Der Wissenschaftsfonds.

The role of protein kinase N1 in hippocampal synapse development

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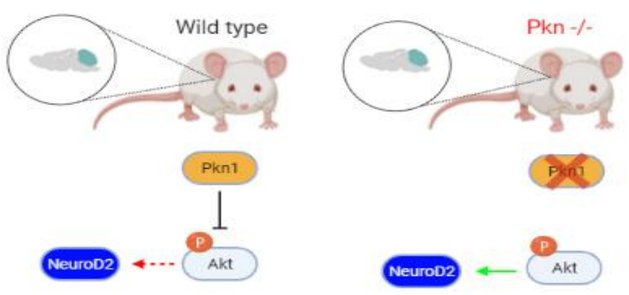
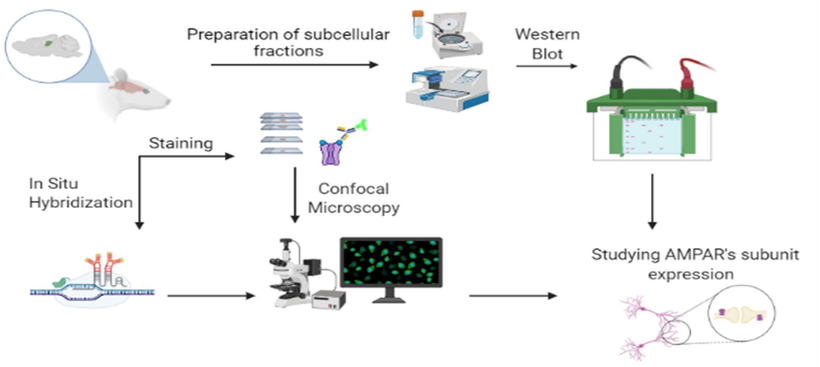
Introduction

Abnormalities in the mechanisms that control α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) expression, assembly and trafficking lead to psychiatric and neurodegenerative disorders. According to our previous findings, the serine/threonine kinase Protein kinase N1 (PKN1) is a developmentally active regulator of cerebellar synaptic maturation by inhibiting AKT and the neurogenic transcription factor neurogenic differentiation factor-2 (NeuroD2). NeuroD2 is implicated in glutamatergic synaptic maturation by regulating expression levels of various synaptic proteins. Here we show how the absence of PKN1 acts on AKT phosphorylation and NeuroD2 levels in the hippocampus and the subsequent expression levels of the NeuroD2 targets and AMPAR subunit glutamate receptor 1 (GluA1). We show that PKN1 is expressed throughout the hippocampus. Postnatal and adult *Pkn1* knockout (*Pkn1*$^{-/-}$) hippocampi showed enhanced AKT phosphorylation, NeuroD2 levels, and also the AMPAR subunit GluA1 expression, particularly in area CA1. In contrast, GluA2/3 levels were not different between both genotypes. Moreover, we showed that the GluA1 content in the membrane fraction of postnatal and adult *Pkn1*$^{-/-}$ hippocampi were enhanced, while GluA2/3 levels remained unchanged. Our results point to a specific regulation of GluA1 expression and/or trafficking by the novel PKN1-AKT-NeuroD2 axis. In view of the important role of GluA1 in hippocampal development as well as the pathophysiology of several disorders, ranging from Alzheimer's, to depression and schizophrenia, our findings establish PKN1 as a target for future studies into neurological disorders related to altered AMPAR subunit expression in the hippocampus.

Novel Pkn1-AKT-NeuroD2 axis[1]

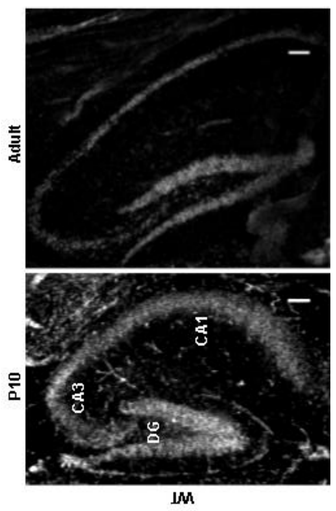


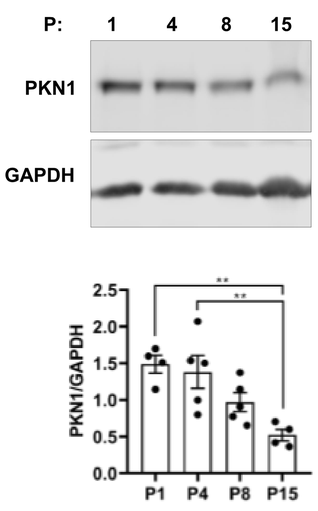
Methods



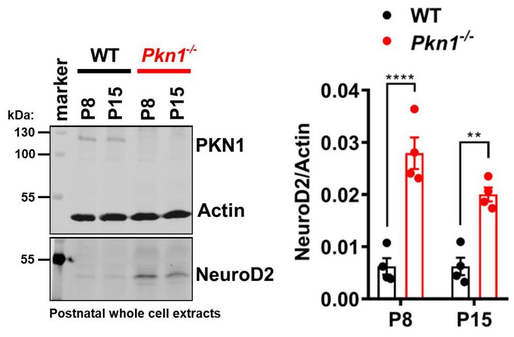
PKN1 is highly expressed throughout the hippocampal formation

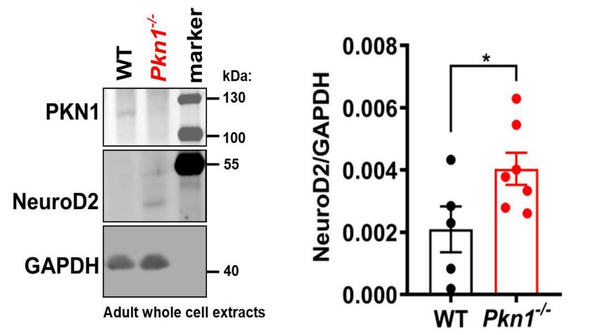
- ✓ Protein kinase N1 *in situ* hybridization revealed that PKN1 is abundantly expressed in the hippocampus in P10 old and adult animals. PKN1 mRNA was found in all hippocampal areas.
- ✓ There was no difference in mean intensity levels between hippocampal layers in P10 old or in adult animals.
- ✓ Western blot analysis of WT whole cell hippocampal protein extracts revealed a significant reduction in PKN1 expression from P1 to P15 , suggesting a vital role of PKN1 during postnatal development.



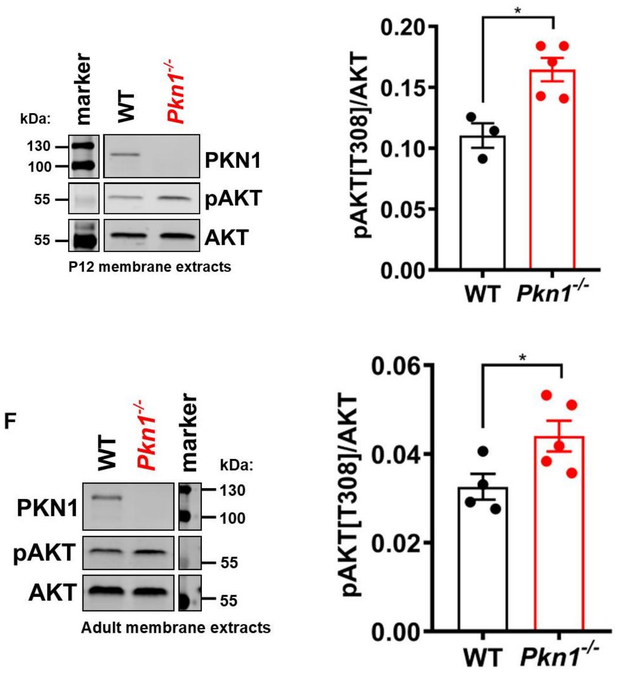


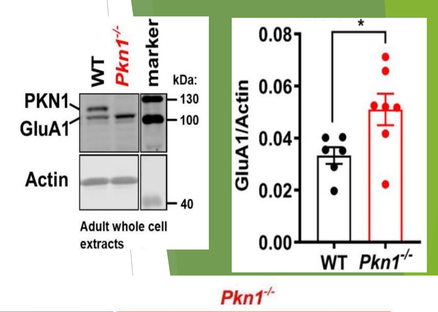
- ✓ We next tested the effect of *Pkn1* knockout on hippocampal AKT phosphorylation and NeuroD2 levels.
- ✓ In agreement with our earlier findings in the cerebellum[1] hippocampal NeuroD2 levels in postnatal *Pkn1*$^{-/-}$ animals were strongly elevated at P8 and P15, suggesting that PKN1-mediated inhibition of NeuroD2 is important in various brain areas during development.

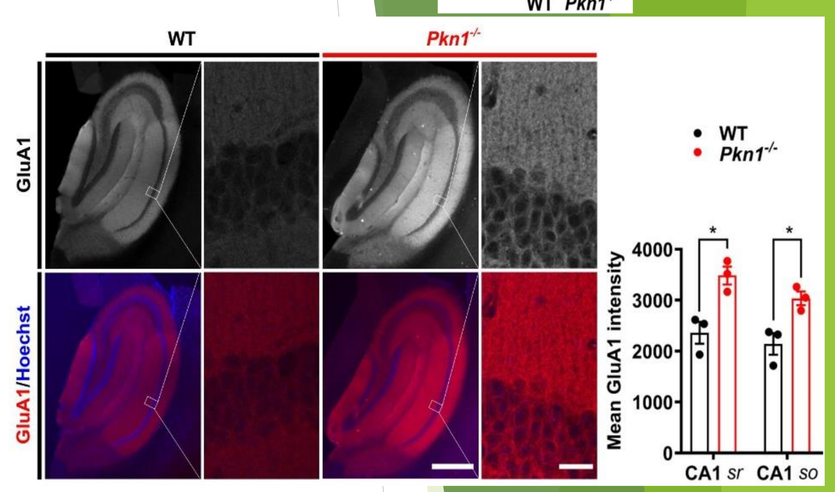




- ✓ AKT phosphorylation in the cytosolic fraction was not different at both ages.
- ✓ To investigate if *Pkn1* knockout results in enhanced AKT phosphorylation, we prepared detergent-soluble (cytosolic) and detergent-insoluble fractions (membrane-associated proteins) of P12 old and adult WT and *Pkn1*$^{-/-}$ hippocampi.
- ✓ Interestingly, we found that particularly membrane-associated AKT was hyper-phosphorylated upon *Pkn1* knockout in P12 old as well as in adult animals.

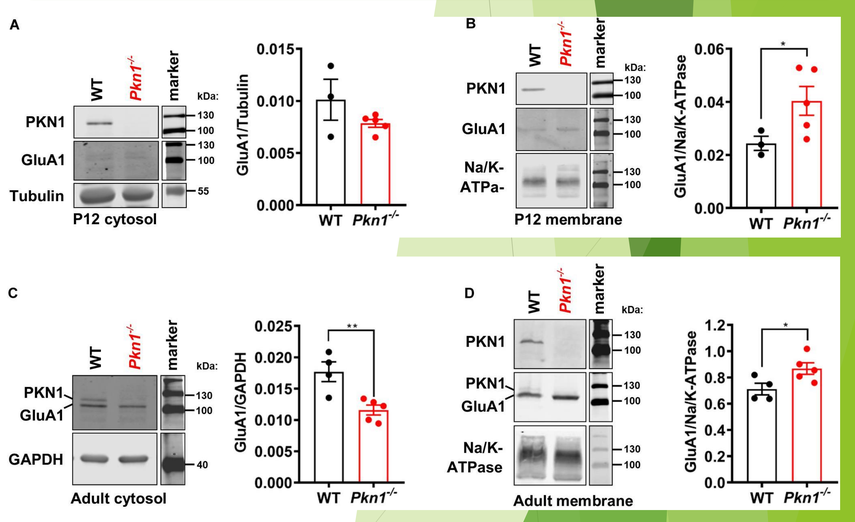






Pkn1$^{-/-}$ Animals Show Higher Membrane-Associated GluA1 Levels

- ✓ In addition, other research shed light to the fact that decreased protein expression levels, *Neurod2* knockout neurons showed a reduction in GluA1 surface expression.
- ✓ To test if hippocampal extracts from young postnatal and adult animals show differences in cytosolic and membrane-associated -GluA1 levels, we analyzed the detergent-soluble and the detergent-insoluble membrane protein fraction (contains plasma membrane-associated proteins and vesicles) of P12 old and adult WT and *Pkn1*$^{-/-}$ hippocampi by immunoblotting.
- ✓ While we did not find a significant difference in the GluA1 content in the cytosolic fraction of P12 old animals (A), we observed a significant increase in GluA1 levels in the membrane fraction of *Pkn1*$^{-/-}$ hippocampi (B).
- ✓ In adult *Pkn1*$^{-/-}$ animals GluA1 levels in the cytosolic fraction were significantly reduced (C), while the membrane-associated content of GluA1 was significantly increased (D).
- ✓ This suggests that besides differences in protein levels, GluA1 trafficking might be affected by *Pkn1* knockout in adult animals.
- ✓ GluA2/3 levels in the cytosolic or membrane fraction of P12 old and adult animals were not different between both genotypes.
- ✓ Likewise, the content of several other synaptic proteins (such as PSD-95, synapsin 1, Znt-3, $GABA_A$ Receptor subtypes) was not significantly affected by *Pkn1* knockout.



Pkn1$^{-/-}$ Hippocampi Have Higher GluA1 Levels

- ✓ It has been previously reported that NeuroD2 controls the excitatory/inhibitory synaptic balance in pyramidal cortical neurons. We therefore probed adult hippocampal extracts from WT and *Pkn1* knockout animals for series of glutamatergic, gamma aminobutyric acid (GABA)-ergic and general synaptic markers.
- ✓ Adult *Pkn1*$^{-/-}$ animals showed significantly elevated GluA1 levels, however, GluA2/3 levels were not different between both genotypes.
- ✓ Immunofluorescence staining revealed that GluA1 levels in *Pkn1*$^{-/-}$ animals were particularly elevated in the CA1 area of the hippocampus.
- ✓ Another protein significantly upregulated upon *Pkn1* knockout was VGlut 1, however, several other synaptic proteins (such as PSD-95, Znt-3, $GABA_A$ receptor subtypes,etc) were not, or only moderately affected by *Pkn1* knockout (such as SNAP-25).

Summary

Taken together our data validate PKN1 as a novel regulator of hippocampal GluA1 levels and GluA1 membrane-association, thereby providing a new tool to understand the functional consequences of this specific subunit as well as raising the potential for the modulation of GluA1 as a possible strategy for therapeutic intervention. In that context, it is worth noting that non-specific PKN1 inhibitors have already been validated for safe use in humans and the development of specific PKN1 inhibitors is a focus of cancer research.

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1 **Protein kinase N1 critically regulates cerebellar development and long-term function**, zur Nedden, S., Eith, R., Schwarzer, C., Zanetti, L., Seitter, H., Fresser, F., et al. (2018). Protein kinase N1 critically regulates cerebellar development and long-term function. *J. Clin. Invest*. 128, 2076-2088. doi: 10.1172/JCI96165