression of the different polypeptides of the neural cell adhesion molecule by cultured oligodendrocytes and myelin. *J Neurosci Res*. 22:369-83.

Umemori, H., Y. Kadowaki, K. Hirosawa, Y. Yoshida, K. Hironaka, H. Okano, and T. Yamamoto. 1999. Stimulation of myelin basic protein gene transcription by Fyn tyrosine kinase for myelination. *J Neurosci*. 19:1393-7.

Umemori, H., S. Sato, T. Yagi, S. Aizawa, and T. Yamamoto. 1994. Initial events of myelination involve Fyn tyrosine kinase signalling. *Nature*. 367:572-6.

Valencia-Sanchez, M.A., J. Liu, G.J. Hannon, and R. Parker. 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev*. 20:515-24.

Veraldi, K.L., G.K. Arhin, K. Martincic, L.H. Chung-Ganster, J. Wilusz, and C. Milcarek. 2001. hnRNP F influences binding of a 64-kilodalton subunit of cleavage stimulation factor to mRNA precursors in mouse B cells. *Mol Cell Biol*. 21:1228-38.

Verhey, K.J., D.L. Lizotte, T. Abramson, L. Barenboim, B.J. Schnapp, and T.A. Rapoport. 1998. Light chain-dependent regulation of Kinesin's interaction with microtubules. *J Cell Biol*. 143:1053-66.

Waxman, S.G., and M.V. Bennett. 1972. Relative conduction velocities of small myelinated and non-myelinated fibres in the central nervous system. *Nat New Biol*. 238:217-9.

Whittard, J.D., T. Sakurai, M.R. Cassella, M. Gazdoiu, and D.P. Felsenfeld. 2006. MAP kinase pathway-dependent phosphorylation of the L1-CAM ankyrin binding site regulates neuronal growth. *Mol Biol Cell*. 17:2696-706.

Wickens, M., and A. Goldstrohm. 2003. Molecular biology. A place to die, a place to sleep. *Science*. 300:753-5.

Wolf, R.M., J.J. Wilkes, M.V. Chao, and M.D. Resh. 2001. Tyrosine phosphorylation of p190 RhoGAP by Fyn regulates oligodendrocyte differentiation. *J Neurobiol*. 49:62-78.

Wolff, J.M., T. Brummendorf, and F.G. Rathjen. 1989. Neural cell recognition molecule F11: membrane interaction by covalently attached phosphatidylinositol. *Biochem Biophys Res Commun*. 161:931-8.

Wood, P.M., M. Schachner, and R.P. Bunge. 1990. Inhibition of Schwann cell myelination in vitro by antibody to the L1 adhesion molecule. *J Neurosci*. 10:3635-45.

Yaffe, M.B. 2002. Phosphotyrosine-binding domains in signal transduction. *Nat Rev Mol Cell Biol*. 3:177-86.

Yokoo, H., S. Nobusawa, H. Takebayashi, K. Ikenaka, K. Isoda, M. Kamiya, A. Sasaki, J. Hirato, and Y. Nakazato. 2004. Anti-human Olig2 antibody as a useful immunohistochemical marker of normal oligodendrocytes and gliomas. *Am J Pathol*. 164:1717-25.

Zeng, L., L. D'Alessandri, M.B. Kalousek, L. Vaughan, and C.J. Pallen. 1999. Protein tyrosine phosphatase alpha (PTPalpha) and contactin form a novel neuronal receptor complex linked to the intracellular tyrosine kinase fyn. *J Cell Biol*. 147:707-14.

Zinszner, H., J. Sok, D. Immanuel, Y. Yin, and D. Ron. 1997. TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling. *J Cell Sci*. 110 (Pt 15):1741-50.

Zisch, A.H., L. D'Alessandri, K. Amrein, B. Ranscht, K.H. Winterhalter, and L. Vaughan. 1995. The glypiated neuronal cell adhesion molecule contactin/F11 complexes with src-family protein tyrosine kinase Fyn. *Mol Cell Neurosci*. 6:263-79.

8 Appendix

8.1 DNA sequences of generated plasmids

8.1.1 pFyn WT

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8.1.2 pFyn K299M (Fyn-)

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8.1.3 pFyn-Y528F (Fyn+)

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8.1.4 pLuciferase-MBP3'UTR+A2RE

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8.1.5 pLuciferase-MBP3'UTR-A2RE

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8.2 Mass Spec Data

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 61 KAGKEPGLQI WRVEKFDLVP VPTNLYGDFF TGDAYVILKT VQLRNGNLQY DLHYWLGNEC
121 SQDESGAAAI FTVQLDDYLN GRAVQHREVQ GFESATFLGY FKSGLKYKKG GVASGFKHVV
181 PNEVVVQRLF QVKGRRVVRA TEVPVSWESF NNGDCFILDL GNNIHQWCGS NSNRYERLKA
241 TQVSKGIRDN ERSGRARVHV SEEGTEPEAM LQVLGPKPAL PAGTEDTAKE DAANRKLAKL
301 YKVSNGAGTM SVSLVADENP FAQGALKSED CFILDHGKDG KIFVWKGKQA NTEERKAALK
361 TASDFITKMD YPKQTQVSVL PEGGETPLFK QFFKNWRDPD QTDGLGLSYL SSHIANVERV
421 PFDAATLHTS TAMAAQHGMD DDGTGQKQIW RIEGSNKVPV DPATYGQFYG GDSYIILYNY
481 RHGGRQGQII YNWQGAQSTQ DEVAASAILT AQLDEELGGT PVQSRVVQGK EPAHLMSLFG
541 GKPMIIYKGG TSREGGQTAP ASTRLFQVRA NSAGATRAVE VLPKAGALNS NDAFVLKTPS
601 AAYLWVGTGA SEAEKTGAQE LLRVLRAQPV QVAEGSEPDG FWEALGGKAA YRTSPRLKDK
661 KMDAHPPRLF ACSNKIGRFV IEEVPGELMQ EDLATDDVML LDTWDQVFVW VGKDSQEEEK
721 TEALTSAKRY IETDPANRDR RTPITVVKQG FEPPSFVGWF LGWDDDYWSV DPLDRAMAEL
781 AA
```

Gelsolin: Accession number P06396; 3% coverage; 1% chance of false positive

```
  1 MSSYSSDRDR GRDRGFGAPR FGGSRTGPLS GKKFGNPGEK LVKKKWNLDE LPKFEKNFYQ
 61 EHPDLARRTA QEVDTYRRSK EITVRGHNCP KPVLNFYEAN FPANVMDVIA RHNFTEPTAI
121 QAQGWPVALS GLDMVGVAQT GSGKTLSYLL PAIVHINHHP FLERGDGPIC LVLAPTRELA
181 QQVQQVAAEY CRACRLKSTC IYGGAPKGPQ IRDLERGVEI CIATPGRLID FLECGKTNLR
241 RTTYLVLDEA DRMLDMGFEP QIRKIVDQIR PDRQTLMWSA TWPKEVRQLA EDFLKDYIHI
301 NIGALELSAN HNILQIVDVC HDVEKDEKLI RLMEEIMSEK ENKTIVFVET KRRCDELTRK
361 MRRDGWPAMG IHGDKSQQER DWVLNEFKHG KAPILIATDV ASRGLDVEDV KFVINYDYPN
421 SSEDYIHRIG RTARSTKTGT AYTFFTPNNI KQVSDLISVL REANQAINPK LLQLVEDRGS
481 GRSRGRGGMK DDRRDRYSAG KRGGFNTFRD RENYDRGYSN LLKRDFGAKT QNGVYSAANY
541 TNGSFGSNFV SAGIQTSFRT GNPTGTYQNG YDSTQQYGSN VANMHNGMNQ QAYAYPVPQP
601 APMIGYPMPT GYSQ
```

DDX5: Accession number Q61656; 16% coverage; 1% chance of false positive

```
  1 MASNDYTQQA TQSYGAYPTQ PGQGYSQQSS QPYGQQSYSG YGQSADTSGY GQSSYGSSYG
 61 QTQNTGYGTQ SAPQGYGSTG GYGSSQSSQS SYGQQSSYPG YGQQPAPSST SGSYGGSSQS
121 SSYGQPQSGG YGQQSGYGGQ QQSYGQQQSS YNPPQGYGQQ NQYNSSSGGG GGGGGGNYGQ
181 DQSSMSGGGG GGGYGNQDQS GGGGGGYGGG QQDRGGRGRG GGGGYNRSSG GYEPRGRGGG
241 RGGRGGMGGS DRGGFNKFGG PRDQGSRHDS EQDNSDNNTI FVQGLGENVT IESVADYFKQ
301 IGIIKTNKKT GQPMINLYTD RETGKLKGEA TVSFDDPPSA KAAIDWFDGK EFSGNPIKVS
361 FATRRADFNR GGGNGRGGRG RGGPMGRGGY GGGGSGGGGR GGFPSGGGGG GGQQRAGDWK
421 CPNPTCENMN FSWRNECNQC KAPKPDGPGG GPGGSHMGGN YGDDRRGRGG YDRGGYRGRG
481 GDRGGFRGGR GGGDRGGFGP GKMDSRGEHR QDRRERPY
```

FUS: Accession number P56959; 8% coverage; 1% chance of false positive

```
   1 MKYLNVLAKA LYDNVAESPD ELSFRKGDIM TVLERDTQGL DGWWLCSLHG
  51 RQGIVPGNRL KILVGMYDKK PAGPGPGPPA TPPQPQPSLP QGVHAPVPPA
 101 SQYSPMLPTA YQPQSDNVYL VPTPSKTQQG LYQAPGPNPQ FQSPPAKQTS
 151 TFSKQTPHHS FPSPATDLYQ VPPGPGSPAQ DIYQVPPSAG IGHDIYQVPP
 201 SLDTRGWEGT KPPAKVVVPT RVGQGYVYEA AQTEQDEYDT PRHLLAPGPQ
 251 DIYDVPPVRG LLPNQYGQEV YDTPPMAVKG PNGRDPLLDV YDVPPSVEKG
 301 LLSSSHHSVY DVPPSVSKDV PDGPLLREET YDVPPAFAKP KPFDPTRHPL
 351 ILAAPPPDSP AAEDVYDVPP PAPDLYDVPP GLRRPGPGTL YDVPRERVLP
 401 PEVADGSVVD DGVYAVPPPA EREAPTDGKR LSASSTGSTR SSQSASSLEV
 451 VVPGREPLEL EVAVESLARL QQGVSTTVAH LLDLVGSASG PGGWRGTSEP
 501 QEPPAQDLKA AVAAVHGAVH ELLEFARGAV SNATHTSDRT LHAKLSRQLQ
 551 KMEDVYQTLV VHGQVLDSGR GSPGFTPEDL DRLVACSRAV PEDAKQLASF
 601 LHGNASLLFR RTKAPGPGPE GSSSLHPNPT DKASSIQSRP LPSPPKFTSQ
 651 DSPDGQYENS EGGWMEDYDY VHLQGKEEFE KTQKELLERG NIMRQGKGQL
 701 ELQQLKQFER LEQEVSRPID HDLANWTPAQ PLVPGRTGGL GPSDRQLLLF
 751 YLEQCEANLT TLTDAVDAFF TAVATNQPPK IFVAHSKFVI LSAHKLVFIG
 801 DTLSRQAKAA DVRSQVTHYS NLLCDLLRGI VATTKAAALQ YPSPSAAQDM
 851 VDRVKELGHS TQQFRRVLGQ LAAA
```

P130CAS: Accession number AAA93248; 20% coverage; Score 67; significant > 63

```
  1 MMLGPEGGEG YVVKLRGLPW SCSIEDVQNF LSDCTIHDGV AGVHFIYTRE
 51 GRQSGEAFVE LESEDDVKLA LKKDRESMGH RYIEVFKSHR TEMDWVLKHS
101 GPNSADSAND GFVRLRGLPF GCTKEEIVQF FSGLEIVPNG ITLPVDPEGK
151 ITGEAFVQFA SQELAEKALG KHKERIGHRY IEVFKSSQEE VRSYSDPPLK
201 FMSVQRPGPY DRPGTARRYI GIVKQAGLDR MRSGAYSAGY GGYEEYSGLS
251 DGYGFTTDLF GRDLSYCLSG MYDHRYGDSE FTVQSTTGHC VHMRGLPYKA
301 TENDIYNFFS PLNPVRVHIE IGPDGRVTGE ADVEFATHEE AVAAMSKDRA
351 NMQHRYIELF LNSTTGASNG AYSSQVMQGM GVSAAQATYS GLESQSVSGC
401 YGAGYSGQNS MGGYD
```

hnRNP F: Accession number AAH33483; 31% coverage; Score 72, significant > 63

```
  1 MEREKEQFRK LFIGGLSFET TEESLRNYYE QWGKLTDCVV MRDPASKRSR
 51 GFGFVTFSSM AEVDAAMAAR PHSIDGRVVE PKRAVAREES GKPGAHVTVK
101 KLFVGGIKED TEEHHLRDYF EEYGKIDTIE IITDRQSGKK RGFGFVTFDD
151 HDPVDKIVLQ KYHTINGHNA EVRKALSRQE MQEVQSSRSG RGGNFGFGDS
201 RGGGGNFGPG PGSNFRGGSD GYGSGRGFGD GYNGYGGGPG GGNFGGSPGY
251 GGGRGGYGGG GPGYGNQGGG YGGGYDNYGG GNYGSGSYND FGNYNQQPSN
301 YGPMKSGNFG GSRNMGGPYG GGNYGPGGSG GSGGYGGRSR Y
```

hnRNP A2/ B1 (section4.5-1):

Accession number AAK9860; 20% coverage; Score 286, significant > 53

Proteins were identified by mass spectrometry in collaboration with different groups (see result section). Different softwares were used for analysis. Hence it is either stated, that there is a probability of 1% for identification of a false positive protein (DDX5, Gelsolin and FUS) or the probability score is listed in addition to the value above which it can be regarded as a significant hit (p130CAS, hnRNP F, hnRNP A2/ B1).

8.3 Small hairpin RNA (shRNA) interference

As mentioned in the Materials and Methods section, shRNA and siRNA was established to silence distinct mRNAs and reduce protein levels in oligodendrocytes.

Although synthetic siRNA duplexes turned out to be more appropriate for the experiments presented in the result section, the shRNA system also led to efficient reduction of protein levels.

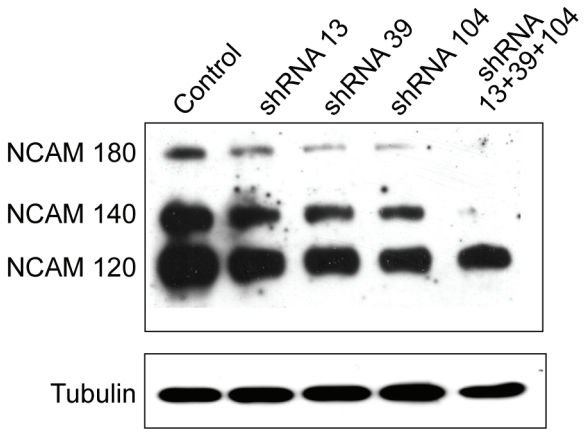

Figure 8.3-1: shRNA reduces NCAM protein levels in Oli-*neu* cells

Oli-*neu* cells were transfected with 3 different pSilencer vectors (Ambion) expressing 3 different target shRNAs (13, 39, 104) or an eGFP control plasmid (control). Additionally, all 3 vectors were cotransfected to target 3 regions in NCAM mRNA simultaneously (shRNA13+39+104). Cells were lysed and analysed by Western Blotting with NCAM antibodies recognising all 3 NCAM isoforms in oligodendrocytes. A subsequent tubulin blot served as a loading control.

The pSilencer system (Ambion) was used to design and express target sequences 13, 39 and 104 directed against NCAM mRNA (see table 8.3). Briefly, target sequences were designed in agreement with ambion's instructions and the synthetically synthesised DNA oligonucleotides were annealed and cloned into the pSilencer 1.0 vector according to the manufacturer's instructions. Plasmids were transfected into Oli-*neu* cells on two consecutive days using fugene6 (Roche) as suggested in the user's manual resulting in ~60% transfection efficiency as assessed by counting eGFP positive control transfected cells. Cells were lysed and proteins were analysed by Western Blotting using NCAM antibodies recognising all 3 NCAM isoforms in oligodendrocytes. A subsequent tubulin blot served as a loading control. Small hairpin RNA expression leads to reduction of NCAM protein levels. Upon individual transfection, target sequence 104 works most efficiently. However, the combined expression of all three shRNAs leads to the highest reduction of

NCAM. Interestingly, NCAM isoforms 140 and 180 seem to be more affected by the treatment than isoform 120. Perhaps the turnover of the NCAM 120 isoform is slower which could perhaps correlate with the fact that in oligodendrocytes this isoform is distributed into lipid raft microdomains by its glycosyl phosphatidyl inosotol anchor (Kramer et al., 1997).

	Target sequence in NCAM
shRNA 13	5'-AACTAAGGATCTCATCTGGAC-3'
shRNA 39	5'-AAAGGCCGAGATGTCATTCTG-3'
shRNA 104	5'-AAGAATCCTGGCATTTCACGT-3'

Table 8.3 : shRNA target sequences

9 List of abbreviations

A	**a**mpere
ATP	**A**denosine 5' **t**ri**p**hosphate
BSA	**B**ovine **S**erum **A**lbumine
C	**C**ontrol or **c**elcius
CDS	**C**o**d**ing **s**ecquence
CAM	**C**ell **a**dhesion **m**olecule
dbcAMP	**d**ibutyryl **c**yclic **a**denosine **m**ono**p**hosphate
DMEM	**D**ulbecco **m**odified **e**agle's **m**edium
DNA	**D**eoxyribo**n**ucleic **a**cid
dNTP	**D**eoxyribo**n**ucleotide **t**ri**p**hosphate
E14	**E**mbryonal day **14**
ECL	**E**nhanced **c**hemo**l**uminescence
EDTA	**E**thylene**d**i**a**mine**t**etra**a**cetic acid
eGFP	**E**nhanced **g**reen **f**luorescent **p**rotein
EM	**E**lectron **m**icroscopy
F	phenylalanine
FCS	**F**etal **c**alf **s**erum
FN	**f**ibro**n**ectin
g	Relative centrifuge force or **g**ram
GFP	**G**reen **f**luorescent **p**rotein
h	**H**our
HBSS	**H**anks' **b**alanced **s**alt **s**olution
hnRNP	**H**eterogeneous **n**uclear **r**ibo**n**ucleo**p**rotein
HRP	**H**orse**r**adish **p**eroxidase
HS	**H**orse **S**erum
Ig	**I**mmuno**g**lobulin
IgSF	**I**mmuno**g**lobulin **S**uper**f**amily
K	lysine
kD	**k**ilo**d**alton
l	**l**itre
LSM	**L**aser **S**canning **M**icroscope
M	**m**ethionine
MAG	**M**yelin **a**ssociated **g**lycoprotein
MBP	**M**yelin **B**asic **P**rotein

min	**min**ute
ml	**M**illi**l**itre
MOBP	**m**yelin-associated **o**ligodendrocytic **b**asic **p**rotein
MW	**M**olecular **w**eight
NF	**n**euro**f**ascin
ng	**n**ano**g**ram
nM	**n**ano**m**olar
P	**P**ost**n**atal
PCR	**P**olymerase **C**hain **R**eaction
PFA	**P**ara**f**orm**a**ldehyde
PLL	**P**oly-**L**-**L**ysine
PLP	**P**roteo**l**ipid**p**rotein
PNS	**P**eripheral **n**ervous **s**ystem or **p**ost **n**uclear **s**upernatant
qRT-PCR	**Q**uantitative RT-PCR
RT	**r**oom **t**emperature
RT-PCR	**R**everse **t**ranscriptase **p**olymerase **c**hain **r**eaction
SDS-PAGE	**S**odium **d**odecyl **s**ulphate **p**oly**a**crylamide gel **e**lectrophoresis
SEM	**S**tandard **e**rror of the **m**ean
TBS	**T**ris **b**uffered **s**aline
U	**U**nit
v/ v	**v**olume per **v**olume
w/ v	**w**eight per **v**olume
Y	T**y**rosine
YFP	**Y**ellow **f**luorescent **p**rotein
µg	microgram
µl	microlitre