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Neuroplastin in Ca^{2+} signal regulation and plasticity of glutamatergic synapses

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The main function of neurons is information transmission in the form of action potentials. To fulfill this duty, neurons are connected functionally with each other via synapses, the microscopic structures where specialized molecular machinery is strategically placed to release and receive neurotransmitters and to generate and extinguish calcium (Ca^{2+}) signals. These synaptic molecular components are highly dynamic and they influence each other to confer structural and functional adaptability (plasticity) to neuronal communication (Biederer et al., 2017). Recently, neuroplastin (Np), a cell recognition molecule, has emerged to play diverse neuronal functions including synapse formation, spine structure, Ca^{2+} signal regulation, excitatory/inhibitory balance, and synaptic plasticity. Evidence from different labs has converged to form a coherent picture; however, the uncovered mechanisms may represent only the tip of Np's iceberg. Many questions remain to be answered. For example, why do neurons need two Np isoforms? How do Np isoforms contribute to Ca^{2+} signal regulation and synaptic plasticity?

Neuroplastin isoforms and their importance: The two Np isoforms Np55 and Np65 are alternatively spliced single-pass transmembrane glycoproteins and members of the immunoglobulin (Ig) superfamily of cell adhesion molecules encoded by the *Nptn* gene. They both contain a transmembrane segment and a short intracellular domain. While Np65 contains three extracellular Ig-like domains (fully glycosydated protein: 65 kDa; deglycosydated backbone: 40 kDa), Np55 lacks the first and most peripheral Ig-like domain (glycosidated: 55 kDa; deglycosidated: 28 kDa) (Langnaese et al., 1997; Owczarek et al., 2011; Herrera-Molina et al., 2014). They are highly abundant in neurons, located in pre- and post-synaptic compartments as well as extrasynaptic membranes. The generation and analysis of $Nptn^{-/-}$ mice and inducible conditional Np mutant mice at Dirk Montag's laboratory have made it evident the importance of the Np expression for hearing, hypothalamic-pituitary-adrenal hormonal axis, male fertility, and learning and memory (Bhattacharya et al., 2017; Herrera-Molina et al., 2017; Lin et al., 2021). Further analysis in cultured $Nptn^{-/-}$ neurons uncovered failed spinogenesis and altered structure of glutamatergic spines (Herrera-Molina et al., 2014). While the expression of Np55 and Np65 is developmentally regulated to foster the density of newly born glutamatergic dendritic protrusions in young neurons (Vemula et al., 2020), Np65 may also promote mushroom-like morphology in spines of mature neurons (**Figure 1A**). The meaning of Np65 promoting this structure in mature spines is unknown, but it could reflect structural plasticity via homophilic trans-synaptic binding of its isoform-specific Ig-like domain (Owczarek et al., 2011) in the context of Ca^{2+} signal regulation or long-term potentiation (LTP) (see below).

Tell me who your friends are, and I will tell you who you are: In the following paragraphs, we discuss recent studies describing novel molecular mechanisms of Np for Ca^{2+} signal regulation and LTP. In particular, we focus on how Np is either directly and/or functionally associated with the four plasma membrane Ca^{2+} ATPases (PMCA1-4), N-methyl-D-aspartate-type ionotropic glutamate receptors (iGluNRs), and GluA1-containing α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type ionotropic glutamate receptors (iGluA1Rs).

Np55 and Np65 bind PMCA to regulate Ca^{2+} signals in neurons: About 98% of the total PMCA1-4 in the rodent brain are found to bind with Np55 or Np65 in native protein complexes biochemically extracted from rodent brain homogenates (Schmidt et al., 2017). PMCA1-4 protein levels are drastically reduced, while their mRNA levels remain unchanged, in the brains of $Nptn^{-/-}$ mice and inducible conditional Np mutant mice evidencing post-translational stabilization of PMCA1-4 by Np binding (Bhattacharya et al., 2017; Herrera-Molina et al., 2017). Np-PMCA complexes have been characterized as dimers of dimers with a 1:1 stoichiometric ratio resulting from the binding interface between the single transmembrane domain of Np55 or Np65 to TM10 of PMCA1 (Gong et al., 2018). Nevertheless, this information does not clarify the heterogeneity of complexes constituted, for example, by a particular Np isoform with different PMCAs with or without intracellular PSD95-binding domains. Interestingly, the distributions of PMCA1 and 2, but not PMCA3 and 4, are altered in lipid raft fractions derived from GM2/GD2 synthase-deficient mouse brains lacking complex gangliosides such as GM1 (Ilic et al., 2021). GM1 co-localized with Np65 in the plasma membrane of cultured hippocampal neurons suggesting that PMCA1 and 2 are selectively stabilized by Np65 in GM1-containing lipid rafts (Ilic et al., 2021). As delineated by recent studies (Herrera-Molina et al., 2017; Schmidt et al., 2017; Gong et al., 2018; Ilic et al., 2021; Malci et al., 2022), proteomic approaches and super-resolution microscopy techniques shall be used to characterize the lipid and protein composition and sub-cellular localization of different native Np-PMCA complexes. Also, biochemical and electrophysiological approaches combined with molecular dynamics and Ca^{2+} imaging may be used to define binding properties, structural and kinetic features, and contribution to Ca^{2+} signal regulation and synapse physiology of each Np-PMCA complex.

PMCA1-4 are P-type integral proteins with ten membrane-spanning regions and ATPase-consuming activity to expel cytosolic Ca^{2+} toward the extracellular space in exchange for $2H^{+}$. Thus, PMCA activity maintains an electrochemically neutral ionic gradient and contributes to perisynaptic alkalinization required for activation of iGluNRs (Chen and Chesler, 2015). A recent study by Malci et al. (2022) demonstrated that Np65 and Np55 are able to promote PMCA expression and enhance PMCA-dependent cytosolic Ca^{2+} clearance of pyramidal hippocampal neurons. In the case of Np65-PMCA complexes, Malci et al. (2022) showed that they may interplay functionally with iGluNRs to regulate Ca^{2+} transients in postsynaptic neurons at different levels. First, Np65 was found to coordinate PMCA and iGluRs' functions to modulate the duration and dynamics of Ca^{2+} transients in spines and dendrites. Second, PMCA activity required iGluR activation to restore basal cytosolic Ca^{2+} levels. Third, the joined activity of PMCA and GluN2A-containing glutamate receptors was needed during the early stages of LTP in hippocampal slices pointing to active functional cross-talk between the pump and the receptor at plastic postsynapses. Interestingly, enhanced Np65-PMCA activity is reflected in decreased Ca^{2+} transient amplitude while the such effect was much lesser evident for Np55-PMCA activity pointing to distinguishable functional signatures for Np65- or Np55-containing PMCA complexes in spines (Malci et al., 2022). Therefore, both Np isoforms impact PMCA-mediated Ca^{2+} extrusion directly but differently as Np65 is additionally talented to link PMCA function with GluN2A activity during the early phase of LTP induction (**Figure 1B**). Np elimination in $Nptn^{-/-}$ mice resulted in reduced PMCA1-4 whereas Np overexpression in cultured hippocampal neurons resulted in increased PMCA levels evidencing the direct dependency of PMCA levels on Np. Interestingly, in both antagonistic conditions, Np elimination and overexpression, GluN2A levels were decreased suggesting that the levels of the receptor might not be dependent correlatively on Np itself but rather on the fine-tuned regulation of Ca^{2+} transients by PMCA. This hypothesis could be tested by identifying the Ca^{2+}-dependent postsynaptic signaling events regulated by Np55-PMCA and Np65-PMCA as well as by confirming whether Np65-PMCA regulates synaptic levels and activity of iGluN2A during LTP induction via PMCA-dependent perisynaptic alkalinization (Chen and Chesler, 2015).

Total and cell surface levels of GluA1 are reduced by Np65 overexpression but not by Np55 overexpression in cultured pyramidal neurons (Malci et al., 2022). Because Np55 and Np65 promote PMCA levels and PMCA-dependent Ca^{2+} extrusion whereas that Np65- but not Np55-promoted PMCA activity resulted in reduced Ca^{2+} transient amplitudes, Malci et al. (2022) concluded that a putative Np65-specific mechanism related to synaptic levels of GluA1 may influence the regulation of the amplitude of Ca^{2+} transients. Interestingly, Jiang et al. (2021) reported that GluA1 levels and synaptic AMPA receptor currents are reduced in CRISPR/Cas9-edited *Nptn* CA1 neurons compared to control neurons in hippocampal slices. This apparent contradiction

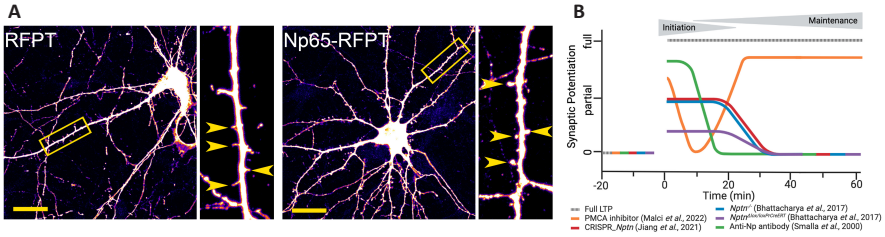

Figure 1 | **Neuroplastin drives structural and functional plasticity in glutamatergic neurons.**
(A) Np65 promotes mushroom-like shape in spines of hippocampal neurons (14 days *in vitro*) that were transfected with control red fluorescent protein (RFPT)- or Np65-RFPT-expressing plasmids, fixed, immunostained with RFPT antibody, and photographed using a confocal microscope as detailed in Malci et al. (2022). Scale bars: 20 µm. (B) The graph is a visual summary of long-term potentiation (LTP) experiments performed in CA1 pyramidal neurons in different studies. Based on Figure 6B from Malci et al. (2022) (orange line), Figure 4C and 4H from Bhattacharya et al. (2017) (blue and purple lines), Figure 4A from Jiang et al. (2021) (red line), and Figure 4 from Smalla et al. (2000) (green line), we illustrated how Np function in Ca^{2+} signal regulation may contribute to synaptic plasticity. Full LTP is represented by a segmented line. The proposed tripartite neuroplastin 65 (Np65)-plasma membrane Ca^{2+} ATPases (PMCA)-GluN2A functional association might be particularly important during the initiation of LTP (Chen and Chesler, 2015; Gong et al., 2018; Malci et al., 2022), whereas that Np65-GluA1 may play a more critical role during the maintenance of LTP (Smalla et al., 2000; Jiang et al., 2021). For further description of this working model, please see the text of this article. Unpublished data.

may reflect some intrinsic differences between the two experimental models. For example, whereas Np65 overexpression in cultured neurons (Malci et al., 2022) may promote sustained enhancement of Np65 binding to GluA1 resulting in its internalization and degradation, CRISPR/Cas9-mediated *Nptn* gene deletion in single neurons (Jiang et al., 2021) eliminates the Np65-GluA1 binding evoked during LTP maintenance (see below). In any case, further studies may also consider evaluating how Np65 influences the abundance of GluA1 as Ca^{2+}-permeable homomers or GluA1/A2 heteromers.

Np65 but not Np55 binds iGluA1 to maintain LTP: LTP of CA1 pyramidal neurons is drastically affected by *Nptn* gene elimination in hippocampal slices derived from *Nptn*$^{-/-}$ mice, inducible conditional Np mutant mice (Bhattacharya et al., 2017), and mice with post-synaptically CRISPR/Cas9-edited *Nptn* gene (Jiang et al., 2021; **Figure 1B**). This evidence confirms a previous report using anti-Np antibodies and Np recombinant proteins to impair LTP maintenance in rat hippocampal slices (Smalla et al., 2000). Interestingly, Jiang et al. show that postsynaptic re-expression of Np65 but not Np55 in the CRISPR/Cas9-edited *Nptn* background was very effective to restore LTP maintenance and provides a molecular mechanism to explain these findings (Jiang et al., 2021). First, the NH_2-terminal domain of GluA1 was found required for LTP maintenance. Second, Np65 but not Np55 co-precipitated with GluA1 NH_2-terminal domain from homogenates of transfected HEK293T cells and activated GluA1Rs in electroporated hippocampal slices to maintain LTP. Third, the expression of a vector encoding a chimeric construct carrying Np65-specific Ig-like domain rescued LTP maintenance in Np-knockout neurons. Jiang et al. concluded that the potential binding of the Np65-specific Ig-like domain to the NH2-terminal domain of GluA1 mediates LTP maintenance.

The results from Jiang et al. (2021) suggest that Np65 but not Np55 contributes to GluA1R anchoring into the glutamatergic postsynapses. If so and considering the large size of GluA1Rs (500–600 kDa), it is fair to ask how Np65 does it. It is proposed that the nano-organization of pre- and post-synaptic proteins may control synaptic transmission (Biederer et al., 2017). In this proposed context, trans-synaptic interactions by several cell adhesion molecules collaborate to create diffusional traps or slots for receptors clustered at the postsynaptic site. Then, the post-synaptic receptors can face the presynaptic active zone where vesicles are fusing the plasma membrane and releasing neurotransmitters. Considering that the Np65-specific Ig1 domain could form such homophilic binding in trans (Owczarek et al., 2011) and that the synaptic abundance of Np65 increases rapidly after high-frequency stimulation in hippocampal spines (Malci et al., 2022) confirming previous observations in PSD-enriched hippocampal preparations from LTP-induced slices (Smalla et al., 2000), we imagine that Np65 in the post-synaptic site may be able to bind another Np65 in the complementary perisynaptic site influencing the anchoring and/or lateral diffusion of GluA1Rs. Alternatively, another pre-synaptic protein may also be able to trans-interact with post-synaptic Np65 and facilitate GluA1R anchoring. In addition, since Ca^{2+} level regulation is essential for neurotransmitter release, specific pre-synaptic Np-PMCA complexes should be identified and evaluated in their capacity to engage with presynaptic Ca^{2+} signal regulation and LTP (see above). These and other similar possibilities are currently under intensive evaluation in our laboratories.

Concluding remarks: Neurons express Np55 and Np65 to bind PMCA1-4 in stable complexes in the plasma membrane, ensuring an efficient extrusion of cytosolic Ca^{2+} raised by neuronal activity, i.e. synaptic transmission. In glutamatergic synapses, additionally, Np65 might coordinate an interdependent functional communication between PMCA activity that "off" Ca^{2+} signals with iGluN2A-containing receptors activity that "on" Ca^{2+} signals during LTP induction. This shall be the focus of future studies. Np65 but not Np55 promotes GluA1-containing receptors activity to maintain LTP. Also, the Np65-specific Ig-like domain seems to be required for GluA1 anchoring to the post-synaptic membrane might be facing the pre-synaptic active zone. Further research may determine the involvement of Np65 at pre- and postsynapses and whether there is a direct binding to GluA1.

Potential connections between Np65-PMCA and Np65-GluA1 and the importance of Ca^{2+} signal regulation and LTP should be investigated more deeply. To understand LTP, we need to decipher the molecular mechanisms underlying the structural and functional changes occurring in glutamatergic synapses. However, it is still difficult to discriminate the specific mechanisms participating in each of the two phases of LTP, namely induction and maintenance. We propose that a clearer understanding of the nanopositioning of Np65 to fine-tune Ca^{2+} entry and extrusion will shed light on the changes in synapse architecture and Ca^{2+}-dependent mechanisms occurring at the different phases of LTP.

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