Identification, characterization and functional analysis of novel protein binding partners of the cell recognition molecule L1 and the polysialylated neural cell adhesion molecule in mouse (*Mus musculus L., 1758*)

Dissertation

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1. Introduction

1.1. Cell adhesion molecules in the nervous system

The establishment of a functional nervous system involves highly concerted organization of cell migration, differentiation, survival and connection between neurons and their appropriate postsynaptic targets (Lee 2005). During early development of the nervous system, neurons elongate their axons towards their targets and establish and maintain synapses through generation of cell-cell adhesions. Formation of cellular polarity and the constitution of synaptic contacts establish a functional and ordered tissue. Even after termination of developmental processes, cellular contacts still remain variable. Significant stages of plasticity in adults include learning, memory consolidation and neuronal regeneration and require structural flexibility of the nervous system. Synaptic plasticity and axonal outgrowth are generated by contact-mediated attraction or repulsion of nerve cells or nerve/glia cell contacts.

Many of these processes are mediated by a variety of integral membrane proteins, collectively termed cell adhesion molecules (CAMs). CAMs play critical roles in all aspects of nervous system development and maintenance. Important phenomena in which CAMs are involved include initial formation of the neural tube and the neural crest, migration of all neurons and glial cells, axonal outgrowth and guidance, target selection, synaptic stabilization and plasticity, myelination and nerve regeneration after injury (Colman and Filbin 1999).

Adhesion molecules interact with each other and with non-adhesive cell surface and/or cytoplasmic molecules thereby trigger signal transduction cascades leading to functional changes. Cell adhesion molecules comprise several “families”. These families are defined by individual members which are related to each other by common primary sequences, structural motifs and binding properties (Dayhoff et al. 1983). The three major groups of CAMs found in the nervous system are the members of the immunoglobulin (Ig) gene superfamily (IgCAMs), the integrins and the cadherins. The cadherins and IgCAMs are engaged in cell-cell or membrane-membrane interactions, while integrins, for the most part, interact with components of the extracellular matrix (ECM). Distinct binding requirements also characterize each family. For example, cadherins interact in a Ca^{2+}-dependent, usually homophilic manner (Angst et al. 2001). Binding of the members of the Ig family is Ca^{2+}-independent and, although frequently homophilic, can be heterophilic (Juliano 2002). Integrin binding is also divalent cation-dependent (Ca^{2+}, Mg^{2+}) but always heterophilic (Clark and Brugge 1995).

1.2. Immunoglobulin gene superfamily of cell adhesion molecules

The best studied group of recognition/adhesion molecules expressed in the nervous system is the IgCAMs, which are defined by regions that have sequence similarity with immunoglobulins, termed the Ig domains (Edelman 1987;Williams and Barclay 1988). Members of this family of molecules may have only one Ig-like domain, as in the case for the myelin protein P0, or, as for most of the family, have many Ig domains (**Figure 1**). There are about 765 members of the Ig superfamily in humans (Alberts et al. 2002a). The amino acids in each Ig-like domain are usually encoded by a separate exon. It seems likely that the entire gene superfamily evolved from a gene coding for a single Ig-like domain that may have mediated cell-cell interactions (Alberts et al. 2002a). There is evidence that such a primordial gene arose before vertebrates diverged from their invertebrate ancestors about 400 million years ago. New family members presumably arose by exon and gene duplications. The multiple gene segments that encode antibodies and T-cell receptors may have arisen when a transposable element, or transposon, inserted into an exon of a gene encoding an Ig family member in an ancestral lymphocyte-like cell (Alberts et al. 2002a).

Ig family members can be broadly divided into three general classes (Kelm et al. 1994): 1) those that have only Ig-like domains; 2) those that have Ig domains and additional domains that resemble regions of the ECM component fibronectin, termed FnIII-like domains (Cunningham 1995); 3) those that have Ig domains and motifs other than Fn-like domains (catalytic cytoplasmic domains). Moreover, anyone Ig family member may have many isoforms, which may differ in the length of the cytoplasmic domain, in their post-translational modifications and whether they are membrane-spanning or glycophosphatidylinositol (GPI)-anchored proteins (Colman and Filbin 1999) (**Figure 1**). The first isolated and characterized IgCAM was the neural cell adhesion molecule (N-CAM) (Thiery et al. 1977) and L1 (Salton et al. 1983;Rathjen and Schachner 1984), representative molecules of two different subgroups. MAG (myelin associated glycoprotein), FGF-R (fibroblast growth factor-receptor), Thy-1 (thymus cell antigen 1) and P0 represent additional subgroups that occur in the nervous system (**Figure 1**).

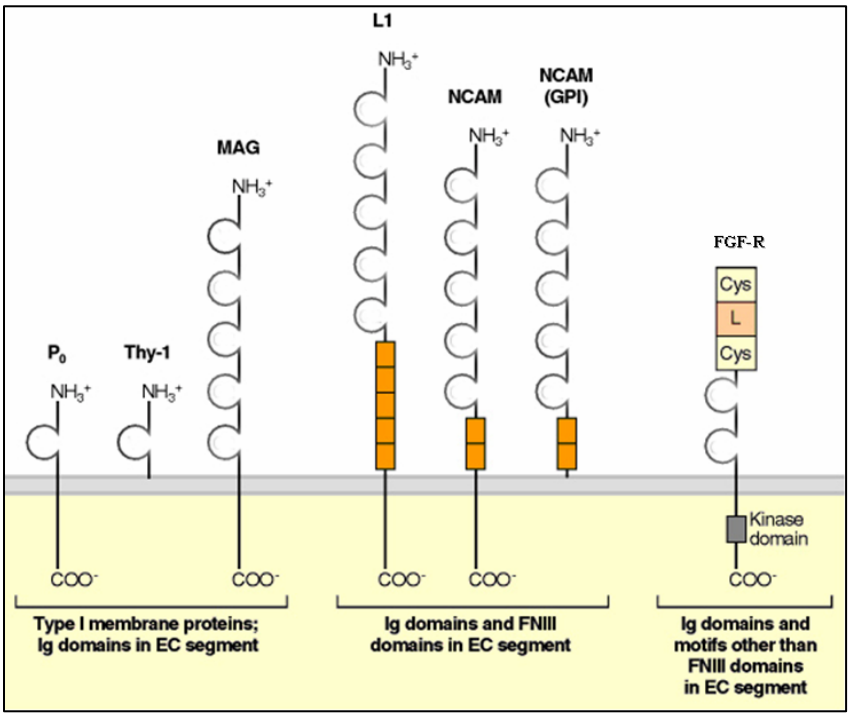


Figure 1: The immunoglobulin (Ig) gene family of molecules.

The majority of molecules of the immunoglobulin superfamily that are found in the nervous system fall into classes depending on the number of Ig-like domains and combination with fibronectin type III (FnIII) repeats or other motifs. All of them are transmembrane proteins or GPI-anchored. One class contains varying numbers of Ig-like domains but no FnIII repeats. Another class represents extracellular part with different number of Ig-like domains and FnIII repeats. The enzymatic cytoplasmic domain (CD) with kinase activity characterises the third class. NCAM (neural cell adhesion molecule); MAG (myelin-associated glycoprotein); FGF-R (fibroblast growth factor receptor); Thy-1 (thymus cell antigen 1); GPI (glycophosphatidylinositol); EC (extracellular) (Colman and Filbin 1999).

1.3. The neural cell adhesion molecule L1

1.3.1. The L1 subfamily of neuronal IgCAMs

The transmembrane cell recognition molecule L1 was originally identified in the nervous system at the beginning of the 1980's (Rathjen and Schachner 1984). It is the founder of neural subfamily of the immunoglobulin superfamily of cell adhesion molecules (IgCAMs), which comprises four vertebrate members - L1, neurofascin, NrCAM (neuronal-glia cell adhesion molecule related cell adhesion molecule) and CHL1 (close homologue of L1) – and two invertebrate members - neuroglian and tractin (Brummendorf et al. 1998). All members of the L1 family display high similarity in the composition and conformation of their modules and are composed of six amino-terminal Ig domains, four to five FnIII repeats, a single

hydrophobic membrane-spanning region and a short, phylogenetically highly conserved cytoplasmic tail at the carboxyl terminus (Brummendorf and Rathjen 1995). These molecules are widespread throughout the developing nervous system and involved in a variety of morphogenetic processes, such as cell migration, axon outgrowth, myelination, pathfinding, fasciculation and synaptic plasticity (Brummendorf et al. 1998;Hortsch 2000). Members of the L1 family are mainly found on the surface of axons and at sites of cell-cell contact and expressed by neurons and glial cells (Hortsch 1996). The sequence similarity among species homologous of different animal classes ranges between 30 to 60%, with the intracellular domain showing the highest degree of interspecies homology (Hortsch 1996;Hortsch 2000).

1.3.2. The neural recognition molecule L1

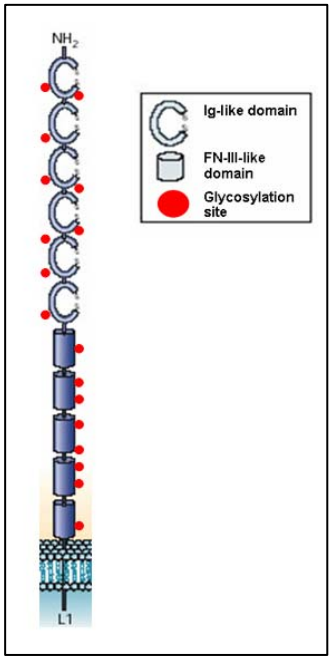
1.3.2.1. Characteristics of L1

L1 is a phosphorylated, integral membrane glycoprotein that can be recovered from mouse brain tissue in a distinct set of polypeptides with apparent molecular masses of 200, 180, 140 and 80 kDa (Sadoul et al. 1988). Mammalian L1 consists of six Ig domains, five FnIII repeats, a single membrane-spanning region followed by a short (~110 residues) cytoplasmic tail. Putative sites for asparagine-N-linked glycosylation are distributed over the extracellular domain of L1 (**Figure 2**). In addition, a substantial portion of the glycans is O-linked as indicated by tunicamycin inhibition of cotranslational N-glycosylation (Faissner et al. 1985). Glycans contribute about 25% to the total molecular mass of L1, since deglycosylation revealed an apparent molecular mass of about 150 kDa (Lindner et al. 1983;Rathjen and Schachner 1984).

L1 molecule, normally membrane-bound, can be released as a soluble proteolytic fragment, which has been described in various forms (Kalus et al. 2003). The 140- and 80 kDa fragments resulting from cleavage within the third FnIII domain have been generated *in vitro* by trypsin or plasmin or protein convertase PC5A (Kalus et al. 2003). Cleavage within this domain by plasmin reduces multimerization and RGD-independent integrin binding (Silletti et al. 2000). The 180- and 50 kDa fragments result from membrane-proximal cleavage of the membrane-spanning 200- and 80 kDa L1 forms, respectively, by a metalloprotease ADAM (a disintegrin and metalloprotease) family (Beer et al. 1999). This cleavage step has been proposed to be required for cell migration (Gutwein et al. 2000).

Figure 2: L1 cell adhesion molecule.

L1 is a cell surface macromolecule that belongs to the immunoglobulin superfamily of cell adhesion molecules. L1 is a transmembrane glycoprotein that contains six Ig-like, five fibronectin type III-like extracellular modules and short, highly conserved cytoplasmic tail. 25% from the molecular mass of L1 are composed by glycans. Potential N-glycosylation sites across L1 molecule are marked by red. L1 is found at regions of contact between neighbouring axons and on growth cones, where it modulates many cellular functions. Spontaneous mutations in L1 produce various neurological syndromes, including mental retardation (Sandi 2004).



L1 is widely expressed in the nervous system and have been implicated in a variety of developmental processes including neuronal differentiation, axon growth and guidance, axon fasciculation, myelination and synaptic plasticity (Panicker et al. 2003). L1 has acquired a special significance since it was identified that mutations in the human L1 gene are responsible for a number of nervous system defects. In humans, these phenotypes include hydrocephalus, mental retardation, spastic paraplegia, adducted thumbs, and agenesis of corpus callosum (Zhao and Hortsch 1998) which have recently been summarized under the acronym CRASH syndrome (Fransen et al. 1995).

The L1 protein is encoded by a single gene located on the *q*28 band on human X-chromosome which is the homologous to the *A*-6B region of the mouse X-chromosome (Djabali et al. 1990). The L1 gene contains 29 exons, 28 exons encodes the protein (designated 1b-28) while one exon contains 5´untranslated sequences (exon 1a) (Kohl et al. 1992;Kallunki et al. 1997). The mRNA provides an open reading frame of 3783 nucleotides. The encoded 1260 amino acids comprise a 19 amino acid signal peptide and a mature protein of 1241 amino acids (Moos et al. 1988).

Up to date about 140 different pathogenic mutations have been identified in virtually all regions of the L1 gene. All types of mutations were found in human patients including missense, nonsense and frame shift mutations, deletion, duplication, insertion, and splice site mutations. Some of them are known to truncate the L1 protein, giving rise to a null mutation. Missense mutations occurring throughout the L1 extracellular and intracellular domains can generate a protein with altered function or cause failure to be transported to the plasma

membrane resulting in degradation (Moulding et al. 2000;De Angelis et al. 2002;Runker et al. 2003). Based on the knowledge that some of the L1 mutations cause disruption of cell surface expression of the protein leading to the neurological disorder in humans, several L1 knock out mouse lines were generated to test as an animal model for the human L1 syndrome CRASH. The various L1 mutants share many of the pathological features observed in human patients independent of their origin. The general appearance of the mutants displayed several characteristics. They were smaller than their wild type littermates. They were also mostly infertile and less viable. Their eyes were sunken and lacrimous. The observed weakness in hind limbs could be the impairment corresponding to the spasticity in human patients (Dahme et al. 1997;Cohen et al. 1998;Rolf et al. 2001;Demyanenko et al. 1999). Thus, L1 may participate in a fundamental way in normal neurodevelopmental mechanisms requiring cell location and axon growth or guidance, and mutation of critical residues within extracellular and intracellular L1 domains may alter neuronal motility and axon growth resulting in developmental abnormalities and altered mental functions (Panicker et al. 2003).

1.3.2.2. Expression and functions of L1 in the nervous system

Different isoforms of L1 are generated by alternative splicing and yield functionally distinct proteins that are expressed in different cell types. L1 isoforms retaining exons 2 and 27 are expressed by neurons, whereas forms lacking these sequences are expressed in certain non-neuronal cells including Schwann cells, hematopoietic cells (T-cells, B-cells, granulocytes), and epithelial cells (Panicker et al. 2003).

In the nervous system L1 expression is temporally and spatially regulated. It is detected from embryonic day 10 onwards in the central nervous system on postmitotic neurons and the distribution in the developing nervous system already indicates its role in late cell migration (Rathjen and Schachner 1984;Fushiki and Schachner 1986). It does not appear to mediate synaptic formation (Mehrke et al. 1984;Schuster et al. 2001) or synaptic or electrical activity of neurons (Kettenmann et al. 1983). L1 has been found to mediate neuron-to-neuron but not neuron-to-astrocyte or astrocyte-to-astrocyte adhesion (Keilhauer et al. 1985). Recently also oligodendrocytes were found to express short isoform of L1 in addition to full-length L1, regulated in a maturation-dependent manner (Itoh et al. 2000). It is not expressed in all postmitotic neurons; for instance, in the mouse cerebellum L1 is not expressed by stellate or bascket cells. On granule and Purkinje cells it is not expressed on dendrites, only transiently on their cell bodies and restricted to the non-myelinated axons and growth cones. Mice showed expression of L1 in the hippocampus is restricted to fasciculating axons forming the stratum moleculare and the hilus where expression increases with age while dendrites and

regions of cell body remain negative for L1 (Persohn and Schachner 1987). In peripheral nervous system L1 is found not only on neurons but also on Schwann cells; expression is disappeared from both, axons and Schwann cells, since myelination starts (Martini and Schachner 1986).

In adulthood expression of L1 is continued on non-myelinated axons; thereby L1 is implicated in such important processes as axonal regeneration (Martini and Schachner 1988), proliferation and differentiation of neurons (Dihne et al. 2003), neuronal cell survival (Chen et al. 1999), learning and memory formation (Rose 1995;Venero et al. 2004) and the establishment of long-term potentiation in the hippocampus (Luthi et al. 1996).

1.3.2.3. Complexity of L1 interactions

L1 participates in a variety of different cellular responses both within and outside of cells and flexibility of its functions was suggested to be related to a multiplicity of binding partners, potential modifications and subsequent signal transduction changes.

Cell surface ligands – A bewildering array of molecules has been identified to bind to the extracellular domain of L1. These ligands can be generally grouped as: 1) neural IgCAMs (such as L1 itself, Nr-CAM, axonin-1, contactin), 2) non-Ig family CAMs (integrins), 3) extracellular matrix constituents (laminin, phosphacan and neurocan) and 4) signalling receptors (Neuropillin-1 (NP-1) and Fibroblast Growth Factor Receptor (FGFR)) (Haspel and Grumet 2003) which are shown in the **Table 1**. The orientation of binding between the L1 extracellular region and its ligands can be described as either *cis*- (within the plane of the cell membrane) or *trans*-position (where the binding partners are situated on opposing cells).

Haspel and Grumet (Haspel and Grumet 2003) have proposed that L1 extracellular region utilizes two distinct strategies for protein-protein interactions. **"Modular mode"** strategy is defined as interactions mediated by single L1 domain that does not require contribution from neighbouring domains to mediate binding. For example, the binding sites for nervous tissue-specific chondroitin sulfate proteoglycan neurocan and membrane receptor NP-1 were localized to Ig I domain (Oleszewski et al. 1999;Castellani et al. 2002). Two binding sites for several integrins also were found to precise domains: first is localized to RGD-containing motif in Ig VI domain (Oleszewski et al. 1999), another RGD-independent sequence in Fn3 domain was shown to bind integrins as well (Silletti et al. 2000). Fn3 domain is also reported to mediate homophilic L1-L1 interaction in *cis*-position (Silletti et al. 2000). **"Cooperative mode"** strategy requires the contribution of multiple L1 domains forming a unique quaternary "horseshoe" structure from Ig I-Ig IV domains that is critical for binding (Su et al. 1998). The prominent example of this strategy is homophilic L1-L1 binding in *trans*-orientation which is

mediated by non-covalent bonds between Ig III domain of one protein with the horseshoe fold of another L1 molecule (Haspel and Grumet 2003). Interactions with other members of neural IgCAMs - axonin, contactin - also require biologically active "horseshoe" conformation of L1 (Haspel and Grumet 2003).

Other important mediators of interaction between L1 and its ligands are carbohydrates. It was established that NCAM possesses carbohydrate recognition domain for oligomannosidic glycans thereby can bind to L1 and promote neurite outgrowth (Horstkorte et al. 1993). Binding of L1 to a small cell surface glycoprotein nectadrin (CD24) is mostly mediated by alpha-2,3-sialic acid expressed on CD24 which determines the CD24-induced and cell type-specific promotion or inhibition of neurite outgrowth (Kleene et al. 2001). Expression of HNK-1 carbohydrate epitope on L1 is responsible for the binding of L1 to extracellular matrix glycoprotein laminin (Hall et al. 1997).

Obviously that L1 extracellular domain accommodates many different binding activities; the function of each interaction is briefly described in the right column of **Table 1**. Influences on cell behaviour imply that L1 ligand binding must be linked to intracellular signalling pathways. How does a cell surface glycoprotein with no self-containing catalytic domains engage a signalling pathway?

Signal transduction events mediated by L1 - Walsh and Doherty (Walsh and Doherty 1997;Doherty et al. 2000) proposed a model for L1-mediated axonal growth and guidance in which L1-type proteins activate type-1 FGF receptors. Their group provided the evidence for a signalling cascade involving phospholipase C, rather than mitogen-activated protein kinase (MAPK). This signalling pathway results in a localized elevation of cytosolic calcium through L- and N-type channels at localized sites in the growth cone and, ultimately, neurite outgrowth. Islam and colleagues (Islam et al. 2004) demonstrated that L1 adhesion activates epidermal growth factor (EGF) receptor tyrosine kinase activity at cell contact sites in the absence of classical EGF-receptor ligands. In addition, there is convincing genetic evidence from the *Drosophila* system that axonal growth and pathfinding is mediated by neuronal FGF and EGF receptors (Garcia-Alonso et al. 2000). The non-receptor tyrosine kinase $pp60^{c\text{-}src}$ has also been implicated in the L1-mediated induction of neurite growth. Cerebellar neurons from $pp60^{c\text{-}src}$-deficient mice have a reduced rate of neurite outgrowth on L1 substrate, suggesting that this tyrosine kinase also has an important role in L1-mediated signalling events (Ignelzi, Jr. et al. 1994). However, it remains unclear whether L1-type proteins directly interact with and activate $pp60^{c\text{-}src}$ (Nagaraj and Hortsch 2006).

Direct connection to cytoskeleton is important regulatory mechanism involving the surface-expressed molecules in intracellular signal cascade events. It is known that all members of L1

subfamily contain an ankyrin-binding site in their highly conserved cytoplasmic domain (Hortsch 2000). Ankyrins are bifunctional linker proteins that tether certain membrane proteins to the membrane-associated actin-spectrin cytoskeleton (Bennett and Chen 2001).

Ankyrin binding to the cytoplasmic domain of L1 protein is regulated by its homophilic adhesive interaction. A highly conserved, intracellular FIGQY motif in L1-type proteins constitutes the core of this ankyrin-binding site. Ankyrin binding enhances the homophilic adhesive activity of L1-type proteins and reduces their mobility within the plasma membrane, whereas phosphorylation of the FIGQY tyrosine residue inhibits ankyrin binding and thereby enhances their ability to induce neurite outgrowth (Nagaraj and Hortsch 2006). Whittard and colleagues (Whittard et al. 2006) now provide new experimental evidence that identifies MAPK as a central regulator of FIGQY phosphorylation. The cytoplasmic domain also houses a tyrosine-based sorting motif, YRSLE, which is required for the correct trafficking of L1 along axons to the growth cones as well as for L1 endocytosis. The YRSLE motif binds to the adaptor complex AP-2 in a pathway of internalization via clathrin-coated pits (Kenwrick et al. 2000) and can also be important for the activation of $pp60^{c\text{-}src}$ function (Nagaraj and Hortsch 2006). Recently it was identified that L1 also binds to ezrin, another linker protein of the membrane cytoskeleton, at a site overlapping that for AP-2 binding (Dickson et al. 2002). This interaction seems to occur predominantly during migration and axon growth suggesting functional importance in early stages of development (Mintz et al. 2003). **Figure 3** shows the mechanisms by which L1 mediates the signal transduction pathways.

Modifications of L1 - L1 is subjected to glycosylation and phosphorylation, both of them may change the functions and binding properties of L1 protein. In addition to tyrosine phosphorylation of the ankyrin-binding domain, serine phosphorylation occurs at several sites of cytoplasmic L1 domain by specific kinases, for instance, casein kinase II, $p90^{rsk}$, ERK2, Cek5 (Kenwrick et al. 2000). These sites are the next to known binding domains (cytoskeleton components or AP-2 complex) suggesting that serine phosphorylation may influence cytoplasmic interactions, L1 mobility and internalization (Kenwrick et al. 2000). The phosphorylation of extracellular domain of L1, involvement of particular ecto-kinases and following functional changes so far are not reported in current literature.

Molecule	Type of interaction	Direct biochemical evidence for protein-protein interaction?	Comments
L1CAM/Ng-CAM (homophilic binding)	Trans	Yes	Mediates neurite outgrowth and adhesion in most primary neurons
	Cis	Yes	Inferred from clustering of recombinant Fn3 in vitro Thought to potentiate neurite outgrowth activity
Nr-CAM	Unclear	Yes	May potentiate L1CAM mediated neurite outgrowth
Axonin-1/TAG-1/TAX-1	Trans	Yes	Axonin-1 can serve as a substrate for neurite outgrowth in vitro, by binding to L1CAM on the neural cell membrane. However, whether this trans interaction occurs in vivo has been called into question.
	Unclear		
	Cis	No	Axonin-1 was proposed to be a co-receptor for L1CAM mediated neurite outgrowth by clustering with it in the plane of the plasma membrane.
Contactin/F11	Unclear	No	A member of the axonin-1 subfamily of IgCAMs.
FAR-2	Unclear	No	A member of the axonin-1 subfamily of IgCAMs.
N-CAM	Cis	No	May synergize with L1CAM to promote neurite outgrowth. Carbohydrate moieties on L1CAM are thought to be critical to this interaction.
SC1/DM-Grasp	Cis	No	Implicated as a co-receptor for L1CAM mediated neurite outgrowth.
Phosphacan	Trans	Yes	Interaction leads to growth cone collapse.
Neurocan	Trans	Yes	Interaction leads to growth cone collapse.
Laminin	Trans	Yes	Interaction possibly mediated through HNK-1 moieties on L1CAM.
Integrins: $\alpha_v\beta_3$ $\alpha_v\beta_1$ $\alpha_v\beta_5$ $\alpha_5\beta_1$ $\alpha_{IIB}\beta_3$ $\alpha_9\beta_1$	Trans	Yes	Cleaved L1CAM extracellular region is thought to be secreted into the extracellular matrix, where it would bind cell surface integrin receptors. The interaction with $\alpha_v\beta_3$ can mediate neurite outgrowth in PC12 cells, and chick DRG neurons. The interaction with $\alpha_5\beta_1$ potentiates epithelial cell migration on fibronectin and laminin.
CD9	Cis	No	May simultaneously interact with L1CAM and the integrin, $\alpha_6\beta_1$.
Nectadrin (CD24)	Cis	Yes	Sialic acid moieties on CD24 are critical for its interaction with L1CAM. CD24 interferes with L1CAM mediated neurite outgrowth in vitro.
Neuropillin-1 (NP-1)	Cis and Trans	No	L1CAM interactions with NP-1are thought to modulate the neuronal response to Sema3A. In L1CAM knockout mice, commissural neurons are not repelled by Sema3A in vitro. Soluble L1-Fc added to spinal cord explant cultures converts the neural response to Sem3A from repulsion to attraction.
Basic FGFR	Cis	No	Inhibitors of FGF receptor signaling interfere with L1CAM mediated neurite outgrowth in contexts when this biological activity is subserved by homophilic binding. Genetic evidence for an interaction between FGFR and Neuroglian has been advanced in Drosophila.

Table 1: Binding partners of the L1 extracellular region.

In this table, evidence of a protein-protein interaction is defined strictly when purified L1 has been shown to interact with a purified preparation of the protein of interest. The molecules bound by the L1 extracellular region are organized into groups. These include neural IgCAMs (L1CAM-SC1), extracellular matrix constituents (phosphacan - laminin), non-Ig family CAMs (integrins - nectadrin) and signalling molecules (Basic FGFR). Names of species homologues for certain proteins are

separated by a backslash. The orientation of binding between the L1 extracellular region and its ligands can be described as either *cis* (within the plane of the cell membrane), or *trans* (where the binding partners are situated on opposing cells). In some cases L1 has been proposed to interact with particular molecules in *cis* and in *trans* (Haspel and Grumet 2003).

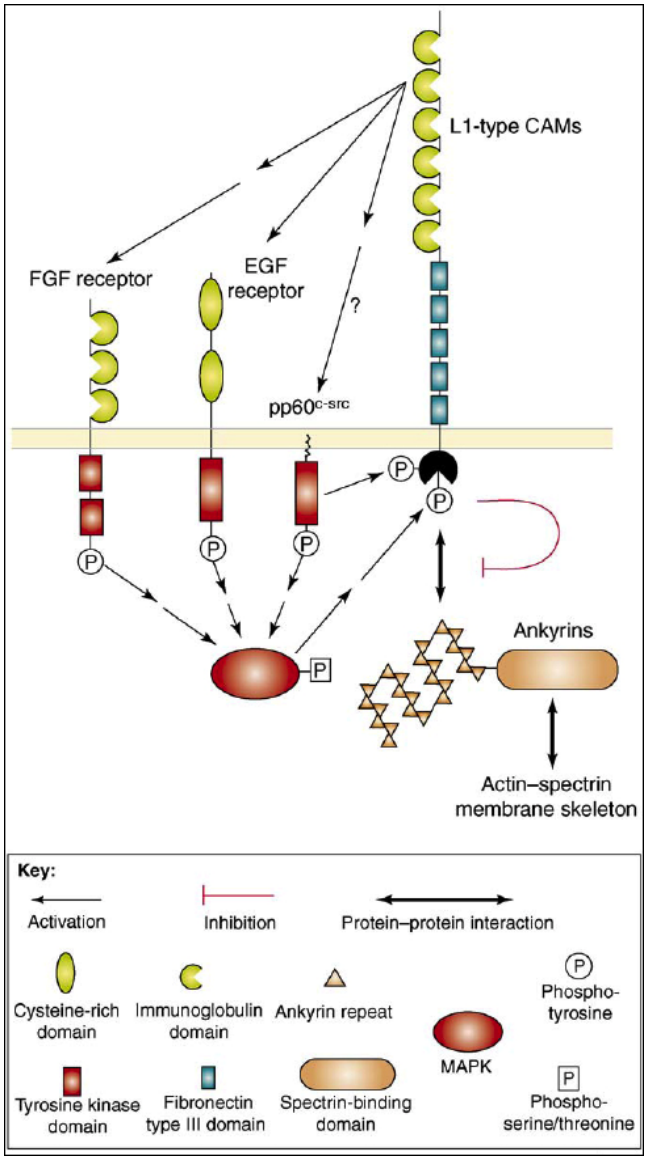


Figure 3: Signal transduction events mediated by L1 cell adhesion molecule.
There is the schematic representation of the regulatory interactions between L1 cell adhesion molecule and several plasma membrane-associated tyrosine kinases which are mainly regulate L1 functions. With the exception of the activation of $pp60^{c\text{-}src}$ by L1 protein, all these interactions are supported by experimental data from various *in vitro* and *in vivo* systems. The MAPK-regulated phosphorylation of the conserved FIGQY tyrosine residue in L1 protein by an as yet unknown tyrosine kinase inhibits the binding of ankyrin proteins to the L1 cytoplasmic domain. $pp60^{c\text{-}src}$ might be responsible for the phosphorylation of a second conserved tyrosine residue (within the endocytosis signal YRSLE) and thereby inhibits the clathrin-mediated endocytosis of vertebrate L1 molecules. Both tyrosine phosphorylation events impact the ability of L1 protein to induce neurite outgrowth in neuronal cells (Nagaraj and Hortsch 2006).

1.4. The neural cell adhesion molecule NCAM

1.4.1. Characteristics of NCAM

Neural cell adhesion molecule NCAM is firstly identified member of the immunoglobulin superfamily and the best characterized within the IgCAMs. It was initially described by Jorgensen and Bock (Jorgensen and Bock 1974). NCAM is expressed as three principal isoforms: two are transmembrane forms with either a short (NCAM-140) or long (NCAM-180) cytoplasmic domains, and another form lacking a cytoplasmic domain (NCAM-120) is anchored to the plasma membrane by a glycophosphatidylinositol (GPI)-linkage (**Figure 4**).

All three NCAM isoforms have five Ig-like domains and two fibronectin type III domains in the extracellular region (Panicker et al. 2003). NCAM-180 differs from NCAM-140 in having a 261-amino acid insert in the cytoplasmic domain, which confers an ability to interact with cytoskeleton protein spectrin (Pollerberg et al. 1986). NCAM-140 is localized to migratory growth cones and axon shafts of developing neurons, whereas NCAM-180 is enriched at sites of cell-cell contact and postsynaptic densities of mature neurons (Dityatev et al. 2000). Unlike transmembrane isoform, GPI-linked NCAM-120 is preferentially expressed in glia. An alternative splice variant of NCAM containing the VASE exon in the Ig IV domain arises postnatally and serves to downmodulate axon growth (Doherty et al. 1992). Among other splice variants, a secreted isoform consisting of most of the extracellular region of NCAM is expressed in brain and skeletal muscle (Gower et al. 1988).

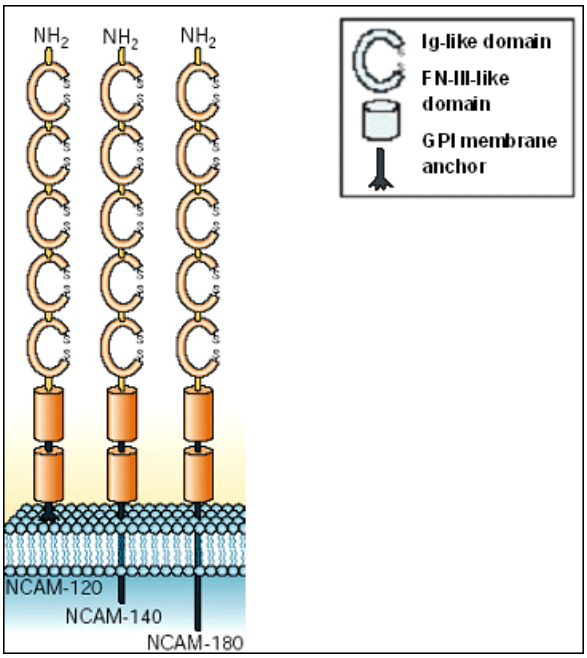


Figure 4: Neural cell adhesion molecule (NCAM)

Neural cell adhesion molecule is a cell surface macromolecule that belongs to the immunoglobulin superfamily of cell adhesion molecules. NCAM is expressed as three main isoforms - NCAM-120, NCAM-140 and NCAM-180 which are generated by alternative splicing of pre-mRNA that is encoded by a single NCAM gene. Extracellularly, all isoforms bear five Ig-like modules and two fibronectin type III modules. NCAM-120 is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) membrane anchor, whereas NCAM-140 and NCAM-180 are transmembrane proteins which differ in a structure of cytoplasmic domain (Sandi 2004).

NCAM plays a role in modulating adhesiveness of neurons and their processes through homophilic and heterophilic binding (Panicker et al. 2003). NCAM-null mutant mice show presynaptic defects in neurotransmission at the neuromuscular junction and retain an immature synaptic vesicle cycling mechanism (Polo-Parada et al. 2004). Molecular dissection of NCAM and identification of splice variants have revealed complex interactions of the extracellular region. The NCAM domains mediating homophilic binding are still debated but dimerization of the third Ig domain and double reciprocal dimerization of the first and second Ig domains have been proposed (Panicker et al. 2003). NCAM binds heterophilically to heparan sulphate proteoglycans (Burg et al. 1995) through heparin-binding sites in the first and second Ig domains, and to the extracellular matrix protein agrin (Storms and Rutishauser 1998) and several chondroitin sulfate proteoglycans, including neurocan and phosphocan (Margolis et al. 1996). Additionally, NCAM is capable of lateral binding to L1 via the NCAM Ig IV domain, which facilitates homophilic binding between L1 molecules apposed in *trans* (Kadmon et al. 1990). The first and second fibronectin type III domains can bind FGFR and contain a putative motif for receptor activation and NCAM-mediated neurite growth, signalling and learning (Kiselyov et al. 2005;Anderson et al. 2005).

In general, signal transduction events mediated by NCAM, can be described as a two different pathways summarized recently by Maness and Schachner (Maness and Schachner 2007). Outside of lipid rafts (functional microdomains within the plasma membrane), NCAM activates cyclic adenine-monophosphate (cAMP)-dependent protein kinase via an unknown mechanism and also interacts with the FGFR in some contexts, which leads to activation of diacylglycerol lipase to generate arachidonic acid and elevate intracellular calcium. The phospholipase Cγ pathway activates arachidonic acid and Ca^{2+}, separately or in combination, leading to the formation of a complex of NCAM-140 and receptor protein phosphatase-α, the association and activation of protein kinase C and the activation of MAPK. Inside lipid rafts, Fyn kinase is attached to the raft membrane compartment via palmitoylation, and is inactivated by tyrosine phosphorylation within its C-terminal regulatory region. Clustering of NCAM-140 induces protein phosphotase mediated dephosphorylation and activation of Fyn, recruiting focal adhesion kinase (FAK), which triggers the G-protein activated cascade. Co-signalling from NCAM inside and outside of rafts is required for cytoskeletal and transcriptional events that culminate in neurite outgrowth. Spectrin binds NCAM-180, and to a lesser extent NCAM-140, enhancing complex formation with receptor protein phosphatase-α and protein kinase C.

1.4.2. The polysialylation of NCAM

Function variations of NCAM molecule can be introduced via post-translation modifications, most prominent from those is specific unique pattern of glycosylation. Glycans are chains of monosaccharides (single sugars) that vary in length from a few sugars to several hundred. Among these glycans, polysialic acid (PSA) is particularly interesting as its function is manifested in cell migration, neurite pathfinding and synaptic plasticity (Nakayama et al. 1998). "Primary" sialic acids are shown in the **Figure 5A**: Neu5Ac is much more common in most vertebrate cell types (Varku et al. 1999). Structurally, polysialic acid is unique because it has a linear homopolymer of α-2,8-linked sialic acid, which contains at least 55 sialic acid residues per chain (Nakayama et al. 1998) (**Figure 5B**). This unique glycan is mainly attached to two N-glycosylation sites within the fifth Ig-like domain of all three NCAM isoforms (Kleene and Schachner 2004). However, the PSA appears not to be restricted to N-linked glycosylations and recent evidence suggests additional O-linked PSA-modification sites in the Fn3 domain region of NCAM (Hinsby et al. 2004). So far, two enzymes that are responsible for the formation of PSA have been identified in the brain - the polysialyltransferase ST8siaIV, which is predominantly expressed in the adult, and ST8siaII, which is more highly expressed during development (Eckhardt et al. 2000). In general, for the synthesis of PSA, N-glycans with terminal sialic acid moieties in α-2,3-linkage serve as the substrate for attachment of the initial α-2,8-linked sialic acid residue. This so-called "initiase" reaction is followed by an "elongation" reaction in which the α-2,8 sialic acid moiety added in the preceding step serves as the attachment site for the next α-2,8-linked sialic acid (Varku et al. 1999) (**Figure 5B**).

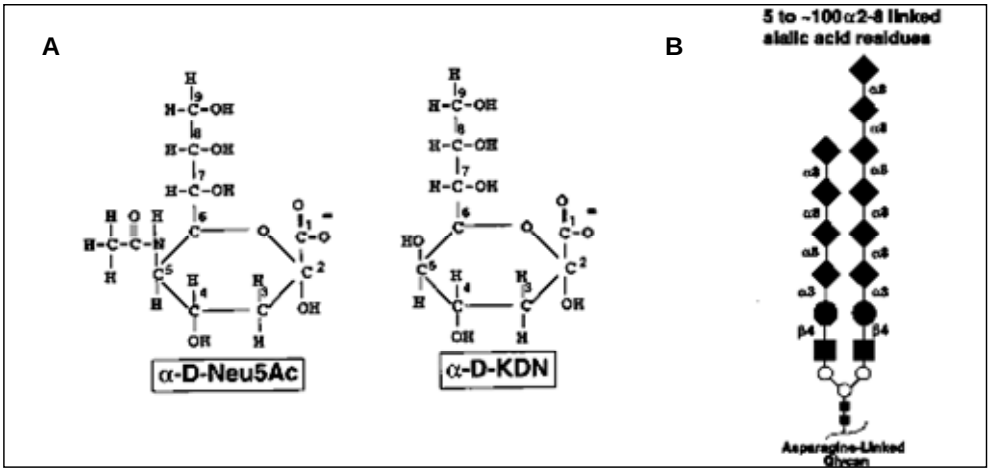


Figure 5: (A) "Primary" sialic acids: 2-keto-5-acetamido-3,5-dideoxy-d-glycero-d-galactononulosonic acid (Neu5Ac) and 2-keto-3-deoxy-d-glycero-d-galactononononic acid (KDN). The only difference is the substitution at the 5-carbon position. Neu5Ac is much more common in most vertebrate cell types. All other sialic acids are thought to be metabolically derived from these two. **(B)** Biosynthesis of

polysialic acid starts from the attachment of initial α-2,8-linked sialic acid residue to asparagine residue with terminal sialic acid moieties in α-2,3-linkage and continues with following attachment of α-2,8-linked sialic acid residues up to 100 (Varku et al. 1999).

PSA is reduced by 85% in NCAM knock-out mice (Cremer et al. 1994); this polysialylation pattern appears to be an almost unique feature of NCAM, with remaining 15% generated mainly by auto-polysialylaton of transferases themselves (Hinsby et al. 2004). PSA has also been detected on voltage-dependent sodium channels, as shown most convincingly in invertebrates (Rutishauser and Landmesser 1996). As expression of PSA is most prominent during development, PSA-NCAM was initially called the embryonic form of NCAM (Kleene and Schachner 2004). Removal of PSA by a specific enzyme, endosialidase N (endo-N), alters neuronal and glial migration, outgrowth or sprouting of axons, and axon branching and fasciculation (Yamamoto et al. 2000;Durbec and Cremer 2001). These results strongly suggest that PSA is critical for neural development and plasticity. *In vitro* studies on purified PSA–NCAM show that PSA can decrease homophilic NCAM-mediated interactions and therefore considered to be a de-adhesive or anti-adhesive component of the NCAM molecule, as a result of its highly negative charge and/or large hydration volume (Sadoul et al. 1983). PSA could also act in a heterophilic mode by affecting interactions of NCAM with other glycan-carrying molecules, such as heparan sulphate proteoglycans (Storms and Rutishauser 1998) and it might also function in the plane of the cell membrane in a *cis*-interaction mode. Fujimoto and co-workers (Fujimoto et al. 2001) provided strong evidence for the effect of PSA on cell adhesion to be independent of binding or signalling properties of NCAM, but through steric inhibition of membrane-membrane apposition solely based on biophysical properties of PSA.

Expression of PSA is reduced when developmental events cease, concomitant with the stabilization of cell interactions. Prolonging the critical period of axon–target interactions also prolongs expression of PSA, whereas pharmacologically induced premature differentiation of these interactions curtails its expression. In adult brain PSA expression retained in the regions with high plasticity such as the hippocampus and olfactory bulbs, hypothalamo - neurohypophysial system and suprachiasmatic nuclei (for the review see: Kleene and Schachner 2004). In hippocampus the population of neurogenic stem cells mature and integrate into the neuronal network of the dentate gyrus in an activity-dependent manner, and they lose PSA on differentiation (Seki 2003). Strikingly, the percentage of PSA–NCAM-positive neuroblasts is enhanced after training in spatial memory tasks (Kempermann et al. 2002), when new connections might have to be formed. Dying olfactory neurons constantly

need to be replaced, so neurogenic activity and PSA expression in the subventricular zone is maintained throughout life. In the hypothalamo-neurohypophysial system the polysialylation increases during physiological conditions that result in enhanced oxytocin release, while in the suprachiasmatic nuclei expression of PSA depends on diurnal activity and regulates the circadian body functions (for the review see: Kleene and Schachner 2004). PSA also has an important role during regeneration of axons and dendrites after a lesion (Muller et al. 1994). PSA is up-regulated after various types of lesion, concomitant with sprouting in the central nervous system and axon re-growth in the peripheral nervous system (Rutishauser and Landmesser 1996). So it is clear that PSA is important modulator of neural plasticity. Side by side with steric inhibition of membrane-membrane apposition, PSA was also proposed to be a ligand or accumulator of ligands for receptors that mediate its own functions. Recently PSA has been suggested to trap and accumulate the neurotrophin BDNF (brain derived neurotrophic factor) and present it to its receptor tyrosine kinase, TrkB. This enhances the biological activity of BDNF for survival and differentiation of neurons in culture (Vutskits et al. 2001). PSA, among other negatively charged carbohydrates, has also been suggested to act as a cell surface receptor for internalization of a homeobox peptide that acts as an intercellular signalling transcription factor (Joliot et al. 1991). In spite of these investigations the knowledge about potential activities of PSA and also ligands and receptors, to which PSA can bind to, remains poor. The application of conventional biochemical approaches might be interesting to answer this important question.

1.4.3. Affinity chromatography using an anti-idiotype scFv "antibody" mimicking PSA obtained by phage display

Anti-idiotype approach was used by Maren von der Ohe in order to identify novel potential binding partners of PSA and described in details in her PhD thesis (von der Ohe 2000). Briefly, the main idea of this investigation was to obtain single chain variable fragment (scFv) "antibody" containing epitope, structurally mimicking PSA, with following application of them for the affinity chromatography column. Single chain variable fragment (scFv) constructs have been developed as more specific alternative to both Fab and Fv fragments of IgG molecule and have the variable regions of a single heavy- and single light chain linked to retain immunoreactivity (Huston et al. 1988). To generate scFv mimicking PSA Griffin.1 library was used (Griffiths et al. 1994). It is the phagemid library of scFv's made by recloning the heavy and light chain variable regions from the lox library vectors into phagemid vector pHEN2. Screening of the library against an immobilized antigen - monoclonal α-PSA 735 antibody – was done using standard panning methodology (Winter et al. 1994). Antigen

specific phages were recloned into bacterial expression vector to enlarge the quantity of the phages and then the phages producing monoclonal scFv were isolated; they structurally mimicked polysialic acid and could bind the proteins normally interacting to PSA. After verification of scFv specificity they were immobilized on the affinity chromatography column and both soluble brain homogenate and membrane-containing Triton X-100 soluble/insoluble fractions were applied. Using mass spectrometry analysis three proteins with apparent molecular weight of 80, 50 and 32 kDa were identified as potential binding partners of PSA. First protein sequence revealed the identity to myristoylated alanine-rich C kinase substrate (MARCKS), the second – to neuronal tissue-enriched acidic protein (NAP-22) and the third – to well known DNA binding nuclear protein Histone H1 (von der Ohe et al. unpublished data).

MARCKS - The members of the myristoylated alanine-rich C kinase substrate family are widely distributed acidic, rod-shaped proteins essential for brain development and survival. Changes in expression, protein kinase C (PKC)-dependent phosphorylation and subcellular localization of MARCKS proteins have been implicated in the regulation of brain development and postnatal survival, cellular migration and adhesion, as well as endo-, exo- and phagocytosis, and neurosecretion (Arbuzova et al. 2002). MARCKS is significantly down-regulated in proliferating, oncogenically transformed cells. Conversely, overexpression of MARCKS strongly depresses the proliferation of such cells (Ramsden 2000). The family comprises MARCKS and the smaller MARCKS related protein (MRP) sharing structural features (Ramsden 2000).

The story of MARCKS begins in 1982 when a protein with an apparent molecular weight of 89 kDa was found to be phosphorylated by PKC upon increasing Ca^{2+} concentration in the cytoplasm (Wu et al. 1982). MARCKS is abundant (in the brain it constitutes 0.2 % of total soluble protein (Albert et al. 1987)) and remarkably widely distributed in different tissues, but its precise physiological role has not yet been convincingly established (Ramsden 2000).

MARCKS proteins contain a highly basic region, the effector domain (also the phosphorylation site domain, or PSD). This domain is phosphorylated following activation of cellular PKC with phorbol esters, binds calmodulin in the presence of calcium with high affinity and cross-links actin filaments *in vitro* (Vergeres and Ramsden 1998). It has been inferred that positively charged residues mediate the binding of the effector domain to negatively-charged phospholipid membranes via electrostatic interactions. These interactions are presumably regulated by PKC, since phosphorylation of the serine residues decreases the affinity of the effector domain of MARCKS for membranes (Vergeres and Ramsden 1998). The N-terminal glycine residue of MARCKS proteins is myristoylated via a reaction

catalysed by protein N-myristoyl transferase; the myristoyl group is also involved in membrane binding (Swierczynski and Blackshear 1995). There is no evidence for a specific protein receptor for MARCKS at the plasma membrane, the knowledge of this intriguing protein still seems far too incomplete (Ramsden 2000).

NAP-22 - neuronal tissue-enriched acidic protein at a biochemical and a cell biological level is related to MARCKS (Wiederkehr et al. 1997), it is also a substrate of protein kinase C, can be modified by myristoylation and bind calmodulin. NAP-22 was discovered during the characterization of membrane components of the growth cones in a Triton-insoluble membrane fraction. Since molecular cloning showed that the molecular mass of this protein is 22 kDa, the protein was termed NAP-22 (Maekawa et al. 2003). The same protein is also called CAP-23 (cortical cytoskeleton-associated protein) or BASP1 (brain acid soluble protein 1) (Maekawa et al. 2003). Further studies showed the effect of calmodulin on phosphorylation of NAP-22 with protein kinase C (Maekawa et al. 2003). From the studies using knockout and knocking mice, Frey and co-workers (Frey et al. 2000) showed the participation of NAP-22 on the membrane dynamics through the regulation of the actin dynamics. In contrast, the biochemical analysis was not enough to explain its cellular function. The protein was assumed to be a hydrophilic because it had no hydrophobic sequence (Maekawa et al. 2003). Since NAP-22 showed a specific localization to the rafts, it provides a good marker to identify raft domains in neurons and in brain sections. NAP-22 immunoreactivity was detected through the whole brain and observed to be associated mainly with pre- and postsynaptic membranes and synaptic vesicles. Investigation on the changes in the localization of NAP-22 during the development of the neuronal polarity *in vitro* and *in vivo*, using cultured hippocampal neurons and developing cerebellum neurons, showed a gradual localization of the protein to the synaptic region (Maekawa et al. 2003). The main function of NAP-22 was hypothesized the organization by NAP-22 of specific lipid species such as cholesterol and others at the raft domains (Maekawa et al. 2003).

Histone H1 - Histones are highly conserved proteins that serve as the structural scaffold for the organization of nuclear DNA into chromatin. Histone H1, the most common form of the linker histone, binds to nucleosomal DNA at the point from which the DNA exits the nucleosome, and is required for higher order packing of chromatin (Alberts et al. 2002b). Histones have strikingly basic properties because a quarter of the residues in each histone are either arginine or lysine. Histone H1 is lysine-rich protein (Alberts et al. 2002b). There are six distinct subtypes of histone H1; H1a–e and H1; that display both developmental and tissue specificities (Lennox 1984). H1a and H1c can be considered as a separate subset to H1b, -d, and –e based on functional differences (Lennox 1984). Although histones are primarily a

nuclear component, the presence of cytoplasmic and plasma membrane pools of histones has been demonstrated. Extranuclear functions of cytoplasmic histone H1 were firstly identified in 1985 (Reichhart et al. 1985); currently the involvement of histone H1 in apoptosis via stimulation of cytochrome C release from outer mitochondrial membrane (Tsoneva et al. 2005) and potential tumor suppression effect (Vani et al. 2006) are investigated.

Non-nuclear isoformes of histone were surprisingly found also at the surface of plasma membrane (Henriquez et al. 2002) and constitutively expressed on neurones in the CNS. Histone H1, detected using the ANA108 antibody, was shown to be localized not to the nucleus, but in the membranes of neurones, where histone H1 revealed strong lipopolysaccharide (LPS) binding properties (Bolton and Perry 1997). Histone H1 was suggested to be an acute phase protein like LPS-binding protein (LBP) and involved in the host defence mechanism against bacterial infection as it has been shown to exhibit antibacterial properties (Hiemstra et al. 1993). There is further evidence that non-nuclear histones act as cell surface receptors as T-cells express histones H2 and H3 on their cell surface which bind to sulphated polysaccharides (Watson et al. 1995).

The up-regulation of histone H1 expression in prion and Alzheimer's disease but not in acute neurodegeneration was observed (Bolton et al. 1999), but the role of histone H1 in the diseases is still obscure. The involvement of endogenous peptide Histogranin with 80% of structural homology to a fragment-(86–100) of histone H4 in ischemic brain injury was recently discovered (Chiang et al. 2006). It was shown that Histogranin non-competitively inhibited the binding of [^{3}H]-CGP-39653, a specific N-methyl-D-aspartate (NMDA) receptor ligand, to membrane preparations of rat brain (Shukla et al. 1995). These data suggest that Histogranin may serve as an endogenous antagonist for the NMDA receptor, antagonizing NMDA-mediated convulsions, and be a model for ischemic brain injury (Chiang et al. 2006).

2. The aim of the study

Study 1

L1 is a neural cell adhesion molecule that is critical for proper nervous system development and maintenance. Mutations in human L1 gene, which is X-chromosome linked, leads to highly variable neurological diseases in hemizygous male children including mental retardation, gross motor defects, hydrocephalus and early mortality (Kenwrick et al. 2000). L1 mediates a myriad of activities important to nervous system maturation, including neurite outgrowth, adhesion, fasciculation, migration, myelination and axon guidance (Haspel and Grumet 2003). It is presumed that L1 promotes these cellular activities by interacting with a diverse group of other cell adhesion molecules, extracellular matrix components, signalling receptors and cytoskeleton elements (Haspel and Grumet 2003), but precise mechanisms by which L1 promotes a bewildering array of functions in the nervous system have not yet been investigated completely.

Therefore, the aim of this study was to isolate and characterize still unknown binding partners of L1 adhesion molecule to better understand the role of L1 in the nervous system and to identify the complexity of protein interactions, disturbed by L1 mutations. For this purpose, L1 molecule was immunoprecipitated using monoclonal α-L1 antibody 555 from L1-enriched membrane subfraction in order to co-precipitate proteins associated with L1 and isolate them by mass spectrometry analysis. After isolation and identification of putative L1 binding partners, the interaction should be confirmed using alternative binding assays and further investigated performing functional analysis in order to predict the role of the interactions in the complexity of signal transduction events.

Study 2

Polysialic acid (PSA) is a large carbohydrate found exclusively on the neural cell adhesion molecule (NCAM) (Maness and Schachner 2007). PSA is abundant in the developing brain and it is limited to areas of neural plasticity in adulthood (Seki 2003). *In vivo*, the presence of PSA is crucial to brain development (Weinhold et al. 2005) and for the regeneration of peripheral axons. Following a lesion in the adult brain, PSA is abundantly expressed by damaged and regenerating axons. The biophysical properties of PSA have been described to cause steric hindrance and shield ligand–receptor interactions (Fujimoto et al. 2001). *In vitro*, PSA can promote or limit neurite outgrowth, depending on the environment (Doherty et al. 1990). Appeared to be an important and mysterious modulator of adhesive properties of

NCAM, PSA itself has been shown to possess the ligand-binding activity (Kleene and Schachner 2004). Regarding to that, the identification of novel potential binding partners of PSA using structurally mimicking anti-idiotype antibody was indeed important confirmation for the presence of still unknown PSA functions in the nervous system (von der Ohe, unpublished data).

In this study, the main question was addressed to the characterization of association between PSA and it's identified potential binding partners MARCKS, NAP-22 and histone H1 using different binding assays. The aim was to investigate the possible direct interaction of PSA to it's ligands *in vitro* and perform the functional analysis in order to determine the involvement of new interactions in the regulation of neuroplasticity in the brain.

3. Materials

3.1. Antibodies

3.1.1. Primary antibodies

Table 2: Primary antibodies and their description.

Name	Description and working dilution	Reference
L1	rabbit polyclonal antibodies raised against the extracellular domain of mouse L1 Immunoblotting (IB): 1:7000 (5% milk/ PBS) Immunoprecipitation (IP): 1:1000 (PBS) ELISA : 1:5000 (1% BSA/ TBS)	lab of Melitta Schachner, Hamburg
L1	rabbit polyclonal anti-mouse antibodies Immunocytochemistry (IC): 1:1000 (1% BSA/ PBS)	lab of Melitta Schachner, Hamburg
L1	monoclonal antibody clone 555 raised against the amino acid sequence between second and third fibronectin type III domains IB: 1:5000 (5% milk/ PBS) IP: 1:1000 (PBS)	(Appel et al. 1993)
L1	monoclonal antibody clone 74-5H7 raised against cytoplasmic domain of L1 molecule ELISA: 1:2000 (1% BSA/ TBS)	Covance (Germany)
NCAM	rabbit polyclonal antibodies 1B2 recognizing all three isoforms of NCAM IB: 1:5000 (5% milk/ PBS) ELISA: 1:5000 (1% BSA/ TBS)	(Niethammer et al. 2002)
APP	mouse monoclonal antibody recognizing amino acids 1-16 on the Aβ of APP molecule, clone W02 ELISA: 1:1000 (1%BSA/ TBS)	Genetics company (Schlieren, Switzerland)

PSA	monoclonal antibody against PSA clone 735 IB: 1300 (5% milk/ PBS) ELISA: 1:150 (1% BSA/ TBS) IC: 1:150 (2% BSA/ PBS)	(Frosch et al. 1985)
GAPDH	mouse monoclonal antibody made against rabbit skeletal muscle GAPDH IB: 1:1000 (5% milk/ PBS) IP: 1:500 (PBS) IC: 1:250 (2% BSA/ PBS) ELISA: 1:500 (1% BSA/ TBS)	Chemicon International (Schwalbach, Germany)
CHL1	rabbit polyclonal antibodies raised against the extracellular domain of mouse CHL1 ELISA : 1:1000 (1% BSA/ TBS)	lab of Melitta Schachner, Hamburg
Thy-1.2	monoclonal mouse antibody 19E12 (cell culture supernatant) IB: 1:200 (5% milk/ PBS) IP: 1:10 (PBS)	lab of Angela Jen, London
MARCKS	Rabbit polyclonal antibodies IB: 1:1000 (5% milk/ PBS)	lab of Perry Blackshear, NIH, New-York
NAP-22 (CAP-23)	Rabbit anti-rat antiserum to the carboxyl-terminal residues [VASSEQSVAVKE] of NAP-22 IB: 1:500 (5% milk/ PBS)	lab of Pico Caroni, Basel
Histone H1	Rabbit anti-human polyclonal antibodies IB: 1:200 (5% milk/ PBS) IC: 1:100 (2% BSA/ PBS)	Santa Cruz (CA, USA)
Fc fragment	Goat anti-human polyclonal antibodies IB: 1:10000 (5% milk/ PBS)	DIANOVA (Hamburg, Germany)

tau	Polyclonal rabbit antibodies clone K9JA IC: 1:1000 (2% BSA/ PBS)	DAKO (Hamburg, Germany)
ANT1	polyclonal antibodies recognizing rabbit heart ANT1 IB: 1:500 (5% milk/ PBS)	lab of Andrea Dörner, Berlin
ANT2	polyclonal antibodies recognizing rabbit heart ANT2 IB: 1:500 (5% milk/ PBS)	lab of Andrea Dörner, Berlin
ANT total	polyclonal antibodies recognizing all isoforms of rabbit heart ANT IB: 1:1000 (5% milk/ PBS) ELISA: 1:1000 (1% BSA/ TBS)	lab of Andrea Dörner, Berlin

3.1.2. Secondary antibodies

All horseradish-coupled secondary antibodies were purchased from Dianova and used in a dilution of 1:10000 or 1:5000. For immunocytochemistry Cy2 and Cy3 secondary antibodies were obtained from Dianova and used in a dilution of 1:100.

3.2. Bacterial strains and cell lines

Different cell lines and bacterial strains which were used are described in the following tables.

Table 3: Bacterial strains

Name	Description	Reference
Escherichia coli BL21(DE3)	F-, *omp*T, hsdSB (rB -mB -), *gal, dcm* (DE3)	Novagen (Bad Soden, Germany)

Table 4: Cell lines

Name	Description
CHO	Chinese Hamster Ovary
N2A	Mouse neuroblastoma cell line established from the spontaneous tumor of a strain A
B104	Rat neuroblastoma cell line

3.3. Expression constructs

(Appel et al. 1993)

These constructs for the production of mouse L1 fragments have been obtained from the Institute for Biosynthesis of Neural Structures, Centre for Molecular Neurobiology, Hamburg.

Table 5: Expression constructs and their description.

Fragment of mouse L1 molecule	(5`)-end	(3`)-end	Size of insert	Sequence in L1 protein
Ig I-VI	Bam HI	Bam HI	1826 bp (bp 62 – 1888)	Amino acids 44-661
Ig I- II	Bam HI	Bam HI	591 bp (bp 126-717)	Amino acids 22-240
Ig III-IV	Bam HI	Bam HI	558 bp (bp 717-1275)	Amino acids 239-427
Ig V-VI	Bam HI	Bam HI	613 bp (bp1275 -1888)	Amino acids 426-661
Fn 1-5	Bam HI	Bam HI	1386 bp (bp 1888 – 3274)	Amino acids 631-1093
Fn 1-2	Bam HI	Bam HI	611 bp (bp 1888 – 2499)	Amino acids 631-826
Fn 2-3	Bam HI	Bam HI	522 bp (bp 2235- 2757)	Amino acids 709-918
Fn 4-5	Bam HI	Bam HI	517 bp (bp 2757-3274)	Amino acids 919-1093
Fn 3-5	Bam HI	Bam HI	775 bp (bp 2499 – 3274)	Amino acids 828-1093

3.4. Plasmids

Table 6: Plasmids and their description.

Plasmide name	Description	Reference
pET31F1P	prokaryotic expression plasmid for recombinant expression of proteins; ampicillin resistant; T7 Promoter	(Appel et al. 1993)
pET28	prokaryotic expression plasmid for recombinant expression of proteins; kanamycin resistant; T7 Promoter	Novagen

3.5. Cell culture/ bacterial media

3.5.1. Bacterial media

Media were autoclaved and antibiotics were supplemented before use.

Table 7: Bacterial media and their supplements.

Name	Amount		Supplements
LB-medium pH 7,4	10	g/l	Bacto-tryptone
	10	g/l	NaCl
	5	g/l	yeast extract
LB/Amp-medium	100	mg/l	ampicilin in LB-medium
LB/Amp-plates	20	g/l	agar in LB-medium
	100	mg/l	ampicillin
LB/Kan-medium	25	mg/l	kanamycin in LB-medium
LB/Kan-plates	20	g/l	agar in LB-Medium
	25	mg/l	kanamycin

3.5.2. Cell culture media

Table 8: Cell culture media and their supplements.

Name	Supplements
CHO cell medium	**Glasgow MEM** (GMEM) (with nucleotides, L-Glutamine) 10 % (v/v) fetal calf serum (FCS) 50 U/ml penicillin/streptomycin 4 mM L-Glutamine
N2A cell medium	**Dulbecco MEM** (DMEM) 10 % (v/v) FCS 50 U/ml penicillin/streptomycin 1 mM pyruvate
B104 cell medium	**DMEM** high glucose 0.4 mg/ml hydromycin 10 % FCS 5 % HS 1 % sodium pyruvate 1 % glutamate
X-1 medium (cerebellar primary neurons)	**Basal medium essential** (BME) 50 U/ml Penicillin 50 µg/ml Streptomycin

	0.1 % (w/v) BSA 10 µg/ml insulin 4 nM L-thyroxin 100 µg/ml transferrin 0.027 TIU/ml aprotinin 30 nM Na-selenite 1 % (w/v) L-glutamine 1 % (w/v) sodium pyruvate
Medium for hippocampal primary neurons	**Neurobasal-A** medium
	0.5 µg/ml gentamycin 5 mg/ml D-glucose 0.1 mg/ml transferrin 25 µg/ml insulin 2 mM glutamax-I
Medium 1 for astrocytes	**DMEM** 10 % FCS 50 U/ml Penicillin 50 µg/ml Streptomycin 50 µg/ml gentamycin
Medium 2 for astrocytes	**50 % DMEM** **50 % Ham's F12** 50 mg/ml insulin 50 mg/ml transferrin 30 nM Na-selenite 30 nM tri-iodothyroxine 10 nM hydrocortisone 100 µM putrescine 20 nM progesterone 100 µg/ml BSA 50 U/ml Penicillin 50µg/ml Streptomycin 50 µg/ml gentamycin
Pyruvate	Invitrogen (CA, USA)
Versene	Invitrogen
Trypsine/EDTA	Invitrogen
HBSS	Invitrogen

3.6. Chemicals

All chemicals were obtained from the following companies in p.a. quality: Gibco BRL (Life technologies, Karlsruhe, Germany), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), VWR (Darmstadt) and Sigma-Aldrich (Deisenhofen, Germany).

Plasmids were obtained from Clontech (CA, USA). Cell culture material was ordered from Invitrogen, Nunc (Roskilde, Denmark), Life Technologies and PAA Laboratories GmbH (Gölbe, Germany). Molecular weight standards were obtained from BioRad (München, Germany).

3.7. Molecular weight standards

1. ***Precision Plus Protein Standard***

10 µl of the *Precision Plus Protein Standard* (BioRad) were loaded on the SDS-PAGE gel

Band No.	apparent molecular weight (kDa)
1	250
2	150
3	100
4	**75 ***
5	**50 ***
6	37
7	25
8	20
9	15
10	10

*75kDa and 50 kDa proteins are more prominent for proper identification

2. ***BenchMark™ Prestained Protein Ladder***

10 µl of the *Prestained Protein* (Invitrogen) were loaded on the SDS-PAGE gel

Band No.	apparent molecular weight (kDa)
1	182.9
2	113.7
3	80.9
4	**63.8***
5	49.5
6	37.4
7	26.0
8	20.5
9	14.9
10	8.4

*Orientation band (pink in colour)

3.8. Solutions and buffers

(In alphabetical order)

Table 9: Solutions, buffers and their components.

Name	Amount		Components
BCA-Reagent A *(BCA kit)*	1	% (w/v)	Bicincholinacid disodium salt
	1.7	% (w/v)	Na_2CO_3 x H_2O
	0.16	% (w/v)	Natriumtartrat
	0.4	% (w/v)	NaOH
	0.95	% (w/v)	$NaHCO_3$ pH 11.25
BCA-Reagent B *(BCA kit)*	4	% (w/v)	$CuSO_4$ x 5 H_2O
Blocking buffer *(ELISA)*	2	% (w/v)	BSA in PBS
Blocking buffer *(Immunoblotting)*	5	% (w/v)	instant milk powder in TBS/PBS
Blocking buffer *(Immunocytochemistry)*	2	% (w/v)	BSA in PBS
	0.1	% (v/v)	Triton X-100
Blotting buffer	25	mM	Tris
(Western Blotting)	192	mM	Glycine
	10	% (v/v)	Methanol
Buffer 1	1	% (v/v)	BSA in TBS/ PBS
(Binding assay, ELISA)	1	mM	$CaCl_2$
	1	mM	$MnCl_2$
	1	mM	$MgCl_2$
Concentrating gel (10%) *(PAGE)*	1.05	ml	deionised water
	0.19	ml	1 M Tris pH 6.8
	0.02	ml	10% SDS
	0.25	ml	30% Acrylamide-Bis 29:1
	10	µl	10% APS
	4	µl	TEMED
Developing solution *(Silver Staining)*	2	% (w/v)	Na_2CO_3

	0.04	% (v/v)	Formaldehyde
Developing solution *(ELISA)*	4.75	ml	Na-acetate pH 5.0
	0.25	ml	ABTS 2% (w/v)
	3.5	µl	H_2O_2 30% (v/v)
Destaining solution *(Coomassie staining)*	45	% (v/v)	Methanol
	10	% (v/v)	Acetic acid
Digestion solution *(Production of primary neurons)*	136	mM	NaCl
	5	mM	KCl
	7	mM	Na_2HPO_4
	25	mM	HEPES
	4	mM	$NaHCO_3$
Dissection solution *(Production of primary neurons)*	240	ml	Hank's solution (Invitrogen)
	0.3	% (w/v)	BSA
	25	mM	$MgSO_4$
Elution buffer *(Affinity chromatography purification)*	0.1	M	Citric Acid pH 3.0
Elution buffer 1 *(Immunoprecipitation)*	0.1	M	Glycine pH 2.7
Elution buffer 2 *(Immunoprecipitation)*	0.5	M	NaCl
	50	mM	Ethanolamine pH 11.5
	150	mM	NaCl
	0.2	% (w/v)	CHAPS
Fixation solution *(Silver Staining)*	50	% (v/v)	Methanol
	5	% (v/v)	Acetic Acid
Fixation solution *(Colloidal Coomassie Staining)*	20	ml	Methanol
	1	ml	o-Phosphoric acid 85%
Fixation solution	1	x	PBS

(Immunocytochemistry)			
	4	% (w/v)	Paraformaldehyde
Homogenisation buffer *(Subfraction preparation)*	0.32	M	Sucrose
	1	mM	$CaCl_2$
	1	mM	$MgCl_2$
	5	mM	Tris-HCl, pH 7.5
Lysis buffer *(cerebellar primary neurons)*	20	mM	HEPES pH 7.4
	100	mM	NaCl
	1	mM	EGTA
	1	mM	Na_3VO_4
	1	% (v/v)	NP-40
	1	% (w/v)	Deoxycholate
	0.01	% (w/v)	SDS
	1	tablet	Cocktail of protease inhibitors (Rosche, Germany)
Phosphate buffered saline/ Tween *(PBS-T)*	1	x	PBS
	0.05	% (v/v)	Tween-20
Phosphate buffered saline *(PBS 2+)* *(cell surface biotinylation)*	1	x	PBS
	0.5	mM	$CaCl_2$
	2	mM	$MgCl_2$
Quenching buffer *(cell surface biotinylation)*	1	x	PBS 2+
	20	mM	Glycine
RIPA-buffer *(N2A, B104 cell lines)*	50	mM	Tris pH 7.4
	150	mM	NaCl
	1	% (v/v)	NP-40
	1	mM	EDTA
Roti-Blue staining solution *(Colloidal coomassie Staining)*	20	ml	Methanol
	20	ml	Roti-Blue 5 x concentrate (Carl Roth, Karlsruhe, Germany)
	60	ml	H_2O

Running Gel 10% *(PAGE)*	1.13	ml	deionised water
	1.5	ml	1M Tris pH 8.8
	40	µl	10% SDS
	1.33	ml	30% Acrylamide–Bis 29:1
	10	µl	10% APS
	4	µl	TEMED
Sample buffer (5x) *(Protein samples for PAGE)*	0.31	M	Tris-HCl pH 6.8
	10	% (v/v)	SDS
	5	% (w/v)	β-Mercaptoethanol
	50	% (v/v)	Glycerol
	0.13	% (w/v)	Bromphenol blue
Sensitive solution *(Silver Staining)*	0.02	% (w/v)	Sodium thiosulfate
SDS running buffer (10x) *(PAGE)*	0.25	M	Tris-HCl, pH 8.8
	1.92	M	Glycine
	1	M	SDS
Stabilizing solution *(Colloidal Coomassie Staining)*	1	M	Ammonium sulphate
Staining solution *(Coomassie staining)*	1	%	Coomassie Blue
	45	% (v/v)	Methanol
	10	% (v/v)	Acetic acid
Staining solution *(Silver staining)*	0.1	% (w/v)	$AgNO_3$
Staining solution *(Neurite outgrowth assay)*	1	% (w/v)	Na-tetraborate
	1	% (w/v)	Toluidine blue
Stopping solution *(Silver staining)*	5	% (v/v)	Acetic acid
Stopping solution *(ELISA)*	1.25	% (w/v)	Sodium fluoride
Storage solution	1	% (v/v)	Acetic acid

(Silver Staining)			
Storage buffer *(Affinity chromatography)*	1	x	PBS
	0.01	% (w/v)	Sodium azide
Stripping buffer *(Immunoblotting)*	0.5	M	NaCl
	0.5	M	Acetic acid
Sucrose stock solution *(Subfraction preparation)*	80	% (w/v)	Sucrose
TE *(10x)*	0,1	M	Tris-HCl, pH 7,5
	0.5	M	EDTA
TFB 1 *(competent E.coli)*	100	mM	RbCl
	50	mM	$MnCl_2$
	30	mM	K_2CO_3
	10	mM	$CaCl_2$
	15	%	Glycerol
TFB 2 *(competent E.coli)*	10	mM	MOPS
	10	mM	RbCl
	75	mM	$CaCl_2$
	15	%	Glycerol
Tris Buffered Saline (*TBS*)	10	mM	Tris-HCl, pH 8.0
	150	mM	NaCl
TBS-T *(ELISA)*	1	x	TBS
	0.05	% (v/v)	Tween-20
Washing buffer *(Coomassie Staining)*	25	ml	Methanol
	75	ml	H_2O
Washing buffer 1 *(Affinity chromatography)*	100	mM	Tris/HCl
	150	mM	NaCl
	1	mM	EDTA pH 8
Washing buffer 2 *(Affinity chromatography)*	25	mM	Tris/HCl pH 6.8

4. Methods

4.1. Protein biochemistry

4.1.1. Brain homogenisation

Brains were prepared from adult mice of different ages varying from 2 weeks up to 3-4 months. Mice were decapitated; brains were removed from skulls and immediately transferred into a homogeniser (Teflon pestle, 0.1μm) (Wheaton, Millville, USA). All following steps were carried out on ice. Each brain was homogenized applying 10-12 up-and-down strokes in 3 ml of homogenisation buffer containing the cocktail of protease inhibitors (Rosche, Germany) diluted to the appropriate concentration. Then brain homogenate was subjected to immunoprecipitation or chemical cross-linking experiments. For the isolation of membrane subfractions brain homogenate was put on sucrose gradient of different molarities and subjected to further centrifugation steps.

4.1.2. Preparation of membrane subfractions

4.1.2.1. Preparation of synaptosomes

The brain homogenate was centrifuged (1000 x g, 10 min, 4°C) and supernatant was further centrifuged at 17500 x g for 15 min. The resulting pellet (P1) was resuspended in homogenisation buffer, the sucrose concentration was adjusted to 0.8 M using 2.34 M sucrose stock solution and then this mixture was applied on the top of sucrose gradient (1.2 M, 1.0 M) while the resulting supernatant (soluble fraction) was collected and stored at -20°C until the further analysis was performed. The 1000 x g pellet (P2) was resuspended in homogenisation buffer and layered between 1.2 M sucrose and homogenisation buffer.

The sucrose gradients were ultracentrifuged at 25000 rpm for 2 hours at 4°C using SW 28 rotor (Beckman, Fullerton, USA). The bands at the 1.0/1.2 M interfaces which contained synaptosomes (Syn1) and synaptosomes from mossy fibers (Syn2) and the band at the 1.0/0.8 M interface deriving from P2 and containing glial membranes were collected, diluted at least two times with homogenisation buffer and subsequently centrifuged at 11000 rpm for 30 min to remove the rest of sucrose.

The pellets were resuspended in cold TE buffer and incubated for 60 min on ice in order to osmotically shock the synaptosomes. Membranes were isolated by centrifugation at 11000 rpm for 1 hour, resuspended in TE buffer pH 7.4, applied to a sucrose step gradient (1.2 M, 1.0 M, 0.85 M,) and centrifuged at 25000 rpm for 2 hours. The bands at the 1.0/1.2 M

interfaces which contained synaptosomal membranes were collected, diluted at least two times with TE buffer and centrifuged at 11000 rpm for 30 min. The half of these membranes were resuspended in TE buffer and incubated for 30 min on ice in presence of 0.15 M $NaHCO_3$, pH=11.

Treated and untreated membranes were diluted to 5 ml with appropriated buffers and centrifuged for 1 hour at 35000 rpm using SW 55i rotor (Beckman). The pellets were resuspended in TE buffer in the presence of 1% of Triton X-100, incubated for 1 hour on ice and then centrifuged at 35000 rpm for 1.5 hours. After centrifugation the supernatant – Triton X-100 soluble membrane subfraction – was collected and stored at -20°C. The pellet - Triton X-100 insoluble membrane subfraction – was resuspended in TE/1% Triton X-100 buffer and stored at-20°C.

4.1.2.2. Preparation of L1-enriched membrane subfraction

"Crude" membrane subfractions were prepared in order to enrich the amount of L1 protein. The brain homogenate was centrifuged (1000 x g, 10 min, 4°C) and supernatant was further centrifuged at 17500 x g for 15 min. The resulting pellet was subjected to one ultracentrifugation step in sucrose gradient (1.2, 1.0, 0.8 M) at 25000 rpm for 2 hours at 4°C. 1.0/0.8 M interface containing peri- and axolemma membranes and 1.2/1.0 M interface containing synaptosomes were collected, resuspended in PBS/1% Triton X-100 buffer and further applied for immunoprecipitation assay.

4.1.2.3. Preparation of rafts

Part of Triton X-100 insoluble membrane subfractions were used to isolate low density Triton-insoluble membrane subdomains (rafts). The sucrose concentration was adjusted to 1.2 M and the suspensions were overlaid with 1.1 M sucrose and 0.3 M sucrose. The gradient was centrifuged at 35000 rpm for 2 hours using SW 55i rotor (Beckman). The protein layer from the 0.3/1.1M interface which contained the lipid rafts and from the 1.1/1.2 M interface which represented the non-raft fraction were collected, diluted at least three times and centrifuged at 35.000 rpm for 1 hour to remove the rest of sucrose. The resulting pellets were used for further analysis.

4.1.3. Determination of protein concentration (BCA kit)

The protein concentration was determined using the BCA kit (Pierce, Rockford, USA). Solution A and B were mixed in a ratio of 1:50 to give the BCA solution. 10 µl of the protein

sample was applied to 200 µl BCA solution in microtiter plates and incubated for 30 min at 37°C. BSA standards ranging from 100 µg/ml to 2 mg/ml were co-incubated. The extinction of the samples was determined at 560 nm in a microtiter plate by an ELISA reader.

4.1.4. SDS-polyacrylamide gel electrophoresis

Separation of proteins was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean III system (BioRad). The size of the concentrating and running gels were followed:

Concentrating gel: height 1 cm, thickness 1 mm
4 % - 5% (v/v) acrylamide solution

Running gel: height 4.5 cm, thickness 1 mm
8-10 % acrylamide solution

10 or 15-well combs

After complete polymerisation of the gel, the chamber was assembled as described by the manufactures protocol. Up to 35 µl of sample were loaded in the pockets and the gel was run at constant voltage at 60 V for 20 min and then for the rest at 130V. The gel run was stopped when the bromphenol blue line had reached the end of the gel. Gels were then either stained or subjected to Western blotting.

4.1.5. Western blot analysis

4.1.5.1. Electrophoretic transfer

Proteins were transferred from the SDS-gel on a Nitrocellulose membrane (Protran Nitrocellulose BA 85, 0.45 µm) (BioRad) using a MINI TRANSBLOT apparatus (BioRad). After equilibration of the SDS-PAGE in blot buffer for 5 min, the blotting sandwich was assembled as described in the manufacture protocol. Proteins were transferred electrophoretically at 4°C in blotting buffer at constant voltage (90 V for 120 min or 40 V overnight). The protein precision marker standard (BioRad) was used as a molecular weight marker and to detect electrophoretic transfer.

4.1.5.2. Immunological detection of proteins on nitrocellulose membranes

After electrophoretic transfer, the membranes were removed from the sandwiches and placed protein-binding side up in glass vessels. Membranes were incubated in 10-15 ml of blocking buffer for 1 hour at RT. Afterwards the primary antibodies were added in the appropriate dilution either for 1.5 hour at RT or overnight at 4°C. The primary antibodies were removed by washing of the membrane 3 x 20 min with PBS-T. The appropriate secondary antibodies were applied for 1 hour at RT. The membrane was washed again 3 x 20 min with PBS-T and immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL).

4.1.5.3. Immunological detection using enhanced chemiluminescence

The antibodies bound to the membrane were detected using the enhanced chemiluminescence detection system (Pierce). The membrane was soaked for 1 min in detection solution (1:1 mixture of solutions I and II). The solution was removed and the blot was placed between two wrap foils. The membrane was exposed to X-ray film (Biomax-MR, Kodak, Germany) for several time periods, starting with a 2 min to 24 hours exposure.

4.1.6. Silver staining of polyacrylamide gels

After SDS-PAGE, gels were fixed with acetic acid/methanol solution for 30 min, washed in distilled water during at least 30 min and quickly rinsed with freshly prepared sensitive solution. Afterwards, gels were silvered for 30 min at 4°C, washed twice in water and developed with developing solution. When a sufficient degree of staining has been obtained, reaction was quenched with 5% acetic acid and the gels were replaced in a storage solution.

4.1.7. Coomassie staining of polyacrylamide gels

4.1.7.1. Standard Coomassie staining

After SDS-PAGE, the gels were stained in staining solution during 1 hour at RT with constant shaking. The gels were then incubated in washing solution until the background of the gel appeared nearly transparent.

4.1.7.2. Colloidal Coomassie staining

The colloidal Coomassie staining of polyacrylamide gels were performed with Roti-Blue kit (Carl Roth). After SDS-PAGE the gels were fixed in fixing solution for 60 min and subsequently incubated with Roti-Blue staining solution for 15 hours with constant shaking.

The gels were then incubated in washing solution during approximately 15 min and then replaced in stabilizing solution.

4.1.8. Drying of polyacrylamide gels

Polyacrylamide gels were incubated in 10% glycerol during 30 min at RT and dried using GelAir Drying Frame (BioRad). The gels were placed between two wet cellophane sheets, fixed using metal frame and plastic clips and dried during 6 hours at 50°C with constant blowing.

4.1.9. Immunoprecipitation

4.1.9.1. Immunoprecipitation using protein A/G magnetic beads

Aliquots of protein A or protein G magnetic beads (Invitrogen) were taken and washed twice with PBS. The appropriate amount of either polyclonal or monoclonal antibodies respectively were applied to magnetic beads and incubated for 1 hour at RT. Then probes were washed with PBS, chemical cross-linker BS-3 (Pierce) was added, and probes were incubated for 1 hour at RT. Several washing steps were done after incubation: twice with TBS, once with 0.1 M glycine pH=2.7 to quench the binding reaction of cross-linker, and twice with TBS. Then magnetic beads coupled to antibodies were resuspended in PBS and ready for use.

Triton X-100 insoluble and soluble membrane subfractions were used for immunoprecipitation. Incubation with antibody-coupled magnetic beads was proceeded overnight at 4°C. Then magnetic beads were washed several times with PBS; specific elution with low or high pH buffers was done to remove bound proteins which were applied then for SDS-PAGE. All incubation steps proceeded with constant rotation. All washing steps were done using magnet.

4.1.9.2. Immunoprecipitation using surface activated Epoxy-270 magnetic beads

Required amount of dry magnetic beads (Invitrogen) was dissolved in sodium phosphate buffer pH=7.4 and washed once. Appropriate concentration of antibodies was mixed with magnetic beads; then 1M ammonium sulphate was added to allow the binding of ligand to the surface via direct covalent binding to primary amino and sulfhydryl functional groups in proteins. Mixture was incubated at 4°C during 24 hours. Then magnetic beads were washed several times with PBS and ready for use.

Triton X-100 insoluble and soluble membrane subfractions were used for immunoprecipitation. After overnight incubation at 4°C magnetic beads were washed twice with PBS. Bound to the beads proteins were then specifically eluted with ethanolamine elution buffer pH=11.5 and applied for the SDS-PAGE. All incubation steps proceeded with constant rotation. All washing steps were done using magnet.

4.1.9.3. Immunoprecipitation using protein A/G agarose beads

Aliquot of protein A/G agarose beads was washed from ethanol with PBS. Triton X-100 insoluble and soluble membrane subfractions or crude brain homogenate were incubated with uncoupled portion of agarose beads during 3 hours at 4°C to reduce the amount of non-specifically bound proteins. Samples were centrifuged at 1000 x g for 5 min at 4°C. Required antibodies were added to the collected supernatant and incubated overnight at 4°C. A new portion of agarose beads was applied to the mixture and after 3 hours of incubation and centrifugation at 1000 x g for 5 min at 4°C the supernatant was gently removed and agarose beads were washed several times. After boiling in SDS-loading buffer bound proteins were applied for SDS-PAGE.

4.1.10. Sample preparation for mass spectrometry analysis

Protein samples were subjected to SDS-PAGE with following colloidal Coomassie staining or silver staining. All reagents and solutions were freshly prepared in order to avoid accidental contamination. Whole colloidal Coomassie stained gel was removed from stabilizing solution and packed in sterile plastic film. Required regions for sequencing were marked and the gel was sent for mass spectrometry analysis to the Cancer Research Center (Heidelberg, Germany).

Silvered gel was placed under an acrylic glass cover to allow safe handling; each stained band to be sequenced was cut out from the gel with a sterile scalpel, placed into the 1.5 ml centrifuge tube and covered with storage solution. Afterwards, the samples were sent for sequencing. MALDI-TOF analysis was done in UKE (Hamburg, Germany).

4.1.11. Methanol-chlorophorm protein precipitation

(Wessel and Flugge 1984)

Using this procedure proteins from solution were precipitated in several approaches. To one volume of solution which contained the proteins of interest four volumes of methanol were added and mixed thoroughly. Then one volume of chloroform was added and mixed

thoroughly. Finally three volumes of water were added, mixed thoroughly and centrifuged at 14000 x g for 5 min at RT. Upper phase was carefully removed. Then three volumes of methanol were added to lower phase, mixed and centrifuged at 14000 x g for 3 min to pellet proteins. Methanol was carefully removed; pellet was dried and dissolved then in appropriate buffers.

4.1.12. Chemical cross-linking using sulfo-SBED reagent

1 mg of sulfo-SBED reagent (Perbio, Bonn, Germany) according to manufacture protocol was dissolved in DMSO and incubated with 400 µg of recombinant L1-Fc and PSA-NCAM-Fc proteins for 30 min at RT and then dialysed against PBS overnight at 4°C with three buffer changes. 300 µl of Protein A magnetic beads were added to the dialysed samples and incubated for 1 hour at RT with constant rotation. Protein A magnetic beads were washed three times in PBS and incubated with 3 mg of BS-3 cross-linker (Pierce) for 1 hour at RT. After six washes with TBS, Triton X-100 solubilized synaptosomal membrane subfraction or crude brain homogenate was added and incubated for 1 h at RT. The samples were transferred to plastic dish (Nunc, Roskilde, Denmark), placed on ice and treated with UV light of 365 nm for 15 min using ultraviolet cross-linker (Amersham Life Science, Piscataway, NJ, USA). After successive washing steps using increasing concentrations of SDS (0.1, 0.5 and 1 % in PBS) proteins were eluted from the magnetic beads by boiling the sample in SDS-loading buffer for 5 min at 96°C and separated by SDS-PAGE. All procedure steps till ultra-violet treatment were done in the darkness because of high sensitivity of sulfo-SBED reagent to the light.

4.1.13. BIACORE surface plasmon resonance technology

Surface plasmon resonance-based biosensor technology allows the real-time detection and monitoring of protein binding events. In a BIACORE experiment the interacting molecule (the bait) is bound to the biosensor surface (sensor chip), whereas the purified analytes or analytes present in soluble fraction are delivered to the surface in a continuous flow through a microfluidic system. Binding to the immobilized molecule is followed by surface plasmon resonance which detects the mass concentrations at the surface. The response is directly proportional to the mass of molecules that bind to the surface. This allows, in combination with mass spectrometry, the identification of binding partners of a given molecular target immobilized on the sensor chip.

BIACORE analysis was performed using Biacore 3000 system (Biacore International AB, Freiburg, Germany). Recombinant L1-Fc, APP-Fc, PSA-NCAM-Fc or human Fc (Dianova)

were diluted to 50 µg/ml in 10 mM sodium acetate, pH 4.0 and immobilized on the surface of a Sensor Chip CM5 (Biacore International AB) using amine coupling. Brain protein extract from 2 months old mice (C57/black), obtained after 17000 x g centrifugation, was suspended in buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) and injected at a flow rate of 15 µl/min at RT. Proteins bound to the ligand were eluted directly onto matrix-assisted laser desorption/ionization (MALDI) target and identified through their peptide fingerprint. Data were analyzed with ProteinProspector sequence database (v 4.0.8) (http://prospector.ucsf.edu/).

4.1.14. Enzyme-linked immunosorbent assay (ELISA)

Several proteins were coated into 96-well microtiter plate (Nunc) in concentration 3-6 µg/ml overnight at 4°C. Non-absorbed proteins were removed; the wells were washed three times for 5 min with TBS-T and blocked with 2% BSA in TBS for 1 hour at RT. After washing wells were subsequently incubated with the putative binding proteins diluted in a wide range (25 ng/ml to 100 µg/ml) in TBS containing 1% BSA, 1mM CaCl2, 1mM MgCl2 and 1mM MnCl2 at RT. Non-bound proteins were removed and the wells were washed three times for 5 min at RT to remove non-specifically bound proteins. Specifically bound proteins were detected with corresponding certain primary antibodies and appropriate HRP-linked secondary antibodies. Protein binding was visualized by the detection reaction of HRP with ABTS reagent that resulted into a coloured product the optic density of which was measured using ELISA reader at 405 nm.

All graphic results are presented in this study as the experiment values of absorbance, where the values of TBS control were subtracted, to the concentration of titrated proteins.

4.1.15. Expression of recombinant proteins in stable transfected CHO cells

Proteins of interest were expressed using glutamine synthetase (GS) gene amplification system (Celltech, UK). The expression vector pEE 14 coding the extracellular parts of mouse L1, NCAM, CHL1, APP fused to human Fc tag was constructed in the lab of Melitta Schachner (Centre for Molecular Neurobiology, Hamburg, Germany). Murine PSA-NCAM-Fc was produced using a stably transfected TE671 cell line kindly provided by Genevieve Rougon (Institute of Developmental Biology of Marseille, Université de la Mediterranee, Marseille, France).

Rapidly thawed aliquots of appropriate cells were transferred to 25cm^2 flasks in GMEM medium with supplements. In three hours the medium was changed to remove DMSO. Then four-five passages of the cells into 75cm^2 flasks were done to enlarge the number of cells.

CHO cells transformed with the vector were selected for lines containing the increasing numbers of copies of the vector using increased level of GS inhibitor methionine sulfoximine (MSX) in a single round of amplification. The medium for the selection should not contain L-glutamine. After selection the cells were passaged into roller bottles with 90 ml of GMEM medium with low density fetal calf serum (FCS). Fc-tagged fusion proteins were secreted into the cell supernatant which was harvested after 4–5 days in culture before medium became yellow. The supernatant was centrifuged (1000 x g, 15 min, 4°C) to remove detached cells and filtered using 0.22 µm filters (Millipore, Massachusetts, USA) to remove cell debris or other particles. Then the supernatant was subjected for purification of Fc-tagged fusion proteins.

4.1.16. Affinity chromatography. Purification of recombinant proteins

The protein A sepharose column (Pfeizer, Wien, Austria) was washed with PBS with at least the 20-fold volume of the column before use. The supernatant containing Fc-tagged fusion proteins was loaded onto the column with a loading velocity of 0.25 ml/min. The column after coupling of ligand was washed with 10-fold volume of PBS and then with washing buffers 1 and 2. Specifically bound fusion protein was eluted with acetate buffer pH=2.7, collected and neutralized with 1M Tris pH=8.0. The buffer was exchanged against PBS and the fusion protein was concentrated using an Amicon ultrafiltration unit. Protein concentration was determined using the BCA protein concentration determination kit.

4.1.17. Expression of recombinant proteins in Escherichia coli

Expression of recombinant proteins in *E. coli* can be obtained by introduction of required cDNA coding for a protein of interest into an appropriate expression vector (Sambrook and Gething 1989). The choice of the vector depends on the desired position of a fusion tag, on the presence of a reporter protein function, whether internal protease sites are required and how DNA fragments will be inserted in the correct reading frame. The expression construct is created by ligation of digested vector and inserted and then transformed into the appropriate host cells carrying a repressor plasmid.

4.1.17.1. Expression of recombinant fragments of L1 extracellular domain in E. coli

(Appel et al. 1993)

Competent bacteria, E. coli strain BL21 (λDE3 lysogen), were transformed with plasmid DNA encoding several fragments of L1 protein, namely Fn 1-5, Fn 1-2, Fn 2-3, Fn 3-5, Fn 4-5, Ig I-VI, Ig I-II, Ig III-IV, Ig V-VI. The constructs were obtained in the Centre for Molecular Neurobiology (Hamburg, Germany). Positive colonies after overnight cultivating were transferred from the LB plates to 3 ml of LB medium with 100 μg/ml ampicilin. Incubation was overnight at 37°C with constant rotation. Then cultivated colonies were transferred to 250 ml of LB medium with ampicilin. Protein synthesis was induced by adding of 0.4 mM of IPTG to cultures in exponential phase (OD between 0.6 and 0.8). The induced proteins were isolated as inclusion bodies by sonifying of the harvested cells for 3 min in 10 ml of PBS on ice. Inclusion bodies collected by centrifugation for 10 min at 10000 x g at 4°C were kept frozen in PBS until use.

4.1.17.2. Expression of recombinant L1 intracellular domain in E. coli

The intracellular domain of L1 molecule was kindly provided by Gerrit Wolters (ZMNH, Hamburg, Germany). The cDNA encoding the intracellular domain of L1 was cloned into the pET28 expression vector. pET28/L1-ICD constructs were transformed into the host strain BL21 (λDE3 lysogen), in which the T7 RNA polymerase is under control of IPTG-inducible lacUV5 promoter. Therefore, the target gene is under control of T7 promoter. L1-ICD was constructed with *his*-tag located in N-terminal. Production and purification were performed as described in the QIAexpressionist handbook.

4.1.18. Preparative SDS-polyacrylamide gel electrophoresis

High amount of untagged recombinant proteins could be purified using preparative electrophoresis instrument which allows simultaneous electro-elution of multiple protein bands separated on polyacrylamide gel.

The samples (recombinant fragments of extracellular domain of L1 protein – inclusion bodies) were separated using Protean® II electrophoresis chamber (BioRad). Around 2 mg of total protein were mixed with SDS-loading buffer and without boiling were subjected onto 10 % polyacryamide gel. The electrophoresis was done with either constant electric power 40 mA or constant voltage 130 V. Then proteins were eluted from the gel using Whole Gel Eluter (BioRad) during 25 min with constant electric power 225 mA. Before collecting of proteins the polarity of eluter system was changed for 20 seconds to avoid of protein stacking to the cellophane. Around 20 fractions were collected according the molecular weight using vacuum harvester. Proteins were concentrated using Vivaspin Concentrators (Vivascience AG, Hanover, Germany) and diluted in appropriate buffers: fragments Fn 1-5, Fn 1-2, Ig I-VI,

Ig I-II, Ig III-IV, Ig V-VI in 20 mM Tris/HCl pH 8.0; Fn 2-3, Fn 4-5 in PBS; Fn 3-5 in 20 mM acetic acid pH 5.5.

4.1.19. In vitro phosphorylation assay

(Laschet et al. 2004)

Several target fusion proteins and recombinant fragments of L1 in different concentrations (0.09–0.25 nmol) were used for the direct *in vitro* phosphorylation assay. Equimolar amount of proteins was incubated with 10 nmol [^{33}P]-ATP (PerkinElmer ISOTOPE), 10-1000 μM Mg^{2+} in HEPES-Tris buffer (50 mM pH 7.3) for 20-40 min at 37°C. GAPDH (rabbit muscle; Sigma) was added in some experiments. To allow protein–protein interaction, each protein was co-dialyzed with 2-5 equimolar excess concentration of GAPDH during three hours at 4°C before phosphorylation assay *in vitro*.

Target proteins after phosphorylation were isolated from the reaction mixture with Protein A magnetic beads or methanol-chlorophorm protein precipitation, resuspended in SDS-loading buffer and subjected to SDS-PAGE. Then the gels were stained with Coomassie and dried using vacuum dryer. Radiolabbelled proteins were visualized using a PhosphorImager.

4.1.20. In vitro ADP-ribosylation assay

(Zhao et al. 2005)

Several target fusion proteins of different concentrations (0.09–0.25 nmol) were used for the *in vitro* ADP-ribosylation assay. Equimolar amount of proteins was incubated with 100 μM [^{32}P]-NAD^+ (specific activity 0.3–0.6 Ci/mmol) (Amersham Pharmacia Biotech ISOTOPE) in PBS with 0.5 mM Mg^{2+} and 1 mM Ca^{2+} for 20-40 min at 37°C. GAPDH (rabbit muscle; Sigma) was added in some experiments. To allow protein–protein interaction, each protein was co-dialyzed with 2-5 equmolar excess concentration of GAPDH during three hours at 4°C before *in vitro* ADP-ribosylation assay.

Target proteins after phosphorylation were isolated from the reaction mixture with Protein A magnetic beads or methanol-chlorophorm protein precipitation, resuspended in SDS-loading buffer and subjected to SDS-PAGE. Then the gels were stained with Coomassie and dried using vacuum dryer. Radiolabbelled proteins were visualized using a PhosphorImager.

4.1.21. Detection of phosphoimages

The dried gels were put into cassette and covered by Fuji imaging plate. The exposure time was between 24 hours and two weeks. Imaging plate was scanned using the fluorescent image analyzer Fujifilm FLA-3000 (Japan) and processed in TINA programme.

4.1.22. Aggregation assay (Fast track)

(Jacob et al. 2002)

10 µl of magnetic beads coupled to Protein A (Invitrogen) were incubated with equimolar concentrations (0.2 nmol) of recombinant L1-Fc and human Fc (Dianova) overnight at 4°C. After washing the beads were resuspended in 50 mM HEPES-Tris buffer with 1mM Mg^{2+}; a 2- or 5-fold molar excess of GAPDH and/or ATP was added to the reaction mixture. The beads were incubated at 37°C for 30 min. The samples were pipetted up-and-down several times, put on a slide and analysed under Kontron microscope (Zeiss, Germany) with 20-fold magnification using AxioVision program (release 4.6) (Zeiss).

4.2. Cell culture of cell lines

4.2.1. Cell culture of stable transfected CHO cells

CHO cells were cultured in GMEM with 10% FCS (fetal calf serum) and 1% penicillin/streptomycin 37°C, 5% CO_2 and 90% relative humidity in flasks of increasing volume (Nunc). Cells were passaged when they were confluent (usually after 3-4 days). Medium was removed and cells were detached by incubation with 2 ml of trypsin/EDTA for 2 min at 37°C. Cells were centrifuged (900 x g, 10 min, RT) and the pellet was resuspended in fresh medium. Cells were split 1:8 for the maintenance and for selection. After selection for production of L1-Fc, NCAM-Fc, CHL1-Fc, APP-Fc cells were seeded in 175 cm^2 flasks or roller bottles and medium was exchanged for GMEM containing 5% ultra low IgG FCS with L-glutamine (final concentration 2 mM) every five days.

4.2.2. Cell culture of N2A cells

Neuroblastoma N2A cells were cultured in DMEM with 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% L-glutamine at 37°C, 5% CO_2 and 90% relative humidity in 75 cm^2 flasks (Nunc) with 12 ml of medium, in 10 cm^2 round plates with 8 ml or in six-well plates (d = 35 mm; area = 9.69 cm^2) with 2 ml medium. Cells were passaged when they were confluent (usually after 3-4 days). Medium was removed and cells

were detached by incubation with 2 ml of Versene for 3 min at 37°C. Cells were split 1:5 for the maintenance or seeded in 10 cm^2 round plates for the cell surface biotinylation.

4.2.3. Cell culture of B104 cells

Neuroblastoma B104 cells were cultured in DMEM with 10% FCS, 5% HS (horse serum), 1% sodium pyruvate, 1% L-glutamine and 0.5% hydromycin at 37°C, 5% CO_2 and 90% relative humidity in 75 cm^2 flasks (Nunc) with 12 ml of medium or in 10 cm^2 round plates with 8 ml. Cells were passaged when they were confluent (twice per week). Medium was removed and cells were detached by incubation with 2 ml of trypsin/EDTA for 3 min at 37°C. Cells were split 1:10 for the maintenance or seeded in 10 cm^2 round plates for the cell surface biotinylation.

4.2.4. Cell surface biotinylation on N2A and B104 cell lines

Cell surface biotinylation was done using sulfo-NHS-LS-biotin (Pierce). This reagent reacts with primary amines at pH 7-9. Negative charge of the sulfo-group prevents the reagent from passing through cell membranes and allows the cell surface specific biotinylation. Sulfo-NHS ester group starts to hydrolyze when it is dissolved in an aqueous buffer and in 30 min the reagent becomes inactive. All these features allow to perform the specific biotinylation of cell surface proteins.

Cells taken from the incubator from 37°C were put on ice and washed twice with cold PBS-2+ to remove all components of medium. Then the cells were incubated with sulfo-NHS-LS-biotin freshly dissolved in PBS-2+ during 10 min on ice to avoid the exocytosis. Part of the cells was incubated in PBS-2+ without sulfo-NHS-LS-biotin for the negative control. The reaction of sulfo-NHS-LS-biotin with surface proteins was quenched during 5 min on ice with quenching buffer. Then the cells were carefully washed twice with PBS-2+ and 1 ml of RIPA-buffer was added to each 10 cm^2 round plate. Lysis of cells occurred at 4°C during 30 min with shaking. Lysate was centrifuged 10 min with 1000 x g at 4°C to remove DNA. Streptavidin-coupled magnetic beads were added to the lysate and this mixture was incubated overnight at 4°C in order to precipitate all biotinylated proteins. Then magnetic beads were washed twice with RIPA-buffer and boiled in SDS-loading buffer. Samples then were subjected to SDS-PAGE and biotinilated proteins were detected with Western blot using either neutravidin-conjugated with HRP or specific antibodies.

4.3. Cell culture of primary neurons

4.3.1. Preparation and cultivation of dissociated cerebellar granule cells

Mice of 6-7 days old were used for preparation. After cutting of the head brains were removed from the skull and put into ice cold dissection solution. The cerebella were pinched off from the whole brains, cleaned under binocular from the meninges, blood vessels and foreign tissues and cut into 1 mm pieces which were transferred into 15 ml falcon tube (Nunc). Tissue pieces were washed three times with dissection solution, and 5 ml of Hank's solution was applied. During this time 6 µg of trypsin and 1.5 µg of DNAse I per each cerebellum were dissolved in digestion solution, filtered and subjected to the tissue. Falcon tube was warmed in the hands during 1 min and then stayed for 5 min at RT to allow trypsin cleavage. After trypsin removing tissue was washed thoroughly three times with dissection solution. 4 µg of trypsin inhibitor were dissolved in 4 ml of dissection solution and filtered. 2.5 ml of trypsin inhibitor were applied to cerebellar pieces for 5 min and then removed. Fresh 1.5 ml portion of trypsin inhibitor was added for 5 min, removed and washed twice with dissection solution. DNAse I solution was subjected to the tissue which was resuspended into separate cells using rounded Pasteur pipettes with decreasing diameter of aperture. At least three volumes of dissection solution were added to cell suspension and centrifuged 15 min at 1000 x g at 4°C to remove dead cells. If supernatant was really turbid the centrifugation was repeated. The pellet was diluted with appropriate amount of X-1 medium. The number of the cells was counted and the cerebellar primary neurons were seeded for different approaches with determinate cell density into appropriate plates.

4.3.1.1. Neuritogenesis of cerebellar granule cells

Neurite outgrowth assay is a functional study showing the effects of different biological compounds on the length of neurites. For measuring of neurite outgrowth the cerebellar primary granule cells have been seeded at a density of $2\text{-}5*10^5$ cells/ml into 96-well plastic plate with glass bottom (PerkinElmer, Germany). The cells were cultured in chemically defined serum-free medium. Previously wells were coated with 10 µg/ml poly-L-lysine (PLL) overnight, washed with distilled water and treated under UV light during 10 min. Then proteins of interest were coated above PLL overnight in different concentrations, washed once, and fresh appropriated medium was added to wells before seeding of the cells.

In order to investigate the effect of soluble compounds they were added in different concentrations 2 hours after the cell seeding to avoid the influence on the cell attachment.

In vitro phosphorylation and ADP-ribosylation assays were performed on the recombinant mouse L1-Fc coated substrate directly to see the effect of modificated L1 on the neurite outgrowth. For that aim wells were coated with PLL (10 µg/ml) overnight and after washing with L1-Fc in the concentration 10 µg/ml also overnight. Uncoated L1-Fc was removed. For *in vitro* phosphorylation, L1-coated wells and control PLL-coated wells were incubated in Hepes-Tris buffer pH=7.4 with 1mM $MgCl_2$, GAPDH 5 µg/ml (0.135 nmol) and/or ATP 5 µg/ml (10 µM) during 40 min at 37°C. For *in vitro* ADP-ribosylation wells were incubated in PBS with 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, GAPDH 5 µg/ml (0.135 nmol) and/or NAD^+ 5 µg/ml (13 µM) during 40 min at 37°C. Then wells were washed once and cerebellar neurons were seeded.

The cells grew during 24 hours, then were washed carefully with warm PBS to remove the medium and fixed in 4% paraformaldehyde for 1 hour at RT. Staining of the fixed cells was done using staining solution for 1-1.5 hours. The dye was washed three times with distilled water. The plate was dried on the air, and the neurite outgrowth was measured at the Kontron microscope with IBAS imaging system.

4.3.1.2. Cell survival assay on cerebellar granule cells

Dissociated cerebellar granule cells were seeded into 48-well plastic plate (Nunc) at the density 10^6 cells/ml. The cells were cultured in chemically defined serum-free medium. Wells were previously coated with PLL (10 µg/ml) and then with L1-Fc for the positive control or with proteins of interest. Soluble compounds were added in different concentrations 2 hours after the cell seeding.

The cells were cultured 24-48 hours. Before cell death induction the medium was changed to fresh and soluble compounds were added again. For induction of cell death 10 µM hydrogen peroxide (cell death through oxidative stress) or 500 nM staurosporine (broad spectrum kinase inhibitor) were added for 6-24 hours. Cell death was determined by counting the numbers of calcein AM (Molecular Probes, Leiden, the Netherlands) and propidium iodide (Sigma, Germany) -positive cells. Calcein AM is a live cell marker; propidium iodide is a dead cell marker. For calcein/propidium iodide staining cells were treated with 1 µg/ml calcein and 1 µg/ml propidium iodide for 20 min at 37°C. The cells from three-four randomly chosen areas of a microscopic field (magnification 10×20) in each well were counted, and for each experimental value of four wells were measured. Cell survival was calculated as a ratio (%) of live cell number to a total cell number.

4.3.1.3. Cell surface biotinylation on cerebellar granule cells

Dissociated cerebellar granule cells were seeded into 6-well plastic plate (Nunc) at the density 10^6 cells/ml. The cells were cultured in chemically defined serum-free medium. Wells were previously coated with PLL (10 µg/ml) overnight, washed with distilled water and treat under UV light during 10 min. Cell surface biotinylation was done after 48 hours in a culture using sulfo-NHS-LS-biotin (Pierce). Cells taken from incubator from 37°C were put on ice and washed twice with cold PBS-2+. Then the cells were incubated with sulfo-NHS-LS-biotin freshly dissolved in PBS-2+ during 10 min on ice. Part of the cells was incubated in PBS-2+ without sulfo-NHS-LS-biotin for the negative control. The reaction of sulfo-NHS-LS-biotin with surface protein was quenched during 5 min on ice with quenching buffer. Then the cells were carefully twice washed with PBS-2+ and 250 µl of lysis buffer were added to each well. Lysis of cells occurred at 4°C during 20 min with shaking. Cells were scratched from plastic using scraper. Lysate was centrifuged 10 min with 1000 x g at 4°C to remove DNA. Streptavidin-coupled magnetic beads were added to the lysate and this mixture was incubated overnight at 4°C in order to precipitate all biotinylated proteins. Then magnetic beads were washed twice with lysis buffer and boiled in SDS-loading buffer. Samples then were subjected to SDS-PAGE and biotinilated proteins were detected using Western blot with either neutravidin-conjugated with HRP or specific antibodies.

4.3.2. Preparation and cultivation of dissociated hippocampal neurons

Mice of 1-2 days old were used for preparation. After cutting of the head brains were removed from the skull and put into ice cold dissection solution. The brains were cut along midline. Each half was fixed with glass needles and hippocampi were extracted, cut into 1 mm pieces and transferred into 15 ml falcon tube. Tissue pieces were washed three times with dissection solution, and 5 ml of Hank's solution was applied. During this time 6 µg of trypsin and 1.5 µg of DNAse I per each hippocampus were dissolved in digestion solution, filtered and subjected to the tissue. Falcon tube was warmed in the hands during 1 min and then stayed for 5 min at RT to allow the trypsin cleavage. After trypsin removing tissue was washed thoroughly three times with dissection solution. 4 µg of trypsin inhibitor were dissolved in 4 ml of dissection solution and filtered. 2.5 ml of trypsin inhibitor were applied to hippocampi for 5 min, removed and the rest 1.5 ml were added for 5 min. 1 mg of DNAse I was dissolved in 2.5 ml of dissection solution and subjected to the tissue which was resuspended into separate cells using rounded Pasteur pipettes with decreasing diameter of aperture. At least three volumes of dissection solution were added to the cell suspension and centrifuged 15 min at 900 x g at 4°C to remove dead cells and cell debris. The pellet was diluted with appropriate amount of

neurobasal medium with supplements. The number of the cells was counted in a Neubauer cell chamber.

Previously coverslips (d=14 mm) were first cleaned by extensive washing with acetone and sonification. Then they were washed thoroughly 20-30 times with distilled water and air-dried. Coverslips were coated with PLL (10 µg/ml) overnight and then with matrigel (Becton Dickinson). Glass cylinders were sterilised by autoclaving and stuck to coverslips with silicon (GE Bayer Silicones, Germany). The hippocampal primary neurons were seeded into cylinders to reduce the useful number of cells per coverslip at the cell density 10^6 cells/ml for immunocytochemistry. The cells were cultured in chemically defined serum-free medium.

4.3.3. Preparation and cultivation of astrocytes from the whole brain

Mice of postnatal day zero to two were used for preparation. After cutting of the head brains were removed from the skull and put into ice cold Hank's solution. The brains were cleaned from meninges, blood vessels and foreign tissues and cut into 2 mm pieces which were transferred into 15 ml falcon tube. Tissue pieces were washed three times with cold Hank's solution. Then brain pieces were homogenized using Pasteur pipette in medium 1. Further tissue was homogenized by pressing through the 200, 150 and 30 µm nylon meshes. The resulting cell suspension was diluted in pre-warm medium 1, cell number was counted and the cells were seeded in appropriate density onto PLL-coated dishes and plates.

In 24 hours medium 1 was carefully aspirated and pre-warmed medium 2 was added to the cultures. Every 2-3 days the medium 2 was changed to fresh and in 7 days astrocyte cultures were ready for use.

4.3.4. Preparation of exosomes from cerebellar primary neurons and astocytes cultures

(Faure et al. 2006)

The cells were seeded at the density 10^6 cells/ml onto PLL-coated flasks (25 cm^2). Before exosome preparation the cells were cultivated overnight in a serum-free medium and then treated with or without 1 µM ionomycin (final concentration) for 2 hours at 37°C. Overnight medium and ionomycin-treated and untreated culture supernatants were collected and centrifuged for 10 min at 1000 x g and for 15 min at 17000 g to remove cellular debris. Membrane vesicles were collected by centrifugation at 100000 x g for 1 hour at 4 °C using a Beckman SW55i rotor. Vesicles (the pellet) were directly dissolved in SDS-sample buffer.

Proteins contained in supernatant were precipitated with methanol-chlorophorm method, dissolved in SDS-loading buffer and subjected for SDS-PAGE.

4.4. Immunocytochemistry

For immunocytochemistry dissociated hippocampal and cerebellar cell cultures were seeded onto coverslips (d=12 mm) at the density 2-5*10^5 cells/ml. Coverslips were first cleaned by extensive washing with acetone and sonification. Then they were washed thoroughly 20-30 times with distilled water and air-dried. Coverslips were coated with PLL (10 μg/ml) overnight and then with matrigel (Becton Dickinson), laminin containing mixture. The cells were cultured in chemically defined serum-free medium during 4-5 days.

4.4.1. Immunocytochemistry of living cells

In order to prove cell surface localization of proteins using immunocytochemistry the staining of living cells was used.

1 hour before experiment the medium was exchanged to fresh. Primary antibodies were added in appropriate concentration to the cells just taken out from the incubator. The cells were immediately put back and incubated with antibodies during 15 min. Then the cells were carefully washed three times with warm PBS and 4% paraformaldehyde (PF) was added for 60 min to fix the cells.

In order to prevent the exocytosis of cytoplasmic proteins and their non-specific binding to the membrane the part of cells were put on ice for 15 min before application of primary antibodies. Then the cells were incubated with primary antibodies of appropriate concentration during 40 min on ice, washed three times with cold PBS and fixed in cold 4% PF.

Fixing solution was washed out three times with PBS. Blocking solution with 0.1% of Triton X-100 was subjected to the fixed cells for 10 min at RT with additional aim to permeabilise cell membranes. Appropriate secondary antibodies coupled to fluorescent dye (Cy2 or Cy3) were applied to the cells in the dilution 1:100 for 30 min at RT in the darkness and then washed three times with PBS. Coverslips with fixed stained cells were put on the slices and embedded into Aqua PolyMount medium (Polysciences, Eppelheim, Germany).

4.4.2. Immunocytochemistry of fixed cells

The medium was removed from the coverslips; the cells were washed with PBS and fixed with 4% PF for 60 min at RT. Fixing solution was removed and washed three times with PBS. Blocking solution was applied to the fixed cells for 15 min and then primary antibodies were added in appropriate concentration for 30 min at RT. After washing with PBS appropriate secondary antibodies coupled to fluorescent dye (Cy2 or Cy3) were applied to the cells in the dilution 1:100 for 30 min at RT and then washed three times with PBS. Coverslips with fixed stained cells were put on the slices and embedded into Aqua PolyMount medium (Polysciences).

4.4.3. Confocal laser-scanning microscopy

All images of dissociated primary neurons were obtained with a Zeiss LSM510 argon-crypton confocal laser-scanning microscope equipped with a 60x oil-immersion objective lens. Images were scanned with a resolution of 512x512. Detector gain and pinhole were adjusted to give an optimal signal to noise ratio.

4.5. Molecular biology

4.5.1. Bacterial expression system

(Sambrook and Gething 1989)

For recombinant expression of proteins in *Escherichia coli* (Sambrook and Gething 1989) the corresponding cDNA of a protein was inserted in the frame with ATG start codon and purification tag of the convenient expression plasmid. The appropriate *Escherichia coli* strain was transformed with the expression construct and streaked on LB plates supplemented with appropriate antibiotic. 20 ml of LB pre-culture were inoculated by a single colony and incubated overnight at 37°C with constant rotation. Afterwards pre-culture was transferred into a 400 ml LB culture and incubated at 37°C under constant rotation until the culture had reached an optical density of 0.6. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration 1 mM) to the culture with further incubation for 2-6 hours at 37°C. Bacteria were collected by centrifugation (4000 x g, 10 min, 4°C) and stored at -20°C. Protein expression was monitored by removing 1 ml aliquots of the culture every hour after IPTG induction. Bacteria were pelleted, lysed in SDS-loading buffer and applied on SDS-PAGE for Coomassie or silver staining.

4.5.2. Production of chemically competent bacteria

BL21 (DE3) bacterial strain was streaked on LB-plates containing the appropriate antibiotic and incubated overnight at 37°C. Single colonies were picked and used for inoculation of 10 ml of an overnight culture. 1 ml of the overnight culture was added to 100 ml of pre-warmed LB medium containing antibiotics and shaked until an optical density of 0.5 was reached (approximately 90–120 min). The culture was cooled down on ice, transferred to sterile round-bottom tubes and centrifuged at low speed (4000 x g, 5 min, 4°C). The supernatants were discarded and the cells were resuspended in cold TFB1 buffer (30 ml for a 100 ml culture). The suspension was kept on ice for additional 90 min. Then the cells were collected by centrifugation (4000 x g, 5 min, 4°C), the supernatant was discarded again and the cells resuspended in 4 ml ice-cold TFB2 buffer. Aliquots of 100 µl were prepared, frozen in dry ice-ethanol mix and stored at –80°C.

4.5.3. Determination of DNA concentration

DNA concentration was determined spectroscopically using an Amersham-Pharmacia spectrometer. The absolute volume necessary for measurement was 50 µl. For determining the concentration of DNA it was diluted 1:50 with water and the solution was pipetted into a 50 µl cuvette. Concentration was determined by measuring the absorbance at 260 nm, 280 nm and 320 nm. Absorbance at 260 nm had to be higher than 0.1 but less than 0.6 for reliable determinations. A ratio of A260/A280 between 1.8 and 2 indicated a sufficient purity of the DNA.

4.5.4. Transformation of bacteria

(Sambrook and Gething 1989)

To 100 µl of competent BL21 (DE3) either 50-100 ng of plasmid DNA or 20 µl of ligation mixture were added and incubated for 30 min on ice. After a heat shock (2 min, 42°C) and successive incubation on ice (3 min), 800 µl LB-medium were added to the bacteria and incubated at 37°C for 30 min. The cells were then centrifuged (10000 x g, 1 min, RT) and the supernatant removed. Cells were resuspended in 100 µl LB medium and plated on LB plates containing the appropriate antibiotics. Plates were incubated overnight at 37°C.

4.6. Computer analysis

4.6.1. Sequence analysis

Protein sequences obtained during BIACORE experiments combined with mass spectrometry were analysed in order to get matched proteins in international sequence database PROTEIN PROSPECTOR (version 4.0.6) used for Proteomics experiments (http://prospector.ucsf.edu/).

4.6.2. Statistical analysis

Difference between groups in discontinuous variables (neuritogenesis, ELISA, cell survival, aggregation assay) was analyzed by Student's t-test for independent groups.

Statistical comparisons between two groups (cell survival assay) were made using the Kolmogorov-Smirnov two sample test (http://www.physics.csbsju.edu/stats/).

Results are expressed as mean ± standard error mean. p-values < 0.05 were classified as statistically significant for all statistical tests.

5. Results

5.1. Study 1:

Identification, characterization and functional analysis of putative binding partners of the neural cell recognition molecule L1

5.1.1. Preparation of L1-containing membrane subfractions

For the immunoprecipitation of L1 protein synaptosomal membrane subfractions were prepared from 2-2.5 months old C57BL/6J wild type mice. Triton X-100 soluble and insoluble and "crude" L1-enriched membrane subractions were subjected for SDS-PAGE and analysed using Western blot with polyclonal α-L1 antibodies (**Figure 6**).

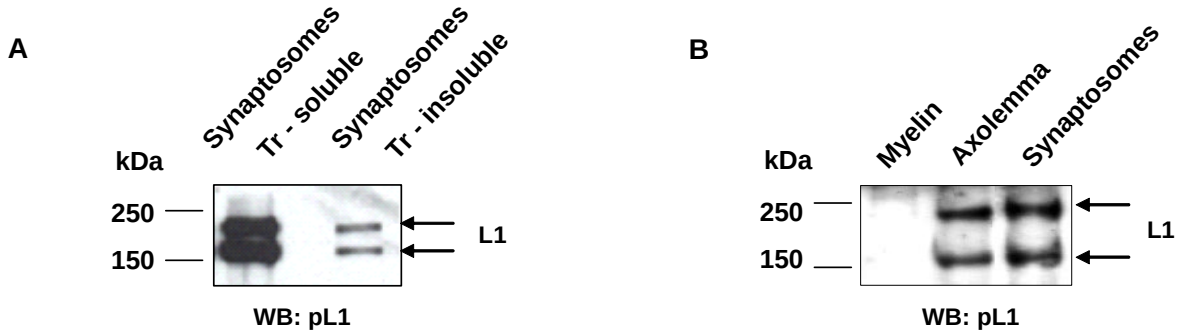


Figure 6: Distribution of L1 protein in different membrane subfractions.

(A) Western blot (WB) analysis showed the presence of L1 protein in both Triton X-100 soluble and insoluble synaptosomal fractions. The amount equivalent to 50 µg of the total protein was applied per each lane. High amounts of L1 are observed in Triton X-100 soluble fraction. **(B)** "Crude" membrane preparation. Myelin, peri- and axolemma and synaptosomal membrane subfractions were obtained using a single ultracentrifugation step in a sucrose gradient (0.8, 1.0, 1.2M). The amount equivalent to 10 µg of the total protein was applied per each lane. Western blot analysis using polyclonal α-L1 antibodies revealed the enrichment of L1 in axolemma and synaptosomal membranes.

5.1.2. Immunoprecipitation of L1 protein from synaptosomal subfraction using surface activated Epoxy-270 magnetic beads

Immunoprecipitation of L1 protein from Triton X-100 soluble synaptosomal or "crude" membrane subfractions was performed in order to identify novel potential binding partners which co-precipitated in a complex with L1. For this purpose surface activated Epoxy-270 magnetic beads which allow direct covalent binding of antibodies to the bead surface without

any cross-linker were used. Monoclonal α-L1 555 antibody was coupled to the beads. Non-specific IgG were used as a control. Antibodies coupled to the beads were then incubated with Triton X-100 soluble synaptosomal or "crude" membrane subfractions. L1 molecule and co-precipitated proteins were specifically eluted using ethanolamine elution buffer, at pH=11.5, and subjected to Western blot analysis using polyclonal α-L1 antibodies (**Figure 7A**) or to silver staining (**Figure 7B**) or to Coomassie staining for mass spectrometry analysis. Western blot and silver staining revealed immunoprecipitation of the 200 kDa full-length L1 and the 140 kDa proteolytic L1-fragment by the monoclonal L1 antibody, while no L1 was detectable when the control antibodies was used. Several silver stained protein bands were only detected in the immunoprecipitate obtained with monoclonal L1 antibody but not in the immunoprecipitate using the control antibodies (**Figure 7B**, marked by red arrows). For sequence analysis colloidal Coomassie staining of the gel was performed; the regions contained proteins of interest were selected and analyzed by mass spectrometry.

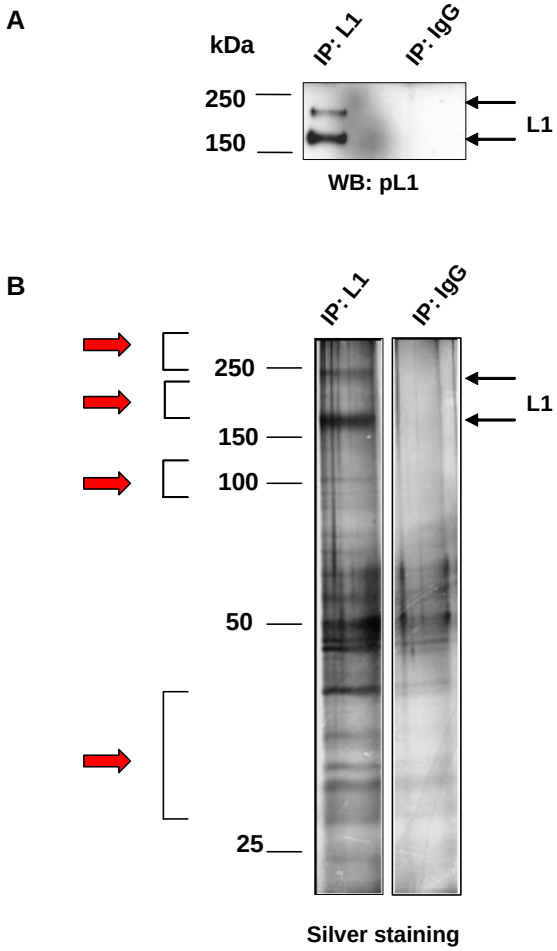


Figure 7: Immunoprecipitation of L1 protein from synaptosomal membrane subfraction using surface activated Epoxy-270 magnetic beads.

L1 protein was precipitated using monoclonal α-L1 555 antibody immobilized on the surface activated Epoxy-270 magnetic beads from synaptosomal membrane subfraction.

(A) Western blot (WB) analysis showed the specific immunoprecipitation (IP) of L1 protein. The bands corresponding to molecular weight of L1 molecule are marked by arrows. Detection was performed with polyclonal α-L1 antibodies.

(B) Silver staining of the proteins co-immunoprecipitated with L1. The bands corresponding to molecular weight of L1 molecule are marked by black arrows. Several bands were only co-precipitated with L1, but not with control non-specific IgG. Regions with proteins of interest were marked (red arrows) and analysed by mass spectrometry.

5.1.3. Mass spectrometric analysis of co-precipitated with L1 proteins

Matrix-assisted laser desorption/ionisation – mass spectrometry (MALDI-MS) (Mortz et al. 1994;Jensen et al. 1997) combined with postsource decay (PSD) fragment ion mass analysis has identified four co-precipitated proteins as possible L1 binding partners. Analysis was performed for all proteins present in the regions. The proteins not detectable in the control were accepted as potential binding partners. One of them is the cytoskeleton protein **ß-spectrin 2** which molecular weight is 251 kDa. It was described previously to be indirectly associated with L1 via ankyrin (Davis and Bennett 1994). No proteins were identified in the region corresponding to molecular weight of 200 and 100 kDa. From the region corresponding to molecular weight of 25-45 kDa three proteins were identified which were not shown previously to be associated with L1 adhesion molecule. The first was the glycolytic enzyme **glyceraldehyde-3-phosphate dehydrogenase (GAPDH),** which showed a molecular weight of 37 kDa. **ATP/ADP carrier,** the member of mitochondrial **adenine nucleotide translocator** family **(ANT)** with a molecular weight of 32 kDa, was the second identified protein. And the third protein, showed a molecular weight of 30 kDa, was GPI-linked integral membrane protein **Thy-1,** a member of Ig superfamily.

5.1.4. Co-immunoprecipitation of L1 adhesion molecule and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from synaptosomal subfraction

Immunoprecipitation of L1 protein from Triton X-100 soluble synaptosomal and "crude" membrane subfractions was performed in order to confirm mass spectrometry results. For this purpose monoclonal α-L1 555 antibody were coupled to the surface activated Epoxy-270 magnetic beads. For the control α-PSA monoclonal antibody 735, non-specific IgG and magnetic beads without antibodies were used. Beads were then incubated with Triton X-100 soluble synaptosomal or "crude" membrane subfractions. L1 molecule and co-precipitated proteins were specifically eluted using ethanolamine elution buffer (pH=11.5) and applied for Western blot analysis. Detection using monoclonal α-GAPDH antibody confirmed the co-precipitation of GAPDH with L1 protein (**Figure 8**).

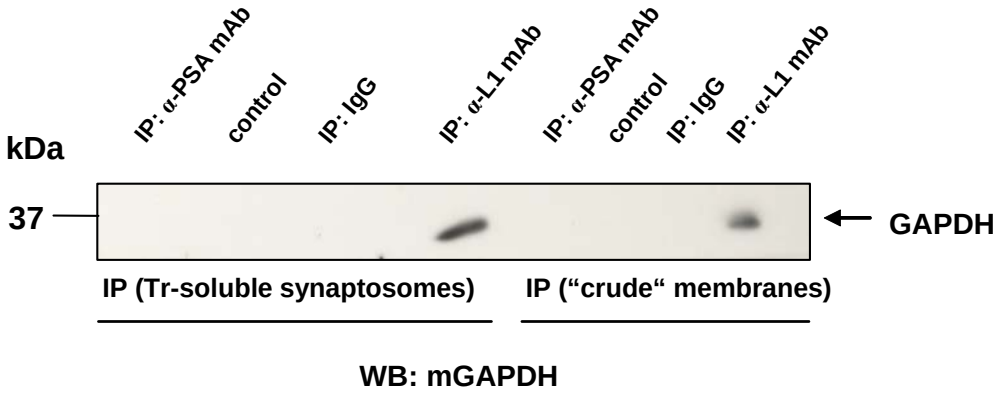


Figure 8: Co-immunoprecipitation of L1 adhesion molecule and GAPDH from synaptosomal subfractions.

L1 protein was precipitated using monoclonal α-L1 555 antibody immobilized on the surface activated Epoxy-270 magnetic beads from Triton X-100 soluble synaptosomal and "crude" membrane subfractions. Western blot (WB) analysis using monoclonal α-GAPDH antibody revealed co-precipitation of GAPDH with L1 protein, but not with control antibodies. (IP – immunoprecipitation; Tr – Triton X-100).

5.1.5. Co-immunoprecipitation of L1 adhesion molecule and adenine nucleotide translocator (ANT) from Triton X-100 soluble synaptosomal subfraction

Immunoprecipitation of ANT protein from Triton X-100 soluble synaptosomal subfraction was performed in order to confirm mass spectrometry results. For this purpose polyclonal antibodies against ANT1 and ANT2 were incubated with Triton X-100 soluble synaptosomal subfraction. Co-precipitated with ANT proteins were isolated using protein A/G agarose beads. For the control non-specific IgG and agarose beads without antibodies were used. Isolated proteins were applied for Western blot analysis. Detection using monoclonal α-L1 antibody showed the co-precipitation of 190 kDa and unknown 100 kDa cleavage fragments of L1 with ANT 1 and ANT2 but not with control antibodies (**Figure 9**).

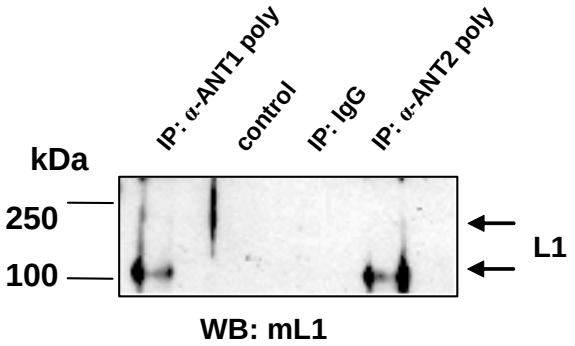


Figure 9: Co-immunoprecipitation of L1 adhesion molecule with ANT1 and ANT2 from Triton X-100 soluble synaptosomal subfraction.

ANT1 and ANT2 were precipitated using polyclonal antibodies from Triton X-100 soluble

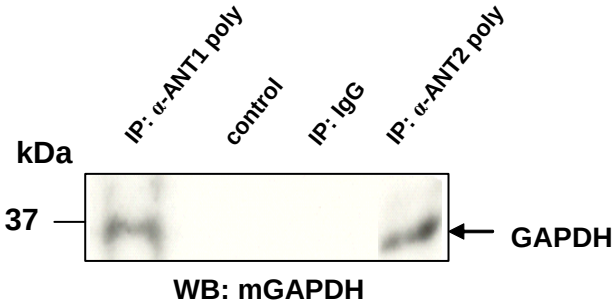
synaptosomal subfraction. Western blot (WB) analysis using monoclonal α-L1 555 antibody showed the co-precipitation of 190 kDa and 100 kDa fragments of L1 with ANT1 as well as with ANT2 but not with control antibodies (IP – immunoprecipitation).

5.1.6. Co-immunoprecipitation of GAPDH and ANT from Triton X-100 soluble synaptosomal subfraction

Since GAPDH and ANT both were identified as co-precipitated in a complex with L1 proteins, possible association between GAPDH and ANT was checked. For that purpose polyclonal antibodies against ANT1 and ANT2 were incubated with Triton X-100 soluble synaptosomal subfraction. Proteins co-precipitated with ANT were isolated using protein A/G agarose beads. For the control non-specific IgG and agarose beads without antibodies were used. Isolated proteins were applied for Western blot analysis. Detection using monoclonal α-GAPDH antibody confirmed the co-precipitation of GAPDH with ANT 1 as well with ANT2 (**Figure 10**).

Figure 10: Co-immunoprecipitation of GAPDH with ANT1 and ANT2 from Triton X-100 soluble synaptosomal subfraction.

ANT1 and ANT2 were precipitated using polyclonal antibodies from Triton X-100 soluble synaptosomal subfraction. Western blot (WB) analysis using monoclonal α-GAPDH antibody revealed co-precipitation of GAPDH with ANT1 as well as with ANT2 but not with control antibodies (IP – immunoprecipitation).



5.1.7. Production of recombinant L1-Fc, NCAM-Fc and PSA-NCAM-Fc

The extracellular domains of L1, NCAM and PSA-NCAM adhesion molecules were produced in order to investigate the protein interaction in different *in vitro* binding assays. For this purpose, stably transfected CHO and TE671 cells were cultured to express recombinant L1, NCAM and PSA-NCAM, fused to human Fc tag, which were secreted into the cell supernatant. Cell culture supernatants were harvested successively and applied for purification via the human Fc-tag using a Protein A affinity column. After purification of the fusion proteins the bands of approximately 200, 150 and 160 kDa corresponding to L1-Fc, NCAM-

Fc and PSA-NCAM-Fc were seen on the Western blot after detection using α-Fc polyclonal antibodies (**Figure 11**).

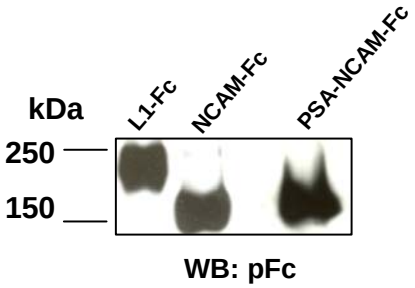


Figure 11: Production of recombinant L1-Fc, NCAM-Fc and PSA-NCAM-Fc proteins.

The supernatants of stably transfected CHO and TE671 cells producing recombinant L1-Fc, NCAM-Fc and PSA-NCAM-Fc were applied for Protein A sepharose column for the purification via affinity chromatography. Bound proteins were specifically eluted with low pH buffer, concentrated using Vivaspin column and analysed by Western blot (WB). After detection using polyclonal α-Fc antibodies the single bands approximately 200, 150 and 160 kDa of molecular weight were seen; they are corresponding to L1-Fc, NCAM-Fc and PSA-NCAM-Fc proteins.

5.1.8. BIACORE technology

In an alternative approach to identify proteins binding to the extracellular domain of L1, BIACORE analysis combined with mass spectrometry was conducted. Extracellular domains of L1, APP and PSA-NCAM fused to Fc or Fc alone were immobilized on the sensor chip and a 17000 x g supernatant of a mouse brain homogenate was applied. The set of binding/regeneration experiments was repeated twice. Proteins bound to the targets were recovered from the chip and analysed by mass spectrometry analysis. GAPDH was identified to bind to L1-Fc, but not to APP-Fc, PSA-NCAM-Fc or Fc. Mass spectrometry analysis (details are shown in **Table 10**) demonstrated that the matched peptides covered 23% (77 from 333 amino acids) of GAPDH protein.

These results suggest that GAPDH binds to the extracellular domain of L1 adhesion molecule.

m/z Submitted	MH^+ Matched	Delta ppm	Start End	Missed Cleavages	Database Sequence
1697.94	1697.89	27	196 213	1	(R)DGRGAAQNIIPASTGAAK (A)
1779.94	1779.79	80	308 321	0	(K)LISWYDNEYGYSNR (V)
2291.09	2291.05	16	85 105	0	(K)WGEAGEAYVVESTGVFTTMEK (A)
2595.35	2595.36	-4.0	161 184	0	(K)VIHDNFGIVEGLMTTVAITATQK (T)

Table 10: BIACORE technology combined with mass spectrometry analysis revealed the GAPDH as a direct binding partner of the L1 extracellular domain.

BIACORE experiments were done using a BIACORE 3000 system. L1-Fc, APP-Fc, PSA-NCAM-Fc and human Fc fragment were immobilized on the sensor chip, and brain soluble fraction was applied.

GAPDH was identified as a protein bound to L1-Fc, but not to other proteins. Mass spectrometry analysis demonstrated that the matched peptides covered 23% (77 from 333 amino acids) of GAPDH protein. On the table the first column is corresponding to fragment mass applied for sequence. Mass of matched fragment is placed in the second column. Position of sequenced peptides within the protein is indicated by start and end points. Identified amino acid sequences are shown in the last column.

5.1.9. Chemical cross-linking experiments using Sulfo-SBED reagent

Chemical cross-linking approach using trifunctional reagent Sulfo-SBED was performed in order to confirm the direct interaction between extracellular domain of L1 and GAPDH and also to investigate the possible interaction between L1 and its potential binding partners Thy-1 and ANT. This technique allows to trap direct interactions between bait and prey binding partner and to isolate the bait/binding partner complex after stringent washing.

L1-Fc and control NCAM-Fc and PSA-NCAM-Fc (around 500 µg of total protein per one experiment) were coupled to Sulfo-SBED cross-linker. Then this complex was immobilzed onto Protein A magnetic beads via BS-3 cross-linker and incubated either with crude brain homogenate or with Triton X-100 soluble synaptosomal membrane subfraction. After exposure to UV light proteins bound to L1-Fc, NCAM-Fc and PSA-NCAM-Fc were isolated and subjected to SDS-PAGE under reducing conditions leading to a transfer of the biotin label from the bait proteins to the bound proteins. Western blot using streptavidin-HRP showed a biotin-labelled band of 37 kDa when using L1-Fc and NCAM-Fc for crosslinking, but not when using PSA-NCAM-Fc (**Figure 12B**). Western blot analysis using monoclonal α-GAPDH antibody confirmed that this 37 kDa band corresponded to GAPDH (**Figure 12A**).

The results confirmed the direct interaction between GAPDH and extracellular domain of L1. Also the interaction between GAPDH and extracellular domain of NCAM was observed. ANT and Thy-1.2 were not detected as proteins cross-linked with L1 or NCAM extracellular domains.

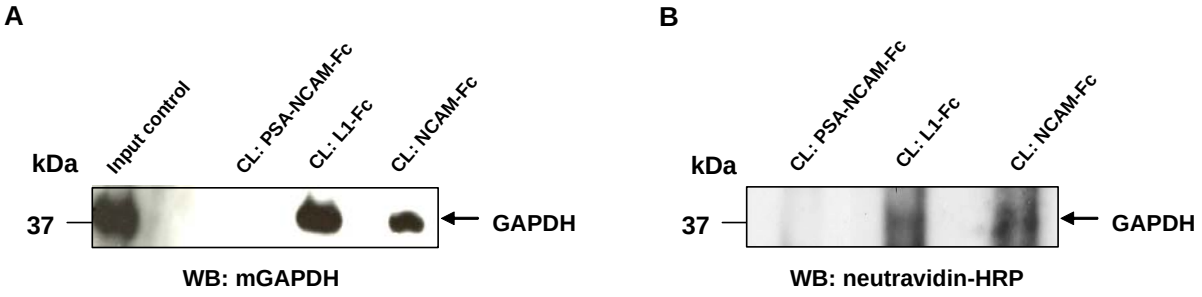


Figure 12: Chemical cross-linking using L1-Fc and control NCAM-Fc and PSA-NCAM-Fc.

L1-Fc, NCAM-Fc and PSA-NCAM-Fc proteins were conjugated with Sulfo-SBED reagent, immobilized onto the Protein A magnetic beads and incubated with either crude brain homogenate or with Triton X-100 soluble synaptosomal membrane subfraction. After exposure to UV light all proteins specifically bound to the targets were subjected to Western blot (WB) analysis and detected either using monoclonal α-GAPDH antibody **(A)** or neutravidin conjugated with HRP **(B)** (CL – cross-linking).

5.1.10. Characterization of the binding of ANT to extracellular domain of L1 using an ELISA approach

Co-immunoprecipitation experiments revealed the association between L1 molecule and ANT. To check the possible direct binding between ANT and the extracellular L1 domain an ELISA assay was performed. ANT, purified from heart, was coated on the absorbent plastic surface and recombinant L1-Fc in different concentrations was incubated with coated ANT. Detection of the potential interaction was done using polyclonal α-L1 antibodies. ELISA assay showed the direct interaction between ANT and extracellular domain of L1 (**Figure 13**).

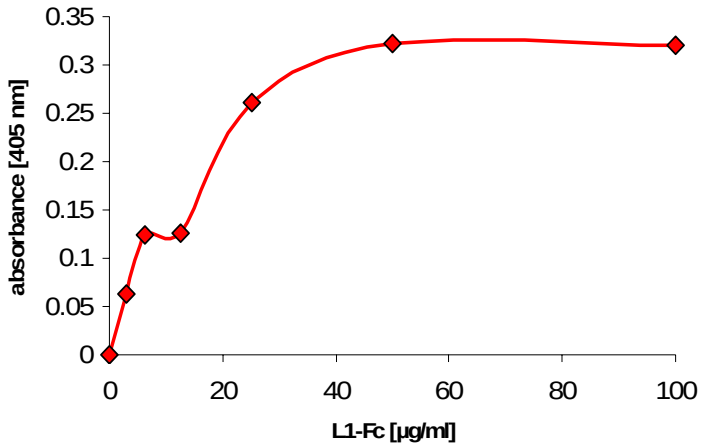


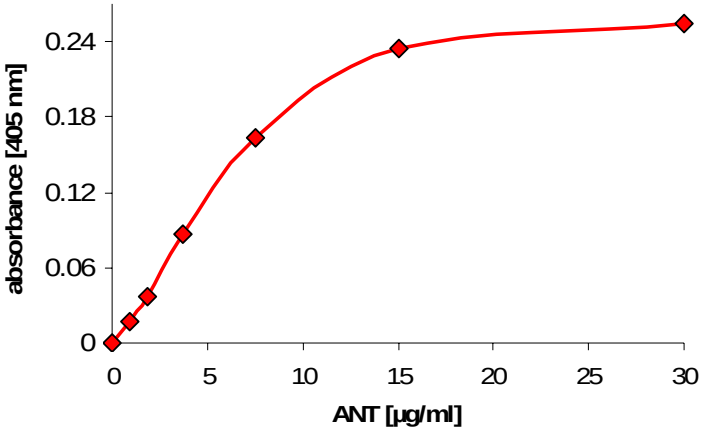
Figure 13: ELISA assay for the evaluation of ANT and L1 extracellular domain interaction. Immobilized ANT (5 µg/ml) was incubated with L1-Fc in different concentrations (1.5 – 100 µg/ml). Detection of the potential interaction was done using polyclonal α-L1 antibodies.

5.1.11. Characterization of the binding of ANT to GAPDH using an ELISA approach

GAPDH molecule and ANT were found to be simultaneously co-immunoprecipitated with L1. The possibility of the direct interaction between GAPDH and ANT was checked using an ELISA assay. GAPDH was coated on the absorbent plastic surface. ANT purified from heart in different concentrations was incubated with coated GAPDH. Detection of the potential interaction was done using polyclonal α-ANT total antibodies. ELISA assay has demonstrated the direct interaction between GAPDH and ANT (**Figure 14**).

Figure 14: ELISA assay for the evaluation of ANT and GAPDH interaction.

Immobilized GAPDH (5 µg/ml) was incubated with ANT purified from heart in different concentrations (0.5–30 µg/ml). Detection of the potential interaction was done using polyclonal α-ANT total antibodies.



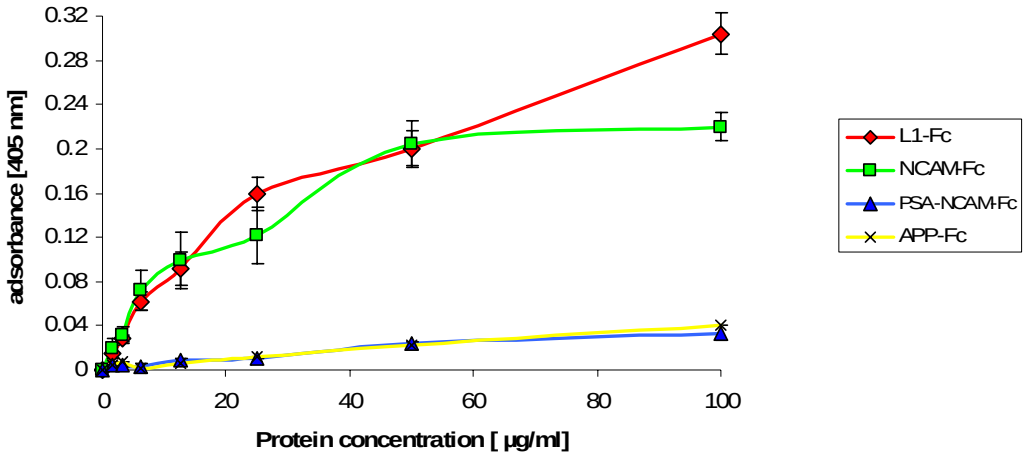
5.1.12. Characterization of the binding of GAPDH to the L1 extracellular domain using an ELISA approach

BIACORE technique and the chemical cross-linking experiment showed that GAPDH can directly bind to the extracellular domain of L1. To further substantiate the interactions an ELISA assay was performed. GAPDH was coated on the absorbent plastic surface. L1-Fc and control NCAM-Fc, APP-Fc and PSA-NCAM-Fc in different concentrations were incubated with coated GAPDH. Detection of the potential interaction was done using polyclonal α-L1, polyclonal α-NCAM, monoclonal α-APP and monoclonal α-PSA 735 antibodies. ELISA assay revealed that GAPDH directly binds to extracellular domains of L1 and NCAM, but not of PSA-NCAM and APP adhesion molecules (**Figure 15A**).

ELISA assay where GAPDH was not a substrate coated but used as a titrated protein never resulted in visualized binding because of the specificity of monoclonal α-GAPDH antibody, used for the detection of the potential binding.

As an additional control for interaction between GAPDH and L1 extracellular domain an ELISA assay was performed with another enzyme of glycolytic pathway - phoshoglucose isomerase (PGI). GAPDH and PGI were coated on the absorbent plastic surface. L1-Fc in different concentrations was incubated with coated GAPDH and PGI. Detection of the potential interaction was done using polyclonal α-L1 antibodies. ELISA assay showed binding of GAPDH, but not of PGI, to L1 extracellular domain (**Figure 15B**).

A



B

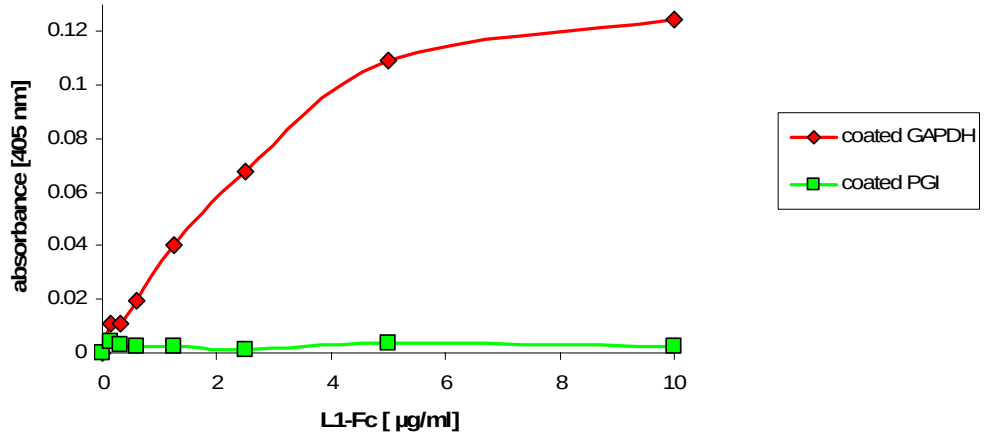


Figure 15: ELISA assay for the confirmation of direct interaction of GAPDH with L1 extracellular domain.

(A) Immobilized GAPDH (5 µg/ml) was incubated with L1-Fc and control NCAM-Fc, APP-Fc and PSA-NCAM-Fc in different concentrations (1.5 – 100 µg/ml). Detection of the potential interaction was done using polyclonal α-L1, polyclonal α-NCAM, monoclonal α-APP and monoclonal α-PSA 735 antibodies. Mean values ± SEM (n = 4) are shown. **(B)** GAPDH and phosphoglucose isomerase (PGI) were coated on the absorbent plastic surface (5 µg/ml). L1-Fc in different concentrations (0.3 – 20 µg/ml) was incubated with GAPDH and PGI. Detection of the potential interaction was done using polyclonal α-L1 antibodies.

5.1.13. GAPDH and the cytoplasmic domain of L1 molecule do not bind in an ELISA approach

In order to check the possible interaction of GAPDH with the cytoplasmic domain of L1 adhesion molecule an ELISA assay was performed with recombinant intracellular domain of L1 (L1-ICD). GAPDH and PGI were coated on the absorbent plastic surface. L1-ICD in different concentrations was incubated with coated GAPDH and PGI. Detection of the potential interaction was done using monoclonal α-L1 antibodies raised against cytoplasmic domain of L1. ELISA assay demonstrated no binding of either GAPDH or PGI to L1-ICD (**Figure 16**).

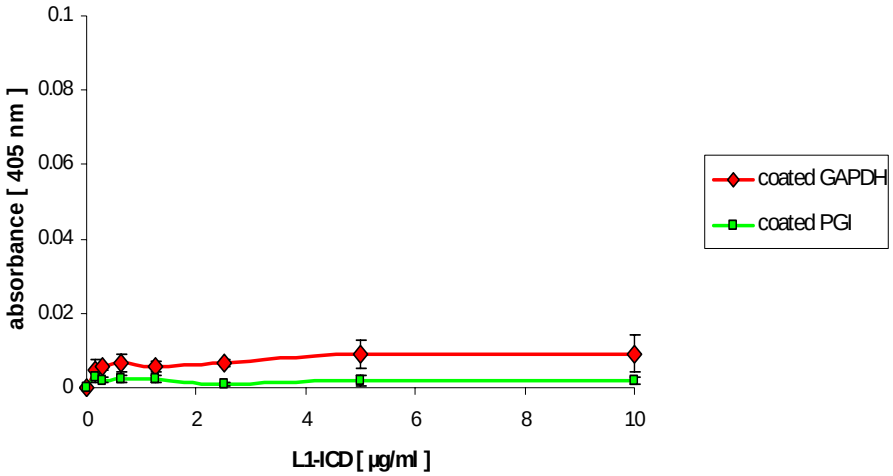
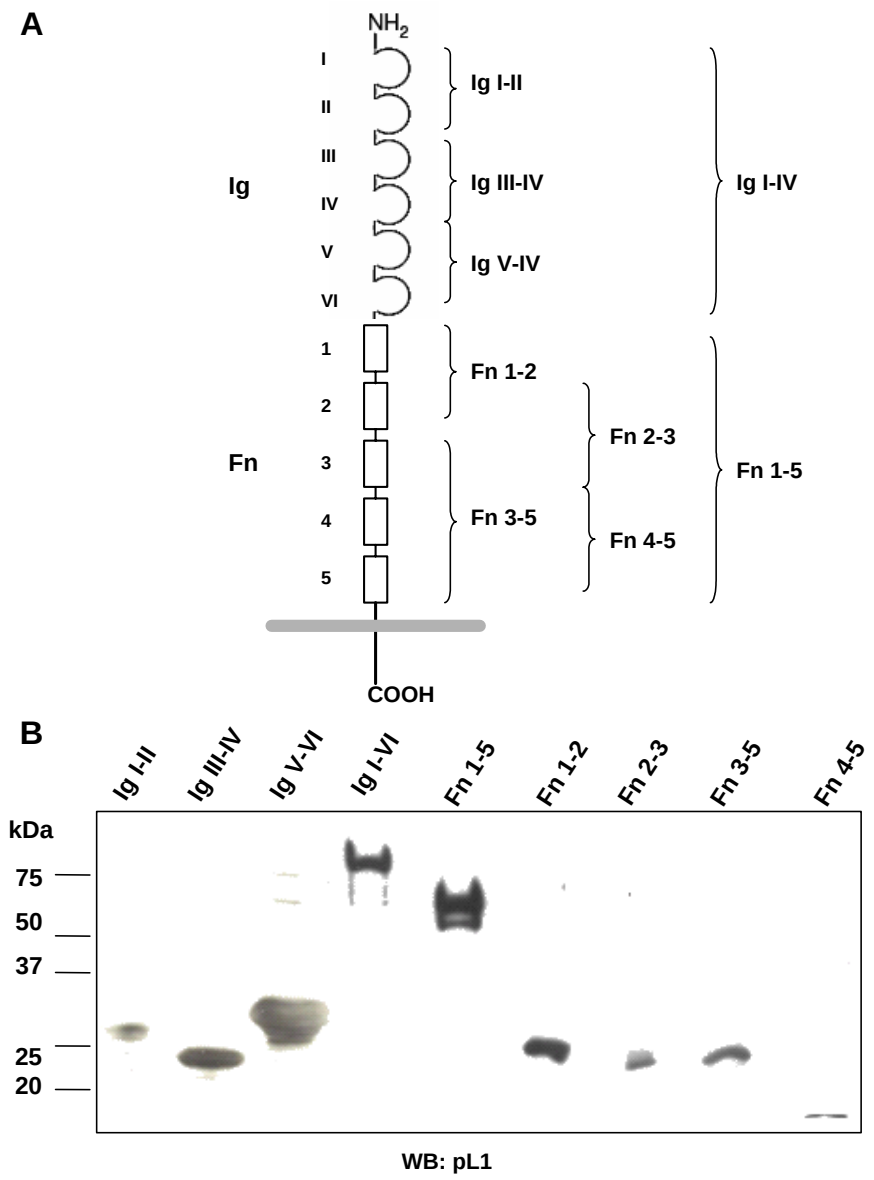


Figure 16: GAPDH and cytoplasmic domain of L1 molecule do not bind in an ELISA assay. Immobilized GAPDH and PGI (5 µg/ml) were incubated with recombinant intracellular domain of L1 molecule (L1-ICD) in different concentrations (0.15 – 10 µg/ml). Detection of the potential interaction was done using monoclonal α-L1 antibodies raised against cytoplasmic domain of L1. Mean values ± SEM (n = 4) are shown.

5.1.14. Production of recombinant fragments of the L1 extracellular domain

The recombinant fragments of extracellular domain of L1 molecule were produced in order to apply them for *in vitro* binding assays. For this purpose *E. coli* strain BL21 (DE3) was transformed with plasmid DNA encoding nine fragments of L1 protein, namely Fn 1-5, Fn 1-2, Fn 2-3, Fn 3-5, Fn 4-5, Ig I-VI, Ig I-II, Ig III-IV, Ig V-VI. Schematic presentation of the L1 adhesion molecule and recombinant fragments used in this study is shown on the **figure 17A**. After the purification proteins were applied for Western blot analysis using polyclonal α-L1 antibodies (**Figure 17B**).

Figure 17: Production of recombinant fragments of L1 extracellular domain



(A) Schematic presentation of the L1 adhesion molecule and recombinant fragments used for this study.

(B) *E. coli* strain BL21 (DE3) was transformed with plasmid DNA encoding several fragments of L1 protein, namely Ig I-VI, Ig I-II, Ig III-IV, Ig V-VI, Fn 1-5, Fn 1-2, Fn 2-3, Fn 3-5, Fn 4-5.

After the purification proteins were analysed by Western blot (WB). Detection using polyclonal α-L1 antibodies showed single bands corresponded to molecular weight of each of nine fragments.

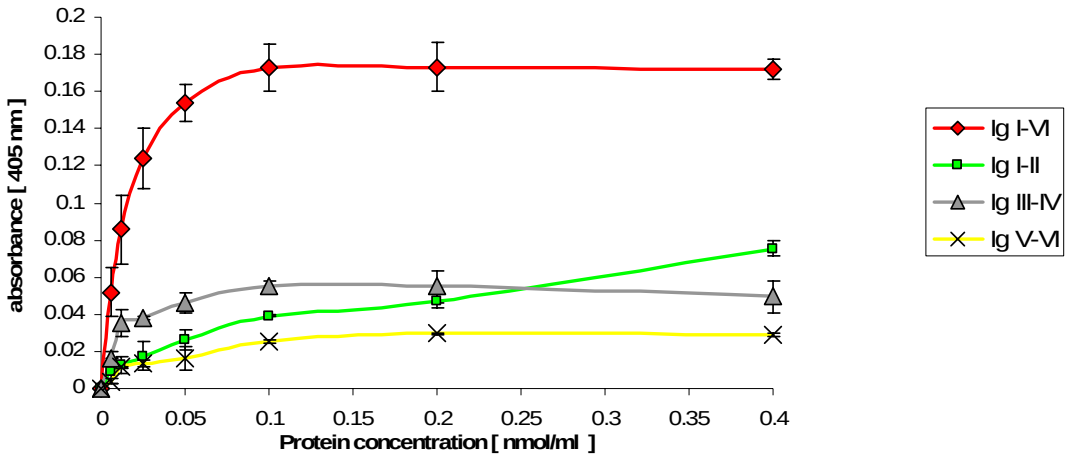
5.1.15. Identification of possible binding site for GAPDH on the L1 extracellular domain using an ELISA approach with recombinant fragments of L1

In order to narrow down the region on the L1 extracellular domain, mediating the binding of GAPDH, an ELISA assay was performed with recombinant fragments Ig I-VI, Ig I-II, Ig III-IV, Ig V-VI, Fn 1-5, Fn 1-2, Fn 2-3, Fn 3-5, Fn 4-5 of the L1 extracellular domain. As a control PGI, which demonstrated no binding to the whole extracellular domain of L1, was used. GAPDH and PGI were coated on the absorbent plastic surface. Recombinant L1 fragments in equimolar concentrations were incubated with coated GAPDH and PGI. Detection of the potential interaction was done using polyclonal α-L1 antibodies.

ELISA assay revealed the binding of the whole Ig domain (I-VI) as well as the whole Fn domain (1-5) to GAPDH (**Figure 18A, 19A**), but not to PGI (**Figure 18B, 19B**). When subfragments of Ig part (Ig I-II, Ig III-IV and Ig V-VI) were applied ELISA has shown no binding of them either to GAPDH (**Figure 18A**) or to PGI (**Figure 18B**) that demonstrated requirement of the whole Ig domain for GAPDH binding.

Application of Fn subfragments (Fn 1-2, Fn 2-3, Fn 3-5 and Fn 4-5) revealed different affinity of binding of all fragments to GAPDH (**Figure 19A**) but not to PGI (**Figure 19B**). It was observed that GAPDH binding to Fn part of L1 is mostly mediated by Fn 4-5 fragment (the strongest affinity) while the presence of the third domain in Fn 3-5 somehow inhibited the binding.

A



B

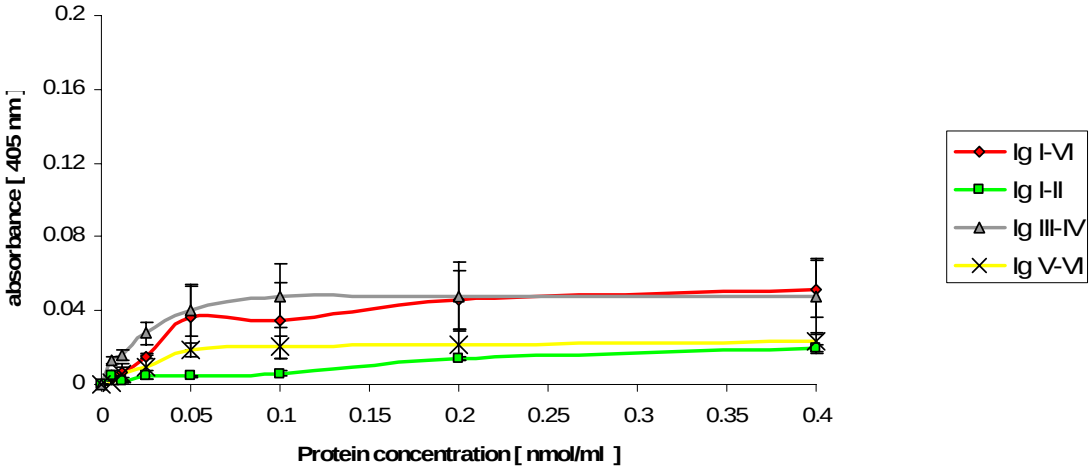
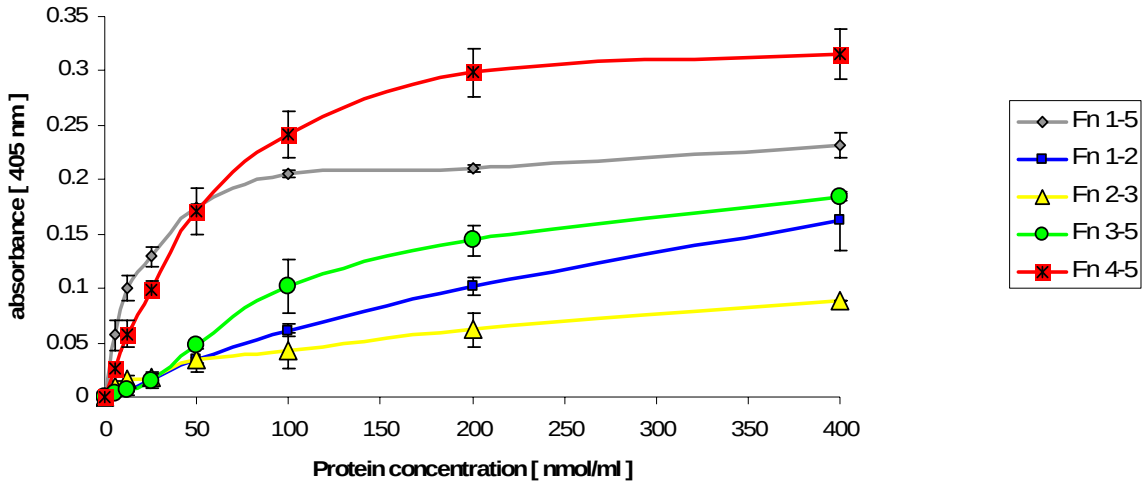


Figure 18: Identification of possible binding site for GAPDH on the L1 extracellular domain. ELISA assay performed with recombinant Ig I-VI, Ig I-II, Ig III-IV and Ig V-VI fragments of L1.

GAPDH **(A)** and PGI **(B)** were coated on the absorbent plastic surface (5 µg/ml). Recombinant Ig I-VI, Ig I-II, Ig III-IV and Ig V-VI fragments of L1 in different concentrations (0.006 - 0.4 nmol) were incubated with coated GAPDH and PGI. Detection of the potential interaction was done using polyclonal α-L1 antibodies. Mean values ± SEM (n = 4) are shown.

A



B

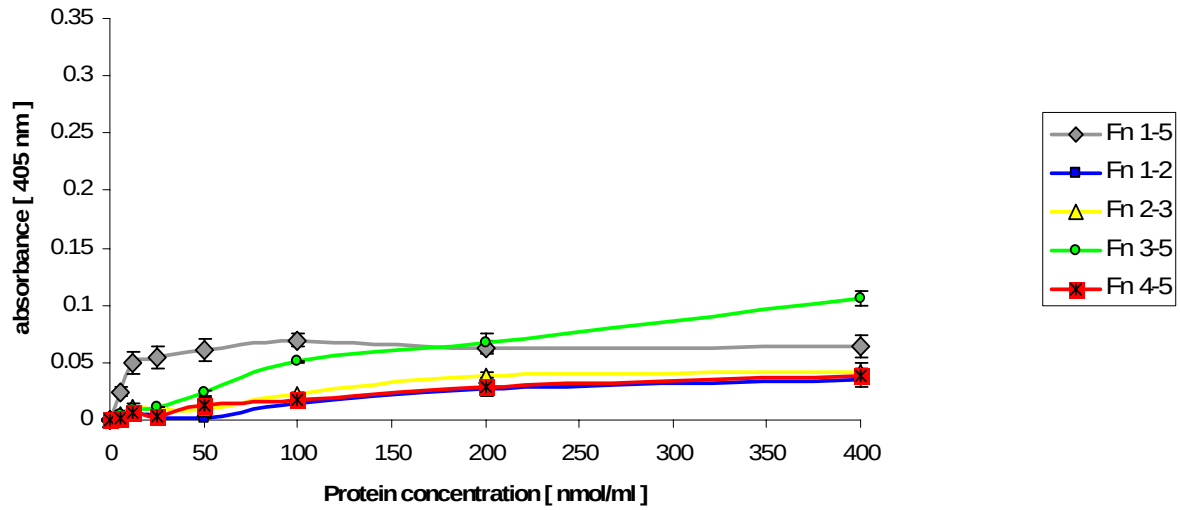


Figure 19: Identification of possible binding site for GAPDH on the L1 extracellular domain. ELISA assay performed with recombinant Fn 1-5, Fn 1-2, Fn 2-3, Fn 3-5 and Fn 4-5 fragments of L1.

GAPDH **(A)** and PGI **(B)** were coated on the absorbent plastic surface (5 µg/ml). Recombinant Fn 1-5, Fn 1-2, Fn 2-3, Fn 3-5 and Fn 4-5 fragments of L1 in different concentrations (6 – 400 nmol) were incubated with coated GAPDH and PGI. Detection of the potential interaction was done using polyclonal α-L1 antibodies. Mean values ± SEM (n = 4) are shown.

5.1.16. Cell surface biotinylation of N2A and B104 cell lines. Establishment of extracellular localization of GAPDH and ANT

Since it has been shown that GAPDH and ANT interact with extracellular domains of L1 one could suppose that GAPDH and ANT may interact with L1 outside of the cell. To test, whether GAPDH and ANT are expressed on the outer surface of neuronal cells, B104 and N2A neuroblastoma cell line were subjected to surface biotinylation. Biotinylated proteins were isolated using streptavidin magnetic beads and separated by SDS-PAGE. Western blot analysis with monoclonal α-GAPDH antibody showed that GAPDH was isolated from B104 (**Figure 20A**) and N2A (**Figure 20B**) as biotinylated protein migrating with an apparent molecular weight of 37 kDa. Western blot with HRP-conjugated neutravidin showed a biotinylated 37 kDa protein band confirming that GAPDH was biotinylated and suggested that it is located on the outer surface of neuronal cells. Estimation of the percentage of GAPDH on the outer surface relative to total cellular GAPDH revealed that GAPDH at the outer cell surface accounts for about 0.01% of total cellular GAPDH.

Western blot analysis with polyclonal α-ANT antibodies showed that from three isoforms detected by antibodies one, migrating with an apparent molecular weight of 32 kDa, was isolated from N2A cells (**Figure 20C**). Western blot with HRP-conjugated neutravidin showed a biotinylated 32 kDa protein band confirming that ANT was biotinylated and suggested that one isoform of ANT is located at the outer surface of neuronal cells.

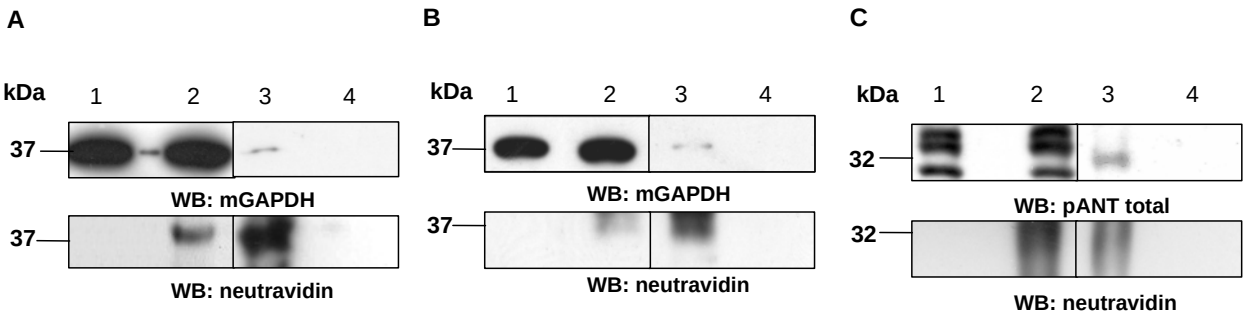


Figure 20: Cell surface biotinylation of N2A and B104 neuroblastoma cell lines. Establishment of extracellular localization of GAPDH and ANT.

Neuroblastoma B104 **(A, C)** or N2A **(B)** cells were treated in the absence (lane 1 and 4) or presence (lane 2 and 3) of biotinylation reagent. Biotinylated surface proteins were precipitated by streptavidin beads (lane 3 and 4) and subjected to SDS-PAGE. Cell lysates (lane 1 and 2) were used as input control. Western blot (WB) analysis using either monoclonal GAPDH antibody (**A, B)** or polyclonal ANT antibodies **(C)** and neutravidin-HRP demonstrated that GAPDH and one isoform of ANT are localized at the outer surface of neuronal cells.

5.1.17. Cell surface biotinylation of primary cerebellar neurons. Confirmation of extracellular localization of GAPDH

Since GAPDH was detected to be associated with outer surface of neuroblastoma cell lines it was interesting also to verify that for primary neurons. Cell surface biotinylation was performed on primary cerebellar granule neurons. Biotinylated proteins precipitated using streptavidin magnetic beads were applied for Western blot analysis. Detection using monoclonal α-GAPDH antibody revealed that GAPDH was precipitated from granular cerebellar neurons as a biotinylated protein which was confirmed by neutravidin-HRP detection (**Figure 21**). It was an additional evidence for the existence of surface-associated GAPDH in neurons.

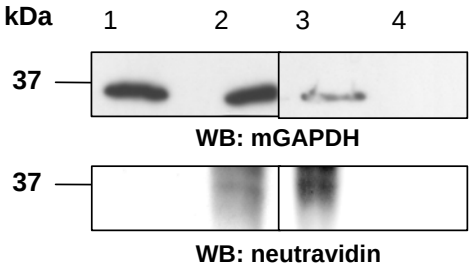


Figure 21: Cell surface biotinylation of primary cerebellar neurons.

Primary cerbellar granular neurons were treated in the absence (lane 1 and 4) or presence (lane 2 and 3) of biotinylation reagent. Biotinylated surface proteins were precipitated by streptavidin beads (lane 3 and 4) and subjected to SDS-PAGE. Cell lysates (lane 1 and 2) were used as input control. Western blot (WB) analysis using monoclonal α-GAPDH antibody and neutravidin-HRP demonstrated that GAPDH is localized at the outer surface of cerebellar neurons.

5.1.18. Confirmation of extracellular localization of GAPDH and co-localization of GAPDH and L1 using immunocytochemistry of primary cerebellar neurons

5.1.18.1. Application of monoclonal α-GAPDH antibody to living cells

To corroborate localization of GAPDH at the outer surface and also to investigate co-localization of GAPDH and L1 adhesion molecule monoclonal α-GAPDH antibody was used for immunocytochemical analysis of living cerebellar neurons. Primary neurons were cultivating in chemically defined serum-free medium for 4-5 days. α-GAPDH monoclonal antibody was applied to living cells and incubated with antibody during 15 min at 37°C. According this procedure only GAPDH associated with the outer membrane could be labelled. Confocal microscopic analysis revealed the specific GAPDH label mostly around plasma membrane and a little bit in the processes (**Figure 22A**, red). After fixation and permeabilisation of cell membrane the neurons were stained with α-L1 polyclonal antibodies. L1 staining showed the typical surface distribution in neurons (**Figure 22A**, green). Overlay

demonstrated co-localization of L1 and GAPDH at the plasma membrane and in neurites (**Figure 22A**, merged).

For the control α-GAPDH monoclonal antibody were added at the same concentration to the already fixed and permeabilised cells for 30 min at room temperature (**Figure 22B**). According this procedure all GAPDH present in the cell should be labelled.

Control GAPDH staining of fixed neurons has shown normal cytoplasmic spreading of GAPDH.

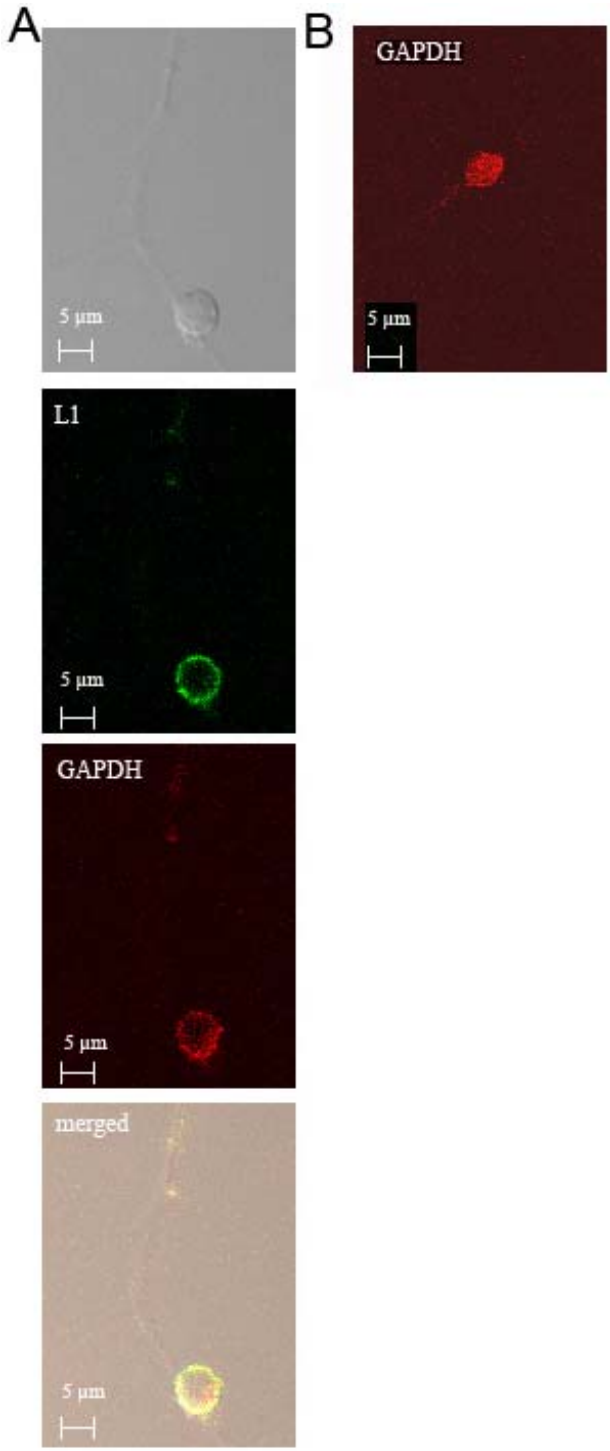


Figure 22: Immunocytochemistry on living primary cerebellar neurons

(A) α-GAPDH monoclonal antibody was applied in final concentration 1-2 µg/ml to living cells for 15 min at 37°C (red label), that revealed membrane-associated localization of GAPDH. After fixation of the cells and staining using α-L1 polyclonal antibodies typical surface distribution of L1 molecule was seen (green label). Overlay showed co-localization of L1 adhesion molecule and GAPDH around the plasma membrane and in neurites. **(B)** Control staining of fixed neurons with α-GAPDH monoclonal antibody for 30 min at room temperature revealed the normal cytoplasmic distribution of GAPDH.

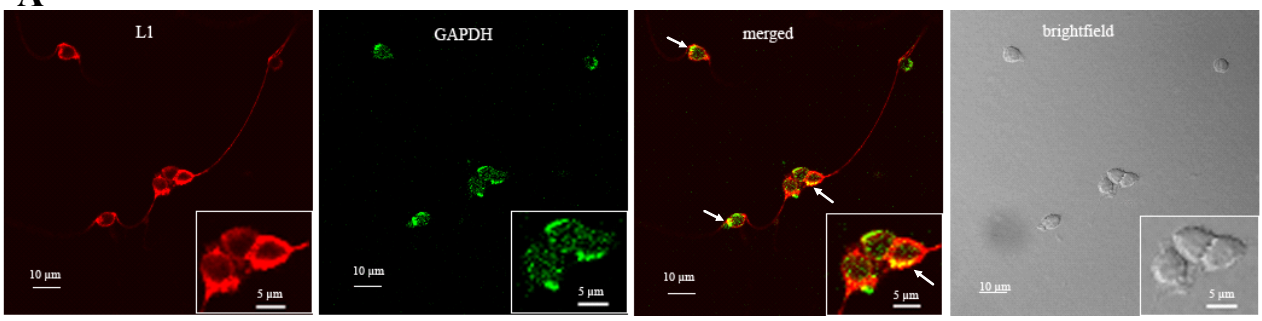
5.1.18.2. Application of α-GAPDH monoclonal antibody to living neurons on ice

To exclude the possibility of endo- and exocytosis during the incubation at 37°C α-GAPDH monoclonal antibody was applied to living cell and incubated on ice for 50 min. To show the absence of endo- and exocytosis the cells were stained with control polyclonal α-tau antibodies because tau was exclusively found in the cytoplasm. After fixation and permeabilisation of cell membrane α-L1 polyclonal antibodies were added.

Application of α-GAPDH monoclonal antibody to living cells on ice revealed the presence of extracellular GAPDH only on neuronal cell bodies (**Figure 23A**, green). L1 staining showed surface distribution at plasma membrane of neurons and axons (**Figure 23A**, red). Overlay demonstrated that L1 and GAPDH co-localized at the surface of neurons but not axons (**Figure 23A**, merged).

Application of control α-tau polyclonal antibodies revealed no staining for tau protein and proved absence of endo- or exocytosis during incubation with antibodies on ice (**Figure 23B**). Staining of fixed neurons with α-tau antibodies showed normal cytoplasmic distribution of tau protein (data are not shown).

A



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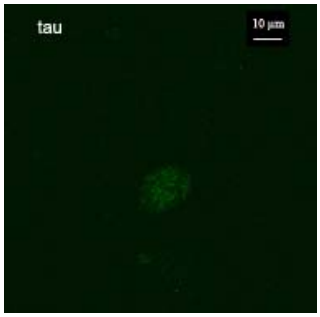


Figure 23: Immunocytochemistry on living primary cerebellar neurons.

(A) α-GAPDH monoclonal antibody was applied in final concentration 1-2 µg/ml to living cells for 50 min on ice (green label). After fixation of the cells and staining with α-L1 polyclonal antibodies typical surface distribution of L1 molecule was seen (red label). Overlay showed co-localization of L1 adhesion molecule and GAPDH at the outer membrane of cerebellar neurons (marked by white arrows). **(B)** Control application of α-tau polyclonal antibodies on ice simultaneously with α-GAPDH antibody demonstrated no staining of tau protein and no exo- and endocytosis during antibodies incubation.

5.1.19. Functional analysis of interaction between L1 adhesion molecule and GAPDH

Since L1 and GAPDH seemed to interact at the extracellular site of the cells, the main question was whether a GAPDH-dependent modification of L1 protein leads to changes in the nervous system functions normally mediated by L1. So far it has not been reported that GAPDH is present outside of the neurons and there interacts with components of extracellular matrix. GAPDH has been well characterized as a glycolytic enzyme with oxidative phosphorylation activity (Berg et al. 2002) and also with several functions unrelated to glycolysis which were identified recently (Sirover 1999). According to these results two hypothesises about the possible role of GAPDH in L1-mediated functions were proposed.

Hypothesis 1: GAPDH is an ecto-protein kinase for L1

Laschet and colleagues (Laschet et al. 2004) have discovered the novel function for GAPDH, namely endogenous kinase activity for α-subunit of GABAa receptor. This novel kinase activity could be one of unknown GAPDH functions outside of the cell because of several reasons. Firstly, the new function of GAPDH was identified in the central nervous system associated with the modulation of functions of the transmembrane protein. Secondly, a sequence **(NXXSR)** similar to the consensus motif **(NXXS/TK)** for phosphorylation by GAPDH on α-subunit of GABAa receptor has been found on the third Ig-like domain of L1. Finally, essential components for the phosphorylation - ATP and Mg^{2+} ions - present outside of the cell. In order to check this hypothesis several experiments were performed.

Hypothesis 2: GAPDH is an ecto-ribosyltransferase for L1

Another function of GAPDH unrelated to glycolysis was found in 1993 by Pancholi and Fischetti. They have shown that GAPDH can act as ADP-ribosylating enzyme on the surface of group A *streptococci* (Pancholi and Fischetti 1993). In 2005 Zhao and colleagues have shown that ADP-ribosylation of extracellular matrix protein integrin can modulate the binding of integrin to another extracellular molecule laminin (Zhao et al. 2005). In the connection to that it was hypothesized that GAPDH, possessing ADP-ribosylation activity, can ribosylate L1 adhesion molecule and modify the interaction of L1 to other extracellular components. Two reasons argue for this hypothesis. Firstly, L1 was shown to carry a RGD-independent integrin-binding motif within the third Fn-like domain (Silletti et al. 2000). The RGD contains an arginine which could be ribosylated. And secondly, NAD^+ - resource of ADP-ribose – was known to be outside of the cell that made the extracellular ADP-ribosylation likely. In order to check this hypothesis several experiments were performed.

5.1.19.1. In vitro phosphorylation assay

For *in vitro* phosphorylation assay L1-Fc, NCAM-Fc and recombinant fragments of L1 – Ig I-VI and Fn 1-5 - were used.

Initially the working concentration of Mg^{2+} ions was established. 0.25 nmol of the L1-Fc was taken per each experiment. To allow protein–protein interaction, target protein was co-dialyzed with **2-fold** molar excess of GAPDH. L1 in the presence and in the absence of GAPDH was incubated with 10 nmol [^{33}P]-ATP and different concentrations of Mg^{2+} ions. After phosphorylation L1-Fc protein was isolated from the reaction mixture with Protein A magnetic beads and subjected to SDS-PAGE and autoradiography (**Figure 24**). The results revealed that in the presence of 10 µM of Mg^{2+} ions no phosphorylation occurred while 1 mM concentration of Mg^{2+} provided a maximal level of phosphorylation in comparison to 100 µM of Mg^{2+}. Additionally it was observed that L1 extracellular domain itself can be phosphorylated without GAPDH.

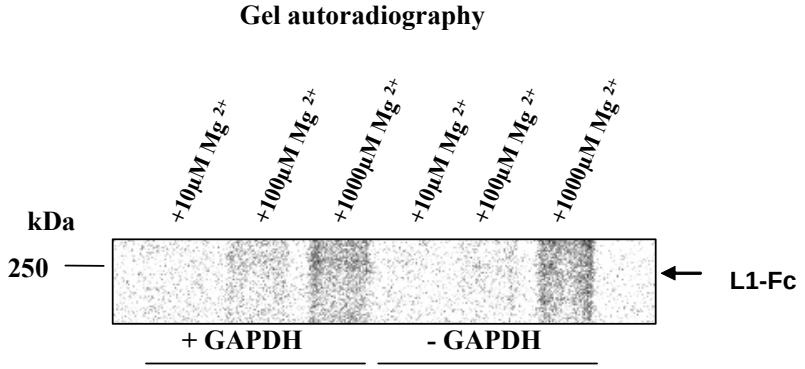


Figure 24: *In vitro* phosphorylation assay with L1-Fc.
0.25 nmol of the L1-Fc was taken and co-dialyzed with **2-fold** molar excess of GAPDH. L1-Fc with or without GAPDH was incubated with 10 nmol [^{33}P]-ATP and different concentrations of Mg^{2+} ions (10µM – 1000 µM). 1mM concentration of Mg^{2+} resulted in a maximal level of phosphorylation. Also auto-phosphorylation activity of L1 extracellular domain itself was observed.

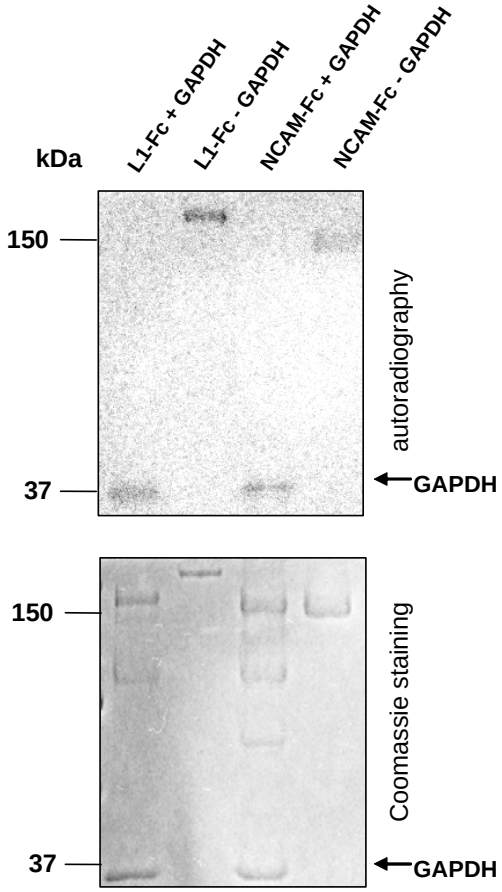
The next experiments were done with equimolar concentration (0.25 nmol) of L1-Fc and control NCAM-Fc and **5-fold** molar excess of GAPDH. The proteins with or without GAPDH were incubated in the presence of 10 nmol [^{33}P]-ATP and 1mM of Mg^{2+}.

Autoradiography (**Figure 25**) showed phosphorylation of L1-Fc and NCAM-Fc in the absence of GAPDH, suggesting auto-phosphorylation of the extracellular domain of L1 and NCAM. In the presence of **5-fold** molar excess of GAPDH L1-Fc and NCAM-Fc were not found to be phosphorylated, while GAPDH was phosphorylated. Coomassie staining (**Figure 25**) revealed a shift in a molecular weight of L1-Fc in the presence of ATP, showing the possible formation of L1-Fc homo-dimer of approximately 320 kDa in comparison to

monomer of L1-Fc of 160 kDa observed in the presence of GAPDH. No such alteration in the apparent molecular weight was observed with NCAM-Fc.

Figure 25: *In vitro* phosphorylation assay with L1-Fc and NCAM-Fc.

Equimolar concentration (0.25 nmol) of L1-Fc and NCAM-Fc and **5-fold** molar excess of GAPDH were taken for the experiment. Proteins in the presence and absence of GAPDH were incubated with 10 nmol [^{33}P]-ATP and 1mM of Mg^{2+} and subjected for SDS-PAGE, Coomassie staining and autoradiography.



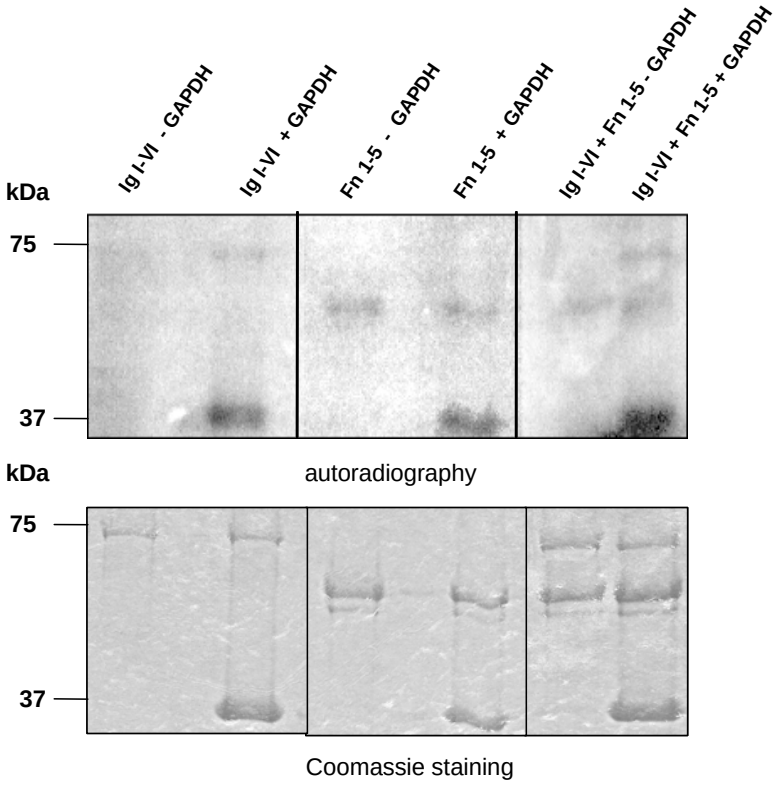
To narrow down sites responsible for auto-phosphorylation of L1 and sites specifically phosphorylated by GAPDH, recombinant fragments Ig I-VI and Fn 1-5 were used. The proteins with or without GAPDH were incubated in the presence of 10 nmol [^{32}P]-ATP and 1 mM of Mg^{2+}, precipitated, applied to SDS-PAGE, stained with Coomassie and subjected to autoradiography.

Coomassie staining (**Figure 26**) revealed equal amount of proteins applied for SDS-PAGE. Autoradiography (**Figure 26**) showed phosphorylation of Ig I-VI domain of L1 only in the presence of GAPDH whereas Fn 1-5 domain was phosphorylated in both situations: with and without GAPDH. When Ig I-VI and Fn 1-5 were applied together, in the absence of GAPDH phosphorylation only of Fn 1-5 was observed, whereas in the presence of GAPDH both fragments were phosphorylated.

Figure 26: *In vitro* phosphorylation assay with recombinant Ig I-VI and Fn 1-5 fragments of L1.

Recombinant fragments Ig I-VI and Fn 1-5 were used in equimolar concentrations (0.25 nmol) and co-dialyzed with 2-fold molar excess concentration of GAPDH. Then proteins were incubated with 10 nmol [32P]-ATP and 1 mM of Mg^{2+}.

Specific GAPDH-dependent phosphorylation of Ig I-VI domain was observed, Fn 1-5 domain was phosphorylated with and without GAPDH.



5.1.19.2. In vitro ADP-ribosylation assay

For the *in vitro* ADP-ribosylation assay L1-Fc, NCAM-Fc and recombinant fragments of L1 – Ig I-VI and Fn 1-5 - were used.

The experiments were done with equimolar concentration (0.25 nmol) of L1-Fc and NCAM-Fc and/or **2** or **5-fold** molar excess of GAPDH. To allow protein–protein interaction, each protein was co-dialyzed with 2 or 5-molar excess of GAPDH. Then proteins were incubated with 100 μM [^{32}P]-NAD (specific activity 0.3–0.6 Ci/mmol) in PBS with 0.5 mM Mg^{2+} and 1 mM Ca^{2+}. Target proteins after ribosylation were isolated using Protein-A magnetic beads and subjected to SDS-PAGE and autoradiography.

In vitro ADP-ribosylation assay revealed that extracellular domains of L1 and NCAM adhesion molecules can bind itself either NAD^+ or ADP-ribose, because the radioactivity was observed in the samples where L1-Fc and NCAM-Fc were present without GAPDH (**Figure 27A, B**). In the presence of 2-fold molar excess of GAPDH the ADP-ribosylation of L1-Fc and NCAM-Fc is higher (**Figure 27A**) in comparison with 5-fold molar excess of GAPDH, which causes auto-ribosylation of GAPDH and decrease of L1-Fc and NCAM-Fc ribosylation (**Figure 27B**).

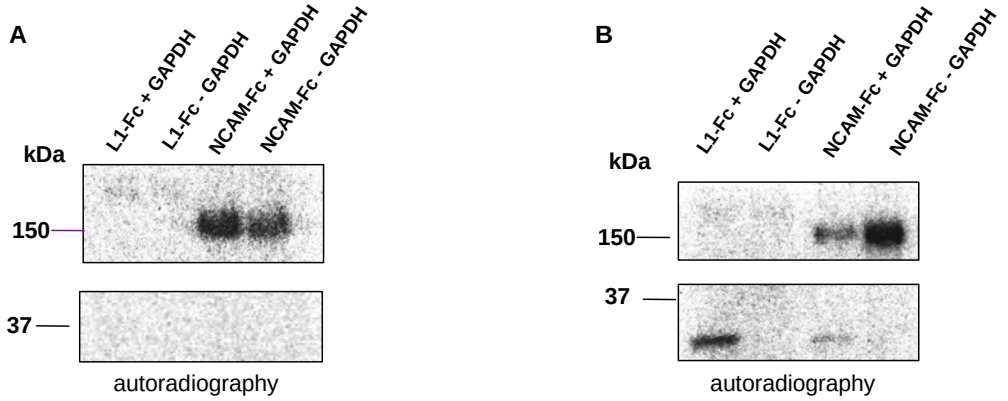


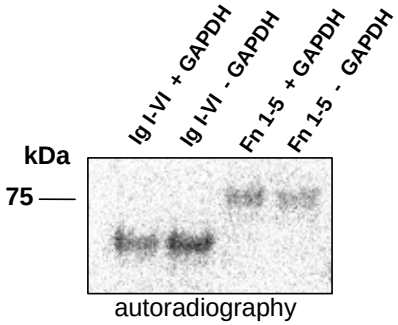
Figure 27: *In vitro* ADP-ribosylation assay with L1-Fc and NCAM-Fc.

Equimolar concentration (0.25 nmol) of L1-Fc and NCAM-Fc and/or 2 **(A)** or 5-fold **(B)** molar excess of GAPDH were co-dialyzed and then incubated with 100 µM [^{32}P]-NAD in PBS with 0.5 mM Mg^{2+} and 1 mM Ca^{2+}. Proteins were isolated, subjected to SDS-PAGE and autoradiography.

Recombinant fragments Ig I-VI and Fn 1-5 were used in equimolar concentrations (0.09 nmol) for *in vitro* ADP-ribosylation assay. Proteins were co-dialyzed with **2-fold** molar excess of GAPDH and incubated with 100 µM [^{32}P]-NAD in PBS with 0.5 mM Mg^{2+} and 1 mM Ca^{2+}. Proteins were precipitated, subjected to SDS-PAGE and autoradiography which revealed that Fn 1-5 fragment is higher ribosylated in the absence of GAPDH but Ig I-VI is slightly higher ribosylated in the presence of GAPDH (**Figure 28**).

Figure 28: *In vitro* ADP-ribosylation assay with recombinant Ig I-VI and Fn 1-5 fragments of L1.

Recombinant fragments Ig I-VI and Fn 1-5 in equimolar concentrations (0.09 nmol) were co-dialyzed with 2-fold molar excess of GAPDH and then incubated with 100 µM [^{32}P]-NAD. Proteins were precipitated, subjected to SDS-PAGE and autoradiography.



5.1.19.3. Stimulation of neuritogenesis of cerebellar granule cells by different concentrations of soluble GAPDH

Neurite outgrowth assay was performed in order to check whether GAPDH has biological function in the activities mediated by L1 in the nervous system. For investigating of GAPDH effect on the length of neurites cerebellar primary granule cells have been used. GAPDH was added in the concentrations 1, 2, 5 and 10 µg/ml. Since L1 adhesion molecule was found to

stimulate neurite outgrowth of neurons (Maness and Schachner 2007), L1-Fc (10 µg/ml) was coated as a positive control.

Neurons were only analysed which had no cell-cell-contacts and when neurite was as least as long as or longer than the diameter of the cell. Three different parameters were measured: length of the longest neurite, total length of neurites and number of neurites per cell. The statistical analysis of stimulation effects compared to control (PLL) was performed using student T-test.

Neurite outgrowth assay on cerebellar primary neurons has shown the dose-dependent stimulation effect mediated by soluble GAPDH with saturation concentration 5 µg/ml (**Figure 29**). PLL control was accepted as 100%. GAPDH stimulated the neurite outgrowth by 30 to 50% comparing to PLL control with maximal effect of 5 µg/ml concentration for the length of the longest neurite and total length of neurites parameters. The number of neurites per cell was significantly increased when 2 and 5 µg/ml concentrations were applied. Significant increase of the total length of neurites was observed also for soluble GAPDH (5 µg/ml) added to coated L1-Fc.

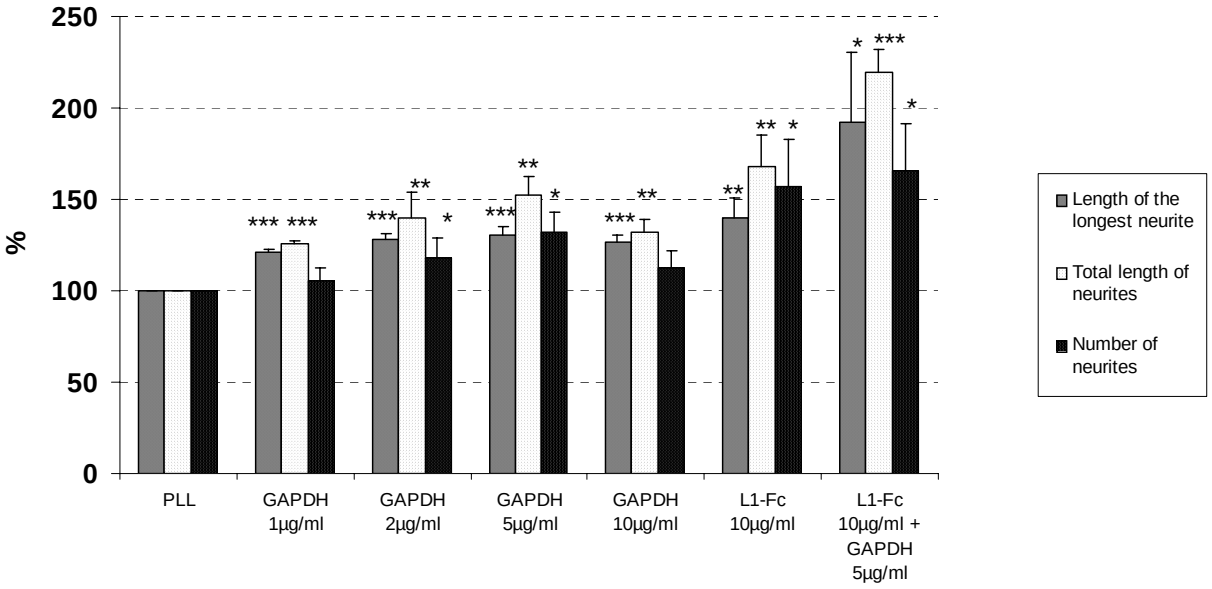


Figure 29: Stimulation of neuritogenesis of cerebellar granule cells by different concentrations of soluble GAPDH

Cerebellar primary granule cells were cultured in chemically defined serum-free medium. GAPDH was added in the concentrations 1, 2, 5 and 10 µg/ml. L1-Fc (10 µg/ml) was coated as a positive control. Three different parameters were measured: length of the longest neurite, total length of neurites and number of neurites per cell. The results of three independent experiments are presented. PLL control was accepted as 100 %. The statistical analysis of stimulation effects compared to control

(PLL) was performed using student T-test. Error bars represent standard error mean, asterisks demonstrate a statistical significance (Student's t-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

5.1.19.4. Stimulation of neuritogenesis of cerebellar granule cells by different concentrations of soluble GAPDH and ATP

According to the results of *in vitro* phosphorylation assay, exogenous ATP can play an important role extracellulary in a complex with GAPDH; it seemed that GAPDH can compete for the phosphate group with adhesion molecules L1 and NCAM. The neurite outgrowth assay was performed in order to check the effect of exogenous soluble GAPDH and ATP together on the elongation of neurites.

Cerebellar primary granule cells have been cultured in chemically defined serum-free medium. Half of wells were coated with L1-Fc (10 µg/ml) at PLL. PLL-coated and L1-Fc-coated wells were taken as controls for the experiment. GAPDH and ATP were added as soluble compounds in different compositions.

For the comparison of groups the length of the longest neurite was determined. The statistical analysis of stimulation effect compared to control (PLL) was performed using student T-test.

Significant elongation of neurites (approximately 20%) was detected when GAPDH and ATP were added alone in the concentration 5 µg/ml. The same effect was observed on neurons seeded on PLL as well as on L1-Fc substrate (**Figure 30**). When GAPDH (5 µg/ml) and ATP (1, 2 and 5 µg/ml) were applied together, no stimulating effect was revealed. This observation confirmed *in vitro* phosphorylation results and an idea that GAPDH can compete for the phosphate group with L1 adhesion molecule. However the same effects were observed both on PLL- and L1-coated substrates. For the next experiments it was necessary to confirm that the involvement of GAPDH and ATP in neurite outgrowth was indeed L1-mediated.

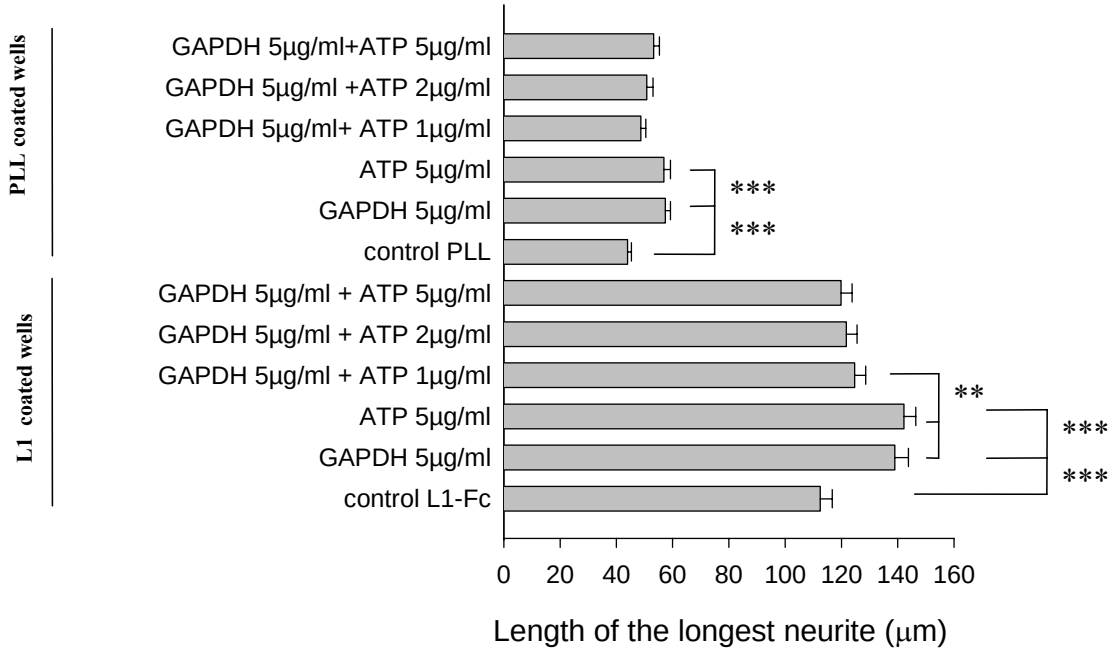


Figure 30: Stimulation of neuritogenesis of cerebellar granule cells by different concentrations of soluble GAPDH and ATP.

GAPDH (5 µg/ml) and ATP (1, 2 and 5 µg/ml) were applied in different compositions to cerebellar primary granule cells. PLL- and L1-Fc-coated wells were accepted as controls. For the comparison of groups the length of the longest neurite was chosen. The statistical analysis of the difference between groups was performed using student T-test. Error bars represent standard deviation, asterisks demonstrate a statistical significance (Student's t-test: ** $p<0.01$; *** $p<0.001$).

5.1.19.5. Neuritogenesis of cerebellar granule cells performed on the substrate modificated by in vitro phosphorylation and ADP-ribosylation

To answer the question whether exogenous GAPDH and ATP, GAPDH and NAD^+ can modify L1 adhesion molecule and influence functional changes, neurite outgrowth with substrate pre-treatment was performed.

96-well plastic plates with glass bottom were coated with PLL. Half of wells was then coated with L1-Fc at PLL. One plate was incubated with GAPDH (5 µg/ml) and/or ATP (5 µg/ml) in HEPES-TRIS buffer with 1mM Mg^{2+} for 40 min at 37°C. Then the reaction mixture was removed, the wells were washed once and cerebellar primary granule cells have been seeded. Another plate was incubated with GAPDH (5 µg/ml) and/or NAD^+ (5 µg/ml) in PBS with 0.5 mM Mg^{2+} and 1 mM Ca^{2+} for 40 min at 37°C. Then the reaction mixture was removed, the wells were washed once and cerebellar primary granule cells have been seeded. Untreated PLL- and L1-Fc-coated wells were taken as controls for the experiment. For the comparison

of groups the length of the longest neurite was determined. The statistical analysis of stimulation effects compared to controls (PLL and L1-Fc) was performed using student T-test. Modification of coated L1 by GAPDH and ATP together demonstrated a stimulation effect on the neurite outgrowth whereas treatment with either GAPDH or ATP had no effect. No elongation of neurons was observed on PLL substrate after any applications (**Figure 31**). The stimulation effect was indeed L1-mediated.

Modification of coated L1 by GAPDH and NAD^+ did not show significant effect on the neurite outgrowth of cerebellar neurons (**Figure 32**). These results were obtained in two independent experiments.

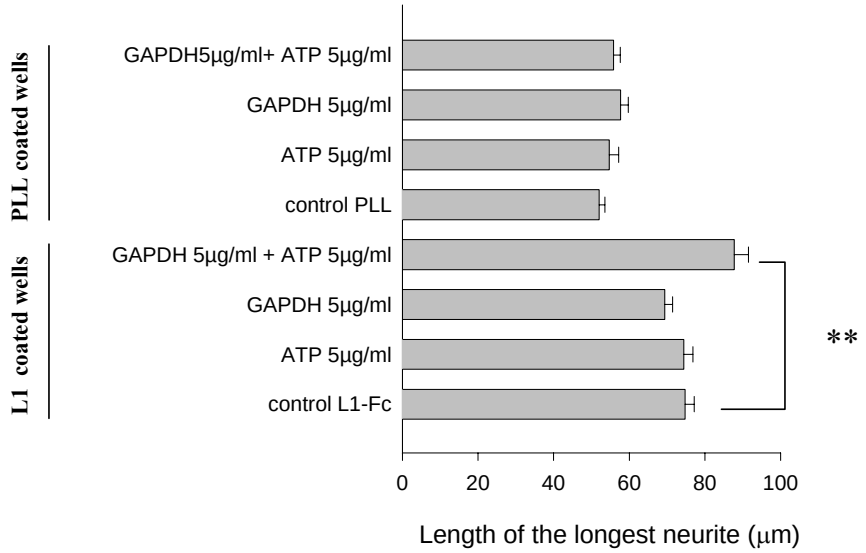


Figure 31: Modulation of the L1-induced neurite outgrowth by pre-treatment of L1 substrate coated with GAPDH and ATP.

Wells were coated with PLL or L1-Fc and treated with GAPDH (5 µg/ml) and/or ATP (5 µg/ml). Upon removal of reaction mixtures cerebellar granule cells were seeded and cultivated. The lengths of the longest neurite were determined. The statistical analysis of stimulation effects compared to controls was performed using student T-test. Error bars represent standard deviation, asterisks demonstrate a statistical significance (Student's t-test: **$p<0.01$).

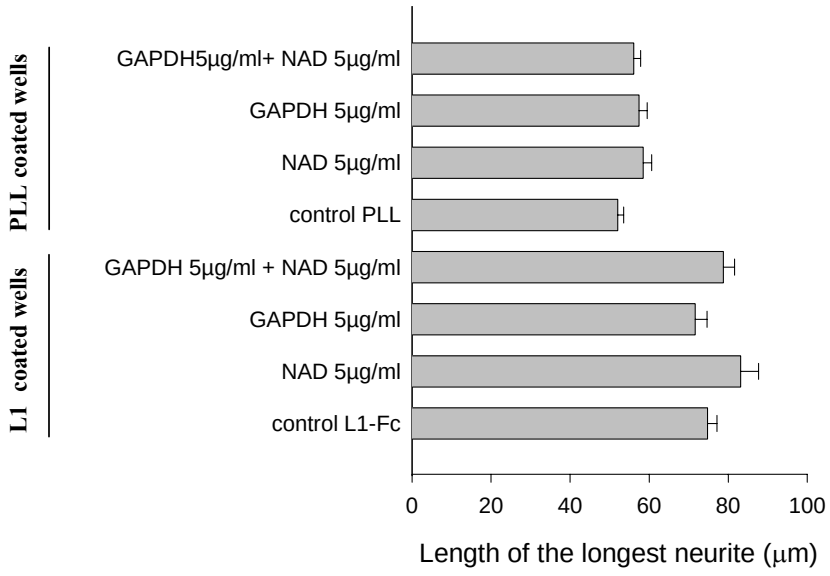


Figure 32: Modulation of L1-induced neurite outgrowth by pre-treatment of L1 substrate coated with GAPDH and NAD^+.

Wells were coated with PLL or L1-Fc and treated with GAPDH (5µg/ml) and/or NAD^+ (5µg/ml). Upon removal of reaction mixtures cerebellar granule cells were seeded and cultivated. The lengths of the longest neurite were determined. Error bars represent standard deviation.

5.1.19.6. Cell survival assay on primary cerebellar granule cells

Cell survival assay was performed in order to check GAPDH involvement in L1-mediated survival effect (Chen et al. 1999).

Dissociated cerebellar granule cells were cultivated in chemically defined serum-free medium. Wells were previously coated with PLL (10 µg/ml) and then with L1-Fc for the control. GAPDH was either coated at PLL or L1-Fc or added as a soluble compound in different concentrations. For induction of cell death 10 µM hydrogen peroxide (cell death through oxidative stress) or 500 nM staurosporine (broad spectrum kinase inhibitor) were added for 6-24 hours. Cell death was determined by counting the numbers of calcein and propidium iodide-positive cells. Calcein is a live cell marker; propidium iodide is a dead cell marker. The cells from 3-4 randomly chosen areas of a microscopic field (magnification 10 × 20) in each well were counted, and for each experimental value of four wells were measured. Cell survival was calculated as a ratio (%) of live cell number to a total cell number.

Cell survival assay showed that GAPDH had a survival effect on H_2O_2-treated cerebellar neurons and this effect is higher when GAPDH is soluble (**Figure 33A**) in comparison to

GAPDH - substrate coated (**Figure 33B**). There was no survival effect of GAPDH observed in staurosporine-treated cerebellar neurons.

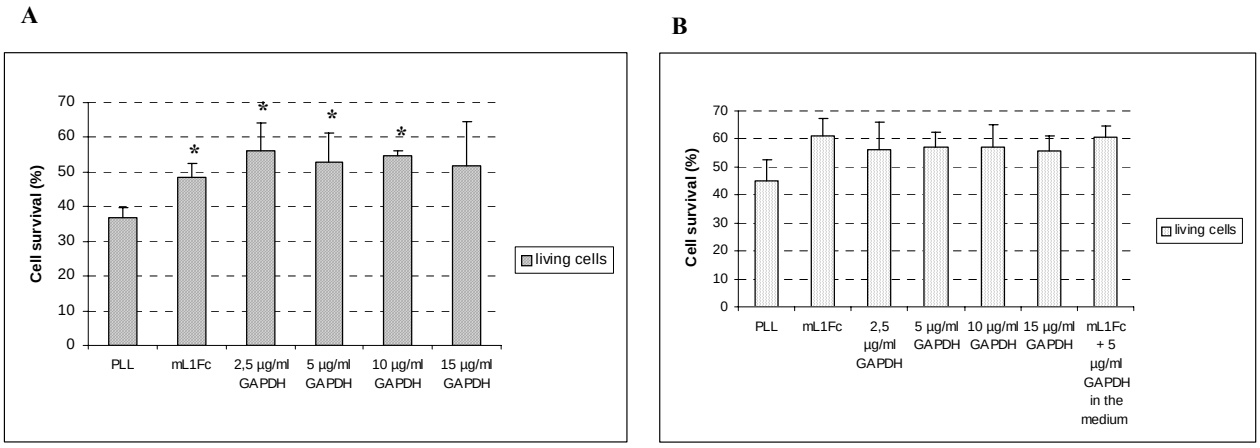


Figure 33: Cell survival assay on primary cerebellar granule cells.

Dissociated cerebellar granule cells were cultivated in chemically defined serum-free medium. Wells were previously coated with PLL and then with L1-Fc for the control. GAPDH (2.5, 5, 10, 15 µg/ml) was either coated at PLL or L1-Fc **(B)** or added as a soluble compound **(A)**. 10 µM hydrogen peroxide was applied for 6-24 hours. Cell survival was calculated as a ratio (%) of live cell number to a total cell number. The statistical analysis of survival effect compared to PLL control was performed using student T-test. Error bars represent standard error mean, asterisks demonstrate a statistical significance (Student's t-test: * $p<0.05$).

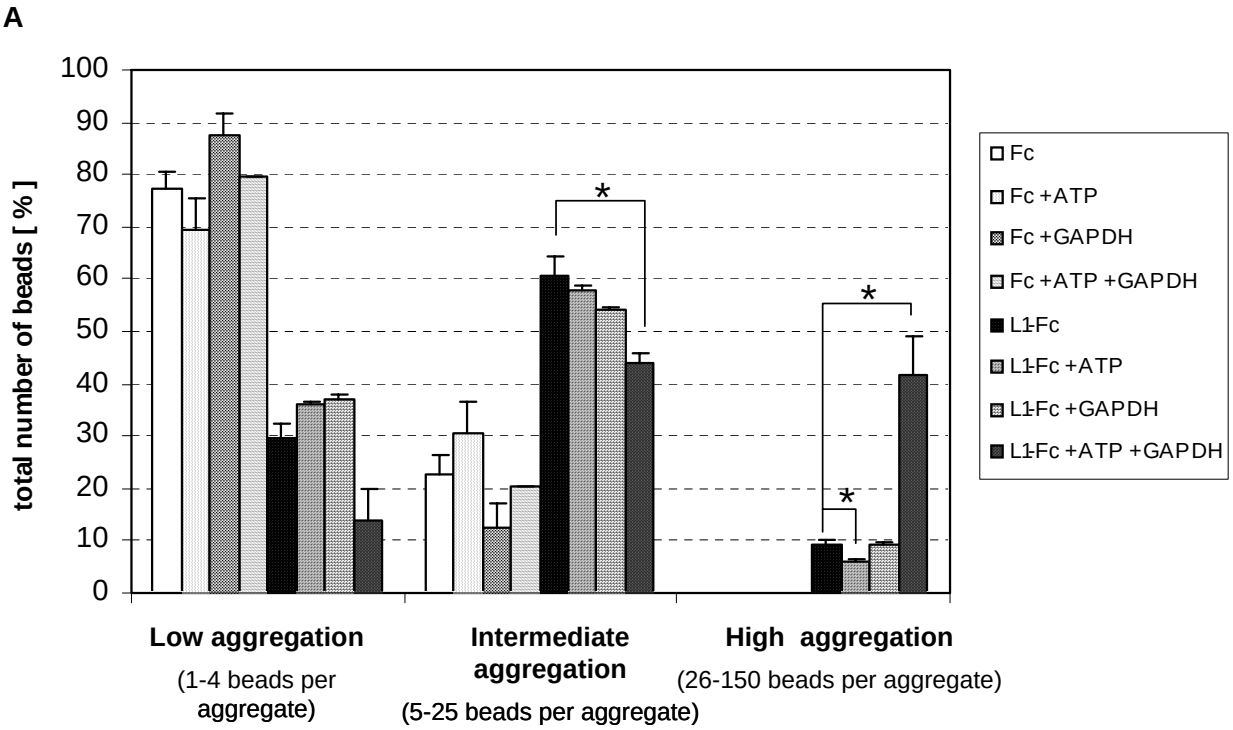
5.1.19.7. Aggregation assay (Fast track)

In phosphorylation assay it was observed that in the presence of high concentrations of GAPDH L1-Fc was not phosphorylated and formed a monomer on SDS-PAGE, while in the absence of GAPDH and presence of only ATP the molecular weight of L1-Fc shifted to a higher molecular weight that corresponded to that of a homo-dimer.

To test whether GAPDH and ATP modulate homophilic L1 interaction aggregation assay was performed. Therefore L1-Fc and Fc (control) were immobilized to beads and these beads were incubated either without or with GAPDH (5 µg/ml) and/or ATP (5 µg/ml).

In the absence of ATP and GAPDH approximately 60% of all beads with immobilized L1-Fc were present in aggregates of intermediate size comprising 5-25 beads, about 30% were found in small aggregates of 1-4 beads and approximately 10% were in large aggregates of 26-150 beads (**Figure 34A, B**). In the presence of ATP or GAPDH 58% or 55% of all beads carrying L1-Fc formed aggregates of intermediate size, 33% or 35% formed small aggregates, and 5% or 10% formed large aggregates, respectively (**Figure 34A, C, D**). When both GAPDH and ATP were present a significant increase in aggregation of L1-Fc-coated beads was observed:

up to 30% of beads were found in large aggregates comprising 26-150 beads, while 45% and 15% were detected in intermediate and small aggregates, respectively (**Figure 34A, E**). Under all conditions 70-90% of all beads with Fc as control were found as single beads or formed very small aggregates consisting of 2-4 beads, while 10-30% formed aggregates of intermediate size consisting of 5-25 beads, but no large aggregates were observed (**Figure 34A, F**).



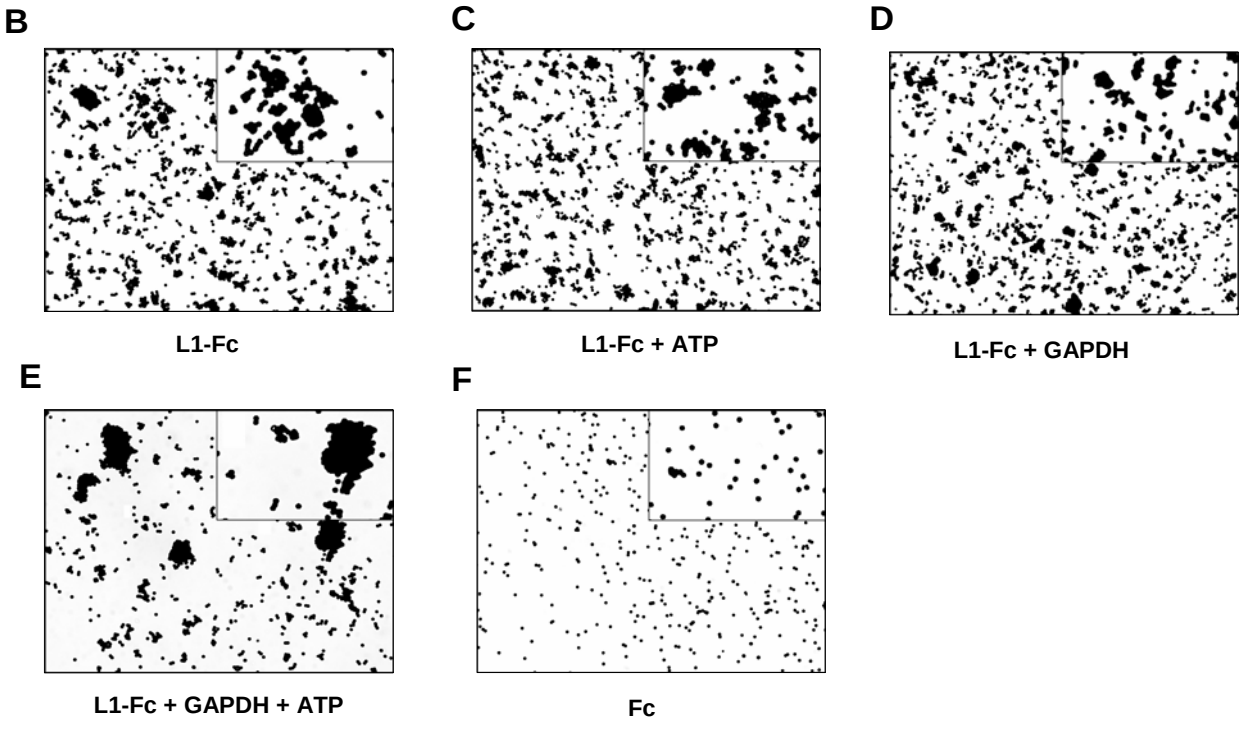


Figure 34: Aggregation assay (Fast track). Promotion of L1 homophilic adhesion by application of GAPDH and ATP.

Protein A beads were coupled to L1-Fc **(B-E)** or human Fc **(F)** and then incubated either with GAPDH **(D)** or ATP **(C)** or GAPDH and ATP **(E)**. Aggregation was measured under Kontron microscope using AxioVision programm (Zeiss).The total number of beads subjected for one experiment was set as 100%; percentage of beads forming different type of aggregates (1-4; 5-25; 26-150) was counted **(A)**. Three independent experiments were done. The statistical analysis was performed using student T-test. Error bars represent standard error mean, asterisks demonstrate a statistical significance (Student's t-test: * $p<0.05$).

5.1.20. Exosomal secretion as a possible origin of the extracellular GAPDH

Since GAPDH was characterized as a soluble cytoplasmic protein it was unclear how GAPDH can reach the extracellular matrix. Recently it was found that the part of multivesicular bodies (MVB's) can fuse with plasma membrane, thereby releasing different proteins in the extracellular milieu (Faure et al. 2006). Secretion of such extracellular vesicles, called exosomes, allows the exchange of membrane proteins and modulate membrane surface properties. Exosome secretion can be regulated by depolarisation (Faure et al. 2006). It has also been shown that in some types of exosomes enzymes such as GAPDH and pyruvate kinase were secreted (Pisitkun et al. 2004). In order to check the possible mechanism of secretion of GAPDH exosomes from asrocyte and neurons were isolated.

For this purpose astrocytes from the whole brain and cerebellar neurons were prepared and the cells were seeded onto PLL-coated flasks (25 cm^2). Before exosome preparation the cells were cultivated overnight in a serum-free medium and then treated with or without 1 μM ionomycin for 2 hours at 37°C to allow the membrane depolarisation calcium influx. Overnight medium and ionomycin-treated and untreated culture supernatants were collected and centrifuged for 10 min at 1000 x g and for 15 min at 17000 g to remove cellular debris. Membrane vesicles were collected by centrifugation at 100000 g for 1.5 hours at 4°C using a Beckman SW55i rotor. Exosomes (the pellet) were directly dissolved in SDS-sample buffer. Proteins contained in supernatant were precipitated and applied for Western blot analysis using α-GAPDH monoclonal antibody (**Figure 35**) demonstrated the presence of GAPDH in astrocytes as well as in cerebellar exosomes released after ionomycin induction but not in exosomes without ionomycin treatment.

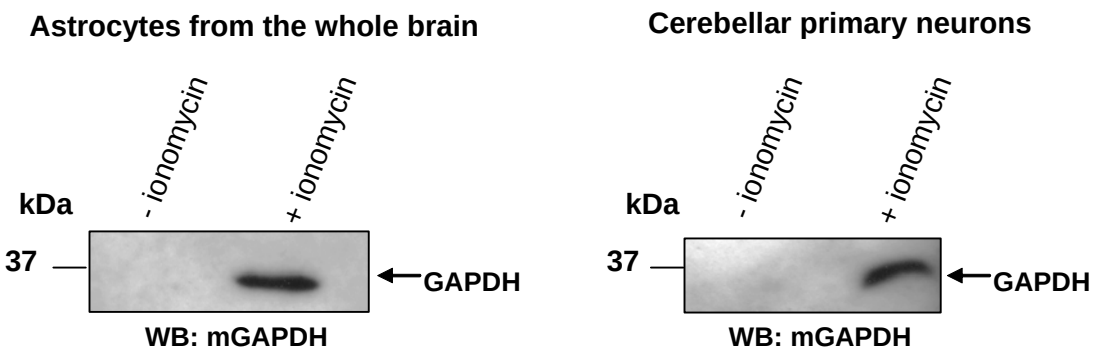


Figure 35: Exosomal secretion as a possible origin of the extracellular GAPDH.

Exosome release was induced by 1 μM ionomycin application for 2 hour to astrocytes and cerebellar primary neurons. Exosomes were collected from the medium by three steps of centrifugation: at 1000 x g (10 min), 17000 x g (15 min) and 100000 x g (1.5 hours). Exosomes (the pellet) were directly dissolved in SDS-sample buffer and applied for Western blot (WB) analysis using monoclonal α-GAPDH antibody.

5.2. Study 2:

Characterization and functional analysis of putative binding partners of polysialylated neural cell adhesion molecule (PSA-NCAM)

5.2.1. Distribution of MARCKS, CAP-23 and histone H1 in different membrane subfractions

Since novel putative binding partners of PSA-NCAM, which were discovered using anti-idiotype approach, have been shown to associate to lipid-enriched membrane regions, distribution of MARCKS, CAP-23 and histone H1 were investigated in Triton X-100 soluble and insoluble membrane subfractions and in postsynaptic densities. For that purpose Western blot analysis using polyclonal α-MARCKS, α-CAP-23 and α-histone H1 was performed (**Figure 36**) which revealed that MARCKS is distributed in all fractions with increased amount in the insoluble subfraction and in postsynaptic densities, CAP-23 is presented by different isoforms also in all membrane subdomains and histone H1 is detectable only in the insoluble fraction and in postsynaptic densities.

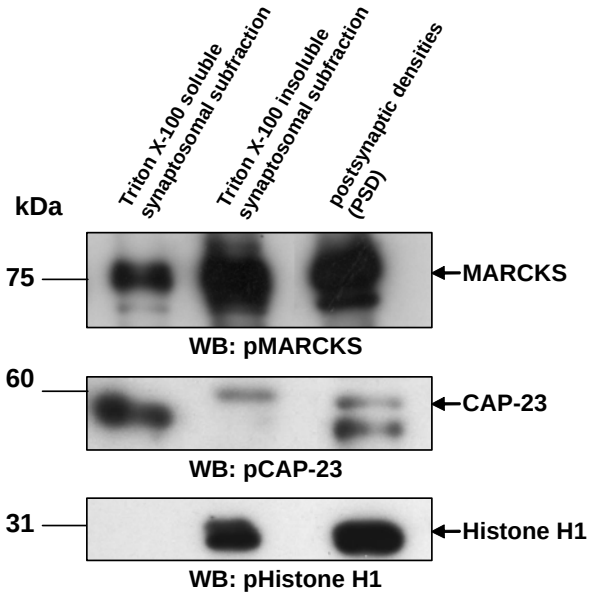


Figure 36: Distribution of MARCKS, CAP-23 and histone H1 in different membrane subfractions.

Triton X-100 soluble, insoluble subfractions and postsynaptic densities were obtained and subjected for Western blot analysis using polyclonal α-MARCKS, α-CAP-23 and α-histone H1 antibodies.

5.2.2. Co-immunoprecipitation of PSA-NCAM and histone H1 from brain homogenate

Since NCAM was identified as a main carrier of polysialic acid in the brain, immunoprecipitation of histone H1 from brain homogenate from NCAM wild type and knockout mice was performed in order to observe the association between PSA-NCAM and

histone H1. For this purpose polyclonal α-histone H1 antibodies were coupled to Protein A/G magnetic beads. After incubation with brain homogenates from NCAM$^{+/+}$ and NCAM$^{-/-}$ mice co-precipitated proteins were specifically eluted using glycine elution buffer (pH=2.7) and applied for Western blot analysis using monoclonal α-PSA antibody (**Figure 37**) which demonstrated the co-precipitation of PSA-NCAM from homogenate of NCAM wild type mice, but not from NCAM knockout mice. No PSA-NCAM co-precipitated with α-MARCKS and α-CAP-23 polyclonal antibodies were detectable.

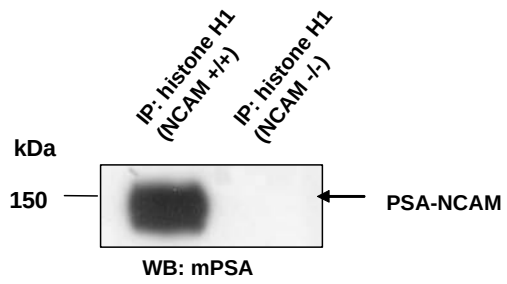


Figure 37: Co-immunoprecipitation of PSA-NCAM and histone H1 from brain homogenate. Histone H1 was precipitated using polyclonal α-histone H1 antibodies immobilized on the Protein A/G magnetic beads from homogenate of NCAM wild type and knockout mice and subjected for Western blot (WB) analysis using monoclonal α-PSA antibody (IP – immunoprecipitation).

5.2.3. Characterization of the binding of histone H1 and MARCKS to PSA-NCAM using an ELISA approach

BIACORE technique and chemical cross-linking experiment using recombinant PSA-NCAM-Fc and brain homogenate did not reveal direct interactions between PSA-NCAM and its potential binding partners (data are not shown). To further investigate possible direct binding between PSA-NCAM and histone H1 or MARCKS an ELISA assay was performed. MARCKS purified from brain and histone H1 purified from calf thymus were coated on the absorbent plastic surface. Recombinant PSA-NCAM-Fc in different concentrations was incubated with coated MARCKS or histone H1. Detection of the potential interaction was done using monoclonal α-PSA antibody. ELISA assay revealed direct binding of PSA-NCAM-Fc to histone H1, but not to MARCKS (**Figure 38**).

5.2.4. Characterization of the binding of histone H1 to PSA-NCAM using an ELISA approach

To exclude the possible association of histone H1 to protein backbone of PSA-NCAM-Fc ELISA was performed with NCAM-Fc as an additional control. Histone H1 purified from calf thymus was coated on the absorbent plastic surface. Recombinant PSA-NCAM-Fc and

NCAM-Fc in different concentrations were incubated with coated histone H1. Detection of the potential interaction was done using monoclonal α-PSA and polyclonal α-NCAM antibodies. ELISA assay revealed that histone H1 directly binds to PSA-NCAM-Fc and to NCAM-Fc as well but the affinity of PSA-NCAM-Fc to histone H1 is 50% higher than the affinity of NCAM-Fc (**Figure 39**).

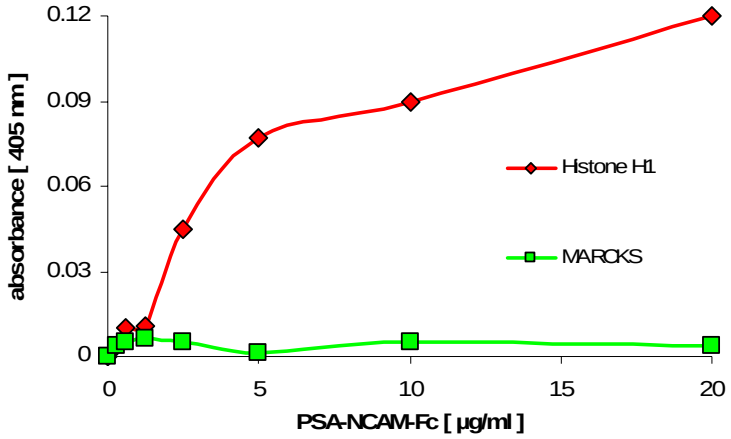


Figure 38: ELISA assay for the evaluation of interaction of histone H1 and MARCKS to PSA-NCAM. Immobilized MARCKS and histone H1 (5 µg/ml) were incubated with PSA-NCAM-Fc in different concentrations (0.45-20 µg/ml). Detection of the potential interaction was done using monoclonal α-PSA antibody.

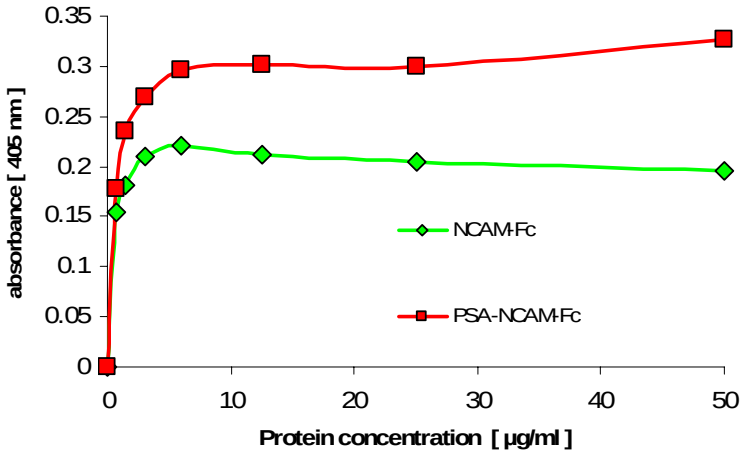


Figure 39: ELISA assay for the evaluation of interaction between histone H1 and PSA-NCAM. Immobilized histone H1 (5 µg/ml) was incubated with PSA-NCAM-Fc and NCAM-Fc in different concentrations (0.8-50 µg/ml). Detection of the potential interaction was done using monoclonal α-PSA and polyclonal α-NCAM antibodies.

5.2.5. Establishment of extracellular localization of histone H1 and MARCKS proteins using cell surface biotinylation of N2A cell line

Since polysialylation of NCAM occurs only extracellulary, proteins able to interact to PSA should be present (temporary or constantly) at the surface of neuronal cells. To test, whether MARCKS, CAP-23 and histone H1 are expressed on the outer surface of neuronal cells, N2A

neuroblastoma cell line was subjected to surface biotinylation. Biotinylated proteins were isolated using streptavidin magnetic beads and separated by SDS-PAGE. Western blot analysis with polyclonal α-MARCKS, α-CAP-23 and α-histone H1 showed that MARCKS (**Figure 40A**) and histone H1 (**Figure 40B**), but not CAP-23, were isolated from N2A cells. Detection using neutravidin-HRP confirmed that proteins with apparent molecular weight of 80 and 32 kDa were indeed biotinylated.

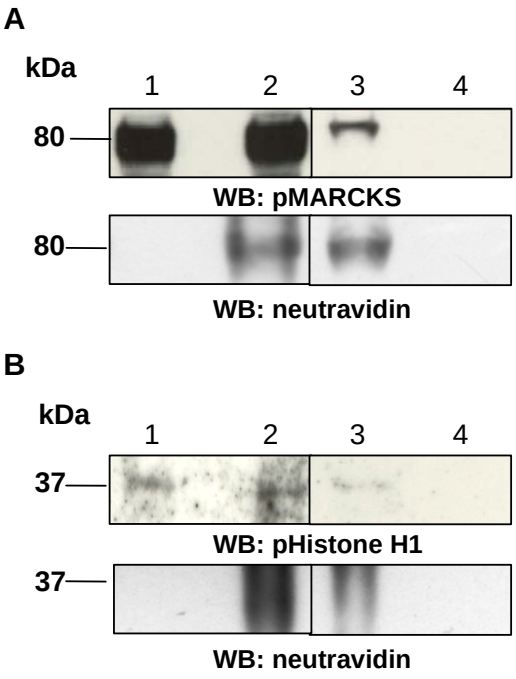


Figure 40: Establishment of the extracellular localization of MARCKS and histone H1 proteins using cell surface biotinylation of N2A neuroblastoma cell line.

Neuroblastoma N2A cells were treated in the absence (lane 1 and 4) or presence (lane 2 and 3) of biotinylation reagent. Biotinylated surface proteins were precipitated by streptavidin beads (lane 3 and 4) and subjected to SDS-PAGE. Cell lysates (lane 1 and 2) were used as input control. Western blot (WB) analysis using either polyclonal α-MARCKS antibodies **(A)** or polyclonal α-histone H1 antibodies **(B)** and neutravidin-HRP demonstrated that MARCKS and histone H1 are localized at the outer surface of neuronal cells.

5.2.6. Stimulation of neuritogenesis of cerebellar granule cells by histone H1

Since the only interaction between PSA-NCAM and histone H1 was proved neurite outgrowth assay was performed in order to check influence of purified histone H1 on neuritigenesis mediated by PSA-NCAM. For investigating of histone H1 effect on the length of neurites cerebellar primary granule cells have been used. Histone H1 was either coated alone (10 µg/ml) or in combination with PSA-NCAM-Fc (10 µg/ml) or added as a soluble compound (5 µg/ml). L1-Fc (10 µg/ml) and PSA-NCAM-Fc (10 µg/ml) were coated as controls.

Total length of neurites was chosen for the comparison of groups. The statistical analysis of stimulation effects compared to PLL control was performed using student T-test.

Neurite outgrowth assay on cerebellar primary neurons has shown the stimulation effect mediated by coated, but not soluble, histone H1 (**Figure 41**). Stimulation effect of coated histone H1 was measured as 25% increase comparing to PLL control. Significant increase of the total length of neurites was observed also when PSA-NCAM and histone H1 were coated together.

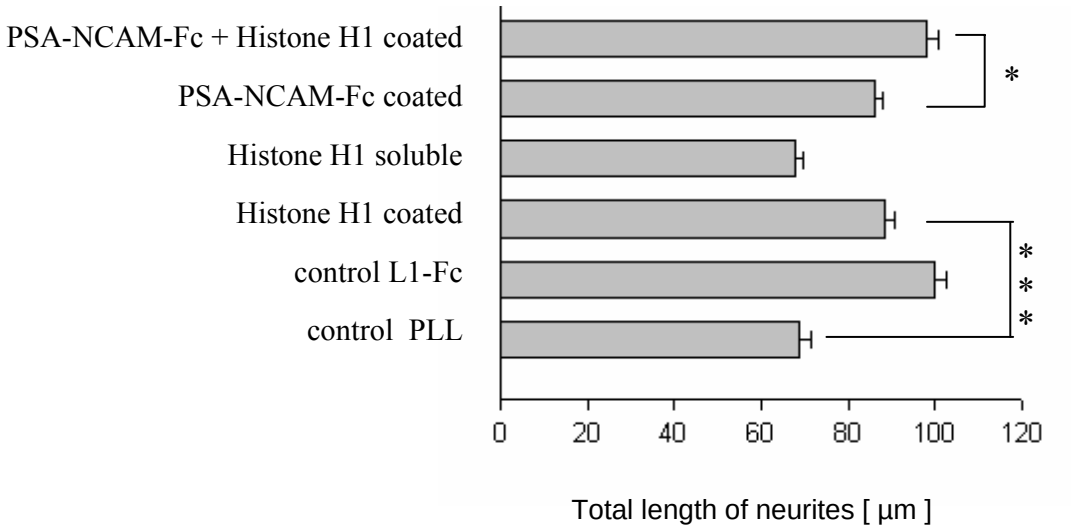


Figure 41: Stimulation of neuritogenesis of cerebellar granule cells by histone H1.

Cerebellar primary granule cells were cultured in chemically defined serum-free medium. Histone H1 was coated alone (10 µg/ml) or in combination with PSA-NCAM or added as a soluble (5 mg/ml). Total length of neurites was measured. The statistical analysis of stimulation effects compared to PLL control was performed using student T-test. Error bars represent standard deviation, asterisks demonstrate a statistical significance (Student's t-test: * $p<0.05$; *** $p<0.001$).

6. Discussion

6.1. Study 1

In the present study, the identification, characterization and functional analysis of putative binding partners of adhesion molecule L1 were carried out. For this purpose, L1 molecule was immunoprecipitated using monoclonal α-L1 antibody 555 from L1-enriched synaptosomal membrane subfraction. Three proteins with apparent molecular weight of 37, 32 and 30 kDa, co-immunoprecipitated in a complex with L1, were isolated from Coomassie stained gel and subjected to mass spectrometry analysis. One protein was identified as a glycolitc enzyme **glyceraldehyde-3-phosphate dehydrogenase (GAPDH)**. Another one was identical to **ATP/ADP carrier,** a member of the mitochondrial **adenine nucleotide translocator family (ANT),** while a third protein was found to be the GPI-linked integral membrane protein **Thy-1,** a member of Ig superfamily.

6.1.1. Characterization of GAPDH as a novel direct binding partner of L1

6.1.1.1. Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered a classical glycolytic protein involved in cytosolic energy production. GAPDH catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD):

D-glyceraldehyde-3-phosphate + NAD^+ + HPO_4^{2-} <--> 1,3-diphosphoglycerate+NADH+ H^+.

Historically, this reverse reaction was the primary assay to quantitate GAPDH glycolytic activity (Berg et al. 2002). GAPDH is comprised of a polypeptide chain of 330 amino acids. As an active glycolytic enzyme it is isolated as a tetramer of approximately 150 kDa composed of four identical 37 kDa subunits whereas the monomer and the dimer are inactive. Each subunit is composed of two domains; three bound substrates lie at an interface between the two domains. GAPDH binds four NAD^+ molecules to individual active sites on each subunit. Critical amino acids include cys^{149} and his^{176}. Structural studies identified two significant regions, the NAD^+ binding site which encompasses amino acids 1-150, and the glyceraldehyde-3-phosphate binding site comprising the C-terminal portion: 151-335 amino acids (Skarzynski et al. 1987) (**Figure 42**).

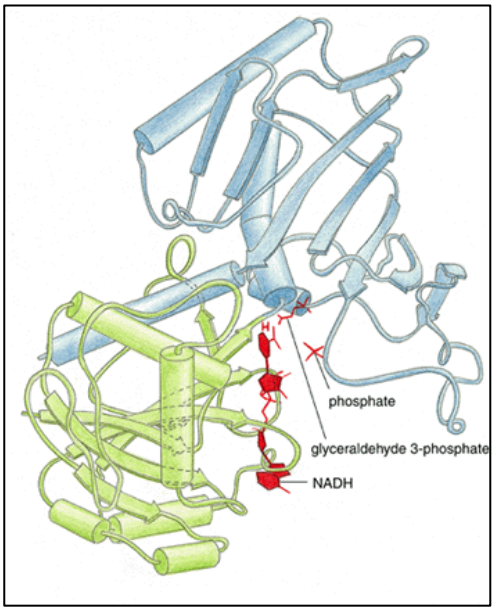


Figure 42: The structure of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
The protein is composed of two domains, each shown in a different color, with regions of a helix represented by cylinders and regions of beta-sheet represented by arrows. Three bound substrates lie at an interface between the two domains (Berg et al. 2002).

GAPDH has been isolated from numerous organisms and it is ubiquitous in nature. Sequences are highly conserved across the phylogenetic scale. Archaeal glyceraldehyde-3-phosphate dehydrogenases are homologous to those from bacteria and eukaryotes (Lebherz and Rutter 1967).

Mammalian GAPDH gene family appears to contain one active gene and diversity of pseudogenes. The GAPDH active gene was localized to human chromosome 12 (Bruns and Gerald 1976) and mouse chromosome 16 (Seipp and Buselmaier 1994). In accord with these findings, several studies identified a single mRNA species in variety of tissues because of that GAPDH was used as an invariant control in studies of gene and protein expression. However, recent evidence suggests revision of this conventional dogma (Sirover 1999).

Number of studies has indicated that GAPDH is not an uncomplicated, simple glycolytic protein, but demonstrated diverse biological properties unrelated to glycolysis. These included roles for GAPDH in membrane transport and membrane fusion, microtubule association, nuclear RNA export, phosphotranferase/kinase reactions, the translation control of gene expression, DNA replication and DNA repair (Sirover 1999).

Functional diversity of GAPDH has been also shown to play an important role in nervous system events. Direct involvement for GAPDH in apoptotic mechanisms was firstly shown in cerebellar granule cell cultures where low extracellular K^+-induced apoptosis resulted in an increase in both GAPDH mRNA and protein that was integral to the induction of apoptosis. By pre-incubation of cultures with GAPDH anti-sense oligodeoxynucleotides evoked not only GAPDH changes, but also prevention of apoptosis (Sunaga et al. 1995). Currently this novel function of GAPDH is intensively investigated (Berry and Boulton 2000). GAPDH has been demonstrated to participate in neurodegenerative diseases via interacting with a number of

proteins implicated in the etiology of diseases, including ß-amyloid precursor protein (Cumming and Schubert 2005) and proteins containing polyglutamine repeats, in particular huntingtin (Koshy et al. 1996). It has recently been reported that in the nervous system GAPDH has a kinase activity modulating GABAa receptor functions and thus regulates neuronal inhibition associated with a GABAa receptor subunit (Laschet et al. 2004).

GAPDH is a very abundant protein and constitutes between 10-20 % of total cellular proteins (Sirover 1999). In spite of "classical" cytoplasmic and accidental nuclear localization, GAPDH was found, carrying particular functions, at the surface of plasma membrane. First indications that GAPDH is also present outside of cells in the extracellular space and may play a role in cell adhesion derived from the observation that GAPDH was found at the outer surface of bacteria like *Streptococci* or fungi like *Candida albicans* and that GAPDH functions as a mediator of bacterial and fungal adherence to mammalian host cells (Seifert et al. 2003;Brassard et al. 2004;Jin et al. 2005). GAPDH was shown to bind cell adhesion molecules in extracellular matrix like fibronectin and laminin (Gozalbo et al. 1998). Recently, it has been shown for the number of different mammalian cell lines, including neuroblastoma N2A cells that secreted GAPDH binds to the outer cell surface and affects the cell spreading (Yamaji et al. 2005). Furthermore, it has been shown that GAPDH is present at the outer surface of human and murine macrophages and acts as a transferrin receptor (Raje et al. 2007). The involvement of GAPDH in neuronal inhibition as well as neurodegenerative diseases, apoptosis and cell adhesion has made GAPDH the unique "moonlighting" protein (Jeffery 1999) with variety of activities in both prokaryotic and eukaryotic cells.

6.1.1.2. Characterization of the L1–GAPDH interaction

Cell-cell adhesion is crucial for brain morphology, regeneration, synaptogenesis and highly coordinated brain functions such as memory and learning (Washbourne et al. 2004). Since L1 was identified as a one of key molecules mediated neuronal adhesion the investigation of the complexity of L1 interaction has become a topical question. Although classical glycolytic enzyme GAPDH has been demonstrated to exhibit multiple functions unrelated to glycolysis, the identification of GAPDH as protein co-precipitated with L1 from synaptosomal subfraction represented the first intriguing evidence for possible direct association between neuronal cell adhesion and enzymes, involved in energy production. Although several glycolytic enzymes, namely GAPDH, brain-specific aldolase C and enolase were previously purified from synaptic vesicles from both bovine and rat brains and from a neuroblastoma cell line (Bulliard et al. 1997), there were no indication about involvement of adhesion molecules as receptors. The attention to that unexpected identification was supported by high reliability

of sequence analysis which revealed GAPDH as a co-precipitated with L1. Mass spectrometry has been combined with postsource decay fragment ion mass analysis, which strongly proved the identity to GAPDH. But so far co-immunprecipitation approach was known to indicate only protein association, but not direct interaction, different binding assays required to be performed in order to prove this interesting finding. For this purpose, recombinant proteins composed of extracellular domains of L1, APP, NCAM and PSA-NCAM fused to Fc tag were produced and used in BIACORE and chemical cross-linking experiments. APP-Fc was used as a control protein because the direct association between cytoplasmic, but not extracellular domain of APP and GAPDH was shown previously (Schulze et al. 1993). Control NCAM-Fc as another member of immunoglobulin superfamily, shared similar features, was applied to assays whereas polysialylated NCAM was chosen to investigate possible influence of carbohydrate modifications. As a result, the first evidence for direct binding between GAPDH and extracellular domain of L1 was obtained in BIACORE approach and further confirmed by chemical cross-linking assay. Application of control proteins revealed no binding of GAPDH to APP-Fc and PSA-NCAM-Fc, but surprisingly showed the interaction between GAPDH and extracellular domain of NCAM, providing the information that common structural features of L1 and NCAM can be involved in the interaction with GAPDH, and prominent carbohydrate modification can disturb the binding. Furthermore, these results were confirmed by application of the same recombinant proteins to ELISA experiments, where purified GAPDH was substrate coated. No binding was observed between GAPDH and APP-Fc and PSA-NCAM-Fc, but L1-Fc and NCAM-Fc demonstrated the strong reproducible interaction to coated GAPDH.

Since GAPDH was co-isolated with the whole immunoprecipitated L1 molecule, the possibility of GAPDH binding also to cytoplasmic domain of L1 was checked in ELISA using recombinantly produced intracellular domain of L1. Results of experiments, demonstrated no binding of GAPDH to cytoplasmic part of L1, finally confirmed the fact that unusual interaction between adhesion molecule L1 and glycolytic enzyme GAPDH situated outside of the cell and attached to extracellular L1 region.

The L1 extracellular region has been shown to bind a range of cell surface and extracellular matrix molecules, demonstrating both homo- and heterophilic binding either in *cis*-, or in *trans* orientation; these interactions influence such important processes like neurite fasciculation and neurite outgrowth, migration, myelination, regeneration of the peripheral nervous system, axon guidance (Holm et al. 1995). Particular regions on L1 extracellular domain, involved in the binding of L1 to other proteins, almost for all known interactions are identified (Haspel and Grumet 2003). Thus, mapping of precise binding site for GAPDH on

L1 extracellular domain was important step to check the possible GAPDH-mediated regulation of L1 binding to other surface receptors. In order to prove this analysis, recombinant fragments of L1 extracellular region were produced and used in the ELISA approach. As a control for those experiments another glycolytic enzyme phosphoglucose isomerase (PGI) was used. PGI was found at the surface of neuronal cells and identified as a neuroleukin, associated with autocrine motility factor (Lagana et al. 2005). ELISA approach revealed the binding between GAPDH, but not PGI, and both the whole Ig I-VI and Fn 1-5 fragments of L1; the affinity of Fn 1-5 binding to GAPDH was at least three times higher in comparison to those of Ig I-VI. Analysis using individual domains demonstrated that only the whole Ig I-VI, but not Ig I-II, Ig III-IV, Ig V-VI, could bind to GAPDH, while the binding of Fn region is mostly mediated by 4-5 domains. The presence of a third domain in Fn 3-5 somehow inhibited its interaction to GAPDH. According to work of several independent laboratories, Fn 4-5 region was not shown previously to mediate any of important L1 interaction, whereas the whole Ig I-VI fragment demonstrated influence on many of L1 functions, including homophilic L1-L1 and heterophilic binding to the majority of surface-expressed adhesion molecules (Holm et al. 1995;Haspel and Grumet 2003). Futhermore, identification of more than one binding sites for GAPDH at the L1 extracellular region could be an indication for dual, perhaps independent, function of GAPDH in regards to adhesion in the brain.

Localization of the binding site within GAPDH molecule, involved in the binding to L1 extracellular domain, appeared to be a more difficult question, because there was no precise function for that interaction known. Active site of GAPDH, responsible for classical glycolytic function and also mediating many of unrelated to glycolysis activities (Sirover 1999) could be involved in this interaction. In order to check this possibility, recombinant human wild type and mutant Cys^{149}-GAPDH were obtained from Dr. Yamaji (Lab of Prof. Nakano, Osaka, Japan). This group has shown that secretion of GAPDH from different cell lines, including neuroblastoma N2A, and inhibition of cell spreading are affected by mutation in GAPDH, substituted Ser instead of active Cys^{149} (Yamaji et al. 2005). Subjection of those molecules to functional neurite outgrowth assay, which will be discussed later, revealed no difference in the effects mediated either by wild type or mutant GAPDH (data are not shown). This finding allowed to conclude that active site of GAPDH enzyme including Cys^{149} is not important for L1 binding.

6.1.1.3. Establishment of surface localization of GAPDH in neuronal cells

L1 adhesion molecule is known to express in neurons at their cell bodies and across non-myelinated axons (Faissner et al. 1984), but not detected in postsynaptic densities, where GAPDH was previously isolated from (Moon et al. 1998;Walikonis et al. 2000). Internalization of L1 molecule, critical regulatory point of L1-mediated signal transduction (Schaefer et al. 2002), and proteolytic processing of L1 spread the potential subcellular localizations for L1-GAPDH interaction. However, in spite of only a few reports describing the presence of GAPDH on the surface of mammalian cells (Chu and Low 2006;Raje et al. 2007) the outer membrane of neuronal cells appeared to be the most possible location for GAPDH-L1 binding. In order to analyse this hypothesis, cell surface biotinylation was performed using sulfo-biotin reagent, providing with high probability the biotinylation only of surface-expressed proteins. In neuroblastoma cell lines N2A and B104 and primary cerebellar granule cells GAPDH was detected as a biotinylated protein, confirmed outer localization and plasma membrane association of glycolytic enzyme GAPDH. Estimation of the percentage of GAPDH at the outer surface relative to total cellular GAPDH revealed that GAPDH at the outer cell surface accounts for about 0.01% of total cellular GAPDH.

Furthermore, application of α-GAPDH monoclonal antibody to living cerebellar granule cells in immunocytochemistry experiments revealed, that GAPDH indeed was detected at the neuronal plasma membrane and partially co-localized with L1 adhesion molecule at cell bodies but not along the axons. However, it was clear from immunoflurescent imaging that not all GAPDH, located at the membrane, is associated with the L1. Here it is appropriate to remember the indication for direct GAPDH binding also to NCAM extracellular domain. Although the GAPDH-NCAM association is not a topical question of the present study, the extended function of surface GAPDH in neural adhesion based on the interaction with at least two cell adhesion molecules of the immunoglobulin superfamily could be an interesting outlook for the future experiments.

Mechanisms, mediated the secretion of “classical” cytoplasmic proteins to the extracellular space, are not known up to date. It has been proposed that these cytosolic proteins may be secreted by utilizing non-classical secretory pathways (Cooper and Barondes 1990;Nickel 2003). The hypothesis for the secretion of functional GAPDH to the surface of plasma membrane via exosome release, checked in this study, presents just one of the possible pathways.

Exosomes are the 60–100-nm membrane internal vesicles of multivesicular bodies (MVBs) that are delivered to the extracellular fluid by fusion of the outer membrane of MVB with the plasma membrane. Exosomes were discovered by R. Johnstone in studies of erythrocyte

maturation two decades ago (Pisitkun et al. 2004). Since the initial description of exosomes, many cell types have been shown to secrete them including B-cells, T-cells, dendritic cells, reticulocytes, mastocytes, enterocytes, and platelets. The exosome secretion was proposed to be a novel mechanism of cell communication, since many cellular proteins, including GAPDH, were defined as a specifically targeted to exosomes (Thery et al. 2002). Release of exosomes was found to be dependent on increasing of intracellular Ca^{2+} and membrane depolarisation (Wubbolts et al. 2003) but factors stimulated release of exosomal content into extracellular space are not identified up to date.

In this study exosomes were obtained from astrocyte and primary neuronal cultures by application of ionomycin. Exosomes, isolated from the cell culture supernatant without application of ionomycin, were used as a control. GAPDH was detected in exosomes obtained from ionomycin treated, but not untreated, both astrocyte and neuronal cultures, that is corresponding to previous finding (Faure et al. 2006). Nevertheless, other secretory pathways should not be excluded as possible mechanisms for GAPDH secretion.

6.1.1.4. Functional analysis of the L1–GAPDH interaction

Since the direct binding between L1 and GAPDH and the surface localization of GAPDH were established, the reasonable question about function for that unusual interaction has appeared. GAPDH demonstration of multiple functions different from the glycolytic dehydrogenase activity in the multiple intra- and extracellular localizations (Yamaji et al. 2005) has addressed the main question whether the GAPDH-dependent modification of L1 extracellular region changes nervous system functions, normally mediated by L1.

Regarding to novel function for GAPDH in the nervous system, namely endogenous kinase activity for α-subunit of GABAa receptor, discovered by Laschet and colleagues (Laschet et al. 2004), it was hypothesized that GAPDH could be an ecto-protein kinase for L1. This hypothesis was supported by identification a sequence **(NXXSR)** on the third Ig-like domain of L1 similar to the consensus motif **(NXXS/TK)** for phosphorylation by GAPDH on α-subunit of GABAa receptor (Laschet et al. 2004). The presence of ATP and Mg^{2+} ions - essential components for the phosphorylation - outside of the cell made the ecto-phosphorylation of L1 likely.

The discovery of neuronal ecto-protein kinases has revealed that the powerful regulatory mechanism of protein phosphorylation operates also in the extracellular environment of the nervous system. The phosphorylation state of surface proteins appears to influence cell communication as well as cell-environment interaction (Ehrlich et al. 1998). Recent studies demonstrated that ecto-kinase activity is not catalyzed by a single enzyme, but is carried-out

by several different ecto-protein kinases that can be blocked by inhibitors. Thus, extracellular phosphorylating events can regulate the function of receptors, ion channel, uptake carries, integrins, adhesion proteins, growth factors in the nervous system, and thus may play critical roles in homeostasis, neuritogenesis, neuronal adhesion, synaptogenesis and maintenance of long-term potentiation (Ehrlich et al. 1998).

Although GAPDH itself is not phosphorylated in its glycolytic reaction, and ATP is not a substrate in this oxidation–reduction cycle, auto-phosphorylation of GAPDH and transfer of phosphate to target proteins under certain conditions have been established (Kawamoto and Caswell 1986;Laschet et al. 2004).

In order to check the possible GAPDH-dependent phosphorylation of L1 extracellular domain, *in vitro* phosphorylation assay was performed using L1-Fc and NCAM-Fc and recombinant fragments of L1 extracellular region. NCAM-Fc was applied as a control to the assay, because NCAM has been shown to bind and hydrolyze extracellular ATP; two potential ATP-binding sites were proposed to localize to Fn 1-2 domains of NCAM (Skladchikova et al. 1999).

In vitro phosphorylation assay using L1-Fc, NCAM-Fc and 2- or 5-fold molar excess of GAPDH has revealed several important observations for further analysis. Firstly, auto-phosphorylation of extracellular L1 region in the absence of GAPDH was observed. This finding could argue either for ATP binding, as it was shown for NCAM, or kinase activity attached to L1 extracellular region itself. Secondly, with 2-fold excess the GAPDH-dependent phosphorylation of L1 extracellular domain was observed, and increasing the GAPDH concentration (up to 5-fold excess) led to abolishment of L1 phosphorylation and increasing of auto-phosphorylation of GAPDH, that can be explained by competition for ATP. And finally, very intriguing shift in the molecular weight of L1, but not NCAM, extracellular domain was observed in the presence of only ATP. After verification by Western blot using α-L1 antibodies, it was established, that dimerization of L1-Fc took place after auto-phosphorylation, but was disturbed by GAPDH (data are not shown). This dimerization of L1-Fc has provided the first evidence, that GAPDH somehow influences the L1 homophilic interaction.

Using Ig I-VI and Fn 1-5 recombinant fragments of L1 extracellular region in phosphorylation assay *in vitro* has demonstrated that GAPDH-dependent phosphorylation localized to Ig I-VI domain. Within this domain the potential phosphorylation sequence **(NXXSR)** was identified in addition to ELISA results revealed GAPDH binding to the whole Ig I-VI domain. Auto-phosphorylation of L1 was established to be Fn 1-5-dependent, since this domain was phosphorylated in the absence of GAPDH. It is interesting to note that within GAPDH

sequence ATP-binding motif (**GXXGXG**) was identified to 7-13 amino acids in N-terminus, which is characterized as a well-known Rossman motif (Kemp and Pearson 1990) necessary for kinase activity. A similar sequence motif **(GXGXG)** was found within L1 corresponding to 899 – 903 amino acids which compose the part of the third and forth Fn domains.

Since L1 adhesion molecule and its homophilic interaction were shown to be strongly involved in neuritogenesis and targeting of axons (Zhao et al. 1998) further confirmation for significance of GAPDH-dependent modification of L1 for its adhesive properties was obtained in neurite outgrowth assay. Promoting concentration-dependent effect of soluble GAPDH on the neuritogenesis of primary cerebellar granule cells showed the importance of exogenous GAPDH, but did not prove that this stimulation effect is indeed mediated by L1 molecule after GAPDH modification. Application of soluble GAPDH and ATP in different combinations to the medium of primary cerebellar cells just confirmed the results of *in vitro* phosphorylation assay showing competition for ATP between GAPDH and unknown targets.

Final improvement that the promoting effect of exogenous GAPDH on the neuritogenesis of cerebellar neurons was indeed L1-mediated was obtained in experiments with pre-treatment of L1 coated substrate by both GAPDH and ATP. Application either GAPDH or ATP alone to coated L1 had no effect on neuritogenesis giving an additional confirmation, that functional GAPDH modification of L1 is an ATP-dependent.

The homophilic L1 interaction triggers the neurite outgrowth in nearly all primary neurons (Haspel and Grumet 2003). Homophilic L1 binding in *trans* has been shown to be dependent on the whole Ig I-VI domain (Lemmon et al. 1989) whereas interaction in *cis* is promoted by the third Fn-like domain (Silletti et al. 2000;Stallcup 2000). Combined results of *in vitro* phosphorylation, neurite outgrowth and additionally performed aggregation assay have finally established that GAPDH modification of L1 in an ATP-dependent manner influences homophilic L1 interaction in *trans* orientation whereas the auto-phosphorylation of L1 stimulates the L1 homophilic binding in *cis* position.

Aggregation assay, performed in order to directly investigate the L1 homophilic interaction, revealed that high clustering of beads was observed after application of GAPDH and ATP together and it corresponds to neurite outgrowth assay results. In phosphorylation *in vitro* it was established that GAPDH-dependent phosphorylation restricted to Ig I-VI domain which is responsible for L1 homophilic interaction in *trans*. So it could be concluded that GAPDH phosphorylation of L1 extracellular domain in Ig region stimulates L1 homophilic interaction in *trans* orientation which is well seen in promotion of the beads aggregation.

Addition of only ATP has caused the inhibition of the beads aggregation, represented in a decreased number of big clusters. Combined with the observed dimerization of L1-Fc under

auto-phosphorylation state of L1 restricted to Fn region one can conclude that auto-phosphorylation of L1 extracellular domain promotes homophilic interaction in *cis* orientation. L1 molecules forming homo-dimers at the surface of one bead are no more available for interaction in *trans*, which caused the inhibition of the aggregation.

In parallel to potential GAPDH kinase activity one more hypothesis have been investigating where GAPDH was suggested to be an ecto-ribosyltransferase for L1. This hypothesis based on the observation of Pancholi and colleagues (Pancholi and Fischetti 1993) that GAPDH can act as an ADP-ribosylating enzyme on the surface of group A *streptococci*. GAPDH within its "classical" glycolytic function can bind NAD^+ that is a source of ADP-ribose. Pancholi and colleagues showed that GAPDH can transfer ADP-ribose group to target proteins under certain conditions.

Mono-ADP-ribosylation is a widely used method by which eukaryotic cells modify protein structure and function. It is covalent, post-translation protein modification in which ADP-ribose moiety of NAD^+ is transferred to a specific amino acid in individual substrate. Zhao and colleagues have shown that ADP-ribosylation of extracellular matrix protein integrin can modulate the binding of integrin to another extracellular molecule laminin (Zhao et al. 2005). In the connection to that it was hypothesized that GAPDH, possessing ADP-ribosylation activity, can ribosylate L1 adhesion molecule and modify the interaction of L1 to other extracellular components, because RGD-independent integrin-binding motif within the third Fn-like domain of L1 (Silletti et al. 2000) contains arginine which could be ribosylated.

To check this possibility, *in vitro* ADP-ribosylation assay was performed which revealed non-specific binding of NAD^+ to L1-Fc, NCAM-Fc and recombinant L1 fragments. Application of combinations of NAD^+ and GAPDH to L1-mediated neurite outgrowth assay has demonstrated no effect. According to those results the hypothesis about potential GAPDH-dependent ribosylation of L1 was not confirmed.

Additional interesting function of GAPDH was established after its application to cell survival assay. Both L1 adhesion molecule and GAPDH were known previously to be involved in apoptosis: L1 exhibited the neuroprotective effect on primary neurons (Chen et al. 1999) whereas GAPDH was established as a pro-apoptotic protein, because its over-expression and translocation to the nucleus were involved in initiation of apoptosis (Chuang et al. 2005). Nevertheless some contradictional data about participation of GAPDH in oxidative stress and apoptosis served as reason for survival assay performance. For those experiments coated L1-Fc and either coated or added as a soluble compound exogenous GAPDH were used. Surprisingly the protective effect on hydrogen peroxide-induced cell death in primary cerebellar neurons was observed for both coated and soluble GAPDH, but the effect was

higher, when GAPDH was added as a soluble compound. No survival effect on staurosporin-induced cell death of neurons was observed after application of exogenous GAPDH. This involvement in protection of cells from the oxidative stress could be explained either by still unknown function of extracellular GAPDH or by already established inactivation of GAPDH by hydrogene peroxide. H_2O_2 was shown to modify GAPDH on its catalytic cysteine residue not only to inactivate the dehydrogenase activity of GAPDH but also to endow GAPDH with the ability to bind to phospholipase D2 that is involved in anti-apoptotic effect (Kim et al. 2003). It became clear that due to multifunctionality of GAPDH enzyme, it can reveal several activities dependent on environmental conditions, which still should be intensively investigated.

To summarize all mentioned above, one could conclude that glycolytic enzyme GAPDH was established to interact directly with the adhesion molecule L1 at the surface of neurons and modulate L1-induced neurite outgrowth in an ATP-dependent manner that provided novel functions for glycolityc enzymes in the connection to the cell adhesion in the nervous system.

6.1.2. Characterization of ANT as a novel direct binding partner of L1

The ADP/ATP carrier, or adenine nucleotide translocator (ANT), is the most abundant mitochondrial protein. In its functional state, it is a homodimer of 32 kDa subunits embedded asymmetrically in the inner mitochondrial membrane (**Figure 43**). The dimer forms a gated pore through which ATP is moved from the matrix into the cytoplasm (Neckelmann et al. 1987). The main function of ANT is to exchange mitochondrial matrix ATP for cytosolic ADP across the inner mitochondrial membrane, utilising the electrochemical gradient. In humans there are three isoforms of this protein, ANT1, ANT2 and ANT3, whereas there are only two isoforms in the mouse (Ant1 and Ant2). The gene Ant1 is predominantly expressed in heart and skeletal muscles and has been designated as a heart/ muscle specific isoform. By knocking out Ant1 in mouse, an ATP deficiency would result in the heart and skeletal muscle tissues dystrophy, effectively creating a tissue specific knockout.

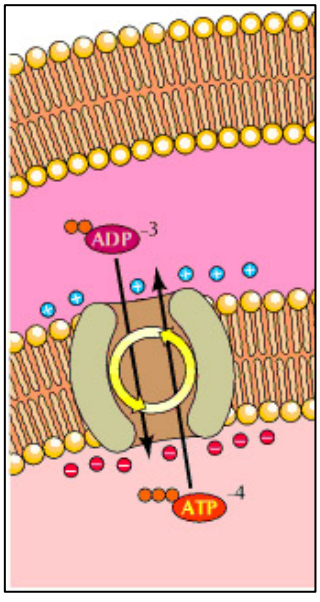


Figure 43: The structure of adenine nucleotide translocator (ANT). The protein represents a homodimer of 32 kDa subunits embedded asymmetrically in the inner mitochondrial membrane.

ANT catalyzes the coupled entry of ADP and exit of ATP into and from the matrix. The reaction cycle is driven by membrane potential (Cooper 2002).

ANT is very intensively investigated, because of the involvement in apoptosis as a part of membrane permeability transition pore (MPTP). MPTP is a protein complex that mediates a Ca^{2+}-dependent sudden increase in inner mitochondrial membrane permeability that is a common feature of apoptosis (Kokoszka et al. 2004).

The identification of ANT co-precipiated with L1 adhesion molecule from membrane fraction was not expectable since no interactions between members of immunoglobulin superfamily of cell adhesion molecules and mitochondrial proteins were previously described.

Nevertheless in this study the evidence for direct binding between ANT and L1 were obtained using co-immunoprecipitation and ELISA approaches. Immunoprecipitation experiments were performed using both α-L1 monoclonal and α-ANT polyclonal antibodies and co-immunoprecipitated ANT and 100 and 190 kDa L1 forms respectively were detected. ELISA approach performed with coated purified from heart ANT and L1-Fc revealed the direct binding. Both these findings require additional improvement, controls and another type of α-ANT antibodies. Here it should be admitted that α-ANT antibodies used in this study were produced against heart-expressed isoform of ANT1 and 2, which are known to give more than one band after application to the brain homogenate for Western blot analysis. Whether these detected bands correspond to different brain isoforms of ANT is not known up to date. To improve the results of direct binding between ANT and L1, it is necessary to produce antibodies against brain specific isoform of ANT.

In spite of mitochondrial localization of ANT, the surface of plasma membrane of neuronal cells could be proposed as potential subcellular localizations for interaction between L1 adhesion molecule and mitochondrial ANT. The possibility of expression of novel, not yet identified, membrane specific isoform of ATP/ADP transporter is not excluded. Results of the surface biotinylation on neuroblastoma cells have provided the first indication for this possibility. The approach has revealed only one biotinylated band, detected by α-ANT polyclonal antibodies. Following sequence analysis of that band could be useful for the identification of brain-specific membrane-expressed isoform of ANT.

6.1.3. Characteristics of Thy-1

Thy-1, the smallest member of the immunoglobulin superfamily (**Figure 1**), is a major cell-surface component expressed by several tissues. The protein, carbohydrate and gene structures of this molecule are known, yet its function is not. It is highly expressed in nervous

tissue, where it appears on virtually all neurons after the cessation of axonal growth. It was shown that expression of Thy-1 by a neural cell line inhibits neurite outgrowth on mature astrocytes, but not on other cellular substrata which include Schwann cells and embryonic glia. This inhibition of neurite extension on astrocytes can be reversed by low concentrations (nanomolar) of soluble Thy-1. If a similar interaction between neuronal Thy-1 and astrocytes occurs *in vivo*, it could stabilize neuronal connections and suppress axonal regrowth after injury in the astrocyte-rich areas of the adult central nervous system (Tiveron et al. 1992).

Transgenic mice with a targeted deletion of the Thy-1 gene have exhibited normal cellular organization, normal anatomical features of the corticospinal and thalamocortical pathways, and basic neurophysiological properties indicating that Thy-1 is not crucial molecule for normal development and maintenance of the nervous system (Barlow et al. 2002).

Appeared as the most probable candidate for direct interaction with L1 adhesion molecule, Thy-1 nevertheless was not proved to bind L1 by any approaches. The most possible explanation for identification of Thy-1, co-precipitated with L1 from synaptosomal subfraction, is the potential regulation of L1 delivery to raft compartments by Thy-1 because the role of Thy-1 in organization of microdomains was previously shown (Taylor and Hooper 2006).

6.2. Study 2

The vast majority of mammalian surface proteins are glycoproteins; the glycan moieties of which frequently act as modifiers and mediators of cell-cell contacts. Unique pattern of NCAM modification by high number of polysialic acid residues considered to destabilize cell-cell interactions and promote neural plasticity (Fujimoto et al. 2001). In spite of identification of many functions for polysialylated form of NCAM, it has remained unclear whether PSA influences signal transduction events because it modifies cell interaction and may act as a receptor itself (von der Ohe M. et al. 2002).

Identification of potential binding partners of PSA using anti-idiotypic approach (von der Ohe, unpublished data) has provided the information about structural, but not charge-dependent, interaction of PSA to other proteins.

In the present study, the characterization of MARCKS, NAP-22 and histone H1 as putative binding partners of PSA were performed using different approaches in order to prove the interaction and possibly highlight the mechanisms of PSA-mediated functions in the nervous system.

Initial distribution analysis of identified putative binding partners of PSA in membrane subfractions has demonstrated that MARCKS presents in Triton X-100 soluble membrane subfraction in fewer amounts compare to insoluble fraction and postsynaptic densities (PSD) whereas NAP-22 is detected as a different isoforms but also presenting in all soluble, insoluble subfractions and PSD. PSD is an electron-dense structure in which neurotransmitter receptors and associated signalling molecules (for instance, protein kinase C) are tightly clustered (Nonaka et al. 2006). So far MARCKS and NAP-22 were described as soluble proteins temporary associating with membrane and appeared to be a substrate for protein kinase C, the detection of MARCKS and NAP-22 in those membrane structures is quit expectable. Although PSA was also detected in these membrane subfractions, no further confirmation for direct interaction between PSA-NCAM and MARCKS or NAP-22 were obtained using different biochemical approaches. Detection of MARCKS, but not NAP-22, at the surface of neuroblastoma cells was a promising indication for potential interaction with PSA, but it seems to be either indirect or required further strong modification of biochemical protocols.

Histone H1, the third protein, identified as putative PSA-binding partner, was initially described as a classical nuclear DNA-packing protein and at the first glance appeared to be an experimental artefact. But discovery of the particular group of neuronal surface-expressed histone H1 with main lipopolysacharide-binding activity (Bolton and Perry 1997) has provided a possibility for interaction with polysialic acid. Distribution analysis has revealed the presence of histone H1 in Triton X-100 insoluble fraction and in PSD, but not in soluble subfraction, that confirmed tight association of histone H1 with plasma membrane. Surface biotinylation of neuroblastoma cells has confirmed the outer membrane expression of histone H1.

Further immunoprecipitaion and ELISA approaches have demonstrated the direct interaction of PSA-NCAM and histone H1. Although ELISA required the additional repetition with other controls, obtained results were promising indication for binding between PSA and histone H1. Application of purified histone H1 in neurite outgrowth assay has revealed the involvement of histone H1 in important functions of nervous system. The stimulation effect of coated, but not soluble, histone H1 on the neuritogenesis of primary cerebellar granule cells was observed. Moreover, the promoting effect on the neuritogenesis, mediated by PSA-NCAM, which has been shown previously (Doherty et al. 1990), was enhanced by simultaneous addition of histone H1. Modification of adhesive properties of polysialylated NCAM by interaction with surface-expressed histone H1 provided a new potential regulatory mechanism in the nervous system, which requires being further investigated.

Histone H1 also is a good candidate to be involved in N-methyl-D-aspartate (NMDA) receptor dependent synaptic plasticity in the adult brain. On one hand, endogenous peptide Histogranin with 80% of structural homology to a fragment-(86–100) of histone H4 was shown to inhibite non-competitively the binding of [^{3}H]-CGP-39653, a specific NMDA receptor ligand, to membrane preparations of rat brain (Shukla et al. 1995) characterizing Histogranin as an endogenous antagonist for the NMDA receptor (Chiang et al. 2006). On the other hand, it was established that application of polysialic acid or polysialylated NCAM, but not the sialic acid monomer or non-polysialylated NCAM can influence 30% reduction of glutamate-elicited NMDA receptor currents in cultured hippocampal neurons (Hammond et al. 2006). Interaction between histone H1, homologous to histone H4, and PSA-NCAM, observed in this study, might be the initial indication for interesting important interplay of these surface expressed molecules in the regulation of NMDA-dependent plasticity in the brain.

7. Summary

The establishment of a functional nervous system involves highly concerted organization of cell migration, differentiation, survival and connection between neurons and their appropriate targets. Many of these processes are mediated by a variety of integral membrane proteins, collectively termed cell adhesion molecules.

The **cell adhesion molecule L1** belongs to the immunoglobulin superfamily of cell adhesion molecules and plays critical roles in development and maintenance of the nervous system. L1 is widely expressed in the nervous system and implicated in a variety of activities including neuronal differentiation, axon growth and guidance, axon fasciculation, myelination, synaptic plasticity and memory formation. L1 has acquired a special significance since it was shown that mutations in the human L1 gene are responsible for a number of nervous system defects, termed the L1 syndrome. L1 is an integral membrane glycoprotein which mediates cell adhesion and cellular activities by Ca^{2+}-independent homophilic binding or by interacting with a diverse group of molecules, including other cell adhesion molecules, extracellular matrix proteins or signalling receptors at the cell surface.

In order to provide new insights into understanding of mechanisms mediating L1 activities, novel putative binding partners of L1 were identified and further characterized in the present study. Using co-immunoprecipitation approach and following mass spectrometry analysis it has been demonstrated that glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mitochondrial inner membrane integral adenine nucleotide transporter (ANT) and the smallest member of the immunoglobulin superfamily Thy-1 were specifically associated with L1 adhesion molecule in synaptosomal membrane subfraction. Further application of different biochemical assays (chemical cross-linking, BIACORE analysis, ELISA) has confirmed the direct interaction of GAPDH and ANT, but not Thy-1, with L1 molecule. Since both GAPDH and ANT showed the specific direct binding to the recombinantly produced extracellular, but not intracellular, domain of L1, the surface of neuronal cells was proposed as a potential localization for these unexpected interactions. Furthermore it was confirmed using surface biotinylation assay that indeed “classical” glycolytic enzyme GAPDH and mitochondrial ANT can be detected at the extracellular side of neuronal cells.

The presence of ANT at the outer surface and it’s interaction with adhesion molecule L1 suggests a completely novel connection between nucleotide transport system and regulation of adhesion in the brain.

In order to establish the functional role of interaction between L1 and GAPDH, the hypothesis whether GAPDH could act as an ecto-protein kinase, able to phosphorylate L1 extracellular region under certain conditions, was investigated in the present study.

Immunocytochemical labelling of living primary cerebellar neurons additionally confirmed the cell surface localization of GAPDH and also demonstrated the co-localization of L1 and GAPDH at neuronal cell bodies but not along the axons. Furthermore, *in vitro* phosphorylation assay revealed GAPDH-dependent phosphorylation of extracellular L1 region within the Ig domains, and auto-phosphorylation of L1, mediated by Fn domains. Moreover, it was observed that auto-phosphorylation of L1 extracellular domain promoted the homophilic dimerization of L1, which could be disturbed by GAPDH binding; this interesting observation was confirmed in beads aggregation assay. Functional significance of this finding was established using pre-phosphorylation of L1 substrate in neurite outgrowth assay, suggesting that GAPDH-dependent phosphorylation of Ig domains promotes homophilic L1 interaction in *trans*-orientation whereas auto-phosphorylation of L1 within Fn domains stimulates homophilic L1 binding in *cis*-position.

To sum up, this study could demonstrate that glycolytic enzyme GAPDH directly interacts with the adhesion molecule L1 at the extracellular cell surface of neurons and modulates L1-induced neurite outgrowth in an ATP-dependent manner.

Another member of immunoglobulin superfamily - **neural cell adhesion molecule in its polysialylated state (PSA-NCAM) -** has been shown to regulate cell migration, neurite outgrowth, and axonal fasciculation in the developing central nervous system. Its expression is not only limited to the period of brain development, but is also observed in discrete brain areas that maintain the ability to undergo structural and functional changes in adulthood.

Recently, three novel potential binding partners of polysialyc acid (PSA) were identified using affinity chromatography with anti-idiotype single chain variable fragment antibody structurally mimicking PSA (von der Ohe, unpublished data).

In the second part of the present study myristoylated alanine-rich C kinase substrate (MARCKS), neuronal tissue-enriched acidic protein (NAP-22) and DNA binding nuclear protein histone H1 were investigated as potential binding partners of PSA-NCAM. Distribution analysis in different membrane subfractions has demonstrated that all three proteins are co-expressed with PSA, although the direct binding using ELISA assay was established only between PSA-NCAM and histone H1, but not MARCKS or NAP-22. Cell surface biotinylation assay has revealed the plasma membrane association of MARCKS and histone H1. Since the group of non-nuclear histones H1 were identified in the brain as major lipopolysaccharide-binding proteins, histone H1 became a good candidate for regulation of

PSA functions in the brain. Demonstration of the histone H1 capacity to modulate PSA-NCAM-dependent neurite outgrowth provided a promising indication that histone H1 may be involved in mechanisms of PSA-mediated neuroplasticity.

8. Zusammenfassung

Die Entwicklung eines funktionellen Nervensystems erfordert eine genau abgestimmte Organisation von Zellmigration, Differenzierung, Zellüberleben und die Vernetzung von Neuronen mit ihren Zielzellen. Viele dieser Prozesse werden durch eine Vielzahl von integralen Membranproteinen vermittelt, welche allgemein als Zelladhäsionsmoleküle bezeichnet werden.

Das Zelladhäsionsmolekül L1 gehört zur Immunglobulin-Superfamilie von Zelladhäsionsmolekülen und spielt eine wichtige Rolle während der Entwicklung und bei der Aufrechterhaltung des Nervensystems. L1 zeigt ein breites Expressionsmuster im Nervensystem und ist an einer Vielzahl von Prozessen beteiligt, unter anderem an neuronaler Differenzierung, Neuritenwachstum, Axonführung und - bündelung, Myelinisierung, synaptischer Plastizität und Gedächtnisbildung. L1 hat in den letzten Jahren an Wichtigkeit gewonnen, da gezeigt wurde, daß Mutationen im humanen L1-Gen für eine Reihe von Defekten des Nervensystems verantwortlich sind, welche man als L1-Syndrom zusammenfaßt. L1 ist ein integrales, membranständiges Glykoprotein und vermittelt Zelladhäsion und zelluläre Prozesse durch Ca^{2+}-unabhängige homophile Interaktion oder durch heterophilen Interaktionen mit diversen Molekülen, wie Zelladhäsionsmolekülen, extrazellulären Matrixproteinen oder Signalrezeptoren an der Zelloberfläche.

Um neue Erkenntnisse für das Verständnis von L1-vermittelten Mechanismen zu gewinnen, wurden in dieser Arbeit mögliche neue Bindungspartner von L1 identifiziert und charakterisiert. Mit Hilfe von Co-Immunpräzipitation und Massenspektrometrie wurde gezeigt, daß das glykolytische Enzym Glyceraldehyd-3-Phosphat Dehydrogenase (GAPDH), der mitochondriale Adenin-Nukleotid-Transporter (ANT) und Thy1, ein Mitglied der Immunglobulin-Superfamilie, in einer synaptosomalen Membranfraktion spezifisch mit L1 assoziiert waren. Darüber hinaus wurde mit verschiedenen biochemischen Untersuchungen (chemisches Cross-Linking, BIACORE-Analyse, und ELISA) die direkte Interaktion von GAPDH und ANT mit L1 aber nicht von Thy1 mit L1 gezeigt. Da sowohl GAPDH als auch ANT eine direkte Bindung mit der rekombinant-produzierte extrazellulären Domäne von L1 eingehen, wurde die neuronale Zelloberfläche als möglicher Ort für die oben genannten, unerwarteten Interaktionen angenommen. Mit Hilfe der Oberflächen-Biotinylierung konnte das „klassische“ glykolytische Enzym GAPDH und das mitochondriale ANT-Protein tatsächlich an der extrazellulären Seite von neuronalen Zellen detektiert werden. Die Anwesenheit von ANT an der Zelloberfläche und seine Interaktion mit dem

Adhäsionsmolekül L1 weist auf eine vollkommen neue Verknüpfung des Nukleotid-Transportsystems mit der Regulation von Zell-Zell-Interaktion im Gehirn hin.

Um die funktionelle Rolle der Interaktion von L1 und GAPDH zu manifestieren, wurde für dieser Arbeit eine Hypothese erstellt, in der GAPDH als Ecto-Protein-Kinase agieren könnte, und damit in der Lage wäre, die extrazelluläre Region von L1 unter bestimmten Bedingungen zu phosphorylieren.

Immunocytochemische Markierungen an lebenden primären Kleinhirnneuronen zeigten eine Zelloberflächenlokalisation von GAPDH und eine Kolokalisation von L1 und GAPDH an der Oberfläche von neuronalen Zellkörpern, jedoch nicht entlang von Axonen. Weiterhin wurden mit Hilfe eines In-vitro-Phosphorylierungsversuches die GAPDH-abhängige Phosphorylierung von extrazellulärem L1 innerhalb der Ig-Domäne und eine über Fn-Domänen vermittelte Autophosphorylierung von L1 entdeckt. Ferner wird durch die Autophosphorylierung der extrazellulären Domäne von L1 die homophile Interaktion von L1 begünstigt. Dieser Prozeß kann durch die Bindung von GAPDH gestört werden. Diese interessanten Beobachtungen wurden mit Hilfe eines Bead-Aggregations-Versuches bestätigt. Die funktionelle Bedeutung der Phosphorylierung von L1 konnte in Neuritenwachstum-Versuchen nach Vorbehandlung des L1-Substrates bestätigt werden. Die GAPDH-abhängige Phosphorylierung der Ig-Domänen unterstützt die homophile L1-Interaktion in Trans-Orientierung, während die Autophosphorylierung von L1 in den Fn-Domänen die homophile L1 Bindung in Cis-Orientierung begünstigt.

Zusammenfassend konnte diese Arbeit zeigen, daß das glykolytische Enzym GAPDH direkt mit dem Zelladhäsionsmolekül L1 an der extrazellulären Oberfläche von Neuronen interagiert und L1-induziertes Neuritenwachstum ATP-abhängig moduliert.

Ein weiteres Mitglied der Immunglobulin-Superfamilie, das neurale Zelladhäsionsmolekül (NCAM) reguliert in seiner polysialysierten Form (PSA-NCAM) Zellmigration, Neuritenwachstum und axonale Faszikulierung im sich entwickelnden Zentralnervensystem. Die Expression von PSA-NCAM ist nicht auf die Entwicklung des Gehirns beschränkt, sondern eine erhöhte Expression kann auch im Erwachsenenalter in Gehirnarealen, die strukturellen und funktionellen Veränderungen unterliegen, gefunden werden. Kürzlich konnten mittels Affinitäts-Chromatographie mit anti-idiotypischen Antikörperfragmenten, die strukturell in der Lage sind PSA zu mimikrieren, drei neue Bindungspartner für PSA identifiziert werden (von der Ohe, nicht publizierte Daten).

Im zweiten Teil der Untersuchung wurden als potentielle Bindungspartner von PSA-NCAM die folgenden Proteine näher untersucht: „myristoylated alanine-rich C kinase substrate“ (MARCKS), „neuronal tissue-enriched acidic protein“ (NAP-22) und „DNA-binding nuclear

protein histone H1“. Die Untersuchung der Expression dieser Proteine in verschiedenen Membransubfraktionen zeigte, daß alle drei Proteine mit PSA ko-exprimiert waren. Die direkte Interaktion zwischen PSA-NCAM und Histon H1 konnte im ELISA-Experiment verifiziert werden, wohingegen eine Interaktion zwischen PSA-NCAM und MARCKS oder NAP-22 unter diesen Bedingungen nicht nachweisbar war. Oberflächen-Biotinylierungsexperimente zeigten eine Assoziation von MARCKS und Histon H1 mit der Plasmamembran. Da die Gruppe der nicht-nukleären Histone H1 wichtige Lipopolysaccharid-bindende Proteine im Gehirn darstellt, ist das Histon H1 ein potentieller Kandidat für eine Regulation der PSA-Funktion im Gehirn.

Wichtige Hinweise auf eine Funktion von Histon H1 bei der Modulation von PSA-vermittelter Neuroplastizität lieferten Neuritenwachstumsexperimente, bei denen Histon H1 modulatorische Effekte auf PSA-NCAM-vermitteltes Neuritenwachstum zeigte.

9. Abbreviations

Amino acids were abbreviated using the one letter code

× g	g-force
°C	grad Celsius
A	Ampear
ADP	adenosine diphosphate
Amp	ampicillin
AMP	Adenosine monophosphate
ANT	Adenine nucleotide transporter
APP	Amiloid precursor protein
APS	ammoniumperoxodisulfate
ATP	adenosine triphosphate
B104	Rat neuroblastoma cell line
BDNF	brain derived neurotrophic factor
bp	base pairs
BSA	bovine serum albumine
Ca	Calcium
CAM	Cell adhesion molecule
CHL1	Close homolog of L1
CHO	Chinese Hamster Ovarian
CL	Cross-linking
Da	dalton
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
EC	extracellular
ECM	Extracellular matrix
EDTA	ethylendiamintetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
endo-N	endosialidase N
f.c.	final concentration
FAK	focal adhesion kinase
FCS	fetal calf serum
FGF-R	fibroblast growth factor-receptor
Fn	fibronectin
g	gram
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPI	glycophosphatidylinositol
h	human, hour
HEPES	2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid
HNK-1	Human natural killer antigen

HS	Horse serum
IC	immunocytochemistry
ICD	Intracellular domain
Ig	immunoglobulin
IgCAM	Immunoglobulin cell adhesion molecule
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactoside
kb	kilo base pairs
l	litre
LB	Luria Bertani
m	milli (10^{-3})
MAG	myelin associated glycoprotein
MALDI-TOF	Matrix-assisted laser desorption/ionisation – mass spectrometry
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C kinase substrate
MEM	minimal essential medium
Mg	Magnesium
min	minute
Mn	Manganese
n	nano (10^{-9}), number
N2A	Mouse neuroblastoma cell line
NAD	Nicotinamine adenine dinucleotide
NAP-22	neuronal tissue-enriched acidic protein
NCAM	Neural cell adhesion molecule
NMDA	N-methyl-D-aspartate
NP-1	Neuropillin-1
OD	optic density
p	pico (10^{-12})
p	statistical significance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphat-buffered saline
PKC	Protein kinase C
PSA	Polysialic acid
PSD	Postsynaptic densities
rpm	rounds per minute
RT	room temperature
scFv	Single chain variable fragment
SDS	sodium dodecyl sulfate
syn	synaptosome
TE	tris-EDTA
TEMED	N,N,N',N'-tetraethylenamine
Thy-1	thymus cell antigen
TM	Trade Mark
Tris	tris(-hydroxymethyl)-aminomethane
TrkB	receptor tyrosine kinase

U	unit (enzymatic)
V	volt
v/v	volume per volume
w/v	weight per volume
WB	Western blot
ZMNH	Zentrum für Molekulare Neurobiologie Hamburg
α	anti
µ	micro (10^{-6})

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As an English native speaker hereby I confirm that the PhD thesis by Tatiana Makhina titled as "Identification, characterization and functional analysis of novel protein binding partners of the cell recognition molecule L1 and the polysialylated neural cell adhesion molecule in mouse (*Mus musculus L., 1758*)" is written in correct grammar and appropriate style.

This is the same in German:
Bestätigung der Korrektheit der Englischen Sprache in der Dissertation von Tatiana Makhina mit dem Thema „Identification, characterization and functional analysis of novel protein binding partners of the cell recognition molecule L1 and the polysialylated neural cell adhesion molecule in mouse (*Mus musculus L., 1758*)".

Hiermit bestätige ich die Korrektheit der englischen Sprache in Wort und Schrift.

Sincerely,

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