The Function of Neurobeachin in the Central Nervous System

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CHAPTER 1

Introduction

A prerequisite for normal synapse formation is precise coordination of trafficking of synaptic proteins and the structural assembly of the presynaptic exocytic machinery and the postsynaptic neurotransmitter units. One of the key players in the formation and functioning of synapses is the protein Neurobeachin. Nbea is an A-kinase anchoring protein (AKAP) that has been implicated in membrane trafficking and belongs to the BEACH family of proteins. In order to understand what this means, this chapter presents a short review of these theoretical concepts, which will accompany us throughout this thesis. The first section is dedicated to protein traffic in general, which is followed by a description of the family of BEACH (<u>be</u>ige <u>an</u>d <u>C</u>hediak-<u>H</u>igashi) proteins. Then the structure and function of AKAPs as dynamic organizers of cellular events is explained. The aim and outline of the thesis comprise the end of this chapter.

1.1 Protein trafficking

Eukaryotic cells have compartmentalized the cytoplasm into distinct membrane-bound organelles, which enable them to regulate delivery of newly synthesized proteins, carbohydrates, and lipids to the cell exterior and to take up macromolecules by the process of endocytosis (Alberts et al., 2002; Derby and Gleeson, 2007). The initial definition of the outward-bound membrane transport pathway as the *secretory pathway* and the inward-bound membrane transport pathway as the *endocytic pathway*, has lost its sharp distinction when it became clear that the two pathways converge at a variety of locations (Derby and Gleeson, 2007).

The components of the secretory and endocytic pathway include the <u>e</u>ndoplasmic

reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), the Golgi apparatus, the *trans*-Golgi network (TGN), a heterogenous set of endosomes, lysosomes, the plasma membrane, and secretory granules in regulated secretory cells (Condon and Ehlers, 2007; Derby and Gleeson, 2007). These membrane-bound organelles must selectively allow the movement of proteins and membrane in a vectorial fashion by a carefully coordinated series of vesicle budding and fusion events and at the same time maintain a unique set of resident proteins that define their structural and functional properties (Rothman, 1996; Teasdale and Jackson, 1996).

1.1.1 The early biosynthetic-secretory pathway

The endoplasmic reticulum

The ER, the first organelle in the secretory pathway, is a polygonal meshwork of membrane tubules and sheet-like cisternae (Palade, 1956) that are connected with the continuous membrane of the outer nuclear envelope (Prinz et al., 2000). It can be divided into the ribosome-bound rough ER (RER) that is constituted primarily by sheets or cisternae, and the ribosome-free smooth ER (SER) that is predominantly composed of tubules where vesicle budding and fusion takes place (Shibata et al., 2006). While in yeast and plant cells, the ER is located close to the plasma membrane (referred to as "cortical ER"), in mammals, the tubular ER network extends throughout the entire cell (Hu et al., 2011). It is responsible for lipid and sterol synthesis, the synthesis and post-translational modifications of most secretory and membrane proteins, and the regulation of Ca^{2+} levels and arachidonic acid release (Ramirez and Couve, 2011).

The ER represents a key quality control step, where proteins are screened for proper folding before being transported by coat protein complex II (COPII)-coated vesicles, emerging from specialized ER-exit sites (ERES), to other organelles (Bannykh et al., 1996; Derby and Gleeson, 2007). Soluble ER-resident proteins are kept in place by preventing them from entering newly formed vesicles and by sorting and retrieving back to the ER those resident proteins that escape (Teasdale and Jackson, 1996). Many soluble ER resident proteins possess a KDEL (Lys-Asp-Glu-Leu)-like motif at their extreme C-terminus (Raykhel et al., 2007). The latter allows them to interact with a KDEL receptor in the ERGIC leading ultimately to their return to the ER via COPI-coated vesicles (Raykhel et al., 2007). Hence, the KDEL motif is often used as a marker for the identification of the ER.

The ER-Golgi intermediate compartment

The ERGIC, also called the vesicular-tubular clusters (VTC) or *cis*-Golgi network, is a complex interconnected membrane system defined by the presence of a 53 kDa membrane protein, i.e. ERGIC-53 (Altan-Bonnet and Lippincott-Schwartz, 2005; Bannykh et al., 1996; Schweizer et al., 1988). It is localized between, but is discontinuous with, the ER and *cis*-Golgi (Bannykh et al., 1996) and functions as the first post-ER sorting station for antero- and retrograde protein traffic between the ER and

the *cis*-Golgi (Appenzeller-Herzog and Hauri, 2006; Derby and Gleeson, 2007). In addition, it also represents another checkpoint for conformation-based quality control of proteins and, possibly protein folding (Appenzeller-Herzog and Hauri, 2006) and is the site of *N*-acetylgalactosamine (GalNAc) addition and palmitylation of proteins (Teasdale and Jackson, 1996).

The Golgi apparatus

The Golgi apparatus, also called the Golgi complex, is in higher eukaryotic cells typically organized as a series of three to ten flattened cisternae arranged as parallel stacks that are traditionally categorized into three compartments: the *cis*-Golgi (in secretory cells facing the ER), *medial-* and *trans*-Golgi (in secretory cells facing the plasma membrane; Mogelsvang et al., 2004). Since the emergence of the ERGIC that comprises the cis-most cisterna of the Golgi stack, there is little to distinguish the *cis* elements of the Golgi apparatus from the ERGIC. Similarly the *trans* side of the Golgi is nowadays referred to as the trans-Golgi network (TGN; see below).

The medial Golgi is a carbohydrate factory engaged in the biosynthesis of glycolipids and of oligosaccharide portions of glycoproteins and proteoglycans (Mellman and Simons, 1992). The most common post-translational sugar modification is the remodeling of the high mannose N-linked glycoproteins that are initially generated in the ER/ERGIC and the initiation and extension of O-linked glycan chains of glycosaminoglycans (Altan-Bonnet and Lippincott-Schwartz, 2005).

The *trans*-Golgi network

The TGN is a unique compartment located at the exit face of the Golgi stack that by EM tomography typically appears to be associated with a large amount of vesicular and tubular membranes (Mogelsvang et al., 2004). Its main function is to sort various cargo proteins and lipids and package them into membrane carriers, which are sent on divergent pathways to different post-Golgi destinations, e.g. distinct domains of the plasma membrane, endosomes, lysosomes (via late endosomes), secretory granules, or backward to earlier Golgi cisternae and possibly directly to the ER (Derby and Gleeson, 2007). At the same time the TGN also receives membrane traffic from the endocytic pathway (Mellman and Simons, 1992) and thereby represents the site at which the secretory and endocytic pathways intersect (Derby and Gleeson, 2007). Like the rest of the Golgi stack, it also plays a role in processing cargo molecules - here "late" protein modification events occur, e.g. galactose α2,6 sialyation (Mellman and Simons, 1992).

1.1.2 The endocytic pathway

While the biosynthetic secretory system delivers newly synthesized transmembrane proteins to the plasma membrane, membrane proteins, but also lipids and extracellular ligands, are removed from the cell surface by endocytosis, a process crucial for rapid

and localized regulation of proteins at the plasma membrane (Lasiecka and Winckler, 2011). The "classical" endocytic pathway that has mainly been studied in non-neuronal cells comprises numerous vesicular organelles, including the early/sorting and recycling endosomes (EE and RE, respectively), multivesicular bodies (MVBs) and late endosomes (LE) and lysosomes, as well as a group of cell-type specific organelles that are collectively referred to as lysosome-related organelles (e.g. melanosomes, platelet bodies; Bonifacino and Rojas, 2006; Gould and Lippincott-Schwartz, 2009; Schmidt and Haucke, 2007). These internal membrane bound structures have distinct roles in the uptake of extracellular molecules and ligands, the internalization of plasma membrane proteins and lipids, the regulation of cell signaling pathways, the recycling of proteins to the Golgi apparatus, TGN, and plasma membrane, as well as the degradation of proteins from the secretory and endocytic pathways (Derby and Gleeson, 2007).

Early and recycling endosomes

Upon internalization of membrane proteins and lipids, including surface receptors and their associated ligands, endocytosed material generally travels in primary endocytic vesicles. These, after uncoating, fuse with each other or pre-existing endosomes by action of Rab5 and the aid of accessory endosomal proteins, such as the early endosome antigen 1 (EEA1), to form EEs (also referred to as sorting endosomes; Arancibia-Carcamo et al., 2006; Huotari and Helenius, 2011; Schmidt and Haucke, 2007). The latter are peripherally located tubular-vesicular structures that represent the first main branch point in the receptor-mediated endocytosis pathway (Maxfield and McGraw, 2004). With a lumenal pH of ~ 6.0, they accept incoming material for only about 5-10 minutes, during which time membrane and fluid is rapidly targeted either to the plasma membrane, directly or via the RE (also called the endocytic recycling compartment - ERC), or to the TGN (Dunn et al., 1989; Grant and Donaldson, 2009). Then they translocate along microtubules, stop fusing with newly endocytosed vesicles, become more acidic and acquire hydrolase activity, i.e. mature into LE and then into lysosomes (Dunn et al., 1989; Maxfield and McGraw, 2004). Finally, remaining contents that did not exit the EE are degraded in lysosomes (Maxfield and McGraw, 2004).

REs are vesiculo-tubular compartments devoid of fluid that are molecularly defined by the presence of Rab11 and/or the Eps15-homology-domain protein EHD1/Rme1 (Grant and Donaldson, 2009). However, it should be noted, that thus far no single protein or lipid species could be identified that would truly be specific for recycling endosomes, i.e. is not found to some extent also on EEs (Johannes and Wunder, 2011).

The direct sorting of proteins from the EE to the plasma membrane, which reflects a rapid recycling route, is believed to mediate constitutive protein recycling, whereas the "slow" recycling route, which involves the transport of cargo proteins from the EE to the RE, and from the RE to the plasma membrane, mediates a targeted and/or regulated recycling pathway (Maxfield and McGraw, 2004; Schonteich

et al., 2008). Quantitative analyses of membrane proteins (such as the transferrin receptor) demonstrated that these two recycling pathways are not mutually exclusive (Sheff et al., 1999).

Multivesicular bodies, late endosomes and lysosomes

While the majority of endocytosed cargo in mammalian cells is recycled back to the plasma membrane via EE, only a relatively small fraction enters the degradative pathway, moving from the early to the late endosomal compartment to end up in lysosomes, the eukryotic cell's main engine for the breakdown of membrane proteins and internalized materials (Huotari and Helenius, 2011). Maturation from early to late endosomes occurs through the formation of MVBs, which contain large amounts of invaginated membrane and multiple internal vesicles (Von Bartheld and Altick, 2011). Eventually MVB either fuse with a late endosomal compartment or undergo homotypic fusion to become LEs. The latter communicate with the TGN and, in addition to ferrying cargo for degradation, also receive newly synthesized lysosomal hydrolases and membrane components from the secretory pathway and mature into lysosomes (Huotari and Helenius, 2011). Alternatively, in some cases, the MVBs fuse with the plasma membrane to shed the intralumenal vesicles into the extracellular space (Derby and Gleeson, 2007).
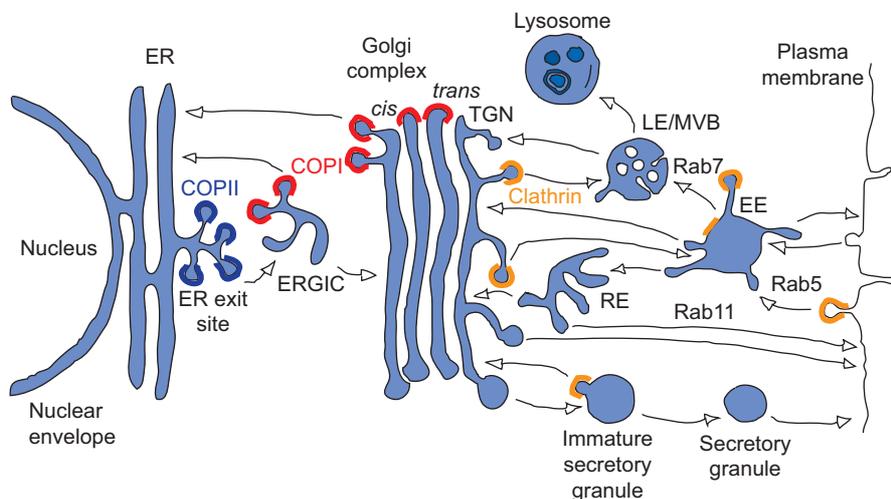

Figure 1.1 Intracellular transport pathways. The scheme depicts the components of the secretory and endocytic pathway. Transport steps are indicated by arrows. Colors indicate the known or the presumed locations of COPII (blue), COPI (red), and clathrin (orange; adapted from Bonifacino and Glick, 2004)

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1.2 The BEACH family of proteins

In the last several years an increasing number of genes, encoding components of the endocytic pathway, have been associated with different human diseases (Dikic and Puertollano, 2006). In 1996, Nagle et al. discovered that the rare autosomal recessive disorder Chediak-Higashi syndrome (CHS), cellularly characterized by giant lysosomes, results from mutations in the *LYST* gene, also known as lysosomal trafficking regulator gene (Kaplan et al., 2008; Nagle et al., 1996). The corresponding protein, CHS1 and its murine orthologue beige contain a highly conserved BEACH domain that is shared by a diverse group of proteins identified in multiple organisms, giving rise to the BEACH family of proteins (Barbosa et al., 1996; Nagle et al., 1996; Wang et al., 2002; see Table1.1).

Table 1.1 Overview of the BEACH family of proteins (Adapted from Kaplan et al., 2008).

Organism	Gene/protein	Function
S. cerevisiae	Bph1	Protein sorting and cell wall formation
D.discoideum	LvsA	Cytokinesis, osmoregulation and organization of early endocytic and phagocytic pathway
	LvsB	Negative regulator of lysosome fusion/involved in the biogenesis of postlysosomes
	LvsC	Unknown
	LvsD	Unknown
	LvsE	Unknown
	LvsF	Unknown
A. thaliana	F16M22.8	Unknown
	At2g45540	Unknown
	F1003.12	Unknown
	T10P11.5	Unknown
	T18124.16	Unknown
C. elegans	SEL-2	Negative regulator of *lin12/Notch* activity/involved in endocytosis from basolateral surface of polarized epithelial cells
	VT23B5.2	Unknown
	T01H10.8	Unknown
	F52C9.1	Unknown
D. melanogaster	rugose/DAKAP550	Protein kinase A anchoring protein/interacts with components of the EGFR- and Notch-mediated signaling pathways
	BCHS/blue cheese	Antagonist of Rab 11/involved in lysosomal/autophagosomal traffic and degradation
	CG6734	Unknown
	CG9001	Unknown
	CG11814	Unknown

Table 1.1 (continued)

Organism	Gene/protein	Function
	CG1332	Unknown
	CHS1/Lyst/Beige	Regulates lysosome-related organelle size
	ALFY/BWF1/WDFY3	PI3P-binding protein involved in macroautophagic degradation of protein aggregates
Mammals	FAN	Binds TNF-R55 activating N-sphingomyelinase/involved in actin remodeling, apoptosis and immune response
	LRBA/LBA/BGL/CDC4L	Involved in polarized secretion, cancer cell growth and EGFR pathway
	NBEAL1	Unknown
	NBEAL2	Involved in thrombocyte generation
	Neurobeachin/Lyst 2/BCL8B	Protein kinase A anchoring protein involved in trafficking of glutamatergic and GABA-ergic receptors to the synapse

In general, BEACH proteins share, in addition to the BEACH domain, two other carboxyl-terminal (C-terminal) domains: a pleckstrin-homology (PH)-like domain (Jogl et al., 2002) and WD40 (tryptophan-aspartic acid) repeats (Nagle et al., 1996). Many of the these proteins are very large (>400 kDa) and quite a few of them have been implicated in membrane trafficking. When analyzing the evolutionary descent of the BEACH proteins based on their BEACH and WD40 domains, one can discern distinct classes, and it is tempting to speculate that they may represent different cellular functions modulated by BEACH proteins (Wang et al., 2002; see Figure 1.2).

For instance, one class of BEACH proteins includes the mammalian proteins ALFY and BWFY1, as well as the *Drosophila* protein blue cheese (BCHS) and the predicted protein VT23B5.2 in *C.elegans*. These are the only BEACH proteins that share, in addition to the "classical" domains, also a FYVE zinc-finger domain at the end of the WD40 repeats (Simonsen et al., 2004). A unique class is represented by the mammalian FAN and the *Dictyostelium* protein LVSF, both are only about a quarter of the size of all the other members of the family (Wang et al., 2002). On the other hand the class including AKAP550, LRBA and NBEA, seems to cluster proteins that are able to anchor protein kinase A (PKA). Based on the phylogeny, it will be interesting to see, whether these different protein classes each really share a common functional denominator.

While CHS1 regulates lysosomal size by affecting fission (Durchfort et al., 2012), other BEACH proteins have been found to be involved at different stages in the organization of the secretory and endocytic pathways. In the following paragraphs their role in different species will be discussed.
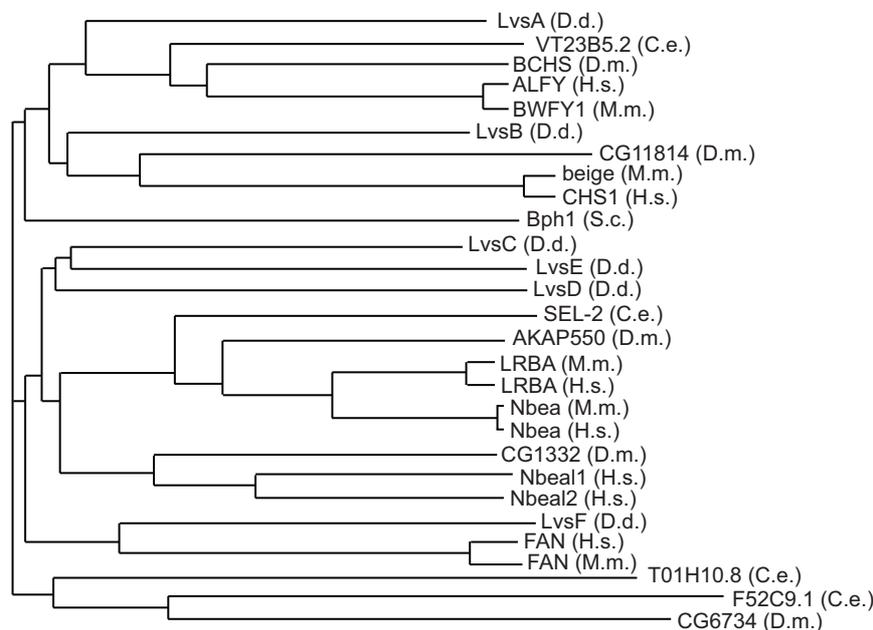
Figure 1.2 Phylogenetic tree of selected BEACH proteins. The carboxy terminal region (extending from the BEACH domain to the end of the protein) of selected proteins were aligned by the ClustalW2 algorithm and the alignment was used to construct this phylogenetic tree.

1.2.1 *Saccharomyces cerevisiae*

Studying the function of yeast homologues of mammalian genes required for vesicular traffic has led to important insights into the function of their mammalian counterparts (Shiflett et al., 2004). The *beige protein homologue 1* (*Bph1*) is the only *LYST* homologue identified in yeast and is probably the ancestral gene for the BEACH family (Shiflett et al., 2004).

Beige protein homologue 1 (Bph1)

Bph1 is not essential, since its disruption is viable (Shiflett et al., 2004). The strongest *Bph1*-null phenotype is a diminished growth on defined synthetic media containing potassium acetate buffered below pH 4.25, and on media containing calcofluor white, suggesting a deficit in cell wall biosynthesis (Shiflett et al., 2004).

While overexpressing CHS1 results in a decreased lysosomal size (Perou et al., 1997), neither disruption nor overexpression of BPH1 affects vacuole morphology, the yeast equivalent of lysosomes (Shiflett et al., 2004). However, in addition to the improperly formed cell wall, also a milder phenotype in sorting vacuolar components is apparent (Shiflett et al., 2004), which manifests in increased secretion of

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carboxypeptidase Y (CPY) and decreased transport of alkaline phosphatase (ALP) to the vacuole (Shiflett et al., 2004).

CPY and ALP both travel through the early stages of the secretory pathway and once they reach the last compartment of the Golgi apparatus (equivalent to the mammalian TGN), they are sorted away from proteins destined for delivery to the cell surface (Bryant and Stevens, 1998). However, the transport pathways of CPY and ALP differ. While the soluble vacuolar protein CPY is delivered to the vacuole via a prevacuolar compartment (PVC), an organelle analogous to the mammalian late endosome (Radisky et al., 1997), the membrane protein ALP is delivered to the vacuole directly, without passing the PVC (Bryant and Stevens, 1998). Since both proteins are glycosylated normally, the secretion defect seems to occurs after Golgi processing (Shiflett et al., 2004). Interestingly, the vacuolar enzyme carboxypeptidase S (CPS), which is a biosynthetic cargo that requires a functional multivesicular body sorting pathway to the lumen of the vacuole, is not affected by BPH1 loss (Shiflett et al., 2004; Shohdy et al., 2005).

That BPH1 plays a role in vesicular traffic from the Golgi, is also supported by the notion that overexpression of VPS9, a gene that encodes a guanyl-nucleotide exchange factor involved in vesicle trafficking between the *trans*-Golgi and the prevacuole, suppresses the CPY secretion defect (Shiflett et al., 2004). In addition, VPS9 is also a high copy suppressor of the growth defect on both low pH potassium acetate and calcofluor white, raising the possibility that the cell wall defect stems from altered protein trafficking (Shiflett et al., 2004).

1.2.2   *Dictyostelium discoideum*

Dictyostelium has six BEACH-domain containing proteins, LVSA through F, none of which are essential for growth or differentiation (Wang et al., 2002).

Large volume sphere A (LvsA)

LvsA is essential for cytokinesis in *Dictyostelium*, the last step in cell division (Kwak et al., 1999). *LvsA*-null mutants fail to grow in suspension and become large and multinucleate, hence the gene name large volume sphere A (Kwak et al., 1999). Several membrane traffic proteins, including clathrin, play crucial roles during cytokinesis (O'Halloran, 2000). The *LvsA* phenotype is similar to the cytokinesis failure of *Dictyostelium* clathrin heavy-chain mutants (Kwak et al., 1999) and although the LVSA mutants can still perform many cellular processes that are defective in clathrin mutants, it is possible that a specific LVSA/clathrin-mediated membrane-trafficking pathway is required for the correct formation of a cleavage furrow during cytokinesis (Kwak et al., 1999). Interestingly, GFP-LVSA does not localize to the cleavage furrow of dividing cells, but in addition to a punctate distribution in the cytosol, also transiently associates with, and is essential for, the function of the contractile vacuole (Gerald et al., 2002).

The cytosol of protozoa living in fresh water is always hypertonic to the environ-

ment, therefore water flows across their plasma membrane (Allen and Naitoh, 2002). In many wall-less protozoa a contractile vacuole complex collects and expels excess water (Allen and Naitoh, 2002). In *LvsA*-null mutants, vacuoles can still form when they are first in contact with water, however the discharge of those vacuoles is often abnormal in that they appear to fuse entirely with the plasma membrane (Gerald et al., 2002). In *LvsA*-null mutants, calmodulin, which localizes almost exclusively to contractile vacuole tubules and cisternae in wild-type (WT) cells, is mislocalized and appears mostly dispersed in the cytoplasm (Gerald et al., 2002). Intriguingly, the same is true for the clathrin mutants (Gerald et al., 2002). Whether the cytokinesis and osmoregulatory defects of *LvsA*-null mutants are associated with each other still remains to be determined.

In addition, loss of LVSA leads to a defect in the organization of the early endocytic and phagocytic pathway, with an increased concentration of vacuolar H^+-ATPase in early endo/phagocytic compartments and a defect in phagocytosis (Cornillon et al., 2002).

For LVSA's function in cytokinesis and osmoregulation, most of its protein structure is required (Wu et al., 2004). Not only deleting the WD40 repeats leads to complete loss of function, but even deleting only one fifth of LVSA's amino acid (AA) sequence at the amino-terminal (N-terminal) domain yields a protein that is only partially functional (Wu et al., 2004). Consistent with this, the LVSA fragment encompassing half of the PH-like domain and the entire BEACH and WD40 domains is not sufficient for function (Wu et al., 2004). Surprisingly, this C-terminal fragment does not produce a dominant negative effect when overexpressed in WT cells, while analogous proteins from other systems do (Wu et al., 2004).

Large volume sphere B (LvsB)

In contrast to *LvsA*, the contractile vacuole system of *LvsB*-null cells is not affected. Instead, they have enlarged lysosomes reminiscent of the ones found in beige and CHS1 mutant cell lines (Cornillon et al., 2002; Harris et al., 2002). This is not surprising, since the BEACH and WD40 domain sequences of LvsB and CHS1 are closely related (Wang et al., 2002). While enlarged lysosomes are not present in any of the other Lvs-null mutants, in *LvsB* mutants they are probably the result of increased homotypic endolysosomal fusion (Harris et al., 2002). Additionally, they also display a minor secretory defect in form of an increased rate of secretion of the mature form of α-mannosidase and two cysteine proteinases from lysosomes (out of > 20 proteins assayed), while endocytosis, phagocytsis or endosomal efflux is intact (Harris et al., 2002).

In *Dictyostelium*, endocytosed material traffics through a system of vesicles that end in the secretion of undigested material (Maniak, 2003). Soon after internalization proton pumps are delivered to early endosomes that acidify their contents. In a process that takes 30-40 min these acidified EEs receive lysosomal enzymes to form early and then late lysosomes, which after a subsequent retrieval of proton pumps and lysomal enzymes mature into a neutral compartment called the postlysosome. The

latter is a secretory organelle that eventually expels its contents by exocytosis and is functionally equivalent to the mammalian fusion-competent lysosome (Charette and Cosson, 2007; Kypri et al., 2007).

Like the lysosomes, also the postlysosomes in *LvsB*-null cells are affected (Kypri et al., 2007). They are decreased in number and differ from WT postlysosomes in their acidity, which is shifted from a neutral to a low pH (Charette and Cosson, 2007; Kypri et al., 2007). This could be due to missorting of the proton pump v-ATPase, which is not removed from postlysosomes in *LvsB*-null mutants, as reported by Kypri et al., 2007. Transfer of particles to the postlysosomes is markedly slower (Charette and Cosson, 2007; Kypri et al., 2007). Whether they are, like CHS1-defficient lysosomes, enlarged, is still controversial. When the postlysosomal marker GFP-vacuolin B, is transfected into *LvsB*-null cells, the postlysosomes display an abnormal size (Kypri et al., 2007). However, when immunocytochemistry is used, no alterations in postlysosomal size can be observed (Charette and Cosson, 2007).

In the current model of LVSB's proposed function, LVSB inhibits the inappropriate fusion between early endosomes and postlysosomal compartments and its loss results in the mixing of early and late compartments of the endolysosomal system (Kypri et al., 2007).

At this time the function of the four other *Dictyostelium* BEACH proteins, i.e. LVSC through F is still not known. While LVSC, D and E are closely related to each other (see Figure 1.2), the C-terminus of LVSF is closely related to the mammalian FAN (De Lozanne, 2003). The latter two form a unique group of BEACH proteins, however, it still needs to be determined whether LVSF, like FAN, functions in apoptosis of *Dictyostelium* (Adam-Klages et al., 1996).

1.2.3 *Caernohabditis elegans*

In *C. elegans* the only described BEACH protein so far is SEL-2 (de Souza et al., 2007). However, at least three other proteins have been predicted to be BEACH family members: VT23B5.2, T01H10.8 and F52C9.1 (de Souza et al., 2007).

Suppressor/enhancer of *lin-12* (SEL-2)

In multicellular organisms the EGFR (epidermal growth factor receptor alias LET-23 in *Drosophila*) and LIN-12 (alias Notch in *Drosophila*) signaling pathways play an important role in cell fate specification and pattern formation (Shamloula et al., 2002). The basolateral EGFR family member LET-23 (lethal protein 23), and the apical LIN-12 regulate cell fate specification in the developing *C. elegans* vulva (de Souza et al., 2007; Greenwald, 2005). When vulval precursor cells (VPCs), which are polarized epithelial cells, undergo patterning, a reciprocal endocytosis and trafficking of both LET-23 and LIN-12 receptors takes place. Maximal activation of LET-23 leads to LIN-12 internalization via endocytosis and degradation via multivesicular endosomes, as well as the production of ligands that activate LIN-12 in neighboring cells

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(de Souza et al., 2007). Vice versa, when LIN-12 is activated, LET-23 is downregulated (de Souza et al., 2007).

Loss of SEL-2 activity enhances LIN-12 activity, hence SEL-2 is a negative regulator of LIN-12, therefore the name suppressor/enhancer of lin-12 (Greenwald, 2005). While In WT cells LIN-12-GFP is localized to the apical surface of the VPCs or in puncta close to the apical surface, in sel-2 non-sense mutants, lacking the BEACH and WD40 domains, LIN-12-GFP is mislocalized (de Souza et al., 2007). Instead of being restricted to the apical surface, it is also present basolaterally, with the general polarization of VPCs being intact (de Souza et al., 2007). Furthermore, the overall level of LIN-12-GFP is elevated in SEL-2 mutants (de Souza et al., 2007). While the endocytic downregulation of LIN12-GFP from the apical surface occurs normally in *Sel-2*(-) mutants, LIN12-GFP-BL, which is mistargeted basolaterally by the addition of a basolateral targeting signal, is downregulated less efficiently in *Sel-2*(-) mutants (de Souza et al., 2007). Additionally, LET-23/EGFR is downregulated less efficiently in *Sel-2*(-) mutants and loss of SEL-2 results in slowed delivery of basolaterally-delivered fluorescent lipid to the lysosomes in the polarized cells of the intestinal epithelium (de Souza et al., 2007). Therefore it seems that SEL-2 plays a role in endocytosis from the basolateral, but not apical surface of polarized epithelial cells. Last but not least, the early and late endosomes, as well as the lysosomes of sel-2(-) worm intestines show no significant perturbations, suggesting that in absence of SEL-2 activity, there is a defect in traffic to late endosomes and lysosomes (de Souza et al., 2007).

VT23B5.2, the orthologue of human ALFY (Simonsen et al., 2004) and *Drosophila* Blue Cheese (Finley et al., 2003), consisting mainly of the BEACH domain and five WD40 motifs, plus a C-terminal FYVE (Fabp1, YOTB, Vac1 and EEA1) domain; de Souza et al., 2007; Khodosh et al., 2006). The VT23B5.2(ok912)-null allele does not have an apparent phenotype on its own nor as a double mutant with sel-2(-) and does not cause a phenocopy of the sel-2(-) phenotype in the combination with the LIN-12 mutant (de Souza et al., 2007). T01H10.8 does not have any predicted WD40 motifs (de Souza et al., 2007). The fourth BEACH-containing protein in *C. elegans*, i.e. F52C9.1, has only two WD40 motifs (de Souza et al., 2007).

1.2.4 *Drosophila melanogaster*

In *Drosophila* AKAP550/rugose, Blue cheese (BCHS) and three additional predicted proteins (CG6734, CG11814 and CG1332) have been identified as members of the BEACH protein family (Wang et al., 2002).

A-kinase anchoring protein 550 (AKAP550/Rugose)

The WT *Drosophila* compound eye is composed of about 800 units called ommatidia, each containing eight specialized sensory neurons (the retinular photoreceptor cells), four cone cells, six pigment cells, and cells of the mechano-sensory bristle complex

(Shamloula et al., 2002). Excess cells are eliminated through apoptosis resulting in precise hexagonal cellular latice (Wech and Nagel, 2005). Normally, the arrangement of ommatidia results in a smooth appearance of the external surface of the eye (Shamloula et al., 2002). As a result of defective ommatidial organization (irregular ommatidial facets and positioning of mechano-sensory bristles), missing retinal photoreceptors and death of cone cells, rugose mutants display a rough eye phenotype (Shamloula et al., 2002). In addition to phenotypes in the eye, rugose mutants also exhibit wing vein defects and an embryonic semilethality (Shamloula et al., 2002).

Just like SEL-2 in *C. elegans*, rugose also seems to genetically interact with components of the *Drosophila* EGFR- and Notch-activated signal transduction cascades, both of which are involved in the maturation and differentiation of the retina at various steps of development (de Souza et al., 2007; Schreiber et al., 2002; Shamloula et al., 2002). While mutations that reduce either EGFR or Notch signaling behave as dominant enhancers of the rough eye phenotype, gain-of-function mutations that increase EGFR or Notch signaling act as dominant suppressors of the rugose phenotype. In line with this, rugose acts as a strong enhancer of the rough eye phenotype induced by the overexpression of the Notch antagonist *Hairless* in the differentiating eye (Schreiber et al., 2002). Vice versa, the rugose eye phenotype is enhanced by the Notch ligand Delta and rescued by *Hairless* mutations (Schreiber et al., 2002).

Rugose encodes a *Drosophila* A kinase anchor protein (AKAP; Han et al., 1997; Shamloula et al., 2002). AKAPs target protein kinase A (PKA) to specific substrates and subcellular compartments (Scott and McCartney, 1994; see next section). Interestingly, reducing the activity of the catalytic subunit of PKA, results in an enhancement of the rough eye phenotype (Wech and Nagel, 2005). This indicates that rugose's importance for correct retinal pattern formation is likely mediated through its effects on the subcellular localization of and activity of PKA. Since the cAMP-PKA signal cascade may play a role in cell fate determination through its interaction with the Notch and EGFR pathway, the major function of rugose might be to integrate the crosstalk between different signaling pathways (Volders et al., 2011; Wech and Nagel, 2005).

Blue cheese (Bchs)

Bchs mutants, while lacking pronounced developmental defects (no defects in axon guidance or growth cone morphology) and displaying normal behaviors in young adults, have a reduced adult life span, indicating that the *Bchs* gene is essential for normal adult survival and longevity (Finley et al., 2003; Khodosh et al., 2006). The latter is closely associated with the progressive formation of CNS-wide protein aggregates, containing insoluble ubiquinated proteins and amyloid precursor-like protein (Finley et al., 2003). BCHS adult mutants show extensive neurodegeneration in form of progressive loss of CNS size and morphology, along with an increase in neuronal apoptotic death (Finley et al., 2003).

BCHS overexpression disrupts the photoreceptor growth cone morphology and eye development, resulting in a smaller, glazed adult eye, accompanied by neural de-

fects, e.g. alterations in the formation of terminal synapses and axonal varicosities containing ubiquitin (Khodosh et al., 2006; Simonsen et al., 2007). This overexpression phenotype is enhanced by mutations in Rab11, a member of the Rab family of small GTPases that plays a role in both the exocytic/biosynthetic, and the recycling pathway (Khodosh et al., 2006). On the other hand, a reduction in, or loss of, BCHS restores viability and normal bristle development in Rab11 mutants, suggesting that *Bchs* and Rab11 functionally antagonize one another (Khodosh et al., 2006). In line with this, loss of BCHS also partially suppresses the rab11 phenotype at neuro-muscular junctions (NMJ), i.e. an increase in number and density of synaptic boutons that display unusual clustering, while *Bchs* loss-of-function mutants have no detectable defects at the NMJ (Khodosh et al., 2006).

In addition to Rab11, mutations in a plethora of other proteins modifies the BCHS overexpression glazed-eye phenotype: mutations in autophagy genes (*Atg1* and *Atg6* moderate eye enhancers, *Atg2* and *Atg8* moderate suppressors), mutations in genes involved in lysosomal and pigment granule biogenesis (four subunits of the *Drosophila* AP-3 adaptor complex mediating trafficking between the TGN and lysosomes), mutations in the lysosome/late endosomal autophagic cell death pathway promoter spinster, mutations in members of the homeotypic vacuole fusion and protein sorting (HOPS) trafficking complex that promote late endosomal/lysosomal fusion events, mutations in the regulator of multivesicular endosome trafficking hook and mutations in SNARE proteins: ROP, syntaxin1A and syntaxin13 (Simonsen et al., 2007). Also defects in cytoskeletal and motor proteins, as well as mutations in the SUMO and ubiquitin signaling pathways behave as modifiers of the Bchs gain-of-function (GOF) eye phenotype (Simonsen et al., 2007).

Among all these potential genetic interactors of *Bchs*, especially mutations in the lysosomal trafficking mutants demonstrate a loss-of-function phenotype similar to the one caused by loss of *Bchs*, i.e. a reduction in adult longevity paired with an alteration of ubiquitinated (UB) protein profiles in young adults (Simonsen et al., 2007). Since both, functional loss and overproduction of bchs result in the accumulation of ubiquinated proteins in neural tissue, and lysosomal mutations suppress the *Bchs* overexpression eye phenotype (Finley et al., 2003; Simonsen et al., 2007), it is possible that BCHS plays a role in lysosomal-autophagic trafficking and degradation, defects in which may alter the turnover of UB proteins and lead to degeneration (Lim and Kraut, 2009). In support of this notion, lysosomal vesicles in *Bchs* mutant and *Bchs*-overexpressing larvae fail to be transported toward motor neuron termini, revealing a link between the lysosomal degradative pathway and transport (Lim and Kraut, 2009). It remains to be determined whether the cargo transported by BCHS is autophagosomal and whether fusion between lysosomes and autophagosomes is affected.

1.2.5 Mammals

Seven mammalian BEACH proteins have been identified so far (see Figure 1.3). Some of them have been studied rather extensively (e.g. CHS1, ALFY, FAN and Nbea), while others only recently emerged as members of this family (NBEAL1 and 2), lacking
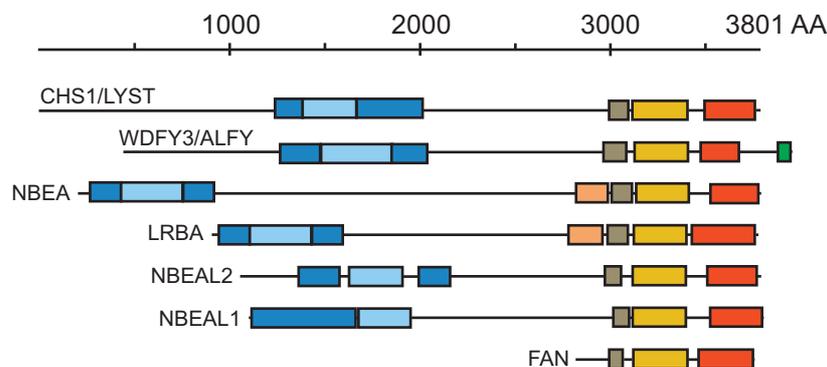
thorough characterization.

Figure 1.3 Primary structure of the human BEACH protein family members.
Schematic drawing of the domain structures of CHS1/LYST, WDFY3/ALFY, Nbea, LRBA, NBEAL1, NBEAL2 and FAN. The Pleckstrin-Homology like domain (PH), the BEACH domain, and the WD40 repeats are shown as gray, yellow and red rectangles, respectively. The predicted armadillo (ARM) repeat-flanked Concavanalin A (Con A)-like lectin domain localized at the N-termini of the BEACH proteins is depicted as blue rectangles. Nbea and LRBA share an additional common feature, i.e. the domain of unknown function (DUF1088: in orange). For details on Nbea's domain structure see Chapter 4. Note that WDFY3/ALFY is the only human paralogue of Nbea that contains a FYVE domain (in green). NCBI reference sequences are: NP_000072.2 (LYST), NP_055806 (WDFY3), NP_056493.3 (Nbea), NP_006717.2 (LRBA isoform 2), NP_055990.1 (NBEAL2), NP_001107604.1 (NBEAL1), NP_003571.2 (FAN).

Lysosomal trafficking regulator (CHS1/Beige/Lyst)

LYST is the causative gene for the CHS syndrome in humans (Nagle et al., 1996). CHS is characterized by severe immunodeficiency, bleeding tendency, frequent bacterial infections, variable oculocutaneous albinism, and progressive neurologic dysfunction (Kaplan et al., 2008; Spritz, 1998). The hallmark of this disease are enlarged organelles of lysosomal origin, including lysosomes, melanosomes, platelet dense δ-granules and cytolytic granules, in various cell types (Kaplan et al., 2008). The severe immunodeficiency is a result of defective cytotoxicity in T lymphocytes, natural killer cells and granulocytes, which is most likely due to a secretory defect that prevents lytic granule exocytosis (Faigle et al., 1998; Spritz, 1998). An alternative explanation would be the absence of cytolytic enzymes from granules due to missorting (Huizing et al., 2001).

The platelet dense δ-granules, which normally contain serotonin, calcium, adenosine diphosphate (ADP), adenosine triphosphate (ATP), and pyrophosphate, are greatly reduced in number, while α-granules are normal (Huizing et al., 2001; Shiflett et al., 2002). Therefore it is not surprising that although CHS patients have normal platelet numbers, the platelets themselves are defective in coagulation (Shiflett et al.,

2002).

Whereas secretory lysosomes are synthesized normally in CHS cells, melanosome, secretory granule- and lysosome-release are functionally impaired (Huizing et al., 2001). This is also in line with a defective plasma membrane repair observed in CHS cells, a process, that requires peripheral small lysosomes to fuse with the plasma membrane (Huynh et al., 2004). Interestingly, other functions such as protein degradation and recycling of transferrin receptors are unaffected (Huizing et al., 2001).

In Epstein-Barr virus (EBV)-infected CHS-B cells, only the lysosomal multilaminar compartments are enlarged, while multivesicular endosomes display normal size and morphology (Faigle et al., 1998). The lysosomal residents, HLA-DM, Lamp1 and 2, CD63, CD82, and β-hexosaminidase, not only accumulate in macrolysosomes (as expected), but also at the cell surface (Faigle et al., 2000). In addition, multivesicular endosomes show reduced levels of these different membrane markers (Faigle et al., 2000), suggesting that transport of these markers from the TGN and/or early endosomes into late endosomes is affected (Faigle et al., 1998). Strikingly, the mannose-6-phosphate receptor, normally excluded from lysosomes, is very abundant in macrolysosomes (Faigle et al., 2000). Taking into account that LYST partially colocalizes with microtubules, which are involved in transport from early to late endosomes, the observed aberrant compartmentalization suggests that *LYST* is implicated in the sorting and trafficking of multiple membrane proteins between endosomes, lysosomes and the plasma membrane (Wang et al., 2000).

The orthologous murine disorder shows a coat-color alteration described as beige and matches the human CHS also in virtually all other aspects, including missorting of proteins (e.g. beige lysosomal membranes contain higher than normal levels of ER proteins; Perou et al., 1997; Zhang et al., 2007). The loss of beige protein results in a clustering of enlarged lysosome-related organelles in the perinuclear region, while beige overexpression leads to lysosomes that are smaller-than-normal and are located near the cell periphery (Perou et al., 1997). Given the fact that beige/CHS giant lysosomes are as capable of fusing with other lysosomes as are WT ones, it seems that the increase in lysosomal size results from a decrease in lysosome fission (Perou et al., 1997). In line with this, loss of the CHS1/beige protein decreases the lysosomal fission rate in macrophages, while overexpression gives rise to a higher fission rate (with an unchanged fusion rate; Durchfort et al., 2012).

Currently, there are different speculations about the underlying biochemical defect in CHS/beige ranging from alterations in cyclic nucleotides, protein kinase C (PKC) levels, to lipid turnover/composition (Shiflett et al., 2002). For instance an increased ceramide production in beige fibroblasts has been linked to the rapid down-regulation of PKC in these cells, which in turn is suggested to give rise to the enlarged lysosome-related organelles (Tanabe et al., 2000). Increasing PKC levels by treating beige fibroblasts with inhibitors of PKC proteolysis (E-64-d), prevents the giant granule formation (Huynh et al., 2004; Tanabe et al., 2000). Furthermore, inhibition of PKC activity in WT cells results in enlarged perinuclear lysosomes, and oral administration of E-64-d to beige mice leads to an improvement in lysosomal enzyme activity and bactericidal activity (Shiflett et al., 2002). Since E-64-d treatment also restores

lysosomal fusion with the plasma membrane in beige cells, the underlying factor interfering with normal lysosomal exocytosis, is probably the abnormal enlargement of CHS/beige lysosomes, rather than a disfunction of the exocytic machinery (Huynh et al., 2004). However, it remains to be determined whether the alteration in PKC levels is the immediate biochemical defect resulting from loss of CHS1/beige or if changes in PKC levels are a more downstream effect.

Autophagy-linked FYVE protein (ALFY/BWF1/WDFY3)

ALFY is the putative mammalian orthologue of the *Drosophila* protein BCHS and the hypothetical *C.elegans* protein VT23B5.2 (Chen et al., 2004a; Simonsen et al., 2004). All these BEACH proteins contain a FYVE domain, i.e. a ∼70-residue zinc finger-like domain (Chen et al., 2004a; de Souza et al., 2007; Finley et al., 2003; Simonsen et al., 2004). Similar to other FYVE fingers, which are phosphatidylinositol-3-phosphate- (PtIns(3)P) binding domains, ALFY binds to, and partially colocalizes with PtIns(3)P in vivo (Simonsen et al., 2004). However, ALFY is not found on early endosomes (as usual for the majority of FYVE-domain proteins), but instead localizes under normal conditions mainly to the nuclear envelope (Simonsen et al., 2004). Upon serum and amino acid starvation (a condition that induces autophagy), or inhibition of the proteasome, ALFY relocalizes to ubiquitin-positive protein inclusions and the early authophagic pathway (Simonsen et al., 2004).

Degradation of cytoplasmic proteins occurs either by proteosomal proteolysis or by macroautophagy. The latter is a process through which cytosolic constituents are taken up into a multimembranous structure known as the autophagosome, which subsequently fuses to endosomal and lysosomal structures, permitting degradation (Filimonenko et al., 2010). Since this process has been implicated in the elimination of aggregated, often polyubiquinated, proteins in various cell types, including neurons (Boland and Nixon, 2006; Filimonenko et al., 2010), and *Bchs* mutant flies accumulate protein aggregates (Finley et al., 2003), ALFY might play a role in regulating selective autophagy for certain cytoplasmic protein aggregates that are inefficiently degraded by proteasomes (Simonsen et al., 2004).

In line with this notion, siRNA knock-down of *Alfy* significantly impedes macrophagic degradation of cytoplasmic protein aggregates associated with Huntington disease (HD; Filimonenko et al., 2010). Via its WD40 repeats ALFY interacts with ATG5 (a protein that is required for the initial sequestration of cytosolic material) and can be found in a complex with other core autophagy proteins like, ATG12 and ATG16L (Filimonenko et al., 2010; Simonsen et al., 2004). Furthermore, ALFY's PH-BEACH region also interacts with the ubiquitin-binding adaptor protein p62 (Clausen et al., 2010; Hocking et al., 2010).

Overexpression of the C-terminal fragment of ALFY decreases the number of protein inclusions and protects cells from expanded polyglutamine toxicity in an autophagy-dependent manner in a primary neuronal HD model (Filimonenko et al., 2010). The same is true for ALFY/BCHS overexpression in a *Drosophila* eye model

of polyglutamine toxicity (Filimonenko et al., 2010).

Taken together, ALFY seems to function as a scaffolding adaptor protein connecting the p62-positive ubiquinated aggregation-prone proteins and the core autophagy effector proteins, thereby bridging cargo to the molecular machinery that builds autophagosomes (Filimonenko et al., 2010; Yamamoto and Simonsen, 2011).

Factor associated with neutral sphingomyelnase activation (FAN)

FAN, the smallest mammalian BEACH protein, physically and functionally interacts with two members of the tumor necrosis factor receptor (TNF-R) superfamily, TNF-R55 and CD40, and the cannabinoid type 1 receptor (Adam-Klages et al., 1996; Sanchez et al., 2001; Segui et al., 1999).

TNF-R55, interacting with the pleiotropic cytokine TNF-α, mediates activation of kinases of the MAPK family, transcription factors such as the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), and caspase cascade, modulating cell survival, proliferation, and apoptosis, as well as inflammatory responses (Montfort et al., 2010). FAN binds to the neutral-sphingomyelinase activation domain (NSD; Adam et al., 1996) of TNF-R55, and as its name suggests, activates the neutral sphyngomyelinases 2 and 3 (N-SMases; Adam-Klages et al., 1996; Montfort et al., 2010). This results in the hydrolysis of sphyngomyelin (SM) to ceramide, an important intracellular signaling molecule for many membrane receptors in diverse tissues (Wiegmann et al., 1994). The binding to NSD occurs through FAN's WD40 domain (Adam-Klages et al., 1996), which also interacts with RACK1 (receptor for activated C-kinase 1), another WD40 repeat protein that modulates the activation of N-SMase (Montfort et al., 2010; Tcherkasowa et al., 2002). Mouse embryonic fibroblasts (MEFs) from FAN-deficient mice show an impaired TNF-induced neutral sphyngomyelinase (N-Smase) activation (Kreder et al., 1999), while COS-1 cells, when transfected with FAN, display enhanced TNF-induced N-Smase activation (Adam-Klages et al., 1996). Expressing a dominant-negative form of FAN in COS-1 cells, human fibroblasts and rat primary astrocytes, abrogates TNF-, CD40L- and Δ9-tetrahydroccannabinol-induced N-Smase activation and ceramide generation from sphingomyelin hydrolysis (Adam-Klages et al., 1996; Sanchez et al., 2001; Segui et al., 1999).

FAN-deficient MEFs show an impaired TNF-induced filamentous actin increase and filopodia formation, revealing FAN's important role in TNF-mediated reorganization of the actin cytoskeleton (Haubert et al., 2007). While modulation of actin is controlled by various members of the Rho family small GTPases (Rho, Rac and Cdc42), the loss of FAN only affects TNF-induced activation of Cdc42 (Haubert et al., 2007; Montfort et al., 2010). Like other actin modulating proteins, FAN is targeted to the plasma membrane via its PH-like domain binding, to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P; Haubert et al., 2007). It is a functionally dormant plasma membrane-associated protein, since it interacts with the actin cytoskeleton via direct filamentous actin binding only upon TNF stimulation (Haubert et al., 2007). Whether and how the N-Smase-ceramide pathway and actin remodeling are linked

remains to be elucidated.

In addition, FAN is also able to regulate TNF- and CD40 ligand- (CD40L) induced apoptosis, which includes lysosomal permeabilization with release of cathepsin B (ctsb), a cystein protease, into the cytosol and activation of caspases and mitochondria-associated death events leading to the release in the cytosol of apoptogenetic proteins such as cytochrome c (cyt c; Segui et al., 2001). This notion is supported by the fact that expression of a dominant negative form of FAN in human fibroblasts attenuates caspase cascade activation and cyt c release from mitochondria and thereby significantly reduces TNF- and CD40L-induced cell death (Segui et al., 1999, 2001). The expression of a dominant-negative FAN in a rat hepatoma cell line, reduces caspase-8 activation and increase in lysosomal permeability and markedly reduces lysosomal release of ctsb (Werneburg et al., 2004). Furthermore, FAN-deficient MEFs are less susceptible to TNF toxicity than their normal counterparts by significantly resisting the TNF-induced caspase activation (Segui et al., 2001). Re-expression of human FAN in FAN-deficient MEFs restores TNF-induced apoptosis (Segui et al., 2001). Finally, loss of FAN in MEFs also protects from CD40-mediated cell death (Segui et al., 1999). How exactly FAN mediates apoptosis is not yet fully established, but since it is required for N-Smase activation, and ceramide has been shown to increases permeability of mitochondrial and lysosomal membranes, it is possible that the N-Smase-cermaide pathway is linked to cell death signaling (Montfort et al., 2010).

It was believed for a long time that TNF's role in inflammatory responses is mediated solely via the death domain (DD) of TNF-R55 (Montfort et al., 2010). TNF induces the association of TRADD (TNF-R55-associated protein with death domain), an adaptor protein enabling the activation of a proinflammatory pathway, with TNF-R55, TRAF2 and the serine-threonine kinase RIP1. This leads to the activation of transcription factors (e.g. NF-κB) that are involved in the expression of genes encoding survival inflammatory proteins (Montfort et al., 2010). However, the expression of some proinflammatory cytokines is mediated partly by NSD, since deletion of NSD selectively perturbs TNF-induced gene expression (Montfort et al., 2009). Moreover, FAN is required for full expression of genes encoding the cytokines CXCL2 and IL-6, which are upregulated upon activation of the transcription factors NF-κB and AP-1 (Montfort et al., 2009, 2010). Interestingly, the TNF-R55 activation of the NF-κB, as well as the ERK, and JNK pathways is unaltered in FAN-deficient MEFs (Kreder et al., 1999; Luschen et al., 2000; Montfort et al., 2009; Segui et al., 2001). Thus, the molecular mechanism coupling FAN to selective gene expression is currently not known.

FAN-deficient mice defective in TNF-induced N-SMase activation are healthy and fertile with no gross phenotypic abnormalities (Kreder et al., 1999). However, they do show a weakened humoral response toward a thymo-dependent antigen and a reduction in several different types of leukocytes within secondary lymphoid organs, indicating that FAN plays a role in the establishment of a specific immune system (Montfort et al., 2009). Additionally, they show a delayed cutaneous wound repair and a reduced epidermal proliferation, suggesting a physiological role of FAN in epidermal

homeostasis (Kreder et al., 1999). This is similar to the described defective wound repair of CHS/beige fibroblasts (Huynh et al., 2004). Although the exact mechanism by which eukaryotic cells reseal plasma membrane lesions is still unknown, the process depends on the Ca^{2+}-regulated exocytosis of lysosomes (Jaiswal et al., 2002; Terasaki et al., 1997). And since in addition to Lyst, cells lacking FAN also show an increase in lysosome size (although less pronounced as Lyst), it is probable that FAN functions in signaling pathways that regulate the lysosomal compartment, however their exact nature is still largely enigmatic (Mohlig et al., 2007).

Lipopolysaccharide-responsive beige-like anchor gene (LRBA/BGL/LBA)

LRBA, the protein, most closely related to Neurobeachin (Figure 1.2 and 1.3), is expressed upon lipopolysaccharide (LPS) stimulation of B cells and macrophages (Wang et al., 2001). After LPS stimulation, the initially cytosolic LRBA-green-fluorescent protein (GFP) fusion protein is targeted to intracellular vesicles, thereby establishing a possible link with protein trafficking (Wang et al., 2001).

LRBA is overexpressed in several different cancers (Wang et al., 2004). Its role in cancer is further supported by the fact that the LRBA promoter is regulated by the transcription factors p53 and E2F1 that play a a crucial role in the action of tumor suppressor proteins (Wang et al., 2004). Both, LRBA knock-down, as well as inhibiting LRBA's activity with a dominant-negative mutant, sensitizes cancer cells to apoptosis and thereby inhibits growth in human cancer cells (Wang et al., 2004). However, the underlying molecular function of LRBA still remains to be elucidated.

Interestingly, like its orthologue rugose/AKAP550, LRBA is also involved in the regulation of EGFR, since antagonizing LRBA activity attenuates activation of EGFR (Wang et al., 2004). However, unlike AKAP550 (and Nbea), LRBA does not have the ability to bind regulatory subunits of PKA in the part corresponding to the AKAP domain containing region in Nbea (Wang et al., 2000). Two other, AKAP domains, distinct from the ones in rugose and Nbea are predicted in LRBA, but the functionality of these yet remains to be determined (Wang et al., 2001).

Neurobeachin-like 1 and 2 (NBEAL1 and 2)

NBEAL1 and NBEAL2 are two BEACH proteins that were identified most recently and which cellular functions are largely unknown (Albers et al., 2011; Chen et al., 2004b; Gunay-Aygun et al., 2011; Kahr et al., 2011). While NBEAL1 is highly expressed in the brain (including in different grades of glioma) and many other tissues, NBEAL2 is absent from the brain, but expressed in megakaryocites (Albers et al., 2011; Chen et al., 2004b). NBEAL1 contains a vacuolar-targeting motif ILPK, which suggests that the protein might be located in lysosomes (Chen et al., 2004b).

NBEAL2 is the causative gene for a bleeding disorder called Gray platelet syndrome (GPS; Albers et al., 2011; Gunay-Aygun et al., 2011; Kahr et al., 2011). GPS is characterized by a low number of platelets, which are large and lack α-granules, but have normal dense δ-granules, lysosomes, mitochondria and peroxisomes (Kahr et al., 2011). α-granules are the most abundant platelet organelles which store large

proteins that, when released, promote platelet adhesiveness, homeostasis and wound healing (Gunay-Aygun et al., 2011). The platelets in GPS appear gray under light microscopy, hence the name GPS (Gunay-Aygun et al., 2011). Probably due to the lack of α granules, proteins that are normally only released upon platelet activation, are spontaneously released from megakaryocytes, precursor cells in the bone marrow (Albers et al., 2011). This constitutive release of platalet-derived growth factor, and other profibrotic substances, might be the underlying cause of two additional hallmarks of GPS, i.e. myelofibrosis, where bone marrow is replaced by scar (fibrous) tissue and a consequential enlargement of the spleen (Gunay-Aygun et al., 2011).

While some platelet α-granule constituents are taken up from the plasma by receptor-mediated endocytosis, others are synthesized in megakaryocytes. The latter are markedly reduced in individuals with GPS, whereas endocytosed α-granule constituents are less affected, suggesting that megakaryocytes in GPS fail to pack their endogenously synthesized secretory proteins into developing α-granules (Gunay-Aygun et al., 2011).

NBEAL2-knock down in zebrafish shows a complete abrogation of platelet (thrombocyte) formation, but normal production of red blood cells (Albers et al., 2011). Furthermore, embryos show spontaneous bleedings in the tail (Albers et al., 2011). These results support the essential role of NBEAL2 in thrombocyte generation.

The only mammalian BEACH protein left to discuss is Neurobeachin. Since this protein is the main topic of this thesis, it will be discussed more extensively in a separate section below.

1.3 Neurobeachin/Lysosomal trafficking regulator 2 (Nbea, Lyst2, BCL8B)

Nbea is a large (327 kDa), multidomain, brain-enriched scaffolding protein that has been suggested to be involved in neuronal post-Golgi membrane traffic (Wang et al., 2000). The Neurobeachin gene, which maps to region 13q13 in humans and encompasses the FRA13A fragile site (Savelyeva et al., 2006), has been linked to idiopathic autism (Smith et al., 2002; Castermans et al., 2003) as well as to multiple myeloma, where it might act as a novel candidate tumor suppressor gene (O'Neal et al., 2009).

Two independent Nbea knock-out (KO) mouse models have been generated, showing the same characteristics. Nbea knock-out (KO) mice die perinatally due to paralysis and subsequent inability to initiate respiration (Medrihan et al., 2009; Su et al., 2004). Electrophysiological examination revealed a complete absence of evoked neurotransmitter release at neuromuscular junctions, while spontaneous release was intact (Su et al., 2004). Also in the central nervous system (CNS) abnormalities in the formation and function of synapses have been described. In fetal Nbea KO brainstem slices, both spontaneous excitatory and inhibitory postsynaptic currents are reduced (Medrihan et al., 2009). Additionally, miniature excitatory postsynaptic currents (mini EPSCs) show a reduction in frequency, whereas miniature inhibitory postsy-

naptic currents (mini IPSCs) are both reduced in frequency and amplitude (Medrihan et al., 2009). In the fetal brainstem, the area density of mature asymmetric contacts is reduced, along with reduced levels of several presynaptic proteins (Medrihan et al., 2009). Also cultured hippocampal neurons from KO mice and cortical slices from heterozygous mice show altered miniature postsynaptic currents (Niesmann et al., 2011). The number of spinous synapses is reduced in these preparations and in KO neurons excitatory synapses terminate mostly at dendritic shafts instead of on spine heads (Niesmann et al., 2011). Interestingly, Nbea KO neurons exhibit a change in the subcellular distribution of actin, with actin accumulating in large clusters in the cell bodies, dendritic shafts and axons (Niesmann et al., 2011).

In the mouse pancreatic cell line β-TC3, when Nbea is knocked down, stimulated secretion of large dense core vesicles (LDCVs) is increased 2-fold, whereas overexpression suppresses secretion, making Nbea a negative regulator of LDCV secretion (Castermans et al., 2010). Furthermore, dense granules, which are counterparts of LDCVs in blood platelets of patients with happloinsuficieny of Nbea are smaller, more irregular, frequently peripherally localized and poorly delineated (Castermans et al., 2010).

Like its orthologue AKAP550, Nbea possesses a PKA binding domain and might therefore act as an AKAP. The latter is an important protein property and is therefore shortly review in the following section.

1.3.1 Neurobeachin as an A-kinase anchoring protein

All brain functions are ultimately mediated by molecular signaling. Central to many signaling responses is the regulation of the opposing actions of adenylyl cyclases (ACs) and phosphodiesterases (PDEs) that control levels of the second messenger 3'5'-cyclic-adenosine-monophosphate (cAMP), of which the principal intracellular target is the cAMP - dependent serine/threonine protein kinase A (PKA; Skalhegg and Tasken, 2000). In order to ensure specificity in the ubiquitous cAMP/PKA system, the scaffolding protein A-kinase anchoring proteins (AKAPs) cluster cAMP signaling enzymes in discrete units, thereby providing the molecular architecture for the cAMP microdomains underlying the spatial and temporal resolution of cAMP signaling (Smith and Scott, 2006). Up to date more than 50 AKAPs have been identified (for review see Skroblin et al., 2010; Tasken and Aandahl, 2004).

The primary effector of the second messenger cAMP, i.e. the PKA holoenzyme, is a tetramer consisting of two catalytic (C) subunits that are held in an inactive state by a regulatory (R) subunit dimer (Scott, 1991). Binding of cAMP to the R subunits leads to the relief of the autoinhibitory contact, causing dissociation of the holoenzyme. The release of active C subunits, which are multifunctional serine/threonine kinases allows phosphorylation of a number of downstream substrates. The C subunits Cα, Cβ, Cγ, PRKX (the human chromosome-encoded protein kinase X) as well as the R subunits (RIα, RIβ, RIIα, RIβ) possess distinct physical and biological properties, are differentially expressed, and are able to form different isoforms of PKA holoen-

zymes (reviewed in Skalhegg and Tasken, 2000). The type I PKA (composed of RI dimers) is most highly expressed in the immune system, whereas type II PKA (composed of RII dimmers) is abundant in the heart and brain (Scott, 1991). While type I is biochemically soluble and therefore assumed to be predominantly cytoplasmic, the type II PKA holoenzyme is particulate and associates with specific cellular structures and organelles through cell- and tissue specific AKAPs (reviewed in Colledge and Scott, 1999; Dodge and Scott, 2000). However, recently several dual specificity AKAPs (D-AKAPs) anchoring both PKA types have been identified (Tasken and Aandahl, 2004).

AKAPs' common properties

Most AKAPs share a PKA R-subunit binding sequence, binding regions that participate in lipid/protein or protein/protein interactions, allowing for the targeting of the PKA/AKAP complex to subcellular environments, additional enzyme-binding sites and recruitment into much larger multiprotein complexes through the interactions with other adaptor molecules (Carnegie and Scott, 2003; Dodge-Kafka et al., 2008):

Figure 1.4 The four properties of AKAPs. Their ability to bind the R-subunit of PKA define them as AKAPs. Unique targeting domains determine their subcellular placement. AKAPs are not only capable to incorporate multiple signaling pathways, but, via association with additional adaptor proteins, they integrate into multi-protein networks (Adapted from Dodge-Kafka et. al., 2008)
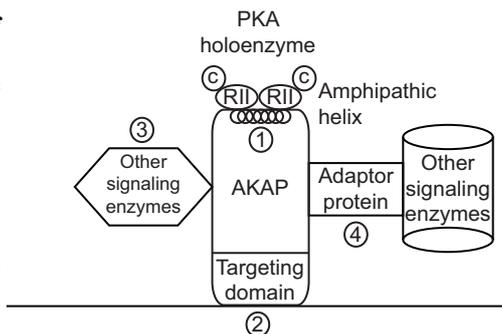
The conserved PKA anchoring domain in AKAPs is composed of 14-18 residues, which form an amphipathic helix with hydrophobic residues being aligned along one face of the helix and charged residues along the other. This tethering domain interacts with hydrophobic determinants located in the extreme NH2 terminus of the regulatory subunit dimer (Carr et al., 1992, 1991), i.e. the hydrophobic side of the helix is inserted into the hydrophobic pocket formed by the two regulatory subunits (Newlon et al., 1999). Techniques to globally disrupt PKA anchoring in a cell have proven quite useful when studying cellular processes where PKA anchoring has been suspected to be involved. A peptide usually referred to as the Ht31 anchoring disruption peptide derived from the PKA anchoring domain of the human thyroid AKAP Ht31 (now called AKAP-Lbc), mimics the R-II binding domain of the AKAP and competes with AKAP/PKA binding (Carr et al., 1991; Lester et al., 1997; Rosenmund et al., 1994).

Specialized targeting regions on each AKAP determine the location of the PKA/ AKAP complex in the cell and thereby allow for compartmentalized cAMP signaling. Often protein-lipid interactions target the PKA/AKAP complex to the correct cellu-

lar environment, while protein-protein interactions precisely orient the kinase towards its substrates. In some cases, however, targeting of AKAP complexes to substrates can be accomplished by protein-protein interactions only (reviewed in Carnegie and Scott, 2003). It is not surprising that multiple AKAPs can mediate targeting to the same organelle, since it has been suggested that in some cells up to 15 different AKAPs are being expressed (Skalhegg and Tasken, 2000), raising the possibility that, within a single cellular compartment, different AKAPs function to target PKA to specific substrates. For instance several AKAPs anchor PKA at mitochondria: Rab 32, AKAP121, AKAP149 (Alto et al., 2002; Chen et al., 1997; Trendelenburg et al., 1996). Similarly multiple AKAPs are found to be associated with the cytoskeleton and centrosome, the nucleus, ion channels, peroxisomes, vesicles, the Golgi, endoplasmic reticulum (ER) and membranes (for review see Colledge and Scott, 1999; Skalhegg and Tasken, 2000; Skroblin et al., 2010; Tasken and Aandahl, 2004). Although the subcellular location of many AKAPs is known nowadays, the precise details of targeting have been determined for only a few molecules. However, with further studies examining AKAP targeting undoubtedly additional sequences will be identified.

The most intriguing feature of AKAPs is their ability to interact with several signaling proteins and thereby coordinate the integration of enzymes from multiple signaling networks onto a specific substrate. Virtually any signal transduction protein, such as protein phosphatases, phosphodiesterases, adenylyl cyclases, G proteins, ion channels and other protein kinases (Coghlan et al., 1995; Dodge et al., 2001; Fraser et al., 2000; Klauck et al., 1996; Westphal et al., 1999) can be integrated by these multivalent anchoring proteins. For instance AKAP79 is attached to the post-synaptic densities and associates with PKA, calcium/phospholipid-dependent kinase (protein kinase C, PKC), the calcium/calmodulin-dependent phosphatase (protein phosphatase 2B, PP2B, calcineurin, CaN) and adenylyl cyclase V/VI (Bauman et al., 2006; Klauck et al., 1996). Simultaneous binding of enzymes with opposing actions highlights the central role of AKAPs in the customized regulation of cAMP signaling.

Due to their ability of associating with additional adaptor proteins through protein-protein interaction domains such as PDZ and SH3, AKAPs can integrate into multi-protein networks (Colledge et al., 2000; Soderling and Scott, 2006), thereby further increasing the specificity required for the precise regulation of the numerous cellular and physiological processes, like synaptic plasticity, sperm motility, T-cell immune responses and several exocytic processes (Skroblin et al., 2010)

In the historical perspective, AKAPs have come a long way, from their first discovery in the early 1980s (Theurkauf and Vallee, 1982; Smith and Scott, 2006), they have long exceeded their role as mere targeting molecules and have emerged as focal points for the convergence and integration of highly organized signaling pathways.

1.4 Synthesis

Phylogenetic analysis suggests that Nbea, its human paralogue LRBA and their common ancestors SEL-2 and AKAP550 form a specific class of BEACH proteins. Given the fact that AKAP550 and Nbea both bind the type II regulatory subunit of protein kinase A, it is possible that this group of BEACH proteins evolved to function as AKAPs (Shamloula et al., 2002; Wang et al., 2000. Although the RII-binding sites identified in AKAP550 and Nbea are not present in LRBA, in the latter, two alternative AKAP domains have been predicted (Wang et al., 2001). Whether the *C.Elegans* homologue SEL-2 also possesses PKA binding sites, or whether this is a feature that only appeared later in the evolution of BEACH proteins, remains to be determined.

Interestingly, these three Nbea homologues have all been linked to EGFR signaling (de Souza et al., 2007; Shamloula et al., 2002; Wang et al., 2004). Activation of EGFR leads to homodimerization/heterodimerization, phosphorylation of specific tyrosine residues and recruitment of several proteins at the intracellular portion of the receptor (e.g. phospholipase Cγ, the <u>s</u>ignal <u>t</u>ransducer and <u>a</u>ctivator of <u>t</u>ranscription (STAT) protein, the adaptor proteins SHC, GRB2 and Gab-1; Mitsudomi and Yatabe, 2010; Scaltriti and Baselga, 2006). This in turn leads to the initiation of signaling via several pathways: Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase/Akt pathway, phospoholipase Cγ pathway, STAT pathway and Src kinase pathways (Mitsudomi and Yatabe, 2010; Scaltriti and Baselga, 2006). These eventually result in cell proliferation, migration and metastasis, evasion from apoptosis, or in angiogenesis, which are all associated with cancer phenotypes (Mitsudomi and Yatabe, 2010). In line with this, EGFR is overexpressed, dysregulated or mutated in many epithelial malignancies, and EGFR activation appears important in tumor growth and progression (Harari, 2004). LRBA, which has a very similar primary structure and is most closely related to Nbea, functions as an oncogene (Wang et al., 2004). The dominant negative form of LRBA (lacking the BEACH-WD40 domains) reduces the phosphorylation of EGFR and MAPK and sensitizes cancer cells to apoptosis (Wang et al., 2004). Similarly, Nbea has also been linked to cancer, but in contrast to LRBA, acts as a potential tumor suppressor gene in multiple myeloma (O'Neal et al., 2009). Unfortunately, Nbea's role in EGFR signaling has not yet been elucidated. However, it has been shown that basal activity of PKA controls EGFR function by modulating the accessibility of receptors to stimuli - inhibition of basal PKA activity induces internalization of inactive EGFR (Salazar and Gonzalez, 2002) and that overexpression of R subunits is associated with aggressive tumor behavior and resistance to treatment (Miller, 2002). Whether EGFR- or PKA pathways are responsible for Nbea's function as a tumor suppressor is not yet clear.

Nbea shares also a functional similarity with FAN. Both proteins have been been shown to be involved in actin distribution (Haubert et al., 2007; Niesmann et al., 2011). However, FAN interacts with F-actin directly upon TNF induced stimulation of Cdc42, while Nbea's influence on actin distribution is an indirect one, via the spine-associated protein with actin-bundling activity, i.e. synaptopodin (Haubert et al., 2007; Montfort et al., 2010), suggesting two different cellular pathways. Also,

an interaction of Nbea with TNF-Rs has not yet been reported.

Neurobeachin is unique among BEACH proteins for several reasons. It is the only BEACH protein that is essential, i.e. its loss results in neonatal lethality (Medrihan et al., 2009; Su et al., 2004). Although some other BEACH protein null-mutants show severe phenotypes, like the *LvsA* mutants, which fail to grow in suspension and become multinucleate (Kwak et al., 1999), or the reduced adult life span in *Bchs* mutants (Finley et al., 2003; Khodosh et al., 2006), all of them are viable. In addition, Nbea is the only BEACH protein that is brain specific (Wang et al., 2000). Also NBEAL1 mRNA is highly detected in the brain, but in addition it is also highly expressed in the kidney, prostate and testis, while weakly in several other tissues (Chen et al., 2004a). FAN, LRBA and LYST are ubiqitously expressed in a variety of tissues including the brain (Adam-Klages et al., 1996; Perou et al., 1997; Wang et al., 2000), while BWFY3 mRNA is strongly expressed in the liver, moderately in the kidney and testis, and only weakly expressed in the brain (Chen et al., 2004a). Last, but not least, until the functionality of the two predicted AKAP domains in LRBA is not determined, Nbea is for now the only mammalian BEACH protein that has the ability to bind regulatory subunits of PKA (Wang et al., 2000). Based on these findings, together with the fact that Nbea is essential for the function of central synapses (Medrihan et al., 2009; Niesmann et al., 2011), it can be deduced that Nbea is involved in membrane trafficking steps specific for the central nervous system that influence synaptic transmission and require protein kinase A activity.

1.5   Aim and outline of this thesis

Although Neurobeachin was identified as a putative regulator of membrane protein trafficking that is essential for evoked transmission at neuromuscular junctions, its role in the central nervous system has not been thoroughly characterized. The research described in this thesis aims to elucidate the function of Neurobeachin in neurons of the CNS, in particular related to synaptic transmission. In order to do so, we made use of a number of different approaches.

- Because the localization of a protein can give valuable insight into its function, in **Chapter 2** we analyzed Nbea's subcellular localization and its developmental expression pattern in dissociated hippocampal neurons. Therefore, a specific Nbea antibody was raised and used for immunocytochemistry in combination with confocal microscopy. Since Nbea has been implicated in membrane protein traffic, we also studied the overall subcellular architecture of Nbea null mice, focusing especially on the morphology of organelles of the early secretory and endosomal pathway. This is important, since Nbea related proteins LvsB and LYST have been shown to regulate the size of lysosomes (Harris et al., 2002; Shiflett et al., 2002).

- To find out whether Nbea is important for neurotransmission in central synapses,

in **Chapter 3** we thoroughly characterized the synaptic transmission of Nbea KO mice at the single neuron level, using a highly standardized preparation which allowed dissection of exactly which aspects of synaptic function are normal in Nbea KO neurons and which are not.

- In order to clarify how Nbea mediates its effect on synaptic transmission, in **Chapter 4** we set out to identify novel interactors of Nbea in the brains of embryonic and adult mice by an immunoaffinity-based proteomics approach.

- Since Vti1a, a protein that has also been implicated in protein transport, colocalized to the same cellular compartment as Nbea, in **Chapter 5** we examined Vti1a's function in more detail given the possibility that these two proteins act in the same cellular pathway. In particular, we studied the impact of Nbea loss on the subcellular localization of Vti1a and vice versa and investigated Vtia's role in large dense core vesicle release from adrenal chromaffin cells from VTi1a null mice.

- In **Chapter 6** we discussed the implications of the main findings of the previous chapters and attempted to generate a model for Nbea's role and suggested future directions.

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