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The neural cell adhesion molecule NCAM promotes maturation of the presynaptic endocytic machinery by switching synaptic vesicle recycling from AP3- to AP2-dependent mechanism in mice *(Mus musculus, strain C57Bl/6J).*

Dissertation

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I. Abstract/Zusammenfassung

The neural cell adhesion molecule NCAM promotes maturation of the presynaptic endocytotic machinery by switching synaptic vesicle recycling from AP3 to AP2-dependent mechanisms

Newly formed synapses undergo maturation during ontogenetic development via mechanisms that remain poorly understood. We show that maturation of the presynaptic endocytic machinery in central nervous system neurons requires substitution of the adaptor protein 3 (AP3) with AP2 at the presynaptic plasma membrane. In mature synapses, AP2 associates with the intracellular domain of the neural cell adhesion molecule NCAM. NCAM promotes binding of AP2 over binding of AP3 to presynaptic membranes, thus favoring the substitution of AP3 for AP2 during formation of mature synapses. The presynaptic endocytotic machinery remains immature in adult NCAM-deficient mice accumulating AP3 instead of AP2 in synaptic membranes and vesicles. NCAM deficiency or disruption of the NCAM/AP2 complex in NCAM+/+ neurons by transfection with a dominant-negative NCAM containing the mutated AP2 binding site leads to less efficient endocytosis of synaptic vesicle membranes. Abnormalities in synaptic vesicle endocytosis and recycling may thus contribute to neurological disorders associated with mutations in NCAM.

Das neurale Zelladhäsionsmolekül NCAM unterstützt den Reifungsprozess des präsynaptischen Endozytosemechanismus durch die Umstellung des synaptischen Vesikelrecyclings von einem AP3- zu einem AP2-abhängigen Prozess

Im Verlauf der ontogenetischen Entwicklung unterliegen neu geformte Synapsen einem Reifungsprozess durch bislang nur unzureichend aufgeklärte Mechanismen. Wir zeigen nun in der vorliegenden Arbeit, dass der Reifungsprozess des präsynaptischen Endozytosemechanismus in Neuronen des zentralen Nervensystems den Austausch des Adapterproteins 3 (AP3) durch das Protein AP2 an der präsynaptischen Plasmamembran erfordert. In ausgereiften Synapsen ist AP2 mit der intrazellulären Domäne des neuralen Zelladhäsionsmoleküls NCAM assoziiert. NCAM unterstützt somit die Bindung von AP2, aber nicht von AP3, an die präsynaptische Membran und begünstigt dadurch die Substitution von AP3 durch AP2 bei der Ausbildung reifer Synapsen. In adulten NCAM- defizienten Mäusen bleibt der präsynaptische Endozytosemechanismus jedoch unausgereift. Anstelle von AP2 wird AP3 in der synaptischen Membran und den Vesikeln akkumuliert. NCAM-Defizienz sowie die Zerstörung des NCAM/AP2-Komplexes in NCAM+/+ Neuronen durch die Transfektion eines dominant-negativen NCAM-Moleküls, welches eine mutierte AP2-Bindestelle enthält, führen zu einer weniger effizienten Endozytose der synaptischen Vesikelmembranen. Abnormalitäten in der Endozytose synaptischer Vesikel und im Recycling könnten daher zu neurologischen Störungen beitragen, die auf eine NCAM- Mutation zurückgehen.

II. Introduction

II.1. Neural cell adhesion molecule

The neural cell adhesion molecule (NCAM) is a cell surface glycoprotein abundantly expressed in the nervous system. NCAM was first discovered in retinal cells in the chick embryo (Thiery et al., 1977a). Although NCAM was initially characterized as a cell adhesion molecule, its role in cell signalling pathways classifies it more accurately as a signal transducing receptor molecule. Despite it somehow misleading name, NCAM is also expressed in non-neuronal cells like glial cells and myofibrils (Rieger et al., 1985). The presence of extracellular immunoglobulin (Ig) domains defines it as a member of the immunoglobulin superfamily of cell adhesion molecules (for general reviews on cell adhesion molecules and their classification, refer to (Chothia and Jones, 1997; Elangbam et al., 1997). NCAM mediates both homophilic and heterophilic adhesion and is a highly conserved molecule throughout the evolution of vertebrates (Hall and Rutishauser, 1985). In invertebrates, Fasciculin II is considered as the Drosophila homologue and SAX-3 (Sensory AXon guidance family member protein) is the nematode homologue of NCAM (Mendoza and Faye, 1999).

Mouse NCAM is encoded by a single gene consisting of 20 exons (Walmod et al., 2004). The full-length transcript encodes five Ig domains, two fibronectin III domains, a transmembrane region and a cytosolic domain. Alternative splicing generates four major isoforms with (apparently desialylated) molecular weights of 180, 140, 120 and 105 kDa (Figure 1). The longer transmembrane products are expressed in neurons (180 kDa, 140 kDa) and muscle cells (140 kDa) (Akeson et al., 1988), whereas the 120 kDa product is GPI-anchored on the surface of oligodendrites (Bhat and Silberberg, 1988) and the 110 kDa product is secreted (Hoffman and Edelman, 1983).

Further splice variation arises from a variable alternatively spliced exon (VASE) in the fourth Ig domain and a muscle-specific domain (MSD) between the two FNIII domains containing an O-glycosylation site (Santoni et al., 1989). Interestingly, although 27 different NCAM transcripts are expressed during cardiac

development, the molecular mechanism of their action is unclear (Reyes et al., 1991). Recent work has shown that vast isoform diversity of another cell adhesion molecule, dscam, is required for correct neuronal wiring (Schmucker, 2007). In depth study of NCAM isoforms is necessary to investigate whether NCAM is involved in similar mechanisms of development.

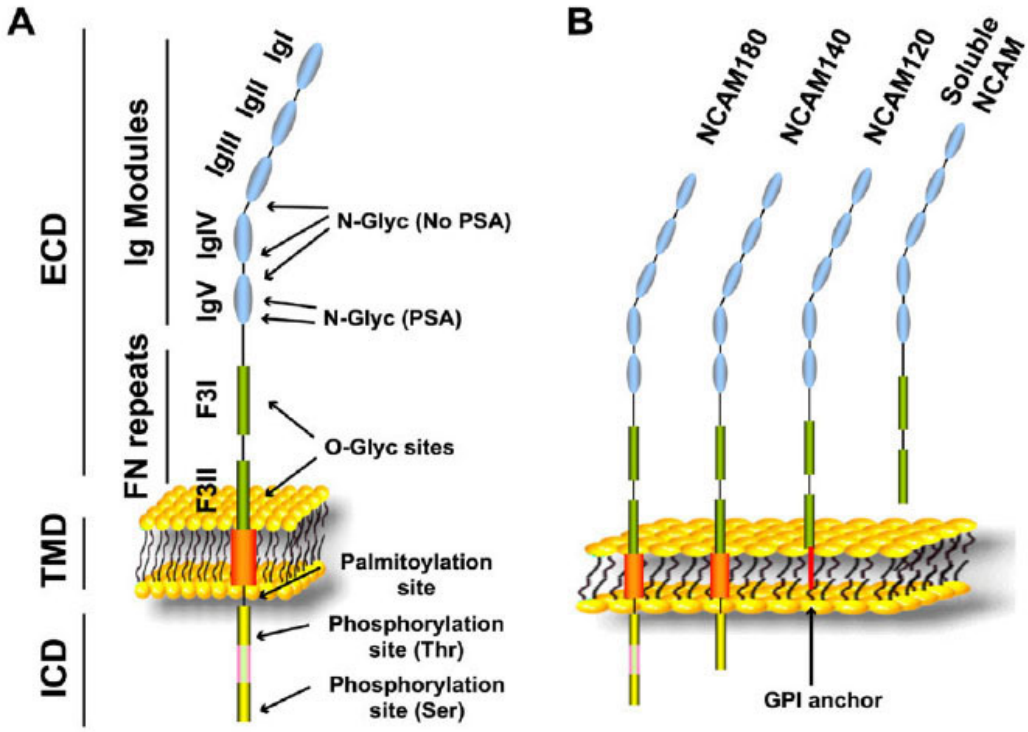

Figure 1. Schematic representation of NCAM and its main isoforms (Gascon et al., 2007). (A) The highly conserved extracellular domain (ECD) mediates cell adhesion while the intracellular region (ICD) interacts with several proteins including spectrin and receptor tyrosine phosphatase RPTPα (not shown). The different posttranslational modification sites including glycosylation, palmitoylation and phosphorylation are indicated. (B) NCAM140 and NCAM180 differ in 261 amino acids in the ICD as illustrated. [TMD: transmembrane domain, FN3I/II: fibronectin type 3 homology domain I and II, Ig I-V: immunoglobulin like domain I-V.]

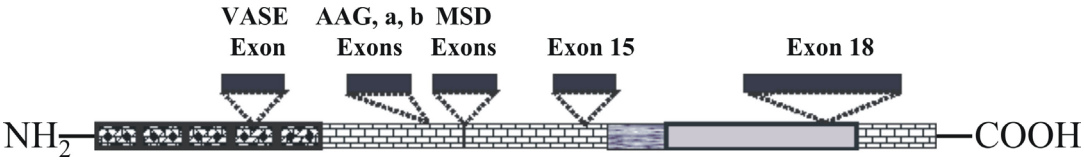

Figure 2. Schematic representation of the NCAM transcript with the alternatively spliced exons. The VASE exon consisting of 10 amino acids increases the adhesive property of NCAM (Chen et al., 1994) and is developmentally upregulated (Small and Akeson, 1990). MSD exon is expressed in NCAM present in muscle facilitating myoblast fusion. Insertion of exon 18 in the NCAM ICD gives rise to NCAM180. The role of exons AAG, a, b and exon 15 is less well understood.

The highly sialylated 140 kDa isoform of NCAM was the first to be characterized as a molecule involved in initial adhesion among neural cells (Thiery et al., 1977b). There are changes in the degree of polysialyation of NCAM (PSA-NCAM) with neural development. Removal of sialic acid from NCAM increases the adhesive properties of NCAM (Rutishauser et al., 1985). Sialyation of NCAM increases with histogenesis of the central nervous system. The decreased adhesive function of NCAM with a high content of polysialic acid provides more plasticity in cell interactions during cell migration, axon outgrowth, and formation of neural circuits (Sunshine et al., 1987). With the progression of neural development, polysialyation of NCAM is reduced. The embryonic to adult conversion of NCAM by the removal of polysialyation occurs at different time points in different brain regions. It is hypothesized that both the embryonic to adult conversion of NCAM and the temporally regulated tissue distribution of NCAM are related to early regulation of neuronal pattern formation and connectivity. NCAM with a low content of polysialic acid functions both to maintain integrity of neuroepithelium during morphogenesis of the early embryo and to stabilize differentiated structures in the adult.

II.1.1. NCAM in disease and pathology

Anti-NCAM antibodies block fasciculation (Rutishauser, 1985) and inhibite neurite outgrowth (Neugebauer et al., 1988; Drazba and Lemmon, 1990) indicating an important role for NCAM during neural development. Hence involvement of NCAM in the etiology of diseases is not surprising (Plioplys et al., 1990; Arai et al., 2004; Sandi and Bisaz, 2007) and has been shown for other cell adhesion molecules

like neuroligins (Comoletti et al., 2004; Tabuchi et al., 2007) and cadherins (Senzaki et al., 1999; Bray et al., 2002). Accordingly, NCAM levels in blood serum of autistic patients are decreased and increased in schizophrenic patients (Plioplys et al., 1990). Interestingly, stressing juvenile mice results in increased conversion of NCAM to PSA-NCAM (polysialylated acid NCAM), contrary to decreased PSA-NCAM expression levels during normal neuronal development (Tsoory et al., 2007). This observation implicates NCAM in depression and anxiety disorders, as childhood trauma is a predisposition to mood disorders. NCAM is also downregulated under demyelinating conditions in mouse mutants, suggesting a role of NCAM in myelination (Bhat and Silberberg, 1990). A recent report shows that a reduction in the levels of PSA on NCAM in mature oligodendrocytes is required for efficient myelination (Fewou et al., 2007). However, the molecular details of how PSA can maintain myelination have not been resolved.

Interestingly, NCAM is expressed on all brain tumours and neuroblastomas and majority of multiple myelomas (Bourne et al., 1991; Tassone et al., 2004). Since NCAM is a cell adhesion molecule, its role is investigated in tumor metastasis (Johnson, 1991). Increased PSA level is shown to correlate with metastasis formation and tumor progression (Suzuki et al., 2005). In line with this observation, removal of PSA results in inhibition of tumor growth and tumour cell differentiation (Seidenfaden et al., 2003). Strong efforts are being made to exploit NCAM as a tumor associated antigen for immunotherapy based on the accumulating evidence for the role of NCAM in tumor formation and progression (Jensen and Berthold, 2007).

II.1.2. NCAM in learning and memory

An exciting discovery was the involvement of NCAM in learning and memory. The role of NCAM in synaptic plasticity was first discovered in Aplysia, a marine mollusc, in 1992 (Mayford et al., 1992). Injection of NCAM antibodies during memory consolidation results in amnesia (Rose, 1995). In the early 1990s, after the generation of the NCAM deficient mouse, evidence started to accumulate that NCAM also plays a role in intracellular signaling by initiating a molecular signaling cascade. Mice lacking the three membrane-bound isoforms of NCAM show deficits in learning and memory. The NCAM deficient (NCAM-/-) mice are

impaired in spatial learning as concluded from tests in the Morris water maze (Cremer et al., 1994). In this behavioural test, the mice or rats are placed in a water pool surrounded by visual cues. In order to escape from the pool, they have to learn to find a hidden platform in the water pool based on these visual cues. The speed at which mice learn how to find the hidden platform is a measure of their spatial memory and learning abilities (Morris, 1984).

The role of NCAM in learning is further reiterated by the observation that learning is impaired after infusion of NCAM antibodies (Arami et al., 1996). Long term potentiation (LTP) is impaired in the hippocampus on the addition of antibodies against NCAM (Luthl et al., 1994). LTP is the enhancement of electrical signaling in synapses that results from repetitive stimulation of neurons. Molecular changes at the synapse leading to LTP are considered the basis of learning and memory. In other words, plasticity of the synapse is thought to result in the formation of learning (Bliss and Lomo, 1973; Sweatt, 1999; Lomo, 2003).

Interestingly, it was shown that the alpha-2,8-linked polysialic acid (PSA) on NCAM in the hippocampus is also involved in learning (Murphy et al., 1996; Venero et al., 2006). Although polysialyation of NCAM decreases in the adult, in some areas of the brain, like the hippocampus, it remains prominent. When hippocampal slices were treated with endo-neuraminidase (endo-N, an enzyme that specifically cleaves PSA), electrical stimulation failed to elicit long term potentiation (LTP) (Becker et al., 1996). Since PSA-NCAM is less adhesive than NCAM, a balance of the two molecules could influence synaptic plasticity.

Interestingly, some forms of LTP in the NCAM-/- mice can be rescued with the application of brain-derived neurotropic factor (BDNF) (Muller et al., 2000). BDNF triggers intracellular signaling by phosphorylation of its receptor, TrkB. Levels of phosphorylated TrkB are reduced in the NCAM-/- mice suggesting a modulatory role of NCAM in BDNF induced signaling. BDNF modulates synaptic activity by eliciting an increase in calcium concentration (He et al., 2005). Interestingly, NCAM derived peptides added to neuronal cultures to mimic homophilic interaction of NCAM induces an increase in calcium levels intracellularly (Kiryushko et al., 2006).

NCAM can increase intracellular calcium levels by the modulation of two separate signalling pathways (Figure 3). The first pathway is associated with activation of fibronectin growth factor receptor (FGFR), phospholipase Cγ, and production of diacylglycerol. All three isoforms of NCAM interact with FGFR on the cell surface (Sanchez-Heras et al., 2006) and can modulate FGF mediated signalling (Anderson et al., 2005; Francavilla et al., 2007). The second pathway that increases intracellular calcium is when NCAM recruits a receptor tyrosine phosphatase (RPTPα) which dephosphorylates a Src-family kinase, (p59 fyn) leading to neurite outgrowth (Bodrikov et al., 2005). Identifying the downstream components following NCAM association with FGFR or tyrosine phosphatase could lead to understanding the role of NCAM in memory and learning.

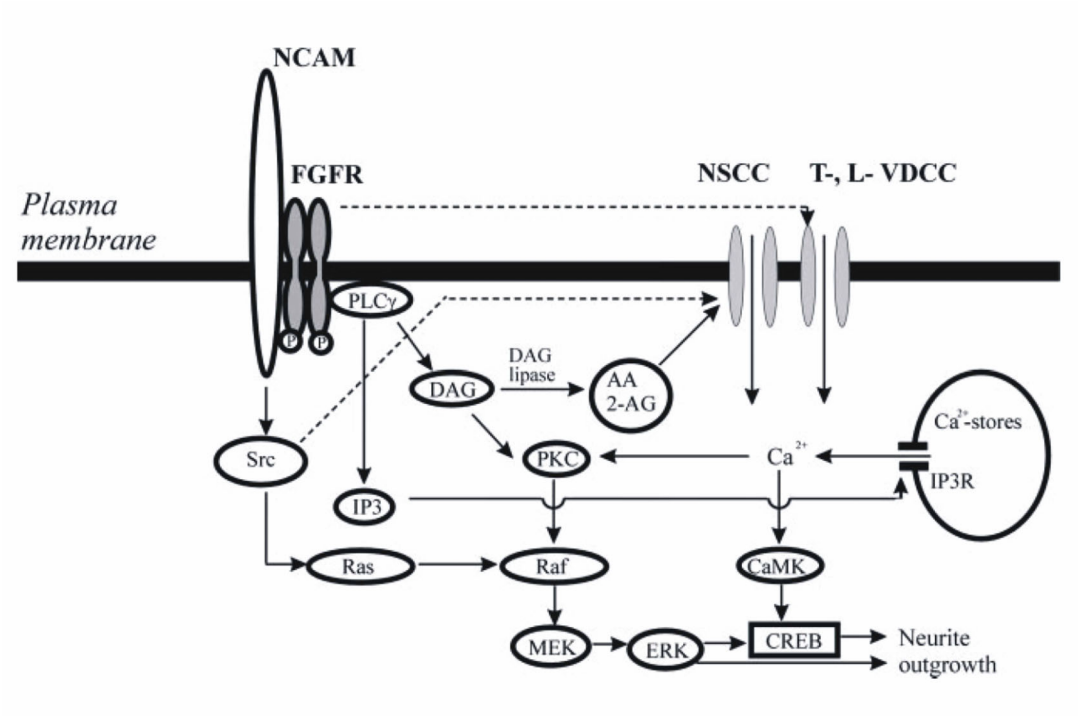

Figure 3. NCAM interacts with FGFR leading to the transduciton of two signaling pathways involving both diacylglycerol production and activation of *Src* kinase pathway leading to neurite outgrowth (Kiryushko et al., 2006).

II.2. NCAM deficient mice

The development of techniques to create specific genetic deletions in mammals opened up an additional approach to elucidate the function of NCAM. The first NCAM deficient mouse to be generated had a deletion in the gene encoding the NCAM180 isoform (Tomasiewicz et al., 1993). The most obvious

phenotype of this mutant is reduced olfactory bulbs and enlarged ventricles in the brain. The granular cells forming one of the layers of the cerebellar cortex are reduced in number and disorganized. In 1994, a mouse model deficient in the three major NCAM isoforms was generated (Cremer et al., 1994). The loss of all NCAM isoforms resulted also in a 10% decrease in brain weight with 36% decrease in the size of olfactory bulbs.

This severe reduction of olfactory lobes was traced to deficiency in cell migration. Neuronal precursors migrate through the forebrain into the olfactory bulb along a well-defined pathway, the rostral migratory stream. In NCAM-/- mice, there is an accumulation of precursor cells in the rostral migratory stream (Cremer et al., 1994). It was seen that the migratory environment for the precursor cells was drastically altered in NCAM-/- mice. In the mutant rostral migratory stream, there is massive accumulation of myelinated axon profiles along with a loss of fasciculation (Chazal et al., 2000). In addition, there is an accumulation of astrocytes in the migratory pathway. These observations lead to the speculation that the loss of NCAM results in perturbation of neuro-glial interactions between the precursors and the environment causing inhibition of migration.

Supporting the role of NCAM in learning, NCAM-/- mice also performed significantly worse than the controls in the Morris water maze. In addition to their learning disabilities, these mice are also less exploratory (Cremer et al., 1994). Interestingly, when fyn kinase, which is now known to be downstream of NCAM mediated signaling, is deleted, a similar behavioural phenotype is exhibited (Miyakawa et al., 1994). NCAM-/- male mice also show increased aggression to an intruder male which is attributed to increased corticosterone levels (Stork et al., 1997). Overexpression of NCAM180 in the NCAM-/- mice rescued the aggressive behaviour indicating that NCAM180 is essential for modulation of aggression (Stork et al., 2000). NCAM-/- mice also exhibits problems associated with locomotor abilities when tested on rotorods at very high speeds (Polo-Parada et al., 2001). In the rotorod test, mice are placed on rods rotating at different speeds. The time the mice can remain on the rotating rod is used as a parameter for locomotor activity. The locomotor defect observed in the NCAM-/- mice is attributed to the inefficient synaptic activity at neuromuscular junction.

II.3. NCAM at the neuronal synapse

New synapses are formed when a migrating axon makes a contact with a dendrite. Growing neurites form a dynamic structure at the tip called the growth cone, which finally finds the right cues and forms a synapse. NCAM is involved in forming new synaptic connections (Mayford et al., 1992; Dityatev et al., 2000; Dityatev et al., 2004; Gascon et al., 2007). NCAM stabilizes transgolgi network (TGN) derived cargo organelles at nascent synapses aiding synaptogenesis (Sytnyk et al., 2002). Studies with hippocampal cultures indicate that postsynaptic NCAM promotes synapse formation (Dityatev et al., 2000). A recent study shows that NCAM assembles the postsynaptic density (PSD) by recruiting spectrin to the PSD. In the PSD, NCAM forms a complex with N-methyl D-aspartate (NMDA) receptor. Activation of NMDA receptor results in recruitment of calcium calmodulin kinase IIα (CaMKIIα) and NCAM assists in this recruitment by forming the PSD complex (Sytnyk et al., 2006).

Ultrastructural analysis of hippocampal synapses show the presence of NCAM both postsynaptically and presynaptically (Schuster et al., 2001). The levels of presynaptic NCAM doubles in the active zone on learning, indicating that NCAM increases synaptic adhesion, and probably leads to memory consolidation (Skibo et al., 1998). The presynaptic function of NCAM in the CNS remains poorly investigated and has been better characterized mostly in the neuromuscular junction (NMJ). The NMJ is a specialized contact point of a skeletal muscle with the peripheral motorneuron terminal. The axonal nerve end forms a pre synaptic terminal, which on activation releases the neurotransmitter acetylcholinevia the fusion of synaptic vesicles. The neurotransmitters bind to receptors present at the motor end plate, the muscle side of the junction that mirrors the presynaptic region. Initial studies in the Drosophila showed that the fasciculin II (Drosophila homologue of NCAM) is important for NMJ stability and size (Schuster et al., 1996a, b). This decrease in size of the NMJs is also observed in NCAM deficient mice.

The neurotransmission parameters in the NCAM null mouse NMJ have been extensively characterized electrophysiologically (Rafuse et al., 2000; Polo-Parada et al., 2001). The “quantal content” (number of vesicles released per stimulus i.e. EPP/MEPP) of NCAM null NMJ is higher. Additionally, the time

required for exocytosis and endocytosis to form vesicles is more rapid in the absence of NCAM. . FM dye uptake experiments on the NCAM-/- NMJs showed endocytosis and exocytosis sites were not limited to the presynaptic terminal but also extended to the preterminal axon (Polo-Parada et al., 2001). This loss of dye along the preterminal axon accounts for the more rapid rates of exocytosis seen in the NCAM-/- NMJs. Despite these observations, the NCAM-/- NMJs showed transmission failure on repetitive electrical stimulus at higher frequencies.

Immature motoneurons release neurotransmitter all along the axon which on maturation is restricted to the presynaptic termini (Chow and Poo, 1985). Polo–Parada and co-workers (Polo-Parada et al., 2001) showed that NCAM null NMJ utilize an immature mode of vesicle recycling in adult NMJs. Brefeldin A (BFA), a fungal metabolite inhibits the process of neurotransmitter release in immature axons but has no effect on mature synapses (Zakharenko et al., 1999). When NMJs from NCAM null mice are pretreated with BFA, FM dye loading probably due to AP3 dependnet endocytosis is inhibited suggesting the retention of the immature mode of endocytosis. The same group also demonstrated that in the absence of NCAM release of neurotransmittors at the NMJs were dependent on a different sub class of calcium channels (Figure 4).

The studies done on the NCAM-/- NMJs indicated that in the absence of NCAM, there is a problem in vesicle mobilzation. Since the immature synaptic vesicle machinery persists in the absence of NCAM, the synaptic vesicles formed after the depletion of the readily releasable pool could be “incompetent”, which would explain the transmission failures seen in electrical conduction on repetitive stimulation of the NCAM-/- NMJs (Polo-Parada et al., 2001).

Synaptic vesicles are made competent for fusion by ATP dependent priming of specialized presynaptic proteins: SNARE (soluble N-ethylmaleimide- sensitive factor attachment protein receptors) and munc family of proteins (Klenchin and Martin, 2000; Martin, 2002; Sudhof, 2004). SNARE protein complexes are responsible for the mechanistic fusion of the vesicle with the plasma membrane. In the absence of NCAM, vesicle fusion and formation seems to occur even faster than normal. Therefore, it is unlikely that NCAM is involved in the structural or mechanistic aspect of endocytosis. A possibility is that synaptic vesicles are “incompetent” in the absence of NCAM because they do not have the right

complement of proteins required to "prime" them. It has already been seen that in the absence of NCAM, the synaptic targeting on SV2, a mature synaptic vesicle protein is delayed (Rafuse et al., 2000).

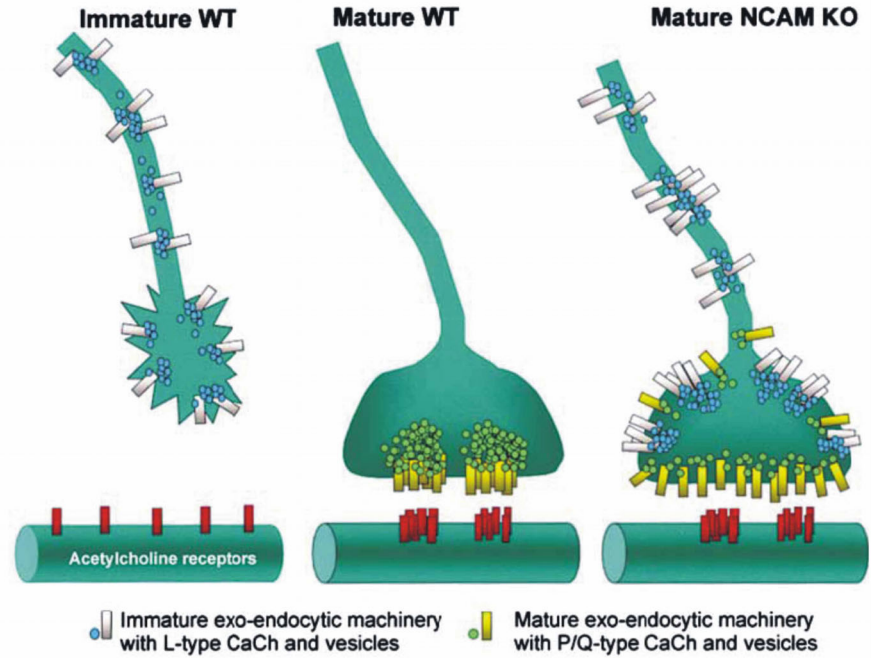

Figure 4. Development at the NCAM deficient NMJ (neuromuscular junction). During development, synaptic vesicle release occurs along the axon and growth cone. On making contact with the muscle fiber, the immature release mechanism along the axon disappears and is replaced by the mature exo-endocytic machinery at the presynaptic termini. In the NCAM-/- NMJ, the immature machinery persists in the axon leading to the presynaptic termini (Polo-Parada et al., 2001).

These observations in the NCAM null NMJs by Polo-Parada et al (2001) were the first indication that NCAM is involved in the process of synaptic vesicle recycling. A possibility is that synaptic NCAM could target proteins required for mature synaptic vesicle recycling during development. In order to understand this potential role of NCAM better, a brief overview of the process of synaptic vesicle recycling is necessary.

II.4. Synaptic vesicle recycling

The process of "retrieval" or "recycling" of synaptic vesicles by endocytosis after fusion at the presynaptic membrane is termed as synaptic vesicle recycling (Figure 5). This process is necessary to maintain equilibrium of membrane area at the active zone after endocytosis of synaptic vesicles. Synaptic vesicle recycling replenishes vesicles which are depleted after stimulation (Model et al., 1975). If this process is inhibited, the synapse is "fatigued" on further stimulation as no further synaptic vesicles are available for fusion resulting in the synapse failing to respond (Ceccarelli et al., 1972).

More than 30 years ago, biochemical experiments with synaptosomes suggested that after exocytosis, a subpopulation of vesicles associated with the active zone undergo endocytosis and re-fill with neurotransmitters rapidly (Fried and Blaustein, 1978). How fast synaptic vesicle endocytosis happens after exocytosis is a critical question. This question was addressed directly in the calyx of Held, the largest synaptic terminal in the central nervous system (CNS) using capacitance measurements. Membrane capacitance studies take advantage of the fact that membrane capacitance is directly proportional to surface area of the membrane. These studies along with others (Morgan et al., 2001; Sun and Wu, 2001; Sun et al., 2002) showed that endocytosis following vesicle fusion can be within 1 s or can be slower taking up to 30 s, leading to the conclusion that there is more than one mode of vesicle recycling.

Ceccarelli and colleagues demonstrated at the frog neuromuscular junction that vesicles endocytose and recycle rapidly without a clathrin-coated intermediate (referred to as kiss-and-run because the vesicles did not remain attached to the active zone). Contradictorily, at the same time, Heuser & Reese described that extensive stimulation of the same frog neuromuscular junctions causes vesicles to endocytose via parasynaptic cisternae and coated pits (endosomal recycling) (Ceccarelli et al., 1973; Heuser and Reese, 1973). On the basis of these observations and subsequent studies, at least three vesicle recycling pathways are proposed: two fast pathways in which the vesicles either remain at the active zone for refilling (kiss-and-stay) or are recycled locally without clathrin-mediated endocytosis (kiss-and-run), and a slower pathway that involves clathrin-mediated endocytosis. The speed of endocytosis measured by Sun and colleagues implies that

vesicle recycling is very rapid, even for the slow component of endocytosis (Sun et al., 2002). Gandhi & Stevens confirmed multiple modes of recycling using fluorescence methods. They demonstrated that after a vesicle fuses at the presynaptic terminus, it could be retrieved within 400-860 ms by the kiss-and-run mode; slower within 8-21 s via the clathrin dependent mode; or a vesicle can be 'stranded' until a nerve impulse triggers its retrieval. Synapses with high release probability utilized the clathrin-mediated mode where as those with low release probability utilized the 'kiss-and-run' mode (Gandhi and Stevens, 2003). After retrieval, not all synaptic vesicles are immediately ready for fusion. Only 10-20% of the total pool of synaptic vesicles forms the recycling pool (Rizzoli and Betz, 2005).

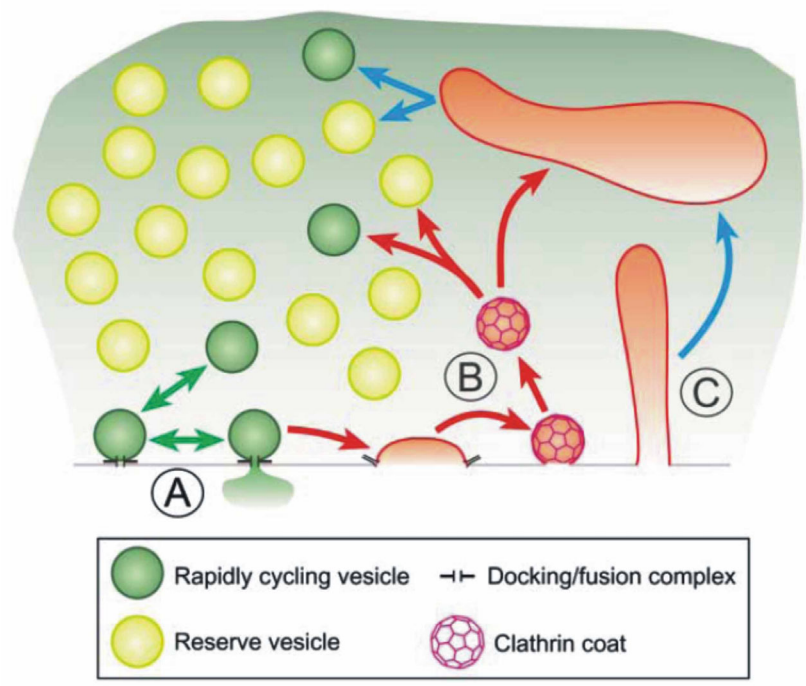

Figure 5. Modes of synaptic vesicle recycling. (A) The "Kiss and run" mode with a transient pore formation. (B) Clathrin mediated endocytosis involving the formation of clathrin coated vesicles. (C) Membrane invaginations forming the endosomes (Matthews, 2004).

The biogenesis of new synaptic vesicles is from endosomal compartments (Kelly et al., 1993; Mundigl et al., 1993) but whether there is an intermediate endosomal compartment during recycling is not clear. Clearly, most vesicles recycle directly without passing through an endosomal intermediate. However,

multiple lines of evidence now unequivocally establish that the endosomal pathway is physiologically relevant for synaptic vesicle recycling under some conditions.

Firstly, purified synaptic vesicles contain as a stoichiometric component (i.e., a component present on all vesicles and not only a small subset) the SNARE protein Vti1aβ (Antonin et al., 2000). Vti1aβ is a neuronal splice variant of the ubiquitous SNARE protein Vti1a, which, like other SNARE proteins, functions in membrane fusion. Vti1a participates in fusion reactions involving endosomes and the trans-Golgi network but not the plasma membrane. Its presence on all synaptic vesicles implies that all vesicles at one point fuse with intracellular membranes and not only the plasma membrane.

Rab5 is an obligatory synaptic vesicle component. Rab5 is also a ubiquitous protein involved in endosomal fusion and recruits effecter proteins such as phosphatidylinositol-3-kinases and rabenosyn to transport vesicles destined to fuse into endosomes. The high concentration of Rab5 on synaptic vesicles suggests a function in endosome fusion during the vesicle cycle. This suggestion is supported by the finding that Rab5 mutations interfere with the efficacy of neurotransmittor release and overexpression of Rab5 results in extended endosomes at the presynaptic area (Wucherpfennig et al., 2003). This is the next evidence to suggest that vesicles go through an intermediate endosomal compartment during recycling. Thirdly, the absence of endosomes from most nerve terminals at steady state is not surprising considering the transient nature of endosomal organelles. For example, in Drosophila neuromuscular junctions, endosomes were revealed as an obligatory component of the vesicle pathway in all synapses using endosome-specific green fluorescent protein (GFP)-labeled markers, even though regular electron microscopy did not detect them easily (Sweeney and Davis, 2002; Wucherpfennig et al., 2003). Next, pharmacologic inhibition of endosome fusion in frog neuromuscular junctions using phosphatidylinositol 3-kinase blockers potently impaired neurotransmitter release and depleted synaptic vesicles (Rizolli et al., 2002).

A more direct functional evidence for endosomal involvement in vesicle recycling comes from a recent paper on the recycling of a vesicle glutamate transporter VGLUT1 (Voglmaier et al., 2006). It was observed that when VGLUT1 recycling via clathrin and a clathrin adaptor protein, AP2 was blocked, VGLUT1

recycled via a pathway that is dependent on another adaptor protein, AP3. This is an adaptor protein which is involved in the formation of vesicles from the endosomes (Horng and Tan, 2004; Peden et al., 2004). Further, Rizzolli and colleagues showed that at least a fraction of recycling synaptic vesicles fuse with the early endosomal compartment in vitro (Rizzoli et al., 2006). Voglmaier and colleagues suggest that the AP3 dependent endosomal pathway is "compensatory". Is the slower AP3 mediated recycling mode for resorting and repackaging of synaptic proteins before every new cycle of release necessary? What is clear is that the AP3 mode is not the predominant recycling pathway and most of the synaptic vesicle recycling is via the clathrin-mediated pathway.

II.4.1. Clathrin mediated vesicle recycling

The nerve terminal is endowed richly with proteins that specifically function in clathrin-mediated endocytosis (Brodin et al., 2000; Slepnev and De Camilli, 2000). Although there are different modes of synaptic vesicle recycling, it is now accepted that clathrin mediated mode is the predominant one at the central nervous system synapses (Morgan et al., 2002; Granseth et al., 2006). The Heuser and Reese model showed that on repetitive stimulus of the NMJ, there is depletion of synaptic vesicles and formation of new electron dense coated vesicles away from the active zone presynaptically (Heuser and Reese, 1973). Clathrin is recruited at the synaptic membrane via adaptor protein, AP2 to initiate budding (Keen et al., 1991; Prasad and Keen, 1991; Gonzalez-Gaitan and Jackle, 1997). The initiation of vesicle budding at specialized endocytic zones is followed by clathrin coat recruitment, vesicle fission and coat uncoating (Slepnev and De Camilli, 2000). AP2 has been shown to interact with synaptic vesicle proteins like synaptotagmin and synaptojanin (Zhang et al., 1994; Haffner et al., 2000; Haucke et al., 2000); and this interaction is shown to be regulated by phosphoinositides (De Camilli et al., 1996; Cremona and De Camilli, 2001). There are several other proteins, which are involved in regulating this process present at the synapse.

Particularly striking is the nerve terminal abundance of proteins that are dedicated to accelerating clathrin-mediated endocytosis, consistent with the relatively fast time constants observed in capacitance measurements (Cochilla et al., 1999). Synaptojanin is a synaptic phosphatase which hydrolyzes

phosphatidylinositol-4,5-bisphosphate (PI(4,5)P), regulating clathrin coat assembly and disassembly (Cremona and De Camilli, 2001; Di Paolo et al., 2004). Endophilin, an SH3 (src homology-3) domain containing protein, targets synaptojanin to synaptic vesicles (Schuske et al., 2003; Verstreken et al., 2003). Endophilin is a lipid curvature modifying protein that is thought to promote synaptic vesicle formation (Schmidt et al., 1999). The SH3 domain on endophilin recruits synaptojanin and another important protein involved in clathrin mediated endocytosis called dynamin (Gad et al., 2000). Dynamin is a GTPase (Guanosine triphosphatase) which mechanically twists clathrin coated pits free from the endocytic zones (Roux et al., 2006).

In spite of the evidence accumulating over the past years emphasizing the role of clathrin mediated synaptic vesicle recycling, it is known that this is not the only pathway that exists. There have been indications of synaptic vesicle recycling occurring independent of clathrin. “Kiss and run” mode of recycling occurs without the formation of the clathrin coat (Ceccarelli et al., 1973). Disruption of synaptic protein synaptophysin and dynamin, a protein involved in endocytosis resulted in blocking clathrin independent retrieval of vesicles (Daly et al., 2000).

As mentioned before, synaptic vesicles are also formed independent of clathrin and AP2 via another adaptor protein AP3 from endosomes (Shi et al., 1998) (Figure 6). AP3 mediated recycling at the synapse is shown to be required for the full recovery of at least one protein, VGLUT1 (Voglmaier et al., 2006). The AP3 dependent pathway of vesicle recycling is not as well characterized as the AP2/clathrin mode. Following is a brief overview of the AP2 dependent and AP3 dependent pathways of synaptic vesicle recycling.

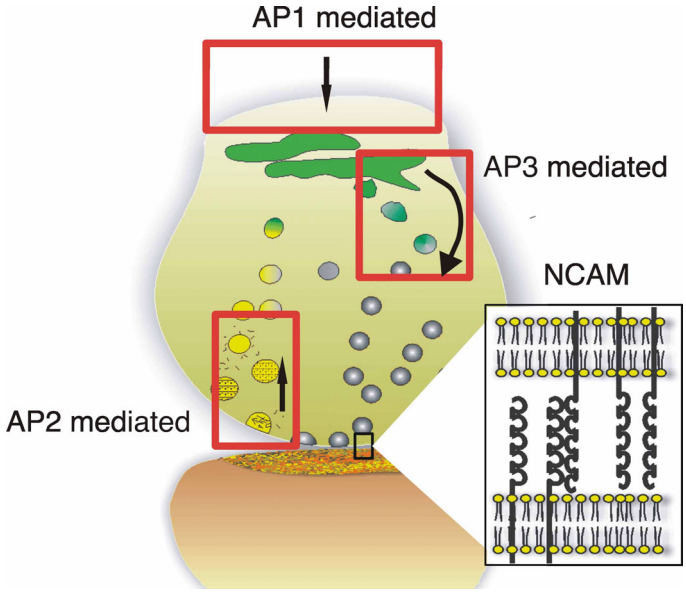

Figure 6. Synaptic vesicles formation is mediated via different adaptor proteins from distinct sub-cellular compartments. AP2 mediates clathrin coated vesicular endocytosis from the synaptic membrane whereas AP1 coated vesicles are trafficked from the Golgi network to the endosomal compartment. AP3 has been shown to be involved in the formation of synaptic vesicles from the endosomes in PC12 cells. **NCAM present at the synapse affects vesicle recycling in neuromuscular junctions.**

II.4.1a. Clathrin and clathrin adaptor AP2

Clathrin was for the first time obtained from coated vesicles (Pearse, 1976). Later it was seen that hexagonal lattice forming the coat in membrane vesicles is actually clathrin (Kartenbeck, 1978). This discovery of clathrin as a coat protein was followed by characterization of the molecule (Nandi et al., 1980) and the assembly process of the coat (Ungewickell and Branton, 1981). It was seen that several subunits of clathrin bind to membranes in hexagons or pentagons to form a coated vesicle (Unanue et al., 1981). Each monomer of the clathrin lattice is made up of three heavy chains and three light chains (Kirchhausen and Harrison, 1981) to form a triskelion or spider like structure. The first time clathrin was detected in the synaptic region, it was thought to contribute to the structural framework of the synaptic junction (Cheng and Wood, 1982). A 50 kDa polypeptide was isolated along with clathrin from clathrin coated pits that could be readily phosphorylated (Keen et al., 1979). Characterization of this polypeptide indicated that it was a subunit of a clathrin assembly or adaptor protein (AP). The first adaptor protein that was discovered was AP2 (Keen, 1987; Keen et al., 1987) .

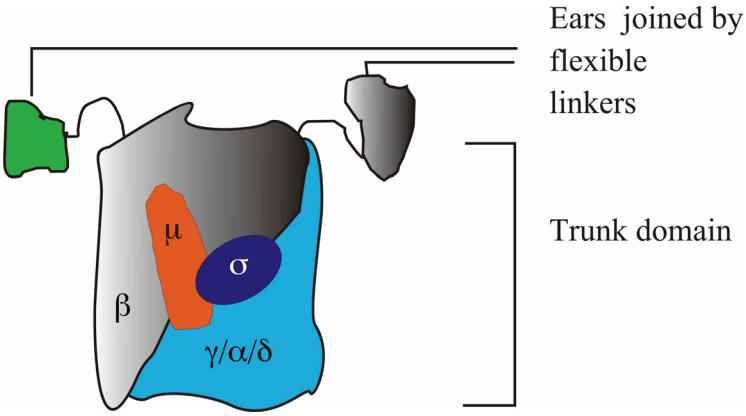

Figure 7. Schematic representation of a heterotetrameric adaptor protein. AP1, AP2 and AP3 all have two large subunits, a medium sized subunit and small subunit that associate to form the functional protein complex of around 300 kDa. AP1 is a complex of subunits δ, β1, μ1 and σ1; AP2 of α, β2, μ2 and σ2; and AP3 of δ, β3, μ3 and σ3. The ears along with the linker usually contain binding motifs for accessory proteins and coat proteins whereas the trunk recognizes cargo proteins or phospholipids at the membrane surface.

Adaptor proteins consist of at least four heterotetrameric members and several monomeric members. All the heterotetrameric adaptors look like " Mickey mouse" under the electron microscope with a large central core forming the "head" joined by flexible linkers to small "ears" (Heuser and Keen, 1988).

The adaptor proteins AP1, AP2, AP3 and AP4 all have 2 large subunits of ~100 to 130 kDa (γ/β1, α/β2, δ/β3, ε/β4 in AP1, AP2, AP3 and AP4 respectively), a medium sized subunit of ~50 kDa (different isoforms of μ) and a small subunit of 17 to 20 kDa (different isoforms of σ) (Robinson, 2004). The large subunits can be divided into a trunk domain, which forms the core, an appendage that forms the ears and a flexible hinge domain which join the trunk and the appendage (Kirchhausen et al., 1989) (Figure 7). Along with the trunk domains of the large subunits, the core is made up of the medium and the small subunits (Collins et al., 2002).

AP2 is the best characterized adaptor protein in mediating clathrin coated vesicles which binds to both the protein cargo and plasma membrane head groups (Gaidarov et al., 1996). The interaction of the trunk domain of α with phosphatidylinositol-4,5-bisphosphate (PIP2) or phosphatidylinositol-3,4,5-

trisphosphate (PIP3) headgroups is thought to cause recruitment of AP2 to the plasma membrane (Gaidarov et al., 1996).

The accepted model for clathrin mediated vesicle endocytosis begins with coat assembly initiated by the oligomerization of AP2 followed by the recruitment of clathrin. AP2 interacts with clathrin via a clathrin box containing LϕD/EϕD/E (Leu-ϕ-Asp/Glu-ϕ-Asp/Glu , where ϕ is a bulky hydrophobic residue) on β2 hinge (ter Haar et al., 2000). With addition of clathrin units, a clathrin coated pit is formed which invaginates to form a clathrin coated vesicle. This vesicle undergoes fission with the help of dynamin to release a clathrin coated vesicle into the presynaptic terminus. The clathrin coated vesicle is a transient structure which is uncoated rapidly (Slepnev and De Camilli, 2000).

Coat assembly at the synaptic membrane is initiated by phosphoinositides. An adenosine diphosphate ribosylation factor, ARF6 activates synaptically enriched phosphotidyl inositol phosphate kinase (PIPKIγ) which results in binding of AP2 to phosphoinositides at the membrane (Krauss et al., 2003). However, since the nucleation of clathrin assembly occurs at specific “endocytic hotspots” close to the active zone, it is postulated that AP2 must have a more specific presynaptic receptor. Synaptotagmin, a multifunctional synaptic membrane protein binds to AP2, indicating a role in regulating synaptic vesicle recycling. Since synaptotagmin binds to a variety of effector molecules including acidic phospholipids, phosphoinositides, SNAREs and calcium channel, it could function as a scaffold or linker molecule in the synaptic endocytic pathway (Grass et al., 2004). However, it is unclear if synaptotagmin is responsible for the initial recruitment of AP2 to the presynaptic membrane leaving open the possibility for other synaptic transmembrane proteins. This is supported by the observation that binding of synaptotagmin to AP2 is stimulated by peptides with specific endocytic motifs (Haucke and De Camilli, 1999).

Most of the transmembrane cargo proteins recognized by AP2 have an endocytic peptide motif, either a dileucine motif (Asp/Glu-X-X-X-Leu-Leu/Ile, D/EXXXLL/I) or a tyrosine motif (Tyr-X-X-ϕ, YXXϕ where ϕ is a bulky hydrophobic amino acid).

II.4.1b. AP180 is an accessory protein involved in clathrin/AP2 mediated synaptic vesicle recycling.

AP180 is an adaptor protein by definition as it can promote clathrin assembly and acts as an accessory protein in recruiting clathrin to the membrane (Ahle and Ungewickell, 1986). It is a brain specific protein enriched in nerve terminals and co-purifies with clathrin. AP180 was discovered independently by several groups and called NP185, phosphoprotein F1-20, AP-3 and 155K protein till it was established that all of them are identical (Murphy et al., 1991). Unlike AP2, AP180 is a monomeric protein with a highly acidic domain, a property that causes the 100 kDa protein to run aberrantly at 180 kDa on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). AP180 is the functional homologue in neurons of the ubiquitously present clathrin assembly lymphoid myeloid leukemia protein (CALM) (Tebar et al., 1999). AP180 binds to clathrin via a clathrin box present at the amino terminus and binds to the ear domains of AP2. It was observed in an in vitro assay that AP2 and AP180 cooperatively act to assemble clathrin to form clathrin coated vesicles more efficiently than either protein alone. The binding of AP180 to AP2 is regulated by its phosphorylation state and this may contribute to the regulation of clathrin mediated endocytosis in synapses (Hao et al., 1999).

Mutation of the Drosophila homologue of AP180 results in defective endocytosis at the presynaptic termini. It is also observed that the loss of AP180 results in reduced number of synaptic vesicles. Most importantly, synaptic vesicle size and quantal size are increased in the AP180 mutants (Zhang et al., 1998). An assay with purified proteins showed that clathrin baskets polymerized in the presence of AP180 are more uniform and smaller in size than without AP180 (Ye and Lafer, 1995). Since AP180 also binds to phosphoinositides, it is one of the factors involved in nucleation of clathrin lattices on the membrane (Ford et al., 2001). Overexpression of the C-terminus of AP180 in neurons blocks clathrin mediated retrieval of synaptic vesicles (Granseth et al., 2006). The C-terminal of AP180 binds to clathrin and promotes clathrin assembly activity *in vivo*. Overexpression of the C-terminal of AP180 in neurons results in a dominant negative phenotype, probably by sequestering clathrin and making clathrin unavailable for mediating endocytosis (Morgan et al., 1999).

Current literature strongly suggests that AP180 assisted AP2 mediated recruitment of clathrin is the predominant mode of vesicle recycling at synapses in the central nervous system. The fact that in the NCAM deficient NMJ there are abnormalities in the process of synaptic vesicle recycling suggests that NCAM is important in this process (Polo-Parada et al., 2001). Additionally there has been a report where NCAM has been shown to associate with α adaptin of AP2 and clathrin in neurons (Minana et al., 2001). Is this association between AP2 and NCAM direct? Is NCAM one of the synaptic transmembrane proteins that target AP2 at the presynaptic termini during synaptic vesicle recycling? If so, then in the absence of NCAM, is there compensation by an immature mode of recycling? A potential candidate for the immature mode of synaptic vesicle recycling is the AP3 mediated mode.

II.4.2. AP3 mediated synaptic vesicle genesis

The existence of AP3 first came into light with the identification of μ3 subunit due to its homology with μ1 and μ2 (Pevsner et al., 1994). The ubiquitously expressed AP3 consists of the large δ and β3 (3βA), the medium sized μ3 (3μA) and the small σ3 subunit. The brain, spinal cord and neuronal cell lines express a neuronal isoform of AP3 which differs in the β3 (3βB referred to as βNAP) and a μ3 (3μB) subunits (Newman et al., 1995). Although AP3 has high degree of structural homology with clathrin adaptors AP1 and AP2, the association of AP3 with clathrin is different in different cell types. βNAP as well as 3βA have a clathrin binding domain and associate with clathrin in in-vitro assays. Although βNAP is found in nerve terminals, it does not purify along with clathrin coated vesicles indicating that neuronal AP3 does not associate with clathrin (Newman et al., 1995). However, there is evidence for both clathrin independent and dependent AP3 mediated trafficking for the ubiquitous isoform. In PC12 and HeLa cells, AP3 is found on clathrin coated vesicles (Dell'Angelica et al., 1998). Studies in protein trafficking in yeast showed that AP3 is involved in transporting certain membrane proteins in a clathrin independent pathway from the Golgi to the vacuoles (Odorizzi et al., 1998).

A GTPase called ARF1 recruits ubiquitous AP3 on membranes to transport cargo. ARF1 is also involved in the formation of AP1 and COP1 positive vesicles and its action is inhibited by the presence of BFA. ARF6, which recruits AP2 to the plasma membrane, is BFA insensitive and is not able to recruit AP3 (Cowles et al., 1997; Ooi et al., 1998).

Mutation of AP3 in Drosophila results in defective pigmentation due to aberrant vesicular trafficking required for biogenesis of pigment granules (Ooi et al., 1997). Similarly, AP3 deficient mice exhibit defective coat pigmentation and prolonged bleeding times. This phenotype is attributed to dysfunctional trafficking of melanin to melanosomes leading to mutant coat phenotype and reduced dense storage granules in platelets causing bleeding phenotype. Both melanosomes and dense granules are specialized form of lysosomes and AP3 mediates trafficking from the endosomes to the lysosomes in a clathrin independent manner (Odorizzi et al., 1998).

In contrast to the ubiquitous AP3, the neuronal isoform has been found only on non-clathrin coated synaptic vesicles at the nerve terminals (Simpson et al., 1996). Although both the isoforms of AP3 are present in the brain, they both have distinct functions (Seong et al., 2005). At the nerve terminals, the neuronal form of AP3 is indicated in the biogenesis of a population of synaptic vesicles. Experiments with cell free assay systems showed that the neuronal isoform of AP3 is required for formation of synaptic vesicles from endosomes (Blumstein et al., 2001). A synaptic vesicle protein, VAMP 2, associates with AP3 and could be one of the vesicle proteins recruiting AP3 on the surface of synaptic vesicle membrane (Salem et al., 1998).

II.4.3. Recruitment of adaptor proteins to the surface of membrane

Soluble adaptor proteins have to be recruited on the membrane to induce coated vesicle formation (Chang et al., 1993; Zhu et al., 1998). This recruitment is mediated and regulated by a family of proteins called ADP ribosylation factors (ARFs) in a GTP dependent manner (Kawasaki et al., 2005). There are six different ARFs with ARF1 recruting AP1 (Stamnes and Rothman, 1993) and AP3 (Ooi et al., 1998) and ARF6 recruiting AP2 on the membrane surface (Krauss et al., 2003; Paleotti et al., 2005). Specificity of recruitment of adaptor proteins at particular

sites is mediated via at least two different interactions on the plasma membrane. The first involves protein-protein interaction with transmembrane cargo proteins and the second protein-lipid interaction with specific lipid moieties on the plasma membrane.

The protein-protein interactions leading to adaptor protein recruitment on the membrane have been extensively investigated. Each of the adaptor proteins has a distinct set of cargo proteins to traffic within the cell. For example AP1 is involved in trafficking of mannose-6-phosphate receptor (Nakagawa et al., 2000), AP2 of transferrin receptor (Banbury et al., 2003), VGLUT1(Voglmaier et al., 2006), L1 (Kamiguchi et al., 1998), MHC II (McCormick et al., 2005); AP3 transports LimpII, tyrosinase (Honing et al., 1998), TI-VAMP (Martinez-Arca et al., 2003), Zn transporter ZnT3 (Salazar et al., 2004a), Chloride channel ClC-3 (Salazar et al., 2004b), to name a few. Analysis of adaptor-cargo protein interactions shows that in most cases the interaction is mediated via either a tyrosine motif or an acidic dileucine motif present on cargo protein. The affinity of the AP complexes to specific binding motifs depends on the context of the motif in the protein, accessory proteins and the phosphorylation state of the AP (McNiven and Thompson, 2006).

II.4.3a. Tyrosine motif

The tyrosine motif has been well characterized and is found in the cytoplasmic domains of many endocytosed proteins. Study of mutations on the LDL (low density liopoprotein) receptors resulting in the inhibition of internalization first revealed the presence of tyrosine motifs (Davis et al., 1986). This internalization motif consists of an essential tyrosine, which is either part of a motif NPXY (where X is any amino acid) or YXXΦ (where Φ is an amino acid with a bulky hydrophobic side group).

II.4.3b. Dileucine motif

The other motif recognized by adaptor proteins is the dileucine motif where one of the leucines can be replaced with a methionine, isoleucine, valine or alanine (Sandoval and Bakke, 1994). X-ray crystal diffraction studies of various peptides containing the dileucine motif (Asp-X-X-Leu-Leu) complexed with an adaptor

protein GGA (Golgi-localized, δ-ear-containing, ADP-ribosylation-factor-binding protein) demonstrated that the aspartate and the dileucine motifs contribute to the binding (Misra et al., 2002). However, it has also been observed that non-canonical dileucine motifs without the aspartate also function as binding motifs for adaptor proteins, as demonstrated by the binding of tyrosinase to AP3 (Honing et al., 1998). The dileucine motif present in tyrosinase comprises of KQPLL with a lysine present instead of the crucial aspartate. In the case of vesicular monoamine transporter (VMAT1), the dileucine motif is AKEEKMAIL, indicating that the basis for the binding is different from those dileucine motifs with aspartate.

The dileucine motif is recognized by the β2 and/or μ2 subunit and the tyrosine motif is recognized by the μ2 subunit (Owen and Evans, 1998). Dileucine motifs are also recognized by AP1 and AP3 but each AP exhibits different preferences.

Remarkably, integral synaptic proteins like synaptotagmin have both a degenerate dileucine motif and a tyrosine motif. The degenerate dileucine motif present in synaptotagmin mediates trafficking to synaptic vesicles, probably via AP3, as concluded from BFA sensitivity (Blagoveshchenskaya et al., 1999). It should be noted that synaptotagmin is trafficked to synaptic vesicles even in the presence of BFA, indicating an AP3 independent pathway. It was shown that AP2 binds to synaptotagmin at a dilysine motif present at its C terminal, which is not an internalization signal, but a regulator of endocytosis (Chapman et al., 1998). Interestingly, endocytosis of synaptotagmin is mediated by a tryptophan containing motif via the AP2 pathway (Jarousse et al., 2003). This complex regulation of trafficking of synaptotagmin by different adaptor proteins mediated by distinct motifs has been revealed only by detailed study. It is possible that trafficking of several other proteins is also mediated via different motifs interacting with the same adaptor proteins.

II.4.3c. Inositol lipid binding motifs

Recognition of cargo proteins by AP2 is dependent on simultaneous binding to phosphatidylinositol-4,5-bisphosphate (PtdIns 4,5P(2)) on the membrane. AP2 is initially recruited on the membrane by weak binding of the α and μ subunits of AP2 to PtdIns 4,5P(2) (Gaidarov et al., 1996). Honing and colleagues have shown

that a conformational change induced by phosphorylation of μ subunit of AP2 facilitates binding to peptide motifs. The initial weak binding of AP2 to phospholipids is potentiated by binding to tyrosine or dileucine motif containing peptides (Honing et al., 2005).

The lipid binding sites on clathrin adaptor proteins have been mapped to N terminal on the α subunit of AP2 (Beck and Keen, 1991; Voglmaier et al., 1992; Gaidarov et al., 1996) and the N terminal ENTH (Epsin N-terminal homology) domain of AP180 (Ye et al., 1995). AP2 shows a high degree of homology with IP_6 receptor and could function as a potassium channel (Timerman et al., 1992). It was also found that AP2 could bind to other phosphinositides like phosphoinositol 4,5-bisphosphate and phosphoinositol 1,4,5-triphosphate, although with differing affinities.

II.5. Use of toxins to study synaptic vesicle recycling

II.5.1. Brefeldin A (BFA)

Several toxins are used as tools to study protein trafficking and synaptic vesicle recycling. Brefeldin A is a fungal metabolite and as mentioned before, inhibits ARF1 mediated recruitment of coat proteins (Sata et al., 1998). The GTD/GDP exchange activity of ARFs is stimulated by guanine nucleotide exchange factors (GEFs). GEFs are multidomain proteins that disassociate the tightly bound GDP-ARF complex. Sec7 is the main catalytic domain present in GEFs. BFA binds to a specific cavity formed at the interface of sec7 in GEF and ARF-GDP (Robineau et al., 2000). Since BFA stabilizes a transient reaction intermediate, ARF1 is no longer available for activation and subsequent recruitment of adaptor proteins. Hence application BFA inhibits AP1 and AP3 mediated coat formation by acting on ARF1.

Sensitivity to BFA is conferred by two residues - an aspartic acid residue and methionine residues in the sec7 domain of GEFs (Sata et al., 1998). Interestingly, the presence of an additional phenylalanine and alanine residue results in a BFA resistant GEF factor. ARF6 is activated by ARF-GEP100 (ARF-guanine nucleotide exchange protein). ARF-GEP100 contains a sec7 domain with phenylalanine at 535 and alanine at 536 position and therefore does not bind to

BFA (Someya et al., 2001). Since AP2 recruitment is by ARF6, it is resistant to the effects of BFA.

Hence, sensitivity to BFA can be exploited to differentiate between AP2 and AP3 modes of vesicle biogenesis. Developing axons and immature NMJs exhibit vesicle recycling that is inhibited by BFA suggesting that the process of synaptic vesicle formation here is AP3 dependent (Zakharenko et al., 1999). Once NMJs are mature and synaptic contacts are made, the BFA sensitivity is lost (Polo-Parada et al., 2001). This suggests that AP3 is involved in formation of synaptic vesicles in immature synapses or during development. After maturation of the synapse, the formation of synaptic vesicles is replaced by AP2/clathrin dependent pathway.

II.5.2. Tetanus toxin

Another toxin used in synaptic vesicle recycling studies is tetanus toxin. Tetanus toxin, derived from *Clostridium tetani*, cleaves vesicle associated membrane protein, VAMP-2, a synaptic protein involved in vesicle fusion (Schiavo et al., 1992). However, it affects only vesicle fusion at mature synapses and not at developing axons (Verderio et al., 1999). The resistance to the action of tetanus toxin is due to the presence of a tetanus toxin insensitive VAMP, TI-VAMP (Galli et al., 1998). In developing axons or in immature synapses resistant to tetanus toxin, TI-VAMP is probably involved in exocytosis of synaptic vesicles formed via AP3. This is supported by the fact that in AP3 deficient hippocampal synapses, exocytosis is tetanus toxin sensitive due to the loss of TI-VAMP targeting to the presynaptic termini (Scheuber et al., 2006). Interestingly, although it is now clear that TI-VAMP functions during developmental processes like neurite outgrowth (Martinez-Arca et al., 2000), it has also been found is some subsets of synapses in the adult brain (Muzerelle et al., 2003).

II.5.3. Latrunculin

Actin is an important cytoskeletal protein at the synapse and is concentrated at developing presynaptic termini (Dai and Peng, 1996). It anchors synaptic vesicles via synapsin, a vesicle protein at the presynaptic termini and has been speculated to have a role in mobilizing and recycling of the vesicles (Ceccaldi et al., 1995; Bernstein et al., 1998). Latrunculin, a toxin derived from red sea sponges

(e.g. *Latrunculia magnifica*) inhibits actin polymerization in-vitro and in-vivo (Spector et al., 1983; Coue et al., 1987).

Use of latrunculin during different stages of synaptogenesis in hippocampal neural cultures showed that actin depolymerization does not affect synaptic vesicle recycling at later stages of synaptic development. However, application of latrunculin in younger cultures disrupted synaptic vesicle recycling (Zhang and Benson, 2001). Hence latrunculin could be potentially used to differentiate between AP3 mediated (probably immature) mode of synaptic vesicle recycling and the mature AP2/clathrin mediated mode of recycling.

III. Rationale and aims of the study

Why is there a need for a parallel pathway for the biogenesis of synaptic vesicles? It is well established that the predominant pathway for synaptic vesicle biogenesis is via AP2 from the synaptic plasma membrane. In yeast, the AP3 dependent pathway exists in parallel to another protein trafficking pathway to the vacuoles (Odorizzi et al., 1998). Similarly, at the presynaptic termini, synaptic vesicles can be generated via both AP2 and AP3. One possibility is that both the pathways are present at the presynaptic termini in the adult but are predominant at different stages of synaptic development.

AP3 could potentially be the main mode of vesicle recycling in immature neurons since BFA inhibits release of neurotransmitters in developing axons (Zakharenko et al., 1999). A study of synaptic recycling of vesicular glutamate transporter, VGLUT1 shows that the AP3 pathway acts like an alternative pathway at mature synapses when endocytosis of VGLUT1 via AP2 is blocked (Voglmaier et al., 2006). An explanation could be that under normal circumstances, the AP3 pathway is downregulated in the mature synapse and only when the predominant mode of recycling via AP2 is blocked, synaptic vesicles are generated via AP3

Since BFA also blocks endocytosis in mature NCAM null NMJs (Polo-Parada et al., 2001), AP3 could be mediating synaptic vesicle recycling here. If NCAM is involved in establishing AP2/clathrin mediated mode of recycling, in the NCAM deficient synapse, AP3 being the “immature” pathway is probably retained resulting in BFA sensitivity. This PhD project tries to validate the above hypothesis.

The possibility of defective synaptic recycling at NCAM-/- CNS synapses is high since NCAM is present abundantly at CNS synapses and involved in an active process like synaptic plasticity (Gerrow and El-Husseini, 2006). Inhibition of paired pulse facilitation in the NCAM-/- Schaffer collateral synapses indicates a presynaptic role of NCAM (Cremer et al., 1994). Additionally, impaired LTP in mossy fiber synapses in the NCAM-/- mice strongly suggests that NCAM is involved in presynaptic functioning (Bukalo et al., 2004). However, the mechanism of action of NCAM in presynaptic region is not well understood.

This study investigates whether NCAM plays a role in establishing and regulating the mature mode of recycling mediated via AP2/clathrin.

To state briefly, the focus of the present study is as follows:

- ❖ To establish whether the loss of NCAM affects vesicle recycling in synapses of the CNS.
- ❖ If so, to understand the molecular mechanism by which NCAM influences synaptic vesicle recycling.

The uptake of fluorescent marker dyes (FM) was used to monitor synaptic vesicle endocytosis in NCAM deficient and wild type hippocampal neurons. Dysregulation of proteins involved in vesicle recycling was checked biochemically. SynaptopHluorin, a pH sensitive EGFP fused to the synaptic protein, synaptobrevin, was used to follow rates of endocytosis in hippocampal synapses. Synaptic and sub-synaptic fractions prepared from whole mouse brain were used for biochemical characterization and hippocampal neuronal cultures for immunocytochemistry. Further techniques used are described in the materials and methods section.

IV. Materials and methods

IV.1. Materials

IV.1.1. List of chemicals, commercial reagents and kits used.

1.	β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
2.	2-propanol	Th. Geyer Hamburg GmbH & Co. KG
3.	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
4.	Acetic acid 100%	Th. Geyer Hamburg GmbH & Co. KG
5.	Acrylamide /Bis solution 29:1 30 % w/v	SERVA Electrophoresis GmbH, Heidelberg, Germany
6.	Adenosine 5'- triphosphate (ATP)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
7.	Ammonium persulphate (APS)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
8.	BC (bicinchoninic acid) Assay protein Quantification Kit	Uptima, INTERCHIM, France
9.	Biotrace polyvinylidene fluoride transfer membrane (PVDF)	VWR International GmbH, Hannover, Germany
10.	Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany

11.	B-PER® Bacterial Protein Extraction Reagent	Pierce, PerBio Science Deutschland GmbH, Bonn, Germany
12.	Brefeldin A (BFA)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
13.	Bromphenol blue	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
14.	Calcium chloride ($CaCl_2$)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
15.	Creatine phosphokinase from rabbit muscle	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
16.	dATP, dCTP, dGTP, dTTP	Invitrogen GmbH, Karlsruhe, Germany
17.	DiMethyl Sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
18.	Dulbecco's modified Eagle's Medium (DMEM)	Invitrogen GmbH, Karlsruhe, Germany
19.	Dynasore	Gift from Dr. Volker Hauke, Berlin
20.	Enhanced chemiluminescence (ECL) western blotting reagents	Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany
21.	Ethanol absolute	Th. Geyer Hamburg GmbH & Co. KG
22.	Ethidium bromide	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
23.	Ethylene diamine tetra acetic acid (EDTA)	Sigma-Aldrich Chemie

		GmbH, Deisenhofen, Germany
24.	Glasgow MEM medium	Invitrogen GmbH, Karlsruhe, Germany
25.	Glucose	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
26.	Glutathione agarose beads	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
27.	Glycerol	Merck Biosciences GmbH, Bad Soden/Ts, Germany
28.	Glycine	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
29.	Guanosine 5'[γ-thio]triphosphate tetralithium salt (GTPγS)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
30.	Guanosine 5'-triphosphate lithium salt (GTP)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
31.	Hydrochloric acid (HCl)	Merck Biosciences GmbH, Bad Soden/Ts, Germany
32.	Latrunculin	BIOMOL GmbH, Hamburg, Germany
33.	L-glutathione reduced	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
34.	Lipofectamine	Invitrogen GmbH, Karlsruhe, Germany
35.	Lipofectamine™2000	Invitrogen GmbH, Karlsruhe, Germany
36.	MagnaBind Streptavidin beads	Pierce, Rockford, IL, USA

37.	Magnesium chloride ($MgCl_2$)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
38.	MEM non–essential amni acids solution (100X)	Invitrogen GmbH, Karlsruhe, Germany
39.	Methanol	Th. Geyer Hamburg GmbH & Co. KG
40.	N,N,N',N'-Tetramethylethylenediamine (TEMED)	SERVA Electrophoresis GmbH, Heidelberg, Germany
41.	NiNTA Agarose beads	QIAGEN GmbH, Hilden, Germany
42.	Opti-MEM® I medium	Invitrogen GmbH, Karlsruhe, Germany
43.	Orange G	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
44.	Penicillin/Streptomycin solution (100X)	Invitrogen GmbH, Karlsruhe, Germany
45.	Phenylmethylsulfonyl flouride (PMSF)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
46.	Phospho creatine disodium salt hydrate enzymatic	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
47.	Plus™reagent	Invitrogen GmbH, Karlsruhe, Germany
48.	Polyethylene glycol 300 (PEG 300)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
49.	Potassium chloride (KCl)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany

50.	Potassium L-glutamate	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
51.	Precision plus Protein all blue standard	Bio-Rad Laboratories GmbH, Munich, Germany
52.	Protease inhibitor cocktail EDTA free	Roche Diagnostics GmbH, Mannheim, Germany
53.	Protein A/G plus agarose	Santa Cruz Biotechnology, Inc., Hiedelberg, Germany
54.	Protran® nitrocellulose membranes	VWR International GmbH, Hannover, Germany
55.	PureLink™HiPure Plasmid Filter Maxiprep Kit	Invitrogen GmbH, Karlsruhe, Germany
56.	QIAfilter Plasmid Maxi kit	QIAGEN GmbH, Hilden, Germany
57.	QIAquick gel extraction kit	QIAGEN GmbH, Hilden, Germany
58.	QIAquick rapid PCR purification kit	QIAGEN GmbH, Hilden, Germany
59.	QuikChange® II XL Site-Directed Mutagenesis Kit	Strategene Europe, Amsterdam Zuidoost, Netherlands
60.	Restriction enzymes	New England Biolabs GmbH, Frankfurt am Main, Germany, Roche Diagnostics GmbH, Mannheim, Germany
61.	Shrimp Alkaline Phosphatase (SAP)	New England Biolabs GmbH, Frankfurt am Main, Germany, Roche Diagnostics GmbH,

		Mannheim, Germany
62.	Sodium azide (NaN_3)	Merck Biosciences GmbH, Bad Soden/Ts, Germany
63.	Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
64.	Sodium Dodecyl Sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
65.	Sodium fluoride (NaF)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
66.	Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
67.	Sodium phospate dibasic (Na_2HPO_4)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
68.	Sodium phosphate monobasic (NaH_2PO_4)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
69.	Sucrose	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
70.	Sulfo-NHS- LC-biotin	Pierce, Rockford, IL, USA
71.	SuperSignal West Dura	PerBio Science Deutschland GmbH, Bonn, Germany
72.	SuperSignal West Pico	PerBio Science Deutschland GmbH, Bonn, Germany
73.	T4 Taq polymerase	New England Biolabs GmbH, Frankfurt am Main, Germany

74.	Tetanus Toxin	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
75.	Tris (trishydroxymethylaminomethane)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
76.	Trypsin-EDTA (0.05% Trypsin, with EDTA 4Na)	Invitrogen GmbH, Karlsruhe, Germany
77.	Turbo Pfu polymerase	Strategene Europe, Amsterdam Zuidoost, Netherlands
78.	Tween® 20	Merck Biosciences GmbH, Bad Soden/Ts, Germany

IV.1.2. List of buffers and solutions

1.	0.4 M sucrose in Tris buffer (For cytosolic synaptic fraction isolation)	400 mM sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 6.5 to 7
2.	0.6 M sucrose in Tris buffer (For cytosolic synaptic fraction isolation)	600 mM sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
3.	0.65 M sucrose in Tris buffer (For synaptosomal fraction isolation)	650 mM sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
4.	0.85 M sucrose in Tris buffer (For synaptosomal fraction isolation)	850 mM sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
5.	1 M sucrose in Tris buffer (For synaptosomal fraction isolation)	1 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4

6.	1.2 M sucrose in Tris buffer (For synaptosomal fraction isolation)	1.2 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.4
7.	30mM sucrose in HEPES (For purified synaptic vesicle fraction isolation)	800 mM sucrose 4mM HEPES-KOH pH 7.4
8.	320 mM sucrose in HEPES (For whole brain homogenization)	320 mM sucrose 4mM HEPES-KOH pH 7.4
9.	50 mM sucrose in HEPES (For purified synaptic vesicle fraction isolation)	50 mM sucrose 4mM HEPES-KOH pH 7.4
10.	800 mM sucrose in HEPES (For purified synaptic vesicle fraction isolation)	800 mM sucrose 4mM HEPES-KOH pH 7.4
11.	Blocking solution in phosphate buffered saline (For immunoblotting)	3-5 % skimmed milk powder in phosphate buffered saline
12.	Buffer A	150 mM NaCl 0.1 mM $MgCl_2$ 4 mM HEPES-KOH pH 7.4
13.	Cytosolic buffer (For synaptic membrane recruitment assay)	2.5 mM HEPES-KOH 25 mM KCl 2.5 mM Mg-acetate 5mM EGTA 150 mM K-glutamate pH 7.4

14.	De-staining solution (For coomassie staining of sodium dodecyl polyacrylamide (SDS-PAGE) gels)	40 % (v/v) ethanol 10% (v/v) acetic acid
15.	DNA-sample buffer (5x) (For agarose gels for DNA)	20% (w/v) glycerol in TAE 0.025% (w/v) orange G
16.	dNTP-stock solutions (For PCR)	20 mM each dATP, dCTP, dGTP, dTTP
17.	Ethidium bromide solution (For agarose gels for DNA)	10 µg/ml ethidium bromide in 1xTAE
18.	Homo buffer (For whole brain homogenization)	320 mM sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl pH 7.5
19.	Modified Radio Immunoprecipitation Assay (RIPA) buffer (For immunoprecipitation)	50 mM Tris 150 mM NaCl 2 mM EDTA 1 mM Na_3VO_4 1 mM NaF 1% Nonidet P-40 100 µM PMSF (Add just before use) Protease inhibitor cocktail-EDTA free. Used according to the manufacture's instructions.
20.	Modified RIPA buffer with 0.2 mM Ca^{2+}, 2 mM Mg^{2+} (For immunoprecipitation)	50 mM Tris 150 mM NaCl 1% Nonidet P-40 0.2 mM $CaCl_2$ 2 mM $MgCl_2$ 100 µM PMSF (Add just

		before use) Protease inhibitor cocktail-EDTA free. Used according to the manufacture's instructions.
21.	Modified RIPA buffer with 2 mM Ca^{2+}, 2 mM Mg^{2+} (For immunoprecipitation)	50 mM Tris 150 mM NaCl 1% Nonidet P-40 2 mM $CaCl_2$ 2 mM $MgCl_2$ 100 µM PMSF (Add just before use) Protease inhibitor cocktail-EDTA free. Used according to the manufacture's instructions.
22.	PBSCM (For cell surface biotinylation)	10 mM Na_2HPO_4 2.5 mM NaH_2PO_4 150 mM NaCl 3 mM KCl 0.2 mM $CaCl_2$ 2 mM MgCl pH 7.3
23.	PBS Tween (PBST) (For immunoblotting)	10 mM Na_2HPO_4 2.5 mM NaH_2PO_4 150 mM NaCl 3 mM KCl 0.2% Tween-20
24.	Phosphate buffered saline (PBS) (For immunoblotting)	10 mM Na_2HPO_4 2.5 mM NaH_2PO_4 150 mM NaCl 3 mM KCl pH 7.3

25.	Resolving gel composition for SDS-PAGE	375 mM Tris-HCl pH 8.8 0.1% SDS 0.025% APS 0.001% TEMED Acrylamide /Bis solution 29:1 (according to % of gel required)
26.	Sample buffer for protein gels (5x)	0.312 M Tris-HCl pH 6.8 10% (w/v) SDS 5%(w/v) β-Mercaptoethanol 25% (v/v) Glycerol 0.13% (w/v) Bromphenol blue
27.	SDS PAGE running buffer	192 mM glycine 25 mM Tris 0.1% SDS
28.	Stacking gel composition for SDS-PAGE	125 mM Tris-HCl pH 8.8 0.1% SDS 0.06% APS 0.025% TEMED 5% Acrylamide /Bis solution 29:1 (according to % of gel required)
29.	Staining solution (For Coomassie staining of SDS-PAGE gels)	40% (v/v) ethanol 10% (v/v) acetic acid 0.1% (w/v) Brilliant blue R250
30.	Transfer buffer (For electrophoretic transfer of SDS-PAGE gels during western blotting)	192 mM glycine 25 mM Tris 0.1% SDS 20% methanol
31.	Tris EDTA (TE) buffer 10x (Solvent for DNA)	0.1 M Tris-HCl, pH 7.5 10 mM EDTA

32.	Tris EDTA acetate (TAE) buffer 50X (Buffer for running DNA agarose gels)	2 M Tris-Acetate, pH 8 100 mM EDTA
33.	Tris EDTA NaCl (TEN) buffer (For GST pull-down assay)	20 mM Tris, pH 7.4 0.1 mM EDTA 100 mM NaCl
34.	TSS buffer in Luria-Bertani broth (To make chemically competent bacteria)	10 mM Tris-HCl, pH 8.0 150 mM NaCl 10% w/v PEG 300 30 mM $MgCl_2$ 5% v/v DMSO Filter sterilize with 0.22 μm filter.

IV.1.3. Primary antibodies

1.	δ_A adaptin (AP1)	Mouse monoclonal IgG_1 from BD Transduction Laboratories, BD Biosciences, Pharmingen.
2.	α_A adaptin (AP2)	Mouse monoclonal IgG_1 from BD Transduction Laboratories, BD Biosciences, Pharmingen.
3.	βNAP (AP3)	Mouse monoclonal IgG_1 from BD Transduction Laboratories, BD Biosciences, Pharmingen.
4.	AP180	Mouse monoclonal $IgGb_2$ from Sigma-Aldrich, USA
5.	Clathrin	Mouse monoclonal IgG_1 from BD Transduction Laboratories, BD Biosciences, Pharmingen.
6.	Dynamin I	Affinity purified goat polyclonal (C-16) from Santa cruz Biotechnology, Inc.
7.	Dynamin II	Affinity purified goat polyclonal (C-18) from Santa cruz Biotechnology, Inc.
8.	EEA1	Mouse monoclonal IgG_1 from BD Transduction Laboratories, BD Biosciences, Pharmingen.
9.	GAPDH	Mouse monoclonal antibodies from Chemicon, Temecula, CA, USA

10.	L1	Rabbit polyclonal antibody against the extracellular domain of mouse L1.This antibody was produced in the lab of Dr. M. Schachner.
11.	NaKATPase - α6F	Mouse monoclonal IgG from Developmental Studies Hybridoma Bank, University of Iowa, USA
12.	NCAM 2b2	Rabbit polyclonal antibody against the extracellular domain of mouse NCAM (produced in the lab of Dr. M. Schachner)
13.	NCAM 5b8	Mouse monoclonal IgG_1 from Developmental Studies Hybridoma Bank, University of Iowa, USA
14.	NCAM H28	Rat monoclonal antibodies against the extracellular domain of mouse NCAM. The hybridoma cell line producing the H28 antibody was developed in the laboratory of Dr. C. Goridis (Centre National de la Recherche Scientifique UMR 8542, Paris, France).
15.	NCAM P61	Mouse monoclonal antibody produced against the C-terminus of the intracellular domain of NCAM140 and NCAM180 (ref)
16.	PSD-95	Mouse monoclonal IgG_1 from BD Transduction Laboratories, BD Biosciences, Pharmingen.
17.	Synaptophysin	Rabbit polyclonal antibody produced in the lab of Dr. R. Jahn.
18.	Synaptophysin	Goat polyclonal antibodies (C-20) from Santa cruz Biotechnology, Inc.
19.	Syntaxin 1B	Rabbit polyclonal antibodies from Synaptic Systems GmbH, Goettingen, Germany.
20.	SV2	Mouse monoclonal IgG_1 from Developmental Studies Hybridoma Bank, University of Iowa, USA

IV.1.4. Secondary antibodies

1.	Peroxidase-conjugated mouse anti-rabbit IgG (H + L)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
2.	Peroxidase-conjugated goat anti-rat IgG + IgM (H+L)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
3.	Peroxidase-conjugated goat anti-mouse IgG + IgM (H + L)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
4.	Peroxidase-conjugated donkey anti-mouse IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Rb, Shp Sr Prot)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
5.	Peroxidase-conjugated donkey anti-rabbit IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Ms, Rat, Shp Sr Prot)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
6.	Cyanine dyes (Cy2, Cy3 or Cy5) conjugated mouse anti-rabbit IgG (H + L)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
7.	Cyanine dyes (Cy2, Cy3 or Cy5) conjugated goat anti-rat IgG + IgM (H+L)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
8.	Cyanine dyes (Cy2, Cy3 or Cy5) conjugated goat anti-mouse IgG + IgM (H + L)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).
9.	Cyanine dyes (Cy2, Cy3 or Cy5) conjugated donkey anti-mouse IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Rb, Shp Sr Prot)	Jacksons ImmunoResearch laboratories, purchased from Dianova (Hamburg, Germany).

IV.1.5. Bacterial and mammalian cell culture medium

Bacterial media were autoclaved and antibiotics were added to warm media later.

1.	LB-medium	10 g/l Bacto-tryptone 10 g/l NaCl 5 g/l yeast extract
2.	LB ampicillin medium	100 mg/l ampicillin in LB-Medium
3.	LB ampicillin plates	20 g/l agar in LB-Medium 100 mg/l ampicillin
4.	LB tetracycline medium	25 mg/l tetracycline in LB-Medium
5.	LB tetracycline plates	20 g/l agar in LB-Medium 25 mg/l tetracycline
6.	LB kanamycin medium	25 mg/l kanamycin in LB-Medium
7.	LB kanamycin plates	20 g/l agar in LB-Medium 25 mg/l kanamycin
8.	CHO cell medium	Glasgow MEM (G-MEM) (with L-Glutamine), 10% fetal calf serum 20 ml/l penicillin/ streptomycin solution (100x) 10 ml/l MEM non essential amino acids solution (100X) 1 mM sodium pyruvate MEM 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

IV.1.6. Bacterial strains and cell lines

I.	Name	Description	Genotype	Company
II.	BL21 (DE3)	*Escherichia coli* strain for protein overexpression	F^-, *ompT, hsdS*$_B$ (r_B^- m_B^-), *gal dcm* (DE3)	Novagene, VWR International GmbH. Darmstadt Germany
III.	CHO-K1	*Chinese Hamster Ovary* for ectopic mammalian protein expression	Dehydrofolatereductase deficient	ATCC (American Type Culture Collection) CCL 61
IV.	DH5α	*Escherichia coli* strain for cloning and sub-cloning.	F-,φ80d*lac*ZΔM15, Δ(*lacZYA-argF*)U169, *deoR, recA1, endA1, hsdR17*(rk^{--},mk^+), *phoA, supE44*, λ-, *thi*-1, *gyrA*96, *rel*A1	New England Biolabs GmbH, Frankfurt am Main, Germany, Roche Diagnostics GmbH, Mannheim, Germany
V.	HEK293 cells	*Human Embryonic Kidney* for ectopic mammalian protein expression		Invitrogen GmbH, Karlsruhe, Germany
VI.	XL1-Blue	Escherichia coli for cloning and sub-cloning.	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F´ *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^r)]	Strategene Europe, Amsterdam Zuidoost, Netherlands

IV.1.7. Plasmid constructs

1. pGEM-T easy	Intermediate vector for TA cloning	Promega GmbH, High-Tech-Park, Mannheim, Germany
2. pQE30	Prokaryotic expression plasmid for histidine tagged recombinant expression of proteins.	QIAGEN GmbH, Hilden, Germany
3. pcDNA3	Mammalian expression vector for transfection.	Invitrogen GmbH, Karlsruhe, Germany
4. NCAM140 pcDNA3	For expression of full length NCAM140 in mammalian cells	From Dr. P. Maness, University of South Carolina, USA
5. NCAM180 pcDNA3	For expression of full length NCAM180 in mammalian cells	From Dr. P. Maness, University of South Carolina, USA
6. NCAM140 ICD pQE30	For bacterial over expression of histidine tagged NCAM140 intracellular domain	(Delling et al., 2002)
7. NCAM180 ICD pQE30	For bacterial over expression of histidine tagged NCAM180 intracellular domain	(Delling et al., 2002)
8. AP2 β pGEX-4T-2	For bacterial over expression of glutathione S transferase tagged β subunit of AP2	(Yao et al., 2002)
9. NCAM140 LA pcDNA3	For expression of mutated full length NCAM140 in mammalian cells	Site directed mutagenesis of leucine 752 for alanine in NCAM140 pcDNA3

10	NCAM180 pcDNA3	LA	For expression of mutated full length NCAM180 in mammalian cells	Site directed mutagenesis of leucine 752 for alanine in NCAM180 pcDNA3
11	NCAM140 ICD pQE30	LA	For bacterial over expression of histidine tagged mutated NCAM140 intracellular domain	Site directed mutagenesis of leucine 752 for alanine in NCAM140 ICD pQE30
12	NCAM180 ICD pQE30	LA	For bacterial over expression of histidine tagged mutated NCAM180 intracellular domain.	Site directed mutagenesis of leucine 752 for alanine in NCAM180 ICD pQE30
13	SynaptopHlourin		PH sensitive EGFP fused to synaptobrevin/VAMP to follow rates synaptic vesicle recycling	(Li et al., 2005)

IV.1.8. Mouse strains

C57BL/6J mice were used as control wildtype mice in all experiments. NCAM deficient mice were provided by H. Cremer (Cremer et al., 1994) and were inbred for at least nine generations onto the C57BL/6J background. Animals used for biochemical experiments were NCAM+/+ and NCAM-/- littermates obtained from heterozygous breeding. For most of the biochemical experiments, when not indicated in the text, animals of 3 months of age were used. To prepare cultures of hippocampal neurons, 1- to 3-day-old C57BL/6J and NCAM-/- mice from homozygous breeding pairs were used.

IV.1.9. Centrifuges and rotors

1. Eppendorf 5804R Refrigerated Centrifuge (Eppendorf AG, Hamburg, Germany)
2. Sorvall Ultracentrifuge (Kendro, Hanau, Germany)
   Rotors used- SA600, HB-6, SLA1500, SLA3000

3. Beckman XL-80 Ultracentrifuge (Beckman Instruments GmbH, Munich, Germany)

   Rotors used- SW28, 80Ti, 45Ti

IV.2. Methods

IV.2.1. Molecular biology

2.1.1.Maintainence of bacterial strains

Strains were stored as glycerol stocks (LB-medium, 25% (v/v) glycerol) at –80°C. An aliquot of the stock was streaked on a LB-plate containing the appropriate antibiotics and incubated overnight at 37°C. A single colony was picked up and inoculated into 2 ml of LB broth with the selection antibiotic and incubated at 37°C either for 4 hours for a starter culture or 8 hours for isolating plasmids.

2.1.2. Production of competent bacteria

(Inoue et al., 1990)

An innoculum of DH5α or BL21 bacteria from a glycerol stock was streaked on a LB plate to obtain a single colony. This single colony was used to obtain a 5ml overnight culture in LB medium. This culture was diluted 100 fold in LB medium. The diluted culture was grown to an OD_{600} of 0.3 - 0.5. The culture was spilt into two 50ml falcon tubes and incubated on ice for 10 min. All subsequent steps were carried out at on ice or at 4ºC in the cold room. The cells were pelleted down by centrifuging at 5000 g for 10 minutes at 4°C. The supernatant was discarded. The pellet of cells was resuspended in chilled 5 ml TSS buffer (refer to materials). The pellet was resuspended thoroughly by vortexing gently to ensure that there are no lumps. The resuspended cells were centrifuged again at 5000 g for 10 minutes at 4°C and the pellet resuspended in 5 ml of pre-chilled TSS buffer. Aliquots of 100 µl of the resuspended cells were made in pre-chilled eppendorf tubes and immediately frozen in liquid nitrogen.

2.1.3. Transformation of bacteria

(Sambrook, 1989)

To 100 µl of thawed competent XL1-Blue, DH5α or BL21 on ice, either 50-100 ng of plasmid DNA or 20 µl of DNA ligation mixture (refer to 2.1.5c) were added and incubated for 30 minutes on ice. After a heat shock (90 seconds, 42°C) and successive incubation on ice (3 minutes), 800 µl of LB medium without antibiotics

was added to the bacteria and incubated at 37°C for 40 minutes. After transformation with a plasmid, then 100 µl of the transformation mixture was plated on LB plates with the selection antibiotic. If a ligation mixture was used for the transformation, then cells were centrifuged (5000 g, 1 minutes., RT) and the supernatant removed. Cells were then resuspended 100 µl LB medium and plated on LB plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight.

2.1.4. Plasmid isolation

All plasmid isolations were done using kits mentioned in materials. Protocols followed were according to the manufacturer's instructions.

2.1.5. Enzymatic modification of DNA

2.1.5a. Restriction digestion of DNA

All restriction digestions were made using restriction enzyme in accordance with the New England Biolabs catalogue and technical reference.

2.1.5b. Dephosphorylation of plasmid DNA

(Sambrook, 1989)

To prevent religation of digested vector ends during ligation, the plasmid DNA was dephosphorylated. After restriction digestion, the plasmid DNA was purified and 1U of Shrimp alkaline phosphatase (SAP) buffer (Boehringer Ingelheim) and 1U SAP (scrimps alkaline phosphatase) per 100 ng plasmid DNA were added. The reaction was incubated at 37°C for 2 h and terminated by incubation at 70°C for 10 minutes. The plasmid DNA was used for ligation without further purification.

2.1.5c. Ligation of DNA fragments

(Sambrook, 1989)

Ligation of DNA fragments was performed by mixing 50 ng vector DNA with appropriate amount of insert DNA depending on the size of the insert. 1 µl of T4 DNA ligase and 2 µl of ligation buffer were added and the reaction mix was brought to a final volume of 20 µl with distilled water. The reaction was incubated either for 2 h at room temperature or overnight at 16°C. The reaction mixture was used directly for transformation without any further purification.

2.1.5d. Polymerase chain reaction

(Saiki et al., 1988)

Amplification of DNA fragments was performed in a 50 µl reaction mix with thin-walled PCR tubes in PCR cyclers. Turbo Pfu Polymerase and the appropriate reaction buffer were obtained from Stratagene. Reaction times used were as described in the enzyme datasheet.

The following reaction mixture was used for a standard PCR:

Template 2-10 ng

Forward primers 10pM

Reverse primer 10pM

Nucleotides (dNTPs) 20 mM

2.1.6. DNA gel electrophoresis

DNA fragments were separated by horizontal electrophoresis in DNA electrophoresis chambers (BioRad) using agarose gels. Agarose gels were prepared by heating 1-2 % agarose (w/v) (depending on the size of DNA fragments) in 1xTAE buffer till all the agarose was dissolved and then poured onto DNA gel trays. The gel was covered with 1xTAE buffer, the DNA samples were mixed with DNA sample buffer and pipetted into the sample pockets. The gel was run at constant voltage (10 V/cm gel length) until the orange G dye had reached the end of the gel. Afterwards, the gel was stained with ethidium bromide staining solution for 20 minutes and documented using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloh, Germany).

2.1.7. Extraction of DNA fragments from agarose gels

For isolation and purification of DNA fragments from agarose gels, ethidium bromide stained gels were illuminated shortly with UV-light and the appropriate DNA band was excised from the gel with a clean scalpel and transferred into an Eppendorf tube. The fragment was isolated using the QIAquick gel extraction kit from Qiagen following the manufacturer's protocol.

2.1.8. Purification of DNA fragments after PCR

For purification of DNA fragments, the Rapid PCR Purification kit from Qiagen was used according to the manufacturer's protocol.

2.1.9. Determination of DNA concentration

DNA concentrations were determined spectroscopically. The absolute volume necessary for measurement was 10 µl. For determining the concentration of DNA in case of large-scale plasmid preparations, the DNA was diluted appropriately with distilled water before taking the reading. Concentration was determined by measuring the absorbance at 260 nm and 280 nm. 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 260nm (50 µg/ml of double stranded DNA has an absorbance of 1 at 260nm). A ratio of A_{260}/A_{280} between 1.8 and 2 indicated sufficient purity of the DNA preparation for either transformation or further enzymatic modifications.

2.1.10. Sequencing of DNA

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

2.1.11. Site directed mutagenesis

Single amino acid changes were made using QuikChange® II XL Site-Directed Mutagenesis Kit from Stratagene as per the protocol given by the manufacturer.

IV.2.2. Cell culture

2.2.1. Maintenance of CHO cells

CHO cells were either cultured in GMEM with 10 % FCS (fetal calf serum) and 2% Penicillin/Streptomycinat 37°C, 5 % CO_2 and 90 % relative humidity in 75 cm^2 flasks (Nunc) with 15 ml medium or in six-well plates (d = 35 mm; area = 9.69 cm^2) with 2 ml of medium. Cells were passaged when they were confluent (usually after 3-4 days). Medium was removed and cells were detached by incubation with 4 ml of trypsin EDTA for 5 minutes at 37°C. Cells were resuspended in 10 ml fresh medium and were split 1:10 for maintenance or seeded in six- well plates for transfection (300 µl per well)

2.2.2. Transient transfection of CHO cells

CHO cells were transfected with plasmids using Lipofectamine with Plus™reagent as per manufacturer's instructions. Transfected cells were maintained for 48 hours for transfected gene expression by adding fresh GMEM every 20 hours.

2.2.3. Lysis of CHO cells

After maintenance of CHO cells in 35 mm-culture dishes, the medium was removed and the cells were washed thrice with ice cold PBS. The cells were lysed in 0.5 to 1 ml of RIPA buffer per 35 mm well with constant agitation (1 h, 4°C). Cells were scraped of the wells and transferred into a 1.5 ml eppendorf tube. Debris was removed by centrifugation (15000g, 4°C, 10 minutes) and the supernatant was used for immunoprecipitation.

2.2.4. Cell surface biotinylation

To observe surface delivered proteins, cell surface biotinylation was performed, essentially as described before (Schmidt et al., 1997). CHO and HEK293 cells were transfected with NCAM constructs in a six well plate with 2 µg of DNA per well. 48 hours after transfection, cells were washed twice with ice-cold PBSCM. Surface proteins were biotinylated by incubating cells with 0.5 mg/ml Sulfo-NHS- SS-biotin (1 ml/well) in PBSCM for 30 min at 4°C. Biotinylation was terminated by incubation with 20mM glycine in PBSCM at 4°C for 10 min followed by washing with PBSCM (1 ml/well 3 times). Biotinylated cells were lysed directly by addition of RIPA-buffer (300 µl/well) followed by incubation on ice for 30 min. The supernatants were collected after centrifuging at 4°C for 15 min at 14,000xg and protein concentrations were determined using the BCA kit (Pierce). The amounts of surface-localized proteins were determined by precipitating biotinylated proteins from the cell lysate with streptavidin coupled magnetic beads (MagnaBind) at 4°C overnight. An aliquote of the supernatant was collected before and after the addition of the beads to capture biotinylated proteins to determine proportion of surface delivered NCAM. The beads were pelleted on a magnetic stand and washed twice with 1 ml RIPA-buffer followed by twice with PBS. Precipitated proteins were solubilized by addition of 75 µl 2X SDS sample buffer to the beads. Proteins were separated by SDSPAGE and were quantified by immunoblot analysis using monoclonal NCAM 5b8 antibodies

IV.2.3. Protein biochemistry

2.3.1. Protein quantification by Bicinchoninic Acid assay method

Protein concentrations were determined with the BC Assay protein Quantification Kit as per manufacturer's instructions.

2.3.2. SDS-PAGE

Protein samples were subjected to 7.5-16 % (depending on the molecular weight of the protein of interest) SDS-PAGE under reducing conditions using the Mini-Protean II system (Bio-Rad) using standard protocols (Laemmli, 1970). Gel composition is given in the materials section. After polymerization of the gels, the chamber was assembled as described by the manufacturer's protocol. Samples were diluted with appropriate amount of 5X SDS sample buffer followed by boiling for 10 minutes at 100ºC and 10 µg of total protein of each sample was loaded per well. The assembly was filled with 1X SDS running buffer and the gel was run at a constant voltage of 60V for approximately 15 minutes and then at 100V till the end of the run. The run was stopped when the bromophenol blue running front had reached the end of the gel. Gels were either stained or subjected to Western blotting.

2.3.3. Coomassie staining of polyacrylamide gels

(Ausrubel, 1996)

After SDS-PAGE, the gels were stained in staining solution (1h, RT) with constant agitation. The gels were then incubated in de-staining solution until the background of the gel appeared nearly transparent.

2.3.4. Electrophoretic transfer of proteins

Proteins were transferred after SDS-PAGE (see above) onto a Protran® nitrocellulose or Biotrace PVDF membrane using a MINI TRANSBLOT-apparatus (Bio-Rad). After equilibration of the polyacrylamide gel in transfer buffer for approximately 5 minutes, a blotting sandwich was assembled as described in the manufacturer's protocol. Proteins were transferred at 4°C in blot buffer at constant voltage (80V for 2 h or 35V overnight). Prestained protein markers from Bio-Rad

Laboratories were used as a molecular weight marker and to monitor successful protein transfer after tank blotting.

2.3.5. Immunochemical detection of electrophoretically transferred proteins

After the electrophoretic transfer, membranes were removed from the sandwiches and placed protein-bound side-up in glass vessels. Membranes were washed once in PBST for 5 minutes and were subsequently blocked for 1 h in PBST with 3% skimmed milk powder under gentle shaking at room temperature. Incubation with an appropriate antibody diluted in PBST with 3% skimmed milk powder, was performed either for 90 minutes at room temperature or overnight at 4°C. The primary antibody solution was removed and membranes were washed 5x10 minutes with PBST under constant shaking. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was applied at a concentration varying from 10 ng/ml to 1 ng/ml in PBST with 5% skimmed milk powder for 90 minutes at room temperature. The membrane was washed six times by incubating with PBST with constant shaking and changing the PBST at five minute intervals. Immunoreactive bands were visualized using the chemiluminescence detection reagents. The membrane was soaked for 5 minutes in detection solution (1:1 mixture of kit-supplied reagent I and II of enhanced chemiluminiscence detection system). The detection solution was drained off and the blot was placed between plastic sheets. The membrane was exposed to BIOMAX ML (Kodak) for several time intervals, starting with a 1-minute exposure to 12 hours. In case of weak signal detection of the desired protein, enhanced chemiluminiscence was used to amplify the signal.

2.3.6. Enhanced chemiluminescence system

The antibody bound to the membrane was detected using the enhanced chemiluminescence detection system (SuperSignal West Dura or SuperSignal West Pico). The membrane was soaked for 5 min in detection solution (1:1 mixture of solutions I and II). The solution was removed and the blot was placed between transparent plastic sheets. The membrane was exposed to BIOMAX ML film (Kodak) for several time periods, starting with a 30 sec exposure to 24 hours.

2.3.7. Densitometric evaluation of band intensity

Chemiluminescence was quantified using the image processing software *Scion Image* (Scion Corporation, Frederick, MD, USA) or TINA 2.09 software (University of Manchester, UK). The developed film was scanned and the digitized picture was exported to the image processing program. The quantified data was analyzed using Microsoft Excel software.

2.3.8. Stripping and re-probing of Western blots

For detection of an additional protein on the immunoblot, the nitrocellulose membranes were stripped from bound antibodies by shaking blots in stripping buffer for 10 minutes at room temperature. Blots were neutralized by incubation for 2x5 minutes in 1 M Tris-HCl (pH 7.5) and again subjected to immunochemical detection as described above.

IV.2.4. Expression of recombinant proteins in Escherichia coli

For recombinant expression of proteins in *E. coli*, the corresponding cDNA of the gene was cloned in frame with the purification tag of the corresponding expression plasmid. The appropriate *E. coli* strain was transformed with the expression plasmid and streaked on LB plates supplemented with the selection antibiotic. A single colony was inoculated in a 2 ml of LB culture with the selection antibiotic and incubated overnight at 37°C with constant agitation. The overnight culture was used as a starter inoculum at a ratio of 1:50 and incubated at 37°C under constant agitation until the culture had reached an optical density of 0.7. Protein expression was induced by adding IPTG (0.1-0.5 mM) to the culture with further incubation for 2-3 hours at 37°C. Bacteria were collected by centrifugation and stored at –20°C. Protein expression was monitored by Western blotting by removing small aliquots of the culture every hour after IPTG induction.

2.4.1. Lysis of bacteria

2.4.1a. Sonication

(Frangioni and Neel, 1993)

The bacterial culture was centrifuged (8000 x g, 4°C, 10 minutes) and the pellet was resuspended in SDS sample buffer. The suspension was lysed using a sonicator

(Branson Sonifier B15, level 6, 50% pulse, 5 x 20 s, in ice) and the debris was removed by centrifugation (10000 g, 4°C, 10 minutes).

2.4.1b. French press

Bacteria were pelleted (8000 g, 4°C, 10 minutes) and resuspended in native lysis buffer (20 ml lysis buffer per 500 ml culture). The suspension was transferred into a pre-cooled French- Pressure-20K-chamber (capacity: 40 ml). Bacteria were compressed (Spectronic Instruments/SLM Aminco, 10000 psi, 5 minutes) and lysed by opening the valve carefully. The procedure was repeated 3 times and then the suspension was centrifuged (15000 g, 10 minutes, 4°C) in a Beckman centrifuge.

2.4.1c. Mild extraction

B-PER® Bacterial Protein Extraction Reagent was used as per manufacturer's instructions to lyse cells where the protein was prone to degradation or denaturing under conditions of extraction used for sonication or/and French press.

2.4.2. Protein purification

Histidine tagged proteins were captured using Ni-NTA agarose beads from Qiagen and the protein purified from the beads according to the manufacturer's protocol. Glutathione S transferase tagged proteins were captured using glutathione agarose beads from Sigma–Aldrich. The captured protein was purified from the beads according to the manufacturer's protocol.

IV.2.5. Protein interaction detection methods

2.5.1. Protein pull down assay

Protein pull-down experiments were done to check for direct interaction between AP-2 β subunit and NCAM intracellular domain. The AP-2 β subunit tagged with GST was bacterially produced and purified on glutathione sepharose beads (bioPLUS™, Germany). GST bound to glutathione sepharose beads (bioPLUS™, Germany) was used as a control. All the buffers were at 4°C and all steps of the experiments were carried out on ice or in the 4°C room. The AP-2 β GST and GST bound beads were treated with TEN buffer (3x volume of beads) and washed once (10 minutes with constant shaking). TEN buffer with 3% BSA was added to the protein bound beads and the beads were incubated at 4°C for 1-2 hours with

constant gentle shaking. Bacterially expressed and purified intracellular domains of NCAM140 and NCAM180 (1.5 µM molar amounts of CHL1 and L1 intracellular domains were used as controls) were added and incubated overnight at 4°C with constant gentle shaking. The incubation was followed by washing the beads with TEN buffer with 0.5% NP40 (4 X 10 minutes) and once with TEN buffer. 5X SDS sample buffer (75µl) was added and samples were boiled at 100°C for 10 minutes for immunoblot analysis.

2.5.2. Co-immunoprecipitation

NCAM immunoprecipitation was conducted with 2b2 polyclonal antibodies against NCAM, using protein A or protein A/G sepharose beads (Santa Cruz Biotechnology, California) to immunoprecipitate. 1 mg of total protein of brain homogenate, synaptosomal fraction or CHO cell lysate was incubated with 1 ml of ice-cold RIPA lysis buffer for 1 hour at 4°C with constant gentle shaking. Samples were centrifuged at 20000 g for 15 minutes at 4°C. The pellet was discarded and the supernatant was pre–cleared with 20 µl of thoroughly resuspended sepharose protein A (A/G) beads by incubating for 3 hours at 4°C with constant gentle shaking. After pre–clearing, the beads were pelleted down by spinning at 500 x g for 5 minutes and supernatant carefully pipetted out into another tube. The supernatant was incubated with corresponding antibodies or Ig control overnight at 4°C with constant mixing. Precipitation of the antibodies was with sepharose beads (1 hour at 4°C) followed by washing 4 times with ice-cold RIPA buffer and once with ice-cold PBS. The samples were analysed by immunoblotting after addition of SDS sample buffer and boiling at 100°C for 10 minutes.

IV.2.6. Isolation of subcellular organelles by differential density gradient centrifugation

2.6.1. Isolation of synaptosomes, synaptic plasma membrane and complete pool of synaptic vesicles from total brain homogenate

Whole brains of C57BL/6J adult mice were dissected quickly on ice and frozen in liquid nitrogen for storage at -80°C. To obtain sufficient amounts of synaptic vesicles at least 3 adult mice brains were used. Each brain was homogenized in 2 ml of ice-cold 0.32 M sucrose in Tris buffer using 12-15 strokes

of a Dounce homogenizer to obtain a homogenate. All tissues and buffers were maintained at 4°C throughout the experiment. All centrifugation steps were done at 4°C using prechilled centrifuges, rotors and tubes. The homogenates of all 3 brains were mixed and a 50 µl aliquot was taken for analysis of brain homogenate protein levels. The mixture of homogenates obtained was centrifuged at 1100 g for 15 minutes to pellet down tissue debris, nuclei and large myelin fragments. This pellet was called P1. The supernatant (S1) was carefully tipped off and collected in a tube, which was placed on ice. The pellet P1 was resuspended in 0.32 M sucrose in Tris buffer and centrifuged again at 1000 g for 11 minutes. The supernatant obtained was pooled with S1. This was repeated once more (resuspension of pellet and centrifugation at 1000 g for 11 minutes) and the supernatant obtained was pooled with S1. This supernatant S1 was centrifuged at 17500 g for 15 minutes to obtain pellet P2 and supernatant S2. The pellet P2 was resuspended in 4 ml of 0.32 M sucrose in Tris buffer. The supernatant S2 contains cytosolic soluble proteins. Protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) was added to this fraction and stored at –20°C for analysis later.

The pellet P1 was resuspended in 1 M sucrose in Tris buffer thoroughly with repeated pipetting to a final volume of 20ml. The resuspended P1 was layered on 10 ml of 1.2 M sucrose in Tris buffer. On top of this gradient, 6ml of 0.32 M sucrose in Tris buffer was carefully layered. This was the first gradient.

The second gradient consisted of 7 ml each of 1 M, 0.85 M and 0.65 M sucrose (concentration in a decreasing gradient from the bottom to the top of the tube) of sucrose on a cushion of 10 ml of 1.2 M sucrose in Tris buffer. The resuspended P2 was carefully layered on top of this second gradient. All gradients were made on ice or at 4°C. Both the sucrose gradients were centrifuged at 82705 g for 2 hours. After 2 hours, both the sucrose gradients had a banded appearance. The interface between 1.2 M and 1 M sucrose was enriched in the synaptosomal fraction. This interface was carefully collected with a Pasteur pipette from both the gradients and was pooled together. Appropriate amounts of protease inhibitors were added and an aliquot was stored at -20°C for analysis later. This was the crude synaptosomal fraction and had to be purified on a gradient again.

The crude synaptosomal fraction was resuspended with 1 M sucrose in Tris buffer to make a total volume of 20 ml. This was layered on 10 ml of 1.2 M sucrose

in Tris buffer to make a discontinuous gradient. As before, 6 ml of 0.32 M sucrose in Tris buffer was carefully layered on top and the gradient was centrifuged at 82705 g for 2 hours. The interface between 1.2 M and 1 M sucrose contained the double enriched synaptosomal fraction. Appropriate amounts of protease inhibitors were added and an aliquot was stored at -20°C for analysis later.

The synaptosomal fraction was resuspended to get a homogeneous mass. To this 10 volumes of ice cold sterile water was added to apply a hypoosmotic shock. An appropriate volume of 1 M Tris at pH 7.5 was added to obtain a final concentration of 7.5 mM Tris and mixed thoroughly with repeated pipetting. The resuspended synaptosomal fraction in hypoosmotic solution was incubated at 4°C with constant stirring for 40 minutes. This was followed by centrifugation at 25500 g for 30 minutes to pellet the synaptic membrane. The supernatant obtained after the centrifugation contained the soluble synaptic proteins and synaptic vesicles. The pellet obtained was resuspended in 0.32 M sucrose in Tris buffer and stored at -20°C after the addition of protease inhibitors.

The supernatant was carefully collected and centrifuged at 186010 g for 2 hours to pellet down the synaptic vesicles. After centrifugation, a glassy pellet was obtained which was resuspended in minimal volume 0.32 M sucrose in Tris buffer.

Appropriate amounts of protease inhibitors were added and the fraction was stored at -20°C. The total protein content of the different fractions obtained was estimated by BCA test. Fractions collected were analyzed by immunoblotting with indicated antibodies.

2.6.2. Isolation of soluble synaptic proteins

(Whittaker and Barker, 1972)

To isolate fractions enriched in soluble synaptic proteins, five brains were homogenized in an appropriate volume of 0.32 M sucrose in Tris buffer to obtain a 10% homogenate. The homogenate was centrifuged at 1000g for 11 minutes to pellet down tissue debris, nuclei and large myelin fragments. This pellet was called P1. The supernatant (S1) was carefully tipped off and collected in a tube placed on ice. The pellet P1 was resuspended in 0.32 M sucrose in Tris buffer and centrifuged again at 1000g for 11 minutes. The supernatant obtained was pooled with S1. This

was repeated once more (resuspension of pellet and centrifugation at 1000g for 11 minutes) and the supernatant obtained was pooled with S1.

The combined supernatant S1 was centrifuged at 17000 g for 60 minutes to obtain supernatant S2 and pellet P2. The supernatant S2 was poured off and the pellet P2 was resuspended in 0.32 M sucrose in Tris buffer (approximately 2 to 3 ml for every gram of original tissue). This was the crude mitochondrial fraction. It contains small myelin and glial fragments, synaptosomes, and mitochondria. The pellet P2 was suspended in ice-cold distilled water (2 ml/g of original tissue) by means of repeated pipetting. The suspension was called fraction W and this was centrifuged at 12000g for 30 minutes. The resulting pellet Wp is myelin and the resulting supernatant Ws was collected for further separation. This supernatant Ws was carefully layered over a sucrose density gradient consisting of equal volumes of 0.4, 0.6, 0.8, 1 and 1.2 M sucrose in Tris buffer. The gradient was made in 32 ml capacity tubes. The supernatant Ws (5 ml derived from about 2 g of tissue) was layered over the gradient consisting of 5 ml volumes of each of the different sucrose solutions. The tubes were then centrifuged at 63000 g for 2 hours.

After centrifugation, the gradient had a banded appearance with a clear top band devoid of particulate material occupying volume corresponding to Ws. This contained 75-80% of soluble synaptosomal components. This was collected carefully and protease inhibitor cocktail was added before storing it at -20°C. The soluble synaptic fraction obtained was analyzed by immunoblotting with indicated antibodies.

2.6.3. Fractionation of different populations of synaptic vesicles from synaptosomes

Highly purified synaptic vesicles were obtained by a modification of a published protocol (Huttner et al., 1983). Five mice were dissected quickly on ice and frozen in liquid nitrogen for storage at -80°C. Each brain was homogenized in 2 ml of ice-cold Homo buffer using 12-15 strokes of a Dounce homogenizer to obtain a homogenate. All tissues and buffers were maintained at 4°C throughout the experiment. All centrifugations steps were done at 4°C using prechilled centrifuges, rotors and tubes. The homogenates of all 5 brains were mixed and a 50 µl aliquot was taken for analysis later. The mixture of homogenates obtained was centrifuged

at 1100 g for 15 minutes to obtain a pellet P1 and supernatant S1. An aliquot of 50 µl of the supernatant was taken for analyses later and rest of the supernatant was discarded. The pellet was resuspended in 10 ml of Homo buffer and centrifuged again at 1100 g for 10 minutes to obtain a pellet (P10) and a supernatant (S10). S10 was then centrifuged at 9200 g to obtain a supernatant (S2) and a pellet (P2). P10 was resuspended in 10 ml of Homo buffer/brain and centrifuged at 10200g for 15 minutes to obtain S3 supernatant and P3 pellet. The pellets obtained after both the centrifugations (P2 and P3) were pooled and resuspended in 5 ml of Homo buffer. This suspension was then referred to as the crude synaptosomal fraction.

The crude synaptosomal fraction was transferred to a glass-Teflon homogenizer. Nine volumes of ice-cold water were added, and the whole suspension was immediately subjected to three up and down strokes. The resulting lysate was poured rapidly into a beaker containing 3.75 ml of a 1 M HEPES-NaOH buffer, pH 7.4 and the suspension was kept on ice for 30 minutes. The final concentration of HEPES in this lysate was 7.5 mM. This treatment causes the lysis of the synaptosomes by osmotic shock resulting in the release of synaptic vesicles. The lysate was then centrifuged for 20 minutes at 25000 g to yield a lysate pellet (LP1), which were the synaptosomal membrane and a lysate supernatant (LS1). LP1 was stored at -20°C. LS1 was collected, transferred into polycarbonate tubes, and centrifuged for 2 h in a Beckman 50Ti rotor at 165000 g. At the end of this centrifugation, vesicles released by the synaptosomes were pelleted. The supernatant contained the soluble proteins present at the synapse. The pellet was then resuspended in 1 ml of 30 mM sucrose in 4 mM HEPES at pH 7.4 and homogenized by passing through a 25 gauge needle 5 times to obtain a total synaptic vesicle fraction. An aliquot (50 µl) of the total synaptic vesicle fraction was collected and stored at -20°C. The finely resuspended synaptic vesicle fraction was loaded on a continuous gradient of 50 –800 mM sucrose in 4 mM HEPES at pH 7.4 and the gradient was centrifuged for 5 hours in a SW40Ti rotor at 71000 g. At the end of the spin, four interfaces were seen in the gradient. Each of the four interfaces was pelleted by centrifuging at 175000 g for 2 hours. The pellets were resuspended separately in buffer A by pipetting up and down gently. 50 µl samples of these 4 interfaces were taken for analysis later. For further separation of these

four interfaces, each of the resuspended pellets was loaded on top of a continuous equilibrium sucrose density gradient (300 mM to 1.5 M sucrose in buffer A). This gradient was centrifuged at 183000 g for 18 hours. 500 µl fractions were collected from the top of the gradient to the bottom and stored at -20°C. Approximately 25 fractions for every gradient were obtained.

2.6.4. Isolation of synaptosomes and synaptic plasma membrane at different ages for the developmental profile

To study the expression pattern of the different adaptor proteins during brain development, synaptosomes and synaptic plasma membrane fractions were isolated from the whole brains of mice at different stages of development. Four ages of mice were chosen; postnatal day 7 (p7), 14 (p14), 21 (p21) and 60 (p60). Two brains of each age group from C57BL/6J and NCAM knockout mice were homogenized in 0.32 M sucrose in Tris buffer to obtain a 10% homogenate. The isolation of the synaptosomes, followed by the isolation of the synaptic plasma membrane was done as described above. The samples were subjected to SDS-PAGE and immunoblotting.

IV.2.7. Synaptic membrane recruitment assay

Synaptic plasma membrane (SPM) was isolated from osmotically lysed density-gradient isolated synaptosomes. The isolated SPM was treated with 0.1 M Na_2CO_3 for 15 minutes in a water bath at 37ºC to strip off peripheral proteins and any cytosolic proteins that may have co purified with the SPM. The efficiency of alkali treatment was checked by immunoblotting stripped SPM for cytosolic proteins AP2 and AP1. Total brain cytosol was isolated by collecting the supernatant after centrifuging total brain homogenate at 10000 g for 1 hour.

10X cytosolic buffer was used to dilute SPM to final concentration of 5mg/ml and cytosol to 2 mg/ml after adding appropriate concentrations of protease inhibitor cocktail. 50 µg of alkali treated SPM was incubated with 500 µg of cytosol in a total assay volume of 500 µl in cytosolic buffer for every reaction. After incubation at 37ºC for 15 minutes or 25ºC for 1 hour, the SPM was collected by centrifugation at 10000 g for 1 hour. The SPM was washed once by resuspending in 500 µl of cytosolic buffer and repeating the centrifugation. The pellets obtained were resuspended in 50 µl of water and 100 µl of 5X SDS sample

buffer was added prior to boiling at 100ºC for 10 minutes. The samples were subjected to SDS-PAGE for analysis. Recruited adaptor proteins were detected by immunoblotting with indicated antibodies.

V. Results

V.1. Synaptic vesicle recycling is abnormal in NCAM deficient synapses of the central nervous system (CNS)

Studies in the neuromuscular junction showed immaturity of the synaptic vesicle recycling machinery in NCAM deficient preparations characterized by increased sensitivity to fungal toxin Brefeldin A (BFA) application of which inhibited synaptic vesicle recycling in these synapses (Polo-Parada et al., 2001). To compare that to CNS synapses, we analyzed uptake of the FM1-43 dye to synaptic boutons in wild type (NCAM+/+) and NCAM deficient (NCAM-/-) neurons in response to membrane depolarization induced by application of the 47 mM K+ containing buffer. A fixable analogue of FM1-43 was used in these experiments, and levels of FM1-43 dye were measured in the synaptic boutons visualized with SV2 antibodies after fixation of the neurons. FM1-43 uptake was reduced in NCAM-/- boutons when compared to NCAM+/+ synapses (Figure 8).

To analyze maturity of these synapses, we compared effects of drugs disrupting mature or immature modes of synaptic vesicle recycling in NCAM+/+ and NCAM-/- synapses. Interestingly, whereas application of BFA slightly decreased FM1-43 uptake in NCAM+/+ synapses probably by interfering with residual immature pathways of synaptic vesicle recycling in these synapses, FM1-43 uptake was increased to wild type level in NCAM-/- neurons treated with BFA. Sensitivity to latrunculin, a sequestering agent for actin monomers that disrupts actin microfilaments, is another hallmark of immature synaptic vesicle recycling machinery relying on actin dependent endocytosis (Zhang and Benson, 2001). Latrunculin treatment had no effect on FM1-43 uptake at NCAM+/+ synaptic boutons, whereas in NCAM-/- synaptic boutons treatment with latrunculin has a similar to BFA effect and resulted in a twofold increase in FM1-43 uptake. In contrast to BFA and latrunculin, tetanus toxin inhibits synaptic vesicle recycling in mature synapses (Elferink and Scheller, 1993) but does not affect immature recycling machinery. Application of tetanus toxin caused a decrease in FM1-43 uptake at NCAM+/+ synapses, but did not affect NCAM-/- synapses. Dynasore is a small molecule, which rapidly inhibits the GTPase activity of dynamin and blocks

dynamin-dependent synaptic vesicle endocytosis (Newton et al., 2006). Application of dynasore, either alone or in combination with BFA, almost completely blocked FM1-43 uptake to NCAM+/+ and NCAM-/- synaptic boutons (Figure 8) indicating that synaptic vesicle recycling depends on dynamin in neurons of both genotypes.

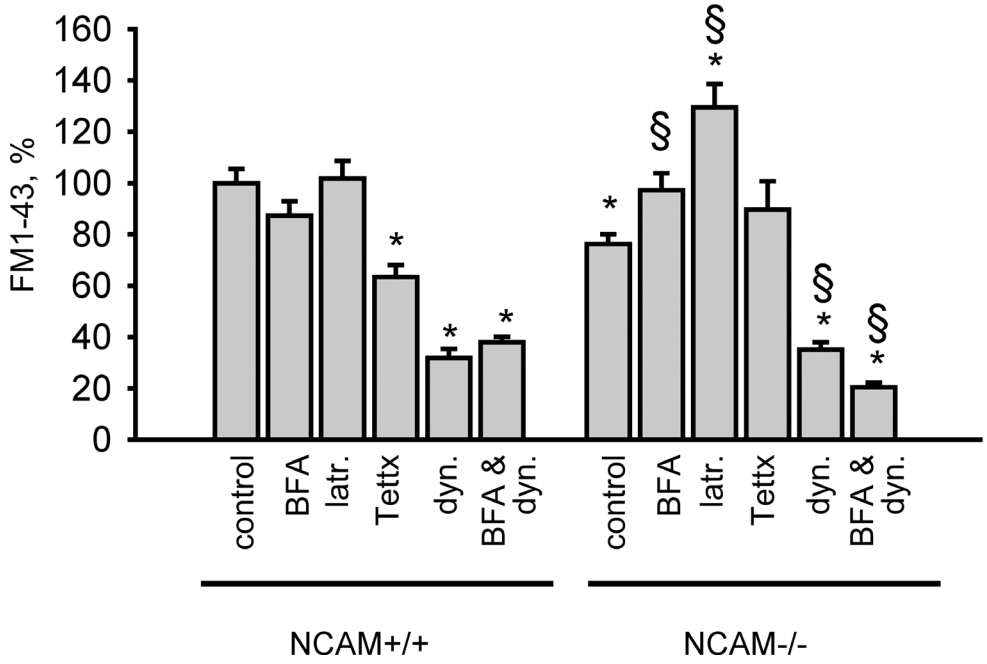

Figure 8. Presynaptic boutons of cultured NCAM+/+ and NCAM-/- hippocampal neurons, that were either non-treated (control) or treated with brefeldin A (BFA), tetanus toxin (Tettx), latrunculin (latr.), dynasore (dyn.) or dynasore with BFA, were loaded with the fixable analogue of FM1-43 applied in the 47 mM K+ containing buffer for 90 seconds. After washing, neurons were fixed and co-labeled with the antibodies against the presynaptic marker-protein SV2 to visualize presynaptic boutons. Graph shows mean values ± SEM of FM1-43 fluorescence in SV2 accumulations with the mean FM1-43 level in control NCAM+/+ neurons set to 100%. Note reduced uptake of FM1-43 in NCAM-/- presynaptic boutons. BFA and latrunculin do not affect FM1-43 uptake in NCAM+/+ presynaptic boutons but increase its uptake in NCAM-/- presynaptic boutons. Tetanus toxin inhibits FM1-43 uptake in NCAM+/+ but not in NCAM-/- presynaptic boutons. Dynasore inhibits FM1-43 uptake both in NCAM+/+ and NCAM-/- presynaptic boutons. *,§ P<0.05, t-test (*-show statistically significant differences when compared to NCAM+/+ control neurons, §-show statistically significant differences between NCAM-/- groups compared to control NCAM-/- neurons; n > 20 images of neurons with N > 200 synapses per image were analyzed). Experiment done by Vladimir Sytnyk.

Altogether, our data indicate that similarly to NCAM-/- neuromuscular junction, NCAM-/- hippocampal synapses rely predominantly on the immature mode of synaptic vesicle recycling. However in contrast to neuromuscular junction

synapses, inhibition of the immature mode of synaptic vesicle recycling in NCAM-/- hippocampal synapses resulted in the potentiation of FM1-43 uptake, probably by reactivating mature modes of synaptic vesicle recycling.

V.2. NCAM is enriched at the synaptic plasma membrane and is present on synaptic vesicles isolated CNS synapses.

While it was shown that postsynaptic NCAM, and particularly its NCAM180 isoform, plays a prominent role in the activity-dependent organization of the postsynaptic machinery in CNS synapses (Dityatev et al., 2000; Sytnyk et al., 2006), the role of the presynaptic NCAM in CNS remained poorly characterized. Immunolabeling of cultured hippocampal neurons with antibodies recognizing all NCAM isoforms shows, however, that NCAM clusters are not only apposed to but also co-localize with accumulations of synaptophysin, an integral membrane protein of synaptic vesicles, indicating that NCAM accumulates in presynaptic boutons (Figure 9) in accordance with previous reports for cultured neurons and brain tissue (Persohn et al., 1989; Schuster et al., 1998; Sytnyk et al., 2002). Since NCAM180 isoform is predominantly postsynaptic both in brain tissue (Persohn et al., 1989) and in cultured hippocampal neurons (Sytnyk et al., 2006), presynaptic labeling thus reflects predominantly the distribution of the second major neuronal isoform of NCAM, NCAM140. To visualize presynaptic localization of NCAM140 with a better resolution that is under conditions when postsynaptic NCAM was absent, we transfected cultured hippocampal neurons from NCAM deficient (NCAM-/-) mice with NCAM140. In transfected neurons, NCAM140 was targeted both to dendrites and axons as observed previously (Dityatev et al., 2004).

To analyze subsynaptic distribution of NCAM, isolated synaptosomes were further fractionated to obtain synaptic plasma membrane and synaptic vesicles. Western blot analysis of these fractions (Figure 10) showed that the synaptic vesicle fraction was enriched in synaptophysin, an integral membrane protein of synaptic vesicles, while synaptic plasma membrane fraction was enriched in Na, K-ATPase, an integral membrane protein localized exclusively to the surface membrane. NCAM140 and NCAM180 were enriched in the synaptic plasma membrane fraction by 50% when compared to the brain homogenate in accordance with previous reports (Pollerberg et al., 1986; Persohn et al., 1989). Interestingly,

NCAM was also present in the synaptic vesicle fraction (Figure 10). One of the possible reasons for that could be contamination of isolated synaptic vesicles with pre- or postsynaptic membranes. However, synaptic vesicle fraction was negative both for Na, K-ATPase and PSD95, a scaffold protein, highly enriched in the synaptic plasma membrane fraction that indicated that isolated synaptic vesicles were free of plasma membrane contaminants. Thus our observation suggested that NCAM is present in synaptic vesicles probably being involved in an uncharacterized aspect of synaptic vesicle recycling in CNS synapses.

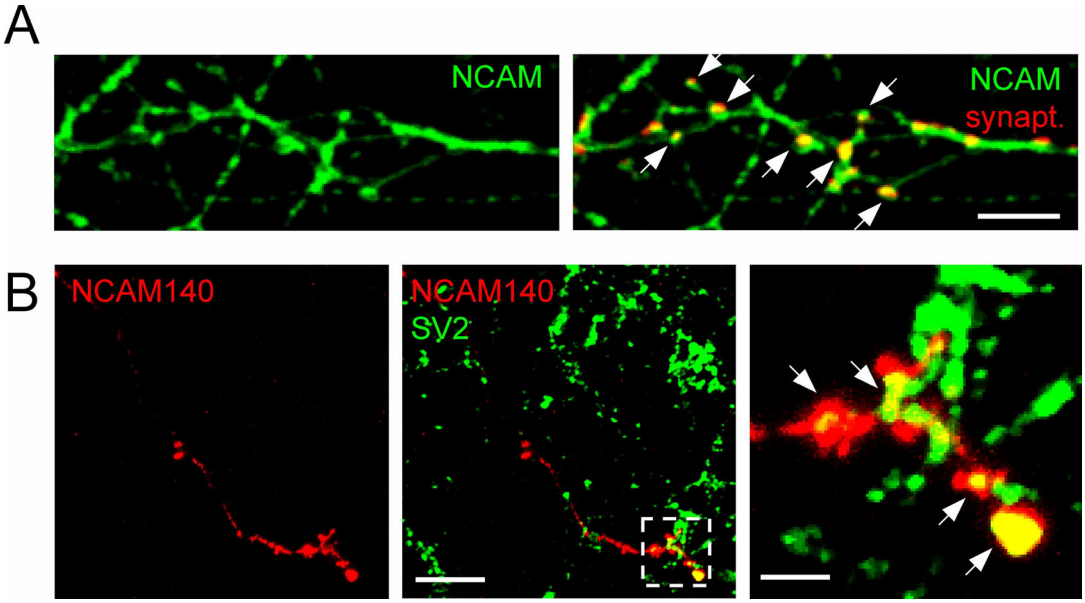

Figure 9. Cell surface NCAM was labeled by indirect immunofluorescence in formaldehyde fixed NCAM+/+ hippocampal neurons (A) or NCAM-/- neurons transfected with NCAM140 (B) maintained in culture for 12 days. Neurons were then permeabilized and labeled with antibodies against the presynaptic marker protein synaptophysin (A) or SV2 (B). In B, high magnification image of the area outlined with dashed lines is shown on the right. Note that clusters of synaptophysin and SV2 co-localize with cell surface accumulations of NCAM (arrows). Bars 10 µm (low magnification), 1 µm (high magnification in B). Experiment done by Vladimir Sytnyk.

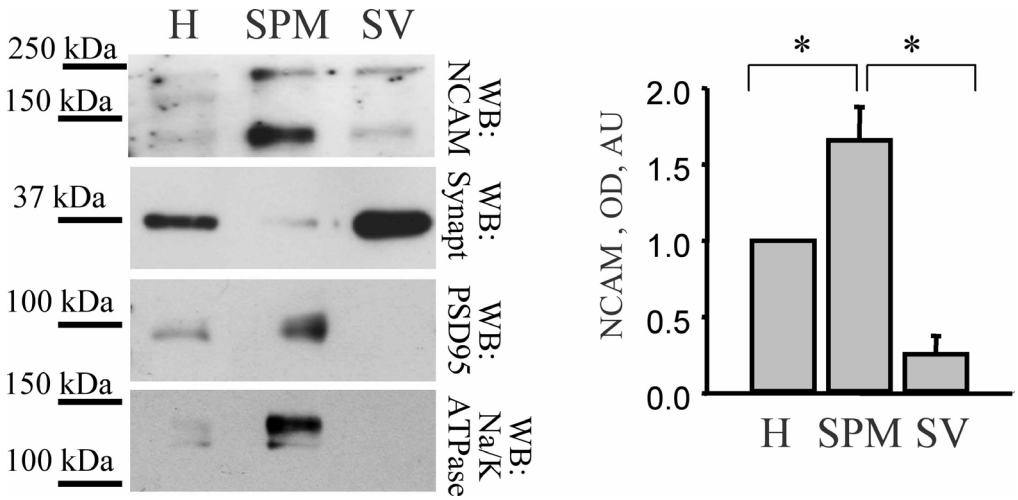

Figure 10. Brain homogenates (H), synaptic plasma membranes (SPM) and crude synaptic vesicle fraction (SV) were probed by Western blot with the indicated antibodies. Note, that NCAM accumulates in the synaptic plasma membrane. Synaptic membranes, containing both pre- and postsynaptic portions, are also enriched in the postsynaptic density marker protein PSD95 and plasma membrane localized Na,K-ATPase. NCAM is also present in synaptic vesicles, which are highly enriched in synaptophysin (synapt.) but are free of PSD95 and Na,K-ATPse. Graph shows quantitation of blots (mean + SEM) with the signal in homogenates set to 100%. *P<0.05, paired t-test (n = 3).

V.3. Deficiency of NCAM at CNS synapses results in aberrant expression of adaptor proteins and clathrin

Immature and mature modes of synaptic vesicle recycling have been suggested to be largely defined by the way new synaptic vesicles are generated within a synaptic bouton – either retrieved from the plasma membrane following synaptic vesicle exocytosis (mature mode), or generated from intracellular organelles of TGN/endosomal origin (immature mode) (Blagoveshchenskaya and Cutler, 2000; Horng and Tan, 2004). Generation of vesicles from different subcellular compartments is mediated by distinct adaptor proteins: adaptor protein 2 (AP2), together with AP180 is involved in clathrin-dependent endocytosis of the synaptic vesicle membrane from the plasma membrane (Hao et al., 1999; Ford et al., 2001), while AP3 has been suggested to mediate clathrin-independent generation of synaptic vesicles from TGN/endosomal compartment. To investigate whether the abnormalities in synaptic vesicle recycling observed in the NCAM-/- synapses (Figure 8) were due to aberrant formation of synaptic vesicles, we first checked the expression levels of the different adaptor proteins and clathrin in the

whole brain. We observed an overall enhancement of AP1, AP2, AP3 and AP180 and clathrin expression in the absence of NCAM in the brain (Figure 11).

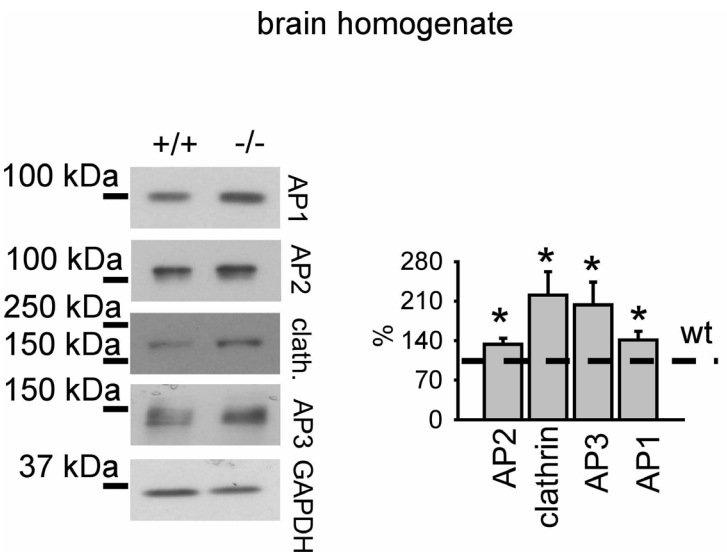

Figure 11. Expression of AP1, AP2, clathrin and AP3 is enhanced in NCAM-/- versus NCAM+/+ brains. NCAM+/+ and NCAM-/- brain homogenates were probed by Western blot with the indicated antibodies. Note increased levels of AP1, AP2, clathrin (clath.) and AP3 in NCAM-/- versus NCAM+/+ homogenates. GAPDH served as loading control. Graphs show the quantitation of the proteins in NCAM-/- mice normalized to the levels in NCAM+/+ mice. are shown. Levels from NCAM+/+ mice were set to 100%. *P<0.05, paired t-test (compared to NCAM+/+ level).

In cultured hippocampal neurons, synaptophysin clusters overlapped with accumulations of AP2 and AP3 adaptor protein complexes. Interestingly, AP2 and AP3 containing synaptic boutons both overlapped with NCAM clusters (Figure 12), indicating that AP2 and AP3 dependent synaptic vesicle generating pathways co-exist in NCAM positive synaptic boutons and suggesting that immaturity of NCAM-/- CNS synapses is related to abnormalities in subsynaptic distribution of these adaptor proteins.

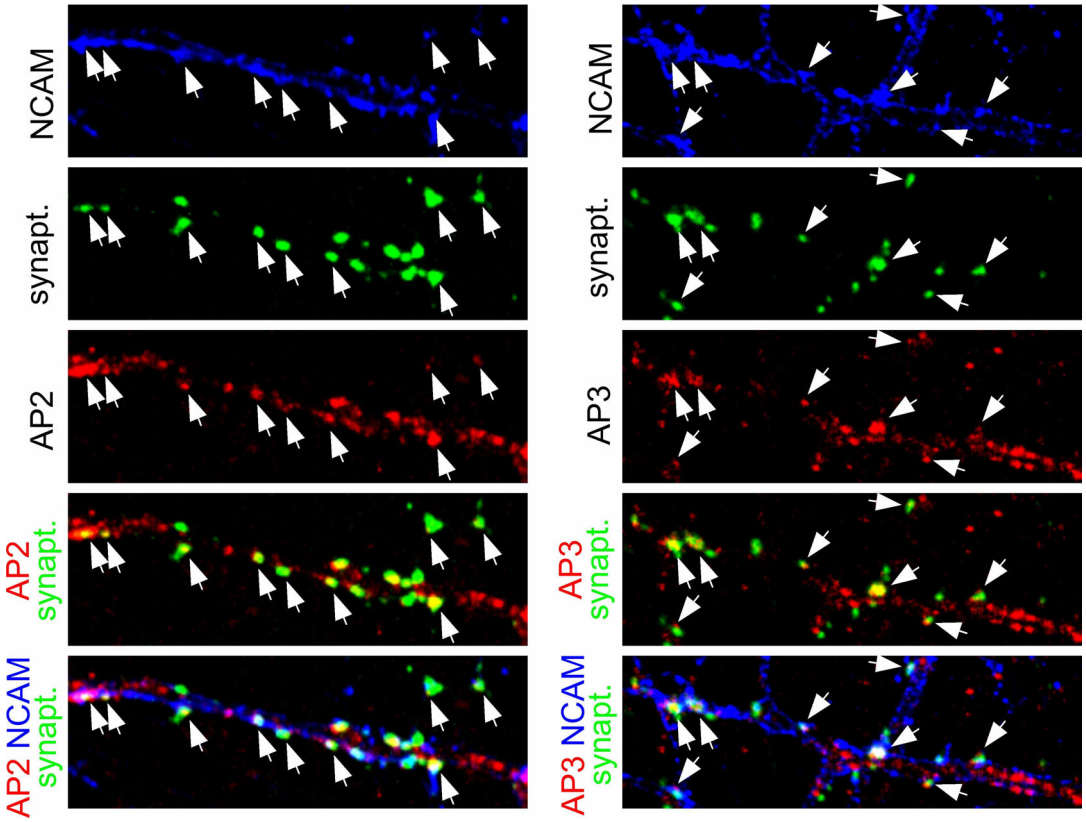

Figure 12. Cell surface NCAM was labeled by indirect immunofluorescence in non-permeabilized hippocampal neurons maintained in culture for 12 days. Neurons were then permeabilized and co-labeled with the antibodies against synaptophysin and AP2 or AP3. Note that NCAM positive presynaptic synaptophysin clusters co-localize both with AP2 and AP3 accumulations (arrows). Bar = 10 μm. Experiment done by Vladimir Sytnyk.

V.3.a. In NCAM deficient synapses, clathrin, AP2 and AP180 show reduced expression at the presynaptic membrane that results in lower number of clathrin coated vesicles formed via AP2/AP180 pathway.

AP2/AP180 and AP3 adaptor protein complexes are expressed in hippocampal synapses (Figure 12) (Granseth et al., 2006), suggesting that immaturity of NCAM-/- CNS synapses may be related to abnormalities in subsynaptic distribution of these adaptor proteins. To verify this idea, synaptosomes from NCAM+/+ and NCAM-/- brains were fractionated to obtain synaptic plasma membranes, synaptic organelles and soluble synaptic protein fraction, which were analyzed then by Western blot (Figure 13,Figure 14). Levels of clathrin, AP2 and AP180 were increased in NCAM-/- synaptosomes (Figure 13), probably because of the overall enhanced expression of these proteins in NCAM-/- brains (Figure 11).

In spite of the overall enhanced expression, the levels of clathrin, AP2 and AP180 were decreased by approximately 50 % in synaptic plasma membranes and synaptic vesicular organelles from NCAM-/- brains when compared to NCAM+/+ mice (Figure 13). Although synaptosomes contain both pre- and postsynaptic plasma membranes and vesicular organelles, AP180 is expressed only presynaptically. Postsynaptically, the average number of clathrin coated vesicles is increased in NCAM-/- synapses (Puchkov et al., submitted). Thus, these observations suggest that recruitment of clathrin, AP2 and AP180 from the cytoplasm to the synaptic plasma membrane and formation of AP2/AP180/clathrin coated vesicles is reduced in NCAM-/- synapses presynaptically. In accordance with this idea, levels of AP2, AP180 and clathrin were increased in the cytosolic synaptic fraction from NCAM-/- synaptosomes when compared to NCAM+/+ mice (Figure 13) again indicating reduced recruitment of these proteins to the surface plasma membrane and vesicular compartments from the cytoplasm.

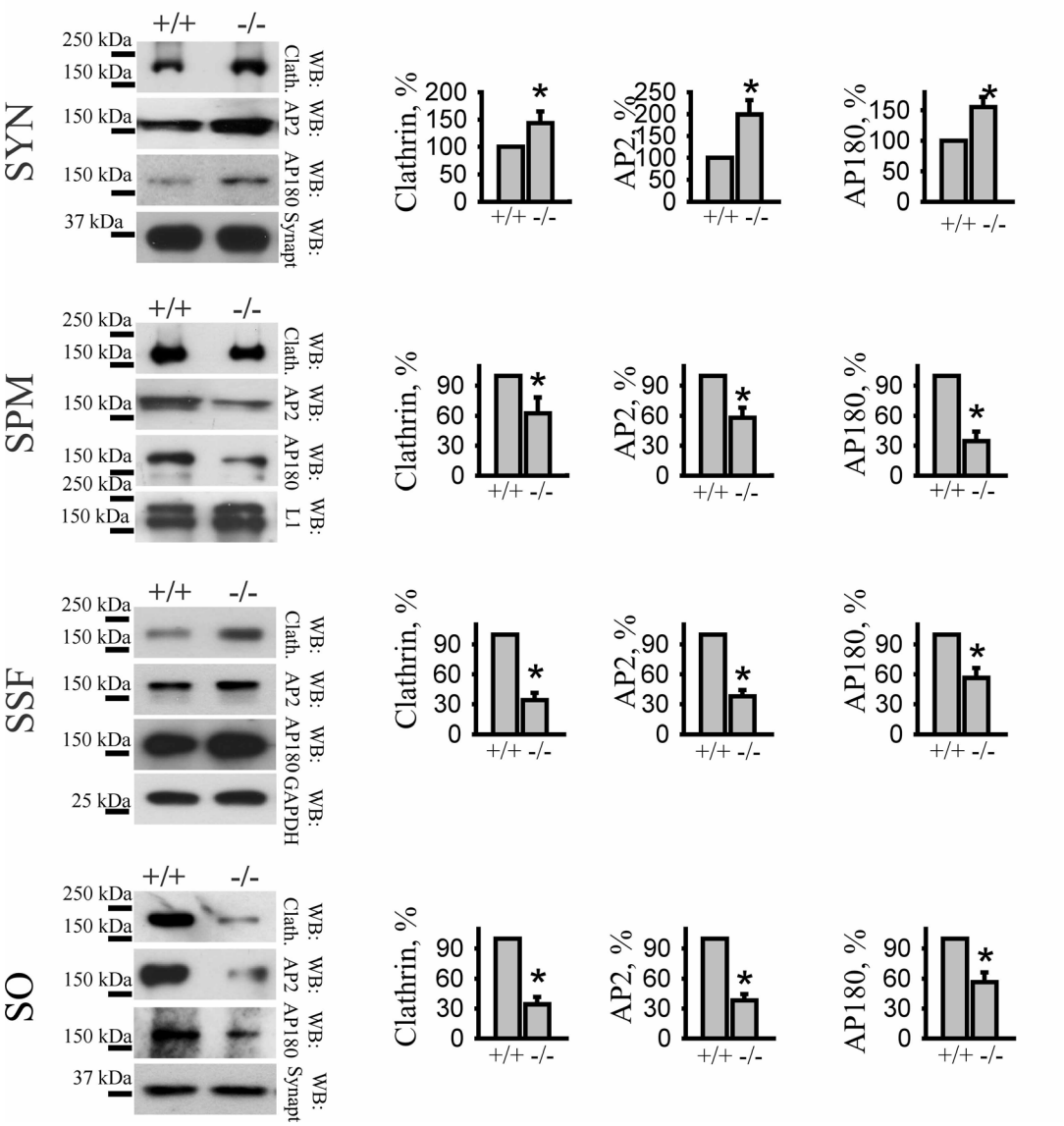

Figure 13. Distribution of AP2, AP180 and clathrin in synaptosomes (SYN), synaptic plasma membrane (SPM), synaptic soluble fraction (SSF) and synaptic vesicular organelles (SO) isolated from NCAM wildtype (+/+) and NCAM deficient (-/-) brains. Note reduced levels of AP2, AP180 and clathri in NCAM-/- synaptic plasma membranes and vesicular organelles. Synaptophysin served as loading control for synaptosomes and vesicular organelles, while labeling with antibodies against the plasma membrane associated cell adhesion molecule L1 and cytosolic marker protein GAPDH verified equal loading in synaptic plasma membranes and soluble fraction, respectively. Graphs show quantitation of blots. Mean levels + SEM in NCAM-/- mice normalized to the levels in NCAM+/+ mice set to 100% (dashed lines) are shown. *P<0.05, paired t-test (compared to NCAM+/+, n=3).

V.3.b. Levels of AP3 are increased in NCAM deficient synaptic membrane leading to the formation of AP3 coated synaptic vesicles.

In spite of abnormalities in subsynaptic levels of AP2, AP180 and clathrin, the total number of synaptic vesicles however was normal in NCAM-/- synapses as indicated by similar levels of synaptophysin in NCAM+/+ and NCAM-/- synaptosomes and synaptic vesicles (Figure 13) suggesting that NCAM-/- synapses compensate for the reduction in AP2/AP180/clathrin pathway by utilizing an alternative AP2/AP180/clathrin independent and BFA sensitive pathway to generate synaptic vesicle.

Supporting this hypothesis, the levels of AP3 and AP1 were increased in NCAM-/- synaptosomes and synaptic organelles isolated from NCAM-/- synaptosomes (Figure 14). Furthermore, in spite of overall increased expression in NCAM-/- synaptosomes the levels of AP1 were not changed while levels of AP3 were reduced in the cytosolic synaptic fraction from NCAM-/- synaptosomes when compared to NCAM+/+ mice suggesting increased recruitment of AP1 and AP3 to vesicular organelles in NCAM-/- synapses. In contrast to AP1, which was not detectable in synaptic plasma membrane fractions of both genotypes, AP3 was present in the synaptic plasma membrane fraction also. Interestingly, the levels of AP3 were six fold higher in the plasma membrane fraction from NCAM-/- synaptosomes when compared to NCAM+/+ mice, raising a possibility that AP3 functions at the plasma membrane in accordance with a recent report (Voglmaier et al., 2006) and that this function of AP3 is potentiated under conditions of reduced recruitment of AP2 to the surface plasma membrane in NCAM-/- synaptic boutons.

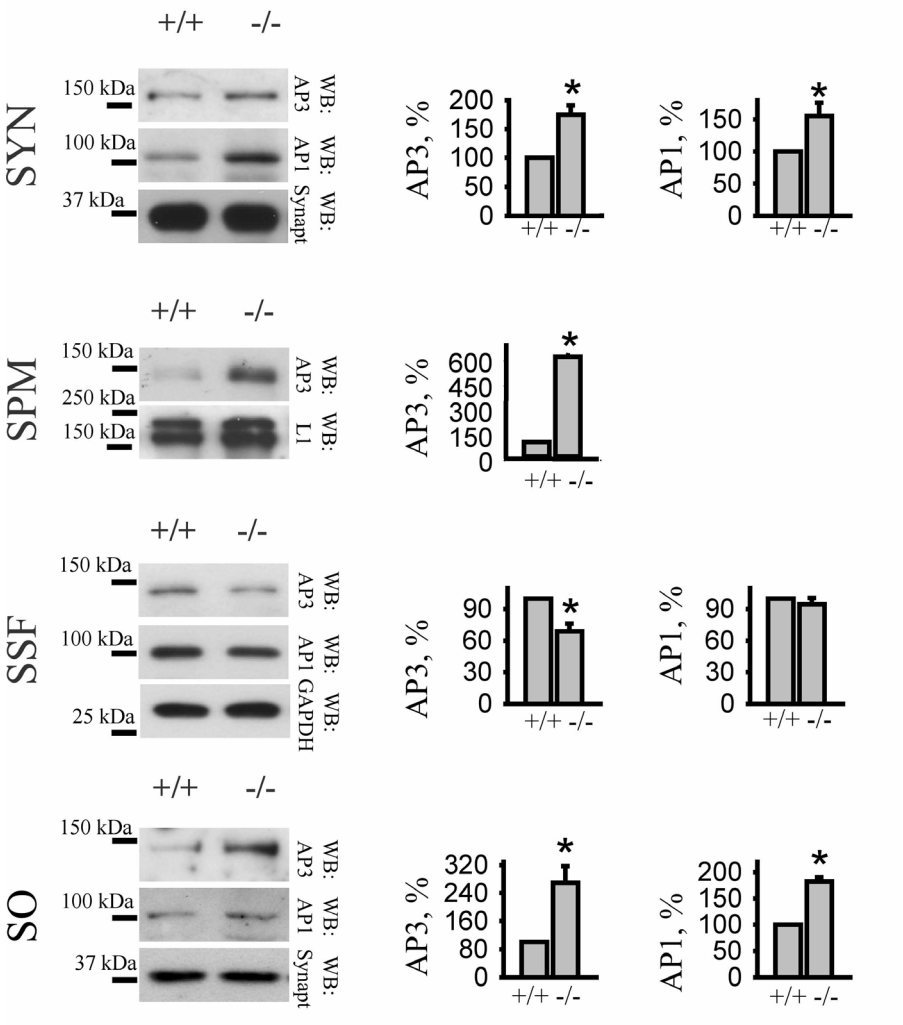

Figure 14. Distribution of AP3 and AP1 in synaptosomes (SYN), synaptic plasma membrane (SPM), synaptic soluble fraction (SSF) and synaptic organelles (SO) isolated from NCAM wildtype (+/+) and NCAM deficient (-/-) brains. Note increased levels of AP3 in NCAM-/- synaptic plasma membranes and vesicular organelles. Synaptophysin served as loading control for synaptosomes and vesicular organelles, while labeling with antibodies against the plasma membrane associated cell adhesion molecule L1 and cytosolic marker protein GAPDH verified equal loading in synaptic plasma membranes and soluble fraction, respectively. Graphs show quantitation of blots. Mean levels + SEM in NCAM-/- mice normalized to the levels in NCAM+/+ mice set to 100% (dashed lines) are shown. *P<0.05, paired t-test (compared to NCAM+/+, n=3).

V.3.c. Levels of AP2/AP180 containing synaptic vesicles are reduced in NCAM-/- synapses

Isolated synaptosomes contain various organelles including synaptic vesicles and endosomes which both are generated via clathrin dependent pathway. To confirm that deficiency in NCAM affects levels of clathrin/AP2/AP180 containing synaptic vesicles, we further fractionated the crude vesicle fraction from

lysed NCAM+/+ and NCAM-/- synaptosomes on a sucrose gradient to purify synaptic vesicles (Huttner et al., 1983; Thoidis et al., 1998). These purified NCAM+/+ and NCAM-/-/- synaptic vesicles contained similar levels of synaptophysin suggesting that the number of synaptic vesicles was unchanged in NCAM-/- synapses when compared to NCAM+/+ synapses. However, levels of AP2, AP180 and clathrin were decreased in the NCAM-/- synaptic vesicles, suggesting that AP2/AP180-mediated formation of clathrin-coated synaptic vesicle was impaired (Figure 15).

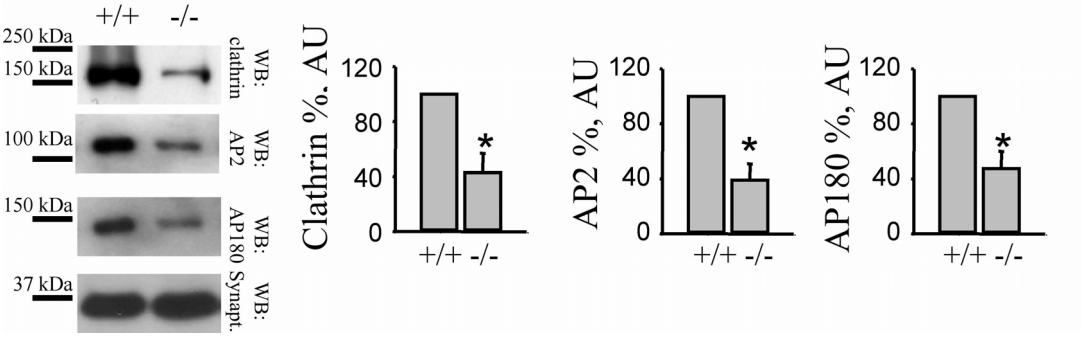

Figure 15. Synaptic vesicles purified in a sucrose gradient were probed by Western blot with the antibodies against clathrin, AP2, AP180 and synaptophysin (synapt.). Note lower levels of clathrin, AP2 and AP180 and unchanged levels of synaptophysin in NCAM-/- synaptic vesicles. Graphs show quantitation of the blots from three independent experiments (n=3 to 6 Western blots) with the signal intensity in wild type set to 1. Mean values ± SEM are shown. *P<0.05, paired t-test.

A similar result was observed for synaptic vesicles that were further purified from the purified synaptic vesicle fraction by immunoprecipitation with antibodies against synaptophysin: AP2 levels in these highly pure NCAM-/- synaptic vesicles were also reduced when compared to the NCAM+/+ synaptic vesicles (Figure 16). Interestingly, AP3 was undetectable in the purified synaptic vesicles. An explanation for this phenomenon could be that the biophysical properties of vesicles formed by AP3, such as their buoyant density, are similar to those of endosomes thus causing these synaptic vesicles not to be separated from endosomes.

Indeed, AP3 has been found on endosomes and implicated in the formation of synaptic vesicles from endosomes (Salem et al., 1998; Blumstein et al., 2001). In agreement, AP3 was present in the total pool of synaptophysin positive organelles immunoprecipitated with antibodies against synaptophysin from the crude fraction

of synaptic vesicular organelles (Figure 17), being the fraction that contains not only synaptic vesicle but also their structural intermediates. The levels of AP3 were strongly increased, while levels of AP2 were reduced in these synaptophysin positive organelles from NCAM-/- synaptosomes. These observations suggest that reduced targeting of AP2, AP180 and clathrin to synaptic membranes in NCAM-/- synapses impairs AP2/AP180-mediated formation of clathrin-coated synaptic vesicles.

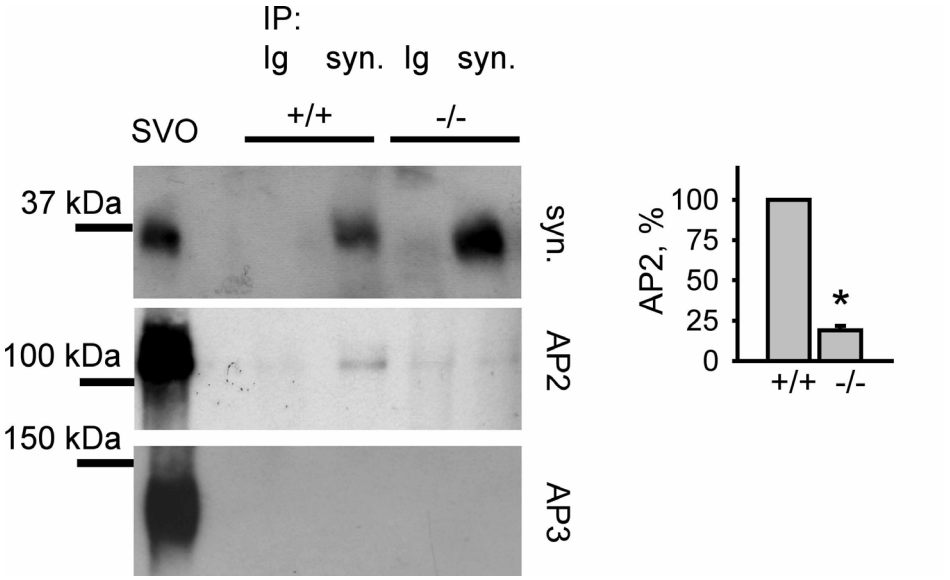

Figure 16. Synaptic vesicles purified in a sucrose gradient were further purified by immunoprecipitation (IP) with antibodies against synaptophysin (syn.) and probed by Western blot for synaptophysin, AP2 and AP3. Mock immunoprecipitation with non-immune rabbit immunoglobulins (Ig) was performed for control. Note unchanged levels of synaptophysin and reduced levels of AP2 in NCAM-/- synaptic vesicles. Synaptic vesicles are negative for AP3. Graphs show quantitation of blots (mean + SEM) with the signal in NCAM+/+ mice set to 100%. *P<0.05, paired t-test (n=3). The immunoisolation was done by Iryna Leshchyns'ka.

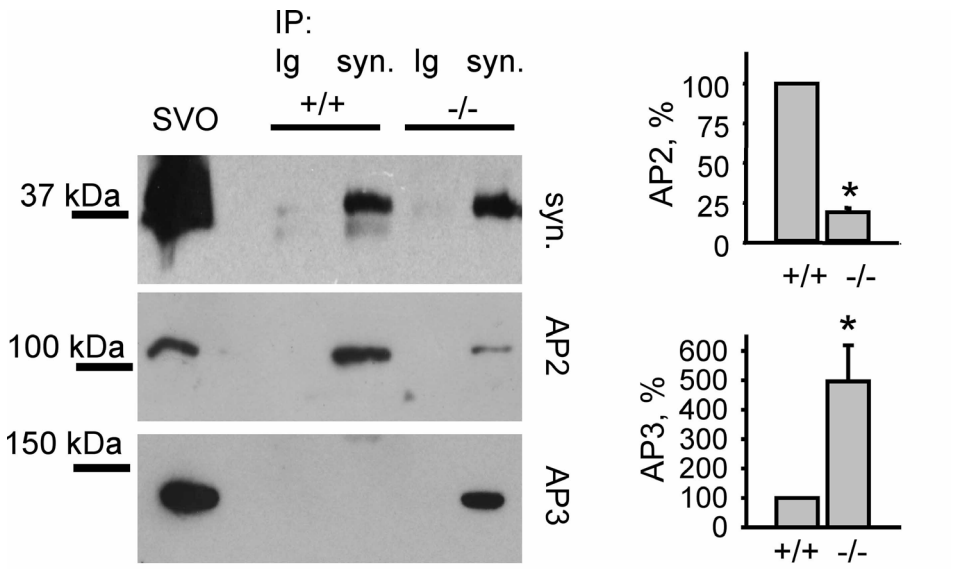

Figure 17. Synaptophysin positive organelles were immunoprecipitated (IP) with antibodies against synaptophysin from the crude synaptic vesicular organelle fraction and probed by Western blot for synaptophysin, AP2 and AP3. Note unchanged levels of synaptophysin, reduced levels of AP2 and increased levels of AP3 in NCAM-/- organelles. Graphs show quantitation of blots (mean + SEM) with the signal in NCAM+/+ mice set to 100%. *P<0.05, paired t-test (n=3). The immunoisolation was done by Iryna Leshchyns'ka.

V.4. NCAM promotes recruitment of AP2 to the synaptic plasma membrane

To verify that NCAM induces attachment of AP2 to the synaptic plasma membrane, we investigated the recruitment of AP2 and clathrin from the synaptosomal cytosol of NCAM+/+ mice, which served as a source of these proteins, to synaptic membranes isolated from NCAM+/+ and NCAM-/- mice. Before incubation with cytosol, synaptic membranes were exposed to higher pH levels to strip peripheral proteins from the membranes. Western blot analysis of the synaptic membranes incubated with cytosol showed that cytosolic AP2 and clathrin bound to NCAM+/+ and NCAM-/- synaptic membranes (Figure 18). However, the efficiency of attachment of these proteins to NCAM-/- membranes was approximately three times lower than to NCAM+/+ membranes, showing that NCAM is an essential component of the machinery recruiting AP2 and clathrin to synaptic membranes. Interestingly, attachment of AP3 from cytosol to NCAM-/-

synaptic membranes was approximately three times higher than to NCAM+/+ synaptic membranes, again suggesting that AP3 substitutes for AP2 in the absence of NCAM.

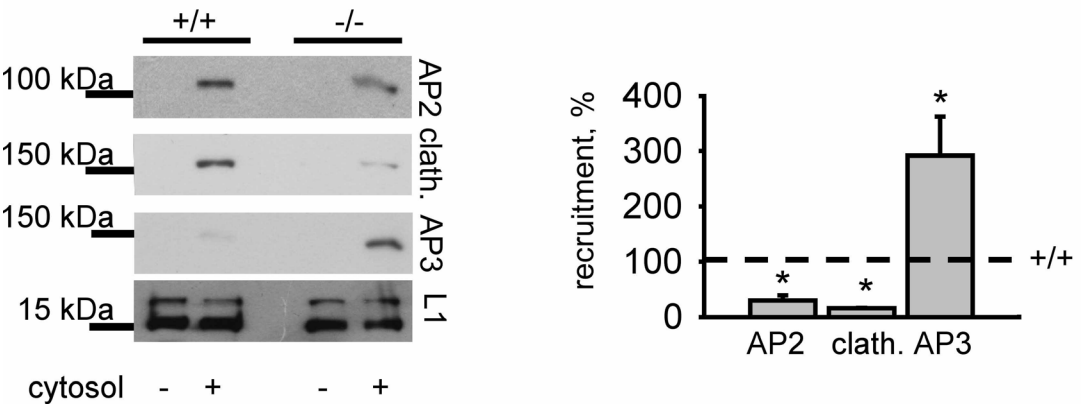

Figure 18. NCAM+/+ and NCAM-/- synaptic plasma membranes were treated with alkali to strip peripheral proteins. The membranes were then incubated with cytosol and recruitment of AP2, clathrin and AP3 from the cytosol to the membranes was assessed by Western blot. Note that alkali removed AP2, clathrin and AP3 from the membranes, but not L1, which served as loading control. Note more efficient recruitment of AP2 and clathrin to NCAM+/+ synaptic membranes and enhanced recruitment of AP3 to NCAM-/- synaptic membranes. Graph shows quantitation of blots. Mean levels + SEM of AP2, clathrin and AP3, recruited to NCAM-/- synaptic membranes, are shown. The signals were normalized to NCAM+/+ levels (dashed line) set to 100%. *P<0.05, paired t-test (compared to NCAM+/+).

V.5. NCAM interacts with the beta subunit of AP2 via a dileucine motif present in its intracellular domain

V.5.1. AP2 co-immunoprecipitates with NCAM from synaptosomes

Cell adhesion molecules that accumulate at synapses due to the homo- and heterophilic interactions play a prominent role in anchoring associated proteins in the pre- and postsynaptic membranes (Sytnyk et al., 2004; Sytnyk et al., 2006; Dalva et al., 2007). Reduced levels of clathrin, AP2 and AP180 associated with the plasma membrane in NCAM-/- synapses suggested a role for NCAM in recruitment of these components of the endocytic machinery to the synaptic surface membrane. To analyze whether NCAM associates with clathrin and AP2 in synapses, NCAM was immunoprecipitated from the isolated synaptosomes. Western blot analysis of the NCAM immunoprecipitates showed that AP2 and clathrin, but not AP3 and

AP1 co-immunoprecipitated with NCAM indicating that NCAM associates with the AP2/clathrin complex in synapses.

Interestingly, however, association of AP2 and clathrin with NCAM was differentially regulated by Ca2+: The highest levels of AP2 co-immunoprecipitated with NCAM in the presence of EDTA, a Ca2+ chelator, in the incubation buffer while an increase in Ca2+ concentration in the incubation buffer reduced levels of AP2 that co-immunoprecipitated with NCAM from synaptosomes in a concentration dependent manner. In contrast, the highest levels of clathrin co-immunoprecipitated with NCAM in the presence of 2 mM Ca2+ in the incubation buffer, the highest concentration that was analyzed, and a reduction in the Ca2+ concentration in the incubation buffer resulted in the reduced association of clathrin with NCAM.

Since clathrin mediated endocytosis is facilitated by the transient Ca^{2+} increase resulting from synaptic activity (Chen et al., 2003), our data suggest that NCAM recruits AP2 to the presynaptic membrane in the absence of synaptic activity. Synapse activation accompanied by an increase in Ca2+ concentration in the presynaptic bouton would then result in the dissociation of NCAM/AP2 complex thereby reducing NCAM internalization to synaptic vesicles. Our observation that only minor levels of NCAM are present in vesicular organelles isolated from synaptosomes (Figure 10) is in agreement with this notion.

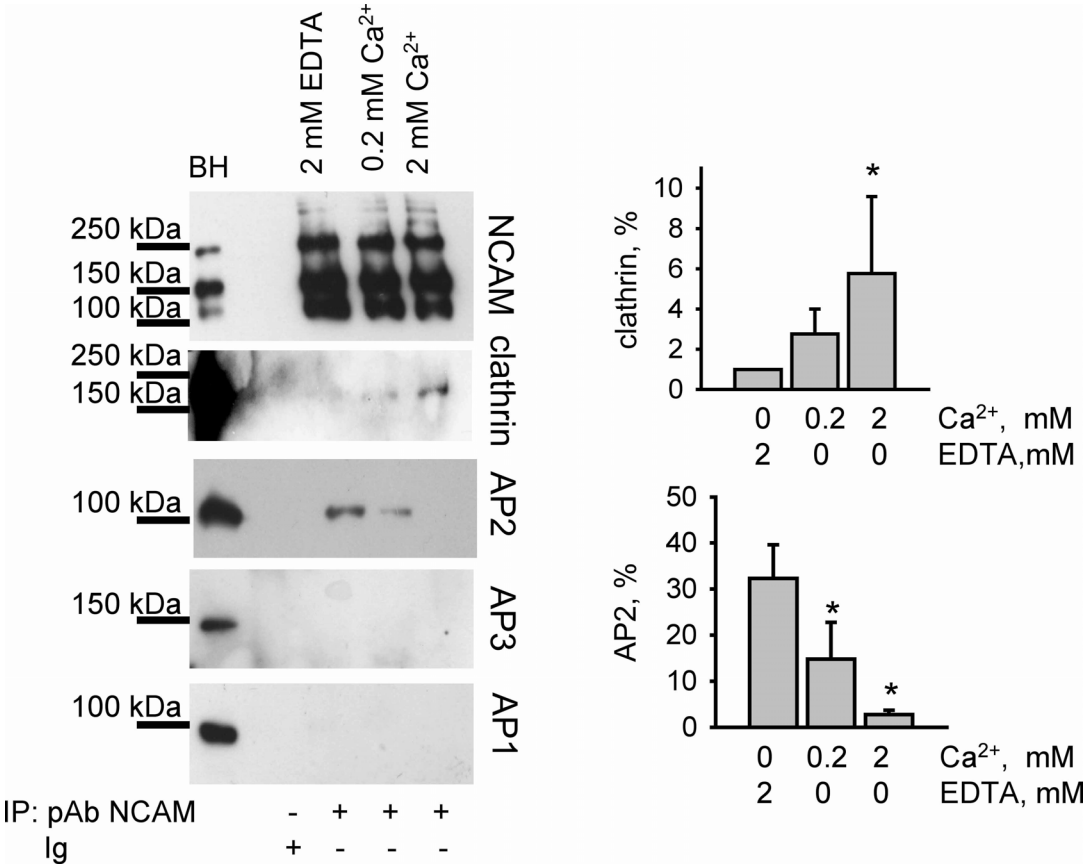

Figure 19. NCAM immunoprecipitates (IP) from synaptosomes obtained in the presence of 2 mM EDTA or in the presence of 0.2 mM or 2 mM Ca2+ were probed by Western blot with the indicated antibodies. Mock immunoprecipitation with non-immune rabbit immunoglobulins (Ig) was performed for control. Note that Ca2+ inhibits co-immunoprecipitation of AP2, and enhances co-immunoprecipitation of clathrin with NCAM. AP3 and AP1 do not co-immunoprecipitate with NCAM. Graphs show percentage of AP2 and clathrin (mean + SEM) co-immunoprecipitating with NCAM with the total pool of these proteins in the input set to 100%. *P<0.05, paired t-test (compared to EDTA incubated group, n=3).

V.5.2. Beta subunit of AP2 interacts with a dileucine motif in the NCAM intracellular domain in-vitro

Analysis of the amino acid sequence of the mouse NCAM showed the presence of two dileucine like motifs at positions 751 and 853 of the NCAM intracellular domain (Figure 20), suggesting that NCAM directly associates with the β2 subunit of AP2 that has been shown to interact directly with the dileucine motifs in other proteins (Greenberg et al., 1998; Rapoport et al., 1998).

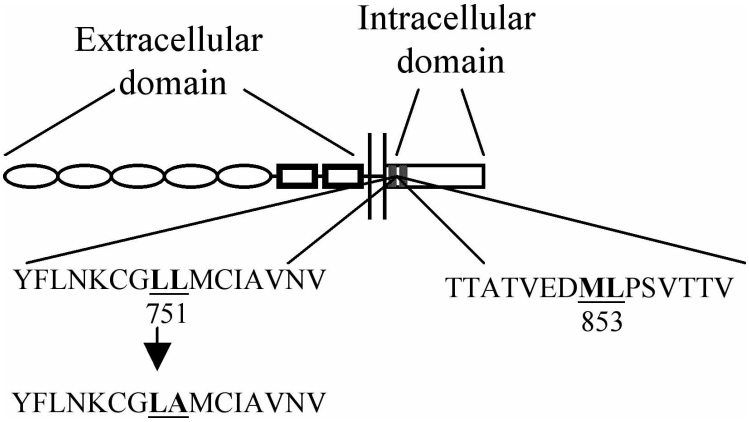

Figure 20. A scheme of NCAM showing two putative AP2-binding motifs within intracellular domain. A single amino acid mutation disrupting the dileucine motif is also shown. The LL motif is present in both NCAM140 and NCAM180 whereas the ML motif is present only in NCAM180.

Confirming this, in *in vitro* pull-down assay recombinant intracellular domains of NCAM (NCAM-ICD) were pulled down with the immobilized on the beads recombinant GST-tagged β2 subunits of AP2 (β2-GST) but not with the GST alone used for control (Figure 21). To investigate whether this interaction was via the dileucine motifs present in NCAM-ICD, pull down of NCAM-ICD with β2-GST was analyzed after beads containing β2-GST were pre-incubated with the peptides corresponding to the amino acid sequences 744-759 ($NCAM_{LL}$) and 846-860 ($NCAM_{ML}$) within NCAM-ICD or a LL-motif containing peptide derived from the viral protein NEF (NEF_{LL}), which is known to interact with the β2 subunit of AP2 and served as a positive control (Greenberg et al., 1998). Pre-incubation with NEF_{LL} or $NCAM_{LL}$ reduced pull-down of NCAM-ICD with β2-GST suggesting that they competed with NCAM-ICD for binding to β2. In contrast, pre-incubation with $NCAM_{ML}$ only slightly inhibited pull-down of NCAM-ICD with β2-GST. Thus our data indicate that β**2** subunit of AP2 directly interacts with

NCAM-ICD via the dileucine motif present between amino acids 744-759 of NCAM-ICD, while ML motif between amino acids 846-860 of NCAM-ICD does not play a major role in the interaction between NCAM and AP2.

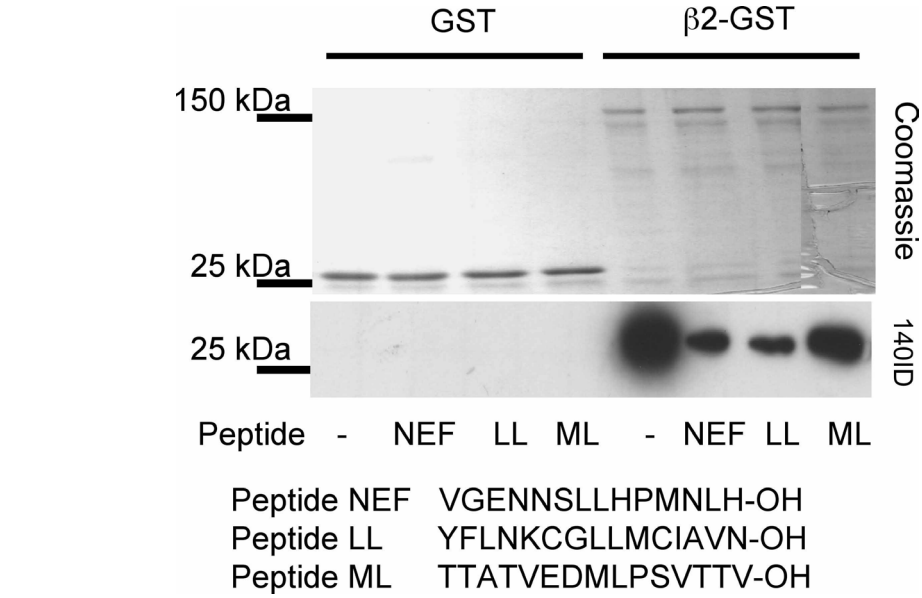

Figure 21. Recombinant GST-tagged β subunit of AP2 (β-GST) or GST alone were immobilized on beads and their binding to recombinant intracellular domains of NCAM140 (140ID), that were either non-treated or preincubated with the indicated peptides, was assessed by pull down assay. Western blot analysis of the eluates from the beads with the antibodies recognizing 140ID showed that 140ID was pulled down with β-GST but not GST alone. Binding of 140ID to β-GST is strongly inhibited by the dileucine containing peptide derived from 140ID and the positive control peptide NEF and only mildly affected by the ML motif containing peptide from 180ID.

V.5.3. Single amino acid mutation in the NCAM intracellular domain reduces interaction of NCAM with AP2 in live cells

To confirm that the LL motif is important for NCAM/AP2 complex formation in live cells, non-mutated rat NCAM140 or mutated rat NCAM140 with leucine 752 exchanged to alanine were transfected into CHO cells and assayed for their ability to associate with AP2 by co-immunoprecipitation. Western blot analysis of NCAM immunoprecipitates showed that non-mutated NCAM140 forms a complex with AP2 (Figure 22). Interestingly, AP2 associated with NCAM140 more strongly than with NCAM180 (not shown). A possible explanation for this phenomenon could be a stronger association of NCAM180 with the spectrin meshwork (Leshchyns'ka et al., 2003), which inhibits assembly of the endocytotic machinery at the surface plasma membrane (Kamal et al., 1998; Hardy et al., 1979; Hardy and Schrier,

1978). Disruption of the LL motif by the LA mutation strongly reduced levels of AP2 co-immunoprecipitating with NCAM140 from transfected CHO cells, indicating that the LL motif is required for efficient formation of the NCAM/AP2 complex in live cells.

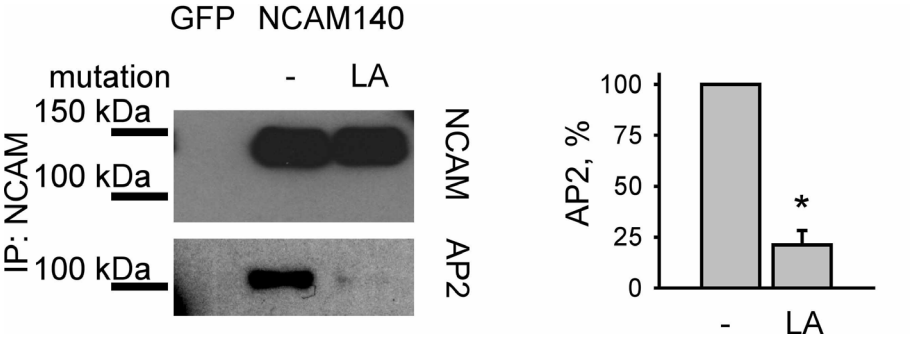

Figure 22. NCAM immunoprecipitates (IP) from CHO cells transfected with NCAM140, non-mutated or mutated in the membrane-proximal LL dileucine motif, were analyzed by Western blot with antibodies against NCAM and AP2. Mock immunoprecipitation with NCAM antibodies from CHO cells transfected with GFP only was performed for control. Note that mutation in the LL dileucine motif inhibits co-immunoprecipitation of AP2 with NCAM140. Graph shows quantitation of blots (mean + SEM) with the signal in non-mutated NCAM140 immunoprecipitates set to 100%. *P<0.05, paired t-test (n = 4).

V.6. NCAM promotes recruitment of AP2 at the synaptic plasma membrane by directly binding to AP2

To analyze the role of the dileucine motif within NCAM-ID in recruitment of AP2 to the synaptic membranes, we analyzed redistribution of AP2 to NCAM+/+ synaptic membranes from cytosol that was pre-incubated with recombinant NCAM140-ID or with mutated NCAM140LA-ID where leucine 752 was exchanged to alanine. Preincubation of cytosol with NCAM140-ID inhibited binding of AP2 to the synaptic membranes (Figure 23A,B) suggesting that recombinant NCAM140-ID competed with endogenous NCAM in the synaptic membranes for AP2. In contrast, NCAM140LA-ID did not affect binding of AP2 to the membranes (Figure 23A) showing that the dileucine motif is essential for NCAM-dependent recruitment of AP2 to synaptic membranes.

Interestingly, when redistribution of AP2 from cytosol to NCAM+/+ synaptic membranes was inhibited by pre-incubation with NCAM140-ID,

redistribution of AP3 to these NCAM+/+ synaptic membranes was enhanced (Figure 23B) resembling NCAM-/- phenotype (Figure 18) and suggesting that AP2 and AP3 compete for the same binding sites at the synaptic membrane with NCAM enhancing the affinity of AP2 - synaptic membrane binding over that of AP3.

To support this idea, we analyzed recruitment of AP2 and AP3 to NCAM+/+ synaptic membranes from cytosol pre-incubated with BFA, which inhibits recruitment of AP3 to membranes. BFA-induced inhibition of AP3 recruitment to synaptic membranes was accompanied by increased attachment of AP2 (Figure 24), further suggesting that AP2 and AP3 compete and can substitute for each other. Importantly, this observation provides an explanation for the enhanced FM1-43 uptake into synaptic boutons of NCAM-/- neurons treated with BFA: under conditions of overall enhanced AP2 and clathrin expression in NCAM+/+ mice, inhibition of AP3 recruitment to synaptic membranes would result in increased AP2 recruitment, thereby resembling the NCAM+/+ phenotype, which was indeed observed (Figure 8).

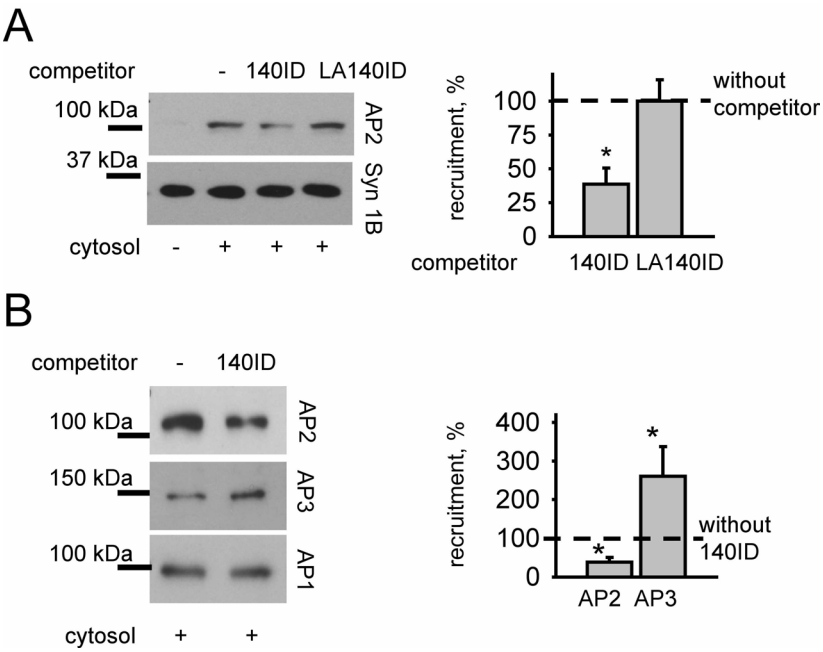

Figure 23. A, B **– Recruitment of AP2 and AP3 to NCAM+/+ synaptic membranes as analyzed by Western blot. When indicated, the cytosol was preincubated with competitors – either non-mutated intracellular domain of NCAM140 (140ID) or 140ID with the mutated membrane-proximal dileucine motif (LA140ID). Note that 140ID, but not LA140ID reduces recruitment of AP2 to the membranes (**A**). Reduced AP2 recruitment results in enhanced AP3 recruitment (**B**). Graphs show quantitation of blots. Mean levels + SEM of AP2 and AP3, recruited to synaptic membranes from cytosol in the presence of competitors, are shown. The signals were normalized to the signal without competitors (dashed lines) set to 100%. *P<0.05, paired t-test (compared to the signal without competitors).**

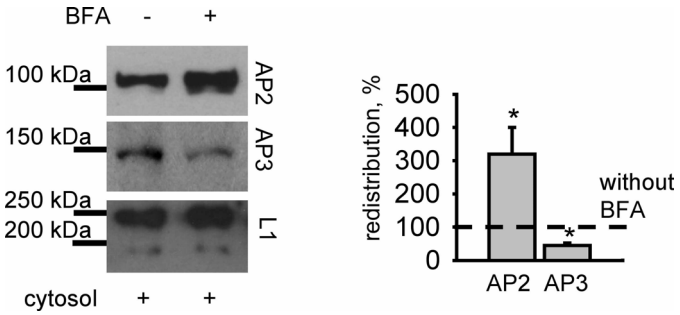

Figure 24. Recruitment of AP2 and AP3 to NCAM+/+ synaptic membranes from the cytosol, that was either non-treated or preincubated with BFA, was analyzed by Western blot. Note that the inhibition of AP3 recruitment by BFA results in enhanced recruitment of AP2. The graph shows mean ± SEM levels of AP2 and AP3, recruited to the synaptic membranes from the cytosol that was preincubated with BFA, normalized to the levels of AP2 and AP3, recruited to the membranes from non-treated cytosol (dashed line). *P<0.05, paired t-test (three independent experiments (n=3 to 6 Western blots)). Labeling for L1 served as a loading control.

V.7. NCAM deficiency impairs age-dependent substitution of AP3 with AP2 at the synaptic membrane

BFA-dependent synaptic vesicle recycling has been suggested to represent an immature mode of synaptic vesicle recycling in isolated motor neuron axons (Zakharenko et al., 1999). To analyze whether the role of AP3 diminishes with age in CNS synapses also, levels of AP3 were analyzed in synaptosomes and synaptic membrane fractions isolated from the brains of postnatal day 7 (P7) and P60 mice. Western blot analysis showed that whereas similar levels of AP3 were present in synaptosomes from NCAM+/+ mice of different age, the levels of AP3 that was co-isolated with synaptic plasma membranes were strongly reduced in adult (P60) versus young (P7) NCAM+/+ mice (Figure 25). In contrast, levels of AP2 and AP180 were strongly increased in synaptic plasma membrane fractions from adult versus young NCAM+/+ mice suggesting that synaptic vesicle membrane retrieval machinery switched from the AP3- to AP2-dependent mechanism. The levels of AP3 and AP2 in synaptosomes and synaptic plasma membranes from P7 NCAM-/- mice were similar to that of P7 NCAM+/+ mice. However, in contrast to NCAM+/+ mice, the levels of AP3 in synaptosomes and synaptic plasma membranes from P60 NCAM-/- mice remained high and were at the same level as in P7 mice, while levels of AP2 only slightly increased with age in these mice. Thus, our data suggest that NCAM deficiency impairs a developmental switch from the AP3- to AP2-dependent synaptic vesicle recycling probably by abolishing NCAM- and age-dependent recruitment of AP2 to synaptic plasma membranes.

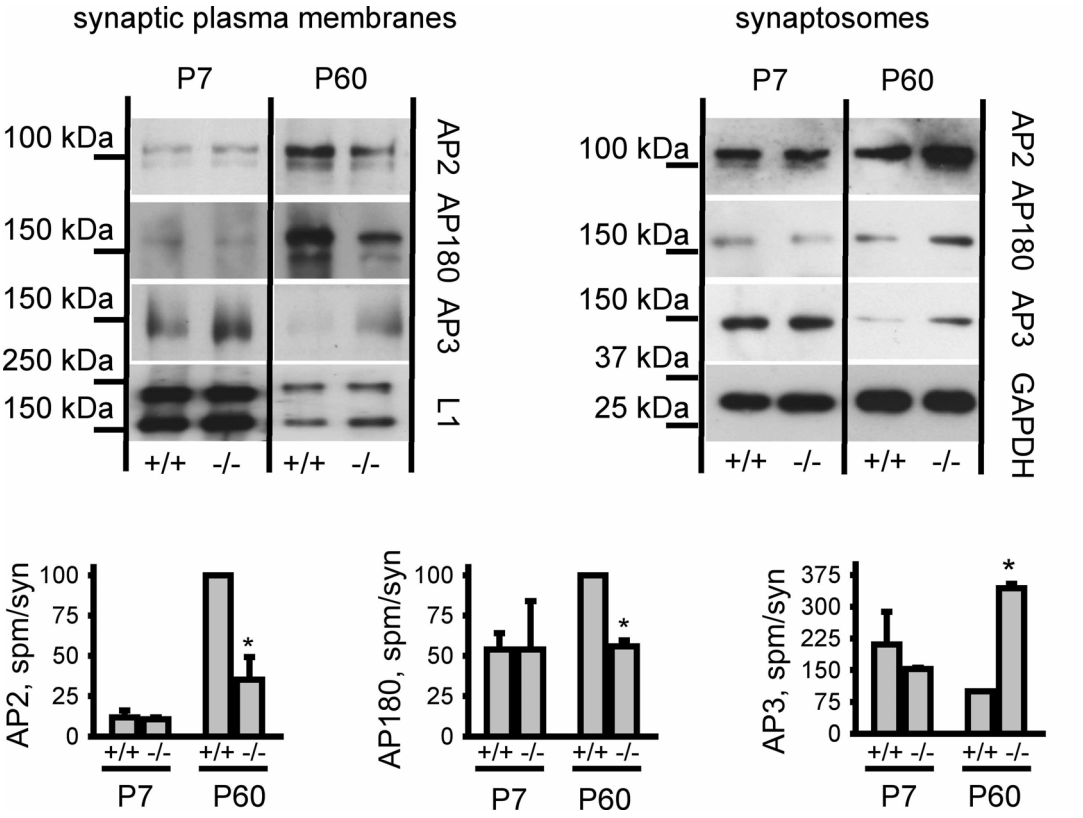

Figure 25. Levels of AP2, AP3 and AP180 in NCAM+/+ and NCAM-/- synaptic plasma membranes and synaptosomes from postnatal day (P) 7 (P7) or P60 mice were analyzed by Western blot. Note that levels of AP2 and AP180 increase while levels of AP3 decrease with age in synaptic plasma membranes from NCAM+/+ mice. Developmental substitution of AP3 by AP2 and AP180 is reduced in NCAM-/- synaptic plasma membranes. Graphs show quantitation of blots. Mean ratios + SEM of protein levels in synaptic plasma membranes to their levels in synaptosomes are shown. Values were normalized to the values for P60 NCAM+/+ mice set to 100%. *P<0.05, paired t-test (compared to P60 NCAM+/+). Labeling for L1 (for synaptic membranes) or GAPDH (for synaptosomes) served as loading controls.

V.8. NCAM enhances the efficiency of the synaptic vesicle membrane endocytosis in hippocampal neurons

To analyze how NCAM deficiency affects synaptic vesicle recycling, NCAM+/+ and NCAM-/- cultured hippocampal neurons were transfected with the pH-sensitive form of green-fluorescent protein (GFP) fused to the lumenal domain of VAMP (synaptopHluorin), which provides a sensitive optical probe to follow exo- and endocytosis of synaptic vesicles, containing this protein, in real time. Transfected neurons were then challenged with the high K+ containing buffer for

90 s to depolarize synaptic membranes and induce Ca2+ influx to presynaptic terminals leading to synaptic vesicle exocytosis. Following stimulation, the buffer was exchanged to 4 mM K+ containing buffer to allow synaptic vesicle membrane endocytosis. Axons of the transfected neurons were continuously monitored before, during and after stimulation using a time series function of the laser scanning microscope.

Application of the high K+ containing buffer resulted in an increase in the synaptopHluorin fluorescence at synaptic boutons due to synaptic vesicle exocytosis and exposure of the lumenal synaptopHluorin tag to the extracellular medium with the fluorescence permissive pH (Figure 28). During recovery phase, synaptopHluorin fluorescence reduced indicating endocytosis of the protein to synaptic vesicles. While the amplitude of the increase in synaptopHluorin fluorescence intensity in response to high K+ was approximately the same in NCAM+/+ and NCAM-/- synaptic boutons, the amplitude of the decrease in synaptopHluorin fluorescence intensity during the recovery time was strongly reduced in NCAM-/- synaptic boutons. In extreme cases, no decrease in synaptopHluorin fluorescence intensity was observed in NCAM-/- synaptic boutons during the whole recovery time indicating impaired endocytosis of synaptopHluorin.

Co-transfection of NCAM-/- neurons with NCAM140 partially improved the efficiency of synaptopHluorin endocytosis. Interestingly, the amplitudes of synaptopHluorin fluorescence increase in response to high K+ tended to be higher in neurons co-transfected with NCAM140 versus neurons transfected with synaptopHluorin only. The incomplete recovery then could be due to higher levels of synaptopHluorin exocytosed during the stimulation in these neurons. Unexpectedly, co-transfection with NCAM140LA also improved synaptopHluorin endocytosis efficiency in NCAM-/- neurons, although this effect was less prominent than that for NCAM140.

Substitution of leucine 752 with alanine in NCAM140LA mutant did not affect the cell surface expression of this protein and its targeting to presynaptic boutons in neurons (Figure 26). To further verify this, cell surface biotinylation experiments were performed on HEK293 cells transfected with constructs encoding full length NCAM140 and NCAM140LA (Figure 27). It was seen also in these

cells that suface delivery of NCAM140LA was similar to that of NCAM140. The LA mutation in NCAM only partially inhibits the association of NCAM140 with AP2 (Figure 22). Therefore, it is possible that even weak association of the overexpressed NCAM140LA with AP2 in NCAM-/- neurons is sufficient to improve synaptopHluorin endocytosis under conditions of higher expression of AP2 and clathrin in NCAM-/- neurons. However, when NCAM140LA was co-transfected with synaptopHluorin to NCAM+/+ neurons, endocytosis of synaptopHluorin during the recovery time was strongly inhibited resembling the NCAM-/- phenotype (Figure 28).

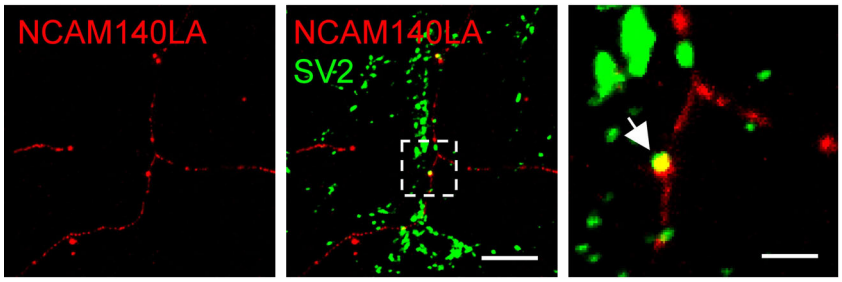

Figure 26. Axon of NCAM-/- cultured hippocampal neuron transfected with NCAM140LA is shown. NCAM at the cell surface was visualized by NCAM antibodies applied to formaldehyde fixed neurons. Neurons were then permeabilized and co-labeled with SV2 antibodies. Note that NCAM140LA accumulate in the presynaptic membrane of SV2 positive synaptic boutons (arrow). High magnification image of the area outlined with dashed lines is shown on the right. Scale bar 10 µm (low magnification images), 1 µm (high magnification image). The experiment was done by Vladimir Sytnyk.

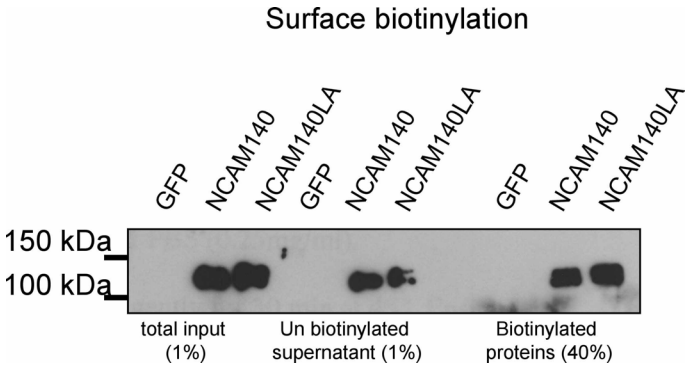

Figure 27. Cell surface biotinylation was performed with HEK293 cells transfected with constructs encoding full length NCAM140 and NCAM140LA. Transfection with GFP was used as a negative control. Proteins at the cell surface were biotinylated. After lysing the cells, the biotinylated proteins are captured on magnetic neuravidin beads. The proteins were separated from the matrix by addition of SDS sample buffer. Immunoblot analysis with NCAM antibodies shows that surface delivery of NCAM140LA is similar to that of wildtype NCAM140.

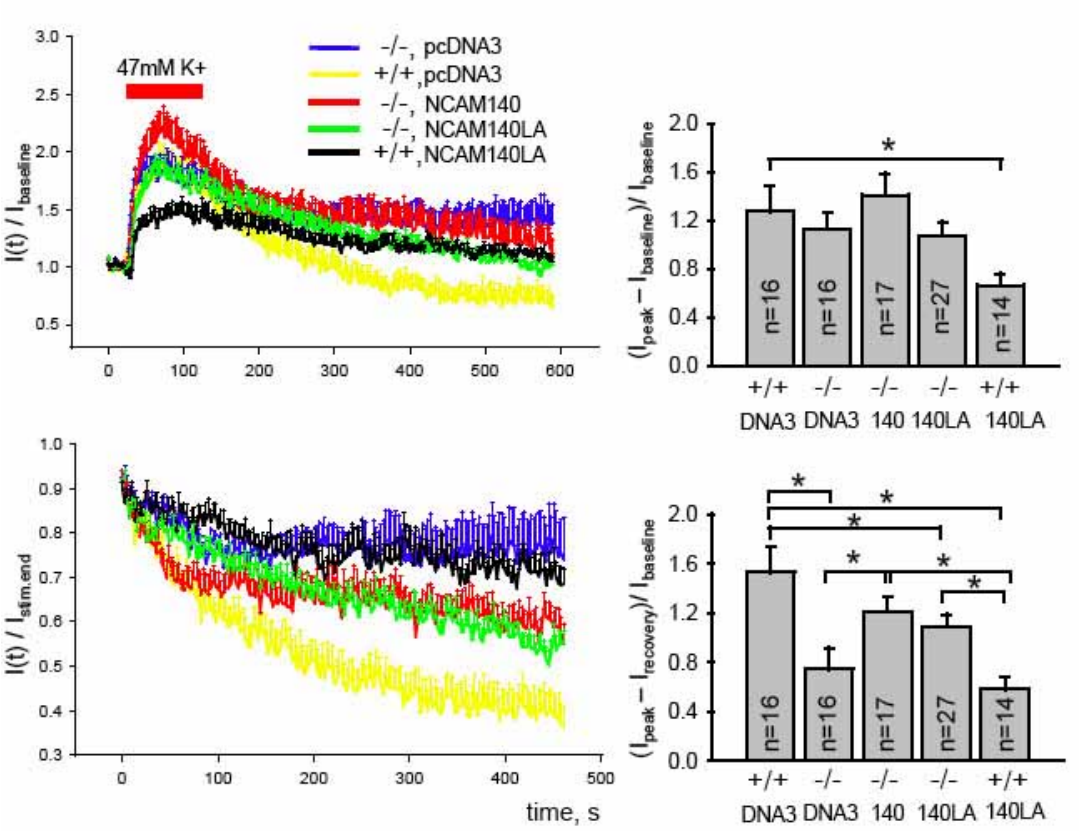

Figure 28. NCAM+/+ and NCAM-/- cultured hippocampal neurons were co-transfected with synaptopHluorin (spH) and NCAM140, NCAM140LA or empty pcDNA3 vector. Changes in spH fluorescence were then monitored in synaptic boutons of transfected neurons before and during stimulation with 47 mM K+ and during recovery in 4 mM K+ following stimulation. The upper left diagram shows fluorescence intensity signals normalized to the mean baseline signal before stimulation. Red bar indicates the time of 47 mM K+ application. To better visualize differences during the recovery time, the lower left diagram shows spH fluorescence intensity signals during the recovery time only, normalized to the spH intensity immediately after stimulation. In both diagrams, mean values ± SEM are shown. The graphs on the right show mean amplitudes + SEM of spH fluorescence intensity increase (peak) following 47 mM K+ application (upper panel) and spH fluorescence intensity decrease during the recovery time (lower panel) normalized to the mean baseline signal before stimulation. Note reduced efficiency of spH endocytosis, characterized by lower amplitudes in the decrease of spH fluorescence signal intensity during recovery time in NCAM-/- neurons and NCAM+/+ neurons transfected with NCAM140LA. In NCAM-/- neurons co-transfected with NCAM140 or NCAM140LA, spH endocytosis is increased when compared to NCAM-/- neurons. The number of synapses (n) taken from 8-10 neurons is shown for each bar. *P<0.05, t-test. The experiment done by Vladimir Sytnyk and quantification was done by Iryna Leshyns'ka.

VI. Discussion

The project was aimed at investigating the role of NCAM in synaptic vesicle recycling at the central nervous system synapses. Therefore we begin by discussing the abnormalities in the endocytic machinery in NCAM-/- synapses that were uncovered in this study. The later part of the discussion deals with establishing a mechanistic model portraying the role of NCAM in regulating synaptic vesicle recycling that is supported by results of this project.

VI.1. Deficiency of NCAM leads to down regulation in AP2 mediated synaptic vesicle synthesis.

Previous studies in NCAM-/- mice showed impaired PPF and abnormal presynaptic form of LTP in hippocampal mossy fibres (Muller et al., 1996; Bukalo et al., 2004). Additionally, the studies of the NCAM-/- NMJ indicated very strongly that NCAM is involved in synaptic vesicle dynamics. Correlating with these findings, the NCAM-/- mice fare poorly in balancing themselves on a rotating rod (rotorod test), implying fatigued synapses (Polo-Parada et al., 2001). Inability of synaptic vesicles to fuse on sustained stimulus is one of the reasons for a failed neurotransmission at a synapse and hence fatigue. This can occur when the synapse is unable to maintain a pool of releasable synaptic vesicles on repetitive stimulation. Decreased recruitment of AP2 on NCAM-/- synaptic plasma membrane (Figure 18) can lead to decreased number of AP2 coated synaptic vesicles that we observe (Figure 15, Figure 16).

It is well established that AP2 mediated clathrin coated synaptic vesicle recycling is an essential way to obtain competent synaptic vesicles after activity induced fusion (Morgan et al., 2002; Granseth et al., 2006). The observation that the expression of AP180 and clathrin, other proteins in this pathway is also decreased on NCAM-/- synaptic plasma membrane and vesicles (Figure 13) rules out the possibility that AP2 dysregulation is a unconnected event to the pathway. The effect on synaptic expression of the downstream (clathrin) and upstream (AP180) components implies that the AP2 mediated vesicle formation pathway is downregulated in the NCAM-/- synapse.

Intricate and complex regulation of gene expression in living organisms leads to compensatory mechanisms when there is flaw in the system. When a gene is knocked out or a pathway is downregulated, the organism tries to rectify the situation by overexpressing related proteins. For example, when PSD-95 and PSD-93, components of the post synaptic density (PSD), are genetically ablated, SAP-102, another PSD protein is overexpressed, compensating for the loss (Elias et al., 2006). Similarly, in the NCAM-/- synaptic terminal, we observe overexpression of AP2, AP180 and clathrin in the total brain (Figure 11) as well as in the whole synapse (as seen in the synaptosomal fraction, Figure 13) to compensate for the downregulated AP2 mediated pathway. However, most of the overexpressed AP2, AP180 and clathrin remain soluble and hence non-functional, as there is decreased recruitment of these proteins on the synaptic plasma membrane in the absence of NCAM at the synapse (Figure 13). Since these proteins have other functions, the overexpressed proteins could be functionally compensating in another pathway.

Previous work has shown that AP2 interacts with NCAM leading to endocytosis and hence regulating surface expression of NCAM in astrocytes and neurons (Minana et al., 2001; Diestel et al., 2007). Although this indicates that NCAM and AP2 interact, immunoprecipitation of NCAM from synaptosomes shows synapse specific interaction (Figure 12, Figure 19). Additionally, we were able to characterize the site of interaction as a dileucine motif present in the NCAM intracellular domain (Figure 20, Figure 21). Contradictory to AP2/NCAM interaction in the cell body and axons, synapse specific interaction does not lead to endocytosis of NCAM, as can be concluded from the relatively low amounts of NCAM present in synaptic vesicles (Figure 10). This indicates that the direct binding of AP2 to NCAM at the synapse is involved not in maintaining NCAM surface expression but in AP2 mediated clathrin coated vesicle endocytosis.

Calcium is involved in the regulation of several protein-protein interactions. Specifically, the transient increase of calcium seen on synaptic stimulation leads to temporal regulation of neurotransmission (Igarashi and Watanabe, 2007). Therefore it is not surprising that calcium also regulates AP2 binding to NCAM at the synapse (Figure 19). Opposite patterns of calcium regulation of AP2 and clathrin binding to NCAM indicates that AP2 initially binds to NCAM on the synaptic plasma membrane in the absence of activity.

Calcium increase on stimulation causes the AP2/NCAM complex to disassociate. Following this, AP2 can bind to an adjacent synaptic protein, which is then endocytosed into vesicles on clathrin recruitment. Synaptotagmin, a synaptic protein has been shown to bind to AP2 regulating endocytosis (Jarousse and Kelly, 2001; Jarousse et al., 2003) and therefore could function downstream to AP2 recruitment by NCAM. The increased levels of clathrin/NCAM complexes seen at higher calcium concentration probably means that localised clathrin is made available by synaptic NCAM to bind to AP2 along with synaptotagmin, inducing endocytosis. Therefore NCAM acts as a docking station for AP2 on the synaptic plasma membrane, keeping the synapse primed to act rapidly on stimulation to form competent AP2/clathrin coated vesicles. The observation that along with numerous proteins like stoned, AP180, endophilin, etc.; lipid components like inositol phosphates are also involved in synaptic endocytosis indicates that different levels of regulation of this precise process exists (Fergestad and Broadie, 2001; Augustine et al., 2006; Jung and Haucke, 2007). This is probably one of the reasons for the diversity and plasticity during neurotransmission at synapses.

VI.2. AP3 mediated synaptic vesicle recycling pathway coexists with the AP2-dependent mode in mature synapses acting as the default mechanism for synaptic vesicle formation in the absence of NCAM

Interestingly, we observed that although expression of proteins involved in AP2 mediated pathway is dysregulated, the effect on endocytosis in the NCAM-/- synapses is not to the expected extend (Figure 8). Additionally, although AP2 is overexpressed in the absence of NCAM, reduced recruitment of AP2 at the NCAM-/- synaptic plasma membrane indicates a compensatory mechanism independent of AP2 and clathrin to generate synaptic vesicles. Accordingly, AP3, a protein characterised in the formation of clathrin independent synaptic vesicles (Salem et al., 1998; Shi et al., 1998) shows increased expression on NCAM-/- on synaptic plasma membrane and synaptic vesicles (Figure 14). The role of AP3 in compensating for AP2 in the absence of NCAM is supported by the fact that we observed increased AP3 recruitment on the NCAM-/- synaptic plasma membrane (Figure 18).

The question raised then is the need for the co-existence of two seemingly redundant pathways, when both result in the genesis of synaptic vesicles. AP3 is involved in vesicle formation from endosomes, which might not be competent for fusion (Faundez et al., 1998). This is supported by the fact that AP3 positive synaptic vesicles are not obtained from the protocol used to isolate purified synaptic vesicles on a sucrose density gradient (Figure 15, Figure 16). However, when the protocol is modified to include larger vesicular organelles of endosomal origin, AP3 positive synaptic vesicles could be obtained. The increased number of AP3 coated vesicles seen in the NCAM-/- synapses (Figure 14,Figure 17) can compensate for the reduction of AP2 coated vesicles (Figure 15, Figure 16). Hence although AP3 also forms synaptic vesicles like AP2, they are larger and probably have to be processed before reuse, resulting in the slight decrease in the rate of endocytosis in the NCAM-/- synapse (Figure 8). Further proof for AP3 compensation in the NCAM-/- synapse was obtained when we were able to mimic increased AP3 recruitment on synaptic plasma membrane containing NCAM on addition of the NCAM intracellular domain (ICD). Competition of the ICD with the endogenous NCAM for AP2 binding results in decreased recruitment of AP2, correlating with increased AP3 recruitment (Figure 23), which is as seen in the NCAM-/- synapse (Figure 18).

VI.3. NCAM plays a role in establishing the AP2 mediated mature mode of synaptic vesicle recycling.

Previous results implied that the absence of synaptic NCAM leads to the retention of an immature mode of vesicle recycling. The NCAM-/- NMJ is sensitive to the effects of BFA, which is a drug that inhibits vesicle fusion in developing immature axons (Polo-Parada et al., 2001). As BFA does not affect recycling in mature NMJs, insensitivity to the drug is used as a property of mature synapses (Zakharenko et al., 1999). Our data, to verify if synaptic immaturity is limited to NCAM-/- NMJ, or extends to the CNS synapses, showed the presence of strong compensatory mechanisms in NCAM-/- hippocampal synapses (Figure 8). BFA and latrunculin increase synaptic uptake in the absence of NCAM, clearly indicating that another pathway compensates for the inhibition caused by these drugs. Discussed above already is how BFA sensitive AP3 compensates for

decreased AP2 recruitment on NCAM-/- synaptic plasma membrane (Figure 14, Figure 17, Figure 18, Figure 23). Addition of BFA enhances recruitment of soluble AP2 on synaptic plasma membrane (Figure 24). Overexpression of AP2 in NCAM-/- synapses hints that compensation of synaptic uptake in NCAM-/- synapses treated with latrunculin and BFA occurs by AP2 recruitment by one of its other binding partners. All these results together indicate that the AP3 pathway compensates for downregulated AP2 pathway in NCAM-/- synapses and increased AP2 recruitment compromises BFA inhibition of AP3 in NCAM-/- synapses.

Complimenting the effect of BFA and latrunculine, NCAM-/- synapses are resistant to tetanus toxin, which inhibits fusion of vesicles preventing FM dye uptake uptake in NCAM+/+ synapses (Figure 8). This further reiterates the fact that in the absence of NCAM, the process of maturation of the synapse slows down with the NCAM-/- synapse retaining the immature AP3 mediated mechanism. Furthermore, the increase in AP2 expression with age on the synaptic plasma membrane clearly indicates that AP2/AP180 recruitment promotes synaptic vesicle maturation (Figure 25). The drastic reduction in rate of endocytosis quantified by following the trafficking of synaptopHluorin in the absence of NCAM, uncovers more precisely the functional fault in synaptic recycling (Figure 28). Furthermore NCAM promotes endocytosis by recruiting AP2, since the mutant form of NCAM (NCAM140LA) competes with wildtype NCAM in a dominant negative fashion, decreasing the rate of endocytosis (Figure 28).

BFA inhibits synaptic recycling in developing axons (Zakharenko et al., 1999), and in PC12 cells (Shi et al., 1998). Since AP3 forms synaptic vesicles in PC12 cells from endosomes (Faundez et al., 1998), it has been assumed that BFA action seen in developing axons is excreted through AP3. Since this assumption had not been confirmed, the developmental profile of adaptor protein expression was analyzed (Figure 25). Our data showing the decreased expression of AP3 on synaptic plasma membrane with age clearly indicates that AP3 is regulated with age. As AP3 is predominantly expressed in young immature synapses that are sensitive to BFA, it is involved in immature recycling. The role of NCAM in this process of synaptic recycling maturation is clarified when the switch in expression is retarded in NCAM-/- synapses. Therefore, AP3 is the predominant mode of synaptic vesicle recycling in immature synapses, which on development gets slowly

replaced by the more efficient AP2, mediated pathway. However, the AP3 pathway is still retained, although not predominantly functional under normal stimulation in the mature synapse, as can be concluded from the slight sensitivity to BFA of synaptic uptake of FM dyes (Figure 8) and the presence of AP3 in adult NCAM+/+ synapses (Figure 14,Figure 25).

VI.4. Proposed model indicating the role of NCAM in synaptic vesicle endocytosis.

To conclude, we predict a model based on the results obtained and discussed in this study (Figure 29). Adaptor proteins have low affinity to inositol phosphates (InPs) present in the synaptic membrane. On further binding to the specific peptide motifs, the weak association of AP2 to the lipid moiety is converted to high affinity recruitment to the membrane. This has been shown specifically in the case of AP2 recruitment (Honing et al., 1998; Honing et al., 2005). NCAM is an ideal candidate to anchor AP2, as it contains a dileucine motif, which binds specifically to AP2, recruiting it. Although the affinity of AP3 to inositol phosphates has never been investigated, it is possible that AP3, like AP2 also binds to InPs since the μ subunits of both adaptors exhibit high degree of homology. Hence this would result in a competition between AP2 and AP3 for synaptic plasma membrane recruitment, albeit via an initial weak binding to InPs. NCAM enriched on the synaptic plasma membrane binds specifically to AP2, resulting in its selection over AP3. Downstream of AP2 recruitment is the formation of clathrin coated vesicles on endocytosis.

However, when NCAM is absent, AP2 recruitment is no longer at an advantage and hence “loses” the competition to AP3. AP3 recruitment then leads to the formation of increased numbers of AP3 coated vesicles, thereby compensating for the decreased synaptic AP2 binding. The formation of AP3 coated vesicles is not as efficient resulting in slower rates of endocytosis that we observe in NCAM-/- synapses.

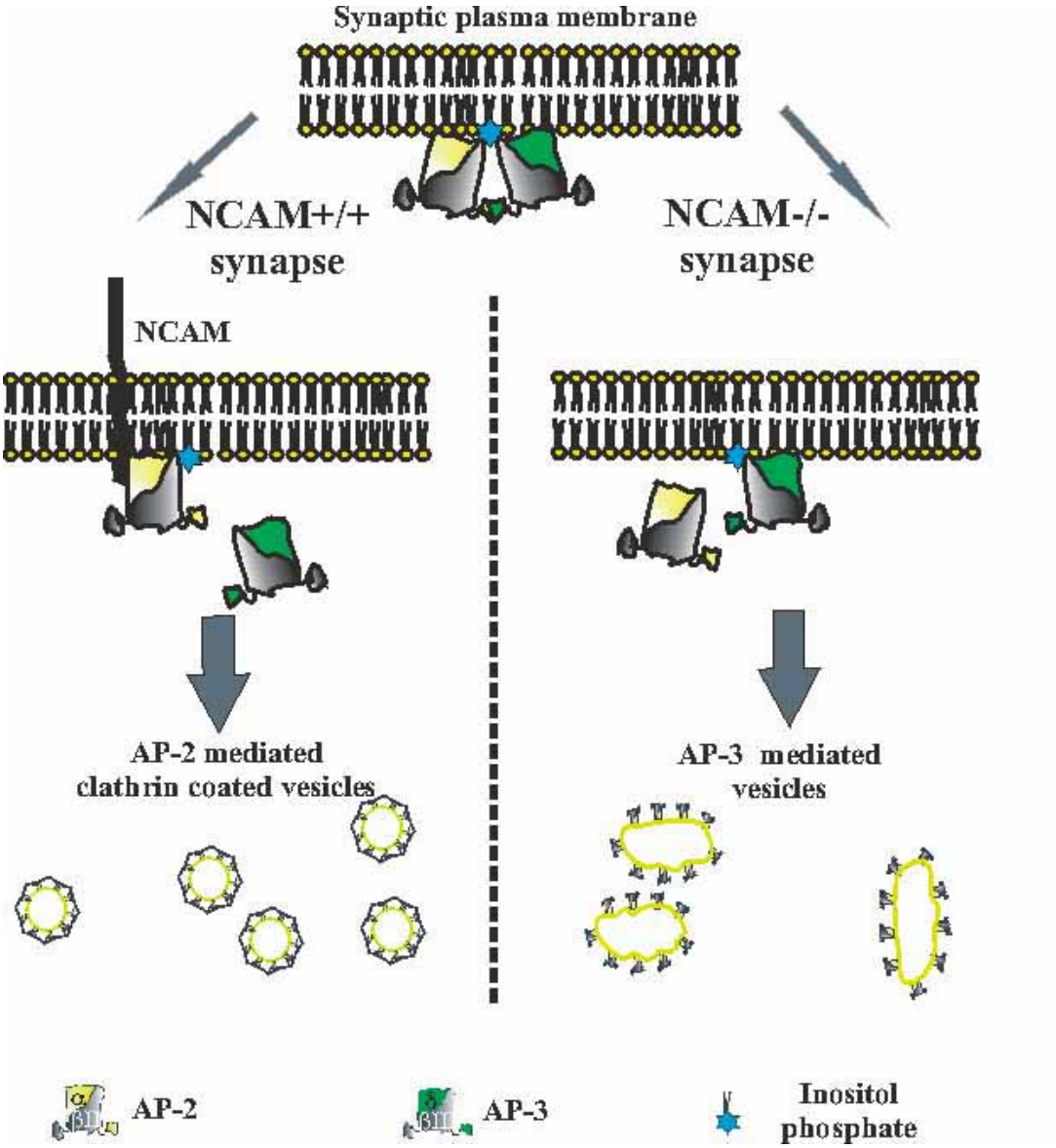

Figure 29. Proposed model depicting competition between AP2 and AP3 for recruitment, leading to the two different modes of synaptic vesicle formation. Both AP2 and AP3 bind weakly to inositol phosphates on the synaptic plasma membrane, thus competing for recruitment. Presence of NCAM potentiates AP2 recruitment, leading to competent clathrin coated vesicles ready for fusion. However, in NCAM-/- synapses, AP2 recruitment is not potentiated, leading to the AP3 recruitment at the synaptic plasma membrane resulting in the formation of AP3 coated vesicles.

The result of this study demonstrates for the first time the co-existence of two modes of synaptic vesicle formation via recycling regulated by a cell adhesion molecule, NCAM. One of them is the clathrin dependent mode mediated by the recruitment of AP2 by NCAM and the other is the clathrin independent pathway involving AP3. The following lines of evidence support this. Firstly, we are able to

observe that synaptic vesicle recycling is not affected drastically in NCAM-/- hippocampal synapses. Additionally, when inhibitors of immature synaptic recycling (BFA and latrunculin) are added to NCAM-/- synapses, there is a compensatory upregulation of synaptic uptake. Secondly, we observe decreased recruitment of AP2 and increased recruitment of AP3 on NCAM-/- synaptic plasma membrane. Thirdly, when AP2 recruitment is artificially inhibited by the addition of exogenous NCAM, we induce AP3 recruitment. We conclude from this that AP2 is the predominant pathway for recycling, and AP3 is the "back-up" pathway.

The AP2 and AP3 are compensatory to each other in terms of cargo selection at the adult synapse (Voglmaier et al., 2006). But why does the adult synapse need the AP3 pathway? We show for the first time that the expression of AP2 and AP3 are developmentally regulated. AP3 mediated synaptic recycling is predominantly functional during early stages of development. The switch in the expression pattern of synaptic AP3 with AP2 during development emphasizes the role of AP2 in mature synaptic vesicle recycling. Although AP3 compensates for AP2 in the adult synapse, decreased rate of endocytosis in the NCAM-/- synapse hints at an "incompetent" recycling pathway. The difference in efficiencies of the two pathways indicates that the AP3 pathway is only retained in the adult, becoming functional in cases when the AP2 pathway is hindered. The AP3 pathway during development is probably necessary and functions optimally. More detailed characterisation of AP3 during neuronal development is needed for better understanding of this pathway.

Most importantly, recruitment of AP2 by NCAM on the synaptic plasma membrane uncovers a new role for the cell adhesion molecule in establishing a vesicle recycling pathway. We show that NCAM recruits AP2 to the synaptic plasma membrane and in the absence of NCAM, there are fewer AP2 coated synaptic vesicles, meaning there is a down regulation of the pathway. The specific binding of AP2 to synaptic NCAM along with the functional irregularities in synaptic vesicle endocytosis in the absence of NCAM uncovers a novel, uncharacterised role for NCAM.

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VIII. Appendix

1. Peptides

1.	NEF peptide 8245	Biotin-VGENNS*LL*HPMNLH-OH	Derived from viral protein NEF
2.	LL peptide 8426	Biotin-YFLNKCG*LL*MCIAVN-OH	Potential LL motif derived from NCAM ICD
3.	ML peptide 8427	Biotin-TTATVED*ML*PSVTTV-OH	Potential ML motif derived from NCAM ICD

2. Oligonucleotides

1.	NCAM 5' primer	gaacaagtgtggcctg**gcc**atgtgcatcgctg	Site directed mutagenesis for NCAM ICD (LL to LA)
2.	NCAM 3' primer	cagcgatgcacat**ggc**caggccacacttgttc	Site directed mutagenesis for NCAM ICD (LL to LA)

3. Abbreviations

1.	μ	Micro (10^{-6})
2.	°C	Degree celcius
3.	A	Adenosine
4.	ADP	Adenosine biphosphate
5.	Amp	Ampicillin
6.	APS	Ammonium per sulphate
7.	ATP	Adenosine triphosphate
8.	bp	Base pairs
9.	BSA	Bovine serum albumin
10.	C	Cytosine
11.	cDNA	Complementary deoxyribonucleic acid
12.	CHL1	Close homologue of L1
13.	DNA	Deoxyribonucleic acid
14.	EDTA	Ethylene diamine tetra acetic acid
15.	FCS	Fetal calf serum
16.	FNIII	Fibronectin III
17.	g	g-force
18.	G	Guanosine
19.	GFP	Green fluorescent protein
20.	GPI	Glycosylphosphotidylinositol
21.	GST	Glutathione S transferase
22.	HBSS	Hank's buffered sodium chloride solution
23.	HEPES	2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid
24.	ICD	Intracellular domain
25.	Ig	Immunoglobulin
26.	IgG	Immunoglobulin subclass G
27.	IPTG	Isopropyl-β-D-thiogalactoside
28.	Kan	Kanamycin
29.	kb	Kilo base pairs

30.	l	liter
31.	LB	Luria Bertani
32.	LTP	Long term potentiation
33.	m	Milli (10^{-3})
34.	MEM	Minimal essential medium
35.	mRNA	Messenger ribonucleic acid
36.	n	Nano (10^{9})
37.	NCAM	Neural Cell Adhesion Molecule
38.	OD_x	Optical density at x
39.	ORF	Open reading frame
40.	p	Pico (10^{12})
41.	PBS	Phosphate buffered saline
42.	PCR	Polymerase chain reaction
43.	PMSF	Phenyl methyl sulfonyl fluoride
44.	RNA	Ribonucleic acid
45.	RNase	Ribonuclease
46.	rpm	Rotations per minute
47.	RT	Room temperature
48.	SDS-PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
49.	T	Thymine
50.	TE	Tris EDTA
51.	Tet	Tetracycline
52.	TM	Transmembrane
53.	Tris	tris(-hydroxymethyl)-aminomethane
54.	V	Volts
55.	v/v	Volume per volume
56.	w/v	Weight per volume

IX . Publications

The present thesis is mainly based on the following article and abstract of poster presented.

Article

- Shetty, A., V. Sytnyk, I. Leshchyns'ka, and M. Schacher, 2008, The neural cell adhesion molecule NCAM promotes maturation of thepresynaptic endocytotic machinery by switching synaptic vesicle recycling from AP3 to AP2-dependent mechanism: (In preperation).

Abstract

- Shetty, A., I. Leshchyns'Ka, V. Sytnyk, and M. Schachner, 2006, Neuronal cell adhesion molecule promotes maturation of synaptic vesicle recycling machinery in the central nervous system.: FENS forum.

X. Acknowledgements

I end this thesis with the acknowledgements. The beginning, however, would not be possible without the ZMNH, Prof. Schachner and Dr. Dutta. The entire project was supervised by Dr. Sytnyk and Dr. Leshchyns'ka. I am grateful to them for the scientific training I have gained during the course of my Ph.D. A special thanks to Ellen, our librarian who always managed to get any literature that I desired. Other people from the centre who I periodically went for help are Fritz and Torsten for never failed to come up with a solution for a problem; the technical support (EDV) who I went to for my frequent computer problems; and the ever friendly administrative staff. Thank you all!

I cannot thank enough all those colleagues who supported me in different ways, some of whom are now my closest friends. I am especially grateful to the innumerable people who I have annoyed in the past years with my questions, complains and problems. I would like to express my immense gratitude to Praveen, who had to bear up with the after affects of the innumerable "bad" lab days. The only reason I have not mentioned the other names is because I would need a few pages to fill in all of them.

I feel obliged to acknowledge caffeine and chocolate for the successful completion of my thesis.

Lastly, I dedicate this thesis to my family, who made me what I am today.

XI. Certificate

UNIVERSITÄT HAMBURG

Fakultät für Mathematik, Informatik und Naturwissenschaften

Frau Aparna SHETTY

Ich eröffne Ihnen hiermit, daß Sie die Doktorprüfung beim Department Biologie gemäß Promotionsordnung vom 05. Juli 1999 mit den folgenden Noten:

Dissertation: „sehr gut“
Gesamtnote: „sehr gut“

bestanden haben.

Nach Ablieferung der Pflichtexemplare der Dissertation wird Ihnen die Doktorwürde durch Aushändigung des vom Dekan für Mathematik, Informatik und Naturwissenschaften unterschriebenen und gesiegelten Doktorbriefes verliehen.

Vorher sind Sie nicht berechtigt, den Doktortitel zu führen.

Hamburg, den 03. April 2008

Universität Hamburg 64 Fakultät Mathematik, Informatik und Naturwissenschaften

Professor Dr. J. Ganzhorn
Leiter des Departments Biologie