Neuroplastin-dependent signaling in neurons

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von M.S. (Pharm.), Sampath Kumar Vemula

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Gutachter: Prof. Dr. rer. nat. Constanze I. Seidenbecher

Prof. Dr. med. Markus Missler

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Summary

Neuroplastins are Ig domain-containing cell adhesion molecules important for brain function. Four neuroplastin isoforms arise from a single gene by alternative splicing. Np55 and Np65, which differ in the presence of an additional Ig domain, can both occur with or without a four amino acids insertion (DDEP) in their intracellular domains. While Np55 is also expressed in various non-nervous tissues, Np65 is expressed primarily in neurons, and it is proposed to mediate *trans*-synaptic cell adhesion by homophilic interaction during formation and stabilization of synapses. Polymorphisms in the human neuroplastin gene promoter are associated with cortical thickness, intellectual ability, and risk to develop schizophrenia. Neuroplastin-deficient mice display severe cognitive deficits as well as reduced number and altered organization of excitatory synapses resulting in an imbalanced excitatory-to-inhibitory synapse ratio. Moreover, in cultured neuroplastin-deficient hippocampal neurons, the actin cytoskeleton is disorganized in excitatory synapses. However, the cellular mechanisms underlying neuroplastin function in excitatory synapse organization and/or stabilization remained unknown.

In my thesis, I demonstrate that neuroplastins bind the tumor necrosis factor receptor-associated factor 6 (TRAF6) via a specific site in their intracellular domain to promote spinogenesis by activating PI3K/Akt and NF-κB signaling in hippocampal neurons. I employed immunoprecipitation, overexpression and knockdown techniques to reveal neuroplastin-TRAF6 interaction and its underlying signaling pathway during early neuronal development. I could demonstrate by co-immunoprecipitation, pull-down assay and Surface Plasmon Resonance that neuroplastins interact with TRAF6 through a TRAF6-binding motif in its intracellular domain. Further, neuroplastin-mediated signaling and cellular phenotypes were studied in heterologous systems, i.e., human embryonic kidney (HEK) 293T cells, and also in hippocampal neurons derived from rat pups and neuroplastin knockout (*Nptn-/-*) mice embryos. I found that overexpression of neuroplastin increases the filopodia number and length in HEK cells and also increases the number of dendritic protrusions in neurons. Neuroplastin deficiency reduced the number of dendritic protrusions in *Nptn-/-* neurons, and this effect was rescued by re-expressing neuroplastin in *Nptn-/-* neurons. Mutantions in the TRAF6 binding site of neuroplastin constructs as well as TRAF6 knockdown decrease the filopodia number in HEK cells and dendritic protrusion number in neurons. Moreover, neuroplastin overexpression leads to recruitment of TRAF6 to the plasma membrane and redistribution into filopodia. Using immunocytochemistry, I showed that inhibition of the PI3K/Akt pathway reduced filopodia formation in neuroplastin over-expressing cells and counteracted the induction of dendritic protrusions in neurons after acute treatment with Enplastin, an Np65 Ig1 domain-specific peptide. Moreover, neuroplastin promotes translocation of nuclear factor-kappa B (NF-κB) from the cytosol to the nucleus in HEK cells as well as in hippocampal neurons. Long-term inhibition of NF-κB translocation to the nucleus reduced filopodia formation in heterologous cells as well as dendritic protrusions in neurons. But, acute inhibition of NF-kB translocation did not affect the Enplastin-induced dendritic protrusion number. Altogether, this thesis shows that neuroplastins interact with TRAF6 at the plasma membrane to initiate downstream signaling i.e., phosphatidylinositol 3-kinase (PI3K)/Akt activation to regulate early synaptogenesis, and NF-κB activation may be needed for recruitment of the synaptic machinery in mature hippocampal neurons.

Zusammenfassung

Neuroplastin ist ein Zelladhäsionsmolekül aus der Immunglobulin-Superfamilie. Es spielt eine wichtige Rolle für die Gehirnfunktion. Für das humane Neuroplastin-Gen wurden Assoziationen mit der Dicke des zerebralen Kortex und der geistigen Begabung bei Jugendlichen sowie mit dem Risiko für schizophrene Erkrankungen berichtet. Es existieren vier Neuroplastin-Isoformen, die alle durch alternatives Spleißen des Primärtranskripts entstehen – nämlich Np55 und Np65, die zwei bzw. drei Immunglobulin-Domänen besitzen, sowie je eine Version mit bzw. ohne ein vier Aminosäuren langes Mini-Exon (DDEP-Sequenz) in der cytoplasmatischen Region. Np65 ist vorwiegend in Neuronen exprimiert und trägt hier zur trans-synaptischen Zelladhäsion und damit zur Bildung und Stabilisierung von Synapsen bei. Np55 hingegen ist auch in einer Vielzahl nicht-neuraler Gewebe exprimiert. Beide Neuroplastine sind an der Regulation des Neuritenwachstums, der Synaptogenese und synaptischer Plastizität im Gehirn beteiligt. Mäuse mit einer Neuroplastin-Defizienz weisen kognitive Defizite und eine verschlechterte Kalziumregulation auf. Hippocampale Primärneuronen von Neuroplastin-Nullmutanten (*Nptn-/-*) zeigen ebenfalls Defizite bei der Organisation und/oder Stabilisierung exzitatorischer Synapsen. Des Weiteren beeinträchtigt der Verlust von Neuroplastin das Zytoskelett exzitatorischer Synapsen. Außerdem ist das Gleichgewicht zwischen inhibitorischen und exzitatorischen Synapsen – die E/I-Balance – bei Neuroplastin-Mutanten gestört. Die zu Grunde liegenden Mechanismen, wie Neuroplastin die Synapsenorganisation und -stabilisierung reguliert, sind allerdings bisher nicht bekannt.

In der intrazellulären Domäne von Neuroplastin konnte eine potentielle Bindestelle des Tumornekrosefaktorrezeptor-assoziierten Faktors 6 (TRAF6) identifiziert werden. Darauf aufbauend wurde die Hypothese entwickelt, dass ein Komplex aus Neuroplastin und TRAF6 durch die Aktivierung des PI3K/Akt- oder NF-κB-Signalweges die dendritische Spinogenese in hippocampalen Prinzipalneuronen fördert.

In meiner Arbeit habe ich unter Verwendung von Immunpräzipitation, Überexpression und Knock-down eine mögliche Interaktion zwischen Neuroplastin und TRAF6 sowie den potentiell angesteuerten Signalweg während der frühen neuronalen Entwicklung untersucht. Ich konnte in einem heterologen Expressionssystem, d.h. in humanen embryonalen Nierenzellen (HEK 293T-Zellen), zeigen, dass Neuroplastin mit TRAF6 über ein in silico identifiziertes TRAF6-Bindemotiv in seiner intrazellulären Domäne interagieren kann. Des Weiteren konnte mittels Plasmon-Resonanz-Technologie eine direkte Assoziation zwischen einem Neuroplastin-Peptid, welches das TRAF6-Bindemotiv enthält, mit TRAF6-Fusionsproteinen nachgewiesen und der Dissoziationskoeffizient ermittelt werden. Die Neuroplastin-vermittelte Signalübertragung und der dazugehörige zelluläre Phänotyp wurden dann in verschiedenen Systemen analysiert: im heterologen Expressionssystem wie den HEK 293T-Zellen und in hippocampalen Primärneuronen. Ich konnte zeigen, dass eine Überexpression von Neuroplastin in HEK-Zellen sowohl zu einem Anstieg in der Filopodienanzahl und -länge, als auch zu einem Anstieg in der Zahl dendritischer Ausstülpungen in Neuronen führt. Im Gegensatz dazu zeigen sowohl Neuroplastin-Isofomen mit mutierter TRAF6-Bindestelle als auch der Knock-down von endogenem TRAF6 in HEK-Zellen eine verringerte Filopodienanzahl und in Neuronen eine verminderte Anzahl an dendritischen Ausstülpungen. Neuroplastin-Defizienz resultiert in *Nptn-/-* -Neuronen in einer verminderten Anzahl von dendritischen Ausstülpungen. Die Expression von rekombinatem Neuroplastin in diesen Neuronen kann dem

entgegenwirken. Zudem führt die Überexpression von Neuroplastin zu einer Rekrutierung von TRAF6 an die Plasmamembran und einer Verlagerung der Komplexe in die neu gebildeten Filopodien. Eine Verlaufsstudie der Filopodienbildung offenbarte eine unterschiedliche Regulation von Filopodienanzahl und -länge. Daraus ergab sich die Hypothese, dass die Anzahl möglicherweise vom PI3K/Akt Signalweg abhängig ist und die Länge von NF-κB-vermittelter Gentranskription gesteuert wird. Mit Hilfe von immuncytochemischen Färbungen konnte gezeigt werden, dass die Blockade des PI3K/Akt-Signalweges in der Tat zu einer verminderten Filopodienbildung in Neuroplastin-expremierenden HEK-Zellen und bei akuter Behandlung zu verringerten dendritischen Ausstülpungen in Neuronen nach Stimulation mit dem Np65-Ig1-spezifischen Peptid Enplastin führt. Des Weiteren kann Neuroplastin die Translokation des Nuclear factor-kappa B (NF-κB) vom Zytosol in den Zellkern sowohl in HEK-Zellen als auch in hippocampalen Primärneuronen vermitteln. Langzeitinhibierung der Translokation von NF-κB zum Zellkern führt zu reduzierter Filopodienformation in HEK-Zellen und zu einer verminderten Anzahl dendritischer Ausstülpungen in Neuronen. Allerdings zeigte die akute Blockierung der NF-κB-Translokation keinen Effekt auf die Anzahl der Enplastin-induzierten dendritischen Ausstülpungen.

Zusammengefasst konnte hier gezeigt werden, dass die Interaktion von Neuroplastinen mit TRAF6 an der Zellmembran einerseits nachgeschaltete lokale Signalwege, wie die Aktivierung des PI3K/Akt-Signalwegs, ansprechen kann. Dies könnte frühe Prozesse der Synaptogenese einleiten. Andererseits könnte die Aktivierung von NF-κB notwendig sein für die Rekrutierung der synaptischen Maschinerie in reifenden hippocampalen Neuronen.

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1. Introduction

The formation of synaptic contacts is a crucial developmental process required for the establishment of neuronal circuits in the brain. Indeed, in the adult brain, formation of new synapses as well as proper function and plasticity of synapses are essential for cognitive functions such as learning and memory (Bozdagi et al., 2000; Radwanska et al., 2011). Cell adhesion molecules (CAMs) play a number of essential roles during extension of axons and dendrites as they help them to find the right targets (Dalva et al., 2007). After making contact between axons and dendrites, CAMs initiate signaling to recruit large molecular machinery during the construction of synaptic structures (Dalva et al., 2007). A dynamic remodeling of actin cytoskeleton along dendrites, especially in the synapses, is one of the central mechanisms that regulate synapse formation and synaptic plasticity (Spence and Soderling, 2015). To date, the mechanisms that link CAM-mediated signaling and regulation of actin cytoskeleton during synapse formation and stabilization are only partially understood.

1.1. Synaptogenesis during development and in mature neurons

In this thesis, I focus on early mechanisms of synaptogenesis during the time when axons and dendrites first contact to create new the specialized subcellular compartment called synapses. In this period, a number of proteins to either side of the contacts are recruited (Washbourne et al., 2004; Dalva et al., 2007; McAllister, 2007) promoting early spinogenesis. Therefore, these molecular events are occurring before to the maturation of synapses, activity-dependent modulation of spine morphology, or even earlier than the synthesis of most pre- and post-synaptic molecular components. Indeed, there are three general proposition of how early spinogenesis may proceed as described by 1) the Sotelo model, 2) the Miller/ Peters model and 3) the filopodial model (Fig. 1).

Based on studies from several mutant mice including *weaver* mutant mice lacking granule cells, the presynaptic partners of Purkinje cells, and *reeler* mutant mice in which the migration of granule cells is perturbed, the Sotelo model (Fig. 1a), proposes that dendritic spines are generated intrinsically from the dendritic shaft and maintained independently of granule cell fiber terminal on Purkinje cells.

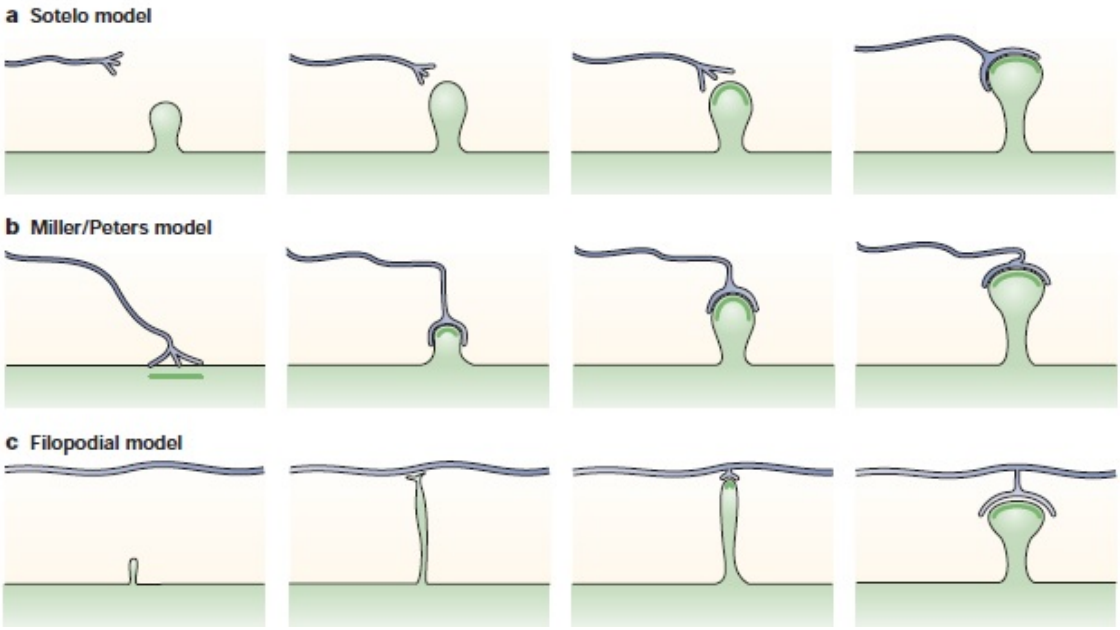


Fig. 1. The schematic representation of three types of models for spinogenesis in neurons. a) Dendritic spine develops intrinsically from dendritic shaft and independent of axon terminals. b) Axonal contact with dendrite induces spine formation on dendritic shaft. c) Dendritic filopodia make a contact with an axonal terminal to become a spine (Image was taken from Yuste and Bonhoeffer, 2004).

Indeed, in these mutant mice, spines are quite normal independent of absence of presynaptic axons demonstrating that initial formation of dendritic spines on Purkinje cells does not dependent on presynaptic terminals of granule cells (Yuste and Bonhoeffer, 2004; Garacia-López et al., 2010). The Miller/ Peters model (Fig. 1b) proposes that 'stubby and immature' spines are developed on the dendritic shaft right after the contact of presynaptic axons showing a swelling as the synaptic vesicles accumulate. Then, mature synapses are formed by mushroom-shaped spines with a clear neck and the axon terminals with developed varicosities. The filopodial model (Fig. 1c) describes that dendritic protrusions, which are long (2-30 µm), thin (>0.3 µm diameter) and highly dynamic structures (Yuste and Bonhoeffer, 2004). actin-based microvilli or filopodia, develop on the dendritic shaft in absence of pre-synaptic contact and then, mature into dendritic spines when contacted by axons (Yuste and Bonhoeffer, 2004; Garacia-López et al., 2010). This model of spinogenesis based on dendritic protrusion formation is largely accepted to explain formation of synaptic contacts in pyramidal neurons during the development of the hippocampal circuit.

Mature synapses are junctions formed between pre-synaptic axon terminals and post-synaptic dendritic spines specialized to mediate the transmission of information from one neuron to another neuron (Dalva et al., 2007). The pre-synaptic axon terminal contains complex membrane trafficking machinery that regulates the secretion of

neurotransmitter packed into vesicles (Südhof, 2012). The neurotransmitter is released in a calcium-dependent manner at the active zone, a specialized region of the pre-synaptic plasma membrane that docks and primes vesicles for exocytosis (Gundelfinger and Fejtova, 2012; Südhof, 2012). The post-synaptic structure opposing to pre-synaptic bouton contains neurotransmitter receptors, ion channels and scaffold proteins. They can associate to the postsynaptic density (PSD) formed by PSD-95 as well as other scaffolding proteins involved in the regulation of synapse transmission (McAllister, 2007). Based on their morphology, dendritic spines are classified into different categories for example, short and thin spines with a bear a small head, stubby spines with a small spine neck, mushroom spines which are having a large spine head with short spine neck. These spines are typically associated with memory formation and traces (Matsuzaki et al., 2004). Cup shaped spines display a cup shaped head (Hering and Sheng, 2001). The morphological dynamics of dendritic spines are regulated by Rho GTPases and thereby regulate the actin dynamics via N-WASP, WAVE, Myosin-X and LIM kinase pathways (Govek et al., 2005; Mattila and Lappalainen, 2008). In general the density and morphology of dendritic spine has been correlated with different synaptic functions as established by correlating several parameters e.g., size of PSD, number of postsynaptic receptors and synaptic strength (Robbins et al., 2010; Bernardinelli et al., 2014). Moreover, neuronal activity regulates dendritic spine and spine morphology in adult brain (Spires et al., 2005; Diering et al., 2011; Hamilton et al., 2012). Interestingly, perturbation in the formation of dendritic spine is associated with several mental disorders such as fragile X, Down and Rett syndromes (Kaufmann and Moser, 2000).

1.2. Cell adhesion molecules (CAMs) in the brain

The diverse processes of cell adhesion play an important role in multiple biological functions like tissue morphogenesis, neuronal cell migration, axon bundle formation, synapse formation and formation of complex of glial networks which surround axons and synapses. This cell adhesion is also crucial for brain morphology and its functions i.e., learning and memory (Washbourne et al., 2004; Dalva et al., 2007; Robbins et al., 2010; Missler et al., 2012). In neurons, this adhesion is mediated by the proteins called cell adhesion molecules (CAMs), which are present in the pre- and post-synaptic membranes. CAMs not only function in cell adhesion, but also mediate homo- or heterophilic interaction across the synaptic cleft through their extracellular domains (Washbourne et al., 2004; Dalva et al., 2007). The stabilization of trans-synaptic contact is a crucial step during the synaptogenesis. Thus, CAMs recruit synaptic scaffolding proteins via their cytoplasmic tails to provide a platform for further

recruitment of post-synaptic neurotransmitter receptor (Scheiffele et al., 2000; Dean et al., 2003; Washbourne et al., 2004). In mature synapse, CAMs modulate the synaptic function by interacting with synaptic proteins like NMDA receptors and thereby synaptic strength in neurons (Dalva et al., 2007). Therefore, CAMs are crucial during the initial stage of synaptogenesis and later to regulate the synaptic function.

Based on their structure and functions, CAMs fall into 4 major families: cadherins, immunoglobulin (Ig) superfamily, integrins and selectins (Lodish et al., 2003). These CAMs contain repeated domains in their extracellular domain of the same molecule. Mostly, these domains mediate homophilic interactions with the same molecule present on the same or opposite cell. Similarly, CAMs also mediate heterophilic interactions with other CAM present on the same or opposite cell. Moreover, the cytoplasmic tail of CAMs recruits the diverse adapter proteins, which are connected directly or indirectly to the cytoskeleton proteins. Most of these families' proteins have been implicated in neuronal differentiation, synapse formation and synaptic plasticity (Washbourne et al., 2004; Dalva et al., 2007; Missler et al., 2012).

Immunoglobulin (Ig) superfamily CAMs are characterized by the presence of one or more extracellular immunoglobulin-like domains (Crossin and Krushel, 2000). Among all CAMs, the neuronal cell adhesion molecule (NCAM) was the first isolated and best characterized prototypic Ig-like CAM (Brackenbury et al., 1977 & 1987; Weledji and Assob, 2014). Apart from Ig-like domains, some CAMs also contain fibronectin III repeats in their extracellular region (Crossin and Krushel, 2000). One of the Ig superfamily members is neuroplastin.

1.2.1. Neuroplastin: An Immunoglobulin (Ig) superfamily member

Neuroplastin is a synaptic cell adhesion molecule, which spans the synaptic cleft to mediate synaptic adhesion of pre- and post-synapses (Herrera-Molina et al., 2014; Beesley et al., 2014). Neuroplastin is also known as stromal cell-derived receptor 1 (SDR1) and stromal cell-derived factor receptor 1 (SDRF1) (http://www.uniprot.org/uniprot/Q9Y639). It occurs in two isoforms termed Np55 and Np65 based on their molecular weights of 55 or 65kDa, respectively. Initially, neuroplastins were identified as of concavalin A-binding bands in synaptic membrane (SM) glycoprotein fractions (Kelly et al., 1977; Fu et al., 1981). Later, both Np55 and Np65 were recognized with a SM glycoprotein65 (SMgp65) monoclonal antibody (Hill et al., 1988). The human neuroplastin (*NPTN*) gene is located on chromosome 15q22. It contains nine exons (Beesley et al., 2014). Its transcripts are alternatively spliced into 4 isoforms, two of them having different initiation sites. Np55 contains two N-terminal

extracellular Ig-like domains, a transmembrane domain and a C-terminal intracellular domain (Fig. 2; Langnaese et al., 1997).

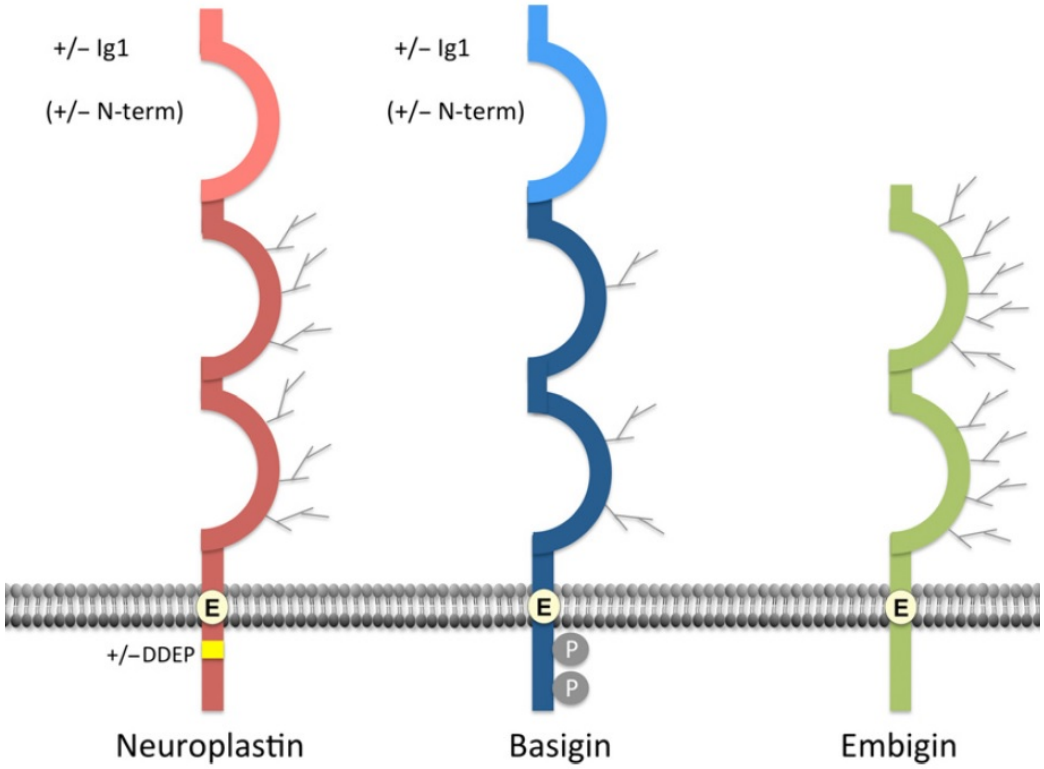


Fig. 2. Schematic representation of Neuroplastin, Basigin and Embigin structures. Neuroplastin, Basigin and Embigin contain two Ig like domains, which are glycosylated, a transmembrane domain with a charged glutamate (E) residue and a short intracellular domain. Np65 and Basigin 2 contain an extra Ig1 domain in its extracellular part. Neuroplastins may contain an additional four amino acid i.e., Asp-Asp-Glu-Pro (DDEP) insertion in their cytoplasmic tail. Phosphorylation sites were predicted in cytoplasmic tail of Basigin (Image modified from Beesley et al., 2014).

Np65 contains an extra Ig1 domain, which mediates homophilic interaction and regulates synaptic plasticity (Smalla et al., 2000; Empson et al., 2006; Owczarek et al., 2011; Herrera-Molina et al., 2014). Neuroplastins have six N-glycosylation sites present on Ig2 and Ig3 domains. Four of six sites have an N-acetylglucosamine moiety. After deglycosylation, the core proteins have a molecular mass of 28 kDa (for Np55) and 40 kDa (for Np65) (Langnaese et al., 1997; Owczarek et al., 2010; Beesley et al., 2014). Additionally, the Ig1 domain in Np65 is predicted to be O-glycosylated at serine 95 (Beesley et al., 2014). By an additional splicing event, both isoforms may contain an additional four amino acid mini exon i.e., Asp-Asp-Glu-Pro (DDEP) insertion in their intracellular domain (Langnaese et al., 1997). Recently, Sakguchi et al showed that neuroplastin interacts with growth factor receptor-bound protein 2 (GRB2). This interaction occurs through the SH3 binding motif (PxxP), which is generated by the four

amino acids insertion in the neuroplastin (Sakaguchi et al., 2016). However, the functional importance of the insertion is not yet clear.

Closest relatives of neuroplastin are the Ig CAMs Basigin (also termed Emmprin) and Embigin (Lagnaese et al., 1997; for review see Beesley et al., 2014). Neuroplastin and basigin protein sequence identity ranges from 40 to 45% with the highest identity in the transmembrane and intracellular regions (Beesley et al., 2014). The conserved charged glutamate residue in the transmembrane domain of basigin (see Fig. 2) plays an important role in trafficking of the protein to the plasma membrane (Manoharan et al. 2006). The lowest primary structure identity is found in the Ig1 domain of Np65 and Basigin 2 with only 20% (Beesley et al., 2014).

1.2.1.1 Neuroplastin expression in the brain

Neuroplastins exhibit a specific pattern of expression and differential subcellular distribution in the brain. Np55 is expressed already during embryogenesis but Np65 expression parallels dendritic outgrowth and synaptogenesis in early postnatal life and peaks after postnatal day 21 (Langnaese et al., 1997; Buckby et al., 2004). Np65 expression was observed mainly in forebrain regions such as cerebral cortex, striatum and hippocampus with lower expression in thalamus and barely detected in brainstem regions. The immunocytochemical analysis of Np65 expression revealed that it is prominently expressed in neuropil regions i.e., layers II, III and Vb/VI of cerebral cortex, stratum radiatum and stratum oriens of hippocampus of the rat and also in glutamatergic pyramidal neurons of layers II, IV and V of human brain (Langnaese et al., 1997; Smalla et al., 2000; Herrera-Molina et al., 2017). Moreover, Np65 expression is higher in CA1 and dentate gyrus (DG) as compared to CA2 and CA3 regions of the hippocampus (Herrera-Molina et al., 2014). Immunostaining and subcellular fractionation studies show that Np65 is enriched in PSD fractions and co-localizes with the post-synaptic marker PSD95 (Smalla et al., 2000; Herrera-Molina et al., 2014).

Recently, Np65 expression was reported in keratinocytes and lesions of atopic dermatitis skin in a complex together with S100A9 and basigin (Sakaguchi et al., 2016).

Np55 is expressed in ubiquitously, but tissue-specific glycosylation may be crucial for neuroplastin function (Beesley et al., 2014). Np55 is present in all regions of the brain as well as in other organs like kidney, spleen, thymus, skeletal muscle, heart, and liver (Langnaese et al., 1997; Smalla et al., 2000; Marzban et al., 2003). In situ hybridization studies show that in synaptic layers of the retina expression of neuroplastin transcripts for Np55 and Np65 lacking the DDEP insertion is higher than that of miniexon-containing transcripts (Kreutz et al., 2001). Increased neuroplastin mRNA and protein

expression was reported in the hippocampus of mice lacking complex gangliosides (Mlinac et al., 2012).

1.2.1.2. Phenotypes of neuroplastin-deficient mice

The human *NPTN* gene has been reported to be associated with schizophrenia, and intellectual ability through cortical thickness and cognitive functions (Saito et al., 2007; Desrivières et al., 2015). Studies of *Nptn-/-* mice have revealed major functions for neuroplastin in the brain. It has been shown that neuroplastin is important for brain function and cognitive abilities in mice (Amuti et al., 2016; Bhattacharya et al., 2016; Herrera-Molina et al., 2017). Recently, it was shown that neuroplastin ablation in glutamateric neurons alters hippocampal, striatal and sensorimotor cortex-dependent functions and leads to cognitive deterioration in mice (Herrera-Molina et al., 2017). Neuroplastin deletion, both constitutive and induced, leads to impaired fear-conditioned associative learning (Battacharya et al., 2016). *Nptn* gene ablation in adult mice causes retrograde amnesia of learned associative memories, which is a remarkable phenotype for this CAM. Thus, it suggests that neuroplastin is necessary to recall previously acquired associative memories. Additionally, this mouse displays a complex swimming and diving behaviors (Battacharya et al., 2016). In contrast, in another study with a Np65-specific deletion (Np65 KO) mutant the authors report improved learning and memory (Amuti et al., 2016).

Further, the neuroplastin full knockout (*Nptn-/-*) mice also exhibit pleiotropic effects, which are related to psychiatric disorders like autism spectrum and depression (Battacharya et al., 2016). Interestingly, rats injected with the Np55-derived peptide Narpin have shown anti-depressant like-behavior in forced swim test (Owczarek et al., 2010). Rats injected with the Np65 Ig1 specific peptide Enplastin showed altered initial phase spatial memory, which was measured by Morris water maze (MWM) paradigm, which is a hippocampus-dependent task. The author explained this effect as a consequence of disturbed or affected Np65-Np65 trans-homophilic interaction (Owczarek et al., 2011). However, neuroplastin deficiency does not affect the general neuronal morphology or survival (Herrera-Molina et al., 2014; Amuti et al., 2016).

1.2.1.3. Neuroplastin binding partners

Over the past two decades of neuroplastin research several binding partners could be identified in different cell types, some of them binding extracellularly, intracellularly and also in the membrane, which suggests involvement of neuroplastin in various cellular functions. Obviously, neuroplastin functions as a chaperone for some membrane proteins like the A-type gamma-amino butyric acid receptor ($GABA_AR$), Xk-related protein 8 (Xkr8) , which is an Xkr family protein playing a pivotal role in phospholipids scrambling during apoptosis, and the mono carboxylate transporter 2 (MCT2) (Sarto-

Jackson et al., 2012; Wilson et al., 2013; Suzuki et al., 2016). One of the first reported neuroplastin binding partners was fibroblast growth factor receptor type-1 (FGFR1). Surface plasmon resonance studies revealed that interaction between Np55 and FGFR1 is mediated via Ig2 domain of Np55. The alpha subunit of $GABA_A$ receptors associates directly with neuroplastin in the cell membrane as shown by affinity chromatography, immunoprecipitation and FRET experiments. Np65 co-localizes with $GABA_A$ receptor α2 subunit at inhibitory synapses in hippocampal neurons. Moreover, hippocampal neurons from *Nptn-/-* mice show increased synaptic inhibitory currents compared to WT neurons because of altered localization of $GABA_A$ receptors in *Nptn-/-* neurons (Sarto-Jackson et al., 2012; Herrera-Molina et al., 2014). Wilson and colleagues showed that Np65 and Np55 can act as chaperones for MCT2 by localizing the transporter in the cell surface and facilitating lactate uptake in COS cells (Wilson et al., 2013). Indeed, the chaperoning function of neuroplastin together with Basigin is also crucial for Xkr8 to localize in the plasma membrane (Suzuki et al., 2016).

It was shown that both Np55 and Np65 form homo and heterodimers and they also form heterodimers with Basigin to regulate keratinocyte proliferation (Sakaguchi et al., 2016). Recently, the downstream interacting partners of neuroplastin were identified in keratinocytes (Sakaguchi et al., 2016). They reported that Np55 and Np65 interact with TRAF2 and GRB2 and mediate keratinocyte proliferation and cytokine induction (Sakaguchi et al., 2016).

1.2.1.4. Signaling pathways downstream of neuroplastin

As discussed above, neuroplastin interacts with several proteins to regulate or modify neuroplastin function. Therefore, signaling pathways influencing neuroplastin function is subject of investigation.

The most prominent signaling pathways triggered by neuroplastin are related to calcium-dependent signaling. Recently, we showed that neuroplastin deficiency in glutamatergic neurons leads to decreased plasma membrane Ca^{2+} ATPase (PMCA) levels and altered calcium homeostasis (Herrera-Molina et al., 2017). Np65 KO mice showed increased levels of phosphorylation of extracellular regulated protein kinase 1/2 (pERK1/2) and transcription factor cyclic-AMP response element binding protein (CREB) in brain homogenates compared to WT mice (Amuti et al., 2016).

Moreover, Empson and colleagues have shown that the treatment of hippocampal neurons with Np65-specific fusion protein activates p38 mitogen activated protein kinase (MAPK) signaling to regulate the surface expression of glutamate receptor subunit GluR1 and synaptic plasticity (Empson et al., 2006). Moreover, application of the Np65-derived peptide Enplastin increased neurite outgrowth in hippocampal

neurons. This effect seems to be related to Np65-Np65 *trans*-homophilic interaction leading to activation of ERK1/2 and p38 MAP kinases. Indeed, this *trans*-homophilic interaction occurs through the F-G loop of Ig1 domain of Np65 (Smalla et al., 2000; Empson et al., 2006; Owczarek et al., 2011). Treatment of hippocampal neurons with recombinant peptides specific to Ig2-3 domains of neuroplastin or with Narpin increased the activation of MAPK and ERK1/2 to regulate neurite outgrowth. Activation of these kinases was dependent on synaptic calcium (Owczarek et al., 2010 & 2011).

Apart from aforementioned proteins, it was reported that in brain homogenates of Np65 KO mice levels of some synaptic proteins were also altered e.g., decreased levels of PSD95 and vGluT, and increased amount of N-Methyl-d-aspartate (NMDA) receptor subunit 2A (NR2A) (Amuti et al., 2016).

Sakaguchi and colleagues showed that neuroplastin activates nuclear factor-kappa B (NF-κB) during keratinocyte proliferation and inflammatory responses (Sakaguchi et al., 2016).

Of note, based on the evidence summerized here, neuroplastin can interact with various proteins to regulate cell specific functions through the activation of downstream signaling molecules.

Hippocampal neurons from neuroplastin-deficient mice revealed increased mismatch of excitatory but not inhibitory synapses and also altered $GABA_A$ receptor localization in inhibitory neurons (Herrera-Molina et al., 2014). Recently, it has been shown that neuroplastin promotes synaptogenesis and mutation of the neuroplastin gene prevents the progression in the functional maturation of the synaptic machinery in mouse inner hair cells (IHCs) (Carrot et al., 2016). Similar morphological defects in synapses of two different cell systems suggest a common synaptic function for neuroplastin that regulates synaptic organization in neurons. In particular, in *Nptn-/-* hippocampus a reduced number of excitatory synapses was especially found in CA1 and DG regions, and also disturbed actin cytoskeleton as compared with wild-type neurons (Herrera-Molina et al., 2014). Moreover, *Nptn-/-* mice also show an imbalance between excitatory and inhibitory function (Battacharya et al., 2016). Recently, another group confirmed the *Nptn-/-* phenotype in mice lacking only Np65 (Amuti et al., 2016). It is possible that elimination of the *Nptn* gene and thus lack of Np65 protein expression is sufficient to relate to the reported phenotype. Nevertheless, I know that acute inactivation of neuroplastin function with Np65 antibodies or Np65 Fc proteins in mature WT neurons mimics the features observed in *Nptn-/-* neurons (Herrera-Molina et al., 2014). This suggests manipulation of Np65 function could also lead to altered

spine or synapse formation via unknown mechanism. Despite this wealth of information, the precise role of neuroplastin remains incompletely understood.

1.2.1.5. The cytoplasmic tail of neuroplastins contains a TRAF6 binding motif

Using the Eukaryotic Linear Motif (ELM) data base and sequence analysis approach, I identified a TRAF6 binding motif i.e., (Basic residues)-X-X-P-X-E-X-X-(Ar/Ac), in the juxtamembrane region of the cytoplasmic domain (362-370 aa for Np65) of neuroplastin (Fig. 3A). Recently, Sakaguchi et al reported that neuroplastin possesses a TRAF binding domain in it's cytoplasmic tail (Sakaguchi et al., 2016). However, the TRAF binding site (as discussed below) is different from what they reported. The TRAF6 binding site is conserved in many species as well as in other proteins (Darnay et al., 1999; Ye et al., 2002; Sorrentino et al., 2008). The TRAF6 binding site was initially identified in CD40 as ^{231}QEPQEINFRANK, which was mapped in the membrane proximal region (Pullen et al., 1998). In the same year, a specific putative TRAF6 binding motif was mapped in receptor activator of nuclear factor kappa-B (RANK) protein as (Basic residues)-X-X-P-X-E-X-X-(Ar/Ac) (Darnay et al., 1999). This motif allows binding of several proteins to the adopter protein TRAF6 through it's TRAF-C domain (Pullen et al., 1998; Khusigara et al., 1999; Mansell et al., 2004; Powell et al., 2009). Considering other TRAF proteins, this motif is different from TRAF1, TRAF2 and TRAF5 binding site as they bind to a common TRAF (PxQxT) binding motif (Darnay et al., 1999; Tsukamoto et al., 1999; Bradley and Pobers, 2001). One of the best examples for it is RANK, which possesses a TRAF6 binding motif as well as a common TRAF binding motif in its cytoplasmic tail ((Darnay et al., 1999; Ye et al., 2002). The TRAF6 binding motif was further characterized by Ye and colleagues using X-crystallography modeling of TRAF6 and CD40 peptide with TRAF6 binding site (Fig. 3B, Ye et al., 2002). They have also shown that glutamic acid (E) in the TRAF6 binding motif and glutamine (Q) in the TRAF binding motif is considered as point of intersection (P_0). Both amino acids occupy similarly, but not in identical manner. Additionally, specific side chain residues in the TRAF6 binding motif contribute most to the physical association with TRAF6 (Fig. 3B). The cell permeable TRAF6 binding peptides inhibited CD40-TRAF6-mediated NF-κB signaling and thereby, blocked TRANCE-induced osteoclast differentiation (Ye et al., 2002). As discussed above, neuroplastin might also interact with TRAF6 through the TRAF6 binding site.

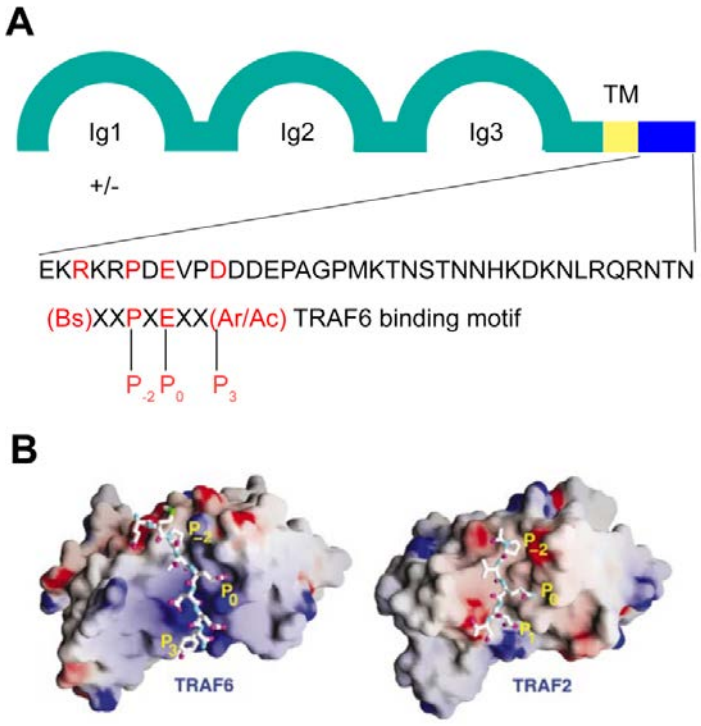


Fig. 3. Both neuroplastins possess a TRAF6 binding motif in their cytoplasmic tail. A) Schematic representation of neuroplastin in which immunoglobulin (Ig) like domains are shown in green, transmembrane domain is shown in yellow and cytoplasmic tail is shown in blue color. Proteins with TRAF6 binding motif i.e., (Bs)-X-X-P-X-E-X-X-(Ar/Ac). The cytoplasmic tail amino acids sequence with TRAF6 binding site shown in the picture (P_0-Glutamic acid (E), P_{-2}-Proline (P), P_3-Aromatic (Ar)/Acidic (Ac) amino acid). B) Cartoon shows c-terminal crystalline surface structure of TRAF6 and TRAF2 binding to peptides with TRAF6 and TRAF2 motif, respectively (Image modified and adopted from Ye et al., 2002).

1.3. Tumor necrosis factor (TNF)-receptor associated factor (TRAF) proteins

1.3.1. TRAF structure

Tumor necrosis factor (TNF)-receptor associated factor (TRAF) proteins play a central role in the immune system and they are very well characterized (Wajant et al., 2001; Xie, 2013). They were identified as major signal transducers for TNF-receptors (TNFR) and function as adopter proteins (Bradley and Pobers, 2001; Xie, 2013). Apart from their role as adopter proteins, TRAFs can function as scaffold proteins to regulate cell structures by forming higher-order oligomers and transducing the extracellular signal to inside of the cell (Wong et al., 1999; Kobayashi et al., 2001; Yin et al., 2009; Wu, 2013). Till date, there are seven known members of TRAF family proteins (TRAF1 to 7) in mammals (Xie, 2013). In general, TRAFs consist of three main domains in their structure. First, all TRAFs (except TRAF7) contain a highly conserved C-terminal TRAF domain, which possesses two subunits: an N-terminal domain called coil-coiled (CC) domain and a C-terminal β-sandwich (TRAF-C). Second, TRAFs contain several zinc finger domains in the N-terminal region. Third, all TRAFs (except TRAF1) contain a unique N-terminal really interesting new gene (RING)-finger domain, which mediates dimerization of TRAFs and most of the cells signaling (Fig. 4; Xie, 2013). The C-terminal domain forms a mushroom shaped trimeric structure, in which TRAF-C binds to receptors and the CC domain mediates TRAF homo and hetero-oligomerization (Yin et al., 2009). Further, small structural differences in the TRAF-C domain will define the

specificity of interaction of individual TRAFs with several receptors (Darnay et al., 1999). Among all TRAFs, TRAF2, 3 and 5 have overlapping receptor binding motifs whereas TRAF6 has a distinct-receptor binding motif (Darnay et al., 1999; Tsukamoto et al., 1999). However, they have overlapping or distinct signaling mechanisms depending on the signal where it is coming from. Manifold evidence shows that removal of the RING-finger domain abolishes the signaling and acts as dominant-negative (Rothe et al., 1995; Bradley and Pobers, 2001; Wajant et al., 2001; Xie 2013).

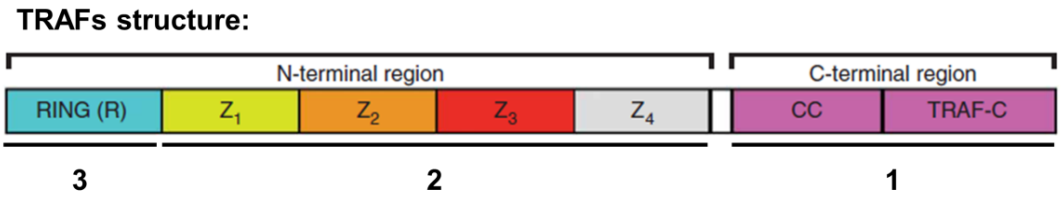


Fig. 4. Schematic representation of TRAFs structure. TRAFs contain a C-terminal region, which possess two subunits i.e., TRAF-C and coil-coiled domain, and a N-terminal region, which possess several zinc finger domains and a RING domain (Image modified and adapted from Yin et al., 2009).

1.3.2. TRAF binds to receptors either directly or indirectly

TRAFs can function downstream of several receptors. In general, all TRAFs mediate the cellular responses through TNFR whereas TRAF6 mediates through TNFR and Toll-like receptors (TLRs). In addition to the TNFR family, there are several other receptors interacting with TRAFs either directly or indirectly (Fig. 5). The receptors or proteins that mediate direct interaction are CD30, CD40, CD27, LT-β receptor, RANK, presenilin 1, and transforming growth factor-β (TGF-β) receptor (Sorrentino et al., 2008; Powell et al., 2009; Xie et al., 2013). Examples for interaction through additional adapter proteins include the TLR family that interacts with TRAFs via myeloid differentiation primary response gene 88 (MyD88) and IL-1 receptor-associated kinase (IRAK) (Fig. 5, Xie, 2013).

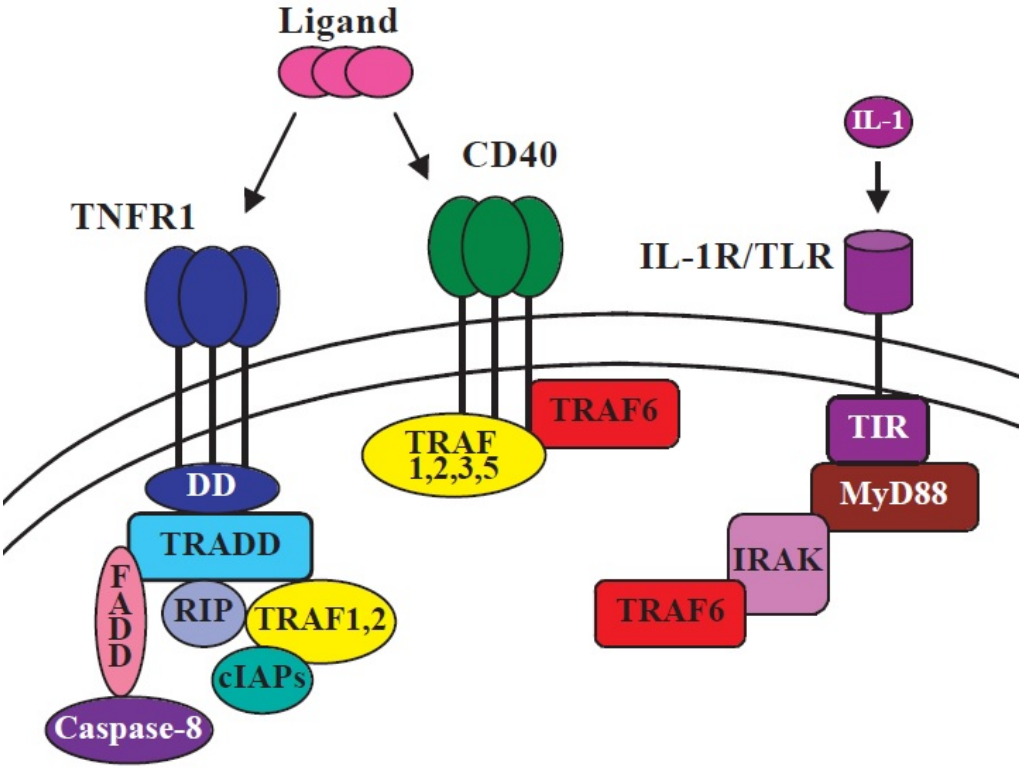


Fig. 5. Pictorial representation of TRAFs interaction with receptors. TNFR family receptors interact directly whereas TLR or IL-1R interacts through adaptor proteins like MyD88 and IRAK with TRAFs (Modified image from Chung et al 2002).

1.3.3. TRAFs act as E3 ubiquitin ligases

Multiple evidence shows that the RING domain acts as E3 ubiquitin ligase and mediates TRAF oligomerization, which further activates E3 ubiquitin ligase activity in cooperation with E2 conjugating complex Uev1A:Ubc13. This ubiquitin ligase catalyzes synthesis of free K48 or K63-ubiquitin chains and attaches the ubiquitin chains to substrate proteins, which are marked for degradation or signal transduction (Yang et al., 2009; Xie, 2013; Zotti et al., 2014). The K48-linked ubiquitination of proteins promotes proteasomal degradation whereas K63-linked ubiquitination leads to activation of downstream signaling molecules (Sorrentino et al., 2008). So far, TRAF2 and TRAF6 have been shown to have E3 ubiquitin ligase activity and catalyze K48 and K63-linked ubiquitination of proteins to elicit biological functions (Lamothe et al., 2007; Pineda et al., 2007; Yin et al., 2009). In contrast, Babu et al show for the first time that K63-linked polyubiquitination of a substrate leads to proteasomal degradation (Babu et al., 2005). Recently, it was shown that HUWE1, is an E3 ubiquitin ligase, generates K48 branches on K63-linked ubiquitin chains formed by TRAF6 i.e., mixed K48-K63 ubiquitination, to activate NF-κB signaling (Ohtake et al., 2016).

1.3.4. TRAF6-mediated downstream signaling

A plethora of studies using primary cultures from TRAF6 knockout (KO) mice have demonstrated that TRAF6 plays a crucial role in IL-1 and lipopolysaccharide (TLR-mediated) signaling (Naito et al., 1999; Lomaga et al., 1999; Kobayashi et al., 2001; Yang et al., 2009). As discussed in section 1.3.2, TRAF6 is known to interact with various receptors to regulate downstream signaling cascades of the receptors. TRAF6 plays an important role in activation of NF-κB, c-Jun N-terminal kinase (JNK), AP-1, p38, ERK1/2, and Akt, and these signaling molecules affect cell survival, apoptosis and stress response (Bradley and Pobers, 2001; Yang et al., 2009). In TGF-β-mediated signaling, TRAF6 interacts with TGF-β receptor (TGF-β) and causes hetero-oligomerization of TGF-βR. This complex further promotes K63-linked auto-polyubiquitination (auto-polyUb) of TRAF6 with cooperation of ubiquitin conjugating enzymes. The polyubiquitinated TRAF6 further activates transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1)-mediated p38 and JNK pathways, which leads to apoptosis (Sorrentino et al., 2009).

1.3.4.1. TRAF6-mediated NF-κB signaling

TRAF6 is a central point or a gatekeeper to divergence of activation of NF-κB and AP-1 signaling pathways. Cell survival and apoptosis occurs through the activation of NF-κB and AP-1 pathways, respectively (Zhang et al., 2009). NF-κB is a transcription factor and plays a paramount role in the regulation of multiple genes, which are responsible for cell proliferation, differentiation, stress and immune response, apoptosis and cell survival as well as neuronal development (Karin and Lin, 2002; Vallabhapurapu and Karin, 2009; Snow et al., 2014). NF-κB is composed of five subunits i.e., RelA (p65), RelB, c-Rel, p50 (p100) and p52 (p105). It forms dimers in different combination. The most predominant form of dimer is p50/p65 in various cell types. Each monomer contains a Rel region in the C-terminal domain, which can bind to DNA to initiate gene transcription whereas p52 and p50 subunits repress gene transcription (Ghosh and Karin, 2002). It binds to a regulatory protein i.e., inhibitor of NF-κB, IκB, through a nuclear localization signal (NLS), as inactive form in the cytoplasm. NF-κB dimers translocate to the nucleus when IκB is phosphorylated and degraded by the 26S proteasome. In the nucleus, NF-κB dimers bind to regulatory elements of the DNA and initiate transcription of genes. The NF-κB-activating signaling has been classified mainly into two types i.e., canonical and non-canonical pathways (Fig. 6; Oeckinghaus et al., 2011). Both NF-κB pathways utilize TRAF family members for activation (Hayden and Ghosh, 2008).

The canonical pathway is the more predominant of the two NF-κB pathways. The NF-κB dimers are present as an inactive form in the cytoplasm bound to regulatory IκB kinase (IKK) proteins (IκBα, IκBβ and IκBγ) through nuclear localization signal (NLS) in NF-κB. Upon stimulation with inflammatory agents such as TNF-α, LPS and IL-1, the corresponding receptors recruit TRAF6, which leads to activation of IKK/NEMO complex. The IκB is phosphorylated by activated kinases and ubiquitinated by 26S proteasome in the cytoplasm. The liberated NF-κB dimers translocate to the nucleus, bind to a regulatory region on DNA and initiate gene transcription to regulate several biological functions (Fig. 6; Hayden and Ghosh, 2008; Oeckinghaus et al., 2011; Ismail et al., 2016; Ji et al., 2016).

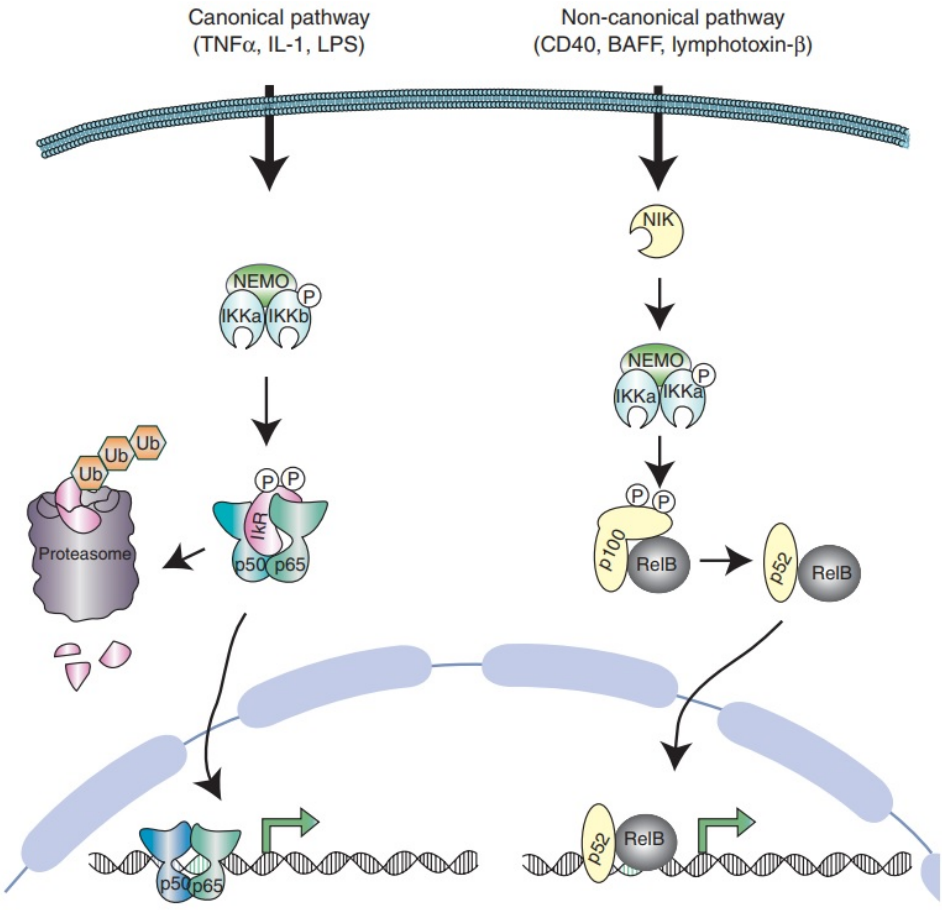


Fig. 6. Schematic representation of NF-κB signaling pathway. The canonical NF-κB pathway induced by TNF, IL-1 and LPS is dependent on activation of IKK. Activation of IKK results in the phosphorylation of IκBα leading to its ubiquitination and subsequent degradation by the 26S proteasome. This causes release of the RelA p50 complex, which translocates to the nucleus and initiates transcription. The non-canonical pathway results in the activation of IKKα by the NF-κB-inducing kinase (NIK), followed by phosphorylation of the NF-κB subunit p100 by IKKα. This results in proteasome-mediated processing of p100 to p52. P52 forms heterodimer with RelB, translocates to the nucleus and initiates transcription (Image taken from Oeckinghaus and Ghosh, 2009).

Moreover, overexpression of TRAF6 also activates NF-κB signaling but RING domain-lacking TRAF6 inhibits NF-κB activation after IL-1 stimulation (Cao et al., 1996).

The non-canonical pathway is activated through B-cell activating factor (BAFF), lymphotoxin-β (LTβ) and CD40. Upon stimulation, in contrast to the canonical pathway, NF-κB inducing kinase (NIK) is activated, which leads to phosphorylation of IKKα in the cytoplasm. Activated IKKα further induces phosphorylation and ubiquitin dependent processing of p100 to p52, which allows translocation of the RelB-p52 heterodimer complex to the nucleus (Fig. 6; Hayden and Ghosh, 2008; Oeckinghaus et al., 2011).

1.3.4.2. TRAF6-mediated phosphatidylinositol 3-kinases (PI3K)/Akt signaling

TRAF6 regulates multiple signal transduction pathways of different receptors. One of the TRAF6 E3 ligase substrates is the serine threonine kinase Akt, also called protein kinase B (PKB) (Wang et al., 2006; Yang et al., 2009). There are three isoforms identified for Akt: Akt1, Akt2 and Akt3 (Yang et al., 2010). Akt regulates a wide range of cellular processes including cell survival, neuronal development, cytoskeletal organization and vesicle trafficking (Wang et al., 2006; Majumdar et al., 2011; Noguchi et al., 2014). Several studies reported that TRAF6 can activate PI3- and Src family kinases and downstream Akt pathway in response to pro-inflammatory stimuli (Wang et al., 2006; Yang et al., 2009 & 2010; Feng et al., 2014). For instance, Wong et al., have shown that stimulation of TRANCE receptor recruits TRAF6 and c-Src and forms a trimeric complex. This complex further potentiates the activation of PI3-kinases leading to membrane localization and activation of Akt (Wong et al., 1999; Arron et al., 2001). Recently, it has been shown that membrane localization of Akt is required to interact with and ubiquitinate through TRAF6 upon stimulation with growth factors (Fig. 7). In the membrane, Akt is phosphorylated and activated through PI3-kinases to regulate downstream signaling (Yang et al., 2009 and 2010; Walsh et al., 2015).

In neurons, TRAF6 signalinging to Akt is not yet clear. One study showed that after traumatic brain injury TRAF6 interacts with GADD34 and inactivates Akt (Farook et al., 2013). Akt is enriched in the plasma membrane of dendritic spines and dendritic shafts in CA1 hippocampal neurons (Znamensky et al., 2003). After receptor activation, Akt translocates to the receptor complexes in the plasma membrane to activate downstream signaling molecules (Znamensky et al., 2003; Kumar et al., 2005; Yang et al., 2009 & 2010). Moreover, activation of PI3K/Akt pathway regulates filopodia like-dendritic protrusions and mushroom-shaped spines in hippocampal neurons from rats (Kumar et al., 2005; Cuesto et al., 2011; Majumdar et al., 2011) and also regulates synapse formation in motor neurons from *Drosophila* (Martin-Pena et al., 2006). The

corresponding data led to the notion that PI3K/Akt pathway is crucial for synaptogenesis in neurons.

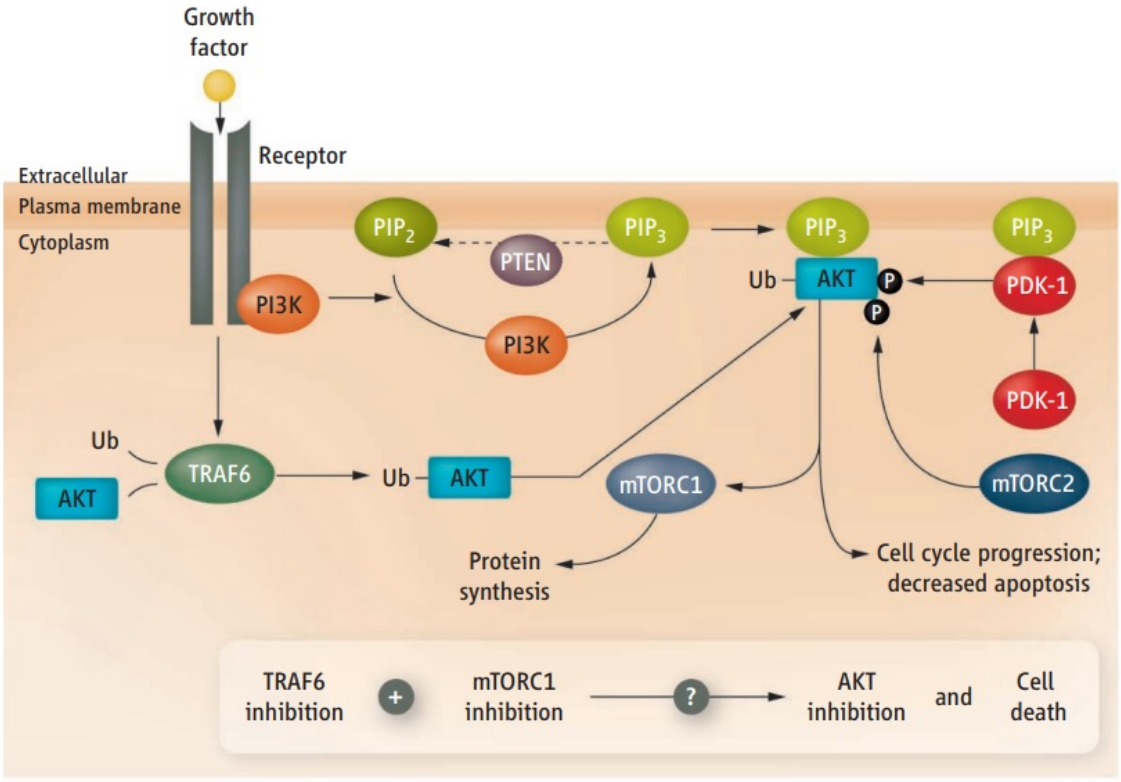


Fig. 7. TRAF6 mediated ubiquitination and membrane localization. Akt resides in cytoplasm and it is activated by interaction with and ubiquitination through TRAF6. Ubiquitinated Akt translocate to membrane, where it is phosphorylated and activate through PI3K (Image taken from Restuccia and Hemmings, 2009).

1.3.5. TRAF6 role in neurons

The role of TRAF6 in neurons is not yet known completely. TRAF6 is expressed in different regions of the brain especially, hippocampus, cerebral cortex, substantia nigra and medulla (Ishida et al., 1996; Zhou et al., 2010; Pranski et al., 2012). TRAF6 KO mice appear normal at birth but die at the age of 17-19 days (Lomaga et al., 1999). TRAF6 deficiency results in impaired perinatal survival and embryogenesis in mice (Lomaga et al., 1999; Naito et al., 1999). In addition, TRAF6 KO mice also show severe osteopetrosis, which is a disorder of bone remodeling caused by impaired bone metabolism, and tooth eruption because of defects in osteoclast differentiation, and impaired RANK, CD40, lipopolysaccharide (LPS) and IL-1 mediated signaling. In the CNS the mice exhibit excencephaly (protrusion of the brain from the skull), which is an embryonic lethal condition (Lomaga et al., 2000). Moreover, TRAF6 KO embryos show reduction in programmed cell death within the developing ventral diencephalon and mesencephalon suggesting that TRAF6 is essential for neuronal development by regulating programmed cell death levels in the brain (Lomaga et al., 2000). TRAF6

binds p75 neurotrophin receptors (p75NTR) to activate NF-κB and JNK signaling pathways to regulate neuronal survival and apoptosis, respectively (Geetha et al., 2005). The p75NTR signaling was impaired in TRAF6 KO mice (Yeiser et al., 2004). In another study, they have shown that nerve growth factor (NGF) ligand binding to p75NTR results in recruitment of TRAF6 leading to ubiquitination of p75NTR in neurons (Geetha et al., 2005). This NGF binding to p75NTR also facilitates the association of TRAF6 to presinillin 1 and degradation of p75NTR (Powell et al., 2009). All this information suggests that TRAF6 mediates p75NTR signaling to regulate NF-κB and JNK activation in neurons.

Moreover, TRAF6 also activates the PI3K/Akt/WASP pathway to promote the formation of filopodia and regulate filopodial extension and axon branching in sensory neurons (Zhou et al., 2007; Wang et al., 2006). The actin-based cytoskeleton is the structural core of dendritic protrusions in young neurons and dendritic spines in mature neurons (Cingolani and Goda, 2008). Thus, TRAF6 may play a role in the regulation of the actin cytoskeleton as a fundamental cellular process in the formation, stabilization and retraction of dendritic spines.

1.3.6. TRAF6 associated with neurodegenerative diseases

TRAF6 is associated with neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington disease (HD) (Zucchelli et al., 2010 & 2011; Vilotti et al., 2012; Popovic et al., 2014). It was found that TRAF6 binds misfolded mutant DJ-1, α-synuclein and N-terminal mutant of huntingtin protein (N-HTT) proteins involved in the pathogenesis of PD, AD and HD, respectively. Mutant DJ-1, α-synuclein and N-HTT proteins are substrates for TRAF6 E3 ubiquitin ligase activity. In the disease conditions, TRAF6 promotes atypical polyubiquitination of K6, K27 and K29 linkage formation instead of conventional K63-polyubiquitination. Thus, TRAF6 promotes polyubiquitination of mutant DJ-1, α-synuclein and N-HTT protein aggregate formation (Zucchelli et al., 2010 & 2011; Vilotti et al., 2012; Popovic et al., 2014). It has been shown that TRAF6 levels were elevated in tissue from PD patients. Increased TRAF6 levels were caused by loss of Parkin, an E3 ligase, in a PD mouse model (Chung et al., 2013). Geetha and co-workers have found that Aβ-impairs the interaction of p75NTR and TRAF6/p62, and thereby impairing TRAF6 mediated polyubiquitination of p75NTR and decreasing NF-κB activity that leads to neuronal death in AD. Overexpression of TRAF6/p62 reversed the neuronal death by ubiquitination of p75NTR and activation of NF-κB (Geetha et al., 2012a and 2012b). In another study, they have shown that tau is a substrate for TRAF6 and interacts with UBA domain of p62 (a shuttling protein to 26S proteasome) after K63-linked polyubiquitination of tau by TRAF6. Thereby, TRAF6 promotes proteasomal

degradation of tau in brain homogenates from AD patients (Babu et al., 2005). Thus, TRAF6 is a versatile and indispensable signal transduction regulator for multiple receptors, and aberrant functions of TRAF6 may contribute to the occurrence and progression of neurodegenerative diseases.

1.4. The role of NF-κB signaling in neurons

NF-κB is one of the downstream signaling molecules of TRAF6 and regulates transcription of several genes (Xie, 2013). NF-κB complexes are present in all cell types of the nervous system including neurons, astrocytes, microglia and oligodendrocytes (Kaltschmidt and Kaltschmidt, 2009). On the subcellular level, NF-κB is enriched in the PSD and dendritic compartment to transmit synaptic signals into gene transcription changes in neurons (Suzuki et al., 1997). Basal NF-κB activation occurs in cerebral cortex during development (Methot et al., 2013). This NF-κB activation is mediated by several endogenous factors such as TNF-α and Fas ligand, NGF, glutamate, depolarization and secreted form of β-amyloid precursor protein (β-APP) in neurons (Kaltschmidt et al., 1995; Barger et al., 1996; Bruce et al., 1996; Carter et al., 1996; Meffert et al., 2003). It is also activated by synaptic transmission and stimulation of various receptors leads to retrograde transport of NF-κB into the nucleus (Klenke et al., 2013; Engelmann and Haenold, 2016). One example for receptor activation is binding of glutamate to NMDA receptors that leads to entry of calcium into synapse and thereby, opening of more calcium channels at the plasma membrane and increase in synaptic intracellular calcium, which can activate local NF-κB signaling (Lilienbaum and Israël, 2003; O'Riorden et al., 2006). Moreover, the basal NF-κB activation also occurs during neuronal development by transient increase in calcium levels (Meffert et al., 2003). Multiple evidence has shown that NF-κB translocates to the nucleus from cytoplasm by calcium elevation in distal dendrites and locally in synapses during the neuronal activation, thereby, communicating the calcium signal from dendrites to nucleus (Meffert et al., 2003; Lilienbaum and Israël, 2003).

NF-κB functions as a messenger for synapse to nucleus communication in neurons (Suzuki et al., 1997). It is required for neuronal development and plasticity-associated synaptogenesis in response to stimuli in hippocampal neurons (Boersma et al., 2011). NF-κB controls circuit formation of mossy fiber pathway and also controls axogenesis by controlling FOXO1 and Protein kinase A (PKA) transcription in hippocampus especially in the dentate gyrus. NF-κB deficient mice also show reduced mossy fiber bouton size and number in brain sections (Imielski et al., 2012). Moreover, overexpression of RelA in hippocampal neurons showed an increase in excitatory

synapse number but unaffected inhibitory synapse numbers (Boersma et al., 2011), which is similar to the *Nptn-/-* phenotype observed in cultured neurons (Herrera-Molina et al., 2014). Recent studies also provide evidence for an involvement of IKK/NF-κB in synaptogenesis in vivo and in vitro (Russo et al., 2009; Christoffel et al., 2011; Imielski et al., 2012; Schmeisser et al., 2012). Moreover, NF-κB ablation increased apoptosis and neurogenesis, and also impaired performance in pattern separation tasks. Phenotypes were restored by reactivation of NF-κB in NF-κB deficient mice (Imielski et al., 2012). RelA knockout mice show severe effects on embryogenesis, which were rescued by concurrent deletion of TNFR1 (Beg et al., 1995; Rosenfeld et al., 2000; Alcamo et al., 2001). Similar effects were found in IκB-α-deficient mice (Beg et al., 1995). Moreover, RelA- and TNFR1-deficient mice show impairment in hippocampus-dependent spatial learning in the radial arm maze task (Meffert et al., 2003).

2. Hypothesis and aims of the thesis

Considering that

1) Neuroplastin deficient mice as well as cultured hippocampal neurons derived from these mutants displayed reduced numbers of excitatory synapses, (Herrera-Molina et al., 2014; Amuti et al., 2016; Bhattacharya et al., 2016)
2) Neuroplastin cytoplasmic domain possesses a binding site for the adapter protein TRAF6,
3) TRAF6 is a clear candidate to mediate neuronal signaling via a number of kinases and NF-κB pathways potentially implicated in synaptogenesis, synaptic plasticity and learning and memory (see Section 1.4.),

the central hypothesis of my PhD thesis is that neuroplastins bind TRAF6 to activate downstream signaling pathways i.e., PI3K/Akt or NF-κB pathways to promote early spinogenesis in young hippocampal neurons.

To test this hypothesis, I aimed

a) investigating neuroplastin and TRAF6 direct interaction. Neuroplastin-TRAF6 interaction was characterized and proven to be direct by performing co-immunoprecipitation and pulled-down assays, immunocytochemistry, Biacore experiments, and in silico modelling.

b) investigating neuroplastin-TRAF6 interaction initiate signaling pathways in heterologous cells and in cultured hippocampal neurons. For this, I performed biochemical and immunocytochemical approaches, mRNA knockdown using siRNA-mediated technology and several pharmacological drugs unravel specific activation of the proposed pathways.

c) defining what is the participation of the neuroplastin-TRAF6 signaling cascades in formation of filopodial-like structures as well as in dendritic protrusions during spinogenetic period *in vitro*. For this, I performed both gain (neuroplastin overexpression) and loss (TRAF6 knockdown) of function experiments to define the role of PI3K/Akt, NF-κB, and other signalling pathways during synaptogenesis in wild type and *Nptn-/-* neurons and in HEK cells.

3. Materials and methods

3.1 Materials

Tab.1 List of primary antibodies

S.No.	Antibody	Species	dilution	CAS/Company
1	anti-GFP	Rabbit	IF & IB: 1:2500	(ab290) Abcam
2	anti-TRAF6	Mouse	IF: 1:100 IB: 1:1000	(sc-8709) SantaCruz
3	anti-TRAF6	Rabbit	IF: 1:100	(sc-7221) SantaCruz
4	anti-Actin	Mouse	IB: 1:1000	(A5441) Sigma
5	anti-GST	Goat	1:1000	GE Healthcare
6	anti-MAP2	Guinea pig	IF: 1:1000	Synaptic systems
7	anti-Flag	Mouse	IB: 1:2000	(F1804) Sigma
8	anti-RelA	Rabbit	IF: 1:500	(sc-372) SantaCruz
9	anti-Np	Sheep	IF: 1:500	(AF5174) R and D systems
10	anti-Np65	Goat	IF: 1:500	R and D systems

Secondary antibodies conjugated to Alexa Fluor® -488, Cy3 and Cy5 secondary antibodies produced in donkey were used in the study and they were purchased from Jackson ImmunoReaserch.

Tab.2 Commonly used buffers and kits:

Material	Composition/company
10X PBS	1.37 M NaCl, 2.7 M KCl, 14 mM KH_2PO_4, 43 mM Na_2HPO_4, dd H_2O, pH 7.3-7.4
10X TBS	0.2 M Tris-base, 1.37 M NaCl, dd H_2O, pH 7.6
µMACS GFP isolation kit	µMACS (#130-091-125)
NucleoBond® Xtra Midi EF Kit	Macherry-Nagel
NucleoSpin® Gel and PCR clean-up kit	Macherry-Nagel
BC assay protein quantification kit	Interchim (#UP40840A)

Tab.3 Peptides used in the study

Peptides	Sequence
Enplastin	DPKRNDLRQNPSITWIR
Neuroplastin cytoplasmic specific peptide	E_{244}KRKRPDEVPDDDEPAG$_{260}$-amide (Numbering according to Np55)
Scrambled peptide	P_{258}AGPMKTNSTNNHKDKNL$_{275}$-amide

3.2. Methods

3.2.1. Molecular biology

Tab.4 Primers used in the study

Construct		Primer sequence
Np65	forward	5'-TCA AGC TTG CCA CCA TGT CG-3'
	reverse	5'- GGC GAT GGA TCC ATT TGT GTT TC-3'
Np65$^{\Delta id}$	reverse	5'-GGA TCC TGG CCT CTT CCT CTT CTC ATA C-3'
Np65 E367A	forward	5'- GAA GAG GCC AGA TGC GGT TCC TG-3'
	reverse	5'- CAG GAA CCG CAT CTG GCC TCT TC-3'
Np65 PED	forward	5'-GAG GAA GAG GGC AGA TGC GGT TCC TGC TG-3'
	reverse	5'-CAG CAG GAA CCG CAT CTG CCC TCT TCC TC-3'
GST-TRAF6	forward	5'-GAC AGG ATC CTC ATG AGT CTC TTA AAC-3'
	reverse	5- TAC GAA TTC CTA CAC CCC CGC ATC AGT A-3'
GST-DN TRAF6 (290-530aa)	forward	5-GCG TCG GAT CCA TAT GGC CGC CTC T-3'
TRAF6-GFP	forward	5'- GTG AAG CTTCTA ATG AGT CTC TTA AAC TGT GA -3'
	reverse	5'- ATA AGG ATC CCT ACA CCC CCG CAT C -3'
DN TRAF6-GFP (290-530aa)	forward	5'- GTG AAG CTT CTA ATG GCC GCC TCT -3'

3.2.1.1. Polymerase chain reaction (PCR) amplification

Constructs were cloned by PCR amplification with a constant program having different Tm and 95°C for 5', 95°C for 45 sec, different Tm, 72°C for 1', 72°C for 10', 4°C for infinite and 35 cycles. The amplicons were separated by ethidium bromide 1% agarose gel electrophoresis with 80mA current for 30 min, visualized by UV-light, cut from the gel and purified using NucleoSpin® Gel and PCR clean-up kit (Macherry-Nagel).The purified amplicons were digested with restriction enzymes for 2 hour (h) at 37°C and separated by gel electrophoresis. The digested amplicons were inserted into a suitable vector using T4 ligase and incubated at room temperature for overnight.

3.2.1.2. Transformation

The cloned DNA (cDNA) was transformed into XL10 GOLD E.coli competent cells (bacteria) by heat shock method. The transformed bacteria were grown on agar plate and incubated at 37°C for overnight.

3.2.1.3. Mini preparation

Single colonies from the agar plate were cultured overnight in 2 ml LB-medium with antibiotic at 37°C and prepared a master plate to purify correct plasmid. Next day, media was transferred into 1.5 ml of eppendorf tube and centrifuged at 1000 x g for 3

min. The supernatant was discarded. The pellet was resuspended in 300 µl P1 buffer and lysed with 300 µl P2 buffer, mixed gently and incubated for 5 min at room temperature.The lysis reaction was neutralized and precipitated the proteins and mRNA by adding 300 µl P3 buffer and incubated for 5min on ice. The precipitated proteins and mRNA were separated by centrifugation at 20800 x g for15 min at 4°C. 800 µl of supernatant was transferred into a new eppendorf tube and added 500 µl of isopropanol and vortexed and incubated at room temperature for 10 min. Again, it was centrifuged at 20800 x g for 15 min at 4°C to separate the precipitate from the tube. The supernatant was discarded and 700 µl of ice cold 70% ethanol was added to pellet. Again, it was centrifuged at 20800 x g for 5 min at 4°C and supernatant was discarded and pellet was dried at 37°C for 45-60 min. The dried pellet was reconstituted with 20-30 ml of 10mM Tris (pH7.5).

For sequencing, the DNA plasmids were sent to SeqLab (Sequence Laboratories, Göttingen) and plasmid sequence was confirmed with nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Tab.5 Mini-preparation (Plasmid isolation) buffers

Buffer	Composition
P1 Buffer	50 mM Tris/HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A, stored at 4°C
P2 Buffer	200 mM NaOH, 1% (w/v) SDS
P3 Buffer	3 M potassium acetate, pH 5.5, stored at 4°C

3.2.1.4. Plasmid purification

For mammalian cells transfection, cDNA plasmids were purified by NucleoBond® Xtra Midi EF Kit (Macherry-Nagel).The plasmid concentration was measured by NanoDrop1000 (peQLab).

3.2.1.5. Plasmids and siRNA

In this study, I used rat Np55 and Np65 with DDEP [Np55(+)-GFP and Np65(+)-GFP], Np55 and Np65 without DDEP [Np55(-)-GFP and Np65(-)-GFP] cDNA's tagged with GFP plasmids. The mouse N-terminal Flag tagged TRAF6 (Flag-TRAF6) mammalian expression plasmid was purchased from Addgene (#21624, GenBank: BAA12705.1). Neuroplastin mutants with HindIII and BamH1 restriction sites were generated from Np65(+)-GFP plasmid. N-terminal GST tagged TRAF6 (GST-TRAF6) and N-terminal GST tagged dominant negative TRAF6 (GST-DN TRAF6) (289-530aa) bacterial expression plasmids with BamH1 and EcoR1 restriction sites were generated by PCR amplification. Scrambled siRNA (sc-37007) or TRAF6 siRNA (sc-36717) were purchased from Santa Cruz.

3.2.2. Cell culture

3.2.2.1. Human embryonic kidney (HEK) cells

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin at 37^{o}C and 5% CO_2.

3.2.2.2. Hippocampal neurons

The rat hippocampal neurons were collected from embryonic day 18 (E18) of pregnant rat and was trypsinized, and washed out trypsin with 1X horse serum in DMEM solution. The hippocampi were then mechanically dissociated into a single-cell suspension using a glass tube with small orifice. 35000 neurons were plated in 12 well plate or 3x10^{6} neurons in a 75 cm^{2} flask. After 1 h, the media was replaced with Neurobasal media containing 2% of B27 supplement and 1% of L-glutamine and 1% of penicillin/streptomycin. Again the media was replaced with fresh media after 24 h and half of the media was replaced with fresh media every 5 days.

3.2.2.3. Transfection

In this study, the cells were transiently transfected with plasmid DNA by Lipofectamine 2000 (#11668-019, Invitrogen) in optiMEM media (GIBCO). Primary neuron cultures were transfected on day 6-7 to check the dendritic protrusions during neuronal development. The siRNA (30 nM) was transfected with siLentFect (Bio-Rad). For knockdown experiments, the cells were transfected either with scrambled siRNA or siTRAF6. After 24 h, the cells were transfected with cDNA plasmids for an additional 24 h.

3.2.3. Biochemical experiments

3.2.3.1. Protein determination

After collecting lysates from the cells as described in section 3.5.2, total protein was determined by bicinchoninic acid assay using BC assay protein quantification kit (#UP40840A, Interchim) according to the manufacturer's instructions.

3.2.3.2. Co-immunoprecipitation (Co-IP) of GFP tagged neuroplastin isoforms from HEK cell lysate

After 24 h of transfection, the cells were washed with cold PBS and incubated with RIPA lysis buffer for 5 min on ice with periodic shaking. The cells were scraped and sheared with a 1-ml syringe, and centrifuged (Eppendorf 5417r) at 13,000 x g for 10 min at 4^{o} C. The supernatant was collected and incubated with GFP antibody coupled magnetic beads (µMACS) at 4° C for 4 h. The immunoprecipitate complexes were eluted using µMACS GFP isolation kit (#130-091-125) according to manufacturer's instructions. The eluted complexes were boiled for 10 min at 95°C and subjected to

sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) as below in section 3.6.6.

Tab.6 RIPA lysis buffer compositions

S.No.	Components	CAS	Company
1	20 mM Tris pH 7.5	77-86-1	Sigma
2	100 mM NaCl	7647-14-5	Sigma
3	1 mM EDTA	60-00-4	Sigma
4	10% glycerin	3783.2	Roth
5	0.1% SDS	2326.2	Sigma
6	1% Triton X-100	T9284	Sigma
7	1 mM AEBSF	A1421,001	Applichem
8	1 mM sodium orthovanadate	S6508	Sigma
9	1 mM sodium molybdate	331058	Sigma
10	1 mM N-Ethylmalemide	04259	Sigma
11	20 mMsodium fluoride	S1504	Sigma
12	20 mM glycerol-2-phosphate	G9422	Sigma
13	10 mM potassium hydrogen phosphate	P749.2	Roth
14	10 mM sodium pyrophosphate	7722-88-5	Sigma
15	Protease inhibitor cocktail	04693159001	Roche

3.2.3.3. GST-TRAF6 and GST-dominant negative TRAF6 (GST-DN TRAF6) expression and purification

GST, GST-TRAF6 and GST-DN TRAF6 were transformed into E.coli BL21 (DE3) bacterial strain with heat shock method at 42°C for 45 sec. Next day, the bacteria were inoculated into LB media and incubated at 37^{o}C for overnight. The 2% over night culture was used for secondary culture and grown for 3 h in fresh LB media containing ampicillin. The bacteria were induced with 0.5 mM isopropyl-beta-D-thiogalactoside (IPTG) when it reached OD_{600}-0.6 and grown at 25^{o}C for additional 6 h. Protein induction was evaluated by SDS-PAGE and followed by coomassie blue staining. I centrifuged the remaining culture at 8000 rpm (Sorvall, SLC4000) for 30 min. The pellet was dissolved in resuspension buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl and protease inhibitor (Roche)). The bacteria were lysed by sonication for 30 min with 30 cycles, 50% power until the lysate become semitransparent. I centrifuged the lysate at 13000 rpm at 4^{o}C for 30 min. Once again, I re-suspended the pellet in resuspension buffer and repeated the centrifugation step. The pellet was dissolved in washing buffer (50 mM Tris-HCl pH 8.0, 2 M Urea, 1 mM EDTA, 0.5% Triton-X100, 0.1 M NaCl, 10 mM DTT) and centrifuged at 13000 rpm for 10 min at 4^{o}C. The pellet was dissolved in

freshly prepared denaturating buffer (50 mM Tris-HCl pH 8.0, 8 M Urea, 20 mM DTT) and kept on ice for 30 min to denature the protein. The dissolved solution, which contains 8 M urea, was gradually diluted with renaturating buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, 1 mM DTT) on ice slowly until the final concentration reached to 0.5 M urea and kept on ice for an additional 24 h to refold the protein properly. The diluted solutions were kept on ice for additional 24 h to fold it properly. The protein solution was concentrated by centrifugation using 20 kDa cut off 20 ml concentrator with manufacturer's instructions (Thermo Scientific).

3.2.3.4. Protein dialysis and purification

Cellulose tubular membrane was boiled for 2 min at 100°C and soaked in 1x TBS buffer (50 mM Tris-HCl pH 8, 50 mM NaCl). The concentrated proteins were added to the membrane and left in the 1x TBS buffer with gentle spinning at 4°C. The proteins were dialyzed for 48 h with dialysis buffer for four changes to remove residual urea in the protein. 2 ml of Glutathione Sepharose 4B (GE Healthcare) beads were washed with 20 bed volumes (bed volume 1.5 ml) of binding buffer provided with purification kit (GE Healthcare) by centrifugation 500x g for 5 min to remove ethanol from the beads. The dialyzed proteins were added to the beads and incubated for 1 h at 4°C. The beads were washed with 10 bed volumes of binding buffer 4-5 times at 500 x g for 5 min. Some parts of the beads were stored at 4°C for pull-down assay and remaining beads were used to elute the recombinant proteins with freshly prepared 10 mM reduced glutathione in 50 mM Tris pH 8.5 overnight at 4°C. The eluted proteins were collected by centrifugation at 500 x g for 10 min. The protein concentration measured using different concentrations of BSA by Bradford assay.

3.2.3.5. GST pull-down assay

The 5 µg of fusion protein coupled beads (GST, GST-TRAF6 and GST-DN TRAF6) were incubated with lysate from HEK cells transfected with Np65(+)-GFP for 1 h at 4°C in 500 µl RIPA lysis buffer. The beads were washed and eluted with pre-warmed SDS sample buffer. The eluted complexes were boiled for 10 min at 95°C and resolved by SDS-PAGE.

Tab.7 Solutions used for pull-down assay

Buffer/Solutions	Composition
Coomassie Brilliant Blue	20% methanol, 10% acetic acid, 0.1% Coomassie R250, dd H_2O
Detainer for Coomassie gels	20% ethanol, 10% acetic acid, dd H_2O
Conserver for Coomassie gels	35% ethanol, 3% glycerin, dd H_2O

3.2.3.6. Immunoblotting

The cell lysates were collected as mentioned in Section 3.5.2. The SDS sample buffer was added to supernatant and boiled for 10 min at 95^{o}C. The samples were resolved by SDS-PAGE. The gel was electro-transferred on to a nitrocellulose membrane and blocked with 5% non-fat milk in TBS containing 0.1% of Tween 20 with 0.01% azide solution for 1 h. Then, the membranes were incubated with mouse anti-Flag, rabbit anti-GFP, mouse anti-TRAF6, mouse anti-β-actin antibodies for indicated time and then, incubated with corresponding secondary antibody conjugated to horseradish peroxidase enzyme. The secondary antibodies were visualized by ECL solution using Intas ECL system (Intas Chemocan ECL Imaging).

Tab.8 Buffers and solutions used for immunoblotting

Buffer	Composition
2x SDS buffer	125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerin, 0.2% bromophenol blue, 10% β-mercaptoethanol, double distilled water (dd H_2O)
1X TBS	10X TBS, dd H_2O
1X TBS-T	10X TBS, Tween-20, dd H_2O
10X TGS buffer	Bio-Rad (161-0772)
10X Blot buffer	0.25 M Tris-base, 1.92 M glycine, 0.2% SDS, dd H_2O
1X Blot buffer	10X blot buffer, methanol, dd H_2O
Restore™ Western Blot Stripping Buffer	Thermoscientific (21059)
Ponceau S	0.5% Ponceau S, 3% TCA, dd H_2O

3.2.3.7. Plasmon Resonance Technology – Biacore

Characterization of the neuroplastin-TRAF6 direct interaction and measurement of the K_d value was performed by Johannes Hradsky in the lab of Dr. Michael Kreutz. The experiment was done essentially the procedure described in Reddy et al., (2014).

3.2.4. Cell biology

3.2.4.1. Immunocytochemistry

HEK cells were fixed with ice cold methanol for 2 min, washed with cold PBS for four times and blocked with 1% horse serum in hank's balanced salt solution (HBSS) for 30 min. Hippocampal neurons were fixed with 4% p-formaldehyde (PFA) for 10 min, permeabilized and blocked with PBS containing 10% fetal bovine serum in 0.1% Triton X-100 for 30 min. The cells or neurons were incubated with anti-GFP rabbit, anti-TRAF6 mouse, anti-TRAF6 rabbit, anti-neuroplastin sheep and anti-MAP2 guinea pig antibodies for overnight at 4°C. The primary antibodies were visualized with fluorescent Alexa-488, Alexa-568 and Cy5 conjugated secondary antibodies produced

in donkey for 1 h and nuclei were stained with DAPI. The coverslips were mounted with Mowiol.

Tab.9 Solutions used for immuncytochemistry

Buffer	Composition
HEK cells blocking buffer	1x Horse serum in hanks balanced salt solution (HBSS)
Neurons blocking buffer	10% fetal bovine serum in 0.1% Triton X-100 in HBSS
4% Paraformaldehyde (PFA)	Paraformaldehyde powder, HCl (Dilute), 1 N NaOH, 1X PBS, dd H_2O
Mowiol	2.4 g Mowiol, 0.2 M Tris pH 8.5, 0.02% Sodium Azide

3.2.4.2. RelA translocation assay

8 DIV hippocampal neurons were used for this assay. Before 1 h of Enplastin treatment, the media was changed to neurobasal media with 1% L-glutamine to suppress the basal stimulation. Then, the neurons were fixed with 4% PFA, washed four times with cold PBS and blocked with PBS containing 10% fetal bovine serum in 0.1% Triton X-100 for 30 min. The neurons were incubated with anti-RelA rabbit and anti-MAP2 guinea pig antibodies for overnight at 4°C. The primary antibodies were visualized with fluorescent Alexa-488 and Alexa-Cy3 conjugated secondary antibodies produced in donkey for 1 h and nuclei were stained with DAPI. The nuclei and their cell body defined as regions and their mean fluorescence was measured using ImageJ-software.

3.2.5. Microscopy and Image analysis

3.2.5.1. Confocal microscopy

Images were acquired from confocal microscope equipped with 63X 1.4 oil immersion objective lens using CCD camera (Leica, Mannheim, Germany). In HEK cells, filopodia number and length were quantified using a MATLAB-based algorithm, FiloDetect, with some modifications (Nilufaret al., 2013). I run the algorithm for every image, and adjusted the image threshold to avoid false filopodia detection and to quantify precise filopodia length and number. The filopodia number per µm was calculated from perimeter of the cell using ImageJ. In neurons, the dendritic protrusions were quantified manually and the dendritic length was measured by ImageJ using maximum intensity projection images from confocal z-stack images. The dendritic protrusions were considered between 0.5 µm and 20 µm and more than 20 µm considered as a branch.

3.2.5.2. Fluorescent microscopy

Fluorescent proteins were visualized using a Zeiss AXIO Imager A2 microscope equipped with a CCD camera (Visitron Systems) using a 63X 1.4 objective. Image acquisition was performed using VisiView software (Visitron Systems, camera binning = 1, pixel size = 0.072 x 0.072 µm, pixel depth = 8 bytes). Image threshold was adjusted using automatic threshold. Middle point was decided in the center of soma and run the image for sholl analysis. Lists of raw data were automatically generated as an Excel-compatible file for further statistical analysis.

Images were prepared using Adobe Photoshop CS6 (Adobe).

3.2.5.3. Fluorescence resonance energy transfer (FRET) and images quantification

For FRET imaging, HEK cells were transfected with Np65-ECFP and Np65-EYFP plasmids 24 h prior to FRET measurement. Cells were maintained in extracellular solution (ECS) buffer (145 mM NaCl, 5 mM KCl, 20 mM glucose, 10 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgSO_4$ pH 7.4) at 37 °C. Imaging was performed with TCS-SP5 confocal microscope using argon laser (excited at 458 nm for CFP and emission was between 462-510 nm; at 514 nm for YFP and emission was between 518-580 nm). Cells were imaged with 63X 1.4 oil immersion inverted objective.

3.2.6. Data and statistical analysis

Data was quantified using GraphPad Prism 5 (GraphPad Software) and statistical analysis was performed using Student's t-test.

4. Results

One main focus of this PhD work was the characterization of the interaction between the CAM neuroplastin with the signaling adaptor protein TRAF6. Also, I describe the activation of signaling pathways downstream of these proteins as well as the role of these mechanisms on the regulation of actin-based cell membrane microstructures. For this purpose, I used primary hippocampal neurons from rat embryos and mouse pups and, as heterologous cell system, HEK cells. The importance of the TRAF6 binding site in neuroplastin for the interaction with TRAF6 is described in Section 4.1. The role of TRAF6 in neuroplastin-induced filopodia formation in HEK cells and dendritic protrusion formation in neurons can we found in sections 4.2 and 4.5, respectively. The participation of signaling pathways regulated by neuroplastin-TRAF6 i.e., PI3K/Akt and NF-κB in is reported in sections 4.4 and 4.6, respectively. In Section 4.7, the NF-κB role in neuroplastin-induced dendritic protrusion formation was characterized further. Additionally, a potential involvement of neuroplastin-TRAF6 in dendritic complexity was explored (Section 4.8).

4.1. Characterization of the binding between Neuroplastins 55/65 and TRAF6

Based on the prediction of a TRAF6 binding site in the cytoplasmic domain of Np55 and 65, binding of both neuroplastins to TRAF6 was evaluated. For this, GFP-tagged Np65 constructs with (Np65(+)) or without (Np65(-)) the DDEP insertion in the C-terminus and GFP as control were co-transfected with Flag-TRAF6 in HEK cells. After 24 h, lysates were prepared and immunoprecipitated with anti-GFP antibody coupled to magnetic beads. The eluted fractions containing immunoprecipitated complexes were resolved using SDS-PAGE. The immunoblotting analysis with anti-Flag antibody revealed that TRAF6 was co-precipitated with both Np65 isoforms, but not with GFP (Fig. 8A). Similar experiments using Np55(+) and Np55(-) plasmids served to demonstrate that both Np55 isoforms also co-precipitate Flag-TRAF6 (Fig. 8B). These results demonstrate that TRAF6 can be in protein complexes with Np55/65 regardless of the presence of the DDEP mini-exon. Unfortunately, attempts to show specific co-immunoprecipitation of neuroplastin and TRAF6 from Triton X-100-extracted rat brain lysates failed, as signals were also detected in the control samples, potentially because the lysis conditions from adult rat brain were not optimal (data not shown). It may be worth in the future to repeat these experiments with endogenous neuronal protein precipitation from primary cultures or brain lysates using different detergents.

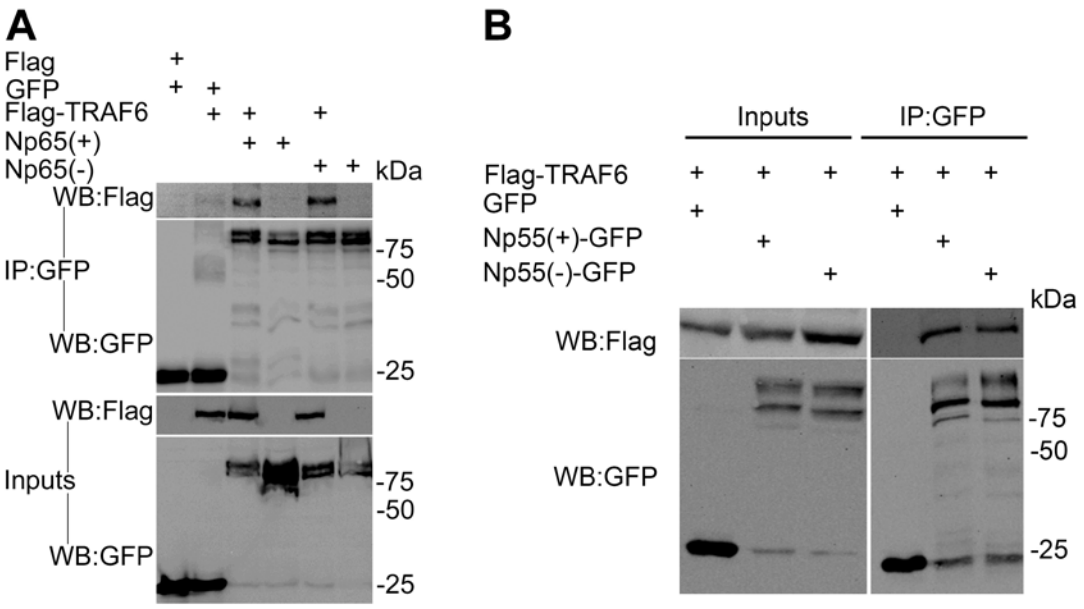


Fig. 8. Np55 and Np65 co-precipitate with TRAF6. A,B) HEK cells were transfected with the indicated constructs and lysed with RIPA lysis buffer. From the extracts neuroplastins were immunoprecipitated with anti-GFP antibody coupled to magnetic beads. Precipitated complexes (IP) were resolved by SDS-PAGE and immunoblotted with anti-Flag or anti-GFP antibodies (n=3 experiments).

As point mutation or deletion of key amino acids in the TRAF6 binding site in IRAK, CD40, p75NTR or TGF-β1 receptor reduces the interaction with TRAF6 (Khursigara G, et al., 1999; Ye et al., 2002; Sorrentino et al., 2008), I generated three different mutants for the TRAF6 binding site in Np65 to characterize the interaction of neuroplastin with TRAF6 as described in Fig. 9A. Wild type (WT) Np65(+)-GFP or Np65 mutants were expressed at comparable levels and localized in the plasma membrane in these transfected HEK cells (Fig. 13). Then, I evaluated co-precipitation of Flag-TRAF6 with GFP, Np65(+)-GFP, $Np65^{\Delta id}$-GFP and $Np65^{PED}$-GFP. Co-precipitation of all three neuroplastin mutants with Flag-TRAF6 was significantly lower compared to WT Np65(+)-GFP (Fig. 9B, D; GFP: 5.17±1.64%, $Np65^{\Delta id}$-GFP: 30.48±8.11%, $Np65^{PED}$-GFP: 46.53±16.29% vs Np65(+)-GFP: 100±1.75%,). In other experiments, the Np65 mutant carrying a single point mutation $Np65^{P}$-GFP also showed reduced co-precipitation of Flag-TRAF6 compared to WT Np65(+)-GFP (Fig. 9C, D; $Np65^{P}$-GFP: 30.96±9.25%) further supporting that neuroplastin interacts with TRAF6 through it's TRAF6 binding motif.

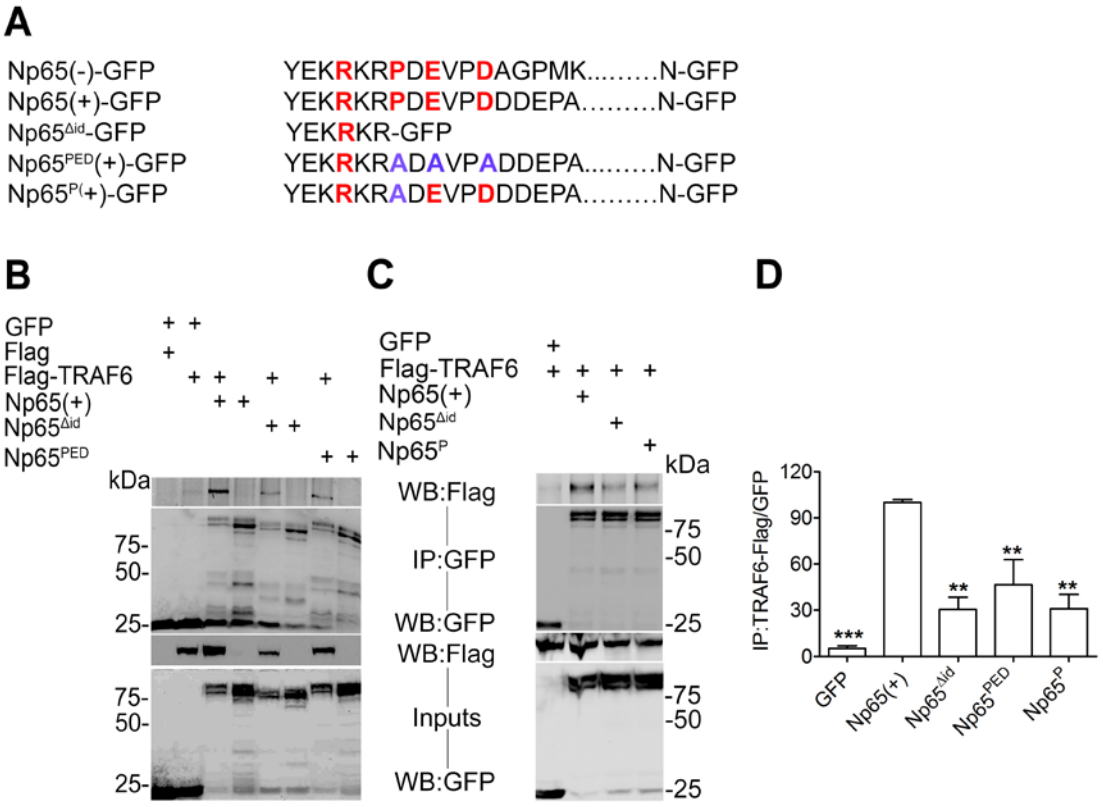


Fig. 9. Mutations in the TRAF6 binding site of neuroplastin reduce co-precipitation of TRAF6. A) Sequence of the TRAF6 binding motif ([basic residue]-X-X-P-X-E-X-X-[aromatic/acidic residue]) in the cytoplasmic domain of Np65 and mutant constructs. Red letters indicate critical amino acids, which were mutated (blue letters). Np65$^{\Delta id}$-GFP: Deletion of intracellular domain (Δ365-397aa) from Np65(+)-GFP isoform. Np65^{PED}-GFP: Replacement of 365 Pro→Ala, 367 Glu→Ala, 370 Asp→Ala. Np65^{P}-GFP: Replacement of 365 Pro→Ala . B, C) HEK cells were either transfected with GFP, wild-type (WT) Np65(+)-GFP, Np65$^{\Delta id}$-GFP, Np65^{PED}-GFP, Np65^{P}–GFP and co-transfected with Flag-TRAF6 or Flag constructs. Co-immunoprecipitations (IP) were performed as mentioned in Fig. 8. D) Densitometric analysis of the ratio between Flag and GFP signals from the precipitated fractions (n=3-6 experiments, Mann-Whitney U test, **p<0.05, ***p<0.001 vs Np65(+)-GFP).

To further characterize the neuroplastin TRAF6 interaction, GST pull-down experiments were performed. First, I generated GST-TRAF6 and a dominant negative version (GST-DN TRAF6), which is the C-terminal domain of TRAF6 and known as sufficient to interact with the TRAF6 binding site in other proteins (Ye et al., 2002; Fig. 10A). The GST plasmids were expressed in *E.coli* and protein production was evaluated using SDS-PAGE before and after IPTG induction. Coomassie staining was used to visualize the proteins. As recombinant GST-TRAF6 and GST-DN TRAF6 are hydrophobic with low solubility, I chose gradual dilution method (see Materials and methods) to purify the recombinant proteins. As expected, the apparent molecular weight of GST-TRAF6 and GST-DN TRAF6 were 86 and 54 kDa, respectively (Fig. 10B,C). These purified fusion proteins were incubated with HEK cell lysis containing Np65(+)-GFP or brain lysates from an adult rat. Immunoblotting analysis revealed that GST-TRAF6 and GST-DN TRAF6 pulled-down Np65(+)-GFP from HEK cell lysates and endogenous neuroplastins from lysate, supporting that TRAF6 binds neuroplastins

through its C-terminus (Fig. 10D, E). Altogether, immunoprecipitation and pull-down assays revealed that the interaction between neuroplastin and TRAF6 involves the predicted TRAF6 binding site of neuroplastin and the TRAF-C domain of TRAF6.

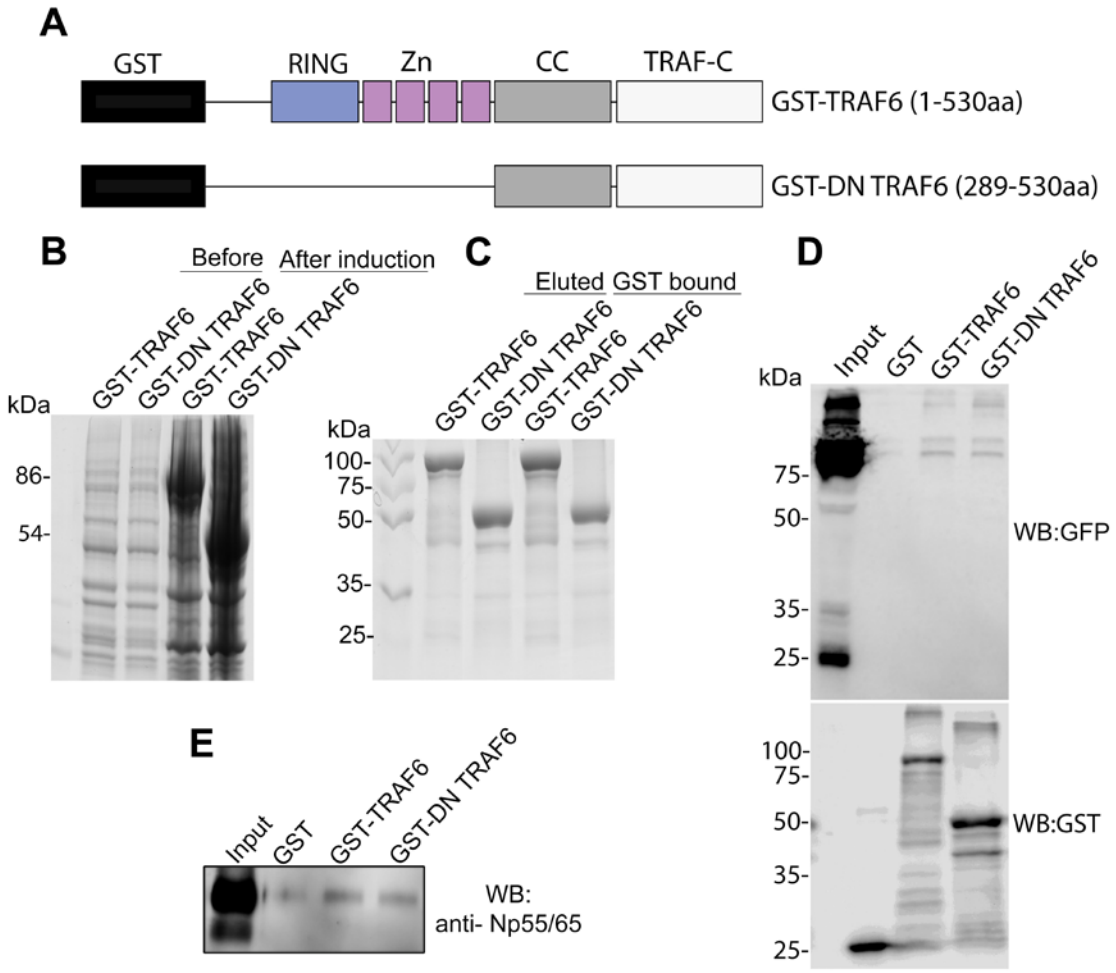
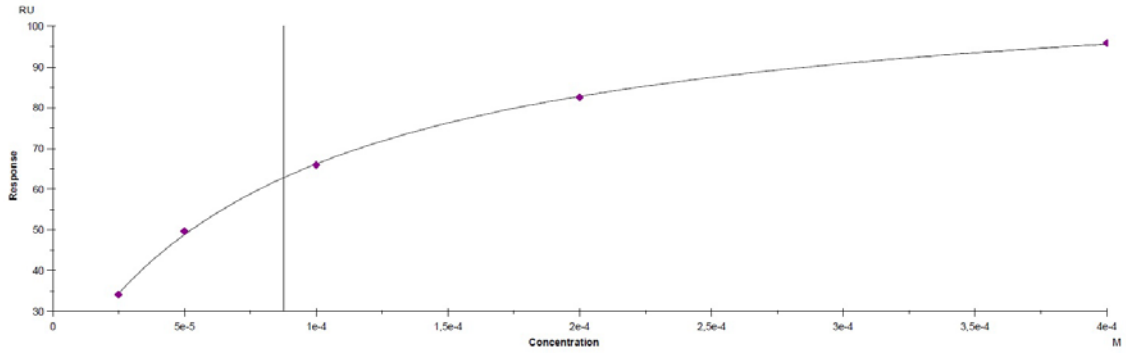


Fig. 10. GST-TRAF6 and GST-dominant negative (DN) TRAF6 recombinant proteins pull-down neuroplastin. A) Schematic representation of GST-TRAF6 and GST-DN TRAF6. B) Before and after induction with IPTG, bacterial proteins were separated on SDS-PAGE and Coomassie-stained. C) Lysates were incubated with GST Sepharose 4B beads for 1 h at 4°C. Coomassie staining shows bound and eluted fusion proteins. D) Lysates of HEK cells transfected with Np65(+)-GFP were incubated with GST-TRAF6 and GST-DN TRAF6 bound to GST-Sepharose 4B beads. Western blot analysis shows both recombinant proteins pulled down Np65(+)-GFP. E) Total rat brain lysates were incubated with GST-TRAF6 and GST-DN TRAF6. Western blot analysis shows both recombinant proteins pulled down brain neuroplastins.

To prove the interaction of neuroplastin with TRAF6 to be direct, I performed surface plasmon resonance analysis (Biacore) experiments in collaboration with Dr. Johannes Hradsky (from Dr. Michael R. Kreutz lab). For this, purified recombinant GST-TRAF6 and control GST proteins were immobilized on CM5 chips via amine-coupling method (Reddy et al., 2014). Binding of 17 aa peptides derived from the intracellular neuroplastin with (aa 244 to 260 according to Np55) or without the TRAF6 binding site (258 to 275) to GST-TRAF6 was analyzed. Specific binding between the neuroplastin peptide containing the TRAF6 binding site and GST-TRAF6 was observed as

compared to the neuroplastin peptide without TRAF6 binding site. The background binding (peptide without TRAF6 site) was subtracted from the specific binding (peptide with TRAF6 site) to generate the final binding curve in Fig. 11. The dissociation constant (K_d) for the neuroplastin-TRAF6 interaction was estimated as 88µM with a stoichiometry of 1:1. These results are strikingly consistent to reported values (K_d=84µM) for the CD40-TRAF6 interaction (Ye et al., 2002).



Parameters table

KD (M)	SE(KD)	Rmax (RU)	SE(Rmax)	offset (RU)	SE(offset)
8,773E-5	7,1E-6				
		102,2	1,4	11,7	2,0

Fig. 11. Binding response curve for binding of neuroplastin peptide containing the TRAF6 binding site to GST-TRAF6 from a Surface Plasmon Resonance experiment. The graph shows the binding of GST-TRAF6 (response units) over different concentrations of neuroplastin intracellular peptide. The vertical bar indicates 50% of neuroplastin peptide binding to GST-TRAF6. The table indicates the final parameters for the specific binding including the KD value. This was performed in collaboration with Johannes Hradsky from the lab of Dr. Michael Kreutz.

Thus I conclude in this section 4.1 from these experiments that neuroplastin binds, via its TRAF6 binding site, with good biologically significant affinity, and directly to, the C-terminal domain of TRAF6.

4.2. Neuroplastin-TRAF6 binding at the plasma membrane triggers the formation of actin-based filopodia in HEK cells

TRAF6 participates in regulation of cell morphology, actin cytoskeleton, and filopodia formation in HEK cells (Wang et.al, 2006). Upon neuroplastin overexpression, I observed drastic morphological changes i.e. clear formation of actin-based filopodia in transfected HEK cells. Thus, I used these filopodia in HEK cells as a read-out for a downstream effect of the neuroplastin-TRAF6 interaction. For this, cells were transfected with GFP and GFP-tagged constructs of all four neuroplastin isoforms. Consistently, a dramatic alteration in cellular morphology (gain of function) i.e., filopodia like-structure formation was observed in more than 90% of neuroplastin-expressing cells (Fig. 12A,B). These filopodia were quantified by FiloDetect (Nilufar et. al, 2013 and collaboration with André Weber from Dr. Werner Zuschratter lab), which is a Matlab-coded algorithm specially programmed to detect filopodia. Images from our

experiments were run one by one and the number of pixels was converted to real physical dimensions (microns).

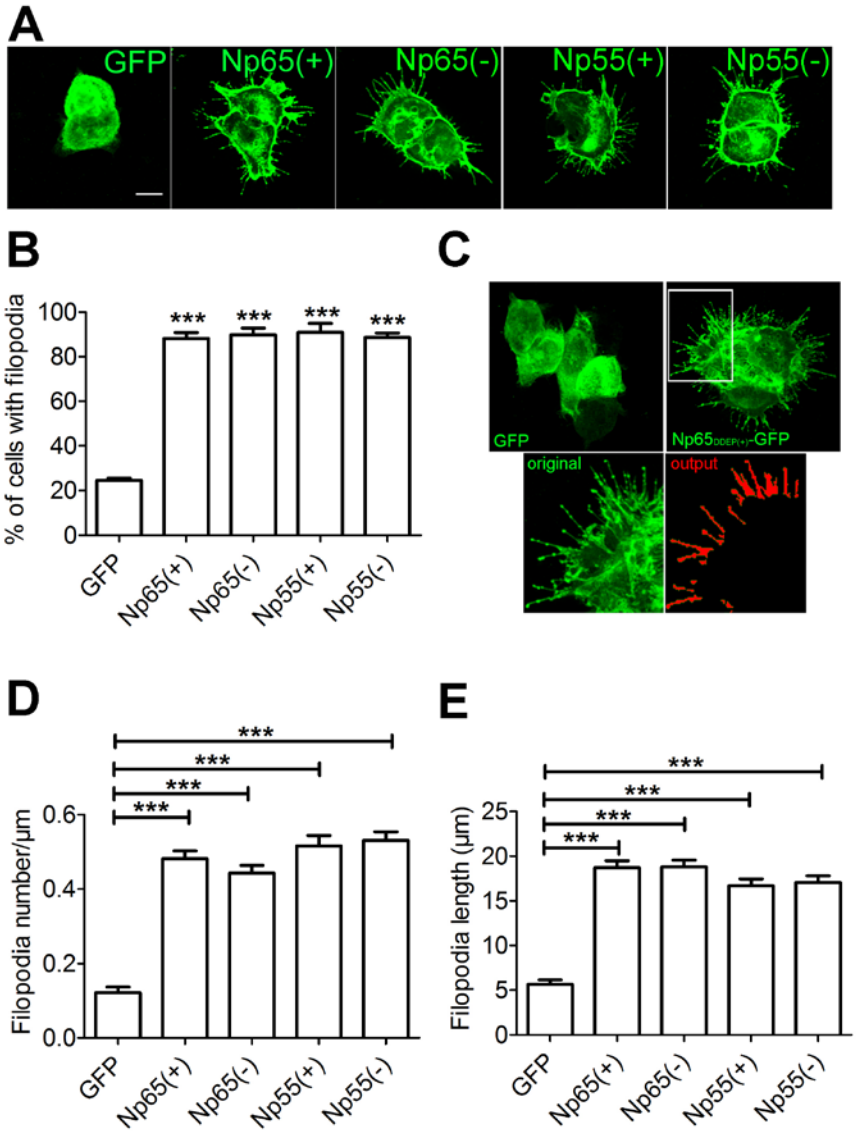


Fig. 12. Neuroplastins increase filopodia formation. A) HEK cells were transfected with GFP, Np65(+)-GFP, Np65(-)-GFP, Np55(+)-GFP or Np55(-)-GFP constructs. After 24h, cells were fixed, immunostained with anti-GFP rabbit antibody followed by an Alexa-488 fluorescent conjugated secondary antibody. Images were acquired by confocal microscope with 3X zoom factor. B) The graph represents the percentage of transfected cells with filopodia. C) Detected filopodia by FiloDetect algorithm are displayed in red color in an example output image. D, E) The graphs show the quantification of filopodia number and length (3 independent experiments, student's t-test, ***p<0.0001 vs GFP) and data shown as mean±SEM. Scale bar = 10 µm.

As result of the image processing, FiloDetect provides output images (Fig. 12C) as well as the number and the average length of detected filopodia (Fig. 12D,E). The filopodia number was divided by the perimeter of the cell using imageJ resulting in a normalized parameter (number of filopodia per µm). Increased number and length of filopodia in HEK cells transfected with each of the four neuroplastin isoforms compared to GFP-transfected cells was quantified (Fig. 12A, D and E; filopodia number -GFP:

0.12±0.015 vs Np65(+)-GFP: 0.48±0.021, Np65(-)-GFP: 0.44±0.02; Np55(+)-GFP: 0.52±0.03, and Np55(-)-GFP: 0.53±0.023 per µm; filopodia length -GFP: 5.66±0.49 vs Np65(+)-GFP: 18.72±0.77, Np65(-)-GFP: 18.79±0.76, Np55(+)-GFP: 16.68±0.76 and Np55(-)-GFP: 17.08±0.72 µm).

I investigated whether the TRAF6 binding site in neuroplastin is necessary for filopodia formation in HEK cells. Cells were transfected with GFP, membrane-anchored GPI-GFP, Np65(+)-GFP or neuroplastin constructs with mutations in TRAF6 binding site: Np65$^{\Delta id}$-GFP, Np65^{PED}-GFP or Np65^{P}-GFP. Consistent with previous experiments, Np65(+)-GFP overexpression triggered a drastic increase in the number and length of filopodia compared to control GFP cells (Fig. 13A,B; filopodia number per µm GFP: 0.18±0.016, GFP-GPI: 0.21±0.016, Np65(+)-GFP: 0.53±0.031; Fig. 13A,C filopodia length in µm GFP: 5.13±0.189, GFP-GPI: 8.907±0.465, Np65(+)-GFP: 16.17±0.625).

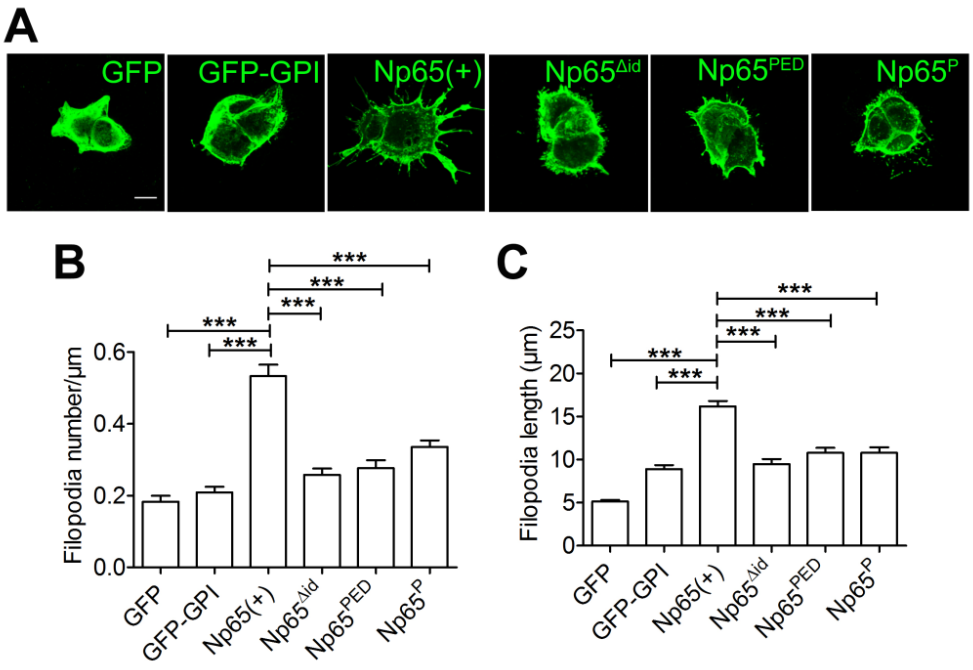


Fig. 13. Mutation of TRAF6 binding site abolishes Np65 capacity to promote filopodia formation. A) HEK cells expressing GFP or membrane-anchored GFP-GPI, Np65(+)-GFP, Np65$^{\Delta id}$-GFP, Np65^{PED}-GFP or Np65^{P}-GFP constructs were immunostained as mentioned in Fig.12. B, C) The graphs show the quantification of filopodia number and length in µm (4 independent experiments, student's t-test, ***p<0.0001 vs Np65(+)-GFP) and data shown as mean±SEM. Scale bar = 10 µm.

In clear contrast to Np65(+)-GFP, the number and length of filopodia were significantly lower in cells transfected with neuroplastin mutants, which were similar to control transfected cells (Fig. 13A,C; filopodia number per µm Np65$^{\Delta id}$-GFP: 0.26±0.017, Np65^{PED}-GFP: 0.28±0.021, Np65^{P}-GFP: 0.34±0.017; filopodia length in µm Np65$^{\Delta id}$-GFP: 9.49±0.575, Np65^{PED}-GFP: 10.79±0.583, Np65^{P}-GFP: 10.78±0.646). These

results indicate that TRAF6 binding site in neuroplastin is indeed necessary to promote both number and length of filopodia potentially via binding of TRAF6 (section 4.1).

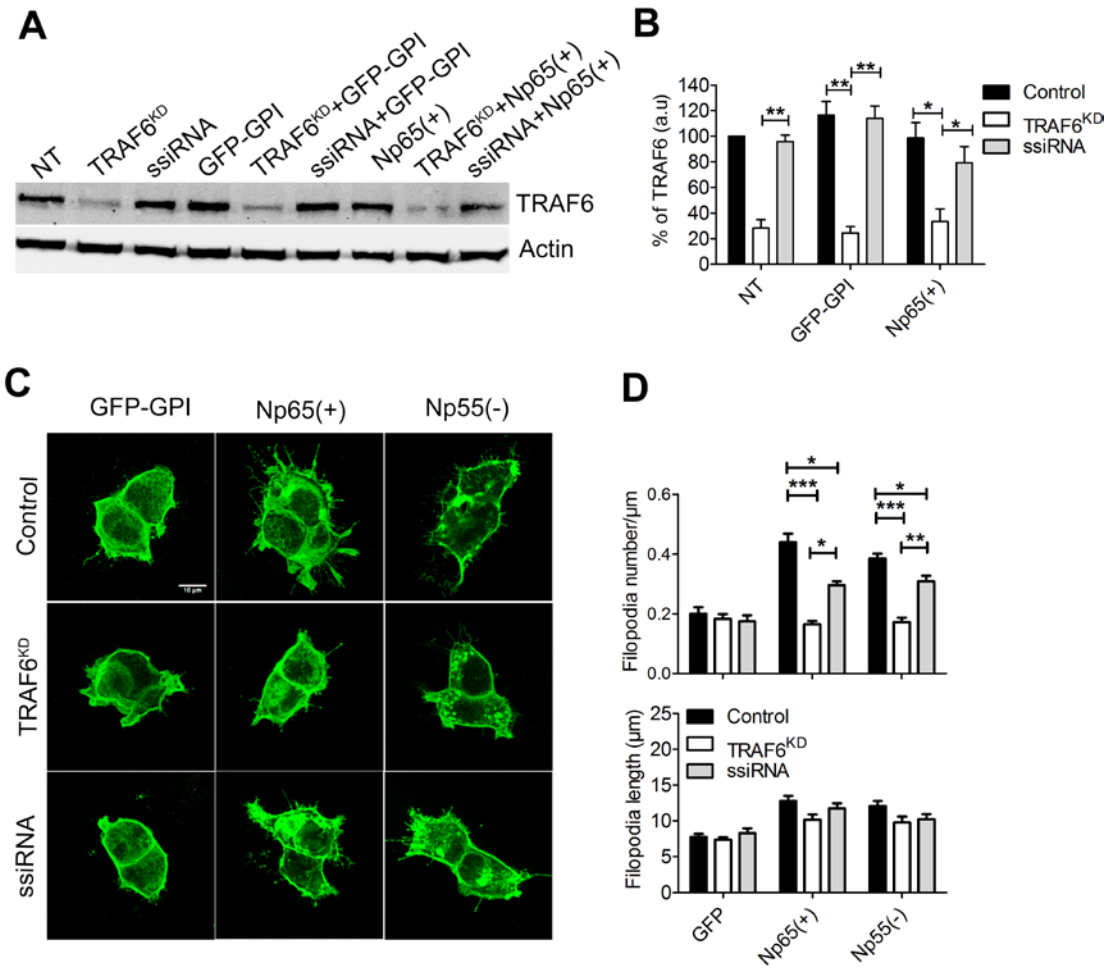


Fig. 14. Knockdown of TRAF6 reduces filopodia in neuroplastin transfected HEK cells. Cells were treated with siRNA (TRAF6^{KD}) or scrambled siRNA (ssiRNA). After 24h, cells were transfected with membrane-anchored GPI-GFP or Np65(+)-GFP or Np55(-)-GFP constructs. A) After 24h of transfection, cells were harvested and lysates were run on SDS-PAGE and immunoblotted for TRAF6 and actin. (B) The graph represents the percentage of TRAF6 levels in HEK cells. C) After 24h, cells were fixed and immunostained as mentioned in Fig. 12. D) The graphs represents the quantification of filopodia number and length (3 independent experiments, n=27-33 cells/condition student's t-test, *p<0.1, **p<0.01, ***p<0.001) and data shown as mean±SEM.

It has already been reported that overexpression of TRAF6 mediates filopodia formation in HEK cells (Wang et.al, 2006). I studied here whether TRAF6 can also mediate neuroplastin-induced filopodia formation using siRNA to knockdown endogenous TRAF6 in HEK cells. For this, cells were transfected either with scrambled siRNA (ssiRNA) or TRAF6 siRNA (TRAF6^{KD}). After 24 h, the cells were transfected with GPI-GFP and Np65(+)-GFP constructs. TRAF6 protein levels were significantly reduced by an average of 75% in TRAF6-knocked down cells compared to non-transfected (NT) or ssiRNA-transfected cells (Fig. 14A,B). TRAF6 knockdown reduced

significantly the filopodia number while ssiRNA did not affect the Np65(+)-GFP- and Np55(-)-GFP-induced filopodia number (Fig. 14C,D). TRAF6 knockdown did not change filopodia length in neuroplastin-transfected cells. Therefore, the experiments in Fig. 13 and 14 support a novel role for TRAF6 in filopodia formation via binding to neuroplastin.

4.3. Neuroplastin recruits TRAF6 to the plasma membrane in HEK cells

When receptors like ILR-1 are activated by their ligands, TRAF6 is recruited to the membrane and binds to receptors through its TRAF-C domain. TRAF6 dimerization occurs through the RING finger domain to form a lattice like-network beneath the plasma membrane (Baud et al., 1999; Yin et al., 2009; Wang et al., 2010).

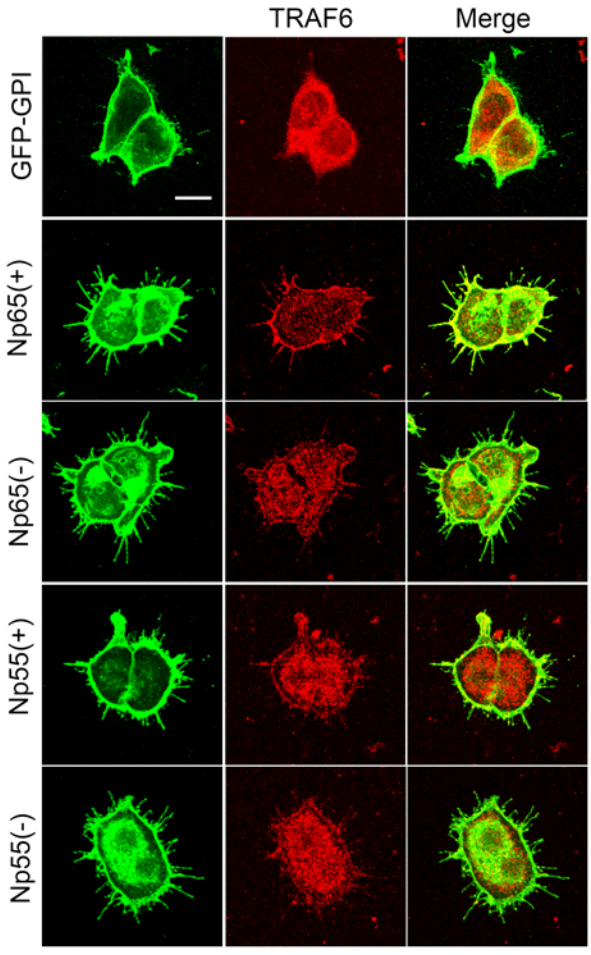


Fig. 15. Neuroplastin recruits TRAF6 to the plasma membrane in HEKcells. Cells were transfected with membrane-anchored GPI-GFP or GFP-tagged neuroplastin constructs. After 24h, cells were fixed and immunostained with anti-GFP rabbit antibody and anti-TRAF6 mouse antibody for overnight and with an Alexa-488 rabbit and Alexa-568 mouse secondary antibodies, respectively. TRAF6 was recruited to the plasma membrane and filopodial structures in neuroplastin transfected cells.

To further investigate whether neuroplastin can indeed recruit TRAF6 to the plasma membrane, cells were transfected with GPI-GFP- and GFP-tagged neuroplastin constructs, and double-stained for GFP and TRAF6 proteins. As predicted from co-immunoprecipitation results, I was able to observe TRAF6 recruitment to the membrane from the cytosol. The TRAF6 immunoreactivity was higher at the

membrane and co-localized with all neuroplastin isoforms in the membrane and filopodia than in GPI-GFP-transfected cells (Fig. 15). This demonstrates that neuroplastin recruits TRAF6 and interacts with it at the plasma membrane to promote filopodia outgrowth.

It has been shown that treatment of cells with cell-permeable peptides comprising a TRAF6-interacting motif or overexpression of dominant-negative (DN) TRAF6 constructs abolishes RANK-mediated cell differentiation and downstream signaling i.e., NF-κB activation, by competing with endogenous TRAF6 (Ye et al., 2002). Here, I have shown that DN TRAF6 was sufficient to bind to neuroplastin in pull-down assay (Fig. 10D,E). To further investigate whether DN TRAF6 is sufficient to promote filopodia formation, TRAF6 and DN TRAF6 were cloned into the GFP vector to directly visualize them in the cellular compartment and also to eliminate antibody cross reactivity. Thus, GFP, TRAF6-GFP and DN TRAF6-GFP were co-transfected with Np55-RFP in HEK cells. While GFP was localized mostly in the cytoplasm TRAF6-GFP and DN TRAF6-GFP were recruited to the plasma membrane as well as into the filopodia of Np55-RFP-transfected cells (Fig. 15).

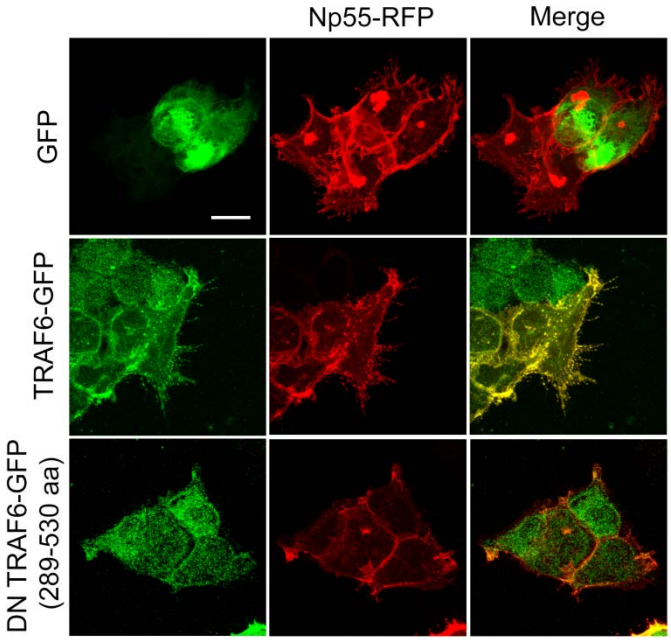


Fig. 16. TRAF6-GFP and dominant negative (DN)TRAF6-GFP colocalize with Np55-RFP in the plasma membrane of transfected HEK cells. Np55-RFP was co-transfected with GFP or TRAF6-GFP or DN TRAF6-GFP. After 24 h, cells were fixed and mounted on a glass slide. In Np55-RFP transfected cells, TRAF6-GFP and DN TRAF6-GFP but not control GFP were strongly associated to the plasma membrane and colocalized with Np55-RFP (see merge) (One experiment, scale bar =10 µm).

But, the filopodia number seems to be reduced in DN TRAF6-GFP- and Np55-RFP-transfected cells. This indicates that DN TRAF6 is recruited to the plasma membrane and acts as a competitor for endogenous TRAF6 to bind to neuroplastin. This confirms the notion that neuroplastin overexpression recruits TRAF6 to the plasma membrane

to induce filopodia. Obviously, this neuroplastin induced filopodia formation also depends on the N-terminal region of TRAF6.

As reported, TRAF6 can stabilize proteins or mark them for degradation by interacting and ubiquitinating them (Powell et al., 2009; Murata et al., 2013; Xie, 2013). In my co-immunopreciptation experiments, neuroplastin protein levels did not change when I co-transfected cells with TRAF6 (see inputs, Fig. 8 and 9), suggesting that interaction with TRAF6 does not lead to neuroplastin degradation. So, it is also important to investigate whether neuroplastin expression alter the endogenous TRAF6 protein levels. For this, HEK cells were transfected with GFP and GFP-tagged neuroplastin constructs. Immunoblotting analysis of cell extracts showed that TRAF6 protein levels were not altered in neuroplastin overexpressing cells (Fig. 17). This result indicates that neuroplastin-TRAF6 interaction does not initiate degradion of either partner.

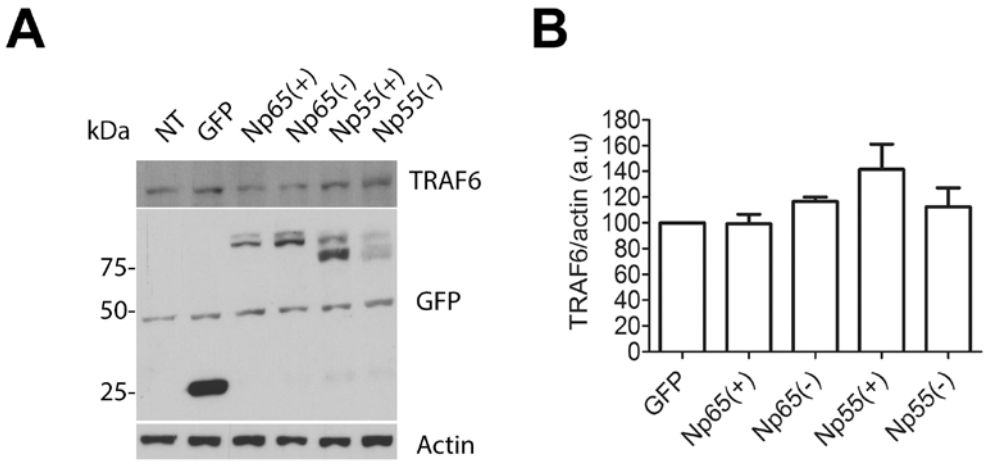


Fig. 17. Neuroplastin overexpression does not affect TRAF6 levels in HEK cells. A) Immunoblotting of lysates from HEK cells transfected with GFP or neuroplastin-GFP constructs and probed for anti-TRAF6 (mouse), anti-β-actin (mouse) and anti-GFP (rabbit,) antibodies. B) Quantification of TRAF6 levels normalized to actin (3 independent experiments).

4.4. PI3K/Akt activity and NF-κB pathway participate in filopodia formation promoted by the neuroplastin-TRAF6 interaction

Wang et al. have shown that TRAF6 mediates activation of signaling cascades, for example PI3K/Akt pathway, to regulate filopodia formation in HEK cells (Wang et al., 2006). Also, PI3K activity regulates filopodia formation in syndecan-2 expressing cells (Lin et al., 2007). Therefore, I tested the hypothesis that neuroplastin interacts with TRAF6 and regulates filopodia formation via PI3K/Akt pathway. For this, HEK cells were transfected with GFP and GFP tagged neuroplastin isoforms and treated with the PI3K inhibitor Wortamannin (100 nM) for 24 h. The quantification of filopodia resulted in a marked decrease in neuroplastin induced filopodia number and length in

Wortmannin treated neuroplastin-expressing cells compared to control DMSO-treated condition (Fig. 18A-C), supporting that PI3K activation is necessary for the formation and elongation of filopodia in neuroplastin-expressing cells.

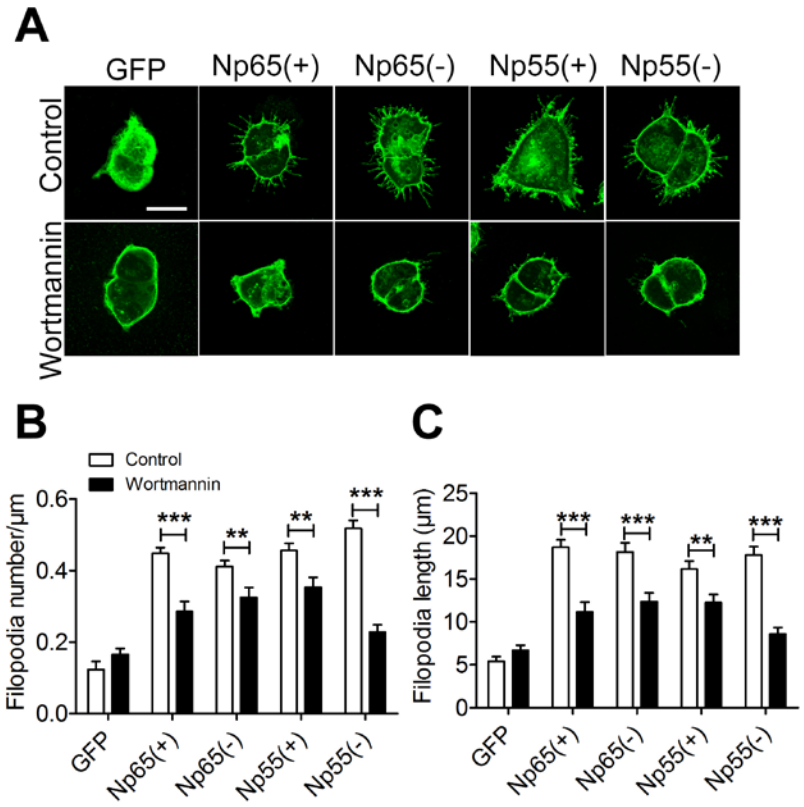


Fig. 18. PI3K inhibition reduces neuroplastin-induced filopodia formation. HEK cells were transfected with GFP or neuroplastin-GFP constructs and treated with Wortmannin (100 nM) or DMSO as control. Quantification of filopodia number revealed that Wortmannin treatment reduced neuroplastin-induced (B) filopodia number and (C) length compared to control neuroplastin-transfected cells. (Mean±SEM from 2 independent experiments, n=18-39 cells,student's t-test, **p<0.01, ***p<0.001; Scale bar =10 µm).

TRAF6 is a well-established canonical activator of the NF-κB pathway in a number of different cell types (Xie, 2013; Kopitar-Jerala, 2015; Walsh et al., 2015). To investigate whether neuroplastin-TRAF6 interaction activates NF-κB signaling pathway, I investigated the translocation of RelA, (a subunit of NF-κB), to the nucleusar translocation in HEK cells. For this, GFP, Np65(+)-GFP and Np65$^{\Delta id}$-GFP constructs were transfected into the cells. After 24 h, I probed with GFP and RelA antibodies, and nuclei were stained with DAPI. I measured the mean nuclear RelA fluorescence, using ImageJ with the criteria the nuclei and their cell cytoplasmic part defined as regions, using ImageJ. Quantification of a mean fluorescence of in the nuclear region resulted in an increase of RelA nuclear translocation in Np65(+)-GFP-expressing as compared to than GFP- and Np65$^{\Delta id}$-GFP- transfected cells (Fig. 19A,B). To confirm that increased levels of nuclear RelA occurred as a consequence of activation and translocation from the cytosol, I treated GFP- and Np65(+)-GFP transfected cells with

SN50, a cell- permeable peptide able to block the nuclear induction of NF-κB by binding to the nuclear localization signal (NLS) in the p50 subunit of NF-κB, for 24 h. As expected, nuclear RelA immunoreactivity was decreased by SN50 treated treatment than as compared to control DMSO-treated Np65(+)-GFP transfected cells (Fig. 19C,D). This means that SN50 treatment efficiently blocks the RelA nuclear translocation downstream of neuroplastin-TRAF6.

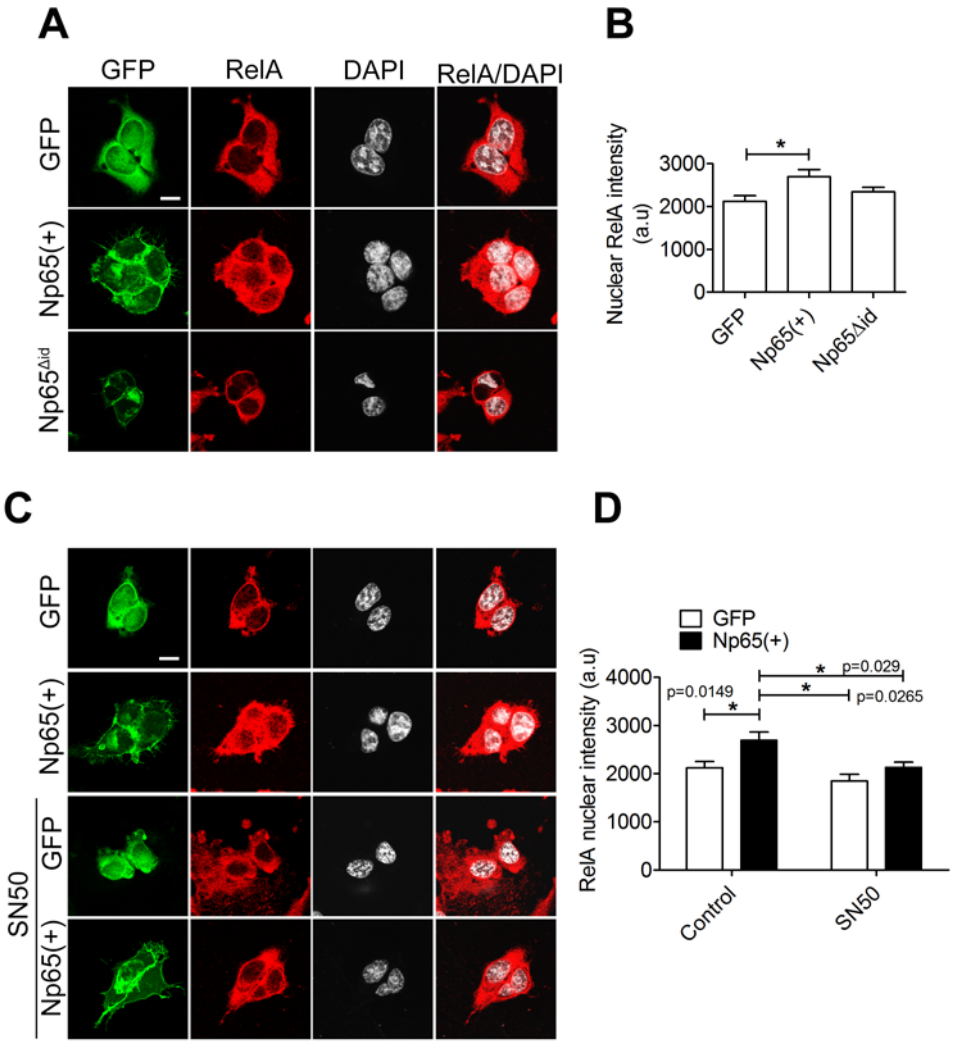


Fig. 19. Inhibition of RelA nuclear translocation in neuroplastin expressing HEK cells. A) GFP, Np65(+)-GFP or Np65$^{\Delta id}$-GFP-transfected HEK cells were fixed and stained for GFP and endogenous RelA. Nuclei were stained using DAPI. B) Quantification of the fluorescent intensity of nuclear RelA from A. C) The cells were transfected with GFP and Np65(+)-GFP, and treated with SN50 (50 µg/ml) or DMSO (control) for 24 h, fixed and stained as in A. D) Quantification of the fluorescent intensity of nuclear RelA from C. (n=8-35 cells/condition, student‘s t-test, Scale bar =10 µm).

Next, I investigated the necessity of RelA activation for filopodia formation. To test this, HEK cells were transfected with GFP and GFP-tagged neuroplastin isoforms and

treated with either SN50 or DMSO. After 24 h, I quantified the filopodia and found both the number and length of filopodia reduced by SN50 as ompared to untreated control neuroplastin-transfected cells (Fig. 20A-C). Altogether, these results suggest that RelA nuclear translocation is necessary to create new and longer filopodia in neuroplastin transfected cells.

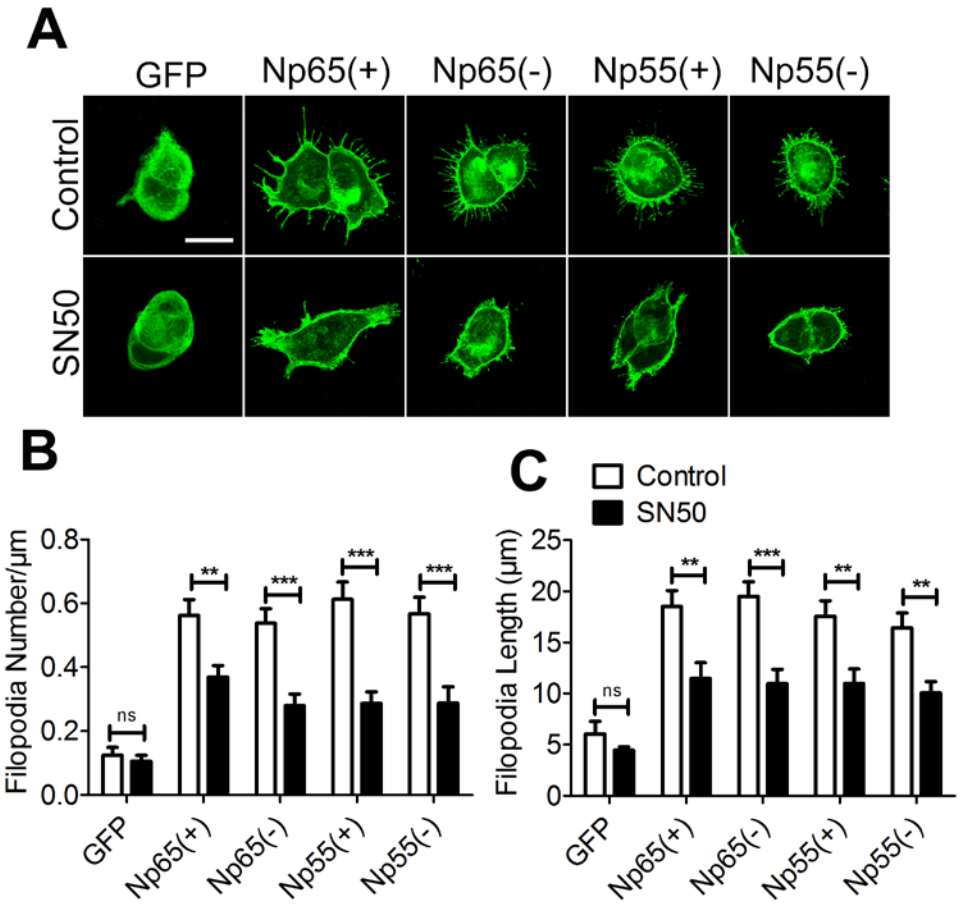


Fig. 20. RelA activation is involved in neuroplastin-induced filopodia formation. A) HEK cells were transfected with GFP or GFP-tagged neuroplastin isoforms, treated with SN50 or DMSO (control) and stained for GFP. (F,G) Quantification of number and length of filopodia from A. (Mean±SEM from 2 independent experiments, n=6-13 cells/condition, student's t-test, **p<0.01, ***p<0.001 vs control; Scale bar =10 µm).

Further, the time-course of neuroplastin-induced filopodia formation was characterized in HEK cells. To this end, cells were cultured in media containing 1% fetal calf serum only to reduce cell proliferation as well as general stimulation of growth factor receptors. Cells were transfected with GFP and Np65(+)-GFP plasmids and fixed at indicated time points. Np65(+)-GFP expression caused morphological changes in the cells rapidly as they displayed a significantly higher number of filopodia 6-8 h after transfection (Fig. 21A,B). In contrast, the filopodia length increased drastically after 12-24 h (Fig. 21C). These distinct time-courses suggest that neuroplastin-induced

formation and grow/ enlargement of filopodial structures could occur in two different phases suggesting the possibility of a differential temporal involvement of intracellular cascades and regulatory proteins involved in actin cytoskeleton regulation downstream of neuroplastin.

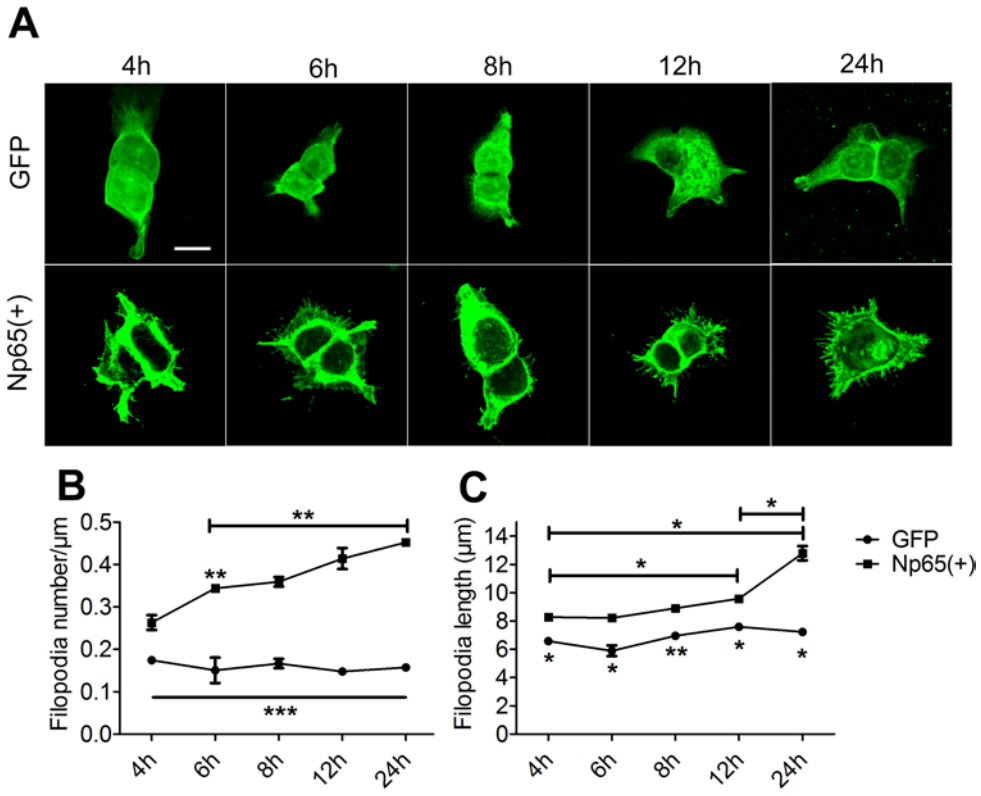


Fig. 21. Time-course study of filopodia formation in neuroplastin-expressing HEK cells. A) Cells were transfected with GFP and Np65(+)-GFP constructs. The cells were maintained with 1% fetal calf serum-containing media. After washing off the transfection mixture, the cells were fixed at 4, 6, 8, 12 and 24 h, as indicated, and immunostained with anti-GFP rabbit antibody. Representative images of filopodia dynamics over 24 h period in Np65(+)-GFP transfected cells. B, C) Quantification of filopodia number and length (2 independent experiments, n=8-10 cells/experiment, student's t-test, *p<0.1, **p<0.01, ***p<0.001; Scale bar =10 µm). Data are shown as mean±SEM.

My above described results provide mechanisms in HEK cells (Fig. 8-20) that neuroplastins interact with TRAF6 at the plasma membrane to initiate the *novo* fomation and promotion of the grow of actin-based filopodia by activating the PI3K/Akt and NF-κB signaling pathways.

4.5. Neuroplastin requires TRAF6 binding site and endogenous TRAF6 to promote early formation of dendritic protrusions and to rescue spinogenesis in *Nptn-/-* neurons.

As neuroplastin deficient mice as well as immature hippocampal neurons derived from these mutants and cultured for 12 DIV displayed reduced numbers of excitatory synapses, I hypothesized that formation of excitatory contacts could be affected by neuroplastin absence (Herrera-Molina et al., 2014). Therefore, for my next experiment, I used 6-9 DIV rat hippocampal neurons. During this stage, neurons acquire cell specific dendritic patterns and produce dendritic protrusions, which are the precursors for excitatory synapses (Ziv and Smith, 1996) and they express both Np55 and Np65 (Buckby et al., 2004; Marzban et al., 2003). First, I investigated whether neuroplastin promotes early spinogenesis in wild type neurons. For this, GFP and GFP tagged neuroplastin isoforms were transfected into 6 DIV neurons. The dendritic protrusions were counted manually on day 8 and the dendritic segment length was measured using ImageJ. The filopodia like-dendritic protrusions were increased by all four neuroplastin isoforms as compared to GFP transfected neurons (Fig. 22A, B; GFP: 1.95 ± 0.19 vs Np65(+)-GFP: 3.23 ± 0.18, Np65(-)-GFP: 3.37 ± 0.18, Np55(+)-GFP: 3.54 ± 0.18 and Np55(-)-GFP: 3.91 ± 0.18 per 10 µm dendritic length).

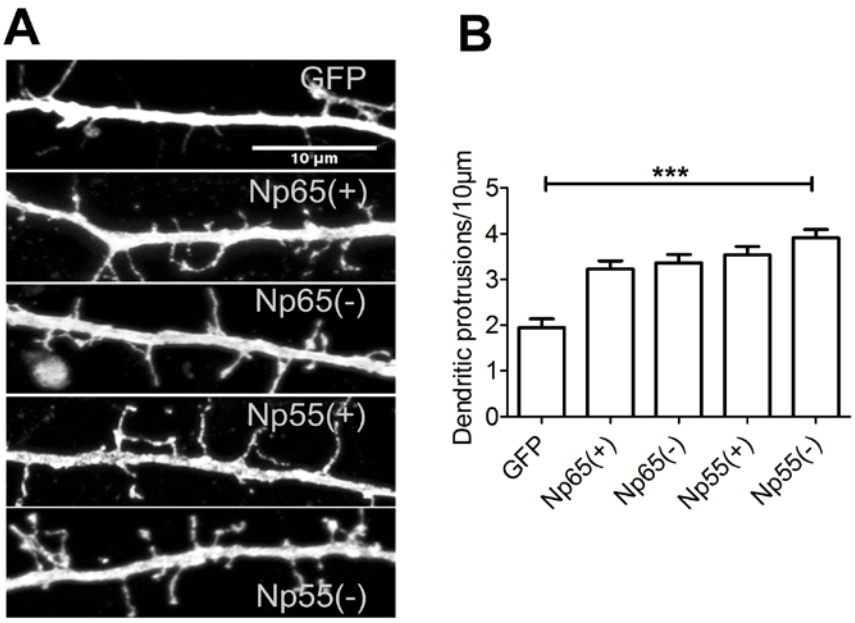


Fig. 22. Neuroplastin increases dendritic protrusions in hippocampal neurons. A) Neurons were transfected with GFP or GFP-constructs for one of the four neuroplastin isofoms at 6 DIV and fixed on day 8. Then, neurons were stained for GFP and a fluorescently conjugated secondary antibody. The pictures show dendritic segments of neurons. B) The graph demonstrates the quantification of numbers of dendritic protrusions per 10 um. Data are shown as mean±SEM (GFP: n=33, Np65(+)-GFP: n=39, Np65(-)-GFP: n=35, Np55(+)-GFP: n=30 and Np55(-)-GFP: n=26 neurons; 3 independent experiments, student‘s t-test, ***p<0.001 vs GFP).

Moreover, larger dendritic protrusions were observed in neurons overexpressing all neuroplastin isoforms. The data suggest that all neuroplastin isoforms have potential synaptogenic capacity.

As shown in Fig. 13, mutation or deletion of the TRAF6 binding site in neuroplastin failed to increase the filopodia number and length in HEK cells. To further investigate the TRAF6 binding site in neuroplastin involvement in dendritic protrusions formation, I overexpressed GFP, Np65(+)-GFP and Np65$^{\Delta id}$-GFP in 7 DIV hippocampal neurons and quantified the dendritic protrusions on day 8. Np65$^{\Delta id}$-GFP failed to increase the dendritic protrusion number compared to Np65(+)-GFP (Fig. 23A,B; GFP: 1.22 ± 0.14, and Np65$^{\Delta id}$-GFP: 1.37 ± 0.16 vs Np65(+)-GFP: 2.93 ± 0.15) suggesting that the TRAF6 binding site in neuroplastin is necessary to promote dendritic protrusions in neurons.

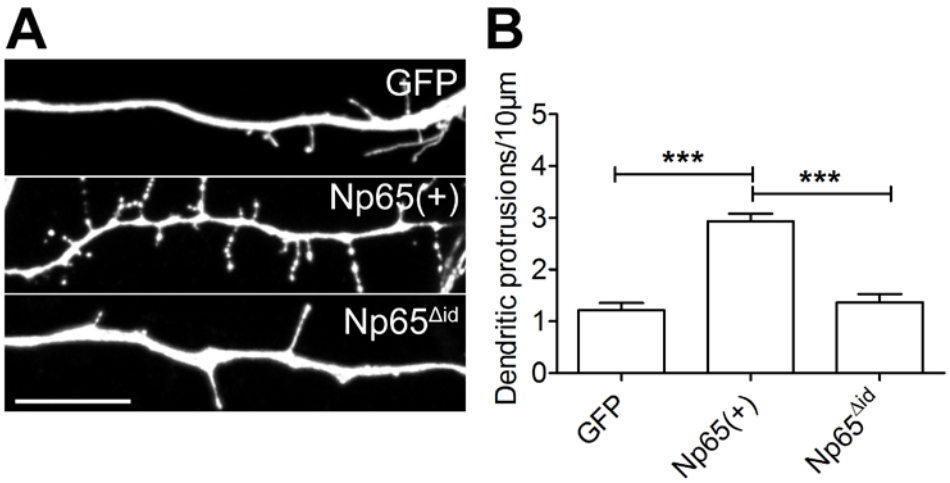


Fig. 23. Neuroplastin mutants failed to increase dendritic protrusion numbers in hippocampal neurons. A) Neurons were transfected with GFP, Np65(+)-GFP or Np65$^{\Delta id}$-GFP at 7 DIV and fixed after 24 h of transfection. Immunostaining was performed as mentioned in Fig. 22. B) Quantitative analysis of dendritic protrusions shows that Np65$^{\Delta id}$-GFP failed to increase dendritic protrusions in neurons. The results are from 3 independent experiments and data are shown as mean±SEM (GFP: n=18; Np65(+)-GFP: n=33; Np65$^{\Delta id}$-GFP: n=27 neurons; student's t-test, ***p<0.001 vs Np65(+)-GFP). Scale bar=10 µm.

Next, to further confirm the TRAF6 role in neuroplastin-induced dendritic protrusion formation, I performed siRNA-mediated knockdown of TRAF6 in cultured neurons. 7 DIV hippocampal neurons were transfected either with TRAF6 siRNA or ssiRNA, and also co-transfected with GFP, Np65(+)-GFP and Np65$^{\Delta id}$-GFP. SiRNA-mediated knock down of TRAF6 resulted in a marked decrease in dendritic protrusion numbers compared to the ssiRNA condition on day 9 in neurons (Fig. 24A,B; TRAF6^{KD}+GFP: 3.63 ± 0.164 vs ssiRNA+GFP: 2.16 ± 0.18). However, this effect was not reversed by overexpression of Np65(+)-GFP and Np65$^{\Delta id}$-GFP in neurons (TRAF6^{KD}+Np65(+)-GFP: 2.09 ± 0.16 vs TRAF6^{KD}+Np65$^{\Delta id}$-GFP: 1.69 ± 0.17). The TRAF6 knock down-

mediated defect in neuroplastin-induced dendritic protrusion formation seems to be loss of endogenous TRAF6 in neurons. This confirms that TRAF6 is crucial for neuroplastin-mediated dendritic protrusions formation in neurons.

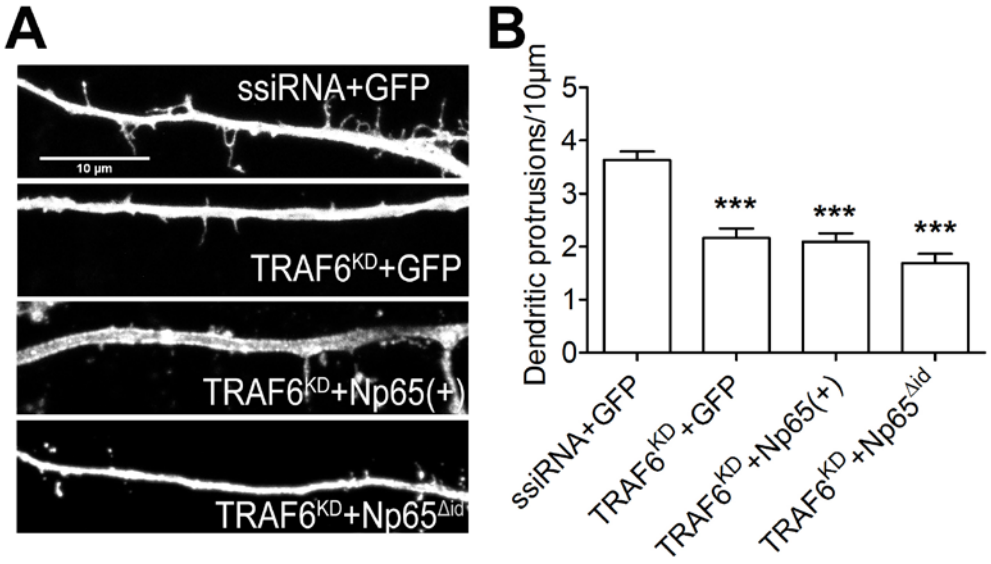


Fig. 24. TRAF6 downregulation decreases dendritic protrusions in hippocampal neurons. A) Co-transfection of either scrambled siRNA or siRNA against TRAF6 with GFP, Np65(+)-GFP and Np65$^{\Delta id}$-GFP in hippocampal neurons on day 7. After 2 days of transfection, the neurons were fixed and stained as mentioned in Fig. 22. B) The graph shows the quantitative analysis of dendritic protrusions from TRAF6 knockdown neurons and scrambled siRNA-treated cells. Data are shown as mean±SEM (3 independent experiments, student's t-test, ***p<0.001) (ssiRNA+GFP: n=49; TRAF6^{KD}+GFP: n=59; TRAF6^{KD}+Np65(+)-GFP: n=31, TRAF6^{KD}+Np65$^{\Delta id}$-GFP: n=24).

As shown in Fig. 22, neuroplastin overexpression increases dendritic protrusions in cultured hippocampal neurons. Neuroplastin deficiency decreased excitatory synapses in *Nptn-/-* mature hippocampal neurons (Herrera-Molina et al., 2014), suggesting a potential role in synaptic development. To further investigate the role of neuroplastin in dendritic protrusions formation, I used *Nptn-/-* hippocampal neurons. To fill the neurons, I overexpressed GFP in 6 DIV neurons from wild-type (WT) and *Nptn-/-* mice. The neurons were stained for GFP and endogenous neuroplastins to confirm the genotypes. In *Nptn-/-* neurons, the density of dendritic protrusions on day 8 was significantly reduced to about 46% than in WT neurons (Fig. 25A, B; WT: 4.12 ± 0.18 vs *Nptn-/-*: 1.92 ± 0.22 per 10 µm dendrite length). Next, I performed an experiment to rescue the phenotype observed in *Nptn-/-* neurons. For this, I overexpressed GFP, Np65(+)-GFP or Np65$^{\Delta id}$-GFP in *Nptn-/-* neurons and GFP alone in WT neurons. Np65(+)-GFP expressing neurons showed a significant increase in density of dendritic protrusions and rescued *Nptn-/-* phenotype but not with Np65$^{\Delta id}$-GFP (Fig. 25C, D; WT_GFP: 4.12 ± 0.18 and *Nptn-/-*_Np65(+)-GFP: 3.67 ± 0.18 vs *Nptn-/-*_GFP: 1.72 ± 0.19 and *Nptn-/-*_Np65$^{\Delta id}$-GFP: 1.79 ± 0.16 per 10 µm dendrite length). This result

demonstrates a specific role for neuroplastin and its TRAF6 binding site in dendritic protrusion formation.

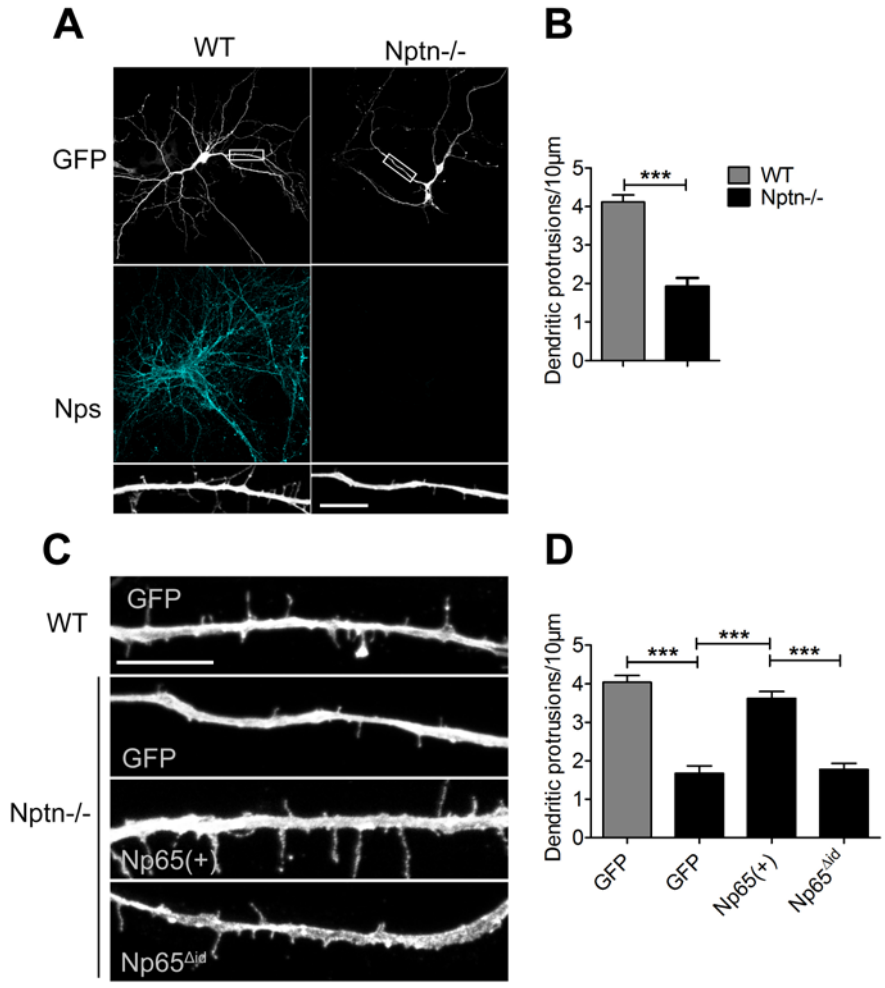


Fig. 25. The number of dendritic protrusions is reduced in *Nptn-/-* hippocampal neurons and rescued by neuroplastin expression. A) 6 DIV WT or *Nptn-/-* hippocampal neurons were transfected with GFP and after 2 days, the neurons were fixed and stained for GFP and neuroplastins. For example magnified images of dendritic segments are shown below. B) Quantification of dendritic protrusion numbers in WT and *Nptn-/-* neurons. C) 6 DIV WT or *Nptn-/-* hippocampal neurons were transfected with GFP or Np65(+)-GFP or Np65$^{\Delta id}$-GFP, cultured for 2 days and stained for GFP. D) Quantification of dendritic protrusion numbers. Data are shown as mean±SEM (3 independent experiments, student‘s t-test, ***p<0.001 vs *Nptn-/-*_GFP, vs Np65$^{\Delta id}$-GFP; Scale bar =10 µm).

TRAF6 mRNA and protein are expessed in different adult brain neurons including mature hippocampal neurons (Ishida et al., 1996; Pranski et al., 2012; Perez, et al., 2015), but expression in inmature neurons is not completely clear. To investigate the expression and localization of endogenous TRAF6 in cultured hippocampal neurons during early development, triple immunofluorescence labeling for Np65, TRAF6 and MAP2 as marker for the somato-dendritic compartment was performed at 3-10 DIV. In general, TRAF6 immunoreactivity was diffuse in soma and dendrites of the neurons. Initially, it was more prominent in the soma and gradually distributed into

dendrites.(Fig. 26). Thus, TRAF6 expression seems to increase gradually during the development of neurons.

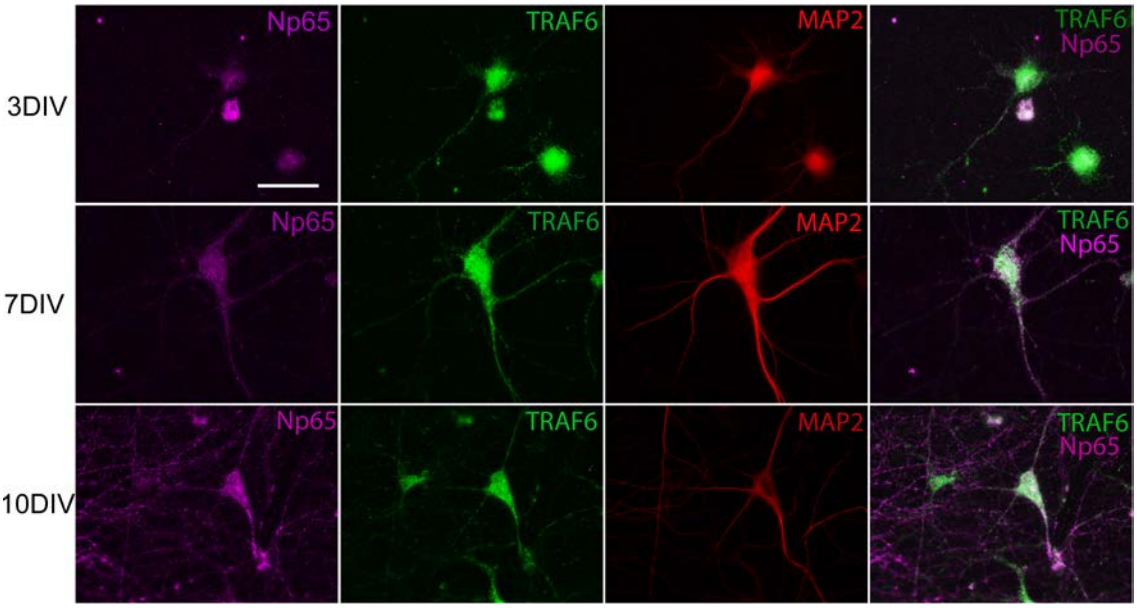


Fig. 26. Immunoreactivity of TRAF6 in cultured hippocampal neurons. Expression of TRAF6 was examined at various intervals during early development between DIV 3 and 10 of hippocampal neurons in culture. TRAF6 expression was also compared to Np65 expression and both proteins seem to increase during neuronal development. Dendrites were immunostained with MAP2 antibody (Scale bar = 100 µm).

4.6. The Np65 Ig1 specific peptide Enplastin increases dendritic protrusions via PI3K/Akt activation in hippocampal neurons

As reported previously, CAMs promote synapse formation in neurons through *trans*-synaptic adhesion either via homophilic or heterophilic interaction (Scheiffele et al., 2000; Biederer et al., 2002; Dean et al., 2003; Kim et al., 2006). Along this line, post-synaptic Np65 can mediate homophilic adhesion in *trans* with Np65 in the pre-synapse. As reported, disruption of this synaptic adhesion with Np65 Ig1 specific fusion proteins or antibodies can regulate synaptogenesis and synaptic plasticity (Smalla et al., 2000; Herrera-Molina et al., 2014). In this study, I used a mimetic peptide derived from a homophilic binding site, the F-G loop, in the Ig1 domain of Np65, which was termed Enplastin (Owczarek et al., 2011), to mimic the homophilic adhesion and to investigate spinogenesis in cultured young neurons. For this, 7 DIV hippocampal neurons were transfected with GFP. After 24 h, the neurons were treated with 500 nM Enplastin for 1 h, which lead to increased numbers of dendritic protrusions compared to unstimulated neurons (Fig. 27A, B; GFP: 1.44 ± 0.18 vs GFP_Enplastin: 3.86 ± 0.23). These protrusions were shorter and some of them beared a mushroom head compared to

neuroplastin-induced dendritic protrusions (arrow head, Fig. 26A). This experiment demonstrates that Enplastin promotes synaptogenesis in cultured neurons.

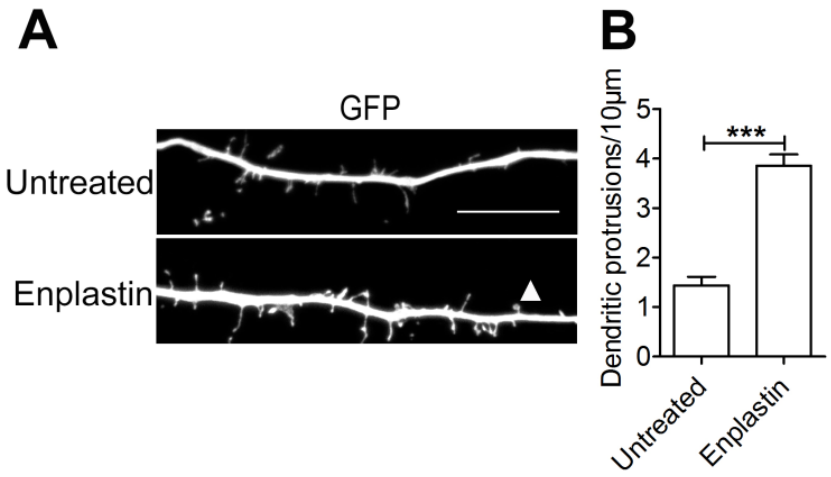


Fig. 27. Enplastin treatment increases dendritic protrusions in hippocampal neurons. A) 7 DIV cultured neurons were transfected with GFP and after 24 h, neurons were treated with Enplastin for 1 h and stained for GFP and an Alexa-488 fluorescently conjugated secondary antibody. B) The graph represents the quantitation of dendritic protrusion numbers. (GFP: n=29 and GFP_Enplastin: n=36; 3 independent experiments, student‘s t-test, ***p<0.001 vs untreated; Scale bar =10 µm). Data are shown as mean±SEM.

To further investigate the neuroplastin-induced downstream signaling after Enplastin treatment, 7 DIV cultured hippocampal neurons were treated with Enplastin for 30 min followed by immunofluorescence detection of RelA and nuclear staining with DAPI. Nuclei and cell body were defined as regions and their mean fluorescence was measured using ImageJ. Enplastin treatment markedly enhanced RelA immunoreactivity in the nucleus compared to untreated neurons (Fig. 28A, B; Untreated: 2024 ± 117.9 vs Enplastin: 3534 ± 175.3). Next, I investigated RelA nuclear translocation in hippocampal neurons treated with TNF-α (as a positive control) for 5 min and Enplastin for 0, 15 and 30 min. Cytosolic, nuclear soluble and insoluble fractions were prepared from the neurons according to a protocol developed for HeLa cells (Schweitzer et al., 2015). The presence and purity of fractions were determined using GAPDH antibody for cytosolic fraction (C), Laminin B2 and HDAC1 for nuclear insoluble fraction (N2), and HDAC1 antibody for nuclear soluble fraction (N1). I found phospho-RelA and IκB levels to be increased in the nuclear soluble fraction after 15 min of Enplastin treatment. But, since the nuclear insoluble fraction was also positive for the post-synaptic marker PSD95, and neuroplastins, indicating that it does not only contain nuclear material but also synaptic proteins, it was not possible to discriminate whether this increase in phospho-RelA and IκB levels in the fraction resulted from synapse or nucleus (Fig. 28C).

In another preliminary experiment results from Enplastin-treated 10 DIV cortical neurons show that phospho-Akt Ser473 levels were increased after 30 min (Fig. 28D). Altogether, it indicates that extracellular engagement of Np activates PI3K/Akt and RelA pathway.

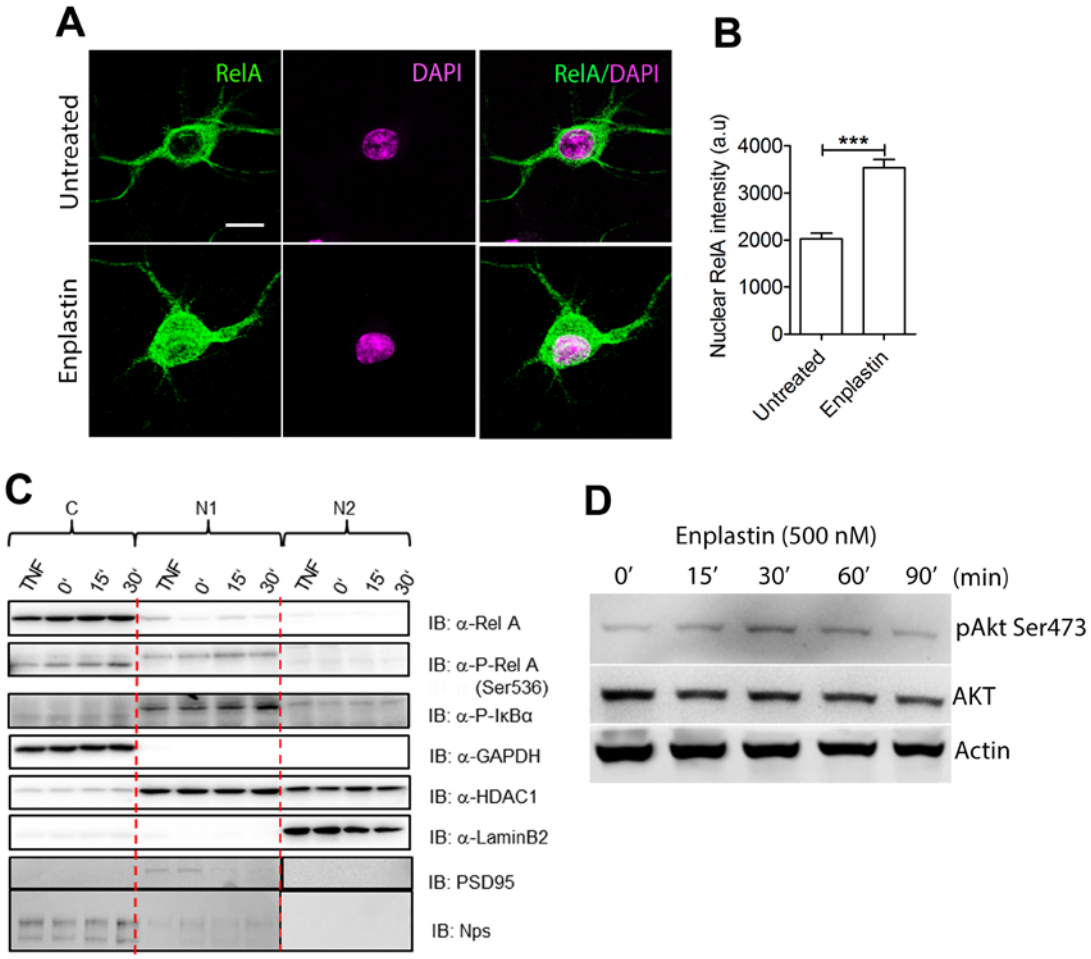


Fig. 28. Enplastin treatment increases RelA nuclear translocation in hippocampal neurons. A) 7 DIV cultured neurons were treated with Enplastin (500 nM) for 30 min. B) The graph represents the quantification of nuclear RelA immunofluorescence in neurons. Data are shown as mean±SEM (3 independent experiments, student's t-test, ***p<0.001 vs untreated; Scale bar = 50 µm). (Untreated: n=46; Enplastin: n=43). C) 9 DIV cultured neurons were treated with Enplastin (500 nM) for 0, 15 and 30 min and TNF-α for 5 min. Cytosolic (C), nuclear soluble (N1) and insoluble (N2) fractions were prepared from Enplastin- or TNF-α-treated neurons. Western blots of all fractions were probed with p-RelA, RelA, p-IκB, IκB, GAPDH, HDAC1, Lamin B2, PSD95 and neuroplastins antibodies. D) 10 DIV cortical neurons were starved for 60 min before stimulation with Enplastin (500 nM) and lysates were prepared from different time points as indicated. Immunoblotting was performed with pAkt Ser473, total AKT and β-actin antibodies (2 independent experiments).

To further dissect the signaling pathways involved in Enplastin-induced increase in dendritic protrusion formation, 8 DIV hippocampal neurons were transfected with GFP. After 24 h, the neurons were acutely treated with inhibitors to block protein synthesis (Cycloheximide (CHX), 100 µM), RelA nuclear translocation (SN50, 50 µg/ml),

PI3K/Akt (LY294002, 10 µM), and proteasome activity (MG132 or lactacystin, 10 µM) for 1 h. Then, the neurons were treated with either Enplastin or Np65-specific antibody (Np65 Ab) coupled to protein A and incubated for 1 h.

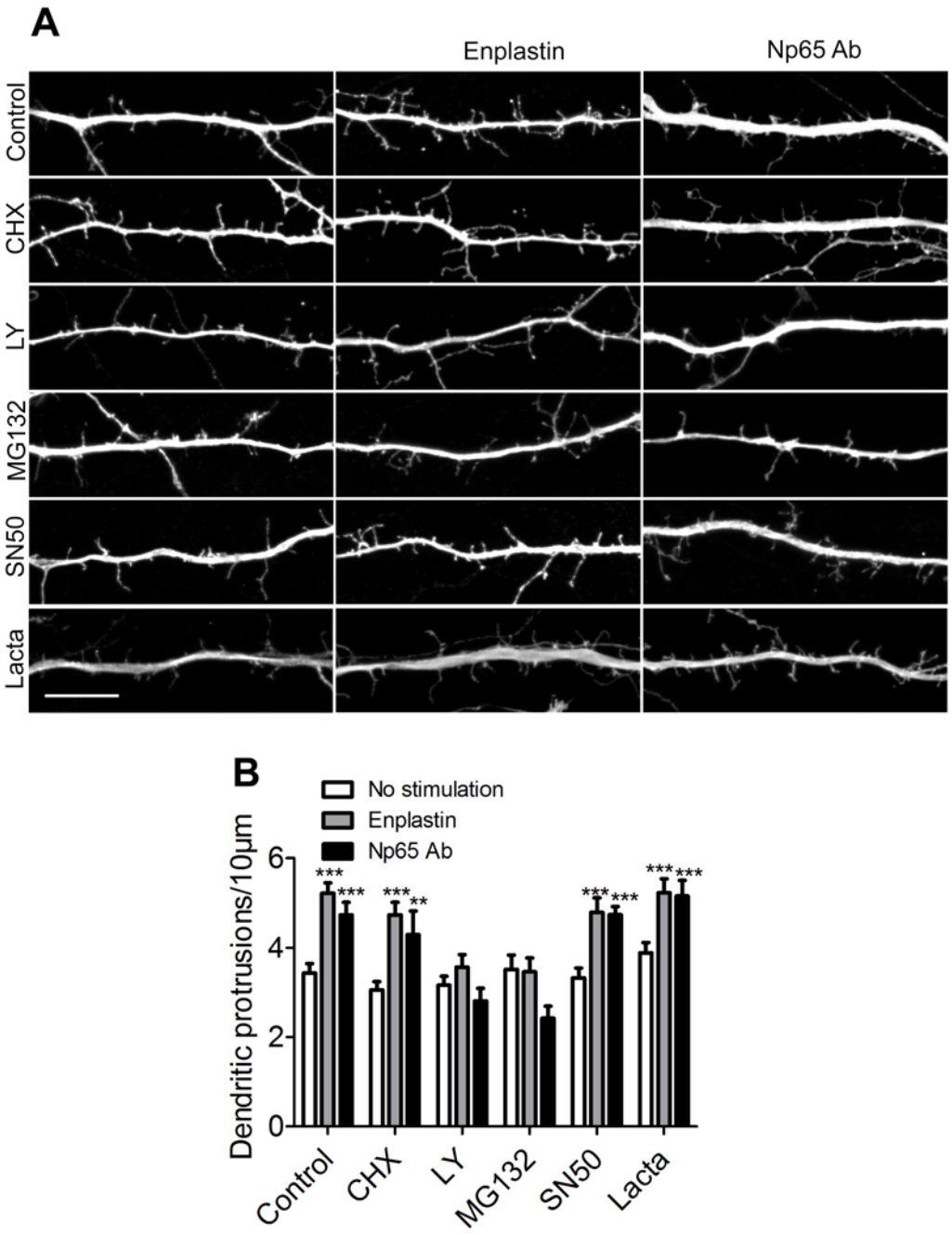


Fig. 29. PI3K/Akt pathway is involved in Enplastin- or Np65 Ab-induced dendritic protrusion formation. A) 8 DIV cultured hippocampal neurons were transfected with GFP. After 24 h, neurons were incubated with DMSO, SN50, MG132, LY294002 (LY) Cycloheximide (CHX) or Lactacystin (Lacta) for 1 h, then neurons were treated with Enplastin or Np65 Ab for 1 h, fixed and stained as mentioned earlier in the legend to Fig. 22. B) The graph represents the quantitative analysis of dendritic protrusions in neurons (3 independent experiments, student‘s t-test, ***p<0.001; Scale bar =10 µm) and data shown as mean±SEM.

The acute inhibition of PI3K/Akt pathway abrogated the dendritic protrusion formation by Enplastin or Np65 antibody (Np65 Ab). While MG123 inhibition of proteasome activity also prevented dendritic protrusion formation, application of the more specific proteasome inhibitor lactacystin (10 µM) as well as of the protein synthesis blocker or of RelA nuclear translocation did not affect dendritic protrusion formation induced by Enplastin or Np65 Ab (Fig. 29A, B). Collectively, these data indicate that Enplastin- or Np65 Ab-induced early synaptogenesis is dependent on PI3K/Akt but neither on protein synthesis nor the NF-κB pathway. These results also confirm that the extracellular engagement of Np65 is sufficient for its synaptogenic activity in neurons.

The aforementioned experiment showed that acute inhibition of RelA did not affect the Enplastin-induced dendritic protrusion formation. Next, I inhibited RelA nuclear translocation chronically for 24 h in neurons. For this, GFP-transfected hippocampal neurons were treated with DMSO or SN50 for 1 day and then Enplastin was applied for 1 h. Interestingly, long-term inhibition of RelA did not affect the Enplastin-mediated increase in dendritic protrusion numbers in neurons (Fig. 30A, B), suggesting that this process is independent of RelA signaling in young neurons.

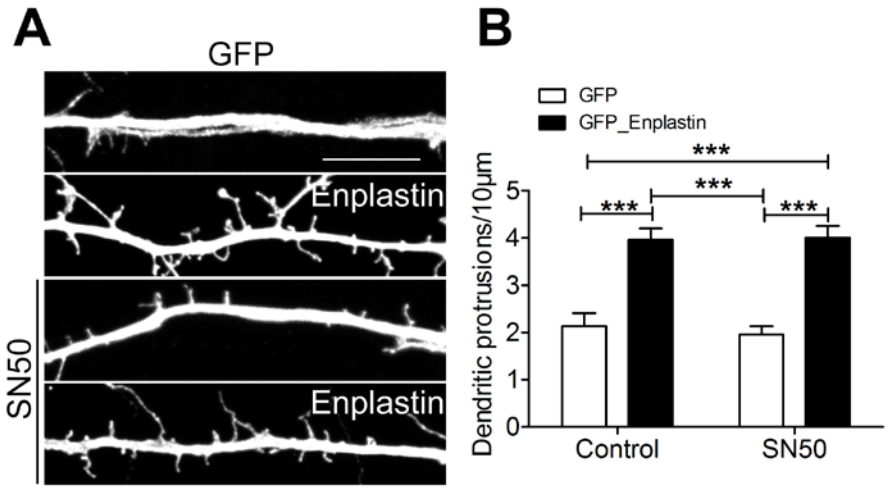


Fig. 30. RelA inhibition does not affect Enplastin-induced increase in the number of dendritic protrusions. A) 7 DIV cultured hippocampal neurons were transfected with GFP and incubated with DMSO or SN50. After 24 h, neurons were treated with Enplastin for 1 h, fixed and stained as mentioned earlier in the legend to Fig. 22. B) Quantitative analysis of dendritic protrusions. (3 independent experiments, student's t-test, ***p<0.001; Scale bar =10 µm) and data shown as mean±SEM.

4.7. Long- but not short-term inhibition of RelA reduces neuroplastin-induced dendritic protrusion formation

As shown, RelA inhibition prevented neuroplastin-induced filopodia formation in HEK cells (Fig. 20A-C). Further, to investigate the role of RelA in neuroplastin-induced dendritic protrusions, I overexpressed GFP and Np65(+)-GFP in 7 DIV hippocampal neurons and treated the neurons either with SN50 or DMSO. Pharmacological inhibition of RelA activation significantly reduced neuroplastin-mediated rise in dendritic protrusion numbers compared to control (Fig. 31A, B; Np65(+)-GFP_SN50: 1.34 ± 0.15 vs Np65(+)-GFP: 3.39 ± 0.19), but there was no difference in GFP control and SN50-treated neurons (GFP_SN50: 1.54 ± 0.17 vs GFP: 1.48 ± 0.13). Thus, this result indicates that Np65 activates RelA to promote dendritic protrusions upon overexpression.

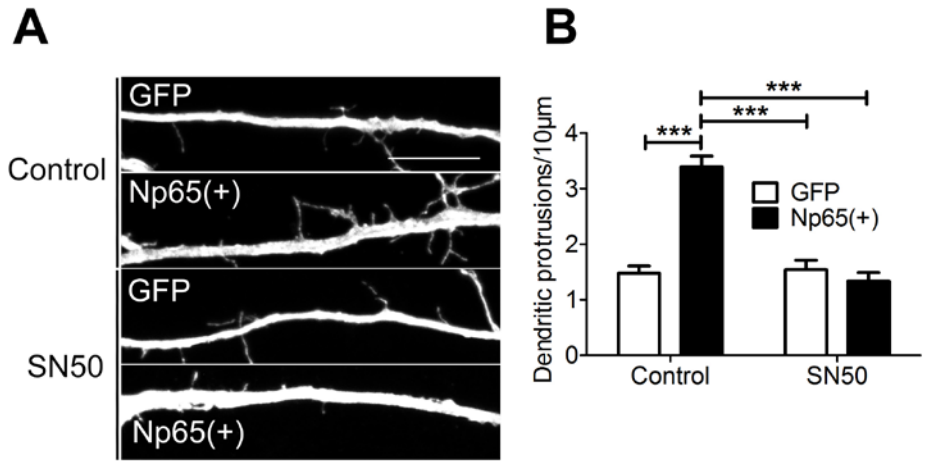


Fig. 31. RelA inhibition reduces Np65-induced dendritic protrusions in hippocampal neurons. A) 7 DIV cultured neurons were transfected with GFP or Np65(+)-GFP and treated with SN50 or DMSO for 24 h. The neurons were fixed and stained with a primary anti-GFP antibody and primary antibody was visualized with a fluorescent conjugated secondary antibody. B) The graph is representing the quantitative analysis of dendritic protrusion. (GFP: n=30; Np65(+)-GFP: n=30; GFP_SN50: n=29; Np65(+)-GFP_SN50: n=30 neurons; 3 independent experiments, student‘s t-test, ***p<0.001; ; Scale bar =10 µm) and data shown as mean±SEM.

4.8. Neuroplastin regulates dendritic arborization in hippocampal neurons via TRAF6

To analyze the function of neuroplastin in dendritic arbor complexity, I transfected WT and *Nptn-/-* hippocampal neurons with GFP and analyzed the dendritic complexity using MAP2 as a marker. The complexity was measured by ImageJ using Sholl analysis. In *Nptn-/-* neurons, dendritic complexity and total number of intersections were significantly decreased between 75-200 µm from the soma compared to WT neurons. This suggests a decreased bifurcation of secondary dendrites, which is a determinant of dendritic length, in *Nptn-/-* neurons (Fig. 32A-C). In contrast, the

dendritic complexity was not changed in Np65 KO mice hippocampal sections (Amuti et al., 2015), suggesting that loss of Np65 might be compensated with higher expression of Np55 in the same mice.

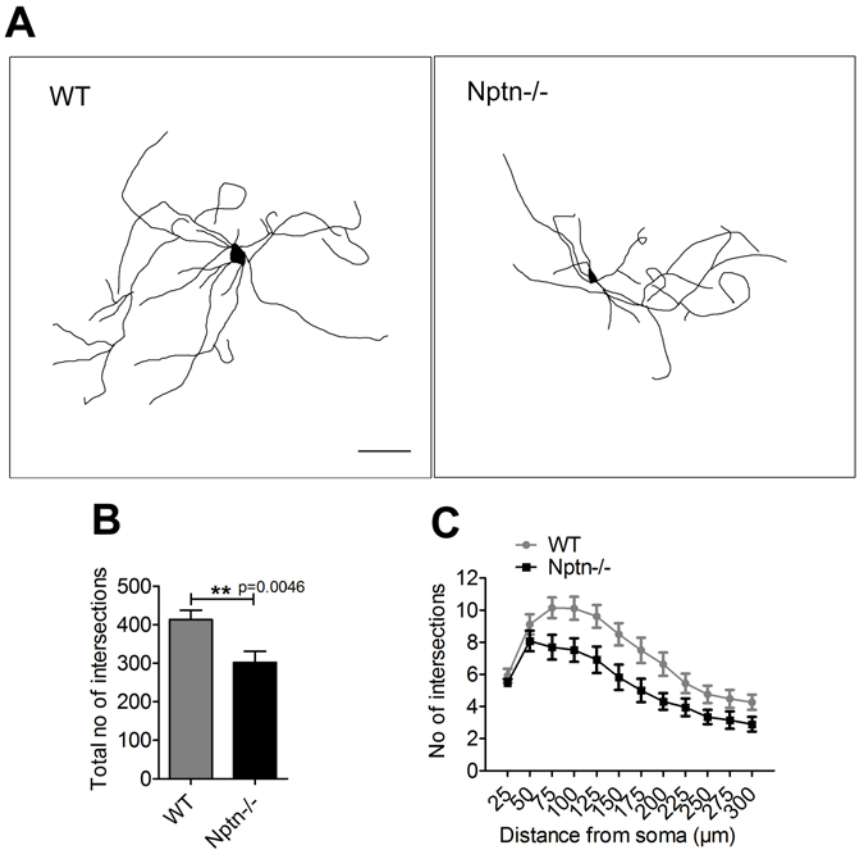


Fig. 32. Neuroplastin deficiency decreases dendritic arborization in hippocampal neurons. A) 6 DIV WT or *Nptn-/-* hippocampal neurons were transfected with GFP and cultured for 2 days. Neuronal morphology was visualized by GFP fluorescence. B, C) Quantitative analysis of total number of intersections and dendritic complexity (n=23) were reduced in *Nptn-/-* neurons compared to WT neurons (n=26). Data were shown as mean±SEM (one experiment, student‘s t-test, Scale bar =100 µm).

To further unravel the TRAF6 role in neuronal development, I knocked down TRAF6 either with TRAF6 siRNA in GFP-transfected hippocampal neurons on day 7. Then, the dendritic complexity was analyzed on day 9. The Sholl analysis revealed decreased dendritic complexity and intersections in TRAF6 knockdown neurons compared to control scrambled siRNA (Fig. 33A-C). This indicates that TRAF6 indeed plays a role in dendrite development in young neurons.

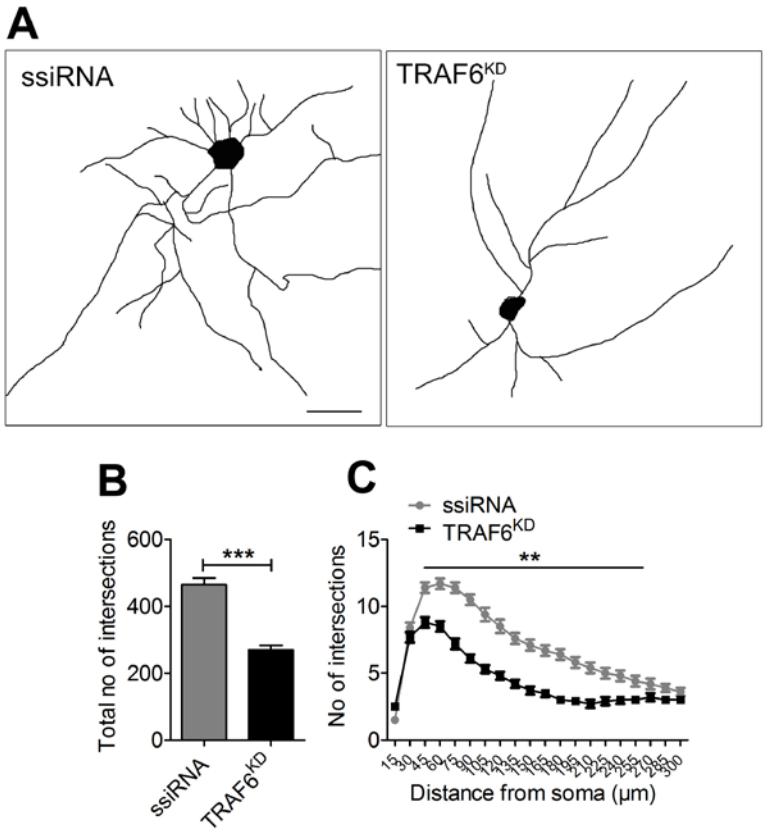


Fig. 33. TRAF6 down regulation reduces dendritic complexity in hippocampal neurons. A) Hippocampal neurons were co-transfected with GFP and either ssiRNA or TRAF6 siRNA at 7 DIV and fixed on day 9. The neuronal morphology was visualized by GFP fluorescence. B, C) The graphs show the Sholl analysis of dendritic complexity and total number of intersections. Data are shown as mean±SEM (3 independent experiments, student's t-test, ***p<0.001; Scale bar =100 µm).

5. Discussion

In the present study, I have investigated the molecular mechanisms of dendritic protrusion formation mediated by interaction of neuroplastin with TRAF6. Neuroplastin is a synaptic cell adhesion molecule, which regulates excitatory synapse number in mature neurons (Herrera-Molina et al., 2014). Here I show that neuroplastin also modulates early spinogenesis in young neurons by interacting with TRAF6 at the plasma membrane. This interaction is evident for the formation of filopodia like-structures in HEK cells as well as in neurons. This dendritic filopodia formation occurs through the activation of PI3K/Akt as well as NF-κB pathways (Znamensky et al., 2003; Wang et al., 2006; Boersma et al., 2011). Together, my results suggest that the interaction between neuroplastin and TRAF6 is a crucial step for the formation of dendritic spines by activating PI3K/Akt and NF-κB signaling in neurons.

5.1. Neuroplastin directly interacts with TRAF6 via a TRAF6 binding site

Here, I showed that both Np55 and Np65 interact with TRAF6. With immunoprecipitation assays, I demonstrated that a potential TRAF6 binding site in the C-terminal domain of neuroplastins is indeed functional and required for the interaction. This binding site is conserved in multiple membrane proteins and species (Ye et al., 2002; Sorrentino et al., 2008). Mutation in this binding site in neuroplastin remarkably reduced the interaction with TRAF6. However, it did not completely abolish the interaction with neuroplastin mutants. The in silico modeling of neuroplastin-TRAF6 showed that basic amino acids from 361st to 364th position in Np65 increase the affinity towards TRAF6. However, the surface plasmon resonance experiment results confirmed the direct interaction between neuroplastin and TRAF6 with good affinity. Moreover, immunoprecipitation and pull-down assays suggest that the interaction between neuroplastin and TRAF6 involves the TRAF6 binding site of neuroplastin and the TRAF-C domain of TRAF6. Several studies reported that regulatory proteins or receptors with TRAF6 binding site mediate the interaction with TRAF6 (Khursigara et al., 1999; Sorrentino et al., 2008; Powell et al., 2009). In contrast to it, it has been shown that basigin also interacts with TRAF6, but via its transmembrane region (Luo et al., 2016). Indeed, another study confirmed that this interaction occurs through Glu218 and Glu230 in the transmembrane and Lys231 and Arg264 in the cytoplasmic domain of basigin (Biswas et al., 2017). This suggests that both neuroplastin and basigin interact with TRAF6, but molecularly different. However, they may still share a common signaling mechanism. Although neuroplastin is reported to interact with TRAF6, there is a growing body of evidence that neuroplastin also associates with TRAF2 and GRB2

in keratinocytes (Sakaguchi et al., 2016). Thus, TRAFs may regulate neuroplastin signaling by physical association.

5.2. Neuroplastin recruits TRAF6 beneath the plasma membrane

Multiple evidence has been reported that TRAF6 trimerization occurs via its TRAF-C domain but dimerization occurs through its RING domain and first zinc finger (Cao et al., 1996; Yin et al., 2009; Wu, 2013). This molecular architecture of the TRAF6 multimeric structure forms a lattice-like network beneath the cell membrane, which allows the execution of cellular functions by mediating signal transduction and allowing more efficient enzymatic reactions (Ferrao et al., 2012; Wu, 2013). Moreover, several papers suggest that TRAF6 helps form, organize and stabilize cell structures via forming higher-order oligomers by interacting with several membrane proteins such as RANK, IL-1R, CD40, p75NTR and presenilins, and thereby recruiting TRAF6 to the inner leaflet of plasma membrane (Khursigara et al., 1999; Schultheiss et al., 2001; Yin et al., 2009; Xie et al., 2013; Yan, et al., 2013). TRAF6 forms not only homo oligomers but also hetero oligomers with TRAF2 and TRAF3 (Cao et al., 1996; Ea et al., 2004; Yin et al., 2009). TRAF6 oligomerization increases the activity of E3 ubiquitin ligase (Ea et al., 2004; Huang et al., 2012) and thereby promotes polyubiquitin synthesis and auto-ubiquitination of TRAF6 (Yin et al., 2009). Here, I found that TRAF6 was translocated to the plasma membrane, where it could associate with neuroplastin, and TRAF6 immunoreactivity was higher at the membrane, which could be the TRAF6 oligomers to induce filopodia. Several CAMs, like neuroligin, NCAM and L1 CAM dimerize in the membrane via cis-interaction as well as trans-interaction with other CAMs present on the opposite cell (Kiselyov et al., 2005). Thus, it is possible that neuroplastins might also dimerize or trimerize and interact with trimeric TRAF6 to regulate formation of supramolecular structures, such as higher-order oligomers, beneath the membrane. In immunostaining experiments, I have shown that N-terminally deleted TRAF6 was also recruited to the membrane but reduced the filopodia, suggesting a functional role of TRAF6 in filopodia formation. However, the results from pull-down assays support the notion that the TRAF-C domain mediates the interaction with receptors but it may fail to form dimers and abolish the signaling. Altogether, it is suggesting that neuroplastin might be forming higher-order oligomeric complexes via TRAF6 with scaffold proteins beneath the plasma membrane to activate downstream signaling mechanisms. Further research will be required to complete understanding of neuroplastin-induced TRAF6 oligomerization.

5.3. TRAF6 is expressed in hippocampal neurons and regulates dendritic protrusion formation

TRAF6 is present in the soma, dendritic shaft (Zhou et al., 2010) and dendritic filopodia of neurons (in this study). TRAF6 acts as an adapter protein and a signaling scaffold to mediate various biological signaling pathways. It possesses several substrates to elicit its functions in normal physiological and pathological conditions (Inoue et al., 2007; Xie et al., 2013; Wu and Aron, 2003). In particular, a major part of TRAF6 function is attributed to ubiquitin-mediated protein signaling or degradation (Yeiser et al., 2004; Lamothe et al., 2007; Powell et al., 2009; Zucchelli et al., 2010; Yan et al., 2013). Moreover, TRAF6 regulates axonal filopodia extension by NGF-mediated ubiquitination of Unc51-like-kinase 1/2 (Ulk1/2) and activation of PI3K/Akt signaling pathway in sensory neurons (Zhou et al., 2007). In this study, I have unveiled the TRAF6 role in formation of dendritic protrusions by knockdown of TRAF6 and overexpression of $Np65^{\Delta id}$ in neurons. However, neuroplastin overexpression did not rescue the decrease in dendritic protrusions in TRAF6 knocked down neurons. It suggests that neuroplastin requires TRAF6 to promote synaptogenesis. These results corroborate an earlier study showing that TRAF6 can lead to filopodia formation in HEK cells (Wang et al., 2006). But, knockdown of TRAF6 did not reduce the filopodia length in HEK cells. This effect might be due to residual TRAF6 levels in the cell, which were sufficient to allow existing filopodia to grow to a certain length but it may not promote new filopodia. A similar function of TRAF6 in two different systems suggests that it can play an important role in the formation of filopodia-like compartments in general and in neuronal synaptogenesis. Moreover, other E3 ubiquitin ligases such as Pam/Highwire/RPM-1 (PHR) proteins regulate presynaptic differentiation and synaptogenesis in *C.elegans* (Grill et al., 2007).

5.4. Neuroplastin increases filopodia-like dendritic protrusions via its TRAF6 binding motif in hippocampal neurons

In my thesis, I showed that ectopic expression of neuroplastin is sufficient to promote dendritic protrusions in neurons. However, this effect is independent of known ligands or growth factors. Further unpublished results indicate that Np65 can associate with Np65 in *cis* in the same membrane as well as at the base of the filopodia in HEK cells, as shown with FRET analysis. It is possible that *cis* dimerization may occur via Ig2-3 domains of two Np65 on the same cell. This process may be a transient phenomenon. Indeed, previously, it has been reported that Np65 can mediate the *cis*-homophilic interaction of both Np65 molecules present on the same cell (Sarto-Jackson et al., 2012). But, how Np65 mediates *cis*-homophilic interaction is not yet known. However, basigin can form *cis* dimers or homo-oligomers through N-terminal Ig2 domain on the

single plasma membrane of several chicken tissues and mouse testis tissue (Fadool and Linser, 1996; Yoshida et al., 2000). Recently, it was shown that basigin is also involved in heterophilic interaction with both neuroplastins to form oligomers in the membrane of cultured keratinocytes (Sakaguchi et al., 2016). These supramolecular structures such as oligomers could regulate the keratinocyte proliferation (Sakaguchi et al., 2016). Moreover, several CAMs like cadherin, L1 and NCAM also mediate both *trans*- and *cis*-homophilic interactions through their Ig-like domains (Boggon et al., 2002; Ditlevsen et al., 2008; Wei and Ryu, 2012). Particularly, *cis*-homophilic interaction occurs between two NCAMs to form nonsymmetrical compact zipper-like structures through the first two Ig domains of NCAM (Ditlevsen et al., 2008).

Moreover, I showed that the Np65$^{\Delta id}$ construct decreased dendritic protrusions. Although Np65$^{\Delta id}$ is able to interact with Np65 in *trans* or *cis* this might not be sufficient to stabilize the synaptic contact. Moreover, TRAF6 knockdown reduced the Neuroplastin-induced filopodia structures in HEK cells and filopodia-like dendritic protrusions in neurons. This suggests that the cytoplasmic domain of neuroplastin is necessary for the interaction with TRAF6 at the plasma membrane, which might play a pivotal role in activating downstream signaling pathways to initiate filopodia/dendritic protrusion formation. However, other synaptic cell adhesion molecules like synCAM 1 regulate filopodial dynamics and synapse number through its cytoplasmic domain via d FERM, Rho/ArhGEF, and Pleckstrin domain protein 1 (Farp1) (Cheadle and Biederer, 2012). Synaptic cell adhesion molecules regulate the initial contact between pre- and post-synapses by producing axonal filopodia and dendritic protrusions (Dalva et al., 2007). Dendritic protrusions are cell membrane extensions by actin filament bundles arranged in parallel beneath the membrane (Cingolani and Goda, 2008). These are thought to be precursors for excitatory synapses in mature neurons (Dailey and Smith, 1996). They are highly dynamic and retract their projections within 15 min in absence of any suitable target. It has been reported that absence of neuroplastin resulted in reduction of excitatory synapses and increase of pre- and post-synaptic mismatch (Herrera-Molina et al., 2014). This was due to loss of neuroplastin, which could mediate adhesion between pre- and post-synapse (Smalla et al., 2000; Empson et al., 2006; Beesley et al., 2014). The lack of neuroplastin might disrupt the recruitment of signaling molecules at the synapses and thereby synapse structure. Based on my results, I propose that neuroplastin can participate both in early stages of synaptogenesis (in this study) and in stabilization of mature excitatory synapses (Herrera-Moilna et al., 2014). Interestingly, it was reported that the heterophilic interaction of Np55 with FGFR1 induces neurite outgrowth by increasing the synaptic calcium concentration in an FGFR1-dependent manner (Owczarek et al., 2010). Thus,

taken together, it is obvious that the homophilic and heterophilic interactions of neuroplastin are critical for its functional role.

In agreement with this, my data suggests that the increased dendritic protrusions and filopodia upon neuroplastin overexpression might occur via regulation of actin polymerization (Wong et al., 1999; Wang et al., 2006), which could be mediated by its interaction partner TRAF6. Indeed, neuroplastin lacking its cytoplasmic domain failed to increase the number of dendritic protrusions, suggesting that TRAF6 is the key downstream molecule of neuroplastin to regulate actin cytoskeleton (Wang et al., 2006). Moreover, short-term treatment of cells with Enplastin increased dendritic protrusions suggesting that neuroplastin may regulate actin cytoskeleton to form dendritic spines in young neurons. In agreement with my results, neuroplastin deficiency alters the actin cytoskeleton in cultured *Nptn-/-* hippocampal neurons (Herrera-Molina et al., 2014). All together this suggests that neuroplastin regulates synapse formation/structure by remodeling of actin cytoskeleton via TRAF6.

5.5. Role of PI3K/Akt activation in dendritic protrusion formation after treatment with Enplastin

Here, I studied if neuroplastin-induced filopodia formation is differentially regulated and involves two different signaling pathways i.e., local signaling to actin and a slower signaling pathway to the nucleus via NF-κB activation. Application of Enplastin or Np65 Ab treatment increased the dendritic protrusion numbers within 1 h time period. However, NF-κB is already in the nucleus after 30 min treatment but inhibition of NF-κB did not affect the Enplastin- or Np65 Ab-induced dendritic protrusion number. In contrast, short-term inhibition of the PI3K/Akt pathway did not promote dendritic protrusions. It is suggesting that extracellular engagement of Np65 activates PI3K/Akt and NF-κB signaling but PI3K/Akt activation is sufficient for dendritic protrusion formation in neurons. NF-κB-dependent gene transcription might not be needed for Enplastin-induced dendritic protrusions, which may occur via local activation of PI3K/Akt in the dendritic compartment (Znamensky et al., 2003). Moreover, neuroplastin activates the PI3K/Akt pathway in Enplastin-treated cortical neurons. It is consistent with published data that the activated PI3K/Akt promotes synaptogenesis in hippocampal neurons (Kumar et al., 2005; Cuesto et al., 2011; Majumdar et al., 2011). Moreover, protein synthesis inhibition does not affect the formation of dendritic protrusions, suggesting Enplastin-induced dendritic protrusion formation being independent of new proteins. In contrast, it has been reported that the PI3K pathway promotes local protein synthesis in dendritic compartments to promote dendritic filopodia formation in neurons (Znamensky et al., 2003). Recently, it has been shown

that Np65 functions as a receptor for the S100A9 protein (Sakaguchi et al., 2016), but S100A9 expression has not been reported in the brain so far. I can speculate that there may be another S100 family protein could bind to Np65. Altogether, extracellular engagement or stimulation of particularly Ig1 domain of Np65 promotes synaptogenesis via activation of PI3K/Akt pathway in neurons.

Because of some limitations in Np65-expressing HEK cells, I could not perform the Np65 stimulation experiments. Np65 overexpression may saturate the system and it may not increase the filopodia number further after Enplastin treatment. Indeed, I performed the experiments in hippocampal neurons at the time point when endogenous Np65 only appears.

5.6. NF-κB activation is necessary for neuroplastin-induced dendritic protrusion formation

In this study, I have shown that overexpression of neuroplastin increased nuclear RelA whereas pharmacological inhibition of RelA using SN50 or Np65$^{\Delta id}$ reduced it. Indeed, I also showed that inhibition of RelA using SN50 reduced dendritic protrusion formation in neurons. Neuronal activity increases the translocation of NF-κB from synapse to nucleus via dynein motor protein along microtubules (Meffert et al., 2003; Shrum et al., 2009). This nuclear translocation of the RelA subunit of NF-κB is needed to interact with Hsc70 and importin-α to reach the nucleus to regulate transcription of genes encoding synaptic proteins related to memory (Mikenburg et al., 2007; Klenke et al., 2013; Kaltschmidt and Kaltschmidt, 2015). Neuronal NF-κB enhances synapse size, spine density and excitatory synapses during neuronal development or in response to neuronal activity (Russo et al., 2009; Boersma et al., 2011; Imielski et al., 2012; Schmeisser et al., 2012). NF-κB also increases the excitatory synaptic function in the CNS (Boersma et al., 2011). These findings in hippocampal neurons suggested that neuroplastin may regulate synaptic function by activating the NF-κB signaling pathway via TRAF6. Canonical NF-κB activation-dependent gene transcription is important for activity-dependent synaptic plasticity and for cognitive functions including learning and memory (Meberg et al., 1996; Meffert et al., 2003; O'Mahony et al., 2006; Boersma et al., 2011; Jarome et al., 2015; Salles et al., 2015). NF-κB signaling mediated by neuroplastin may contribute to gene transcription to recruit the molecular machinery to form functional synapses. But, NF-κB signaling is not necessary for the formation of dendritic protrusions. However, LTP, a cellular correlate of learning and memory, was not maintained in hippocampal slices, which were treated with Ig1-3 specific fusion proteins or Np65 specific antibody (Smalla et al., 2000; Empson et al., 2006). The maintenance phase or late phase of LTP is dependent on protein synthesis, which

provides the building blocks necessary for the changes occurring at synapses during memory formation (Nayak et al., 1999; Alberini, 2009). Recently reported data show that RelA levels were elevated after MWM training in animals (Snow et al., 2015), suggesting that NF-κB activation might be needed for long-term memory formation. Indeed, NF-κB p50 deletion impaired late LTP and altered long-term memory formation in mice (Oikawa et al., 2012). Recently, *Nptn-/-* mice displayed a clear deficit in associative learning and memory tasks (Bhattacharya et al., 2016). Hence, the results presented here delineate a molecular mechanism responsible for the phenotype of *Nptn-/-* mice and cultured hippocampal knockout neurons i.e., increased pre- and post-synapse mismatch and reduced excitatory synapse number (Herrera-Molina et al., 2014), which may require neuroplastin-mediated NF-κB activation to regulate transcription of several genes to form a proper synapse and to maintain long-term potentiation and memory formation. However, I did not exclude other signaling pathways that act simultaneously after neuroplastin activation. L1CAM, a neuronal adhesion molecule essential for neurogenesis and axon guidance, has been shown to interact with integrin and mediate NF-κB signaling in cancer cell lines (Kiefel et al., 2011). The results are consistent with my findings that cell adhesion molecules are able to activate NF-κB signaling to regulate biological functions.

5.7. Conclusion

Finally, this study provides new insights into molecular mechanisms that contribute to neuroplastin-regulated excitatory synapse number in neurons (Herrera-Molina et al., 2014). Therefore, I propose a model (Fig. 34) summarizing molecular mechanisms potentially responsible for neuiroplastin-mediated synaptogenesis in neurons. Both Np55 and Np65 are expressed on the plasma membrane of neurons. Neuroplastin-mediated synaptogenesis occurs in two different ways: 1) Formation of neuroplastin *cis* homo- or heteromultimers 2) Extracellular engagement of Ig1 domain of Np65 leads to TRAF6 binding and recruitment to the plasma membrane (Fig. 34, step 1). This interaction promotes the activation of PI3K/Akt signaling to initiate dendritic protrusion formation via regulation of actin cytoskeleton (Fig. 34, step 2). Neuroplastin-TRAF6 interaction also activates NF-κB nuclear translocation to regulate gene expression, which might be needed for synapse formation (Fig. 34, step 3). However, the gap between neuroplastin-TRAF6 interation and activation of PI3K/Akt or NF-κB pathways, and post-translational modifications of neuroplastin needs to be studied in the future. It will be interesting to study if neuroplastin not only activates PI3K/Akt or NF-κB pathways but may also be capable to mediate other signaling events by multiple

signals emanating from neuroplastin and thus potentially cooperating to induce biological events.

Further studies will be needed to deepen the understanding of the molecular mechanisms behind neuroplastin contribution to synapse formation and thus to its role in cognition and intellectual ability in mice and men.

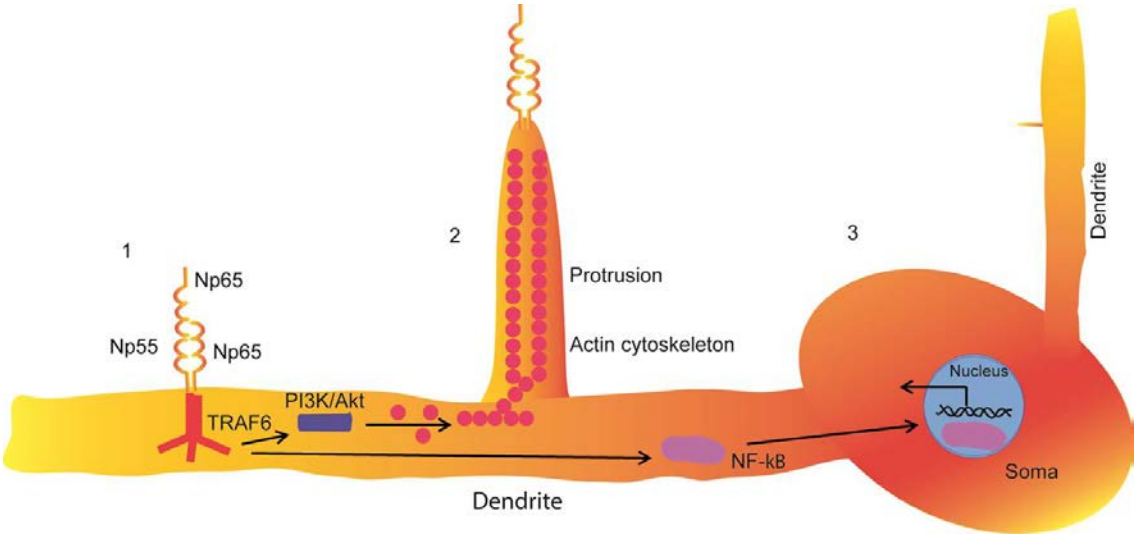


Fig. 34. A model for neuroplastin-TRAF6 interaction and involved signaling pathways during synaptogenesis. In dendrites, neuroplastin cis-multimers or Np65 extracellular engagement recruits TRAF6 to plasma membrane (1). This interaction can send a signal to the actin cytoskeleton via TRAF6 to regulate formation of dendritic protrusions via PI3K/Akt pathway (2) and also to the nucleus via NF-κB pathway to induce adhesion-mediated changes in gene expression (3).

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7. List of Abbreviations

AP-1	Activator protein-1
CAM	Cell adhesion molecules
cDNA	Complementary deoxyribonucleic acid (DNA)
C-terminus	Carboxy terminus
DIV	Day in vitro
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.coli	Escherichia coli
EDTA	Ethylenediamine-N,N,N`,N-tetraacetic Acid
ERK1/2	Extracellular signal-regulated protein kinases ½
FGFR1	Fibroblast growth factor receptor type-1
FRET	Fluorescence resonance energy transfer
$GABA_AR$	A-type of gamma-amino butyric acid receptor
GFP	Green fluorescent protein
GPI-GFP	Glycosylphosphatidylinositol-anchored green fluorescent protein
GRB2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
h	Hours
HEK	Human embryonic kidney cells
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
Ig	Immunoglobulin
IL-1	Interleukin-1
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
KO	Knockout
LB-medium	Luria-Bertani-medium
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCT2	Monocarboxylate transporter 2
NCAM	Neuronal cell adhesion molecules
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
N-HTT	N-terminal mutant of huntingtin protein
p75NTR	p75 neurotrophin receptor
PCR	Polymerase chain reaction
PD	Parkinson's disease
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PSD	Post-synaptic density
RANK	Receptor activator of nuclear factor kappa-B
RING	Really interesting new gene
ssiRNA	Scrambled short interfering ribonucleic acid
TAK1	Transforming growth factor beta-activated kinase 1
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	Tumor necrosis factor receptor-associated factor
WT	Wild-type

8. List of Figures

9. List of Tables

10. Curriculum Vitae

Name:	Sampath Kumar Vemula
Date of Birth:	15.05.1986
Place of Birth:	Indurthy, Karimnagar, Telangana, India
Nationality:	Indian
Professional affiliation:	Department of Neurochemistry and Molecular Biology, Leibniz Institute for Neurobiology, Brenneckestrasse 6, 39118, Magdeburg, Germany
Telephone:	+49-391-626393351
Email:	svemula@lin-magdeburg.de
	sampathniper@gmail.com

Education and experiences

May 2013 - Present	PhD student under the supervision of Prof. Dr. Constanze I. Seidenbecher, Department of Neurochemistry and Molecular Biology, Leibniz Institute for Neurobiology, Magdeburg, Germany.
Mar 2012 - Apr 2013	Worked as an In-charge of Biomedical Services, Shree Dhootpapeshwar Limited, Navi Mumbai, India.
Nov 2011 - Mar 2013	Worked as a Research Associate Trainee in Clintox Bioservices, Hyderabad, Telangana, India. (For 4 months)
June 2009 – May 2011	Master of Science [M.S (Pharm.)] in Pharmacology & Toxicology from National Institute for Pharmaceutical Education and Research (NIPER)-Guwahati, Assam, India. (PI: Prof. Mangala Lahkar)
Sep 2006 – May 09	Bachelor of Pharmacy (B.Pharm) from Kakatiya University, Warangal, Telangana, India.
Aug 2004 – Mar 06	Diploma in Pharmacy (D.Pharm) from Krishnadevaraya (KDR) Govt. Polytechnic, Wanaparthy, Telangana, India.
June 2002 – Mar 04	Board of Intermediate (10+2 Class), Telangana, India
March 2002	Board of Secondary Education of Andhra Pradesh (10th Class), India.

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12. Erklärung

Hiermit erkläre ich, Sampath Kumar Vemula, dass ich die von mir eingereichte Dissertation zum dem Thema

Neuroplastin-dependent signaling in neurons

selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollstandig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, 16.02.2018

Ort, Datum Unterschrift

Sampath Kumar Vemula