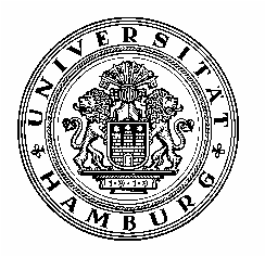

Characterization of carriers and receptors of the Lewis^{x} glycan in the nervous system of mice (*Mus musculus* L., 1758)

DISSERTATION

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vorgelegt von

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Contents

I. Abstract

Carbohydrate carrying cell surface and extracellular matrix molecules have been ascribed with roles in development, regeneration and synaptic plasticity in the nervous system. Lewis^x is one such carbohydrate that is recognized by the monoclonal antibody L5 and is expressed in the developing and adult nervous system, both central and peripheral. It mediates recognition among neural cells at early embryonic stages and has been implicated in neural tube closure and neuritogenesis but the molecular carriers of Lewis^x and its receptors are currently not known. The following findings of the present study can enable further elucidation of the functional roles of Lewis^x: 1) contactin-1 a cell surface adhesion molecule was identified as a novel Lewis^x carrying molecule by antibody affinity chromatography experiments, 2) the lectin galectin-3 and an isoform of myelin basic protein were identified as probable receptors for Lewis^x by phage display experiments and 3) a cyclic peptide was identified as a mimetic of the Lewis^x glycan by phage display experiments. Different aspects of the biological roles of Lewis^x were investigated by introducing the glycomimetic in *in-vitro* assays measuring neurite outgrowth and *in-vivo* assays in mice like regeneration of peripheral nerve and spinal cord after injury. Evidence from these experiments indicates that while the interactions of the glycan and its receptors may be true, a specific *in-vivo* function of the Lewis^x glycan is yet to be identified.

II. Zusammenfassung

Glykosylierte Zelloberflächen- und extrazelluläre Matrix-Moleküle übernehmen wichtige Funktionen im Nervensystem während der Entwicklung, der Regeneration und synaptischen Plastizität. Lewis^x ist ein Kohlenhydrat, welches durch den monoklonalen Antikörper L5 erkannt wird, und es wird sowohl im peripheren als auch zentralen Nervensystem während der Entwicklung und im adulten Organismus exprimiert.

In frühen embryonalen Stadien vermittelt es die Zellerkennung neuraler Zellen und ist allgemein an Prozessen wie Neuritogenese und Neuralrohrbildung beteiligt. Die Trägermoleküle von Lewis^x und ihre Rezeptoren, die bei diesen Vorgängen eine wichtige Rolle spielen, sind bisher nicht bekannt.

Die folgenden Resultate dieser Studie ermöglichen eine weiterführende Charakterisierung bzw. Aufklärung der Funktion von Lewis^x:

1. Durch Immun-Affinitäts-Chromatographie konnte das Zelladhäsionsmolekül *Contactin-1* als neues Lewis^x–Trägermolekül ermittelt werden.
2. Das Lektin *Galectin-3* und eine Isoform von MBP (*myelin basic protein*) wurden mittels *Phage-Display* als mögliche Lewis^x-Rezeptoren identifiziert.
3. Ein zyklisches Peptid, welches als Glycomimetikum des Lewis^x-Glykans wirksam ist wurde durch *Phage-Display* isoliert.

Das Lewis^x-Glycomimetikum wurde *in-vitro* in Neuritenwachstum-Versuchen eingesetzt und *in-vivo* in Regenerations-Experimenten nach Läsion von Rückenmarksnerven und peripheren Nerven verwendet, um die biologische Funktion von Lewis^x zu untersuchen. Obwohl eine Interaktion des Glycans mit den genannten Rezeptoren experimentell nachgewiesen werden konnte, bleibt die *in-vivo*-Funktion des Lewis^x-Glycans weiterhin ungeklärt.

III. Introduction

Glycans are found attached to proteins and lipids at the cell surface forming a Glycocalyx and play important roles in cell-cell and cell-substrate interactions in normal brain development and also in pathologically altered brain tissue (Jessell et al., 1990; Kleene and Schachner, 2004). The Lewis^x is a terminal trisaccharide on cell surface glycans and its carbohydrate determinant α1,3-fucosyl-N-acetyl-lactosamine was originally identified in fluids from ovarian cysts (Lloyd et al., 1966; 1968; 1968a). Lewis^x is also known as CD15 or SSEA-1 (stage specific embryonic antigen-1) and was initially described on blastomeres in pre-implantation mouse embryos and in embryonal carcinoma cells (Solter and Knowles, 1978). It mediates a unique mechanism of cell-to-cell adhesion in the embryo and in the cancer cells involving a homophilic interaction with itself (Eggens et al., 1989). The molecular basis of this carbohydrate-carbohydrate interaction has been elucidated by its crystal structure and also nuclear magnetic resonance studies (Perez et al., 1996; Miller et al., 1992). The adhesion forces mediating Lewis^x-Lewis^x interactions have also been determined by atomic force microscopy and isothermal titration calorimetry (de la Fuente et al., 2005).

In the developing and adult murine central nervous system the carbohydrate epitope is expressed at the surface of astrocytes and in certain sub-populations of neurons (Bartsch and Mai, 1991; Gocht et al., 1994; Lagenaur et al., 1982). It is expressed as early as during embryonic day-9 in the neural tube and in the ventricular zone of the cerebral cortex at embryonic day-11 of mice (Yamamoto et al., 1985). During neural tube closure in the chick embryo, antibodies against Lewis^x inhibit this important morphogenetic event (Roberts et al., 1991; Streit et al., 1997). A region specific expression pattern of Lewis^x is observed in the cerebrum and cerebellum in mature brains of human, monkey, rat and mouse and is maintained throughout adulthood (Niedieck and Lohler, 1987; Gocht et al., 1992; Lagenaur et al., 1982; Gocht et al., 1994; Mai and Reifenberger, 1988; Marani and Mai, 1992). The highest expression of Lewis^x is observed during postnatal day-7 (Streit et al., 1990) which is a period when developmental fate of neurons and their synapses are decided. Lewis^x is also expressed by adult mouse central nervous system stem cells which are the source of

new neurons in adult brains and thus may play a role in the maintenance of stem cell properties (Capela and Temple, 2002). This spatial and temporal expression pattern of Lewis^x, elaborated later in this chapter, suggests its probable involvement in nervous system development and morphogenesis.

In the mature mammalian brain Lewis^x is expressed on astrocytic and neuronal glycoproteins, like by the recognition molecules of the immunoglobulin super family, Thy-1 and L1 (Streit et al., 1990), by the extracellular matrix chondroitin sulphate proteoglycan phosphacan (Garwood et al., 1999), by the mucin-like recognition molecule CD24 (Lieberoth et al., submitted), by dystroglycan (Smalheiser et al., 1998) a cell surface component involved in formation of the nerve muscle synapse and on certain glycolipids (Dasgupta et al., 1996). The fact that it is expressed by these important molecules during various morphogenic processes and at important structures makes it likely that Lewis^x is not only a decoration on these molecules, but supports their functions in cellular adhesion and recognition.

In the mouse brain the Lewis^x epitope is most likely to be synthesized by an alpha1,3-fucosyltransferases IX encoded by the FUT9 (Nishihara et al., 2003), which by itself may be controlled by the transcription factor Pax6 in the embryonic forebrain (Shimoda et al., 2002) adding complexity to the expression of Lewis^x in time and space to modulate cellular interactions.

Lewis^x expression is not limited to the nervous system but is found in other tissues originating from all the germ layers such as the digestive tract, reproductive system, urinary tract, skin and the hematopoietic cells (Fox et al., 1981; Gocht et al., 1996; Combs et al., 1984). Apart from normal tissue it has been shown to be expressed by various tumours (Fox et al., 1983; Hakomori et al., 1981). Pathogenic bacteria like *Helicobacter pylori* that can cause gastritis, peptic ulcer and gastric cancer and helminthes like schistosoma that can cause a chronic and often fatal schistosomiasis are also known to interact with human hosts via their cell surface Lewis^x (Remoortere et al., 2000).

Glycans are also involved in intracellular processes of folding and targeting of many proteins (Molinari, 2007); these functions though important are not the focus of this

study. Rather the focus is on the involvement of glycans occurring on various glycoproteins that are involved in different stages of development, synaptic plasticity and regeneration in the nervous system. In the next few pages, a more detailed perspective outlining the biological significance of the Lewis^x glycan is provided, starting from its original identification, role in neuronal induction, its presence in the nervous system and the molecules that carry it, Lewis^x as a stem cell marker and briefly about its involvement in cancer and host pathogen interactions.

1. The Lewis^x molecule and its first identification

The Lewis^x epitope is determined by the carbohydrate α1,3fucosyl-N-acetyl-lactosamine (Galβ1, 4[Fucα1, 3] GlcNAc) (PubChem-CID: 4571095) (Figure 1). It is a terminal trisaccharide that was first detected by chemical and immunochemical methods amongst oligosaccharides obtained from ovarian cyst fluids (Lloyd et al., 1966; 1968; 1968a) as a Lewis blood group related antigen. The name 'Lewis' relates to the family of individuals who carried antibodies against a related blood group antigen (Mourant, 1946). Lewis^x is now also known as stage specific embryonic antigen 1 (SSEA-1), CD15, LeX, FAL and Forse-1 amongst other names.

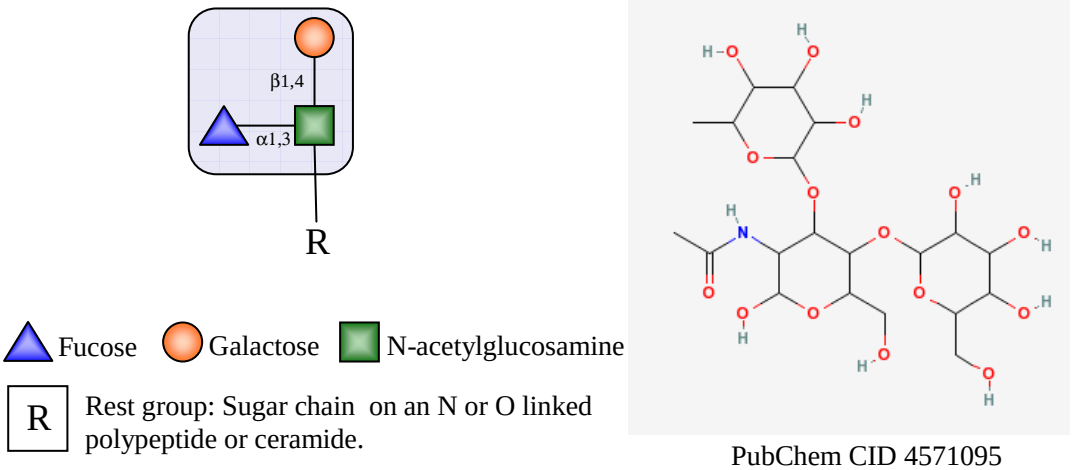


Figure 1: Schematic representation of the Lewis^x epitope.

The biological synthesis of the Lewis^x epitope is synthesized by an enzyme mediated transfer of fucose on type 2 (Galβ1, 4-GlcNAc-) oligosaccharide chains. This reaction is mediated alpha1,3-fucosyltransferases of which thirteen isozymes are known in the

mammalian tissue (Oriol et al., 1999; Becker and Lowe, 2003). Amongst these, the alpha1,3-fucosyltransferases IX encoded by the FUT9 gene is the most likely enzyme that aids the synthesis of Lewis^x in the mouse brain (Nishihara et al., 2003). In the Golgi apparatus fucosyltransferases utilize a nucleotide-activated form of fucose, GDP-fucose, as a fucose donor in the construction of fucosylated oligosaccharides, Figure 2, (Becker and Lowe, 2003). The oligosaccharide chains that are fucosylated to form the Lewis^x glycans are discussed later.

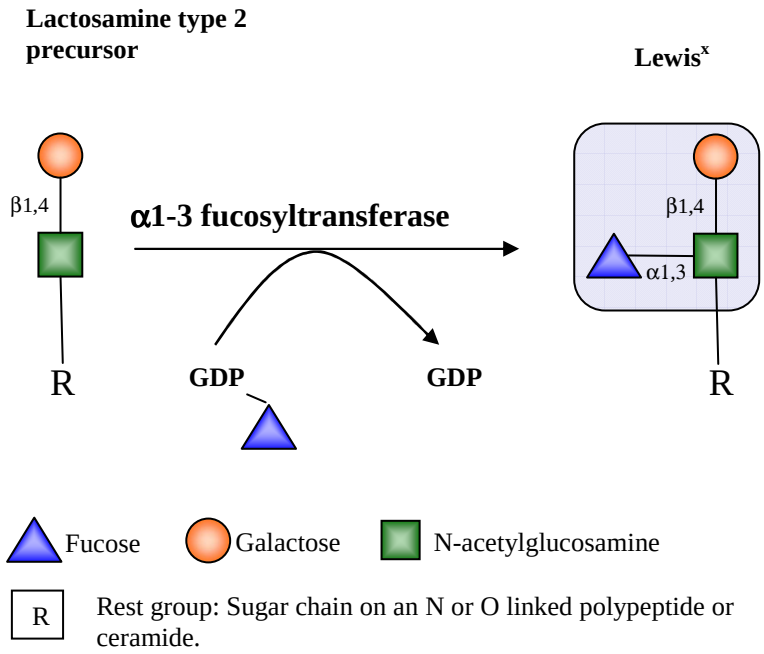


Figure 2: A generalised representation of the fucosylation process. A type 2 lactosamine structure is fucosylated by fucosyltransferase 9 in the brain tissue of mice.

Other glycan members of the Lewis blood group are the Lewis^a, Lewis^b, Lewis^y and the sialyl and/or sulfo forms of Lewis^x and Lewis^a, Figure 3, (Varki et al., 1999). The sialylated forms are well characterised for their essential contribution in the functioning of leukocyte homing by their interactions with selectins in various pathological processes including cancer (Lowe, 2002). Though these Lewis blood group antigens too have not been characterised in terms of normal brain development and function, they are not emphasised in this study.

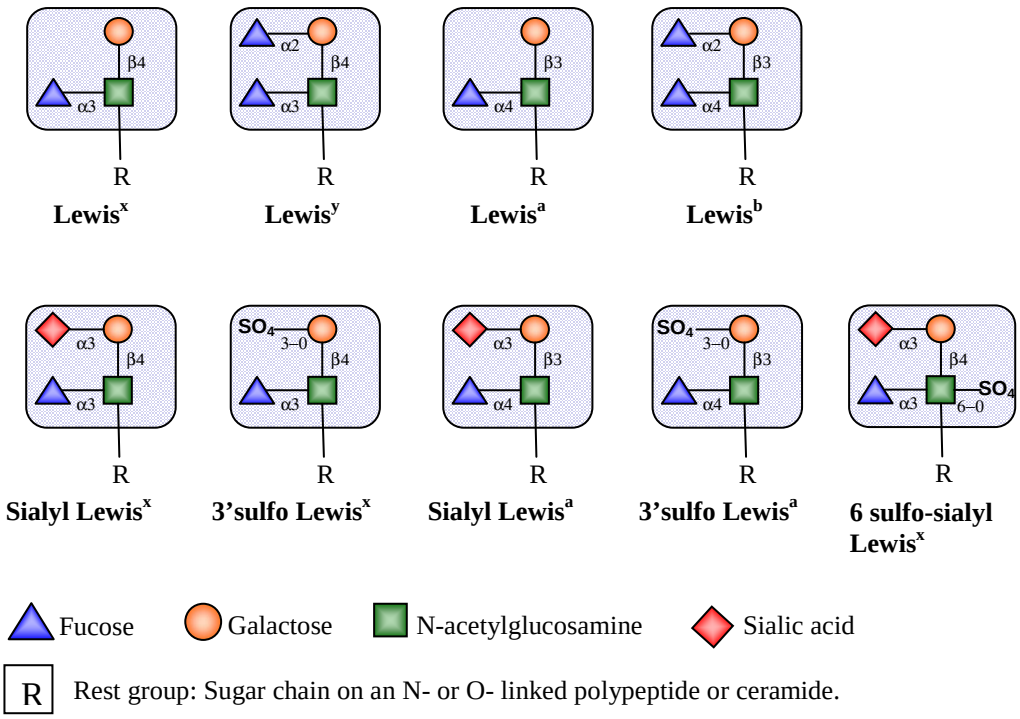


Figure 3: Different types of type 1 and type 2 Lewis structures. The highlighted portions are the characterising terminal modifications on similar rest groups of oligosaccharides occurring on either N- or O-linked proteins or lipids**.**

2. Lewis^x interactions during embryogenesis and neuronal induction

The first intracellular interactions of fertilisation in mammals involves the O-linked glycans of the zona pellucida 3 glycoprotein on the egg that acts as a receptor for the sperm (Florman and Wassarman, 1985). This specific interaction can be inhibited by Lewis^x glycans in a competing manner with high affinity (IC_{50}: 0.5 µM) (Johnston et al., 1998; Kerr et al., 2004; Hanna et al., 2004) highlighting its function in adhesion. Lewis^x containing neoglycoproteins were capable of inducing an acrosome reaction in a dose dependent, calcium dependent and capacitation dependent manner indicating its likely involvement in signalling during the process of zygote formation.

Earlier the Lewis^x or SSEA 1 antigen was shown to be present on preimplantation mouse embryos i.e., starting from the blastomeres of 8-cell stage when the embryo starts undergoing compaction to the 16-32 cell stage and then in the inner cell mass of the blastocyst (Solter and Knowles, 1978). At this stage of the embryo, ultrastructural studies show the presence of Lewis^x preferentially on the edge of low protuberances and microvilli that form clusters (Cui et al., 2004) which may be important for

implantation. The treatment of the compacted embryos with soluble Lewis^x glycoconjugates leads to their decompaction (Fenderson et al., 1984), implying its role in adhesion and thus in modulating the developmental process. These cellular interactions during compaction were explained by Lewis^x-Lewis^x homophilic adhesion which is a novel carbohydrate-to-carbohydrate interaction without involving proteins (Eggens et al., 1989).

Lewis^x recognized by the monoclonal antibody L5 (Streit et al., 1996) was detectable during gastrulation of the chick embryo, at mid-primitive streak stage (Roberts et al., 1991) and persists until at least 3.5 days of development. When they transplanted a Hensen's node from a donor embryo into a host embryo, Lewis^x immunoreactivity appeared in the epiblast surrounding the graft. And when hybridoma cells secreting the L5 antibody were grafted together with Hensen's node into the host chick embryo, the induction of a supernumerary nervous system was inhibited, suggesting that the Lewis^x epitope is an early and general marker for neural induction and that it may be involved directly in inductive interactions. A particular 220kd protein carrying the Lewis^x epitope was shown to be involved in these neuronal inductive interactions or maintaining competence of the epiblast in the chick embryo (Streit and Stern, 1997). In mammals, a little later in development, an increase of Lewis^x expression during early pregnancy is seen at the site of implantation in the uterus (Isaacs and Murphy, 1998), again demonstrating that it may be involved in adhesion during implantation.

3. Lewis^x in the nervous system

The epitope is expressed on all the components of the developing nervous system, both central and peripheral as revealed by immunohistochemical investigations. The Lewis^x epitope was first shown to be present in the external granular layer and prospective white matter of 13-day old mouse cerebellum and expressed by a sub-population of astrocytes (Lagenaur et al., 1982). Similarly a region specific Lewis^x expression was observed in the cerebral cortex, predominantly present in layers II, III, and V of the occipital cortex or in the molecular layer and Purkinje cell layer of the cerebellum (Gocht et al., 1994). It is expressed as early as embryonic day-9 (E9) in the neural tube and in the ventricular zone of the embryonic cerebral cortex at

embryonic day-11 (Yamamoto et al., 1985) indicating the expression on proliferating cells. This spatiotemporal distribution of its expression implies a role for Lewis^x in the development of the brain morphology. Again, for instance in the hippocampus of rats it first appears at embryonic day-10 at the ventricular surface of the hippocampal primordium, then a group of cells in the marginal zone of the hippocampus are labelled at E12 and E13, followed by three strong bands at the hippocampal fissure, stratum oriens and the ventricular surface at E19 (Ashwell and Mai, 1997) indicating a function of outlining the boundaries. Lewis^x demarcates the prosencephalic region by being expressed on radial glial cells and also divides the cerebellum into distinct zones suggesting that it plays an important role in compartmentalization and development of functionally distinct brain areas (Mai et al., 1998; Mai et al., 1995).

At E12 the glial roof plate of the spinal cord and optic tectum express Lewis^x, here its expression is localised along with a proteoglycan that regulates the development of an axon barrier (Snow et al., 1990). Lewis^x is expressed by neuroblasts which organize the dorsal horn of the spinal cord (Oudega et al., 1992), then, the expression becomes strong on the radial glia along the sulcus limitans and the dorsal root entry zone again suggestive of compartmentalization and creating boundaries for incoming afferent nerves.

The expression of Lewis^x has not only been shown to be critical in various stages of development of the sense organs but is present on their specialised receptor cells: on the olfactory cilia and olfactory pathways (Plank and Mai, 1992; Mai et al., 1999), on a subset of amacrine, bipolar and ganglion cells in the retina (Koso et al., 2006; Andressen and Mai, 1997; Sun and Kalloniatis, 2006) and in the inner ear (Meyer and Mai, 1997) on its hair cell sensory tips in the organ of Corti (Hozawa et al., 1993). The presence of Lewis^x on these receptor cells implies that it is not involved in only adhesion as discussed before but may be involved in receptor or physiological functions.

The cellular localisation of Lewis^x has been at the outer cell surface of certain astrocytes, especially on Bergman glia and in the extracellular space (Lagenaur et al., 1982; Gocht et al., 1994; Sajdel-Sulkowska, 1998). At the ultrastructural level it is seen at points of attachment between astrocyte-astrocyte, astrocyte-oligodendrocyte,

astrocyte-axon myelin and astrocyte-blood vessel contacts. Selective adhesion of neural cells via the Lewis^x carbohydrate has been observed in different regions of the developing telencephalon (Gotz et al., 1996) and has been shown to be involved particularly in the adhesion of cerebellar neurons to astrocytes (Sajdel-Sulkowska, 1998). In line with the suggestion that Lewis^x is involved in establishing barriers or boundaries for outgrowing axons, it has been found that a synthetic carbohydrate compound containing the Lewis^x epitope inhibits outgrowing axons in the mouse optic system (Marcus et al., 1995; Lin et al., 2005). A synthetic carbohydrate compound containing the Lewis^x epitope has been shown to inhibit the proliferation of neuroblastoma cells *in-vitro* (Santos-Benito et al., 1992) again suggesting its barrier and signalling functions.

4. Lewis^x carrying molecules in the nervous system and their functions

4.1. Extracellular-matrix proteoglycans like phosphacan and tenascin-R

Extracellular-matrix forms a large portion of brain volume and coordinate synaptogenesis and synaptic activity (Dityatev and Schachner, 2003) of which both phosphacan and tenascin-R are important constituents and are important during synaptogenesis and synaptic activity (Bandtlow and Zimmermann, 2000; Saghatelyan et al., 2001). Phosphacan (RPTPζ; receptor-type protein tyrosine phosphatase) is an extracellular matrix chondroitin sulphate proteoglycan and carries Lewis^x (Garwood et al., 1999). Phosphacan expression level peaks during postnatal week 2 in murine brains (Ripellino et al., 1989) and its ultrastructural localization is mainly at the membrane of migrating neurons and radial glia (Hayashi et al., 2005). This expression is similar to the expression of Lewis^x which also peaks around the postnatal week 2 in murine brains and also in its ultrastructural localisation as seen at membranes between astrocyte-astrocyte, astrocyte-oligodendrocyte, astrocyte-axon myelin (Gocht et al., 1994).

It has been demonstrated that phosphacan binds to neural cell adhesion molecules NCAM (neural cell adhesion molecule) and tenascin by its N-linked glycans (Milev et

al., 1998). Tenascin-C and phosphacan functionally depend on each other in that neurite outgrowth on tenascin-C is modulated by phosphacan and vice versa, neurite outgrowth on phosphacan is modified by tenascin-C (Xiao et al., 1997). Phosphacan has opposing effects on the process of neurite outgrowth depending on the cells neuronal lineage (Garwood et al., 1999) and this could be due to the differences in the glycosylation status of phosphacan.

Tenascin-R, another component of the extracellular matrix and a deficiency of tenascin-R as in mutant mice leads to improper formation of perineuronal nets (Bruckner et al., 2000). It is involved along with its carbohydrate epitope HNK-1 (an epitope first recognised on human natural killer cells, hence the name) in long term potentiation which is a parameter that provides physiological correlates of learning and memory (Saghatelyan et al., 2001). It is also suggested that tenascin-R can either activate or inhibit neurite outgrowth based on the glycosylation it carries (Woodworth et al., 2004). An analysis of glycans on tenascin-R has shown the presence of the Lewis^x epitope (Zamze et al., 1999). A Lewis^x dependence of any of the functions of both phosphacan and tenascin are not known.

4.2. Neural cell recognition molecules of the Ig family like L1, NCAM, Thy1 and MAG.

The cell adhesion molecules L1 and NCAM are molecules critical for neuronal development and normal functioning of the adult brain, mutations in them or absence of either of them leads to malformation of brain and disease such as the L1 syndrome, for a review see (Maness and Schachner, 2007). They are known to mediate neuronal adhesion, granule cell migration, axon growth, synaptogenesis and synaptic plasticity (Dahme et al., 1997). These functions of both L1 and NCAM are modulated or mediated by the carbohydrates they carry that include oligomannose, HNK-1 and polysialic acid (PSA; which is present exclusively on NCAM in the brain) (Kleene and Schachner, 2004). The immense versatility the glycans confer to these proteins has been demonstrated by numerous experiments. For instance, removal of PSA from NCAM in the hippocampus leads to reduced learning and memory to perform a particular task by mice (Becker et al., 1996; Senkov et al., 2006). In an *in-vitro* assay

PSA carried by NCAM has been shown to be important for synapse formation and their remodelling (Dityatev et al., 2004). NCAM apart from being a glycoprotein is a lectin for oligomannose (Horstkorte et al., 1993). It is shown that this carbohydrate binding activity is important for its association with L1 in a *cis* interaction to bring about neurite outgrowth. L1 is also capable of *trans* homophilic interactions binding to itself similar to Lewis^x that is involved in homophilic binding to itself. Since both L1 and NCAM act as lectins and have been shown to carry Lewis^x (Wing et al., 1992) we can hypothesize that Lewis^x could be assisting the *cis* and *trans* binding interactions.

Myelin associated glycoprotein (MAG) is also a cell adhesion molecule and a member of the sialic acid binding Ig-like lectins (siglec) family present in myelin (Martini and Schachner, 1986; Schnaar et al., 1998). Up to 30% of its weight can be made up of carbohydrates (Burger et al., 1993) and carries both the HNK-1 and the Lewis^x epitopes. The MAG activity as an inhibitor of axon regeneration (Mukhopadhyay et al., 1994) and overcoming of this inhibition (Domeniconi and Filbin, 2005) has been subject of clinical relevance in treating neuronal injuries. Its abilities to be involved in myelination and ability to inhibit axon outgrowth can be brought about by its binding to sialic acid on certain glycolipids (Vyas et al., 2005). The HNK-1 decorated MAG on Schwann cells is implicated in specific reinnervation of motor branches after femoral nerve injury (Martini, 1994) showing that a carbohydrate moiety can act as guidance cue for growing axons. MAG and tenascin-R expression overlap in oligodendrocytes to modulate neurite outgrowth (Yang et al., 1999), it would be interesting to know if the Lewis^x present on both of them, modulate any of these interactions?

Thy1, a cell surface immunoglobulin identified in the thymus has been used to characterise cerebellar cells and regions (Schnitzer and Schachner, 1981). In the developing cerebellum they show it is enriched specifically at contact points in the granular, molecular and Purkinje cell layers and is more prominent at the time after the axonal growth reduces and connections are stabilized (Schnitzer and Schachner, 1981; Tiveron et al., 1992). This expression pattern is similar to Lewis^x as discussed before. Whether the specific expression of Lewis^x particularly on Thy1 (Parekh et al., 1987) coincides with the Thy1 expression pattern, is not well resolved.

4.3. Mucin like molecules CD24 and Dystroglycan

CD24 is a mucin like cell surface receptor molecule of only 27 amino acids in mouse but depending on the glycosylation with different molecular weights of 27, 30 and 33 kDa. CD24 via its α2,3 sialic acid binds to L1 in *trans* and influences neurite outgrowth (Kleene et al., 2001). A recent observation made in our lab that CD24 dependent outgrowth is inhibited by the monoclonal antibody L5 or synthetic Lewis^x glycans (Lieberoth et al., submitted) may be related to a ligand and its interaction with CD24. This Lewis^x dependent modulation of neurite outgrowth indicates its ability to be involved, directly or indirectly, in signal transduction processes. Interestingly, only the 33 and 27kDa glycoforms of CD24 express Lewis^x.

Dystroglycan is an essential cell surface glycoprotein component of the dystrophin complex in muscle synapse and brain (Moore et al., 2002). They show that it interacts with the extracellular matrix proteins like laminin via the glycans it carries and helps muscle synapse formation and granule cell migration. Defects in glycosylation of dystroglycan underlie all dystroglycanopathies characterized by muscular dystrophy (Martin, 2006). The existence of Lewis^x on O-linked mannose of dystroglycan was demonstrated (Smalheiser et al., 1998) but its functional involvement is not known.

4.4. Prion protein

The misfolded prion proteins are infectious agents causing scrapie or transmissible spongiform encephalopathy (Prusiner et al., 1981). It has two N linked glycosylation sites and both sites carry oligosaccharides with Lewis^x as the most abundant modification followed by sialyl Lewis^x (Endo et al., 1989; Stimson et al., 1999). The role of this N-linked glycosylation in normal functions of prion are not known but are dispensable for the formation and transmission of the infecting particle (Ikeda et al., 2008; Tuzi et al., 2008). However, the glycosylation does impede fibril formation of prions (Bosques and Imperiali, 2003). In terms of Lewis^x functions, in an *in-vitro* experiment it was required for prion binding to selectins (Li et al., 2007) and they also show that this binding is prevented by sialyl Lewis^x. Though this is contrary to known

sialyl Lewis^x and selectin binding in the literature and that selectins are not found in brain. However, this interaction mechanism may have some pathological relevance.

5. Lewis^x carrying oligosaccharide structures on glycoproteins and glycolipids

Characterization of both N- and O- glycans obtained from murine brain tissue reveal that Lewis^x is abundantly present on neutral carbohydrates (Chen et al., 1998; Chai et al., 1999; Comelli et al., 2006). The Lewis^x can be present on complex di- tri- and tetra-antennary glycans making it a versatile structure to be in various positions to bring about the modulation of functions of the proteins that carry them. A few structures are selected are represented in Figure 4.

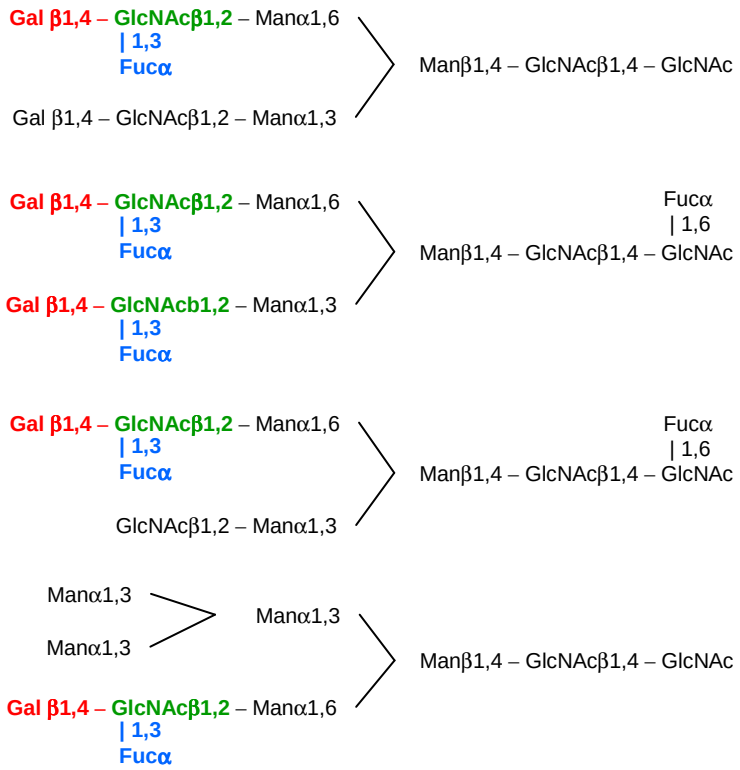


Figure 4: Representations of selected Lewis^x carrying oligosaccharides from brain (Chen et al., 1998, Chai et al., 1999 ;Comelli et al., 2006).

Lipid carriers obtained from brain tissue and elsewhere have the Lewis^x modification on neutral sphingolipids (Dasgupta et al., 1996; Kannagi et al., 1982) of the kinds represented in Figure 5.

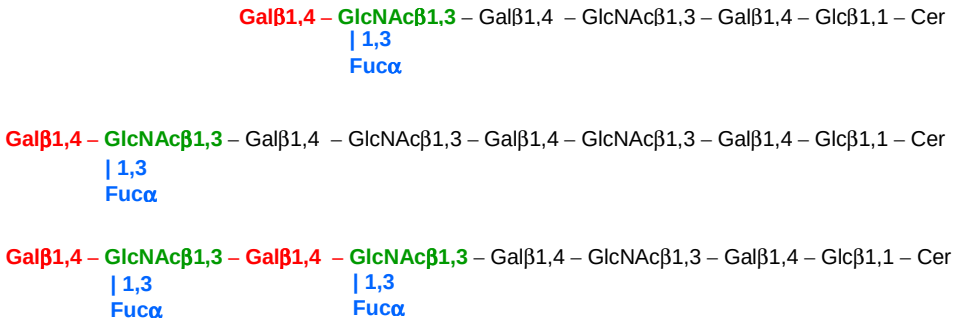


Figure 5: Representations of Lewis^x oligosaccharide structures on lipid molecules (Dasgupta et al., 1996; Kannagi et al., 1982).

6. Lewis^x and stem cells of the nervous system

Lewis^x is expressed by embryonic and adult mouse central nervous system stem cells and thus may play a role in the maintenance of stem cell properties (Solter and Knowles, 1978; Capela and Temple, 2002). The Lewis^x localization in the proliferative ventricular zone of the developing nervous system (Yamamoto et al., 1985) and its strong expression in regions with prolonged neurogenesis, e.g., the olfactory epithelium, hippocampus, basal forebrain cerebellum and retina (Koso et al., 2006; Capela and Temple, 2006) suggests it to be a potential marker for stem cell identification. Imura et al., 2006, demonstrated that glial fibriallary acidic protein (a marker for astrocytes) expressing neural stem cells that were Lewis^x positive had the ability to differentiate into neurons. The progeny of certain stem cells with the attributes of radial glia maintain neurogenic potential (Gotz and Barde, 2005) and contribute to neurogenesis. Taking this and that Lewis^x is expressed on stem cells and by radial glia in embryos (Mai et al., 1998) into consideration, Lewis^x can be attributed to have a role in differentiation. FGF-2 which is a widely used mitogen in culturing stem cells, (Dvorak et al., 1998; Jirmanova et al., 1999) showed that Leiws^X oligosaccharides are able to modulate the FGF-2 mitogenic activity on stem cells and this is not by interfering of its binding to the FGF-2 receptor. Similarly, (Koso et al., 2006) proposed that Lewis^x on a subpopulation of mouse retinal cells is regulated by Wnt signals. A direct interaction between Lewis^x and Wnt-1 was shown by immunoprecipitation experiments by (Capela and Temple, 2006). Based on this and immunohistochemical studies showing proximal positions of FGF-2, FGF-8 and Wnt with Lewis^x positive stem cells in the embryo, they propose an inductive role for

Lewis^x. Most important of all, is that the surface presence of Lewis^x and its localisation to certain populations of stem cells has made it a popular marker for isolating stem cells (Capela and Temple, 2002; Imura et al., 2006; Lanctot et al., 2007).

7. Lewis^x glycans in host pathogen interactions

Helicobacter pylori are pathogenic bacteria infecting about half the worlds population and cause diseases like gastritis, peptic ulcer and gastric cancer (Bergman et al., 2006) in about 20% of them (Duck et al., 2004). Its presence has also been proposed to protect some people from oesophageal cancer (Peek, Jr. and Blaser, 2002). *H. pylori* express blood group antigens on their lipopolysaccharide (LPS) that includes Lewis^x (Appelmelk et al., 1998). The functions of Lewis^x or the other blood group antigens on *H. pylori* are not clear. Lewis^x in particular is used to promote adhesion to the gastric epithelium aiding colonisation (Taylor et al., 1998; Edwards et al., 2000) or mediate persistence by compromising host immunity via the dendritic cell specific ICAM3-grabbing non-integrin (DC-SIGN; a Lewis^x binding C-type lectin on dendritic cells) (Bergman et al., 2006). Another possible mechanism is that the blood group antigens on *H. pylori* are shared by the host and this prevents the host from producing antibodies against them, thus mimicking the host antigens and evading the hosts' immune attack to enable the bacteria to persist (Appelmelk et al., 2000). This molecular mimicry of *H. pylori* has also been implicated in autoimmunity induced gastric disorders (Bergman et al., 2006). It should be noted that *H. pylori* in which Lewis^x structure is ablated, infect and colonise mice gut as good as the bacteria with Lewis^x (Takata et al., 2002). This leaves the importance of Lewis^x in *H. pylori* yet to be assessed.

Schistosomes are parasitic helminthic worms and were first shown to express Lewis^x by immunohistochemical methods (Ko et al., 1990) and it has been later seen that its expression varies depending on the life cycle stage and gender of the worm (Wuhrer et al., 2006). Schistosomes can cause chronic, debilitating and sometime fatal disease called schistosomiasis and infect about 200 million people in the world (Ko et al., 1990). The immune mechanisms of host pathogen interactions involved in

schistosomiasis (Velupillai et al., 2000) are similar to that of *H. pylori* and again involves the molecule DC-SIGN (van, I et al., 2003). This theme of DC-SIGN interacting with pathogens is a recurring one (Naarding et al., 2005) and has been shown that its binding to HIV-1 can be inhibited by Lewis^x present on the glycoprotein bile salt-stimulated lipase (Naarding et al., 2006).

8. Lewis^x and cancer

As described before the demonstration of the very first Lewis^x carbohydrate was from ovarian cyst fluid and the first monoclonal antibodies to identify Lewis^x were also from antigens that were derived from embryonal carcinoma cells. It has been subsequently shown to be present on many different types of tumours, for example adenocarcinomas of the colon and uterus, breast, kidney, lung, brain and various instances of Hodgkin's lymphomas to mention a few (Fox et al., 1983; Gocht et al., 1996; Dinand et al., 2008; Pellegrini et al., 2007). The increased presence of Lewis^x can be responsible for the enhanced adhesion like the homophilic forces involved in embryonic compaction (Handa et al., 2007). Alternatively, in the brain for example, in human gliomas the staining intensity for CD15 inversely correlates with the grade of malignancy (Reifenberger et al., 1992) and this is speculated to result in reduced cellular interactions and loss of contact inhibition. Like in the case of Lewis^x expressing pathogens, the glycan may also be protecting the cancerous tissue from the immune system by mimicking normal host tissue.

IV. Aims of the study

The aim of the study is to elucidate the role of the Lewis^x glycan in the processes of development, synaptic plasticity and regeneration in the nervous system of mice. To do so, we first want to identify the molecules carrying Lewis^x at the cell surface and in the extracellular matrix and also its yet unknown receptor(s). Then the functional roles of these identified molecules were investigated in *in-vitro* and *in-vivo* assay systems measuring neurite outgrowth and regeneration.

1. Approaches used to identify the Lewis^x carrier molecules and its receptors

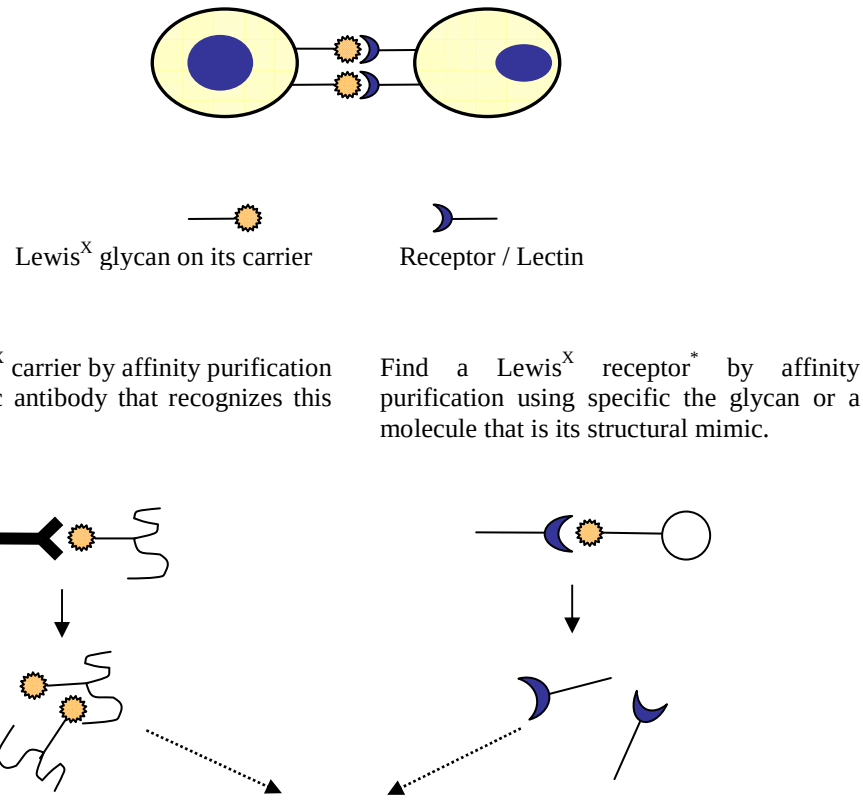


Figure 6: Schematic representation of the approaches used to identify Lewis^x carrier and receptor.
* It is assumed that the Lewis^x glycan has a specific cognate receptor and one that is probably present on the outer cell surface.

1.1. Identification of Lewis^x carriers at the cell surface and the extracellular matrix

The rat monoclonal antibody L5 is an IgM that recognises the Lewis^x carbohydrate (Streit et al., 1996). The L5 antigenic determinant has been demonstrated to be present on murine cell recognition molecules and also to be an early neural marker in chick embryos (Roberts et al., 1991). This antibody can be used to isolate novel molecules that carry the Lewis^x in the brain by affinity chromatography.

1.2. Identification of binding partners or receptors of Lewis^x

To isolate the lectin or receptor for Lewis^x by affinity purification experiments, reasonable quantities of the pure defined glycan as well as a closely related structure that can serve as a negative control is required. A possible alternative from using the carbohydrate would be to develop a peptide mimic that has the same structure and hence could serve the same function.

Peptide mimics have been successfully identified for the glycans like L2/HNK-1 (Simon-Haldi et al., 2002) and Lewis^y (Hoess et al., 1993). The L2/HNK-1 peptide mimic has further been used as a pharmacological agent to promote recovery after peripheral nerve injury (Simova et al., 2006) or demonstrate the HNK-1 glycan's involvement in long term potentiation (Bukalo et al., 2007). These experiments demonstrate the feasibility of selecting peptides that mimic glycans using large libraries of random peptides displayed on the surface of filamentous phage. To obtain a peptide analogous to the Lewis^x glycan, I decided to use such a phage display library and screen it against the Lewis^x carbohydrate recognising antibody L5. Once identified, this mimicking molecule can be synthesised in reasonable quantities to be used for lectin identification and also use it in nerve regeneration experiments that might reveal the glycans function.

V. Materials

All reagents obtained from Sigma-Aldrich, Inc., unless mentioned.

1. Buffers and solutions

Isotonic buffer solution without detergent for brain homogenisation:

50 mM Tris·HCl pH 7.5, 150 mM NaCl, 0.32 M sucrose, 5 mM EDTA (ethylenediaminetetraacetic acid) and Complete protease inhibitor cocktail from Roche Diagnostics (EDTA left out when 1 mM $MgCl_2$ and 1 mM $CaCl_2$ are included).

Brain homogenisation buffer with detergents for obtaining soluble membranes:

50 mM Tris·HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, Complete protease inhibitor cocktail from Roche Diagnostics, 1 % CHAPS and 60 mM Octyl β-D-glucopyranoside (detergents added to the homogenate or membranes to this final concentration after homogenisation to avoid frothing)

Phosphate buffered saline (PBS):

150 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, adjust pH to pH 7.4

Sodium phosphate buffer (0.1 M, pH 7.4):

Per litre: 2.62 g NaH_2PO4 x H_2O, 14.42 g Na_2HPO_4 x $2H_2O$, pH adjusted if necessary.

Tris buffered saline (TBS):

50 mM Tris·HCl (pH 7.5), 150 mM NaCl; autoclaved and stored at room temperature.

Buffer conditions for N-Glycosidase F treatment:

20 mM Sodium phosphate buffer, pH 7.4 and SDS added to a final concentration of 1 % for denaturing step and the SDS diluted to 0.1 % during incubation with enzyme.

Buffer conditions for Chondroitinase ABC-1 treatment:

Tris, pH 8.0, 40 mM Sodium acetate

Enzyme-Linked ImmunoSorbent Assay (ELISA):

Blocking buffer: 1 % (w/v) BSA in PBS.

Detection reagent: For 5 ml; 0.1 N Na Acetate pH 5 (4.75 ml), 2 % ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) in H_20 (0.25 ml) and 30 % H_20_2 (3.5 µl).

Wash buffer: PBS/Tween-20: 0.5 % (v/v) Tween-20.

Ion exchange chromatography

Buffer A: 20 mM Tris pH 7.5 and 4 M Urea

Buffer B: 20 mM Tris pH 7.5, 4 M Urea and 1.5 M NaCl

2. Antibody affinity chromatography experiments

a) Coupling of antibodies to CNBr- activated Sepharose 4B (GE Healthcare) beads:

Rehydration solution: 1 mM HCl.

Coupling buffer: 100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3(Adjust pH with 100 mM Na_2CO_3, 500 mM NaCl; 100 ml solution is sufficient for 1 L of coupling buffer).

Blocking buffer: 200 mM Glycine, 500 mM NaCl, pH 8.0

Wash buffer: 100 mM Sodium Acetate, 500 mM NaCl, pH 4.0

b) Wash buffers:
A: 25 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4

B: 25 mM Tris·HCl, 150 mM NaCl, 1 % Triton X-100, 0.02 % NaN_3, pH 7.4

C: 25 mM Tris·HCl, 500 mM NaCl, 0.1 % Triton X-100, 0.02 % NaN_3, pH 7.4

c) Elution buffer: 50 mM Ethanolamine, 150 mM NaCl, 0.2 % CHAPS, pH 11.5

d) Neutralisation buffer: 1 M Tris·HCl, pH 6.8

e) Regeneration buffers:
A: 100 mM Tris·HCl, 500 mM NaCl, pH 8.5.

B: 100 mM Sodium acetate, 500 mM NaCl, pH 4.5

C: PBS with 0.02 % NaN_3

3. Phage display

a) Blocking buffer: 0.1 M NaHCO3 (pH 8.6), 5 mg/ml BSA, filter sterilized and stored at 4 °C for not more than three days.

b) Wash buffers: PBS/Tween-20: 0.1-0.5 % (v/v) Tween-20

c) Elution buffer: 0.2 M glycine·HCl (pH 2.2), 1 mg/ml BSA.

d) Neutralisation buffer: 1 M Tris·HCl, pH 9.1

e) PEG/NaCl: 20 % (w/v) polyethylene glycol-8000, 2.5 M NaCl, autoclaved and stored at room temperature.

f) Iodide Buffer: 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, 4 M NaI, stored at room temperature in dark.

4. Gel electrophoresis and analysis

4.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Running buffer: 250 mM Tris·HCl, 1.92 mM Glycine, 1% (w/v) SDS.

Resolving gel (8.0 %):
For 12 ml;
4.2 ml deionized water,
4.5 ml of 1 M Tris·HCl, pH 8.8,
3.2 ml of 30 % (w/v) Acrylamide-Bis (29:1),
120 µl of 10 % (w/v) SDS,
30 µl 10 % (w/v) APS and 12 µl TEMED.

Stacking Gel (5%):
For 4.7 ml;
3.2 ml deionized water,
0.6 ml of 1 M Tris·HCl, pH 6.8,
800 µl of 30 % (w/v) Acrylamide-Bis (29:1),
60 µl of 10 % (w/v) SDS,
30 µl 10% (w/v) APS and 12 µl TEMED.

4.2. Western blot

a) Blocking buffer: 5 % (w/v) non fat dry milk in PBS.

b) Antibody dilution buffer: 5 % (w/v) non fat dry milk, 0.2-0.5 % (v/v) Tween-20 in PBS.

c) Wash buffer: PBS/Tween-20: 0.5 % (v/v) Tween-20 in 100 ml PBS.

4.3. 2D gel electrophoresis

a) Immobilised pH gradient strips (IPG strips from BioRad) for 2D gel electrophoresis.

b) Rehydration solution: 5 M urea, 2 M thiourea, 65 mM DTT, 0.8 % ampholyte and 4 % CHAPS.

4.4. SDS-PAGE staining reagents

a) Alcian blue staining:

Fixative/destaining solution:
25 % (v/v) ethanol, 10 % (v/v) acetic acid in water.

Staining solution:
0.025 % (w/v) Alcian Blue 8GX (Sigma) in fixative.

b) Silver staining:

Fixative:
30 % (v/v) ethanol, 10 % (v/v) acetic acid in water.

Sensitising solution:
0.3 % (w/v) potassium tetrathionate, 0.5 M potassium acetate and 30 % (v/v) ethanol in water.

Silvernitrate:
0.2 % (w/v) AgNO3 in water.

Developing reagent:
3 % (w/v) Potassium carbonate, 12.5 µl per 100 ml sodiumthiosulfate pentahydrate (10%) and 30µl per 100ml formaldehyde (37% solution).

Stopping solution:
4 % (w/v) Tris, 2 % (v/v) acetic acid in water.

Storing solution:
30 % (v/v) ethanol, 2 % (v/v) glycerol in water.

5. Medium for bacterial culture and phage display experiments

Luria Bertani (LB) medium:

Per litre; 10 g bacto-tryptone, 5 g NaCl, 5 g yeast extract. Autoclaved and stored at room temperature.

LB/Amp medium or Kan medium:

Antibiotics added to obtain a final concentration of 100 µg/ml ampicillin in LB-Medium or 50 µg/ml kanamycin in LB medium.

LB/Amp plates, Kan plates, Tet plates:

15 g agar per litre LB medium, autoclaved and supplemented with either 100 µg/l ampicillin or 25 µg/l kanamycin, or Tetracycline 10 µg/ml and poured plates stored in dark (not more than 15 days) until use.

LB/IPTG + Gal plates:

15 g agar per litre LB medium, autoclaved, supplemented with 1 ml of IPTG/Gal solution. Poured plates stored in dark (not more than 15 days) until use. IPTG/Gal stock solution prepared with 1 mg IPTG (isopropyl-β-D thiogalactoside) and 1g Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) in 25 ml Dimethyl formamide and stored at -20 °C in dark.

Top Agarose:

Per litre; 10 g bacto-tryptone, 5 g NaCl, 5 g yeast extract, 1 g $MgCl_2.6H_20$, 7 g agarose. Autoclaved, dispensed into 50 ml tubes and stored at room temperature.

IPTG stock solution for induction of expression:

1 M IPTG (isopropyl-β-D thiogalactoside) stored at -20 °C.

6. Medium and solutions for primary cell culture experiments

X-1 medium for primary culture of cerebellar granule cells:
Per 100 ml X-1; 2.2 mg/ml $NaHCO_3$, 1 ml Penicillin/Streptomycin (100x concentrate from PAA), 0.1 % Bovines serum albumin, 10 µg/ml Insulin, 4 nM L-thyroxine, 100 µg/ml Transferrin, holo, 0.027 TIU/ml Aprotinin, 30 nM sodium selenite, 1 ml sodium pyruvate (100x concentrate), 1 ml L-glutamine (100x concentrate), made up to 100 ml with Basal Medium Essential (BME), filter sterilised and equilibrated to 37 °C, 5 % CO_2 and 90 % relative humidity before use.

7. DNA primers

To amplify galectin-3:

Galapet: 5’ cagccatatgatggcagacaatttttcgctc 3’

Galare: 5’ attcctcgagtatcatggtatatgaagcac 3’

To sequence phage coat protein III in M13 phage

–96 gIII sequencing primer: 5’ccctcatagttagcgtaacg 3’

8. Kits and reagents

Reagents and materials from Thermo Fisher Scientific Inc.

a) BCA Protein Assay Kit
b) Imject Bovine Serum Albumin
c) SulfoLink Coupling Resin
d) Immobilized TCEP [Tris(2-carboxyethyl)phosphine hydrochloride] Disulfide Reducing Gel
e) Sulfo SBED Biotin Label Transfer Reagent [Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'-dithioproprionate].
f) Mts-Atf-Biotin Label Transfer Reagent .
g) Zeba Micro Spin Desalting Columns
h) MagnaBind Streptavidin Beads

Reagents from QIAGEN GmbH.

a) QIAprep Spin Miniprep Kit

b) QIAquick Gel Extraction Kit
c) QIAquick PCR Purification Kit
d) QIAGEN Plasmid Maxi Kit
e) EndoFree Plasmid Maxi Kit

Reagents from New England Biolabs.
a) Ph.D.™-12 Phage Display Peptide Library
b) Ph.D.™-C7C Phage Display Peptide Library

Reagents from Novagen, (Merck KGaA).
a) T7Select® Human Brain cDNA Library

Reagents from Dynal Bead Based Separations (Invitrogen).
a) Dynabeads® M-270 Epoxy

Reagents from Dextra Laboratories, Reading, UK.
a) Lewis^a trisaccharide
b) Lewis^x trisaccharide
c) Lacto-N-fucopentaose III-BSA (LNFP III-BSA)
d) Lacto-N-fucopentaose II-BSA (LNFP II-BSA)

9. Custom Synthesis

9.1. Synthetic oligosaccharides

To isolate the lectin or receptor for Lewis^x, reasonable quantities of the pure defined glycan as well as a closely related structure that can serve as a negative control is required. These were custom synthesised and made available to me by collaborators; Prof. Seeberger PH and Dr. Werz DB from the Laboratory for Organic Chemistry, Swiss Federal Institute of Technology (ETH) Zürich, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich, Switzerland. The Lewis^x glycan and the control glycan (Figure 7) were synthesised with a spacer and a sulfhydryl moiety that can be functionalised to be immobilised on appropriate substrates.

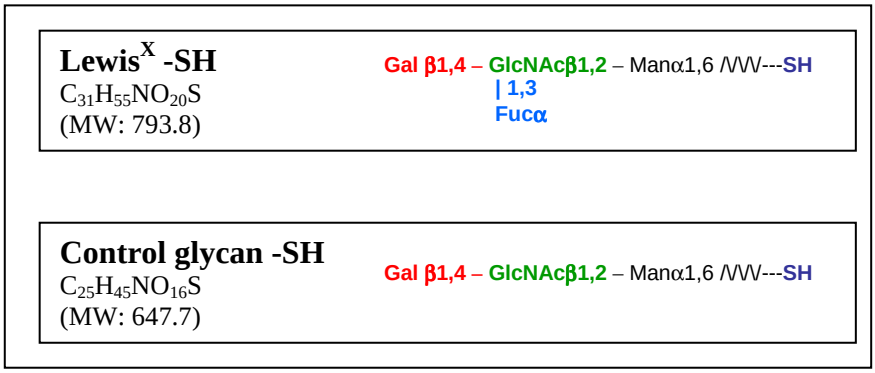


Figure 7: Custom synthesised Lewis^x and control glycans

9.2. Synthetic peptides

The following peptides were synthesised by Schafer-N, Lersø Parkallé 42 DK-2100 Copenhagen, Denmark:
Lewis^x peptides with the amino acid sequences H-SACSRLNYLHC-OH and H-KACSRLNYLHCK-OH.
Scrambled peptides with the amino acid sequences H-SACNHLLRSYC-OH and H-KACNHLLRSYCK-OH.

10. Primary Antibodies

Table 1: Primary antibodies used in the study

Nomenclature	Epitope	Species	Reference
L5	Lewis^x epitope	Rat	(Streit et al., 1996)
L1 555	Extracellular domain of mouse L1	Rat	(Appel et al., 1995)
Contactin	Extracellular domain of mouse Contactin-1	Rabbit	Our lab, Schachner M
473HD	Chondroitin sulfate	Rat	(Ito et al., 2005)
KAF13	Phosphacan	Rat	(Garwood et al., 2003)
M13/HRP	M13 phage coat proteins	-	GE Healthcare
Galectin-3 (H-160)	Galectin-3	Rabbit	Santa cruz biotechnology (sc-20157)

11. Secondary Antibodies

Horseradish peroxidase (HRP)-conjugated to either rat, rabbit or mouse anti IgG + IgM (Dianova, Hamburg, Germany), diluted appropriately before use for ELISA and Western blot experiments.

12. Bacterial strains

1) *Escherichia coli* BLT5615: F-*omp*T [lon] *hsd*SB (rB-mB-) *gal dcm lac* pAR5615 (ampR); used to propagate T7 bacteriophage.

2) *E. coli* DH5a: F- φ80dlacZM15 (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r k-, m k+) phoA supE44 thi-1 gyrA96 relA1 λ-; used to propagate DNA plasmids with ColE1 origin of replication.

3) *E. coli* ER2738: F' lacIq Δ(lacZ)M15 proA+B+ zzf::Tn10(TetR)/fhuA2 supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (rk – mk – McrBC–); used to propogate M13 phage.

4) *E. coli* BL21(DE3): F' *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3); used for recombinant expression of proteins using pET vectors.

VI. Methods

1. Biochemical methods

1.1. Brain homogenate and membrane preparation

Brain tissue from 7-days or 28-days old mice were isolated quickly from decapitated mice and immediately transferred into a Dounce tissue homogenizer (Wheaton, Millville, USA). All subsequent steps were performed on ice or at 4 °C. Each brain was homogenized in about 3 ml of isotonic buffer with 12 strokes of the pestle. The homogenate was subjected to a 1000 x g centrifugation for 10 min to separate the nuclei and un-lysed material. The supernatant was further centrifuged at 100000 x g for 1 hour to obtain a crude membrane fraction as pellet and the soluble fraction as supernatant. The membranes from the pellet were resuspended in the homogenization buffer and detergents were added to a final concentration of 1 % CHAPS and 60 mM Octyl β-D-glucopyranoside and mixed overnight on a head over heel mixer at 4 °C. This suspension was again centrifuged at 100000 x g for 1 hour to obtain a detergent solubilised membrane fraction in the supernatant.

1.2. Affinity chromatography

The L5 monoclonal antibodies were immobilized on activated CNBr Sepharose 4B beads (GE Healthcare) by covalent conjugation via primary amino groups of the proteins. The coupling procedure was carried out according to the instruction manual. The required amount of freeze-dried powder was suspended in 1 mM HCl and the active CNBr-Sepharose 4B was allowed to hydrate completely. 25 mg of the L5 antibody was taken in sodium carbonate buffer pH 8.3 and incubated with 3 ml of the beads as a slowly agitating suspension overnight at 4 °C. The remaining active groups of the gel were blocked by adding glycine buffer. Coupling was finished by alternate washing with basic carbonate buffer or acidic acetate buffer to remove an excess of absorbed protein. For affinity chromatography, coupled sepharose gel was transferred

into an empty glass column and placed at 4°C. For immunoprecipitations, beads were stored in Falcon-tubes at 4°C and applied in aliquots to the experiment.

To isolate the Lewis^x carrying molecules an affinity chromatography experiment with the immobilized L5 monoclonal antibodies (L5 antibodies recognise the Lewis^x epitope) was performed. In brief, Triton X-100 1 % (v/v) or Octyl β-D-glucopyranoside (60 mM) and CHAPS 1 % (w/v) solubilised membrane material from homogenised brains (from 7day old mice) was passed over the L5 antibody column. The column was washed with 10 column volumes of two different buffers, containing either high detergent or high salt. The bound proteins were then eluted using a shift in pH (with Ethanolamine pH 11.5 buffer). The bound and eluted molecules were further analysed by various methods such as Western blotting and mass spectrometry.

1.3. Ion-exchange chromatography

A 1 ml column of an anion exchange matrix; Q-Sepharose, in conjunction with a BioLogic FPLC control station was used and the experiment was carried out at room temperature. The L5 proteins were dialysed and resuspended in 20 mM Tris pH 7.5 and 4 M Urea. This material was pre-filtered using a 0.22 µm syringe filter and applied using a super-loop to the 1 ml Q-Sepharose column that is equilibrated with 20 mM Tris pH 7.5 and 4 M Urea. The bound molecules were eluted using a linear gradient of 0 M to 1.5 M NaCl and 20 column volumes in the same buffer. 500 ml fractions were collected and analysed by Western blot and silver staining.

1.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of proteins was performed by means of the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli, 1970) using the Mini-Protean II system (Bio-Rad laboratories). Samples were prepared in sample buffer and denatured for 5 min at 95 °C before loading. Gel

concentrations were between 6 % and 14 % depending on the molecular weight of the proteins of interest to be analysed and with a height of 5.5cm for the resolving gel and 5 % and a height of 1 cm for the stacking gel. Gels between 0.75 mm and 2 mm thick with 10 or 15-wells were used. After complete polymerization of the gel, the chamber was assembled as described in the manufacturer's protocol. The gels were run in Tris/Glycine/SDS running buffer at constant voltage of 100V until the dye front from the gel runs out. Gels were then subjected to Western blotting or staining procedures as required for the analysis.

1.5. Preparative SDS–PAGE and whole gel elution

To obtain large amounts the Lewis^x carrying proteins, the eluate from the affinity chromatography experiment with immobilized L5 antibodies separated according to their molecular size, large preparative gels in a Protean II system (Bio-Rad Laboratories) were run and subjected to whole gel elution. Whole gel elution is a process of simultaneous electroelution of separated proteins from whole SDS-PAGE into narrow chambers each containing a single or a few proteins of similar molecular weight, thus fractioning the proteins based on their molecular weights. 4 ml of the affinity purified material was precipitated using the chloroform/methanol method, resuspended in 1 ml sample buffer and denatured for 5 min at 95 °C before loading onto the gel. The proteins were separated by electrophoresis at a constant current of 40 mA. The proteins were eluted using the Whole Gel Eluter (Bio-Rad Laboratories) during 25 min by applying a constant current of 225 mA. At the end of the run the polarity of the current is reversed for 15 seconds to dislodge any proteins that are stuck to the cellophane and the eluted fractions are harvested from the chamber. The fractions are subsequently analysed by Western blotting or staining procedures.

1.6. 2-D gel electrophoresis

The precipitated protein samples were solubilised in rehydration buffer (5 M Urea, 2 M thiourea, 4 % CHAPS, 0.8 % ampholyte, and 65 mM diththiothreitol [DTT]), loaded onto an appropriate immobilized pH gradient (Ready IPG strips from Bio-Rad,

pH 3-10 or pH 5-8, 7 cm) as an overnight process. The isoelectric focusing was performed in a paraffin oil coated Multiphor II chamber (Amersham Pharmacia) that was maintained at 17 °C with the following program: 200 V and 2 mA for 1 min, 3500 V and 2 mA for 1.5 hours and 3500 V and 1 hour. The IPG strips were then equilibrated for 10 min with equilibration solution (1 M Tris-base, pH 8.8, 6 M urea, 2 % [w/v] SDS, 2 % [w/v] DTT, 20 % [v/v] glycerol and 0.02 % [w/v] bromophenol blue). Each IPG strip was loaded onto the appropriate percentage SDS-PAGE, sealed with 1 % agarose in running buffer and electrophoresed using a Protean mini gel apparatus (Bio-Rad) and Tris/Glycine/SDS running buffer at 100 V until the dye front runs out. After electrophoresis the proteins are either visualized using silver-staining or subjected to Western blot analysis.

1.7. Western blot analysis

Proteins were transferred from a SDS-PAGE gel onto a PROTEAN® Nitrocellulose Transfer Membrane (Schleicher & Schüll, Dassel, Germany) using the Mini-Protean II system. The blotting sandwich was assembled as described in the manufacturer's protocol. Proteins were transferred electrophoretically in blotting buffer at constant voltage of 80 V for 3 hours at 4°C. The Precison Plus Protein All Blue Standards (Bio-Rad Laboratories), a prestained molecular weight marker used in the SDS-PAGE was used to monitor the efficiency of the electrophoretic transfer.

Following electrophoretic transfer, the membrane was removed from the sandwich, placed with the protein-binding site upwards into a glass vessel, washed once in PBS and the nonspecific binding of antibodies was blocked with 5 % (w/v) non fat milk in PBS for 1 h at room temperature. The membrane was washed with PBS/Tween-20 (0.05 % Tween-20) and incubated overnight at 4°C with the primary antibody that is diluted in an appropriate dilution. The primary antibodies were removed and membranes were washed three times for 10 min with PBS/Tween-20. The membrane was subsequently incubated with an appropriate horse radish peroxidise (HRP) conjugated secondary antibody for 1 hour at room temperature. The membrane was washed again three times for 10 min with PBS/Tween-20. The immune reactive bands

were detected using a chemiluminescence detection system (Pierce). The membrane was soaked for 1 min in detection solution and then the blot was placed between two transparent plastic foils and placed in an Amersham Hypercassette™ autoradiography cassette. The membrane was exposed to Biomax-MR X-ray film (Kodak, Stuttgart) for varying time periods.

1.8. Colloidal Coomassie staining of polyacrylamide gels

The colloidal Coomassie staining of polyacrylamide gels was performed with Roti-Blue kit (Carl Roth GmbH). After SDS-PAGE, the gels were fixed in fixing solution for 60 min and subsequently incubated with Roti-Blue staining solution for 2-15 h with constant agitation. The gels were then incubated in destaining solution until the background of the gel appeared nearly transparent.

1.9. Alcian Blue staining of glycosaminoglycans

Gels were fixed for 4 hours or overnight (with several changes) in 25 % (v/v) ethanol, 10 % (v/v) acetic acid in water. The gels were washed extensively to remove the SDS from the gel. Fixed gels were stained overnight in the 0.025 % (w/v) Alcian Blue 8GX stain prepared in the fixative. The gels were destained with multiple changes of the fixing solution. Adequate destaining was necessary to avoid high background when performing subsequent silver staining.

1.10. Silver staining

After SDS-PAGE, gels were fixed with 30 % (v/v) ethanol, 10 % (v/v) acetic acid in water for 4 hours (with changes at each hour). The gel was then exposed to the sensitising solution for 45 min and washed six times x 10 min with distilled water. The gels were allowed to be impregnated with silver in a silver nitrate solution for an hour. The gel is washed for 15 seconds with distilled water and developing reagent

containing formaldehyde/potassium carbonate. When a sufficient degree of staining was obtained (about 30 to 40 min), reaction was stopped with a 4 % (w/v) Tris, 2 % (v/v) acetic acid in water.

1.11. Immunoprecipitation

Homogenates or detergent solubilised membrane fractions from mouse brains were prepared in 50 mM Tris·HCl pH 7.5, 150 mM NaCl, 0.32 M sucrose, 5 mM EDTA and Complete protease inhibitor cocktail from Roche Diagnostics (1 % CHAPS and 60 mM Octyl β-D-glucopyranoside if used). Samples containing 1 to 2 mg of total protein were cleared with protein A/G-agarose beads (Santa Cruz Biotechnology) (3 h at 4°C) and incubated with antibodies of interest or non-specific control Ig (overnight, 4°C), followed by precipitation with protein A/G-agarose beads (1 h, 4°C). The beads were washed 3 times with buffer containing either 1 % (v/v) Nonidet P-40 and bound proteins were eluted by boiling in SDS-PAGE loading buffer. Eluted material was analysed by Western blot experiments.

1.12. Protein precipitation with methanol and chloroform

This rapid method based on a defined methanol-chloroform-water mixture for the precipitation of soluble as well as hydrophobic proteins from dilute solutions was developed by Wessel and Flugge (Wessel and Flugge, 1984). It was used to concentrate proteins and also remove detergents and lipids from the samples. To one volume of sample protein solution, four volumes of methanol were added and vortexed to mix followed by one volume of chloroform and vortexed again. To separate the phases, three volumes of water was added, vortexed for 10 seconds and centrifuged at 10000 x g for 5 min. The upper phase was carefully removed and discarded. A further three volumes was added to the interphase with proteins and chloroform phase. The samples are mixed again and centrifuges at 10000 x g for 5 min at room temperature to pellet the proteins. The supernatant was removed and the pellet was air dried and dissolved in an appropriate buffer.

1.13. Estimation of protein concentration

The protein concentration of cell lysates was determined using the BCA Protein Assay Reagent Kit. Solution A and B were mixed in a ratio of 1:50 and 200 µl of the resulting solution were added to 10 µl of the cell lysate 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 in a microtiter plate at 562 nm using an ELISA reader and protein concentrations were determined from their relative extinction compared to BSA standards.

1.14. Chondroitinase-ABC treatment

Chondroitinase-ABC (Seikagaku Corporation) is an enzyme that catalyses the cleavage of N-acetylhexosaminide linkages in chondroitin 4-sufate, chondroitin 6-sulfate, dermatan sulfate and chondroitin. In a typical reaction, 5 milli units of enzyme (enzyme diluted in Tris, pH 8.0, 40 mM Sodium acetate, and 0.1 % BSA) was incubated with 50 µg of Lewis^x carrying proteins (antibody affinity purification experiment) in Tris, pH 8.0, 40 mM Sodium acetate buffer and incubated for 4 hours at 37 °C. The removal of chondroitin sulphate was analysed by Western blot analysis using chondroitin sulphate antibodies.

1.15. PNGase F treatment

PNGase F (Roche) cleaves all types of N-glycans present on aspargines in a glycopeptide producing the oligosaccharide/s, aspartic acid (in the peptide) and ammonia. Lewis^x carrying proteins were precipitated and resuspended in 20 mM Sodium phosphate buffer, pH 7.2 and 1 % SDS and denatured by heating to 100 °C for 5 min. The protein solution is diluted to reduce the SDS concentration to 0.1 % during incubation with enzyme. In a typical reaction 1 unit of enzyme was incubated with this 100 µg of denatured Lewis^x carrying proteins and incubated overnight at

37 °C. The removal of Lewis^x carrying N-glycans was analysed by Western blot analysis using the L5 antibodies.

2. Conjugation and cross-linking procedures

2.1. Conjugating Lewis^x to Imject Maleimide Activated BSA

To conjugate the sulfhydryl linked Lewis^x oligosaccharide, 1 mg of Lewis^x was incubated with 1 mg of maleimide activated BSA in 100 µl of 0.1 M sodium phosphate buffer for 2 hours at room temperature. The remaining binding sites were quenched with a solution of 50 mM L-Cysteine·HCl solution. The BSA conjugated to Lewis^x was recovered using Zeba desalt spin columns. The control sugar with the sulfhydryl handle was conjugated to BSA in a similar reaction

2.2. Immobilisation of proteins onto epoxy activated M270 magnetic beads

BSA-Lewis^x (150 µg) in 200 µl of sodium phosphate buffer was mixed with appropriate amounts of prepared epoxy activated M270 epoxy beads (Dynal Biotech), then ammonium sulfate was added to a final concentration of 1 M (from a 3 M stock) and incubated at room temperature for 24 hours to allow covalent linking of BSA-Lewis^x to the beads. The beads were washed with sodium phosphate buffer and blocked with a solution of 1 % BSA. After washing again the BSA-Lewis^x beads mixed with detergent solubilised membrane fractions from three 7-day old mouse brain and incubated overnight at 4 °C to precipitate probable Lewis^x receptors. The interacting complexes along with the magnetic beads were collected each time with a magnet along the sides of the tubes and the non-specifically bound material was washed in three successive rounds with PBS/Tween-20 (0.05 %). The bound proteins were eluted by boiling the beads in appropriate amounts of SDS-PAGE loading buffer and separated on a SDS-PAGE. The gel was then subjected to silver staining and bands of interest were excised and analysed by mass spectrometry analysis.

For phage display experiments the L5 antibody was immobilised to the M270 epoxy activated M270 epoxy beads in a procedure similar to the immobilization of BSA-Lewis^x.

2.3. Coupling of custom made Lewis^x oligosaccharide to SulfoLlink resin

SulfoLink coupling resin was used to immobilise the custom made Lewis^x that is linked to a sulfhydryl molecule by a spacer. 100 µg of the Lewis^x-SH sugar in 100 µl of 50 mM Tris (pH 8.5) was first subjected to reducing conditions using gel immobilised TCEP on gel to obtain the Lewis^x with free sulfhydryls. The reduced material was immediately incubated with appropriate amounts of the drained SulfoLink gel at room temperature for 20 min with constant mixing followed by 30 min without shaking. The beads were washed with the 50 mM Tris buffer and then the remaining binding sites were quenched with a solution of 50 mM L-Cysteine·HCl solution (prepared in50 mM Tris, pH 8.5). The beads were washed with 1 ml of 1M NaCl and then by PBS. These beads were used in pull-down experiments in a procedure identical to the experiment using BSA-Lewis^x to M270 epoxy beads.

2.4. Chemical crosslinking using Sulfo SBED Biotin label transfer reagent

BSA-Lewis^x conjugate was coupled to 250 µg of Sulfo-SBED using the ProFound Sulfo-SBED biotin label transfer reagent from Pierce Chemicals (Rockford, IL). The Sulfo-SBED is a trifunctional cross-linker containing an NHS group linked by a cleavable disulfide bond, a photoreactive aryl azide group and a biotin side group that can be used in a label-transfer method to capture a protein interacting with another protein that has been biotinylated.

Sulfo-SBED was dissolved in dimethyl sulfoxide, and 0.25 mg was added to 100µl of PBS with 0.5 mg BSA-Lewis^x. The reaction was allowed to proceed in the dark for

30 min at room temperature to enable the NHS group to react with the primary amines on the BSA. Residual un-reacted cross-linker was removed using 2 ml Zeba desalting columns. Sulfo-SBED conjugated BSA-Lewis^x was mixed with detergent solubilised membrane fractions from mouse brain for 1 hour at room temperature. Interacting proteins (receptors) were captured by the photoreactive aryl azide moiety when the conjugated bait protein and solubilised membrane mixture was exposed to ultraviolet irradiation on a parafilm lined petri-plate. This crosslinking was performed with a 365nm UV lamp placed 5cm above the sample for 10 min. The interacting complexes were then captured using streptavidin coated magnetic beads and the non-specifically bound material was washed in three successive rounds with PBS/Tween-20 (0.025 %). The disulfide bond was subsequently reduced during denaturation by heating for 5 min at 95 °C with sample buffer containing DTT. Upon reduction of the disulfide bond, the biotin label was transferred only to the interacting protein. The biotin modified interacting protein is detected by Western blot using Streptavidin-HRP and an appropriate substrate or visualised by silver-staining.

2.5. Chemical crosslinking using MTS ATF biotin label transfer reagent

The Mts-Atf-Biotin Label Transfer Reagent is similar to the Sulfo-SBED but instead of the amino reactive NHS group it has a sulfhydryl-reactive methanethiosulfonate (Mts) group. This was conjugated to a custom made Lewis^x sugar that was linked to a sulfhydryl group linked by a spacer molecule. About 100□g of this Lewis^x-SH was mixed with 100□g of Mts-Atf-Biotin and the reaction was allowed to proceed in the dark for 30 min at room temperature. Residual un-reacted cross-linker was removed using 2 ml Zeba desalting columns. Mts-Atf-Biotin conjugated Lewis^x was mixed with detergent solubilised membrane fractions from mouse brain for 1 hour at room temperature. The subsequent steps were identical to the Sulfo-SBED protocol.

3. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Antigens of interest were immobilized on polyvinylchloride surface in 96-well microtiter plate in concentration 5-10 µg/ml for overnight at 4 °C. Non-absorbed

proteins were removed and the wells were blocked for one hour at room temperature with 1 % BSA in PBS. After washing once with PBS, the wells were subsequently incubated for an hour at room temperature with the putative binding proteins diluted in the range of 4 µg/ml to 10 µg/ml in PBS containing 1 % BSA. Unbound proteins were removed and the wells were washed five times for 5 min at room temperature to remove non-specifically bound proteins. Bound proteins were detected with streptavidin coupled to horseradish peroxidise (HRP) or with certain primary antibodies and the appropriate HRP-conjugated secondary antibodies. Protein binding was visualized by the detecting the reaction of HRP with ABTS reagent that results into a coloured product. The absorptions of the samples were quantified in a microtiter plate at 405 nm using an ELISA reader.

4. Cloning of genes and their recombinant expression

4.1. Polymerase chain reaction (PCR)

The *in-vitro* amplification of DNA fragments using the polymerase chain reaction (PCR) was performed in a MJ PTC-200 DNA ENGINE™ Peltier Thermal Cycler. Standard PCR reactions contained the following ingredients: template DNA (typically plasmid or first strand cDNA), primers (flanking the region to be amplified), dNTPs (25 mM each), DNA polymerase buffer and DNA polymerase. Primer sequences were selected manually.

Table 2: PCR cycling parameters

Cycle Step	Temperature	Time	No. of cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	
Annealing	58°C	25 s	25
Extension	72°C	30 s	
Final Extension	72°C	5 min	1

50 µl reactions were performed in 0.2 ml thin-walled tubes (Biozym, Hessisch Oldendorf, Germany). iProof High-Fidelity DNA polymerase (Bio-Rad) was employed to amplify DNA or full-length genes. Table 2 lists the cycling parameters

used. Following cycling, typically 5-10 µl aliquots up to complete reactions were analyzed by agarose gel electrophoresis to detect amplified products.

4.2. PCR/DNA fragment purification

For purification of DNA fragments, the silica matrix-based MiniElute™ PCR Purification Kit was used according to the manufacturer's protocol. The DNA was eluted from the column by addition of 50 µl elution buffer. The DNA concentration was determined using the 1:10 – 1:100 dilutions of the eluate.

4.3. Restriction enzyme digest of DNA

Restriction enzyme digests were performed by incubating double-stranded DNA with an appropriate amount of restriction enzyme(s), the respective buffer as recommended by the supplier and at the optimal temperature for the specific enzyme(s), usually at 37°C. General digests were set up as 20 µl total volume reactions. For preparative restriction digests, the reaction volume was scaled up to 100 µl. After incubation at the optimal temperature for a 1-3 h, enzymes were inactivated by incubation for 20 min at 65 °C. If reaction conditions of enzymes were incompatible to each other, DNA was digested successively with the individual enzymes. Between individual reactions, the DNA was purified.

4.4. DNA agarose gel electrophoresis

To analyze PCR products and restriction digested fragments and DNA preparations, horizontal agarose gel electrophoresis was performed. Gels of 0.8-1.5 % (w/v) agarose (electrophoresis grade) in 1x Tris-acetate buffer (TAE) were used; the agarose concentration depended on the size of fragments to be separated. DNA samples were adjusted to 1x DNA sample buffer and were subjected to

electrophoresis at 10 V/cm in BioRad gel chambers in 1x TAE running buffer. After the samples were run gels were stained in a staining bath containing 0.5 µg/ml ethidium bromide in 1x TAE for approximately 30 min. Gels were documented using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloh, Germany) or when required, bands were made visible on an UV screen (λ = 360 nm) and fragments were excised out with a scalpel for further manipulation.

4.5. DNA fragment extraction from agarose gels

For isolation and purification of DNA fragments from agarose gels, ethidium bromide-stained gels were illuminated with UV light and the appropriate DNA band was excised from the gel with a clean scalpel and transferred into an Eppendorf tube. The fragment was isolated utilizing Qiagen's silica matrix-based QIAquick™ or MiniElute™ Gel Extraction Kits following the manufacturer's protocol. The fragment was eluted from the column by addition of 50 µl elution buffer.

4.6. Ligation of DNA fragments

Ligation of DNA fragments was performed by mixing 100 ng vector DNA with the five-fold molar excess of insert DNA. 1 µl of T4 DNA Ligase and 2 µl of 10x ligation buffer were added and the reaction mix was brought to a final volume of 20 µl. The reaction was incubated overnight at 16 °C and subsequently was used directly for transformation without any further purification.

4.7. Maintenance of bacterial strains

Strains were stored as glycerol stocks (LB broth with 25 % (v/v) glycerol at –80°C). To regrow particular strains, an aliquot of the stock was streaked on an LB agar plate

containing the appropriate antibiotic and incubated overnight at 37°C. Plates were stored up to 4 weeks at 4 °C.

4.8. Production of competent bacteria

E. coli bacteria were streaked on LB agar plates and grown overnight at 37 °C with constant shaking. 50 ml of LB broth were inoculated with 5 colonies and grown at 37 °C under constant shaking (~200 rpm) until the culture had reached an optical density (OD_{600}) of 0.35-0.45. Growth of bacteria was stopped by incubation for 5 min on ice. Cells were pelleted at 1000 x g for 15 min (4 °C) and, after removal of the supernatant, resuspended in 17 ml prechilled RF1 (4 °C). Following a 15 min incubation step on ice, the centrifugation was repeated. The cell pellet was resuspended in 4 ml prechilled RF2 (4 °C) and incubated again for 15 min on ice. Bacteria were frozen in liquid nitrogen in 50 – 100 µl aliquots and stored at –80 °C. Transformation capacity/efficacy of cells was tested by a transformation with a distinct quantity of purified supercoiled plasmid DNA.

4.9. Transformation of DNA into bacteria

Ligation mixture (10 µl)were added to 100 µl of competent DH5α and incubated for 30 min on ice. After a heat shock for 60 s at 42 °C and successive incubation on ice (1 minute), 800 µl of LB broth was added to the bacteria suspension, transferred to a culture tube and incubated at 37 °C for 60 min with constant shaking. 100 µl cells were plated on LB plates containing the appropriate antibiotic. Colonies picked for further analysis after incubation at 37 °C for 12-16 h.

4.10. Small scale plasmid isolation (Miniprep)

LB broth (2 ml, containing 100 µg/ml ampicillin or 25 µg/ml kanamycin) were inoculated with a single colony and incubated over night at 37°C with constant agitation. The culture was transferred into a 2 ml Eppendorf tube and cells were

pelleted by centrifugation (12000 rpm, 1min, RT). Plasmids were isolated from the bacteria using the Qiagen Plasmid Prep Kit, according to the manufacturer's protocol. The DNA was eluted from the columns by addition of 50 µl DNAse and RNAse free water and the plasmid DNA was stored at -20°C.

4.11. Large scale plasmid isolation (Maxiprep)

For preparation of large quantities of DNA, the Qiagen Maxiprep kit was utilized. A single colony was inoculated in 2 ml LB broth (containing 100 µg/ml ampicillin or 25 µg/ml kanamycin) and grown at 37 °C for 3 h with constant agitation. This culture was added to 250 ml LB broth (containing 100 µg/ml ampicillin or 25 µg/ml kanamycin) and the culture was incubated at 37°C with constant agitation overnight. Cells were pelleted in a Beckmann centrifuge (6000 x g, 15 min, 4°C) and the DNA was isolated as described in the manufacture's protocol. Finally, the DNA pellet was resuspended in 500 µl of DNAse and RNAse free water and the plasmid DNA was stored at -20 °C.

4.12. Sequencing of DNA

Sequence determination of double-stranded DNA was performed by the sequencing facility of the ZMNH (Dr. W. Kullmann). Fluorescent dye-labelled chain termination products (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Wellesly, MA, USA) were analyzed with an ABI Prism 377 DNA Sequencer (Perkin Elmer). For preparation, 0.8-1 µg of DNA was diluted in 7 µl ddH_2O and 1 µl of the appropriate sequencing primer (10 pM) was added.

4.13. Recombinant expression and purification of proteins from E. coli

For recombinant expression of proteins in *E. coli*, the corresponding cDNA of the protein was cloned in frame with the purification tag of the expression plasmid

pET28a. The *E. coli* strain BL21 (DE3) was transformed with the expression plasmid and streaked on LB plates supplemented with the Kanamycin (50 µg/ml) and incubated at 37 °C overnight. A single colony (from plates not older than 16 hours of incubation) was inoculated in a 5 ml LB medium supplemented Kanamycin (50 µg/ml) and incubated overnight at 37 °C with constant agitation. This 5 ml starter culture was used to scale up to cultures of the required volume and incubated at 37 °C with constant agitation. When the culture reached an optic density of 0.6, protein expression was induced by adding IPTG to a final concentration of 0.1 mM/ml. The culture was incubated and agitated further for 3 hours at 37 °C. Bacteria were harvested by centrifugation and stored at –80 °C. Protein expression was monitored by removing 250 µl aliquots of the culture every hour after IPTG induction. Bacteria were pelleted, lysed in sample buffer and applied to a SDS-PAGE gel and checked by Coomassie staining.

Purification of the proteins via Ni-NTA column was performed according to the *QIAexpressionist* handbook.

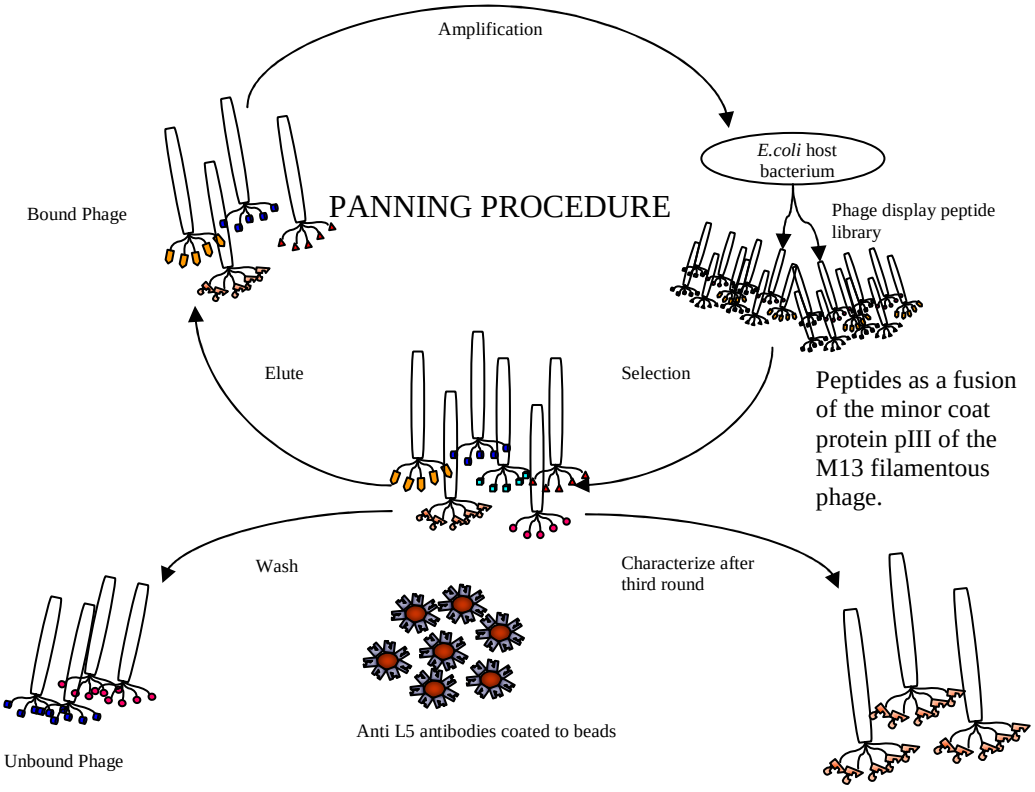
5. Phage display experiment using random peptide phage library

The Ph.D.™-12 Phage Display Peptide Library based on a combinatorial library of random dodecapeptides fused to a minor coat protein (pIII) of M13 phage was used. The displayed peptide is expressed at the N-terminus of pIII. A second library, Ph.D.-7, consisting of a disulfide-constrained heptapeptide library was also used. The randomized segment of the Ph.D.-C7C library is flanked by a pair of cysteine residues, which are oxidized during phage assembly to a disulfide linkage, resulting in the displayed peptides being presented to the target as loops.

L5 monoclonal antibody (10 mg) was coupled to M270 epoxy magnetic beads were blocked with 5 % BSA in PBS and used as target molecule substance. These beads were incubated with 2 x 10^{11} phage (10 ml of the library) in 100µl PBS and Tween-20 (0.025 to 0.050 % v/v) for 25 min at room temperature to allow for binding. The unbound phage discarded and the beads washed for 10 times with PBS and Tween-20 (0.25 %). The bound phage were eluted with either 100 ml of 100 mg/ml L5

monoclonal antibodies in PBS for 20 min or a nonspecific elution with 0.2M Glycine-HCL (pH 2.2) for 10 min. The eluted phage was amplified in the *E. coli* ER2738 host strain and subsequently 2 x 10^{11} phages were used for subsequent rounds of panning. After four rounds of the panning experiments, the DNA sequence of the pIII region of the binding clones was determined to reveal the identity of the coded peptides. Phages that had consensus sequence amongst themselves were selected after assaying by ELISA experiments. For the ELISA, again L5 antibodies were immobilized on immunosorbent microtitre plates and probed with amplified individual phage for their binding followed by detection with anti M13-horse radish peroxidase antibodies. DNA from the binding phage prepared and sequenced using the -96 primer to deduce the encoded peptide sequence. The phage display experiments were performed as per the protocols of the Ph.D.-12 or the Ph.D.-C7C Phage Display Peptide Library manual.

Schematic representation of the Phage Display experiment



5.1. Phage display experiment using T7 phage library

BSA-Lewis^x conjugate, 500 µl at a concentration of 100 mg/ml was coated overnight at 4 °C onto 12 well tissue-culture plates to be used as target molecule. The wells are blocked with 5 % BSA and incubated with 1 x 10^7 phage (10 ml of the library) in 250 ml PBS and Tween-20 (0.05 %) for 30 min at room temperature to allow for binding. The unbound phage discarded and the wells are washed for 10 times with PBST. The bound phage were eluted with either 500 ml of 100 mg/ml L5 monoclonal antibodies in PBS for 20 min or a nonspecific elution with 0.2 M Glycine-HCL (pH 2.2) for 10 min. The eluted phages were incubated with wells coated with BSA-Lewis^a to adsorb and remove non-specific binding phages. Then the phages were amplified and subjected to amplification in the *E. coli* BLT 5615 host strain and 3 x 10^8 phages were used for subsequent rounds of panning. After four rounds of the panning experiments individual clones were selected, DNA prepared from them and sequenced to deduce the identity of the binding polypeptide. The phage display experiments were performed as per the protocols of the Pre-Made T7 Select Library of normal human brain tissue (Catalogue number 70637-7, Novagen). Procedures to select phage from the T7 phage library are similar to the above protocol.

5.2. Isolation of DNA of the identified phage and its sequencing

A 1 ml freshly growing culture of the appropriate *E. coli* host was inoculated with a phage plaque and allowed to grow at 37°C with shaking for about 4.5 hours (till the cells are lysed) to amplify the phage. The supernatant was obtained from the culture by centrifuging and transferred to a new microcentrifuge tube and re-spun, the upper 80 % of this supernatant contained the phage. 1 ml from this supernatant was mixed with 400 ml of PEG/NaCl and let to stand for 10 min. The mixture was centrifuged for 10 minutes at 16000 x g and the pellet was re-suspended in 100 ml of iodide buffer, 250 ml ethanol added and incubated for 10 min at room temperature to selectively precipitate the single stranded phage DNA. The DNA was pelleted by centrifuging for 10 min at 16000 x g and washed with 70 % ethanol. The pellet was

dried and re-suspended in 30 µl of autoclaved distilled water. 7 µl of this DNA (3.5 µl in case of the T7 phage) was used for sequencing using an appropriate primer.

6. Cell Culture

6.1. Primary cell culture of dissociated cerebellar neurons

Dissociated cerebellar neurons were prepared using a procedure as described in Schnitzer and Schachner, 1981. Cerebella of 6-7 day old mice were quickly isolated from decapitated mice and placed into petri dishes containing cold containing HBSS. The meninges along with blood vessels removed using a fine forceps under a dissecting microscope. The cerebella are cut into three pieces and transferred to a 15 ml plastic centrifuge tube in which all the subsequent procedures are performed and in a laminar flow hood. Then tissue from a maximum of three cerebella is incubated in 1 ml of 1 % trypsin and 0.05 % DNase solution for 12 min at room temperature. The tissue pieces are washed thrice with about 5 ml of HBSS and resuspended in 1 ml of 0.05 % DNase solution. The tissue pieces are then triturated by passing through fire polished Pasture pipettes of narrowing bores, about 5-8 passes through each of the 1mm diameter bore pipette followed by a 0.5mm and then a 0.1-0.2 mm bore pipettes. The cells are suspended in 5 ml of cold HBSS and placed on ice for about five min to allow for clumps to settle. The suspension without the clumps are carefully transferred to a new centrifuge tube and centrifuged for 10 min at 4 °C at 100g. The cell pellet is re-suspended in 5 ml of X-1 medium (equilibrated to 37 °C, 5 % CO_2 and 90 % relative humidity). Cell concentrations and viability were simultaneously determined using 10 µl of this cell suspension mixed with 10 µl of a 0.4 % Trypan blue solution and counted in a haemocytometer.

6.2. Plating and analysis of neuritogenesis of the cerebellar neurons

Single cell suspensions prepared as described above were seeded on poly-L-lysine coated, 15 mm diameter coverslips. Coverslips were first cleaned by extensive

washing with 3 M HCl, methanol, acetone and then heat sterilized. Coverslips were coated with poly-L-lysine by constant agitation at 4°C overnight in a 50µg/ml poly-L-lysine solution in PBS. Finally, they were washed twice with sterile distilled water and dried under a sterile laminar flow hood. Proteins of interest at concentrations of 10-20 mg/ml were coated on coverslips (60 or 100 ml per coverslip) overnight at 4 °C. The coverslips were then placed in 12 well cell culture plates and washed twice with cold HBSS. As a positive control, laminin was coated and as a negative control only poly-L-lysine was given as substrate for neurite outgrowth. 1 ml of the dissociated cerebellar neurons suspension were seeded with densities of 1 to 1.5 x 106 cells per well. Cells were allowed to grow for 24 hours at 37 °C and 5 % CO_2. After this time period, cells were fixed by adding 100 µl 25 % glutaraldehyde and stained with 1 % toluidine blue in 1 % borate buffer for 2 hours at room temperature. Cells were washed twice with water and dried at room temperature. Cells were imaged with a Kontron microscope (Zeiss) and analysed with Carl Zeiss Vision KS 400 V2.2 software. For each experimental value, neurites of at least 100 cells (50 from each of the duplicates) with neurites longer than the cell body diameter were measured.

7. In-vivo experiments

7.1. Surgical procedure for femoral nerve injury experiment

The femoral nerve injury experiment and its analysis was performed according to methods established by (Simova et al., 2006). Female C57BL/6J mice were obtained from Taconic (Copenhagen, Denmark) at the age of 3 months. All experiments were conducted in accordance with the German and European Community laws on protection of experimental animals. For surgery, the animals were anaesthetized by intraperitoneal injections of 0.4 mg.kg-1 fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany), 10 mg.kg-1 droperidol (Dehydrobenzperidol, Janssen) and 5 mg.kg-1 diazepam (Valium 10 Roche, Hoffman – La Roche, Grenzach-Wyhlen, Germany).

The femoral nerve bifurcates into two major branches, a quadriceps muscle branch containing motor and sensory axons and a sensory branch innervating the skin, the saphenous branch. After lesion of the common nerve trunk, motor axons regrow at

random into both the proper quadriceps branch and the improper saphenous branch. The left femoral nerve was exposed by a skin incision. Nerve transection was performed using a scissors at a distance of about 3 mm proximal to the bifurcation of the muscle and the cutaneous branches. The cut ends of the nerve were inserted into a polyethylene tubing (3 mm length, 0.38 mm inner diameter, Becton Dickinson, Heidelberg, Germany) and fixed with single epineural 11-0 nylon stitches (Ethicon, Norderstedt, Germany) so that a 2-mm gap was present between the proximal and distal stump. The tube was filled with vehicle substance phosphate buffered saline (PBS) containing scaffold peptide that forms a gel matrix support (0.5 % PuraMatrix Peptide Hydrogel, 3DMatrix, Cambridge, MA) for control group, PBS/scaffold peptide supplemented with either the cyclic Lewis^x peptide (200 µg/ml) or cyclic Lewis^x scrambled peptide (200 µg/ml) for the experimental group of animals. Ten animals in each group were subjected to each of these treatment protocols. After peptide or vehicle application, the skin wound was closed with sutures (Ethicon). Functional analysis was performed over a time-period of 12 weeks.

7.2. Analysis of motor function recovery after femoral nerve injury experiment

Mice were trained to walk on a horizontal wooden beam (1000 mm long, 38 mm wide) leading to their home cage. After the learning phase and prior to operation, one beam-walking trial was video recorded for each animal. A rear view of the walking along the beam was captured. Each animal was video recorded again at 1, 2, 4, 8 and 12 weeks post operation after nerve transection. For analysis, selected video sequences were examined using the frame grabber VirtualDub, a video capture/processing utility written by Avery Lee (free software available at http://www.virtualdub.org). Selected frames in which the animals were seen in defined phases of the step cycle (see below) were used for measurements performed with UTHSCSA ImageTool 2.0 software (University of Texas, San Antonia, TX, USA, http://ddsdx.uthscsa.edu/dig/). The video sequences were analysed using the frames in which the animals were seen in phases of the gait cycle, meeting the criteria for measurements of the:

Heels–tail angle (HTA, Figure 8) is formed by the lines connecting the heels with a clearly discernible sagittal point on the animal's body and the external urethral orifice. The angle is measured with respect to the dorsal aspect.

Foot-base angle (FBA, Figure 8) is measured at toe-off position at which the sole is parallel to the transverse plane as shown in the figures below. The angle is measured with respect to the medial aspect.

Protraction length ratio (PLR, Figure 8), was evaluated from video recordings of voluntary movements of the mice performed during the "pencil" test. An intact mouse, when held by its tail and allowed to grasp a pencil with its forepaws, tries to catch the object with its hind paws and performs cycling flexion–extension movements with the hind limbs. After lesion, the limb cannot be completely extended, so the PLR is the ratio of the relative length of the intact to the lesioned limb. The distance measured between the most distal mid-point of the extremity to a fixed, well-discernible point in the sagittal plane on the animal's body is measured to estimate limb lengths. In intact animals the relative length of the two extremities, as estimated by lines connecting the most distal mid-point of the extremity with the anus, is approximately equal and the PLR (ratio of the right to left limb length) is close to 1.

For each of the parameters an average of five measurements per animal, extremity, and trial was found to be representative for the individual animals. The HTA and FBA are directly related to the ability of the quadriceps muscle to keep the knee joint extended during contralateral swing phases. As a relative measure of functional recovery at different time-point after nerve injury, we calculated the stance recovery index, which is a mean of the recovery index (RI) for the HTA and the FBA. The recovery index (RI) is an individual animal estimate for any given parameter described above and is calculated (percentage) as follows:

$$RI= [(X_{7+n} - X_7)/(X_0 - X_7)]*100$$

where X0, X_7, and X_{7+n} are values before operation, 7 days after injury, and a time point n days after the femoral nerve injury, respectively. These measures reflect the

post injury normalisation of function during locomotion which in turn indicates the proper reinnervation of the femoral nerve.

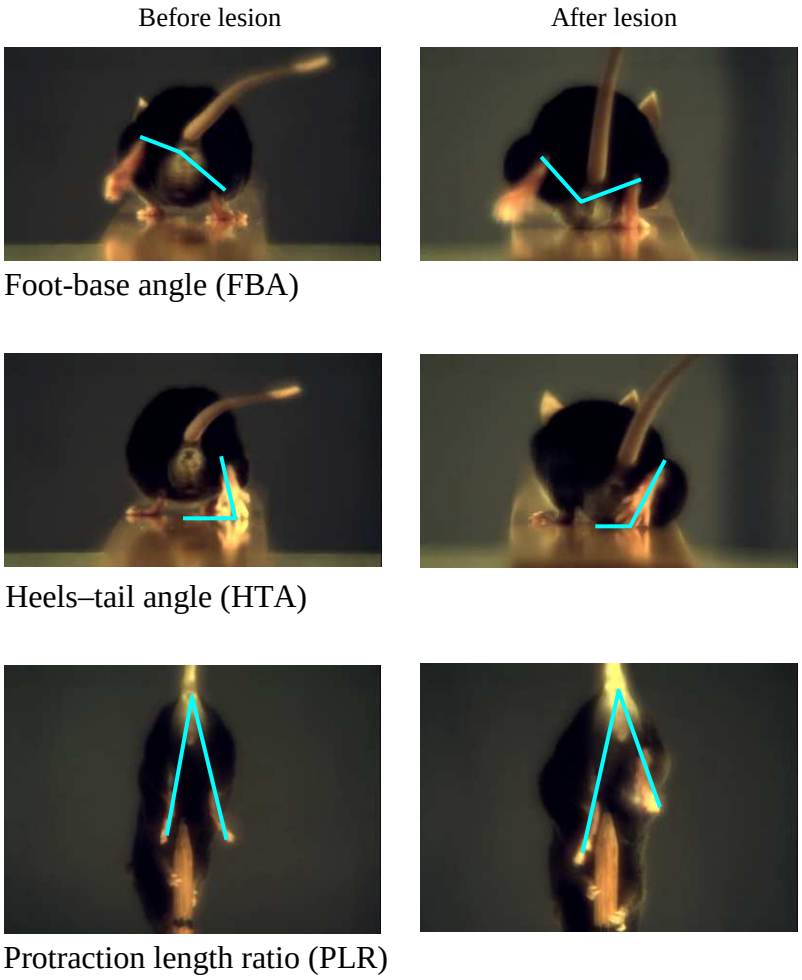


Figure 8: Parameters analysed to assess recovery after femoral nerve injury.

7.3. Surgical procedure for spinal cord injury experiment

The spinal cord injury and analysis of recovery were performed according to methods established by (Apostolova et al., 2006). Female C57BL/6J mice were obtained from Taconic (Copenhagen Denmark) at the age of 4 months. The animals were treated and housed as in the femoral nerve injury experiment.

Implantation of alzet micro-osmotic pumps, model 1002 (Durect Corporation) and delivery of peptide: For surgery, the animals were anaesthetized by intraperitoneal injections of 0.4 mg.kg-1 fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany), 10 mg.kg-1 droperidol (Dehydrobenzperidol, Janssen) and 5 mg.kg-1 diazepam

(Valium 10 Roche, Hoffman – La Roche, Grenzach-Wyhlen, Germany). Lewis^x mimicking peptide (400µg/ml), scrambled peptide (400µg/ml), or vehicle substance-PBS was applied using alzet osmotic pumps (implanted on the back subcutaneously) with a catheter delivering the peptide throw a hole in the region of the cauda equine. The catheter is stabilized into position with sutures at three places along its length to avoid displacement over the period of the experiment.

Spinal cord injury: Laminectomy was performed at the T7–T9 level with mouse laminectomy forceps. A mouse spinal cord compression device was used for compression injury. The device consisted of a pair of watchmaker forceps mounted in a metal block attached to a stereotaxic frame. Compression force (degree of closure of the forceps) and duration were controlled by an electromagnetic device. In most cases, the spinal cord was maximally compressed for 1s by a time-controlled current flow through the electromagnetic device. The skin was then surgically closed using nylon stitches (Ethicon, Norderstedt, Germany). After the operation, mice in individual cages were kept in a warm room (35°C) for several hours to prevent hypothermia and thereafter singly housed in a temperature-controlled (22°C) room with water and standard food provided *ad libitum*. During the postoperative time period, the bladders of the animals were manually voided twice daily. Functional analysis was carried out over a period of 6 weeks time.

7.4. Analysis of motor function recovery after spinal cord injury experiment

Like in the femoral nerve experiment mice were trained to walk on a horizontal wooden beam (1000 mm long, 60 mm wide) leading to their home cage. After the learning phase and prior to operation, one beam-walking trial was video recorded for each animal. A left- and right-side view of each animal during two consecutive walking trials was captured before the operation. The recordings were repeated 1, 3, and 6 weeks after spinal cord lesion. Two parameters that were designated foot-stepping angle and rump-height index were measured in selected frames in which the animals were seen in defined phases of locomotion.

The foot-stepping angle (FSA) is defined by a line parallel to the dorsal surface of the hind paw and the horizontal line (Figure 9, A). The angle is measured with respect to the posterior aspect at the beginning of the stance phase. The average of five measurements per animal, extremity, and trial was found to be representative for the individual animals. After spinal cord injury and severe loss of locomotor abilities, the mice drag behind their hind limbs with dorsal paw surfaces facing the beam surface (Figure 9, B). The angle is increased to >150°. The values for three left and three right leg measurements of individual mice were averaged.

The second parameter, the rump-height index (RHI), was estimated from the recordings used for measurements of the foot-stepping angle. The parameter is defined as height of the rump, i.e., the vertical distance from the dorsal aspect of the animal's tail base to the beam (Figure 9, C and D), normalized to the thickness of the beam (Figure 9, *) measured along the same vertical line. For each animal and trial, at least three frames in which the rump height was maximal during different step cycles, defined according to the stepping ability of the animal as described above, were used for measurements. Recovery indices (RI) were calculated as done before in the femoral nerve experiments.

$$RI= [(X_{7+n} - X_7)/(X_0 - X_7)]*100$$

where X0, X_7, and X_{7+n} are values before operation, 7 days after injury, and a time point n days after the femoral nerve injury, respectively.

(A) Before lesion (B) After lesion

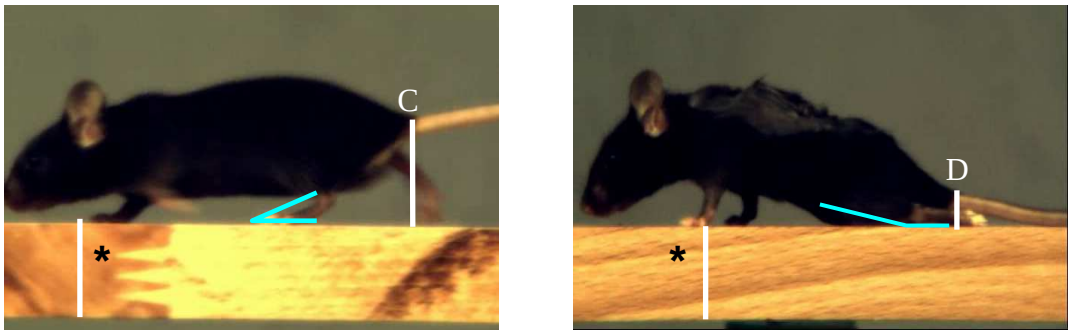


Figure 9: Foot stepping angle (FSA) and Rump height index (RHI)

A third parameter, the limb extension–flexion ratio, was evaluated from video recordings of voluntary movements of the mice performed during the "pencil" test

described for the femoral nerve injury paradigm. For the spinal cord injury paradigm, left- and right-side view videos were recorded for each animal. The extension and flexion length of the extremity (distance from the most distal midpoint of the paw to a fixed, well discernible point on the animal's body, e.g., the tail base) were measured for at least three extension–flexion cycles per animal and time point. Mean values for the two extremities from one animal were averaged. The extension–flexion ratio is a numerical estimate of the animal's ability to initiate and perform voluntary, non-weight bearing movements.

Ladder climbing test: mice climb up rapidly an inclined ladder with rare explorative stops and never turn back to descend the ladder. This behaviour is preserved even in severely disabled animals that climb, although slowly, up to the top of the ladder using their forelimbs. As a ladder, we used a 4-mm-thick frame made of a Resopal plate (96 cm long, 12 cm wide, with central incision of 88 x 6 cm; Resopal, Gross-Umstadt, Germany) to which 43 round wooden rungs (100 x 2 mm) were glued at equal intervals (2 cm). The ladder was fixed in an inclined position (55°) using a Plexiglas platform. The mice were placed at the bottom rungs of the ladder, and climbing was video recorded from a position "below" the ladder, i.e., viewing the ventral aspect of the animals. The video recordings were observed at slow-speed playback, and the number of correct steps (correct placing of the hind paw and sustained position until the next forward move) over 36 rungs were counted.

VII. Results

1. Identification of Lewis^x carriers at the cell surface and in the extracellular matrix

1.1. Affinity chromatography using monoclonal antibody L5 directed against Lewis^x

To isolate Lewis^x carrying molecules an affinity chromatography experiment with immobilized L5 monoclonal antibodies that recognise the Lewis^x epitope was performed. Detergent solubilised membrane fraction from brains of 7-day old mice was passed over an L5 antibody column. The column was washed with buffers containing detergent and salt and then bound molecules were eluted using a shift in pH using ethanolamine buffer, pH 11.5. I now designate these bound and eluted molecules as Lewis^x proteins. Aliquots of this were precipitated and separated on a low percentage SDS-PAGE to enable analysis of large glycoproteins by either silver-staining or Western blot using L5 antibodies.

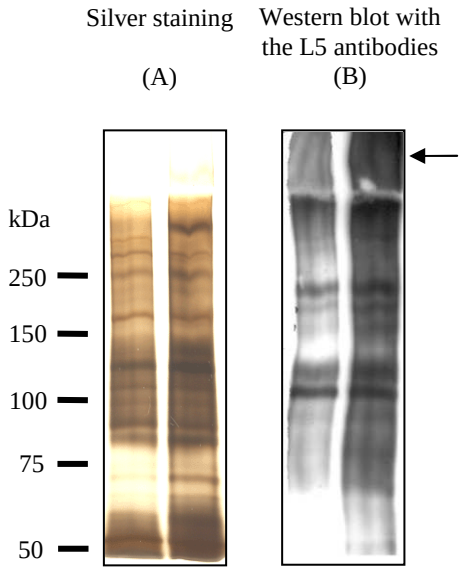


Figure 10: Lewis^x carrying proteins obtained by affinity chromatography on L5 antibody column. (A) Silver-staining and (B) Western blot with L5 antibodies that recognise the Lewis^x epitope. Numerous Lewis^x carrying proteins are present as reflected by the Western blot. A large amount of staining can be noted in the stacking region of the gel in the Western blot (arrow) and these glycoproteins are not visible in the silver stained gel. The two lanes in each depict different quantities of protein loaded.

The Western blot reveals that a number of Lewis^x carrying proteins are present in the high molecular weight region (Figure 10, B). The highest intensity of L5 reactivity is in the region 250 kDa and above and is observed even in the stacking region of the

gel. A streaking or a diffuse band pattern is observed in the Western blot due to the proteins being heavily glycosylated and these proteins are not easily stained by silver-staining as observed in the stacking region (Figure 10, A). The high molecular weight protein that is greater than 400kDa that is L5 positive could be the protein Astrochondrin (Streit et al., 1990). An enrichment of Lewis^x carrying protein has been obtained by the affinity chromatography experiment. Since we see more bands in the silver-stained gel as compared to the number of L5 positive bands on the Western blot, a further purification was necessary to isolate discernable specific proteins.

1.2. Re-purification of Lewis^x proteins by affinity chromatography

Re-purification of the Lewis^x carrying proteins was performed by passing the eluate obtained from the previous affinity chromatography experiment again over the L5 antibody column. The binding of the Lewis^x protein was in the presence of a higher detergent concentration of 1 % CHAPS so as to reduce non-specific binding and also disrupt protein complexes that are known to be present in a complex in membrane microdomains such as lipid-rafts. The bound proteins were eluted and analysed like before.

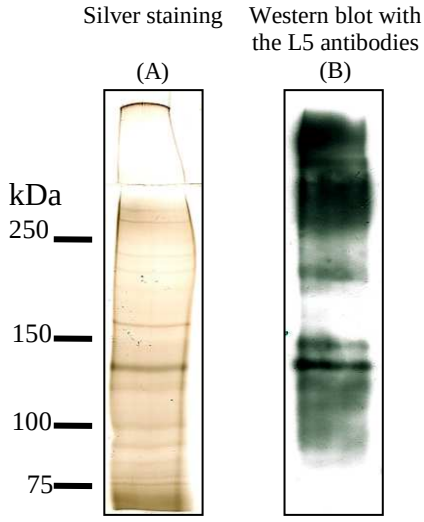


Figure 11: Lewis^x carrying proteins after re-purifying on the L5 antibody column. (A) Silver-staining and (B) Western blot with L5 antibodies that recognise the Lewis^X epitope. A high amount of the large 400 kDa and a 140 kDa protein are enriched. The corresponding heavily glycosylated proteins in the silver-stained image reveal the resistance of these proteins to impregnate silver.

A further enrichment of Lewis^x proteins, especially of the 400 and 140 kDa proteins was obtained but the silver-stained gel reveals a need for further purification to produce discernable bands corresponding to the L5 reactivity in the Western blot (Figure 11).

1.3. 2-D gel electrophoresis of the Lewis^x carrying proteins

A 2-dimensional gel electrophoresis experiment was performed to overcome the problem of resolving the Lewis^x proteins on a one dimensional gel. The gels were analysed by silver-staining and Western blot with L5 antibodies.

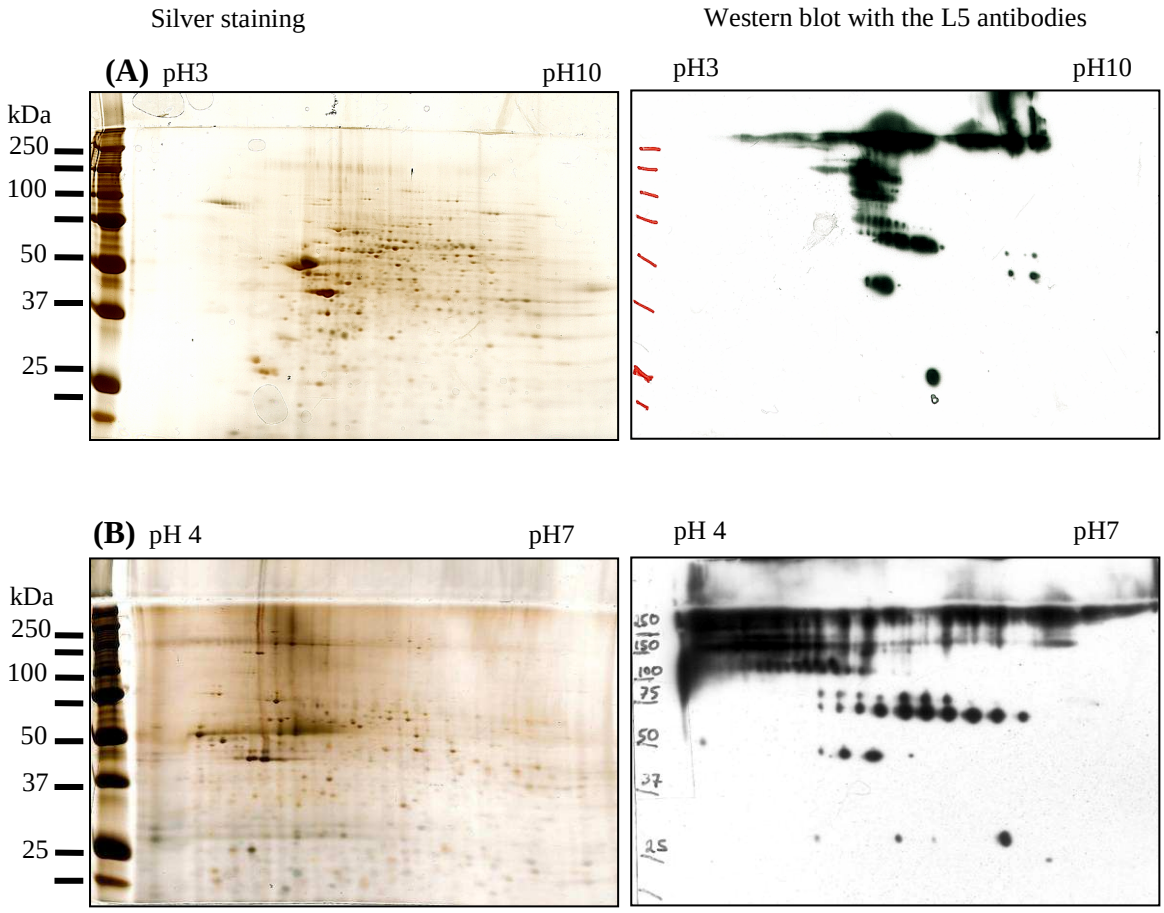


Figure 12: 2-D gel electrophoresis of Lewis^x carrying proteins First dimension of isoelectric focusing was performed on IPG strips in the pH range of (A) 3-10 and (B) 4-7. In (B) twice the amount as in (A) was loaded onto the IPG strips to increase the amount of protein localised at a spot and aid better detection and identification.

The spots corresponding to the L5 positivity in the Western blot are not distinguishable on the silver stained gel (Figure 12). An attempt to obtain better resolution by using an IPG strip of a narrower pH range of 4-7 from 3-10 proved insufficient to overcome the problem as can be noted in the 150 or 250 kDa region (Figure 12, B and A respectively). The 2D-gel method has an added limitation that the loading and resolution of the high molecular weight and glycosylated proteins on an IPG strip is inefficient. A subset of molecules in the range of 50 and 100 kDa could be resolved but the protein was distributed as a number of small spots, probably due

to differences in glycosylation or phosphorylation states of the same protein. The success of the 2D-gel electrophoresis to obtain large amounts of proteins resolved as independent spots was limited.

1.4. Ion exchange chromatography of the Lewis^x carrying proteins

To concentrate and separate the Lewis^x carrying proteins based on the isoelectric point (pI) of the proteins, an ion exchange chromatography experiment of the Lewis^x proteins was performed with a strong anion exchange matrix.

The Lewis^x proteins were denatured in buffer containing 4 M Urea and applied to a Q-Sepharose column. The bound molecules were eluted using an FPLC system with a linear salt gradient of 0 M to 1.5 M NaCl in 20 column volumes of buffer. A chromatogram of the run is shown in figure (Figure 13, A). Fractions were collected and analysed by SDS-PAGE followed by silver-staining or Western blot with L5 antibodies (Figure 13, B). Most of the proteins eluted in a narrow range, between fractions 29 and 37 which is at a low salt concentration (blue trace in the chromatogram, Figure 13, A).

Fractions of interest were analysed by SDS-PAGE followed by combined alcian blue and silver-staining so as to visualise the proteoglycans (Figure 14). The high molecular proteoglycans are normally difficult to stain with a simple staining procedure due to the glycosoaminoglycans but with the modification of using alcian blue staining before silver-staining are now visible as dark bands/streaks. This Lewis^x carrying proteoglycan especially enriched in fraction 37 (Figure 14) could be astrochondrin since it correlates with the L5 reactivity and molecular weight.

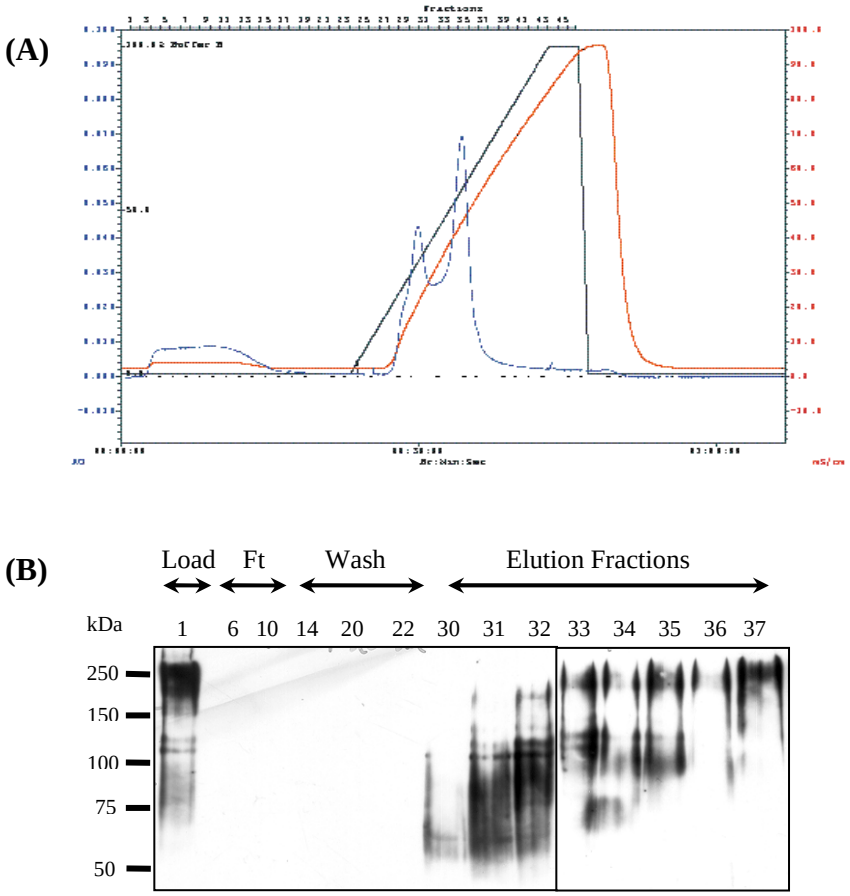


Figure 13: Lewis^x carrying membrane proteins after ion-exchange chromatography (A) Chromatogram of the ion-exchange chromatography experiment with the red trace indicating the salt concentration gradient and the blue trace indicating the eluted proteins as detected by the UV detector at an absorbance of 280 nm. Most of the proteins elute in a narrow range, between fractions 29 and 37. (B) Western blot analysis of the fractions with the L5 antibodies of the 1-Loaded protein, 6 and 10 flow through, 14 to 22 are early and late wash fractions and 30 to 37 are selected eluted fractions.

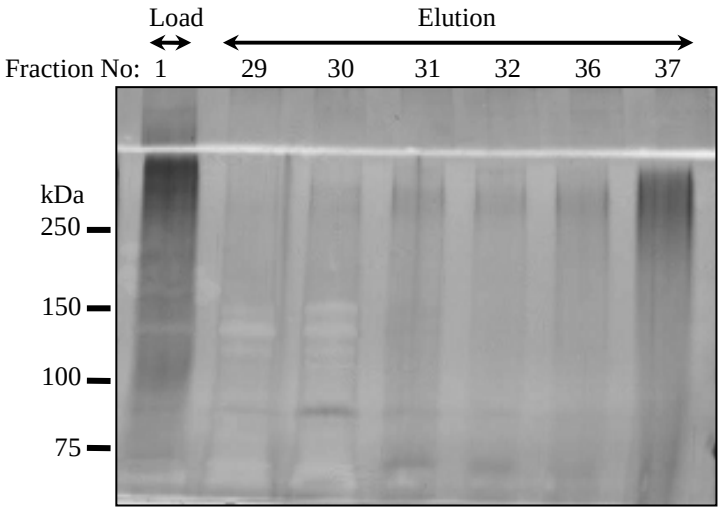


Figure 14: Combined Alcian blue and silver-staining of Lewis^x carrying membrane proteins after ion-exchange chromatography. Selected fractions from the ion-exchange chromatography experiment. Fraction 37 is specifically enriched with a high molecular weight protein, probably a proteoglycan like astrochondrin.

The proteoglycan astrochondrin was purified with an established protocol (Streit et al., 1990), instead of using detergent solubilised membrane, soluble fraction of the homogenised mouse brain was first subjected to affinity chromatography on an L5 antibody column . Then the bound and eluted molecules were denatured in buffer containing 4 M Urea and applied to a Q-Sepharose column. The bound molecules were eluted using an FPLC system with a linear gradient of 0 M to 1.5 M NaCl in 20 column volumes of buffer. Fractions were collected and aliquots were precipitated and analysed on a low percentage SDS-PAGE by combined alcian-blue and silver-staining or Western blot using L5 antibodies.

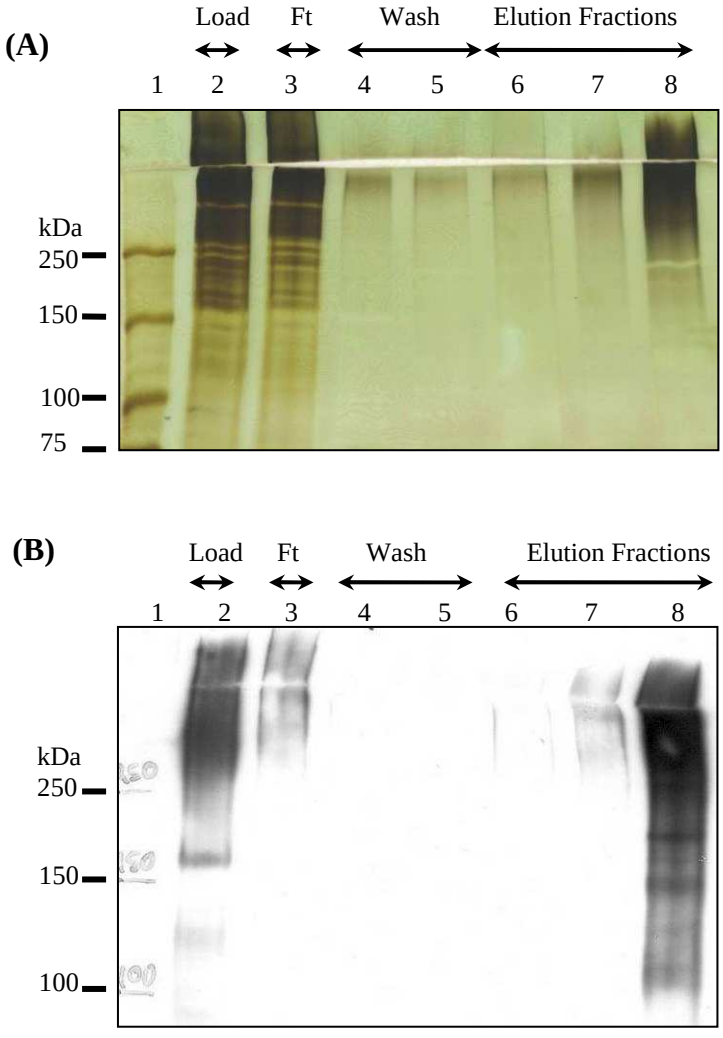


Figure 15: Lewis^x carrying soluble proteins after ion-exchange chromatography. (A) Combined Alcian blue and Silver-staining, (B) Western blot with L5 antibodies that recognise the Lewis^x epitope. Fraction 8 reveals the enrichment of the large molecular weight Lewis^x carrying protein.

The ion-exchange chromatography was useful in isolating the Lewis^x carrying proteoglycan but the problem of separating the other Lewis^x carrying membrane proteins remained (Figure 15).

1.5. Preparative SDS–PAGE and Whole Gel Elution of the Lewis^x carrying proteins

The Lewis^x proteins obtained by affinity chromatography with immobilized L5 antibodies followed by procedures like 2D-gel electrophoresis or ion-exchange chromatography were difficult to be resolved as discrete independent bands or spots in SDS-PAGEs. A number of proteins were also present as contaminants. These two problems were to be overcome to enable the identification of novel Lewis^x carrying protein by mass spectrometric analysis. Whole gel elution is a process of simultaneous electroelution of separated proteins from whole SDS-PAGE into narrow chambers, each containing a single or few proteins of similar molecular weight, thus fractioning the proteins based on their molecular weights. Preparative scale SDS-PAGE electrophoresis followed by whole gel elution of the Lewis^x proteins was performed. The fractions thus obtained were analysed by Western blotting with L5 antibodies and silver-staining procedures.

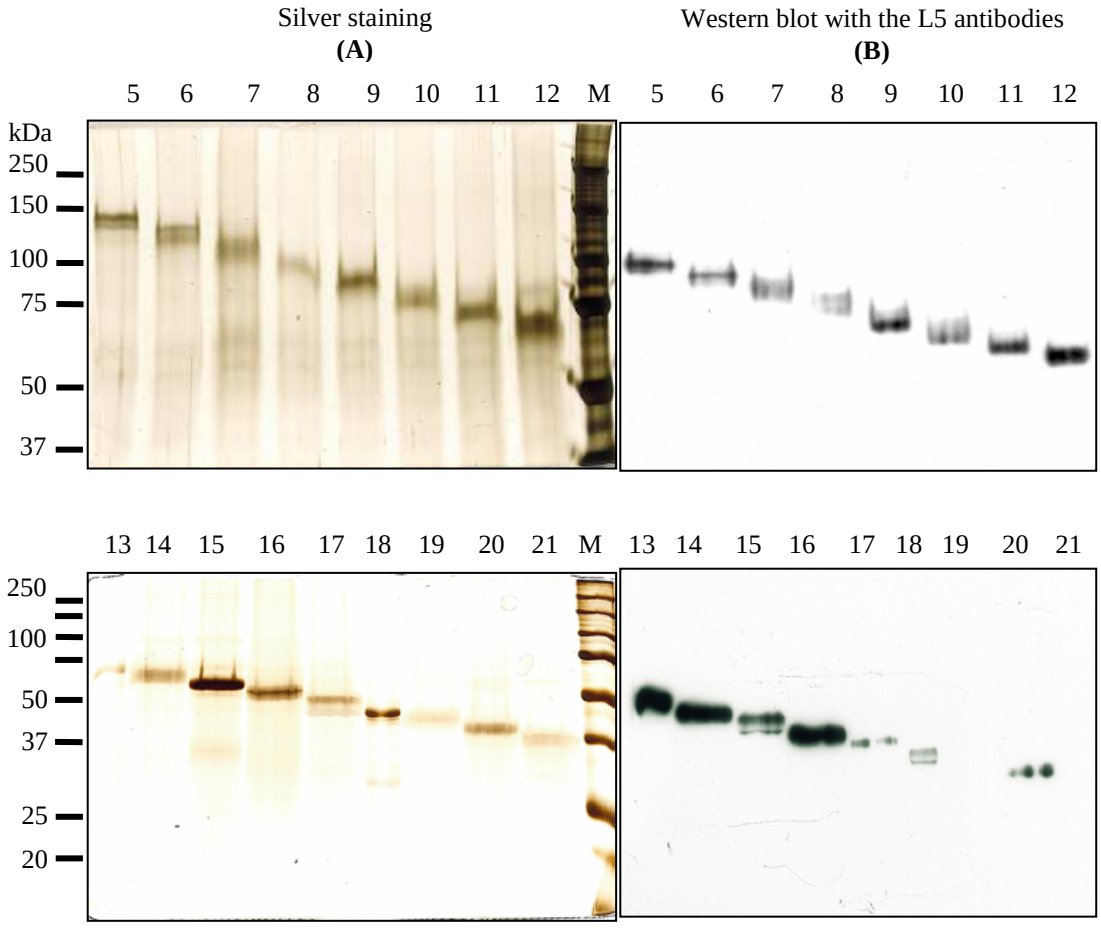


Figure 16: Protein fractions from whole gel elution. (A) Silver staining and (B) Western blot with L5 antibodies. The proteins are separated as discrete bands based on their molecular weight.

Preparative scale SDS–PAGE electrophoresis followed by whole gel elution helped separation of the Lewis^x proteins according to their molecular weights. The gel bands from the silver stained gels (Figure 16, A) corresponding to those that are positive on the Western blot with L5 antibodies (Figure 16, B) were excised and analysed by mass-spectrometry.

1.6. Mass-spectrometry analysis

The mass-spectrometry (MS) analysis of the gel bands containing Lewis^x carrying proteins (Figure 16) was performed at the lab of Prof. Bruce Macher and Dr Robert Yen, Dept of Chemistry and Biochemistry, San Francisco State University. The results from this analysis are summarised below in Table 3 along with their major functions and predicted number of glycosylation sites. Details of the identified peptides and ion scores can be found in the Appendix-Table 9.

Table 3: The proteins from bands in gels Figure 16 that were identified by mass spectrometry analysis along with the predicted O- and N- glycosylation sites.

	Name	**Localisation and functional association**	**Band No.**	**O-gly sites**	**N-gly sites**
1	Contactin-1/F3/F11	GPI anchored membrane protein, cell surface interactions, and cerebellar organisation.	5, 6	3	9
2	L1 cell adhesion molecule	Membrane, adhesion, neurite outgrowth, neuron fasciculation, migration and long term potentiation (LTP).	10, 11,12, 15, 16	4	17
3	CRMP2, (Dihydropyrimidinase related protein-2)	Cytoplasmic, tubulin associated and axon growth.	12	8	4
4	Noelin-1	Secreted and regulates the production of neural crest cells by the neural tube.	11, 12	1	7
5	Neuronal pentraxin-1	Secretory vesicles, secreted, associated with Narp and may mediate uptake of degraded synaptic material.	16	0	2
6	GAP 43	Cytoplasmic surface of growth cone and synaptic plasma membranes and associated with regeneration and synaptic plasticity	17	24	0
7	Tenascin-R	Extracellular matrix, adhesion and de-adhesion, development and regeneration.	20	2	10
8	Phosphacan (RPTPζ)	Extracellular matrix, ligand for Contactin-1, Tenascin-R, NCAM. Laminin, Fibronectin and inhibits neurite outgrowth.	1	9	20
9	Guanine nucleotide-binding protein G (o)	Membrane fraction, G-protein (signal transducing) and knock out mice show its involvement motor control.	20	0	1

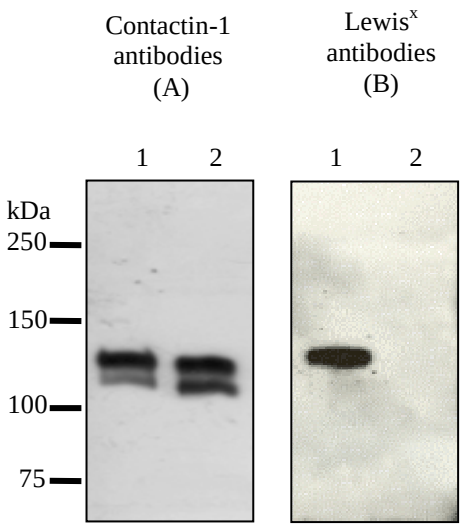
10	ATP synthase beta chain mitochondrial	Involved in establishing the mitochondrial proton gradient.	16	0	0
11	HSP-90	Cytosolic chaperones	9	0	4
12	HSP-70	Cytosolic chaperones	11	3	6
13	Tubulin (alpha-1,-2,-6, beta –2 chains)	Cytoskeletal element	-	-	-
14	Actin	Cytoskeletal element	-	-	-

It is encouraging to note the presence of peptides from known Lewis^x carrying proteins like the cell adhesion molecule L1, tenascin-R and RPTPζ in the mass spectrometry analysis. The molecules like contactin-1, neuronal pentraxin-1 and noelin-1 seem to be novel molecules that could be carriers of the Lewis^x glycans and need further verification. CRMP-2 and GAP-43 are tightly associated to the membrane but have not been shown to be localised on the extracellular surface. Other molecules like G (0), HSP-90, HSP-70, tubulin and actin could be either co-purified components that are functionally coupled to the carriers or contaminants.

1.7. Contactin-1 is a novel Lewis^x carrying molecule

Contactin-1 is implied to be a Lewis^x carrying molecule by the mass-spectrometry analysis of the proteins obtained from the affinity chromatography experiment with L5 antibodies. To verify the presence of the Lewis^x glycans on contactin-1 an immuno-precipitation experiment was performed. Detergent solubilised membrane fraction from a 7-day old mouse was incubated overnight at 4 °C with polyclonal contactin-1 antibodies. The antibodies along with bound proteins were precipitated using protein A/G-agarose beads. Half the beads that carry the complex of the antibodies with the captured contactin-1 protein were treated overnight with the N-glycan cleaving enzyme PNGase F. The bound proteins were washed and eluted in the SDS-PAGE loading buffer by boiling for 5 min, then subjected to SDS-PAGE followed by Western blot analysis with antibodies against contactin-1 and in a parallel experiment the Western blot was performed with Lewis^x antibodies.

Figure 17: Contactin-1 is a Lewis^x carrying molecule.



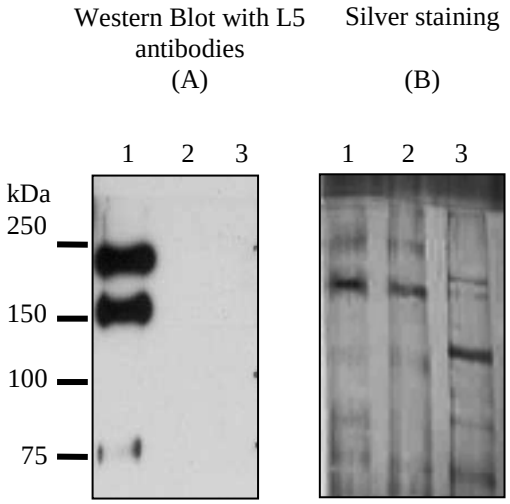
(A) Western blot with Contactin-1 antibodies and (B) Lewis^x antibodies. Immunoprecipitated Contactin-1 in lane 1 and the same after PNGaseF treatment in lane 2.

The immunoprecipitated material is confirmed to be contactin-1, (Figure 17, A). I observed that only a larger glycoform corresponding to the higher band of contactin-1 showed a positive reaction with L5 antibodies (Figure 17, B) also confirming the presence of Lewis^x glycans on contactin-1. Contactin-1 on treatment with PNGase F to cleave the N-linked glycans loses the Lewis^x glycans as shown by the loss of L5 reactivity (lane 2 of Figure 17, B). I, thus conclude that only the larger glycoform of contactin-1 carries the Lewis^x glycans and that it is present on N-linked oligosaccharides.

1.8. Cell adhesion molecule L1 carries the Lewis^x glycans on its N-linked oligosaccharides

L1 is a known carrier of the Lewis^x glycans. To verify the same by and to detect if the Lewis^x glycan is present on either O-linked or N-linked glycans a Western blot experiment was performed with antibody-affinity purified L1 (purified from mouse brain tissue homogenate). It was treated with either PNGase F to cleave all N-linked glycans or α1, 3-fucosidase to remove the fucose from the termini of the oligosaccharides that characterises the Lewis^x glycan. The untreated and treated samples were subjected to SDS-PAGE followed by Western blot analysis with the L5 antibodies or visualised by silver staining.

Figure 18: L1 is a Lewis^x carrying molecule.



(A) Western blot with L5 antibodies and
(B) silver-staining. Affinity purified L1 in lane 1, the same after α1, 3-fucosidase treatment in lane 2 and after PNGase F treatment in lane 3.

The antibody affinity purified L1 material showed a positive reaction with L5 antibodies confirming the presence of Lewis^x glycans on L1 shown in lane 1 (Figure 18, A). Both the 200 and 180 kDa isoform of L1 and the 80 kDa cleaved form of L1 (Kalus et al., 2003) are immunoreactive towards the L5 antibodies. L1 on treatment with PNGase F or α1, 3-fucosidase loses the Lewis^x glycans as shown by the loss of L5 reactivity, shown in lane 2 and 3 (Figure 18, A). The silver stained gel image (Figure 18, B), shows presence of L1 isoforms and a shift in its mobility on losing its N-linked glycans can be observed in lane 3 of the same image. It can be concluded that the cell adhesion molecule L1 carries the Lewis^x glycans on its N-linked oligosaccharides.

1.9. Is Phosphacan identical to the protein Astrochondrin?

Phosphacan was immunoprecipitated using the phosphacan antibody KAF13 (Garwood et al., 1999). Half of the immunoprecipitated material was treated overnight with Chondroitinase ABC at 37 °C to ascertain the presence of the chondroitin sulphate proteoglycans on phosphacan. The treated and untreated samples were subjected to SDS-PAGE followed by Western blot or silver-staining.

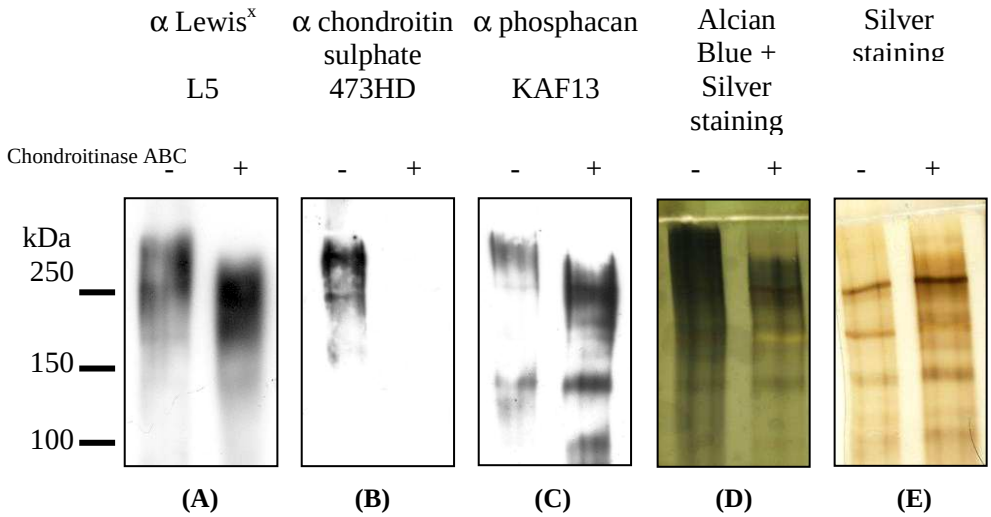


Figure 19: Analysis of the immunoprecipitated Phosphacan. Western blot with (A) α Lewis^x antibodies L5, (B)with αchondroitin sulphate antibodies 473-HD, (C) αPhosphacan antibodies KAF13, (D) combined Alcian blue and silver-staining and (E) silver-staining. Before (-) and after (+) Chondroitinase-ABC treatment.

Western blot analysis confirmed the precipitation of phosphacan with KAF13 antibodies, Figure 19 C. The positive reactivity with L5 antibodies (Figure 19 A) and 473HD (Figure 19, B) reveal the presence of Lewis^x glycans and the presence of chondroitin sulphate respectively. The treatment of Chondroitinase-ABC leads to a shift in the immunoreactivity and increased reactivity, probably due to better access of the antigen to the antibodies on removal of glycans in (Figure 19, A and C). A concomitant loss of 473HD reactivity can be noted due to the removal of chondroitin sulphate in (Figure 19, B +). Combined Alcian blue and silver-staining and silver-staining alone (Figure 19, D and E) confirmed the proteoglycan nature of the immunoprecipitated phosphacan. Taking into account that phosphacan has properties similar to astrochondrin; being a large molecular weight close to 400 kDa, is a chondroitin sulphate proteoglycan and carries the Lewis^x glycans, it can be proposed that they could be one and the same protein.

2. Identification of a Lewis^x mimicking peptide

To isolate the lectin or receptor for Lewis^x by affinity purification experiments, reasonable quantities of the pure defined glycan as well as a closely related structure that can serve as a negative control is required. Apart from using the glycan, a possible alternative to the use of the carbohydrate would be to develop a peptide mimic that could serve the same function.

2.1. Phage display experiments to identify a Lewis^x mimicking peptide.

To obtain a peptide analogous to the Lewis^x glycan, phage display libraries expressing either random dodecapeptide linear peptides (Ph.D.™-12) or heptapeptides constrained between two cysteines (Ph.D.-C7C library) were biopanned against L5 antibodies as target. The unbound phage was washed with detergent containing buffer and bound phage was eluted with 100µg/ ml L5 antibodies to obtain specific binding phages. After four rounds of panning, the DNA sequence of the N-terminus region of the minor coat protein (pIII) from the binding clones was determined. This was translated to reveal the identity of the coded peptides that are expressed as a fusion of the coat protein. The alignment of such sequences from a few of the clones is shown in Figure 20.

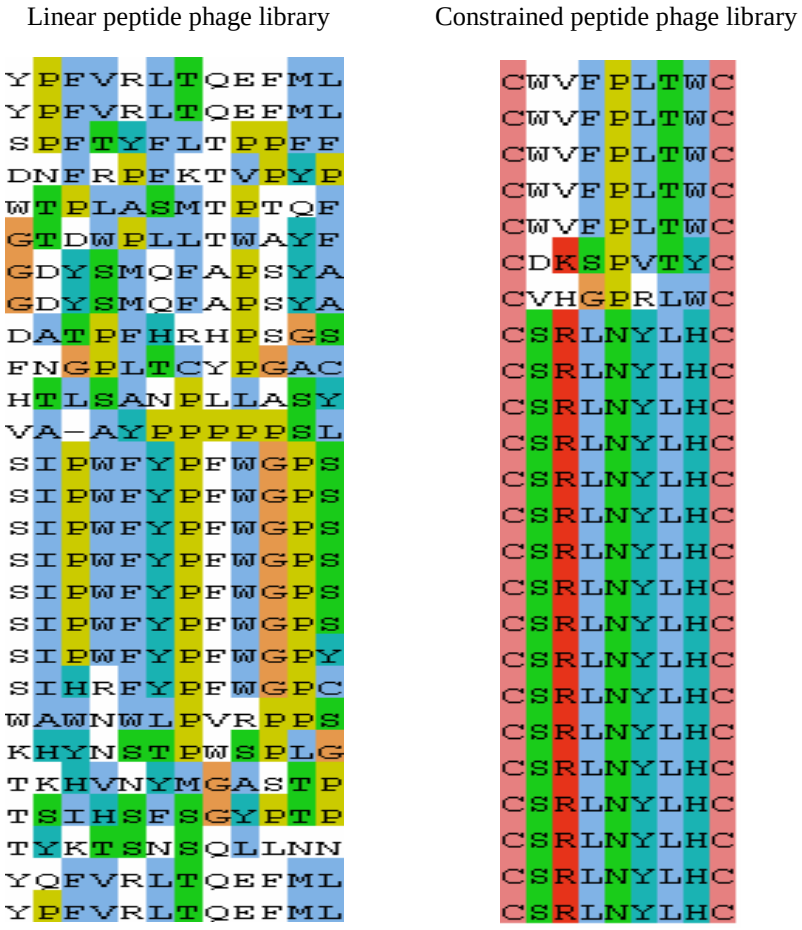


Figure 20: Peptide sequences derived from DNA sequences of the Lewis^x binding phages. The dodecapeptide SIPWFYPFWGPS from the Ph.d.-12 library and the cysteine flanked peptide SRLNYLH from the Ph.D.-C7C library occur at a high frequency revealing the consensus motif that binds the L5 antibody.

The two phage display libraries containing ~2.7 x 10^9 different peptide sequences to start with, after four successive rounds of selection, gave phages encoding particular of peptides that had a consensus motif. The dodecapeptides SIPWFYPFWGPS from the Ph.d.-12 library and the cysteine flanked peptide SRLNYLH from the Ph.D.-C7C library occur at a high frequency (Figure 20). These phages were further analysed for the ability to bind L5 antibodies by ELISA and Western blot experiments.

2.2. ELISA to demonstrate the binding of phages to Lewis^x antibodies

The phages occurring frequently with similar sequences were selected and analysed for their binding to L5 antibodies by ELISA experiments. Purified phage from each clone was allowed to bind the substrate coated L5 antibodies. The unbound phages were washed and bound phage was detected with anti M13 antibodies conjugated to horse radish peroxidase. Results of binding from a few of the phages are shown in Figure 21.

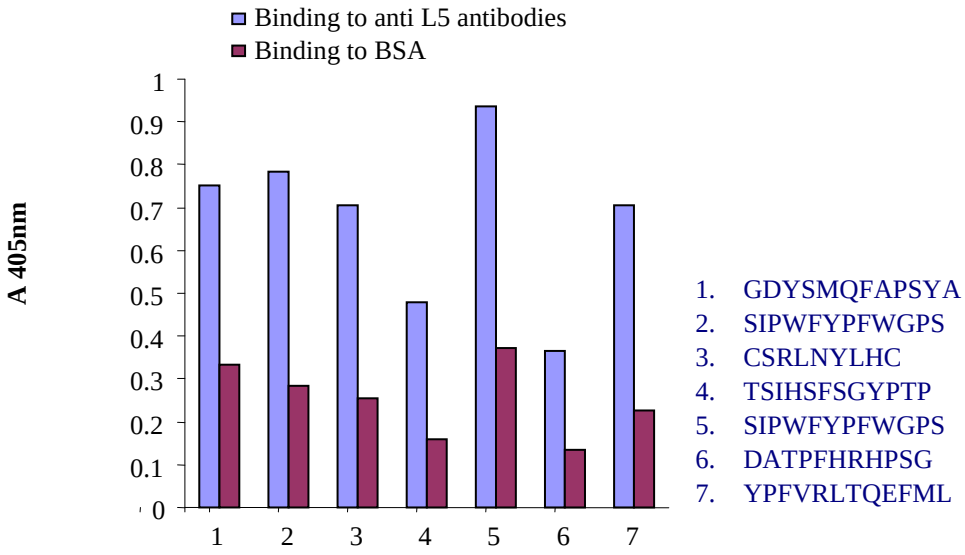


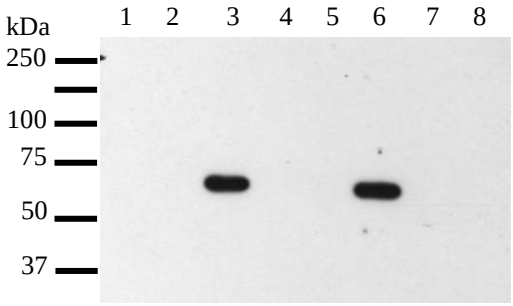
Figure 21: ELISA showing the binding of phage to L5 antibodies. Phages with various peptides sequences bind with higher affinity to the L5 antibody.

A number of phages that occurred with consensus peptide sequences and also some phages with dissimilar sequences bind the L5 antibody in an ELISA (Figure 21).

Phages that occurred at high frequency with sequences CSRLNYLHC and SIPWFYPFWGPS bind with high affinity.

2.3. Western blot analysis of the L5 antibody binding phage

The phages with the fusion of different peptides in their coat proteins bind the L5 antibodies. We posited that in a Western blot experiment using the L5 antibodies; it should be possible to detect the Lewis^x mimicking phage coat proteins by their immunoreactivity towards the antibodies. Total protein from purified phage of individual clones was denatured by boiling in SDS-PAGE loading buffer and separated on an SDS-PAGE and subjected to Western blot analysis with the L5 antibodies.



1. CLSRELPLC
2. CDKSPVTYC
3. CSRLNYLHC
4. CSPTGPQSC
5. CVHGPRLWC
6. CTRLNYLHC
7. CWVFPLTWC
8. CWVFPLTWC

Figure 22: Western blot of total phage proteins with L5 antibodies. Only phages with peptide sequences CSRLNYLHC and CTRLNYLHC show positive immunoreactivity with the L5 antibodies.

The minor coat protein (pIII) of the M13 phage runs in the 70 kDa molecular weight region in and SDS-PAGE (Jazwinski et al., 1973). This protein with the fusion of peptides either CSRLNYLHC or CTRLNYLHC is detected by the L5 antibodies in the Western blot (Figure 22). Other peptide fusions are negative to the L5 immunoreactivity. The evidence that they are recognized by the L5 antibody which normally has the Lewis^x glycans as its antigen reveals an important characteristic of these peptides, that these peptides mimic the Lewis^x glycan. Table 4 below summarizes the characteristics of the interesting clones obtained from the phage display experiments and their binding in phage ELISA and Western blot analysis.

Peptide Sequence		Experimentally determined			Theoretically determined *	
Constrained library		ELISA-Relative binding	Western blot	Frequency of clones in %	Hydropathy	Theoretical pI
1	CSRLNYLHC	0.11	Yes	55 (eluted by antibody)	-0.08	8.07
2	CTRLNYLHC	0.17	Yes	18.33 (eluted by DTT)	0.22	8.07
3	CWVFPLTWC	0.82	No	8 (eluted by antibody)	1.3	5.51
Linear Library						
4	SIPWFYPFWGPS	0.098	No	30 (eluted by antibody)	0.02	5.24
5	GDYSMQFAPSYA	0.14	No	6 (eluted by antibody)	0.41	3.80
5	TSIHSFSGYPTP	nd	Yes	nd	nd	nd

Table 4 Characteristics of the Lewis^x mimicking peptides obtained by phage display experiments. * http://www.bioinformatics.org/sms2/protein_gravy.html (Kyte and Doolittle; 1982) and http://www.bioinformatics.org/sms2/protein_iep.html.

2.4. ELISA to test the binding of synthetic peptides that mimic Lewis^x glycan

Phage coat protein with the peptides sequence CSRLNYLHC binds to the L5 antibody in an ELISA and Western blot. A synthetic form of this peptide was obtained in a cyclised form. The peptide had a sequence H-SACSRLNYLHC-OH and was cyclised between the cysteines and the additional flanking amino acids were added to aid synthesis. The amino acid composition was retained but the sequence was scrambled to H-SACNHLLRSYC-OH in a control peptide. The peptides were coated overnight in varying concentrations on microtire plates. The unbound peptide removed, blocked with BSA and incubated with L5 antibodies. The unbound antibody was washed and the bound antibody was detected with horse radish peroxidase conjugated anti rat antibodies.

In the ELISA the binding curve of the L5 antibodies to the Lewis^x peptide showed a binding that saturated at a substrate coat concentration of about 20 µg/ ml (Figure 23). The control peptide showed no reactivity. This ability of the synthetic cyclic peptide with the sequence H-SACSRLNYLHC-OH binding shows its ability in mimicking the Lewis^x glycan.

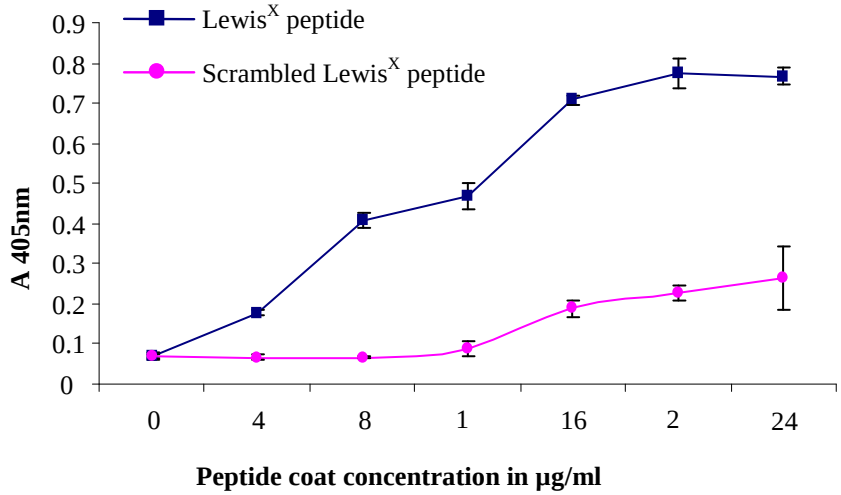


Figure 23: Lewis^x mimicking peptide binding to L5 antibodies. The Lewis^x peptide shows a binding to L5 antibodies that saturates at about a coating concentration of 20µg/ml and the scrambled peptide shows no binding.

2.5. Inhibition of L5 antibody binding to the Lewis^x peptide

The antibody binding to the substrate coated Lewis^x peptide and its inhibition by the Lewis^x glycan was tested in an ELISA. The ELISA was performed as before but before the L5 antibodies were added they were incubated with the inhibitor Lewis^x/BSA conjugate at 5µg/ ml for 10 min at room temperature. Untreated L5 antibodies or with Lewis^x /BSA was treated with fucosidase were used as controls.

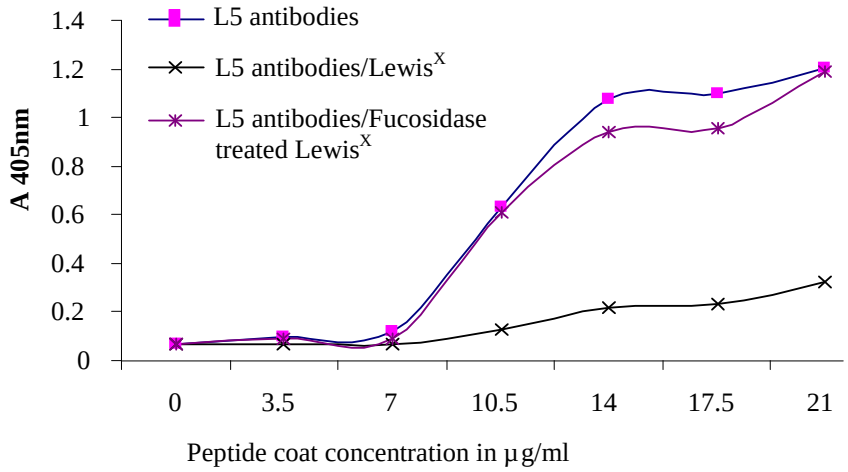


Figure 24: Inhibition of L5 antibody binding to the Lewis^x peptide. The binding of the antibodies is inhibited in the presence of Lewis^x/BSA but not by the fucosidase treated Lewis^x/BSA.

This further evidence of the peptide binding to L5 antibodies being inhibited by Lewis^x/BSA and not by the fucosidase treated Lewis^x/BSA along with the binding of the L5 antibodies in the Western blot (Figure 22) makes the peptide with the sequence H-SACSRLNYLHC-OH a molecule that mimics the Lewis^x glycan.

2.6. In vitro cerebellar neurite outgrowth experiment to test the Lewis^x peptide mimic

Lewis^x glycans on CD24 has been shown to be required for inducing of neurite outgrowth by cerebellar neurons in our lab (Lieberoth et al., submitted). By applying Lewis^x antibody or by addition of soluble Lewis^x carrying oligosaccharides to cultures grown on substrate coated CD24, the carbohydrate-dependent neurite outgrowth was show to be abolished. This served as a functional test system to evaluate the ability of the peptides to mimic the Lewis^x glycan. Dissociated cerebellar neuron cultures were seeded on PLL-coated coverslips (PLL) or on PLL plus CD24. Test substances i.e., Lewis^x peptide, scrambled Lewis^x peptide and Lewis^x (synthetic Lewis^x oligosaccharide) were added to a final concentration of 20 µg/ml to the cultures, 90 min after seeding. The cultures were grown for 24 hours and the total length of neurites per cell was determined.

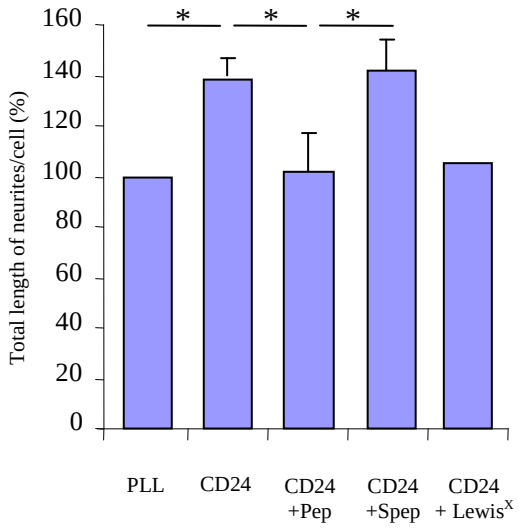


Figure 25: Lewis^x peptide modulates CD24 mediated neurite outgrowth of cerebellar neurons. Dissociated cerebellar neuron cultures were grown on PLL-coated coverslips (PLL) or on PLL plus CD24. Test substances Pep (Lewis^x peptide), Spep (scrambled Lewis^x peptide) and Lewis^x (synthetic Lewis^x sugar). Total length of neurites per cell was determined and shown as percent of control (PLL). Error bars indicate SD from three independent experiments. Bars marked by asterisks ($p<0.05$, Student's t-test) are significantly different from the controls (PLL or CD24).

The Lewis^x mimicking peptide with the sequence H-SACSRLNYLHC-OH was able to inhibit the CD24 induced neurite outgrowth like the Lewis^x oligosaccharide while the scrambled peptide with the sequence H-SACNHLLRSYC-OH and no effect (Figure 25). This ability to functionally substitute the Lewis^x glycan further evidences the Lewis^x peptides ability to mimic the carbohydrate.

3. Identification of binding partners or receptors of Lewis^x

The use of the above confirmed Lewis^x mimicking peptide in pull-down experiments to identify its receptors was not successful due to problems of solubility of the peptide and also non-specific binding of proteins to the peptide immobilization substrate. The peptide was useful in functional assays to simulate the functions of Lewis^x glycan to interfere in the lectin-glycan interactions. This necessitated the use of the real carbohydrate Lewis^x to identify its receptors.

3.1. Identification of Lewis^x receptors by pull-down experiments using BSA-Lewis^x

An affinity pull-down experiment to identify Lewis^x receptors was performed by using Lewis^x conjugated to BSA that was coupled to CNBr activated Sepharose beads (as control BSA coupled to similar beads were used). This 'bait' i.e., the BSA-Lewis^x was incubated with detergent solubilised mouse brain homogenate to capture or precipitate the receptors. The beads were then separated and washed with detergent containing buffer and the bound proteins were eluted in three different steps:

a) synthetic Lewis^x sugar (100µg/ ml,) this first step of elution was done in the hope of obtaining very specific binding partners,
b) followed by low pH elution (Glycine 200mM, pH 2.5) and
c) then, by boiling the beads in reducing and denaturing conditions.

The specific elution using free Lewis^x lead to low protein recovery, therefore the experiment was repeated and all the bound proteins were eluted by boiling in

SDS-PAGE loading buffer under reducing and denaturing conditions. The eluted proteins were separated on an SDS-PAGE and visualised by silver staining. These experiments were verified to be reproducible over three times (results from one experiment shown in Figure 26).

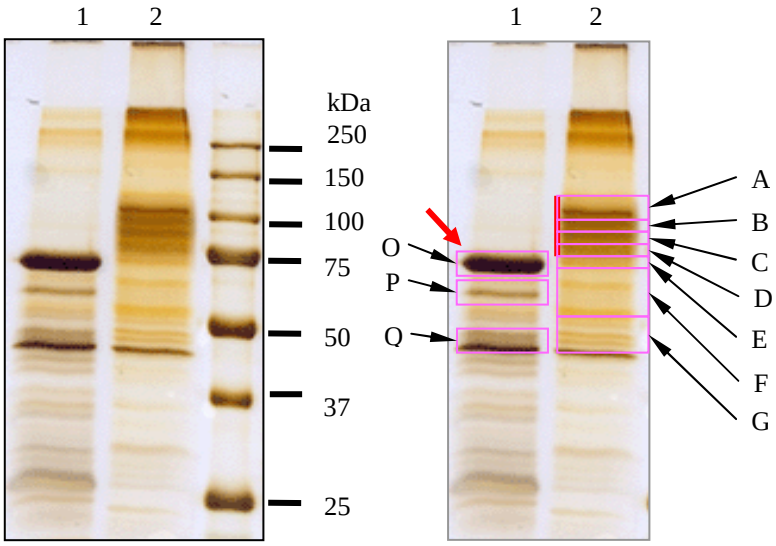


Figure 26: Pull-down experiment with BSA-Lewis^x. BSA-Lewis^x binding proteins in lane 2 and the control BSA binding proteins in lane 1. The two images are the same, the second image used to show that the bands marked A to G are proteins that are different from the proteins pulled-down by the control BSA alone.

The marked bands were excised from the gel (Figure 26) and analysed by mass spectrometry at the lab of Prof. Bruce Macher and Dr Robert Yen, Dept of Chemistry and Biochemistry, San Francisco State University. The results from this analysis along with the number of peptides observed and the percentage of the protein sequence they cover are listed in Table 5 on the next page.

Table 5: Lewis^x binding proteins identified by mass spectrometry analysis from the pull down experiment.

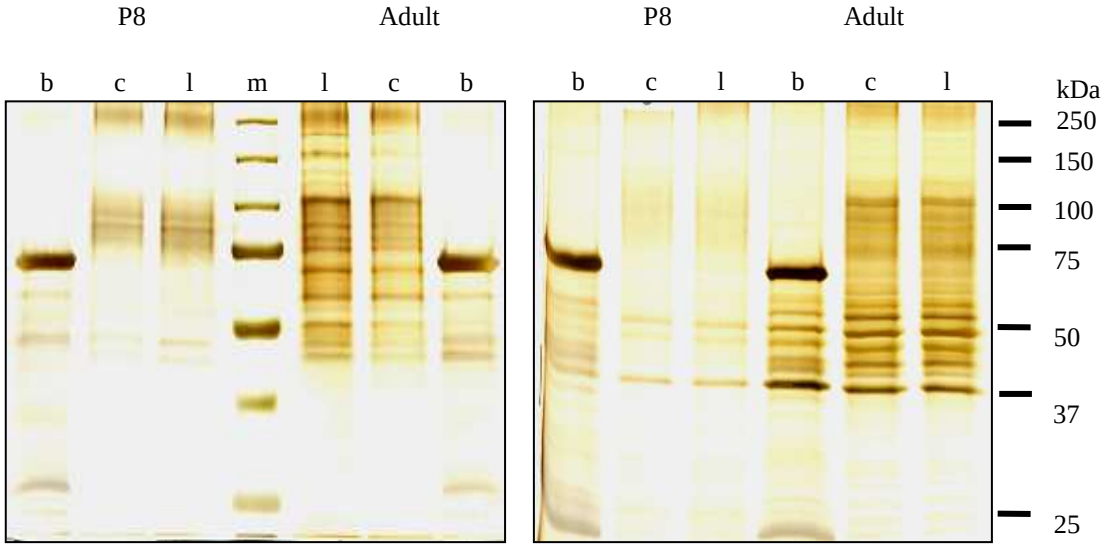
Gel Band	Protein Identity	Mass (KDa)	No. of Peptides Detected	Sequence Coverage (%)
A	Kif 5C	113	19	24
A	Adaptor-related protein complex 2 alpha 2	104	17	27
A	Bovine serum albumin	66	15	21
A	PTB-associated splicing factor	76	6	11
A	Heterogeneous nuclear ribonucleoprotein U	88	5	8
B	Bovine serum albumin	66	21	24
B	Elongation factor 2 (EF-2)	96	6	12
B	PTB-associated splicing factor	76	2	5
B	Adaptor-related protein complex 2 alpha 2 subunit	104	2	3
C	Bovine serum albumin	66	16	26
C	Hypothetical protein (ATP-dependent helicase DDX1)	84	7	17
C	Neural activity-related RING finger protein	82	5	13
C	Leucine zipper-EF-hand containing transmembrane protein	83	4	11
D	Bovine serum albumin	66	10	22
D	Neural activity-related RING finger protein	82	2	5
D	Collapsin response mediator protein 1	62	2	6
E	dnaK-type molecular chaperone hsc73	71	16	40
E	Similar to microtubule-associated protein 2	50	6	21
E	Neural activity-related RING finger protein	82	5	13
E	Dihydropyrimidinase-related protein 2	63	4	14
E	Sclerostin domain containing protein 1 [Precursor]	53	5	7
F	Tubulin alpha-1 chain	51	20	42
F	Tubulin	50	9	30
F	Q8BMK4_MOUSE similar to P63 PROTEIN [fragment]	61	10	27
F	Dihydropyrimidinase-related protein 2	63	9	26
F	Dihydropyrimidinase-related protein 3	63	7	20
G	ACTB_MOUSE Actin, cytoplasmic 1	42	11	32
G	Elongation factor 1-alpha 1	50	7	25
O	Bovine serum albumin	66	42	70
P	Bovine serum albumin	66	18	31
Q	Bovine serum albumin	66	16	27

Sclerostin domain containing protein-1 was the only molecule amongst the mass-spectrometry identified molecules that can be present at the cell surface. It is a secreted BMP (Bone Morphogenetic Protein) inhibitor and is homologous to sclerostin that is a secreted BMP antagonist (Laurikkala et al., 2003). Though this

could be an important molecule for morphogenesis they did not show any gross morphological changes in the brains of sclerostin domain containing protein-1 deficient mice. The known normal localisation of the rest of the detected proteins was either cytoplasmic or nuclear which does not fulfil the requirement to be at the cell surface, hence, were thought to be less probable receptors of Lewis^x.

3.2. Repetition of the Lewis^x pull-down experiments using additional controls

The pull-down experiments were repeated using an additional negative control other than unconjugated BSA. The BSA-control-sugar is similar to the BSA-Lewis^x but without the α1, 3-linked fucose. The pull-down was performed in the presence of either Octyl β-D-glucopyranoside or CHAPS so as to increase solubilisation of membrane proteins and also reduce the binding of non-specific proteins. Detergent solubilised membranes from 8-day old and 28-day old mouse brains were used as prey/source of probable receptors. The eluted proteins were separated on an SDS-PAGE and visualised by silver staining.



(A) 60mM Octyl β-D-glucopyranoside as detergent.

(B) 1% CHAPS as detergent.

Figure 27: Pull-down experiment with BSA-Lewis^x and the BSA-control-sugar. Experiment performed in the presence of the detergent (A) 60 mM Octyl β-D-glucopyranoside and (B) 1 % CHAPS. In both (A) and (B), lane b: BSA, lane l: BSA-Lewis^x, lane c: BSA-control-sugar and m: molecular weight marker. P8 and Adult: membranes from 8-day old and 28-day old mouse brains respectively.

The pattern of silver-stained bands obtained in the precipitate by both the BSA-Lewis^x conjugate and the BSA-control-sugar are similar (Figure 27). However we can note that the proteins these BSA conjugated glycans precipitate are different form the proteins that precipitate with BSA alone. The BSA in the conjugates could be binding to some of the proteins in a non-specific manner. The next experiment was performed with the Lewis^x directly coupled to a matrix without the BSA to enable specific precipitation of proteins that bind the glycan alone.

3.3. Pull-down experiment with Lewis^x oligosaccharide coupled directly to SulfoLink beads

The BSA was thought to be precipitating proteins along with the glycans in the pull-down experiments and needed to be remedied. To eliminate the BSA, the custom made Lewis^x sugar that carried the sulfhydryl group linked by a spacer molecule was coupled directly to SulfoLink beads and used as bait in pull down experiments like before. The precipitated protein were visualised by separating them on an SDS-PAGE followed by silver staining.

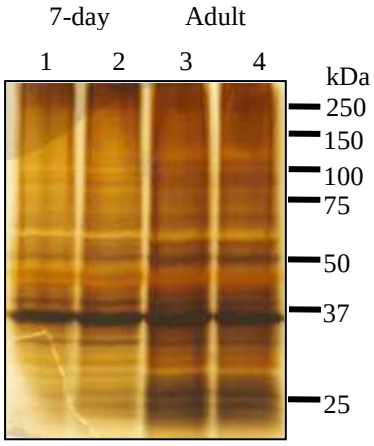


Figure 28: Pull-down experiment with Lewis^x oligosaccharide coupled directly to SulfoLink beads. Mouse tissue homogenates from 7-day old or adult mouse brain tissue. Lanes 1and 3: control-sugar and lanes 2 and 4: Lewis^x oligosaccharide

The problem of non-specific binding of proteins could not be remedied using Lewis^x without the BSA in the pull-down experiment (Figure 28). Though a specific Lewis^x receptor/s could be present amongst the proteins in the BSA-Lewis^x precipitate (Figure 28, lane 2), it is difficult to discern them from other proteins in the gel. There

was a need for enriching and visualising the receptors specifically. Chemical cross-linking methods could provide a means of fulfilling these requirements.

3.4. Sulfo-SBED crosslinking experiment to identify Lewis^x receptor/s

The use of the chemical cross-linker Sulfo-SBED allows the covalent trapping of interacting proteins and simultaneously transferring of a biotin label onto it. This covalent trapping lends itself to stringent wash conditions (of high salt and detergent) without losing the interacting protein. In a second step, the biotin labelled proteins can be recaptured using streptavidin coated beads and washed with stringent conditions to remove non interacting proteins enabling enrichment of binding proteins. Lewis^x/BSA conjugate was coupled to the Sulfo-SBED crosslinker and about 100 µg of it was mixed with detergent solubilised membrane fractions from 7−day old old mouse brain and incubated for 1 hour at room temperature. The mixture was exposed to ultraviolet irradiation and the interacting complexes were captured by streptavidin coated magnetic beads. The beads were washed with detergent containing buffer and the bound proteins were denatured in SDS-PAGE loading buffer and analysed by SDS-PAGE followed by silver staining or detected in a Western blot with horse radish peroxidase coupled streptavidin. The experiment was also performed in parallel with soluble fractions of 7-day old mouse brains.

Western blot analysis with HRP coupled streptavidin reveals that a number of proteins are crosslinked and labelled with biotin that could be Lewis^x receptors (Figure 29, C). However, when these proteins are visualized by silver staining, it is difficult to distinguish the corresponding proteins as discrete bands. The quantity of protein present in each band is not sufficient for a successful mass spectrometry analysis that would be essential to reveal the identity of the proteins. A similar result was obtained when Mts-Atf-Biotin was coupled directly to Lewis^x sugar without the BSA and used in the pull-down experiment (data not shown).

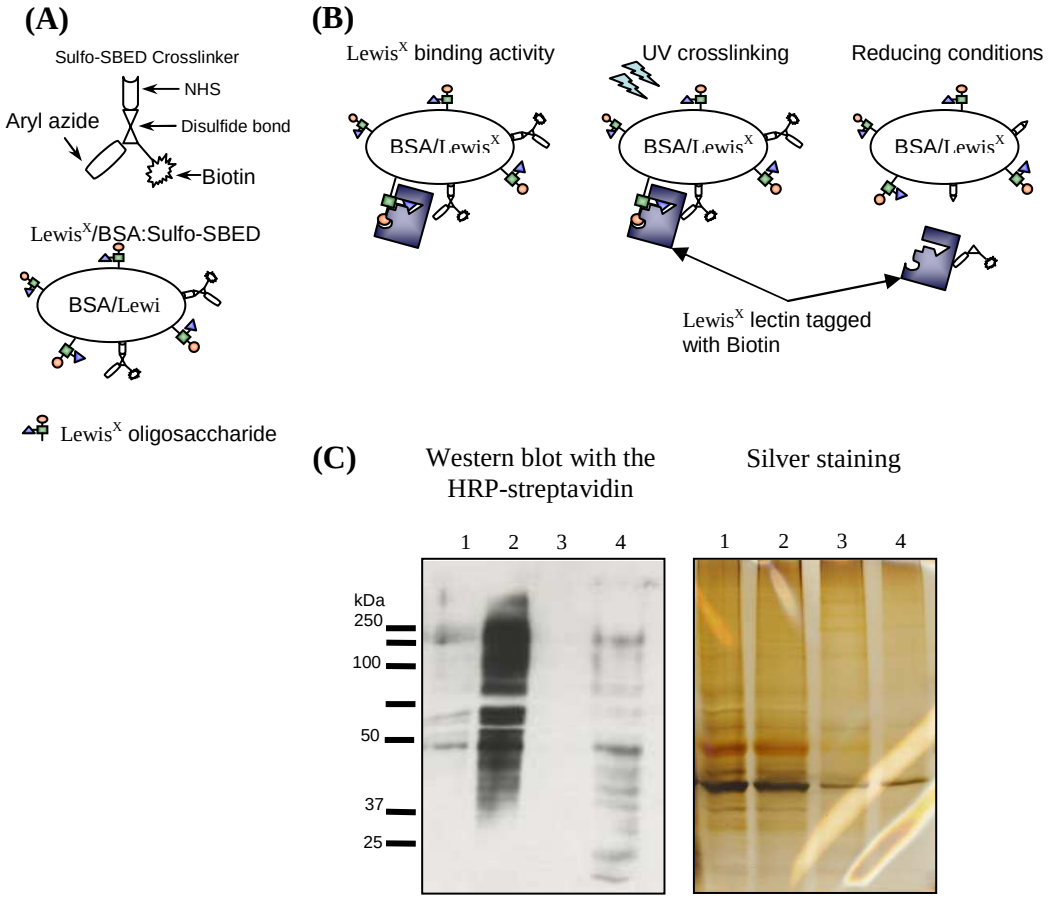


Figure 29: Sulfo-SBED crosslinking experiment to identify Lewis^x receptor/s. (A) Sulfo-SBED trifunctional crosslinker conjugated to BSA-Lewis^x. (B) The crosslinker labelled BSA-Lewis^x is incubated with mixture carrying receptors, on UV incidence the bound receptor due to its proximity is covalently linked and is subsequently released on exposing to reducing conditions. (C) Lanes 2 and 4: Proteins pulled down with BSA-Lewis^x from membrane proteins and soluble mouse brain fractions respectively. Lanes 1 and 3: control pull-down without UV irradiation.

4. Identification of receptors of the Lewis^x carbohydrate by phage display experiment

4.1. Lewis^x receptor identification by phage display experiment

The pull-down experiments to identify a Lewis^x receptor were not successful despite using a number of strategies. Instead of using mouse brain homogenate as a source of probable receptors, the brain proteins presented as phage coat proteins in a phage display library can be used. The phage display library and panning experiments

provides a means of screening a number of proteins and selectively amplify those that bind Lewis^x.

A pre-made T7 phage library expressing human brain cDNA as a fusion of their coat proteins was biopanned for four rounds with Lewis^x conjugated to BSA as the target. The elution was performed with the L5 antibodies at a concentration of 100µg/ ml to obtain specific phage and amplified for further rounds of panning. In a second experiment the eluted phages from each round were incubated in wells coated with BSA-Lewis^a to capture the non-specifically bound phage and increase the specificity. At the end of four rounds the eluted clones were identified by sequencing their DNA. The identity of the proteins present in the phage clones that had their coding DNA sequences in frame are listed in Table 6 and Table 7.

Target	Lewis^x -BSA in Nunc immunosorbent wells
Specificity filter	None
Starting phage titre	$3x10^8$ pfu
Wash conditions	0.02 % Tween-20 in PBS - 0.05 % Tween-20 in PBS
Elution Conditions	100µg/ ml L5 antibodies
4 rounds of panning	

1. BCL2-antagonist of cell death protein
2. Dystrobrevin alpha isoform 1
3. gi|113426619|ref|XP_001130568.1|
4. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
5. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
6. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
7. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
8. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
9. Myelin basic protein (MBP) (Myelin A1 protein) Golli-mbp isoform 1
10. Myelin basic protein (MBP) (Myelin A1 protein) Golli-mbp isoform 1
11. Myelin basic protein (MBP) (Myelin A1 protein) Golli-mbp isoform 1
12. Myelin basic protein (MBP) (Myelin A1 protein) Golli-mbp isoform 1
13. Myelin basic protein (MBP) (Myelin A1 protein) Golli-mbp isoform 1
14. Neurofilament-66
15. Paralemmin isoform 2
16. Poly(A) binding protein, nuclear 1
17. Similar to transcription elongation factor B polypeptide

Table 6: Phages that bind Lewis^x after four rounds of biopanning.

Target	$Lewis^x$ -BSA in Nunc immunosorbent wells
Specificity filter	BSA-$Lewis^a$ in Nunc immunosorbent wells
Starting phage titre	$3x10^8$ pfu
Wash conditions	0.02 % Tween-20 in PBS - 0.05 % Tween-20 in PBS
Elution Conditions	100µg/ ml L5 antibodies
4 rounds of panning	

1. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
2. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
3. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
4. Lectin, galactoside-binding, soluble, 3 (Galectin 3)
5. Myelin basic protein (MBP) (Myelin A1 protein) Golli-mbp isoform 1
6. Neuronal calcium-binding protein 2
7. Proline-rich nuclear receptor coactivator 2
8. PRP8 pre-mRNA processing factor 8 homolog
9. Ribosomal protein L15 isoform 1
10. Complement component 1

Table 7: Phages that bind $Lewis^x$ but not $Lewis^a$ after four rounds of biopanning.

The phage display experiment reveals galectin-3 and a golli isoform of myelin basic protein (MBP) as two probable interacting partners of $Lewis^x$. These two interesting protein that have been shown to have lectin like properties but their ability to be receptors for the glycans $Lewis^x$ needs to be explored.

4.2. Cloning, expression and purification of Galectin-3

To verify the interactions of galectin-3 with $Lewis^x$ by ELISA experiments it was necessary to obtain isolated galectin-3. A recombinant approach was used to express and purify galectin-3. The gene was PCR amplified from the DNA derived from one of the $Lewis^x$ binding phage (obtained from the phage display experiment) and cloned into the pET28a vector. The resulting plasmid has both an amino and carboxyl terminal HIS tag. Protein expression was performed in *E. coli* BL21 strain and the recombinant proteins were purified using Ni-NTA affinity chromatography.

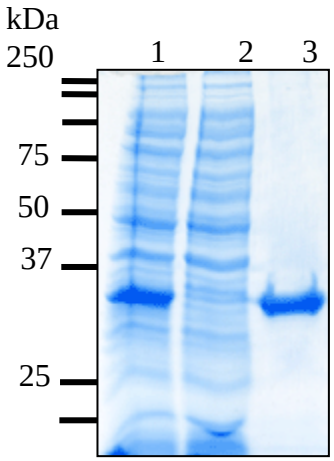


Figure 30: Ni-NTA affinity purification of Galectin-3. Lane 1: Total lysate of cells expressing Galectin-3. Lane 2: Flow through proteins after lysate was allowed to bind Ni-NTA agarose. Lane 3: His-tagged Galectin-3 eluted from the Ni-NTA matrix

Galectin-3 was cloned, expressed and purified using a His-Tag (Figure 30). ELISA and functional experiments like neurite-outgrowth experiments were performed using this recombinant galectin-3.

4.3. Galectin-3 binding to Lewis^x shown by ELISA

BSA-Lewis^x was coated overnight in varying concentrations on microtire plates. The unbound BSA-Lewis^x was removed, blocked with BSA and incubated with 10 µg/ ml galectin-3. The unbound protein was washed and the bound galectin-3 was detected with galectin-3 antibodies followed by secondary antibodies of horse radish peroxidase conjugated anti rabbit antibodies.

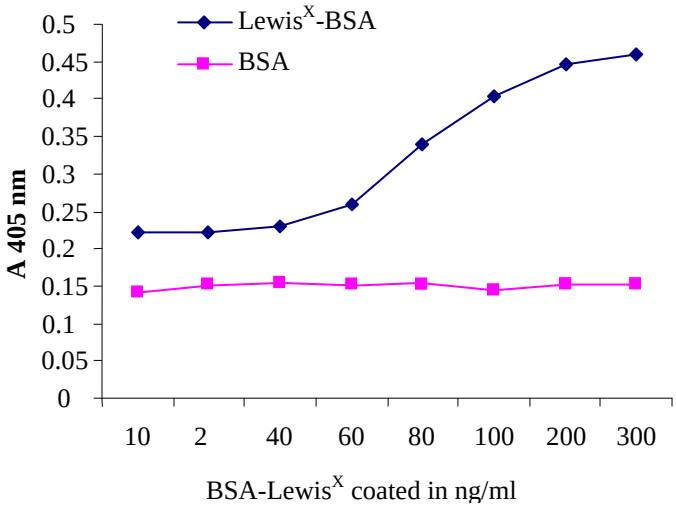


Figure 31: Galectin-3 binding to substrate coated BSA-Lewis^x.

The ELISA experiment shows a binding of galectin-3 to BSA-Lewis^x that saturates at a Lewis^x concentration of about 200 ng (Figure 32). Its binding to Lewis^a saturates at a ten times higher substrate coat concentration of about 2 µg (data not shown).

To test the functional effect of galectin-3, an *in-vitro* cerebellar neurite outgrowth experiment was performed. Galectin-3 when used either as a substrate coat or as a soluble agent had no Lewis^x dependent effects on neurite outgrowth (data not shown). The endogenous function of galectin-3 as a Lewis^x receptor may not be involved in cerebellar neurite outgrowth.

4.4. Screening for potential Lewis^x interacting partners using a protein array

The protein array screens were performed at by the collaborators group of Dr. Zoltan Konthur. The protein array consists of a PVDF membrane strip spotted with more than 10000 different human proteins that are recombinantly produced in *E. coli*. The membrane was first blocked with buffer containing both BSA and fat free milk and then incubated with FITC labeled Lewis^x. The membrane was washed and the bound Lewis^x was detected using alkaline phosphatase conjugated anti FITC antibody. The arrays were scanned to identify the positive spots. About 50 genes from a possible 537 clones were identified to be probable interacting partners of Lewis^x. The list of 50 genes can be found in Hits from protein array screen to identify Lewisx receptors: Table 10 in the Appendix. A short abstracted list of proteins that are known to have important functions in the nervous system and were identified in the protein array experiment are listed below in Table 8.

Table 8: Potential Lewis^x binding partners as identified by protein array experiments.

1	34/67 kDa Laminin receptor, Uniprot/SWISSPROT;Acc:P08865
2	Neuronal migration protein doublecortin (Lis-X), Uniprot/SWISSPROT;Acc:O43602]
3	SPARC precursor (Osteonectin), Uniprot/SWISSPROT;Acc:P09486]
4	Presenilin- associated metalloprotease (PAMP)Uniprot/SWISSPROT;Acc:Q96TA2]
5	60S ribosomal protein L29 (Cell surface heparin-binding protein HIP), SWISSPROT;Acc:P47914
6	Paraneoplastic antigen like, RefSeq peptide;6A Acc:NP_116271
7	Nonhistone chromosomal protein HMG-14 Uniprot/SWISSPROT;Acc:P05114
8	Cold-inducible RNA-binding protein (A18 hnRNP), Uniprot/SWISSPROT;Acc:Q14011

5. In-vivo assays to ascertain Lewis^x functions

5.1. Role of the Lewis^x mimicking peptide in regeneration of the femoral nerve after injury

Glycans like the HNK-1 and polysialic acid (PSA) have been shown to provide molecular cues for proper reinnervation and axon growth in a femoral nerve injury model (Simova et al., 2006; Franz et al., 2005). Peptide mimics of the HNK-1 glycan helped peripheral neurons to regenerate their axons after nerve injury and reinnervate proper peripheral targets. We evaluated if the Lewis^x mimicking peptide played a similar role in providing guidance cues and if it had therapeutic effects for better functional recovery in a femoral nerve injury model. Groups of ten animals in each were subjected to lesion followed by immediate treatment with the Lewis^x peptide (H-KACSRLNYLHCK-OH) or the scrambled peptide (H-KACNHLLRSYCK-OH) or the carrier vehicle PBS. Functional analysis was performed over a time-period of 12 weeks novel single-frame motion analysis approach recently developed in our laboratory. The recovery indices and the time course of the degree of motor function recovery are shown in Figure 32 and Figure 33.

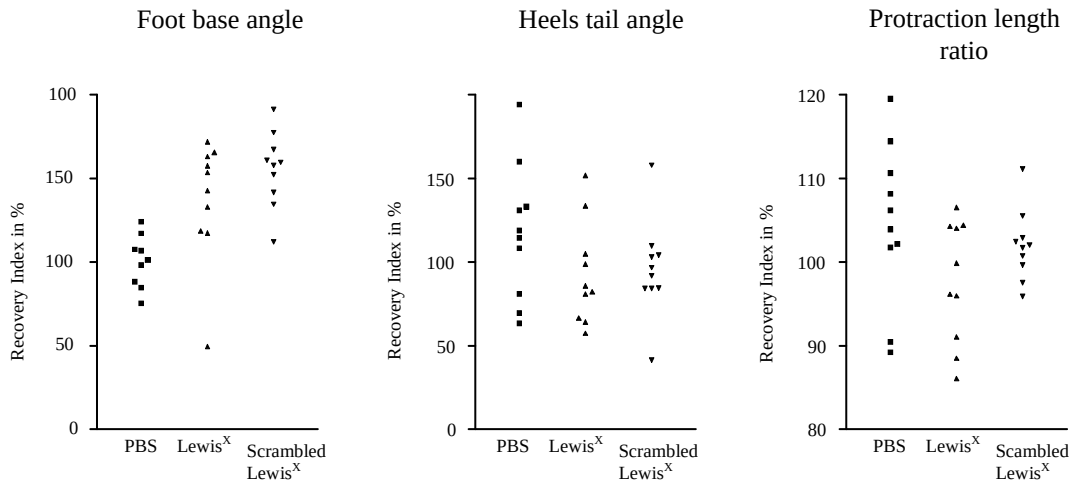


Figure 32: Recovery indices, 12 weeks after femoral nerve transection. Shown are recovery indices of foot-base angles, heels-tail angles and limb protraction length ratios in mice treated with Lewis^x mimicking peptide, scrambled peptide, and PBS.

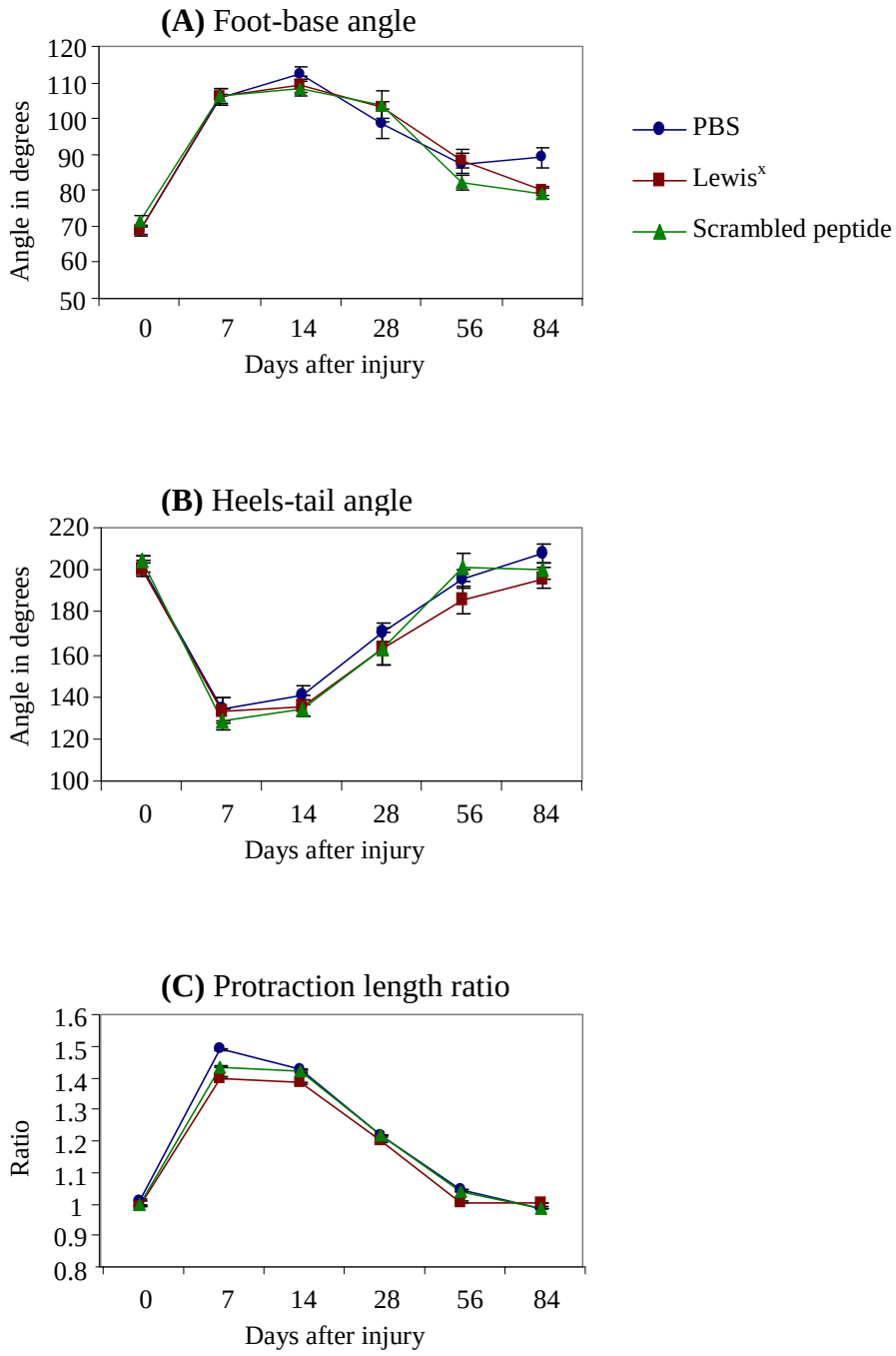


Figure 33: Time course and degree of motor function recovery after femoral nerve lesion. Shown are mean values + SEM of foot-base angles (A), heels-tail angles (B) and limb protraction length ratios (C). Prior to surgery (day 0) and at different time-points after nerve transection in mice treated with Lewis^x mimicking peptide, scrambled peptide, and PBS.

The functional analysis of the mice treated with the Lewis^x mimicking peptide showed no better or quicker recovery as compared to the scrambled peptide or vehicle treated animals (Figure 32 and Figure 33). We can note that in all the three parameters tested; foot-base angles, heels-tail angles and limb protraction length ratios, the

degree of impairment is the maximum at 7-days after injury and by day-84, mice recover completely. The recovery indices shown in figure revealed no significant difference amongst the different groups treated with different test substances after an analysis of variance (ANOVA) and subsequent post hoc analysis (data not shown). This indicates that unlike the HNK-1 and PSA glycans the Lewis^x glycan does not provide an advantageous cue for femoral nerve reinnervation.

5.2. Role of the Lewis^x mimicking peptide in regeneration of the spinal cord after injury

The recovery of function after spinal cord injury can be enhanced by neutralisation of inhibitory cues in the adult central nervous system or with interventions promoting axon regeneration, neuronal survival and synaptic plasticity (Chen et al., 2007). Glycans, proteoglycans in particular along with the astrocytes and macrophages form an inhibiting complex for the axons to regrow. Lewis^x is also upregulated in the reactive astrocytes at sites of lesion in the nervous system (Gocht 1992). We wanted to test if the mimicking peptide can compete for the Lewis^x binding sites and influence the functions of the astrocytes and change an outcome of spinal cord injury. At the same time we evaluated if the Lewis^x mimicking peptide had a neurotrophic effect for better axon regeneration or increased neuronal survival to enhance functional recovery after injury in the central nervous system. Groups of twelve animals in each were subjected to treatment with the Lewis^x peptide (H-KACSRLNYLHCK-OH) or the scrambled peptide (H-KACNHLLRSYCK-OH) or the carrier vehicle PBS immediately after injury. Functional analysis was performed over a time-period of 6 weeks novel single-frame motion analysis approach recently developed in our laboratory. The time course of the degree of motor function recovery and the recovery indices were analysed.

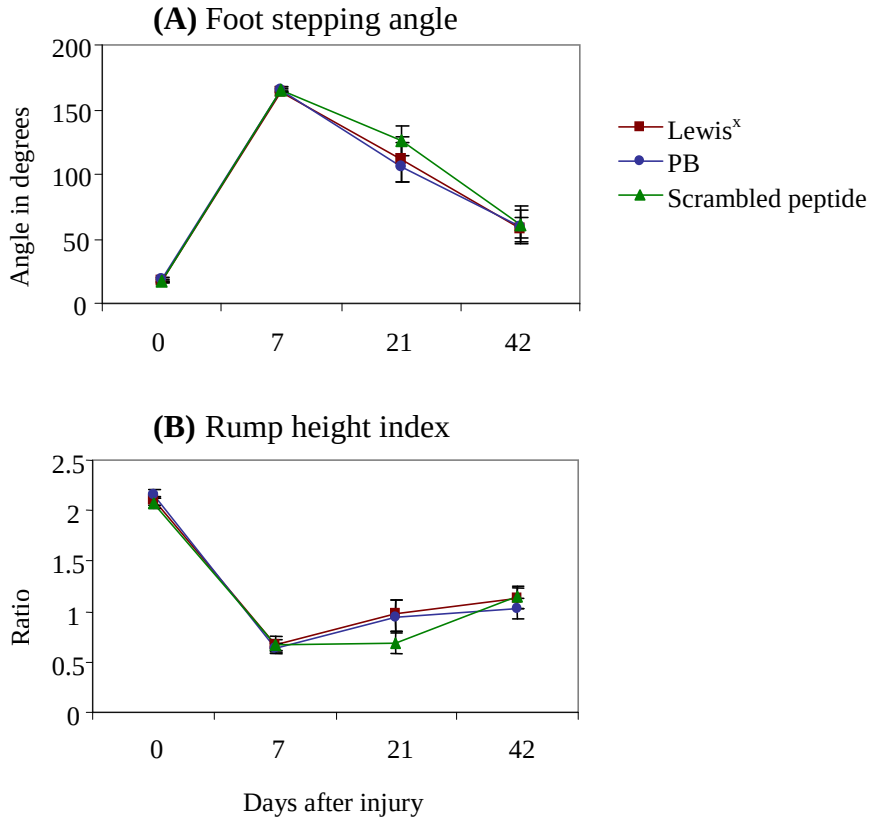


Figure 34: Time course and degree of functional recovery after spinal cord compression injury. Shown are SEM values of foot stepping angle (A) and rump height index (B). Prior to surgery (day 0) and at different time-points after nerve transection in mice treated with Lewis^x mimicking peptide, scrambled peptide and PBS.

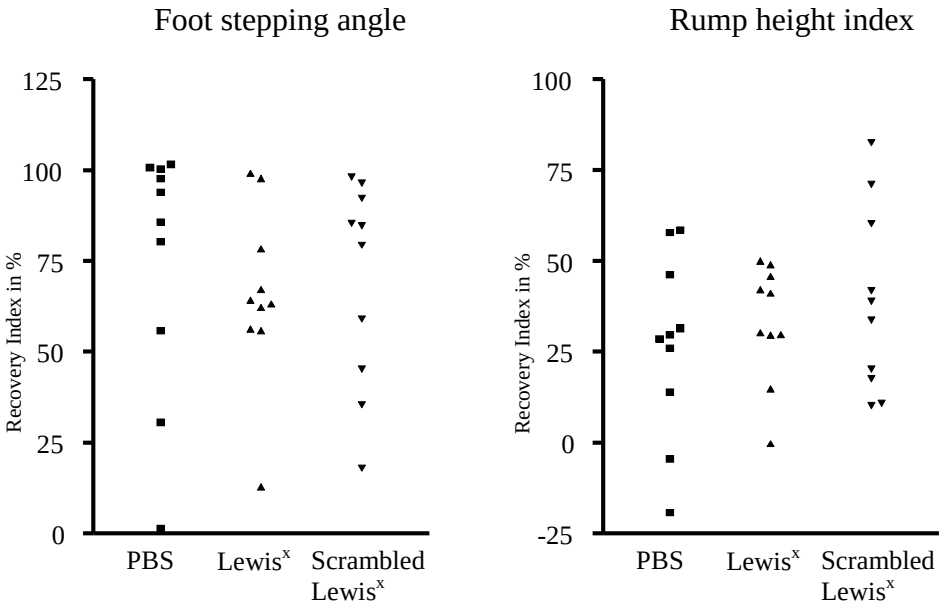


Figure 35: Recovery index 6 weeks after spinal cord compression injury. Shown are recovery indices of foot-base angles, heels-tail angles and limb protraction length ratios after nerve transection in mice treated with Lewis^x mimicking peptide, scrambled peptide and PBS.

The functional analysis of the mice treated with the Lewis^x mimicking peptide had no effect on the recovery after spinal cord injury as compared to the scrambled peptide or vehicle treated animals. We can note that in the two parameters tested and shown here; foot-stepping angles and rump height index, the degree of impairment is the maximum at 7-days after injury and mice recovered by day-42 (Figure 34 and Figure 35). The recovery indices shown in figure revealed no significant difference amongst the different groups treated with different test substances after an analysis of variance (ANOVA) and subsequent post hoc analysis (analysis not shown). This indicates that the Lewis^x glycan does not influence the astrocytes or the outcome of a spinal cord injury.

6. Do α1,3-fucosyltransferase deficient mice lack Lewis^x?

Ablation of the Lewis^x glycan can be a useful means of understanding its function. The Lewis^x epitope is synthesised by the transfer of fucose on type 2 oligosaccharide chains and this reaction is mediated by α1,3-fucosyltransferases. Amongst a possible thirteen, the enzymes α1,3-fucosyltransferases-4, -7 and -9 have been implicated to aid the synthesis of Lewis^x. To identify the enzyme responsible for this fucosylation in the brain, we obtained brain tissue from mice of different ages with gene knock outs of α1,3-fucosyltransferases-4, -7, a double knockout of both -4 and -7 (Maly et al., 1996; Homeister et al., 2001) and a α1,3-fucosyltransferases-9 (Nishihara et al., 2003) and tested them for the presence of Lewis^x. Brain tissue from FX epimerase knock out mice (Smith et al., 2002) that were on either fucose supplemented or depleted diet were also obtained to check for its effects on Lewis^x synthesis. Individual brain tissue was homogenised and about 100 mg of this was subjected to SDS-PAGE and analysed by Western blot with the L5 antibodies.

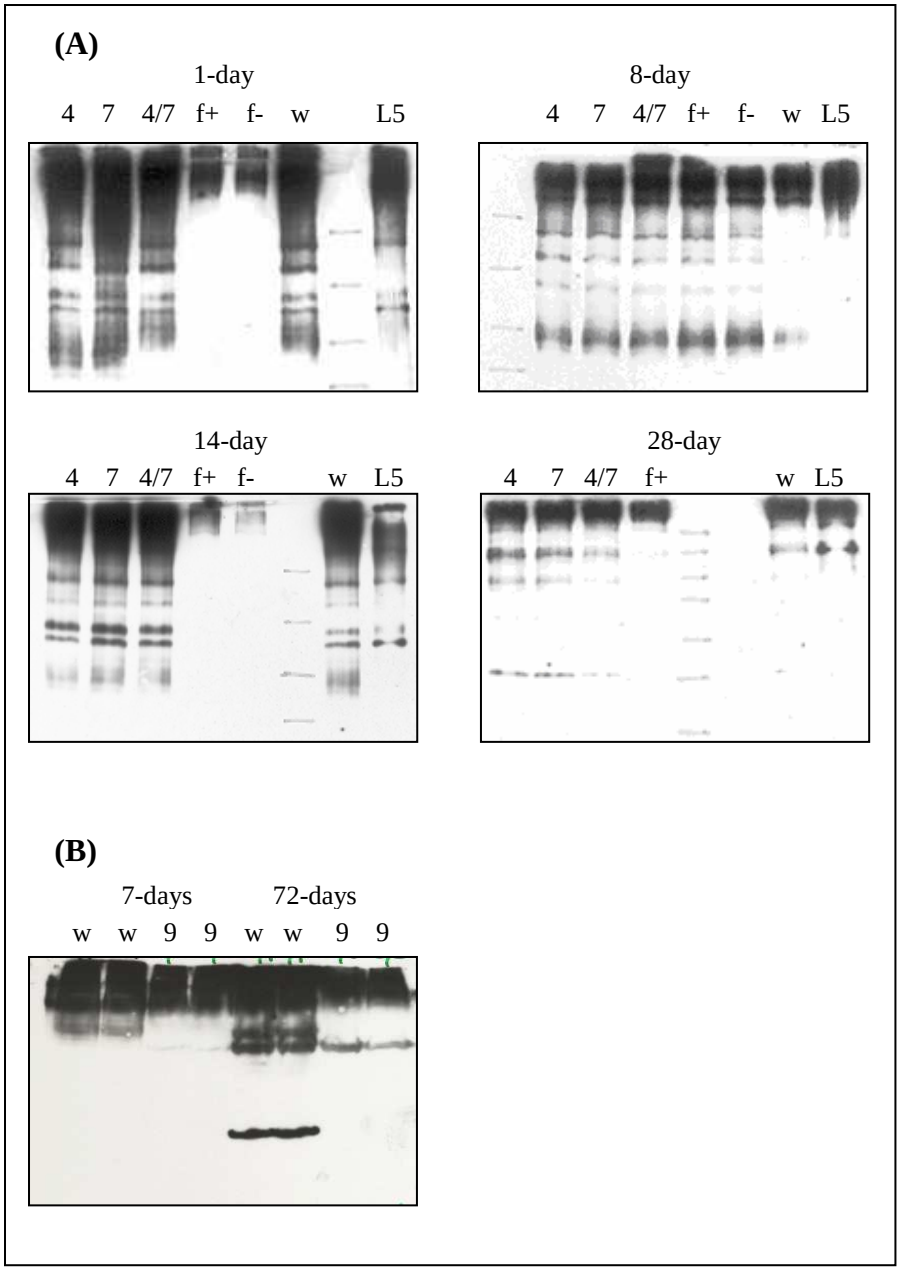


Figure 36: Western blot analysis using αLewis^x antibodies L5, of brain samples from different fucosyltransferase knockout mice. Fucosyltransferase-4, 7, 4/7 double knockout and FX knockout of four age groups of 1, 8, 14 and 28 days (A) and Fucosyltransferase-9 knockout and their wild type littermates (B). f+: FX knockout mice on fucose supplemented diet and f-: without fucose in the diet.

The Western blot analysis of the brain tissue samples from the different fucosyltransferase knockout mice show no loss of immunoreactivity with the L5 antibodies indicating the presence of the Lewis^x glycans (Figure 36). The presence of the Lewis^x glycan in the different fucosyltransferases deficient mice shows that there is redundancy amongst the enzymes and knocking out the gene for one of them is compensated by the activity of the other. The FX locus knockout mouse brain retains the L5 immunoreactivity until the fucose is available for the synthesis of Lewis^x and a

reduction of the reactivity is observed when the fucose is depleted. This only demonstrates the need for fucose for the synthesis of Lewis^x. A deficiency of fucosyltransferase-4 or -7 or -9 does not lead to loss of the Lewis^x glycan. These results suggest that a mouse model with the Lewis^x glycan deficiency to understand its function still needs to be developed.

VIII. Discussion

Lewis^x recognized by the monoclonal antibody L5 (Streit et al., 1996) is expressed on all the components of the developing nervous system, both central and peripheral. It has been shown to be important in early development (Roberts et al., 1991) and in neuron-astrocyte interactions as in cell migration and adhesion (Streit et al., 1993). A novel molecule carrying Lewis^x at the cell surface and also its probable receptors were identified, these were investigated for their Lewis^x dependent roles in the processes of neurite outgrowth and regeneration in the nervous system of mice.

1. Identification of Lewis^x carrying molecules

Lewis^x carrying molecules were identified by performing an affinity chromatography experiment with immobilized L5 monoclonal antibodies that recognise the Lewis^x epitope. Initial experiments led to co-purification of a number of contaminating proteins and the purified proteins could not be resolved as distinct bands in a one dimensional SDS-PAGE. To further enrich the Lewis^x carrying proteins a number of methods were tried like 1) pre-separating the cytosolic and membrane fractions before doing the affinity chromatography experiment, 2) re-purifying by repeating the affinity chromatography with the eluate from the L5 antibody column and 3) 2D-gel electrophoresis. Finally, preparative scale SDS–PAGE electrophoresis followed by whole gel elution enabled the isolation of Lewis^x carrying proteins. Whole gel elution is a process of simultaneous electroelution of separated proteins from whole SDS-PAGE into narrow chambers, each containing proteins of similar molecular weight, thus fractioning the proteins based on their molecular weights. The isolated Lewis^x carrying proteins were identified by mass spectrometry at the lab of Prof. Bruce Macher and Dr Robert Yen, Dept of Chemistry and Biochemistry, San Francisco State University. It was encouraging to note the presence of peptides from known Lewis^x carrying proteins like the cell adhesion molecule L1, tenascin-R and RPTPζ in the mass spectrometry analysis (Wing et al., 1992; Garwood et al., 1999). The molecules like contactin-1, neuronal pentraxin-1 and noelin-1 were novel molecules that could be carriers of the Lewis^x glycans.

2. Contactin-1 is a novel carrier of Lewis^x

Contactin-1 was purified as a Lewis^x carrying protein in an antibody affinity chromatography experiment. Mass spectrometry analysis from a ~130 kDa band in the SDS-PAGE of the purified Lewis^x carrying proteins consisted of peptides belonging to contactin-1. Immunoprecipitation of contactin-1 with contactin-1 antibodies and its Western blot analysis with the L5 antibodies confirmed the presence of Lewis^x glycan on it. Further, glycosidase (PNGaseF) treatment revealed that only the high molecular glycoform of contactin-1 carries the Lewis^x epitope on its N-glycans.

Contactin-1 is involved in the formation of paranodal axo-glial junctions in myelinated peripheral nerves via its association with contactin associated protein (Caspr) and Neurofascin-155 (NF155) (Boyle et al., 2001; Gollan et al., 2003). A glycosylation dependent mechanism has been proposed for these three molecules to interact; the low molecular weight endoglycosidase H-sensitive isoform of contactin-1 associates with Caspr and not NF155 (Gollan et al., 2003) implying that the oligomannose glycans mediate the binding to Caspr resultantly making contactin-1 unavailable to interact with other molecules. Conversely, they show that the high molecular weight glycoform of contactin-1 with complex type glycans is present independent of Caspr at the cell surface, this would make it available for interactions with NF155 and other interacting proteins like phosphacan (Peles et al., 1995), Notch1 (Hu et al., 2003) or the tenascin-R and –C. Contrary to this, the interaction mechanism has been challenged by showing that the low molecular weight contactin-1 can also interact with NF155 (Bonnon et al., 2007). The high molecular weight form of contactin-1 carrying Lewis^x might be able to fine tune the interactions of contactin-1 with its various interacting partners, either to allow them or restrict them from interacting.

Interaction of contactin-1 with a Lewis^x carrying molecule like tenascin-R to induce a repulsion of neurons and an inhibition of neurite outgrowth has been shown to be independent of glycosylation (Pesheva et al., 1993). But the interactions of that of a similar molecule like tenascin-C and contactin-1 are inhibited using glycans (Weber et al., 1996). Lewis^x present on contactin-1 may not mediate direct interactions but

again may only modulate these interactions. Similarly, Lewis^x may modulate formation of functional complex of contactin-1 with the fucose carrying Notch1 or another Lewis^x carrying molecule phosphacan to modulate differentiation and development. Lewis^x which has been demonstrated to be involved in homophilic Lewis^x-Lewis^x interactions (de la Fuente et al., 2005) may bring about adhesion between these molecules.

3. Astrochondrin is the proteoglycan phosphacan

In immunoblots the L5 antibody recognizes a major component of about 400 kDa and several more minor components of lower molecular mass. The 400 kDa molecular component was recognized to be a chondroitin sulphate proteoglycan called astrochondrin (Streit et al., 1990). The extracellular matrix chondroitin sulphate proteoglycan phosphacan also carries Lewis^x and has a similar molecular weight (Garwood et al., 1999).

To test the hypothesis that phosphacan is the protein astrochondrin, a mass spectrometry analysis of the high molecular weight Lewis^x carrying proteins was performed. Indeed, this analysis showed the presence of peptides from RPTPζ which is the parent molecule of phosphacan. Further, immunoprecipitation of phosphacan with phosphacan antibodies KAF13 and its Western blot analysis with L5 antibodies revealed the presence of Lewis^x glycans on it. It could also be ascertained that this immunoprecipitated astrochondrin was a chondroitin sulphate proteoglycan by Western blot with the chondroitin sulphate antibodies 473HD. This reactivity to 473HD could be abolished by removal of the chondroitin sulphate by chondroitinase-ABC treatment.

Both phosphacan and astrochondrin show similar expression levels that peak during postnatal week 2 in murine brains (Ripellino et al., 1989; Streit et al., 1990). Phosphacan's ultrastructural localization is mainly at the membrane of migrating neurons and radial glia (Hayashi et al., 2005). This is similar to the ultrastructural localisation of Lewis^x as seen at membranes between astrocyte-astrocyte, astrocyte-oligodendrocyte, astrocyte-axon myelin (Gocht et al., 1994). Taking these facts into

account that phosphacan has properties similar to astrochondrin as in, both have a large molecular weight close to 400 kDa, both are chondroitin sulphate proteoglycans and carry the Lewis^x glycan, it can be proposed that they could be one and the same protein. However, the non-finding of other proteins in the mass spectrometry analysis of the high molecular weight L5 carrying proteins does not rule out the possibility that a second protein with the same properties is present in the brain.

Astrocytes have been characterised to express both astrochondrin and Lewis^x. The binding of L5 antibodies to astrochondrin on the astrocytes leads to a reduction in the formation of astrocytic processes that were growing on the extracellular matrix glycoproteins laminin and collagen type IV (Streit et al., 1993). They also demonstrate that L5 antibodies and hence the Lewis^x epitope is able to reduce the migration of granule cells in early postnatal mouse cerebellar cortex. Based on these observations they conclude that the Lewis^x epitope contributes to morphogenetic processes and also support astrocyte interactions with the extracellular matrix components.

4. Identification of receptors for Lewis^x

To isolate the lectin or receptor for Lewis^x, pull-down experiments with Lewis^x-BSA or BSA were performed and the obtained molecules were identified by mass spectrometry at the lab of Prof. Bruce Macher and Dr Robert Yen, Dept of Chemistry and Biochemistry, San Francisco State University. Sclerostin domain containing protein-1 was the only molecule amongst the mass-spectrometry identified molecules that was cell surface protein. It is a secreted BMP (Bone Morphogenetic Protein) inhibitor and is homologous to sclerostin that is a secreted BMP antagonist (Laurikkala et al., 2003). Though this could be an important molecule for morphogenesis they did not show any gross morphological changes in the brains of sclerostin domain containing protein-1 deficient mice. The known normal localisation of the rest of the detected proteins was either cytoplasmic or nuclear which did not fulfil the requirement to be at the cell surface, hence, were thought to be less probable receptors of Lewis^x.

When the pull down experiment with Lewis^x-BSA and control sugar–BSA was performed, problems of non-specific protein binding arose that could not be remedied by altering detergent conditions or using the glycan without BSA. To visualise and enrich for specifically binding proteins a biotin tagging method was used, the Lewis^x-BSA conjugate was crosslinked to Sulfo-SBED and used in pull down experiments. The method helped to identify the trapped interactions on a Western blot using Streptavidin/HRP. This method, though helped identify interactions that could be identified in a Western blot it did not provide sufficient amounts of discrete proteins to enable their identification by mass spectrometry analysis. Experiments to eliminate the problem of non specific binding to BSA in the BSA-sugar conjugate like directly coupling the Lewis^x sugar MTS-ATF-Biotin or to an immobilising matrix and using in pull-down experiments were not successful in getting any specific receptors.

The affinity purification experiments to identify a Lewis^x receptor were not successful despite using a number of strategies. Instead of using mouse brain homogenate as a pool of probable receptors, the same proteins were presented as phage coat proteins in a phage display library. The phage display library and panning experiments provided a means of high through put screening of a number of proteins for their binding to Lewis^x. The advantage of using this system was that it eliminated the probable Lewis^x-Lewis^x homophilic interactions that may quench the available Lewis^x for binding in the pull-down assays. It also was simpler to identify the interacting proteins, they could be identified by DNA sequencing and no mass spectrometry was required. It also had disadvantages like 1) the limitations in the size of the cDNA that can be expressed as a fusion of the phage coat protein, 2) the reading frame of the inserted cDNA may produce nonsense fusion proteins, 3) some proteins may not lend themselves to form the phage coat protein or can be toxic to *E. coli* that is used to propagate the phage, and 4) improper folding of eukaryotic proteins in *E. coli.*

A pre-made T7 phage library expressing human brain cDNA as a fusion of their coat proteins was biopanned for four rounds with Lewis^x conjugated to BSA as the target. Specific binding proteins were obtained by performing an elution with the L5 antibodies and further elimination of the non-specific phage was achieved by adsorbing them with BSA-Lewis^a. The phage display experiment revealed galectin-3

and a Golli isoform of myelin basic protein (MBP) as two probable interacting partners of Lewis^x.

5. Galectin-3 is a receptor for Lewis^x

Galectin-3 is a soluble protein belonging to the family of lectins that binds to the carbohydrate moiety β-galactoside (Barondes et al., 1994). It is a S-type lectin (does not require Ca+ ions to bind carbohydrates) consisting of a carbohydrate recognition domain in the C-terminal followed by a proline-, glycine-, and tyrosine-rich region that is homologous to proteins of the heterogeneous ribonuclear protein complex and a short N-terminal domain (Barondes et al., 1994; Leffler et al., 2004). Galectins are suggested to be involved in signal transduction events as well as extracellular matrix adhesive interactions mediating processes such as kidney development, angiogenesis, neuronal functions, tumour metastasis, autoimmune disorders, endocytosis and exocytosis (Nakahara and Raz, 2006). Cells, particularly epithelial cells which lack galectin-3 expression, interact poorly with their extracellular matrices (Ochieng et al., 2004).

Galectin-3 is expressed in the nervous system in the spinal cord and dorsal root ganglia (DRG) (Regan et al., 1986; Dodd and Jessell, 1986). At the cellular level, galectin-3 is expressed by ensheathing cells surrounding nerve fascicles in the submucosa and nerve fibre layer of the olfactory epithelium (Storan et al., 2004), microglia cells (Pesheva et al., 1998b), a subpopulation of DRG (Regan et al., 1986; Dodd and Jessell, 1986) and on injury, in both spinal neurons (Cameron et al., 1993) and the Schwann cells (Reichert et al., 1994). It has been shown to specifically bind to neural recognition molecules like L1, MAG, NCAM and tenascin-C (Probstmeier et al., 1995), all of which are Lewis^x carrying molecules. These neural cell surface molecules that are involved in cell recognition and signal transduction (Maness and Schachner, 2007) interact with galectin-3 raising the question of the effects it has on the cells that express these molecules with or without Lewis^x. L1, NCAM and galectin-3 are all in the DRG or with tenascin-C in the spinal cord. NCAM interacts with galectin-3 via the glycans in the olfactory epithelium to bring about fasciculation of the axons (Storan et al., 2004). The amount of glycans including the Lewis^x present

on the neuronal surface proteins vary with the time in development and cell type and along with galectins they are involved in targeting of the sensory axons (Jessell et al., 1990) and the Lewis^x by its presence or absence may be involved in modulating their interactions with galectin-3. The binding of galectin-3 to carbohydrate back-bones has been shown to be enhanced when the termini are modified to Lewis^x (Ideo et al., 2002). We can speculate that the Lewis^x may be fine tuning these kind of interactions by promoting clustering of certain molecules with the help of galectin-3 and bring about adhesion or signal transduction.

The binding of galectin-3 to neural cells like the cerebellar granule cells and DRG is glycan dependent to bring about adhesion and neurite outgrowth (Pesheva et al., 1998a), however when free Lewis^x sugar was tested for its ability to perturb this function of galectin-3 to induce neurite outgrowth, the Lewis^x had no effect. This still does not rule out the possibility that the Lewis^x glycan, in an *in-vivo* situation, is only modulating the binding of the protein backbone it is present on. It could also be that the endogenous function of galectin-3 as a Lewis^x receptor may not be involved in cerebellar neurite outgrowth.

6. Golli isoform of Myelin Basic Protein (MBP) as a Lewis^x interacting protein

The non-classic group of MBP isoforms may preferentially have a role in the early developing brain before myelination as components of transcriptional complexes (Pribyl et al., 1993) and may also be involved in signalling pathways in T-cells (Feng et al., 2004) and neural cells (Paez et al., 2007) implying that it has a role in places other than just myelin. MBP acts as a mitogen on astrocytes in culture (South et al., 2000) thus indicating a role it could play on the outer cell surface, at least during pathological conditions. It also carries a four amino acid motif ‘WGAE’ that is present on galactose binding lectins and cross reacts with antibodies that bind these lectins (Abbott et al., 1989). These characteristics of golli suggest that it could have lectin like functions. Golli was identified as an interacting protein of Lewis^x in the phage display screen; however, a functional relevance for this interaction is yet to be established.

7. Peptide mimicking the Lewis^x glycan

Apart from using the glycan to precipitate receptors, a possible alternative was to use a peptide mimic that has the similar structure and hence could serve the same function. To select peptides that mimic the Lewis^x glycan a large library of random peptides displayed on the surface of filamentous phage was biopanned against the Lewis^x carbohydrate recognising antibody L5. After four successive rounds of panning and amplification, phage coat protein with the peptides sequence CSRLNYLHC bound to the L5 antibody. This phage binding to L5 antibodies was further verified by ELISA and Western blot experiments. A synthetic form of this short peptide was obtained in a cyclised form and this peptide could also bind the L5 antibody and also functionally substitute for the Lewis^x carbohydrate in an *in-vitro* neurite outgrowth experiment. The use of the this Lewis^x mimicking peptide in pull-down experiments to identify Lewis^x receptors was not successful due to problems of solubility of the peptide and also non-specific binding of proteins. The peptide was useful in functional assays to simulate the functions of Lewis^x glycan to interfere in the lectin-glycan interactions involving CD24.

8. In-vivo assays to ascertain Lewis^x functions

We hoped to ascertain the functions of the Lewis^x glycan using an *in-vivo* assay since assessment using an *in-vitro* assay for the large number of Lewis^x carrying molecules and their lectins proved less useful. The specific peptide mimic of Lewis^x was introduced in neuronal injury models hoping that it will either assist or interfere with a specific function, generating an interpretable phenotype and reveal the functions of Lewis^x. Glycans like the HNK-1 and polysialic acid (PSA) have been shown to provide molecular cues for proper reinnervation and axon growth in a femoral nerve injury model (Simova et al., 2006; Franz et al., 2005). Peptide mimics of the HNK-1 glycan had been shown to help peripheral neurons to regenerate their axons after nerve injury and reinnervate proper peripheral targets. Galectin-3, the probable Lewis^x receptor is expressed among the different components of the peripheral nerves but on injury it is induced transynaptically in dorsal horn neurons (Cameron et al., 1993) and can be released from the cells to interact with membrane or extracellular glycoconjugates on adjacent cells (Dodd and Jessell, 1986), thus could be an

important factor involving recovery after injury. We evaluated if the Lewis^x mimicking peptide played a role in providing guidance cues and if it had therapeutic effects for better functional recovery in a femoral nerve injury model.

Similarly in the central nervous system, an injury model of the spinal cord could provide clues for the functions of Lewis^x. Glycans, proteoglycans in particular along with the astrocytes and macrophages form an inhibiting complex for the axons to regrow (Busch and Silver, 2007; Chen et al., 2005). Activation of astrocytes along with the macrophages and degeneration of neurons and oligodendrocytes are the cellular responses that characterise spinal cord injury. Recovery from injury can be enhanced by neutralisation of inhibitory cues or with interventions promoting axon regeneration (Chen et al., 2005). Lewis^x is upregulated in the reactive astrocytes at sites of lesion in the nervous system (Gocht, 1992). Galectin-3 is localized at the outer cell surface of macrophages (Ho and Springer, 1982) and this may interact with the Lewis^x and get activated during injury. MBP that we propose as a Lewis^x lectin, has been shown to act as a mitogen on astrocytes in culture and thus is suggested to be a factor in stimulation of astrogliosis after demyelination in neuronal pathology (South et al., 2000) though in conjunction with other factors. We hypothesised that 1) the homophilic interaction of Lewis^x with Lewis^x contributes to the formation of the inhibitory complex and that this could be perturbed or decompacted by introducing the Lewis^x glycan or the mimicking peptide like the embryos (Fenderson et al., 1984) and 2) that it could also quench the mitogenic activity of the MBP and the macrophage stimulating factors.

The outcome of the both the *in-vivo* assays of the femoral nerve and spinal cord regeneration did not implicate the Lewis^x glycan in either providing a guiding molecular cue or reducing the inhibitory environment for growing axons.

9. Fucosyltransferases in Lewis^x glycan synthesis

Ablating the Lewis^x glycan in a mouse model could have yielded insights with regard to its function. The analysis of brain tissue from mice with deletions of the enzymes fucosyltransferase-4, 7, 4/7 double knockout or the fucosyltransferase-9 [that has been reported to lead to loss of Lewis^x, (Nishihara et al., 2003)] showed that there was a

redundancy in the functions of these enzymes to synthesise the Lewis^x glycan. Thus, an interpretation of a phenotype due to the loss of Lewis^x is yet to be established. Establishing such a model would require the creating of a mouse with knockout of multiple fucosyltransferases but a more plausible way would be to inhibit the fucosyltransferases by drug like molecules and this is yet to be identified.

10. Lewis^x in carbohydrate interactions

The cellular interactions of adhesion inducing cell activation, motility and growth that are initiated at the cell surface are brought about by complex protein-protein and protein-ligand interactions. The interactions of the proteins at the cell surface and their ability to transform it into activity are influenced by specialised and organised lipid microenvironments called lipid rafts (Simons and Toomre, 2000). In a similar concept, cell surface microdomains have been classified into a framework called 'glycosynapses' (Regina and Hakomori, 2008). In these domains glycolipids and glycoproteins interact with complementary carbohydrates or lectins mediating cell signalling leading to changes in cellular phenotype. The carbohydrate epitopes may be clustered with specific signal transducers, tetraspanins, adhesion receptors or growth factor receptors in these glycosynapses and amongst these, the type 1 glycosynapse is characterised by carbohydrate-carbohydrate interactions like those brought about by Lewis^x (Hakomori, 2004). The molecules that have been identified as Lewis^x carrying molecules like contactin-1 and receptor like galectin-3 can be considered in the light of such carbohydrate microdomains. For example, contactin-1 which is known to partition into lipid microdomains (Kramer et al., 1999), when carrying Lewis^x it may be present in certain microdomains to interact in *trans* with Neurofascin-155 or in other domains without the Lewis^x to interact with Caspr and both these interactions are required for proper myelination. Galectin-3 with its ability to bind carbohydrates and proteins can help in formation of lattices between contactin-1 and other Lewis^x carrying proteins like NCAM or phosphacan and modulate their localisation and biological function.

It is important to remember that Lewis^x may have other functions that are not mediated by a specific receptor interactions like that of maintaining stability, structure

and modulate function involving the glycan itself or its carrier protein and need investigation. I have investigated different aspects about the biological roles of the Lewis^x glycan and proposed a few possible interactions. Evidence from these experiments indicates that while the interactions of the glycan and its receptors may be true, a specific *in-vivo* function of the Lewis^x glycan is yet to be identified.

IX. References

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X. Appendix

1. Peptides identified by mass spectrometry of the Lewis^x carrying proteins: Table 9

Gel Band	Protein Identified	Protein Molecular Weight (Da)	Peptide Detected	Ion Score
20	Guanine nucleotide-binding protein G(O), alpha subunit 1	40498	LLLLGAGESGK	35
			EYQLNDSAK	38
			TTGIVETHFTFK	53
			LWGDSGIQECFNR	70
			QYKPVVYSNTIQSLAAIVR	54
	Tenascin-R	151807	LEGLSENTDYTVLLQAAQEATR	104
18	Murine mRNA for cytoplasmic gamma-actin. sp P63260 ACTG_MOUSE Actin; sp P60710 ACTB_MOUSE Actin	41335	AGFAGDDAPR	30
			EITALAPSTMK + Oxidation (M)	28
			DSYVGDEAQSK	70
			AVFPSIVGRPR	60
			IWHHTFYNELR	42
			SYELPDGQVITIGNER	65
			VAPEEHPVLLTEAPLNPK	54
			KDLYANTVLSGGTTMYPGIADR + Oxidation (M)	62
			LCYVALDFEQEMATAASSSSLEK + Oxidation (M)	112
17	Neuromodulin – Axonal membrane protein GAP-43	23732	QADVPAAVTDAAATTPAAEDAATK	76
16	Atp5b protein	56632	VVDLLAPYAK	43
			IMDPNIVGNEHYDVAR + Oxidation (M)	52
			VLDSGAPIKIPVGPETLGR	50
			DQEGQDVLLFIDNIFR	69
			AIAELGIYPAVDPLDSTSR	45
			FLSQPFQVAEVFTGHMGK + Oxidation (M)	48
			EGNDLYHEMIESGVINLK + Oxidation (M)	27
			SLQDIIAILGMDELSEEDKLTVSR + Oxidation (M)	42
			QFAPIHAEAPEFIEMSVEQEILVTGIK + Oxidation (M)	28
	Neuronal pentraxin-1 precursor	47601	KGSITSVQAIXVPADDLTDPAPATTFAHLDATTV	68
			LSR	27
			ETILSQK	38
			LPFVINDGK	54
			TRLENLEQYSR	75
			KLTPGEVYNLATCSSK	89
			TPAAETLSQLGQTLQSLK	80
	Tubulin, alpha 1	49831	ALSGNVIAWAESQIEIFGGATK	31
			LHFFMPGFAPLTSR + Oxidation (M)3	120
			MSATFIGNSTAIQELFK + Oxidation (M)	105
	L1 cell adhesion molecule	50804	GHYTEGAELVDSVLDVVR	65
			LIGQIVSSITASLR3	44
			NLDIERPTYTNLNR2	38
			VGINYQPPTVVPGGDLAK	74
			TIGGGDDSFNTFFSETGAGK2	55

		141798	FDGALNVDLTEFQTNLVPYPR2	64
			THNLTNLNPDLQYR1	64
			FQLQATTQQGPGEAIVR1	
15	Beta-2-tubulin class II isotype	50345	IREEYPDR	33
			FPGQLNADLR	34
			LAVNMVPFPR + Oxidation (M)	48
			ISEQFTAMFR	75
			KLAVNMVPFPR + Oxidation (M)	60
			LHFFMPGFAPLTSR + Oxidation (M)3	38
			NSSYFVEWIPNNVK	47
			ALTVPELTQQMFDSK + Oxidation (M)	54
			EIVHIQAGQCGNQIGAK	72
			MSATFIGNSTAIQELFK + Oxidation (M)	119
			GHYTEGAELVDSVLDVVR	118
			FWEVISDEHGIDPTGSYHGDSDLQLER	41
			EAESCDCLQGFQLTHSLGGGTGSGMGTLLISK + Oxidation (M)	62 52
			TIGGGDDSFNTFFSETGAGK2	61
	Tubulin alpha-1 chain;	50788	AVFVDLEPTVIDEVR	71
	Tubulin alpha-6 chain;		QLFHPEQLITGK	32
	Tubulin alpha-2 chain		QLFHPEQLITGKEDAANNYAR	35
			EIIDLVLDR	29
			LADQCTGLQGFLVFHSFGGGTGSGFTSLLMER	35
			KLADQCTGLQGFLVFHSFGGGTGSGFTSLLMER	47
			LSVDYGKK	50
			NLDIERPTYTNLNR2	35
			LIGQIVSSITASLR3	43
			FDGALNVDLTEFQTNLVPYPR2	83
			IHFPLATYAPVISAEK2	86
			AYHEQLSVAEITNACFEPANQMVK Oxidation (M)	67 46
			DVNAAIATIK2	26
			TIQFVDWCPTGFK	31
			VGINYQPPTVVPGGDLAK	34
			AVCMLSNTTAIAEAWAR	90
			AFVHWYVGEGMEEGEFSEAR Oxidation (M)	34
			MREIVHIQAGQCGNQIGAK + Oxidation (M)	
			LTTPTYGDLNHLVSATMSGVTTCLR	
14	Tubulin alpha-6 chain;	49909	DVNAAIATIK2	92
	Tubulin alpha-2 chain		NLDIERPTYTNLNR2	57
			IHFPLATYAPVISAEK2	40
			VGINYQPPTVVPGGDLAK	42
			AVCMLSNTTAIAEAWAR + Oxidation (M)	53
			TIGGGDDSFNTFFSETGAGK2	86
			FDGALNVDLTEFQTNLVPYPR2	53
	L1 cell adhesion molecule	141798	THNLTNLNPDLQYR1	77
			FQLQATTQQGPGEAIVR1	75
			YGPGEPSPVSETVVTPEAAPEK	70
13	ND			
12	DPYL2_MOUSE	62170	IVLEDGTLHVTEGSGR	36
	Dihydropyrimidinase related protein-2		MDENQFVAVTSTNAAK + Oxidation (M)	31
			FQLTDSQIYEVLSVIR	44
			FQMPDQGMTSADDFFQGTK + 2 Oxidation (M)	42
			IVNDDQSFYADIYMEDGLIK + Oxidation (M)	35
	Neuronal olfactomedin-related ER localized protein; Olfactomedin		ILDLGITGPEGHVLSRPEEVEAEAVNR	25
	1;Pancortin	55398	DLQYVEK4	39

			LTGISDPVTVK4	39
			FGSWMTDPLAPEGDNR4 Oxidation (M)	28
	L1 cell adhesion molecule	141798	FQLQATTQQGPGEAIVR1	86
11	Heat shock 70 protein	71021	ARFEELNADLFR	39
			NQVAMNPTNTVFDAK + Oxidation	54
			STAGDTHLGGEDFDNR	21
			TVTNAVVTVPAYFNDSQR	20
	Neuronal olfactomedin-related ER localized protein Olfactomedin 1 Pancortin	55398	LTGISDPVTVK4	40
			FGSWMTDPLAPEGDNR4 + Oxidation (M)	39
			DLQYVEK4	34
	L1 cell adhesion molecule	141798	FQLQATTQQGPGEAIVR1	100
10	L1 cell adhesion	141798	FQLQATTQQGPGEAIVR1	66
9	Heat shock 90kDa protein 1, beta	83606	NPDDITQEEYGEFYK	43
			HFSVEGQLEFR	44
			GVVDSEDLPLNISR	33
			GFEVVYMTEPIDEYCVQQLK	16
8	ND			
7	ND			
6	Contactin 1	113388	VLEPMPSTAEISTSGAVLK + Oxidation (M)	48
			IKTDGAAPNVAPSDVGGGGGTNR	49
			FVSQTNGNLYIANVESSDR	37
			IFNIQLEDEGLYECEAENIR	50
			STEATLSFGYLDPFPPEERPEVK	25
			GDGPYSLVAVINSAQDAPSEAPTEVGVK5	34
5	L1 cell adhesion molecule	141798	AQLLVVGSPGPVPHLELSDR	19
			NQHGLLLANAYIYVVQLPAR	56
			LVALQGQSLILECIAEGFPTPTIK	62
			AFGAPVPSVQWLDEEGTTVLQDER	61
	Contactin 1	113388	ASPFPVYK	11
			VVATNTLGTGEPSIPSNR	46
			VLEPMPSTAEISTSGAVLK + Oxidation (M)	51
			GDGPYSLVAVINSAQDAPSEAPTEVGVK5	57

Footnotes:

[1] Peptide found in bands 16, 14, 12, 11, 10 and 5.

[2] Peptide found in bands 16, 15 and 14.

[3] Peptide found in bands 16 and 15.

[4] Peptide found in bands 12 and 11.

[5] Peptide found in bands 5 and 4.

2. Hits from protein array screen to identify Lewis^x receptors: Table 10

Ensembl gene ID	Protein name(s) and SWISSPROT ID	Screening hit frequency	Transcript hit frequency
ENSG00000116148	Cyclin-L2 [Source:Uniprot/SWISSPROT;Acc:Q96S94]	3	3
ENSG00000104859	Splicing factor, arginine/serine-rich 16 [Source:Uniprot/SWISSPROT;Acc:Q8N2M8]	3	1
ENSG00000161016	60S ribosomal protein L8 [Source:Uniprot/SWISSPROT;Acc:P62917]	3	1
ENSG00000168028	34/67 kDa laminin receptor [Source:Uniprot/SWISSPROT;Acc:P08865]	3	1
ENSG00000174996	Kinesin light chain 2 [Source:Uniprot/SWISSPROT;Acc:Q9H0B6]	3	1
ENSG00000065978	Nuclease sensitive element-binding protein 1 [Source:Uniprot/SWISSPROT;Acc:P67809]	2	16*
ENSG00000197907	RcNSEP1 (Fragment) [Source:Uniprot/SPTREMBL;Acc:Q2VIK8]	2	15*
ENSG00000137497	Nuclear mitotic apparatus protein 1 [Source:Uniprot/SWISSPROT;Acc:Q14980]	2	2
ENSG00000089157	60S acidic ribosomal protein P0 [Source:Uniprot/SWISSPROT;Acc:P05388]	2	1
ENSG00000099821	DNA-directed RNA polymerase [Source:Uniprot/SWISSPROT;Acc:O00411]	2	1
ENSG00000108344	26S proteasome non-ATPase regulatory subunit 3 [Source:Uniprot/SWISSPROT;Acc:O43242]	2	1
ENSG00000115875	Splicing factor, arginine/serine-rich 7 [Source:Uniprot/SWISSPROT;Acc:Q16629]	2	1
ENSG00000140319	Signal recognition particle 14 kDa protein [Source:Uniprot/SWISSPROT;Acc:P37108]	2	1
ENSG00000169093	N-acetylserotonin O-methyltransferase-like protein [Source:Uniprot/SWISSPROT;Acc:O95671]	2	1
ENSG00000178338	Zinc finger protein 354B [Source:RefSeq_peptide;Acc:NP_478137]	2	1
ENSG00000187837	Histone H1.2 [Source:Uniprot/SWISSPROT;Acc:P16403]	2	1
ENSG00000161547	Splicing factor, arginine/serine-rich 2 [Source:Uniprot/SWISSPROT;Acc:Q01130]	1	6
ENSG00000140988	40S ribosomal protein S2 [Source:Uniprot/SWISSPROT;Acc:P15880]	1	4
ENSG00000196084	Ubiquitin [Source: Uniprot/SWISSPROT P62988]	1	3
ENSG00000063177	60S ribosomal protein L18 [Source:Uniprot/SWISSPROT;Acc:Q07020]	1	2
ENSG00000099622	Cold-inducible RNA-binding protein (A18 hnRNP) [Source:Uniprot/SWISSPROT;Acc:Q14011]	1	2
ENSG00000100429	Histone deacetylase 10 [Source:Uniprot/SWISSPROT;Acc:Q969S8]	1	2
ENSG00000036257	Cullin-3 [Source:Uniprot/SWISSPROT;Acc:Q13618]	1	1
ENSG00000067248	Putative ATP-dependent RNA helicase DHX29 [Source:Uniprot/SWISSPROT;Acc:Q7Z478]	1	1
ENSG00000077279	Neuronal migration protein doublecortin [Source:Uniprot/SWISSPROT;Acc:O43602]	1	1

ENSG00000089009	60S ribosomal protein L6 [Source:Uniprot/SWISSPROT;Acc:Q02878]	1	1
ENSG00000100650	Splicing factor, arginine/serine-rich 5 [Source:Uniprot/SWISSPROT;Acc:Q13243]	1	1
ENSG00000105058	Protein FAM32A [Source:Uniprot/SWISSPROT;Acc:Q9Y421]	1	1
ENSG00000105447	Glutamate-rich WD repeat-containing protein 1 [Source:Uniprot/SWISSPROT;Acc:Q9BQ67]	1	1
ENSG00000108848	Cisplatin resistance-associated overexpressed protein [Source:RefSeq_peptide;Acc:NP_057508]	1	1
ENSG00000113140	SPARC precursor [Source:Uniprot/SWISSPROT;Acc:P09486]	1	1
ENSG00000115875	Splicing factor, arginine/serine-rich 7 [Source:Uniprot/SWISSPROT;Acc:Q16629]	1	1
ENSG00000116350	Splicing factor, arginine/serine-rich 4 [Source:Uniprot/SWISSPROT;Acc:Q08170]	1	1
ENSG00000124380	U4/U6.U5 tri-snRNP-associated protein 3 [Source:Uniprot/SWISSPROT;Acc:Q8WVK2]	1	1
ENSG00000125846	Zinc finger protein 133 [Source:Uniprot/SWISSPROT;Acc:P52736]	1	1
ENSG00000136450	Splicing factor, arginine/serine-rich 1 [Source:Uniprot/SWISSPROT;Acc:Q07955]	1	1
ENSG00000136527	Arginine/serine-rich-splicing factor 10 [Source:Uniprot/SWISSPROT;Acc:P62995]	1	1
ENSG00000136758	ATP-dependent metalloprotease YME1L1 [Source:Uniprot/SWISSPROT;Acc:Q96TA2]	1	1
ENSG00000139233	C12orf31 ENST00000266604	1	1
ENSG00000140320	Bromo adjacent homology domain containing 1 [Source:RefSeq_peptide;Acc:NP_055767]	1	1
ENSG00000162244	Cell surface heparin-binding protein HIP. [Source:Uniprot/SWISSPROT;Acc:P47914]	1	1
ENSG00000165819	N6-adenosine-methyltransferase 70 kDa subunit [Source:Uniprot/SWISSPROT;Acc:Q86U44]	1	1
ENSG00000168710	Putative adenosylhomocysteinase 2 [Source:Uniprot/SWISSPROT;Acc:O43865]	1	1
ENSG00000174748	60S ribosomal protein L15 [Source:Uniprot/SWISSPROT;Acc:P61313]	1	1
ENSG00000175183	Cysteine and glycine-rich protein 2 [Source:Uniprot/SWISSPROT;Acc:Q16527]	1	1
ENSG00000186111	Pphosphatidylinositol-4-phosphate 5-kinase, type I, gamma [Source:RefSeq_peptide;Acc:NP_036530]	1	1
ENSG00000187260	WD repeat domain 86 [Source:RefSeq_peptide;Acc:NP_938026]	1	1
ENSG00000198013	Paraneoplastic antigen like 6A [Source:RefSeq_peptide;Acc:NP_116271]	1	1
ENSG00000205581	Nonhistone chromosomal protein HMG-14 [Source:Uniprot/SWISSPROT;Acc:P05114]	1	1
ENSG00000205751	Mitochondrial 39S ribosomal protein L23 [Source:Uniprot/SWISSPROT;Acc:Q16540]	1	1

Footnotes:

Screening hit frequency, i.e. how often found in 3 independent screens. Transcript hit frequency, i.e. how many clones were found to contain the same gene in all the screens. *Probably unspecific, comes up in almost every screen independent of screening material.

3. Abbreviations

°C	grad celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
Acc	accession number
Amp	ampicillin
APS	ammoniumperoxodisulfate
bp	base pairs
BSA	bovine serum albumine
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propansulfonate
cm	centimeter
CNBr	cyanogenbromide
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleotide-5'-triphosphate
DTT	dithiotreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Fuc	fucose
g	gram
Gal	galactose
Galβ1-4GlcNAc	N-acetyllactosamine
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
GPI	glycosylphosphatidylinositol
h	hour
HBSS	Hanks buffered saline solution
HNK-1	human natural killer cell glycan
HRP	horseradish-peroxidase
IEF	isoelectric focussing
IgG	immunoglobulin subclass G
IgM	immunoglobulin subclass M
IPG	immobilized pH gradient
IPTG	Isopropyl-β-D-1-thio-galactopyranoside
Kan	kanamycin
kDa	kilodalton
l	liter
LB	Luria Bertani
M	Molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter

mm	millimeter
mM	millimolar
Man	mannose
Neu5Ac	N-acetylneuraminic acid
ng	nanogram
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
pfu	plaque forming units
PLL	poly-L-Lysine
pM	picomolar
pmol	picomol
PMSF	Phenylmethylsulfonylfluoride
PNGase F	N-Glycosidase F
PSA	polysialic acid
RPTPζ	receptor-type protein tyrosine phosphatase ζ
SDS	sodiumdodecylsulphate
siglec	sialic acid Ig-like lectin
TAE	Tris/Acetat/EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N`,N`-Tetramethylethylendiamine
TIU	trypsin inhibitor unit
Tris	Tris-(hydroxyl)-aminomethan
U	units (enzyme)
UV	ultraviolett
V	Volt
v/v	volume per volume
w/v	weight per volume
x g	9,81 m/s^2
ZMNH	Zentrum für Molekulare Neurobiologie Hamburg

DNA

A	adenine
C	Cytosine
G	guanosine
T	thymine

Aminoacids

A	alanine
C	cysteine
H	histidine
L	leucine
N	aspargine
R	arginine
S	serine
Y	tyrosine

4. Risk and Safety statements for the compounds used in the study.

Name of the reagent, abbreviation, molecular formula, molecular weight	Risk Phrases	Safety Phrases	WHC	RTECS
2,2'-azino-bis(3-ethylbenzthiazoline-6-Sulphonic acid, ABTS	8	17-36		
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS, $C_{32}H_{58}N_2O_7S$, 614.88	61	53-45		
Acetic acid, CH_3CO_2H, 60.05	10-35	23-26-45	1	AF1225000
Acrylamide-Bis (29:1)	45-46-20/21-25-36/38-43-48/23/24/25-62	53-26-36/37-45	3	
Ammonium peroxodisulfate, APS, $(NH_4)_2S_2O_8$	8-22-36/37/38-42/43	22-24-26-37	1	SE0350000
Ampicillin	42/43	23-36/37-45	2	XH8400000
BCA Protein Assay Kit	25/42/43	22/36/37/45		
Bovines serum albumin			3	
Calcium chloride $CaCl_2$ 110.98	36	22-24/25	1	EV9800000
Chloroform, $CHCl_3$, 119.38	22-38-40-48/20/22	36/37	3	FS9100000
Coomassie® Brilliant Blue G, $C_{47}H_{48}N_3NaO_7S_2$, 854.02		22-24/25	3	
Diazepam, $C_{16}H_{13}ClN_2O$, 284.74	21/22	36/37	2	DF1575000
Dimethylformamide	61-20/21-36	53-45	1	LQ2100000
Disodium hydrogen phosphate dihydrate, $Na_2HPO_4 \cdot 2H_2O$, 177.99		22-24/25	1	
Dithiothreitol, DTT, $HSCH_2CH(OH)CH(OH)CH_2SH$, 154.25	22-36/37/38	26-36	3	EK1610000
Droperidol, $C_{22}H_{22}FN_3O_2$, 379.43	22	36	3	DE2100000
EndoFree plasmid maxi kit	10-35/36/38/11-36-67/42/43	13/26/26/46		
Ethanol, CH_3CH_2OH, 46.07	11	7-16	1	KQ6300000
Ethanolamine solution, C_2H_7NO, 61.08	10-23/24/25-34-39/23/24/25	26-36/37/39-45	1	
Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTAdisodium salt, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, 372.24	36/37/38	26-36/37/39	2	AH4410000
Fentanyl, $C_{22}H_{28}N_2O \cdot C_6H_8O_7$, 528.59	26/27/28-42/43	36/37/39-45	3	UE5600000
Formaldehyde(37% solution), HCHO, 30.03	23/24/25-34-39/23/24/25-40-43	26-36/37/39-45-51	2	
Glutaraldehyde, $OHC(CH_2)_3CHO$, 100.12	22-23-34-42/43	23-26-36/37/39-45	3	

Glycerol, $HOCH_2CH(OH)CH_2OH$, 92.09			1	MA8050000
Glycine·HCl, $H_2NCH_2CO_2H \cdot HCl$, 111.53			2	MC0560000
Hydrochloric acid, HCl, 36.46	34-37	26-36/37/39-45	1	MW4025000
Imject Bovine Serum Albumin	22-36/37/38			
Immobilized TCEP [Tris(2-carboxyethyl)phosphine hydrochloride] Disulfide Reducing Gel	20/22/26/36	36/37/38		
Insulin		22-24/25	3	NM8895000
Isopropyl-β-D thiogalactoside, IPTG, $C_9H_{18}O_5S$, 238.30	19-40-66	36/37	3	
Kanamycin sulfate, $C_{18}H_{36\text{-}37}N_{4\text{-}5}O_{10\text{-}11} \cdot H_2SO_4$		22-24/25	3	
L –Cysteine, $HSCH_2CH(NH_2)CO_2H$, 121.16	22		2	HA1600000
L-glutamine		22-24/25	1	MA2275100
L-thyroxine		22-24/25	3	YP2833500
Magnesium chloride hexahydrate $MgCl_2 \cdot 6H_20$		22-24/25	1	OM2975000
Magnesium chloride $MgCl_2$ 95.21		22-24/25	1	OM2800000
Methanol, CH_3OH, 32.04	11-23/24/25-39/23/24/25	7-16-36/37-45	1	PC1400000
Mts-Atf-Biotin Label Transfer Reagent {2-[*N*2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)–*N*6-(6-biotin-amidocaproyl)-L-lysinyl]ethyl methanethiosulfonate}.	2/20/21/22	36/37		
Nonidet P-40	22-41	26-39	2	
Octyl β-D-glucopyranoside, $C_{14}H_{28}O_6$, 292.37			3	
Paraffin oil	36	26	2	PY8030000
Pierce ECL Western Blotting Substrate	22/53	60/61		
Polyethylene glycol-8000			1	TQ4105000
Potassium acetate, CH_3COOK, 98.14			1	AJ3325000
Potassium carbonate, K_2CO_3,138.21	22-36/37/38	26-36	1	TS7750000
Potassium chloride, KCl, 74.55		22-24/25	1	TS8050000
Potassium dihydrogen phosphate, KH_2PO_4, 136.09			2	TC6615500
Potassium tetrathionate, $KOSO_2SSSO_3K$, 302.45	36/37/38	26-36	3	XF6450000
QIAGEN Plasmid Maxi Kit	10-35/36/38/ 11-36-67/42/43	13/26/26/46		
QIAprep Spin Miniprep Kit	10-35/36/38/ 11-36-67/42/43	13/26/26/46		
QIAquick Gel Extraction Kit	10-35/36/38/ 11-36-67/42/43	13/26/26/46		
QIAquick PCR Purification Kit	10-35/36/38/ 11-36-67/42/43	13/26/26/46		
Reagents from Novagen, (Merck KGaA).				

Silver nitrate, $AgNO_3$, 169.87	34-50/53	26-45-60-61	3	VW4725000
Sodium Acetate, CH_3COONa, 82.03		22-24/25	1	AJ4300010
Sodium azide, NaN_3, 65.01	28-32-50/53	28-45-60-61	2	VY8050000
Sodium bicarbonate, $NaHCO_3$, 84.01			1	VZ0950000
Sodium carbonate, Na_2CO_3, 105.99	36	22-26	1	VZ4050000
Sodium Chloride, NaCl, 58.44			1	VZ4725000
Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \cdot H_2O$, 137.99			1	
Sodium Dodecyl Sulfate, $CH_3(CH_2)_{11}OSO_3Na$, 288.38	11-21/22-36/37/38	26-36/37	2	WT1050000
Sodium iodide, NaI, 149.89	36/38	26	1	WB6475000
Sodium phosphate, Na_2HPO_4, 141.96			1	WC4500000
Sodium pyruvate, $CH_3COCOONa$, 110.04		3		
Sodium thiosulfate pentahydrate, $Na_2S_2O_3 \cdot 5H_2O$, 248.18			1	WE6660000
Sulfo SBED Biotin Label Transfer Reagent [Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'-dithioproprionate].	26/36			
T7Select® Human Brain cDNA Library				
TEMED [1,2-Bis(dimethylamino)ethane], $(CH_3)_2NCH_2CH_2N(CH_3)_2$,116.20	11-20/22-34	16-26-36/37/39-45	1	KV7175000
Tetracycline	22	22-36	3	QI8750000
Thiourea, NH_2CSNH_2, 76.12	22-40-51/53-63	36/37-61	2	YU2800000
Tris(hydroxymethyl)aminomethane, Tris base, $NH_2C(CH_2OH)_3$, 121.14,	36/37/38	26-36	1	
Triton X-100	22-41-51/53	26-36/39-61	1	MD0907700
Tween 20			1	TR7400000
Urea, NH_2CONH_2, 60.06			1	YR6250000
X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside), $C_{14}H_{15}BrClNO_6$, 408.63			3	
Yeast extract			3	ZF6610000

WHC: Water Hazard Class
RTECS: Registry of Toxic Effects of Chemical Substances

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Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig angefertigt zu haben. Hilfe anderer Personen ist, soweit sie in Anspruch genommen wurde, im Text vermerkt.

Diese Arbeit ist zuvor keiner Prüfungsbehörde, weder in dieser noch in geänderter Form, eingereicht worden.

Hamburg, Oktober 2008

Nainesh Katagihallimath