Unraveling the interactions of 14-3-3 with the neuronal proteins L1 and alpha II spectrin

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So läuft diese Wissenschaft schließlich auf eine Hypothese hinaus, die Klarheit versinkt in einer Metapher, die Ungewißheit löst sich in einem Kunstwerk auf... *Absurd ist der Zusammenstoß des Irrationalen mit dem heftigen Verlangen nach Klarheit, das im tiefsten Inneren des Menschen laut wird.*

Albert Camus (1913 – 1960)

<u>Erklärung zur Dissertation</u>

Hiermit erkläre ich, dass ich die Dissertation

Unraveling the interactions of 14-3-3 with the

neuronal proteins L1 and alpha II spectrin

selbstständig verfasst habe und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hamburg, den 18.02.09

(Elisa M. Ramser)

<u>Summary</u>

14-3-3 proteins are abundant in the brain and involved in various neurological disorders in the central nervous system of mammals, suggesting a critical role for these proteins in neuronal function. In search of 14-3-3-binding partners, with the long term goal of gaining a better understanding of the cellular functions of 14-3-3 proteins in the brain, non-erythroid alpha II spectrin and the cell adhesion molecule L1 were identified as potential 14-3-3-target proteins in this thesis work.

Alpha II spectrin from mouse brain membrane fractions was identified as a 14-3-3β-binding partner using affinity chromatography and mass spectrometry. Pull-down experiments using adult mouse brain homogenates confirmed that 14-3-3β associates with alpha II spectrin. An *in vivo* association of these two proteins was shown by co-immunoprecipitation from mouse brain membrane fractions. Alpha II spectrin possesses a putative mode-2 14-3-3-binding motif encompassing Ser^{1302} in its spectrin repetitive unit 12. By mutagenesis analyses, a binding motif in spectrin repetitive unit 12 of alpha II spectrin was identified as the likely binding site for 14-3-3. The identified 14-3-3-binding site is a predicted target for casein kinase II (CK II), suggesting that the interaction is phosphorylation-dependent. Consistent with this prediction, binding *in vitro* of 14-3-3 to an alpha II spectrin fragment encompassing repetitive units 10-14 was more efficient in the presence of CK II. 14-3-3β-binding to alpha II spectrin fragment 10-14 was also enhanced in the presence of calmodulin. It is postulated that calmodulin binding to alpha II spectrin enhances the 14-3-3β – alpha II spectrin interaction by inducing conformational changes in alpha II spectrin. The enhancing effect of calmodulin was somewhat reduced in the presence of EDTA, suggesting that calmodulin is also able to interact with alpha II spectrin at low Ca^{2+} concentration. The ability of 14-3-3β and alpha II spectrin to associate with NCAM in the brain of mice suggests that 14-3-3β might play a role in NCAM-mediated molecular dynamics of cell recognition via the spectrin cytoskeleton.

Two 14-3-3 genes are overexpressed in GFAP/L1 transgenic mice, and overexpression of 14-3-3 in hippocampal neurons causes a specific reduction of L1-mediated neurite outgrowth. It was thus hypothesized that 14-3-3 is involved in downstream signaling of L1 and thereby influences L1 function. In this thesis, co-immunoprecipitation studies confirmed an

association of 14-3-3 with L1 in the brain of mice and suggested that 14-3-3 may directly bind to the intracellular domain of L1 (L1 ICD). ELISA experiments demonstrated that the β and ζ 14-3-3 isoforms directly interact with L1 and pull-down experiments demonstrated that both non-phosphorylated and phosphorylated L1 ICD can bind 14-3-3ζ. A putative 14-3-3-binding motif, RSLESD, was identified in L1 ICD. The second Ser residue in this motif can be phosphorylated by CK II. Using site-directed mutagenesis, this Ser residue was identified as the principal mediator of the L1 ICD interaction with 14-3-3ζ. Notably, phosphorylation of the Ser by CK II was profoundly promoted by 14-3-3ζ. Given evidence that L1 phosphorylation by CK II is required for proper endocytotic trafficking of L1, the distribution of 14-3-3ζ in L1 immunoprecipitates from endosomal fractions was also analyzed. I could show that 14-3-3ζ was enriched only in those immunoprecipitates where L1 amounts were reduced, suggesting a possible role of 14-3-3ζ in the sorting machinery of L1 trafficking from the plasma membrane to endosomes.

Given that alpha II spectrin and L1 have been identified as 14-3-3-binding partners, it is important to further investigate the cellular consequences of these interactions in neurons. Future functional studies based on the findings of this thesis should lead to a better understanding of brain physiology and pathophysiology.

Key words: 14-3-3 proteins, the cell adhesion molecule L1, L1 trafficking, alpha II spectrin

Zusammenfassung

Mehrere Studien haben gezeigt, dass 14-3-3 Proteine in eine Vielzahl an physiologischen und pathologischen Prozessen des Zentralnervensystems involviert sind. Die hohe Abundanz dieser Proteine im Gehirn lässt unter anderem darauf schließen, dass die Mitglieder der 14-3-3-Proteinfamilie eine wichtige Rolle im Zentralnervensystem ausüben. Deswegen wurde mittels der Affinitätschromatographie nach neuen Bindungspartnern im murinen Gehirn gesucht. Als Ergebnis der affinitätschromatographischen und massenspektrometrischen Analyse wurde das nicht-erythrozytäre Alpha II-Spectrin als ein potentieller 14-3-3β-Bindungspartner aus der murinen Membranfraktion identifiziert. Hierbei handelt es sich um ein Protein, das den wesentlichen Bestandteil des neuronalen Membranskeletts ausmacht. Im Rahmen dieser Arbeit wurde die Alpha II-Spectrin – 14-3-3β-Interaktion mittels Pull-down-Assay und Co-Immunpräzipitation bestätigt und die 14-3-3β-Bindungsstelle im Alpha II-Spectrin Molekül näher charakterisiert. Des weiteren konnte gezeigt werden, dass die Proteinkinase Casein kinase II (CK II) die 14-3-3β-Bindung an Alpha II-Spectrin fördert. Weiterhin wurde eine erhöhte Bindung von 14-3-3β an die Alpha II-Spectrin-Untereinheiten 10-14 in Anwesenheit von Calmodulin beobachtet. Calmodulin ist ein bekannter Bindungspartner von Alpha II-Spectrin, der durch die Bindung an die Spectrin-Untereinheit 11 Veränderungen in der Konformation des Spectrins hervorruft. Da eine Komplexbildung von 14-3-3 mit NCAM und Alpha II-Spectrin beobachtet wurde, liegt der Schluss nahe, dass 14-3-3 bei NCAM-vermittelten Strukturveränderungen des Zytoskeletts während des Neuritenwachstums eine Rolle spielt.

In dieser Arbeit wurde auch die mögliche Bindung von 14-3-3ζ an das Zelladhäsionsmolekül L1 untersucht. Vorausgehende Studien der 14-3-3 – L1-Interaktion aus dem Institut Schachner hatten auf einen funktionalen Zusammenhang zwischen den beiden Proteinen hingedeutet. Mittels Co-Immunpräzipitation wurde eine Assoziation dieser Proteine im Gehirn der Maus gezeigt. Mit Hilfe des ELISA-Bindungstests konnte eine direkte Interaktion zwischen der intrazellulären Domäne von L1 (L1 ICD) und 14-3-3β bzw. ζ gezeigt werden. Des Weiteren konnte ich mittels Pull-down-Assay zeigen, dass sowohl phosphoryliertes als auch nicht-phosphoryliertes L1 ICD an 14-3-3ζ bindet. Das Zelladhäsionsmolekül L1 verfügt über einen Serinrest an Position 1181, der von der CK II phosphoryliert wird. Zusammen mit

den benachbarten Aminosäuren bildet er das potentielle Bindungsmotiv RSLESD, welches bekanntermaßen für die L1-Endozytose und somit auch für das L1-vermittelte Neuritenwachstum wichtig ist. Mutationsanalysen ergaben, dass Ser^{1181} die L1 ICD – 14-3-3ζ-Interaktion vermittelt. Weiterhin konnte gezeigt werden, dass in Anwesenheit von 14-3-3ζ die CK II-vermittelte Phosphorylierung von L1 ICD verstärkt wurde. Die verstärkte L1 ICD-Phosphorylierung in Anwesenheit von 14-3-3ζ und die Assoziation von 14-3-3ζ mit L1 in endosomalen Fraktionen sprechen für eine wichtige Funktion von 14-3-3-Proteinen bei der Regulation der Endozytose von L1.

Weitere funktionale Untersuchungen der im Rahmen der vorliegenden Arbeit entdeckten und analysierten Interaktionen von 14-3-3 mit Alpha II-Spectrin bzw. L1 dürften zu einem besseren Verständnis der Physiologie und Pathophysiologie des Gehirns beitragen.

Schlagwörter: 14-3-3-Proteine, das Zelladhäsionsmolekül L1, L1-*trafficking*, Alpha II-Spectrin

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<u>Abbreviations</u>

β	beta
μ	micro (10^{-6})
ζ	zeta
°C	degrees Celsius
Amp	ampicillin
APS	ammonium per sulphate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
FNIII	fibronectin III
g	g-force
GST	glutathione S transferase
ICD	intracellular domain
Ig	immunoglobulin
IgG	immunoglobulin subclass G
IPTG	isopropyl-β-D-thiogalactoside
Kan	kanamycin
kb	kilobase
kD	kilodalton
l	liter
LB	Luria Bertani
m	milli (10^{-3})
min	minute
mRNA	messenger ribonucleic acid
n	nano (10^{-9})

NCAM	neural cell adhesion molecule
OD	optical density
p	pico (10^{-12})
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TEMED	N,N,N',N'-tetraethylenamine
TM	transmembrane
Tris	tris(hydroxmethyl)aminomethane
V	volt
v/v	volume per volume
w/v	weight per volume

Amino acids are abbreviated using the 1- and 3-letter amino acid codes:

- **G** - Glycine (Gly)
- **P** - Proline (Pro)
- **A** - Alanine (Ala)
- **V** - Valine (Val)
- **L** - Leucine (Leu)
- **I** - Isoleucine (Ile)
- **M** - Methionine (Met)
- **C** - Cysteine (Cys)
- **F** - Phenylalanine (Phe)
- **Y** - Tyrosine (Tyr)
- **W** - Tryptophan (Trp)
- **H** - Histidine (His)
- **K** - Lysine (Lys)
- **R** - Arginine (Arg)
- **Q** - Glutamine (Gln)
- **N** - Asparagine (Asn)
- **E** - Glutamic Acid (Glu)

- **D** - Aspartic Acid (Asp)
- **S** - Serine (Ser)
- **T** - Threonine (Thr)

1. Introduction

Signal transduction events in eukaryotic cells regulate the dynamics of cell behaviour by reversible assembly of large multiprotein complexes. Signaling cascades integrate and transmit the information that controls cell cycle progression, patterns of gene expression, programmed cell death and cytoskeletal rearrangements. The orchestration of many signal transduction events is tightly regulated by protein-protein interactions and posttranslational modifications of the signaling pathway components. The most prevalent means of posttranslational modification is probably reversible protein phosphorylation (Cohen, 2002). Within the past few years, the 14-3-3 protein family, whose members bind specific phosphorylated sites on diverse target proteins, has emerged as major regulator of cellular processes in all eukaryotes.

1.1. 14-3-3 protein family

14-3-3 proteins were the first molecules to be recognized as distinct pSer/Thr binding proteins (Fu et al., 2000). The term "14-3-3" denotes a family of dimeric α-helical molecules with diverse cellular functions (Fu et al., 2000). The 14-3-3 proteins were discovered and named during a systematic classification of brain proteins that was based on their fraction number after diethylaminoethyl (DEAE)-cellulose chromatography and their position after subsequent starch gel electrophoresis (Moore and Perez, 1967). However, their potential importance was not recognized until they were identified as activators of neurotransmitter synthesis (Ichimura et al., 1987). The most important step towards understanding 14-3-3 protein action was the discovery that these proteins bind to specific phosphorylated motifs in target proteins (Muslin et al., 1996). Since then, several interacting partners have been identified that participate in cellular processes as diverse as cell cycle control, apoptosis and protein trafficking (Dougherty and Morrison, 2004; Aitken, 2006), demonstrating that 14-3-3 proteins are key mediators of intracellular signaling.

In mammals, 14-3-3 proteins exist as a family of several highly similar yet distinct protein isoforms. At present, seven isoforms, β, ε, γ, η, σ, τ, and ζ, each encoded by a distinct gene, have been identified. Two additional isoforms, α and δ, are the phosphorylated forms of β and ζ (Aitken et al., 1995). The τ and σ isoforms have been found in T-cells and epithelial cells,

respectively (Nielsen, 1991; Prasad et al., 1992; Leffers et al., 1993), whereas the other five isoforms were originally found in the mammalian brain (Ichimura et al., 1988). 14-3-3 proteins occur in the cytoplasmic compartment, at the plasma membrane and in intracellular organelles (Fu et al., 2000). In humans, cattle, rats, and mice, 14-3-3 proteins are abundant in most areas of the central nervous system (CNS). In particular, 14-3-3 isoforms are highly expressed in the pyramidal cells of the hippocampus, the neurons of the cerebral cortex, olfactory bulb neurons, and Purkinje cells of the cerebellum (Watanabe et al., 1991; Watanabe et al., 1993, 1993; Watanabe et al., 1994; Toyooka et al., 2002). Remarkably, each isoform shows a specific distribution in the brain. The ζ isoform is present at high levels in the gray matter of rat brain. The β, γ, η, and τ isoforms are localized in similar areas. The γ isoform is specifically expressed in the nervous system and the β, γ, and η isoforms are enriched in the Purkinje cells of the cerebellum. In contrast, the ε isoform is enriched in the pineal gland and present in significant amounts in the retina, whereas the τ isoform is only found in glia-like cells of the white matter (Watanabe et al., 1993). These varations in distribution and amount of the 14-3-3 isoforms may reflect functional differences or participation in distinct signal transduction pathways in different cell types.

1.1.1. Structure of 14-3-3 proteins

14-3-3 proteins are small, acidic polypeptides with a monomeric molecular mass of 28-33 kDa (Aitken, 2006). Crystallographic studies of the ζ isoform have shown that 14-3-3 proteins form helical, cup-shaped dimers, with each monomer containing nine α-helices organized in an antiparallel manner (Fig. 1). Homo- and heterodimers can be formed from the multiple 14-3-3 isoforms that are present in a given eukaryotic cell. However, only certain combinations are generally observed, most likely a result of steric compatibility (Chaudhri et al., 2003). The three N-terminal helices of one monomer interact with those of the opposing monomer through a combination of salt bridges, hydrogen bonds and van der Waals contacts, forming a central pocket suitable for protein-ligand interactions. The phosphopeptide-binding pocket is the most highly conserved region within and across species. The residues equivalent to Lys[49], Arg[56], Arg[127], and Tyr[128] of human 14-3-3ζ, which bind to the phosphorylated residues of the bound protein, are completely conserved in all 14-3-3 proteins known (Yaffe et al., 1997; Petosa et al., 1998; Rittinger et al., 1999; Obsil et al., 2001; Wurtele et al., 2003). Although the conserved peptide binding groove is principally required for binding, other

regions of the 14-3-3 surface may also influence substrate specificity. For example, the acidic C-terminal region probably prevents non-specific ligand interaction with 14-3-3, by inhabiting the groove in the absence of ligand (Truong et al., 2002; Obsilova et al., 2004).
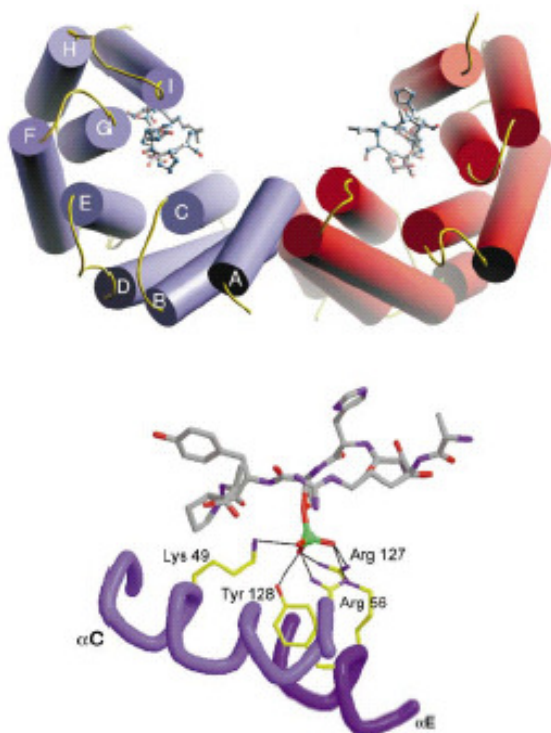
Figure 1: 14-3-3 proteins bind phosphoserine peptides within the binding groove. *Top panel:* Each monomeric subunit of the dimeric 14-3-3 molecule (blue and red, respectively) is composed of nine α helices (αA-αI), with each monomer capable of binding a phosphopeptide (shown in a stick representation). *Bottom panel:* The phosphoserine phosphate from the peptide is coordinated by a basic pocket formed by the critical residues Lys^{49}, Arg^{56}, Arg^{127}, and Tyr^{128}. These 4 residues are located within the αC and αE helices (adapted from (Yaffe and Elia, 2001).

1.1.2. 14-3-3-binding motifs

Amino acid sequence comparisons of 14-3-3-binding partners have revealed two consensus sequence motifs that bind 14-3-3 proteins, **RSXpSXP (mode-1)** and **RXXXpSXP (mode-2)** (where X is any amino acid and pS is phosphoserine) (Muslin et al., 1996; Yaffe et al., 1997). In some 14-3-3-binding partners, phosphothreonine (pT) can replace pS (Muslin et al., 1996; Yaffe et al., 1997). The binding of 14-3-3 to many of its target proteins occurs in a phosphospecific manner. However, some of the identified 14-3-3-binding motifs, for example the sequence KGQSTpSRG of human p53 (Waterman et al., 1998), diverge from the consensus sequences. 14-3-3-binding sites have also been identified that do not require phosphorylation (Yaffe et al., 1997), for example the VTPEER sequence of the amyloid β-protein precursor intracellular domain fragment (Sumioka et al., 2005). More recently, Coblitz *et al.* identified the C-terminal sequence **SWpTX** as a **mode-3** 14-3-3-binding motif (Coblitz et al., 2005). They showed that this motif, when present on the C-terminus of the

Kir2.1 potassium channel, was able to override endoplasmatic reticulum localization signals and facilitate transport of the channel to the plasma membrane. Similar findings with a related, though not identical, C-terminal sequence were also made for members of the TASK family of potassium channels (Rajan et al., 2002).

Target proteins of 14-3-3 containing binding motifs that diverge from the consensus sequence bind the same domain of 14-3-3 proteins as their phosphorylated counterparts (Yaffe et al., 1997). Co-crystal structures of 14-3-3 with peptide fragments containing non-phosphorylated motifs show that the same binding pocket is involved (Petosa et al., 1998), suggesting that non-phosphorylated target proteins can compete with phosphorylated ones for binding to 14-3-3, which increases the level of complexity in 14-3-3-target protein recognition (Wang et al., 1999).

1.1.3. Role of 14-3-3 proteins in physiological and pathological processes of the CNS

Since the first description of 14-3-3 proteins, the biological function of these proteins has been the subject of intense investigation. The first study to assign a specific role to 14-3-3 proteins was that of Ichimura et al. (Ichimura et al., 1987). They showed that 14-3-3 proteins activate tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca2+/calmodulin protein kinase II. Tryptophan 5-monooxygenase and tyrosine 3-monooxygenase are rate-limiting enzymes in catecholamine biosynthesis, and are known targets of different regulatory factors (Ichimura et al., 1987). Structural and biochemical investigations have revealed a crucial role of 14-3-3 proteins in various physiological processes, such as cell growth, adhesion, differentiation, and apoptosis (Aitken, 1996; Pawson et al., 1997; Fu et al., 2000; Toska et al., 2002). 14-3-3 proteins may influence such processes by changing the activity of target proteins, altering protein-protein interactions or redirecting the intracellular localization of certain proteins.

As mentioned above, 14-3-3 proteins are most abundant in the central CNS of mammals, not only during ontogenetic development but also in the adult brain. This points to a critical role of 14-3-3 in neuronal function. The importance of 14-3-3 isoforms in different physiological and pathological processes of the CNS has been shown in a couple of studies.

For example, significantly decreased levels of 14-3-3γ have been observed in the cortex of human embryos with Down's syndrome, suggesting an important role of this isoform in neuronal differentiation and synaptic plasticity (Peyrl et al., 2002). The 14-3-3ε isoform also seems to have an important influence on neuronal migration. Migration of post-mitotic neurons from the ventricular zone to the cortical plate during embryogenesis comprises one of the most critical stages in brain development (Crome, 1956). Deficiency of this process often results in major brain malformations, for example human lissencephaly (smooth brain) (Crome, 1956). In patients with severe lissencephaly, extensive deletions of the chromosome segment that contains 14-3-3ε have been found (Kato and Dobyns, 2003) and mice lacking 14-3-3ε show defects of neuronal migration (Toyo-oka et al., 2003), suggesting that the absence of 14-3-3ε may result in lissencephaly. The precise mechanisms by which 14-3-3ε regulates neuronal migration remain to be fully elucidated, but appear to involve the protein complexes Lis1/Nudel/dynein and Cdk5/p35 (Toyo-oka et al., 2003).

14-3-3 proteins are probably also involved in neurodegenerative diseases caused by the expansion of polyglutamine stretches, in particular spinocerebellar ataxia type 1 (SCA1). In a *Drosophila* model of SCA1, 14-3-3 mediates the neurotoxicity of ataxin-1 by binding to and stabilizing ataxin-1, thereby slowing its normal degradation (Chen et al., 2003). The Chen *et al.* study also showed that the association of ataxin-1 with 14-3-3 is regulated by Akt phosphorylation of ataxin-1 and that both 14-3-3 and Akt modulate neurodegeneration.

The 14-3-3 protein family also appears to play a role in the pathogenesis of Parkinson's disease (PD) and Alzheimer's disease. Xu *et al.* (2002) could show that dopamine-dependent neurotoxicity is mediated by soluble protein complexes that contain α-synuclein and 14-3-3, which are elevated selectively in the substantia nigra in PD. The observation that 14-3-3 binds to α-synuclein in a protein complex suggests that elevated levels of this complex might increase neuronal vulnerability to apoptosis. 14-3-3 inhibits apoptosis by binding and inactivating pro-apoptotic proteins, such as the mitochondrial Bcl-2 family member BAD (Yuan and Yankner, 2000). It has been hypothesized that increased binding to α-synuclein may sequester 14-3-3 and reduce its anti-apoptotic activity (Xu et al., 2002), which, in turn, might increase neuronal vulnerability to reactive oxygen species generated by endogenous dopamine metabolism.

In Alzheimer's disease, the Tau protein becomes abnormally phosphorylated and aggregates into neurofibrillary tangles. Abnormal phosphorylation may prevent Tau from performing its microtubule-related functions, causing cytoskeletal dysfunction and perhaps neurodegeneration (Goedert, 1993). Layfield *et al.* (1996) investigated the localization of 14-3-3 in postmortem Alzheimer's disease brains and detected 14-3-3 in neurofibrillary tangles.

Further investigations showed that Tau interacts with 14-3-3β and 14-3-3ζ. 14-3-3ζ also stimulates Tau phosphorylation at certain Ser residues by protein kinase A. This phosphorylation is sufficient to block Tau-microtubule interaction, causing microtubule instability. These observations suggest that 14-3-3ζ may be a Tau-specific co-factor involved in the abnormal phosphorylation of Tau during Alzheimer's disease development (Hashiguchi et al., 2000).

Studies with *Drosophila* mutants lacking *Leonardo*, the fly ortholog of 14-3-3ζ, revealed an impairment of learning and synaptic plasticity. *Leonardo* is a typical member of the 14-3-3 protein family, which shows 88% amino acid identity to its closest mammalian homolog, 14-3-3ζ (Skoulakis and Davis, 1996). Several data support this study and suggest that 14-3-3 proteins are involved in synaptic function and plasticity (Broadie et al., 1997; Philip et al., 2001; Simsek-Duran et al., 2004).

Taken together, these data from *in vitro* studies, animal models, and *post mortem* analyses of the brains of patients with different neurological disorders point to an important role of the 14-3-3 proteins in CNS development and function.

1.2. Spectrins

Spectrins are a class of multifunctional proteins associated with the cortical cytoplasm of most cells and were first identified at the intracellular surface of the erythrocyte plasma membrane (Goodman et al., 1987). Spectrins are ubiquitous among simple metazoans and vertebrate tissues, and play an important role in cellular functions and maintenance of cell structure (De Matteis and Morrow, 2000; Gascard and Mohandas, 2000; Kordeli, 2000; Bennett and Baines, 2001; Giorgi et al., 2001). Mammalian spectrins are grouped in two classes: erythroid (αSpI or βSpI) and non-erythroid (αSpII or βSpII) spectrins. One of the first identified members in the class of non-erythroid spectrins was fodrin (Levine and

Willard, 1981). Fodrin was first identified in neurons and, as a result, was previously termed brain spectrin. At present, fodrin is more commonly known as alpha II spectrin.

1.2.1. Localization and molecular structure of spectrins

Spectrins are scaffolding proteins that act in association with a variety of adaptor proteins to organize membrane microdomains on the plasma membrane as well as on intracellular organelles. They are present at the plasma membrane, in the Golgi apparatus, in cytoplasmic vesicles, and also in the nucleus (De Matteis and Morrow, 1998; Stankewich et al., 1998; McMahon et al., 1999). Erythroid spectrins structure the erythrocyte its stability and viscoelastic properties. However, the role of spectrins in non-erythroid cells is less clear. They are not required for global membrane support, since they are often highly polarized and lacking in large areas of the plasma membrane. This polarization of spectrin isoforms within neurons has been best characterized in the cerebellum of the mouse (Zagon et al., 1984; Riederer et al., 1986; Zagon et al., 1986; Clark et al., 1994). In murine neurons, two different isoforms of spectrin are distributed to distinct cellular compartments. The existence of an erythroid β and non-erythroid α and β subunit has been shown by immunostaining studies, whereas the presence of the erythroid α subunit in neurons has not been widely accepted (Malchiodi-Albedi et al., 1993; Winkelmann and Forget, 1993).

Non-erythroid spectrins are localized to axons and to presynaptic terminals. For example, immunoelectron microscopy studies by Zagon *et al.* (1986) demonstrated the association of non-erythroid spectrins with the cytoplasmic surface of the plasma membrane and of synaptic vesicles within the presynaptic terminal. Non-erythroid spectrins have also been found in postsynaptic compartments. Alpha II spectrin (previously termed α-fodrin or brain α-spectrin) is expressed in the neuronal dendritic compartment showing strong enrichment toward dendritic spines and postsynaptic densities (Bockers et al., 2001). In fact, alpha II spectrin has been shown to be a major constituent of postsynaptic density (Carlin et al., 1983).

Like erythroid spectrins, most non-erythroid spectrins, in their simplest form, are heterodimers of non-identical α and β subunits, with molecular weights of 280 and 246 kDa, respectively (Bignone and Baines, 2003). The human spectrin family includes two α subunits and five β subunits (De Matteis and Morrow, 2000). Spectrin dimers self-associate head-to-

head into tetramers at the so-called tetramerization site. The α I and β I chains that form spectrin tetramers found in red blood cells interact with lower affinity than the α II and β II chains in non-erythroid cells (Bignone and Baines, 2003).

The basic structural unit of spectrin is a triple helical repeat (Speicher and Marchesi, 1984). The α spectrin chains are usually made up of 21 repeats and β spectrin chains of 17 repeats, except βV, which has 30 repeats (Fig. 2).
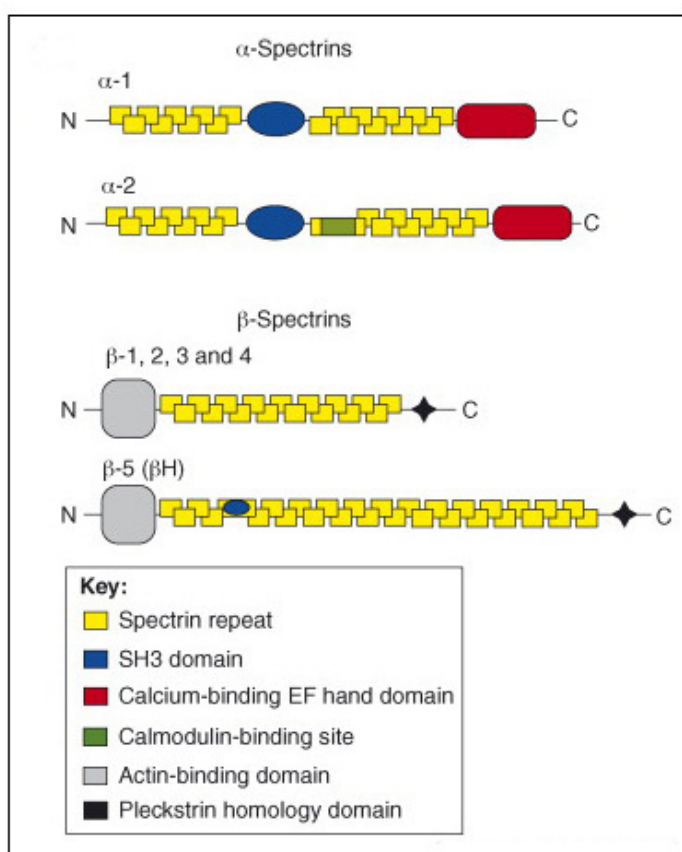
Figure 2: Domain structure of spectrins. Schematics of the two α spectrins and five β spectrins are shown here. Spectrins comprise repetitive units called spectrin repeats (yellow squares). Other domains, such as the Src-homology domain (SH3, blue), EF-hand domain (a protein fold associated with calcium-binding activity) (red), and the calmodulin-binding domain (green) promote interactions with binding partners that are important for spectrin function. The pleckstrin-homology domain (black) promotes association with the plasma membrane via PI lipids, and the actin-binding domain (grey) tethers the spectrin-based membrane skeleton to short actin filaments, which are stabilized by accessory proteins. All spectrins are subject to alternative splicing, which further increases their functional diversity (Bennett and Baines, 2001) (Figure adapted and modified from (Bennett and Healy, 2008)).

The structural units are characteristic of spectrin family members (Bennett and Baines, 2001). They are ~106 amino acids long and folded in a coiled-coil structure made up of three helices (Speicher and Marchesi, 1984; Davison et al., 1989; Parry et al., 1992). In addition to these repeat units, spectrin isoforms can also contain several functional domains, such as an SH3

domain, EF hands, PH domains, and binding domains for ankyrin, actin, protein 4.1, and calmodulin (Bennett and Baines, 2001).

Non-erythroid alpha II spectrin exhibits a low degree of homology (58% amino acid identity) with erythroid alpha I spectrin. The main difference is a non-homologous sequence inserted into the 11th repeat unit of the vertebrate alpha II spectrin subunit. This insertion is absent in erythroid alpha I spectrin. The non-homologous sequence is located near the SH3 domain and calmodulin binding site, and contains several cleavage sites for different proteases, including μ-calpain and caspases (Harris et al., 1988; Rotter et al., 2004).

1.2.2. Function of spectrins

The involvement of spectrins in many diverse cellular processes can be explained by their distinct patterns of distribution and by their modular structure that combines numerous protein-interacting domains. As the major component of the cytoskeletal network associated with the plasma membrane of vertebrate cells, spectrin, together with actin and ankyrin, controls the distribution of many integral and peripheral membrane proteins. Mutations in spectrin or its associated adapter protein ankyrin often destabilize the membrane, and their absence is embryonically lethal (Peters et al., 1992; Deng et al., 1995).

Spectrins are tethered to cellular membranes by protein–protein interactions. The best studied spectrin-membrane adaptors are the ankyrins. Ankyrins are a family of peripheral membrane proteins controlling the interactions of spectrins with various transmembrane proteins (Bennett, 1992). They are known to bind to the beta spectrin subunit (Goodman et al., 1988). Ankyrins link spectrins to a number of physiologically important transmembrane proteins, including Na,K-ATPase, and the voltage-gated Na^{+} channel (Bennett, 1992).

Ankyrins can also bind cell adhesion molecules (CAMs) of the CD44 family (Kalomiris and Bourguignon, 1988) and the L1 CAM family (e.g., L1, neurofascin, NrCAM, NgCAM in the vertebrate nervous system) (Davis and Bennett, 1994; Dubreuil et al., 1996). Evidence for L1 CAM-ankyrin interactions includes findings that L1 CAM-members co-localize with ankyrin at nodes of Ranvier and axon initial segments (Davis et al., 1996) and are missorted in ankyrin-G-deficient-mice (Zhou et al., 1998).

Ankyrin-independent association of spectrins with membrane proteins is also possible. Spectrins can directly bind to NMDAR and acetylcholine receptors, and anchoring them within the postsynaptic density (Bloch and Morrow, 1989; Daniels, 1990; Bloch et al., 1997; Wechsler and Teichberg, 1998; Hirai and Matsuda, 1999). Spectrin-NMDAR interactions might form the basis for the actin-mediated regulation of NMDAR channel activity and represent one of the events leading to the plasticity-induced changes in spine morphology (Wechsler and Teichberg, 1998). Thus, receptor anchoring via spectrin at postsynaptic sites may regulate synaptogenesis and/or synaptic plasticity. Another example for an ankyrin-independent binding partner of spectrins is NCAM 180, which previously has been demonstrated to bind brain spectrin (Pollerberg et al., 1987). This interaction is thought to limit the mobility of NCAM 180 within the plasma membrane (Pollerberg et al., 1986) and might also play a role in the control of NCAM-mediated signaling (Leshchyns'ka et al., 2003).

Spectrin function is regulated by proteolysis, which is catalyzed by Ca^{2+}-dependent proteases, in particular μ-calpain, as well as calmodulin, and phosphorylation. Calcium-dependent proteolytic modification of the alpha II spectrin subunit, particularly by μ-calpain, is linked to several physiological processes, such as the onset of long-term potentiation in hippocampal neurons, dendritic and postsynaptic density remodeling, and receptor-mediated endocytosis (Lynch and Baudry, 1984; Sheppard et al., 1993; Bahr et al., 1995; Bednarski et al., 1995; Dosemeci and Reese, 1995; Vanderklish et al., 1995; Faddis et al., 1997; Kamal et al., 1998). Cleavage of spectrin by μ-calpain is not only part of important physiological processes but it also follows hypoxic or ischemic injury (Seubert et al., 1989) and has been observed during apoptosis in neurons (Nath et al., 1996). The molecular basis for these divergent consequences of μ-calpain action, and their relationship to spectrin proteolysis, is not well understood.

Calmodulin plays, among other things, an important role in synapse formation by binding to the alpha II spectrin subunit. Specifically, calmodulin binds to the above-mentioned non-homologous sequence inserted in the 11th repeat unit of the vertebrate alpha II spectrin subunit (Harris et al., 1988; Simonovic et al., 2006). Calmodulin binding has been shown to enhance the susceptibility of a peptide bond (Tyr^{1176}–Gly^{1177}) near the calmodulin-binding site on alpha II spectrin to cleavage by μ-calpain and also renders the adjacent beta II spectrin subunit susceptible to μ-calpain cleavage (Glantz et al., 2007). The sensitivity of the cleavage site on the beta II spectrin subunit (Gln^{1441}–Ser^{1442}) is also regulated at the substrate level by

the phosphorylation of Tyr^{1176}, which is mediated by src-family kinases and low-molecular-weight phosphotyrosine phosphatase (Nicolas et al., 2002; Nedrelow et al., 2003).

Thus, two Ca^{2+}-dependent processes, calmodulin binding and calpain proteolysis, unless suppressed by Tyr phosphorylation of alpha II spectrin, act synergistically to regulate the proteolysis of spectrin and the organization and integrity of the cortical membrane skeleton. These events can occur in response to an elevation in Ca^{2+}-ion levels (Harris and Morrow, 1990) and synaptic activity (Vanderklish et al., 1995). Morphological alterations of the cytoskeleton during synaptic plasticity allows a rapid remodeling of synapses after stimulation.

1.2.3. Spectrin and 14-3-3

Several studies have indicated the involvement of 14-3-3 proteins in cellular processes and various neurological disorders in the brain. Based on these studies, we previously sought to identify further 14-3-3-binding target (Ramser, 2005). Using affinity chromatography and ESI tandem mass spectrometry (MS/MS) analysis, non-erythroid alpha II spectrin was identified as a potential 14-3-3-binding protein (Ramser, 2005). However, experiments to confirm the mass spectrometry data and characterize the possible interaction between 14-3-3 and alpha II spectrin were not performed previously.

1.3. Neural cell adhesion molecules of the immunoglobulin superfamily

Neural CAMs are key mediators of neuronal interactions (Hortsch, 1996; Kenwrick et al., 2000; Chen et al., 2007; Maness and Schachner, 2007). Their roles during the developmental stages of the nervous system include cell migration, axon guidance, synaptic targeting, and synapse formation. In the mature nervous system, their principal role is preserving synaptic connections, cell-cell contacts, and neuron-glial interactions. One group of the well-studied CAMs in the nervous system is the immunoglobulin (Ig) superfamily, which includes L1 and NCAM.

1.3.1. L1 CAM

L1 is a member of a subfamily of vertebrate CAMs that are related by structure and sequence (e.g., L1, NgCAM, NrCAM, neurofascin, CHL1, and neuroglian). These molecules mediate cell-cell adhesion through Ca^{2+}-independent homophilic and heterophilic binding at the cell surface (Rathjen and Schachner, 1984; Grumet, 1991). L1 was first identified as a transmembrane glycoprotein in the CNS of mice (Lindner et al., 1983; Rathjen and Schachner, 1984).

1.3.2. Molecular structure and localization of L1

The L1 molecule is comprised of an extracellular region of six Ig-like domains, five fibronectin type III repeats, a single membrane-spanning region, and a highly conserved cytoplasmic domain (Fig. 3). L1 or L1-like molecules with this structural architecture have been identified in a variety of species. In all species investigated so far, these molecules have proved to be important components of the ligand-receptor network of guidance forces that influence axonal growth (Rathjen and Schachner, 1984; Grumet, 1991; Brummendorf and Rathjen, 1995; Hortsch, 1996).
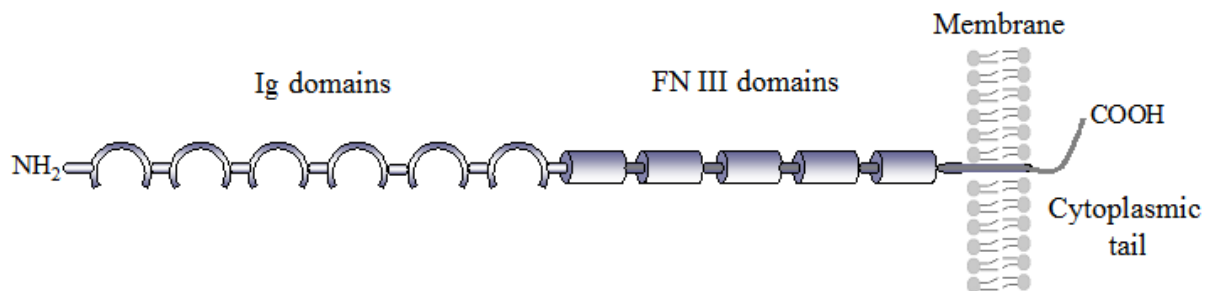
Figure 3: Modular structure of L1 CAM. L1 belongs to the Ig superfamily. It is a transmembrane glycoprotein that contains six Ig-like, five fibronectin type III-like extracellular domains, a single membrane-spanning region and a cytoplasmic tail.

In mammals, L1 expression has been observed throughout the nervous system in developing neurons and on the axons of many differentiated nerve cells. Axonal expression of L1 seems to be concentrated at the surface that makes contact with neighboring axons, suggesting that L1 may be involved in the development of axon bundles. However, L1 is also found on growth cones, which are responsible for sensing extracellular guidance cues. Although primarily neuronal, L1 is also found on the Schwann cells of the peripheral nervous system

when they make contact with axons, suggesting that L1 is implicated in the onset of myelination (Seilheimer and Schachner, 1988; Martini et al., 1994). L1 is also expressed by a subclass of leukocytes and on intestinal cells, indicating that L1 also has functions outside the nervous system (Thor et al., 1987; Kadmon and Altevogt, 1997). Because L1 is a cell adhesion molecule, its role in tumor metastasis has also been investigated (Johnson, 1991).

1.3.3. Function of L1 in axonal outgrowth and neuronal migration

L1 mediates cell migration, axon outgrowth and guidance, branching, and synaptogenesis in the nervous system (Hortsch, 1996; Chen et al., 2007). The ICD of neuronal L1 contains four additional amino acids (RSLE) compared to the L1 ICD expressed in non-neuronal cells (Miura et al., 1991). These extra residues are encoded by the alternatively spliced exon 27 and are important in several L1 functions. Several L1-associated cytosolic molecules participating in axon outgrowth and guidance have also been identified (Davis and Bennett, 1993; Kamiguchi et al., 1998; Dickson et al., 2002). However, the structural basis for L1-mediated intracellular signaling and cell remodelling has still not been unravelled.

The function of L1 in regulation of axonal outgrowth and neuronal migration is dependent on CAM-mediated signaling to the inside of the neuron, rather than passive adhesion, and also requires coordination with the actin cytoskeleton. L1 does not bind directly to the actin cytoskeleton; rather, its interaction is regulated by membrane-cytoskeletal linker proteins, such as ankyrin and the ezrin, radixin, and moesin (ERM) protein family. Ezrin interacts directly with the L1 ICD and this interaction plays an important role in neurite branching (Turunen et al., 1994; Chishti et al., 1998; Dickson et al., 2002; Cheng et al., 2005). The interaction between L1 and ezrin involves two major sites in L1, one of which is the [1176]YRSLE region within the neuronal isoform of L1 ICD (Dickson et al., 2002; Cheng et al., 2005). Dynamic regulation of the cell surface expression of adhesion molecules is also an important mechanism for controlling neuronal growth cone motility and guidance. L1-controlled endocytosis plays a crucial role in the motility of the nerve growth cones (Kamiguchi and Yoshihara, 2001).
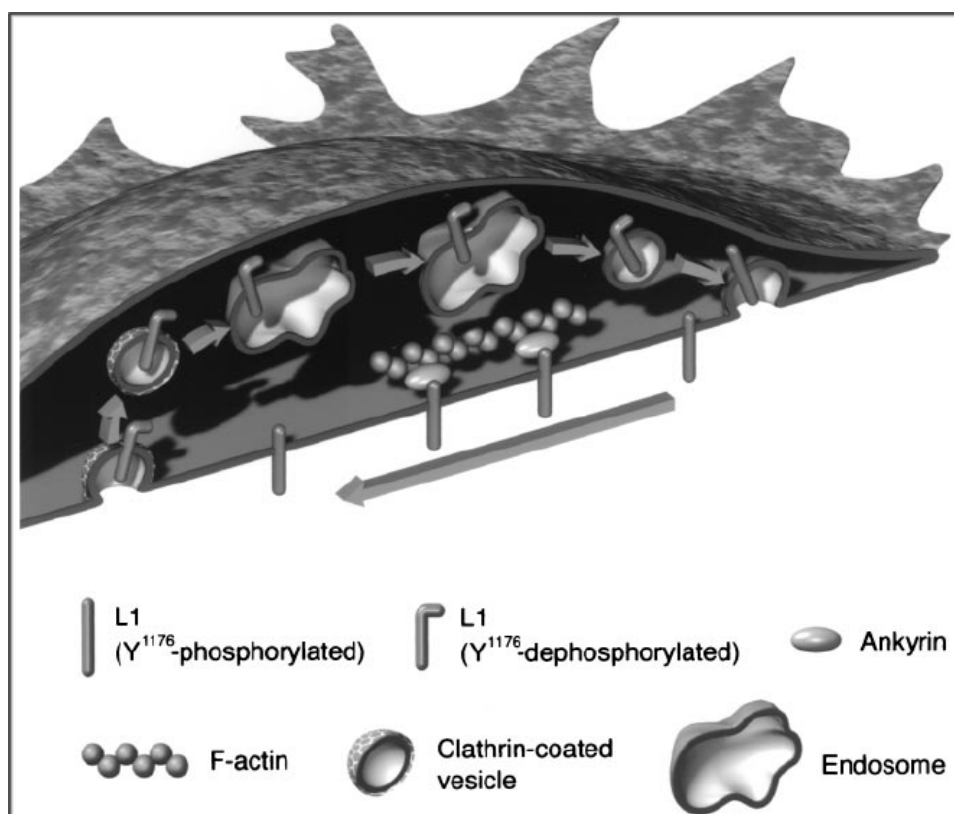
Figure 4: A model of L1's internalization and trafficking in the axonal growth cone. L1 is internalized from the plasma membrane (*left*) at the C-domain via clathrin-mediated pathways. Endocytosed L1 is transported into the P-domain via sorting and recycling endosomes. Recycled L1 is reinserted into the plasma membrane at the leading edge (*right*). The ability of L1 to interact with clathrin adaptors is regulated by phosphorylation. Recycled L1 on the cell surface moves toward the C-domain by coupling to the retrogradely moving actin filaments via ankyrin or other linker proteins (Figure adapted from (Kamiguchi and Lemmon, 2000)).

The amino acid sequence ^{1176}YRSLE within the L1 ICD enables recruitment of L1 to the AP2-clathrin adapter for endocytosis (Kamiguchi et al., 1998). L1 internalization by clathrin-mediated endocytosis within the central domain of the growth cone leads to recycling of L1 to the front, promoting motility through new adhesive contacts at the leading edge and the detachment of old adhesions (Fig. 4) (Kamiguchi and Lemmon, 2000). Endocytosis of L1 is regulated by pp60c-src, which can phosphorylate the cytoplasmic ^{1176}YRSLE motif, thus inhibiting L1 binding to AP2-clathrin (Schaefer et al., 2002). The L1 ICD contains a Ser residue (Ser^{1181}) adjacent to the AP-2 binding site that can be phosphorylated by casein kinase II (CK II) (Wong et al., 1996). Although the possible role of Ser^{1181} in L1 function has not been investigated so far, there is evidence that L1 phosphorylation at this site by CK II is required for proper endocytic trafficking of L1 and for L1-stimulated axon outgrowth (Nakata

and Kamiguchi, 2007). The findings by Nakata and Kamiguchi also suggested that sorting pathways following L1 internalization might be regulated by Ser[1181] phosphorylation.

1.3.4. L1 and 14-3-3

The GFAP/L1 mouse is a transgenic mouse expressing L1 ectopically in glial fibrilliary acidic protein (GFAP)-expressing astrocytes. This transgenic mouse model was generated to study the effects of L1 on learning and synaptic plasticity (Wolfer et al., 1998). Results from several experiments performed with this model led to the hypothesis that L1-expression in astrocytes alters gene expression, thereby leading to increased flexibility and selectivity in spatial learning. Serial analysis of gene expression (*SAGE*) in GFAP/L1 mice hippocampi also revealed an overexpression of 14-3-3β and ζ genes (R. Löbbert, U. Wirkner, and O. Kreft, Lion BIOSCIENCE AG, Heidelberg; unpublished observations). The observation that 14-3-3 genes were overexpressed in this transgenic mouse model led to the hypothesis that 14-3-3 could be involved in downstream signaling of L1 and thereby influence L1 function. Data from the Schachner laboratory also suggested a possible functional connection between L1 and 14-3-3: overexpression of 14-3-3 led to a specific reduction of neurite length in L1-mediated neurite outgrowth (T. Tilling, unpublished results). Previously, attempts were made to demonstrate an interaction between L1 and 14-3-3 but results were inconclusive (Ramser, 2005).

1.4. Aim of this study

14-3-3 proteins are most abundant in the CNS of mammals, not only during ontogenetic development but also in the adult brain, pointing to a critical role for 14-3-3 in neuronal function. Results from previous experimentation had suggested that non-erythroid alpha II spectrin and L1 may interact with 14-3-3. The aim of this study was to demonstrate and specifically characterize the interactions of these two neuronal proteins with 14-3-3, by employing molecular biological and biochemical approaches.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals were obtained in analytical grade quality from the following companies: BioRad Laboratories (Munich, Germany), PerBio Science (Bonn, Germany), Santa Cruz Biotechnology (Heidelberg, Germany), SERVA Electrophoresis (Heidelberg, Germany), Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) and Th. Geyer (Hamburg, Germany).

Plasmids and molecular cloning reagents were obtained from Invitrogen (Karlsruhe, Germany), Stratagene (Waldbronn, Germany) and Qiagen (Hilden, Germany). Restriction enzymes were obtained from New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were ordered from metabion (Munich, Germany). All nucleotides used in this thesis work are listed in the Appendix section. Plasmid Maxi Kit and QIAquick Gel Extraction Kit were obtained from Qiagen (Hilden, Germany). GFX Micro Plasmid Prep Kit was obtained from GE Healthcare (München, Germany). Cell culture materials were obtained from PAA Laboratories GmbH (Cölbe, Germany).

2.1.2. Buffers and solutions

0.25 M sucrose in Tris buffer (For isolation of endosomes)	0.25 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
0.32 M sucrose in Tris buffer (For whole brain homogenization and membrane fraction isolation)	0.32 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4

0.5 M sucrose in Tris buffer (For isolation of endosomes)	0.5 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
0.8 M sucrose in Tris buffer (For isolation of endosomes)	0.8 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
1 M sucrose in Tris buffer (For membrane fraction isolation)	1 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
1.1 M sucrose in Tris buffer (For isolation of endosomes)	1.1 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
1.2 M sucrose in Tris buffer (For membrane fraction isolation)	1.2 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
1.3 M sucrose in Tris buffer (For isolation of endosomes)	1.3 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$

1 mM NaHCO$_3$

5 mM Tris-HCl pH 7.4

2 M sucrose in Tris buffer (For isolation of endosomes)	2 M sucrose 1 mM MgCl$_2$ 1 mM CaCl$_2$ 1 mM NaHCO$_3$ 5 mM Tris-HCl pH 7.4
Blocking solution (For ELISA)	1% BSA in TBS
Blocking solution (For protein immunostaining)	5% skimmed milk powder in TBST or PBST
Buffer A (For ELISA)	TBS 1% BSA 1 mM CaCl$_2$ 1 mM MgCl$_2$ 0.05% Tween
μ-Calpain proteolysis buffer (μ-Calpain proteolysis assay)	20 mM Tris pH7.4 25 mM NaCl 0.15 mM CaCl$_2$ 0.03 µg μ-Calpain (Sigma)
DNA-sample buffer (5x concentrated) (For DNA agarose gels)	20% (w/v) glycerol in TAE 0.025% (w/v) orange G
DNA elution buffer	10 mM Tris-HCl pH 8.0
Elution buffer	50 mM Tris-HCl pH 7.4

(For GST protein purification)	20 mM glutathione, reduced
Elution buffer (For His protein purification)	50 mM NaH_2PO_4 300 mM NaCl 250 mM imidazole pH 8.0
Ethidium bromide solution (For DNA agarose gels)	10 µg/ml ethidium bromide in 1xTAE
Lysis buffer (For GST protein purification)	50 mM K_2HPO_4 400 mM NaCl 100 mM KCl 10% (v/v) glycerol 0.5% (v/v) Triton X-100 10 mM imidazole Protease Inhibitor Cocktail EDTA-free (Roche)
Lysis buffer (For His protein purification)	50 mM NaH_2PO_4 300 mM NaCl 10 mM imidazole pH 8.0
Modified RIPA buffer (For brain homogenates and membrane fractions)	50 mM Tris-HCl pH 7.4 150 mM NaCl 2 mM EDTA 1 mM NaF 1 mM Na_3VO_4 1% NP-40 0.5% SDS 100 µM PMSF (added just before use) Protease Inhibitor Cocktail EDTA-free (Roche)

Phosphatase Inhibitor Cocktail I (Sigma-Aldrich)

Modified RIPA buffer (For cell lysates)	50 mM Tris-HCl pH 7.4
	150 mM NaCl
	1 mM EDTA
	1 mM NaF
	1 mM Na_3VO_4
	1% NP-40
	100 µM PMSF (add just before use)
	Protease Inhibitor Cocktail EDTA-free (Roche)
	Phosphatase Inhibitor Cocktail I (Sigma-Aldrich)
PBS	150 mM NaCl
	10 mM Na_2HPO_4
	2.5 mM NaH_2PO_4
	3 mM KCl
	pH 7.4
PBST (For protein immunostaining)	150 mM NaCl
	10 mM Na_2HPO_4
	2.5 mM NaH_2PO_4
	3 mM KCl
	0.05% Tween-20
PBSCM	PBS with Ca^{2+} and Mg^{2+} (PAA)
Pull-down buffer	20 mM Tris pH 7.4
	100 mM NaCl
Quenching solution (For cell surface biotinylation)	20 mM glycine in PBSCM

Sample buffer with DTT for protein gels (4x concentrated)	8% SDS 320 mM Tris-HCl pH 6.8 40% Glycerol 10 mg/ml DTT 0.1% bromphenol blue
SDS-PAGE running buffer	192 mM glycine 25 mM Tris 0.1% SDS
Staining solution (For Coomassie staining of SDS-PAGE gels)	40% (v/v) ethanol 10% (v/v) acetic acid 0.1% (w/v) Coomassie Brilliant blue R250
Stripping buffer (For protein immunostaining)	25 mM glycine 1 % SDS pH 2.0
Substrate solution (For ELISA)	100 mM sodium acetate pH 5.0 2% ABTS 30 % H_2O_2
Sulfo-NHS-LC-Biotin (Pierce) (For cell surface biotinylation)	in PBSCM
TBS (10x concentrated)	100 mM Tris 1.5 M NaCl pH 7.4
TBST (For protein immunostaining)	10 mM Tris 150 mM NaCl 0.05% Tween-20

pH 7.4

Transfer buffer	192 mM glycine
(For Western blotting	25 mM Tris
of SDS-polyacrylamide gels)	10-20% methanol

Tris EDTA acetate (TAE)	2 M Tris-Acetate, pH 8.0
DNA gel running buffer	100 mM EDTA
(50x concentrated)	

Tris buffer	5 mM Tris-HCl pH 7.4
(For membrane fraction	Protease Inhibitor Cocktail EDTA-free (Roche)
isolation)	

Washing buffer	PBS
(For GST protein	1% Triton X-100
purification)	1 mM DTT

Washing buffer	50 mM NaH_2PO_4
(For His protein	300 mM NaCl
purification)	20 mM imidazole
	pH 8.0

2.1.3. Primary antibodies

Primary antibodies used in this thesis, their characteristics, and sources:

Name	Host	Epitope	Source
14-3-3 (H8)	Mouse	N-terminus of human 14-3-3β	Santa Cruz Biotechnology
14-3-3β	Rabbit	N-terminus of human 14-3-3β	Immunobiological Laboratories

14-3-3ζ	Rabbit	N-terminal part of human 14-3-3ζ (residues MDKNELVQK)	Immunobiological Laboratories
Alpha II spectrin (non-erythroid)	Mouse	C-terminus of non-erythroid alpha II spectrin (15-20 repetitive units)	Chemicon International
c-Myc (9E10)	Mouse	C-terminal domain of human c-Myc (residues 408-439)	Santa Cruz Biotechnology
GST	Goat	Unknown	GE Healthcare
L1 557	Rat	Fibronectin type III repeats 2 and 3 (FN_{III}2/3) of the L1 extracellular domain	Schachner laboratory
L1 Cell Adhesion Molecule Ab-1 (Clone UJ127)	Mouse	Unknown	Dianova GmbH
L1 CAM	Rabbit	Extracellular domain of murine L1	PINEDA
L1 Monoclonal Antibody	Mouse	Cytoplasmic domain of murine L1 (adjacent to the YRSL sorting signal)	HISS Diagnostics GmbH
NCAM 1β1	Rabbit	Extracellular domain of murine NCAM	Schachner laboratory
NCAM 1β2	Rabbit	Extracellular domain of murine NCAM	Schachner laboratory
Penta-His	Mouse	N-terminal, C-terminal, and internal 6xHis tags	Qiagen GmbH

2.1.4. Secondary antibodies

Secondary antibodies used in this thesis and their sources:

Name	Source
Peroxidase-conjugated mouse anti-rabbit IgG (H + L)	Jackson ImmunoResearch laboratories; purchased through Dianova GmbH (Hamburg, Germany).
Peroxidase-conjugated goat anti-mouse IgG + IgM (H + L)	Jackson ImmunoResearch laboratories; purchased through Dianova GmbH (Hamburg, Germany).
Peroxidase-conjugated donkey anti-mouse IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Rb, Shp Sr Prot)	Jackson ImmunoResearch laboratories; purchased through Dianova GmbH (Hamburg, Germany).
Peroxidase-conjugated donkey anti-rabbit IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Ms, Rat, Shp Sr Prot)	Jackson ImmunoResearch laboratories; purchased through Dianova GmbH (Hamburg, Germany).

2.1.5. Bacterial and mammalian cell culture medium

Bacterial media were autoclaved and antibiotics were supplemented prior to use.

DMEM	Dulbecco's MEM, high glucose
(For HEK 293 cells)	10% fetal calf serum
	20 ml/l penicillin/streptomycin solution (100x)
	10% L-Glutamine
	10% sodium pyruvate

LB medium	10 g/l Bacto-tryptone
	10 g/l Na Cl
	5 g/l Yeast extract
LB ampicillin medium	100 mg/l ampicillin in LB medium
LB ampicillin plates	20 g/l agar in LB medium
	100 mg/l ampicillin
LB kanamycin medium	25 mg/l kanamycin in LB medium
LB kanamycin plates	20 g/l agar in LB medium
	25 mg/l kanamycin
LB ampicillin/kanamycin medium	100 mg/l ampicillin
	25 mg/l kanamycin in LB medium
LB ampicillin/kanamycin plates	20 g/l agar in LB medium
	100 mg/l ampicillin
	25 mg/l kanamycin
LB chloramphenicol/ ampicillin plates	20 g/l agar in LB medium
	34 mg/l chloramphenicol
	100 mg/l ampicillin

2.1.6. Bacterial cells

E. coli BL21(DE3)   F⁻, *ompT, hsdSB ($r_b^- m_b^-$), gal dcm* (DE3)
(For protein overexpression) Novagene, VWR International GmbH (Darmstadt, Germany)

E. coli BL21(DE3)pLysS   F⁻, *ompT, hsdSB ($r_b^- m_b^-$), gal dcm* (DE3) pLysS (Cam^R)
(For protein overexpression) Invitrogen GmbH (Karlsruhe, Germany)

E. coli BL21-AI^{TM} (For protein overexpression)	Invitrogen GmbH (Karlsruhe, Germany)
E. coli DH5α (For cloning)	F$^-$, φ80d*lac*ZΔM15, Δ(*lacZYA-argF*)U169, *deoR, recA1, endA1, hsdR17*(rk^{--},mk$^+$), *phoA*, *supE44*, λ-, *thi*-1, *gyrA*96, *rel*A1 New England Biolabs GmbH (Frankfurt am Main, Germany)
E. coli TOP10F' (For cloning)	F' *lac*Iq, Tn*10*(TetR) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
E. coli M15 (For protein overexpression)	derived from *E. coli* K12 NaI^S, Str^S, Rif^S, Thi$^-$, Lac$^-$, Ara$^+$, Gal$^+$, Mtl$^-$, F$^-$, RecA$^+$, Uvr$^+$, Lon$^+$ Qiagen GmbH (Hilden, Germany)

2.1.7. Cell lines

Mammalian cell lines were utilized for ectopic protein expression.

HEK293 cells	*Human Embryonic Kidney* (HEK) epithelial cell line
SH-SY5Y cells	Derivative of neuroblastoma cell line SK-N-SH

2.1.8. Plasmid constructs

pcDNA3-L1	For expression of full length neuronal L1 in mammalian cells. Obtained from A. Rünker, ZMNH, Germany

pcDNA3/6xmyc	Mammalian expression vector for 6x myc-tagged proteins. Derived from pcDNA3 vector (Invitrogen) and generated in the Schachner laboratory
pcDNA3/6xmyc – 14-3-3β	For expression of 6x myc-tagged full-length 14-3-3β in mammalian cells
pcDNA^{TM}3.1/myc-his – alpha II spectrin 10-14	For expression of myc-histidine-tagged alpha II spectrin 10-14 in mammalian cells
pcDNA^{TM}3.1/myc-his – alpha II spectrin 10-14	For expression of myc-histidine-tagged alpha II spectrin 10-14 mutant S1302A in mammalian cells. Generated by S1302A site-directed mutation of Ser^{1302} to Ala in pcDNA^{TM}3.1/myc-his – alpha II specrin 10-14
pCI - alpha II spectrin	For expression of flag-tagged full length non-erythroid alpha II spectrinin mammalian cells. Provided by Dr. J. Morrow, Yale University School of Medicine, New Haven, CT, USA
pDEST15 - 14-3-3β	For overexpression of glutathione S transferase (GST)-tagged full length 14-3-3β protein in bacteria. Obtained from Dr. H. Fu, Emory University, Atlanta, GA, USA
pDEST15 – 14-3-3ζ	For overexpression of GST-tagged 14-3-3ζ protein in bacteria Obtained from Dr. H. Fu, Emory University, Atlanta, GA, USA
pDEST26 - 14-3-3ζ	For expression of histidine-tagged 14-3-3ζ in mammalian cells Obtained from Dr. H. Fu, Emory University, Atlanta, GA, USA
pDEST26 - 14-3-3ζ K49E	For expression of histidine-tagged 14-3-3ζ K49E in mammalian cells. Obtained from Dr. H. Fu, Emory University, Atlanta, GA, USA

pGEX-2T - 14-3-3β	For overexpression of GST-tagged 14-3-3β in bacteria. Provided by Dr. Y. Takihara, Osaka University, Osaka, Japan
pGEX-4T-2	Expression vector for GST-tagged recombinant proteins in bacteria
pGEX-4T-2 – alpha II spectrin 10-14	For overexpression of GST-tagged alpha II spectrin 10-14 in bacteria
pGEX-4T-2 – alpha II spectrin 10-14-S1302A	For overexpression of GST-tagged alpha II spectrin 10-14 in mutant S1302A in bacteria. Generated by mutating Ser^{1302} to Ala in pGEX-4T2 – alpha II spectrin 10-14
pQE30 – µ2	For overexpression of histidine-tagged µ2 subunit of the adaptor protein AP2 in bacteria. Provided by Dr. V. Haucke, Freie Universität Berlin, Germany
pQE30 – L1 ICD	For overexpression of histidine-tagged L1 ICD in bacteria. Obtained from G. Wolters, ZMNH, Germany
pQE30 – L1 ICD-S1181A	For overexpression of histidine-tagged L1 ICD mutant S1181A in bacteria. Generated by of Ser^{1181}-> Ala mutation in pQE30 – L1 ICD
pQE30 – L1 ICD ΔRSLESD	For overexpression of histidine-tagged L1 ICD with a RSLESD-motif deletion in bacteria. RSLESD sequence deleted in pQE30 – L1 ICD generated by PCR

2.1.9. DNA and protein standards

1 kb DNA ladder	Invitrogen GmbH
Precision Plus Protein ^{TM}	Bio-Rad Laboratories GmbH

Dual Color Standards

2.2. Methods

2.2.1. Molecular biology

2.2.1.1. Transformation of chemically-competent bacteria

(Sambrook, 1989)

To 100 µl of competent BL21(DE3), BL21(DE3)pLysS, DH5α, M15 or TOP10F' cells, either 50-100 ng of plasmid DNA, 10 µl of DNA ligation mixture (see 2.1.3b), or 10 µl of DpnI-treated PCR-mutagenesis mixture (see 2.1.8) were added and incubated on ice for 30 minutes. After a heat shock (2 minutes, 42°C) and a brief incubation period on ice (3 minutes), 800 µl of either LB or SOC medium (without antibiotics) were added to the bacteria and the cells were incubated at 37°C for 1 h. The cell suspension was then centrifuged (1,000 x g, 1 minute, RT) and the supernatant removed. The cells were resuspended in 50 µl of LB medium and aliquots of suspension were plated on LB plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight.

2.2.1.2. Plasmid isolation

All plasmids were isolated using Qiagen or GE Healthcare plasmid preparation kits (see 2.1.1) according to the respective manufacturer's instructions.

2.2.1.3. DNA restriction and ligation

2.2.1.3a. Restriction digestion of DNA

All restriction digestions were carried out using restriction enzymes supplied by New England Biolabs (NEB) in accordance with the supplied instructions and technical references (http://www.neb.com).

2.2.1.3b. Ligation of DNA fragments

(Rapid ligation Kit, Roche)

Ligation of DNA fragments was performed by mixing 10 fmol of vector DNA with either 30 or 50 fmol of insert DNA in 1x concentrated DNA dilution buffer. 1 µl of T4 DNA ligase and 10 µl of ligation buffer were added to a final volume of 40 µl. The reaction was incubated overnight at RT. 10 µl of the ligation mixture were used for transformation of bacteria.

2.2.1.4. DNA gel electrophoresis
(Sambrook et al., 1989)

DNA fragments were separated in agarose gels by horizontal electrophoresis in DNA electrophoresis chambers (Bio-Rad Laboratories). To prepare the gels, agarose (1% w/v) was dissolved in 1x concentrated TAE buffer by heating, poured into gel electrophoresis trays, and allowed to solidify. Thereafter, the gels were placed in appropriate electrophoresis chambers and covered with 1x concentrated TAE buffer. DNA samples for electrophoresis were prepared by mixing the necessary volume of DNA with DNA sample buffer. The mixtures were pipetted into the agarose gel sample pockets and the gel was run at constant voltage (10 V/cm gel length). Afterwards, the gel was stained with ethidium bromide staining solution for 20-30 minutes and documented using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloch, Germany).

2.2.1.5. Extraction of DNA fragments from agarose gels

To extract DNA fragments from agarose gels, ethidium bromide-stained gels were illuminated shortly with UV-light and the appropriate DNA band was excised from the gel with a clean scalpel. The excised band was placed into an Eppendorf tube. The DNA was extracted from the agarose using the QIAquick gel extraction kit (Qiagen) following the manufacturer's protocol. The extracted DNA was resuspended in elution buffer.

2.2.1.6. Determination of DNA concentration

DNA concentrations were determined by UV spectroscopy. In general, an aliquot of the DNA sample was diluted with distilled water for analysis. The DNA concentration of the diluted sample was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). A_{260} readings between 0.1 and 1.0 were considered reliable. The DNA concentration of the sample was calculated from the A_{260}, taking into account the dilution factor of the analyzed sample (A_{260} = 1 corresponds to a concentration of 50 µg/ml of double stranded DNA). A A_{260}/A_{280} ratio of between 1.8 and 2.0 was used as a measure of adequate purity of the DNA preparation for further experimentation.

2.2.1.7. DNA sequencing

DNA sequencing was performed by the ZMNH sequencing facility in Hamburg. For sequencing, 1 µg of DNA was diluted in 7 µl double distilled water along with the appropriate sequencing primer (10 pM final concentration).

2.2.1.8. DNA amplification

Amplification of DNA fragments was performed in a 50 µl reaction mixture using the HotStarTaq Master Mix (Qiagen). The following composition was used for a typical amplification reaction:

Component	Volume/amount
HotStarTaq Master Mix	25 µl
Forward primer	20 pmol
Reverse primer	20 pmol
Template DNA	50 ng (< 1 µg/50 µl reaction)
Total volume	50 µl

2.2.1.9. Site-directed mutagenesis

Single amino acid mutations were made using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) following the protocol provided by the manufacturer. The following composition was generally used for site-directed mutagenesis reactions:

Component	Volume/amount
10x reaction buffer	5 µl
double-stranded DNA template	50 ng
mut primer-5' (0.1 µg/µl)	2.5 µl
mut primer-3' (0.1 µg/µl)	2.5 µl
dNTP mix (proprietary mixture)	1 µl
QuikSolution	3 µl
H_2O	Adj. to 50 µl
Pfu Ultra HF polymerase (2.5 U/µl)	1 µl

2.2.2. Protein biochemistry

2.2.2.1. Protein quantification (Bicinchoninic Acid assay)

Protein concentrations were determined with the bicinchoninic acid (BCA) protein quantification kit (Pierce) following the manufacturer's instructions.

2.2.2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were subjected to SDS-PAGE under reducing conditions using the Mini-Protean II system (Bio-Rad) and following standard SDS-PAGE protocols (Laemmli, 1970). The percentage of acrylamide to be used (8-14 %) was determined based on the molecular weight of the protein being analyzed. The following gel composition was used for the running gel: 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.025% APS, and 0.001% TEMED, plus acrylamide/bis solution 29:1 based on the required acrylamide content. For the stacking gel, the following composition was used: 125 mM Tris-HCl pH 8.8, 0.1% SDS, 0.06% APS, and 0.025% TEMED, plus acrylamide/bis solution 29:1 required for 5% (w/v) acrylamide. After

polymerisation of the gels, the electrophoresis chamber was assembled as described by the manufacturer. Samples were diluted with appropriate amounts of 2x concentrated SDS sample buffer then boiled for 5 min at 95°C in order to completely denature the proteins. After loading the samples onto the gel, the gel chamber was filled with 1x concentrated SDS running buffer. Electrophoresis was performed at a constant voltage of 90 V for approximately 15 minutes to allow the proteins to fully enter the stacking gel, and then at 120 V until the bromophenol blue running front had reached the bottom of the gel. Gels were either stained with Coomassie or subjected to Western blotting.

2.2.2.3. Coomassie staining of SDS-polyacrylamide gels

Gels were stained in Coomassie staining solution for 1 h at RT under constant agitation. Afterwards, the gels were incubated in de-staining solution until the background of the gel appeared nearly transparent.

2.2.2.4. Western blotting

Proteins separated by SDS-PAGE were transferred onto a Protran® nitrocellulose membrane using a MINI TRANSBLOT-apparatus (Bio-Rad Laboratories). After equilibration of the polyacrylamide gel in transfer buffer, a blotting sandwich was assembled as described in the manufacturer's protocol. Proteins were transferred at 4°C in blotting buffer at constant voltage (90 V for 1-2 h). Prestained protein markers from Bio-Rad Laboratories were used as molecular weight indicators and to monitor successful protein transfer to the nitrocellulose membrane.

2.2.2.5. Protein immunostaining

After electrophoretic transfer, membranes were removed from the blotting sandwiches and placed with the protein-bound side up in glass or plastic vessels. Membranes were washed once with TBST for 5 minutes and then incubated under gentle shaking for 1 h at RT in blocking solution to block the unreacted sites on the membranes to reduce the amount of non-specific binding of proteins in subsequent steps. After the blocking step, membranes were incubated overnight at 4°C with an appropriate primary antibody diluted in either blocking

solution or PBST with 3-5% (w/v) BSA. The primary antibody solution was removed the next day and membranes were washed 6x 5 minutes with TBST under constant shaking. The membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody, diluted 1:10,000 in blocking solution, for 1-3 h at RT, then washed 6x 5 minutes as before. Immunoreactive bands were visualized using the SuperSignal West Dura or SuperSignal West Pico enhanced chemiluminescence detection system (Pierce). The membranes were soaked for 5 min in detection solution (1:1 mixture of solutions I and II). The solution was removed and the blots were placed between transparent plastic sheets. The membranes were exposed to BIOMAX ML (Kodak) for several time periods (between 1 min and 6 h) to visualize protein bands.

2.2.3. Expression of recombinant proteins in *Escherichia coli*

For recombinant expression of proteins in *E. coli*, the appropriate *E. coli* strain was transformed with the expression plasmid encoding the construct of interest and transformants were selected on LB plates containing the proper selection antibiotic. LB medium (20 – 50 ml) with the selection antibiotic was inoculated with a single colony and incubated overnight at 37°C with constant agitation. The overnight bacterial culture was used as a starter inoculum at a ratio of 1:20 – 1:50 and incubated at 37°C under constant agitation until the culture had reached an optical density (A_{600}) of 0.4 – 0.6. Protein expression was induced by adding IPTG (0.5-1.0 mM) or L-arabinose (0.2%) to the culture with further incubation for 3-4 h at 37°C. Bacteria were collected by centrifugation (4,000 x g, 4°C, 20 minutes) and pellets stored at – 20°C. Protein expression was examined by Western blot analysis of small samples taken from the culture supernatant before and after IPTG/L-arabinose induction.

2.2.3.1. Bacterial lysis by French press

Bacterial pellets were resuspended in lysis buffer (10- 20 ml lysis buffer per 500 ml culture). The suspension was transferred to a pre-cooled French-Pressure-20K-chamber (capacity 40 ml). Bacteria were compressed (Spectronic Instruments/SLM Aminco, 10000 psi, 3 minutes) and lysed by opening the valve carefully. This procedure was repeated 3 times. The lysate was then centrifuged (10,000 x g, 20 minutes, 4°C) in a Beckman centrifuge to pellet the cellular debris.

2.2.3.2. Protein purification (native conditions)

Glutathione S transferase- (GST-) tagged proteins were purified using glutathione agarose beads (Sigma-Aldrich). The captured protein was eluted from the beads according to the manufacturer's protocol. Histidine-tagged proteins were purified using Ni-NTA agarose beads (Qiagen), and protein purification was carried out following the manufacturer's protocol.

2.2.4. Protein-protein interaction detection methods

2.2.4.1. GST-pull down assay

Protein pull-down experiments were performed to investigate the direct interaction between 14-3-3ζ protein and the ICD of L1. Recombinant 14-3-3ζ protein tagged with GST was expressed in bacteria and purified on glutathione sepharose bead columns (Sigma-Aldrich). GST alone was bound to glutathione sepharose beads and used as a control. All steps of the experiments were carried out on ice or at 4°C. GST-14-3-3ζ and GST bound to beads (50 µl) were washed once with pull-down buffer (~150 µl). Pull-down buffer containing 5% (w/v) BSA was added to the protein-loaded beads and the beads were incubated at 4°C for 2 h with constant shaking. Recombinantly expressed and purified L1 ICD constructs were added to the mixture and the samples allowed to incubate overnight at 4°C with constant gentle shaking. The beads were washed once with pull-down buffer supplemented with 0.5% NP-40 and once with pull-down buffer. 2x concentrated SDS sample buffer (75 µl) was added to the bead mixture and samples were boiled at 95°C for 5 minutes to elute bound proteins for Western blot analysis.

2.2.4.2. <u>E</u>nzyme-<u>l</u>inked <u>I</u>mmunosorbent <u>A</u>ssay (ELISA)

ELISA experiments served to investigate the direct interaction between 14-3-3 proteins and the ICD of L1. 14-3-3β or ζ protein tagged with GST- and histidine-tagged L1 ICD were expressed in bacteria and purified. Purified L1 ICD (5 µg/ml in TBS) was immobilized overnight at 4°C on a polyvinyl chloride surface in a 96-well plate (Nunc-Immunomodule Maxisorb, Nunc, Roskilde, Denmark) under constant gentle shaking (50 µl/well). Recombinant NCAM 180 intracellular domain was used as a control. The subsequent

incubation and washing steps of the ELISA experiments were performed at RT. All incubation steps were performed with 50 µl/well and washing steps with 100 µl/well. First, blocking of unreacted sites on the membrane was performed with blocking solution for 1 h. GST-14-3-3β or ζ protein diluted in buffer A was added and incubated for 1 h. The wells were then washed 3 times with TBST. Primary antibody and its respective HRP-coupled secondary antibody were used for the detection of bound protein. For visualization of protein binding, 50 µl of freshly prepared ABTS substrate solution was applied to the wells. The product of the HRP-catalyzed reaction was quantified by measuring the absorbance at 405 nm using an ELISA reader (Kcjunior).

2.2.4.3. Co-immunoprecipitation

Immunoprecipitation experiments were conducted with several different antibodies using protein A or protein A/G sepharose beads (Santa Cruz Biotechnology). 1-2 mg of total protein from brain homogenates, total membrane fractions, or HEK 293 cell lysates were incubated with 1-2 ml of ice-cold radio immunoprecipitation assay (RIPA) lysis buffer for 1 h at 4°C under constant gentle shaking. Samples were centrifuged at 10,000 x g for 20 minutes at 4°C. The pellet was discarded and the supernatant was pre-cleared with 15 µl of resuspended sepharose protein A or A/G beads by incubating 3 hours at 4°C with constant gentle shaking. After pre-clearing, the beads were pelleted down by centrifugation at 500 x g for 5 minutes. The supernatant was carefully transferred to a clean tube and then incubated with corresponding antibodies or antibody control overnight at 4°C with constant mixing. Antibody-target protein-complexes were captured with the respective sepharose beads by incubating the samples for up to 3 h at 4°C, followed by washing 3 times with ice-cold RIPA buffer and once with PBS. Captured proteins were eluted by adding 2x concentrated SDS sample buffer and boiling at 95°C for 5 minutes. The eluted proteins were analyzed by Western blotting.

2.2.4.4. Casein kinase II phosphorylation *in vitro*

To phosphorylate recombinant GST- or histidine-tagged fusion proteins in vitro, purified proteins were incubated with 1 unit of CK II (500 Units/ml, NEB), 200 µM ATP, and 1x

concentrated casein kinase II buffer (NEB) for 3 h at 30°C in a total reaction volume of 50 µl. The phosphorylated proteins were used for further experiments.

2.2.4.5. Calpain cleavage assay

Calpain cleavage of recombinant GST-alpha II spectrin 10-14 was performed by incubating the protein with 0.03 µg of µ-calpain (Sigma), 150 µM $CaCl_2$, 20 mM Tris (pH 7.5), 25 mM NaCl, and 10 mM DTT for 30 min at 30°C in a total reaction volume of 25 µl. The cleavage reaction was stopped by adding 1x concentrated SDS sample buffer.

2.2.5. Subcellular fractionation of mouse brain homogenates by differential density gradient centrifugation

2.2.5.1. Isolation of total membrane fraction

Whole brains of 3-weeks-old mice (C57BL/6J) were dissected quickly on ice and either frozen in liquid nitrogen for storage (–80°C) or immediately processed. To obtain sufficient amounts of total membrane fraction, at least 2 mouse brains were used. Each brain was homogenized in 2 ml of ice cold 0.32 M sucrose in Tris buffer using 15 strokes of a Dounce homogenizer to obtain a homogenate. All tissues and buffers were maintained at 4°C throughout the experiment. All centrifugation steps were done at 4°C using pre-chilled centrifuges, rotors and tubes. The homogenates of 2 brains were mixed and a 50 µl aliquot was taken for analysis of brain homogenate protein levels. The mixture of homogenates obtained was centrifuged at 1,400 x g for 10 minutes to pellet tissue debris, nuclei and large myelin fragments (pellet P1). The supernatant (S1) was carefully decanted and placed in a tube on ice. The pellet was resuspended and centrifuged again at 700 x g for 10 minutes. This centrifugation step was performed two more times. The pellet fractions were pooled and resuspended in 1 M sucrose in Tris buffer by repeated pipetting (final volume of 20 ml). The pooled pellet fractions were layered on 10 ml of 1.2 M sucrose in Tris buffer. On top of this first layer, 6 ml of 0.32 M sucrose in Tris buffer was carefully layered. Pellet P5, obtained by centrifugation of S4 at 17,500 x g for 15 min and resuspended in 0.32 M sucrose in Tris buffer to a final volume of 5 ml, was carefully layered on top of a second sucrose gradient. This second gradient consisted of 20 ml 1 M sucrose in Tris buffer on a cushion of 10 ml of

1.2 M sucrose in Tris buffer. Both sucrose gradients were centrifuged at 82,705 x *g* for 2 h. The interface between 1 M and 1.2 M sucrose was enriched in the crude membrane fraction. This interface was carefully collected with a Pasteur pipette from both gradients and pooled. The collected crude membrane fraction was purified on a gradient again. The fraction thus obtained was resuspended with 1 M sucrose in Tris buffer (20 ml total volume). The suspension was layered onto 10 ml of 1.2 M sucrose in Tris buffer to create a discontinuous gradient. As before, 6 ml of 0.32 M sucrose in Tris buffer was carefully layered on top and the gradient was centrifuged at 82,705 x *g* for 2 h. The interface between 1 M and 1.2 M sucrose contained the 2-fold enriched membrane fraction. The enriched membrane fraction was resuspended in 5 mM Tris buffer containing protease inhibitor cocktail (Roche) and centrifuged again at 82,705 x *g* for 30 minutes. Appropriate amounts of protease inhibitors were added and the fraction was stored at –80°C until further use.

2.2.5.2. Isolation of endosomes

To isolate endosomal fractions, 7-days-old mice (C57BL/6J; n = 20-40) were dissected quickly on ice and immediately homogenized in ice cold 0.32 M sucrose in Tris buffer. The brain homogenates were centrifuged at 17,000 x *g* for 1 h. The 17,000 x *g*-supernatant thus obtained was centrifuged at 100,000 x *g* for 1 h. The subsequent 100,000 x g-pellet was homogenized in 0.32 M sucrose in Tris buffer and loaded onto a step gradient comprising layers of 2, 1.3, 1.1, 0.8, 0.5 and 0.25 M sucrose. The gradients were centrifuged at 100,000 x *g* for 2 h. Nine 1 ml-fractions were collected from the top of the gradient and homogenized in 0.32 M sucrose in Tris buffer. After another centrifugation step at 100,000 x g for 30 min, each of the nine fractions, enriched in distinct endosomes, was collected and the total amount of protein was estimated by the BCA test. These endosomal fractions were used for further experiments.

2.2.6. Mammalian cell culture

2.2.6.1. Maintenance of HEK 293 and SH-SY5Y cells

HEK 293 or SH-SY5Y cells were cultured in DMEM with 10% fetal calf serum (FCS) and 2% penicillin/streptomycin at 37°C, 5% CO_2 and 90% relative humidity in 75 cm^2 flasks (10

ml medium) or in six-well plates with 2 ml of medium. Cells were passaged when they were confluent (usually after 2-3 days). Medium was removed and cells were detached by incubation with 2 ml trypsin/EDTA solution (0.05%/0.02%) for up to 5 minutes at RT. Cells were resuspended in 8 ml fresh medium and were split 1:10 for continued culturing or they were seeded into 6-well plates for transfection.

2.2.6.2. Transient transfection of HEK 293 and SH-SY5Y cells

HEK 293 cells or SH-SY5Y cells were transfected transiently with plasmids using FUGENE 6 (Roche) as per the manufacturer's instructions. Transfected cells were grown for 24-48 hours to allow for recombinant protein expression.

2.2.6.3. Lysis of HEK 293 cells

After growing transiently-transfected HEK 293 cells, medium was removed from the cells and they were washed twice with ice-cold PBS. Cells were scraped into PBS, transferred to a 1.5 ml Eppendorf tube and pelleted at 1,000 x g for 10 minutes at 4°C. The cells were lysed in 0.3-0.6 ml of RIPA buffer per well with constant agitation for 1 hour at 4°C. Cellular debris was removed by centrifugation (10,000 x g, 4°C, 15 minutes). The supernatant was used for immunoprecipitation.

2.2.6.4. Cell surface biotinylation

To investigate the presence of L1 at the surface of transfected SH-SY5Y cells, cell surface biotinylation was performed as described before (Baqui et al., 2003). SH-SY5Y cells, expressing L1 endogenously, were transfected with 14-3-3ζ constructs in a 6-well plate (2 µg of DNA per well). Two days after transfection, cells were washed twice with ice-cold PBSCM. Surface proteins were biotinylated by incubating cells with 0.5 mg/ml cell-impermeant Sulfo-NHS-LC-Biotin (Pierce) (1 ml/well) in PBSCM for 10 min on ice. Biotinylation was quenched by incubation with 20 mM glycine in PBSCM on ice followed by washing with PBSCM (1 ml/well, 2 times). Biotinylated cells were lysed by addition of RIPA buffer (350 µl/well), followed by incubation at 4°C for 30 min. The supernatants were collected after centrifugation (700 x g) at 4°C for 10 min and protein concentrations were determined using the BCA kit (Pierce). The amount of surface-localized proteins was

determined by precipitating biotinylated proteins from the cell lysate with streptavidin coupled magnetic beads (DYNAL Biotech, Invitrogen) at 4°C overnight. To determine the proportion of surface L1, aliquots of the supernatant were collected before and after the addition of streptavidin beads. The beads were pelleted on a magnetic stand, washed twice with 1 ml of RIPA buffer, then once with PBS. Precipitated proteins were solubilized by addition of 50 µl of 2x concentrated SDS sample buffer to the beads. Proteins were separated by SDS-PAGE and Western blot analysis was performed with an anti-L1 (human) mouse monoclonal (Dianova) and the isoform-specific anti-14-3-3ζ monoclonal antibody (Immunobiological Laboratories).

3. Results

The results section consists of two parts. The first part describes the finding that brain alpha II spectrin is a direct binding partner of the signaling protein 14-3-3β, and the characterization of the interaction between 14-3-3 and alpha II spectrin (see pages 49-57). In the second part, the discovery of the cell adhesion molecule L1 as a 14-3-3-binding partner is documented. This part includes an analysis of the L1 - 14-3-3-interaction and its implications for L1 phosphorylation (see pages 58-68).

3.1. Alpha II spectrin interacts with the signaling molecule 14-3-3

3.1.1. Identification of alpha II spectrin as a potential 14-3-3β-interacting protein

14-3-3 proteins are known to regulate a variety of both general and specialized signaling pathways by binding to specific phosphoserine- and phosphothreonine-containing motifs within target proteins (Muslin et al., 1996). The functional complexity of 14-3-3 proteins is reflected in the increasing number of binding partners that have been identified so far (Fu et al., 2000). Although 14-3-3 proteins are highly abundant in the brain, the number of identified 14-3-3-binding partners in brain is small so far.

In the course of my diploma thesis (Ramser, 2005), I used affinity chromatography to identify cellular proteins from the brain that may interact with 14-3-3 proteins. Human GST-tagged 14-3-3β was coupled to GSH-agarose and incubated with soluble and membrane fractions isolated from 3 week-old wild-type mouse brains. 14-3-3-interacting proteins were purified from both fractions by elution with R18 peptide (Petosa et al., 1998; Wang et al., 1999). Proteins from the membrane fraction bound to GST-14-3-3β were compared to the eluate from a GST-tag column to control for non-specific protein interactions. Among the eluted proteins, a protein of ~280 kDa was specifically enriched in the GST-14-3-3β eluate (Fig. 5). Analysis of this 280 kDa protein by ESI tandem mass spectrometry (MS/MS), which was performed by Dr. Fritz Buck (Universitätsklinikum Hamburg-Eppendorf), identified it as the cytoskeletal protein alpha II spectrin.
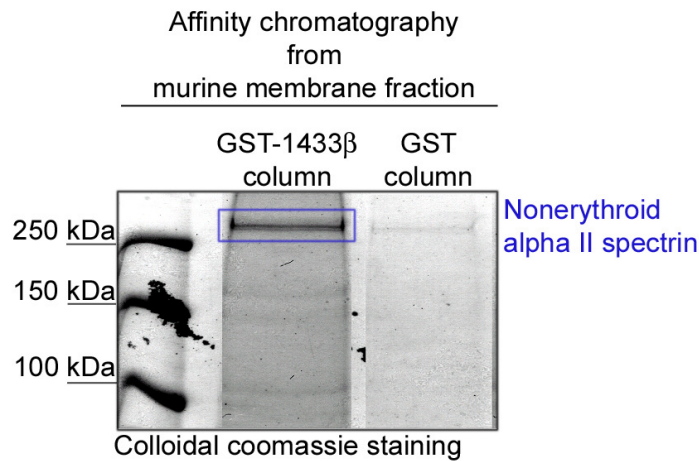
Figure 5: Identification of non-erythroid alpha II spectrin as a 14-3-3 interacting protein from murine brain membrane fraction. Colloidal Coomassie-stained SDS-PAGE gel of membrane fraction eluates from the GST-14-3-3β affinity column and GST-only column (control).

The interaction of non-erythroid alpha II spectrin with 14-3-3β protein was further investigated in the present study.

3.1.2. 14-3-3β binds endogenous alpha II spectrin from mouse brain

To validate the interaction of 14-3-3β with alpha II spectrin, a GST-14-3-3β pull-down experiment was conducted. The experiment was performed with murine brain membrane fractions to confirm the association between the two proteins. As shown in Fig. 6, full-length alpha II spectrin (~280 kDa) was efficiently pulled down with beads on which recombinant GST-tagged 14-3-3β had been immobilized, but not with beads loaded with GST alone (control). C-terminal breakdown products (BDP: ~150 kDa and ~145 kDa), resulting from calpain and caspase cleavage during post-lysis proteolysis (Wang et al., 1998; Nedrelow et al., 2003), could also be detected via the monoclonal anti-mouse alpha II spectrin antibody (Fig. 6), which binds to the C-terminal region of the alpha II spectrin molecule (Wang et al., 1998).

GST-pull down

WB:
anti-alpha II spectrin
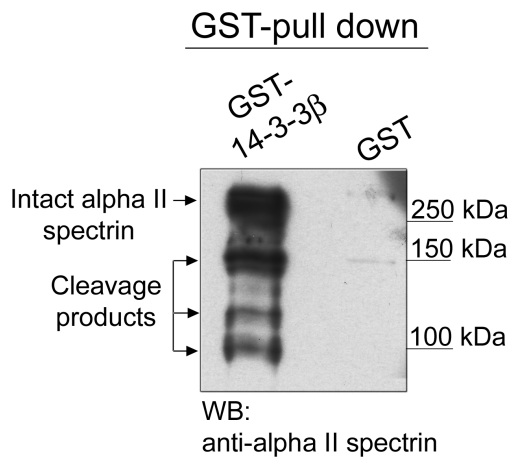
Figure 6: 14-3-3β binds endogenous alpha II spectrin from crude brain membrane fraction. Murine brain membrane fraction was incubated with GST-14-3-3β coupled to glutathione beads or GST control beads. Bound protein was resolved by SDS-PAGE and analyzed by Western blotting with the indicated antibody. Cleavage products probably generated by calpain and caspase were also detectable with the monoclonal anti-alpha II spectrin antibody (indicated by arrows).

3.1.3. Co-immunoprecipitation of alpha II spectrin with 14-3-3β

Co-immunoprecipitation was chosen as a further method to confirm the interaction of alpha II spectrin with 14-3-3 proteins. For this experiment, a 14-3-3β isoform-specific antibody was used to co-precipitate alpha II spectrin from mouse brain membrane fractions solubilized in detergent-containing buffer. Western blot analysis of the 14-3-3β immunoprecipitates revealed the presence of full-length alpha II spectrin (280 kDa) (Fig. 7; upper panel). Spectrin cleavage products were also detected in the 14-3-3β precipitate, but no co-immunoprecipitation of alpha II spectrin was observed when a control IgG was used for immunoprecipitation (Fig. 7; upper panel). These results strongly suggest that alpha II spectrin is associated with 14-3-3β in the brain, and further confirm the interaction shown in the chromatography and pull-down assay.

WB: anti-alpha II
spectrin

WB: anti-14-3-3β
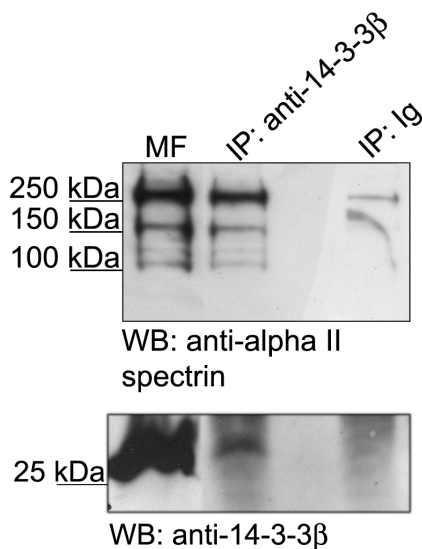
Figure 7: Co-immunoprecipitation reveals 14-3-3β - alpha II spectrin complexes in mouse brain. *Upper panel:* Co-immunoprecipitation from crude brain membrane fractions (MF) was performed using an antibody against 14-3-3β. Proteins were resolved by SDS-PAGE and analyzed by Western blotting (WB) with an anti-alpha II spectrin antibody. *Lower panel:* Western blot analysis of the co-precipitates was also performed with an anti-14-3-3β antibody, in order to show that 14-3-3β was successfully precipitated from the membrane fraction.

3.1.4. NCAM – spectrin - 14-3-3 complexes in mouse brain

Alpha II spectrin forms heterotetramers with the beta I spectrin subunit (Goodman et al., 1995), which is known to bind NCAM 180 and 140 (Leshchyns'ka et al., 2003). I therefore wished to analyze whether alpha II spectrin and 14-3-3β also associate with NCAM. The 1β1 NCAM polyclonal antibody preparation was used to precipitate NCAM from membrane fractions isolated from 3 week-old wild-type mouse brains. Western blot analysis of the NCAM immunoprecipitates with an anti-14-3-3β isoform-specific antibody and an anti-alpha II spectrin monoclonal antibody revealed the presence of 14-3-3β and alpha II spectrin in these immunoprecipitates (Fig. 8; left, upper panel; right panel), indicating that these two proteins are indeed associated with NCAM in mouse brain.
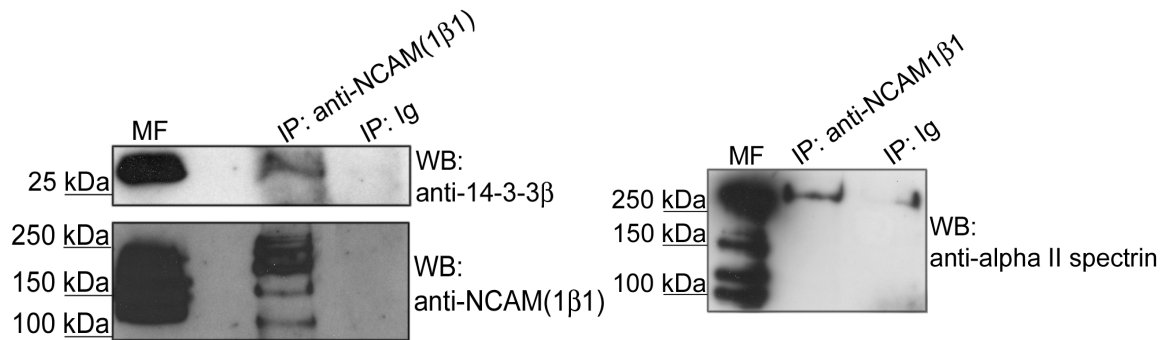
Figure 8: Immunoprecipitation reveals NCAM – spectrin - 14-3-3 complexes in mouse brain. *Left, upper panel:* Immunoprecipitation (IP) of NCAM from crude brain membrane fractions (MF) was performed with an antibody to NCAM. Proteins were resolved by SDS-PAGE and analyzed by Western blotting (WB) with an anti-14-3-3β antibody. *Left, lower panel:* Western blot analysis of the co-precipitates was also performed with an anti-NCAM antibody in order to show that NCAM was successfully precipitated from membrane fractions. *Right panel:* IP of NCAM from brain membrane fractions (MF) was performed with an antibody to NCAM. Western blot was performed with an anti-alpha II spectrin antibody. Precipitation with rabbit non-immune IgGs (IP: Ig) served as a negative control.

3.1.5 S1302A mutation in alpha II spectrin repetitive units 10-14 disrupts 14-3-3β binding

It is generally believed that 14-3-3 proteins function through their ability to bind specific phosphoserine sites (Muslin et al., 1996; Yaffe et al., 1997). Two main consensus 14-3-3-binding motifs have been identified: RSXpSXP and RXXXpSXP (pS represents the phosphorylated Ser residue) (Yaffe et al., 1997). A 14-3-3-binding motif (RLIQS[1302]HP)

within the alpha II spectrin repetitive unit 12 corresponds to the consensus 14-3-3-binding motif RXXXpSXP and is the only consensus 14-3-3-binding motif found in the alpha II spectrin molecule, as predicted by the PATTINPROT algorithm of the Network Protein Sequence Analysis (Combet et al., 2000). To determine whether alpha II spectrin requires phosphorylated Ser^{1302} to interact with 14-3-3 (Fig. 9), binding assays were performed with GST-14-3-3β.
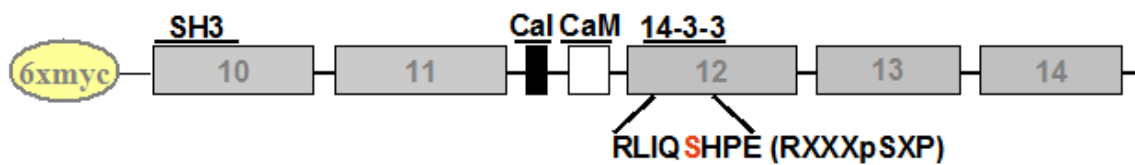
Figure 9: Scheme representing the recombinant fusion protein alpha II spectrin repetitive units 10-14. The calpain cleavage site (Cal) and the calmodulin-binding domain (CaM) are sequences inserted into the spectrin repeat 11. The novel 14-3-3-binding site (RLIQS^{1302}HP) is located within the spectrin repeat 12. Ser^{1302} (highlighted in red) is the phosphorylation target within a consensus phosphorylation site for casein kinase II. The alpha II spectrin 10-14 construct was overexpressed as 6xmyc-tagged protein in HEK 293 cells and used in pull-down experiments.

Recombinant alpha II spectrin repetitive units 10-14 and alpha II spectrin 10-14 with a Ser^{1302}->Ala substitution were compared as 14-3-3-interaction partners. The alpha II spectrin constructs were expressed as myc-tagged proteins in HEK 293 cells, precipitated with anti-c-myc-agarose conjugate from HEK 293 cell lysates, and incubated with equal amounts of recombinant GST-14-3-3β protein. Western blot analysis revealed that the Ser^{1302} to Ala mutation resulted in substantially reduced alpha II spectrin – 14-3-3β-interaction (Fig. 10; upper panel), indicating that Ser^{1302} of alpha II spectrin is important for the interaction with 14-3-3β.
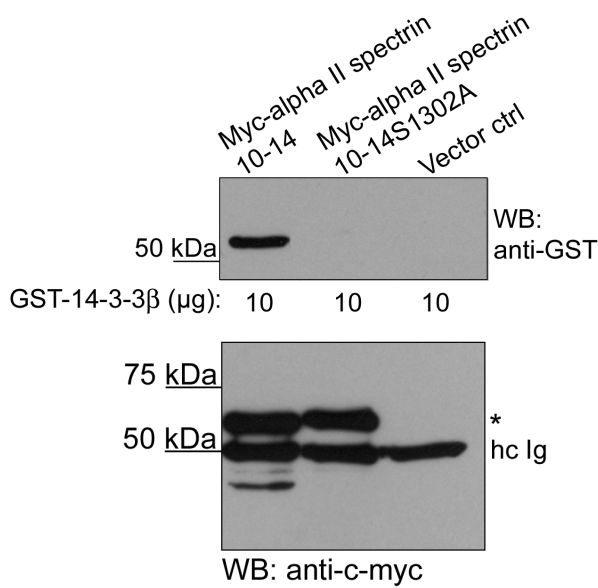
Figure 10: Ser^{1302} -> Ala substitution in alpha II spectrin repetitive units 10-14 disrupts binding to 14-3-3β. Wild-type and mutant alpha II spectrin constructs were transfected into HEK293 cells. Myc-tagged proteins from cell lysates were precipitated with anti-myc-conjugated agarose beads and subjected to *in vitro* binding assays. Binding of GST-14-3-3β to precipitated myc-tagged wild-type and mutated alpha II spectrin 10-14 was analyzed by Western blotting. *Upper panel:* GST-14-3-3β protein bound to myc-tagged alpha II spectrin 10-14 was detected by using an anti-GST antibody. *Lower panel:* Western blot analysis of the precipitated myc-tagged wild-type and mutated alpha II spectrin 10-14 was performed with an anti-c-myc antibody (*precipitated myc-alpha II spectrin 10-14 and Ser^{1302}Ala mutant; **hc Ig**, Immunoglobulin heavy chain).

3.1.6. *In vitro* casein kinase II phosphorylation of alpha II spectrin repetitive units 10-14 stimulates 14-3-3 binding

The Ser^{1302} residue in alpha II spectrin domain 12, which was shown above to be important for the interaction with 14-3-3, is part of a consensus phosphorylation site for casein kinase II (CK II). To determine whether phosphorylation is required for the interaction of alpha II spectrin repetitive units 10-14 and 14-3-3, recombinant GST-alpha II spectrin 10-14 bound to GSH agarose beads was preincubated with or without CK II in the presence of 200 µM ATP, followed by an incubation with crude brain membrane fractions isolated from 3 week-old wild-type mice. Immunoblot analysis (Fig. 11; left panel) revealed that preincubation of GST-alpha II spectrin 10-14 with CK II was indeed necessary for 14-3-3 binding.

A dot blot analysis performed with an anti-pSer antibody confirmed that GST-alpha II spectrin 10-14 was phosphorylated after CK II treatment (Fig. 11; right panel).
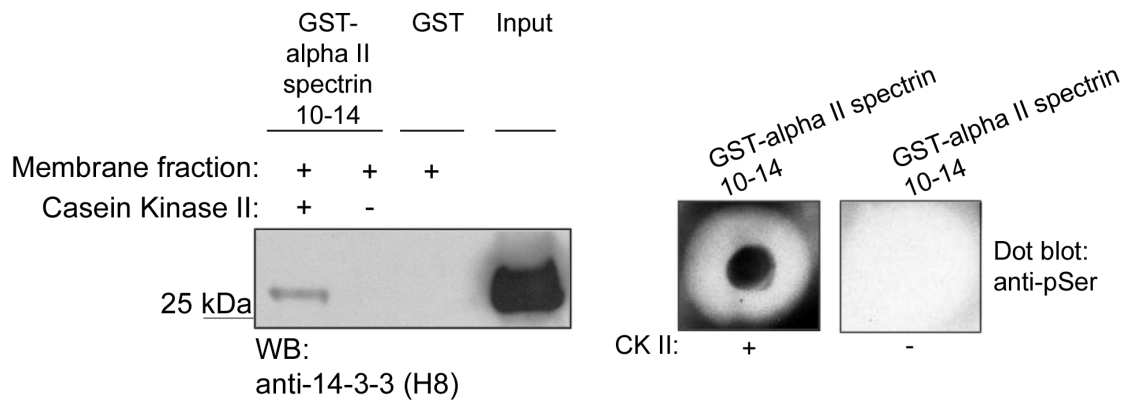
Figure 11: *In vitro* **CK II phosphorylation of alpha II spectrin 10-14 stimulates 14-3-3 binding.** *Left panel:* Alpha II spectrin repetitive units 10-14 were expressed as a GST fusion protein in bacteria and immobilized on glutathione-Sepharose 4B beads. The beads were incubated in the presence or absence of CK II and then washed. Murine membrane fractions were incubated with the beads. Western blot analysis of the eluates from the beads with the antibody H8 recognizing 14-3-3 showed that 14-3-3 was pulled down with CK II-treated GST-alpha II spectrin 10-14 but not with either GST-alpha II spectrin 10-14 preincubated in the absence of CK II or GST alone. *Right panel:* Dot blot analysis of GST-alpha II spectrin 10-14 after incubation with or without CK II was performed with an anti-pSer antibody. Phosphorylation of GST-alpha II spectrin 10-14 was evident after CK II treatment.

3.1.7. Calmodulin enhances 14-3-3β binding to alpha II spectrin 10-14

Having investigated the phosphorylation-dependent interaction of alpha II spectrin 10-14 with 14-3-3β above, I wished to investigate whether other proteins might influence the interaction of 14-3-3β with alpha II spectrin 10-14. Calmodulin is known to bind at the end of the non-homologous sequence inserted in the 11th repeat unit of the vertebrate alpha II spectrin subunit (Harris et al., 1988; Simonovic et al., 2006). Calmodulin binding to alpha II spectrin induces conformational changes in the molecule (Simonovic et al., 2006) and may influence binding of interaction partners to alpha II spectrin. To investigate whether calmodulin influences 14-3-3β binding to alpha II spectrin 10-14, a binding assay was performed. Recombinant GST-alpha II spectrin 10-14 (Fig. 12) bound to GSH agarose beads was preincubated with CK II and then washed. Afterwards, beads were incubated with lysates from HEK293 cells transiently expressing 6xmyc-14-3-3β in the presence of increasing concentrations of histidine-tagged calmodulin. As shown by Western blot analysis (Fig. 13; left, upper panel;

right panel), myc-14-3-3β-binding to alpha II spectrin 10-14 was enhanced in the presence of calmodulin.
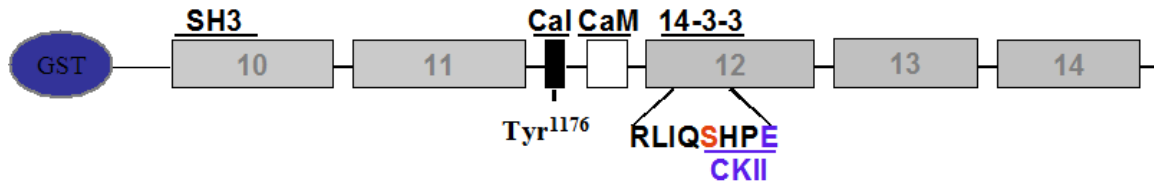
Figure 12: Scheme representing the recombinant fusion protein alpha II spectrin repetitive units 10-14. The calpain cleavage site (Cal) and the calmodulin-binding domain (CaM) are sequences inserted into the spectrin repeat 11. The novel 14-3-3-binding site (RLIQS^{1302}HP) is located within the spectrin repeat 12. Ser^{1302} (highlighted in red) is the phosphorylation target within a consensus phosphorylation site for casein kinase II. The alpha II spectrin 10-14 construct was recombinantly expressed as a GST-tagged protein, purified from bacterial lysates and used in pull-down experiments.
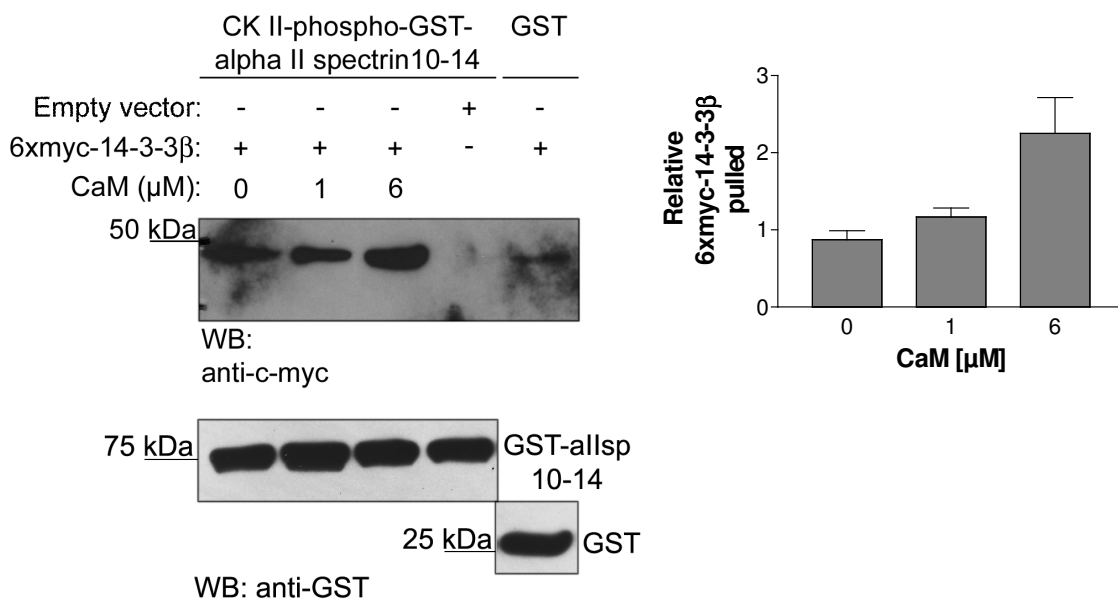
Figure 13: Influence of calmodulin on 14-3-3β-binding to alpha II spectrin 10-14. *Left, upper panel:* Recombinant GST-alpha II spectrin 10-14 encompassing the calmodulin-binding domain and the 14-3-3-binding site was preincubated with CK II followed by incubation with 6xmyc-14-3-3β from HEK 293 cell lysates and increasing concentrations of recombinant calmodulin (CaM). Western blot analysis was performed with an anti-c-myc antibody to detect bound 14-3-3β. *Left, lower panel:* GST and GST-alpha II spectrin 10-14 (GST-aIIsp10-14) were detected by Western blot (WB) with an anti-GST antibody. *Right panel:* Quantification of pulled 6xmyc-14-3-3β, with the value for pulled 6xmyc-14-3-3β in the absence of CaM normalized to 1. Error bars denote standard deviation from 4 independent experiments.

In order to confirm that the enhancement of 14-3-3β-binding to alpha II spectrin 10-14 by calmodulin was a specific effect, the binding assay was performed in the presence of EDTA to inhibit calmodulin activity. Western blot analysis of bound 14-3-3β revealed that calmodulin only slightly stimulated the interaction between 14-3-3β and alpha II spectrin 10-14 if EDTA was present (Fig. 14; left, upper panel; right panel). These results indicate that only active calmodulin enhances 14-3-3β – alpha II spectrin 10-14 binding.
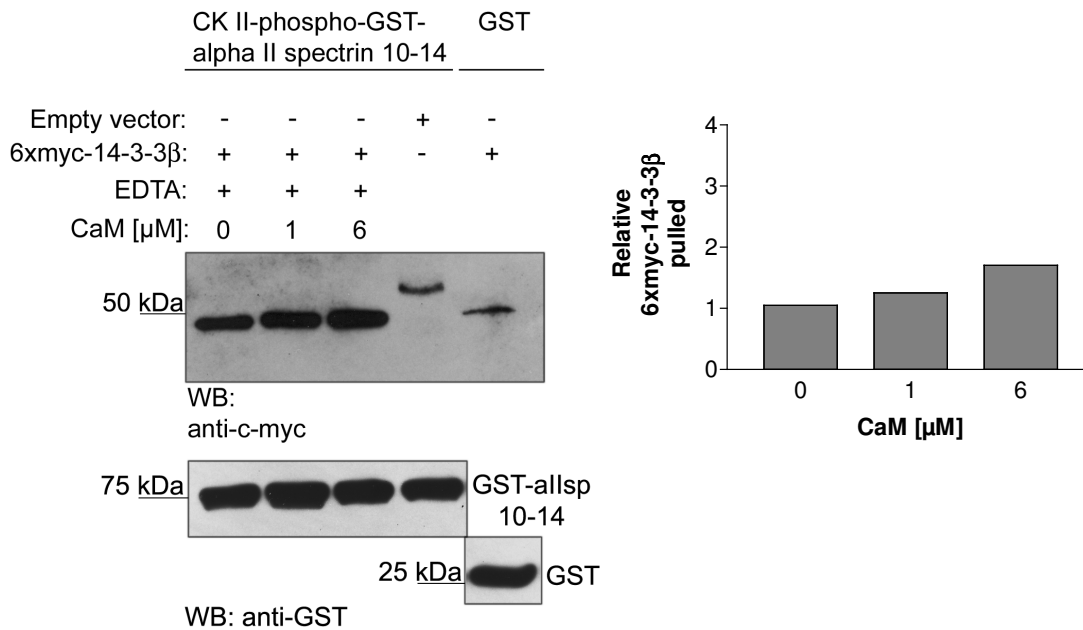
Figure 14: Effect of Calmodulin on 14-3-3β binding to alpha II spectrin 10-14 is slightly reduced in the presence of EDTA. *Left, upper panel:* Recombinant GST-alpha II spectrin 10-14 encompassing the calmodulin-binding domain and 14-3-3-binding site was preincubated with CK II followed by incubation with 6xmyc-14-3-3β from HEK 293 cell lysates and increasing concentrations of recombinant calmodulin in the presence of 2 mM EDTA. Western blot analysis was performed with an anti-c-myc antibody. *Left, lower panel:* GST and GST-alpha II spectrin 10-14 were detected by Western blot (WB) with an anti-GST antibody. *Right panel:* Quantification of pulled 6xmyc-14-3-3β, with the value for pulled 6xmyc-14-3-3β in the absence of CaM normalized to 1. This experiment was performed only twice.

3.2. Cell adhesion molecule L1: a novel 14-3-3-binding protein

Serial analysis of gene expression (*SAGE*) in hippocampi of GFAP/L1 mice revealed an overexpression of 14-3-3β and ζ genes. These transgenic mice, which ectopically express the cell adhesion molecule L1 in astrocytes, were generated to study the effects of L1 on nerve regeneration, synaptic plasticity and learning (Wolfer et al., 1998). The observation that 14-3-3 genes were overexpressed in transgenic animals relative to wild-type littermates suggested an involvement of 14-3-3 in signaling downstream of L1, thereby influencing L1 function. Furthermore, unpublished data from the laboratory of Melitta Schachner (T. Tilling, pers. communication) revealed a possible functional connection between L1 and 14-3-3 proteins. 14-3-3 overexpression in hippocampal neurons led to a specific reduction of L1-mediated neurite outgrowth. Therefore, in the present study, molecular and biochemical approaches were used to investigate and characterize the L1 – 14-3-3-interaction in order to understand 14-3-3's function in L1 downstream signaling.

3.2.1. 14-3-3 associates with L1

Immunoprecipitation experiments were performed to investigate whether L1 and 14-3-3 form complexes in the brain. An L1 polyclonal antibody preparation was used to precipitate L1 from membrane fractions isolated from 3 week-old wild-type mice brains. Western blot analysis of the L1 immunoprecipitates with an anti-14-3-3 monoclonal antibody revealed the presence of 14-3-3 in these immunoprecipitates (Fig. 15; upper panel), indicating that 14-3-3 is associated with L1 in mouse brain.
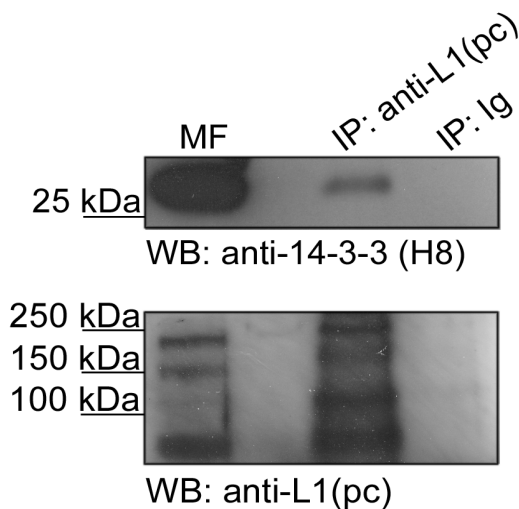
Figure 15: Immunoprecipitation reveals 14-3-3 – L1 complexes in mouse brain. *Upper panel:* Immunoprecipitation (IP) of L1 from crude brain membrane fractions (MF) was performed using an antibody to L1. Proteins were resolved by SDS-PAGE and analyzed by Western blotting (WB) with the H8 anti-14-3-3 antibody. *Lower panel:* Successful immunoprecipitation of L1 was shown by Western blot analysis of the precipitates with an anti-L1 antibody.

3.2.2. 14-3-3 isoforms β and ζ directly interact with the intracellular domain of L1

Based on the finding that 14-3-3 co-immunoprecipitates with L1, it was hypothesized that 14-3-3 may directly bind to the intracellular domain of L1 (L1 ICD). To test this hypothesis, an ELISA-based direct binding assay was performed. Recombinantly expressed L1 ICD was immobilized on microtiter plate wells, and its ability to bind 14-3-3ζ and β was measured in a semiquantitative manner. GST-14-3-3ζ and GST-14-3-3β bound in a concentration-dependent manner to L1 ICD (Fig. 16). There was no binding of GST-14-3-3ζ and β to the intracellular domain of a non-homologous cell adhesion molecule, NCAM180 (Fig 16; left and right panel), indicating that the interaction between L1 ICD and 14-3-3 is specific. Correction for potential GST background signals was performed by subtracting absorbance values in wells incubated with GST only (not shown). Taken together, these data demonstrate that 14-3-3ζ and β directly bind L1 ICD.
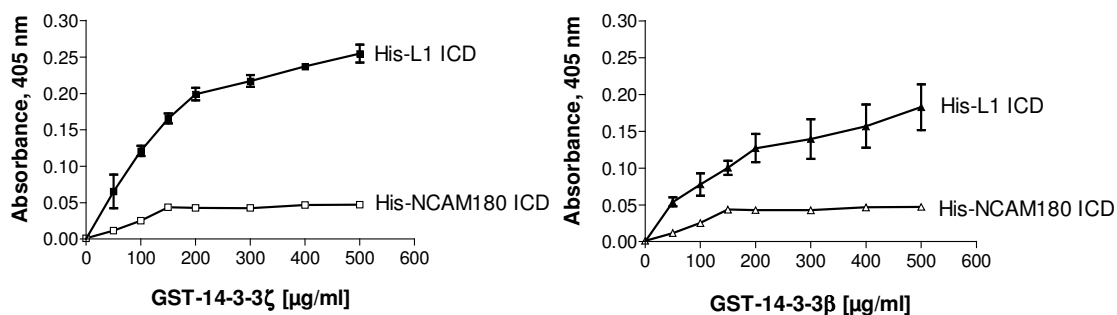
Figure 16: Direct binding of 14-3-3 isoforms β and ζ to L1 intracellular domain (L1 ICD). Recombinantly-expressed L1 ICD was immobilized on microtiter plate wells and assayed by ELISA for its ability to bind GST-14-3-3ζ (left panel) and GST-14-3-3β (right panel). Binding to the intracellular domain of a non-homologous cell adhesion molecule, NCAM180, served as a negative control. Specific absorbance values were calculated by subtracting total observed absorbance from absorbance in wells incubated with GST only. Error bars denote standard deviation based on 3 independent experiments.

3.2.3. Location of the 14-3-3ζ-binding region/site within the L1 intracellular domain

In the above experiments, it was observed that the 14-3-3ζ isoform binds the L1 ICD more strongly than 14-3-3β. Therefore, further experiments were performed with 14-3-3ζ. The next aim was to investigate which part of L1 ICD 14-3-3ζ binds preferentially. To this end, an ELISA was performed with peptides spanning the entire L1 intracellular domain (Fig. 17).
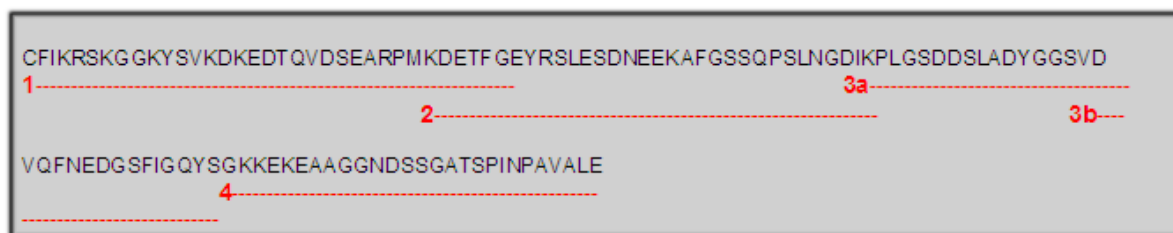
Figure 17: Synthetic peptides representing parts of the L1 intracellular domain. The L1 intracellular domain (L1 ICD) is shown in the single-letter amino acid code. Sequences of the L1 ICD peptides, which span the entire L1 intracellular domain, are indicated by the red dotted lines, with the respective number given at the N terminus. The synthetic peptides were used in an ELISA binding assay (Fig. 18).

The L1 peptides were immobilized, and GST-14-3-3ζ was applied in increasing concentrations to the coated plates. GST-14-3-3ζ bound specifically and in a concentration-dependent manner to the L1 peptides 2, 3a and 3b (Fig. 18), which comprise the central part of L1 ICD. Weak binding of GST-14-3-3ζ was observed to L1 peptides 1 and 4, encompassing the N- and C-termini of the L1 ICD, respectively. These results suggest that GST-14-3-3ζ preferentially binds to the central portion of the L1 ICD.
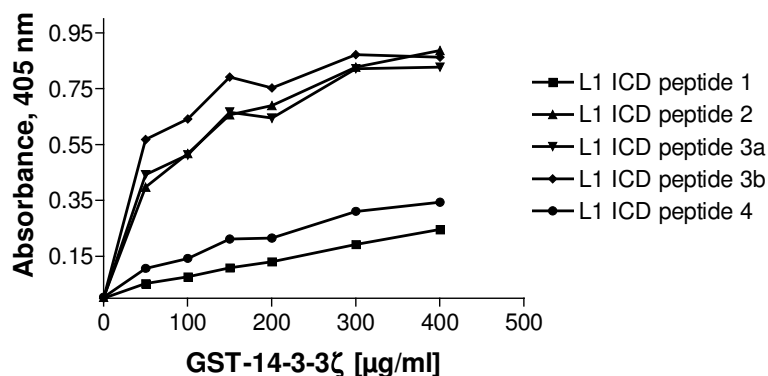
Figure 18: Concentration-dependent binding of GST-14-3-3ζ to the central part of the L1 ICD. Fixed amounts of peptides comprising the L1 ICD were immobilized and the ability of GST-14-3-3ζ to bind these peptides in ELISA was determined. GST-14-3-3ζ was applied at increasing concentrations to the plates.

3.2.4. 14-3-3ζ interacts with both non-phosphorylated and CK II-phosphorylated L1 ICD

In the majority of cases documented so far, 14-3-3 interacts with phosphoproteins. However, the above experiments showed that 14-3-3ζ is able to interact with non-phosphorylated recombinant L1 ICD and synthetic peptides representing the ICD. I therefore sought to more closely investigate whether phosphorylation of L1 ICD affects its interaction with 14-3-3ζ. To this end, L1 ICD was preincubated with CK II. This kinase was used here because the L1 ICD is specifically phosphorylated at Ser[1181] *in vitro* by CK II (Wong et al., 1996). Furthermore, [1177]RSLES[1181]D, containing the Ser[1181] residue, was hypothesized as a potential 14-3-3-binding site in the L1 ICD. In order to monitor the specificity of CK II phosphorylation, 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK II (Sarno et al., 2001), was utilized. We observed that 14-3-3ζ is indeed able to bind non-phosphorylated L1 ICD (Fig. 19; left, upper panel). However, L1 phosphorylation resulted in a stronger interaction with 14-3-3ζ compared to non-phosphorylated L1 (Fig. 19; left, upper panel; right panel), supporting the preferential interaction of 14-3-3 with phosphoproteins. We also observed that in the presence of TBB, CK II activity was partially inhibited because non-phosphorylated L1 ICD was present in the GST-14-3-3ζ eluate (Fig. 19; left, upper panel, lane 3: L1 ICD, CK II and TBB).
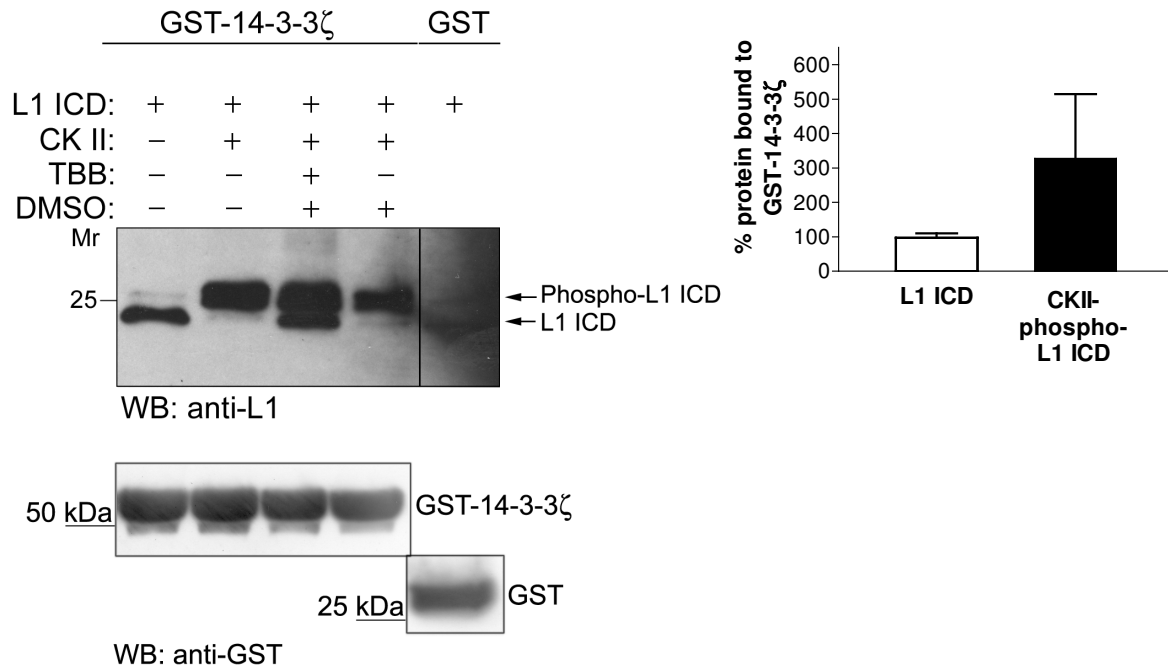
Figure 19: 14-3-3ζ pulled down phosphorylated and non-phosphorylated L1 ICD. *Left, upper panel:* Recombinant His-tagged L1 ICD was incubated in the presence or absence of CK II. To specifically inhibit CK II, 5 μM TBB was used. After treatment, a GST-14-3-3ζ pull-down was performed to investigate direct binding of L1 ICD to 14-3-3. Pull-down eluates were analyzed by Western blotting (WB) with the 74-5H7 anti-L1 antibody. *Left, lower panel:* GST and GST-14-3-3ζ were detected by Western blot (WB) with an anti-GST antibody. *Right panel:* L1-immunoreactive bands in lane 1 (L1 ICD only) and lane 2 (L1 ICD and CK II) were quantified and the percentage of bound L1 ICD and CK II-phospho-L1 ICD bound to GST-14-3-3ζ were graphed. Error bars denote standard deviation based on 3 independent experiments.

3.2.5. Ser[1181]->Ala substitution and RSLESD sequence deletion in the L1 ICD disrupt 14-3-3ζ binding

Based on the above observations, I wished to identify the 14-3-3-binding site in L1 ICD. The central part of the L1 ICD contains the amino acid sequence RSLESD. The second serine within this sequence, Ser[1181] (Fig. 20), can be phosphorylated by CK II (Wong et al., 1996) and $RX_{2-3}pS$ is a potential 14-3-3-binding motif (Fu et al., 2000). To determine whether the RSLESD sequence and, in particular, Ser[1181], are important for 14-3-3ζ binding to L1 ICD, pull-down assays were performed by comparing the ability of GST-tagged 14-3-3ζ to interact with *in vitro* CK II-phosphorylated L1 ICD and mutants L1 ICD-S1181A and L1 ICDΔRSLESD (Fig. 21). Both mutations, S1181A and ΔRSLESD, substantially reduced L1

ICD binding to 14-3-3ζ (Fig. 21; upper panel). These data suggest that 14-3-3 binding to L1 requires the amino acid sequence RSLESD, in particular the second serine, Ser[1181].
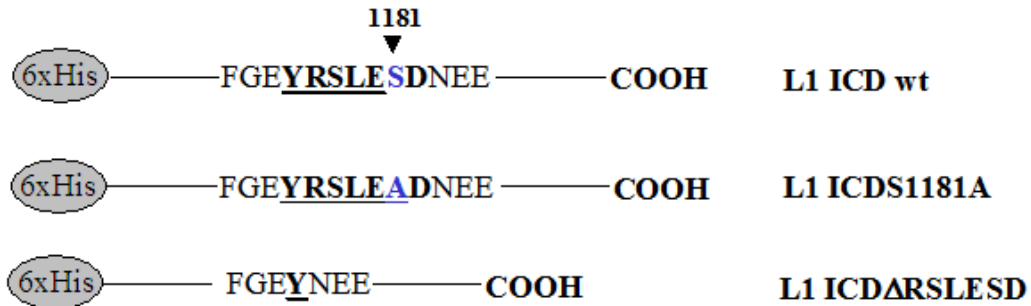
Figure 20: Scheme of recombinant L1 intracellular domain constructs. The full-length L1 intracellular domain construct contains the RSLESD sequence (**boldface**). L1 ICDS1181A has a single amino acid substitution (S1181A) of a serine residue in the motif. S1181 can be phosphorylated by casein kinase II. The RSLESD sequence, a potential 14-3-3 binding motif, is deleted in L1 ICDΔRSLESD. Wild-type and mutated L1 ICD constructs were recombinantly expressed as 6xHis-tagged proteins, purified from bacterial lysates and used in pull-down experiments.
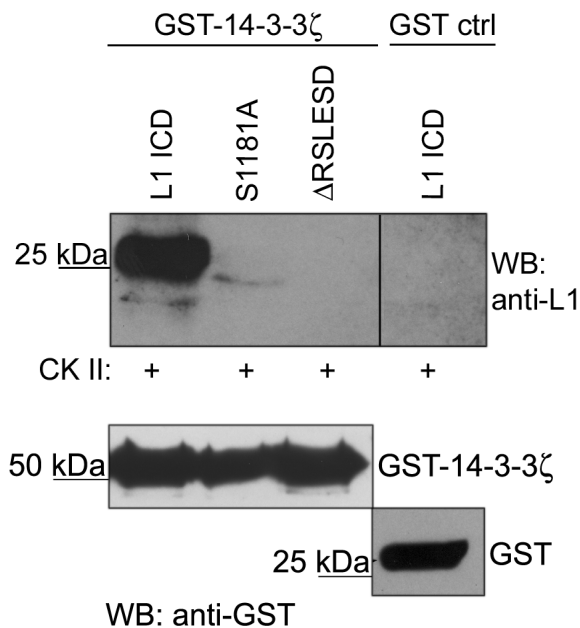
Figure 21: Ser[1181]->Ala substitution and RSLESD deletion in L1 ICD disrupt binding to 14-3-3ζ. *Upper panel:* 6xHis-tagged proteins, purified from bacterial lysates, were subjected to GST-14-3-3ζ pull-down assays after treatment with CK II. GST was used as a control. Pull-down eluates were analyzed by Western blotting (WB) with the 74-5H7 anti-L1 antibody. *Lower panel:* GST and GST-14-3-3ζ were detected by Western blot (WB) with an anti-GST antibody.

3.2.6. 14-3-3ζ stimulates CK II-catalyzed L1 ICD phosphorylation

CK II is known to phosphorylate Ser[1181] in the L1 ICD (Wong et al., 1996) and mutation of Ser[1181] had shown that this residue is important for L1 ICD interaction with 14-3-3ζ (see above). To gain further insight into the function of the L1 ICD - 14-3-3ζ-interaction, the influence of 14-3-3ζ on CK II-catalyzed L1 ICD phosphorylation was examined. To this end, L1 ICD was preincubated overnight with GST-14-3-3ζ (or GST only as a negative control) and then subjected to CK II phosphorylation. At various time points, the CK II-catalyzed phosphorylation reaction was stopped by adding SDS-PAGE loading buffer. The effect of GST-14-3-3ζ on L1 ICD phosphorylation was analyzed by Western blotting using an anti-L1 monoclonal antibody. Phosphorylation of L1 ICD was evident by an upward shift in mobility in SDS-PAGE relative to non-phosphorylated L1 ICD (Fig. 22). Western blot analysis with anti-L1 revealed that the band intensities of phosphorylated L1 increased over time when L1 ICD was preincubated with GST-14-3-3ζ (Fig. 22; upper panel).
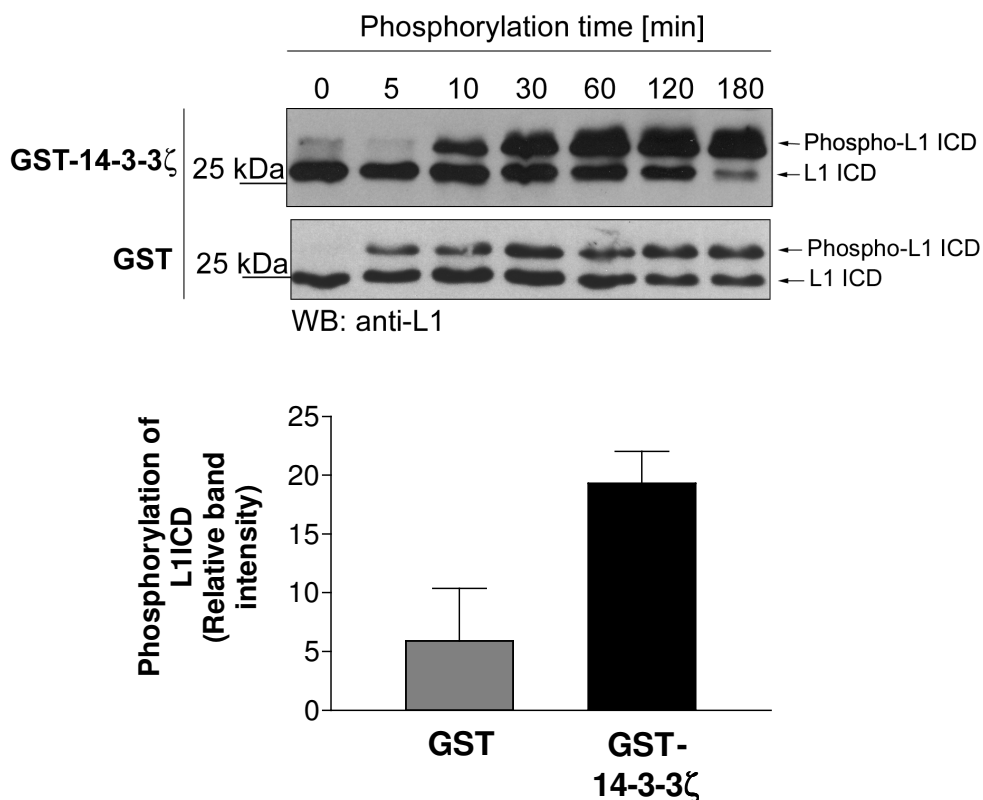
Figure 22: Time-dependent phosphorylation of L1 ICD by casein kinase II in the presence of 14-3-3ζ.
Upper panel: L1 ICD was preincubated in the presence or absence of 14-3-3ζ followed by incubation with casein kinase II. At different time points, CK II phosphorylation was stopped, and the level of L1 ICD phosphorylation was determined by comparing the intensities of the upper band, which presumably represents phosphorylated L1 ICD, and the lower band, most probably non-phosphorylated L1 ICD. *Lower panel:* Blot bands at time points 0 and 180 min were quantified and the relative intensities were graphed. Error bars denote standard deviation based on 3 independent experiments.

When L1 ICD was preincubated with GST, there was no significant change in the band intensities over time (Fig. 22; upper panel). A quantitative comparison of band intensities at time points 0 and 180 min indicated that at the latter time point, L1 ICD phosphorylation was ~15 times greater in the presence of 14-3-3ζ than in its absence (Fig. 22; lower panel). These data indicate that 14-3-3ζ promotes phosphorylation of L1 ICD by CK II.

To investigate whether the time-dependent CK II-catalyzed phosphorylation of L1 ICD requires Ser[1181], the above experiment was repeated with an L1 ICD-S1181A mutant. In contrast to wild-type L1 ICD, no band shift was observed over time with the mutants (Fig.

23), indicating that Ser^{1181} is the specific CK II phosphorylation site on L1 ICD and is stimulated by 14-3-3ζ.
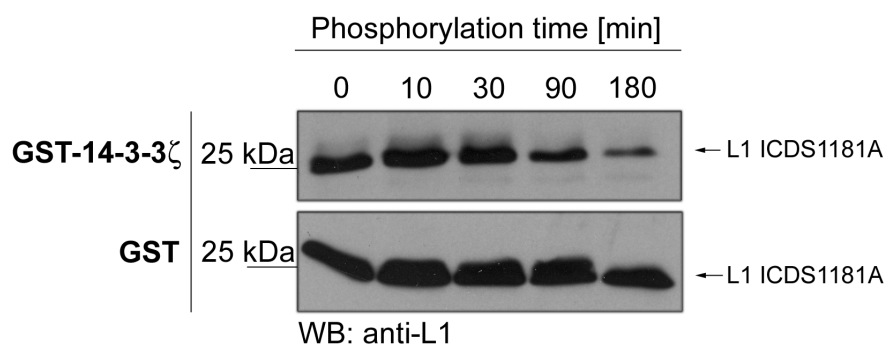
Figure 23: Time-dependent phosphorylation of L1 ICDS1181A by CK II in the presence of 14-3-3ζ. L1 ICDS1181A mutant was preincubated in the presence or absence of 14-3-3ζ followed by incubation with casein kinase II. At different time points the CK II phosphorylation was stopped, and a possible L1 ICDS1181A phosphorylation was investigated by checking for an additional L1-specific band at slightly higher molecular weight (compare Fig. 21).

3.2.7. The adaptor protein μ-AP2 does not affect L1 ICD binding to 14-3-3ζ

Having investigated the phosphorylation-dependent interaction of L1 with 14-3-3ζ above, I next wished to investigate whether other proteins might influence the interaction of 14-3-3ζ with L1. The adaptor protein μ-AP2 is known to bind Tyr^{1176}, immediately preceding the RSLESD sequence. The underlined sequence forms a Tyr-based sorting motif that is necessary for endocytosis of L1 via the AP-2/clathrin-mediated pathway (Kamiguchi and Lemmon, 1998). To investigate whether μ-AP2 competes with 14-3-3 for the RSLESD binding site, a competition pull-down assay was performed. CK II-preincubated L1 ICD was incubated in the presence of 14-3-3ζ and increasing concentrations of μ-AP2. There was no difference in the level of 14-3-3-bound L1 in the presence or absence of μ-AP2, suggesting that μ-AP2 does not compete with 14-3-3ζ for L1 binding (Fig. 24; upper panel). Thus, the results revealed that μ-AP2 does not affect L1 ICD binding to 14-3-3ζ.
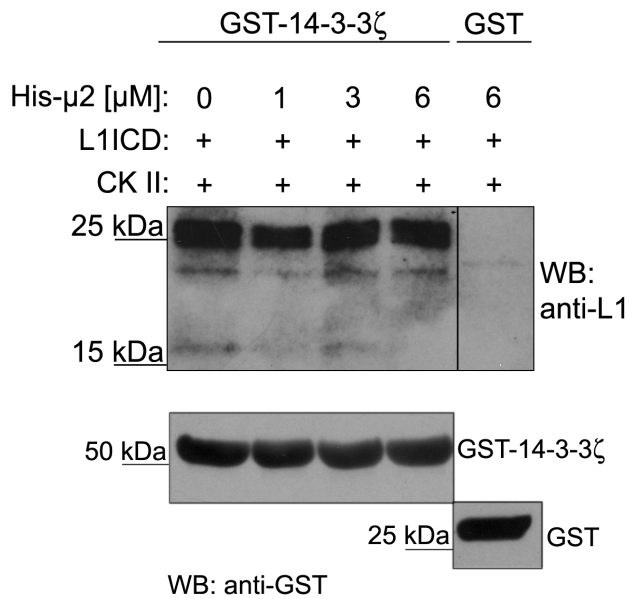
Figure 24: µ-AP2 does not compete with 14-3-3ζ for L1 binding. *Upper panel:* Recombinant L1 ICD encompassing the µ-AP2-binding domain and 14-3-3-binding site was preincubated with CK II followed by incubation with increasing concentrations of recombinant µ-AP2 and in the presence of GST-14-3-3ζ. Bound L1 ICD from GST-14-3-3ζ pull-down eluates was analyzed by Western blot with the 74-5H7 anti-L1 antibody. *Lower panel:* GST and GST-14-3-3ζ were detected by Western blot (WB) with an anti-GST antibody.

3.2.8 14-3-3ζ is enriched in endosomal fractions

Endosomes are intracellular vesicles important for the organization of intracellular membrane dynamics (Schmidt and Haucke, 2007). Recycling of endocytosed L1 occurs via sorting and recycling endosomes (Kamiguchi and Lemmon, 2000). Previous studies by Nakata *et al.* demonstrated that Ser^{1181} phosphorylation by CK II is implicated in normal endocytic trafficking of L1 (Nakata and Kamiguchi, 2007). Having shown the direct interaction of L1 with 14-3-3ζ above, we wished to analyze the distribution of 14-3-3ζ in endosomal fractions. To this end, crude membrane preparations from P7 wild-type mice were fractionated to obtain endosomal fractions. The 557 anti-L1 rat monoclonal antibody was used to precipitate L1 from these endosomal fractions. Western blot analysis of the L1 immunoprecipitates showed that full length L1 (~200 kDa) was succesfully precipitated and enriched in certain endosomes (Fig. 25; upper panel). Western blot analysis with an isoform-specific anti-14-3-3ζ antibody revealed the presence of 14-3-3ζ (~30 kDa) in L1 immunoprecipitates. These results indicated that 14-3-3ζ is associated with L1 in certain endosomes. Interestingly, we also observed that 14-3-3ζ was most strongly associated with L1 in the endosomal fraction 6, which contains relatively low L1 amounts (Fig. 25; upper panel), suggesting an influence of 14-3-3ζ on the distribution of L1 in endosomes during L1 endocytosis.
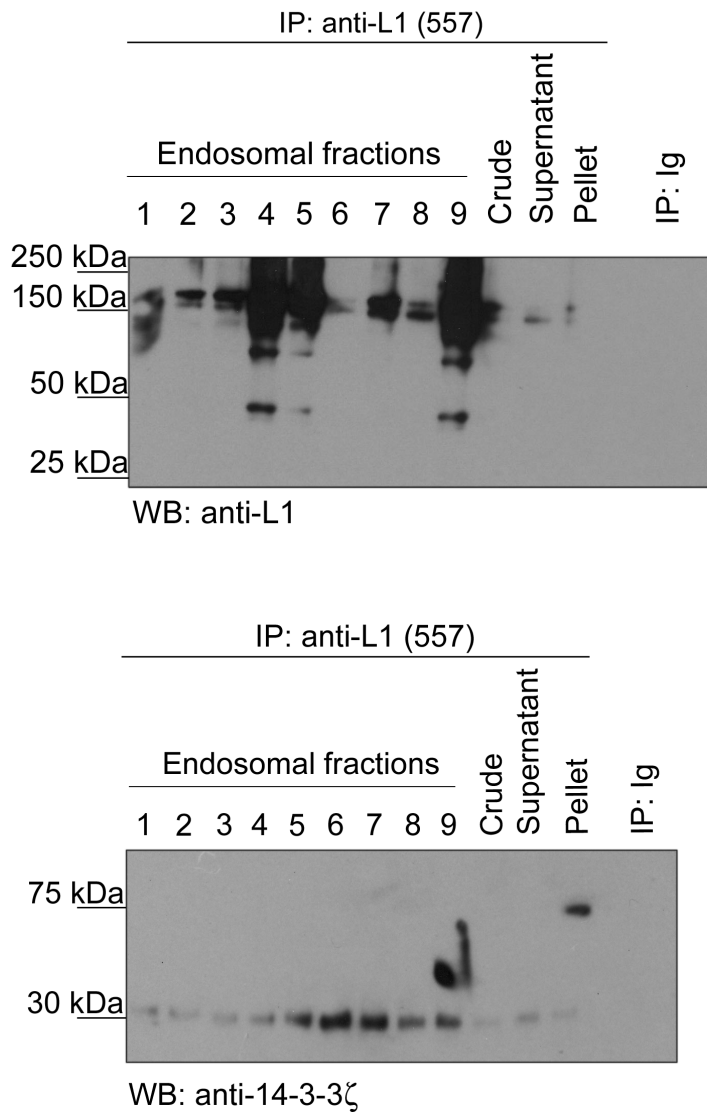
Figure 25: Co-immunoprecipitation of 14-3-3ζ with L1 from endosomal fractions. Preparation of endosomal fractions was performed from crude membrane fractions, followed by immunoprecipitation of L1. *Upper panel:* Immunoprecipitation (IP) of L1 from endosomal fractions was performed using the anti-L1 monoclonal antibody 557. Proteins were resolved by SDS-PAGE and analyzed by Western blotting (WB) with an anti-L1 antibody (74-5H7), showing that L1 was successfully precipitated. *Lower panel:* Western blot analysis of the L1 immunoprecipitates was also performed with an isoform-specific anti-14-3-3ζ antibody, revealing the presence of 14-3-3ζ in L1 eluates of endosomal fractions. This experiment was performed with the help of Gerrit Wolters. Crude: crude endosomal preparation; supernatant: cytosolic compounds; pellet: crude membrane fraction; Ig: control IgG used for immunoprecipitation.

4. Discussion

The aim of this thesis was to investigate and specifically characterize neuronal 14-3-3-binding partners, with the long-term goal of gaining a better understanding of the cellular functions of 14-3-3 proteins in the brain. The first major finding was the identification of alpha II spectrin as a 14-3-3β-binding partner. Alpha II spectrin was found to associate with 14-3-3β in the mouse brain and a mode-2 14-3-3-binding motif was identified within alpha II spectrin repeat unit 12. A Ser residue at position 1302 within this binding motif was discovered as a critical mediator of alpha II spectrin interaction with 14-3-3β. This interaction was more efficient in the presence of CK II, indicating the necessity for phosphorylation. The presence of calmodulin was found to enhance 14-3-3β-binding to an alpha II spectrin 10-14 fragment.

The second major finding was the observation that 14-3-3ζ associates with L1 in the mouse brain and profoundly stimulates CK II-catalyzed *in vitro* phosphorylation of the L1 ICD. 14-3-3ζ was found to interact directly with both phosphorylated and non-phosporylated L1 ICD; a Ser residue located at position 1181 within the sequence RSLESD, a putative 14-3-3-binding site, was found to mediate the L1 ICD interaction with 14-3-3ζ. 14-3-3ζ co-purified with L1 from endosomal fractions and was enriched in L1 immunoprecipitates when L1 amounts were reduced.

4.1. Alpha II spectrin interaction with 14-3-3

4.4.1. Phosphorylation-dependent interaction of alpha II spectrin with 14-3-3β

The 14-3-3 protein family is ubiquitous and known to play an important role in many intracellular processes (Aitken et al., 2002). Given that 14-3-3 proteins exert their functions by binding other proteins, identifying target proteins that interact with 14-3-3 has proven to be useful in characterizing the molecular mechanisms of these cellular events (Darling et al., 2005). 14-3-3 proteins are highly abundant in the brain, and although only a few binding partners have been postulated so far, 14-3-3 proteins are clearly critical for brain development, memory, and learning, and have been implicated in neurological disorders as

well (Berg et al., 2003; Mackintosh, 2004). This study provides clear evidence of an interaction between alpha II spectrin and 14-3-3β in the brain.

In an attempt to identify new 14-3-3-binding proteins, several groups have performed affinity chromatography and mass spectrometry analysis (Suginta et al., 2001; Jin et al., 2004; Meek et al., 2004; Angrand et al., 2006). In the present study, affinity chromatography also proved useful for identifying additional 14-3-3β-binding proteins located in the brain. Using mouse brain membrane fractions, alpha II spectrin, a major constituent of the cytoskeletal network that is associated with the plasma membrane of cells in the brain (De Matteis and Morrow, 2000; Bennett and Baines, 2001), was identified as a 14-3-3β target (Fig. 5). This result mirrors that from a previous study by Angrand et *al.*, in which alpha II spectrin was identified as a putative 14-3-3ζ-binding partner using a tandem affinity purification (TAP)-MS method to look for 14-3-3ζ-binding targets in the brain of transgenic mice (Angrand et al., 2006). However, the interaction between alpha II spectrin and 14-3-3ζ was not investigated further in the Angrand et *al.* study. Here, the interaction of 14-3-3β with alpha II spectrin was confirmed by GST-14-3-3β pull-down experiments using adult mouse brain homogenates and co-immunoprecipitation of alpha II spectrin with 14-3-3β from mouse brain membrane fractions (Figs. 6 and 7).

14-3-3 proteins typically interact with specific phosphoserine residues on their binding partners. A putative 14-3-3-binding motif, ^{1298}RLIQ$\underline{S}$HP^{1304}, (putative phosphoserine, Ser^{1302}, underlined) within the alpha II spectrin repetitive unit 12 corresponds to the consensus 14-3-3-binding motif RXXXpSXP (Yaffe et al., 1997). The RLIQSHP sequence is the only consensus 14-3-3-binding motif in the entire alpha II spectrin molecule, as predicted using the PATTINPROT algorithm of the Network Protein Sequence Analysis web server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html; (Combet et al., 2000)). Loss of 14-3-3β binding upon mutating Ser^{1302} to Ala in alpha II spectrin repetitive units 10-14 confirmed Ser^{1302} as an important mediator of the 14-3-3β interaction (Fig. 10). A more detailed characterization of the interaction between alpha II spectrin and 14-3-3β was not achievable during this thesis work. For example, it might be of interest to determine the affinity of the interaction between these two proteins by performing surface plasmon resonance (SPR), as has been done in other studies (Muslin et al., 1996; Sadik et al., 2009).

Such analyses would allow determination of the specificity of the two partners, and compare the strength of this binding with other known interactions of alpha II spectrin.

4.1.2. CK II phosphorylation is required for efficient 14-3-3β binding to alpha II spectrin

The Ser[1302] residue noted above is located within a consensus site (S[1302]HPE) for CK II phosphorylation. CK II, which is highly abundant in neurons, is suggested to perform essential roles during neurite outgrowth in developing neurons (Blanquet, 2000). The general determinants of a CK II substrate site are acidic residues C-terminal to the Ser/Thr residue to be phosphorylated, with a crucial acidic residue at the +3 position (Pinna, 1990). The ability of CK II to promote 14-3-3β binding to alpha II spectrin fragment 10-14 *in vitro* (Fig. 11) demonstrates the importance of Ser[1302] phosphorylation for binding. It should be mentioned that the phosphorylation-dependent interaction was shown only for alpha II spectrin fragment 10-14, which encompasses 5 of the 21 spectrin repeats of alpha II spectrin and includes the predicted 14-3-3-binding motif. The possibility of further 14-3-3-binding motifs within the native alpha II spectrin molecule that may not be predictable by the PATTINPROT algorithm used here cannot be fully excluded. Therefore, it may be of interest for future studies to investigate the interaction of 14-3-3β with full-length alpha II spectrin. To further define the extent to which 14-3-3β depends on alpha II spectrin phosphorylation for binding, it might also be interesting to analyze the interaction of the two proteins *in vitro* after alkaline phosphatase treatment or inhibition of CK II-mediated phosphorylation in cells with the specific inhibitor TBB (Battistutta et al., 2001; Sarno et al., 2001; Pagano et al., 2004). We hypothesize that CK II could be one of several protein kinases that function through the assembly of cytoskeleton-associated multiprotein complexes together with 14-3-3 to coordinate signaling mechanisms underlying synaptic plasticity in neurons.

4.1.3. 14-3-3β as a possible target of NCAM-mediated cell dynamics

It is known that alpha II spectrin forms heterotetramers with the beta I spectrin subunit (Goodman et al., 1995), which binds to the 180 and 140 kDa isoforms of the cell adhesion molecule NCAM (Pollerberg et al., 1985; Leshchyns'ka et al., 2003; Sytnyk et al., 2006). The NCAM 180 – spectrin association may be important for the organization of membrane

proximal signaling complexes, for example in the postsynaptic density (Ditlevsen et al., 2008). The ability of 14-3-3β and alpha II spectrin to co-precipitate with NCAM (Fig. 8) from the brain suggests that these two proteins form complexes with NCAM in neuronal cells.

One might conclude from this observation that 14-3-3β plays a role in NCAM-mediated molecular dynamics of cell recognition via the spectrin cytoskeleton. Because it is assumed that CK II is involved in the regulation of spectrin (Clari and Moret, 1985), phosphorylated alpha II spectrin might recruit 14-3-3β, which may help to reorganize the cytoskeleton during NCAM-mediated neurite outgrowth. From previous studies, it is known that NCAM 180 and the growth-associated protein, GAP-43, form a functional complex with spectrin that may control cytoskeleton dynamics to induce neurite outgrowth (Korshunova et al., 2007). The phosphorylated form of GAP-43 and GAP-43-like proteins are implicated in the reorganization of the cytoskeleton (Aarts et al., 1999; Frey et al., 2000; Laux et al., 2000). Therefore, I hypothesize that 14-3-3β, like GAP-43, may play a role in the reorganization of the spectrin cytoskeleton by binding directly to CK II-phosphorylated spectrin during NCAM-mediated neurite outgrowth. More studies will be required to better comprehend the participation of 14-3-3β in NCAM-mediated neuronal processes, e.g. neurite growth and synaptic plasticity.

4.1.4. Calmodulin activity affects 14-3-3β - alpha II spectrin interactions

Calmodulin is known to bind to a sequence contained in the 11th repeat unit of the vertebrate alpha II spectrin subunit, thereby influencing the interaction of alpha II spectrin with other binding partners (Harris et al., 1988; Simonovic et al., 2006). For example, calmodulin has been reported to stimulate the proteolytic modification of alpha II spectrin by calpain during postsynaptic density remodeling (Harris and Morrow, 1990; Dosemeci and Reese, 1995). Other studies have shown that calmodulin, together with 14-3-3, controls the subcellular distribution of the Ras-related GTPase subfamily members Kir/Gem that are involved in cytoskeletal reorganization (Beguin et al., 2005). In the present study, calmodulin's influence on the alpha II spectrin – 14-3-3β-interaction was shown by calmodulin-enhanced 14-3-3β binding to alpha II spectrin 10-14 (Fig. 13). The enhanced binding of 14-3-3β might be due to conformational changes in the alpha II spectrin molecule caused by the calmodulin - spectrin-interaction, resulting in a better exposure of the 14-3-3β-binding motif on alpha II spectrin.

Addition of EDTA only led to a slight reduction of 14-3-3β binding to the alpha II spectrin fragment 10-14 in the presence of calmodulin, suggesting that calmodulin is able to bind alpha II spectrin at low Ca^{2+} concentrations. Future studies will need to address this question more critically by mutating the calmodulin-binding site within alpha II spectrin.

4.1.5. Influence of 14-3-3β on alpha II spectrin proteolysis by μ-calpain

The proteolysis of alpha II spectrin by μ-calpain may be physiologically relevant to synaptic remodeling, long-term potentiation, and memory formation (Dosemeci and Reese, 1995; Vanderklish et al., 1995; Faddis et al., 1997). Alpha II spectrin proteolysis by μ-calpain is regulated by phosphorylation, Ca^{2+} and calmodulin (Croall and DeMartino, 1991; Chan and Mattson, 1999; Nicolas et al., 2002; Nedrelow et al., 2003; Simonovic et al., 2006; Glantz et al., 2007). Therefore, the potential influence of 14-3-3β on alpha II spectrin proteolysis by μ-calpain was also investigated in this work. Initial studies showed that proteolysis of GST-tagged alpha II spectrin 10-14 by μ-calpain can occur *in vitro* (data not shown). However, no effect of 14-3-3β on proteolysis of alpha II spectrin 10-14 by μ-calpain was observed. A possible avenue of further experimentation to answer this question would be to express an alpha II spectrin construct with two different tags, one at the N- and one at the C-terminus. The use of tags at each end of the protein might allow better assaying of proteolytic fragments in the presence or absence of 14-3-3β.

4.1.6. 14-3-3 – alpha II spectrin binding: implications for synapse formation

In summary, this study has shown that alpha II spectrin from mouse brain membrane fractions associates with 14-3-3β. This work also provides evidence of residue Ser[1302] in the alpha II spectrin site as the critical site for 14-3-3β binding. Finally, a more efficient binding of 14-3-3β to alpha II spectrin was observed in the presence of CK II, suggesting a phosphorylation-dependent interaction. In this context, it is noteworthy that alpha II spectrin is able to form heteromeric complexes with beta I spectrin, which is suggested to occur postsynaptically in hippocampal neurons (Ursitti et al., 2001).

Previous studies by Carlin *et al.* suggested that alpha II spectrin is a major constituent of the postsynaptic density (Carlin et al., 1983). Selective phosphorylation- and calmodulin-

dependent processing of alpha II spectrin in response to synaptic activity might lead to a structural rearrangement of the synapse, allowing a rapid remodeling of synapses after stimulation (Harris and Morrow, 1990; Vanderklish et al., 1995). Therefore, it would be highly interesting to investigate whether the alpha II spectrin – 14-3-3β-interaction is involved in synapse formation. By selective binding to CKII-phosphorylated alpha II spectrin, 14-3-3β may exert a significant influence on cytoskeletal remodeling during synaptogenesis. It is tempting to speculate that the 14-3-3β – spectrin-interaction functions as a molecular switch, regulating binding of other ligands to alpha II spectrin in the postsynaptic density. Thus, further investigations to elucidate the physiological interaction of alpha II spectrin with 14-3-3β will likely contribute to the characterization of alpha II spectrin function in the brain, in particular in the synaptic context.

4.2. L1 interaction with 14-3-3

L1 is a cell adhesion molecule that plays an important role in mediating cell migration and axon outgrowth during neural development and regeneration (Hortsch, 1996; Kenwrick et al., 2000; Chen et al., 2007; Maness and Schachner, 2007). L1 is able to function as a mediator of signal transduction via its highly conserved ICD. One important aspect of the L1 ICD is the regulation of L1 recycling via endocytic pathways during axon outgrowth (Kamiguchi and Yoshihara, 2001). Prior to the work presented here, experiments performed with 14-3-3-transfected hippocampal neurons showed a specific reduction of neurite length in L1-mediated outgrowth (T. Tilling, unpublished data), suggesting the possible involvement of 14-3-3 as an intracellular mediator of L1 downstream signaling. This study provides the first evidence that 14-3-3 interacts *directly* with L1 and thereby possibly influences L1 function. The direct association between L1 and 14-3-3 was demonstrated by

- o Co-precipitation of 14-3-3 and L1 from mouse brain membrane fractions (Fig. 15),
- o Direct binding of a GST-14-3-3ζ fusion protein to L1 ICD (Fig. 16), and
- o Co-precipitation of 14-3-3ζ protein and L1 from mouse brain endosomal fractions (Fig. 25)

Direct association of L1 and 14-3-3ζ depends on Ser^{1181} within the ICD of L1. Mutation of Ser^{1181} to Ala (S1181A) resulted in greatly reduced binding to 14-3-3ζ (Fig. 21). Furthermore,

I found that phosphorylation of L1 ICD at Ser[1181] by CK II was profoundly stimulated by 14-3-3ζ (Fig. 22 and 23).

4.2.1. 14-3-3 proteins interact directly with L1

14-3-3 was found to co-immunoprecipitate with L1 form mouse brain membrane fractions (Fig. 15), suggesting a physiological association between 14-3-3 and L1. ELISA binding studies with histidine-tagged L1 ICD protein and GST-tagged 14-3-3ζ or 14-3-3β demonstrated the direct interaction of L1 with both 14-3-3 isoforms (Fig. 16). Interestingly, the interaction between the ζ isoform of 14-3-3 and L1 ICD appeared to be stronger than the one between 14-3-3β and L1 ICD. How might this isoform specificity be explained? 14-3-3 proteins interact with target proteins via a highly conserved binding groove (cf. Fig. 1). Although the residues that form the binding groove are conserved in all seven 14-3-3 isoforms, their binding intensities differ for the same ligand. For example, all 14-3-3 isoforms bind tryptophan hydroxylase with the same affinity, whereas only the β and ζ isoforms bind Raf (Freed et al., 1994; Reuther and Pendergast, 1996; Aitken, 2002; Truong et al., 2002). Furthermore, ELISA experiments with L1 ICD-derived peptides and GST-14-3-3ζ localized the 14-3-3 – L1-interaction to the central part of the ICD of L1 (Fig. 18). The ICD of L1 contains several amino acids that are negatively charged, most of them occuring in the central part of L1 ICD (Fig. 17), which may mediate the interaction with 14-3-3ζ.

4.2.2. Enhanced binding of phosphorylated L1 ICD to 14-3-3ζ

The above-mentioned experiments showed that 14-3-3ζ interacts with non-phosphorylated recombinant L1 ICD and synthetic peptides encompassing the ICD. Although most known 14-3-3 ligands possess phosphoserine- or phosphothreonine-based motifs, several interactions between 14-3-3 and non-phosphorylated motifs within ligand proteins have been described. Examples include the sequences VTPEER of the amyloid β-protein precursor ICD fragment (Sumioka et al., 2005), WLDLE of the synthetic peptide R18 (Petosa et al., 1998; Wang et al., 1999), and LDSLDL of the exoenzyme S cytotoxin from *Pseudomonas aeruginosa* (Masters et al., 1999; Henriksson et al., 2000). A common feature of these non-phosphorylated 14-3-3-binding motifs is that they contain negatively charged residues that mediate the interaction

with positively charged residues in the 14-3-3 amphipathic groove. Thus, the observed interaction of 14-3-3 with non-phosphorylated L1 was not entirely surprising. However, by performing pull-down experiments with L1 ICD and *in vitro* CK II phosphorylated L1 ICD, I could demonstrate that the L1 phosphorylation resulted in a stronger interaction with 14-3-3ζ compared to non-phosphorylated L1 (Fig. 19), thus, supporting the preferential interaction of 14-3-3 with phosphoproteins.

These results are in line with a previous study by Hashiguchi *et al.* (2000), who showed that 14-3-3ζ is able to interact with non-phosphorylated and phosphorylated forms of the Tau protein from bovine brain. However, their observations suggested that phosphorylation of Tau does not improve 14-3-3ζ binding. Hashiguchi *et al.* (2000) proposed that 14-3-3ζ is a Tau protein effector and may be involved in the abnormal Tau phosphorylation occuring during Alzheimer's disease. To further examine the interaction of L1 ICD with 14-3-3ζ under phosphorylation-dependent and phosphorylation-independent conditions, it might be fruitful to determine the equilibrium dissociation constants (K_d) for these interactions by performing SPR.

The central part of the L1 ICD contains the amino acid sequence RSLESD. The second serine within this sequence, Ser[1181] (Fig. 20), can be phosphorylated by CK II (Wong et al., 1996) and $RX_{2-3}pS$ is a potential 14-3-3-binding motif (Fu et al., 2000). This CK II-phosphorylation site is evolutionarily well-conserved among L1 orthologs and L1 family CAMs and, therefore, is likely to be required for subsequent interactions of L1 proteins in signaling cascades and to serve a significant role in L1 function. In this thesis, I have shown that Ser[1181] in the amino acid sequence [1177]RSLESD[1182] of the L1 ICD mediates the 14-3-3ζ interaction (Fig. 21). Substitution of Ser[1181] with Ala and deletion of the entire RSLESD motif resulted in abolition of 14-3-3ζ binding. It is worth noting that the RSLE sequence occurs only in the neuronal isoform of L1 (Miura et al., 1991), and that the CK II phosphorylation site in L1 is adjacent to this alternatively spliced, neuron-specific motif. Given that the general determinants for a CK II substrate site are acidic residues C-terminal to the Ser and a crucial acidic residue at the +3 position, it was not expected that the RSLE domain itself would have an effect on the phosphorylation of Ser[1181]. Thus, it is arguable whether 14-3-3ζ also interacts with the non-neural form of L1. Further studies will be needed to elucidate whether 14-3-3ζ specifically interacts with L1 only in neurons.

Based on the observation that 14-3-3ζ interacts with non-phosphorylated and phosphorylated L1 ICD, the question arose whether 14-3-3ζ exclusively utilizes the identified binding site (RSLESD) for phosphorylation-dependent and –independent interaction with L1, or whether the L1 ICD contains more than one binding site for 14-3-3ζ. Notably, previous studies showed that 14-3-3ζ interacts with phosphorylated and non-phosphorylated Tau protein and that 14-3-3ζ utilizes two distinct binding sites for these interactions (Hashiguchi et al., 2000; Sadik et al., 2009). Both studies also demonstrated that the phosphorylation of the Tau protein by PKA increased its affinity for 14-3-3ζ. This observation is reminiscent of the influence of CK II on the 14-3-3ζ - L1-interaction, as CK II phosphorylation enhanced binding of 14-3-3ζ to L1 ICD. These observations might reflect a more general mechanism of regulating 14-3-3 – ligand-interactions: in a first step, 14-3-3 could bind to its non-phosphorylated ligand with low affinity. Phosphorylation of the ligand, e.g. as a consequence of a physiological stimulus, could then tighten the 14-3-3 – ligand-interaction.

Where might the putative additional 14-3-3ζ-binding site be located within the L1 ICD? In ELISA experiments performed with L1 peptides, I observed a preferential binding of 14-3-3ζ to the L1 peptides 2, 3a and 3b (Fig. 18), which comprise the central part of the L ICD. The Ser[1181] residue described in this study as important for the L1 ICD interaction with 14-3-3ζ is located only within the L1 peptide 2 and, thus, further 14-3-3ζ-binding sites within the L1 peptide 3a and 3b may be possible. Additional studies are warranted to further examine these binding sites within L1 ICD that may mediate the phosphorylation-independent interaction with 14-3-3ζ.

4.2.3. 14-3-3ζ stimulates CK II-catalyzed L1 ICD phosphorylation

Previous studies by Nakata *et al.* suggested that CK II regulates endocytic L1 trafficking in the axonal growth cone via phosphorylation of the L1 ICD (Nakata and Kamiguchi, 2007). Growth cones need regulatory mechanisms to select the correct endocytic pathway for L1 molecules. One possibility is a regulated CK II phosphorylation of L1 ICD, allowing subsequent binding of molecules at this site or other residues in the vicinity. Regulation is also likely to be mediated by cytosolic molecules that bind to L1 ICD and influence endocytosis (Kamiguchi, 2003). As demonstrated above, 14-3-3ζ binds to L1 ICD, and their

phosphorylation-dependent interaction is mediated by Ser[1181], which is phosphorylated by CK II. Results from a time-dependent CK II phosphorylation assay performed with L1 ICD that has been preincubated with 14-3-3ζ demonstrated that phosphorylation of L1 ICD on Ser[1181] by CK II is profoundly enhanced by 14-3-3ζ (Fig. 22 and 23). These data suggest that 14-3-3ζ is an effector of CK II-mediated L1 ICD phosphorylation. This modulation of L1 ICD phosphorylation may play a role in controlling L1 sorting and trafficking between plasma membranes and endosomes in neuronal growth cones.

How might 14-3-3ζ enhance phosphorylation of L1 by CK II? One explanation is that L1 phosphorylation is promoted by initial interactions between 14-3-3ζ and (non-phosphorylated) L1 ICD, resulting in L1 ICD conformational changes and, consequently, increased susceptibility for CK II phosphorylation. From the literature it is known that 14-3-3 behaves in essence like a molecular anvil, deforming its bound ligands while undergoing only minimal structural alterations itself. For example, in the case of serotonin N-acetyl transferase, and presumably exoenzyme S, 14-3-3 binding deforms the catalytic residues so as to promote substrate binding and product formation, perhaps through inducing a conformation that stabilizes the transition state of the enzyme:substrate complex (Henriksson et al., 2000; Obsil et al., 2001). For other proteins, 14-3-3-mediated conformational changes might facilitate the interaction with their binding partners, leading for example to enhanced phosphorylation (Yaffe, 2002).

Another possible explanation for 14-3-3-enhanced phosphorylation of L1 by CK II is that 14-3-3ζ acts as a scaffolding protein by recruiting CK II to L1 ICD. Even though there is no direct evidence at present for an association between 14-3-3ζ and CK II, there is some evidence suggesting that 14-3-3ζ needs to dimerize in order to act as a scaffolding protein. It is possible that GST-14-3-3ζ forms dimers as a result of dimerization by the glutathione S-transferase (Wilce and Parker, 1994), thus, allowing 14-3-3ζ to function as a scaffold for CK II.

Several earlier studies have suggested that the ability of 14-3-3 to bind its target proteins is independent of 14-3-3 dimerization (Ichimura et al., 1995; Luo et al., 1995; Yaffe et al., 1997; Tzivion et al., 1998; Rittinger et al., 1999). However, other studies, including more recent ones, have shown that 14-3-3 dimerization is important for the phosphorylation-dependent

binding of cellular proteins (Jones et al., 1995; Shen et al., 2003; Sumioka et al., 2005). Interestingly, Woodcock *et al.* (2003) showed that the dimeric status of 14-3-3 can be regulated *in vivo* by site-specific phosphorylation at the dimer interface, yet phosphorylation-induced monomerization does not prevent 14-3-3 binding to a phosphopeptide target. Therefore, further experiments investigating the CK II-catalyzed phosphorylation of L1 in the presence of a 14-3-3ζ dimer and a dimerization-deficient 14-3-3ζ may help to further elucidate how the dimeric structure of 14-3-3ζ may influence L1 phosphorylation.

4.2.4. 14-3-3ζ is enriched in endosomal fractions

Results obtained here from studying the interaction between L1 ICD and 14-3-3ζ have suggested that 14-3-3ζ might play a role in controlling L1 sorting and trafficking between endosomes and plasma membranes (cf. section 4.2.3). I therefore investigated the possible interaction between L1 and 14-3-3ζ in endosomal fractions from mouse brain. I could show that 14-3-3ζ associates with L1 in endosomes and observed that 14-3-3ζ was enriched in L1 immunoprecipitates from endosomal fractions with rather low amounts of L1 (Fig. 25). The association of 14-3-3ζ and L1 in endosomal fractions further supports an involvement of 14-3-3ζ in L1 sorting and trafficking. It would be beneficial to characterize the endosomal fractions to determine the type of endosome (i.e., early, late, or recycling endosome) in which the co-localization of 14-3-3ζ with L1 ICD occurs more precisely. This might be done by using antibodies against marker proteins specifically expressed in distinct endosome populations.

Cell surface biotinylation was performed in an attempt to investigate whether 14-3-3ζ overexpression in SH-SY5Y neuroblastoma cells results in reduced L1 amounts at the cell surface due to increased L1 endocytosis. However, we were unable to analyze the amount of L1 at the cell surface in the presence of 14-3-3ζ. Unfortunately, 14-3-3ζ overexpression in SH-SY5Y cells could not be achieved, even after repeated attempts. Attempts to co-express 14-3-3ζ constructs with L1 construct in HEK 293 cells also failed. It is not clear why 14-3-3 overexpression in the presence of endogenous or exogenous L1 was not possible in these cell lines.

4.2.5. The adaptor protein μ-AP-2 does not affect L1 ICD binding to 14-3-3ζ

The adaptor protein μ-AP-2 is known to bind Tyr[1176], immediately preceding the RSLESD sequence in L1 ICD; [1176]YRSLESD[1182] forms a tyrosine-based sorting motif that is necessary for endocytosis of L1 via the AP-2/clathrin-mediated pathway (Kamiguchi and Lemmon, 1998). Given that we could show binding of 14-3-3ζ to almost the same region of L1 ICD, suggesting an involvement in L1 trafficking control, we investigated whether μ-AP-2 competes with 14-3-3ζ for L1 binding. We found that L1 ICD is able to bind 14-3-3ζ also in the presence of μ-AP-2 (Fig. 24), implying that the adaptor protein does not measurably affect L1 ICD binding to 14-3-3ζ. μ-AP-2 and 14-3-3ζ might therefore bind independently of each other to L1 although their binding regions overlap. Alternatively, they might interact with L1 at different stages of L1 endocytosis. Further studies will need to clarify whether 14-3-3ζ might impair the interaction between L1 and AP-2.

4.2.6. A model of L1 – 14-3-3-interaction

Endocytosis is a basic cellular process that is used by cells to internalize a variety of molecules. Because these molecules can be quite diverse, understanding the different pathways that mediate their internalization and how these pathways are regulated is important in many areas of cell and developmental biology.

The highly conserved ICD of the L1 molecule plays an important role in its endocytic trafficking (Kamiguchi et al., 1998), yet the molecular mechanisms that regulate L1 trafficking have not been fully unraveled. Previous investigations suggest that L1 internalization in neuronal growth cones may be induced by the spatially restricted activation of the clathrin endocytic machinery and its associated molecules (Kirchhausen et al., 1997; Kamiguchi et al., 1998; Hinshaw, 2000; Schmid et al., 2000). Most probably, there are several critical points at which the endocytosed L1 might follow distinct pathways after internalization from the plasma membrane and during trafficking through endosomal compartments within the growth cone. Such a critical point is located at sorting endosomes, where during axonal growth, most of the endocytosed L1 should be sorted into recycling endosomes, but not into late endosomes/lysosomes to be degraded (Kamiguchi, 2003).

14-3-3 proteins have been shown to modulate the trafficking of ATP-sensitive K^+ channels, TASK-1 and TASK-3 channels (O'Kelly et al., 2002; Rajan et al., 2002; Yuan et al., 2003; O'Kelly and Goldstein, 2008). Furthermore, Efendiev *et al.* could show that 14-3-3 provides the signal to initiate endocytosis of the Na^+, K^+ -ATPase (Efendiev et al., 2005). It has been reported more recently that 14-3-3 in complex with other molecules plays a role in modulating epidermal growth factor receptor endocytosis (Tomassi et al., 2008). The examples mentioned here illustrate that it is reasonable to assume that 14-3-3 plays a role in L1 endocytic trafficking.

The results obtained in this thesis work are not sufficient to provide full detail of the mechanistic control of the L1 – 14-3-3-interaction. Nevertheless, the following model is proposed to illustrate the findings from this study in the greater context of L1 trafficking (Fig. 26).
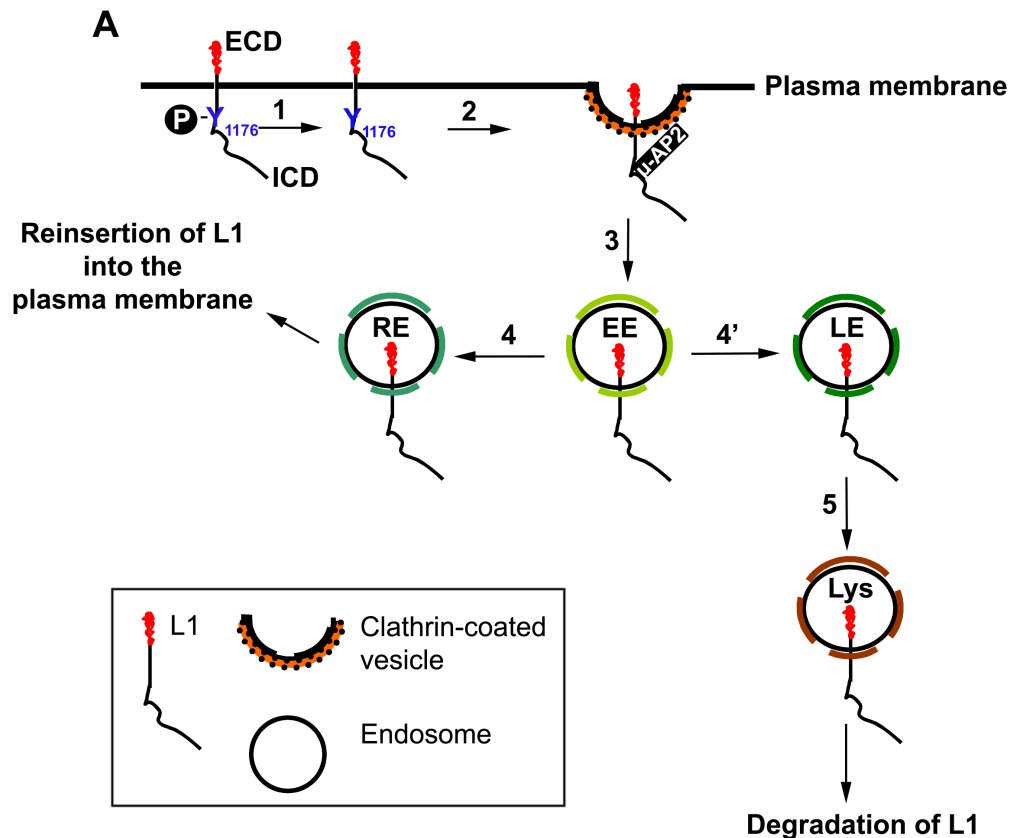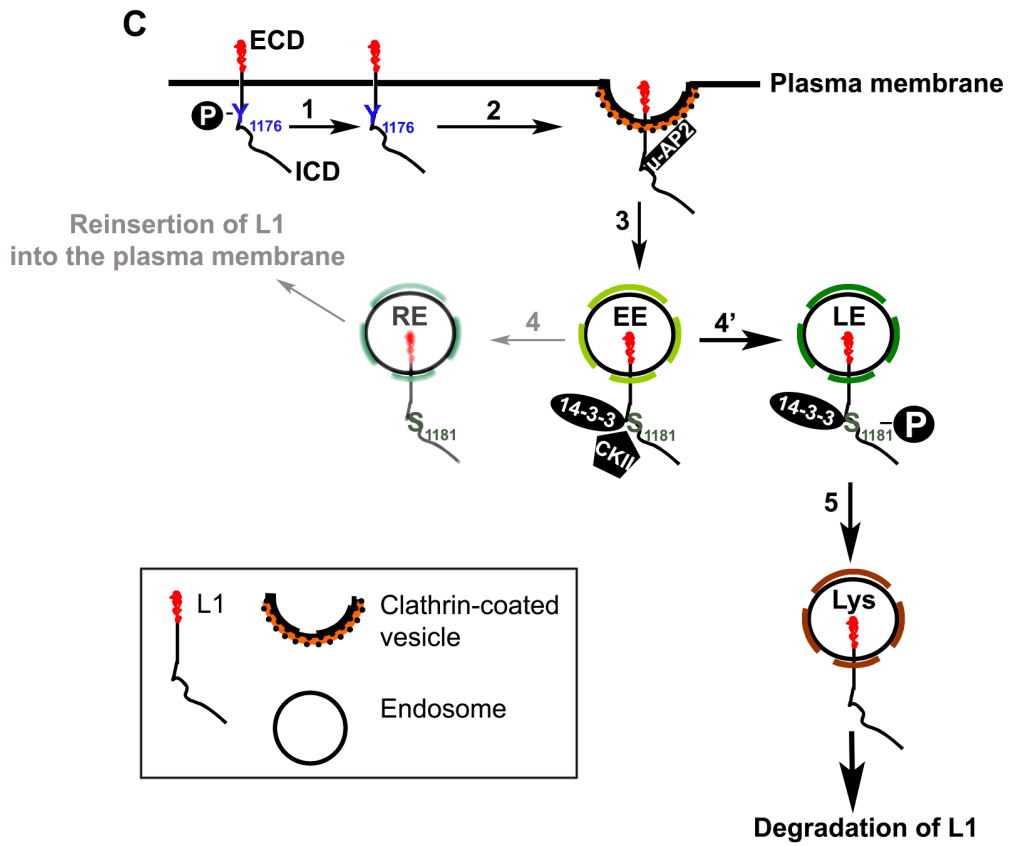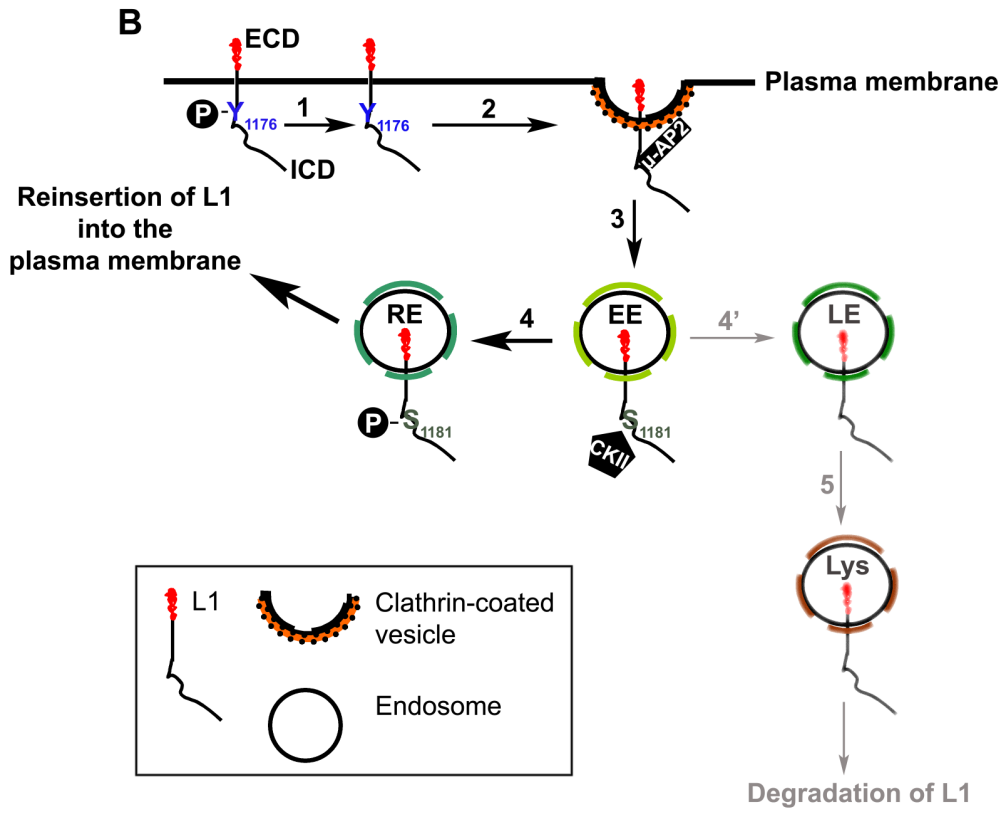
Figure 26: Model of 14-3-3 association with the intracellular domain (ICD) of L1 in the context of L1 endocytic trafficking. L1 molecules (extracellular domain (ECD), red [knob]; ICD, black [coil]) on the cell surface are phosphorylated at Tyr^{1176} ($P-Y^{1176}$) in the ICD. Dephosphorylation of Tyr^{1176} (step 1) may trigger L1 endocytosis (step 2). Dephosphorylated L1 molecules are then internalized via the AP-2/clathrin-dependent pathway and sorted into early endosomes (step 3). (A) Known pathways of L1 endocytic trafficking indicating potential endosomal routes for reinsertion of L1 into the plasma membrane (step 4) and L1 degradation (steps 4' and 5). The particular pathway taken by L1 is postulated to be regulated by 14-3-3. (B) Postulated pathway of L1 endocytic trafficking under conditions of low intracellular 14-3-3 concentrations. Due to low levels of 14-3-3 relatively few L1 molecules are phosphorylated by CK II at Ser^{1181} in early endosomes. As a consequence, L1 is routed to recycling endosomes (step 4) and reinserted into the plasma membrane. (C) Postulated pathway of L1 endocytic trafficking at high levels of 14-3-3. The binding of 14-3-3 to L1 molecules in early endosomes enhances CK II phosphorylation of endocytic L1 ICD. Strong phosphorylation of the L1 ICD is postulated to result in L1 sorting into late endosomes (step 4') and then lysosomes for degradation (step 5). μ-AP-2: μ subunit of adaptor protein AP-2; CKII: casein kinase II; EE: early endosome; LE: late endosome; Lys: lysosome; RE: recycling endosome. The different coloring of the endosomes is to represent different marker proteins that are specific to each endosomal stage.

It has been shown that a post-translational modification in the L1 ICD could be responsible for region-specific endocytosis of L1 ICD. The L1 ICD is subject to phosphorylation/dephosphorylation *in vivo* at Tyr^{1176}, the critical Tyr residue for AP-2 binding (Schaefer et al., 2002). Phosphorylation of Tyr^{1176} prevents L1 from interacting with AP-2, thus preventing clathrin-mediated endocytosis of L1. L1 endocytosis may be triggered by dephosphorylation of Tyr^{1176} (step 1) (Fig. 26). This hypothesis is supported by a study by Schaefer *et al.* (2002*)* showing that in growth cones Tyr^{1176}-dephosphorylated L1 is found in vesicle-like structures. μ-AP-2 binding to the $^{1176}YRSLE^{1180}$ sequence in L1 ICD then enables L1 to be endocytosed via the clathrin-dependent pathway (step 2), as demonstrated in a study by Kamiguchi *et al.* (1998). The endocytosed L1 molecules are sorted into early endosomes (step 3). These early endosome-sorted L1 molecules can then follow two different pathways: reinsertion into the plasma membrane via recycling endosomes (step 4) or degradation via late endosomes and lysosomes (steps 4' and 5) (Fig. 26A). In the present study, I provide evidence to suggest that 14-3-3 orchestrates the trafficking of L1 molecules after endocytosis. *In vitro* binding assays revealed that 14-3-3 interacts with L1 ICD, and co-immunoprecipitation experiments demonstrated that 14-3-3 associates with L1 *in vivo*. It was also shown that 14-3-3 associates with L1 in distinct endosomal fractions, suggesting the presence of 14-3-3 – L1-complexes during trafficking/sorting through endosomal compartments such as early and late

endosomes. Furthermore, I hypothesize that only L1 and AP-2 interact at the plasma membrane, with 14-3-3 interaction occurring further downstream. Support for this hypothesis is provided by results obtained in this study showing that AP-2 does not influence binding of L1 ICD to 14-3-3.

Enhanced L1 phosphorylation of L1 ICD by CK II was observed in the presence of 14-3-3. Although the physiological relevance of this finding in the context of L1 sorting is not entirely clear at present, the level of phosphorylation, presumably regulated by 14-3-3, determines whether L1 is recirculated to the plasma membrane or is degraded. Independent results show that overexpression of 14-3-3 in hippocampal neurons results in a specific reduction of L1-mediated neurite outgrowth (T. Tilling, unpublished data). I speculate that in the absence of 14-3-3, less L1 is phosphorylated by CK II and reinsertion of L1 into the plasma membrane occurs (step 4) (Fig. 26B). In contrast, enhanced phosphorylation of L1 due to the interaction with 14-3-3 leads to sorting into late endosomes, then lysosomes and degradation of L1 (steps 4' and 5) (Fig. 26C).

Taken together, these data suggest that 14-3-3 is a key molecule in regulating CK II-catalyzed phosphorylation of L1, thereby directing endocytic L1 trafficking. 14-3-3 interacts directly with L1 ICD and this interaction is required for CK II-dependent phosphorylation of L1. More studies will be required to investigate the function of 14-3-3 in L1 trafficking during neurite outgrowth in detail. Also, the identification of additional molecules that are part of critical sorting points would greatly enhance our understanding of how cells are able to regulate intracellular cell adhesion molecule trafficking.

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6. Poster presentation

Elisa M. Ramser, Thomas Tilling, Melitta Schachner. **Alpha II spectrin from brain binds to the crucial signaling protein 14-3-3**. 6th FENS FORUM of European Neuroscience. July 12–16, 2008. Geneva, Switzerland

7. Curriculum vitae

Persönliche Daten

Geburtsort:	Klausenburg, Rumänien
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Schulausbildung

1982 – 1989	Grundschule und Gymnasium George Cosbuc, Klausenburg
1989 – 1996	Dietrich-Bonhoeffer Gymnasium, Wiehl
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Berufliche Ausbildung

09/1996 – 07/1999	Ausbildung zur Biologielaborantin am Forschungszentrum Borstel, Leibniz-Zentrum für Medizin und Biowissenschaften
07/1999	**Abschluss: Biologielaborantin**

Universitäre Ausbildung

10/1999 – 07/2005	Studium der Biochemie, Gottfried Wilhelm Leibniz Universität Hannover
09/2002 – 11/2002	Praktikum an der Medizinischen Hochschule Hannover, Institut für Neuroanatomie; Betreuung durch Frau Prof. Dr. Claudia Grothe
07/2003 – 09/2003	Praktikum am Zentrum für Molekulare Neurobiologie Hamburg Institut für Biosynthese Neuraler Strukturen; Betreuung durch Frau Prof. Dr. rer. nat. Melitta Schachner
10/2003 – 12/2003	Praktikum an der Medizinischen Hochschule Hannover, Institut für Molekularbiologie; Betreuung durch Prof. Dr. Achim Gossler
01/2005 – 07/2005	Externe Diplomarbeit am Zentrum für Molekulare Neurobiologie Hamburg mit dem Thema: „Untersuchung der Wechselwirkung von 14-3-3 mit neuralen Proteinen" Betreuung durch Frau Prof. Dr. rer. nat. Melitta Schachner
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9. Appendix

A. Oligonucleotides

1. Sequencing primers	Sequence (5'-3')
1. pQ30 T7 promoter fwd	ccc gaa aag tgc cac ctg
2. pcDNA3	ctg ctt act ggc tta tcg aa
3. L1 ICD 3431 fwd	gct cta tca aag gca gca a
4. GST 615 fwd	gat gcg ttc cca aaa tta gtt tg
5. Alpha II spectrin 2605 fwd	gga cat gaa cca gcg atc aa
6. Alpha II spectrin 3236 fwd	ggc agg agc aga ttg aca at
7. Alpha II spectrin 3650 fwd	aaa ctg att cca aga cag cc
8. Alpha II spectrin 3885 fwd	gga cat gat ctc gcc agt gt
9. Alpha II spectrin 4537 fwd	gcg tag agg ctc tga tca aa
10. 14-3-3 β 413 fwd	ggg caa aga gta ccg tga
11. 14-3-3 β 834 fwd	cag ctg ctc agg gac aat ctc ca

2. Mutagenesis primers	Sequence (5'-3')
1. Alpha II spectrin S1302A fwd	aca gca gag cgc ctg acc cag gcc cat ccc gag tca gca gaa gac
2. Alpha II spectrin S1302A rev	gtc ttc tgc tga ctc ttt atg ggc ctg ggt cag gcg ctc tgc tgt
3. L1 ICDS1181A fwd	ggc gag tac agg tcc ctg gag gct gac aat gaa gag aag gcc ttt
4. L1 ICDS1181A rev	aaa ggc ctt ctc ttc att gtc agc ctc cag gga cct gta ctc gcc
5. L1 ICDΔRSLESD fwd	gac gag acc ttc ggc gag tac aat gaa gag aag gcc ttt ggc
6. L1 ICDΔRSLESD rev	gcc aaa ggc ctt ctc ttc att gta ctc gcc gaa ggt ctc gtc

3. PCR primers	Sequence (5'-3')
1. 14-3-3 β fwd	ata ctc gag cat gga taa gag tga gct ggt a
2. 14-3-3 β rev	ata tct aga tgg ttc tct ccc tct cca gca t
3. Alpha II spectrin 10-14 Kozak fwd	ata gaa ttc gcc acc atg ttt atg ttg ttc cgt gaa gcg aat
4. Alpha II spectrin 10-14 Kozak rev	ata ctc gag gac ctc att gcg ccg gct aga
5. Alpha II spectrin 10-14 fwd	ata gaa ttc cct tta tgt tgt tcc gtg aag cga at

6. Alpha II spectrin 10-14 rev	ata ctc gag tgc cca aga gct ggc tcc gtt c

B.1. Generation of pcDNA3/6xmyc-14-3-3β

pcDNA3/6xmyc-14-3-3β was generated for expression of 6xmyc-tagged 14-3-3β in mammalian cells. cDNA encoding the open reading frame of the 14-3-3β isoform was amplified by PCR using pGEX-2T-14-3-3β as a template and the above listed primers 3.1 and 3.2 (see A3). The PCR product was digested with Xba I-Xho I and then ligated into pcDNA3/6xmyc vector.

B.2. Generation of pGEX-4T-2-alpha II spectrin 10-14

For overexpression of glutathione S transferase- (GST-) tagged alpha II spectrin 10-14 in bacteria, alpha II spectrin 10-14 was amplified using pCI - alpha II spectrin (cDNA kindly obtained from Jon Morrow, Yale University) as a template and the above listed primers 3.5 and 3.6 (see A3). The PCR product, encompassing nucleotides 3376-4683 of the human alpha II spectrin sequence, was digested with EcoR I-Xho I and then ligated into the EcoR I/Xho I cloning sites of pGEX-4T-2 vector.
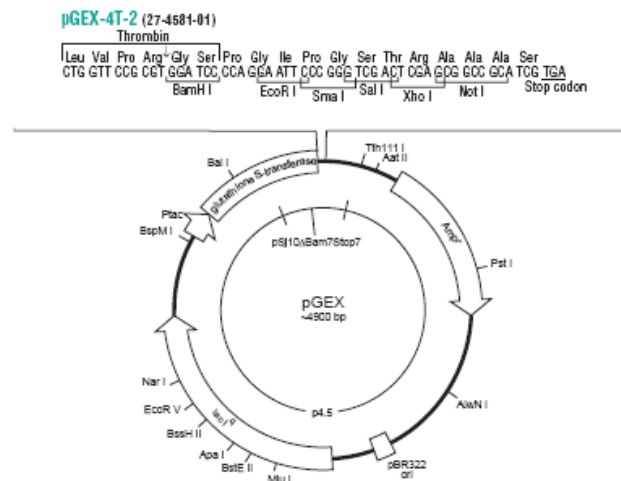

Figure 1: pGEX-4T-2 vector map (GE Healthcare; www.gehealthcare.com)

B.3. Generation of pcDNA^{TM}3.1/myc-his – alpha II spectrin 10-14

For mammalian expression of myc-histidine-tagged alpha II spectrin 10-14, alpha II spectrin 10-14 was amplified using pCI - alpha II spectrin (cDNA provided by Jon Morrow, Yale University) as a template and the above listed primers 3.3 and 3.4 (see A3). The PCR product, encompassing nucleotides 3376-4683 of the human alpha II spectrin sequence, was digested with EcoR I-Xho I and then ligated into the EcoR I/Xho I cloning sites of pcDNA^{TM}3.1/myc-his vector.
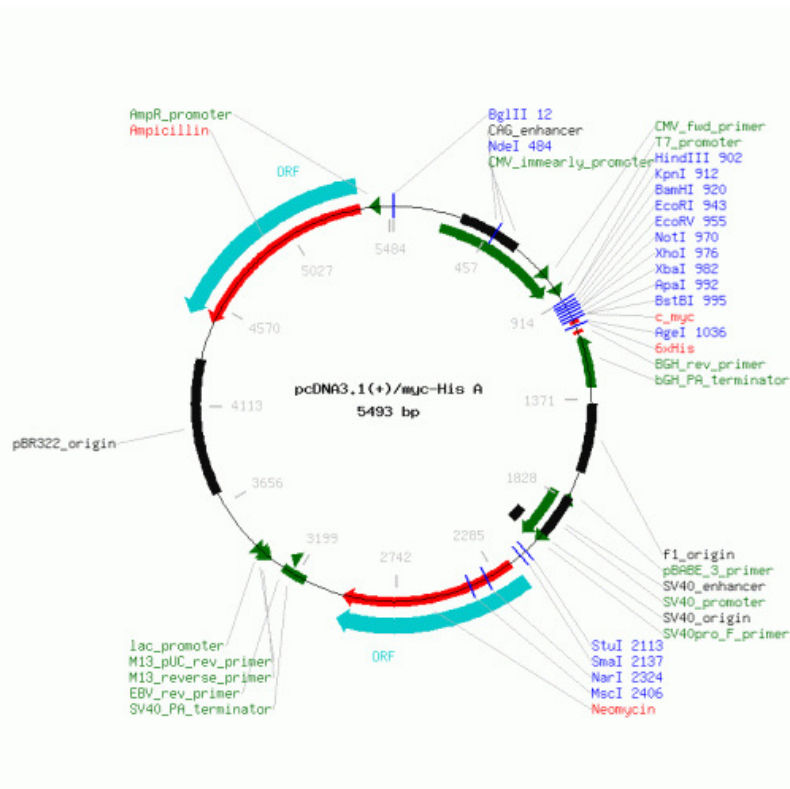

Figure 2: pcDNA^{TM}3.1/myc-his A vector map (Invitrogen; www.invitrogen.com)

B.4. Mutations in L1 ICD

Mutants of histidine-tagged L1 ICD were generated using a QuikChange Site-directed mutagenesis kit (Stratagene) and primers listed in A2. Mutation sites were: Ser[1181] to Ala and deletion of RSLESD sequence (positions 1177-1182). The specific base changes in all mutants were confirmed by DNA sequence analysis.

B.5. Mutations in alpha II spectrin 10-14

A Ser to Ala mutant (Ser1302 to Ala) of myc-histidine-tagged alpha II spectrin was generated using a QuikChange Site-directed mutagenesis kit (Stratagene) and primers listed in A2. The specific base changes were confirmed by DNA sequencing.