Synapse associated protein 102 (SAP102) binds the C-terminal part of the scaffolding protein Neurobeachin

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Abstract

Neurobeachin (Nbea) is a multidomain scaffold protein abundant in the brain, where it is highly expressed during development. Nbea-null mice have severe defects in neuromuscular synaptic transmission resulting in lethal paralysis of the newborns. Recently, it became clear that Nbea is important also for the functioning of central synapses, where it is suggested to play a role in trafficking membrane proteins to both, the pre- and post-synaptic sites. So far, only few binding partners of Nbea have been found and the precise mechanism of their trafficking remains unclear. Here, we used mass spectrometry to identify SAP102, a MAGUK protein implicated in trafficking of the ionotropic glutamate AMPA- and NMDA-type receptors during synaptogenesis, as a novel Nbea interacting protein in mouse brain. Experiments in heterologous cells confirmed this interaction and revealed that SAP102 binds to the C-terminal part of Nbea that contains the DUF, PH, BEACH and WD40 domains. Furthermore, we discovered that introducing a mutation in Nbea’s PH domain, which disrupts its interaction with the BEACH domain, abolishes this binding, thereby creating an excellent starting point to further investigate Nbea-SAP102 function in the central nervous system.
Neurobeachin (Nbea), a large (327 kDa), brain-enriched, multi-domain protein is essential for synaptic transmission (Medrihan et al., 2009; Niesmaan et al., 2011; Su et al., 2004). Nbea was initially discovered in an attempt to isolate novel synaptic proteins, but was subsequently found to associate with tubulovesicular endomembranes near the trans-Golgi network and throughout the neuronal cell body and dendrites (Niesmaan et al., 2011; Wang et al., 2000, see Chapter 2). Its membrane association is stimulated by GTP and antagonized by brefeldin A (Wang et al., 2000, see also Chapter 2). Hence, Nbea may play a role in post-Golgi sorting or targeting of neuronal membrane proteins and vesicle trafficking (Wang et al., 2000).

Nbea knock-out (KO) mice lack spontaneous and reflexive movement (i.e. movement elicited by tail pinch) and die immediately after birth due to their inability to breathe (Medrihan et al., 2009; Su et al., 2004). This primary asphyxia is probably the result of the absence of evoked neurotransmitter release at neuromuscular junctions (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, spontaneous and miniature excitatory postsynaptic currents (mini EPSCs) show a reduction in frequency, whereas spontaneous and miniature inhibitory postsynaptic currents (mini IPSCs) are both reduced in frequency and amplitude (Medrihan et al., 2009). Along with a reduced number of asymmetric contacts in the fetal brainstem, reduced levels of several presynaptic proteins were observed (Medrihan et al., 2009). Also, altered miniature excitatory and inhibitory postsynaptic currents were reported in cultured hippocampal neurons from KO mice and cortical slices from heterozygous mice, accompanied by reduced numbers of spine-localized synapses (Niesmaan et al., 2011). In addition, in KO neurons excitatory presynaptic terminals are mostly on dendritic shafts instead of on spine heads and actin in these synapses is less enriched (Niesmaan et al., 2011).

Nbea belongs to a family of BEACH (Beige and Chediak-Higashi) proteins, which share three carboxyl-terminal (C-terminal) domains: a Pleckstrin-Homology like domain (PH) (Jogl et al., 2002), a highly conserved BEACH domain (Nagle et al., 1996) and tryptophan-aspartic acid (WD40) repeats. In addition, some of the BEACH proteins share a domain of unknown function 1088 (DUF 1088).

Pleckstrin-homology domains, first identified as an internal repeat in the phosphoprotein pleckstrin (a substrate of protein kinase C in platelets; Haslam et al., 1993; Mayer et al., 1993), comprise a well-defined class of phospholipid-binding protein domains. More than 500 different PH domain-containing proteins have been found (Hurley and Misra, 2000), many of which are involved in signal transduction and cytoskeletal organization (Bottomley et al., 1998). While generally binding to inositol lipids, a subset of PH domains respond to up-stream signals by targeting the host protein to the correct cellular site (Blomberg et al., 1999). In addition, they can also function in phosphotyrosine binding and mediating protein-protein interaction (Jogl et al., 2002).

The BEACH domain that is adjacent to the PH-like domain is highly conserved
among the BEACH proteins (50-60 % sequence identity; De Lozanne, 2003). With approximately 280 amino acids, it is much larger than a simple protein-protein interaction domain and it has been hypothesized to contain enzymatic activity (Wang et al., 2000). However, no catalytic sites have been found (Jogl et al., 2002). Structural analysis revealed that the BEACH domain is in extensive association with the PH domain (Jogl et al., 2002). Protein binding assays, using purified PH domain fused with glutathione S-transferase and the purified His-tagged BEACH domain of the protein FAN, clearly demonstrated strong interactions between the PH and BEACH domains of FAN (Jogl et al., 2002). Moreover, specific single-site mutations in FAN’s PH-BEACH interface not only disrupted the interactions between these two domains, but also reduced FAN’s signaling, showing that the two domains may function as a single unit (Jogl et al., 2002).

The WD40 repeat domain (also called the beta-transducin repeat (Duronio et al., 1992) or the GH-WD repeat domain (Neer et al., 1993)) was first identified in the β-subunit of trimeric G-proteins (Fong et al., 1986). A common feature of WD40 repeat domains is that their propeller structures create a stable platform for reversible interactions with multiple other proteins to form complexes (Smith et al., 1999).

The amino-terminal (N-terminal) region of Nbea contains an armadillo repeat-flanked Concanavalin A (ConA)-like lectin domain (Figure 4.1A) that is shared by other mammalian BEACH proteins, e.g. CHS (Cheidak-Higashi syndrome), LRBA and ALFY (Burgess et al., 2009). Unfortunately, also the function of this domain is still unidentified. Furthermore, apart from the Drosophila AKAP550, Nbea is the only BEACH protein with a binding site for the RII regulatory subunit of the 3’5’-cyclic-adenosine-monophosphate (cAMP)-dependent protein kinase (also called Protein kinase A; PKA), classifying it as an A-kinase anchoring protein (AKAP; for graphical representation see Figure 4.1A; Han et al., 1997; Wang et al., 2000). AKAP's cluster cAMP signaling enzymes in discrete units, creating cAMP microdomains that underlie the spatial and temporal resolution of cAMP signaling (Smith and Scott, 2006).

Figure 4.1 Schematic representation of mouse Nbea (NCBI Reference Sequence: NP_085098.1). The predicted armadillo (ARM) repeat-flanked Concanavalin A (Con A)-like lectin domain is localized at the N-terminus of the protein (blue). The region the Nbea antibody was raised against is depicted by the purple rectangle, encompassing also the PKA binding site (pink stripe). At the C-terminus the domain of unknown function 1088 (DUF; in orange), the Pleckstrin-Homology like domain (PH; in gray), the BEACH domain (yellow) and the WD40 repeats (red) are depicted.

Taken together, Nbea is a complexly organized multidomain protein, and therefore, likely acts as a scaffold for binding of many proteins. So far, only a few binding
partners of Nbea have been reported (Table 4.1) and it has not yet been possible to deduce a role of Nbea in cellular function. In this study we used protein interaction proteomics technology to identify novel Nbea interactors. Here we show that SAP102, a scaffolding protein that has been implicated in trafficking of AMPA and NMDA receptors during synaptogenesis (Elias and Nicoll, 2007), binds to Nbea’s C-terminal part, and that this encompasses the DUF1088, PH, BEACH and WD40 domains. In addition, we describe a mutation in the PH domain that abolishes this binding, creating a solid base to further dissect Nbea-SAP102 function in the CNS.

Table 4.1 Previously identified binding partners of Nbea.

<table>
<thead>
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<th>Interactor</th>
<th>Alternative names</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
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<td>Wang et al., 2000</td>
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<tr>
<td>PRKAR2B</td>
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<td>Pull down</td>
<td>Hassel et al., 2004</td>
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<td>STRN4</td>
<td>Striatin calmodulin binding protein 4; Zinedin</td>
<td>Two hybrid pooling approach</td>
<td>Stelzl et al., 2005</td>
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<td>Ywhab</td>
<td>Protein kinase C inhibitor protein 1; 14-3-3 protein beta</td>
<td>Co-sedimentation through density gradient</td>
<td>Collins et al., 2006</td>
</tr>
<tr>
<td>FYN</td>
<td>Proto-oncogene Syn; Src-like kinase; Proto-oncogene-cFyn; p-59-Fyn</td>
<td>Peptide array</td>
<td>Wu et al., 2007</td>
</tr>
<tr>
<td>ABL1</td>
<td>Abelson murine leukemia viral oncogene homolog1; Abelson tyrosine-protein kinase 1; Proto-oncogene c-Abl; p50</td>
<td>Peptide array</td>
<td>Wu et al., 2007</td>
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<td>DTNBP1</td>
<td>Dysbindin-1; Hermansky-Pudlak syndrome 7 protein; Dystrobrevin-binding protein 1</td>
<td>Two-hybrid</td>
<td>Camargo et al., 2007</td>
</tr>
<tr>
<td>glpD</td>
<td>Y3891; YP_2499; q8cwg4 yerpe</td>
<td>Two hybrid pooling approach</td>
<td>Dyer et al., 2010</td>
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<tr>
<td>BA0681</td>
<td>BAS0647; GBAA0681</td>
<td>Two hybrid pooling approach</td>
<td>Dyer et al., 2010</td>
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<tr>
<td>GlyRβ</td>
<td>Glycine receptor beta subunit</td>
<td>Pull down</td>
<td>del Pino et al., 2011</td>
</tr>
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Materials and Methods

Ethics statement

These studies were approved by the institutional ethic committee of the VU University (Protocol FGA 06-11-2). All animals were housed and bred according to the institutional and Dutch governmental guidelines for animal welfare.

Use of human embryonic kidney cells 293T (HEK293T/17; ATCC No: CRL-11268) was approved according to the institutional and Dutch governmental regula-
Laboratory animals and cell lines

Nbea KO mice have been described before (Su et al., 2004). Mouse embryos were obtained at embryonic day 18 by caesarian section of pregnant females from timed mating of Nbea heterozygous animals (C57/B16 background). For experiments involving older animals, 12 week old C57/B16 mice were used. For mass spec experiments mice were decapitated, the brain was removed and immediately frozen. The tissue was stored at -80°C until further use. For rat glia preparations newborn P0-P1 pups from pregnant female Wistar rat (Harlan or Charles River) were used. For immunoprecipitation experiments in heterologous cell lines, human embryonic kidney cells 293T (HEK293T) were used.

Mass spectrometry

Preparation of brain lysates

For immuno-precipitation Triton X-100 (TX-100) and n-Dodecyl β D-maltoside (DDM) extracts were prepared by adding extraction buffer (25 mM HEPES pH7.4, 150 mM NaCl, 1% detergent, protease inhibitor (complete Mini, EDTA-free; Roche Applied Science) to E18 WT or Nbea KO mouse brains followed by homogenization at 900 rpm on ice using a Potter homogenizer (B. Braun Biotech International). We used 2 brains per IP and 1ml of extraction buffer per brain. The homogenate was then left to slowly rotate for 20 min at 4°C, before it was centrifuged at 20000 rcf for 20 min at 4°C. Afterwards, the supernatant was transferred to a new Eppendorf tube and centrifuged again at 20000 rcf for 20 min at 4°C. Finally, after discarding the pellet, the supernatant was used directly in the immunoprecipitation with the α-Nbea antibody.

Preparation of brain subcellular fractions

Hippocampi were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 5 mM HEPES, pH 7.4) containing protease inhibitor (Roche Applied Science). Cell debris and nuclei were removed by 1.000 g centrifugation for 10 min. The supernatant was either spun at 12.000 g for 20 min resulting in supernatant and pellet P2 (crude membrane, synaptosomes, mitochondria and myelin) or at 100.000 g for 2 hrs to obtain a fraction enriched in crude membranes with microsomes (pellet 2+ microsomes).

The P2 pellet was fractionated by centrifugation (100.000 g, 2 hrs) in a sucrose step gradient to purify synaptosome, an organelle that contains both pre- and post-synaptic compartments. Synaptosome was lysed in hypotonic solution to release the cytoplasmic proteins and organelles such as mitochondria and small synaptic vesicle, and the resulting synaptic membrane was recovered by centrifugation using the sucrose gradient as stated above.

Further synaptic membranes were mixed with an equal volume of 2% Triton X-100, 5 mM HEPES, pH 7.4 containing protease inhibitor and stirred for 30 min over ice. Obtained lipid rafts and PSD were recovered on a sucrose gradient consisting of
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1 M, 1.5 M and 2 M sucrose. Lipid rafts were collected at the top of 1 M sucrose and PSD at the interphase 1.5 M and 2 M.

Immunoprecipitation
The fetal brain or P2+ microsome fraction from adult mice were solubilized with 1% detergent. For E18 IPs we employed the commonly used detergent, Triton X-100 for solubilization. In the adult mice brain, especially proteins located in the post-synapse are tightly packed and may be difficult to extract in Triton X-100. We therefore used a stronger detergent, the n-Dodecyl β-D-maltoside (DDM). The extracts were incubated with either 7 µg of the α-Nbea antibody or 10 µg of α-SAP102 antibody at 4°C on a mechanical rotator. 30 µl slurry of protein A/G PLUS-Agarose beads (Santa Cruz) was washed four times with washing buffer (150 mM NaCl, 25 mM HEPES, 0.1% detergent) before it was added to the samples for 1 h at 4°C.

Afterwards, the beads were washed four times with washing buffer to washout unbound proteins. The buffer was completely removed using an insulin syringe before storing the samples at -20°C until further use.

Sample preparation for Mass Spectrometry
The ID PAGE LC-MS/MS analysis of protein was carried out as described previously (Chen et al., 2011). In short, the immuno-isolated samples were resuspended in reducing SDS loading buffer and heated for 5 min 98°C before being separated on a 8% SDS polyacrylamide gel. After coomassie staining the sample lane was cut into five slices, each was subjected individually to trypsin digest. The slices were cut into small pieces and washed for 20 min at room temperature sequentially with 600 µl 25 mM NH₄HCO₃/50% acetonitrile, 100% acetonitrile and 25 mM NH₄HCO₃. The whole cycle was repeated three times; except the last one, which was stopped at the washing step with 100% acetonitrile. The gel pieces were dried in a Speedvac for 30 min and afterwards rehydrated with trypsin solution. About 200 µl of extra 25 mM NH₄HCO₃ was added to each gel slice, and then incubated at 37°C over night. The supernatant containing tryptic peptides were transferred into a new tube. Peptide mixtures were dried in a Speedvac and subsequently stored at -20°C until mass spectrometric analysis.

LC-MS/MS analysis
The peptides were redissolved in 15 µl 0.1 % acetic acid, and injected into the loop of an Eksogent nano LC-ultra 1D plus HPLC system equipped with a C18 column (200-nm homemade Alltima C18 analytical column, 100 µm ID 3 µm particle size). Peptides were separated using a linear gradient of 5 % solvent A (0.1 % acetic acid, 5 % acetonitrile) and 45 % solvent B (0.1 % acetic acid, 80 % acetonitrile) in 50 min. The LC system was directly coupled in-line with a LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific).

The LTQ-Orbitrap was set to data dependent mode to switch automatically between MS and MS/MS. MS spectra range from 330 till 2000 m/z can be acquired in the Orbitrap at a FWHM resolution of 30,000 after accumulation to 500,000 in the
Identification of proteins from IP samples
The ID PAGE LC-MS/MS approach was used for protein identification as described previously (Chen et al., 2011). MS/MS spectra were searched against a mouse database (uniprot_sprot_101020) with the ProteinPilot™ software (version 3.0; AB-Sciex) using the Paragon™ algorithm (version 3.0.0.0; Shilov et al., 2007) as the search engine. The search parameters were set to cysteine modification by acrylamide and digestion was done with trypsin. The detected protein threshold (unused protscore (confidence)) in the software was set to 0.10 to achieve 20% confidence, and identified proteins were grouped to minimize redundancy.

Proteins with “unused” value <1.3 have low confidence and were excluded from the analysis. The “unused” value is defined in the handbook of ProteinPilot as a summation of peptide scores from all the non-redundant peptides matched to a protein. Peptides with confidence of ≥ 99% would have a peptide score of 2; ≥ 95% a peptide score of 1.3, and ≥ 66% a peptide score of 0.47, etc. Tryptic peptide shared by multiple proteins will be assigned to the winner protein.

Plasmids and generation of Nbea constructs
The full-length Nbea was generated by using a yeast-two-hybrid cDNA library (Clontech Cat# ML408AH) and a partial image clone (Kazusa mKIAA1544). First, the N-terminal part of Nbea was obtained from the yeast-two-hybrid cDNA library and subcloned in pCR-Script (Stratagene Cat# 211190) using the following primers: rz62 5’TGCACAGCTCCTCAGCAGCG3’; rz63r 5’GCTGGGTGTTCTGACATTAGAGCC3’ and rz64 5’CAGCTCATATTAAAGGATCGAGG’3’; rz65r 5’GGATGAGGAGATGATGGTATGACC3’. The resulting subclones were ligated at PstI and Scal sites. Then, the C-terminal part from the Kazusa image clone was connected to the N-terminal part using NotI and ScaI resulting in a full-length Nbea in a pCR-Script backbone. A fusion of EYFP and Nbea was made by digesting the Nbea full-length pCR-Script with Sall & KspI and ligation this into the pEYFP-C1, digested with the same enzymes.

For creating the EGFP-Nbea C-terminal fusion construct (containing the DUF1088, PH, BEACH and WD40 domains) the Kazusa image clone was used as template and a C-terminal Nbea fragment containing AA 1956 - 2936 was amplified using rz106 5’AAAGAATTCACCATGGCGGAAGGAAGGTTGTTGTGCCATGC3’ (adding an EcoRI site) and rz118r 5’TCTGGATCCCACCTGAATGTTGCTTCTTGCTGC3’ (adding a BamHI site) which was subcloned into pCR-Script. EcoRI and BamHI sites were used for cloning into pEGFP-C3. In order to create the GFP-Duf-PH-Beach (encompassing AA1956-2553 of Nbea), the following primers were used: RZ550 5’AAACTCTGGAGGAAGGATTCTTGTGCGC3’ and RZ111r 5’TCTGGATCCCACCTGAATGTTGCTTCTTGCTGC3’ which was subcloned into pCR-Script. EcoRI and BamHI sites were used for cloning into pEGFP-C3. For the GFP-PH-Beach-WD40 (AA 2140-
SAP102 interacts with Nbea

2925) RZ562 5'AAACTCGAGGGCCCTGTGGTTCTCAGCACC3' and RZ549r 5'TTTGAATTCTCAATCTATATTAAAAGCTACAATGCTGCC3' were used. For the GFP-PH-Beach (AA 2140-2553) RZ90 5'AAAGAATTCAGGGCCCTGTGGTTCTCAGCACC3' and RZ87r 5'CCTAGGCGGATGTGGCTCAATGAGC3' were used. For the GFP-PH (AA 2140-2247) RZ90 and RZ88r 5'AACCCGAGGCAAGCTCTGAGAC3' were used. For GFP-Beach (AA2215-2553) the primers RZ91 5'AAAGAATTCACACTCGAGGGCCCTGTGGTTCTCAGCACC3' and RZ87r were used.

To create point mutations in the C-terminus of Nbea the standard protocol of QuickChange™ Site-Directed Mutagenesis Kit was used. The mutated inserts were subsequently cloned in expression vector and fused to GFP. The point mutations were created using the following primers: E2090K 5'GCACCTTGGAGAGGGAGGCTGTGGTTATG3' and E2090Kr 5'CAGATCTTACCCAGAAGCTGAGAGTGGTTATG3', E2218R 5'CTTCTGGAGAGGTGGTTATG3' and E2218Rr 5'CTTCTGGAGAGGTGGTTATG3'.

The FLAG-tagged SAP102 was obtained from the yeast-two-hybrid cDNA library, by using the following primers to create mouse SAP102 with 5'EcoRV and 3'SalI restriction sites: 5'GATATGCGAGAATCTTACACTCGAGGGCCCTGTGGTTCTCAGCACC3' and 5'GCTACATCGAGGCGGATGTGGCTCAATGAGC3'. It was first cloned into a pCR-blunt vector (Invitrogen), before it was subcloned, using the EcoRV and SalI sites, into a pCMV3TAG1A vector (Invitrogen).

pEGFP and pmCherry were both purchased from Clontech (CAT#PT2039-5, CAT#PT3973-5). All created constructs were sequence-verified.

Construct expression and co-immunoprecipitation in HEK cells

For expression of DNA constructs, HEK293T cells were cultured in DMEM medium (Invitrogen) containing 10% fetal calf serum (FCS), 1% non-essential amino acids (NEAA) and 1% penicillin/streptomycin (all Gibco). They were plated at equal density in 10 cm dishes one day before transfection. Cells were transfected with calcium phosphate transfection.

For one 10 cm dish of HEK293T cells (80% confluency) a total of 10 µg of DNA (5µg of each construct) was mixed with 400 µl of 2X Hepes-buffered saline (HBS; containing: 140 mM NaCl, 1.5 mM Na₂HPO₄·2H₂O, 50 mM HEPES adjusted to
pH 7.05 with NaOH). Under constant vortexing, 400 µl of 2M CaCl$_2$ solution was added dropwise and let to precipitate for 30 min at RT. Afterwards, this mixture was pipetted in a drop-wise-manner onto the plated cells, which were subsequently placed back into the incubator at 37°C for 17 hrs. Next, the medium was replaced with warm fresh culture medium and incubation was resumed for another 24 hrs prior to assaying.

Cells were grown for 41 hrs after transfection before they were lysed in 800 µl of lysis buffer (containing 50 mM Tris pH 7.5, 1% Triton X-100, 1.5 mM MgCl$_2$, 5.0 mM EDTA, 100 mM NaCl, protease inhibitor). After centrifugation at 14000 rpm for 10 min at 4°C, the supernatant was used for immunoprecipitation assays. The lysates were precleared of immunoglobulin by incubation for 1h at 4°C with Protein A Agarose beads (Sigma). The latter were washed three times with lysis buffer before usage and were afterwards removed by centrifugation. The precleared cell lysates were then incubated with Protein A Agarose beads that were preblocked in 1% chicken egg albumin (Sigma) and were removed after incubation with cell lysate by centrifugation. In the different co-immunoprecipitations we either added the α-Nbea antibody (rabbit polyclonal, 0.18 µg), the α-GFP antibody (rabbit polyclonal, Abcam ab290/50, 0.25 µg) or the α-FLAG antibody (mouse monoclonal clone M2, Sigma, 1.25 µg) for 2 h at 4°C. Afterwards the beads were washed five times with lysis buffer and resuspended in SDS-PAGE loading buffer, and samples were subjected to gel electrophoresis and immuno-blotting.

In short, samples were loaded into 5%-10% SDS-PAGE gels and run on 25 mA per gel until satisfactory mass separation. Proteins were then transferred to PVDF membranes (Bio-Rad) at 350 mA for 2 hrs. Blocking with 2% milk (Merck) and 0.5% bovine serum albumin (BSA; Sigma) for 1 h was used to circumvent unspecific binding. Primary antibodies (α-Nbea rabbit polyclonal, SySy, 1:500; α-GFP rabbit polyclonal, Abcam, 1:5000 and α-FLAG mouse monoclonal clone M2, Sigma, 1:5000) were applied for 2 hrs or overnight at 4°C. After substantial washing, alkaline phosphatase labeled secondary antibodies (goat α-mouse AP 1:10000 or goat α-rabbit AP 1:10000; both DAKO) were applied for 1 hour at 4°C. Blots were then washed and scanned using ECF substrate for immuno-blot (GE Healthcare) on a Fujifilm FLA-5000 Reader. All solutions for blocking, staining or washing were prepared in PBS (pH 7.4) containing 0.1% Tween-20 (Sigma). Immuno-blotting were stripped using Re-blot Plus Strong Antibody Stripping Solution (Millipore).

**Immuno-blot data analysis**

For quantification of immuno-blot band intensities the GelAnalyzer tool in ImageJ (NIH; Bethesda, MD) was used. The bar graphs in Figure 4.4B and Figure 4.5B depict average intensities of at least 3 separate experiments. Since there might be a difference in the affinity of the α-GFP antibody for GFP and YFP, we did not decide to normalize the data to the full-length Nbea fused to YFP. Instead, we normalized it to the intensity of the band obtained with the C-terminal domain fragment (encompassing the Duf, PH, BEACH and WD40 domain) fused to GFP.
Dissociated hippocampal cultures and immunofluorescence staining

Hippocampi were dissected from embryonic day 18 (E18) wild type C57/Bl6 mice and collected in ice-cold Hanks Buffered Salt Solution (HBSS; Sigma), buffered with 7 mM HEPES (Invitrogen). They were incubated in Hanks-HEPES with 0.25% trypsin (Invitrogen) for 20 min at 37°C. After washing, neurons were triturated using a fire-polished Pasteur pipette and counted in a Fuchs-Rosenthal chamber. The cells were plated in pre-warmed Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% HEPES, 0.25% glutamax (Invitrogen) and 0.1% Pen/Strep (Invitrogen) at a density of 25k/well on 18 mm glass coverslips coated with a mixture of 0.1 mg/ml poly-D-lysine (Sigma), 0.2 mg/ml rat tail collagen (BD Biosciences) solution and 10.2 mM acetic acid solution (Sigma) containing a glial feeder layer, and allowed to grow for 14 days before fixation. At DIV14-15 they were fixed in 100% methanol (Interchema) for 4 min before being rinsed with Dulbecco’s Phosphate Buffered Saline (D-PBS, Gibco). They were incubated for 2 hrs in the primary antibody mixture containing α-Nbea (rabbit polyclonal, SySy, 1:1000) and α-MAP2 (chicken polyclonal, Abcam ab5392, 1:1000) and α-SAP102 (mouse monoclonal, NeuroMab clone N19/2, 1:100) diluted in D-PBS, washed 3 times with D-PBS and incubated in corresponding secondary antibodies (all Molecular Probes, 1:1000) diluted in D-PBS for 1 h. After additional 3 washes they were mounted on microscopic slides with ProLong® Gold (Invitrogen) and imaged with a Zeiss 510 Meta Confocal microscope (Carl Zeiss b.v. Weesp) on a Zeiss 510 Meta Confocal microscope (Carl Zeiss).

Confocal imaging and quantification of transfected HEK293T cells

For confocal imaging HEK293T cells were transfected as described above and subsequently fixed in 3.7% formaldehyde (Electron Microscopy Sciences) in Dulbecco’s phosphate buffered saline (D-PBS; Gibco) for 20 min. Afterwards cells were washed with D-PBS, mounted on microscopic slides with ProLong® Gold (Invitrogen) and imaged with a Zeiss 510 Meta Confocal microscope. To determine the fraction of cells exhibiting a compartmentalized expression pattern, images of 5 different fields of view were analyzed. In every field of view first all the cells expressing the corresponding construct (regardless of the pattern of expression) were counted and then the cells showing the compartmentalized pattern were counted and the fraction of these cells in regard to the total number of expressing cells was calculated. The fractions in the bar graph in Supplementary material Figure 4.10B represent the average fractions from the 5 different fields of view.
Results

**SAP102 is a potential interactor of Nbea**

To identify novel interaction partners of Nbea, we performed a proteomics interaction analysis in fetal (embryonic day 18; E18) and adult mice (postnatal day 84; P84) using the α-Nbea antibody to immunoprecipitate (IP) potential binding proteins. Since the Nbea KO mice present with a lethal phenotype, we first carried out 3 IPs on E18 brain homogenates. IPs were performed on WT and KO brain homogenates, using α-Nbea antibody (n=3), and on WT brain homogenates using either beads coated with pre-immune serum (n=1) or non-coated, empty-beads (EB; n=1) served as controls for non-specific binding. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by coomassie. Proteins separated on gel, were trypsin digested, and identified by LTQ-Orbitrap mass spectrometry. Table 4.2 shows the list of proteins that were present in at least 2 of the 3 IP experiments, and were enriched at least 20-fold in the IP samples compared to the controls. In total, 8 proteins were identified, among which the Discs large homolog 3 (Dlg3), also called Synapse Associated Protein 102 (SAP102) was repeatedly identified (Table 4.2).

<table>
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<tr>
<th>Protein name</th>
<th>Genes name</th>
<th>Exp 1</th>
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<th>Exp3</th>
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<td>209.2</td>
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<td>Eml2</td>
<td>5.0</td>
<td>4.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Echinoderm microtubule-associated protein-like 4</td>
<td>Eml4</td>
<td>2.0</td>
<td>12.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Lipopolysaccharide-responsive and beige-like anchor protein</td>
<td>Lrba /</td>
<td>/</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Serine/threonine-protein kinase Nek9</td>
<td>Nek9</td>
<td>1.7</td>
<td>6.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>

The experiments were performed on brain homogenates of Nbea WT and KO E18 mice using Triton X-100 as detergent for protein extraction. Proteins listed in this table were present in at least 2 of the 3 IP experiments, and were enriched at least 20-fold in the IP samples compared to the controls. The “unused” value is a summation of protein scores from all non-redundant peptides matched to a single protein. Proteins with “unused” value <1.3 have low confidence and were excluded from analysis.

To examine the Nbea protein interactome in adult mice, we next performed IPs on a crude membrane with microsomes fraction (P2+M) from 12 week old mice (n=3). In addition to the non-coated, empty beads (EB; n=2), we also used the IP from E18 KO mice as controls. Using the same analysis as for the E18 condition, 29 proteins were identified. Most of the proteins identified from the E18 IP samples were also present in the adult IP samples. Importantly, Dlg3/SAP102 was repeatedly identified (Table 4.3).
Table 4.3 List of proteins identified from IPs on P2+microsomes fraction from P84 mice.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene name</th>
<th>Unused values</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobeachin</td>
<td>Nbea</td>
<td></td>
<td>108.7</td>
<td>126.1</td>
<td>150.4</td>
</tr>
<tr>
<td>Discs large homolog 3; Synapse-associated protein 102</td>
<td>Dlg3</td>
<td>2.0</td>
<td>12.1</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Complement component 1Q subcomponent binding protein,</td>
<td>C1qbp</td>
<td>8.0</td>
<td>6.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>mitochondrial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAP-Gly domain-containing linker protein 2</td>
<td>Clip2</td>
<td>12.3</td>
<td>4.0</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Dihydrolipoyl dehydrogenase, mitochondrial</td>
<td>Dld</td>
<td>6.0</td>
<td>6.5</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Dipeptidyl aminopeptidase-like protein 6</td>
<td>Dpp6</td>
<td>6.0</td>
<td>6.2</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic dynein 1 intermediate chain 2</td>
<td>Dyncl2</td>
<td>2.0</td>
<td>/</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Elongation factor 1-alpha1</td>
<td>Eef1a1</td>
<td>4.0</td>
<td>/</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Echinoderm microtubule-associated protein-like 2</td>
<td>Eml2</td>
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<td></td>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>ERC protein 2</td>
<td>Erc2</td>
<td></td>
<td></td>
<td>3.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Similar to Heat shock protein 1</td>
<td>Gm12141</td>
<td></td>
<td></td>
<td>6.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Heat shock 70kDa protein 12A</td>
<td>Hspa12a, Kisao417</td>
<td>16.6</td>
<td>2.0</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Potassium voltage-gated channel subfamily D member 2</td>
<td>Kcnd2</td>
<td>2.4</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Similar to hCG45299</td>
<td>LOC100045958</td>
<td>5.7</td>
<td>2.0</td>
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<tr>
<td>Serine/threonine-protein kinase Nek9</td>
<td>Nek9</td>
<td></td>
<td></td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>6-Phosphofructokinase type C</td>
<td>Pfkp</td>
<td>3.4</td>
<td></td>
<td>2.2</td>
<td></td>
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<tr>
<td>cAMP-dependent protein kinase type II-beta regulatory subunit</td>
<td>Prkar2b</td>
<td>14.4</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein quaking</td>
<td>Qk</td>
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<td></td>
<td>4.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Mitochondrial glutamate carrier 1</td>
<td>Sclc25a22</td>
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<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptosomal-associated protein 25; protein</td>
<td>Snap25</td>
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<td>2.8</td>
<td>2.0</td>
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</tr>
<tr>
<td>Sjogren syndrome/scleroderma autoantigen 1 homolog</td>
<td>Sscca1</td>
<td>2.0</td>
<td>1.6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Striatin</td>
<td>Strn</td>
<td>6.0</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synapsin-2; Synapsin II</td>
<td>Syn2</td>
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<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptopyrin-1</td>
<td>Syngr1</td>
<td></td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>TBC1 domain family member 5</td>
<td>Tbc1d5</td>
<td>16.8</td>
<td></td>
<td>4.8</td>
<td></td>
</tr>
<tr>
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<td>Tubb4a</td>
<td>1.3</td>
<td>3.3</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic dynein 1 light intermediate chain 2;</td>
<td>Dyncl1a2</td>
<td></td>
<td>2.0</td>
<td>4.0</td>
<td></td>
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<tr>
<td>Excitatory amino acid transporter 2</td>
<td>Sclc1a2</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin alpha-4A chain</td>
<td>Tuba4a</td>
<td>3.3</td>
<td>3.0</td>
<td>4.1</td>
<td></td>
</tr>
</tbody>
</table>

The experiments were performed on P2+microsomes fraction from P84 WT mice using DDM as detergent for protein extraction. Proteins listed in this table were present in at least 2 of the 3 IP experiments, and were enriched at least 20-fold in the IP samples compared to the controls. The proteins in bold were also identified in IPs performed on brain lysates of E18 Nbea mice. The "unused" value is a summation of protein scores from all non-redundant peptides matched to a single protein. Proteins with “unused” value <1.3 have low confidence and were excluded from analysis.
In line with this, a reverse IP using two different α-SAP102 antibodies on P2+M fractions from adult WT mice, identified Nbea (Supplementary material Figure 4.6). These data show that SAP102 and Nbea are part of the same complex in vivo in young (E18), as well as in adult (P84) mice.

**Limited co-precipitation and co-localization of SAP102 and Nbea**

![Diagram](image)

**Figure 4.2 Subcellular localization of Nbea and SAP102.** (A) Hippocampi from adult WT mice were used to obtain different subcellular fractions, which were analyzed by immunoblotting for the presence of Nbea (using a rabbit polyclonal antibody; SySy, 1:1000), SAP102 (using a mouse monoclonal antibody; NeuroMab clone N19/2, 1:1000) and PSD-95 (using a rabbit polyclonal antibody; Genescript, 1:1000). (B) Dendritic Nbea immunoreactivity shows limited overlap with SAP102. DIV14 hippocampal neurons (E18) fixed in methanol and stained for endogenous SAP102 (in green), endogenous Nbea (in red) and MAP2 (not shown in the merge). Top scale bar = 20 µm, lower scale bar = 5 µm.

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In comparison to the abundance of identified Nbea peptides (highest unused value 353.8), only a relatively low number of SAP102 peptides (highest unused value 13.6) were identified by mass spectrometry (Table 4.2 & Table 4.3). This indicates that only a fraction of SAP102 interacts with Nbea. Vice versa, as shown by the reverse IP (Supplementary material Figure 4.6), only a subset of Nbea was immunoprecipitated by SAP102. This notion was further supported by subcellular fractionation (Figure 4.2 A), which revealed that only small amount of both proteins is present in the same fractions, and that the two proteins in general localize to different compartments within a cell. Whereas the largest proportion of SAP102 was found in the postsynaptic density (PSD) fraction, Nbea was mostly enriched in P2, and P2+M fractions, but was also found in synaptosomes. Only a small proportion of Nbea was present in the PSD-enriched fraction.

In line with these findings, Nbea and SAP102 staining patterns show only very limited co-localization in DIV14 cultured hippocampal neurons (Figure 4.2B). Whereas SAP102 was not detectable in the soma, but was only present as puncta in dendrites, a large fraction of Nbea staining was found in the cell soma. Additionally, we detected a prominent punctate Nbea pattern throughout dendrites. This is in line with Nbea’s reported localization throughout the cell, especially near the trans-Golgi (del Pino et al., 2011; Wang et al., 2000). As established in Chapter 2, Nbea did not colocalize with postsynaptic markers (Chapter 2, Figure 2.3, Supplementary material Figure 2.8). Although, Nbea’s lack of immunoreactivity at postsynaptic sites might result from the inaccessibility of the epitope to the antibody, these data indicate that only a small proportion of SAP102 and Nbea engage in the same complex.

Nbea and SAP102 co-immunoprecipitate from heterologous cells

To further confirm the interaction of SAP102 and Nbea, we performed co-immunoprecipitation (co-IP) assays using heterologous (HEK293T) cells transfected with DNA constructs expressing full-length Nbea-YFP, FLAG-SAP102 or empty control vector. An α-GFP antibody, which also binds YFP, pulled down FLAG-SAP102 in lysates of cells expressing full-length Nbea, but not in empty vector control (Figure 4.3A). Although we also used the α-Nbea antibody successfully (see Supplementary material Figure 4.7C), we decided to use α-GFP instead. The rationale for this is that the Nbea fragments used in later experiments did not contain the epitope to which the Nbea antibody was raised. Thus, GFP-tagging allowed us to use the same antibody for co-IPs throughout our study (for additional control experiments see Supplementary material Figure 4.7). Similarly, in the reverse co-IP assay, α-FLAG pulled down full-length Nbea-YFP in lysates of cells expressing FLAG-SAP102, but not in the empty vector control (Figure 4.3B). These data suggest that the interaction between Nbea and SAP102 originates either from direct binding or alternatively, that the expressed full-length Nbea-YFP interacts with endogenous HEK cell proteins forming a complex with SAP102 and thereby interacting with SAP102 indirectly.
Figure 4.3 Nbea interacts with SAP102 in HEK293T cells. (A) Co-immunoprecipitation of Nbea and SAP102. HEK 293 cells were co-transfected with full length Nbea tagged with YFP and FLAG-tagged SAP102 or an empty vector and were immunoprecipitated (IP) with α-GFP antibody before immunoblotting (IB) with α-Nbea and α-FLAG antibody. In the control condition non-coated, empty beads (EB) were used for the IP. (B) Reverse IP to the ones in A. HEK 293 cells were co-transfected with full-length Nbea tagged with YFP and FLAG-tagged SAP102 or an empty vector, but this time they were immunoprecipitated (IP) with α-FLAG antibody before immunoblotting (IB) with α-Nbea and α-flag antibody. In the control condition non-coated, empty beads (EB) were used for the IP.

SAP102 binds to the C-terminal part of Nbea

To define the domains of Nbea involved in its interaction with SAP102, we transfected HEK293T cells with constructs expressing various deletions of Nbea (Figure 4.4) along with FLAG-SAP102 and conducted a co-IP assay with α-GFP antibody. In addition to full length Nbea, only the fragment containing all four C-terminal domains (DUF, PH, BEACH, WD40) immunoprecipitated SAP102 (Figure 4.4). As all constructs, apart from the full-length, lack the N-terminal part of Nbea, the latter does not seem to be a prerequisite for the interaction with SAP102. Immuno-blot analysis of the input, as well as confocal microscopy (Supplementary material Figure 4.8) of the transfected HEK cells demonstrated that all constructs were of the expected molecular mass and adequately expressed (Figure 4.4A, Supplementary material Figure 4.8). None of the Nbea fragments showed the same subcellular expression pattern as full-length Nbea. Whereas the latter displayed a punctate pat-
tern, the Nbea fragments exhibited an overall diffuse localization, similar to mCherry (Supplementary material Figure 4.8). These results imply that SAP102 binds to different domains in Nbea’s C terminus and that the PH and BEACH domains alone are not sufficient for this interaction. In addition, at least in HEK cells, the N-terminus is necessary for proper localization of full-length Nbea.

**A mutation within the PH domain of Nbea disrupts the binding to SAP102**

![Figure 4.4 SAP102 binds to the C-terminal part of Nbea.](image)

(A) HEK293 cells were co-transfected with flag-tagged SAP102 and either full-length Nbea tagged with YFP or various Nbea deletions encompassing different domains fused to GFP. In the control condition SAP-102 was co-transfected with YFP and GFP. IPs were performed using the α-GFP antibody, before immune-blotting with α-flag and α-GFP antibodies. (B) Quantification of flag and GFP protein levels using the immuno-labelled bands. Error bars indicate the standard error of the mean (SEM).
The sub-cellular expression patterns of the mutated Nbea proteins did not differ from the non-mutated protein, although a fraction of cells also showed a compartmentalized expression of the proteins (Supplementary material Figure 4.10), which might reflect targeting of these proteins, probably for degradation.

Figure 4.5 The E2218R mutation within the PH domain abolishes Nbea’s binding to SAP102. (A) HEK293 cells were co-transfected with flag-tagged SAP102 and either the non-mutated C-terminal part of Nbea (encompassing the Duf, PH, Beach and WD40 domains) fused to GFP or the mutated versions of this protein. The following mutations were used: E2090K in the DUF domain (1), E2218R in the PH domain (2), N2302A in the BEACH domain (3), V2773I in the WD40 domain (4), and additional three mutations within the BEACH domain V2346Q (5), E2447R (6) and P2499S (7) (B) Quantification of flag-tagged and GFP-tagged protein levels of the immuno-blot. Error bars indicate the standard error of the mean (SEM).
To further characterize the interaction between SAP102 and Nbea, we engineered mutations in the different domains of Nbea, some of which have previously been analyzed and shown to have functional consequences (Jogl et al., 2002; Karim et al., 2002; Rudelius et al., 2006). The Nbea mutants created included E2090K in the DUF domain (a missense mutation causing a milder form of Chediak-Higashi Syndrome in adults; Karim et al., 2002), E2218R in the PH domain and N2302A in the Beach domain (equivalent mutations in FAN: E256R and N328A, respectively, disrupt the interactions between the PH and Beach domain; Jogl et al., 2002), V2346Q, E2447R and P2499S in the Beach domain and V2773I in the WD40 repeats (corresponds to a missense mutation within the murine LYST gene that causes severe progressive Purkinje cell degeneration; Rudelius et al., 2006; Supplementary material Figure 4.9).

Introducing the E2218R mutation in the PH domain prevented the interaction with SAP102, whereas the other mutations did not show loss of binding (Figure 4.5). Thus, either this amino acid is at the interaction interface with SAP102, or more likely, the lost interaction of PH and BEACH domains by this mutation, causes the loss of interaction. This emphasizes the importance of the local orientation of PH and BEACH domains in Nbea with respect to interaction with SAP102.

Discussion

Nbea was initially identified and characterized as an essential player in synaptic transmission in the peripheral nervous system (Su et al., 2004), and further studies confirmed also its vital role in the formation and functioning of central synapses (Medrihan et al., 2009; Niesmaan et al., 2011). Considerable evidence has accumulated confirming Nbea’s importance for trafficking cargo to pre- and post-synaptic compartments (del Pino et al., 2011; Medrihan et al., 2009; Niesmaan et al., 2011; Su et al., 2004; Wang et al., 2000). Still, the precise role of Nbea in this has remained unclear. Using an immunoaffinity-based proteomics approach, we identified SAP102 as a novel, Nbea interacting protein in the brains of embryonic and adult mice. Experiments in heterologous cells demonstrated that Nbea bound to SAP102 via the C-terminal part of the protein and that introducing the E2218R mutation in the PH domain disrupted this binding. Because we confirmed this binding by multiple independent approaches, we concluded that Nbea and SAP102 interact.

The Nbea interacting SAP102 is a scaffold protein in excitatory synapses and it belongs to the PSD-95 (post-synaptic density protein of 95 kDa) family of membrane-associated guanylate kinases (MAGUKs). These include in addition to SAP102/Dlg3, also SAP-90 (also known as PSD-95 or Dlg4), PSD93 (also known as Chapsyn-110 or Dlg2) and SAP97 (also known as Dlg1; Zheng et al., 2011), which all share three PDZ (PSD-95/Discs large/Zona occludens 1) domains, a src-homology 3 (SH3) domain and a C-terminal guanylate kinase (GUK) domain (Gardoni et al., 2009). These large scaffolding proteins are important for clustering and anchoring receptors at the postsynaptic site (Kim et al., 1996; Zheng et al., 2011), and through this they can
significantly affect synaptic plasticity, i.e. keep or modulate the strength of synaptic transmission between neurons (Zheng et al., 2011).

SAP102 has been shown to associate with NR2A- and NR2B-subunit containing NMDA receptors (NMDARs) in synapses (Al-Hallaq et al., 2007; Cousins et al., 2008; Lau et al., 1996; Muller et al., 1996; Sans et al., 2000). In addition to its scaffolding function, SAP102 has also been implicated in transport and membrane insertion of NMDA receptors preceding synapse formation (Sans et al., 2005; Washbourne et al., 2004. In mice, its expression is highest before P10 and then gradually declines (Sans et al., 2000). NR2B receptors exhibit a similar expression pattern (Sans et al., 2000). They are trafficked by SAP102 during synaptogenesis (Elias et al., 2008), before the maturational switch from NR2B- to NR2A-type NMDARs occurs and during synapse maturation when NMDAR trafficking is taken over by PSD95 (Elias et al., 2008). Nbea, which also shows a high level of expression during synaptogenesis (Wang et al., 2000), and SAP102 might be involved in the same pathway of trafficking NMDA receptors.

Only small proportions of the total amount of SAP102 and Nbea engage in the same complex. Since very little Nbea is present in the PSD, where SAP102 is enriched in, it is likely that the interaction of the two proteins takes place predominantly somewhere else in the cell. It has been shown that the MAGUK SAP97 selectively associates with a subset of AMPA receptors early in their biosynthetic pathway (Sans et al., 2001). Given Nbea’s localization at the trans-Golgi, its enrichment in the P2+M fraction and SAP102’s role in NMDA receptor trafficking, one might speculate that a similar scenario is possible for SAP102 and Nbea, interacting in the early secretory pathway.

Different studies that used expression constructs to dissect the functional domains of BEACH proteins indicate that the various domains can be simultaneously involved in different cellular actions (see Adam-Klages et al., 1996; Kaplan et al., 2008; Wu et al., 2004), e.g. the PH-BEACH region in Alfy is involved in the direct interaction with the autophagy receptor p62 (Clausen et al., 2010), while the WD40 is essential for its colocalization and interaction with the autophagic marker Atg5 (Filimonenko et al., 2010).

We found that SAP102 interacts with the C-terminal part of Nbea that contains the DUF, PH, BEACH and WD40 domains. Like the BEACH domain of BGL (Wang et al., 2001), the BEACH domain of Nbea contains a predicted SH3 binding site. Hence, we expected SAP102 to bind to the BEACH domain and since the PH and BEACH domains might function as a single unit (Jogl et al., 2002), we expected the PH-BEACH fragment to be sufficient for the interaction with SAP102. However, no SAP102 binding was observed when using this construct. This suggests that the DUF and WD40 domains are also necessary for binding or for the correct conformation of the PH-BEACH domains.

We discovered that mutation of E2218R within the PH domain of Nbea compromised SAP102 interaction. This mutation is located within the β6 strand that forms the PH portion of the conserved interface between the PH and BEACH domains together with strands β1, β5 and β7. A similar mutation in FAN disrupts the inter-
action between these two domains (Jogl et al., 2002), indicating that preservation of this interaction is vital for the association of SAP102.

In addition to SAP102, other interactors of Nbea could also be part of the complex. We identified the RII regulatory subunit of the cAMP-dependent protein kinase (Table 4.3) in adult mice, confirming earlier findings (Wang et al., 2000). Activation of PKA induces synaptic targeting of NMDA receptors (Crump et al., 2001). Nbea might also indirectly regulate glutamate receptors via its association with SAP102-NR2B in analogy to the way the AKAP79/150 binds PSD95 and indirectly regulates NMDARs (Colledge et al., 2000).

In our mass spectrometry experiments using young, as well as adult mice, several proteins were identified multiple times, i.e. dipeptidyl aminopeptidase-like protein 6 (DPP6), Echinoderm microtubule-associated protein-like 2 (EML2) and Serine/threonine-protein kinase Nek 9 (Nek9; Table 4.2 and Table 4.3). None of these proteins have been linked to Nbea before and might form a part of the SAP102/Nbea complex. Further studies will be necessary to reveal their role in this context.

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References


SAP102 interacts with Nbea

and identified by two-dimensional gel electrophoresis and mass spectrometry. Proteomics, 4:1346–1358.


Supplementary Material

Figure 4.6 Nbea interacts with a fraction of SAP102 in vivo. Co-immunoprecipitation of SAP102 and Nbea from crude membrane with microsomes fraction (P2+M) of P84 WT mice. Proteins were immunoprecipitated (IP) with two different α-SAP102 antibodies, i.e. a mouse monoclonal (NeuroMab clone N19/2; left lane) and a rabbit polyclonal one (GenScript; right lane), respectively. In the control condition non-coated, empty beads (EB) were used for the IP. The Input lane represents the crude membrane with microsomes fraction that was used for immunoprecipitation. Immuno-blotting (IB) was performed with α-Nbea and α-SAP102 antibody (NeuroMab clone N19/2).
Figure 4.7 Control IPs confirming Nbeas interaction with SAP102 in HEK293 cells. (A) Co-IP of Nbea and SAP102. HEK293 cells were co-transfected with full length Nbea-YFP and FLAG-SAP102 or an empty vector and were immuno-precipitated (IP) with α-GFP antibody before immunoblotting (IB) with α-GFP and α-FLAG antibody (AB). In the control condition non-coated, empty beads (EB) were used for the IP. (B) Reverse IPs of IPs performed in A. (C) HEK293 cells were co-transfected with full length Nbea-YFP and FLAG-SAP102 or an empty vector and were immuno-precipitated with α-Nbea AB before immunoblotting (IB) with α-Nbea and α-FLAG AB. (D) Reverse IP of IPs performed in C.
Figure 4.8 Subcellular localization of Nbea deletion constructs in HEK293T cells. HEK293T cells co-transfected via calcium transfection with either full length Nbea-YFP or different GFP-fused Nbea deletions and mCherry. Scale bar = 5 µm.
Figure 4.9 The mutations introduced in the C-terminal amino acid sequence of Nbea. The shaded areas represent the amino acid sequence of the domain of unknown function 1088 (DUF; in orange), the Pleckstrin-Homology like domain (PH; in gray), the BEACH domain (yellow) and the WD40 repeats (red). The red squares depict the amino acids that have been mutated in our study. The numbers on top of the squares are used for identification of the mutations (see also Figure 4.5 and Supplementary material Figure 4.10).
Figure 4.10 Subcellular localization of Nbea mutation constructs in HEK293 cells. (A) HEK293 cells co-transfected via calcium transfection with either the non-mutated form of the C-terminal part of Nbea (encompassing the Duf, PH, Beach and WD40 domains) fused to GFP or the mutated versions of this construct and mCherry (not shown). Scale bar = 5 µm. (B) Quantification of the proportion of cells exhibiting a compartmentalized pattern. Error bars indicate the standard error of the mean (SEM).