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The regulation of the expression of PMCA isoforms by neuroplastin has an impact on the calcium clearance in cultured hippocampal neurons

D i s s e r t a t i o n

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Zusammenfassung

Die Wiederherstellung des basalen Calciumspiegels nach einer Exzitation ist entscheidend für die Funktionalität aller Neuronen. Ein wichtiger Bestandteil der dafür verantwortlichen Maschinerie ist die Plasma membrane calcium ATPase (PMCA), eine Calciumpumpe, die mit hoher Affinität die Calciumionen vom Zytosol in den extrazellulären Raum pumpt. Vier Gene kodieren die vier PMCA-Isoformen, die für verschiedene Zelltypen und Funktionen spezifisch sind. Es wurde bereits etabliert, dass PMCAs mit Neuroplastin Komplexe bilden und die Expression der Isoformen durch diese Interaktion unterschiedlich beeinflusst wird. Um diesen Sachverhalt genauer zu untersuchen, wurden in der vorliegenden Arbeit die PMCAs gemeinsam mit Neuroplastin in verschiedenen Zelllinien überexprimiert, um festzustellen, ob sie in Bezug auf Proteinexpression unterschiedlich beeinflusst werden. Die Experimente zeigten, dass in COS-7-Zellen die Überexpression gemeinsam mit Neuroplastin die Expression der neuronenspezifischen PMCA2 stärker erhöhte als die der anderen PMCAs. Da sowohl Neuroplastin als auch alle PMCAs eine Bindungsstelle für das Signalmolekül TRAF6 haben, lag die Vermutung nahe, dass TRAF6 für die Interaktion zwischen den zwei Proteinen relevant ist. Mittels einer Mutation von Neuroplastin, der die TRAF6-Bindungsstelle fehlt, konnte gezeigt werden, dass sowohl in HEK-Zellen als auch in Hippocampusneuronen die Steigerung der PMCA2 Expression durch Neuroplastin unabhängig von TRAF6 geschieht. Um den Einfluss der Überexpression von Neuroplastin auf die Zellphysiologie zu untersuchen, wurde Neuroplastin in hippocampalen Neuronen überexprimiert und die zytosolische Calciumkonzentrationsänderungen nach einer elektrischen Stimulation quantifiziert.

Die Tatsache, dass die Überexpression von Neuroplastin zu einer Verlangsamung der Calcium-Clearance führte, statt sie durch die Erhöhung der PMCA-Expression zu beschleunigen, deutet darauf hin, dass der Einfluss von Neuroplastin auf den Calciumhaushalt komplexer zu sein scheint und weitere Faktoren berücksichtigt werden müssen, um die Auswirkung der Interaktion von Neuroplastin und PMCA auf die Zellfunktion beurteilen zu können.

Documentation

Bibliographic account

Abstract

The restoration of basal cytosolic calcium levels after excitation is essential for neurons. An important part of the machinery responsible for calcium clearance is the Plasma Membrane ATPAse (PMCA), which pumps calcium ions from the cytosol to the extracellular space with high affinity. Four PMCA paralogs are specific for distinct cell types and functions. As suggested recently, PMCAs form complexes with neuroplastin (Np) and their expression levels are affected differently by this interaction. To test the hypothesis that the ability of neuroplastin to stimulate PMCA surface expression and to increase the speed of the calcium clearance depends on the neuroplastin interaction partner TRAF6, the PMCAs were overexpressed together with neuroplastin in cell lines and neurons.

The experiments showed that in COS-7 cells the overexpression of neuroplastin increased the protein level of the neuron-specific PMCA2 more than of the other PMCAs. Since neuroplastin and all PMCAs have a binding site for the signal adapter TRAF6, I tested if this protein might be relevant for their interaction. By employing a neuroplastin mutant construct missing the TRAF6 binding site I could show, that the increase of PMCA2 expression levels caused by neuroplastin in HEK cells and hippocampal neurons is TRAF6-independent.

Against our expectation, in calcium imaging experiments I could show that increased PMCA levels due to overexpression of neuroplastin lead to slowing down calcium extrusion after stimulation, indicating that the influence of neuroplastin on the calcium balance is more complex. Additional factors such as the time and duration of the transfection need to be considered in order to understand the influence of the interaction of PMCA and neuroplastin on the calcium balance.

Contents

1. List of Abbreviations

AD	Alzheimer's disease
A.U.	Arbitrary units
ATP	Adenosine triphosphate
CAM	Cell adhesion molecules
CHO	Chinese Hamster Ovary
COS	CV-1 (simian) in Origin, and carrying the SV40 genetic material
Cy	Cyanine
DIV	Days in vitro
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCaMP	Fusion protein of green fluorescent protein (GFP), calmodulin and M13
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
h	Hour
HBSS	Hank's Balanced Salt Solution
HEK cells	Human embryonic kidney cells
HRP	Horseradish peroxidase
Ig	Immunoglobulin
Il	Interleukin
IP3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor
IRAK	Interleukin-1 receptor-associated kinase
kDA	Kilodalton

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MAP2	Microtubule-associated protein 2
MCT1	Monocarboxylate transporter 1
mRNA	Messenger RNA
NCKX	Na/Ca-K exchanger
NCX	Na/Ca$^+$ exchanger
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
Np	Neuroplastin
PBS	Phosphate-buffered saline
PDZ	PSD-95, DlgA, ZO1
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP	Phosphatidylinositol phosphate
PIP2	Phosphatidylinositol Biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane calcium ATPase
RyR	Ryanodine receptor

2. Introduction

2.1 Role of the intracellular calcium signaling in neurons

Calcium (Ca^{2+}) plays a crucial role in all cells as most cell functions depend on it and it is recognized as the most universal carrier of signals (Brini et al., 2013a). In all processes from development through cell adaptation to cell death, calcium has a substantial function (Berridge, 2012).

A variety of molecular systems interact with each other in a complex manner to regulate the calcium concentrations within and between the various cellular organelles, both temporally and locally through processes as extrusion, chelation and compartmentalization, since the calcium ion itself cannot be chemically altered or inactivated. The speed and intensity of signal transduction by calcium are highly dependent on the enormous 20,000-fold gradient that cells build up across the cell membrane at high cost, while in contrast the concentrations of other ions differ less between the intracellular and extracellular space. The maintenance and restoration of cytoplasmic calcium levels depend primarily on the Na^+/Ca^{2+}-K exchanger NCKX, the Na^+/Ca^{2+} exchanger NCX, and Ca^{2+} -activated K^+ and Cl^- channels (Clapham, 2007). The Plasma membrane calcium ATPase PMCA extrudes calcium from the cytosol to the extracellular space and the Sarcoplasmic Reticulum Calcium ATPase SERCA pumps calcium into the sarcoplasmic reticulum. The increase of the cytoplasmic calcium level depends on voltage-gated and ligand-gated calcium channels, which allow calcium to flow in from the extracellular space, and the calcium-sensitive ryanodine receptor RyR and the ligand-dependent inositol trisphosphate receptor IP_3R from the ER (Guerini et al., 2005) (Figure 1).

The changes in the cytoplasmic calcium level trigger a series of signal cascades responsible for the signal transduction of the calcium signal. Many effects of calcium are facilitated by the protein calmodulin, whose conformation is altered by calcium and can interact with a variety of proteins that incorporate a calmodulin recruitment site. Calmodulin itself is highly conserved and plays a prominent role in calcium signaling in many organisms and is necessary for the transduction of changes of the intracellular calcium concentration into a cellular response (Clapham, 2007).
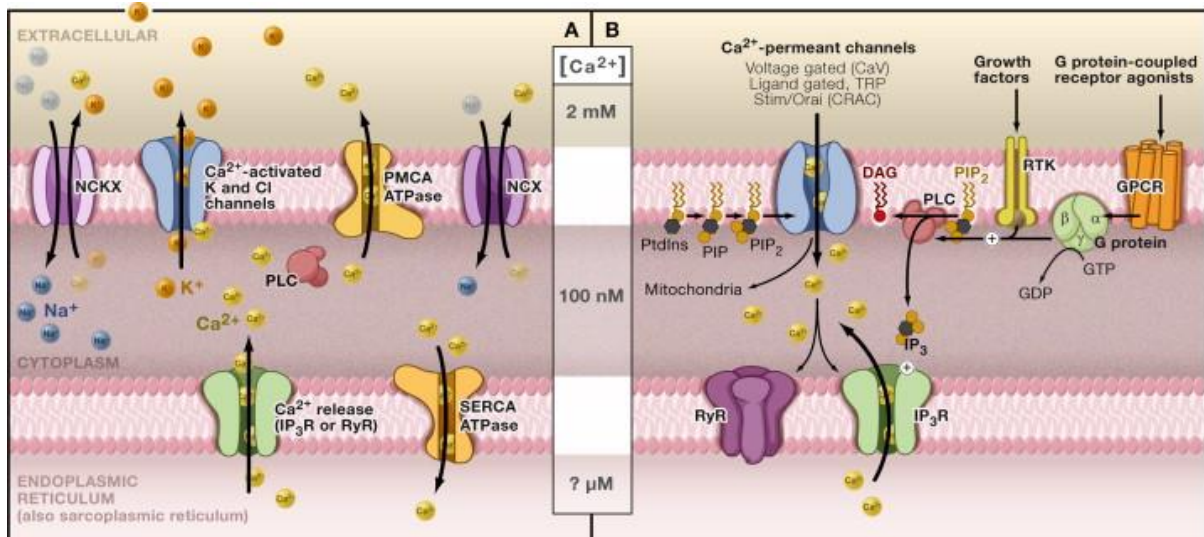
Figure 1: Components of the system of maintenance and modulation of intracellular calcium *(Clapham, 2007)*

A) The cytoplasmic calcium level is maintained strictly and at high costs at ~100nM in resting cells mainly by the plasma membrane Ca^{2+} ATPase (PMCA), the smooth endoplasmic reticular Ca^{2+} ATPase (SERCA), the Na^+/Ca^2-K^+ exchanger NCKX and the Na^+/Ca^{2+} exchanger (NCX).

B) The main mechanism of signal transduction by calcium is the opening of voltage-dependent or ligand-activated calcium channels, which in turn cause the release of calcium from the endoplasmic reticulum (ER). Ultimately, this process increases the intracellular calcium up to 20,000 fold, which triggers a number of signaling processes and is terminated by the calcium extruding transmembrane proteins.

Due to its importance it is not surprising that aberrations of the calcium system are linked to a large number of diseases and malfunctions (Berridge, 2010). Especially neurons as excitatory cells require a particularly precise control of the intracellular calcium levels in order to function. A dyshomeostasis of the calcium household or an increase of the basal calcium level in hippocampal neurons are associated with neuronal aging (Thibault et al., 2007; Raza et al., 2007). The variety of possible diseases linked to an imbalance in the calcium household ranges from neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease to neuropsychological disorders such as schizophrenia, bipolar disorders and depression (Lisek et al., 2017). Therefore, for successful treatment and understanding of many diseases and disorders, it is necessary to understand the mechanisms involved in the complex system regulating the calcium homeostasis.

2.2 Plasma membrane calcium ATPase

As members of the large family of ion-transport ATPases with a high affinity to calcium (Kd 0,2 µM), PMCAs constitute an important part of the extrusion system for calcium (Strehler and Thayer, 2018; Hajieva et al., 2018).The PMCAs derive from four separate genes encoding the paralogs PMCA 1 to 4 but the number of PMCA isoforms with distinct functions is increased manifold by various splice variants (Brini et al., 2013b). There are two main isoforms of each PMCA paralog, which differ in their regulatory properties, namely the A and B isoform (Chicka and Strehler, 2003). During the maturation of neurons, a switch from the B to the A type takes place. PMCA2 alone remains the B-type and the A-type is never detectable (Kip et al., 2006). All these variants harbor ten transmembrane domains (Figure 2) and transport one calcium ion for the cost of one ATP with a 1:1 stoichiometry (Siegel, 1998).
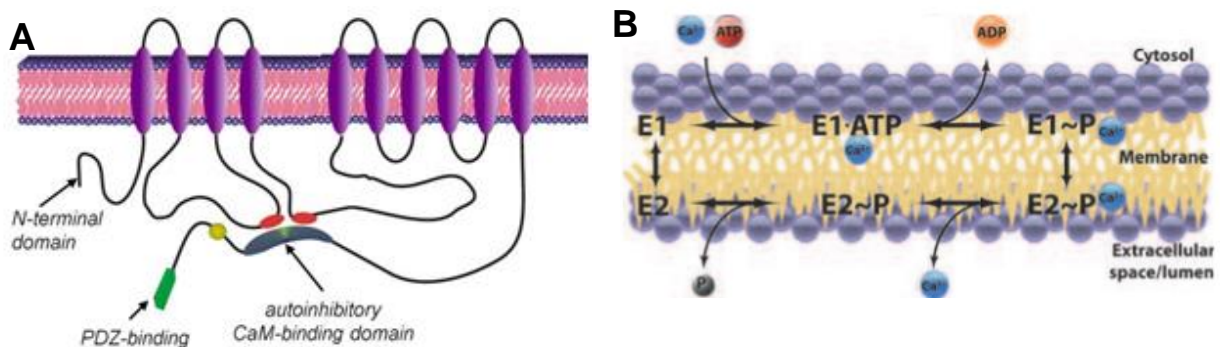
Figure 2: PMCA structure and function

A)   All PMCA isoforms contain ten transmembrane domains, an autoinhibitory calmodulin-binding domain and a PDZ-binding site. (Scheme taken from Bruce 2013)

B)   The cycle of calcium extrusion by PMCAs can be split into two different states. In the high affinity state E1 the PMCA binds the calcium in the cytosol and releases the ion in state E2 to the extracellular space with low affinity, due to a change of the protein conformation. The cost of the extrusion of one calcium ion is the hydrolysis of one adenosine triphosphate (ATP). (Scheme from Brini et al., 2013b)

The PMCAs are auto-inhibited and become activated by high calcium concentrations via calmodulin, except for PMCA2, which does not need to be activated by calmodulin since its autoinhibitory site seems to be ineffective. The cycle of the calcium extrusion consists of two states: the E1 state with high affinity for calcium which binds cytoplasmic calcium and the E2 state with low affinity to calcium which releases the ion to the extracellular space (Brini et al., 2017). The C-terminal domain of the PMCA interacts with the PDZ domain of various calcium-dependent proteins and modulates their interaction and the location of the pump, creating

microdomains specialized for calcium signaling (Brini et al., 2013b). While the isoforms 1 and 4 are expressed ubiquitously, PMCA2 is primarily found in the nervous system and the epithelial cells of the mammary gland, whereas PMCA3 is mainly expressed in the nervous system and skeletal muscle tissue (Brini et al., 2013b). As the different isoforms each have a distinct function, polymorphisms and ablations within the genes cause a variety of specific phenotypes. Thus, an ablation of the ubiquitous PMCA1 is already lethal during the embryonic phase, while PMCA2 mutations lead to deafness, motility problems, impaired retinal function and a decrease of motoneurons in the spinal cord (Kurnellas et al., 2005). This can be explained by its high expression in the nervous system, especially in the cerebellum and the outer hair cells of the inner ear. A successful knockdown of PMCA3, however, has not been successfully performed yet, suggesting that it might be lethal. Ablation of PMCA4 is known not to be lethal but to cause male sterility (Brini et al., 2013c).

2.3 Neuroplastins

Neuroplastins are cell adhesion molecules (CAMs) and members of the immunoglobulin superfamily. The four isoforms of neuroplastin consist of Np55 with two Ig-like domains and Np65 with an additional N-terminal Ig1 domain, both either with or without an additional four amino acids insert in their cytoplasmic tail: Asp-Asp-Glu-Pro (DDEP) (Smalla et al., 2000; Kreutz et al., 2001) (Figure 3). Neuroplastin can be tissue-dependently glycosylated at six sites (Langnaese et al., 1998). By complete deglycosylation the apparent molecular weight of Np65 and Np55 is reduced from 65 and 55 kDa to 40 and 28 kDa, respectively (Willmott et al., 1992). Whereas Np65 is neuron-specific, enriched in postsynaptic densities and can be mostly found in the cerebral cortex, the hippocampus, the amygdala and the striatum, Np55 can be found in a wide range of tissues and cells including neurons (Smalla et al., 2000). Because of its specific Ig1 domain Np65 is capable of engaging in trans-homophilic interaction over the synaptic cleft to mediate synaptic plasticity (Herrera-Molina et al., 2014) (Figure 3). Neuroplastin interacts with a variety of binding partners either via its cytosolic tail, the extracellular Ig domains or the transmembrane domain suggesting an involvement in several cellular functions.

It has long been known that CAMs are important for accurate tuning of the neuronal communication. New studies now show the importance of neuroplastin as a member of the CAM family in this context (Dalva et al., 2007). Neuroplastin is necessary for the establishment of excitatory synapses, thus being important for a balance of inhibitory and excitatory signals in the central nervous system (Herrera-Molina et al., 2017). This balance is of great importance for brain health and contributes to cognitive capabilities like learning and memory (Bird and

Burgess, 2008). A variety of recent studies has shown the multiple phenotypes caused by an ablation of the neuroplastin gene. Thus, the loss of one allele in Np+/- mice leads to a change in spatial memory performance and anxiety-like behavior (Amuti et al., 2016). Loss of both alleles also leads to altered sensorimotor capabilities, complex changes in diving and swimming behavior, and other behaviors associated with autism and schizophrenia (Bhattacharya et al., 2017). Studies have underlined that neuroplastin is necessary for long-term potentiation and regulating synaptic plasticity, by showing that neuroplastin deficient mice have severe neurological deficits in associative hippocampus-dependent learning and memory, and that neuron-specific neuroplastin inactivation leads to retrograde amnesia of associative memories (Herrera-Molina et al., 2017; Bhattacharya et al., 2017). In humans, a nucleotide polymorphism in neuroplastin is linked to the lower cortical thickness and impaired intellectual abilities. The neuroplastin expression accounts for 0.5 % of the variance of cognitive capacities tested in a cohort of adolescents (Desrivières et al., 2015).
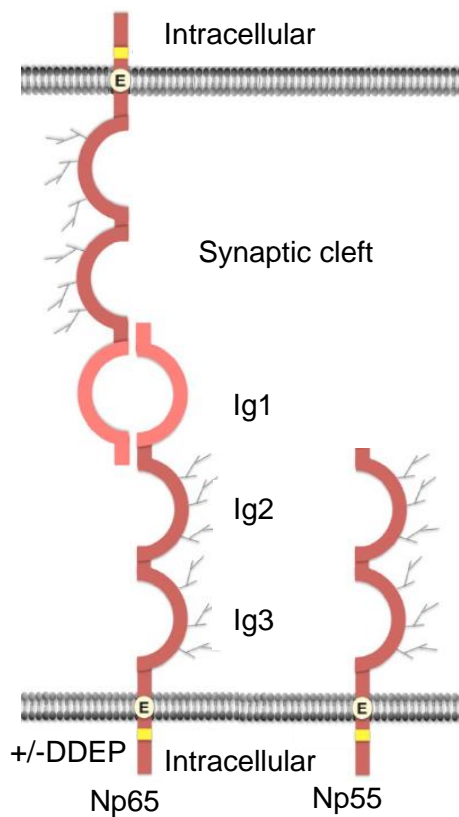
Figure 3: Schematic representation of neuroplastin isoforms

Np65 and Np55 have three and two Ig-domains, respectively. The glycosylation sites are shown in grey. Both isoforms have a hydrophilic glutamate (E) in their transmembrane domains. The optional amino acids DDEP encoded by a mini-exon (yellow box) in the cytoplasmic domains create four splice variants in total. Np65 can engage in a trans-homophilic binding through the Ig1 domain crossing the synaptic cleft. Modified from (Beesley et al., 2014)

2.4 The PMCA-neuroplastin interaction

Unpublished evidence suggests that the two transmembrane proteins PMCA and neuroplastin form complexes in synaptic preparations (Smalla, Kähne unpublished results) and that the amount of protein of PMCA depends on neuroplastin as it is reduced in Np-knockout mice (Bhattacharya et al., 2017). Recently it has been discovered that PMCA and neuroplastin strictly form heterotetramers consisting of two neuroplastins and two PMCA subunits (Schmidt et al., 2017; Gong et al., 2018). The CAM Basigin, which is a close relative to neuroplastin, can also form heterotetramers with PMCA, but does so to a lesser extent than neuroplastin. However, if neuroplastin is knocked out, Basigin can partially compensate the reduction of the PMCAs. When neuroplastin is knocked out, the resulting decrease of PMCAs leads to an increase of the basal calcium levels as the cells' capacity to extrude calcium is impaired (Figure 4B). This effect can be further intensified by knocking out Basigin as well (Schmidt et al., 2017). Experiments with HEK cells, which are a relatively clean environment as non-excitatory cells for the proteins of interest have shown that neuroplastin promotes both endogenous and exogenous expression of PMCAs, leading to the assumption that there is a direct interaction between those proteins (Herrera-Molina et al., 2017). Experiments with a neuroplastin mutation, which is retained in the endoplasmic reticulum (ER), show that the interaction of the proteins probably takes place at an early stage of protein biosynthesis, since PMCAs do not reach the plasma membrane under these conditions and are most likely degraded in the ER. The fact that the interaction takes place during protein biosynthesis is backed up by experiments demonstrating the reduction of PMCAs evenly in all membrane compartments in Np -/- cells and by the finding that PMCA mRNA levels are not altered by the knockout of neuroplastin (Schmidt et al., 2017). Since PMCAs depend on neuroplastin and are the only system that can completely restore the basal calcium level, the interaction between those proteins is required for the control of intracellular calcium in all cell types.

In order to characterize the interaction of PMCAs and neuroplastin more precisely, the exact location of the interaction within the proteins is of particular interest. Working with a truncated neuroplastin consisting of the transmembrane part with adjacent N- and C-terminal residues has shown that this part of the protein alone is already sufficient to increase the PMCA levels in cell membranes and to affect calcium levels, indicating that neither the Ig domains nor the intracellular part of neuroplastin are important for this interaction (Schmidt et al., 2017). A glutamate residue within the transmembrane domain could be crucial for this interaction as it is at an unusual location for a hydrophilic amino acid and can also be found in another binding partner of the PMCA - Basigin (Figure 3).
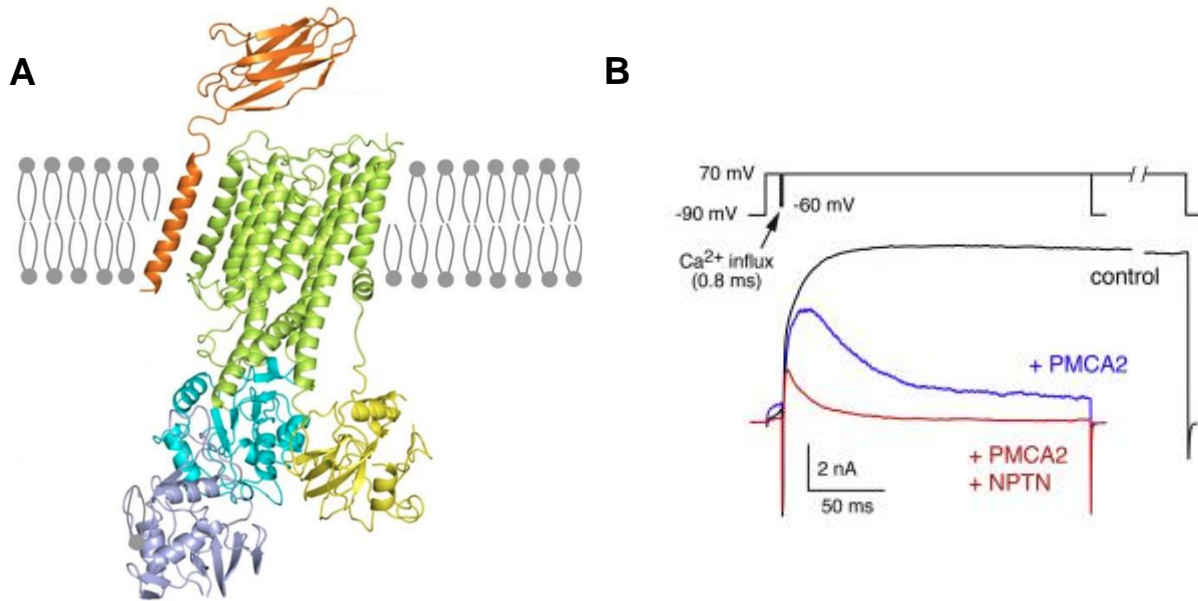
Figure 4: Complex formation of neuroplastin and PMCA and the impact on the cell physiology

A)   Model of the PMCA- neuroplastin interaction based on cryo-electron microscopy. The transmembrane domain of neuroplastin (orange) forms a complex with the transmembrane domain of the PMCA (green) in the plasma membrane. The intracellular part of the PMCA consists of the actuator (yellow), the nucleotide-binding site (purple) and the phosphorylation site (blue). Modified from (Gong et al., 2018)

B)   Setup and result of patch clamp experiments in CHO cells showing the effect caused by a transfection with PMCA2 (blue), co-transfection of PMCA2 + neuroplastin (red) and an untransfected control (black) on the extrusion capacity of Ca^{2+} after stimulation. (Schmidt et al., 2017)

2.5 TRAF6 as a neuroplastin interaction partner

TRAF6 is, as a member of the TNF receptor associated factor (TRAF) family, a cytoplasmic adaptor protein with N-terminal E3-ligase activity. It mediates signal transduction from receptors of the TNF receptor superfamily as well as of the TOLL/IL-1 family (Bradley and Pober, 2001; Zotti et al., 2014). TRAF6 is part of distinct pathways via its interaction with protein kinases like IRAK1/IRAK, SRC and is recognized as the main regulator of NFkappaB, PI3K and ERK 1/2 (Wang et al., 2006; Xie, 2013; Amuti et al., 2016). Because TRAF6 is part of many pathways through these interactions, it has already been identified as a necessary component of many cellular functions, ranging from a role in inflammatory responses to the migration of cancer cells (Wang et al., 2006; Kiefel et al., 2011). Recently an interaction between TRAF6 and neuroplastin via the TRAF6 binding motif (Figure 5 A,B) within the cytoplasmic tail of neuroplastin has been discovered to be indispensable for the spinogenic capacity of neuroplastin and for a balanced ratio between excitatory and inhibitory synapses (Herrera-Molina et al., 2014). Recent results show a significant increase of the filopodia number in HEK cells and dendritic protrusions in neurons caused by an overexpression of neuroplastin (Vemula et al., 2020). A mutation of the TRAF6 binding site in neuroplastin or a knockdown of TRAF6 causes a decrease of both. The interaction of neuroplastin and TRAF6 is also visible in the recruitment of TRAF6 to the plasma membrane and filopodia in cells overexpressing neuroplastin (Figure 5 C).

A) Np65 with three Ig domains shown in green, the transmembrane domain in yellow and the cytoplasmic tail containing the TRAF6-binding motif (Bs-X-X-P-X-E-X-X-(Ar/Ac)) in blue.

B) In silico reproduced docking model of a peptide (green) binding to TRAF6 (Vemula et al., 2020)

C) Effect of the overexpression of the mutant lacking the TRAF6 binding motif (ΔNp65) on the number of dendritic protrusions in hippocampal neurons compared to the number of dendritic protrusions of neurons overexpressing Np65 and GFP (Vemula et al 2020).

Figure 5: Neuroplastin contains a TRAF6 binding motif, which affects its ability to promote the growth of dendritic protrusions

3. Hypothesis and Aims

On the grounds of the evidence presented, the following hypothesis and aims of the thesis were derived.

3.1 State of the art evidence underlying the hypothesis

- Neuroplastin and PMCA form heterotetramers (Schmidt et al., 2017)
- PMCA is important for the extrusion of cytoplasmic calcium (Strehler and Thayer, 2018)
- Neuroplastin promotes endogenous and exogenous expression of PMCA in HEK cells (Herrera-Molina et al., 2017)
- A knockdown of neuroplastin affects the PMCA isoforms differently in murine neurons (Herrera-Molina et al., 2017)
- A treatment with a TRAF6 activator does not change the expression level of PMCA in murine leukemic macrophage cells and marrow-derived macrophage precursor cells (Son et al., 2012)

3.2 Hypothesis

Neuroplastin interacts with all isoforms of the PMCA. This interaction occurs isoform-specific and is independent on the known neuroplastin interaction partner TRAF6. The neuroplastin-PMCA interaction affects the calcium homeostasis of hippocampal neurons.

3.3 Aims

a) To quantify the influence of a cotransfection with Np65 on the protein levels of different isoforms of the PMCA in COS-7 cells.

b) To quantify the influence of the cotransfection of Np65 and PMCA isoforms on their staining intensity and co-localization in the plasma membrane in COS-7 cells.

c) To clarify, if TRAF6 plays a role in the Np65-PMCA2 interaction in hippocampal neurons by using a Np65 mutant with deleted TRAF6-binding site.

d) To analyze the impact of Np65 on the calcium extrusion capacity of PMCA in hippocampal neurons after electrical stimulation.

All these aims should contribute to a better understanding of the interaction between those transmembrane proteins and its possible impact on the intracellular calcium balance.

4. Material and methods

4.1. Animals

All Wistar rats used in this work were housed in the animal facilities of the Leibniz Institute for Neurobiology Magdeburg. Experimental methods and animal housing were carried out according to the institutional and federal regulations of the State of Saxony-Anhalt.

4.2 Cell culture

4.2.1 COS-7 and HEK 293 cell lines

The COS-7 cells are derived from kidney fibroblasts of the Chlorocebus aethiops and immortalized by a replication-deficient Simian virus (Gluzman, 1981). HEK293 cells are derived from human embryonic kidney cells and immortalized by transforming them with parts of the human adenovirus 5 (Graham et al., 1977).

4.2.2 Hippocampal primary neuron cultures

The hippocampal neurons were obtained as mixed neuron-glia cultures from rats at embryonic day 18 and prepared according to (Herrera-Molina and Bernhardi, 2005). The transfection was performed at day 8 or 9 (DIV 8 or 9) (Herrera-Molina et al., 2017).

4.2.3 Passage of cell lines COS-7 and HEK293

Both cell lines were kept in a growth medium at 37 °C with 5 % CO_2. To split the cells, they were washed with 10 ml of pre-warmed HBSS and then incubated with 0.5 ml trypsin and 3.5 ml HBSS for 5 minutes to detach the cells from the bottom of the flask. The obtained cells were examined under the microscope, stained with trypan blue and counted under the microscope. For Western blots, 300,000 cells were then plated in a 6 well dish, while for microscopic studies, 30,000 cells were plated on poly-D-lysine-coated cover slips in 12 well dishes.

Table 1: Media, buffers, solutions and reagents for cell culture

Reagent	Supplier
DMEM	Gibco by Life Technologies
Optimem reduced serum medium	Gibco by Life Technologies
Neurobasal medium	Gibco by Life Technologies
HBSS	Gibco by Life Technologies
Lipofectamine	Invitrogen
Trypan blue	Invitrogen
Penicillin	Invitrogen
B27	Gibco by Life Technologies
Fetal bovine Serum	Gibco by Life Technologies
Growth medium HEK and COS-7	DMEM+ 10 % FBS, 1 % Penicillin, 1 % Glutamate
Growth medium neurons	Neurobasal medium containing 2 % of B27 supplement and 1 % of L-glutamine and 1 % of penicillin/streptomycin
10x PBS	1.37 M NaCl, 2.7 mM KCL, 14 mM KH2PO4, 43 MM Na2HPO4, ddH20 pH 7.4
1x PBS	10 % 10x PBS, 90 % ddH20

4.2.4 Transfection of HEK293 and COS-7 cells

70 % confluent cells were used the day after the cell splitting. The growth medium was aspirated and incubated in a Falcon tube for the duration of the transfection at 37 °C with   5 % CO_2. The plates were washed once and refilled with Optimem. The transfection solution consisted of Optimem with 4 µl of Lipofectamine and 1 µg of the respective plasmids (Table 2) for the transfection. After an incubation time of 4 hours the plates were washed once with DMEM and afterwards refilled with the preserved growth medium mixed with 10 % of fresh growth medium.

Table 2: Plasmids

Construct	Species	Supplier
pMM2-hPMCA2z/b	Mammalian	Addgene
pMM2-hPMCA1b	Mammalian	Addgene
pMM2-hPMCA4b	Mammalian	Addgene
EGFP-hPMCA2z/b	Mammalian	Addgene
Np65Δid-GFP	Mammalian	Ramya Rama/LIN
GFP	Mammalian	Addgene
GFP-GPI	Mammalian	Martin Heine/LIN
Np65(+)-GFP	Mammalian	Ramya Rama/LIN
Np55(+)-GFP	Mammalian	Ramya Rama/LIN
Np55-GCaMP-RFP	Mammalian	Xiao Lin/LIN
RFPT-P2a-GCaMP5	Mammalian	Xiao Lin/LIN
Np65(+)-RFPT-P2A-GCaMP5	Mammalian	Xiao Lin/LIN

4.2.5 Transfection of hippocampal neurons

The protocol of the transfection of the hippocampal neurons was the same as for COS-7 and HEK cells, but with a neurobasal growth medium instead of Optimem and DMEM (Table 1). The neurons were only incubated for 1 hour with only 0.5 µg of each plasmid and only 2 µl of Lipofectamin per well (Herrera-Molina et al., 2017).

4.3 Preparation for SDS-PAGE

48 hours after the transfection, the cells were washed with cold PBS and mixed with 70 µl of 2x SDS lysis buffer. After 5 minutes, the material was collected in Eppendorf tubes.
Lysates were kept cooled at all time during the procedure. After the samples were sonicated for 40 seconds and centrifuged, the supernatant was collected, boiled at 98 °C for 10 minutes and centrifuged again for 4 minutes at 13,000 rpm.

4.4 Staining for confocal microscopy

The protocol for the staining was the same for all used cell types.

The cells were fixed with 4 % PFA for 8 minutes, washed 3x with PBS and blocked for 30 minutes with PBS with 10 %   serum and 0.1 % Triton X-100. Afterwards, the cells were incubated for 1 hour with the respective primary antibodies at room temperature in a humidified box. After washing the plates 3 times with PBS for 5 minutes the cells were then incubated with the fluorescently labelled secondary antibodies. The coverslips were finally washed once in water and mounted with moviol on the microscope slides.

4.5 Electrophoresis and Western blot transfer

For SDS PAGE either 10 % gels or gradient (8-20 %) gels were used with 5 % stacking gel. Electrophoresis was performed with a constant current of 10 mA. The proteins were then transferred from the gel to a nitrocellulose membrane over 90 minutes at a constant voltage of 200 mA.

4.6 Immunoblotting

Table 3: Primary antibodies

Antibody	Species	Supplier	Dilution
Actin	mouse	Abcam	1/4000
panPMCA	mouse	Abcam	1/2000 WB; 1/400 staining
GFP	mouse	Abcam	1/2000
GAPDH	rabbit		1/4000
MAP2	guinea pig	Synaptic systems	1/1000
Np	sheep	R&D systems	1/200

Table 4: Secondary antibodies

Antibody	Species	Company	Dilution
anti-gp Cy5	donkey	Jackson Immuno Research	1/1000
anti-ms CY3	donkey	Jackson Immuno Research	1/1000
anti-mouse Cy5	donkey	Jackson Immuno Research	1/1000
anti-mouse-HRP	goat	Dako	1/4000
anti-sheep Cy3	donkey	Jackson Immuno Research	1/1000
anti-rabbit-HRP	mouse	Sigma Aldrich	1/8000

The membranes were blocked with TBST-T containing 5 % milk powder at room temperature, incubated with the primary antibodies (Table 3), and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Table 4) under constant agitation. The membranes were washed after every antibody 3 times 10 minutes with TBS-T. The secondary antibodies were then visualized using a chemiluminescent HRP substrate and an ECL ChemoCam Imager from Intas. The images were quantified with ImageJ. To normalize the amount of the protein of interest, intensities of actin or GAPDH in the cell lysate were used.

Table 5: Buffers and reagents for SDS-Page

Reagent	Compostion/ Supplier
2x SDS	125 mM Tris-HCl, pH 6.8, 4 % SDS, 20 % glycerin, 0.2 % bromophenol blue, 10 % β-mercaptoethanol, double distilled water (ddH2O)
Electrophoresis buffer	10x TGS ddH20
10x Blot buffer	0.25 M Tris-base, 1.92 M glycine, 0.2 % SDS, dd H2O
1x Blot buffer	10X blot buffer, methanol, ddH2O
Protein ladder	Thermo Scientific
Separation gel 10%	2.91 ml ddH2O, 1.5 ml TRIS 1.5 M, 30 µl 20 % SDS, 1.5 ml PAc 40 %, 60 µl APS 10 %, 2.4 µl TMED
Stacking gel	1.83 ml ddH2O, 0.75 ml TRIS 1.5 M, 15 µl 20 % SDS, 375 µl PAc 40 %, 30 µl APS 10 %, 3 µl TMED
Gradient gel	17.94 ml ddH2O, 8.25 ml TRIS 1.5 M, 330 µl 10 % SDS, 118 µl APS 10 %, 22 µl TMED, 1.89 ml glycerol 87 %, 330 µl EDTA 0.2 mol/l, 4.12 mlPAc
NC membrane	GE Healthcare Life Sciences
10xTBS	0.2 M Tris-Base, 1.37M NaCl, ddH2O, pH 7.6
1xTBS	10 % 10x TBS, 90 % ddH2O
TBS-T	10 % 10xTBS, 89.9 % ddH2O, 0.1 % Tween 20

4.7 Confocal microscopy

All images of the COS-7 cells and neurons were recorded on a Leica TCS SP5 confocal microscope. A 63X 1.4 oil immersion objective lens was used.

4.8 Calcium imaging

The cells were stimulated at DIV 14-16 with one train of 10 pulses in 1 second with 14 mV. The cells were kept in Tyrodes medium for the time of the stimulation (Table 6). The temperature in the microscope was kept at 30 °C and the CO_2 at 5 %.

A fluorescence microscope was used to measure the intensity of the GCaMP fluorescence over time. A 63X 1.4 oil immersion objective lens was used.

Table 6: Buffer for calcium imaging

Reagent	Composition
Tyrodes buffer	119 mM NaCl 2.5 mM KCL 25 mM Hepes pH 7.4 30 mM Glucose 2 mM $MgCl_2$ 2 mM $CaCl_2$

## 4.9 Data Processing and Statistical analysis

The western blot intensities, confocal pictures and recordings of the calcium imaging were quantified with Fiji Image J. To measure the co-localization of two channels the tool "coloc2" of Image J was used to obtain a Pearson correlation coefficient. The data of the calcium imaging was first normalized to the RFP channel and then to the average of the first two seconds of baseline. The decay constant Tau was determined with "GraphPad Prism 9" by analyzing the one phase decay. Student`s t-test were performed to determine statistical significance.

5. Results

5.1 The expression levels of PMCA 1, 2 and 4 are differently affected by cotransfections with Np65

To analyze the influence of Np65-GFP on the protein expression of the PMCA isoforms 1, 2 and 4, COS-7 cells were cotransfected with one isoform of the PMCA and Np65. GFP-GPI was the first control condition to rule out that potential changes of the protein level of the PMCA were caused by the tagged GFP or simply by the overexpression of a membrane bound-protein. In a second control condition the cells were only transfected with the plasmids of the respective PMCA isoforms in order to normalize the data. The cells were transfected with 1 µg of each plasmid for four hours and a full cell lysate was obtained the next day followed by an SDS PAGE. The membranes were incubated with the respective primary antibodies for the proteins of interest and for actin as a loading control. Following this, the membranes were incubated with the corresponding secondary antibodies coupled to HRP. The pictures were obtained with an Intas ECL machine and analyzed with ImageJ (Figure 6A). The signal intensity of the PMCA of each condition was first normalized to the loading control actin and the cotransfected conditions then normalized to the single transfection condition with PMCA. The results show that the total protein level of PMCA1 was not increased when cotransfected with Np65 (1.07 ± 0.17) compared to the control condition (1.08 ± 0.17, p=0.93). The total protein level of PMCA2 was significantly increased when cotransfected with Np65 (1.44 ± 0.25) compared to the control (0.91 ± 0.08, p=0.23). PMCA4 protein levels show a trend towards increase when cotransfected with Np65 (1.52 ± 0.38) compared to the control (1.13 ± 0.14, p=0.33), but not reaching significance (Figure 6B).

A

| PMCA2 | | | |
| Np65 | GFP | - | - |

~130 kDa — PMCA

~90 kDa — Np65-GFP

Actin

~30 kDa

GFP
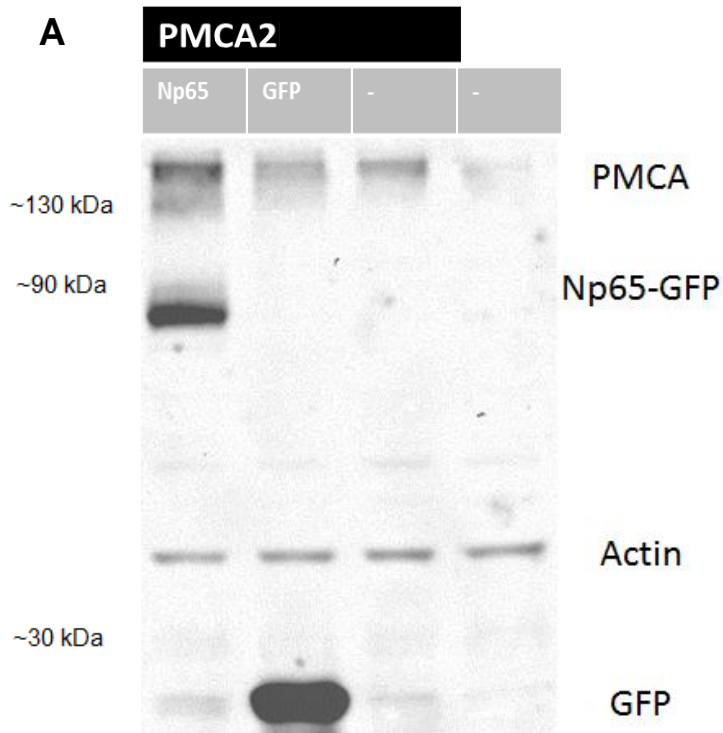
Figure 6A:

Expression levels of PMCA isoforms in heterologous cells when cotransfected with Np65

Representative Western blot from total cell lysates of COS-7 cells transfected with PMCA2 and either Np65 or GFP-GPI or no additional construct. Actin was used as loading control and for normalization.

B
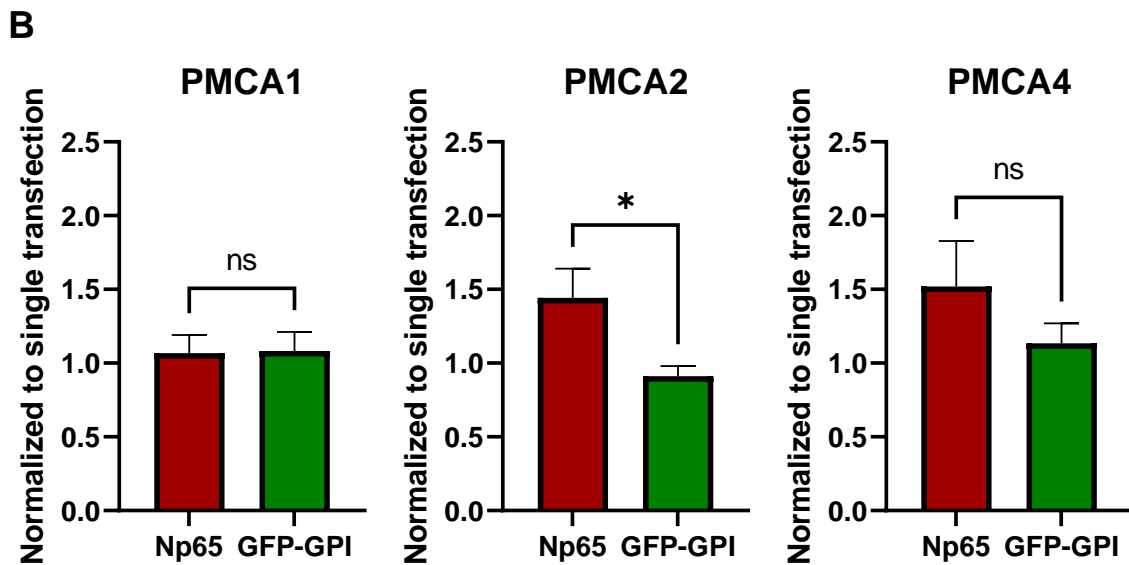
Figure 6B:

Expression levels of PMCA isoforms in heterologous cells when cotransfected with Np65

Differentially affected expression of PMCA protein levels by cotransfection with Np65. Data of 6 independent experiments combined and normalized to the single transfection condition. Two-tailed unpaired t-tests were performed and $p<0.05$ was considered significant. Data is presented in mean ± SEM, *$p<0.05$, ns= not significant

5.2 Cotransfection with Np65 increases PMCA expression in the plasma membrane and co-localization with Np65 in COS-7 cells

To confirm the results of 5.1 and to analyze this phenomenon further, confocal imaging and an ImageJ analyzing tool were used to compare the amount of the PMCA isoforms 1, 2 and 4 in the plasma membrane in stainings of COS-7 cells cotransfected either with Np65 or GFP-GPI in the control condition (Figure 7).
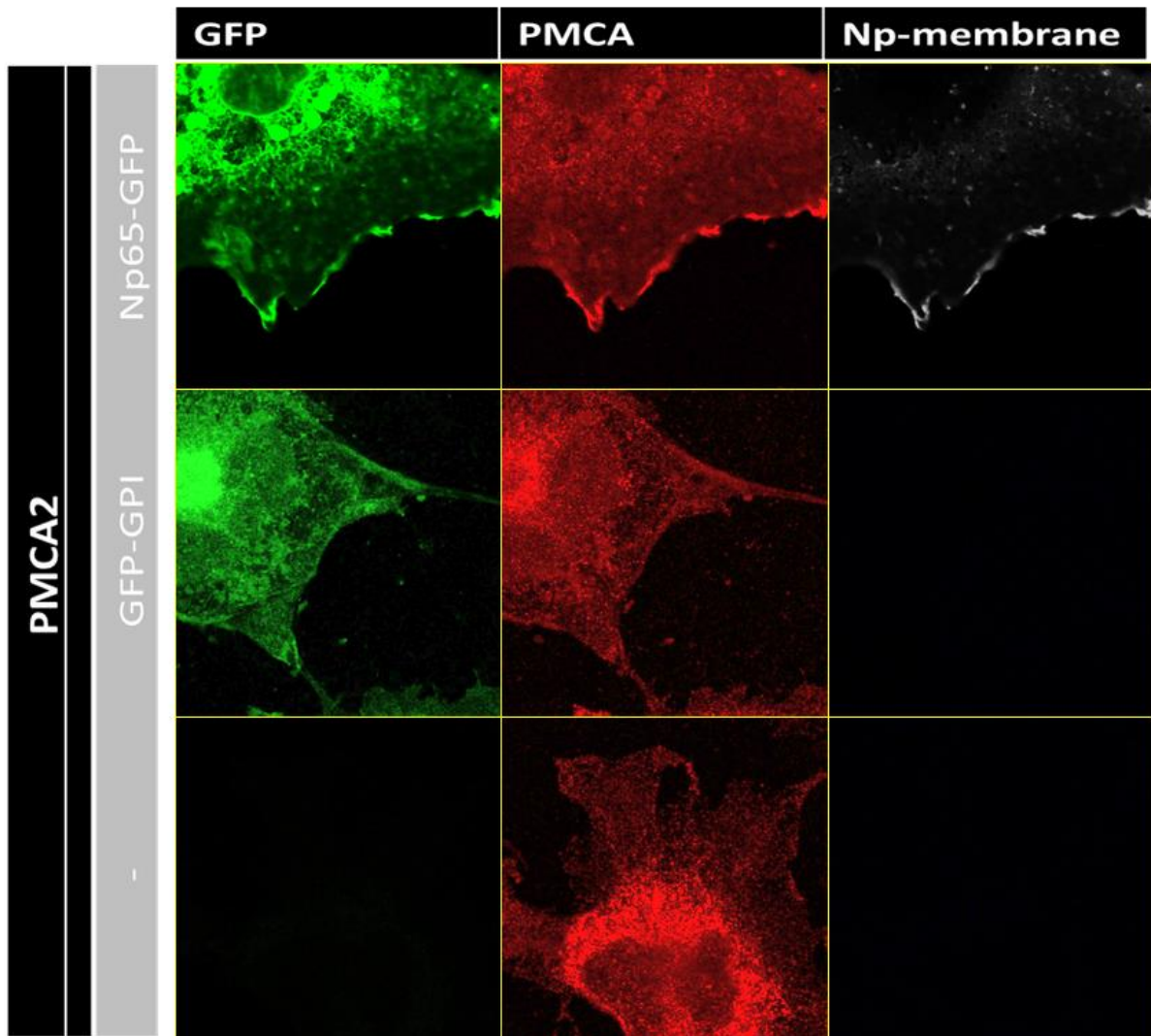
Figure 7: Neuroplastin increases PMCA levels in the plasma membrane and co-localizes with it in COS-7 cells

Representative pictures showing COS-7 cells, which were transfected with PMCA2 combined either with Np65-GFP or GFP-GPI as a control. In the bottom row a single transfection with PMCA2 is shown. A live cell staining with anti-Np antibodies (white) was performed first, which stained only the neuroplastin in the plasma membrane, then the cells were permeabilized with Triton X-100 and stained for PMCA. Np65 and GPI were not stained but visualized via GFP autofluorescence. A Leica TCS SP5 confocal microscope with a 63X 1.4 oil immersion objective lens was used to obtain the pictures.

After fixing, the cells were incubated with a primary antibody for all PMCA isoforms and a corresponding fluorophore-tagged secondary antibody. After obtaining the confocal images, a 2 µm wide region of interest (ROI) containing the plasma membrane was created and the intensity of the PMCA isoforms measured with ImageJ (Figure 8). The average intensities of the ROIs of the PMCA-channel were compared between the isoforms cotransfected with Np65 and their respective controls cotransfected with GFP. The signal intensity was presented by ImageJ in arbitrary units. The signal intensity of PMCA1 in the ROI was significantly increased in the condition cotransfected with Np65 (38046 ± 4481 A.U.) compared to the condition cotransfected with GFP-GPI (24907 ± 3651 A.U., p=0.42). The cotransfection with Np65 increased the signal intensity of PMCA2 in the ROI significantly (54626 ± 6806 A.U.) compared to the control (28981 ± 6240 A.U., p=0.18). The same tendency was visible when PMCA4 was cotransfected with Np65 (52274 ± 5593 A.U.) when compared to cotransfection with GFP-GPI (38006 ± 5139 A.U.), but the increase was statistically not significant (p=0.09) (Figure 8).
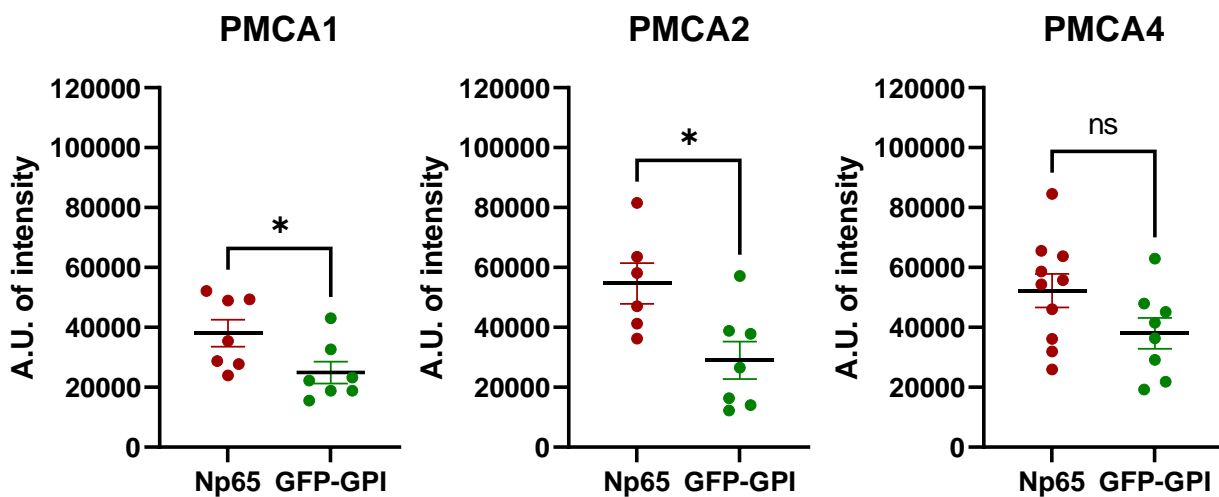
Figure 8: Amount of PMCA isoforms in the plasma membrane when co-transfected with Np65

The fluorescence of the channel showing the panPMCA antibody intensity in the plasma membrane is shown in arbitrary units for PMCA1, PMCA2 and PMCA4. The preliminary result is shown in charts summarizing the results of 6-10 cells per condition. Two-tailed unpaired t-tests were performed and $p<0.05$ was considered significant. Data is presented in mean ± SEM, *$p<0.05$, ns= not significant

The same confocal images and their respective ROIs were used to also calculate the co-localization between the channel showing the PMCA and the one showing either Np65 or GFP-GPI. The tool "coloc2" of ImageJ was used to analyze the ROIs of the channels to create a Pearson correlation of the co-localization. The preliminary result shows that all investigated isoforms of the PMCA co-localize significantly stronger with Np65 than with the corresponding control (PMCA1+Np65=0.48 ± 0.04 vs PMCA1+GFP-GPI=0.32 ± 0.06, p=0.04; PMCA2+Np65=0.60 ± 0.07 vs PMCA2+GFP-GPI=0.28 ± 0.05, p=0.003; PMCA4+Np65= 0.56±0.04 vs PMCA4+GFP-GPI= 0.35 ± 0.04, p=0.002) (Figure 9).
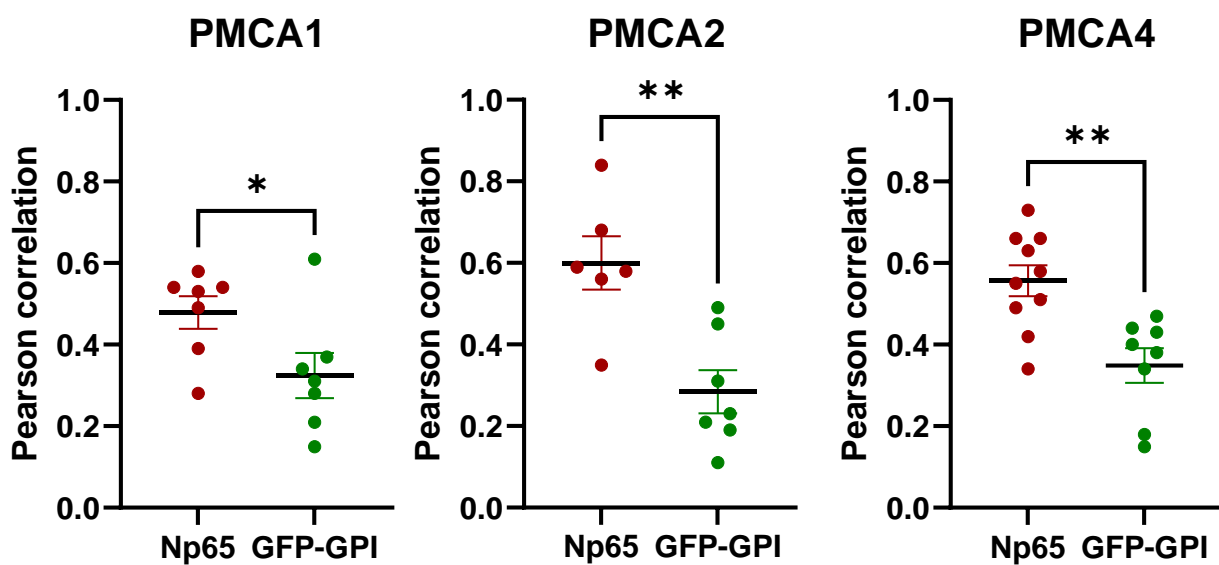
Figure 9: Preliminary result of the correlation of PMCA and Np65 in the plasma membrane

The charts show the co-localization of the channel of PMCA1, PMCA2 and PMCA4 with either the channel showing Np65 or GFP-GPI as a Pearson correlation. The Pearson correlation is determined by the tool "coloc2" of ImageJ. Data of 6-10 cells of each condition. Two tailed unpaired t-tests were performed and p<0.05 was considered significant. Data is presented in mean ± SEM, *p<0.05, **p<0,005
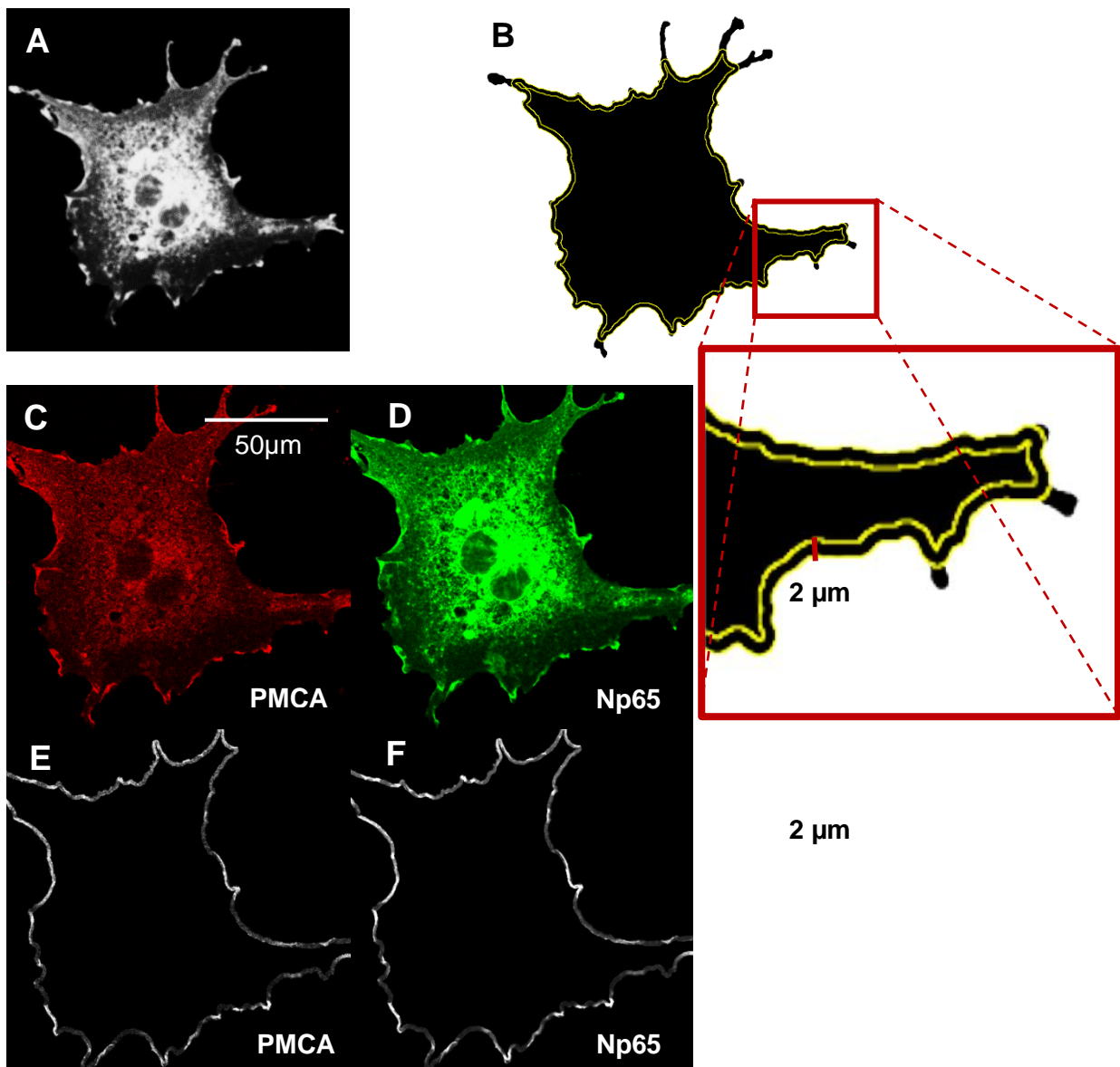
Figure 10: Visualization of the procedure to measure signal intensities in the plasma membrane and to estimate the co-localization of two channels with the tool "coloc2" in ImageJ

Figure 10 shows the workflow to define the region of interest (ROI) containing the plasma membrane to measure the signal intensities and to calculate the correlation between two channels in order to estimate the co-localization of the two proteins of interest. An image with sufficient signal intensity (A) is used to determine the outline of the cell using a threshold. Along the outline, a selection was defined and extended 2 µm inwards, creating a ROI containing the plasma membrane (B). This ROI is then applied to the two channels of interest to determine their signal intensity (C, D). In order to create a Pearson correlation the tool "coloc2" is used to estimate the co-localization of the two signals within the ROIs (E,F).

5.3 The TRAF6 binding site of Np65 is not necessary for its impact on the expression of PMCA2

To characterize the mechanism by which Np65 increases PMCA protein levels, TRAF6 was considered as a possible mediator, since recent studies have shown that Np65 induces neurite outgrowth via TRAF6 (Vemula et al., 2020). The database "Eukaryotic Linear Motive resource" (www.elm.eu.org) was used to check, whether the PMCA isoforms had also a predicted TRAF6 binding site, which could act as modulator for the PMCA neuroplastin interaction. The search revealed that human and rat PMCA isoforms 1, 2 and 4, but not PMCA3, contain a putative TRAF6 binding motif (.P.E.[FYWHDE].) in the ATP-binding domain in their 3rd intracellular loop (Table 7 and Figure 11).

PMCA gene product	Species	TRAF6 binding site predicted	Localization in protein structure	Sequence	Probability
ATP2B1 (PMCA1)	human	566-574	3. intracellular loop	EIPEEALYK	1,715e-03
	rat	566-574	3. intracellular loop	EIPEEALYK	1,715e-03
ATP2B2 (PMCA2)	human	590-598	3. intracellular loop	HPPGEGNEG	1,715e-03
	rat	590-598	3. intracellular loop		1.715e-03
ATP2B4 (PMCA4)	human	556-564	3. intracellular loop	EVPEEKLYK	1.715e-03
	rat	556-564	3. intracellular loop	SNPEEDEEG	1.715e-03

Table 7: Three out of four PMCA isoforms contain predicted TRAF6 binding sites.

According to the ELM prediction algorithm, PMCA isoforms 1, 2 and 4 contain a TRAF6 binding motif. The table summarizes the results obtained from a search in the data base "Eukaryotic Linear Motive resource".
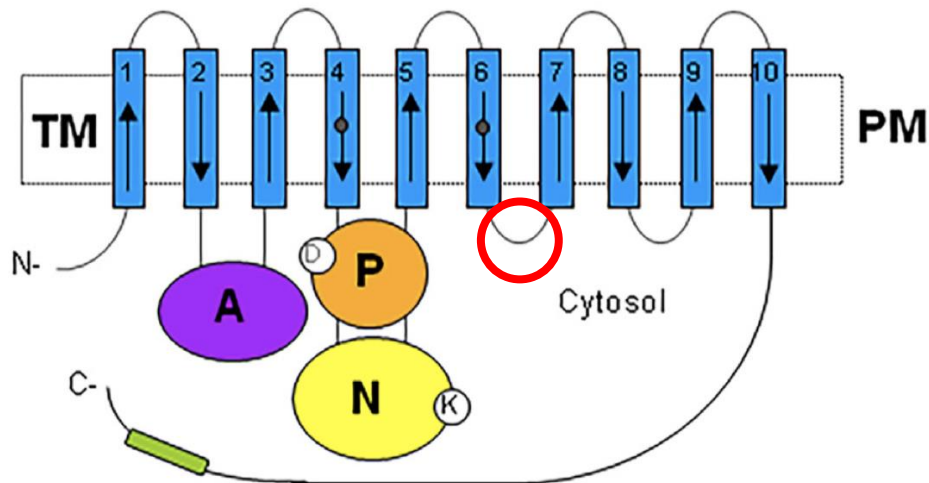
Figure 11 Structure of PMCA and localization of the predicted TRAF6 binding side (Pérez-Gordones et al., 2017)

The figure shows the model of the PMCA consisting of 10 transmembrane domains (TM) passing the plasma membrane (PM), the Actuator domain (A), the phosphorylation domain (P) with the amino acid aspartic acid (D), the ATP-binding-domain (N), with the amino acid Lysine (K) and the putative C-terminal calmodulin binding-domain (green box). PMCA isoforms 1, 2 and 4 possess a predicted TRAF6-binding site in their third intracellular loop marked with a red circle.

Since this search proved that both proteins had a binding site for TRAF6 at a potentially accessible location, further experiments were done to investigate this possible connection in more detail.

To analyze the potential involvement of the TRAF6 binding site in neuroplastin on the increase of PMCA2 protein levels, HEK293 cells were cotransfected with PMCA2 and wildtype Np65, an Np65 mutant lacking theTRAF6 binding site (ΔNp65) (Herrera-Molina et al, 2017), and GFP as a control to compare the PMCA protein levels (Figure 12A).

The HEK cells were transfected 24 hours after the cell splitting with 1 μg of each plasmid and the SDS PAGE was performed 24 hours later.

All conditions were normalized to the condition PMCA2 cotransfected with GFP. The results show that the total protein level of PMCA2 immunoreactivity was increased significantly when it was cotransfected either with Np (2.75 ± 0.66, p=0.02) or ΔNp65 (3.07 ± 0.89, p=0.04). The conditions transfected with Np or ΔNp showed no significant difference regarding the PMCA2 protein levels (p=0.78) (Figure 12B).
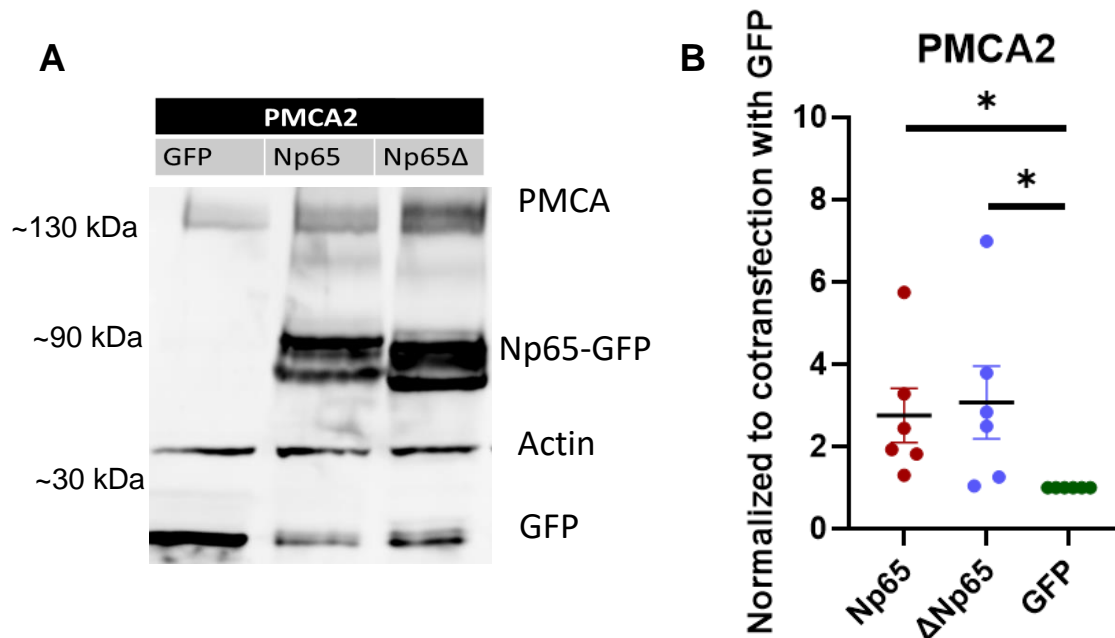

Figure 12: The TRAF6 binding site in Np65 does not affect the ability of Np65 to promote PMCA levels in HEK 293 cells

A)   Representative western blot performed with cell lysates of HEK 293 cells cotransfected with the plasmids of PMCA2 and either GFP, Np65, or ΔNp65. The blot was subsequently probed with three different antibodies: anti-PMCA (pan), anti-actin and anti-GFP. Normalization was done as a two-step process, first to actin levels and then to the GFP control.

B)   The chart shows the values of PMCA2 normalized to actin as a control protein in HEK cells transfected with PMCA2 with either Np65, ΔNp65, or GFP as a control. (Data of seven independent experiments). Two-tailed unpaired t-tests were performed and p<0.05 was considered significant. Data is presented as mean ± SEM, *p<0.05

To verify that the TRAF6 binding site of Np65 has no influence on its interaction with PMCA in neuronal cells, the same constructs have then been used to perform the same transfection with a reduced amount of cDNA and incubation time in murine hippocampal primary neurons at DIV 8. After the fixation of the neurons the cells were stained for MAP2 and panPMCA with fluorophore-tagged antibodies and confocal microscopy was used to obtain the pictures (Figure 14). The intensity of the PMCA signal was then measured in ImageJ with seven line scans along three dendrites of 15 cells of each condition in three independent experiments. The combined data of the experiments show that the average intensity of the PMCA channel is significantly lower in the control condition cotransfected with GFP (335018±16378 A.U.) than in the conditions transfected with Np65 (432219 ± 20120 A.U., p=0.0003) or ΔNp65 (419294 ± 22084 A.U., p=0.003) and show no significant difference between the conditions overexpressing either Np or ΔNp65 (p= 0.67) (Figure 13).
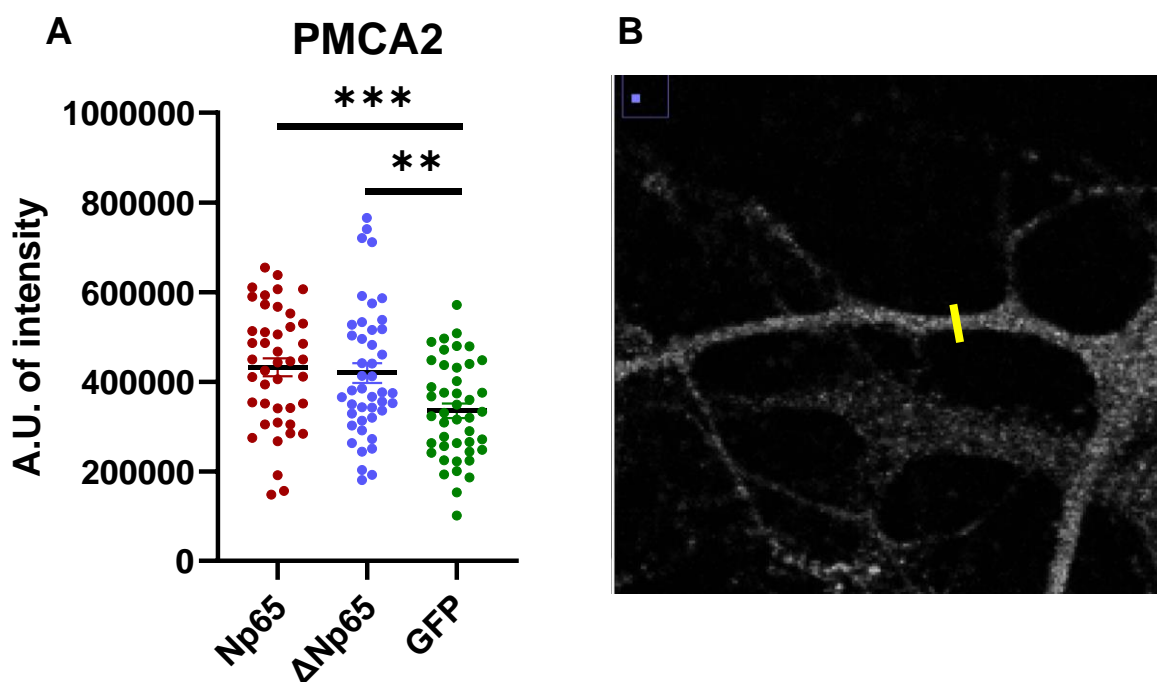
Figure 13: Np65 promotes the PMCA protein levels in the dendrites of hippocampal neurons irrespective of its TRAF6-binding domain

A) The chart shows the results of cotransfections of PMCA2 with either Np65, ΔNp65 missing the TRAF6 binding motif or GFP. ImageJ was used to analyze line scans on representative dendrites to measure the intensity of PMCA. Data are from three independent experiments with seven line scans of three dendrites of 15 cells. A Leica TCS SP5 confocal microscope with a 63X 1.4 oil immersion objective lens was used to obtain the pictures. Two-tailed unpaired t-tests were performed and p<0.05 was considered significant. Data is presented in mean ± SEM, **p<0.005, ***p<0,0005

B) Visualization of a linescan (symbolized by yellow bar) across a dendrite of a hippocampal neuron DIV 9 transfected with ΔNp65 in the channel showing the intensity of PMCA
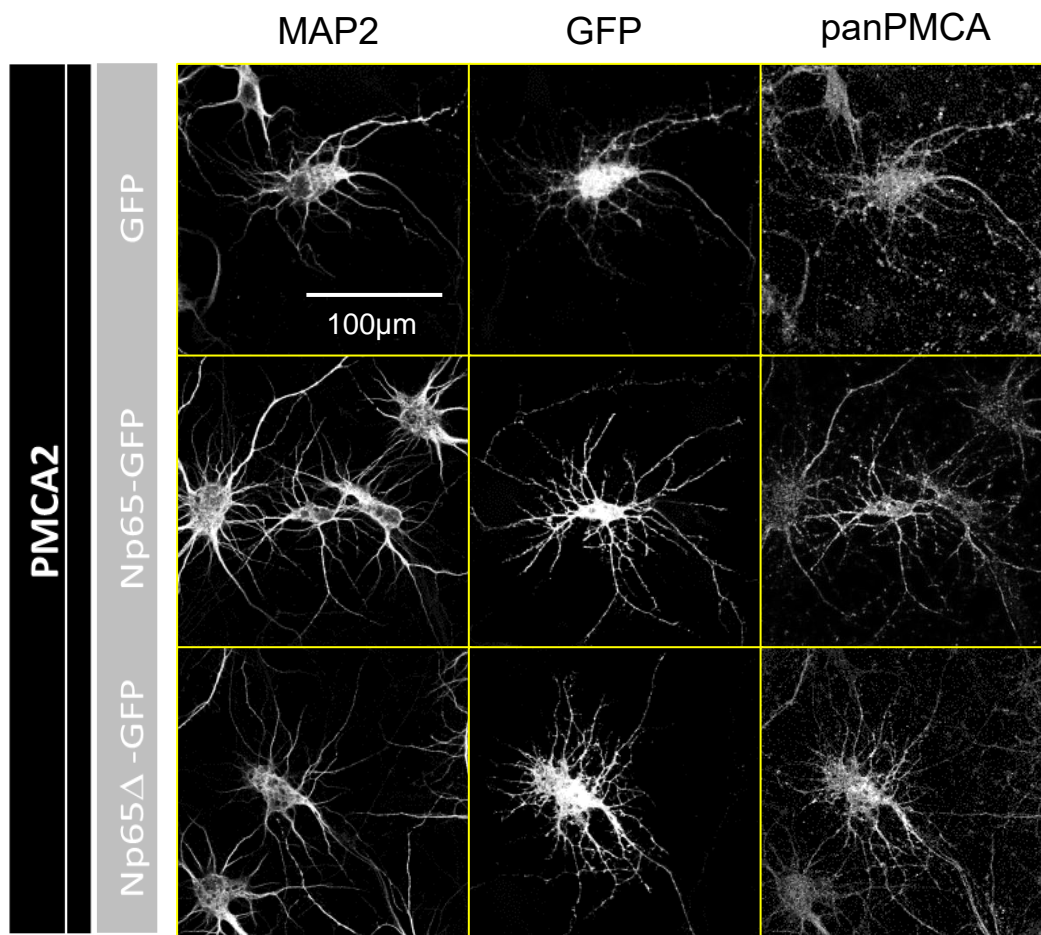
**Figure 14: Representative images of hippocampal neurons cotransfected with PMCA2 with either Np65, ΔNp65 or GFP**

The representative confocal images of the cotransfections of hippocampal neurons with PMCA2 with either Np65, ΔNp65 missing the TRAF6 binding site or GFP show that the intensity of the PMCA channel is visibly higher, when the cells are cotransfected with either Np65 or ΔNp65 compared to the control condition cotransfected with GFP. MAP2 was used to choose the cells unbiased by the fluorescence of the channels of interest and to precisely determine the outline of the dendrites. The cells were transfected at DIV 8, fixing and staining them with fluorescent antibodies at DIV 9. The images shown here have been selected, because they are closest to the average of their respective condition.

Since these experiments showed that the TRAF6 binding site in neuroplastin does not play a role in the expression of PMCA2 in hippocampal neurons or HEK 293, experiments aimed at investigating the necessity of the TRAF6 binding site within the PMCA for the interaction were not performed.

5.4 Overexpression of Np65 increases the basal intracellular calcium level and slows down its restoration in hippocampal neurons after an electrical stimulation

Since the overexpression of Np65 leads to an increase of the protein level of the PMCA, which is an important component of calcium machinery (Strehler and Thayer, 2018), the question arose whether the overexpression of Np65 would also lead to changes in calcium homeostasis in the transfected cells. To analyze the influence of a transfection with Np65 on the cells' basal calcium level and their capacity to extrude calcium, hippocampal neurons were transfected with a construct of Np65 fused to the fluorophore RFP and the calcium indicator GCaMP5 (RFP-Np65-GCaMP). A GCaMP5-RFP fusion protein was used as a control (GCaMP5-RFP). The transfection of rat primary neurons was performed at DIV 9 as described in "Material and methods". The RFP channel was used to select the neurons unbiased by their resting calcium level (Figure 16A). The stimulation was performed at DIV 14-16 with one train of 10 pulses per second with 14 mV. The channels showing GCaMP5 and RFP were recorded with a fluorescence microscope. ImageJ was used to create ROIs of as many dendrites as possible of each cell and to create specific values of the calcium intensity over time (Figure 16B). Only cells, in which the calcium signal returned completely to the baseline after stimulation, were used. In general, electrical stimulation with the parameters used did not seem to damage the cells, but neurons transfected with (RFP-Np65-GCaMP) showed more fluctuations of the basal calcium level than the control condition (Figure 16B). Excel was used to normalize the data first to the RFP channel and then to the baseline of calcium concentration, which was the average intensity of the GCaMP channel of the first two seconds before the stimulation. "GraphPad Prism 9" was used to calculate the decay constant τ to quantify the speed of the extrusion of calcium. The τ values of the neurons transfected with Np65 were then compared to the control condition. The combined data of three independent experiments show that the baselines of the cytosolic calcium levels of the cells transfected with the RFP-Np65-GCaMP construct (1.66 ± 0.33 A.U.) have the tendency to be higher than in the cells transfected with GCaMP-RFP (1.00 ± 0.10, p=0.07) (Figure 15).

The comparison of the τs of the same cells after electrical stimulation according to protocol showed that the capacity to reach the baseline of their original calcium level of the cells transfected with RFP-Np65-GCaMP (1296.64 ± 72.97 ms) was significantly reduced compared to that of the control condition transfected with GCaMP-RFP (868.48 ± 49.53 ms, p<0.0001) (Figure 17).
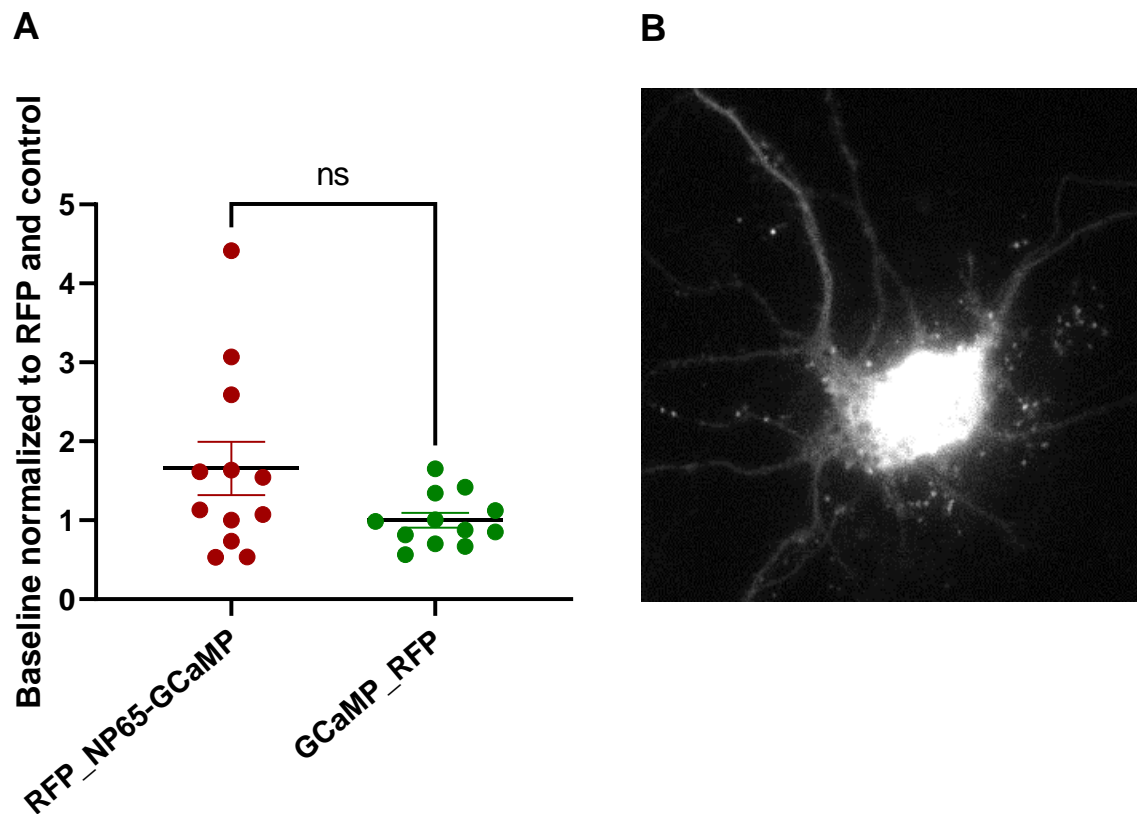
Figure 15: Effect of Np65 on the basal cytosolic calcium level of hippocampal neurons

A)   The graph shows the combined data of three independent experiments. In each experiment three to five neurons per condition were evaluated. It shows the intensity of the first two seconds of the GCaMP channel first normalized to the RFP channel and second to the control condition as an average of the dendrites of a single neuron. The data is presented as average with SEM. Two-tailed unpaired t-tests were performed and p<0.05 was considered significant.

B)   The picture shows an example of the GCaMP channel of a hippocampal neuron at DIV 14, which was transfected with RFP-Np65-GCaMP at DIV 9, before stimulation, where the baseline was measured.
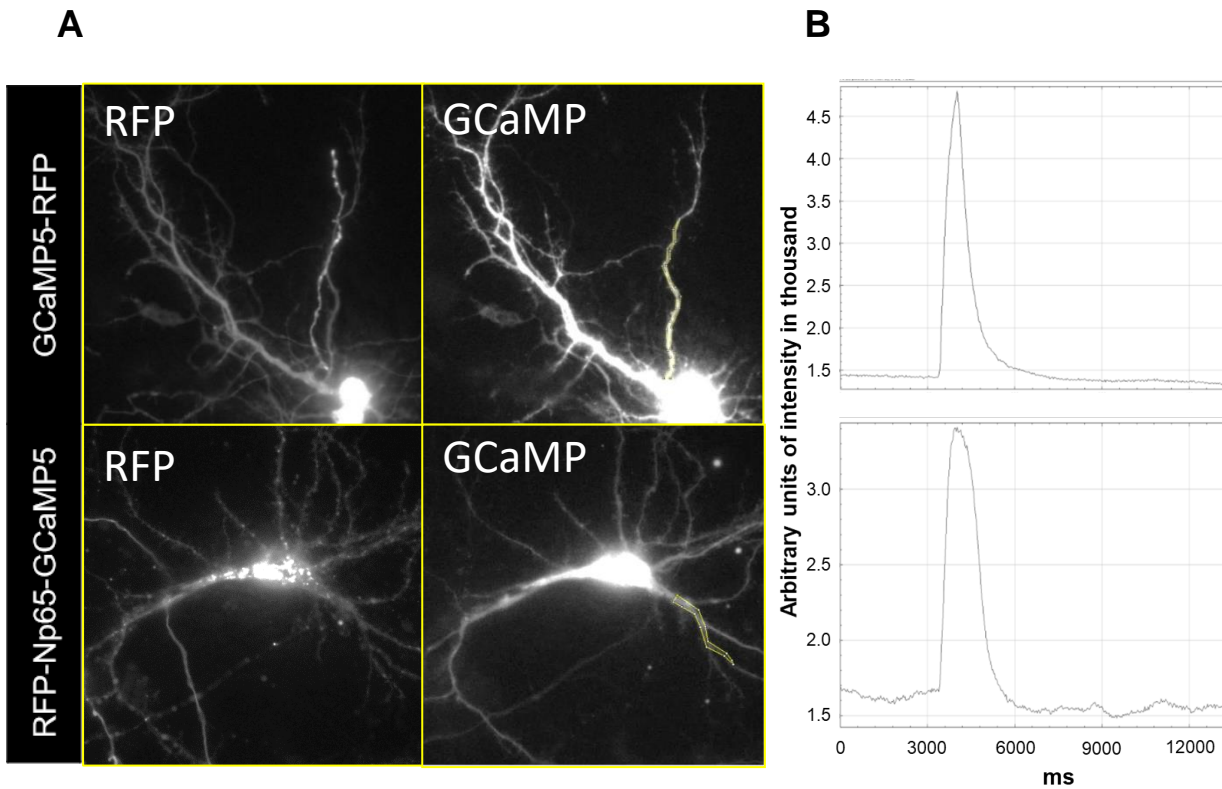
Figure 16: Effect of Np65 on the calcium extrusion capacity of hippocampal neurons after electrical stimulation

A) The representative pictures show rat primary hippocampal neurons transfected with Np65 linked to the calcium indicator GCaMP5 and RFP or transfected with the GCaMP5-RFP as a control. The cells were transfected at DIV 9 and stimulated at DIV 15 with one train of 14 V. The left channel showing RFP was used to select transfected cells unbiased by their calcium level; the right channel shows the GCaMP channel after stimulation. The ROI of one dendrite is shown in yellow

B) The graphs show the intensity of the GCaMP channel, which correlates with the amount of intracellular calcium of the selected dendrite over the number of the frame taken every 30 ms.
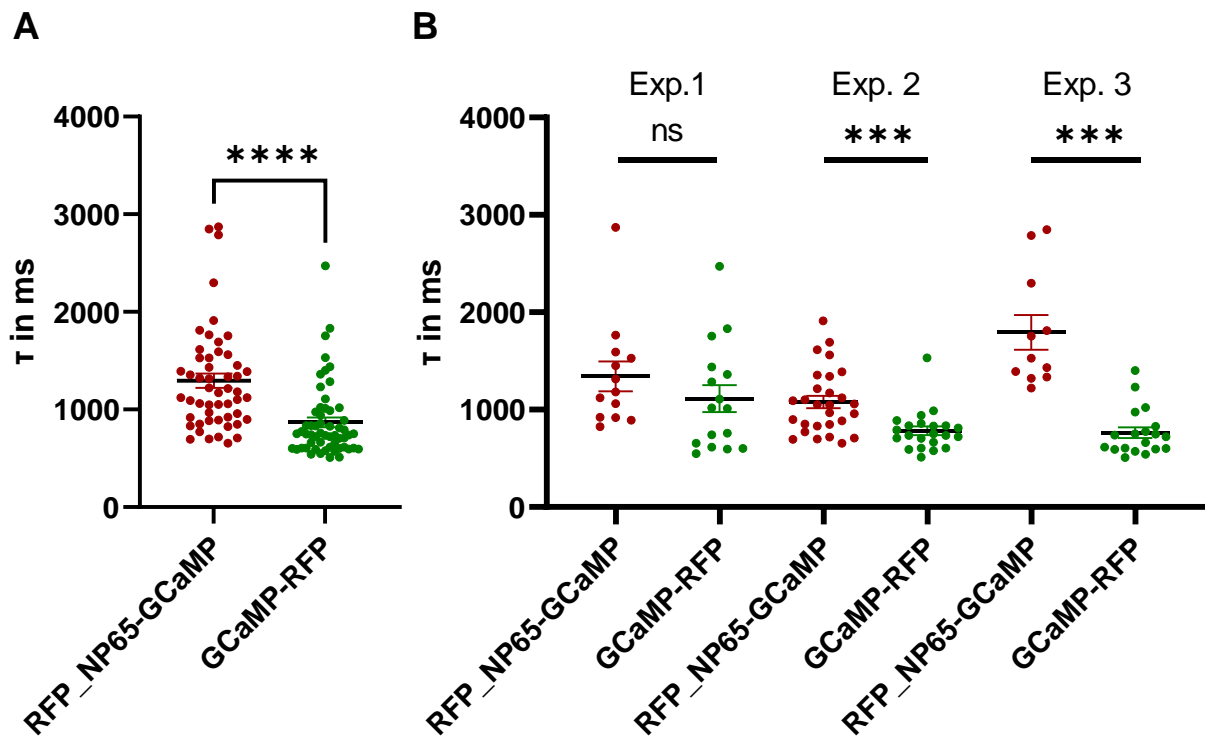
Figure 17: Overexpression of Np65 slows down calcium extrusion in hippocampal neurons

A) The cells were transfected at DIV 9 and stimulated at DIV 14-16 with one train of 10 pulses per second with 14 mV. ImageJ was used to obtain the data of the calcium signal over time of as many dendrites as possible of each cell. "GraphPad Prism 9" was used to calculate the τ of each dendrite. The graph shows the summary of three independent experiments.

B) The graph depicts the individual data of three experiments showing their variability. Each dot represents the τ in ms of the dendrites of three to four cells per experiment and condition. Two tailed unpaired t-tests were performed. The data is presented in mean ± SEM and $p < 0.05$ was considered significant. ***$p < 0.0005$, p***< 0.0001, ns= not significant

6. Discussion

6.1 Neuroplastin co-localizes with all PMCA isoforms investigated and affects their protein levels differentially

In this thesis the influence of neuroplastin on the expression, membrane localization and function of PMCA isoforms was investigated. The COS-7 and HEK cell lines used for this research are particularly suitable for this study as they do not display detectable levels of the glycoprotein neuroplastin. According to the human protein atlas HEK293 cells do not express PMCA2 and 4 and only small amounts of PMCA1, 4 and neuroplastin. In figure 6 it is shown that COS-7 cells only express a small amount of PMCA and it has been published that COS-7 cells do not express neuroplastin (Wilson et al., 2013).

They were therefore used to provide a relatively clean environment for the experiments. This work shows that neuroplastin increases the heterologous amount of PMCA2 in COS-7 and HEK293 cells significantly. A similar effect is visible in COS-7 cells for PMCA4 (Figure 6). This increase might be significant if the number of experiments was increased. Only the amount of protein of PMCA1 does not seem to be strongly influenced by the presence of neuroplastin in the Western blots performed with COS-7 cells. In the preliminary results (Figure 8) all three isoforms of the PMCA seem to show a higher intensity in the plasma membrane than their respective control condition, when cotransfected with neuroplastin. This increase however was just significant in the conditions, where PMCA1 and PMCA2 were used.

The fact that all isoforms of PMCA and neuroplastin co-localize in transfected cells, that they form complexes in synaptic preparations (Smalla, Kähne unpublished results) and the fact that the amount of some PMCA isoforms was significantly increased in the plasma membrane, where it is naturally located, could indicate that they build similar complexes in COS-7 cells as in wild-type cells and that these complexes could potentially have an influence on the calcium homeostasis in the transfected cells.

To further examine how neuroplastin influences PMCA levels in vivo imaging could be used to specify the time and location of the interaction. In addition, patch clamp or calcium imaging could be used to assess the influence of the higher PMCA concentration in the plasma membrane on the calcium homeostasis in COS-7 cells in order to deduce the actual influence of the transfection on the physiology of the cells. Since a construct in which neuroplastin is coupled to the GFP was used in this work, the GFP was used in the control condition to prove that the effect actually originated from the neuroplastin and not from the GFP.

However, it has been reported that proteins coupled to GFP can have other properties than their untagged form, which could be responsible for the effect of the used construct (Thomas and Maule, 2000; Tamamaki et al., 2003). Since it has been shown that the transmembrane domain of neuroplastin itself increases the PMCA protein levels (Schmidt et al., 2017), it is rather unlikely that the phenomenon is caused by the coupling of GFP and neuroplastin.

Further experiments with untagged neuroplastin would be necessary to completely rule out that GFP could be responsible for an alteration of the properties of neuroplastin, which in turn could cause its effect on the PMCA protein levels.

There are several possible explanations for the fact that the protein levels of the PMCA isoforms change differently when they are overexpressed with neuroplastin. One possible explanation could be, that transmembrane domain ten (TM10), which is mainly responsible for the interaction with neuroplastin (Gong et al., 2018), differs between the different isoforms of the PMCA (Gong et al., 2018). The affinity of neuroplastin and PMCA for each other could therefore vary.

That could explain the varying effect on the protein levels of the PMCA caused by alterations of neuroplastin. An obvious candidate responsible for the difference of the interaction between neuroplastin and the respective PMCAs could be the amino acid at position 14 in the transmembrane domain 10, which differs in PMCA1 (containing the neutral amino acid threonine) from PMCA2 and PMCA4 (harboring an acidic glutamate residue at this position) (Figure 18). However, since the sequence of the transmembrane domains of the PMCA isoforms differs in several residues, a more detailed comparison taking the exact structures of the respective PMCA and neuroplastin into account, would be necessary in order to get more detailed information about the influence of the transmembrane domain on the interaction. A further explanation could be, that the COS-7 cells used in these experiments with their specific protein composition could influence the interactions differently and thus not the affinity of the two proteins but the environment itself would determine the outcome of the experiments. An example in which the PMCA protein levels of neurons in the hippocampus and cortex are differently influenced by the knockout of neuroplastin has already been published (Herrera-Molina et al., 2017). It would therefore be possible that the use of a heterologous cell line could have led to a different result.

Position In trans-membrane domain	Np	PMCA1	PMCA2	PMCA4
1	L	I	L	L
2	W	E	D	S
3	P	Q	Q	Q
4	F	W	W	W
5	L	L	M	L
6	G	W	W	W
7	I	S	C	C
8	L	I	I	L
9	A	F	F	F
10	E	L	I	I
11	I	G	G	G
12	I	M	L	I
13	I	G	G	G
14	L	T	E	E
15	V	L	L	L
16	V	L	V	L
17	I	W	W	W
18	I	G	G	G
19	V	Q	Q	Q
20	V	L	V	F
21	Y	I	I	I
22		S	A	S

Figure 18: Sequence of the transmembrane domain 10 (TM10) PMCA1, PMCA2 and PMCA4 in comparison to the neuroplastin transmembrane domain

The table shows the sequence of the transmembrane domains of human neuroplastin (grey box) and the TM10 of PMCA1, PMCA2 and PMCA4. The amino acids labeled in red are acidic, while the rest are neutral. As PMCA1 seems to be differently affected by neuroplastin than PMCA2 and PMCA4, the amino acid at position 14 could be responsible since it is neutral in PMCA1 instead of acidic as in PMCA2 and PMCA4.
(Source: UniProt)

6.2 The TRAF6 binding site of neuroplastin does not affect its ability to promote PMCA2 protein levels

Since it is known that the TRAF6-neuroplastin interaction is important for dendritic protrusions during spinogenesis via NF-κB and PI3K/Akt/WASP pathways (Vemula, 2018), it was conceivable that the TRAF6 binding site was also key for the PMCA- neuroplastin interaction. The experiments in this thesis show that this is not the case since the neuroplastin mutant lacking the binding site had the same effect on PMCA protein levels in COS-7 cell lysates and the dendrites of rodent neurons as the Np. These findings are in line with those recently published, which show that not the TRAF6 binding site-containing intracellular part of

neuroplastin, but only the transmembrane domain is necessary for neuroplastins ability to affect PMCA levels (Schmidt et al., 2017). Another indication that TRAF6 has no influence on PMCA expression levels is the fact, that the stimulation with RANKL, which is an activator of TRAF6, has no impact on the PMCA expression levels in a murine monocyte cell line and bone marrow-derived monocyte/macrophage precursor cells (Son et al., 2012). But even though the protein levels do not depend on TRAF6 it might still be possible that it exerts an influence on PMCA in a different way. It could, for example, be possible that the turnover or the functionality of the pump is affected by a changed ubiquitination pattern due to the absence of the TRAF6 binding site. A relatively simple experiment to find out whether the ubiquitination profile of the PMCA is affected by the absence of the TRAF6 binding site in neuroplastin, would be to perform an immune precipitation and an SDS PAGE with antibodies for lysine 63- and lysine 48-linked poly-ubiquitin chains. If the ubiquitin profile of the PMCA was changed, its influence on cell physiology should be further investigated either with patch clamp or calcium imaging. The ubiquitination profile of other interaction partners of neuroplastin could also be examined using the same setup to further map the influence of the TRAF6 binding site of neuroplastin. Taking into consideration the work of Vemula et al. which has recently shown that the PMCAs have no effect on the neuroplastin-TRAF6-mediated formation of dendritic protrusions during synaptogenesis (Vemula et al., 2020) it is tempting to speculate that neuroplastin has at least two different cell biological functions – an early one in the synapse formation in young neurons, depending on TRAF6, and a late one on the calcium homeostasis in mature neurons, depending on PMCAs.

6.3 Neuroplastin overexpression has an impact on the calcium regulatory system of neurons

This work shows that an overexpression of neuroplastin has an influence on the PMCA protein expression levels and on the calcium balance in neurons. Since it is the function of PMCA to remove calcium ions from the cytosol, the most obvious effect of increasing their expression would be a reduction of cytosolic calcium levels and an acceleration of the restoration of the resting membrane potential after an electrical stimulation (Figure 19).
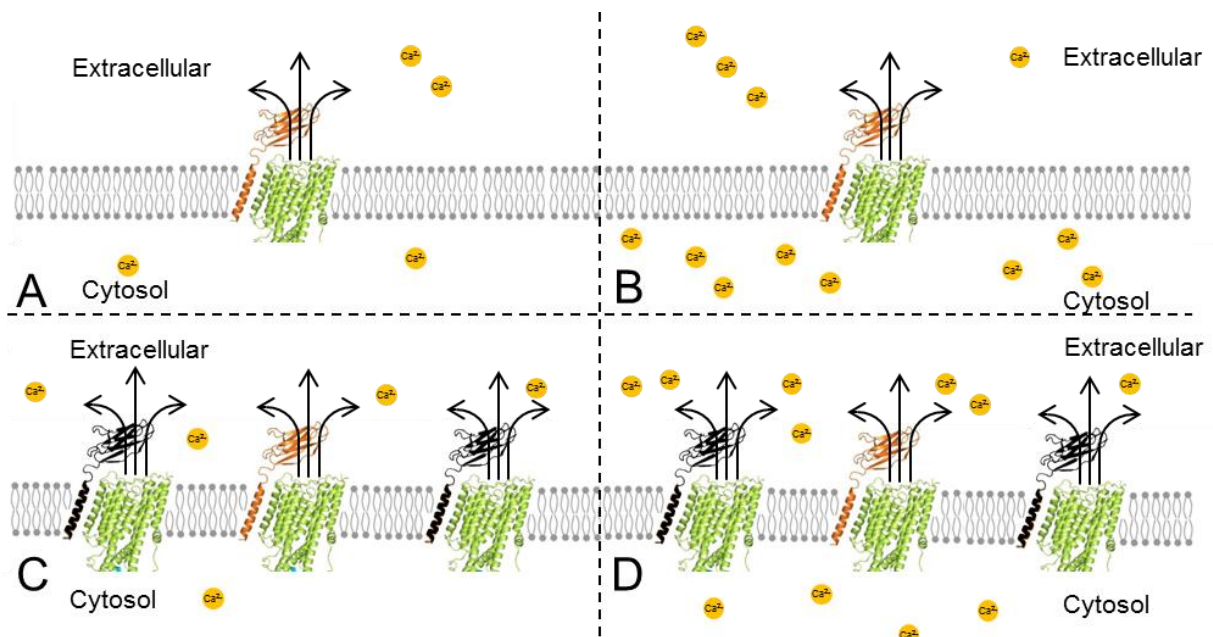
Figure 19: Impact of the overexpression of neuroplastin on the amount of PMCA in the plasma membrane and the expected influence on the extrusion capacity after an electrical stimulation

The cartoon summarizes my model on how the presence of overexpressed neuroplastin (black) increases the amount of PMCA in the plasma membrane (C, D) compared to native conditions (A, B) with only endogenous neuroplastin (orange). A and C show the resting calcium level, which should be lower in conditions with more neuroplastin-PMCA complexes (C). B and D show the conditions after the influx of calcium ions (yellow) following an electrical stimulation. In condition (D) overexpressed neuroplastin should be able to decrease the cytosolic calcium level faster than in the untransfected condition (B). Modified from (Gong et al., 2018)

However, in the experiments performed here the overexpression of neuroplastin increased the basal cytosolic calcium level compared to the control condition and lead to more fluctuations. These results, and the fact that the restoration of the basal cytosolic calcium level after an electrical stimulation took longer in the neurons transfected with neuroplastin, point towards a more complex influence of neuroplastin on the calcium machinery. Those findings are in contrast to published results, which show that artificially elevated levels of PMCA lead to an accelerated removal of calcium in Chinese hamster ovary (CHO) cells and a slower removal of calcium in cortical neurons, when a knockdown of neuroplastin is performed (Schmidt et al., 2017). Possible reasons why the results of this work differ from those of Schmidt et al. are manifold. For example, the patch clamp recordings by Schmidt et al. were done in CHO cells that have a different molecular machinery than the hippocampal neurons used in this work. The approach to the calcium imaging is also fundamentally different, since in the work by Schmidt et al. cortical neurons were used and a knockdown of neuroplastin was performed

and not an overexpression as in this thesis. It is conceivable that the quantity of neuroplastin can be crucial for the calcium household, thus a minimum amount is necessary for an effective calcium extrusion, but an overexpression could damage the calcium machinery. In general, overexpression of proteins may cause artifacts since it can dramatically affect protein mobility within cells (Kobler, PhD thesis OVGU 2020).

Furthermore, the time of calcium imaging could also be decisive, since Schmidt et al. performed their studies between DIV 21 and DIV 30 and in this thesis I used cells between DIV 14 and DIV 16, as the PMCA levels remain stable from this point onwards (Kip et al., 2006). It is known that the expression of PMCA in hippocampal cultures is highly variable during the first weeks in vitro. The expression of PMCAs reaches its maximum at the end of the second week in vitro and remains relatively stable from DIV 21 onwards (Kip et al., 2006). It is therefore plausible that the cells were more vulnerable at the time they were transfected with neuroplastin in this thesis compared to the time when Schmidt et al. performed their experiments.

Another factor that could account for the different results of the experiments, could be the fact that in this thesis hippocampal neurons were used and not cortical neurons. Since neurons express PMCA and neuroplastin differently, depending on their type and localization in the hippocampus, this could influence the experiments. The overexpression of neuroplastin could have a different effect on the cells, depending on how much neuroplastin and which PMCA isoforms are expressed naturally (Herrera-Molina et al., 2017). For example, it is known that the amount of Np65 is much higher in subfields CA1 and the dentate gyrus than in the subfield CA2 and CA3 in the murine hippocampus (Herrera-Molina et al., 2014). In addition, differences in calcium balance between different regions in the hippocampus were described. For instance, the subfield CA1 has a higher calcium extrusion capacity than the subfields CA2 and CA3 and a greater endogenous capacity to buffer calcium via calcium binding proteins (Simons et al., 2009). Since neurons of all types and hippocampal regions grow together in the culture and their morphological discrimination is difficult, neuron-specific markers like Ctip2, Prox1 (Herrera-Molina et al., 2014) would have to be used subsequently to differentiate where the investigated cell originated from in order to map the influence of neuroplastin overexpression on the different types of hippocampal neurons. The difficulty with this procedure would have been that the corresponding staining could only have been performed after the calcium imaging and the assignment of the cell would have to be done solely on its morphology.

Furthermore, it would be interesting to learn how the PMCA isoforms expressed by the different types of neurons are influenced by the overexpression of neuroplastin, because published results have shown that a knockout of neuroplastin affects the isoforms of PMCA differently depending on the type of neuron they are expressed in (Herrera-Molina et al., 2017). Stainings

with specific antibodies for the different PMCA isoforms could be used to analyze this phenomenon.

The fact, that the kinetics of the calcium removal are changed and more fluctuations of the baseline occur, could also indicate that the cells could have been affected by the transfection process, despite the fact that they were still able to restore their respective calcium baselines. This question could be further investigated by modifying transfection parameters such as the time of the transfection, the incubation time, the amount of cDNA used, control protein, calcium indicator or used cell line. An interesting experiment to compare the functionality of the calcium machinery of the cells overexpressing neuroplastin and the control condition would be to stress them with multiple stimulations or by raising the extracellular calcium concentration to check if their general resilience was affected. It would also be interesting to see if a transfection with Basigin at the same time under similar conditions would cause a similar effect, as it is also known to increase PMCA levels and has a highly similar structure to neuroplastin (Beesley et al., 2014; Suzuki et al., 2016; Schmidt et al., 2017).

It would also be possible that the transfection with only the transmembrane part of neuroplastin would lead to accelerated calcium elimination, since the transmembrane part interacts with fewer proteins than the whole neuroplastin molecule. The reduced number of interaction partners, for example, due to the absence of the TRAF6-binding site, could make the interaction between the transmembrane part and PMCA more efficient than the interaction between the whole neuroplastin and PMCA, where more proteins could interfere with the interaction.

Other calcium pumps could lead to a more differentiated view of the function of neuroplastin. Another possible research aspect arising from the results of this thesis could be to test whether different subcellular compartments of the cells are affected differently by a transfection with neuroplastin. For example, it would be well conceivable that the calcium regulation of synapses, dendrites and cell bodies are influenced differently by neuroplastin. In this context, a comparison between the two isoforms Np65, Np55 and their relative Basigin would be interesting, since they are all able to upregulate PMCA levels but their localization differs (Beesley et al., 2014; Muramatsu, 2016).

In conclusion, overexpression of neuroplastin leads to an alteration of the neuronal calcium balance. This alteration was in contrast to what was expected as the calcium extrusion decelerated and the basal calcium concentration increased, even though an overexpression of neuroplastin reportedly leads to an increase of the PMCA concentration in the plasma membrane. In order to gain a better insight into this phenomenon, further experiments would have to be performed to investigate the influence of the respective transfection parameters. In addition, it is necessary to perform more differentiated experiments to evaluate the possible

differences of the impact the overexpression of neuroplastin has on the expression of the different PMCA isoforms and the calcium balances of the respective types of neurons. All this would contribute to a better understanding of the influence the interaction between neuroplastin and PMCA has on the brain.

6. 4 The relevance of the interaction between neuroplastin and PMCA for possible applications

Since changes of neuroplastin and PMCA proteins are associated with severely altered phenotypes, it is important to examine their interactions more closely in order to better understand them (Tempel and Shilling, 2007; Desrivières et al., 2015)

The importance of PMCAs in the pathogenesis of many diseases has been the subject of research for many years. Since the PMCAs are responsible not only for calcium homeostasis in excitatory cells but in all cells, the influence of PMCAs is not limited to excitatory cells or the nervous system, but potentially affects every cell of the body. This explains why alterations of PMCAs are associated with such a broad spectrum of diseases like deafness, cancer and cerebellar ataxia (Brini et al., 2013b).

However, it is usually difficult, to differentiate whether the alterations of the PMCAs are an adaptation to adjust to the conditions of the particular disease and to have corrective influence on the calcium balance, or whether PMCA defects are causal for the problem itself.

Nevertheless, PMCAs are already seen as promising targets for a number of therapies in a variety of diseases. One major example is cancer, since PMCAs reduce the chances of initiating apoptosis in cancer cells by lowering intracellular calcium levels, as elevated calcium levels are required to precede a regulated apoptosis. For instance, PMCA isoforms 2 and 4 are overexpressed in some types of breast cancer and would thus be predestined as targets of the therapy by increasing the likelihood of the apoptosis of cancer cells through the increased calcium levels resulting from the inhibition of the PMCAs (Brini et al., 2013c).

However, no drug has yet been found that can be successfully used to target the PMCA to exert its effect. The reasons for this are that PMCAs do not express a binding site for an extracellular ligand at which an agonist or antagonist could act, and generally the largest part of the protein is located intracellularly. Thus, they offer an extremely reduced target surface. Another complicating factor is that, in order to be able to make a differentiated therapy via the

PMCAs, one would have to find a drug or antibody that is specific enough not to affect all but only the intended isoform (Strehler, 2013).

Since its first characterization, the involvement of the transmembrane protein neuroplastin in the development of a number of pathologies has been discussed in recent years.

For example, a polymorphism of the protein, which is linked to slightly increased expression of the NPTN gene in the human brain, has been associated with reduced cortical thickness and impaired intellectual abilities. (Desrivières et al., 2015). Furthermore, a mutation in the neuroplastin promoter has been linked to the development of schizophrenia in a small Japanese cohort (Saito et al., 2007). Consistent with this, animal experiments have shown that Np-/- mice exhibit behavior associated with depression, autism, and affective disorders (Bhattacharya et al., 2017). In the pathologies, which are associated with alterations in PMCA or neuroplastin, it is noteworthy that there is an overlap in these where the disease is associated with alterations in both neuroplastin and PMCA. Since this work examines the interaction of these proteins, some of these pathologies are elaborated below and a potential association with the interaction of PMCA and neuroplastin is discussed. An apparent possible reason why changes in the amount or structure of neuroplastin could lead to an altered phenotype is that they directly affect the amount of PMCA in the cell membrane and thus indirectly affect the calcium homeostasis of the cell. It is very likely that an altered calcium balance plays an important role in the pathologies linked to alterations of neuroplastin and PMCA. Experiments in which one intervenes in the calcium balance of animals with drugs could substantiate this assumption more precisely. For example, it would be interesting to see whether inhibitors of the PMCAs are able to cause phenotypes similar to the ones of mice with a reduced expression of neuroplastin. Studies on the effects of knockout of PMCA in mice have already been conducted showing tremendous differences depending on the isoform (Stafford et al., 2017). A knockout of both alleles of PMCA 1, for example, is not compatible with life, again providing evidence that either is indispensable as a housekeeping protein or is at least required for organogenesis. However, heterozygous mice in which only one allele of PMCA 1 was affected do not show an obviously altered phenotype. In vitro experiments have shown, by combining heterozygous loss of PMCA 1 with homozygous loss of PMCA 4, increased apoptosis of portal vein smooth muscle (Okunade et al., 2004). This indicates that at least the ubiquitous housekeeping isoform PMCA1 can at least partially compensate for the loss of the housekeeping isoform PMCA 4, which is not the case the other way around (Keeton et al., 1993). In fact the only major difference of the phenotype of PMCA 4 deficient mice is male infertility since it accounts for more than 90% of total amount of PMCA in sperm (Okunade et al., 2004). In the case of a knockout of the non-ubiquitous isoform PMCA2, the situation is different. In this case, the mice appear normal at birth but develop difficulties in maintaining a balance within the first two weeks. Histological examination of PMCA2-deficient

mice demonstrated that both the auditory system and the vestibular system had defects explaining the balance defect as well as the observed deafness (Kozel et al., 1998). In addition to these knockout mice two spontaneous null mutations of the PMCA2 carrying gene have been reported leading to the deafwaddler and the Wriggel mouse Sagami showing similar pathologies like the knockout mice (Street et al., 1998; Takahashi and Kitamura, 1999). It was hypothesized that the deafness, due to an alteration PMCA 2 is caused by its effect on the calcium household, since PMCAs are crucial for maintaining a low calcium level in the endolymph surrounding the hair cells, which is necessary for signal transduction during the hearing process (Yamoah et al., 1998). Here it is noteworthy that the absence of neuroplastin also leads to a profound hearing loss (Carrott et al., 2016; Zeng et al., 2016) and cognitive impairment (Herrera-Molina et al., 2017). Of course strict local and temporal control of intracellular calcium levels is also especially important for excitatory cells of the nervous system, which are thus particularly susceptible to changes that affect capacity to control calcium influx and extrusion (Strehler, 2015). But experiments with T-cells have shown that the interaction of PMCA and neuroplastin, and its effect on the calcium machinery, is also of particular importance for the development of immune cells, since here, too, the strict control of calcium levels is crucial. Np-/- T-cells favor the Th1 profile over the Th2 profile, because of an elevated calcium level. Through this mechanism, neuroplastin and PMCAs exert a direct influence on the balance between cellular and humoral immune responses (Korthals et al., 2017). In light of the fact that PMCAs are inhibited by tau and Aβ accumulation in AD, it should be investigated whether neuroplastin is relevant for this phenomenon, especially since it is known that the basal calcium levels in AD-affected cells are elevated and that the PMCA is the only known pump that is inhibited by those proteins, which is not the case for the SERCA or the Secretory Pathway Ca^{2+} ATPase SPCA. The fact that the PMCA levels are not lowered in total cell lysates but only in membrane compartments and that the affinity of the PMCA for calcium is not affected could point towards a disturbed transport of PMCA to the plasma membrane (Berrocal et al., 2009; Berrocal et al., 2015). Here, further experiments could show whether the overexpression of tau has an influence on the localization or expression of neuroplastin and whether the inhibitory influence on PMCA by tau can be compensated by an overexpression of neuroplastin or even just the transmembrane part of neuroplastin. Also, further experiments could be performed to test, whether Aβ has an influence on the expression or localization of neuroplastin. This knowledge could help to better understand AD and neuronal aging and expand the range of possible therapeutic approaches. A recent study compared the amount and distribution of Np65 in the human hippocampus between Alzheimer patients and control patients using immunohistochemistry. According to this study, increased expression of Np65 occurs in all regions of the hippocampus during the course of AD. However, the amount of Np65 is greatest at the beginning of AD and then decreases again in

the course of the disease. Interestingly, Np65 was also found to be clustered in intracellular structures (Ilic et al., 2019). Regarding this, further experiments would be useful to examine whether neuroplastin and PMCA already co-localize within the described intracellular structures. It is hypothesized that the increase of neuroplastin is an adaptation reaction to the disease in order to be able to react to the neuropathological changes caused by AD. At this point, further studies would be interesting to investigate the interaction of neuroplastin and PMCAs during AD, as it is conceivable that their alteration may be an adaptation to increased intracellular calcium levels, which in turn negatively affect plasticity (Kuchibhotla et al., 2008). This is especially interesting since therapeutic approaches that directly target amyloid plaque have been proven to be difficult to implement, a therapy that targets the calcium balance would be a possible approach to positively influence the course of the disease. For example, the use of dantrolene, an inhibitor of the ryanodine receptor, as a therapy for AD is currently being investigated as a viable option (Shi et al., 2019). Since dantrolene blocks the calcium-induced calcium release from the ER by blocking the ryanodine receptor, it lowers the intracellular calcium level (Parness and Palnitkar, 1995). Therefore, an interesting consideration would be whether the use of dantrolene in mice in which a knockdown of neuroplastin is performed would be able to influence the resulting altered phenotype to test whether this is indeed due to a higher intracellular calcium concentration caused by a lower amount of PMCA in the plasma membrane. Another disorder in which the interaction of neuroplastin and PMCA might play a role is autism. Since a lower level of PMCA2 has been associated with autism (Carayol et al., 2011), the possibility that the interaction of neuroplastin and PMCAs may have an impact on the development of the disease is conceivable, because neuroplastin knockout mice exhibit a behavior associated with this disease (Bhattacharya et al., 2017). The behavior of the neuroplastin knockout mice is also very interesting because it has been linked not only to autism but to schizophrenia too. Since schizophrenia has been linked to a mutation of the NPTN gene in humans (Saito et al., 2007) as well as to an altered regulation of PMCA (Martins-de-Souza et al., 2009), it would be another interesting approach to further investigate whether the interaction of these two proteins could play a role in the development of the disease. Since neuroplastin and PMCA have already been described in various pathologies and their interaction seems likely in at least some of them, it seems worthwhile to investigate their interaction in more detail. This interaction could be used in both directions to develop therapeutic strategies. Drugs targeting neuroplastin could possibly be developed to compensate for changes in PMCA and thereby in the calcium machinery, since PMCA itself has been proven to be a challenging target (Strehler, 2013). Conversely, one could possibly use drugs that have an influence on PMCAs or the calcium balance itself to compensate for changes in neuroplastin. Thus, a more detailed knowledge of the interaction of these two proteins and a potential manipulation of this interaction could potentially provide an opportunity

to develop therapeutic approaches for the various diseases associated with neuroplastin and PMCA.

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8. List of figures

9. List of tables

10. Ehrenerklärung

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

The regulation of the expression of PMCA isoforms by neuroplastin has an impact on the calcium clearance in cultured hippocampal neurons

im Leibniz-Institut für Neurobiologie Magdeburg

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

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