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NCAM induces caspase-3 activation to reorganize the membrane cytoskeleton to enhance neurite elongation in mice (*Mus musculus* L., 1758)

Dissertation

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I. ABSTRACT

The neural cell adhesion molecule (NCAM) promotes neurite outgrowth *in vitro*. This process relies on the remodeling of the cytoskeleton in growth cones. Previously, it has been shown that NCAM is associated with the spectrin-actin meshwork, but it has remained unclear how NCAM induces cytoskeletal rearrangements to mediate neurite outgrowth. Our data indicate that NCAM activates cysteine proteases called caspases that proteolytically disassemble the spectrin meshwork at the growth cone. Immunocytochemical analysis revealed that caspases-3 and -8 were present in growth cones of cultured hippocampal neurons. Moreover, NCAM formed a complex with active caspase-8, a known activator of caspase-3, in brains of young 1- to 3-days old mice. Antibody induced clustering of NCAM at the plasma membrane of NCAM140 expressing CHO cells led to the rapid activation of the initiator caspase-8 but not of caspase-9. Clustering of NCAM at the cell surface of 1 day-old live neurons *in vitro* resulted in an increased level of active caspase-3 in neurons. These data indicate that NCAM induces caspase-8 and caspase-3 activation. Levels of proteolytic β1-spectrin, β2-spectrin and α2-spectrin fragments in growth cones isolated from NCAM deficient brains were decreased, suggesting that spectrin meshwork remodeling is reduced in the absence of NCAM. Interestingly, clustering of NCAM in isolated growth cones enhanced proteolytic cleavage of α2-spectrin. This enhancement was completely abolished in the presence of caspase-3 and caspase-8 inhibitors. Since NCAM clustering at the cell surface results in its redistribution into lipid rafts we analyzed levels of caspases in these membrane microdomains. Levels of caspases-3 and -8 but not of caspase-9 were reduced in lipid rafts isolated from NCAM deficient brains. NCAM, therefore, might be involved in the recruitment of these caspases into lipid rafts. Finally, in a functional assay we also analyzed the effects of caspase inhibitors on neurite elongation of hippocampal neurons stimulated with soluble NCAM-Fc. We observed that inhibitors of caspases-3 and -8 but not of caspases-9 and -10 abolished the NCAM mediated increase in neurite outgrowth rate. Thus, our data suggest an important role for caspases-3 and -8 in the NCAM mediated reorganization of the cytoskeleton. We hypothesize that locally restricted activation of caspases initiated by NCAM occurs in lipid rafts.

II. ZUSAMMENFASSUNG

Das neurale Zelladhäsionsmolekül NCAM fördert *in vitro* das Neuritenwachstum. Dieser Wachstumsprozess basiert auf einer kontinuierlichen Umgestaltung des Zytoskeletts in den Wachstumskegeln der Neuriten. In vorangegangenen Studien wurde gezeigt, dass NCAM intrazellulär mit dem stabilisierenden Spektrin-Aktin-Netzwerk assoziiert ist. Allerdings ist bislang ungeklärt, wie NCAM Strukturveränderungen des Zytoskeletts induziert, um das Neuritenwachstum zu fördern. Unsere Daten lassen darauf schließen, dass NCAM Caspasen aktiviert, die das Spektrin-Netzwerk des Wachstumskegels proteolytisch abbauen. Mittels immunzytochemischer Analysen wurden Caspase-3 und Caspase-8 in den Wachstumskegeln kultivierter hippokampaler Neurone nachgewiesen. Außerdem bildete NCAM in Gehirnen von jungen Mäusen einen Komplex mit aktiver Caspase-8. Clustering von NCAM-Molekülen an der Zelloberfläche von NCAM140-exprimierenden CHO-Zellen mit Hilfe von spezifischen Antikörpern führte zu einer schnellen Aktivierung der Initiator-Caspase-8, aber nicht der Caspase-9. NCAM-Clustering an der neuronalen Plasmamembran führte außerdem zu einem erhöhten Level an aktiver Caspase-3 in den Nervenzellen. Dies lässt darauf schließen, dass NCAM die Aktivierung der Caspasen-3 und -8 induziert. In Wachstumskegeln, die aus NCAM-defizienten Gehirnen isoliert wurden, war interessanterweise der Anteil proteolytisch gespaltener α2-, β1- und β2-Spektrinfragmente reduziert. Diese Beobachtung lässt vermuten, dass die Umbildung des Spektrin-Zytoskeletts in Abwesenheit von NCAM verringert ist. In diesem Zusammenhang ist es interessant zu erwähnen, dass NCAM-Clustering in isolierten Wachstumskegeln zu einer erhöhten Proteolyse von α2-Spektrin führte. Diese gesteigerte Proteolyse wurde allerdings vollständig durch die Zugabe von Inhibitoren der Caspase-3 und der Caspase-8 blockiert. Clustering von NCAM auf der Zelloberfläche durch spezifische Antikörper führt dazu, dass NCAM seine zelluläre Lokalisation verändert und vermehrt in Mikrodomänen der Plasmamembran, sogenannten *Lipid rafts*, zu finden ist. Wir untersuchten daher die Lokalisation von Caspasen in *Lipid rafts*. Im Rahmen dieser Untersuchungen stellte sich heraus, dass *Lipid rafts* von Gehirnen, in denen kein NCAM exprimiert wird, deutlich weniger Caspase-3 und Caspase-8 enthielten als *Lipid rafts* des Wildtyps. NCAM könnte somit an der Rekrutierung beider Caspasen in die Mikrodomänen beteiligt sein. Wir untersuchten schließlich die Effekte der Caspaseinhibitoren auf das Neuritenwachstum hippokampaler Neurone, die mit löslichem NCAM-Fc stimuliert wurden. NCAM-Stimulation mit Hilfe dieser Fusionsproteine förderte das Wachstum hippokampaler Neuriten. Die Inhibitoren der Caspasen-3 und -8, nicht jedoch der Caspasen-9 und -10, blockierten diese

NCAM-induzierte Förderung des Neuritenwachstums. Zusammenfassend ist festzustellen, dass die Caspasen-3 und -8 eine wichtige Funktion in der NCAM-induzierten Umgestaltung des Zytoskeletts besitzen. Wir vermuten zudem, dass die von NCAM hervorgerufene, lokal begrenzte Aktivierung der Caspasen in *Lipid rafts* stattfindet.

III. INTRODUCTION

The development and maintenance of the nervous system involves complex processes which require precise regulation and intercellular communication. During development neurons migrate along diverse pathways from their origin of birth to their final destination in the brain. Thereby, migrating cells adhere to other cells and to the extracellular matrix (ECM). Cell adhesion is therefore a fundamental process during nervous system development. Molecules functioning in cell adhesion in the nervous system belong to the groups of selectins, cadherins, integrins and the cell adhesion molecules (CAM) of the immunoglobulin superfamily (IgCAMs). The group of selectins is especially important in the immune system. Integrins mediate primarily cell-ECM interactions, whereas cadherins and IgCAMs are mainly involved in cell-cell interactions (Juliano, 2002; Lee & Benveniste, 1999). The involvement of CAMs in cell adhesion has been directly demonstrated *in vitro* by application of specific CAM antibodies to cultured cells which blocked adhesiveness between the cells (Akiyama et al, 1989; Beug et al, 1970; Rutishauser et al, 1978; Seilheimer & Schachner, 1988; Thiery et al, 1977).

During the last decades, CAMs have been extensively studied and their numerous roles in developmental processes in the nervous system have been revealed. Besides migration CAMs are involved in neurulation (neural tube formation), neurite outgrowth, synapse formation and plasticity, myelination as well as nerve regeneration after injury. It is now widely accepted that CAMs are not just adhesive molecules, but function also as signal transducing receptors at the cell surface (Crossin & Krushel, 2000; Santuccione et al, 2005; Schuch et al, 1989). The importance of CAMs is underlined by the fact that CAM dysfunctions often give rise to neuropsychiatric and neurodegenerative disorders. For instance, a famous member of the immunoglobulin superfamily, NCAM, is implicated in schizophrenia, anxiety disorders, depression and bipolar disorders (Arai et al, 2004; Gillian et al, 1994; Sullivan et al, 2007; Tsoory et al, 2008). The association between CAMs and various neuropsychiatric disorders emphasizes the urgent need to dissect the cellular functions of these molecules in detail.

III.1. THE NEURAL CELL ADHESION MOLECULE NCAM

NCAM was the first cell adhesion molecule intensively characterized in the brain. It was discovered independently by different groups and is therefore also known as D2, BSP-2 or CD56 (Jorgensen & Bock, 1974; Rutishauser et al, 1976; Thiery et al, 1977). NCAM belongs to the IgCAM superfamily and shares their common features. Its five immunoglobulin-like domains (Ig) carry a stabilizing disulfide bond that connects the two β-sheets and makes the Ig-like domains relatively resistant to proteolysis. Additionally, NCAM carries two domains that resemble regions of the ECM component fibronectin termed fibronectin type 3-like domains (FNIII) (see Fig. 1).

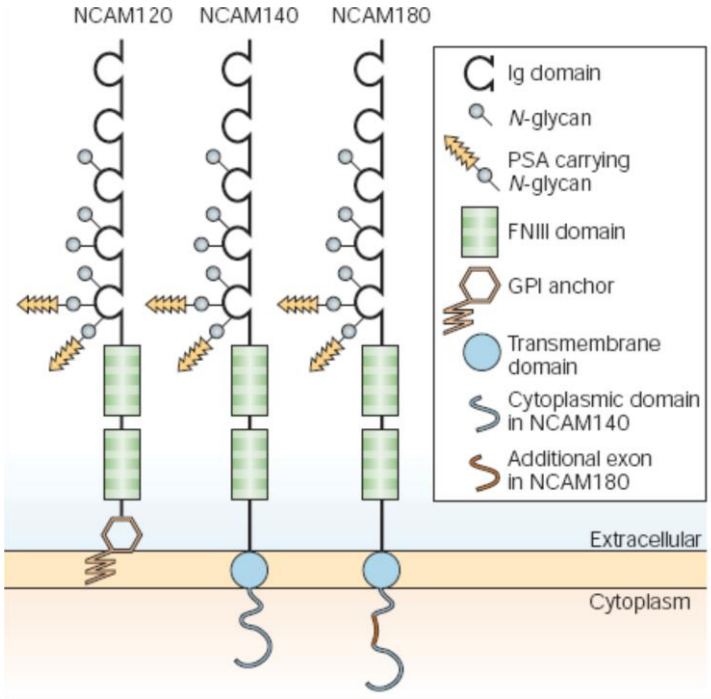

Fig. 1: Major NCAM isoforms (Kleene & Schachner, 2004). NCAM120, NCAM140 and NCAM180 consist of five immunoglobulin-like (Ig) domains and two fibronectin type 3-like (FNIII) domains in the extracellular part. NCAM120 is anchored to the membrane owing to a glycosylphosphatidylinositol (GPI) anchor, whereas NCAM140 and NCAM180 are integral proteins that span the membrane. The cytoplasmic domains of NCAM180 and NCAM140 differ in length due to an additional exon insert in NCAM180. All three isoforms can carry different glycans at several glycosylation sites. See text for details.

NCAM is encoded by a single gene, NCAM1, which is located on chromosome 9 in the mouse genome (D'Eustachio et al, 1985) and on chromosome 11 in the human genome (Nguyen et al, 1986; Walsh et al, 1986). In mice the gene contains twenty exons and six supplementary small exons. Alternative splicing of the transcript gives rise to several

isoforms. The three major isoforms NCAM180, NCAM140 and NCAM120 are named after their apparent molecular weight when separated by SDS-PAGE (Goridis et al, 1983). These isoforms are similar in their extracellular domain but differ in their intracellular structure. The NCAM120 isoform even lacks a cytoplasmic part and is tethered to the membrane via a glycosylphosphatidylinositol (GPI) lipid anchor (Hemperly et al, 1986a). In contrast, NCAM140 and NCAM180 are transmembrane isoforms with a cytoplasmic domain (Hemperly et al, 1986b). NCAM180 contains a larger intracellular part due to a 40 kDa cytoplasmic domain insert. Further isoforms can be generated by splicing at two sites called 'a' and 'π'. For example, the variable alternatively spliced exon (VASE) can be inserted into the splicing site π in the fourth Ig-module (Small & Akeson, 1990; Small et al, 1988). In addition to the transmembrane isoforms, secreted and soluble isoforms of NCAM were also reported (Bock et al, 1987; Gower et al, 1988; He et al, 1986; Hinkle et al, 2006). The soluble isoforms have been demonstrated in human cerebrospinal fluid (CSF), serum and amniotic fluid (Ibsen et al, 1983; Krog et al, 1992). Interestingly, increased levels of soluble NCAM in the CSF, the prefrontal cortex and the hippocampus were implicated in schizophrenia (Poltorak et al, 1995).

The transmembrane isoforms can be posttranslationally modified by palmitoylation. Up to four cysteine residues in the cytoplasmic part, adjacent to the transmembrane domain, can be palmitoylated by attachment of palmitate via thioester linkage (Little et al, 1998; Ponimaskin et al, 2008). Palmitoylation increases the hydrophobicity and enhances the attachment to special microdomains in the membrane, termed lipid rafts. However, this modification is reversible and can be removed. Besides, NCAM is posttranslationally modified by addition of glycans in the endoplasmatic reticulum and the Golgi apparatus (Kiss & Rougon, 1997). Thus, NCAM is part of the glycocalyx and can carry either N-glycosylations (Albach et al, 2004) or O-glycosylations, with the latter found on muscle specific isoforms (Ong et al, 2002; Walsh et al, 1989).

NCAM appears early during embryo development and persists into adulthood in the nervous system (Edelman, 1985). In the central and peripheral nervous system NCAM is expressed in neurons as well as in glial cells. The NCAM140 isoform is present in both cell types, whereas NCAM120 is mainly expressed in glial cells (Bhat & Silberberg, 1988; Noble et al, 1985) and NCAM180 occurs predominantly in neurons (Persohn et al, 1989). Besides, NCAM is expressed in non-neuronal tissues such as muscle (Sanes et al, 1986), heart (Burroughs et al, 1991), pancreas and gonad (Moller et al, 1991). Moreover, the subcellular localization differs among the NCAM isoforms. NCAM140 is the major isoform which is expressed in neurites

and growth cones of developing neurons. In mature neurons NCAM140 is localized to pre- and postsynaptic membranes, whereas NCAM180 is predominantly present in postsynaptic densities in the mature neuron (Persohn et al, 1989; Pollerberg et al, 1987; Schuster et al, 1998; Sytnyk et al, 2006). The differences in their localization probably reflect the diverse functions that different NCAM isoforms support.

NCAM molecules interact with each other in a homophilic manner. The current model of homophilic binding mechanisms is reviewed by Kiselyov and colleagues (Kiselyov et al, 2005). Briefly, homophilic binding of NCAM leads to the formation of so-called zipper structures which seem to be a very common feature of CAMs undergoing homophilic interactions. These homophilic interactions in turn induce the clustering of signaling molecules bound to CAMs. However, NCAM also undergoes heterophilic interactions with various molecules both extracellularly and intracellularly. Homophilic and heterophilic interactions are not always mutually exclusive. Instead they are very likely to occur simultaneously. Extracellular interaction partners comprise other IgCAMs such as L1, the GPI anchored proteins TAG-1 and PrP, growth factors and growth factor receptors (FGFR, GDNF, GFRα, BDNF, PDNF) as well as extracellular matrix molecules (Horstkorte et al, 1993; Kiselyov et al, 2003; Milev et al, 1996; Paratcha et al, 2003; Probstmeier et al, 1989; Santuccione et al, 2005; Schmitt-Ulms et al, 2001; Vutskits et al, 2001; Zhang et al, 2004). Intracellularly NCAM associates with scaffolding molecules and cytoskeleton associated proteins such as spectrin, tubulin and GAP-43 (Buttner et al, 2003; He & Meiri, 2002; Leshchyns'ka et al, 2003; Pollerberg et al, 1986). Non-receptor tyrosine kinase $p59^{fyn}$ has also been described as intracellular interaction partner of NCAM (Beggs et al, 1997). Also the receptor protein tyrosine phosphatase RPTPα binds to NCAM via its intracellular domain (Bodrikov et al, 2005). The local availability of the binding partners may thereby determine the actual interactions of a given NCAM molecule.

To elucidate crucial NCAM functions different mouse models were generated by gene targeting leading to constitutive or conditional disruption of the NCAM gene (Bukalo et al, 2004; Cremer et al, 1994; Tomasiewicz et al, 1993). Cremer and colleagues created a mouse line which is deficient in the three major isoforms of NCAM (Cremer et al, 1994). In a series of studies the phenotype of this homozygous and heterozygous mutant was analyzed in comparison to the C57BL/6J wild type mice (Cremer et al, 1998; Cremer et al, 1997; Cremer et al, 1994). The NCAM deficient mouse is viable and fertile and displays a relative mild phenotype that might be explained by compensatory mechanism of other CAMs. However,

the mutant has a decreased brain size and develops a smaller olfactory bulb (Cremer et al, 1994). A similar phenotype was already discovered in a mutant mouse that does not express the NCAM180 isoform (Tomasiewicz et al, 1993). This phenotype might result from disturbed cell migration of the neuronal precursor cells (Chazal et al, 2000; Tomasiewicz et al, 1993). The precursors normally migrate from the subventricular zone along the rostral migratory pathway, where NCAM is expressed, into the olfactory bulb where they differentiate into interneurons. But when NCAM is not expressed, the precursor cells accumulate at their origin and along the pathway.

Furthermore, the NCAM deficient mouse shows impaired axonal growth and reduced axon fasciculation of mossy fibers in the hippocampus (Cremer et al, 1997). Granule cells of the dentate gyrus form unmyelinated axons which grow to the CA3 region in the hippocampus. Normally these mossy fibers fasciculate and form different axon bundles which merge into a common tract, but in the mutant the division into the different bundles is impaired.

Besides, the laminated distribution of mossy fiber terminals is disturbed in the NCAM mutant (Cremer et al, 1998). In the wild type mouse mossy fibers are organized in a laminated pattern (Amaral & Witter, 1989) and a clear distinction between the pyramidal cell layer and the stratum lucidum is visible. But in the NCAM mutant, in which the laminated expression of NCAM in the stratum lucidum is eliminated, the clear distinction between the pyramidal cell layer and the stratum lucidum is lost. Mossy fiber terminals, that are normally restricted to the stratum lucidum, are also found in the pyramidal cell layer in the mutant. These studies underscore the importance of NCAM in the complex regulation of axonal growth, path finding and axonal fasciculation.

III.2. NCAM IN THE PROCESS OF NEURITE OUTGROWTH

The development of the nervous system involves coordinated processes such as cell migration, neurite outgrowth, the fasciculation of axons and the formation as well as the stabilization of synapses. NCAM participates in these processes during the development of the brain (Chung et al, 1991; Cremer et al, 1997; Lindner et al, 1986; Neugebauer et al, 1988; Seilheimer & Schachner, 1988; Sytnyk et al, 2002). Initial evidence that NCAM promotes neurite extension results from antibody perturbation experiments. Application of NCAM antibodies interfered with neurite outgrowth of retinal neurons grown on astrocytes (Neugebauer et al, 1988) and dorsal root ganglion neurons grown on schwann cells and fibroblast monolayers (Seilheimer & Schachner, 1988). These studies suggested that NCAM stimulates axonal growth due to its adhesive properties. However, later it was shown that

NCAM induces also second messenger systems (Doherty et al, 1991). Moreover, some CAMs such as L1 and NCAM can induce axonal growth when added as soluble fragments (Doherty et al, 1995; Meiri et al, 1998). This strengthens the idea that CAMs exert their neurite outgrowth promoting effects independently of adhesion. CAMs are thus not just adhesive molecules but rather signal transduction molecules promoting axonal growth.

Different experimental approaches were applied in order to investigate the role of NCAM as signal transduction molecule. Crosslinking with NCAM antibodies raised against the extracellular domain was successfully employed to cluster NCAM molecules at the cell surface (Beggs et al, 1997). Besides, recombinant proteins such as Fc-fusion proteins containing the extracellular domain of NCAM fused to the human IgG1 Fc-region were also shown to be valuable techniques for investigating NCAM related signal transduction pathways (Doherty et al, 1995; Niethammer et al, 2002). Signaling cascades downstream of NCAM were successfully analyzed by means of pharmacological inhibitors of potential downstream signaling molecules. Thus, from these studies it can be inferred that NCAM signaling depends on the induction of downstream signal transduction pathways. NCAM can evoke different signaling cascades depending on its interaction partner. Both homophilic (Doherty et al, 1991; Doherty et al, 1990) as well as heterophilic (Williams et al, 1994a) interactions of NCAM lead to the induction of intracellular signaling pathways promoting neurite outgrowth. End points of NCAM mediated signaling, however, not only comprise neurite outgrowth but also involve neuronal survival (Ditlevsen et al, 2003; Doherty et al, 1990; Vutskits et al, 2001).

It is well established that NCAM induces intracellular signaling via the fibroblast growth factor receptor (FGFR), an IgSF receptor tyrosine kinase (Niethammer et al, 2002; Saffell et al, 1997; Williams et al, 1994a). FGFR represents a cofactor for NCAM directed neurite outgrowth. All three isoforms of NCAM are able to interact with FGFR (Kiselyov et al, 2003; Sanchez-Heras et al, 2006). Since NCAM is present at very high concentrations at the cell surface, most of the FGFR molecules are supposed to interact transiently with it (Kiselyov et al, 2003). To induce the activation of FGFR, NCAM interactions between molecules on opposing surfaces (*trans* homophilic interactions) are necessary (Kiselyov et al, 2005). These interactions enhance the formation of zipper structures that cluster NCAM which in turn may cluster bound FGFR molecules at the plasma membrane. Clustering results in the dimerization of FGFR which is a prerequisite for its activation via *trans* autophosphorylation. Thereby, its tyrosine kinase activity leads to the phosphorylation of regulatory tyrosine

residues (reviewed in Schlessinger, 2000). Further interaction partners can now bind to the phosphorylated residues in the cytoplasmic FGFR domain, such as phospholipase C_γ (PLC_γ) which binds via its SH2 domain and gets subsequently activated (Saffell et al, 1997). Activated PLC_γ cleaves in turn its substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2), into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These and further downstream second messengers trigger calcium influx from intracellular stores and extracellular space into the cytoplasm (Doherty et al, 1991; Kiryushko et al, 2006; Ronn et al, 2002; Williams et al, 1994b) as well as the activation of additional signaling molecules, e.g. the serine/threonine protein kinase C (PKC) (Kolkova et al, 2000; Leshchyns'ka et al, 2003). The increasing cellular calcium concentration induces further downstream signaling steps.

The discovery that neurons from $p59^{fyn}$-deficient mice, grown on fibroblasts expressing NCAM, show an inhibition of neurite elongation, suggests a function of non-receptor tyrosine kinases in neurite outgrowth (Beggs et al, 1994). In subsequent studies NCAM was shown to associate with $p59^{fyn}$ (Beggs et al, 1997; Kramer et al, 1999). This association is assumed to occur via the receptor-like protein tyrosine phosphatase RPTPα (Bodrikov et al, 2005) which is a known activator of src family tyrosine kinases (Ponniah et al, 1999; Zheng et al, 1992). The increase in calcium concentration in response to NCAM signaling via FGFR, in turn, strengthens the complex between NCAM140 and RPTPα via spectrin. Upon NCAM activation and palmitoylation the complex redistributes into lipid rafts (Bodrikov et al, 2005; Niethammer et al, 2002). This event triggers the activation of downstream signaling pathways, e.g. the activation of the focal adhesion kinase (FAK) (Niethammer et al, 2002) which in turn leads to the activation of the mitogen activated protein kinase (MAPK) pathway (Schmid et al, 1999). MAPKs are serine/threonine specific protein kinases that regulate the activity of several transcription factors such as c-fos, NFκB and CREB. The kinases translocate to the nucleus and alter the transcription of their target genes (reviewed in Pawson & Scott, 1997). In the course of NCAM directed signaling, the protein kinase C (PKC) is activated as mentioned above. Activated $PKC\beta_2$ binds via spectrin to NCAM140 and NCAM180 (Leshchyns'ka et al, 2003; Rodriguez et al, 1999). Spectrin which forms a submembrane matrix serves as a crosslinking platform for NCAM and PKC. Upon NCAM activation this complex redistributes into lipid rafts where PKC affects various substrates that are implicated in the regulation of the cytoskeleton, for instance the growth associated protein 43 (GAP-43) (Botto et al, 2007; Sheu et al, 1990). This membrane associated and palmitoylated glycoprotein, which is enriched in axons and growth cones, functions eventually in the regulation of actin polymerization (He et al, 1997; Meiri et al, 1998).

III.3. THE ROLE OF LIPID RAFTS IN NEURITE OUTGROWTH

It is evident that lipid rafts exert a crucial role in NCAM signaling and the subsequent regulation of structural changes. The lipid raft hypothesis, originally described by Simons & Ikonen in 1997 (Simons & Ikonen, 1997), presents an update of the fluid mosaic model from Singer & Nicolson (Singer & Nicolson, 1972). According to the lipid raft hypothesis the plasma membrane is not just a continuous and homogenous fluid of lipids with proteins inserted like a mosaic. Instead, it appears that microdomains in the plasma membrane exist that differ in their lipid and protein composition from the rest of the bilayer. Thus, the current definition of lipid rafts characterizes them as dynamic and heterogeneous membrane microdomains with different sizes ranging from 10 nm to 200 nm (Pike, 2006). Due to their enrichment in sphingolipids and sterols they resist to cold non-ionic detergent extraction. These biochemically isolated detergent resistant membranes (DRMs) have been operationally defined as equivalent to lipid rafts (Brown & London, 1997; Brown & London, 1998). Many signaling molecules were co-fractionated with DRMs such as GPI anchored proteins and src family kinases (Simons & Ikonen, 1997). These findings strongly point to a general role of lipid rafts in signaling events. It is supposed that they amplify signal transduction events by concentrating key proteins of the signaling machinery.

NCAM120 is enriched in lipid rafts owing to its GPI anchor, whereas the transmembrane isoforms NCAM180 and NCAM140 are present mostly outside of lipid rafts (He et al, 1986; Niethammer et al, 2002). The localization of the transmembrane isoforms, however, is dynamically regulated and due to palmitoylation they can redistribute into microdomains (Leshchyns'ka et al, 2003; Niethammer et al, 2002). The cellular prion protein was shown to support this recruitment since it stabilizes NCAM in lipid rafts (Santuccione et al, 2005). The importance of lipid rafts in the NCAM mediated signaling is underlined by the observation that mutation of palmitoylation sites in NCAM140 inactivates its ability to induce neurite outgrowth (Niethammer et al, 2002).

Moreover, the tight regulation of structural changes during neurite growth implies the function of lipid rafts. Neurite growth requires a fine tuned communication between the plasma membrane and the underlying cytoskeleton. Cytoskeletal proteins are linked to the membrane via adaptor proteins such as PDZ-domain containing proteins and ankyrin (Bennett & Baines, 2001; Garner et al, 2000; Ponting & Phillips, 1995; Xia et al, 1997). In addition, they can also interact directly with lipids. For instance, brain spectrin binds to anionic phospholipids such as phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Diakowski et al, 1999; Diakowski & Sikorski, 1995). The binding to PIP_2 is supposed

to be mediated by the pleckstrin homology (PH) domain in the β-subunit of spectrin (Fig. 3) (Wang et al, 1996). Since PIP_2 is enriched in lipid rafts (Hope & Pike, 1996; Liu et al, 1998) it is not surprising that spectrin was also found to be localized to lipid rafts and, moreover, to be recruited into the microdomains by NCAM (Leshchyns'ka et al, 2003; von Haller et al, 2001). In addition, spectrin is supposed to be subjected to palmitoylation similar to NCAM which supports its anchoring to the microdomains (Das et al, 1997). Interestingly, it was shown that PIP_2 containing lipid rafts regulate cytoskeletal changes by controlling actin dynamics (Caroni, 2001; Lassing & Lindberg, 1985; Laux et al, 2000; Takenawa & Itoh, 2001; Tolias et al, 2000). They can act as nucleation sites for the polymerization of actin and thus influence the assembly of cytoskeletal components. Proteins that regulate the cytoskeleton downstream of NCAM, e.g. GAP-43, localize to lipid rafts which strengthens the suggestion that microdomains operate as local organizers of the cytoskeleton (del Pozo et al, 2004; Laux et al, 2000; Michaely et al, 1999).

III.4. THE NEURONAL GROWTH CONE AND THE GROWTH CONE CYTOSKELETON

As it was underlined in the preceding chapters, NCAM is an important regulator of neurite growth in the developing brain. During development neurites grow out from the neuronal cell body and extend toward their target region in order to form synapses to either receive or transmit synaptic signals. Upon reaching its target the neurite forms terminal branches and the motile growth cone differentiates into a presynaptic or postsynaptic terminal. This results eventually in the formation of a complex neuronal network (Haydon & Drapeau, 1995). In 1890, Cajal published the first description of a neuronal growth cone in chick embryos (y Cajal, 1890a; y Cajal, 1890b). He described the growth cone as highly dynamic structure that guides the neurite to its target. In fact, the growth cone is very specialized and possesses different structural and functional domains as illustrated in Fig. 2.

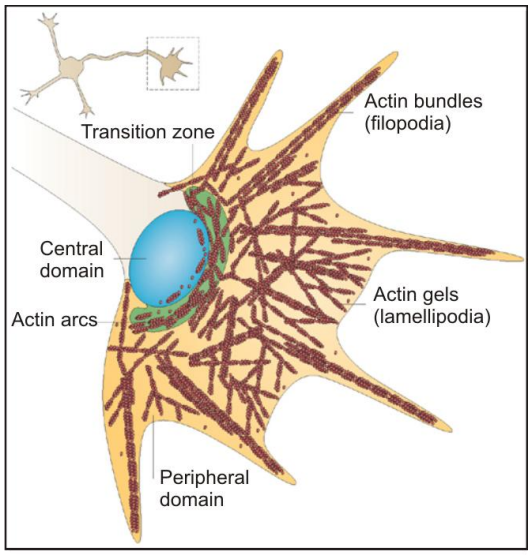

Fig. 2: Cytoskeletal organization of a growth cone (Pak et al, 2008). The growth cone can be divided into three distinct regions: the peripheral domain (in yellow), the central domain (in blue) and the transition zone (in green). In the peripheral domain, linear actin bundles comprise filopodia (microspikes), which protrude outward, and mesh-like gels comprise lamellipodia (veils), which are located between the filopodia. The central domain is rich in microtubules (not shown). In the transition zone, transverse bundles of actin filaments (actin arcs) are present.

The growth cone explores the environment by transient interactions with neighboring cells or the extracellular matrix (Rehder et al, 1996). It recognizes environmental cues via receptors on its cell surface. Fixed or diffusible cues such as components of the extracellular matrix, peptide trophic factors and guidance molecules can either attract or repel growth cones (Tessier-Lavigne & Goodman, 1996). Cell adhesion molecules also constitute important cues that promote neurite growth. When the growth cone interacts with its substrate, an association between the respective receptor and the underlying cytoskeleton is formed (Suter & Forscher, 2000). Downstream of the receptors, intracellular signaling cascades regulate the dynamics of the cytoskeleton. Subsequently the growth cone undergoes structural changes, especially at 'decision points', where a growth cone grows into new directions. Thus, the growth process requires continuous structural changes and a precise regulation of the cytoskeleton in the neurite and the growth cone. It depends on a dynamic assembly, stabilization but also breakdown of cytoskeletal components (Dent & Gertler, 2003). For instance, axon growth requires the extension of the plasma membrane by continuous insertion of new membrane material into the growth cone and along the axon (Harel & Futerman, 1996; Martenson et al, 1993; Pfenninger & Friedman, 1993). This insertion requires a structural rearrangement of the cytoskeleton. For example, the destabilization of microtubules was shown to be a prerequisite for the addition of new membrane into the growth cone (Zakharenko & Popov, 1998). NCAM

is very likely to be involved in the regulation of the cytoskeleton since it interacts with several cytoskeletal components. The intracellular domains of the transmembrane NCAM isoforms were found to associate with α- and β-tubulin (Buttner et al, 2003) as well as brain spectrin (Leshchyns'ka et al., 2003; Pollerberg et al., 1987; Pollerberg et al., 1986). The submembrane spectrin network is, in addition to the actin and microtubule meshwork, an important component of the growth cone cytoskeleton (Gordon-Weeks & Lang, 1988). It links NCAM and other cell adhesion molecules in the plasma membrane to the intracellular actin cytoskeleton. Spectrin is a flexible rod-like protein with binding sites for F-actin at each end. As illustrated in Fig. 3 α- and β-spectrin form heterodimers that assemble into a heterotetramer (Bennett & Baines, 2001; Winkelmann et al, 1990; Winkelmann & Forget, 1993), though, β-spectrin homodimers or heterotetramers without an α-spectrin partner were also described (Bloch & Morrow, 1989; Porter et al, 1997; Pumplin, 1995). These spectrin tetramers form a crosslinked network underneath the plasma membrane.

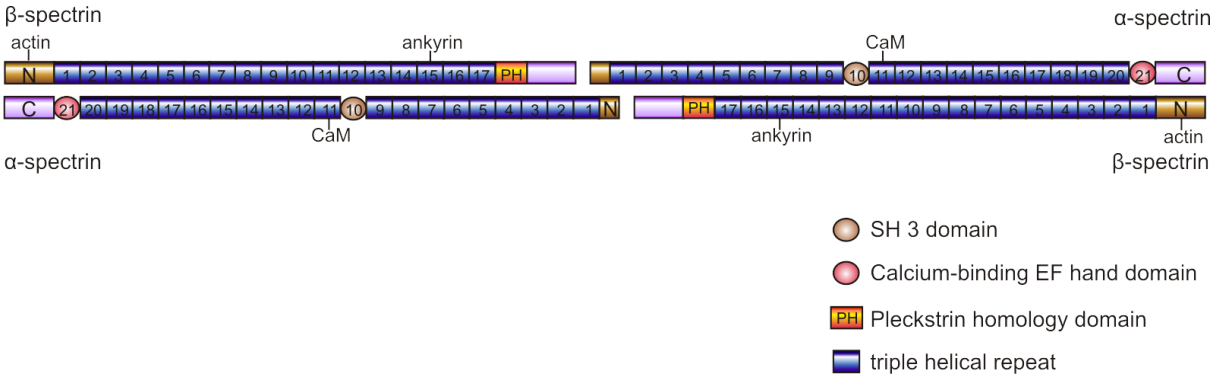

Fig. 3: Structure of the spectrin tetramer. The α- and β-subunits are assembled antiparallel and laterally to form heterodimers (Broderick & Winder, 2002). These heterodimers associate head-to-head to form a tetramer. Thereby, the N-terminus of the α-subunit associates with the C-terminus of the β-subunit. The modular subunits of spectrin are triple helical repeats (blue) (Djinovic-Carugo et al, 2002). The pleckstrin homology domain (PH, orange) in β-spectrin mediates its association with acidic phospholipids (Diakowski et al, 1999; Diakowski & Sikorski, 1995). Repeat 15 of β-spectrin contains the ankyrin binding site (Kennedy et al, 1991). At the N-terminal end β-spectrin is associated with short actin filaments (Broderick & Winder, 2002). The α-spectrin contains instead a calcium binding domain (pink) and a src homology domain (SH 3, brown) (Trave et al, 1995; Wasenius et al, 1989). Repeat 11 contains the calmodulin (CaM) binding site in α-spectrin (Leto et al, 1989). The same repeat represents a caspase-3 cleavage site in α-and β-spectrin (Lee et al, 2001).

Spectrin function is indispensable for axonal outgrowth (Hammarlund et al, 2000; Hammarlund et al, 2007). In *C. elegans* β-spectrin mutation disrupts axonal outgrowth (Hammarlund et al, 2000). Similarly, mutation of β-spectrin in *Drosophila* leads to defects in the architecture of the neuronal growth cone (Hulsmeier et al, 2007). Furthermore, the

spectrin meshwork was shown to be implicated in growth cone adhesion and motility (Sobue & Kanda, 1989). Local proteolysis of the spectrin network is a crucial step in growth cone formation and it is suggested that a transient and localized increase in the calcium concentration activates certain proteases that cleave the spectrin meshwork as well as the actin and microtubule based cytoskeleton (Gitler and Spira, 1998; Spira et al., 2001). Proteolysis of spectrin is attributed to calpains, a class of calcium dependent cysteine proteases. However, a second class of cysteine proteases, called caspases, seems also to be implicated in the rearrangement of the spectrin cytoskeleton, as discussed in more detail in the next chapter.

III.5. APOPTOTIC AND CELLULAR FUNCTIONS OF CASPASES

III.5.1. Caspases in apoptosis

Apoptosis plays a critical role in the developing embryo during morphogenesis (Jacobson et al, 2007). Tissue modeling such as formation of digits (Milligan et al, 1995) and the removal of vestigial structures, e.g. the tail of the tadpole, are crucial functions of apoptosis (Kerr et al, 1974). Moreover, neurons and oligodendrocytes which are generated in excess during brain development but fail to make proper connections to their targets are eliminated by apoptosis (Barres et al, 1992; Oppenheim, 1991).

In 1992, the interleukin-1β-converting enzyme (ICE) was described (Cerretti et al, 1992; Thornberry et al, 1992). This protease was supposed to be involved in the activation and maturation of interleukin-1β. However, one year later the enzyme was shown to be similar to CED-3, a gene product required for cell death in the nematode *Caenorhabditis elegans* (Yuan et al, 1993). Due to this discovery ICE-like proteases have been implicated in apoptosis for the first time. In 1996, the term ‘caspases’ was introduced and a standardized nomenclature was recommended that numbers caspases according to their chronological order of publication (Alnemri et al, 1996). Thus, ICE constitutes the prototype of caspase family, now referred to as caspase-1. In the following years a growing number of caspases in mammalian and non-mammalian species have been characterized (Lamkanfi et al, 2002). Surprisingly, caspase-like proteins were also identified in slim molds, plants, fungi and protozoa and are termed as paracaspases and metacaspases (Aravind & Koonin, 2002).

Caspases constitute cysteinyl dependent aspartate specific proteases. This specificity is rare among protease families and just shared with the serine protease granzyme B (Thornberry et al, 1997). Caspases involved in apoptosis can be grouped into initiator (or apical) and effector (or executioner) caspases. Initiator caspases contain a long N-terminal prodomain whereas

caspases with short prodomains belong to the group of effector caspases (see Fig. 4A). The large prodomains, containing special protein-protein interaction motifs like caspase recruitment domains (CARD) or death effector domains (DED), promote the recruitment of initiator caspases to multiprotein activation complexes (Hofmann et al, 1997; Muzio et al, 1996; Vincenz & Dixit, 1997). A third but phylogenetically distinct class of caspases with long prodomains is involved in inflammation and cytokines maturation (e.g. caspase-1, -4, -5, -11, -12) (reviewed in Martinon & Tschopp, 2004).

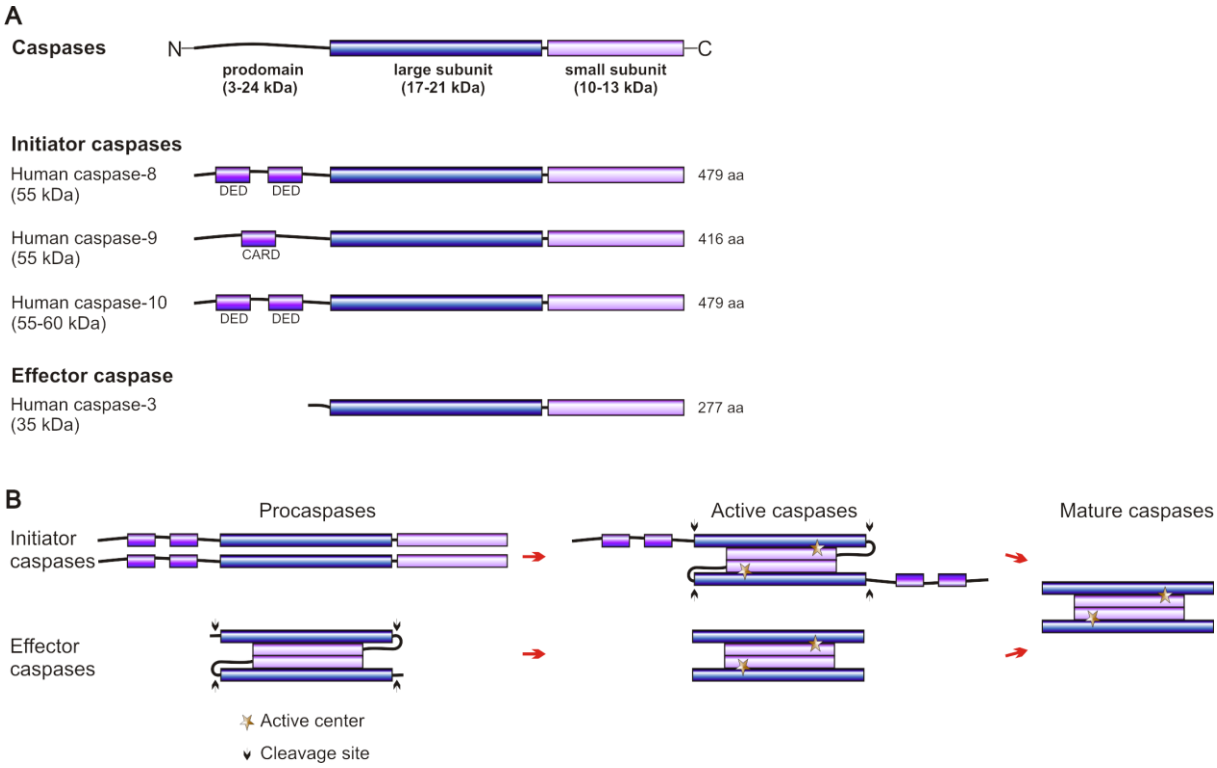

Fig. 4: Caspase organization. (A) Caspases are expressed as latent precursors composed of an N-terminal prodomain and two catalytic subunits (large and small subunit). Initiator caspases possess long prodomains containing FADD-homology domains (DED, CARD), whereas effector caspases contain short prodomains. (B) Initiator and effector caspases are activated differently. Initiator caspases require dimerization for activation which is followed by autoproteolytic cleavage. In contrast, effector caspases require proteolytic cleavage for activation. Processing leads to cleavage within the large and small subunit at certain aspartate residues and subsequently to removal of the prodomain. The mature caspase is arranged as tetramer assembled by two processed precursors and contains two large and two small subunits providing two active centers. See text for details.

Activity of caspases needs to be carefully regulated to prevent inappropriate induction of apoptosis. Thus, caspases are activated in a hierarchical process in which the initiator caspases activate downstream effector caspases. The latter directly cleave irreversibly

regulatory and structural proteins in the cytosol, nucleus and proteins of the cytoskeleton. Within the cell, caspases are stored as inactive precursors (Salvesen & Dixit, 1997; Thornberry & Lazebnik, 1998). The precursors are processed by sequential cleavage at two internal aspartate residues. The mature enzyme constitutes a tetrameric complex composed of two large and two small catalytic subunits with two catalytic centers which operate independently of each other (Walker et al., 1994; Wilson et al., 1994) (see Fig. 4B).

It is interesting in this context that initiator and effector caspases undergo distinct activation mechanism. Initiator caspases are present in the cytosol as monomeric proenzymes, whereas the proenzymes of effector caspases exist as preformed dimers (Fig. 4B) (Boatright et al, 2003; Mittl et al, 1997). Initiator caspases such as caspase-8 and caspase-9 require dimerization for activation. Subsequently they are autoproteolytically processed (Martin et al., 1998; Srinivasula et al., 1998; Yang et al., 1998). For instance, caspase-8 can be recruited by its prodomain to multicomponent complexes such as death inducing signaling complexes (DISCs). This event strengthens dimerization of the proenzymes. It has been suggested that dimerization or oligomerization allows the procaspases to cleave each other in *trans* within the dimer. An inherent activity in the procaspases is possibly responsible for this cross-cleavage. This activation process is described by the 'induced proximity model' (Muzio et al., 1998; Salvesen and Dixit, 1999). Chang and colleagues proposed an 'interdimer processing mechanism' regarding the activation of caspase-8 (Chang et al, 2003). In this model it is assumed that one dimer cleaves its adjacent dimer. Intriguingly, the formation of the active site requires just the dimerization of the proenzyme (Boatright et al, 2003; Donepudi et al, 2003). Dimerization of the procaspases allows translocation of the activation loop thereby inducing the enzymatic activity. Internal cleavage of proenzymes occurs in a subsequent step and involves the separation of the large and the small subunit by cleavage within the linker region. Removal of the prodomain from the large subunit might allow eventually the release of the active caspase into the cytosol.

In contrast, effector caspases like caspase-3 are activated by cleavage between the large and the small subunit exerted by the upstream initiator caspases (Fernandes-Alnemri et al., 1996; Muzio et al., 1997; Srinivasula et al., 1998; Stennicke et al., 1998) or granzyme B which is released by cytotoxic T-lymphocytes (Darmon et al, 1995; Martin et al, 1996). The prodomain of executioner caspases is reported to be removed subsequently in an autocatalytic step (Fernandes-Alnemri et al, 1996; Martin et al, 1996). The short prodomain of caspase-3 is suggested to prevent autoactivation of the protease (Meergans et al, 2000).

Caspase-3 is very abundant in the brain. In mouse brain, caspase-3 mRNA is expressed in all brain nuclei, especially in areas where neurogenesis occurs, such as the olfactory bulb and the dentate gyrus (de Bilbao et al, 1999; Madden et al, 2007). During postnatal development its expression is down regulated. In contrast to caspase-3, the expression of the initiator caspase-9 remains unchanged during postnatal development (Madden et al, 2007). However, the level of caspase-8, another initiator caspase, decreases from postnatal day (pnd) 9.5 to 17.5 in the mouse embryo (Sakamaki et al, 1998). Caspase-10 is a homolog of caspase-8 and in structure and function during apoptosis closely related to caspase-8 (Fernandes-Alnemri et al, 1996; Vincenz & Dixit, 1997). Its expression level is very low in human brain (Fernandes-Alnemri et al, 1996), but abundant in embryonic tissue of lung, kidney and skeletal muscle (Ng et al, 1999). However, existence of caspase-10 in mouse is controversially discussed and remains to be established (Janicke et al, 2006).

Within the cell, caspases are distributed in the cytosol, the ER, the mitochondrial intermembrane space and the nucleus (Kamada et al, 2005; Krebs et al, 2000; Mancini et al, 1998; Qin et al, 2001; Susin et al, 1999). Moreover, caspases are also found on the extracellular surface of the plasma membrane (Singer et al, 1995). The selective localization of caspases in different subcellular compartments might contribute to different functions in the brain (Shimohama et al, 2001). In this regard, it is interesting to mention the phenotype observed in caspase-3 deficient mice. Kuida and coworkers generated caspase-3 deficient mice by targeted gene disruption (Kuida et al, 1996). They observed that mice that do not express caspase-3 die during the early postnatal period because of severe defects in brain development. This includes hyperplasia of the embryonic ventricular zone and enlargement and malformation of the cerebrum. The targeted mutation of caspase-3 was performed in 129/Sv embryonic stem cells and chimeric mice were generated in C57BL/6J recipients (Kuida et al, 1996). Homozygous caspase-3 mutants were obtained by sibling mating. Although most mutant mice died, few survived and reached adulthood. It became apparent that the phenotype of caspase-3 deficient mice is strain dependent (Leonard et al, 2002). Since previous studies were performed with mice on mixed genetic backgrounds, Leonard and colleagues backcrossed the mutation onto the pure 129X1/SvJ and C57BL/6J genetic backgrounds. They observed that caspase-3 deficient mice on the 129X1/SvJ background are embryonic lethal, whereas they were phenotypically normal on the C57BL/6J background. These differences might be attributed to compensatory activation of other executioner caspases (Zheng et al, 2000) that occurs in one strain but not in the other. Moreover, endogenous inhibitors of apoptosis proteins (IAPs) might be differently expressed in both

strains. IAPs bind to active executioner caspases and prevent apoptosis (Roy et al, 1997). However, the hyperplasia observed in caspase-3 deficient mice brains is supposed to be caused by decreased apoptosis during brain development.

Executioner caspases are genuine 'cellular demolition experts' (Creagh & Martin, 2001). Especially caspase-3 is the major effector protease that disassembles the cytoskeleton during apoptosis. A cell undergoing apoptosis shrinks, rounds up and detaches from the ECM (Kerr et al, 1972). Contacts with neighboring cells and the ECM are interrupted by break-down of cell-matrix focal adhesion sites and cell-cell adhesion complexes (Brancolini et al, 1997; Levkau et al, 1998; Steinhusen et al, 2001; Weiske et al, 2001). For instance, the focal adhesion kinase FAK is cleaved by the executioner caspase-3 (Levkau et al, 1998). The cell obviously undergoes cytoskeletal changes during the apoptotic process. Scaffolding proteins and cytoskeleton associated proteins, such as actin, spectrin and gelsolin, can be proteolyzed by caspase-3 (Kothakota et al, 1997; Mashima et al, 1997; Pike et al, 1998; Wang et al, 1998b). Formation of plasma membrane protrusions, known as blebs, is ascribed to actin reorganization. Blebs are preferentially formed in areas where the actin cytoskeleton is weakened, e.g. due to fodrin disassembly by caspases. In addition, intermediate filaments like keratin are proteolyzed (Byun et al, 2001; Ku et al, 1997). The dissection of cytoskeletal components by caspases ensures the orderly dismantling of the dying cell. Hence, caspases are remarkable effectors of the cytoskeleton since cytoskeletal proteins are among their preferred targets.

Caspases recognize their substrates by means of a four amino acids comprising motif (P_1 to P_4). The most C-terminal amino acid (P_1) of this motif within the substrate is usually an aspartate residue where the caspase mediated cleavage is performed (Earnshaw et al, 1999; Howard et al, 1991; Sleath et al, 1990). The amino acids at positions P_2 to P_4 vary among the different caspases. Especially the residue at P_4 accounts for the different substrate specificities. However, not all proteins possessing the cleavage site are cleaved (Thornberry & Lazebnik, 1998). Thus, the substrate recognition seems also to be influenced by the tertiary protein structure. In principle, caspases do not degrade their target proteins, but rather cleave their substrates at single or very few sites, which often results in the loss or alteration of the protein function (Nicholson, 1999; Salvesen and Dixit, 1997). The individual substrate specificity which was analyzed in detail by Thornberry and colleagues provided the basis for the design of specific, synthetic caspase inhibitors (Thornberry et al., 1997). These inhibitors contain a peptide recognition site that corresponds to the preferred cleavage site of the individual caspase within its substrates. This peptide recognition site is attached to a

functional group such as an aldehyde (CHO), fluoromethylketone (FMK) or a chloromethylketone (CMK). Thus, caspase inhibitors are modified pseudosubstrates which operate by binding to the active center of caspases either in a reversible or irreversible manner (Nicholson, 1999). Coupling to ketones gives rise to irreversible inhibitors since they covalently bind to the cysteine residue in the active site while aldehyde coupled inhibitors are reversible and competitive inhibitors.

III.5.2. Novel cellular functions of caspases and implications of caspases in the reorganization of the cytoskeleton

There is emerging evidence that caspases exert physiological functions even in the absence of cell death. Different groups report that these versatile cysteine proteases are involved in normal neuronal functions. Dash and colleagues suggested that non-apoptotic neuronal caspase-3 cleavage occurs during long term potentiation processes (Dash et al, 2000). Infusion of caspase-3 inhibitors directly into the rat brain blocks activity of the protease in the hippocampus and results in inhibition of long term spatial memory storage (Dash et al, 2000). Moreover, incubation of hippocampal slices with a caspase-3 inhibitor blocks also the formation of long term potentiation in the CA1 region (Gulyaeva et al., 2003). The same group shows that infusion of caspase-3 inhibitor into the rat brain impairs active avoidance learning (Stepanichev et al, 2005). Thus, a modest activation of caspase-3 might contribute to memory formation either due to remodeling of active synapses by cleavage of structural proteins or due to cleavage of synaptic junction proteins. Also direct alterations of glutamate receptors are conceivable. The GluR1 subunit of the AMPA receptor is indeed a known caspase-3 substrate (Lu et al, 2002). Moreover, activation of glutamate receptors triggers activation of caspase-3 in dendrites (Mattson et al, 1998). Thus, it is suggested that caspase-3 cleavage of the GluR1 subunit reduces calcium influx via the AMPA receptor. This event results in a reduced synaptic response to glutamatergic stimulation (Lu et al, 2002). In other words, it reduces the sensibility for glutamate and therefore modulates neuronal excitability.

Caspase-3 not only contributes to the regulation of synaptic plasticity, but also affects the cytoskeleton as protease in a non-apoptotic context. For instance, caspase-3 is important for cytoskeletal remodeling in activated astrocytes (Acarin et al, 2007). Acarin and coworkers induced lesions in the cortex by injection of N-methyl-D-aspartate (NMDA) in order to provoke astrogliosis. During astrogliosis astrocytes undergo cytokeletal rearrangements by disassembly of intermediate filaments, e.g. vimentin. Activated astrocytes show an increased activity of caspase-3 without further apoptotic signals. Moreover, vimentin is an identified

substrate of caspase-3 (Byun et al, 2001). The protease might therefore contribute to the reorganization of the cytoskeleton by means of vimentin cleavage. Caspase-3 is also implicated in remodeling of the spectrin membrane skeleton during lens development and aging (Lee et al, 2001). During late embryonic lens development the spectrin cytoskeleton is dissected and discrete spectrin fragments are generated. A-spectrin fragments of approximately 150 kDa and β-spectrin fragments of 120 kDa and 80 kDa agglomerate in the lens. This fragmentation leads to a less efficient membrane association and therefore to a lasting rearrangement of the spectrin cytoskeleton.
Interestingly, Verma and colleagues suggested a role for caspase-3 in growth cone formation (Verma et al, 2005). Inhibition of caspase-3 abrogates the formation of new growth cones of dorsal root ganglion cells and retinal cells after axotomy. Protein degradation by caspase-3 in concert with synthesis of new proteins within the axon support the regeneration of growth cones. In addition, guidance cues are shown to induce caspase-3 activation in retinal growth cones (Campbell & Holt, 2003). Addition of the guidance cues netrin-1 and lysophosphatidic acid (LPA) results in a rapid increase of active caspase-3 in growth cones. Moreover, active caspase-3 is necessary for the chemotropic responses to LPA and netrin-1, since LPA-induced growth cone collapse and netrin mediated growth cone attraction can be blocked by specific caspase-3 inhibitors.

In addition to caspase-3 the initiator caspase-8 is implicated in non-apoptotic functions. Integrin mediated adhesion and cell migration are known to promote $p60^{src}$ activation (Mitra & Schlaepfer, 2006). In neuroblastoma cells caspase-8 promotes the $p60^{src}$ mediated cell adhesion to fibronectin, an ECM glycoprotein that binds to integrin receptors at the cell surface (Finlay & Vuori, 2007). Caspase-8 associates with $p60^{src}$ via its DED domains and promotes the activation of the Erk pathway in a $p60^{src}$ dependent way. Surprisingly, caspase-8 performs this function independently of its catalytic activity. Furthermore, Barbero and coworkers reported that caspase-8 functions in cell migration (Barbero et al, 2008). The integrin mediated adhesion induces phosphorylation of caspase-8 at Tyr 380. Phosphorylated caspase-8 is recruited to the leading lamellae or pseudopodia of migrating neuroblastoma cells. It is intriguing that the catalytic activity seems to be unnecessary for this localization. Since Tyr 380 is localized in the linker region between the large and the small subunit, the phosphorylation might block the cleavage within the linker. However, the phosphorylation site is not directly located in the two recognition sites within the linker. It is suggested that the association occurs between Tyr 380 of caspase-8 and the SH2-domain of $p60^{src}$. Another

group reported that $p60^{src}$ mediates the phosphorylation of caspase-8 on Tyr 380 (Cursi et al, 2006). Thus, caspase-8 increases cell migration of neuroblastoma cells in a $p60^{src}$ dependent manner that is independent of its proteolytic activity. Helfer and colleagues supported the idea that caspase-8 is involved in cell migration of fibroblasts and neuroblastoma cells (Helfer et al, 2006). However, they argue that caspase-8 promotes motility by means of Rac and calpain activation. Cell motility in fibroblasts depends on calpains (Dourdin et al, 2001), which are for instance involved in detachment of the trailing edge and lamellipodia extension or retraction (Franco & Huttenlocher, 2005).

Alltogether, there is compelling evidence that both initiator and effector caspases perform non-traditional functions besides their role in apoptosis. These functions comprise dynamic or permanent cytoskeletal changes, cell adhesion as well as cell migration. Detailed investigations of the underlying mechanisms will be required to understand the ambivalent character of caspases.

IV. AIMS OF THE STUDY

The development of the nervous system involves the formation of complex neural networks which requires the generation of cells in the right temporal and spatial locations, the outgrowth of neurites and eventually the formation of synapses with their proper targets. These fundamental processes are based on a coordinated regulation and communication between the cells of the nervous system. Cell adhesion and recognition molecules are part of this regulatory machinery. The neural cell adhesion molecule NCAM is a well established mediator of neurite outgrowth and elongation which induces intracellular signaling cascades (Doherty et al, 1991; Williams et al, 1994b). Dysregulation of NCAM is supposed to be involved in neuropsychiatric and neurodegenerative disorders, such as schizophrenia, mood disorders and Alzheimer's disease (Brennaman & Maness, 2008). Elucidation of NCAM functions is therefore valuable to understand its contribution to cognitive dysfunctions. Neurite growth includes not only the protrusion of processes, but also their guidance by growth cones and results finally in the formation and stabilization of synapses. Elongation and guidance of neurites require a constant reshaping of the growth cone cytoskeleton, especially at decision points (Dent & Gertler, 2003). This study investigates how NCAM directed neurite growth is regulated intracellularly. NCAM is known to associate with the spectrin-actin meshwork and to influence its dynamics. Special cysteine proteases, termed caspases, are implicated in the proteolysis of the cytoskeleton. Since caspases are able to disassemble the spectrin meshwork we were interested to investigate whether NCAM is involved in their regulation. Moreover, NCAM is redistributed to lipid rafts to induce signal transduction pathways. Interestingly, it was also shown that remodeling of the cytoskeleton can occur in lipid rafts. Thus, these specialized microdomains can function as local organizers of the cytoskeleton. Therefore we analyzed whether lipid rafts are involved in the NCAM mediated regulation of caspases. And finally we wanted to answer the question whether NCAM promoted neurite outgrowth requires a caspase specific rearrangement of the spectrin cytoskeleton. In our study, we demonstrate a novel function of caspases in the regulation of neurite growth besides of their traditional role in apoptosis.

V. MATERIALS AND METHODS

V.1. MATERIALS

V.1.1. Chemicals and materials

All chemicals (in p.a. quality) and materials were purchased from the following companies and are listed below in alphabetical order:

Abcam (Cambridge, UK); Amersham Pharmacia Biotech Europe GmbH (Freiburg, Germany); BD Biosciences (Heidelberg, Germany); Beckman Coulter GmbH (Krefeld, Germany) including Beckman Instruments, Inc.; BIOCAT GmbH (Heidelberg, Germany); Biochrom AG (Berlin, Germany); BIOMOL Feinchemikalien GmbH (Hamburg, Germany); Bio-Rad Laboratories GmbH (Munich, Germany); BioVision, Inc. (Mountain View, California, USA) via BIOCAT GmbH; Calbiochem (EMD Chemicals, Inc., Gibbstown, New Jersey, USA) via Merck Biosciences GmbH; Carl Roth GmbH & Co. KG (Karlsruhe, Germany); CHEMICON via Millipore GmbH; Cell Signaling Technology, Inc. (Danvers, Massachusetts, USA) via New England Biolabs GmbH; Dianova GmbH (Hamburg, Germany); Frema Reform (Lüneburg, Germany); Herolab GmbH Laborgeräte (Wiesloh, Germany); Invitrogen GmbH (Karlsruhe, Germany); Jackson ImmunoResearch laboratories, Inc. via Dianova GmbH; KMF Laborchemie Handels-GmbH via VWR International GmbH; MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany); Mallinckrodt Baker (Griesheim, Germany); Merck Biosciences GmbH (Bad Soden am Taunus, Germany); Millipore GmbH (Eschborn, Germany); New England Biolabs GmbH (Frankfurt am Main, Germany); Nunc GmbH & Co. KG (Thermo Fischer Scientific, Wiesbaden, Germany); PAA Laboratories GmbH (Cölbe, Germany); Pierce/Perbio Science Deutschland GmbH (Bonn, Germany); Pineda Antikörper-Service (Berlin, Germany); Polysciences Europe GmbH (Eppelheim, Germany); QIAGEN GmbH (Hilden, Germany); R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany); Roche Diagnostics GmbH (Mannheim, Germany); Santa Cruz Biotechnology, Inc. (Heidelberg, Germany); SERVA Electrophoresis GmbH (Heidelberg, Germany); Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany); Tebu-bio (Offenbach, Germany); Th. Geyer Hamburg GmbH & Co. KG (Hamburg, Germany); Upstate via Millipore GmbH; VWR International GmbH (Hannover, Germany).

Cell culture material was ordered from Greiner Bio-One GmbH (Frickenhausen, Germany) via Hassa-Laborbedarf GmbH (Lübeck, Germany).

V.1.2. Buffers and solutions

General buffers and stock solutions are listed below. Bi-distilled water (ddH_2O) was used for preparation unless indicated otherwise.

Blocking buffer *(for immunoblotting)*	4 % (w/v) skimmed milk powder (Frema Reform) in PBS
Dilution buffer (2x) *(for growth cone stimulation assay)*	200 mM sucrose 400 mM NaCl 4.8 mM $MgCl_2$ 20 mM KCl 88 mM HEPES 4.8 mM NaH_2PO_4 40 mM glucose
DNA sample buffer (5x) *(for DNA agarose gels)*	20 % (v/v) glycerol in 1x TAE 0.025 % (w/v) Orange G
Ethidium bromide staining solution *(for DNA agarose gels)*	10 µg/ml ethidium bromide in 1x TAE (0.025 mM in 1x TAE)
Homogenization buffer *(for whole brain homogenization and membrane fraction isolation)*	0.32 M sucrose 5 mM Tris-HCl, pH 7.4 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 0.1 mM PMSF
Homogenization buffer *(for growth cone fraction isolation)*	0.32 M sucrose 1 mM $MgCl_2$ 5 mM Tris-HCl, pH 7.4

Phosphate buffered saline (PBS)	136 mM NaCl 10 mM Na_2HPO_4 2.7 mM KCl 1.8 mM KH_2PO_4 pH 7.3
Phosphate buffered saline/TWEEN (PBST) *(for immunoblotting)*	PBS 0.1 % (v/v) TWEEN® 20
Radio immunoprecipitation assay (RIPA) buffer *(for cell lysis, co-immunoprecipitation)*	50 mM Tris-HCl, pH 7.5 150 mM NaCl 10 mM KCl 1 mM $Na_4P_2O_7$ 1 mM NaF 2 mM Na_3VO_4 1 % (v/v) NP-40 1 mM PMSF (2 mM EDTA)
Resolving gel *(for SDS-PAGE)*	375 mM Tris-HCl, pH 8.8 0.1 % (w/v) SDS 0.02 % (w/v) APS 0.1 % (v/v) TEMED Acrylamide/Bis solution (29:1) according to % of gel required
SDS-PAGE running buffer *(for SDS-PAGE)*	25 mM Tris 200 mM glycine 0.1 % (w/v) SDS

SDS sample buffer (5x) *(for SDS-PAGE)*	310 mM Tris-HCl, pH 6.8 25 % (v/v) glycerol 10 % (w/v) SDS 4.5 % (v/v) β-mercaptoethanol 0.015 % (w/v) bromphenol blue
Stacking gel *(for SDS-PAGE)*	125 mM Tris-HCl, pH 6.8 0.13 % (w/v) SDS 0.05 % (w/v) APS 0.2 % (w/v) TEMED Acrylamide/Bis solution (29:1) according to % of gel required
Stripping buffer *(for immunoblotting)*	25 mM glycine-HCl, pH 2.2 1 % (w/v) SDS
Sucrose, 0.75 M *(for growth cone fraction isolation)*	0.75 M sucrose 1 mM $MgCl_2$ 5 mM Tris-HCl, pH 7.4
Sucrose, 1.0 M *(for growth cone fraction isolation)*	1 M sucrose 1 mM $MgCl_2$ 5 mM Tris-HCl, pH 7.4
80 % Sucrose stock solution in ddH_2O *(for growth cone isolation)*	80 % (w/v) sucrose in ddH_2O
80 % Sucrose stock solution in Na_2CO_3 *(for lipid raft isolation)*	80 % (w/v) sucrose in 0.2 M Na_2CO_3
10 % Sucrose in TBS *(for lipid raft isolation)*	10 % (v/v) sucrose (from sucrose stock in Na_2CO_3) in TBS

30 % Sucrose in TBS *(for lipid raft isolation)*	30 % (v/v) sucrose (from sucrose stock in Na_2CO_3) in TBS
1 % Triton® X-100 *(for lipid raft isolation)*	1 % (v/v) Triton® X-100 (Sigma-Aldrich Chemie GmbH) in TBS
Transfer buffer *(for SDS-PAGE)*	25 mM Tris 200 mM glycine 0.001 % (v/v) SDS 10 % (v/v) Methanol pH 8.3
Tris buffered saline (TBS) *(for lipid raft isolation)*	50 mM Tris-HCl, pH 7.4 150 mM NaCl
Tris acetate EDTA (TAE) buffer (50x) *(for DNA agarose gels)*	2 M Tris-acetate, pH8.0 100 mM EDTA

V.1.3. Primary antibodies

Anti-actin (20-33)	Rabbit polyclonal antibody (Sigma-Aldrich Chemie GmbH), Raised against a synthetic actin N-terminal peptide, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-caspase-3	Rabbit polyclonal antibody (Cell Signaling Technology, Inc.), Raised against a synthetic peptide corresponding to residues surrounding the cleavage site and the N-terminus of the large fragment of human caspase-3, Recognizes full length non-active and cleaved active enzyme, Immunoblotting: 1:1000 in 4 % milk in PBS

Anti-caspase-3, cleaved (Asp 175) (5A1)	Rabbit monoclonal antibody (Cell Signaling Technology, Inc.), Raised against a synthetic peptide corresponding to N-terminal residues adjacent to Asp 175 in human caspase-3, Recognizes endogenous levels of the large fragment of activated caspase-3 but does not recognize full length caspase-3, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-caspase-8	Chicken polyclonal antibody (Abcam), Raised against recombinant full length human caspase-8, Recognizes the proform of caspase-8 as well as the cleaved forms, Immunoblotting: 1:1000 in 5 % milk in PBS
Anti-caspase-8	Rabbit polyclonal antibody (BD Biosciences), Raised against a synthetic peptide comprising amino acids 2-20 of human caspase-8, Recognizes the proform of caspase-8 as well as the cleaved forms, Immunoblotting: 1:1000 in 5 % milk in PBS
Anti-caspase-9	Rabbit polyclonal antibody (Cell Signaling Technology, Inc.), Raised against a synthetic peptide corresponding to residues surrounding the cleavage site of mouse caspase-9, Recognizes full length mouse-caspase-9 and the large fragment resulting from cleavage at Asp 353 and/or Asp 368, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-caspase-10	Rabbit polyclonal antibody (Abcam), Raised against a synthetic peptide, corresponding to amino acids 505-521 of human Caspase 10, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-contactin/F3	Goat polyclonal antibody (R&D Systems GmbH), Raised against a recombinant human contactin-1, Immunoblotting: 1:500 in 4 % milk in PBS

Anti-cleaved α-fodrin (Asp1185)	Rabbit polyclonal antibody (Calbiochem), Raised against a synthetic peptide corresponding to N-terminal amino acids adjacent to Asp 1185 of human α-fodrin, Recognizes the ~150 kDa fragment of α-fodrin cleaved by caspase-3, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-GAPDH	Mouse monoclonal antibody (CHEMICON), Raised against GAPDH from rabbit muscle, Immunoblotting: 1:2000 in 4 % milk in PBS
Anti-L1	Rabbit polyclonal antibody (produced in the laboratory of Prof. M. Schachner), Raised against the extracellular domain of mouse L1-Fc (Rolf et al, 2003), Immunoblotting: 1:5000 in 4 % milk in PBS
Anti-NCAM	Rabbit polyclonal antibody (produced in the laboratory of Prof. M. Schachner), Raised against NCAM from mouse brain, Immunoblotting: 1:5000 in 4 % milk in PBS
Anti-NCAM (clone H28)	Rat monoclonal antibody, Raised against the extracellular domain of mouse NCAM, the hybridoma cell line producing the H28 antibody was developed in the laboratory of Dr. Goridis (Centre National de la Recherche Scientifique UMR 8542, Paris, France), Used as cell culture supernatant, for co-immunoprecipitation
Anti-NCAM (clone P61)	Rat monoclonal antibody (Dr. Goridis, Developmental Biology Institute of Marseille, Marseille, France), Recognizes an intracellular determinant expressed by NCAM180 and NCAM140, Immunoblotting: 1:500 in 4 % milk in PBS

Anti-NCAM chicken (Pineda)	Chicken polyclonal antibody (Pineda Antikörper-Service), Raised against the extracellular domain of mouse NCAM-Fc, Stimulation: 1:400
	Chicken preimmune serum (Pineda Antikörper-Service), Stimulation: 1:400
Anti-NCAM rabbit (Pineda)	Rabbit polyclonal antibody (Pineda Antikörper-Service), Raised against the extracellular domain of mouse NCAM-Fc, Stimulation: 1:400
	Rabbit preimmune serum (Pineda Antikörper-Service), Stimulation: 1:400
Anti-PrP (M-20)	Goat polyclonal antibody (Santa Cruz Biotechnology, Inc.), Raised against a peptide mapping near the C-terminus of mouse prion protein
Anti-spectrin α1β1	Rabbit polyclonal antibody (Sigma-Aldrich Chemie GmbH), Raised against α1β1-spectrin from human erythrocyte ghosts, delipidized whole antiserum, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-spectrin β2 (clone 42/B)	Mouse monoclonal antibody (BD Biosciences), Raised against amino acids 2101-2189 of human β2-spectrin, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-α-tubulin (clone DM1A)	Mouse monoclonal antibody (Sigma-Aldrich Chemie GmbH), Raised against chick brain tubulin, Immunoblotting: 1:1000 in 4 % milk in PBS
Anti-β-tubulin (clone E7)	Mouse monoclonal antibody (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, Iowa, USA)

IgG, rabbit	IgG from rabbit serum (Sigma-Aldrich Chemie GmbH)
IgG, rat	IgG from rat serum (Sigma-Aldrich Chemie GmbH)

V.1.4. Secondary antibodies

Secondary antibodies were purchased from Jackson ImmunoResearch laboratories, Inc. The antibodies were coupled to horse radish peroxidase (HRP) and purified by immunoaffinity chromatography. To analyze immunoblots of co-immunoprecipitation assays preabsorbed antibodies were used to minimize cross-reactions to serum proteins of other species. The antibodies were used in dilutions of 1:20,000 or 1:50,000 in 4 % milk in PBS.

V.1.5. DNA and protein standards

1 kb DNA Ladder	Invitrogen GmbH
Precision Plus Protein™ All Blue Standards (10 bands of 10-250 kDa, prestained in blue)	Bio-Rad Laboratories GmbH
Precision Plus Protein™ Dual Color Standards (10 bands of 10-250 kDa, prestained in blue and red)	Bio-Rad Laboratories GmbH

V.1.6. Bacterial strains and mammalian cell lines

Bacterial strains were made chemically competent for transformation with plasmid DNA.

Strain	***Description***	***Origin***
Escherichia coli DH5α	F-,φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk^{--},mk^{+}), phoA, supE44, λ-, thi-1, gyrA96, relA1	New England Biolabs GmbH
CHO-K1	Chinese hamster ovary, Dehydrofolate reductase deficient hamster cell line	ATCC (American Type Culture Collection) CCL 61

V.1.7. Bacterial media

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

LB-medium	10 g/l bacto-tryptone, pH 7.4 10 g/l NaCl 5 g/l yeast extract Antibiotics: 100 mg/l ampicillin
LB-agar plates	20 g/l agar in LB-medium Antibiotics: 100 mg/l ampicillin

V.1.8. Cell culture media and material

Sterile polystyrene cell culture plates and flasks were purchased from Greiner Bio-One GmbH. The material was free from heavy metal, non-pyrogenic and non-cytotoxic.

CHO cell culture media and solutions

Hank's BSS (HBSS)	Without Ca^{2+} and Mg^{2+}, with phenol red (PAA Laboratories GmbH)
Trypsin/EDTA (1:250)	0.5 mg/ml trypsin, 0.22 mg/ml EDTA, without Ca^{2+} and Mg^{2+} (PAA Laboratories GmbH)
CHO cell growth medium	Glasgow Minimum Essential Medium (G-MEM, BHK-21) with L-glutamine (Invitrogen GmbH), Supplemented with: 5 % (v/v) fetal calf serum (FCS) (PAA Laboratories GmbH) 1x MEM non essential amino acids solution (100x) (Invitrogen GmbH) 1 mM sodium pyruvate MEM (Invitrogen GmbH) 0.4 mM L-glutamic acid 0.45 mM aspartic acid 0.026 mM adenosine 0.025 mM guanosine 0.029 mM cytidine 0.029 mM uridine 0.029 mM thymidine 10 ml/l penicillin/streptomycin solution (PAA Laboratories GmbH)
CHO cell freezing medium	CHO cell growth medium Supplemented with: 10 % (v/v) FCS (PAA Laboratories GmbH) 10 % (v/v) DMSO (Fluka, Sigma-Aldrich Chemie GmbH)

Primary cell culture media and solutions

Culture medium *(hippocampal neurons)*	Neurobasal™-A medium (Invitrogen GmbH) Supplemented with: 1x B-27 supplement (Invitrogen GmbH) 2 mM GlutaMAX™-supplement (Invitrogen GmbH) 2 ng/ml basic FGF-2 (R&D Systems GmbH)
Dissection solution *(hippocampal neurons)*	Neurobasal™-A medium (Invitrogen GmbH) Supplemented with: 1x B-27 supplement (Invitrogen GmbH) 2 mM GlutaMAX™-supplement (Invitrogen GmbH)
Digestion solution *(hippocampal neurons)*	Neurobasal™-A medium (Invitrogen GmbH) Supplemented with: 1x B-27 supplement (Invitrogen GmbH) 0.2 mg/ml DNase I (Sigma-Aldrich Chemie GmbH) 2 mg/ml Papain (Sigma-Aldrich Chemie GmbH)
Dissociation solution *(hippocampal neurons)*	Neurobasal™-A medium (Invitrogen GmbH) Supplemented with: 1x B-27 supplement (Invitrogen GmbH) 0.2 mg/ml Dnase I (Sigma-Aldrich Chemie GmbH)
Fixing solution (10x)	4 % (v/v) PFA in PBS

V.1.9. Inhibitors

Aprotinin	Lyophilized powder from bovine lung (Sigma-Aldrich Chemie GmbH), serine protease inhibitor (3-7 TIU/mg)
Caspase-3 inhibitor	Specific peptide inhibitor for caspase-3 (Ac-DMQD-CHO) (Calbiochem), cell permeable, reversible

Caspase-8 inhibitor	Specific peptide inhibitor for caspase-8 (z-IETD-FMK) (BioVision, Inc.), cell permeable, irreversible
Caspase-9 inhibitor	Specific peptide inhibitor for caspase-9 (LEHD-CHO) (Calbiochem), cell permeable, reversible
Caspase-10 inhibitor	Specific peptide inhibitor for caspase-10 (z-AEVD-FMK) (BioVision, Inc.), cell permeable, irreversible
Complete protease inhibitor cocktail, EDTA-free (50x)	Protease Inhibitor Cocktail Tablets, EDTA-free (Roche Diagnostics GmbH), 1 tablet in 1 ml ddH_2O
Leupeptin	Leupeptin hydrochloride (Sigma-Aldrich Chemie GmbH), serine and cysteine protease inhibitor

V.1.10. Plasmids

pcDNA3.1	Mammalian expression vector (Invitrogen GmbH)
Rat NCAM140/ pcDNA3.1	For expression of full length NCAM140 (GenBank accession number X15051) in mammalian cells (received from Dr. P. Maness, University of South Carolina, USA)

V.1.11. Mouse strains

NCAM deficient mice were provided by Harold Cremer (Cremer et al, 1994) and were inbred for at least nine generations onto the C57BL/6J background. For biochemical experiments 1- to 3-days-old wild type and NCAM deficient littermates obtained from heterozygous breeding were used. To prepare cultures of hippocampal neurons, 1- to 3-days-old C57BL/6J and NCAM deficient mice from homozygous breeding pairs were used. The animals were housed under standard conditions, namely a 12 h:12 h light/dark cycle (light on at 06:00); 21 ± 2°C room temperature and free access to food and water.

V.1.12. Centrifuges

Beckman Optima XL-80 Ultracentrifuge with SW32Ti, SW40Ti, SW55Ti and SW80Ti rotors	Beckman Instruments GmbH (Munich, Germany)
Eppendorf Centrifuge 5804 R	Eppendorf AG (Hamburg, Germany)
Eppendorf Centrifuge 5810 R	Eppendorf AG (Hamburg, Germany)
Sorvall RC50*plus* centrifuge with SLA3000, SLA 1500, SA600 and HB-6 rotors	Kendro (Hanau, Germany)

V.2. METHODS

V.2.1. Molecular biological methods

2.1.1. Transformation of bacteria

Competent bacteria were transformed with plasmid DNA according to Sambrook and coworkers (Sambrook et al, 1989). To 100 µl of bacteria 100 ng of plasmid DNA were added and incubated for 30 min on ice. After a heat shock at 42°C for 1 min and subsequent incubation on ice for 3 min, 1 ml of cold LB-medium was added and incubated at 37°C for 40 min. Cells were centrifuged at 10,000 g for 1 min at room temperature and the supernatant removed. Cells were resuspended in 100 µl LB-medium and plated on LB-agar plates containing the appropriate antibiotic. Plates were incubated at 37°C overnight.

2.1.2. Maintenance of bacterial strains

Bacterial strains were maintained according to Sambrook and colleagues (Sambrook et al, 1989). Bacteria were stored as glycerol stocks in LB-medium containing 25 % (v/v) glycerol at -80°C. An aliquot of this stock was streaked on an LB-plate including the appropriate antibiotic and incubated overnight at 37°C. Plates were stored for up to 6 weeks at 4°C.

2.1.3. Plasmid isolation

For preparation of large quantities of DNA, the QIAGEN® Plasmid Maxi Kit (QIAGEN GmbH) was used. To prepare the starter culture a single colony was inoculated in 2 ml LB-medium containing the appropriate antibiotics and grown at 37°C for 8 h with constant agitation. Afterwards, 1 ml of the starter culture was added to 400 ml LB-medium containing antibiotics and the culture was incubated at 37°C with constant agitation overnight for 12-16 h. Cells were harvested in a Sorvall centrifuge at 6,000 g for 15 min at 4°C and DNA was isolated as described in the manufactures protocol. Finally, the DNA pellet was resuspended in 600 µl of warmed (70°C) Tris-HCl (10 mM, pH 8.0) and the DNA concentration was determined.

2.1.4. Restriction digestion of DNA

Restriction enzyme digests were performed by incubating the DNA with an appropiate amount of enzymes, the respective buffer as recommended by the supplier, and at the optimal temperature for the specific enzyme for 2 h. The digest assay was set up as reactions with a total volume of 20 µl. If two enzymes were incompatible with each other, the DNA was

digested successively with the enzymes. The DNA was purified between the two digestions using the MinElute® Reaction Cleanup Kit (QIAGEN GmbH). The restriction was terminated by addition of sample buffer and applied on an agarose gel.

2.1.5. DNA gel electrophoresis

DNA fragments were separated by horizontal electrophoresis in DNA electrophoresis chambers (Bio-Rad Laboratories GmbH) using agarose gels. The percentage of agarose in the gel varied according to the expected size of DNA fragments. Agarose gels were prepared by heating agarose in 1x TAE buffer until agarose was dissolved. After agarose was cooled down to approximately 60°C it was filled into DNA gel trays. The polymerized gel was covered with 1x TAE buffer and the DNA samples mixed with DNA sample buffer were pipetted into the sample pockets. The gel was run at constant voltage at 100 V until the Orange G dye had reached the end of the gel. Subsequently, the gel was stained in an ethidium bromide staining solution for 15-20 min and documented using the E.A.S.Y. UV light documentation system (Herolab GmbH Laborgeräte).

2.1.6. Determination of DNA concentrations

DNA concentrations were determined by spectrophotometric measurements in quartz cuvettes. The absolute volume necessary for measurement was 50 µl. For determining the concentration of DNA preparations, the eluate was diluted appropriately with sterile distilled water. Concentrations were determined by measuring the absorbance at 260 nm (for DNA) and 280 nm (for proteins). Absorbance at 260 nm had to be higher than 0.1 but less than 0.6 for reliable determinations. The concentration of DNA was calculated from the absorbance at 260 nm, given that 50 µg/ml of double stranded DNA has an absorbance of 1 at 260 nm. A ratio of 260 nm and 280 nm in the range of 1.8-2 monitored a sufficient purity of the DNA preparation.

V.2.2. Protein biochemical methods

2.2.1. Preparation of brain homogenate

1- to 3-days-old mice were decapitated and brains were removed from skulls. If not further processed the brains were immediately frozen in liquid nitrogen and stored at -80°C. To obtain uniform homogenates brains were homogenized using a Potter homogenizer with 15 strokes in 2x volume of ice cold homogenization buffer. The buffer was supplemented with a

protease inhibitor cocktail unless indicated otherwise. Homogenates were either used immediately or stored at -80°C.

2.2.2. Determination of protein concentration

The protein concentrations of cell lysates or brain homogenates were determined by using the BCA protein Assay Kit (KMF Laborchemie Handels-GmbH). The reagent was prepared according to manufacturer's instructions. 200 µl of reagent were applied to 10 µl of cell lysate or brain homogenate in a microtiter plate and incubated for 30 min at 37°C. BSA standards ranging from 200 µg/ml to 2 mg/ml were simultaneously incubated. Samples and standards were analyzed in triplicate. Copper ions in the reagent are reduced by proteins in the sample proportional to the amount of protein. The reduced copper ions form a purple colored complex with bicinchoninic acid (BCA). Its absorbance was measured at 562 nm using the µQuant™ microplate spectrophotometer (BioTek Instruments Inc., Winooski, Vermont, USA, KC junior software). Protein concentration was then determined by correlating the relative absorbance to the BSA standards.

2.2.3. SDS-PAGE

Proteins were separated by discontinuous SDS-PAGE using the Mini-PROTEAN® II system (Bio-Rad Laboratories GmbH). Either 1 mm or 1.5 mm thick gels, which were composed of a resolving gel with 8 %, 10 %, 12 % or 16 % acrylamide and a narrow stacking gel with 5 % acrylamide, were used for discontinuous SDS-PAGE. After complete polymerization of the gel, the chamber was assembled as described in the manufacturer's protocol. Samples were mixed with 5x SDS sample buffer followed by boiling at 100°C for 10 min. In a next step, protein marker and samples were loaded. The gel was run in SDS-PAGE running buffer at constant voltage of 60 V with a Bio-Rad PowerPac power supply (Bio-Rad Laboratories GmbH) until the bromphenol blue line had reached the end of the gel. Gels were then subjected to Western blotting.

2.2.4. Electrophoretic transfer of proteins (Western blotting)

Proteins separated by SDS-PAGE were transferred from the gel onto a 0.2 µm Protran® BA83 nitrocellulose membrane (VWR International GmbH) using a Mini Trans-Blot® apparatus (Bio-Rad Laboratories GmbH). The blotting sandwich was assembled according to the manufacturer's protocol. Proteins were electrophoretically transferred in transfer buffer at constant voltage (80 V for 2 h or 35 V overnight at 4°C) using the Bio-Rad PowerPac power

supply (Bio-Rad Laboratories GmbH). The protein standard served as molecular weight marker and was also used for monitoring the efficiency of the protein transfer.

2.2.5. Immunochemical detection of electrophoretically transferred proteins

After electrophoretic transfer membranes were removed from the sandwich, placed with the protein binding side up into glass vessels, washed once in PBST and incubated in blocking buffer for 1 h at room temperature. Afterwards, the primary antibody was added at the desired dilution and incubated for 2 h at room temperature or overnight at 4°C. The primary antibody solution was removed and membrane was washed five times for 6 min with PBST under constant shaking. The appropriate secondary antibody was applied for 1.5 h at room temperature. The membrane was washed again five times for 6 min with PBST. Immunoreactive bands (complexes composed of protein bound to nitrocellulose membrane, primary antibody, and secondary HRP-coupled antibody) were visualized using the ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech Europe GmbH), SuperSignal® West Pico or SuperSignal® West Dura reagents (Pierce, Perbio Science Deutschland GmbH). Membrane was soaked in detection solution (1:1 mixture of solutions I and II) and placed between transparent plastic foils. Afterwards it was exposed to Kodak® BioMax™ light-1 films (Kodak, Sigma-Aldrich Chemie GmbH) in the dark for varying time intervals. Signals on the film were developed and fixed with Kodak® GBX processing chemicals for autoradiography films (Kodak, Sigma-Aldrich Chemie GmbH).

2.2.6. Densitometric evaluation of band intensity

To quantify the signal intensity the developed films were scanned with a resolution of 400 dpi. The digitized pictures were analysed using the image processing software TINA 2.09 (open source, University of Manchester, UK) or Scion Image for Windows (Scion Corporation, Frederick, Maryland, USA). Data were analyzed with the student's two-tailed *t*-test. The results are shown as the mean ± SEM (standard error of the mean).

2.2.7. Stripping and reprobing of Western blots

To detect consecutively proteins on an immunoblot, it was stripped from bound primary and secondary antibodies. Therefore membranes were incubated with stripping buffer for 15 min at room temperature under constant shaking followed by washing twice in PBST. Before incubation with antibodies membranes were blocked.

2.2.8. Co-immunoprecipitation

For co-immunoprecipitation experiments, samples containing 1 mg of total protein were lysed with cold RIPA buffer, containing protease inhibitor cocktail, for 1 h at 4°C. When indicated, 2 mM of EDTA were added to the lysis buffer. Lysates were centrifuged for 15 min at 20,000 g at 4°C. Supernatants were cleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) for 3 h at 4°C to reduce unspecific binding. Afterwards, beads were removed by centrifugation at 600 g for 5 min at 4°C. The supernatant was incubated with the corresponding antibodies or non-specific IgG overnight at 4°C, followed by precipitation with protein A/G-agarose beads for 3 h at 4°C. The beads were pelleted and washed four times with lysis buffer and three times with PBS. The proteins were finally eluted from beads with 5x SDS sample buffer by boiling at 100°C for 10 min. The samples were analysed by Western blotting. Co-immunoprecipitation was performed in cooperation with Dr. Leshchyns'ka.

2.2.9. Subcellular fractionation by differential density gradient centrifugation

2.2.9.a. Isolation of soluble fractions and membrane fractions from total brain homogenates

Whole brains of NCAM wild type and NCAM deficient mice were dissected on ice, frozen in liquid nitrogen and stored at -80°C. All experimental steps were performed at 4°C. Brain homogenates were prepared in homogenization buffer containing protease inhibitor cocktail using a Potter homogenizer. The brain homogenates were centrifuged at 1,400 g for 10 min to spin down mitochondria and nuclei. Pellets and supernatants were carefully separated. Thereafter, the supernatants were centrifuged at 100,000 g for 30 min. The finally obtained supernatants were enriched in cytosolic proteins and termed soluble fractions. The pellets were resuspended in a minimal volume of TBS buffer and used as total membrane fractions. Protease inhibitor cocktail was added to all fractions and the total protein content was estimated (see 2.2.2). Samples for SDS-PAGE were prepared (see 2.2.3) and membrane fractions were further processed to isolate lipid raft fractions (see 2.2.9.b).

2.2.9.b. Isolation of lipid raft fractions from total brain homogenates

Lipid raft fractions were isolated as described (Leshchyns'ka et al, 2003). Lipid rafts were obtained from the membrane fractions of NCAM wild type and NCAM deficient mice brains. All steps were performed at 4°C. Protein concentrations of wild type and NCAM deficient membrane fractions were estimated and equal protein amounts of both fractions were used as starting material. Maximum 500 µl of membrane fractions were mixed with the 4x volume of

ice-cold 1 % Triton® X-100 in TBS in polyallomer tubes (14x95 mm, V=14 ml; Beckman Instruments GmbH) for the SW40Ti Beckman rotor and incubated for 20 min. During this incubation time it is crucial to maintain the material at 4°C. The extracted membranes were mixed with an equal volume of 80 % sucrose in 0.2 M Na_2CO_3 to a final sucrose concentration of 40 %. To create discontinuous gradients the material was overlaid with 2 ml 30 % sucrose in TBS and 1 ml 10 % sucrose in TBS. Tubes were filled up with TBS buffer and centrifuged at 230,000 g for 17 h. After centrifugation the lipid raft fractions were collected at the top of the gradient at 10 % sucrose, resuspended in TBS buffer and pelleted down by centrifugation at 100,000 g for 1 h. The pellets were resuspended in minimum volume of TBS which contained protease inhibitor cocktail. The total protein content was estimated (see 2.2.2) and samples for SDS-PAGE were prepared (see 2.2.3).

2.2.9.c. Isolation of growth cone fractions from total brain homogenates

Isolation of growth cone fractions was performed accoding to Pfenninger and coworkers (Pfenninger et al, 1983). Growth cones were isolated from 3 brains of 1- to 3-days-old mice. All experimental steps were performed at 4°C. Mice brains were dissected on ice and homogenized with few strokes in 10 ml homogenization buffer for growth cone isolation containing protease inhibitor cocktail. The homogenates were centrifuged at 1,660 g for 15 min. The low speed supernatants were carefully tipped off and used further, whereas the low speed pellets, containing nuclei and cell perikarya whose neurites have been sheared off, were discarded. The discontinuous sucrose density gradients were prepared in thinwall polyallomer tubes (13x51 mm, V=5 ml; Beckman Instruments GmbH) for the SW55Ti Beckman rotor. The supernatants were centrifuged on a gradient of 0.75/1.0/2.66 M at 242,000 g for 30 min. The interface between the load and 0.75 M sucrose consisted of growth cone particles. This layer was collected, resuspended in homogenization buffer and centrifuged at 100,000 g for 40 min to pellet down the growth cone fraction. The pellet was resuspended in homogenization buffer containing protease inhibitor cocktail.

2.2.9.d. Analysis of cytoskeleton cleavage in response to NCAM clustering in isolated growth cones

Isolation procedure of growth cone fractions used for stimulation experiments required few modifications. Since the protease inhibitor cocktail added to the homogenization buffer contains aspartate and cysteine inhibitors which interfere with caspase activity it was replaced by the serine protease inhibitors aprotinin and leupeptin. Mice brains were homogenized in homogenization buffer containing 0.027 TIU/ml aprotinin and 0.2 mM leupeptin. The growth

cone fraction was isolated as described in chapter 2.2.9.c. All experimental steps were performed at 4°C if not indicated otherwise. The isolated growth cone fraction was carefully mixed with 2x dilution buffer. To 1.3 ml of growth cone fraction 0.5 ml of 2x dilution buffer were added (Pfenninger et al, 2003). After 20 min of incubation 0.8 ml of 2x dilution buffer were added. After an additional incubation time of 20 min, the fractions were added to assay tubes and preincubated with either 1 mM of caspase-8 inhibitor, caspase-3 inhibitor or with the corresponding vehicle for 2 h on ice. To induce clustering of NCAM at the growth cone surface, chicken or rabbit polyclonal antibodies against NCAM or preimmune serum (Pineda) were added to the samples and samples were equilibrated for 15 min. Samples were then warmed up in a water bath to 37°C for 5 min, and subsequently chilled in ice slurry for 5 min. Samples were resuspended in 60 µl 5x SDS sample buffer and boiled at 100°C for 10 min. Afterwards, the samples were subjected to SDS-PAGE (2.2.3).

V.2.3. Cell culture of CHO cells

2.3.1. Maintenance of CHO cells

CHO cells were either grown in 75 cm^2 flasks (Greiner Bio-One GmbH) with 20 ml CHO cell growth medium or in six-well plates (d = 35 mm; area = 9.69 cm^2, Greiner Bio-One GmbH) with 3 ml medium under constant conditions at 37°C, 5 % CO_2 and 90 % relative humidity. Cells were passaged as they reached confluence after 3 to 4 days. The medium was removed and the cell layer was washed once with HBSS and detached by trypsin/EDTA treatment for 1-3 min at 37°C. Detached cells were resuspended in fresh medium. For maintenance cells were split 1:10 in fresh medium and seeded in new flasks or six-well plates.

In the presence of 10 % DMSO cells were cryoconserved and stored at -80°C. Therefore, the cell layer was washed with HBSS, trypsinized and collected in CHO cell growth medium. The cell pellet, obtained by centrifugation at 700 g for 10 min at room temperature, was resuspended in CHO cell freezing medium containing 15 % FCS and 10 % DMSO and transferred into cryotubes (Biochrom AG). The cryotubes were frozen in a freezing container (Nunc GmbH & Co. KG) filled with 100 % isopropyl alcohol. Isopropyl alcohol ensures a continuous cooling to -80°C with a cooling rate of 1°C per minute. After 24 h the cryotubes were removed from the freezing container and stored at -80°C. To recultivate cryoconserved cells thawed cell solutions were immediately transferred into flasks or plates with prewarmed medium. When cells were adherent medium was exchanged.

2.3.2. Transient transfection of CHO cells

CHO cells were transfected using the Lipofectamine™ Transfection Reagent combined with Plus™ Reagent (Life Technologies, Invitrogen GmbH). Transfection was performed according to the manufacturer's protocol. One day prior transfection cells were seeded in six-well plates. When cell density had reached 80-90 %, the cells were washed with HBSS and serum-free CHO cell growth medium was added to prevent DNA precipitation. Cells were transfected with 1.5 µg total DNA per well. 6 µl Plus™ Reagent per well were used to pre-complex plasmid DNA and 4 µl Lipofectamine™ per well in order to form a lipid-DNA complex. Finally, the six-well plate was centrifuged at 1,000 rpm for 5 min at room temperature. The transfection was completed after 3 h by addition of an equal volume of CHO cell growth medium which contained serum. The medium was replaced after 24 h and 48 h after transfection cells were used for biochemical analysis.

2.3.3. Stable transfection of CHO cells

Transfection was carried out as described in section 2.3.2. CHO cells were stably transfected with a construct containing NCAM140 full length cDNA in a pcDNA3 vector. The vector encodes a neomycin resistance as selection marker for stably transfected cells. 24 h after transfection cells were split into CHO cell growth medium containing 1 mg/ml Geneticin® Selective Antibiotic (Invitrogen GmbH) such that cells were no more than 25 % confluent. The medium was exchanged every second to third day to remove dead cells and to add fresh antibiotic. After two weeks of selection single colonies were grown. For isolation cells were picked using sterile pipette tips. The cell clones were passaged four times using always the same procedure, and afterwards maintained in CHO cell growth medium containing Geneticin®. Aliquots of cells were frozen at several steps (see 2.3.1), after transfection and after isolation of individual clones. The stably transfected cells were maintained in culture for maximal fifth passages. Afterwards fresh aliquots were thawed from the stock.

2.3.4. Stimulation of CHO cells

Transfected cells cultured in six-well plates were stimulated with rabbit polyclonal antibodies against NCAM (10 µg/ml) or non-specific rabbit IgG (10 µg/ml) which were applied into culture medium for 1 min, 5 min, 10 min or 30 min at 37°C in a CO_2 incubator. After stimulation medium was removed immediately and cells were lysed with cold RIPA buffer as described in section 2.3.5.

2.3.5. Lysis of CHO cells

Before lysis cells were washed once with cold HBSS. Then cells were lysed in 150 µl RIPA buffer (+ 1 % NP-40) containing protease inhibitor cocktail for 30-40 min with constant agitation at 4°C. Subsequently cells were scraped off the wells. Cell lysates were centrifuged at 19,000 g for 15 min at 4°C to remove cell debris. The supernatant contained the solubilized proteins. The total protein content was estimated (2.2.2) and samples for SDS-PAGE prepared (2.2.3). Lysates were stored at -20°C if not further processed.

V.2.4. Cell culture of primary neurons

The experiments with primary cultures of hippocampal neurons were performed in cooperation with Dr. Leshchyns'ka and Dr. Sytnyk.

2.4.1. Coating of coverslips

Primary hippocampal neurons were grown on glass coverslips (15 mm, Carl Roth GmbH & Co. KG) coated with poly-D-lysine (PDL). The coating procedure required several incubation and washing steps which were performed with gentle shaking in a glass Erlenmeyer flask in solutions with a volume of 100 ml each. Coverslips were incubated for 30 min with 3 M HCl at room temperature. Afterwards they were washed twice for 10 min with sterile distilled water and incubated overnight with acetone at 4°C. This was followed by five washing steps for 10 min with sterile distilled water and two times for 10 min with absolute ethanol. To sterilize coverslips they were heated for 2 h at 200°C. After cooling down to room temperature coverslips were incubated overnight in sterile 0.01 % PDL in PBS at 4°C and afterwards washed three times with distilled water. Thereafter, coverslips were dried uncovered in the sterile laminar flow hood by exposing them to UV light for 30 min. The coated coverslips were stored in a sterile tube at room temperature until use.

2.4.2. Preparation and cultivation of hippocampal neurons

Cultures of hippocampal neurons were prepared from 1- to 3-days-old mice. Mice were decapitated and brains were removed from the skull. Hippocampi were extracted, placed in cold HBSS and cut into 1 mm thick pieces. Hippocampi were washed once with dissection solution and treated with the digestion solution containing papain and DNase I for 5 min at room temperature. The digestion solution was removed and hippocampi suspended in dissection solution. Hippocampi were carefully dispersed into a homogeneous suspension by trituration with glass Pasteur pipettes having successively smaller diameters. The suspension

was centrifuged at 80 g for 10 min at 4°C. The pelleted cells were resuspended in warmed culture medium. Cell number was estimated using a Neubauer counting cell chamber (Carl Roth GmbH & Co. KG). The cells were seeded on coverslips coated with 0.01 % PDL (see 2.4.1) and incubated at 37°C in a constant CO_2 atmosphere of 5 % and with 90 % relative humidity.

2.4.3. Clustering of NCAM at the neuronal cell surface

To cluster NCAM or PrP molecules at the cell surface of live neurons, rat monoclonal NCAM antibody (H28), goat polyclonal PrP antibody (M-20) or non-immune rat IgG (10 µg/ml) were applied to live neurons for 10 min and clustered by secondary antibodies applied for 5 min, followed by washing for 5 min, all in a CO_2 incubator.

2.4.4. Immunofluorescence labeling

Indirect immunofluorescence labeling was performed as described previously (Sytnyk et al, 2002). All steps were performed at room temperature. Neurons were fixed in 4 % PFA in PBS for 15 min to crosslink and preserve proteins in their native conformation. This fixation procedure does not permeabilize membranes (Sytnyk et al, 2002). Subsequently cells were washed and blocked in 1 % BSA in PBS for 20 min. Antibodies against NCAM and PrP were applied in 1 % BSA in PBS to fixed but non-permeabilized cells for 30 min and detected with fluorochrome coupled secondary antibodies applied for 30 min. To detect the intracellular proteins caspase-3, caspase-8 and tubulin, cells were treated with detergent to permeabilize the cell membrane. The neurons were postfixed for 5 min in 2 % PFA in PBS, washed with PBS, permeabilized with 0.25 % Triton® X-100 in PBS for 5 min, blocked with 1 % BSA in PBS for 20 min. Antibodies were applied in 1 % BSA in PBS for 2 h and then detected with corresponding secondary antibodies applied for 45 min.

2.4.5. Image acquisition and manipulation

Coverslips were embedded in Aqua-Poly/Mount (Polysciences Europe GmbH) and fixed on the microscope slide over night at room temperature. Immunofluorescence images were acquired at room temperature using a confocal laser scanning microscope LSM510, LSM510 software (version 3) and oil Plan-Neofluar 40x objective (numerical aperture 1.3) at 3x digital zoom (Zeiss, Jena, Germany). Contrast and brightness of the images were further adjusted in Corel Photo-Paint 9 (Corel Corporation, Ottawa, Ontario, Canada).

2.4.6. Immunofluorescence quantification

To quantify levels of active caspase-3, neurons were manually outlined in the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and mean immunofluorescence intensities were measured within these outlines using ImageJ. To analyze correlation coefficients, profiles of immunofluorescence intensity along neurites were measured using ImageJ and correlation coefficients between thus obtained immunofluorescence intensity distributions were calculated in Excel (Microsoft Corporation).

2.4.7. Quantification of neurite length (neurite outgrowth assay)

Neurons seeded on coverslips coated with PDL were incubated for 1 h before further components were added. When indicated 1 mM caspase inhibitors were applied to the culture medium 1 h before stimulation with either 8 µg/ml NCAM-Fc or 8 µg/ml human Fc for 24 h. After incubation cells grown on the coverslips were treated with 4 % PFA in PBS for 30 min at room temperature and washed twice with PBS. Coverslips were fixed on the microscope slide over night at room temperature. The cells were imaged using Axiophot 2 microscope equipped with Plan-Neofluar 40x objective (numerical aperture 0.75), AxioCam HRc digital camera and AxioVision software version 3.1 (Zeiss, Jena, Germany). Neurite length was measured with the image processing software ImageJ version 1.34s (open source, National Institutes of Health, USA). For each experimental value at least 250 neurons with neurites longer than the cell body diameter were measured. Results were statistically evaluated with *t*-test (two-tailed). Results are presented as mean ± SEM.

VI. RESULTS

VI.1. NCAM STIMULATION INDUCES THE ACTIVATION OF CASPASE-3

We started our work by analyzing levels of apoptotic markers in NCAM deficient and wild type mouse brains. We decided to compare the levels of caspase-3 in both genotypes because caspase-3 constitutes the major executioner of apoptosis, whereas caspase-6 and caspase-7 play only minor roles during the destruction phase of apoptosis (Creagh & Martin, 2001; Slee et al, 2001; Walsh et al, 2008). Therefore, we performed Western blot analysis of brain homogenates with caspase-3 antibodies recognizing both inactive procaspase-3 and active caspase-3 (Fig. 6). In our Western blots procaspase-3 appears as double band with molecular weights of approximately 33 kDa and 31 kDa (see Fig. 6). This double band was previously described, though it is not known whether it represents different isoforms (Altznauer et al, 2004; Blomgren et al, 2001). The procaspase-3 constitutes a heterotetramer containing two large and two small catalytic subunits as depicted in Fig. 5. This inactive procaspase is cleaved by initiator caspases between the large (approx. 20 kDa) and small (approx. 12 kDa) catalytic subunits which leads to the activation of caspase-3. During sample preparation the active heterotetramer is destroyed and the catalytic subunits can be detected by means of Western blot analysis.

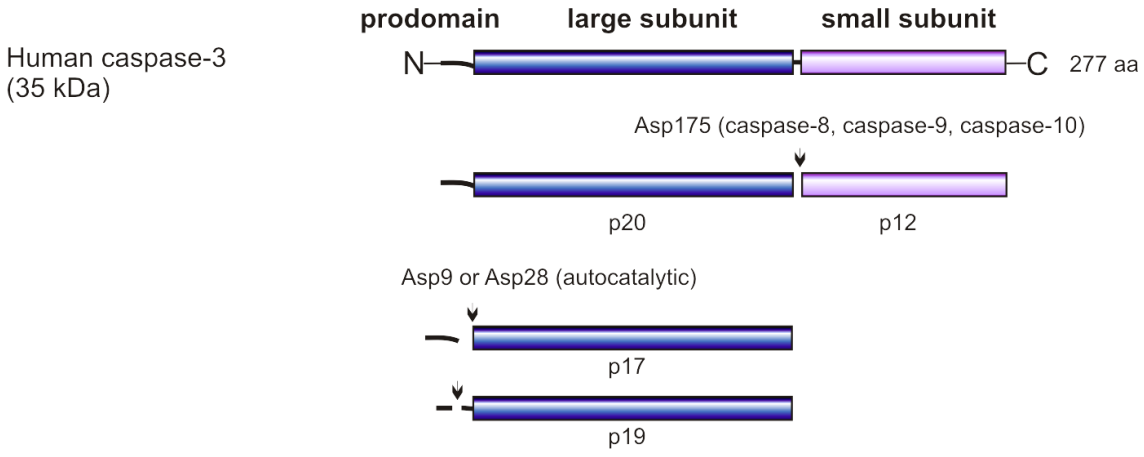

Fig. 5: Cleavage sites in effector caspase-3. Illustration shows the human effector procaspase-3 (monomer) and cleavage sites within the procaspase. Caspase 3 is processed and activated by initiator caspases such as caspases-8, -9 and -10 within the large and small subunit (Fernandes-Alnemri et al., 1996; Li et al., 1997; Stennicke et al., 1998). The prodomain of caspase-3 can be removed autocatalytically in a subsequent event (Fernandes-Alnemri et al., 1996; Martin et al., 1996).

Our Western blot analysis revealed that the levels of procaspase-3 were increased by approximately 20 % in NCAM deficient brain homogenates when compared to wild type brain homogenates (Fig. 6). At longer exposure time, the caspase-3 immunoreactive band at 12 kDa area, which represents active caspase-3, was also detectable. Surprisingly, the amount of active caspase-3 was approximately three times lower in NCAM deficient brain homogenates (4.4 ± 1.1 %) when compared to wild type brain homogenates (11.2 ± 2.7 %).

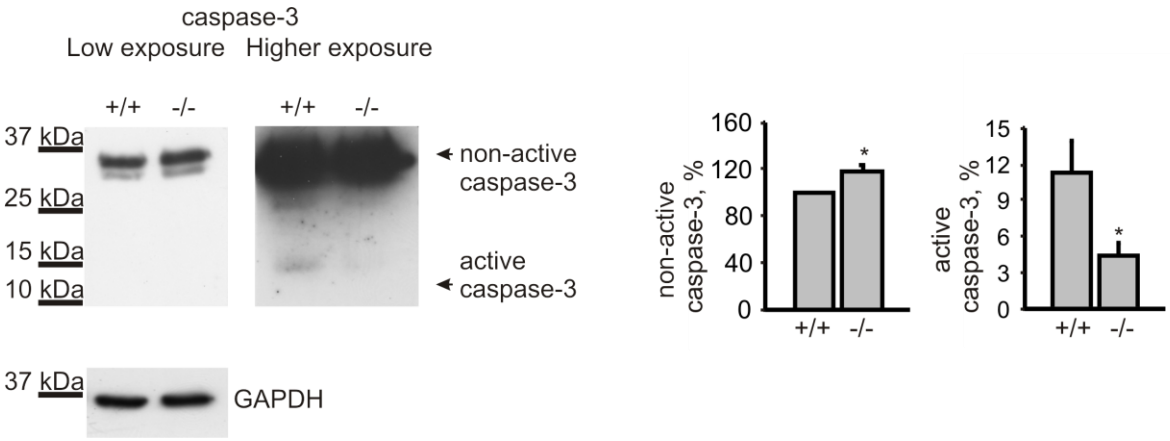

Fig. 6: Activation of effector caspase-3 is impaired in NCAM deficient brains. Wild type (+/+) and NCAM deficient (-/-) brain homogenates were probed by Western blot with antibodies against caspase-3. Arrows indicate procaspase and cleaved activated forms of caspase-3. The level of cleaved activated caspase-3 were reduced in NCAM deficient brain homogenates. GAPDH served as a loading control. Graphs show quantitation of blots (mean ± SEM, n = 6). The sum of optical densities of the bands in NCAM deficient samples which represented the total protein level was set to 100 %. *, $P < 0.05$, paired *t*-test.

The decrease in the level of active caspase-3 was probably the consequence of a diminished processing of the procaspase in mice brains deficient in NCAM. The reason for such a reduced processing and activation of caspase-3 could be lower expression levels of caspase-3 activators, i.e. caspases-8, -9 and -10, which cleave procaspase-3 (see Fig. 5) thereby producing active caspase-3 (Fernandes-Alnemri et al, 1996; Muzio et al, 1997; Srinivasula et al, 1998; Stennicke et al, 1998). Hence, we analyzed in addition the levels of initiator caspases in wild type and NCAM deficient brain homogenates. Similar to caspase-3, cleaved forms of initiator caspases represent active caspases although initiator caspases are mostly cleaved in an autocatalytic process as illustrated in Fig. 7.

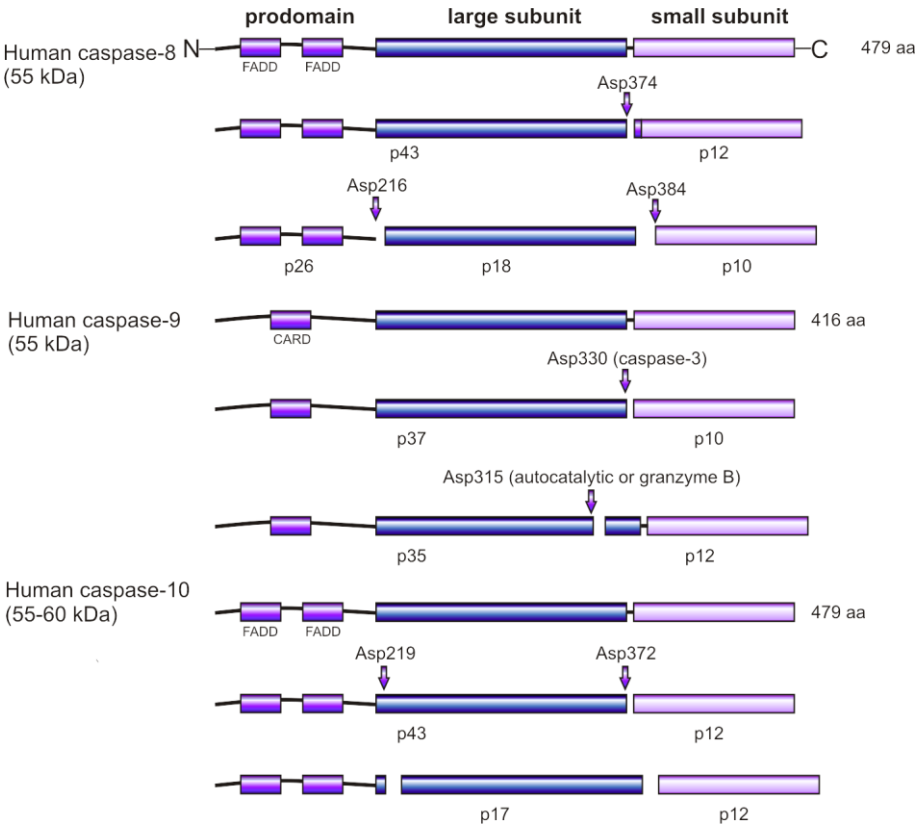

Fig. 7: Cleavage sites in initiator caspases. Illustration shows cleavage sites within human initiator caspases. Initiator caspases are cleaved either autoproteolytically or in a feed-back loop by the effector caspase-3 (Martin et al, 1998; Srinivasula et al, 1998; Yang et al, 2006).

While our Western blot analysis did not reveal any statistically significant differences in the levels of procaspase-8, procaspase-9 and procaspase-10 in wild type and NCAM deficient brain homogenates, the levels of cleaved capase-8 were reduced in NCAM deficient brain homogenates (Fig. 8). This indicates a lower activation of caspase-8, similar to caspase-3, when NCAM is absent. In conclusion, these findings suggest that NCAM is involved in the regulation of caspase-3 activation via the initiator caspase-8.

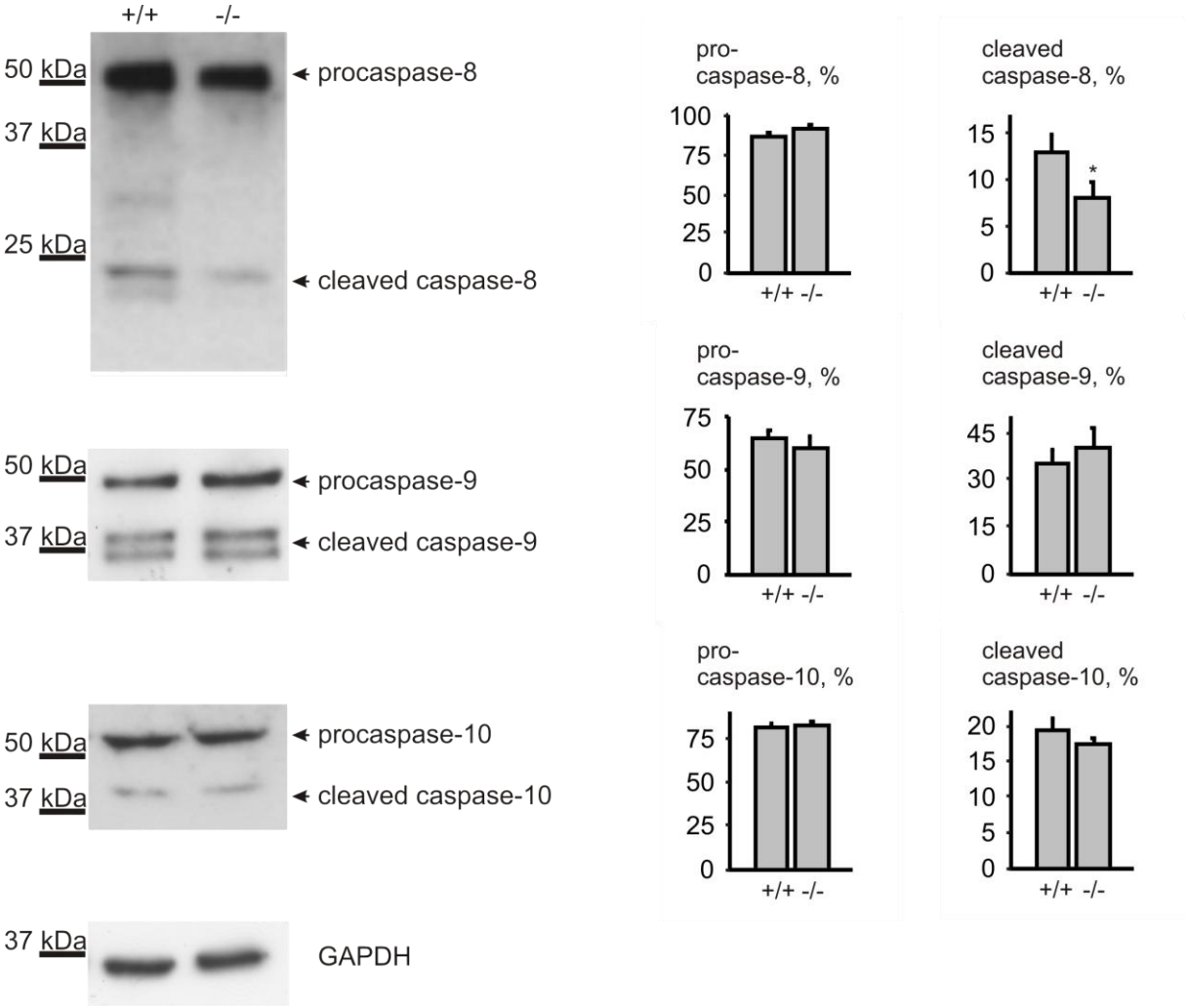

Fig. 8: Activation of caspase-8 is impaired in NCAM deficient brains. Wild type (+/+) and NCAM deficient (-/-) brain homogenates were probed by Western blot with antibodies against caspases-8, -9 and -10. Arrows indicate procaspases and cleaved forms of caspases. Level of activated cleaved caspase-8 was reduced in NCAM deficient brain homogenate. Note similar levels of caspase-9 and caspase-10 in wild type and NCAM deficient brain homogenates. GAPDH served as loading control. Graphs show quantitation of blots (mean ± SEM, n = 6). The sum of optical densities of the bands in wild type samples which represented the total protein level was set to 100 %. *, $P < 0.05$, paired *t*-test.

VI.2. NCAM ASSOCIATES WITH CASPASE-8

To explore possible mechanisms of NCAM dependent caspase-8 activation, we analyzed whether NCAM associates with caspase-8 (Fig. 9). We immunoprecipitated NCAM from wild type brain homogenates with NCAM antibodies that bind NCAM in the extracellular domain. Thereby, the intracellular domains of NCAM140 and NCAM180 remain accessible to interact with intracellular proteins. Afterwards, we analysed the immunoprecipitates for the presence of caspase-8 by Western blot analysis. Interestingly, only cleaved caspase-8 but not procaspase-8 was detected in the NCAM immunoprecipitates.

Calcium is involved in the regulation of many protein interactions. Moreover, it is an important second messenger which is involved in the NCAM mediated signaling (Doherty et al, 1991; Kiryushko et al, 2006; Williams et al, 1994b). Therefore, we analyzed whether the association between NCAM and caspase-8 is calcium dependent. Addition of EDTA, a calcium sequestering agent, reduced the amount of caspase-8 in NCAM containing complexes suggesting that this association can be in fact regulated by calcium (Fig. 9). However, the co-immunoprecipiation assay confirms an association between NCAM and caspase-8 but does not indicate whether the binding between both proteins occurs in a direct manner.

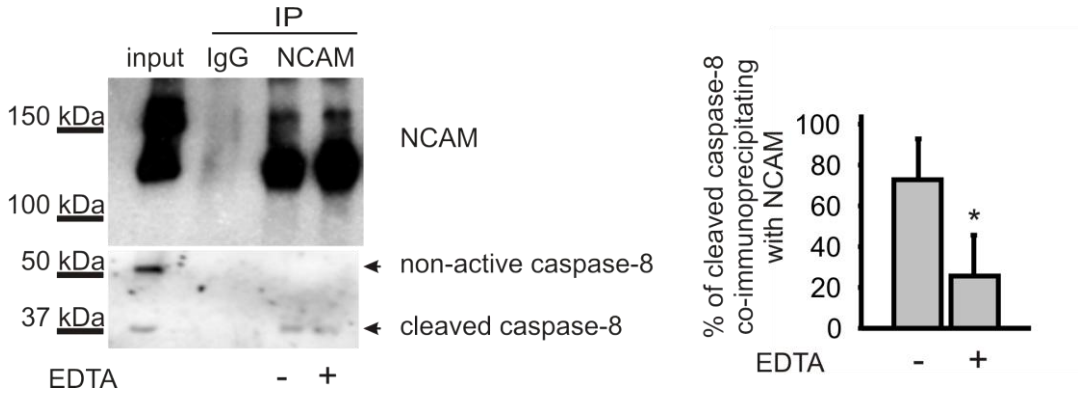

Fig. 9: NCAM forms a complex with caspase-8 in brain tissue. NCAM immunoprecipitates from wild type brain lysates and input were probed by Western blot with the indicated antibodies. Cleaved caspase-8 co-immunoprecipitated with NCAM. Co-immunoprecipitation of caspase-8 with NCAM was reduced in the presence of EDTA. Note that similar levels of NCAM were immunoprecipitated in all groups. Diagrams show levels of cleaved caspase-8 that co-immunoprecipitated with NCAM (mean ± SEM, n = 3) normalized to the total levels of cleaved caspase-8 in the input material. *, $P < 0.05$, paired *t*-test. The experiments were performed in cooperation with Dr. Leshchyns'ka.

We established in the preceding experiment that NCAM and caspase-8 associate with each other by performing a co-immunoprecipitation assay using brain lysate. Thus, in the next step we were interested to investigate whether both molecules are indeed co-localized in live

neurons and, moreover, whether NCAM association with caspase-8 depends on NCAM clustering at the cell surface. Therefore, we cultured hippocampal neurons from 1- to 3-days-old mice for 24 h on substrate coated poly-D-lysine. The cells were treated for 10 min with non-specific immunoglobulins or with NCAM antibodies to cluster NCAM at the cell surface (Fig. 10). Thereafter, we used indirect immunofluorescence labeling to analyze distributions of NCAM and caspase-8 in the hippocampal neurons. In control neurons NCAM and caspase-8 showed diffuse and partially overlapping distributions. Clustering of NCAM with NCAM antibodies induced a pronounced aggregation of caspase-8 in NCAM clusters. Furthermore, the overall overlap between distributions of NCAM and caspase-8 was significantly enhanced after NCAM clustering as shown by the strongly increased correlation coefficient between distributions of these proteins. Neurons treated with non-specific immunoglobulins displayed an overlap of approximately 38 % between distributions of NCAM and caspase-8, whereas cells stimulated with NCAM antibodies showed an overlap of approximately 74 %. In contrast, tubulin labeling showed a diffuse distribution in control neurons and in neurons stimulated with NCAM antibodies. Our combined observations suggest that NCAM associates with caspase-8 at the neuronal surface membrane. Furthermore, clustering of NCAM induces the aggregation of NCAM associated caspase-8. Interestingly, oligomerization of initiator caspases results in their activation (Boatright & Salvesen, 2003; Muzio et al, 1998; Salvesen & Dixit, 1999). Therefore, it was also of interest for us to investigate whether NCAM clustering results in fact in the activation of caspase-8 (see VI.4).

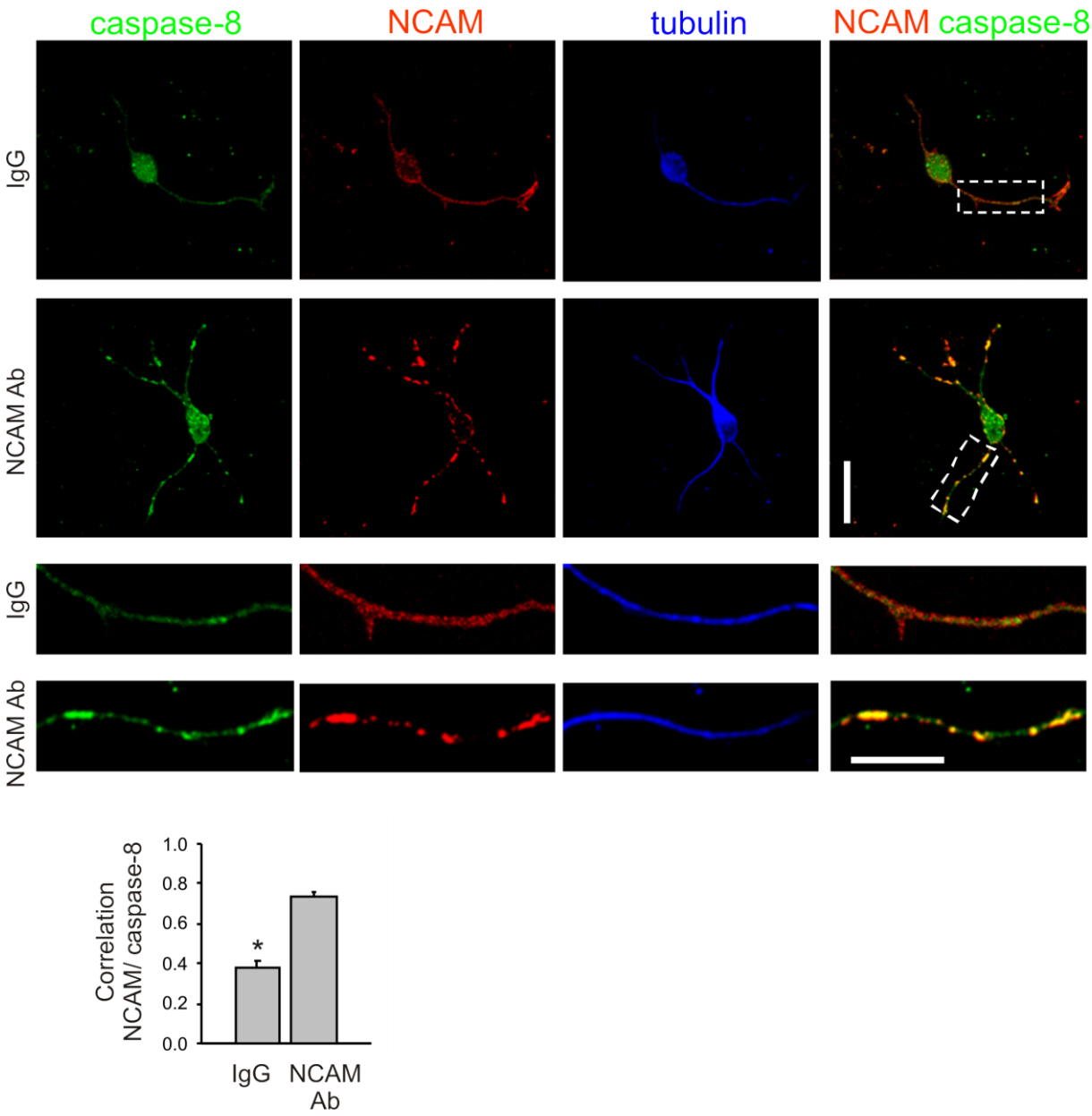

Fig. 10: Caspase-8 associates with NCAM. NCAM was clustered at the cell surface of 1-day-old cultured hippocampal neurons by NCAM antibodies. Alternatively, neurons were stimulated with non-specific immunoglobulins (IgG). Neurons were then fixed and co-labeled with antibodies against caspase-8 and tubulin. Images of the representative neurons (upper panels) and high magnification images of neurites outlined by dashed lines (lower panels) are shown. NCAM clustering induced aggregation of caspase-8 in NCAM clusters and enhanced overlap between distributions of NCAM and caspase-8. Bar = 10 µm. Graph shows coefficients of correlation between distributions of NCAM and caspase-8 (mean ± SEM, n > 30 neurites analyzed in each group). *, $P < 0.05$, *t*-test. The experiments were performed in cooperation with Dr. Leshchyns'ka and Dr. Sytnyk.

VI.3. NCAM ENHANCES ASSOCIATION OF CASPASES-8 AND -3 WITH LIPID RAFTS

Clustering of NCAM enhances its association with lipid rafts and can direct NCAM associated proteins to the plasma membrane and to lipid rafts (Bodrikov et al, 2005; Leshchyns'ka et al, 2003; Niethammer et al, 2002). To analyze whether NCAM deficiency affects the subcellular distribution of caspases we estimated levels of caspases-3, -8, -9 and -10 in soluble cytosolic, total membrane and lipid raft fractions isolated from wild type and NCAM deficient brain homogenates. To isolate lipid raft fractions we used a biochemical method that is based on the observation that lipid rafts are resistent to cold non-ionic detergent extraction, e.g. with Triton® X-100 (Brown & Rose, 1992). Whereas non-raft fractions are solubilized, lipid raft fractions resist to this treatment. After extraction these detergent resistant membranes (DRMs) can be isolated by differential centrifugation. Due to their enrichment in sterols and sphingolipids DRMs float to the top of a buoyant density gradient (Brown, 1994).

In our experiments we used brain homogenates from wild type and NCAM deficient mice to isolate soluble cytosolic fractions and total membrane fractions. Total membrane fractions we used afterwards to isolate detergent resistant microdomains by treatment with 1 % ice-cold Triton® X-100 and subsequent sucrose gradient centrifugation at 4°C. Afterwards, we compared caspase levels in all fractions obtained from brain homogenate of wild type and NCAM deficient mice (Fig. 11A). The fractions were additionally analyzed for lipid raft markers, defined by their enrichment in DRMs, such as the GPI anchored protein contactin (Funatsu et al, 1999; Olive et al, 1995). The cytosolic protein GAPDH was enriched in the soluble fractions and absent of the lipid raft fractions, whereas the GPI anchored protein contactin accumulates in lipid raft fractions. Thus, we concluded that the isolated lipid raft fractions were free of cytosolic contamination. Furthermore, Western blot analysis showed that all analyzed caspases were present at high levels in the cytosolic fractions (Fig. 11A). Caspase-8 and caspase-3 were also enriched in the total membrane fractions while caspase-9 and caspase-10 showed lower preference for these fractions.

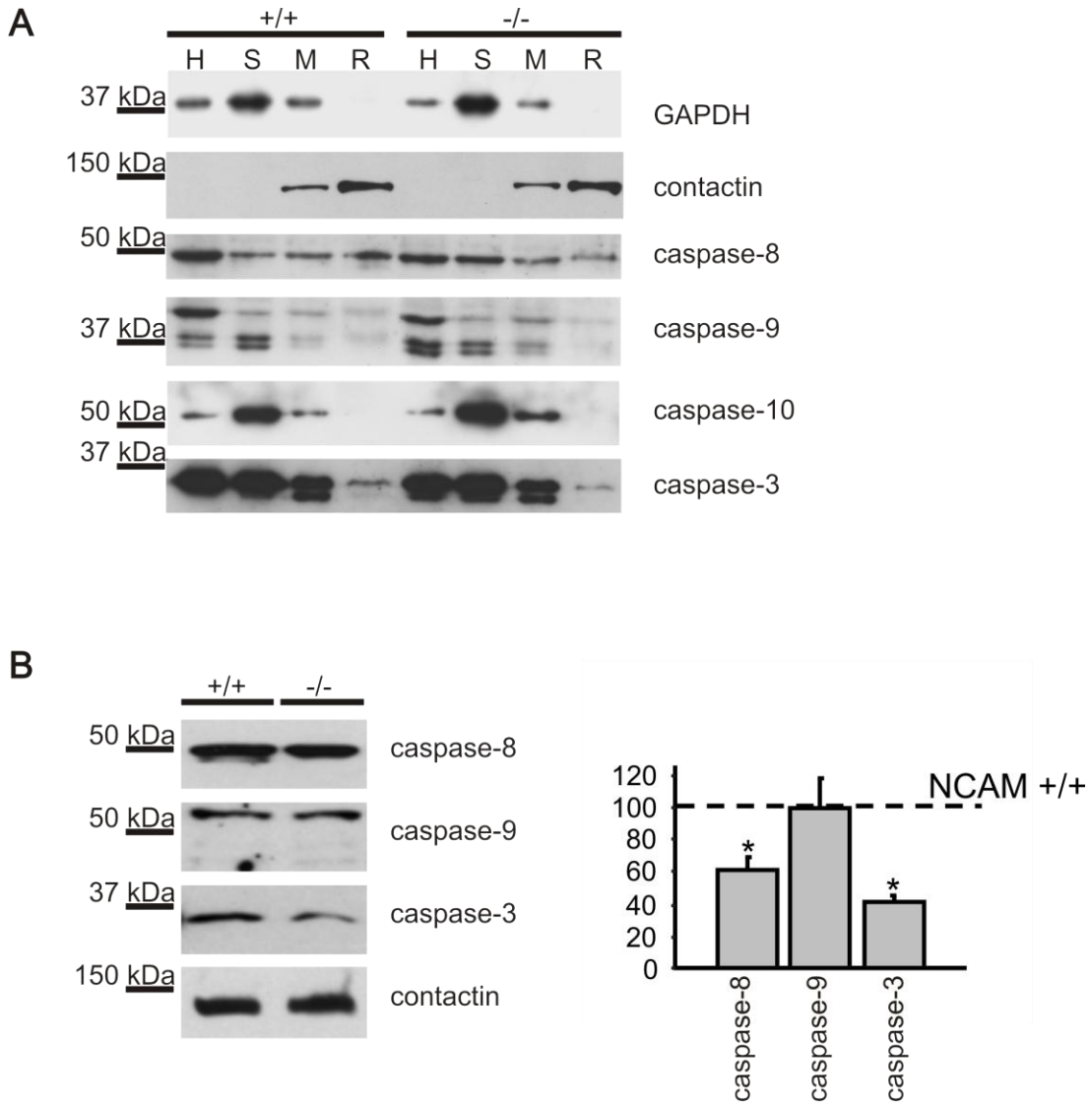

Fig. 11: Levels of caspase-3 and caspase-8 are reduced in NCAM deficient lipid rafts. (A) Brain homogenates (BH), soluble fractions (S), membrane fractions (M) and lipid raft fractions (R) from wild type (+/+) and NCAM deficient (-/-) brains were probed by Western blot with antibodies against caspases-3, -8, caspase-9 and caspase-10. Labeling for cytosol enriched GAPDH and lipid raft marker contactin was performed to check the purity of the isolated fractions. Levels of caspases-3 and -8 were reduced in NCAM deficient lipid rafts when compared to wild type lipid rafts, whereas levels of caspase-9 were similar in both genotypes. Caspase-10 was not detected in lipid rafts. (B) Wild type and NCAM deficient lipid rafts were probed by Western blot with antibodies against caspases-3, -8, and -9. Contactin served as loading control. Graph shows quantitation of blots (mean ± SEM, n = 6). Caspase levels in wild type lipid rafts were set to 100 %. *, $P < 0.05$, paired *t*-test (compared to wild type samples).

Among all caspases, caspase-8 showed the highest association with lipid rafts while only low levels of caspases-3 and -9 were present in lipid rafts. Moreover, caspase-10 was undetectable in lipid raft fractions (Fig. 11A). Interestingly, the levels of caspase-3 and caspase-8 but not levels of caspase-9 were reduced by approximately 50 % in the lipid raft fractions from

NCAM deficient brains when compared to wild type brains (Fig. 11B). The lower level of caspase-8 in the NCAM deficient lipid raft fraction might explain its increase of approximately 50 % in the soluble fraction (Fig. 11A). However, such an effect was not detected for caspase-3. But this might be due to a general lower amount of caspase-3 in lipid rafts and due to minor differences of caspase-3 in lipid raft fractions of wild type and NCAM deficient mice. Thus, it is likely that binding to NCAM enhances the association of caspase-3 and caspase-8 with lipid rafts.

Application of NCAM antibodies induces clustering of NCAM at the cell surface and enhances its association with lipid rafts (Leshchyns'ka et al, 2003). The intracellular domains of NCAM140 and NCAM180 can be palmitoylated at four cysteine residues adjacent to the transmembrane domain (Little et al, 1998; Ponimaskin et al, 2008). This modification increases the hydrophobicity of both transmembrane proteins and targets them to lipid rafts. Upon stimulation NCAM was shown to be localized to PrP containing lipid rafts (Santuccione et al, 2005). The cellular prion protein PrP stabilizes NCAM in lipid rafts, thereby promoting NCAM dependent neurite outgrowth. Thus, we investigated whether NCAM clustering with antibodies leads to a redistribution of caspase-3 into lipid rafts (Fig. 12). We cultured hippocampal neurons of 1- to 3-days-old wild type mice on substrate coated poly-D-lysine for 24 h (Fig. 12). NCAM and PrP, which we considered as marker of NCAM containing lipid rafts, were co-clustered at the cell surface of cultured hippocampal neurons by incubating live neurons for 10 min with NCAM and PrP antibodies. Afterwards, we visualized both proteins at the cell surface with fluorochrome coupled secondary antibodies. Antibodies against caspase-3 which recognize its full length and active form were applied after fixation and permeabilization of cells. As seen in Fig. 12 NCAM labeling revealed a patchy distribution of NCAM and PrP along neurites. Moreover, these PrP containing NCAM clusters co-localized with caspase-3 accumulations along neurites as indicated by arrows in Fig. 12. In summary, these data show that caspase-3 is present to some extent in NCAM containing lipid rafts.

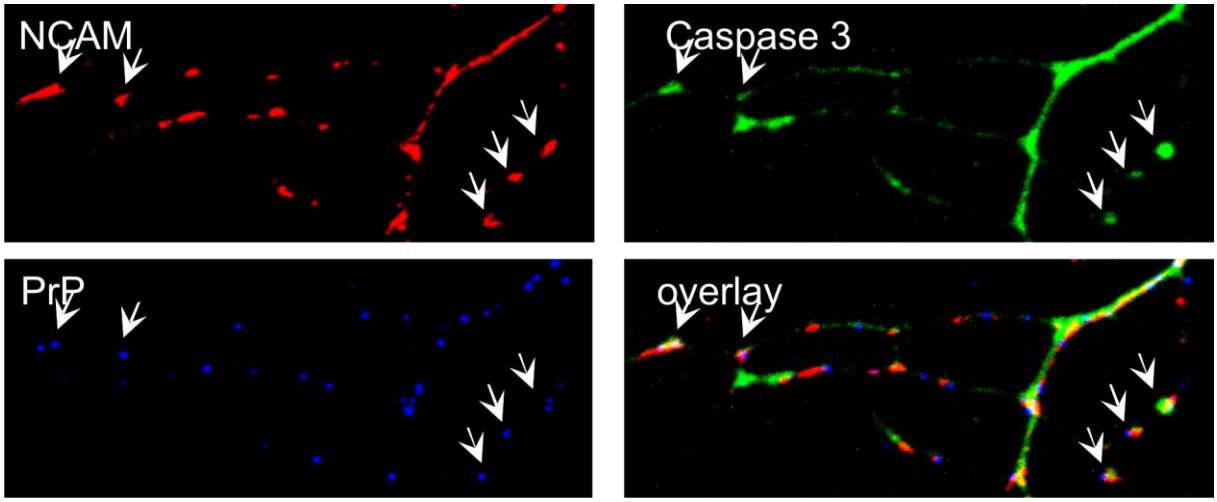

Fig. 12: The complex of NCAM and caspase-3 redistributes into lipid rafts upon clustering of NCAM and PrP. 1-day-old wild type cultured hippocampal neurons were incubated live with NCAM and PrP antibodies to cluster NCAM and PrP. Neurons were then fixed and co-labeled with caspase-3 antibodies. The caspase-3 specific antibodies recognize the full length (inactive) as well as the cleaved (active) forms of caspase-3. Labeling with PrP antibodies served as marker for PrP containing lipid rafts. Upon antibody clustering of NCAM and PrP, caspase-3 partially co-localized with NCAM/PrP clusters indicating that caspase-3 and NCAM redistributed into PrP containing lipid rafts. The experiments were performed in cooperation with Dr. Leshchyns'ka and Dr. Sytnyk.

VI.4. CLUSTERING OF NCAM AT THE CELL SURFACE INDUCES ACTIVATION OF CASPASE-3 AND CASPASE-8

Aggregation of caspase-8 is known to result in its activation (Boatright & Salvesen, 2003; Muzio et al, 1998; Salvesen & Dixit, 1999). To analyze whether clustering of NCAM, which is accompanied by caspase-8 aggregation in NCAM clusters (Fig. 10), results in activation of caspase-8 and consequent activation of caspase-3, we clustered NCAM at the cell surface of live 1-day-old cultured hippocampal neurons grown on substrate coated PDL. Therefore, neurons were incubated with monoclonal antibodies against NCAM for 10 min or with non-immune IgGs for control which were clustered thereafter by incubation with secondary antibodies. Fixed cells were permeabilized and labeled by indirect immunofluorescence with antibodies recognizing only the active form of caspase-3 (Fig. 13). Our experiments showed that application of NCAM antibodies resulted in an approximately 40 % increase in levels of activated caspase-3 along neurites and in cell bodies of NCAM antibody treated neurons when compared to neurons treated with control non-specific immunoglobulins (Fig. 13). Thus, our findings indicate that NCAM induces the activation of the effector caspase-3 in cultured hippocampal neurons.

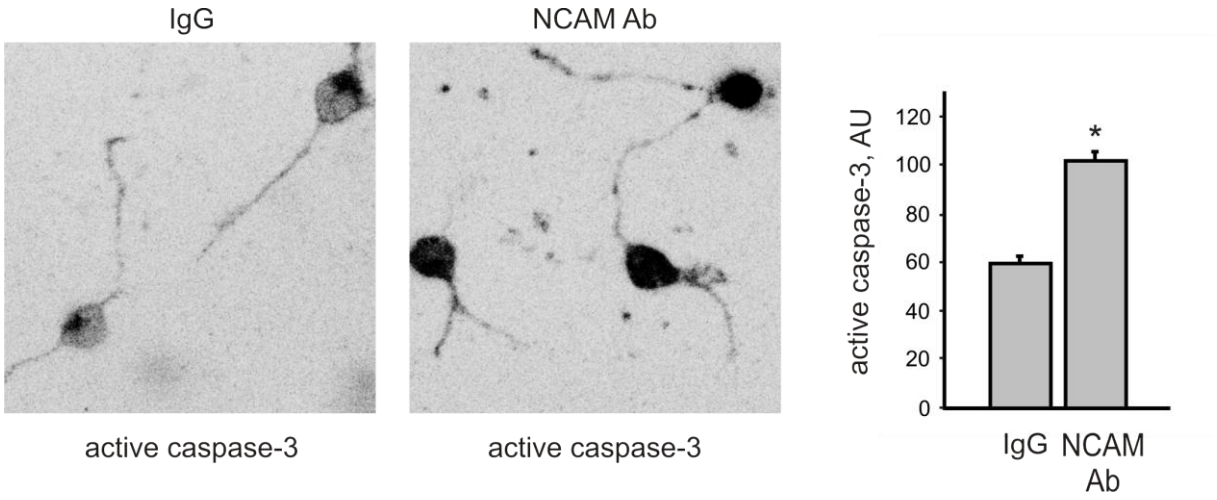

Fig. 13: Clustering of NCAM induces transient activation of caspase-3. 1-day-old wild type cultured hippocampal neurons were incubated live for 10 min with control immunoglobulins (IgG) or NCAM antibodies applied to cluster NCAM at the neuronal cell surface. Neurons were then fixed and labeled with antibodies against active caspase-3. Note that levels of active caspase-3 were higher in NCAM antibody treated neurons when compared to neurons incubated with control IgG. Graph shows levels of the immunofluorescence of active caspase-3 in neurons (mean ± SEM, n = 40 neurons analyzed per group) in arbitrary units (AU). *, $P < 0.05$, *t*-test. The experiments were performed in cooperation with Dr. Leshchyns'ka and Dr. Sytnyk.

Because antibodies recognizing only the activated form of caspase-8 were not available, we used a mammalian immortal cell line, Chinese hamster ovary (CHO) cells, which does not express NCAM, to analyze NCAM dependent caspase-8 activation in detail. The cell line was transfected to express NCAM140 (see Fig. 14A), since NCAM140 is the major isoform involved in neurite outgrowth (Niethammer et al, 2002) whereas NCAM180 is mainly present at postsynaptic densities in the mature neuron (Pollerberg et al, 1987; Schuster et al, 1998; Sytnyk et al, 2006). CHO cells were stably transfected with either NCAM140 in a pcDNA3 vector or mock transfected with the pcDNA3 vector alone. The CHO cells were treated with control non-specific immunoglobulins or NCAM antibodies applied for different time intervals. Lysates of the cells were then analyzed by Western blot with antibodies recognizing procaspase-8 and cleaved activated caspase-8 (Fig. 14B). This analysis showed that clustering of NCAM with NCAM antibodies resulted in a transient increase in levels of cleaved caspase-8 in NCAM140 transfected CHO cells. The peak in caspase-8 activation we observed within 5 min after NCAM antibody application with levels of cleaved caspase-8 being approximately 20 % higher in NCAM antibody treated CHO cells when compared to control immunoglobulin treated CHO cells. Levels of cleaved caspase-8 then gradually declined reaching the baseline level at 30 min after NCAM antibody application. Application of NCAM antibodies did not induce any changes in levels of cleaved caspase-8 in mock

transfected CHO cells. Likewise, it did not have any effect on caspase-9 cleavage and activation in NCAM140 transfected or mock transfected CHO cells.

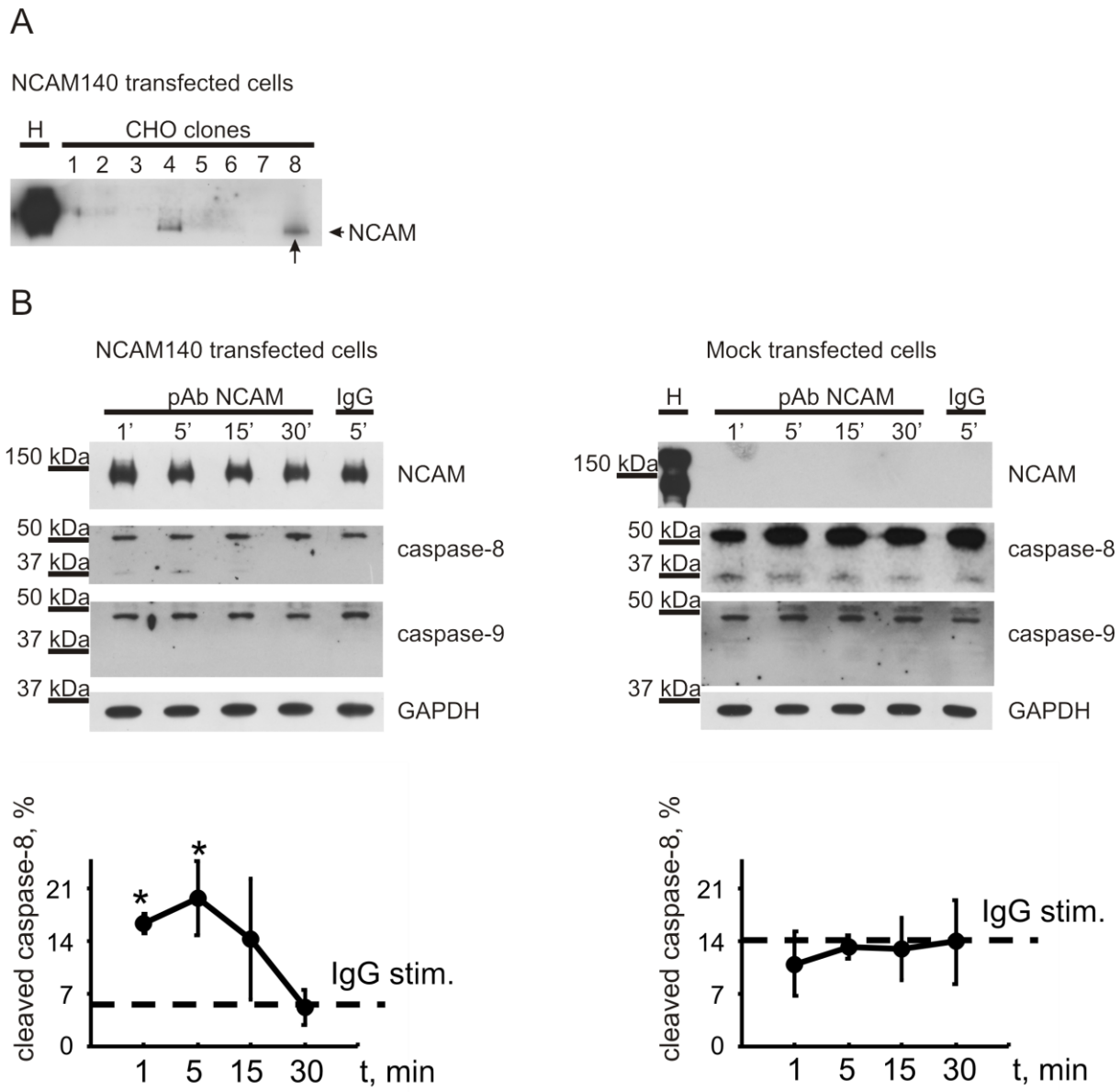

Fig. 14: Clustering of NCAM induces transient activation of caspase-8 but not of caspase-9. (A) CHO cells were transfected with NCAM140. Clone 3 and 8 were successfully transfected and selected. Clone 8 was choosen for further experiments. (B) CHO cells stably transfected with NCAM140 in a pcDNA3 vector, or mock transfected with pcDNA3 vector alone were incubated with NCAM antibodies for 1-30 min or with nonspecific IgG for control. Labeling with NCAM antibodies confirmed expression of NCAM in NCAM140 transfected cells, whereas no NCAM staining was detected in mock transfected cells. Lysates of these cells were then analyzed by Western blot with antibodies against caspase-8 and caspase-9. Note an increase in levels of active cleaved caspase-8 at 5 min after NCAM antibody application to NCAM140 transfected CHO cells. GAPDH labeling served as loading control. Brain homogenate (BH) was applied to the gel in parallel with the samples from mock transfected cells to show bands at the expected molecular weight. Graphs show quantitation of blots (mean ± SEM, n = 3) with optical density in IgG treated cells set to 100 %. *, $P < 0.05$, paired *t*-test.

In summary, our data show that NCAM stimulation leads to an activation of caspase-8 within 5 min and hence support the idea that the NCAM induced activation of caspase-3 is caused by caspase-8.

VI.5. NCAM INDUCES SPECTRIN PROTEOLYSIS IN GROWTH CONES BY ACTIVATING CASPASE-3

NCAM promotes axonal growth by activating signal transduction cascades in growth cones (Walsh & Doherty, 1997). The dynamic reorganization and restructuration of the spectrin cytoskeleton is a key determinant of neurite elongation downstream of NCAM signaling. Since caspases were shown to function in the reorganization of the cytoskeleton in a non-apoptotic context (Acarin et al, 2007; Lee et al, 2001), we were interested to investigate the role of caspases in the NCAM mediated reorganization of the cytoskeleton and the process of neurite outgrowth.

VI.5.1. The proteolytic cleavage of spectrin by caspase-3 is decreased in NCAM deficient growth cones

We used indirect immunofluorescence labeling of 1-day-old cultured hippocampal neurons from wild type mice to investigate the subcellular distribution of caspase-3. The cells were grown on substrate coated PDL, subsequently fixed and labeled for the cell surface proteins NCAM and PrP. After permeabilization the cells were additionally co-labeled with antibodies which recognize the full length as well as the active forms of caspase-3 (Fig. 15). In developing neurons, NCAM accumulated in growth cones of growing neurites (Fig. 15) where it is involved in the regulation of neurite outgrowth via modulation of the cytoskeleton and activation of intracellular signaling cascades (Bodrikov et al, 2005; Leshchyns'ka et al, 2003). Moreover, our indirect immunofluorescence labeling showed that caspase-3 was broadly distributed along growing neurites and also accumulated in growth cones (Fig. 15). Furthermore, distribution of caspase-3 along the fine fingerlike filopodia of the growth cone could be observed. Thereby, caspase-3 distribution partially overlapped with distributions of NCAM and its lipid raft anchoring molecule PrP.

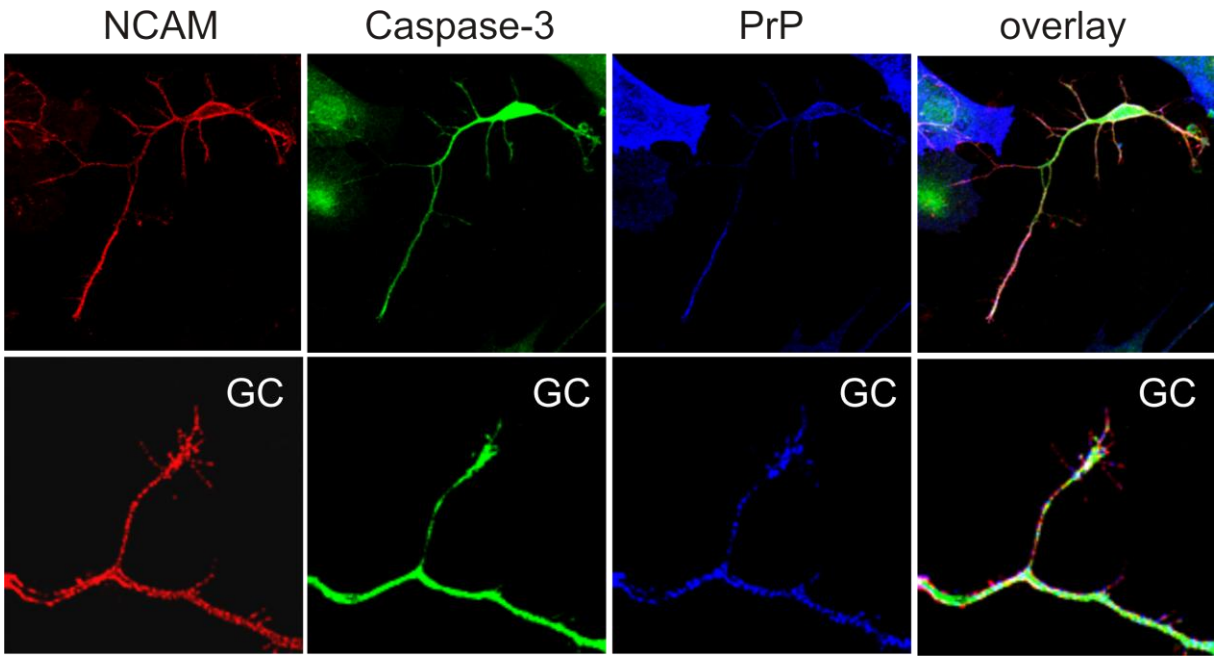

Fig. 15: NCAM and caspase-3 accumulate in growth cones. 1-day-old wild type cultured hippocampal neurons were co-labeled with antibodies against NCAM, caspase-3 and PrP. Images of a representative neuron (upper panel) and a neurite with a growth cone (GC, higher magnification, lower panel) are shown. Note that caspase-3 co-localized with NCAM and PrP in the growth cone and along neurites. Bar = 10 µm. The experiments were performed in cooperation with Dr. Leshchyns'ka and Dr. Sytnyk.

As mentioned above, growth cone migration and elongation requires a dynamic reorganization of the cytoskeleton. This is well established for the actin-microtubule cytoskeleton (Challacombe et al, 1996; Lin & Forscher, 1993; Tanaka et al, 1995). Changes in the direction of axon outgrowth during growth cone turning for instance depend on the reorganization of the microtubule and actin cytoskeleton. However, also spectrin is found at high levels in growth cones (Gordon-Weeks & Lang, 1988; Sobue & Kanda, 1989) and is as well implicated in axonal growth and growth cone behavior (Gitler & Spira, 1998). Moreover, NCAM is known to associate with the spectrin-actin cytoskeleton (Leshchyns'ka et al, 2003; Pollerberg et al, 1987; Pollerberg et al, 1986). Interestingly, actin and spectrin are substrates of caspase-3 (Mashima et al, 1997; Wang et al, 1998b). This prompted us to analyze whether the spectrin-actin cytoskeleton is affected in NCAM deficient growth cones. Caspase-3 cleaves both erythrocyte spectrin (α1/β1) and brain spectrin (α2/β2). Thereby, caspase-3 produces erythrocyte spectrin fragments with the molecular weight of 150 kDa and 120 kDa (Berg et al, 2001) and major β2-spectrin fragments with the molecular weight of 165 kDa and 110 kDa (Wang et al, 1998b) as depicted in Fig. 16. The major cleavage products of α2-spectrin (α2-fodrin) are fragments of 150 kDa and 120 kDa (Wang et al, 1998b).

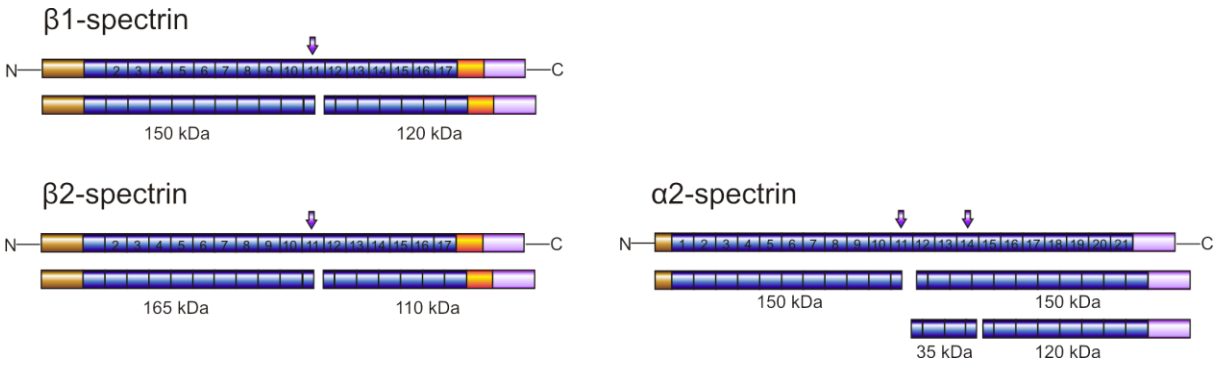

Fig. 16: Spectrin cleavage by caspase-3. Caspase-3 cleaves human spectrin isoforms at the indicated cleavage sites (see arrows) (Berg et al, 2001; Wang et al, 1998b). See text for details.

Therefore, we isolated growth cones from wild type and NCAM deficient brain homogenates by sucrose gradient centrifugation (Fig. 17) as described earlier (Pfenninger et al, 1983). L1 which is concentrated in growth cones (Cypher & Letourneau, 1991; Letourneau & Shattuck, 1989) served as loading control to ensure that comparable amounts of protein were loaded. Since different α- and β-spectrin isoforms are expressed in neuronal cells (Bennett & Baines, 2001; Dhermy, 1991), the fractions were labeled with antibodies recognizing erythrocyte spectrin (α1/β1), β2-spectrin and α2-spectrin. Since α1-spectrin is predominantly expressed in erythrocytes but either nonexistent or present at very low levels in neurons (De Matteis & Morrow, 2000; Harris et al, 1986), we considered the immunoreactive bands obtained by the antibodies raised against erythrocyte spectrin (α1/β1) as β1-spectrin labeling. In accordance with our previous finding of a reduced overall expression of β1-spectrin in NCAM deficient brains (Leshchyns'ka et al, 2003), Western blot analysis of growth cones isolated from wild type and NCAM deficient brains showed that levels of full length β1-spectrin and its degradation products were reduced in NCAM deficient growth cones (Fig. 17). To estimate the efficiency of β1-spectrin proteolysis, levels of β1-spectrin proteolytic fragments were normalized to the total levels of β1-spectrin expression. This analysis showed that while in wild type growth cones approximately 31 ± 4.5 % of all β1-spectrin protein were present as proteolytic fragments with the molecular weight of approximately 150 kDa, only 12 ± 5.4 % of all β1-spectrin protein were present as such proteolytic fragments in NCAM deficient growth cones. Interestingly, levels of full length β2-spectrin were approximately 20 % higher in NCAM deficient growth cones in comparison to wild type growth cones. This reflects probably a compensatory reaction to the reduced β1-spectrin expression.

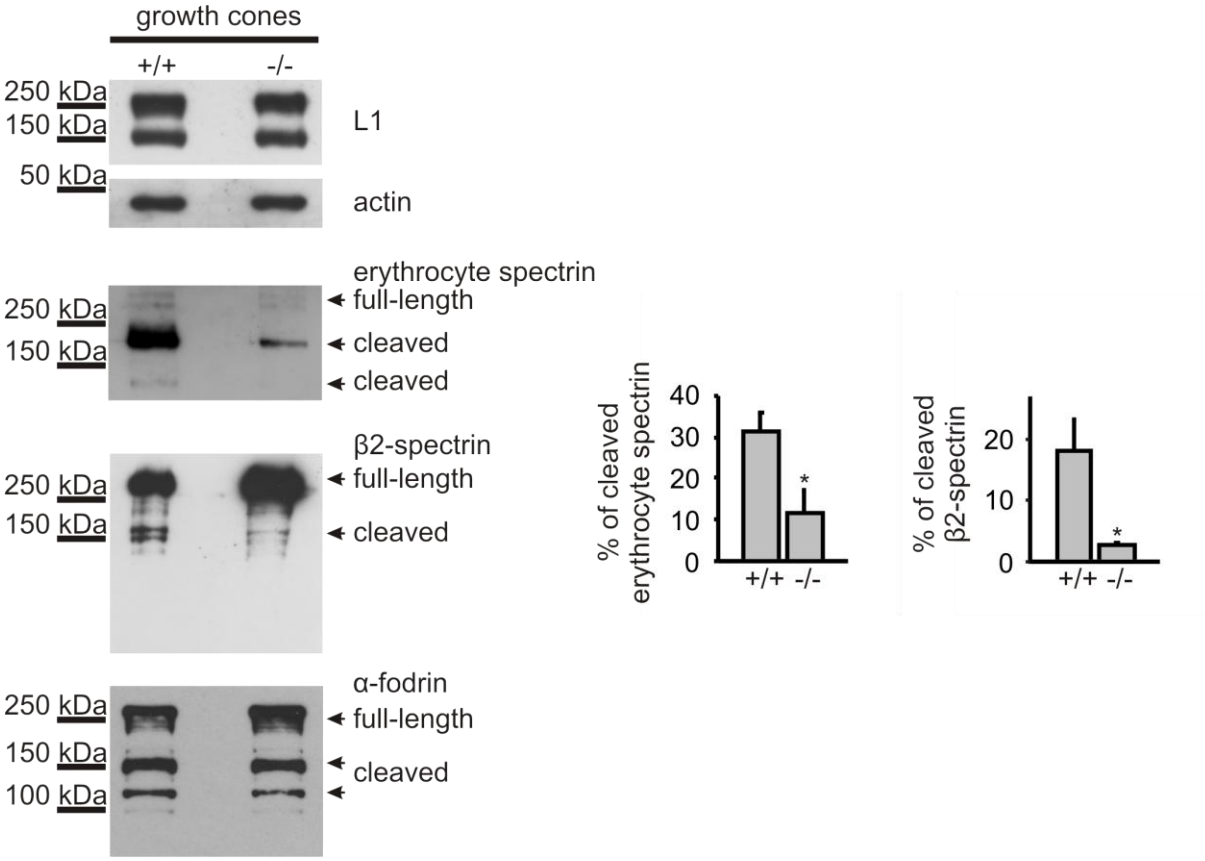

Fig. 17: The proteolytic cleavage of spectrin is decreased in NCAM deficient growth cones. Wild type (+/+) and NCAM deficient (-/-) growth cones were probed by Western blot with antibodies against actin, erythrocyte spectrin (here predominantly β1), β2-spectrin and α2-spectrin (α-fodrin). Arrows indicate bands representing full lengths proteins and their proteolytic fragments. Note that proteolysis of spectrin but not actin was decreased in NCAM deficient growth cones. Labeling for L1 served as loading control. Graphs show levels of cleaved β1- and β2-spectrin normalized to the total levels of β1- and β2-spectrin (mean ± SEM, n = 6). *, $P < 0.05$, paired *t*-test.

Similar to β1-spectrin, proteolysis of β2-spectrin was drastically reduced in NCAM deficient growth cones with approximately 13 ± 1.3 % and 1.3 ± 0.8 % of all β2-spectrin protein being present as proteolytic fragments with the molecular weight of approximately 160 kDa and 110 kDa in wild type and NCAM deficient growth cones, respectively. When we compared levels of α-fodrin in growth cones of both genotypes we observed again a tendential decrease in the level of cleaved α-fodrin (fragments at 150 kDa and 120 kDa) and an increase in the full length form of α-fodrin in NCAM deficient growth cones. In contrast, wild type and NCAM deficient growth cones contained similar levels of actin. Thus, NCAM deficiency specifically affects proteolysis of spectrin isoforms suggesting that clustering of NCAM in growth cones is necessary for spectrin proteolysis.

VI.5.2. The NCAM induced cleavage of spectrin by caspase-3 is inhibited by caspase-3 and caspase-8 inhibitors

In the next step we analyzed whether proteolysis of spectrin upon NCAM stimulation is specifically perfomed by caspase-3. For this experiment we made use of antibodies which recognize only the cleaved form of α2-spectrin (cleaved α-fodrin) that is specifically generated by caspase-3. We isolated growth cone fractions of wild type brains with a protocol that maintains isolated growth cones under physiological conditions (see V.2.2.9.d). The preparation was done in absence of cysteine protease inhibitors which would interfere with caspase activity. Thus, the protease inhibitor cocktail Complete (Roche Diagnostics GmbH) could not be employed since it inhibits a broad spectrum of cysteine protease. The widely used serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) was also shown to impede cysteine protease activity (Carlson et al, 2000; Inanami et al, 1999). Instead, the growth cone fractions were isolated in presence of the protease inhibitors leupeptin and aprotinin. The successfully isolated growth cone fractions were preincubated with either caspase-3 specific (Ac-DMQD-CHO) or caspase-8 specific (z-IETD-FMK) inhibitors or the respective vehicle. Afterwards, the growth cone fractions were incubated for 5 min at 37°C with control non-specific immunoglobulins or NCAM antibodies to cluster NCAM at the surface of growth cones. The immunoblots were analyzed for proteolysis of α2-fodrin with antibodies which recognize only the 150 kDa fragment of α2-fodrin that is generated by caspase-3. Though, the 120 kDa fragment is not recognized by the antibody. Western blot analysis showed indeed that application of NCAM antibodies resulted in a 20 % increase in levels of cleaved α2-fodrin in growth cones when compared to non-specific immunoglobulin treated growth cones (Fig. 18). Application of NCAM antibodies did not have any effect on levels of tubulin, actin and the cell adhesion molecule L1. Furthermore, NCAM antibody induced α2-fodrin proteolysis was completely abolished in the presence of caspase-8 and caspase-3 inhibitors. Thus, the elevated proteolysis of spectrin can be attributed to caspase-3 specific cleavage. The involvement of calpains can be excluded due to the application of leupeptin which inhibits calpain activity (Zimmerman & Schlaepfer, 1982) but not caspase-3 activity (Kaushal et al, 1998; Thibodeau et al, 2003). Hence, we concluded that clustering of NCAM at the surface of growth cones induces caspases-8 and -3 dependent proteolysis of the spectrin meshwork in growth cones.

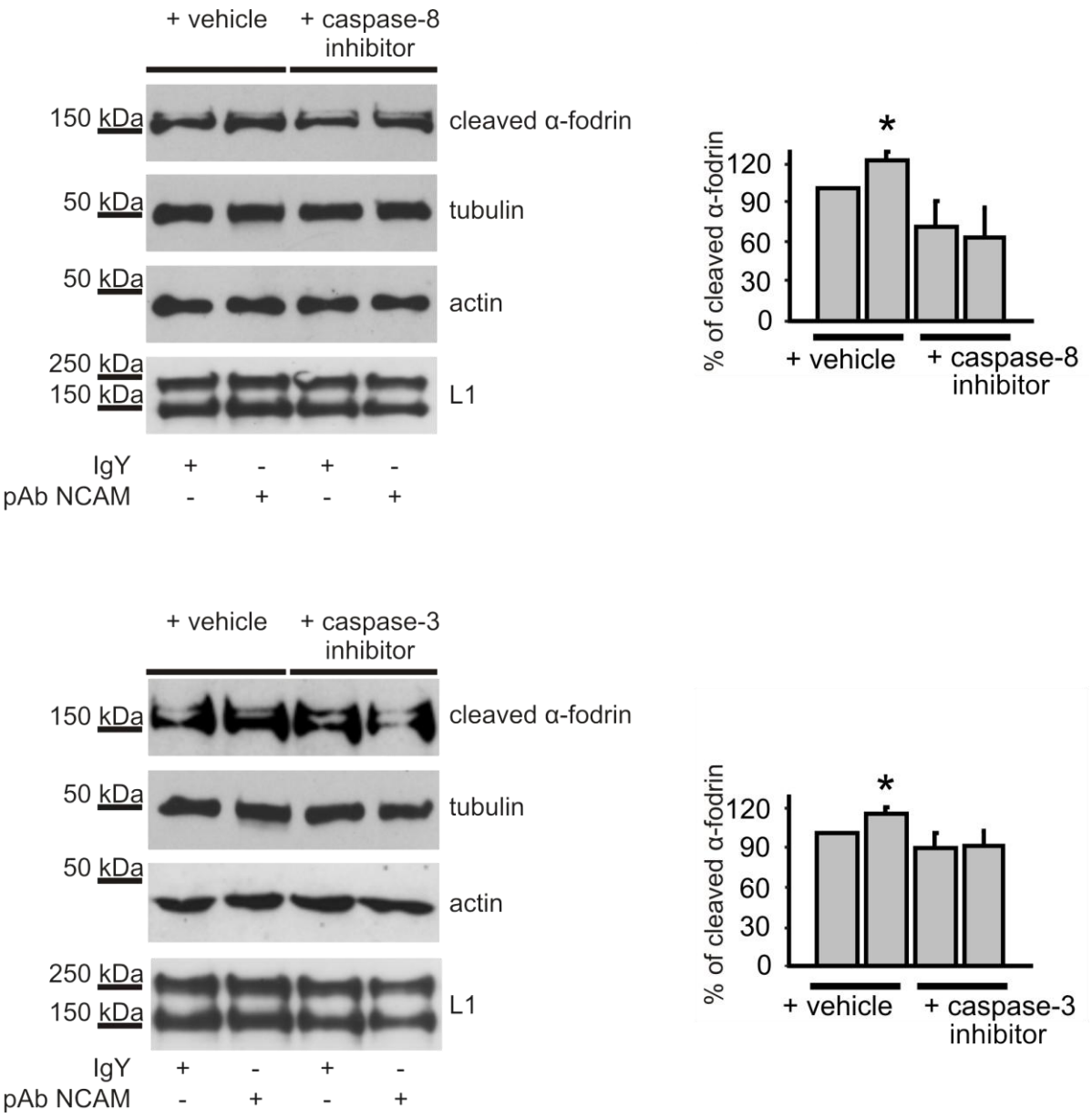

Fig. 18: Clustering of NCAM induces caspases-3 and -8 dependent proteolytic processing of spectrin. Wild type growth cones preincubated with either inhibitors of caspases-3 or -8 or with vehicle were incubated for 5 min with NCAM antibodies (pAb) or nonspecific immunoglobulins (IgY). Growth cones were then analyzed by Western blot with antibodies against cleaved α2-spectrin (cleaved α2-fodrin), actin and tubulin. Note increased levels of cleaved α2- fodrin in growth cones treated with NCAM antibodies in the absence of inhibitors when compared to IgY treated growth cones. This effect was blocked by inhibitors of caspases-3 and -8. L1 served as loading control. Graphs show quantitation of blots (mean ± SEM, n = 3) with the levels in growth cones treated with IgY in the absence of inhibitors set to 100 %. *, $P < 0.05$, paired *t*-test.

VI.5.3. The NCAM directed elongation requires the proteolytic activities of caspase-3 and caspase-8

Ligand induced clustering of NCAM at the neuronal cell surface is known to result in enhanced neurite outgrowth rates (Bodrikov et al, 2005; Leshchyns'ka et al, 2003; Santuccione et al, 2005). To analyze the role of caspases in NCAM dependent neurite outgrowth, cultured wild type hippocampal neurons maintained on substrate coated poly-D-lysine were stimulated with the recombinant extracellular domains of mouse NCAM fused to the Fc-portion of human IgG (NCAM-Fc). NCAM-Fc was applied to the culture medium in the presence or absence of caspase-3 and caspase-8 inhibitors. Hippocampal neurons establish *in vitro* several processes. One neurite becomes the faster extending axon and the remaining neurites grow at slower rate and differentiate into dendrites (Dotti et al, 1988). Accordingly, we estimated in our neurite outgrowth assay neurite elongation as the length of the longest individual neurite, which represents the putative axon, because this correlates with the lengths of all neurites. Incubation of neurons with NCAM-Fc for 24 h resulted in approximately two fold higher neurite lengths when compared to control neurons incubated with human Fc (Fig. 19). However, caspase-3 and caspase-8 inhibitors blocked this NCAM dependent increase in neurite length. But in contrast, inhibitors of caspase-9 and caspase-10 did not have any effect on NCAM dependent neurite elongation. Moreover, none of the inhibitors affected basal neurite outgrowth of Fc treated neurons grown on poly-D-lysine. So we draw the conclusion that the activities of caspase-3 and caspase-8 are required for the NCAM dependent neurite outgrowth. In support of this we observed a pronounced expression of caspase-3 in the early postnatal development of the mouse brain. By means of Western blot analysis we compared the levels of caspase-3 in brain homogenate of young (1-3 days) and adult (8 weeks) wild type and NCAM deficient mice (Fig. 20). We found the levels of caspase-3 decreased to approximately 16 % in brains of adult wild type and NCAM deficient mice compared to young mice brains. This finding indicates a particularly important role of caspase-3 during the early postnatal development of the mouse brain when neurite outgrowth is pronounced.

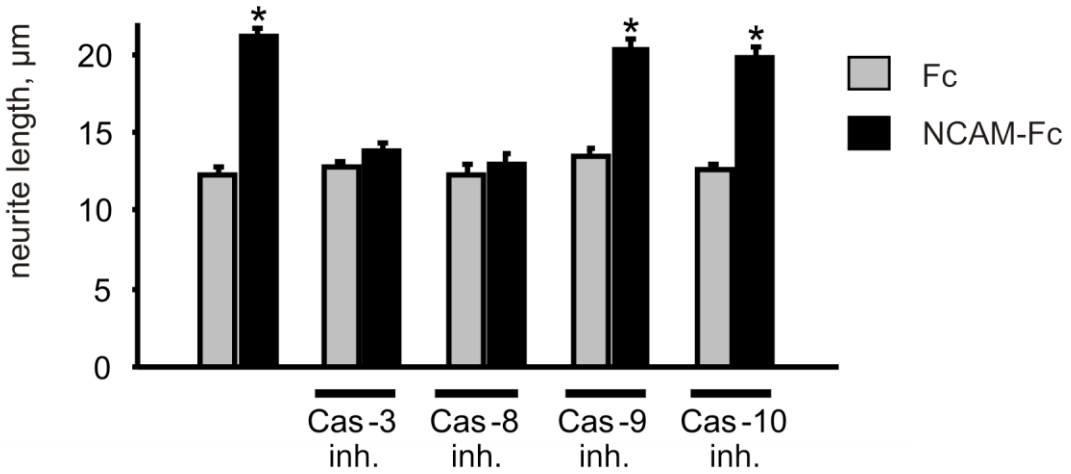

Fig. 19: NCAM dependent neurite outgrowth is blocked by caspase-3 and caspase-8 inhibitors. Cultured hippocampal neurons maintained on poly-D-lysine (PDL) were incubated for 24 h with NCAM-Fc or Fc in the presence or absence of inhibitors of caspases-3, -8, -9 or -10. Graph shows lengths (mean ± SEM, n > 250) of the longest neurites of neurons representing putative axons. Neurite lengths were increased in NCAM-Fc treated neurons when compared to control Fc treated neurons. Caspase-3 and caspase-8 inhibitors abrogated the NCAM-Fc-stimulated elongation of neurites. Caspase-9 and caspase-10 inhibitors did not affect NCAM dependent neurite outgrowth. *, $P < 0.05$, *t*-test. The experiments were performed in cooperation with Dr. Leshchyns'ka and Dr. Sytnyk.

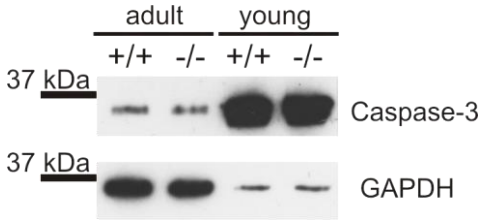

Fig. 20: Caspase-3 expression is increased in young mouse brains. Brain homogenates of young (1-3 days) or adult (8 weeks) wild type (+/+) and NCAM deficient (-/-) mice were labeled with caspase-3 antibodies. Note the prominent protein expression of caspase-3 in young brains compared to the decreased expression in adult brains. GAPDH served as loading control.

VII. DISCUSSION

NCAM promotes neurite outgrowth by activating signaling cascades in growth cones (Walsh et al, 2008). Transduction of NCAM induced signals into the cell regulates downstream the reorganization of the cytoskeleton. The rearrangement of the submembrane spectrin cytoskeleton is required to allow rapid changes in cell shape and structure during migration of the growth cone and neurite elongation. The aim of this study was to analyze the mechanisms that underlie these cytoskeletal changes during the NCAM induced neurite growth. Hence, we examined the influence of caspases, enzymes known to proteolyse the spectrin cytoskeleton (Acarin et al, 2007; Lee et al, 2001; Wang et al, 1998b), on the NCAM induced neurite outgrowth. Using biochemical and immunocytochemical analysis we investigated whether NCAM is involved in the regulation of caspase activities. In fact, we could establish that NCAM induces a transient activation of caspases-3 and -8 in hippocampal neurons and in CHO cells. Moreover, we were able to demonstrate that NCAM recruits caspase-8 and caspase-3 into lipid rafts where the activation of caspase-8 and caspase-3 is likely to occur. Activity of both caspases was necessary for the proteolytic cleavage of the spectrin cytoskeleton induced by NCAM. In a functional assay we confirmed that NCAM mediated neurite outgrowth of hippocampal neurons requires the activity of caspases-3 and -8. In conclusion, the data indicate that caspases-3 and -8 contribute to the NCAM mediated rearrangement of the spectrin cytoskeleton during neurite outgrowth in the developing neuron.

VII.1. NCAM INDUCES THE ACTIVATION OF CASPASE-3

At first we studied the levels of various caspases in wild type and NCAM deficient mice brains. For all analysis we used mice of postnatal day 1-3 since neuritogenesis in the brain is pronounced in this period. When we examined the levels of effector caspase-3 by Western blot analysis we found less active caspase-3 in mice brains that do not express NCAM (Fig. 6). In support of this we noticed that NCAM crosslinking at the cell surface by antibodies raised against the extracellular part of NCAM results in a mild activation of caspase-3 in cultured hippocampal neurons without indication of further apoptotic signs (Fig. 13). Likewise, previous studies applied soluble NCAM antibodies or NCAM-Fc to induce activation of NCAM signaling cascades to enhance neurite outgrowth (Beggs et al., 1997; Bodrikov et al., 2005; Niethammer et al., 2002; Schmid et al., 1999).

However, Azizeh and coworkers argue that surface bound NCAM antibodies promote neurite outgrowth of cortical neurons, whereas crosslinking of NCAM with soluble monoclonal

antibodies induces cell death (Azizeh et al, 1998). The authors treated cells with NCAM specific 5B8 antibodies and monoclonal 5A5 antibodies against PSA-NCAM (Developmental Studies Hybridoma Bank, University of Iowa) for various time intervals. They evaluated cell death and cell viability of cultured cortical neurons by means of morphological criteria and by staining with the vital stain Trypan blue that labels both apoptotic and necrotic cells. In addition they analyzed hallmarks of apoptosis such as nuclear condensation and fragmentation, but activation of caspases was not addressed in this study. Azizeh and colleagues show that antibody treatment for 24 h results in massive cell death, whereas treatment for 2 h gives rise to only few cells with morphological changes (Azizeh et al, 1998). Interestingly, whereas the 5A5 antibodies recognize an epitope at the PSA chain of NCAM, the 5B8 antibodies are produced against the intracellular domain of NCAM140 and NCAM180 (Dodd et al, 1988). Therefore the 5B8 antibodies can not be employed to cluster NCAM at the cell surface. Another group observed that treatment of a non-neuronal cell line, human salivary gland (HSG) tumor cells, with monoclonal NCAM antibodies for 24 h leads to the activation of caspase-3, caspase-7 and caspase-9 (Fukuda et al, 2005). However, the authors declare that induction of cell death in this study is probably attributed to the inhibition of NCAM signaling.

Not merely caspase-3 but also caspase-6 and caspase-7 belong to the group of effector caspases. Among this group caspase-3 and caspase-7 have a high degree of homology (Fernandes-Alnemri et al, 1995). Hence, they exhibit a very close substrate and inhibitor specificity (Fuentes-Prior & Salvesen, 2004). Nonetheless, it was reasonable for us to focus on caspase-3 because of several reasons discussed below. (1) Caspase-3 constitutes the major effector caspase in the demolition phase of apoptosis whereas caspase-6 and caspase-7 play only minor roles (Nicholson, 1999; Slee et al, 2001; Walsh et al, 2008). Using cell-free extracts from Jurkat cells immuno-depleted of either caspase-3, caspase-6 or caspase-7 Slee and colleagues show that caspase-3 is primarily responsible for the proteolysis of proteins that are cleaved during apoptosis, i.e. structural and nuclear proteins (except PARP) (Slee et al, 2001). Moreover, caspase-3 is supposed to be the most important executioner caspase involved in neuronal apoptosis (Bredesen, 2000). Accordingly, caspase-3 is specifically implicated in the cleavage of cytoskeleton associated proteins like actin, spectrin, gelsolin, vimentin and lamin (Byun et al, 2001; Kothakota et al, 1997; Mashima et al, 1997; Orth et al, 1996; Pike et al, 1998; Takahashi et al, 1996; Wang et al, 1998b). (2) Interestingly, caspase-3 deficient mice on the 129X1/SvJ background are embryonically lethal and show severe abnormalities in brain development (Leonard et al, 2002). Likewise, mice deficient in

caspase-7 die early during embryogenesis (Kuida & Flavell, unpublished data) whereas mice deficient in caspase-6 develop normally (Zheng & Flavell, unpublished data). While their phenotype has still to be determined, the phenotype of caspase-3 deficient mice is mainly attributed to decreased apoptosis in the brain. Mice deficient in caspase-3 have an increased number of cells in cortical areas, the cerebellum and the retinal neuroepithelium (Kuida et al, 1996). Mice deficient in caspase-9 display a similar phenotype (Kuida et al, 1998), indicating that apoptosis in the brain is mainly performed by activation of caspase-3 in a caspase-9 dependent pathway. As more and more non-apoptotic functions of caspases become revealed it is tempting to speculate that also defects in neuritogenesis or other non-apoptotic functions might contribute to the phenotype observed in caspase-3 deficient mice. (3) Nevertheless, the main argument for us was the fact that caspase-3 is the effector caspase whose activity is most strikingly implicated in several non-apoptotic functions such as proliferation and cell differentiation, synaptic plasticity, growth cone regeneration and lens fiber development (Campbell & Holt, 2001; Gulyaeva, 2003; Kuranaga & Miura, 2007; Lamkanfi et al, 2007; Launay et al, 2005; Lee et al, 2001; Nhan et al, 2006). In contrast, non-apoptotic functions for other effector caspases were hardly suggested. In conclusion, we decided to focus on caspase-3 and to neglect further effector caspases.

VII.2. THE NCAM INDUCED ACTIVATION OF CASPASE-3 IS MEDIATED BY CASPASE-8

We established that the effector caspase-3 is activated by NCAM upon NCAM stimulation (Fig. 13). Activation of procaspase-3 requires cleavage of the proenzyme within the linker region, which separates the large and small subunits, and a second cleavage to remove the prodomain (Fernandes-Alnemri et al, 1996; Muzio et al, 1997; Srinivasula et al, 1998; Stennicke et al, 1998). The mature enzyme constitutes a tetramer assembled of two large and two small subunits which contains two active centers (Walker et al, 1994; Wilson et al, 1994). Caspase-3 is not known to be activated in an autoproteolytical process just as described for initiator caspases (Martin et al, 1998; Srinivasula et al, 1998; Yang et al, 1998). Because NCAM does not possess an intracellular proteolytic activity the NCAM induced activation of caspase-3 requires an upstream activator of caspase-3. We analyzed the known activating initiator caspases of caspase-3 including caspases-8, -9 and -10 (Li et al, 1997; Stennicke et al, 1998). Among these initiator caspases we found the level of cleaved caspase-8 but not caspases-9 and -10 to be down regulated in NCAM deficient mice brains (Fig. 8). Thus, we considered caspase-8 as a possible candidate to activate caspase-3 upon NCAM activation. To

confirm that caspase-8 is involved in the NCAM mediated activation of caspase-3 we stimulated NCAM140 expressing CHO cells with NCAM antibodies. The experiment revealed that NCAM clustering at the cell surface leads to the activation of caspase-8, whereas caspase-9 is not activated in the course of NCAM stimulation (Fig. 14).

To investigate the idea that NCAM crosslinking results in caspase-8 activation in more detail, we analyzed whether NCAM is able to associate with caspase-8. In fact, in a co-immunoprecipitation assay we verified an association between NCAM and the cleaved form of caspase-8 (Fig. 9). Since Boatright and colleagues showed that aggregation of caspase-8 is sufficient to induce its activation (Boatright & Salvesen, 2003), we suggest that clustering of caspase-8 by NCAM might likewise result in the activation of this caspase. Accordingly, we only detected the cleaved form of caspase-8 in NCAM immunoprecipitates. This cleaved form is processed between the large and small catalytic subunits and indicates that the caspase is activated. But it still contains the prodomain that carries the protein-protein interaction motifs.

Interestingly, the association between NCAM and caspase-8 is effected by calcium. Other NCAM interactions were also shown to be influenced by calcium such as the complex formation between NCAM and RPTPα via spectrin (Bodrikov et al, 2005). The direct binding between NCAM and RPTPα is not dependent on calcium as indicated by ELISA experiments. However, the complex formation by spectrin is enhanced in the presence of calcium. The complex formation between NCAM and caspase-8 might be as well indirectly effected by the presence of calcium whose concentration is increased in the course of NCAM induced signaling (Doherty et al, 1991; Kiryushko et al, 2006; Williams et al, 1994b).

NCAM and caspase-8 might associate with each other directly or indirectly. The N-terminal prodomain of caspase-8 contains protein-protein interaction domains (see Fig. 4). These regions are homologous to the death effector domains (DED) of FADD, which are called FADD-homology domains (Muzio et al, 1996). Via these domains caspase-8 undergoes direct interactions with other proteins. In the course of the extrinsic apoptotic pathway FADD recruits caspase-8 to multiprotein complexes in which caspase-8 becomes clustered and activated (Muzio et al, 1998). Both proteins interact with each other via their death effector domains. Thus, FADD is an adaptor protein that links caspase-8 to death receptors at the plasma membrane. NCAM is not known to possess a DED domain. Instead, NCAM might bind directly to caspase-8 via other binding motifs or in an indirect manner via an adaptor protein. Association via adaptor proteins is known for other proteins. For instance, the cell adhesion molecule L1 is known to interact with the adaptor protein ankyrin (Davis & Bennett,

1994). Interestingly, ankyrin contains a death domain (DD) (Bennett & Baines, 2001). This death domain is also present in FADD (Berglund et al, 2000; Jeong et al, 1999). Via its death domain FADD interacts with the death domain of the Fas receptor (Chinnaiyan et al, 1995; Zhang & Winoto, 1996). To verify a direct binding between NCAM and caspase-8 further experiments are necessary such as enzyme linked immunosorbent assays (ELISA) or pull-down experiments. ELISA techniques can even give detailed information about binding kinetics.

Another question that might appear is whether NCAM is a substrate of caspases. There is no evidence for proteolysis by caspases in literature. Furthermore, the amino acid sequence of the intracellular domains of NCAM180 and NCAM140 do not contain the characteristic cleavage motif of caspase-3 (Asp-X-X-Asp). Besides of caspases there is another class of cysteine dependent proteases, named calpains (calcium dependent protease with papain-like activity). However, both proteases differ in their substrate specificity. While caspases prefer cleavage sites after an aspartate residue, calpains cleave preferentially after residues next to valine, leucine or isoleucine (Wang, 2000). Previous findings indicate that calpains are able to cleave NCAM. Covault and coworkers show that calpains cleave the intracellular domain of chicken NCAM180 and produce NCAM fragments between 160 kDa and 120 kDa (Covault et al, 1991). Most probably calpain I which is activated by calcium concentrations in the micromolar range is responsible for the proteolysis. In contrast, the 140 kDa and 120 kDa isoforms were unaffected. Another group argues that both transmembrane isoforms, NCAM180 and NCAM140, are cleaved by calpains whereas NCAM120 is unaffected (Sheppard et al, 1991a; Sheppard et al, 1991b). This may disrupt the linkage between the intracellular domain of NCAM and the cytoskeleton. Cleavage of the intracellular domain of NCAM would interfere with its signaling function and thus we would anticipate reduced instead of enhanced NCAM dependent neurite outgrowth.
However, calpains and caspases are interacting with each other. Calpains are regulated by the endogenous inhibitor calpastatin which can be cleaved by caspase-3 (Porn-Ares et al, 1998; Wang et al, 1998a). In turn, calpain can also cleave caspases which can result either in activation or inactivation of caspase function (Chua et al, 2000; Nakagawa & Yuan, 2000). For instance, the procaspase-3 can be directly cleaved and inactivated by calpains (Neumar et al, 2003).

VII.3. NCAM RECRUITS CASPASE-8 AND CASPASE-3 INTO LIPID RAFTS

Lipid rafts are in the focus of research interest since they are involved in outside-in signal transduction across the plasma membrane (Simons & Toomre, 2000). They constitute platforms wherein certain proteins such as kinases of the src family, GPI anchored proteins, cytoskeletal components and dual acylated proteins are concentrated (Caroni, 2001; Leshchyns'ka et al., 2003; Simons and Ikonen, 1997, 2000). Moreover, the signaling molecule NCAM is also present in lipid rafts (He et al, 1986; Niethammer et al, 2002). NCAM120 resides constitutively in these microdomains whereas NCAM140 and NCAM180 redistribute into lipid rafts upon stimulation. In their intracellular domain NCAM140 and NCAM180 contain four cysteine residues, of which three represent putative palmitoylation sites, in close proximity to the membrane (Little et al, 1998). Ponimaskin and colleagues recently identified the palmitoyl transferases involved in the palmitoylation of NCAM140 and NCAM180 (Ponimaskin et al, 2008). Interestingly, palmitoylation enhances the association of NCAM with lipid rafts and is crucial for NCAM signaling via the nonreceptor tyrosine kinase pathway (Niethammer et al, 2002). Within the microdomains NCAM140 and NCAM180 are additionally stabilized by the GPI anchored protein PrP (Santuccione et al, 2005). Thus, it was of particular interest for us to investigate whether caspases are present in lipid rafts and, moreover, whether they are localized to NCAM containing lipid rafts. Therefore, we made use of a biochemical method to isolate lipid raft fractions (Brown & Rose, 1992). An important point we have to consider first of all is the still ongoing discussion about the equivalence of detergent resistant membranes (DRMs) and lipid rafts in living cells (Brown, 2006; Lichtenberg et al, 2005; Lingwood & Simons, 2007). Initially, detergent resistant membranes have been operationally defined as equivalent to lipid rafts (Brown & London, 1998; Brown & London, 2000; London & Brown, 2000). Treatment with cold non-ionic detergents is the only available biochemical method to investigate potential lipid raft affinity (Lingwood & Simons, 2007). However, the use of a variety of detergents (e.g. lubrol, Triton X-100) led to the isolation of DRMs which are distinct in their composition (Delaunay et al, 2008; Roper et al, 2000). It should be noted that the detergent concentration and particularly the ratio of detergent to proteins and lipids are critical parameters which influence the efficiency of the solubilization (Lingwood & Simons, 2007). A low detergent efficiency might give misleading results, e.g. the detection of non-raft proteins in DRMs. Hence, it is crucial to use a negative control that is not affine to lipid rafts. Therefore, we decided to

include GAPDH labeling as negative control in order to exclude contaminations in the isolated lipid raft fractions.

In our experiment we used a mild extraction with the non-ionic detergent Triton® X-100 at 4°C. The detergent incorporates into the membrane and forms micelles. Parts of the membrane are totally solubilized whereas other parts, enriched in cholesterol, sphingolipids and lipid raft proteins, remain insoluble. The soluble and the insoluble material we separated afterward in a sucrose density gradient. The low density fraction at the top of the density gradient at 10 % sucrose constitutes the lipid raft fraction. When we compared the levels of caspases in lipid raft fractions of NCAM deficient brains to wild type brains, we found the levels of caspases-3 and -8 reduced by approximately 50 % in NCAM deficient brains (Fig. 11). Our experiments revealed that only the levels of caspases-3 and -8 but not of caspase-9 were reduced in lipid rafts of NCAM deficient mice brains. Moreover, caspase-10 was not detectable at all in lipid raft fractions. In addition we could answer the question whether caspases co-localize with NCAM in NCAM containing lipid rafts (Fig. 10, Fig. 12). We found caspase-3 as well as caspase-8 to be present in NCAM containing microdomains upon NCAM stimulation with antibodies. Our observation that caspase-8 and caspase-3 are present in lipid rafts is consistent with previous publications. In mouse thymocytes caspase-8 was reported to be recruited into lipid rafts by FADD upon activation of the Fas receptor (Aouad et al, 2004; Hueber et al, 2002). Interestingly, caspase-3 is even present in lipid rafts of non-apoptotic Jurkat cells and T-lymphocytes (Aouad et al, 2004). Moreover, also in cells of the central nervous system caspases were found to be localized in lipid rafts. Recently, Davis and colleagues detected caspases-3 and -8 in lipid rafts isolated from crude membrane fractions of rat spinal cords (Davis et al, 2007). Spinal cord injury is known to induce the activation of caspases and to cause apoptosis (Crowe et al, 1997; Emery et al, 1998). Davis and coworkers analyzed the localization of caspases in low density (lipid rafts) and high density fractions (probably cytoskeleton associated membranes) of spinal cord injured and control animals which were shame-operated. Interestingly, they detected caspase-3 in the low and high density fractions and thus confirm our observation that caspase-3 is membrane associated and, moreover, present in lipid rafts (Fig. 11A, B). Whereas caspase-3 was constitutively present in lipid rafts, caspase-8 was translocated into the microdomains after spinal cord injury where it associates with the components of the death inducing signaling complex (DISC) (Davis et al, 2007). Davis and colleagues interprete their data only in the context of apoptosis, since it is known that spinal cord injury leads to the assembly of the DISC and the activation of caspase-8 and subsequently of caspase-3 (Crowe et al, 1997;

Emery et al, 1998). However, it would be also interesting to investigate whether the activation of caspases, and also their presence in lipid rafts, might contribute to regeneration after spinal cord injury. In this regard it is interesting to mention the findings obtained by Verma and colleagues (Verma et al, 2005). They observed that inhibition of caspase-3 activity abrogates the formation of new growth cones of dorsal root ganglion cells and retinal cells after axotomy which supports our idea that caspases are involved in promotion of neurite outgrowth.

We now established that NCAM recruits caspase-8 and caspase-3 into lipid rafts upon NCAM stimulation with NCAM antibodies in live neurons (Fig. 10, Fig. 12). This explains the reduced level of caspase-8 in lipid raft fractions that do not contain NCAM compared to the lipid raft fractions where NCAM is present. Our idea is further supported by the finding that caspase-8 and NCAM associate with each other (Fig. 9). Moreover, we also find caspase-3 levels reduced in NCAM deficient microdomains. Since caspase-3 is a substrate of caspase-8 it might be in turn recruited by caspase-8 into lipid rafts and subsequently activated. However, we were not able to detect cleaved caspases-3 and -8 in lipid raft fractions. This might reflect that active caspases are just transiently present in the microdomains. Upon activation caspase-3 might be rapidly released into the cytosol. Lipid rafts are supposed to be short-lived in the range of milliseconds (Guirland & Zheng, 2007; Hancock, 2006). Accordingly, the composition of the membrane changes dynamically as well as the location of proteins within the membrane. This allows lipid rafts to function in the dynamic regulation of signaling processes such as the NCAM mediated signaling. Moreover, recruitment of caspase-8 into NCAM containing lipid rafts and subsequent activation of caspase-8 within lipid rafts might allow a spatially restricted and mild activation of caspase-8 and, in turn, activation of caspase-3. This in turn results in a restricted proteolysis of the NCAM associated spectrin cytoskeleton.

VII.4. CASPASES-3 AND -8 ARE INVOLVED IN REMODELING OF THE SPECTRIN SCAFFOLD IN GROWTH CONES DURING NCAM MEDIATED NEURITE OUTGROWTH

Among the different NCAM isoforms NCAM140 is enriched in growth cones (Persohn et al, 1989). Interestingly, stimulation of this isoform with NCAM antibodies leads to the activation of caspase-8 and caspase-3 in CHO cells (Fig. 14) as discussed above. This encouraged us to analyze the subcellular localization of caspase-3 in hippocampal neurons. By means of indirect immunofluorescence labeling we detected caspase-3 in hippocampal growth cones and, moreover, even in the fingerlike protrusions of the growth cone (Fig. 15). Interestingly,

caspase-3 co-localized with NCAM and PrP along neurites and growth cones and, hence, caspase-3 might be present at sites of NCAM signaling. Our observation that caspase-3 is localized to growth cones is in agreement with a previous finding (Campbell & Holt, 2003). Campbell and Holt detected active caspase-3 in retinal growth cones of *Xenopus* embryos. They discovered that the level of active caspase-3 in the growth cone was increased in the presence of some guidance cues such as netrin-1 (Campbell & Holt, 2003). Moreover, caspase-3 activity was necessary for the chemotropic responses of growth cones, i.e. collapse or attraction, in response to these guidance cues.

The observation that caspase-3 is localized in growth cones prompted us to analyze whether it contributes to cytoskeletal rearrangements in growth cones of developing neurites as suggested previously (Gilman & Mattson, 2002). We compared growth cone fractions of wild type and NCAM deficient mice brains regarding the proteolysis of the spectrin cytoskeleton. We found the levels of cleaved erythrocyte and β2-spectrin significantly reduced in NCAM deficient growth cones (Fig. 17). This tendency we also observed for α2-spectrin. Thus, we concluded that in the absence of NCAM the proteolysis of spectrin in the growth cone is reduced. Spectrin can not only be cleaved by caspases but also by calpains (Gitler & Spira, 1998; Gitler & Spira, 2002; Nixon, 1986; Ohbayashi et al, 1998). Calpains which were shown to contribute to neuronal apoptosis as well (Nath et al, 1996) were previously implicated in cytoskeletal changes due to cleavage of the submembranous spectrin meshwork (Gitler & Spira, 1998; Gitler & Spira, 2002; Ohbayashi et al, 1998; Siman et al, 1984). It is suggested that spectrin proteolysis by calpains causes morphological alterations of cells because spectrin cleavage uncouples the membrane from the underlying structural matrix which supports structural changes.

Break down products of human spectrin generated by caspase-3 (see Fig. 16) appear to be in part distinct from those produced by calpains. Cleavage of β2-spectrin by caspase-3 results in the major fragments of 165 kDa and 110 kDa (Wang et al, 1998b). But there are reports about different calpain proteolyzed β2-spectrin fragments which describe a 218 kDa fragment (Hu & Bennett, 1991) or a 110 kDa fragment (Wang et al, 1998b). Berg and colleagues observed erythrocyte spectrin cleavage by calpains, but do not describe the fragments (Berg et al, 2001). However, they show that caspase-3 cleaves erythrocyte spectrin *in vitro* into fragments of 150 kDa and 120 kDa size (Berg et al, 2001). Calpains and caspase-3 cleave α2-spectrin within repeat 11 thereby producing the 150 kDa breakdown product (Nath et al, 1996; Wang et al, 1998b). Caspase-3 cleaves additionally in an adjacent repeat which gives rise to the 120 kDa fragment, although it is not clear whether the cleavage occurs in repeat 14 (Wang et

al, 1998b) or between repeat 12 and 13 (Nath et al, 1996). In our experiments we observed the 165 kDa fragment of β2-spectrin and an erythrocyte spectrin fragment which runs at an apparent molecular weight of more than 150 kDa in SDS-PAGE and the 120 kDa fragment which is specific for caspase-3 cleavage. To verify that NCAM activation leads to an increased spectrin cleavage in growth cones in a caspase specific manner we performed stimulation assays in isolated growth cone fractions (Fig. 18). We used antibodies which recognize α2-spectrin (α-fodrin) that is specifically cleaved by caspase-3. These antibodies are raised against the caspase cleavage site which is distinct from the calpain cleavage site. Cleavage by caspase-3 results in the exposition of the antibody epitope. NCAM stimulation by means of NCAM antibodies enhanced the cleavage of α-fodrin in growth cone fractions. Furthermore, the proteolysis was dependent on the activity of caspase-3 and caspase-8 but not of calpains since we applied leupeptin, a potent inhibitor of calpains (Zimmerman & Schlaepfer, 1982). It is tempting to speculate that caspase dependent spectrin cleavage induced by NCAM contributes to the restructuring of the spectrin meshwork underneath the membrane during neurite growth. As mentioned above, spectrin cleavage by calpains was previously supposed to contribute to cytoskeletal rearrangements. Gitler and coworker showed that proteolysis of spectrin enables regeneration of growth cones after axotomy of neurons (Gitler & Spira, 1998; Gitler & Spira, 2002). We suggest that a similar function in the reorganization of the spectrin cytoskeleton might hold true for caspases-3 and -8 upon activation of NCAM. Active caspase-3 cleaves structural proteins in the growth cone such as spectrin on a limited scale. In principal, caspase cleavage does not lead to the complete degradation of proteins (Salvesen & Dixit, 1999). Accordingly, spectrin is cleaved by caspase-3 into two major fragments. Proteolysis of one subunit might result in the release of the other subunit from the heterodimer or heterotetramer, respectively. Subsequently this allows a reorganization and reassembly of the cytoskeleton. Moreover, spectrin crosslinks actin filaments (Stromqvist, 1987) and hence proteolysis of spectrin influences as well actin dynamics.

Since we established that caspases-3 and -8 are involved in the NCAM induced restructuring of the spectrin cytoskeleton, we wanted to confirm in a functional assay whether caspases-3 and -8 are in fact required for the NCAM mediated elongation of hippocampal neurons. Indeed, we were able to verify that the activities of both caspases were necessary for the NCAM dependent neurite outgrowth. In contrast, caspases-9 and -10 were not involved in NCAM induced neurite growth. To reveal the requirement of caspases in this process we applied specific inhibitors to block their activity. An alternative strategy to block caspase

function would have been the use of small interfering RNA (siRNA) which down regulates protein expression by induction of specific mRNA degradation. We decided to employ specific caspase inhibitors since siRNA inhibits only protein expression whereas the use of inhibitors blocks specifically caspase activity. Thus, we were able to clearify whether the proteolytic activities of caspases are required for their function in neurite outgrowth. Indeed, we established that caspase-3 and caspase-8 are involved in NCAM mediated neurite outgrowth and moreover that their proteolytic activity is necessary for their function.

However, there are contradictory results concerning the effect of caspase-3 inhibitors on neurite outgrowth. Oshitari and coworker argue that inhibitors of caspases-1, -3, -8 and -9 promote retinal ganglion cell survival and neurite outgrowth in retinal explants from adult mice (Oshitari & Adachi-Usami, 2003). They analyzed the effect of caspase inhibitors on regenerating neurites of damaged retinal ganglion cells. Cells treated with different caspase inhibitors showed a higher number of regenerating neurites than the controls grown in serum-free medium. In contrast, Fernando and coworkers observed an increased caspase activity during differentiation of neurospheres derived from the striatum of mouse embryos without further apoptotic signs such as PARP cleavage (Fernando et al, 2005). Treatment with caspase-3 but not with caspase-8 inhibitors attenuates neurite elongation and branching during neurosphere differentiation. Moreover, application of the caspase-3 inhibitor reduced the activity of the kinase PAK1 which is known to promote neurite outgrowth (Daniels et al, 1998). The contradictory results indicate that caspase functions are different depending on the stage of development and the cell type. Whereas Oshitari and Adachi-Usami investigated adult mice, we were analyzing caspase function in developing neurons. In support of this we observed that caspase-3 expression is strongly increased during the early postnatal period when neurite outgrowth is pronounced and down regulated in adulthood as demonstrated in Fig. 20. In adult mice brains caspase-3 expression was decreased to approximately 15 % of the level observed in young mice brains. This indicates that caspases might perform different functions in the young and adult brain, respectively. During brain development caspase-3 activity seems to be particular important for neurite outgrowth. Later on, in adulthood caspase-3 function might be restricted to apoptosis. Alltogether, we support a novel function of caspases in NCAM mediated neurite growth during brain development besides of their traditional role in apoptosis. And it has to be emphasized that the employment of caspase inhibitors as chemotherapeutics, e.g. to support putatively nerve regeneration, should be reconsidered now that a growing number of novel, non-apoptotic functions of caspases are revealed.

VII.5. PROPOSED MODEL OF THE NCAM INDUCED ACTIVATION OF CASPASES-3 AND -8 LEADING TO SPECTRIN CYTOSKELETON REARRANGEMENTS

On the basis of previous and our findings we propose a model that is illustrated in Fig. 21. NCAM promotes neurite outgrowth via homophilic and heterophilic interactions with molecules on adjacent cells and the ECM which activates intracellular signaling cascades (Doherty et al, 1991; Doherty et al, 1990; Williams et al, 1994a). As indicated in Fig. 21 (1), NCAM associates intracellularly with the spectrin cytoskeleton (Leshchyns'ka et al, 2003) which can be associated with lipid rafts owing to a pleckstrin homology domain in the β-subunits (Wang et al, 1996). Besides, caspase-3 and caspase-8 are not only localized in the cytosol but also associated with the plasma membrane. Homophilic and heterophilic NCAM interactions induce clustering of NCAM at the cell surface which activates intracellular signaling cascades. In our study we induced clustering of NCAM with NCAM specific antibodies or with soluble extracellular domains of NCAM fused to human Fc which has similar effects on NCAM dependent signal transduction and neurite outgrowth (Beggs et al, 1997; Leshchyns'ka et al, 2003; Niethammer et al, 2002). Clustering of NCAM results in dimerization or oligomerization of NCAM associated caspase-8. Palmitoylation of NCAM at intracellular cysteine residues by a palmitoyl transferase (Little et al, 1998; Ponimaskin et al, 2008) enhances the association of NCAM with lipid rafts (Niethammer et al, 2002). Thus, the complexes of NCAM and spectrin and/or NCAM and caspase-8 as well as caspase-3 redistribute into lipid rafts (3). Clustering of caspase-8 leads to its activation due to conformational changes (Boatright & Salvesen, 2003). Subsequently, caspase-8 is cleaved owing to an inherent proteolytic activity within the linker region which separates the large and small subunits. Activated caspase-8 cleaves and activates caspase-3 which presumably occurs within lipid rafts (4). Now, transiently activated caspase-3 cleaves spectrin subunits (5). Cleavage of one subunit within the heterodimer or heterotetramer might result in the release of the other subunit. Thus, caspase-3 cleavage of spectrin allows the spectrin cytoskeleton to be reassembled in a new configuration which contributes to morphological changes of the growth cone and supports neurite growth.

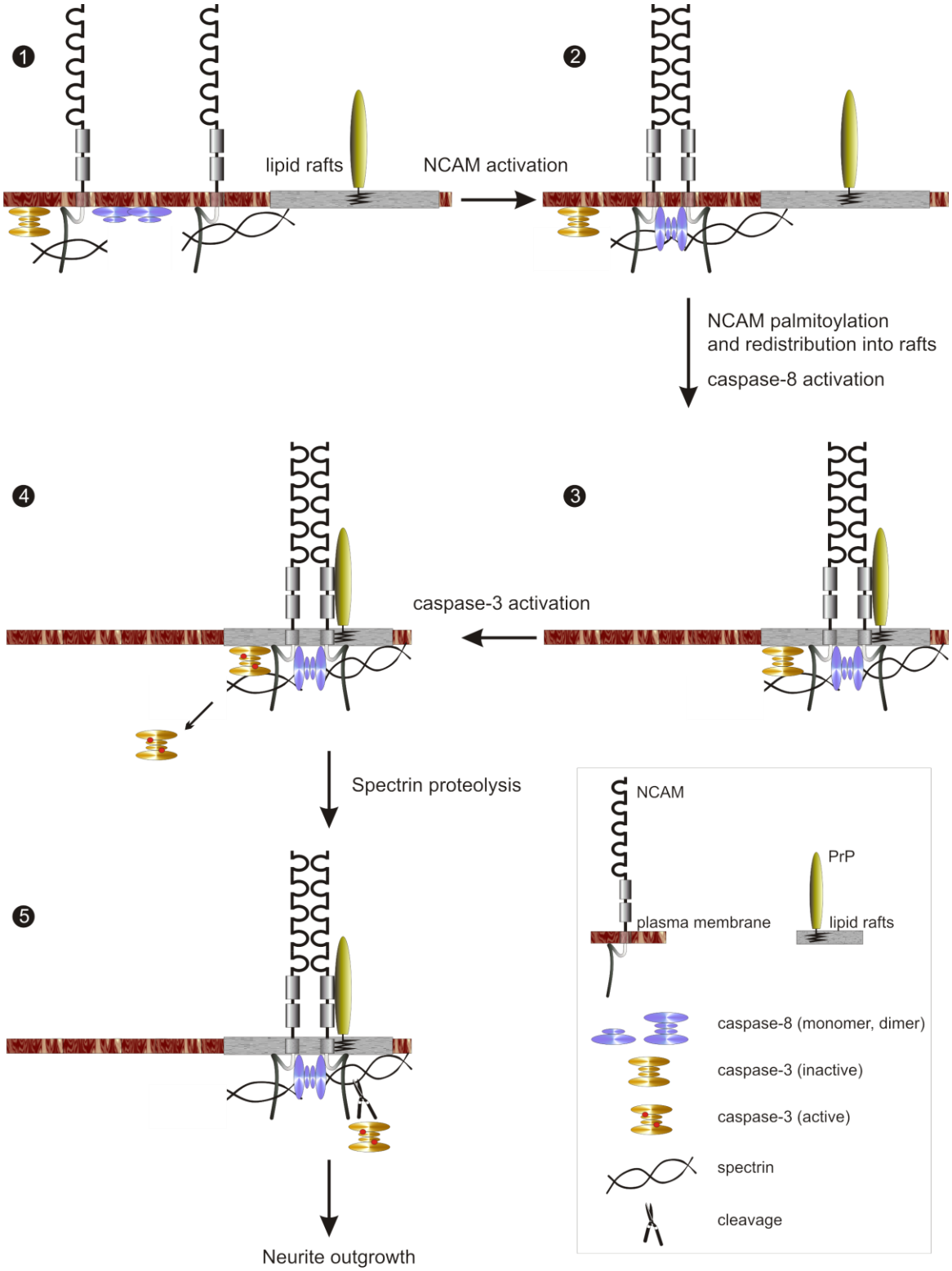

Fig. 21: Proposed model illustrating the activation of caspases-3 and -8 upon NCAM stimulation. NCAM stimulation (2) results in clustering of NCAM at the cell surface and in aggregation of caspase-8 which in turn leads to its activation. Upon stimulation NCAM is palmitoylated and the complex of NCAM and caspase-8 and in turn caspase-3 redistributes into lipid rafts (3). Within lipid rafts NCAM is stabilized by PrP. Activated caspase-8 activates caspase-3 which is released into the cytosol (4). Subsequently caspase-3 cleaves spectrin which leads to a rearrangement of the spectrin cytoskeleton which supports neurite outgrowth (5). See text for details.

VIII. ABBREVIATIONS

approx.	approximately
Asp	Aspartate
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumine
CA	Cornu ammonis
Calpain	Calcium dependent protease with papain like activity
CAM	Cell adhesion molecule
CaMKII	Calcium/calmodulin dependent protein kinase II
CARD	Caspase recruitment domain
Caspase	Cysteinyl dependent aspartate specific protease
CED	Cell death defective
CHO cells	Chinese hamster ovary cells
CHO	Aldehyde
Co.	Compagnie
Corp.	Corporation
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
ddH_2O	Bi-distilled water
DED	Death effector domain
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinase
et al.	*Et alii* (and others)
F3	Fibronectin type 3 like domain
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
Fc	Fragment crystallizable
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fig.	Figure
FNIII	fibronectin type 3 like domain
g	G-force

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell derived neurotrophic factor
GFRα	GDNF family receptor α
GPI	Glycosylphosphatidylinositol
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HRP	Horse radish peroxidase
IAPs	Inhibitors of apoptosis proteins
ICE	Interleukin-1β converting enzyme
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IP_3	Inositol 1,4,5-trisphosphate
LB	Luria Bertani
MAPK	Mitogen activated protein kinase
NCAM	Neural cell adhesion molecule
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NP-40	Nonidet™ P-40, Nonylphenyl-polyethylene glycol (Nonidet is a trademark of Shell Chemical Co.)
p.a.	*Pro analysi* (per analysis)
PAGE	Polyacrylamide gel electrophoresis
PAK1	P21-activating kinase 1
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline /TWEEN
PDL	poly-D-lysine
PDNF	Platelet derived neurotrophic factor
PDZ	PSD-95/Discs large/Z0-1
PFA	Paraformaldehyde
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC_γ	Phospholipase C_γ
PMSF	Phenylmethylsulphonyl fluoride
pnd	Postnatal day
PrP	Prion protein
PS	Phosphatidylserine
PSA	Polysialic acid
®	Registered trademark
RIPA	Radio immunoprecipitation assay
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean

SH2 domain	Src homology 2 domain
siRNA	Small interfering RNA
TAE	Tris acetate EDTA
TAG-1	Transient axonal glycoprotein-1
TBS	Tris buffered saline
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TIU	Trypsin inhibitor unit (1 TIU will decrease the activity of 2 trypsin units by 50 % where one trypsin unit will hydrolyze 1.0 μmole of N-α-benzoyl-DL-arginine p-nitroanilide (BAPNA) per min at pH 7.8 at 25°C)
Tris	Trishydroxymethylaminomethane
Triton® X-100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol (Triton is a registered trademark of Union Carbide Corp.)
TWEEN® 20	Polyethylene glycol sorbitan monolaurate (TWEEN is a registered trademark of Uniqema)
Tyr	Tyrosine
UV	Ultraviolet
VASE	Variable alternatively spliced exon

Units and prefixes were used according to the International System of Units (SI).

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X. DANKSAGUNG

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