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CHAPTER 2

Subcellular localization and cytochemical characterization of Neurobeachin

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Abstract

Using a novel α -Nbea antibody we describe the characterization of Neurobeachin, a brain enriched putative A-kinase anchor protein that is a member of the BEACH family of proteins. Nbea localizes throughout the somatic cytoplasm and dendrites. Its somatic immunoreactivity concentrates near the ERGIC and Golgi complex and shows highest colocalization with the SNARE protein Vti1A. Nbea is also found in the dendrites, where it shows partial overlap with recycling endosomal markers (Rab 11 and TfR), but no substantial overlap with pre- or postsynaptic markers. Nbea is expressed early in cultured neurons and is sensitive to BFA treatment. Unlike the prototype member of the BEACH domain proteins, i.e. LYST, Nbea does not seem to be of vital importance for the overall subcellular architecture, since Nbea null mutant hippocampal neurons do not show any gross perturbations in the ER, *cis*-Golgi, recycling endosomes or lysosomes. Nbea's localization and its BFA sensitivity suggest an involvement in neuronal post-Golgi membrane traffic, where it might function in recruiting protein kinase A to discrete intracellular locations.

Introduction

The knowledge of where a protein is located, often plays a significant role in characterizing the function of newly discovered or little-studied proteins. Therefore, in order to gain more insight into Nbea's role in the CNS, we set out to investigate Nbea's subcellular localization in dissociated hippocampal neurons. Although a few earlier studies reported on Nbea's localization in dissociated neuronal cultures (del Pino et al., 2011; Niesmann et al., 2011, none of them thoroughly investigated its association with organelles of the secretory and endocytic pathway.

In the past, on the subcellular level, using light microscopy, Neurobeachin was found to be located in the cytoplasm of neurons and in their proximal dendrites, but not in nuclei of rat brain sections (Wang et al., 2000). When investigating Nbea's subcellular localization at higher resolution by using electron microscopy of rat cerebellar neurons, the neurobeachin immunoreaction product was found to decorate tubulovesicular endomembranes near the *trans* sides of Golgi complexes and throughout neuronal cell bodies (Wang et al., 2000). Only in 1% of synapses in the molecular layer and 5% of synapses in the granule cell layer decoration of postsynaptic plasma membranes was observed, while presynaptic localization was very rare (Wang et al., 2000). Nbea was also found at subpopulations of inhibitory synapses of dissociated spinal cord neurons at 10-13 days *in vitro* (DIV; del Pino et al., 2011).

Nbea's localization immediately adjacent to the TGN structures was also observed by immunofluorescence microscopy in dissociated hippocampal neurons cultured for 14 (del Pino et al., 2011) or 21 DIV (Niesmann et al., 2011) and PC12 rat neuroendocrine cells (Wang et al., 2000). Here immunofluorescence visualized small puncta, which were densest around the nucleus, where they formed clusters, but were also scattered throughout the cytoplasm (Wang et al., 2000). Although various marker antibodies were used in order to explore which membrane compartments Nbea might be associated with, none of the markers tested displayed significant colocalization with Nbea, particularly not in the cell periphery of PC12 cells (Wang et al., 2000).

Subcellular fractionation of mouse brain homogenate revealed that neurobeachin is primarily a cytosolic protein that peripherally associates with the membranes that it decorates in immunomorphology, but a subpool is more firmly bound to a cytoskeletal-like subcellular fraction (Wang et al., 2000).

In some cases Nbea's localization is reminiscent of other BEACH proteins. For instance SEL2, like Nbea was also found to be distributed throughout the cytoplasm, but particularly concentrated close to the nucleus (de Souza et al., 2007). Although Rugose is expressed in a variety of tissues, it is especially enriched in the neuronal cells such as photoreceptor neurons of the developing eye and in the larval brain. Like Nbea it is expressed in early embryonal stages (Han et al., 1997). The subcellular localization of endogenous LRBA, a paralogue of Nbea, has not yet been determined. However, a BEACH-WD40 GFP fusion construct of this protein showed a cytosolic staining, with rare cells showing a vesicular staining pattern, that was shifted to lysosomes, the TGN, ER and endocytic vacuoles upon LPS stimulation (Wang et al., 2001).

Materials and Methods

Laboratory animals

Nbea KO mice were generated as described previously (Su et al., 2004). Mouse embryos were obtained at embryonic day 18 (E18) by caesarian section of pregnant females from timed mating of Nbea heterozygous animals (C57/Bl6 background). Heterozygous mice display no obvious phenotypic changes in their viability or fertility. However, Nbea-deficient mice can be easily distinguished from wild-type or heterozygous littermates - they exhibit hunched posture and an omphalocele, i.e. an abdominal defect where the intestine is not withdrawn into the abdominal cavity during embryonic development. In addition, they don't show any response to tactile stimuli and die perinatally (Medrihan et al., 2009; Su et al., 2004), most likely due to defective neuromuscular synaptic transmission and concomitant breathing failure (Su et al., 2004). Newborn P₀-P₁ pups from pregnant female Wistar rat (Harlan or Charles River) were used for rat neuronal cultures and glia preparations. All animals were housed and bred according to the institutional and Dutch governmental guidelines for animal welfare.

Nbea antibody production

For the Nbea antibody production a fragment containing the Nbea AA 953-1318 was amplified from the Y2HcDNA library using rz60 5'TGAGGAGTACCAGCGACAA-GAGGAG3' and rz59r 5'CCGAAACATGGTGGTCC3' and subsequently subcloned into the His-tag vector pQE31 (Qiagen, Hilden, Germany) using SphI and PstI. In order to create the correct reading frame the construct was digested with SphI, overhangs were modified into blunt ends and selfligated. His-tag fusion protein was expressed in bacteria, purified on nickel agarose, and used for immunization of rabbits. Serum was affinity-purified using the same fusion protein coupled to CNBr-activated Sepharose (GE-Healthcare) according to the manufacturers instructions.

Plasmids and antibodies

pmCherry was purchased from Clontech (CAT#PT3973-5). The ERGIC53-GFP marker was a generous gift from Bertrand Kleizen, while the Rab5-GFP and Rab11-GFP were a kind gift from Birte Soennichsen. The venus-NPY construct was a cordial gift from Atsushi Miyawaki.

Specific primary antibodies were used against ankyrinB (mouse monoclonal 2.20, Santa Cruz, 1:250), EEA1 (mouse monoclonal 14, BD Transduction Laboratories, 1:100), GM130 (mouse monoclonal 35, BD Transduction Laboratories, 1:400), KDEL (mouse monoclonal 10C3, Stressgen, 1:100), lamp1 (mouse monoclonal LY1C6, Stressgen, 1:100), lamp1 (rat monoclonal 1D4B, Abcam, 1:100), MAP2 (chicken polyclonal, Abcam, 1: 20000), MAP2 (mouse monoclonal AP20, Chemicon, 1:1000), mitochondrial Hsp70 (mouse monoclonal MA3-028, Affinity Bioreagents, 1:1000), Nbea (rabbit polyclonal, Synaptic Systems, 1:1000), PSD95 (mouse monoclonal 6G6-1C9, Abcam,

1:200), TGN38 (mouse monoclonal, kind gift from Jan van Minnen, 1:100), transferin receptor (mouse monoclonal H68,4, Zymed, 1:500), VAMP2 (mouse monoclonal 69.1, Synaptic Systems, 1:1000) and Vti1A (mouse monoclonal 45, Transduction Laboratories, 1:200).

We obtained suitable secondary antibodies from Molecular Probes (1:1000).

Dissociated neuronal culture and transfection

Hippocampi and cortices from embryonic day 18 (E18) mouse embryos were dissected free of meninges and separately 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 25.000 (25k) cells/well on 18 mm glass coverslips and allowed to grow for 14-15 days before fixation.

For the characterization of the α -Nbea antibody, neurons were plated on class coverslips coated with a mixture of 30 μ g/ml poly-L-lysine (Sigma) and 2 μ g/ml laminin (Sigma) in Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco).

For all other immunocytochemical experiments hippocampal neurons were plated on 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. All experiments were performed on mice neurons, except for colocalization experiments with KDEL, EEA1 and Lamp1 (due to lack of reactivity), which was performed on rat neurons. If necessary, neurons (colocalization experiments including ERGIC53-, Rab5- and Rab11-GFP) were transfected at day-in-vitro (DIV) 10 using the calcium-phosphate precipitation method as described previously (Köhrmann et al., 1999). For large dense core vesicle labeling, neurons were transfected via a 10 h incubation with NPY-venus semliki forest virus.

Immunofluorescence and BFA treatment

Neurons were fixed in 4% paraformaldehyde (Sigma) or in 3.7% formaldehyde (Electron Microscopy Sciences) in Dulbecco's phosphate buffered saline (D-PBS; Gibco) for 20 min before being rinsed with D-PBS. Subsequently, they were permeabilized for 5 min in D-PBS containing 0.5% Triton X-100, followed by a 30 min incubation in PBS containing 0.1% Triton X-100 and 2% normal goat serum to block aspecific binding. The same solution was used for diluting antibodies. Neurons were incubated for 2 h in primary antibodies at room temperature (RT), washed 3 times with D-PBS and incubated in secondary antibodies for 1 h. After additional 3 washes they were mounted with ProLong[®]Gold (Invitrogen) on microscopic slides and imaged with a 63x Plan-Neofluar lens (Numerical aperture 1.4, Carl Zeiss b.v. Weesp) on a Zeiss 510 Meta confocal microscope (Carl Zeiss). This protocol was followed for all the

standard immunocytochemistry except for PSD95 when cells were fixed by a 4 min 100% methanol (Interchemia) incubation at -20°C and for EEA1 that required a 30 min 0.1% saponin permeabilization.

For treatment of neurons with Brefeldin A (BFA), BFA (final concentration of 50 μ M) was added to the culture medium and the cells were further incubated at 37°C and 5%CO₂ for either 3, 5, 10, 30 or 60 min, before the medium was replaced with fresh, pre-warmed culture-medium and the neurons were allowed to recover for 30 or 90 min, before fixation.

Electron microscopy

Hippocampal and cortical high-density cultures of wild type and Nbea null mutant mice (E18) grown on glass coverslips were fixed (DIV14) for 45 min at RT with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) 1,2. After fixation cells were washed three times for 5 min with 0.1M cacodylate buffer (pH 7.4), post-fixed for 2 h at RT with 1% OsO₄/1% K₂Ru(CN)₆ in bidest, washed and stained with 1% uranyl acetate for 40 min in the dark. Following dehydration through a series of increasing ethanol concentrations, cells were embedded in Epon and polymerized for 24 h at 60°C. After polymerization of the Epon, the coverslip was removed by alternatively dipping it in liquid nitrogen and hot water. Cells of interest were selected by observing the flat Epon embedded cell monolayer under the light microscope, and mounted on the pre-polymerized Epon blocks for thin sectioning. Ultrathin (~ 90 nm) were cut parallel to the cell monolayer and collected on single-slot, formvar-coated copper grids, and stained in uranyl acetate and lead citrate. We used the JEOL 1010 electron microscope to take electron micrographs of the Golgi apparatus for a qualitative analysis in each condition.

Data analysis and statistics

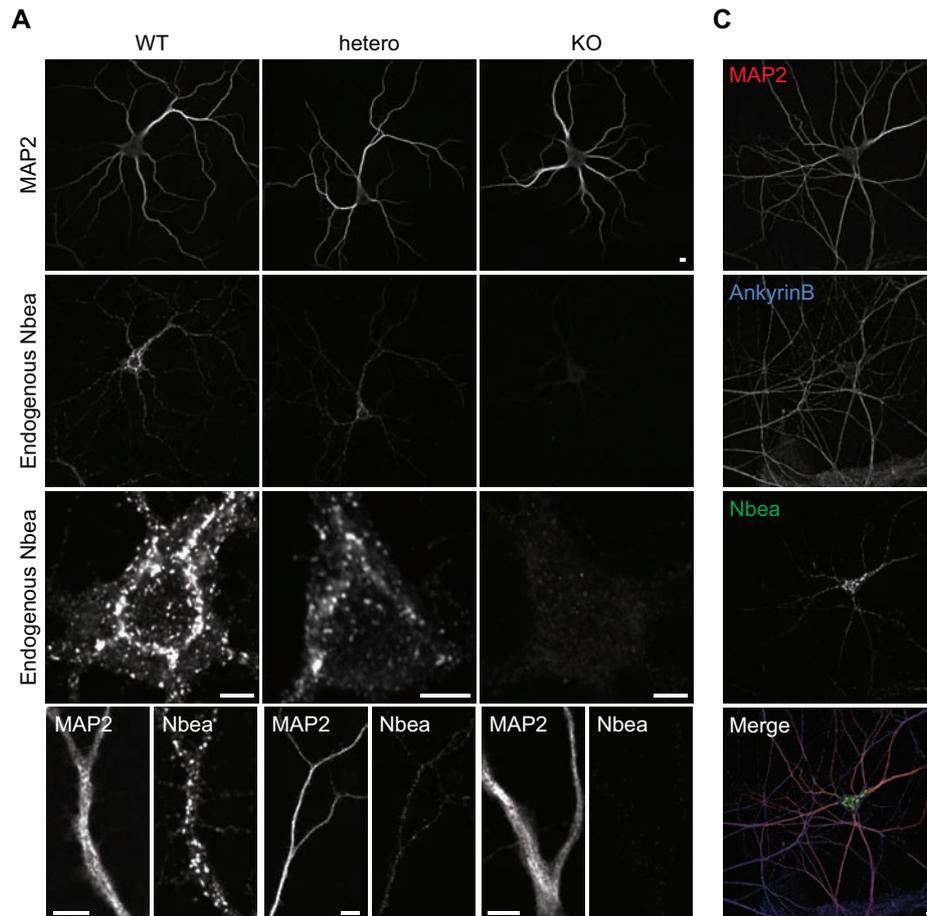
Nbea's developmental expression pattern was assessed by making maximum projections, setting z plane limits for acquisition (0.29 - 0.37 μ m per slice, 15-20 slices) on the basis of the fluorescence signal for Nbea.

For colocalization analysis only confocal images of somas and dendrites with a resolution of 1024 by 1024 pixels were used. Each image was analyzed using ImageJ program (Version 1.44I, National Institutes of Health, USA). First the background was subtracted using the *Subtract Background* function and then the *Intensity Correlation Analysis* plugin was used to obtain the Pearson's correlation coefficient and Manders Overlap coefficient (Manders et al., 1993).

For the analysis of the number and size of lysosomes, single plane images of neurons (1024 x 1024) were analyzed using SynD software (Schmitz et al., 2011). MATLAB R2007a (The MathWorks Inc, Natick, Massachusetts) was used to test our datasets for normality with Lilliefors goodness-of-fit and heterogeneity of variance with Bartlett's test for equality of variance. If data allowed, a two-tailed unpaired Student's t test was performed. Alternatively, the Wilcoxon-Mann-Whitney test was used.

Results

Neurobeachin immunoreactivity shows a punctate pattern throughout the soma and dendrites



B

hetero WT KO

Nbea

250 kD

VCP

Figure 2.1 Characterization of a novel α -Nbea antibody. (A) Immunostaining of Nbea and MAP2 in DIV14 Nbea WT, heterozygous and KO hippocampal neurons plated on poly-D-lysine/laminine. Scale bar = 5 μ m. (B) Characterization of α -Nbea antibody by immuno-blotting. Valosine-containing protein (VCP) served as a loading control. (C) Immunostaining of DIV14 hippocampal neurons for Nbea (in green), AnkyrinB (in blue) and MAP2 (in red). Scale bar = 5 μ m.

Using a novel, Nbea-specific antibody (Figure 2.1A and B) we confirmed previous findings (del Pino et al., 2011; Wang et al., 2000) that Nbea immunoreactivity accumulates predominantly in neuronal cell bodies as small perinuclear puncta, which form clusters, but also scatter throughout the cytoplasm (Figure 2.1A). Immunoreactivity also accumulates within intensely stained punctate structures along neurites (Figure 2.1A and C).

In order to distinguish between dendritic and axonal processes we employed the markers MAP2 and ankyrinB, which revealed that these discrete clusters are found in dendrites, but are excluded from the axons (Figure 2.1C). It should be noted that these dendritic clusters are present not only in proximal, but also in distal dendrites and that they do not exhibit a preferential accumulation at dendritic branching points, but instead, seem to be evenly distributed.

Neurobeachin concentrates near the ERGIC and Golgi complex

In order to explore which subcellular compartments Nbea might be associated with, we used double-immunofluorescence microscopy of WT hippocampal neurons employing various marker antibodies. While somatic Nbea showed a clearly different pattern from the one produced by the ER marker KDEL (Figure 2.2A), it was found in close apposition to the ERGIC, as well as to the *cis*- and *trans*-Golgi markers GM130 and TGN38, respectively. This is in line with earlier findings (del Pino et al., 2011; Wang et al., 2000), which reported enrichment of Nbea at the *trans* side of the Golgi. Interestingly, Nbea showed a qualitatively similar pattern to, and prominent overlap with, Vti1A, a SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) protein that has been linked to an alternative ER to Golgi traffic route (Flowerdew and Burgoyne, 2009).

For a better estimate of Nbea's overlap with the different markers, we used Pearson's correlation coefficient (PCC) and Manders' overlap coefficient (MOC) for colocalization quantification. We decided to use both coefficients, because they have certain advantages but also drawbacks. Although Pearson's coefficient ranges from -1 to 1 and a value close to 1 does indicate reliable colocalization, negative PCC values for fluorescent images can be difficult to interpret. Manders' coefficient on the other hand, ranges from 0 (low colocalization) to 1 (high-colocalization) and is easier than the PCC to comprehend. However, the overlap coefficient is strongly influenced by the ratio of red to green pixels and should therefore be used only if the number of objects in both channels of the image is roughly equal (Manders et al., 1993). As seen in Figure 2.2 B and C, both coefficients show the same trend - highest overlap with Vti1A, followed by the overlap with TGN38 and ERGIC-53.

Nbea's dendritic puncta showed partial overlap with the recycling endosomal markers Rab11 and transferrin receptor (TfR), and low overlap with pre- and postsynaptic markers (VAMP2 and PSD95, respectively), markers for mitochondria (Hsp70), lysosomes (Lamp1), early endosomes (Rab5 and EEA1) and large dense core vesicles (NPY; Figure 2.3 and Supplementary material Figure 2.8 - 2.10).

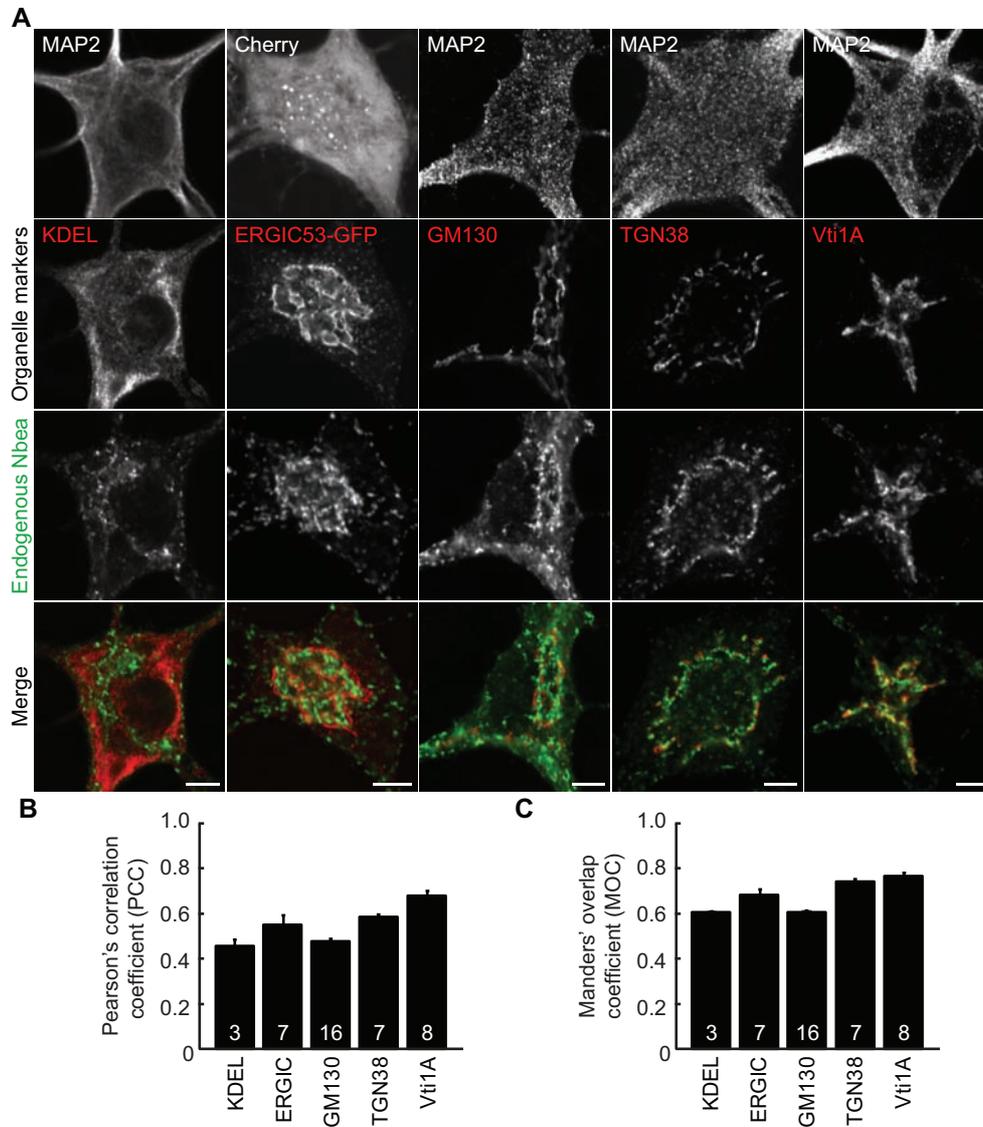


Figure 2.2 Somatic Nbea localization.(A) From left to right: DIV14 WT hippocampal neurons (E18) co-transfected via calcium transfection at DIV10-11 with ERGIC53, an early secretory pathway marker for ER (in red) and Cherry (not shown in the merge). After PFA fixation cells were stained for endogenous Nbea levels with a rabbit polyclonal antibody (in green). DIV14 hippocampal neurons stained for the ER marker KDEL, *cis*-Golgi marker GM130, *trans*-Golgi marker TGN38 or Vti1A (in red), Nbea (in green) and MAP2 (not shown in the merge). Scale bar = 5 μ m. (B) Quantification of colocalization using Pearson's correlation coefficient (PCC). (C) Quantification of colocalization using Manders' overlap coefficient (MOC). Error bars indicate SEM.

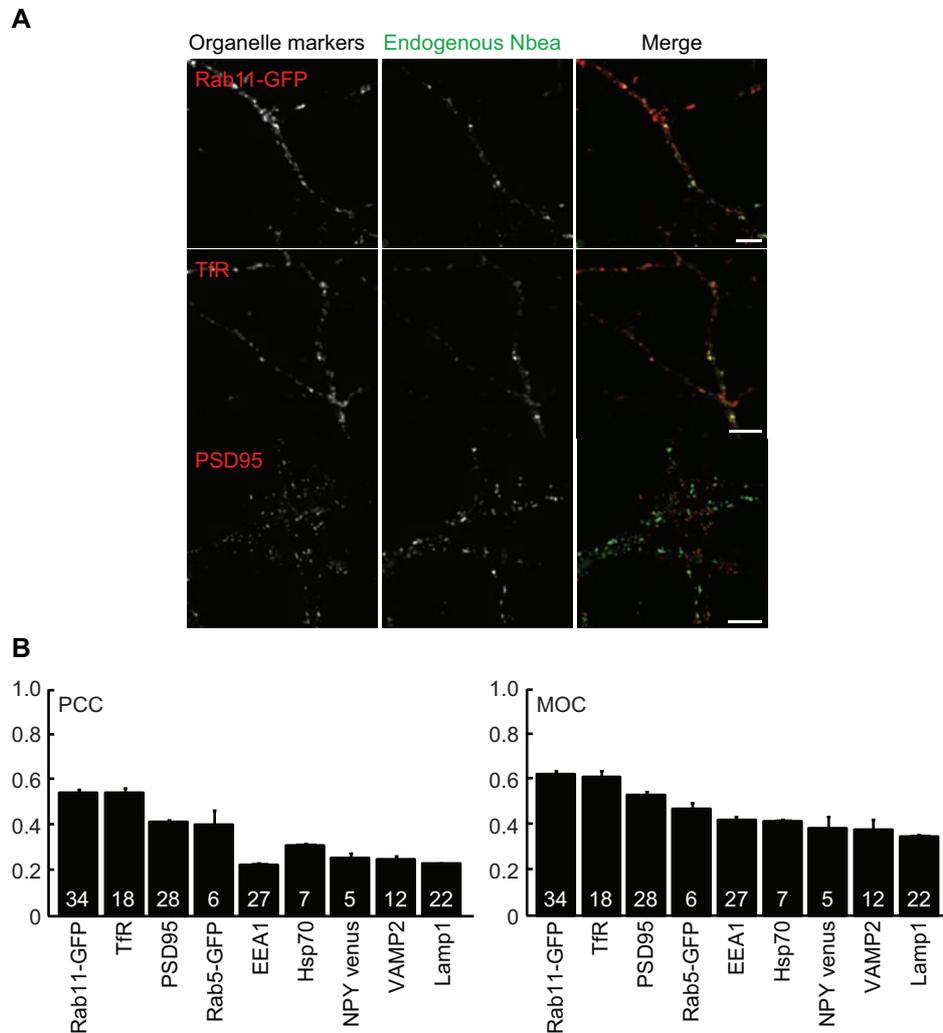


Figure 2.3 Dendritic Nbea localization.(A) Top: DIV15 WT hippocampal (E18) neurons transfected via calcium transfection at DIV10 with Rab11-GFP (in red) and Cherry (not shown in the merge) and after PFA fixation stained for Nbea (in green). Middle: DIV15 hippocampal neurons stained for transferrin receptor (in red) and endogenous Nbea (in green). Bottom: DIV17 hippocampal neurons fixed in methanol and stained for PSD95 (in red) and endogenous Nbea (in green). Scale bar = 5 μ m. (B) Quantification of colocalization using Pearson's correlation- and Manders' overlap coefficient (PCC and MOC, respectively). Error bars indicate SEM.

For further details see Supplementary material.

Neurobeachin is expressed early in cultured neurons

Nbea expression was already observed at DIV3, where it was mainly localized in the soma. However, already by DIV5, before the appearance of functional synapses, Nbea immunoreactivity could also be detected in dendrites and increased progressively during development *in vitro* (Figure 2.4). Such an early expression pattern is expected, given the fact that Nbea mRNA abundance is highest in neonatal brain and declines to reach adult levels (~50% of neonatal) at postnatal day 25 (Wang et al., 2000).

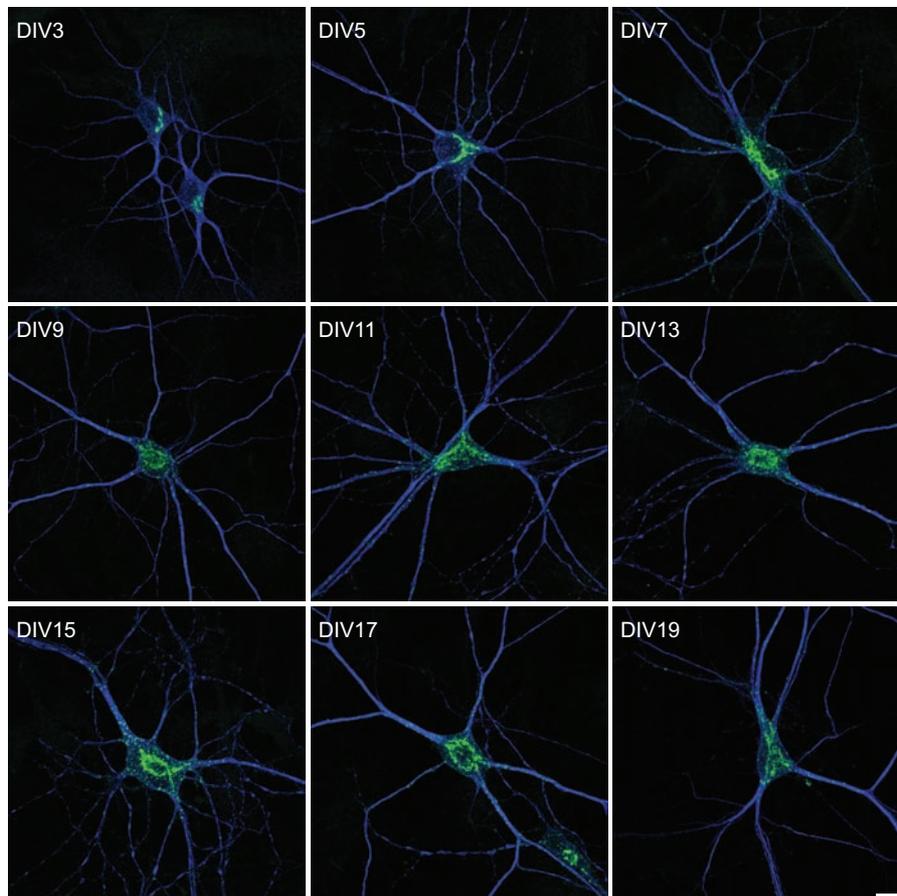


Figure 2.4 Nbea's developmental expression pattern *in vitro*. Z-stack maximum projections of WT hippocampal neurons fixed at different time points (DIV3-19). After PFA fixation cells were stained for endogenous Nbea (in green) and MAP2 (in blue). Scale bar = 20 μm .

Neurobeachin is BFA sensitive

Addition of the fungal metabolite brefeldin A (BFA), a standard tool for studying protein trafficking, to WT hippocampal neurons, causes not only the typical disassembly of the Golgi apparatus, but also dramatically alters the distribution of endogenous Nbea (Figure 2.5).

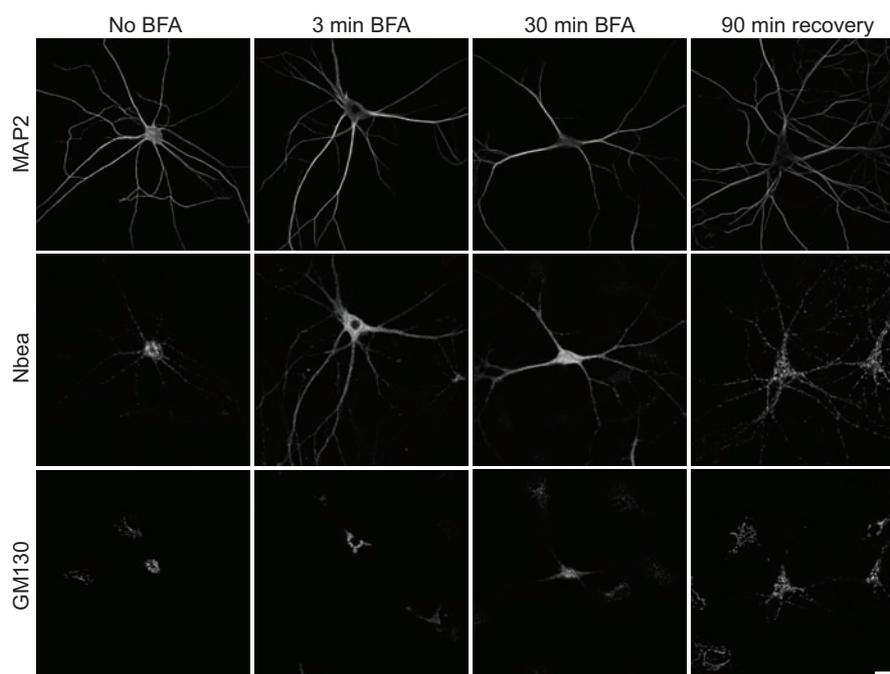


Figure 2.5 Effect of BFA on Nbea's punctate immunofluorescence pattern in hippocampal neurons. BFA rapidly disperses punctate Nbea in the soma and dendrites, while GM130 shows a much slower time course of dispersion by BFA. Scale bar = 20 μm .

Already within 3 min (the earliest fixation time-point) Nbea's circumnuclear and dendritic punctate immunofluorescence dispersed (Figure 2.5). This effect on Nbea was much faster than the effect on the Golgi, which was after 3 min still largely intact. Golgi disintegrated within 30 min of BFA treatment, as visualized by GM130 immunofluorescence. The punctate nature of Nbea's immunofluorescence was fully reversible within 90 min after BFA was washed out. However, in this period the Golgi apparatus did not yet fully recover, showing a fragmented appearance with the existence of Golgi outposts in proximal dendrites (Figure 2.5). This is in line with previous findings in live PC12 cells, where Nbea also dispersed extremely rapidly, as fast as the coat protein β -COP, while full Golgi disintegration was also observed only after 30 min (Wang et al., 2000).

The subcellular architecture of Nbea null mutant hippocampal neurons is intact

Since the immediately evident hallmark of cells lacking the BEACH protein LYST is the presence of giant lysosomes with a clustered perinuclear distribution, and FAN deficiency also affects lysosomal size (Kaplan et al., 2008; Möhlig et al., 2007), we next investigated whether Nbea played a role in the regulation of the lysosomal compartment. Quantification of Lamp1 positive puncta revealed no difference in the size nor number of Lamp1 puncta per μm dendrite (Figure 2.6).

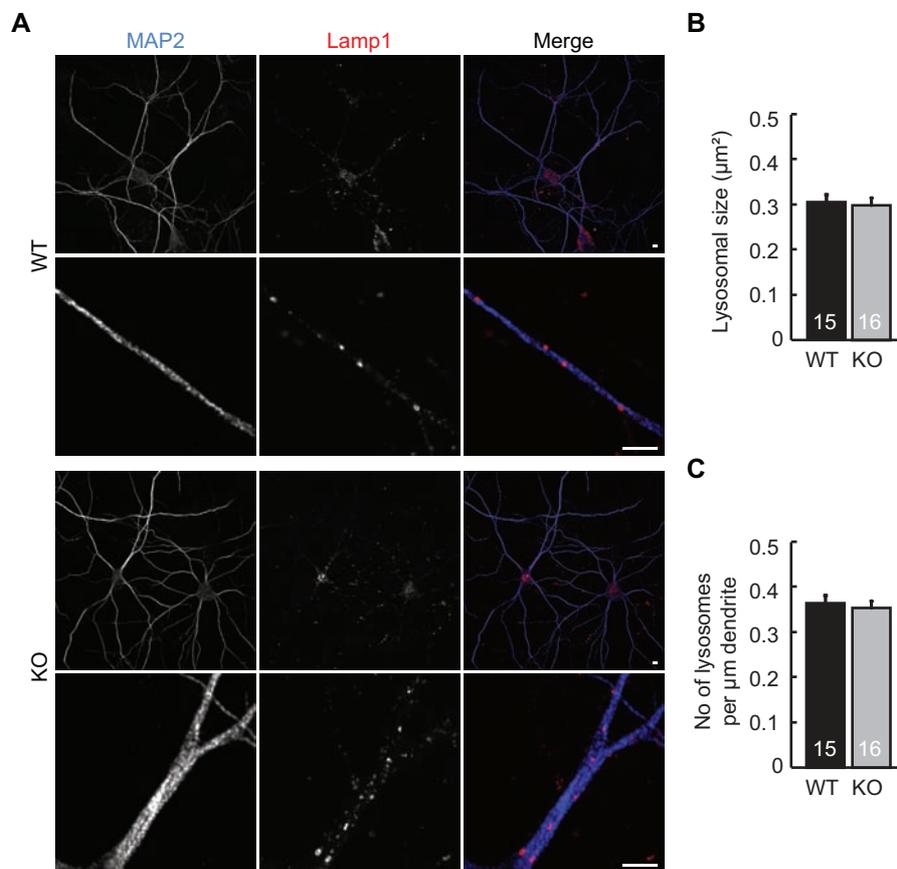


Figure 2.6 Neurobeachin does not regulate lysosome size. (A) DIV14 WT and KO neurons stained for Lamp1 using a rat monoclonal antibody (in red), and MAP2 (in blue). Scale bar = $5 \mu\text{m}$. Quantification of the number of lysosomes (B) and their average size (C) in Nbea WT and KO neurons. Error bars indicate SEM.

Similarly, the immunostainings of KDEL, GM130 and TfR in Nbea KO neurons did not show defects in the morphology of the ER, Golgi apparatus and recycling en-

dosomes (Figure 2.7A). Also when assessing the Golgi morphology of cultured Nbea WT and KO neurons in greater detail by electron microscopy, no major visible differences between the two genotypes could be observed (Figure 2.7B).

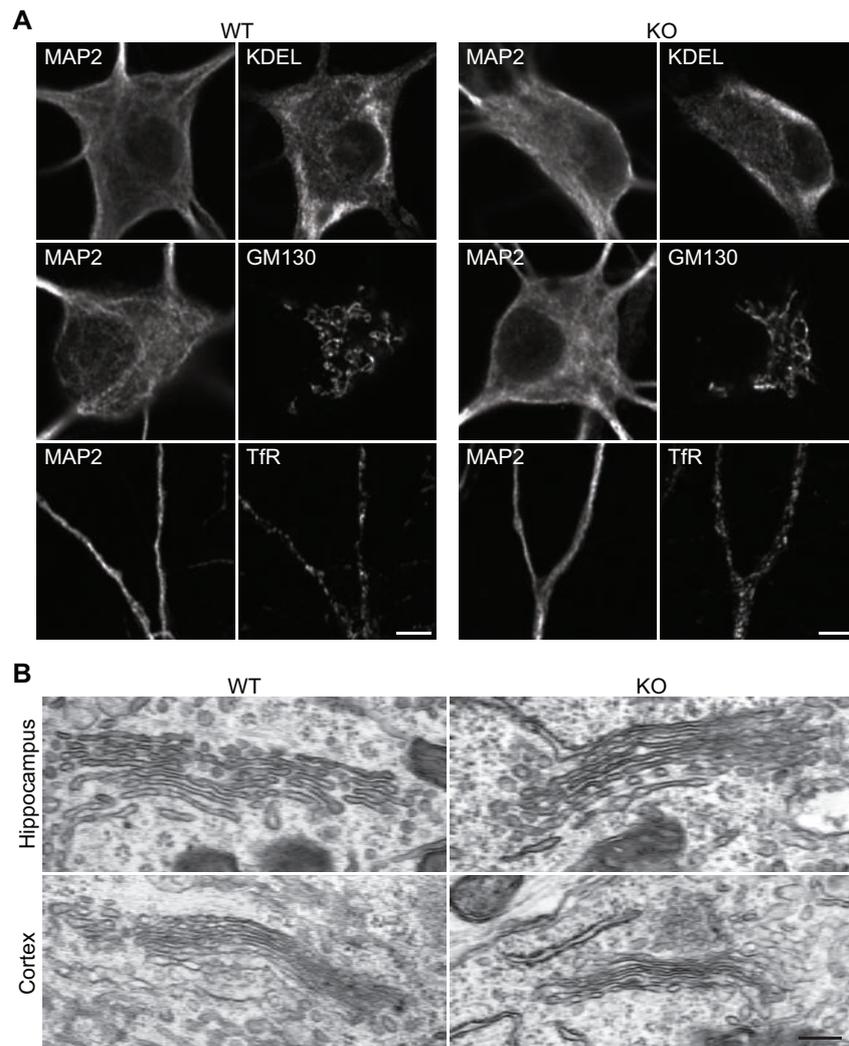


Figure 2.7 Different markers in Nbea WT and KO neurons. (A) Immunostainings of DIV14 WT and KO hippocampal neurons for the ER marker KDEL, the *cis*-Golgi marker GM130 or human transferrin receptor and MAP2. Scale bar = 5 μm . (B) Electron micrographs of the Golgi apparatus of the indicated genotypes in hippocampal and cortical neuronal cultures (DIV14-15). Scale bar = 200 nm.

Discussion

Using a novel α -Nbea antibody we showed that Nbea localizes throughout the somatic cytoplasm and dendrites. Nbea's somatic immunoreactivity concentrates near the ERGIC and Golgi complex and shows highest colocalization with the SNARE protein Vti1A (the implications of the latter colocalization are thoroughly discussed in Chapter 5). Dendritic Nbea shows partial overlap with recycling endosomal markers (Rab 11 and TfR), but no substantial overlap with pre- or postsynaptic markers. Nbea is expressed early in cultured neurons and is sensitive to BFA treatment. It does not seem to be of vital importance for the overall subcellular architecture, since Nbea null mutant hippocampal neurons do not show any gross perturbations in the ER, *cis*-Golgi, recycling endosomes or lysosomes.

Nbea's close apposition to the ERGIC and Golgi complex, together with its sensitivity to BFA, indicates that it might play a role in the early secretory pathway. BFA blocks protein transport into the Golgi apparatus, which breaks down into numerous tubule processes that move along microtubules carrying membrane into ER, thereby rapidly redistributing most of the Golgi into the ER (Lippincott-Schwartz et al., 1991). These effects, which occur at relatively low concentrations of BFA, are completely reversible (Lippincott-Schwartz et al., 1991). The current model of BFA action proposes that Golgi disassembly and the appearance of tubular processes is related to the altered interaction of structural elements of this organelle (Lippincott-Schwartz et al., 1991). Within 30 s of adding BFA to cells, β -COP, part of the coat structures involved in anterograde movement into the Golgi apparatus, dissociates from the Golgi membranes, and it has been therefore suggested that cytosolic coat proteins control the structure and traffic patterns of the Golgi (Lippincott-Schwartz et al., 1991). BFA also inhibits the association of the GTP-binding protein ARF (ADP-ribosylation factor) with Golgi membranes (Klausner et al., 1992), which is required for β -COP binding to Golgi membranes (Palmer et al., 1993). Since upon BFA treatment, Nbea immunoreactivity in PC12 cells disperses as fast as the coat protein β -COP, recruitment from cytosol to Golgi-near-membranes is stimulated by the presence of the stable GTP analogue GTP γ S, and a rapid dispersion is also observed in hippocampal neurons (Figure 2.5), it is likely that neuronal Nbea associates with Golgi membranes in a coat protein-like fashion (Wang et al., 2000).

The lack of colocalization of Nbea's dendritic puncta with pre- or postsynaptic markers (Figure 2.3, Supplementary material Figure 2.8 and Figure 2.10B) is somewhat surprising, given the fact that Nbea was initially discovered in an attempt to isolate novel synaptic proteins (Wang et al., 2000). However, it has to be taken into account that Nbea's immunoreactivity has previously been observed near postsynaptic plasma membranes only in a subset of synapses (Wang et al., 2000), some of which were identified as GABAergic synapses between Golgi and granule cells in the cerebellum, and inhibitory synapses of spinal cord neurons (del Pino et al., 2011; Wang et al., 2000). In contrast, we used hippocampal cultures that contain mostly excitatory synapses. In addition, also earlier studies very rarely detected Nbea presynaptically (Wang et al., 2000). The minor overlap with the recycling endosomal markers (Rab11

and transferrin receptor) links Nbea to the endocytic pathway. This differs from the findings in PC12 cells, where the antibody against transferrin receptor (TfR) produced a clearly different pattern from Nbea with no notable overlap (Wang et al., 2000). If Nbea is involved in protein trafficking it would be of great interest to determine whether these dendritic puncta are mobile or stationary. Unfortunately, the full-length Nbea fused to GFP accumulates in the soma and does not get transported to the dendrites, making live-cell imaging experiments of this kind rather impossible.

Though the somatic Golgi structure in neurons is similar to that of non-neuronal cell types, neurons possess a more elaborate, dispersed network of Golgi elements for protein processing in the dendrites, termed the Golgi outposts (Condon and Ehlers, 2007). Nbea's close alignment with the *trans*-Golgi in the soma makes it reasonable to speculate whether the dendritic Nbea puncta could be Golgi outposts. However, several lines of evidence suggest that Nbea puncta are not Golgi outposts. The most important evidence is the fact that Golgi outposts are readily detectable in only a subset of neuronal dendrites and are most prevalent in primary dendrites (Horton and Ehlers, 2003), while Nbea puncta can always be found in dendrites of hippocampal neurons - in proximal as well as in distal ones. Secondly, presumptive Golgi membranes in dendrites have been immunogold-labelled for the Golgi protein TGN38 (Gardiol et al., 1999), while our ample Nbea dendritic puncta were negative for TGN38 immunoreactivity (data not shown).

Nbea being a PKA anchoring protein and its localization near the TGN, suggests a possible function in the generation of transport vesicles. In fact, it has been shown earlier that PKA activity is required for the budding of constitutive transport vesicles from the TGN (Muniz et al., 1997) and that particularly transport from the TGN to the cell surface was dependent on PKA activity (Muniz et al., 1996). Moreover, PKA has been shown to exert a key regulatory role in the recruitment of ARF1 onto Golgi membranes, as following an increase in the intracellular level of cAMP, ARF proteins redistribute from cytosol to the perinuclear Golgi region of intact cells (Martin et al., 2000).

Since Nbea mRNA expression occurs early (by embryonic day E10.5) and its presence can be observed in cultured neurons already at DIV3 (Figure 2.4) it is likely that Nbea plays an important role during brain development.

Since Nbea null mutant hippocampal neurons do not show any gross perturbations in the ER, *cis*-Golgi, recycling endosomes or lysosomes, Nbea, unlike other BEACH domain proteins, such as LYST, does not control the overall subcellular architecture or integrity of neurons.

Taken together, the results listed above indicate that Nbea might be involved in post-Golgi sorting or targeting of neuronal membrane proteins and vesicle trafficking.

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Supplementary Material

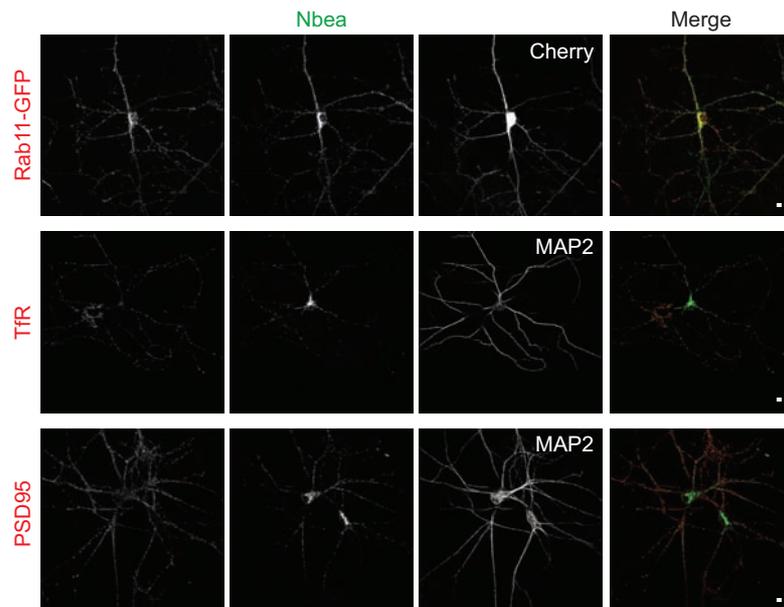


Figure 2.8 Images of full neurons presented partially in **Figure 2.3**. Top: DIV15 WT hippocampal (E18) neurons transfected via calcium transfection at DIV10 with Rab11-GFP (in red) and mCherry (not shown in the merge) and after PFA fixation stained for Nbea (in green). Middle: DIV15 hippocampal neurons stained for transferrin receptor (in red) and endogenous Nbea (in green). Bottom: DIV17 hippocampal neurons fixed in methanol and stained for PSD95 (in red) and endogenous Nbea (in green). Scale bar = 5 μm .

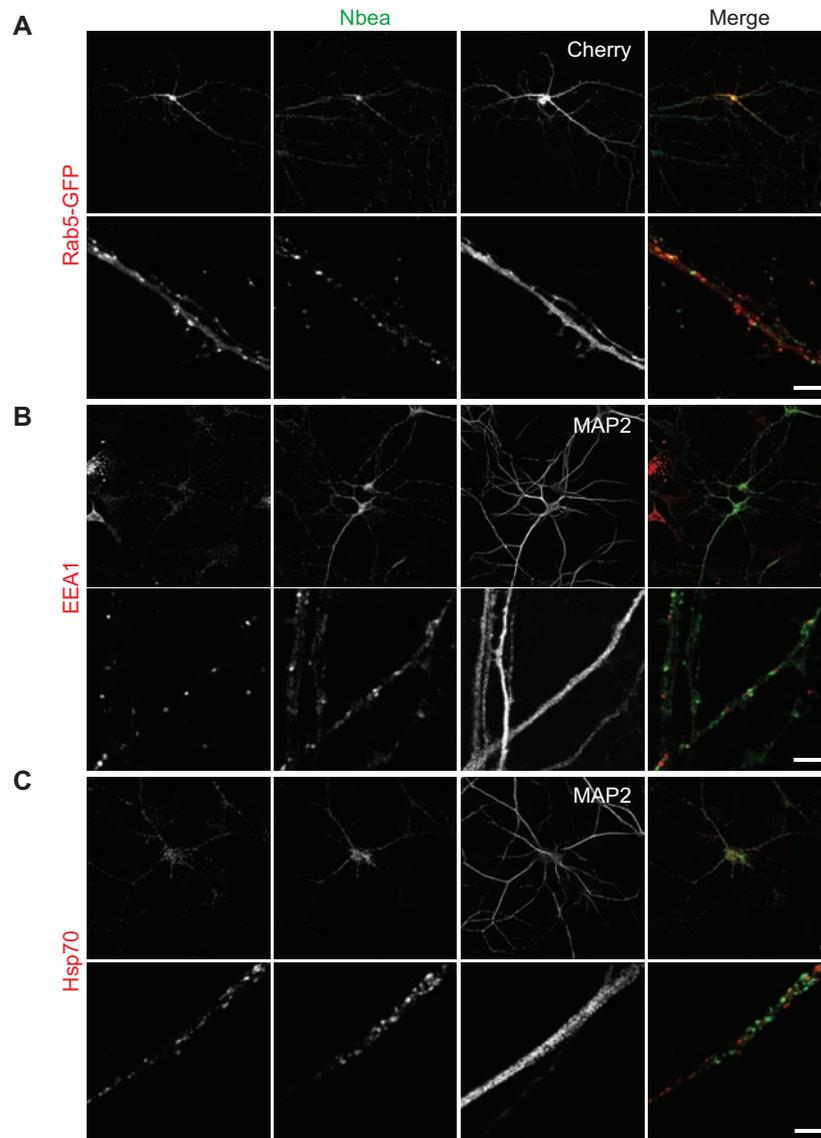


Figure 2.9 Examples of dendritic Nbea colocalization with various markers. (A) DIV15 WT hippocampal neurons co-transfected via calcium transfection at DIV10 with Rab5-GFP (in red) and mCherry (not shown in the merge), after PFA fixation stained for Nbea (in green). (B) DIV13 rat cortical neurons stained for EEA1 (in red), endogenous Nbea (in green) and MAP2 (not shown in the merge). (C) DIV15 WT hippocampal neurons stained for mitochondrial Hsp70 (in red), endogenous Nbea (in green) and MAP2 (not shown in the merge). Scale bar = 5 μ m.

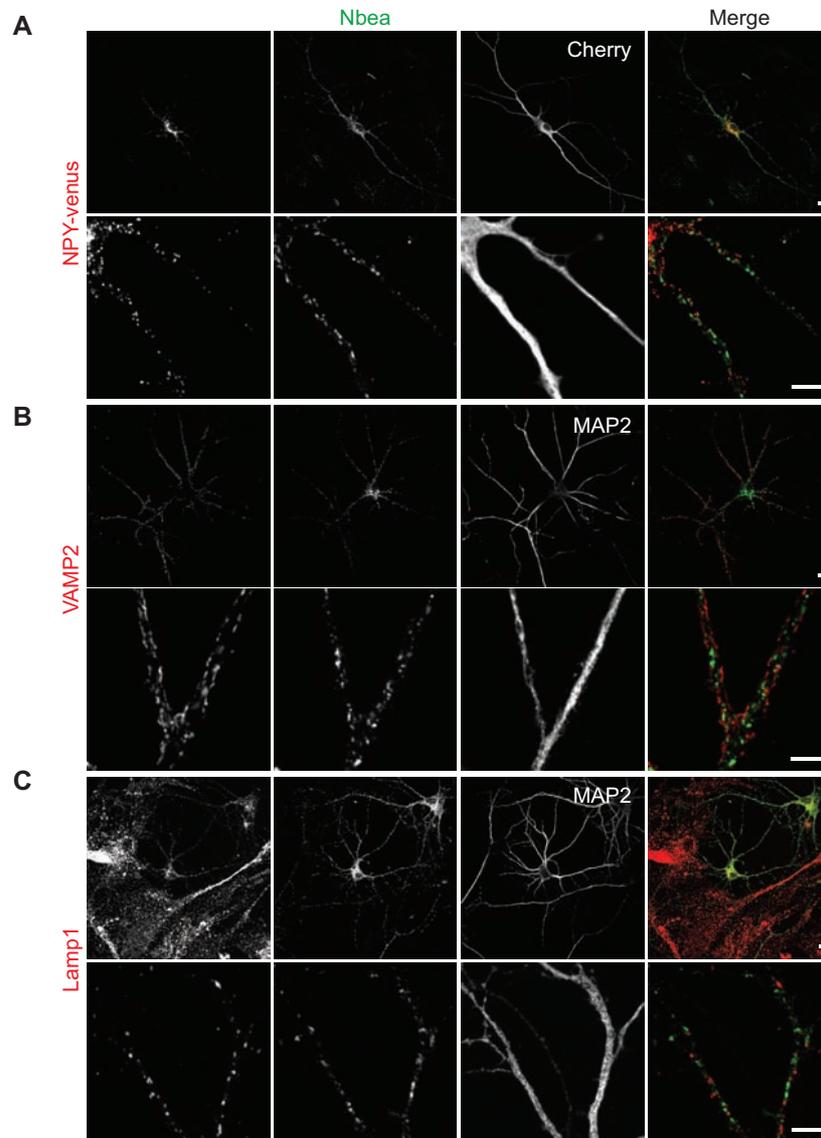


Figure 2.10 Examples of dendritic Nbea colocalization with various markers. (A) DIV14 WT hippocampal neurons transfected with NPY-venus (in red) via semliki forest viral infection (10 h incubation). After PFA fixation cells were stained for endogenous Nbea (in green) and MAP2 (not shown in the merge). (B) DIV15 WT hippocampal neurons stained for VAMP2 (in red), endogenous Nbea (in green) and MAP2 (not shown in the merge). (C) DIV14 WT hippocampal neurons stained for Lamp1 (in red), endogenous Nbea (in green) and MAP2 (not shown in the merge). Scale bar = 5 μ m.

