Universität Stuttgart
Institut für Zellbiologie und Immunologie

Regulation of basal and activity-mediated AMPAR endocytosis by Protein Kinase D (PKD)

Von der Fakultät Energie-, Verfahrens- und Biotechnik

der Universität Stuttgart zur Erlangung der Würde eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigte Abhandlung

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Tag der mündlichen Prüfung:

26. Mai 2021

Institut für Zellbiologie und Immunologie

Universität Stuttgart

2021

Affidavit

I hereby confirm that my thesis entitled “Regulation of basal and activity-mediated AMPAR endocytosis by the Protein Kinase D family (PKD)” is the result of my own work. All sources and/ or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Zusammenfassung

AMPARs sind eine von vier verschiedenen Arten von Glutamat-gesteuerten Ionenkanälen im Gehirn von Säugetieren, die den Einstrom von Na + und den Ausstrom von K + und damit die Depolarisation der neuronalen Membran ermöglichen. Sie sind entscheidend für die korrekte Funktion des Gehirns, da sie die Hauptmediatoren der exzitatorischen synaptischen Übertragung darstellen und als solche für die interzelluläre Kommunikation, die Entwicklung des Gehirns und vor allem für das Lernen und das Gedächtnis von entscheidender Bedeutung sind. AMPARs sind hoch motil und werden sowohl konstitutiv als auch aktivitätsvermittelt innerhalb der Plasmamembran durch laterale Diffusion als auch zwischen der Plasmamembran und intrazellulären Pools durch Endozytose und Recycling transportiert. Die Regulation dieses Transports ist von entscheidender Bedeutung, um die Langzeitpotenzierung (LTP) und die Langzeitdepression (LTD), zelluläre Mechanismen der synaptischen Plastizität, die eine dauerhafte Verstärkung bzw. Abschwächung der synaptischen Übertragung fördern und ein neurophysiologischer Mechanismus für Lernprozesse und Gedächtnis sind, zu vermitteln. Die Mechanismen, die dieser Regulation zugrunde liegen, sind jedoch noch nicht vollständig verstanden.

Die Proteinkinase D Familie der Serin / Threonin Kinasen besteht aus drei Isoformen in Säugetierzellen und wird stromabwärts von DAG und PKC aktiviert, um an der Regulation von Prozessen wie der Vesikelabschnürung am trans-Golgi-Netzwerk und dem Umbau des Aktin-Zytoskeletts teilzunehmen. Alle drei Isoformen werden in Neuronen von einem frühen embryonalen Stadium an exprimiert, wo sie gewebespezifische Prozesse wie die Etablierung und Aufrechterhaltung der neuronalen Polarität, den Neuroprotektionsschutz gegen oxidativen Stress oder die synaptische Plastizität modulieren. Letzteres wird durch Stabilisierung des filamentösen Aktins durch PKD-vermittelte Cofilin-Inaktivierung während der Induktion der LTP und der Phosphorylierung verbleibender Oberflächen-NMDARs während der Induktion von LTD. Trotz dieser Beobachtungen wurde PKD noch nicht mit der Regulierung des AMPAR Transportes in Verbindung gebracht. Daher wollte ich in dieser Arbeit untersuchen, ob PKD den AMPAR Transport in primären neuronalen Zellen unter basalen und / oder unter aktivitätsvermittelten Bedingungen kontrolliert, und den molekularen Mechanismus beleuchten, der dieser Regulation zugrunde liegt.

Diese Arbeit zeigt, dass PKD als Promotor der AMPAR-Endozytose in primären Hippocampus-Neuronen sowohl unter basalen als auch unter aktivitätsvermittelten Bedingungen wirkt. Die kurzfristige pharmakologische Hemmung von PKD-Aktivität durch Behandlung mit dem niedermolekularen Inhibitor CRT0066101 führte zu einem Anstieg der Plasmamembran- und synaptischen AMPAR-Konzentration und zu einer Verlangsamung der

Dynamik des AMPAR-Transportes an der Plasmamembran. Umgekehrt förderte die Expression einer konstitutiv aktiven PKD-Mutante eine Abnahme der synaptischen AMPAR-Konzentration, während gleichzeitig eine erhöhte Lokalisation an frühen Endosomen festgestellt wurde. Darüber hinaus wurde gezeigt, dass die PKD-Aktivität für die Abnahme der Plasmamembran-AMPAR-Konzentration als Reaktion auf Agonisten- und NMDA-Behandlungen notwendig ist. Schließlich lege ich Beweise dafür vor, dass die Phosphorylierung des PKD-Substrats und des Rab5-Effektors Rabaptin-5 an S407, der PKD-Phosphorylierungsstelle, für die korrekte Regulierung der synaptischen AMPAR-Menge und für die Endozytose von AMPAR als Reaktion auf die NMDA-Behandlung erforderlich ist. Zusammen identifizieren diese Ergebnisse PKD als einen neuen Regulator der AMPAR-Endozytose, vermutlich durch die Phosphorylierung von Rabapatin-5 und die anschließende Aktivierung von Rab5.

Abstract

AMPARs are one of the four different types of glutamate-gated ion channels in the mammalian brain, allowing influx of Na^+ and efflux of K^+ and thus depolarisation of the neuronal membrane. They are critical for correct brain functioning, as they represent the main mediators of excitatory synaptic transmission and as such are key for intercellular communication, brain development and, importantly, learning and memory. AMPARs are highly motile, undergoing constitutive and activity-mediated endocytosis, recycling and surface lateral diffusion among their different cellular pools. Notably, regulation of this trafficking is instrumental to mediate long-term potentiation (LTP) and long-term depression (LTD), cellular mechanisms of synaptic plasticity, which promote a long-lasting enhancement or decrease in synaptic strength, respectively, and are widely believed to be the main contributors to learning and memory. However, the mechanisms behind that regulation are not fully understood yet.

The protein kinase D family of serine/threonine kinases consists of three isoforms in mammalian cells, and is activated downstream of DAG and PKC to participate in the regulation of processes such as vesicle fission from the trans-Golgi network and actin cytoskeleton remodelling. All three isoforms are expressed in neurons from an early embryonic stage, where they modulate tissue-specific processes such as the establishment and maintenance of neuronal polarity, neuroprotection against early oxidative stress and, importantly, synaptic plasticity through stabilisation of filamentous actin via cofilin inactivation during the expression of LTP and the phosphorylation of remaining surface NMDARs during the expression of LTD. Despite these observations, PKD has not been linked yet to AMPAR trafficking regulation. Therefore, in this thesis I aimed to elucidate whether PKD controls AMPAR trafficking in primary neuronal cells in basal and/or in activity-mediated conditions, and to shed light on the molecular mechanism that underlies this regulation.

This work presents compelling evidence that PKD acts as a promoter of AMPAR endocytosis in primary hippocampal neurons in both basal and activity-mediated conditions. Short-term pharmacological inhibition of PKD via treatment with the small-molecule inhibitor CRT0066101 led to an increase in surface and synaptic AMPAR levels and slowed down AMPAR surface trafficking dynamics. Conversely, expression of a constitutively active PKD mutant promoted a decrease in AMPAR synaptic levels while increasing its localisation at early endosomes. Moreover, it was found that PKD activity is necessary for the decrease in surface AMPAR levels in response to both agonist- and NMDA treatments. Finally, I provide evidence that phosphorylation of the PKD substrate and Rab5 effector Rabaptin-5 at S407, the PKD phosphorylation site, is necessary for the correct regulation of basal AMPAR synaptic levels and for the endocytosis of AMPAR in response to NMDA treatment. Together, these findings

identify PKD as a novel regulator of AMPAR endocytosis, presumably through the phosphorylation of Rabapatin-5 and subsequent Rab5 activation.

Abbreviations

AA	Aminoacid
ABP	AMPAR-binding protein
AD	Alzheimer’s disease
AKAP	A-kinase anchor protein
ALS	Amyotrophic lateral sclerosis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ASD	Autism Spectrum Disorders
ASK	Apoptosis signal-regulating kinase
ATTEC	Autophagosome-tethering compound
AUTAC	Autophagy-targeting chimera
CA	Cornu Ammoni
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
caPKD	Constitutively-active PKD
CAR	Cytosin-arabinofuranoside
CFA	Complete Freund’s adjuvant
cLTD	Chemically-induced long-term depression
CNS	Central nervous system
CP-AMPAR	Ca^{2+}-permeable AMPAR
CREB	cAMP-responsive element-binding protein
CRT	CRT0066101
CTD	Carboxy-terminal domain
DAG	Diacylglycerol
DG	Dentate gyrus
DRG	Dorsal root ganglia

EC	Entorhinal cortex
EE	Early endosome
EEA1	Early endosome antigen 1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EZ	Endocytic zone
FBS	Foetal bovine serum
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GABA	*gamma*-Aminobutyric acid
GABAR	*gamma*-Aminobutyric acid receptor
GBM	Glioblastoma multiforme
GCL	Granule cell layer
GEF	Guanosine exchange factor
GluR	Glutamate receptor
GPCR	G protein-coupled receptor
GRIP	Glutamate receptor-interacting protein
HD	Huntington’s disease
HDAC	Histone deacetylase
HSP	Heat shock protein
IEG	Immediate early gene
iGluR	Ionotropic glutamate receptor
KAR	Kainate receptor
kdPKD	Kinase-dead PKD
LBD	Ligand binding domain
LE	Late endosome

LFS	Long-frequency stimulation
LRP	Low density receptor-related protein
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
LYTAC	Lysosome-targeting chimera
MAGUK	Membrane-associated guanylate kinase
mGluR	Metabotropic glutamate receptor
NMDAR	N-methyl-D-aspartate receptor
NSF	N-ethylmaleimide-sensitive fusion protein
NTD	Amino-terminal domain
p38MAPK	p38 mitogen-activated kinase
PA	Phosphatidic acid
PBSCM	Ca^{2+}- and Mg^{2+}-containing PBS
PC	Phosphatidylcholine
PD	Parkinson’s disease
PdbU	phorbol 12,13-dibutyrate
PDZ	PSD95/disc large/zona occludens-1
PH	Pleckstrin homology
PICK1	Protein interacting with C-kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PM	Plasma membrane
PROTAC	Proteolysis-targeting chimera

PSD	Post-synaptic density
PTM	Post-translational modification
RE	Recycling endosome
rER	Rough endoplasmic reticulum
RIN1	Ras and Rab interactor 1
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SEP-GluA1	Super ecliptic pHluorin-tagged GluA1
SGZ	Subgranular zone
SM	Sphingomyelin
SSH1	Slingshot 1
STM	Short-term memory
TARP	Transmembrane AMPAR regulatory protein
TfR	Transferrin receptor
TGN	Trans-Golgi network
TMD	Trans-membrane domain
TRPV1	Transient receptor potential V1
ULD	Ubiquitin-like domain

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1. Introduction

1.1 The hippocampus

The hippocampal region constitutes a key part of the limbic system and the mammalian brain. It includes two sets of cortical structures: the parahippocampal region and the hippocampal formation, both of which differ in terms of structure, connectivity and function. The parahippocampal region forms part of the periarchicortex and is comprised by 5 different regions: presubiculum, parasubiculum, entorhinal, perirhinal and postrhinal cortices, all of which are composed by more than three cellular layers. In contrast, the hippocampal formation belongs to the archicortex, and therefore its three regions, the dentate gyrus (DG), the hippocampus and the subiculum, are formed only by three cellular layers (Witter, 2012; Haines and Mihailoff, 2018).

1.1.1 Anatomy and connectivity

In mice, the hippocampal formation is an elongated structure which extends from the septal nuclei of the basal forebrain rostrodorsally, over and behind the diencephalon, into the temporal portions of the brain caudoventrally (Witter, 2012). Each hippocampal region is different in terms of the specific characteristics of its cellular layers, cell types and connectivity.

-The DG lies at the core of the hippocampal region, and is comprised by three layers: the outermost molecular layer, the granule cell layer (GCL) and the innermost hilus or polymorphic layer (Witter, 2012). The molecular layer is bordering the hippocampal fissure and is relatively cell-free. In contrast, the granule cell layer is composed of densely packed excitatory granule cells, the most abundant cell type in the DG (Stanfield and Cowan, 1984). These two layers present a “V” shape, thus enclosing the hilar region (Witter, 2012). The subgranular zone (SGZ) is located between the CGL and the hilus, and constitutes one of the few places where neurogenesis can take place in the adult mammalian brain (Cameron et al., 1993; Kuhn et al., 1996). The DG receives its main input from the entorhinal cortex (EC), while the unmyelinated axons of granule cells (known as mossy fibres) innervate both the hilus and the CA3 area of the hippocampus (Witter, 2012).

-The hippocampus proper has traditionally been divided into three areas according to fine morphological criteria, namely CA1, CA2, and CA3. CA stands for Cornu Ammoni, a terminology introduced by Lorente de Nó (Lorente de Nó, 1934). The hippocampus has the shape of two arcs, the upper one of which merges with the subiculum and transitions into the cerebral cortex, while the lower one transitions into the DG (Reznikov, 1991). From the lateral

ventricle wall upwards, the hippocampus consists of the following layers: the ependymal layer, consisting of ependymal cells which border the ventricle wall; the alveus, which contains the myelinated axons of the pyramidal neurons; the stratum oriens, containing the basal dendrites of the pyramidal neurons; the stratum pyramidale, formed by the densely packed bodies of excitatory pyramidal neurons (the predominant cell type); the stratum lucidum, only existing in the CA3 area and constituted by mossy fibres; the stratum radiatum, containing the unbranched primary shafts of the pyramidal neurons and most of the CA3-CA3 associational connections and CA3-CA1 Schaffer collaterals; and lastly the stratum lacunosum-moleculare, formed by the branched apical dendrites of the pyramidal neurons (Reznikov, 1991). The most important inputs of the hippocampus originate in the EC for the CA1 area and in the septal nucleus for the CA3 area. While the CA1 area projects to numerous cortical and subcortical areas, the CA3 area projects intrinsically to the ipsi- and contralateral CA1-CA3 areas (associational connections or Schaffer collaterals) (Swanson et al., 1987; van Groen et al., 2003; Witter, 2012).

-The subiculum is considered to be the output structure of the hippocampal formation. It borders with the CA1 area of the hippocampus on the one side and with the presubiculum on the other (Aggleton and Christiansen, 2015). The subiculum is comprised by an innermost polymorphic layer, containing small cells; the pyramidal cell layer, consisting of large pyramidal cells; and the molecular layer, containing mainly medium-sized pyramidal neurons as well as the apical dendrites of the cells in the pyramidal layer (Burwell and Agster, 2008). This area is primarily connected to the EC in a reciprocal manner (Witter, 2012).

1.1.2 Physiology and pathology of the hippocampal formation

The primary function of the hippocampal formation is the conversion of short-term memory (STM) into long-term memory (LTM), a remarkable process that can take place due to the redundancy and feedback characteristic of its circuits (Haines and Mihailoff, 2018) and the connections it possesses with the EC (Buzsáki and Moser, 2013). Additionally, it plays a key role in learning and memory, spatial navigation, emotional behaviour (in association with the amygdala) and in the regulation of hypothalamic functions (Toyoda et al., 2011; Anand and Dhikav, 2012; Stella et al., 2012). Interestingly, while memories are encoded at the hippocampus, they are stored and retrieved from the frontal lobe, with the connections between the hippocampus and the neocortex being key for awareness of conscious knowledge (Morgado-Bernal, 2011). Lesions in the hippocampal region, or the presence of hallmark traits of neurodegenerative diseases at the EC or subiculum (such as neurofibrillary tangles and neuritic plaques in Alzheimer's disease (AD)), have been described to promote

an impairment in memory conversion and cognitive deficiency (Avila et al., 2010; Mueller et al., 2010; Haines and Mihailoff, 2018). Additionally, the limbic system is particularly sensitive to seizure damage, with epilepsy being related to hippocampal malfunction (Haines and Mihailoff, 2018).

1.2 The neuron

Neurons are the defining cell type of the brain, allowing the acquisition, manipulation and storage of information. Their role is heavily dependent on their location within the central nervous system (CNS), the connections they present and the neurotransmitter they employ. Neurons receive information either from the environment or from another neuron, process that information and forward it to either another neuron or to effector tissues like skeletal muscle fibres or secretory glands.

1.2.1 Structure

Neuronal architecture, according to different parameters like cell body shape or number and arrangement of its processes, can be incredibly diverse. These factors depend on the function performed by the cell and the brain region where the cell is located. However, there are certain features that define the archetypical (multipolar) neuron, consisting of a cell body, multiple dendrites and a single axon:

-The soma contains a large nucleus surrounded by multiple mitochondria, abundant ribosomes and an extensive rough endoplasmic reticulum (rER), thus being the cell's metabolic centre.

-Dendrites conform relatively short processes sprouting from the cell body that branch extensively and progressively become thinner. On the surface of distant branches they present dendritic spines, small protuberances with varying shapes that constitute the sites of synaptic contact with other neurons. While the cytoplasm of the primary dendritic branches is similar to the somatic cytoplasm, that of more distant branches also presents ER clusters and ribosomes to allow for local protein synthesis, in addition to the microtubule- and microfilament-based cytoskeleton.

-The axon is a single process departing from the cell body at the elevation known as axon hillock. After its proximal part, known as the initial segment of the axon, it extends for a long distance keeping a constant diameter and rarely branching until its distalmost region, where it

forms the terminal arbor. The distal parts of this arbor end in the terminal boutons, the presynaptic component of a typical synapse. In contrast to the somatic cytoplasm, axonal cytoplasm lacks certain structures (such as the endosomal system), having instead the specialized machinery needed for its function (Ehlers, 2000). First, multiple vesicles containing neurotransmitters are present. Additionally, axonal boutons contain mitochondria to provide the necessary energy for the synaptic function. While these boutons are usually located at the end of the axon, boutons en passant, which are located along the axon, have also been observed. Notably, non-bouton like enlargements known as varicosities can also act as information senders. Lastly, the axonal cytoskeleton is composed of microtubules and microfilaments, which act both as structural elements and facilitators of protein and organelle transport (Haines and Mihailoff, 2018).

1.2.2 Bioelectrical features and synaptic transmission

Neurons are able to process and store information through modification of their bioelectrical properties, a highly metabolically demanding process involving active trafficking and concentration of certain ions (typically Na^+, K^+ and Cl^-) at one of the sides of the plasma membrane (PM). During the resting state, Na^+ and Cl^- are more concentrated extracellularly, whereas concentration of K^+ is higher intracellularly. These accumulations of ions against the electrical and concentration gradients are possible due to the semi-permeable nature of the PM and the existence of ion pumps and channels. As a consequence, the membrane potential of resting neurons results in -70 mV. Changes in the concentrations of these ions (e.g. as some ion channels open upon binding to their ligand) give rise to local voltage changes that can cause a net gain of positive or negative charges, referred to as membrane depolarization and hyperpolarization, respectively. When the sum of all local events reaches the depolarization threshold (ca. -55 to -50 mV) at the initial segment of the axon, the voltage-gated sodium channels open, and an action potential is generated. This electrical impulse propagates in anterograde direction through the axon reaching its termination, and promoting calcium influx into the bouton. This releases neurotransmitter vesicles to the synaptic cleft, which in turn promotes the stimulation of the postsynaptic neuron and the transmission of information (Haines and Mihailoff, 2018).

1.3 Synapses and synaptic transmission

The location at which a neuron communicates with another cell constitutes the synapse, a specialized structure whose subcellular architecture and molecular composition are tailored

towards fulfilling their specific role. Synapses can be broadly classified into chemical and electrical, with the vast majority of CNS synapses belonging to the former category. As such, I will focus on chemical synapses for the rest of this introduction. For more information on electrical synapses, please see (Meriney and Fanselow, 2019).

Chemical synapses usually consist of a terminal bouton and a postsynaptic spine as their pre- and postsynaptic element, respectively; the synaptic cleft, which is a space between them of around 20-50 nm; and, frequently, supporting structures such as astrocytic processes. All chemical synapses share the properties of unidirectionality (information travels from the presynaptic to the postsynaptic terminal only) and proportionality (the stronger the stimulus promoting the release of neurotransmitter vesicles, the more vesicles are released), with communication being based on neurotransmitters and neuromodulators. These are synthesized, stored in vesicles at the presynaptic terminal, from which they are released in a tightly regulated manner. In the same manner as a presynaptic terminal can release different neurotransmitters, postsynaptic terminals can express different neurotransmitter receptors. Their classification can respond to multiple criteria, such as the type of response they elicit (excitatory or inhibitory) or the speed of the signal transduction (ionotropic for fast transducing ion channels and metabotropic for the slower G protein-coupled receptors (GPCRs)). Importantly, the postsynaptic density (PSD) is a disc-like structure comprised of hundreds of proteins and located at the postsynaptic membrane (Haines and Mihailoff, 2018). The PSD plays a crucial dual role in synaptic transmission: on the one hand, it anchors neurotransmitter receptors at the place of synaptic transmission; on the other hand, it facilitates receptor interaction with their downstream effectors, thus ensuring the efficient transduction of the signal (Banerjee et al., 2016). EM-tomography has allowed for visualization of its structure, which consists of a network of vertical and horizontal protein filaments of varying length (Chen et al., 2008). While the composition and role of the proteins forming the PSD are very dynamic and dependent on synaptic activity, PSD95 (a member of the membrane-associated guanylate kinase family (MAGUK)) is a prominent component of the vertical type, while GKAP/SAPAP, Shank and Homer have been described as components of the horizontal filaments (Hayashi et al., 2009). Neurotransmitter receptors, their downstream effectors, ion channels and cytoskeletal proteins have been described to bind to the PSD, where they can undergo further fine-tuning by a myriad of processes including phosphorylation (Trinidad et al., 2006) and ubiquitin-proteasome-mediated degradation (Ehlers MD, 2003).

1.3.1 Chemical glutamatergic synapses

The synapses employing glutamate as their main neurotransmitter are called glutamatergic synapses, and they account for approximately 80-90% of all brain synapses. Glutamate is a predominantly excitatory neurotransmitter that mediates most of the fast excitatory neurotransmission in the CNS, and as a central element of the cellular metabolism, is present in all cells in the brain. It has been involved in multiple processes, such as transmission of sensory information, motor coordination, emotions and cognition (Hassel and Dingledine, 2012). Ionotropic glutamate receptors (iGluRs) can be observed at both the presynaptic and postsynaptic terminals, albeit fulfilling different roles (see below). Due to its multiple protein binding domains (PSD95/disc large/zona occludens-1 (PDZ), Src homology and guanylate kinase), PSD95 is able to bind all types of glutamate receptors and some of their key downstream effectors, either directly (GluN2 and GluK1/2 subunits) or indirectly through a number of other proteins (e.g. A-kinase anchor protein (AKAP), glutamate receptor-interacting protein (GRIP), protein interacting with C-kinase 1 (PICK1), AMPAR-binding protein (ABP), Homer and Shank) (Chen et al., 2008; Tunquist et al., 2008; Haines and Mihailoff, 2018). Thus, PSD95 has been postulated as a key facilitator of glutamate signal transduction at glutamatergic PSDs.

1.4 Glutamate receptors

Glutamate receptors (GluRs) is a term englobing all receptors whose natural agonist is glutamate, and they are expressed by every cell in the CNS. Six functional classes of GluRs exist, and they can be further classified into ionotropic or metabotropic (mGluRs). Three of these classes correspond to the iGluRs, namely the N-methyl-D-aspartate receptors (NMDAR), the kainate receptors (KARs) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), while the other three classes simply constitute the groups I to III of the mGluRs. Whereas metabotropic glutamate receptors constitute dimers, all ionotropic glutamate receptor are typically tetrameric assemblies of their individual subunits, which present a differential expression pattern across multiple brain regions. Interestingly, different subunit combinations entail functional differences. On top of this, splice variants that mediate functional differences or have a different cellular expression pattern have also been described for most of the glutamate receptor subunits. All of these mechanisms are used by neurons to finely regulate the properties of their receptors (Hassel and Dingledine, 2012; Haines and Mihailoff, 2018).

1.4.1 Metabotropic glutamate receptors

mGluRs are G-protein coupled receptors which act as modulators of synaptic transmission and plasticity in the CNS. Based on sequence homology, pharmacological properties and their canonical transduction pathways, mGluRs can be subdivided into three distinct functional families. Group I consists of mGluR1 and 5, subunits coupled to Gq-like proteins that lead to Phospholipase C (PLC) stimulation, elevation of intracellular Ca^{2+} and activation of Protein Kinase C (PKC). Groups II and III include mGluRs 2 and 3 and mGluRs 4 and 6-8, respectively, and are coupled to Gi-like proteins that promote adenylate cyclase inhibition, cyclic adenosine monophosphate (cAMP) reduction and Protein Kinase A (PKA) inactivation. The structure of mGluR subunits includes an extracellular amino terminal domain (NTD) containing the glutamate-binding site, seven transmembrane domains (TMDs) and a carboxy-terminal domain (CTD) that modulates receptor signalling, trafficking and G protein coupling while also being the site for phosphorylation and protein-protein interactions. The subcellular localization of mGluRs is highly group-specific: while mGluR6 is exclusively restricted to the retina, group I mGluRs are typically localized to the postsynaptic membrane, and members of the groups II and III are mainly located at the presynaptic terminal. Postsynaptic mGluRs acts as modulators of many ligand- and voltage-gated ion channels, such as NMDARs, KARs and *gamma*-Aminobutyric acid $_A$ receptors ($GABA_ARs$), in a highly tissue-specific fashion, thus directly affecting neuronal excitability. However, presynaptic mGluRs have also been described to block glutamatergic and GABAergic synaptic transmission upon activation, in a mechanism that is hypothesized to involve inhibition of presynaptic Ca^{2+} channels (Hassel and Dingledine, 2012; Suh et al., 2018).

1.4.2 NMDARs

NMDARs were first described by Curtis and Watkins on 1963 (Curtis and Watkins, 1963), and are unique among glutamate receptors in that they need glycine as an additional agonist for activation. This process is very strictly regulated through multiple mechanisms, such as the Mg^{2+} or the H^+ block that influence their opening probability, and this is due to the fundamental role of NMDARs for a normal CNS function (Hassel and Dingledine, 2012; Mori et al., 2017).

1.4.2.1 Structure

NMDAR subunits are composed by a large extracellular NTD, the ligand-binding domain (LBD), three TMDs, a re-entry loop that forms the pore-lining region, and an intracellular CTD

(Lau and Zukin, 2007). The CTD is the domain mediating protein-protein interactions, and thus it promotes PSD localization via interaction with scaffolding proteins (Husi et al., 2000). In addition, it regulates membrane targeting, stabilization and degradation, and constitutes the site for various post-translational modifications (PTMs) (Mori et al., 2017). The subunits can be divided into three families: the first one is represented by the glycine-binding subunit GluN1; the second consists of the glutamate-binding GluN2A-D subunits that are responsible for characteristics such as ligand affinity and sensitivity to modulators; and the third is composed of the glycine-binding subunits GluN3A and B (Mori et al., 2017). While NMDAR subunit composition is age-, region- and even synapse-specific, NMDAR heterotetramers usually present two GluN1 and two GluN2 subunits (Lau and Zukin, 2007; Mori et al., 2017).

1.4.2.2 Synthesis and trafficking

Subcellular localization and trafficking of NMDARs are dynamically influenced by neuronal activity and sensory experience, through mechanisms involving PTMs and protein-protein interactions (Lan et al., 2001; Vissel et al., 2001; Fong et al., 2002; Li et al., 2002; Nong et al., 2003; Prybylowski et al., 2005; Lin et al., 2006). Briefly, NMDAR assembly takes place at the ER, where trafficking to and insertion at synapses are promoted upon GluN1/2 subunit assembly and interaction with scaffold proteins such as synapse-associated protein 102 (SAP-102) and PSD95 (Mu et al., 2003; Hawkins et al., 2004; Sans et al., 2005; Lin et al., 2006). Chronic impairment of neuronal activity has also been described to promote NMDAR accumulation at the PM (Rao and Craig, 1997). Once at the PM, NMDARs are able to laterally diffuse into the PSD, thus being able to perform their role. Interestingly, this lateral diffusion is also an important process for NMDAR endocytosis, due to the presence of specialized endocytic zones (EZ) located at the perisynaptic region (Blanpied et al., 2002). NMDAR endocytosis is clathrin-dependent, mediated by the interaction between the CTD of GluN2 subunits and the clathrin adaptor protein AP2 (Lavezzari et al., 2004). While this interaction typically directs receptors to recycling endosomes, other evidence reports additional motifs that can promote NMDAR degradation (Scott et al., 2004; Lau and Zukin, 2007).

1.4.2.3 Physiology and pathology

Two agonists are required for NMDAR activation: glutamate and glycine. In addition, the presence of the Mg^{2+} block and of multiple endogenous inhibitors must be overcome (Traynelis et al., 1995; Amico-Ruvio et al., 2011). Once NMDARs are open, Na^{+} and K^{+} can flow through the channel following concentration and electronic gradients, with Ca^{2+} influx also taking place

and potentially activating multiple intracellular signalling cascades through Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), PKA and PKC, phospholipases A_2 (PLA_2) and C, calcineurin and nitric oxide synthase. GluN1-KO mice die immediately after birth, proving that correct NMDAR expression is necessary for life. In addition, there is increasing evidence that these receptors are involved in intercellular signalling and neural network development and refinement (Forrest et al., 1994; Iwasato et al., 2000; Mori et al., 2017). Because NMDARs are both voltage- and ligand-gated ion channels they act as one of the main regulators of synaptic plasticity, learning and memory due to their ability to detect coincident activation of the pre- and postsynaptic terminals (Lau and Zukin, 2007; Haines and Mihailoff, 2018).

Due to its multifaceted role, both enhanced and impaired NMDAR activity are involved in many disorders. Neurodegenerative diseases occupy a prominent place among them, as excessive Ca^{2+} influx can activate Ca^{2+}-dependent proteases and lipases and promote depolarization of the mitochondrial membranes, ultimately causing cell death in a sequence of events observed in amyotrophic lateral sclerosis (ALS), epilepsy, schizophrenia, Huntington's disease (HD), Parkinson's disease (PD) and, more prominently, in AD (Choi, 1988; Lau and Zukin, 2007; Ghasemi and Schachter, 2011; Zhou and Sheng, 2013).

1.4.3 Kainate receptors

The kainate subfamily of iGluRs is composed of five different members, GluK1-5. They can be divided into the low-affinity subunits GluK1-GluK3, those able to constitute functional homomeric receptors; and the high-affinity subunits GluK4 and GluK5, subunits acting as modulators that require heteromerization to constitute functional KARs. These receptors share many structural and functional characteristics with AMPARs, such as alternative splice variants, Q/R RNA editing on the GluK1/2 subunits, presence of synaptic KARs with different subunit combinations and conductance of Na+ and K+ (and potentially Ca2+) ions. Despite these similarities, KARs possess certain unique characteristics. In contrast to other iGluRs, KARs signal transduction is very likely to undergo the shared metabotropic, non-canonical signalling pathway of iGluRs, involving PLC and PKC as downstream effectors. In addition to this, KARs are very commonly present in both presynaptic and postsynaptic terminals, thus being in a unique position to regulate the release of neurotransmitters such as GABA and glutamate. Furthermore, they present a small current amplitude and slow activation and deactivation kinetics, which allows the regulation of input integration, short-term plasticity and brain rhythms. Thus, KARs are able to fill a specific niche and fulfil specific roles in the brain. For more information on KARs, please refer to (Lerma and Marques, 2013; Valbuena and Lerma, 2020).

1.4.4 AMPARs

AMPARs are glutamate-gated ion channels whose main role is to mediate the vast majority of excitatory synaptic transmission in the mammalian brain, and therefore, are critical for correct brain function. Imbalances in AMPAR physiology have been correlated with multiple neurological syndromes (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Ashby et al., 2008).

1.4.2.1 Structure

Mammalian cells express four AMPAR subunits (GluA1-4) encoded by the GRIA1-4 genes, and averaging 850 to 900 amino acids (AAs). They are composed by an extracellular NTD, the LBD, four TMDs termed M1-4 forming the ion channel pore and an intracellular CTD (Figure 1).

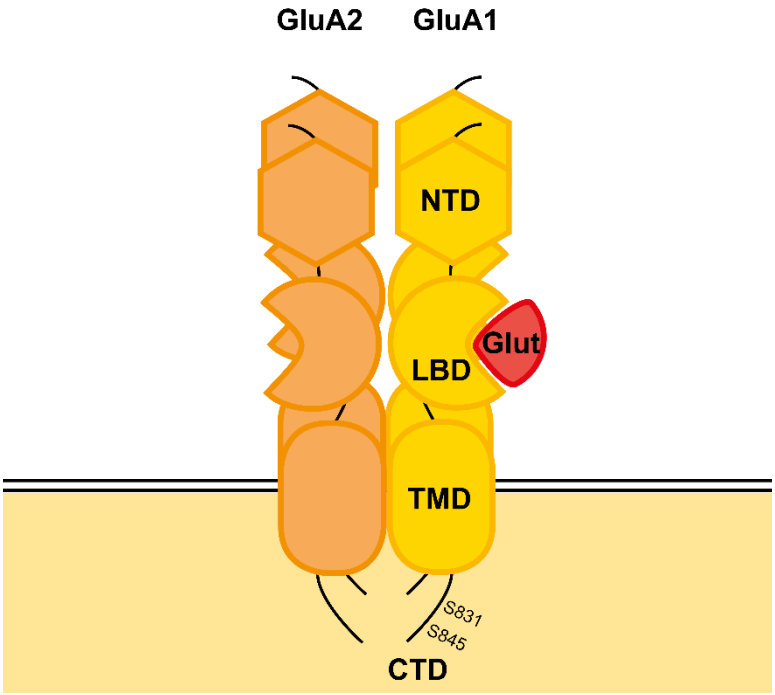

Figure 1 AMPAR structure. For details please refer to text. NTD: N-terminal domain; LBD: ligand-binding domain; TMD: trans-membrane domain; CTD: carboxy-terminal domain. Adapted from (Diering and Huganir, 2018)

The NTD participates in receptor assembly, modulation of channel kinetics, trans-synaptic interactions and spine morphology (Leuschner and Hoch, 1999; Passafaro et al., 2003), while the CTD presents the sites for PTMs and protein-protein interactions (Malinow and Malenka, 2002; Diering and Huganir, 2018). Mature functional AMPARs are tetramers whose specific subunit composition and function are highly dependent on the developmental stage, brain

region and cell type. AMPAR subunits can additionally undergo mRNA editing and alternative splicing, processes which further boost diversity as different subunit variants present distinct structural and functional properties. This is the case for the “flip” and “flop” isoform variants (Sommer et al., 1990), as well as for the different splicing variants of the C-terminal region of subunits GluA1, 2 and 4. The most relevant AMPAR mRNA editing takes place on GluA2 subunits, where the encoded glutamine at position 607 is edited to an arginine (Q/R editing) (Sommer et al., 1991). In the hippocampal region, the edited GluA2 subunit is the most abundant, with GluA3 and 4 expression being much more restricted (Tsuzuki et al., 2001). Neuronal cells typically present a majority of GluA1/2 and GluA2/3-containing receptors, with a small contribution of GluA1 homomers (Wenthold et al., 1996; Henley and Wilkinson, 2013).

1.4.2.2 Synthesis and trafficking

The synthesis, degradation and trafficking of AMPARs are processes tightly regulated by the subunit composition of the different tetramers, as well as by a myriad of protein interactors (e.g. molecular motors, chaperones, auxiliary AMPAR subunits and PSD scaffolding proteins). AMPARs are highly mobile molecules, undergoing constitutive and activity-dependent trafficking between the synaptic, extrasynaptic and intracellular pools present in neuronal cells in a way carefully regulated to cater to the needs of the cell. In hippocampal neurons, only about 25 to 40% of the total AMPAR population is present at the PM at any one time (Parkinson and Hanley, 2018), with exocytic and endocytic events being balanced during basal activity (Figure 2). This, however, is not the case during activity-mediated events, leading to an increase or decrease in the overall amount of synaptic AMPARs, and to synaptic plasticity (Moretto and Passafaro, 2018). The Rab family of small monomeric GTPases stands out as a central regulator of AMPAR trafficking. Briefly, Rab proteins bind different membranes, acting as molecular switches that ensure correct vesicle trafficking and cargo delivery from one compartment to another through multiple effector proteins (Sheperd and Huganir, 2007; Hausser and Schlett, 2017; Parkinson and Hanley, 2018).

AMPAR assembly can take place at either the somatic or dendritic ER, regulated by subunit specific interactions and editing of the Q/R site (Greger et al., 2002; Ayalon et al., 2005; Pick and Ziff, 2018). It is a multi-step mechanism, initiated by the interaction of two monomers through their NTDs (Ayalon and Stern-Bach, 2001) and followed by contact of their LBDs, which enables the union of two dimers to form a proto-tetramer. Lastly, tetramer stabilization occurs upon interaction between the TMDs. AMPAR assembly and ER exit via COPII vesicles is assisted by protein chaperones, Rab39B and specific AMPAR- and COPII protein-interactors such as cornichon proteins, stargazin and PICK1 (Tomita et al., 2003;

Vandenberghe et al., 2005; Budnik and Stephens, 2009; Brockie et al., 2013; Lu et al., 2014; Mignogna et al., 2015; Pick et al., 2017; Pick and Ziff, 2018). Upon ER exit, AMPARs traffic to the PM in a subunit-specific, Rab-8 mediated manner. On the one side, GluA2-containing AMPARs are accumulated at the ER and trafficked directly to the synaptic membrane during constitutive trafficking. On the other side, GluA1-containing receptors rapidly exit the ER and traffic to the PM in response to activity-mediated processes in a two-step process. This involves initial incorporation of AMPARs to the extrasynaptic membrane and subsequent lateral diffusion into the PSD via interaction with scaffolding proteins (Passafaro et al., 2001; Shi et al., 2001; Greger et al., 2002; Brown et al., 2007; Hausser and Schlett, 2017; Pick and Ziff, 2018).

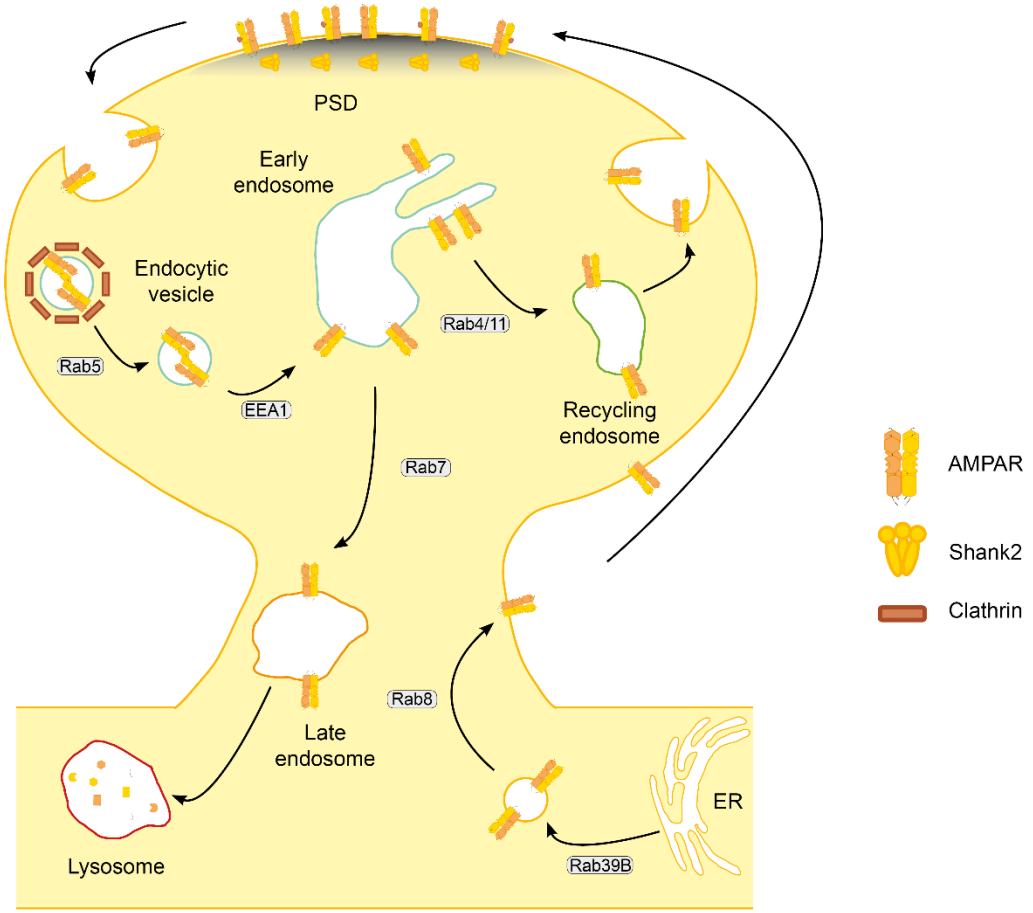

Figure 2 Basal trafficking of AMPARs, including some of their main regulators. For details please refer to text. ER: endoplasmic reticulum; PSD: post-synaptic density

The first prerequisite for AMPAR endocytosis is the release of the receptors from the PSD scaffolding proteins (e.g. GRIP1 and PSD95) and their lateral diffusion to the EZs (Blanpied et al., 2002; Bats et al., 2007). AMPAR endocytosis in response to activity is primarily

mediated by clathrin and dynamin, whereas it has been suggested that basal internalization occurs through a clathrin-independent, actin-mediated mechanism (Carroll et al., 1999; Man et al., 2000; Glebov et al., 2014; Parkinson and Hanley, 2018). Following internalization, endocytic vesicles are trafficked to early endosomes (EE). In clathrin-mediated endocytosis, two proteins stand out as key regulators: Rab5 and EEA1. While Rab5 regulates the uncoating of endocytic vesicles and directs them into EEs for fusion and cargo transfer (Brown et al., 2005; Szíber et al., 2017), the early endosome antigen 1 (EEA1) is a vesicle-tethering protein, which associates with phosphatidylinositol-3-phosphate at EE membranes and directs Rab5-mediated fusion of endocytic vesicles with early endosomes (Simonsen et al., 1998; Gaullier et al., 2000). Inside EEs, the acidic pH promotes ligand disassociation from the receptors, and AMPARs are primed to continue their trafficking through one of three different routes: a recycling path that delivers AMPARs back to the PM via recycling endosomes (RE), the degradation path that transports AMPARs to lysosomes via late endosomes (LE); and a path that returns AMPARs to the biosynthetic machinery for further fine-tuning via PTMs (van der Slujis and Hoogenraad, 2011; Scott et al., 2014; Hu et al., 2015; Parkinson and Hanley, 2018). Different signals promote AMPAR trafficking through these routes, with GluA2 typically determining basal and activity-mediated endocytosis and recycling, and GluA3 rather promoting trafficking through the degradative pathway (Shi et al., 2001; Lee et al., 2004). The degradative pathway, a route also promoted by AMPAR activation (Ehlers, 2000), involves receptor localization in intraluminal multivesicular bodies, from where receptors are subsequently trafficked into Rab7-positive LEs and lysosomes for their degradation. On the other hand, the GluA1 subunit typically mediates recycling of AMPARs in both basal and activity-mediated conditions following one of two different paths: the “short loop”, in which proteins are directly trafficked from the EEs back to the PM; and the “long loop”, in which proteins are returned to the membrane through the central pericentriolar endosomal system. Anterograde RE trafficking involves the microtubule motor KIF1C and the actin motor myosin V, in a process mediated by Rab4 and 11 (and potentially Rab8 and Rab35). Interestingly, retrograde trafficking of REs is mediated by myosin VI instead (Ullrich et al., 1996; Esteves da Silva et al., 2015; Gu et al., 2016; Hausser and Schlett, 2017; Moretto and Passafaro, 2018).

The numerous proteins that associate with AMPARs to regulate their synthesis, trafficking and degradation can be classified into two main groups: auxiliary proteins and interacting proteins. On the one hand, AMPAR auxiliary subunits encompass a group of proteins in which the transmembrane AMPAR regulatory proteins (TARPs) γ-2 (stargazin), γ-3, γ-4, γ-5, γ-7 and γ-8; and the cornichon-like proteins CNIH2 and 3 stand out (Tomita et al., 2003; Schwenk et al., 2009). The main roles of TARPs are to allow for AMPAR stabilisation at the PM and the PSD via their PDZ domain-containing C-termini (Nicoll et al., 2006; Bats et al., 2007; Sheng et al.,

2018) and to slow down AMPAR desensitization kinetics (Cho et al., 2007; Menuz et al., 2008). Similarly, cornichon proteins promote AMPAR surface targeting and slow down AMPAR decay kinetics (Boudkkazi et al., 2014). Interestingly, cornichon and TARP proteins have been described to form tripartite complexes with AMPARs, but also to compete for the same binding sites within AMPARs in a brain region- and tetramer-specific manner, thus allowing for differential regulation of AMPAR trafficking and kinetics in different neuronal cells. In hippocampal neurons specifically, binding of CNIH-2/-3 to GluA1 allows GluA1/2 heterotetramers to reach the PM by displacing TARP γ-8 (Kato et al., 2010; Gill et al., 2011; Herring et al., 2013; Diering and Huganir, 2018). On the other hand, AMPAR-interacting proteins include, among others, GRIP, PICK1, the N-ethylmaleimide-sensitive fusion protein (NSF) and the MAGUK family. GRIPs encompass the proteins GRIP1, GRIP2 and ABP (Dong et al., 1997; Dong et al., 1999), and mediate AMPAR trafficking in both basal and activity-mediated conditions chiefly through regulation of AMPAR insertion at the synaptic membrane, but also through regulation of its removal (Osten et al., 2000; Kim et al., 2001; Tan et al., 2015). This is achieved through the binding of GRIP1 to the motor protein kinesin, thus transporting AMPARs into the dendrites (Setou et al., 2002; Heisler et al., 2014). Interestingly, the role of GRIPs is complemented by PICK1, a protein regulating the endocytosis (and potentially the recycling) of AMPARs in both basal and activity-mediated conditions (Kim et al., 2001; Lin and Huganir, 2007; Sossa et al., 2007; Citri et al., 2010). This role is mediated by PKCα, a protein also binding to PICK1, and highly brain region- and stimulus-dependent. PKCα phosphorylates GluA2 on 880 serine, which decreases its affinity for GRIP1 but not for PICK1, thus leading to AMPAR endocytosis (Chung et al., 2000; Perez et al., 2001; Seidenman et al., 2003). Another protein interacting with GRIP and PICK to regulate the synaptic levels of AMPARs is NSF. Its main role is to act as a protein chaperone involved in the disassembly of SNARE complexes (Lin and Scheller, 2000), and it has been described as a key regulator of AMPARs (Song et al., 1998; Noel et al., 1999) (Nishimune et al. 1998; Osten et al. 1998; Song et al. 1998; Noel et al. 1999). NSF stabilises newly delivered AMPARs at the synapse and prevents their endocytosis by disassembling GluA2-PICK1 complexes, which in turn allows AMPAR binding to GRIP (Hanley et al., 2002; Hanley, 2007). Lastly, MAGUKs are proteins playing an essential role in the development and plasticity of synapses by acting as PSD scaffolds to which AMPARs can bind. Specifically, several members of the Discs Large Homologs subfamily have been found to associate with receptor complexes and regulate AMPAR trafficking and plasticity (Schwenk et al., 2012), with PSD95 being its prototypical member and acting as a regulator of spine density and morphology, playing a role in the maturation of glutamatergic synapses and in plasticity by means of stabilising AMPARs at specific nanodomains within the PSD where they can additionally bind to the TARP γ2 (Migaud et al., 1998; Schnell et al., 2002; Ehrlich et al., 2007; Nagura et al., 2012). Contrarily,

SAP102 is highly mobile and can be found throughout the whole spine, which suggests that it may be more implicated in the regulation of AMPAR trafficking to the synapse rather than in the stabilisation at the synapse itself (Zheng et al., 2010; Zheng et al., 2011). In the same line, SAP97 has been described to bind GluA1 in a specific manner and regulate its early trafficking to the dendritic membrane, as well as at the extrasynaptic and synaptic pools of AMPAR (Sans et al., 2001; Rumbaugh et al., 2003; Goodman et al., 2007). The regulation of these two pools is mediated by its two isoforms: SAP97 α and SAP97 β. While the former one increases the synaptic pools of AMPARs by means of increasing the size of the PSD, and consequently the number of slots at which AMPARs can bind; the latter directs AMPARs to the perisynaptic area, therefore reducing its synaptic levels. It is important to keep in mind that these proteins bind other AMPAR auxiliary proteins, interactors and scaffolding proteins on top of binding the AMPARs themselves, and it is through all of these interactions that they perform their actions. For a more in depth review of these and other proteins, please check (Bissen et al., 2019).

In addition to these proteins, PTMs are another major way to regulate trafficking among the different AMPAR pools. AMPARs may undergo modification of their C-terminal part through phosphorylation, palmitoylation, ubiquitination, S-nitrosylation and potentially O-GlyNAcylation (Diering and Huganir, 2018). Phosphorylation of GluA subunits has been identified as a key regulator of AMPAR trafficking, especially during activity-mediated plasticity, and will be described in detail later. Ubiquitination (the covalent addition of a 76-AA tag to certain lysine residues of a protein) can take place in all GluA subunits, and it has been described to promote AMPAR sorting through the degradative pathway (Widagdo et al., 2015; Diering and Huganir, 2018).

1.4.2.3 Physiology and pathology

Upon glutamate binding, the ion channel of AMPAR opens, allowing for Na^+ (and potentially Ca^{2+}) influx and K^+ efflux, promoting local membrane depolarisation. AMPAR subunit composition, presence of specific splice variants and RNA editing on the channel influence the kinetics and desensitization of the receptor. AMPAR channel kinetics are in general notably fast, while simultaneously presenting a small conductance and a comparatively low glutamate affinity. These characteristics can still be regulated by additional factors such as agonist concentration (Smith and Howe, 2003), specific protein-protein interactions or certain PTMs (Ashby et al., 2008; Diering and Huganir, 2018). Due to their Q/R editing GluA2 subunits are key determinants of the biophysical properties of the channel. Edited GluA2-containing channels are impermeable to Ca^{2+} and present linear current to voltage relationship, whereas

channels lacking this subunit show inward rectification and a considerably higher conductance (Ashby et al., 2008).

As the mediators of excitatory synaptic transmission, AMPARs are key for intercellular communication, brain development and adaptability, being the *de facto* main mediators of synaptic plasticity. On the flip side, and because of the paramount function fulfilled by AMPARs in the CNS, impairments in AMPAR physiology invariably lead to deficits in brain function and subsequently to neuropathological syndromes. Animal models of neurodegenerative diseases including HD, AD and PD have shown deficits in AMPAR trafficking and synaptic transmission, which in turn leads to memory and learning deficits and an impairment of motor function. In addition to this, deletion of the GRIA2 gene and a decrease in AMPAR synaptic density have also been observed in patients with Autism Spectrum Disorders (ASD) (Lee et al., 2016).

1.5 Learning, memory and synaptic plasticity

The ability to learn is a basic feature of animals. The gained knowledge is stored in the nervous system in the form of memories. However, not all memories are the same: they rely on different neural systems and have different attributes. They can be classified, for instance, into declarative (or explicit) and procedural (or implicit) memories (Graf and Schacter, 1985; Squire and Zola, 1996). On the one hand, declarative memories are those that can be consciously remembered (data such as events or facts), and they are known to rely on hippocampal activity. On the other hand, procedural memories store information about learned skills, and specially involve the cerebellum (Bisaz et al., 2014). Similarly, memories can be classified into STMs (those able to hold and recall information only for a short period of time) and LTMs (those able to store information for a prolonged period of time) (Bisaz et al., 2014).

1.5.1 Memory engrams: definition and physical substrate

While the involvement of the hippocampus in memory formation and early retention has been known for quite some time (Kandel et al., 2014), the neural substrate of the encoded memories and the specific mechanisms underlying memory encoding, storage, retrieval or deletion have been the focus of intense debate over the last decades. The term engram was coined by the biologist Richard Semon, and defines the physical entity that contains the stored information resulting from past experience, allowing organisms to reflect that knowledge in their behaviour (Josselyn et al., 2017). However, what is the physical substrate of engrams? It has been demonstrated that memories in the rodent brain are encoded by small and sparsely distributed

groups of neurons typically called memory trace cells (Silva et al., 2009; Josselyn, 2010; Liu et al., 2012). These cells can be identified by their elevated expression of immediate early genes (IEGs), such as cFOS and arc (Guzowski and Worley, 2001), form tightly connected groups, and their activation is necessary and sufficient for memory retrieval (Han et al., 2009; Liu et al., 2012). Despite this evidence, it is now known that the basic building block of memory is not at the neuronal level, but at the synaptic level. This idea was initially brought forth by Santiago Ramón y Cajal (Ramón y Cajal, 1894), and in 1949, Donald Hebb proposed that the associations that constitute a memory are stored by means of activity-dependent changes in the strength of synapses (Hebb, 1949). Nowadays there is considerable evidence, obtained from different species using multiple learning paradigms, that as a result of learning the synapses and dendritic spines of certain neurons undergo chemical and structural changes, which in turn result in either strengthening or weakening of synaptic transmission (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004; Hofer et al., 2009). Studies have proven that changes in the synaptic strength of these synapses alter the information stored by the engram (Whitlock et al., 2006; Nabavi et al., 2014; Mansvelder et al., 2019) and in a sophisticated study, the group of Haruo Kasai was able to show that specific ablation or reduction in size of spines potentiated during a learning process leads to the loss of that specific memory (Hayashi-Takagi et al., 2015). Thus, memory engrams can be defined as the set of alterations in the synaptic weights of an activated neuronal population that change upon the acquisition of information (Poo et al., 2016; Bliss et al., 2018).

1.5.2 Memory consolidation, retrieval and deletion

During memory formation, synaptic connections between the neurons and dendritic spines involved in the memory trace undergo a strengthening process, which will eventually fade unless synapses undergo the process of memory consolidation, the mechanism by which the recently strengthened synaptic connections transition from its initial weak and unstable state to its long-lasting state. This process can take place for a certain period of time after the memory is formed, during which it is still susceptible to modification through two main mechanisms: synaptic potentiation (further described below) and systems consolidation. The latter represents the process by which information initially encoded in the hippocampus is transferred to cortical areas. Interestingly, it takes place during the rest and sleep periods following learning, when 100 ms events called sharp-wave ripples occur in the hippocampus, replaying recent memory. This replay has been demonstrated to be crucial for the formation of stable memories (Wilson and McNaughton, 1994; Nádasdy et al., 1999; Kandel, 2001; Ryan et al., 2015; Lisman et al., 2018). Interestingly, work in amnesic mice proved that memory

consolidation is not necessary for memory storage due to information being encoded in the synaptic connections among memory trace cells, whereas accessibility to that memory was dependent on synaptic weight (Ryan et al., 2015; Tonegawa et al., 2015). Consequently, memory deletion takes place when the spines generated or strengthened during a learning event undergo shrinkage or ablation (Hayashi-Takagi et al., 2015; Poo et al., 2016).

1.5.3 Synaptic plasticity

The search for the mechanisms underlying memory formation, consolidation and retrieval has shown the importance of short- and long-lasting changes in the synaptic strength of the engram cell neuronal circuits (Poo et al., 2016). The term synaptic plasticity, introduced by the Polish neuropsychologist Jerzy Konorski (Konorski, 1948), refers to the process by which both excitatory and inhibitory synapses in the CNS undergo bidirectional changes in synaptic strength, affecting both the pre- and post-synaptic terminals, and is widely believed to be the main mediator of learning, memory encoding and consolidation (Malenka and Bear, 2004; Ho et al., 2011; Kaldun and Sprecher, 2019; Mansvelder et al., 2019). Additionally, it has been described to regulate other processes such as extinction of fear conditioning (Kim et al., 2007a) or the development of the visual cortex (Smith et al., 2009). Multiple pathologies have been associated with synaptic plasticity, such as AD (Rowan et al., 2003), multiple sclerosis (Nisticò et al., 2014), Fragile X syndrome (Lundbye et al., 2018) and psychiatric disorders such as depression or schizophrenia (Coyle and Tsai, 2004; Pittenger and Duman, 2008; Collingridge et al., 2010; Bliss and Collingridge, 2019).

Synaptic plasticity can take place either at a synaptic level, giving rise to the processes of Long-Term Potentiation (LTP) and Long-Term Depression (LTD) (also known as Hebbian plasticity); or at a network level, a process known as homeostatic scaling (Turrigiano, 2008; Huganir and Nicoll, 2013; Diering and Huganir, 2018). The common consensus is that changes in synaptic strength through LTP and LTD form the cellular basis of learning and memory, while homeostatic scaling provides bidirectional regulation of neuronal excitability with the objective of maintaining synaptic strength within a dynamic range (Diering and Huganir, 2018).

1.5.3.1 Long-Term Potentiation

LTP comprises a family of plasticity related phenomena that lead to long-lasting enhancement in signal transmission of excitatory synapses in an input-specific, co-operative and associative manner (Lynch, 2004; Lisman and Raghavachari, 2006; Lagali et al., 2010; Bliss et al., 2018).

Multiple LTP types can be distinguished according to the induction stimuli (tetanic-, pairing-, chemically-induced LTP or spike timing-dependent LTP) or to the transduction initiator [mGluRs, KARs or Ca^{2+}-permeable-AMPARs (CP-AMPARs) (Bashir et al., 1993; Bortolotto et al., 1999; Plant et al., 2006; Bliss and Collingridge, 2013; Park et al., 2016)]. NMDAR-dependent LTP is the best understood variant (Bliss et al., 2018; Diering and Huganir, 2018), presenting three main components:

-Short-Term Potentiation usually lasts for around 30 min, and its duration is shortened by activity. It has been suggested to be a largely presynaptic mechanism, mainly mediated by an increase in the probability of neurotransmitter release (Volianskis and Jensen, 2003; Bliss et al., 2018).

-Long-Term Potentiation (LTP), which can be subdivided into LTP1 (or independent of *de novo* protein synthesis) and LTP2 (dependent of *de novo* protein synthesis) (Bliss et al., 2018). LTP1 can last from one to several hours, whereas LTP2 is always long-lasting. Whether LTP takes place though LTP1 alone or a combination of LTP1 and 2 is dependent on the timing and strength of the triggering mechanism: LTP will not require protein synthesis as long as the process is triggered by a single episode of high frequency stimulation, or by several episodes delivered close in time. However, when the same stimuli are spaced in time, with several minutes in between episodes, a substantial component of the LTP response will require protein synthesis (Reymann and Frey, 2007; Bliss et al., 2018).

NMDAR-LTP is typically induced by high-frequency stimulation, which promotes postsynaptic depolarization via binding of glutamate to AMPARs. This, when added to a transient reduction in GABA-mediated inhibition, allows temporary removal of the NMDAR Mg^{2+} block (Mayer et al., 1984; Nowak et al., 1984; Herron et al., 1986), thus enabling the entry of Ca^{2+} into the postsynaptic spine (Alford et al., 1993; Bliss et al., 2018; Bliss and Collingridge, 2019). Consequently, multiple protein kinases are activated, depending on the developmental stage of the animal, specific synaptic pathway and LTP subtype, with the best studied ones being CaMKII, PKC and PKA (Malinow et al., 1989; Matthies and Reymann, 1993; Bliss et al., 2018).

Expression of NMDAR-LTP is mainly mediated by two mechanisms: an increase in the number of postsynaptic AMPARs (trafficked from the extrasynaptic and intracellular pools, Figure 3), and an increase in AMPAR conductance (Benke et al., 1998; Huganir and Nicoll, 2013; Bliss et al., 2018). Importantly, some AMPAR PTMs, mainly on the GluA1 subunit, are crucial for the changes in AMPAR trafficking and function during LTP, and they are regulated by the aforementioned kinases. PKC phosphorylates GluA1 on cysteine 811 (C811), which increases its interaction with the 4.1N cytoskeletal scaffold protein and thus enables GluA1-containing AMPAR activity-dependent exocytosis from intracellular endosomes, a prerequisite for LTP

maintenance (Lin et al., 2009). PKC, in combination with CaMKII, also phosphorylates GluA1 on serine 831 (S831), promoting an increase in single channel conductance and trafficking of GluA1-containing AMPARs to the PSD (Roche et al., 1996; Barria et al., 1997; Derkach et al., 1999; Ren et al., 2013). Lastly, PKA is known to phosphorylate GluA1 on S845 thus promoting an increase in single-channel open probability and in PM targeting and retention by enhancing GluA1 recycling and reducing internalization (Banke et al., 2000; Ehlers, 2000; Man et al., 2007; Diering and Huganir, 2018). Other mechanisms, such as IEG transcription, structural plasticity, regulation of presynaptic neurotransmitter release and monoamine neurotransmitters also mediate LTP expression (Bliss et al., 1983; Cole et al., 1989; Choi et al., 2003; Lisman and Raghavachari, 2006; Huganir and Nicoll, 2013; Bliss et al., 2018).

1.5.3.2 Long-Term Depression

LTD, in direct contrast to LTP, encompasses a group of mechanisms that promote a long-lasting decrease in synaptic strength. LTD can be categorized according to multiple criteria: it can be homosynaptic (when induced in the conditioned input) or heterosynaptic; it can be induced by various stimuli [prolonged low-frequency stimulation (LFS), pairing-, spike timing-dependent- or chemically-induced LTD (cLTD)] and both its transduction (NMDAR-LTD, mGluR-LTD or Gq-LTD) and its expression [LTD(A), LTD(N), LTD(K) and LTD(mGluR)] can be mediated by multiple receptors (Staubli and Lynch, 1990; Dudek and Bear, 1992; Park et al., 2006; Kim et al., 2007c; Collingridge et al., 2010; Bliss et al., 2018; Bliss and Collingridge, 2019). While all of these variants present different transduction mechanisms and functions, I will focus here on the two most prevalent forms of hippocampal LTD namely NMDAR-LTD(A) and mGluR-LTD(A) (Collingridge et al., 2010; Bliss et al., 2018; Bliss and Collingridge, 2019).

NMDAR-LTD(A) is most commonly induced by LFS and spike-time dependent plasticity, although NMDA addition can also promote cLTD, and is very dependent on protein synthesis for its long-term stability (Kim et al., 1996; Xu et al., 1997; Manahan-Vaughan et al., 2000; Kemp and Manahan-Vaughan, 2007; Collingridge et al., 2010). NMDAR activation leads to Ca^{2+} entry in the cell, activating calmodulin and subsequently calcineurin, which then promotes the release of Ca^{2+} from intracellular storage sites (Nishiyama et al., 2000). Multiple kinases and phosphatases have been implicated in NMDAR-LTD(A), such as PKA, p38 mitogen-activated kinase (p38MAPK), cyclin-dependent kinase, glycogen synthase kinase-3, and protein tyrosine kinases of the sarcoma family (Brandon et al., 1995; Zhu et al., 2002; Ohshima et al., 2005; Peineau et al., 2007; Collingridge et al., 2010). In order to promote AMPAR endocytosis, PSD95 is one of the main targets of these enzymes (Kim et al., 2007b), with the other one being AMPARs themselves. On the GluA1 subunit, protein phosphatase 1-mediated

dephosphorylation of S845 downstream of calcineurin is critical for LTD expression (Lee et al., 2003; Lee et al., 2010); and in contrast to its role during LTP, CaMKII phosphorylation of S567 constitutes another signal for AMPAR removal from synaptic sites (Lu et al., 2010; Coultrap et al., 2014; Diering and Huganir, 2018). PTMs on the GluA2 subunit also are important for LTD expression, with PKC phosphorylation of S880 affecting AMPAR membrane trafficking. This occurs through regulation of the protein-protein interactions between GluA2 and GRIP1/2 or PICK1 (Chung et al., 2000; Seidenman et al., 2003; Steinberg et al., 2006), two protein whose involvement in LTD transduction has been thoroughly explored (Rocca et al., 2008; Collingridge et al., 2010; Emond et al., 2010). The outcome of these signalling cascades affects both the pre- and postsynaptic terminals. On the one hand, the postsynaptic terminal becomes less sensitive to glutamate mainly due to removal of AMPARs from the PSD, but also as a result of changes in receptor conductance (Figure 3) (Collingridge et al., 2010). On the other hand, there is a reduction in the release of glutamate from the presynaptic terminal, which can be due to changes in the postsynaptic terminal communicated through a retrograde messenger or to changes directly on the presynaptic terminal (Stanton et al., 2003; Enoki et al., 2009). AMPAR removal from the PSD occurs through lateral diffusion to EZs, where endocytosis takes place in a clathrin- and dynamin- dependent manner mediated by the clathrin adaptor AP2 (Sheperd and Huganir, 2007; Collingridge et al., 2010; Diering and Huganir, 2018).

The second major form of hippocampal LTD, mGluR-LTD(A), is usually induced by paired pulse LFS or cLTD and is not as dependent on protein synthesis (Palmer et al., 1997; Huber et al., 2000; Massey and Bashir, 2007; Lüscher and Huber, 2010). Transduction via mGluR5/1 involves the hydrolysis of phosphatidylinositol to generate inositoltriphosphate and diacylglycerol (DAG), which in turn can activate PKC (Collingridge et al., 2010). In addition, other proteins such as PICK1, p38MAPK, the extracellular signal-regulated kinase (ERK) and several phosphatases are involved (Rush et al., 2002; Gallagher et al., 2004; Jo et al., 2008; Moult et al., 2008; Collingridge et al., 2010). PTMs also play an important role in this mechanism, as phosphorylation of tyrosine 876 (Y876) in GluA2 is required for Arf6-mediated AMPAR endocytosis (Moult et al., 2006; Gladding et al., 2009; Scholz et al., 2010). Despite the differences between the two processes, expression of mGluR-LTD is also mediated by pre- and postsynaptic changes involving alterations in the postsynaptic receptors and the glutamate release, in a way similar to that described for NMDAR-LTD (Feinmark et al., 2003; Enoki et al., 2009).

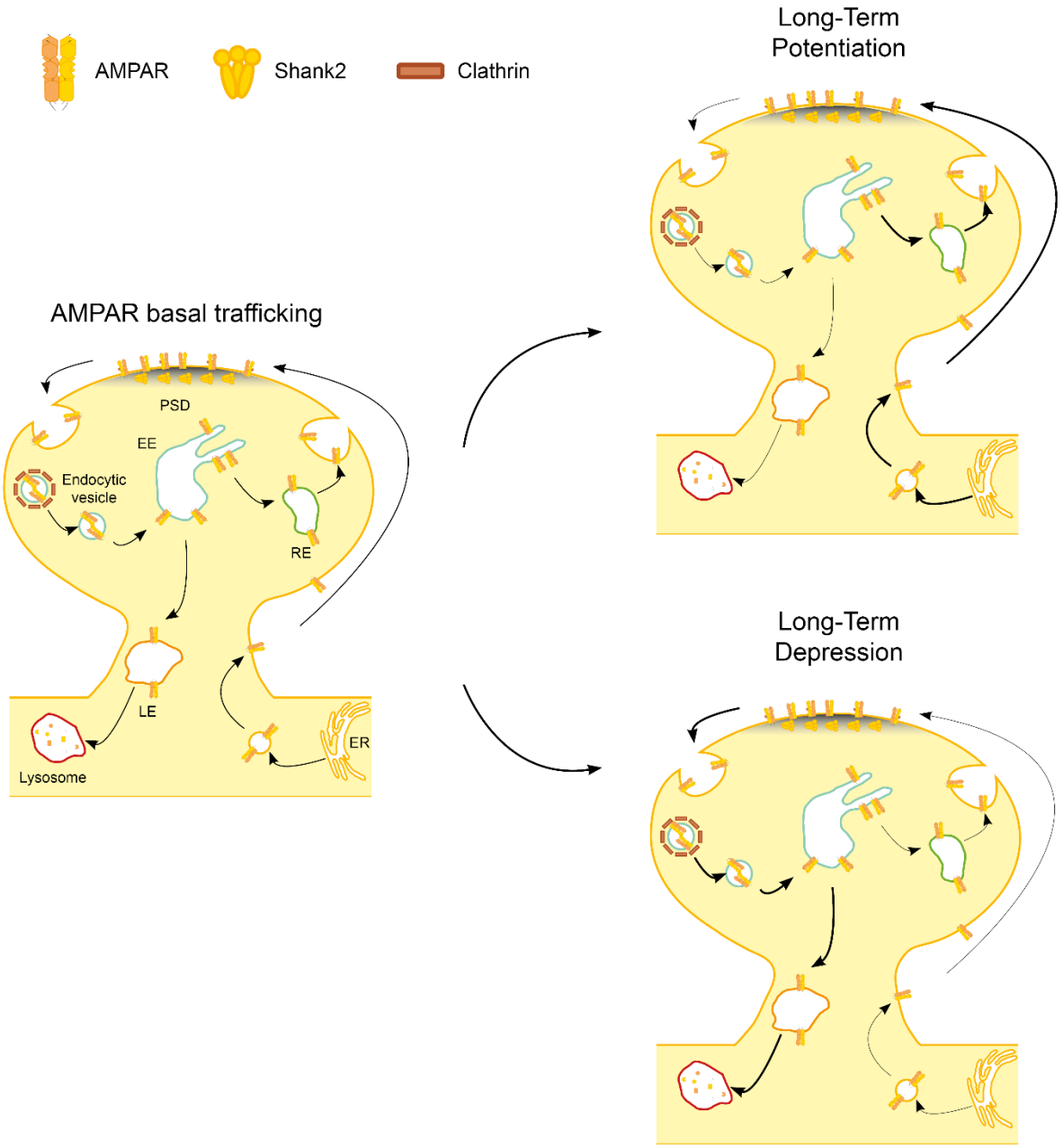

Figure 3 AMPA receptor trafficking during synaptic plasticity: AMPAR exo- and endocytic pathways under basal conditions and during LTP and LTD. For details please refer to text. PSD: post-synaptic density; EE: early endosome; RE: recycling endosomes; LE: late endosome; ER: endoplasmic reticulum.

1.5.3.3 Homeostatic plasticity

In order to counteract the destabilizing changes in neuronal excitability that take place as a consequence of learning and memory and maintain firing rate within a desired range, homeostatic plasticity encompasses a series of mechanisms that allow neurons to sense their activity and modify their properties and excitability accordingly in a graded and transcription-

dependent manner (Turrigiano, 1999; Davis and Bezprozvanny, 2001; Marder and Prinz, 2003; Turrigiano and Nelson, 2004; Turrigiano, 2008; Keck et al., 2013). To this end, neurons can regulate their excitation/inhibition balance (Goel et al., 2006; Goel and Lee, 2007; Maffei and Turrigiano, 2008; Keck et al., 2013), inward and outward voltage-dependent conductance balance ("intrinsic excitability") (Desai, 2003; Zhang and Linden, 2003; Marder and Goaillard, 2006), synapse numbers (Kirov et al., 2004) or the ease with which other forms of plasticity are induced (metaplasticity) (Abraham and Bear, 1996).

The best described form of homeostatic plasticity is synaptic scaling, a process by which all synapses of a neuron are balanced up or down (Turrigiano et al., 1998). It has been reported that chronic hyper- or hypoactivity induces, over a time period of hours, an uniform decrease or increase in the entire amplitude distribution of all miniature excitatory postsynaptic currents in a cell, effectively scaling synaptic strength up or down (Turrigiano et al., 1998; Sutton et al., 2006; Ibata et al., 2008). Various molecules, including BDNF (Rutherford et al., 1998), TNF-α (Stellwagen and Malenka, 2006; Kaneko et al., 2008), intracellular Ca^{2+} (Thiagarajan et al., 2005; Ibata et al., 2008), CaMKs (Thiagarajan et al., 2002; Ibata et al., 2008) and Arc (Rial Verde et al., 2006; Sheperd et al., 2006), take part in the multiple transduction mechanisms involved in synaptic scaling. These mechanisms can lead to regulation of neurotransmitter receptor accumulation, overall protein content of the postsynaptic terminal, number of release sites and probability of neurotransmitter release of the presynaptic terminal, with these mechanisms scaling excitatory and inhibitory synapses in opposite ways (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998; Watt et al., 2000; Murthy et al., 2001; Ehlers, 2003; Thiagarajan et al., 2005; Wierenga et al., 2005; Wierenga et al., 2006; Turrigiano, 2008). As a result, neurons adjust their synaptic strength in a slow, cumulative and dynamic fashion, allowing for the regulation of their firing rate without modifying the relative strength of their synaptic inputs. This allows them to preserve information relying on differences on synaptic weights, such as the ones encoded by LTP or LTD (O'Brien et al., 1998; Turrigiano et al., 1998; Ibata et al., 2008; Turrigiano, 2008).

1.5.3.4 Other mechanisms involved in synaptic plasticity

In addition to these three main mediators of synaptic plasticity, some other mechanisms involved in memory and learning regulation are shortly mentioned below:

-CREB, the cAMP-responsive element-binding protein, is a transcription factor primarily activated downstream of the adenyl kinase-cAMP-PKA axis upon LTP induction (Impey et al., 1996; Dudek and Fields, 2002; Sakamoto et al., 2011; Kandel, 2012; Zhai et al., 2013; Ma et al., 2014; Lisman et al., 2018; Kaldun and Sprecher, 2019). Due to the observation that

bidirectional modification of CREB function leads to a memory deficit or enhancement, the involvement of CREB in memory regulation was proposed. Interestingly, it was observed that overexpressing CREB in certain cells increases the chance of them being incorporated into the forming memory trace (Han et al., 2007; Yiu et al., 2014), probably through a mechanism involving changes in neuronal excitability (Lopez de Armentia et al., 2007; Zhou et al., 2009) thus pointing to CREB as a regulator of memory allocation.

-Structural plasticity encompasses the rearrangements taking place at the spine and dendritic levels as a result of their dynamic nature, a process that is involved in the induction and maintenance of learning and memory (Moser et al., 1997; Matus, 2000; Matsuzaki et al., 2004; Kozorovitskiy et al., 2005; Yasumatsu et al., 2008; Hill and Zito, 2013; Bernardinelli et al., 2014a; Bailey et al., 2015). These structural modifications consist of spine morphological changes (formation, growth, (de)stabilisation, shrinkage or pruning) that affect different cells or cell regions (postsynaptic spines, presynaptic terminals or astrocytic processes) and take place on a scale that ranges from minutes to days or years (Bernardinelli et al., 2014a). Some of them can be observed during LTP expression, such as an increase in the turnover of dendritic spines (Engert and Bonhoeffer, 1999), the selective stabilisation of activated synapses (De Roo et al., 2008) or the activation of silent synapses (Isaac et al., 1995; Bailey et al., 2015); while others mediate LTD, for instance, spine shrinkage and pruning (Wiegert and Oertner, 2013) or the structural changes taking place at the presynaptic terminals (Becker et al., 2008). It is very likely that these processes are regulated independently, with transduction pathways that are activated upon activity downstream of NMDARs, the main ones being a restructuring of the actin cytoskeleton within dendritic spines (Chen et al., 2007) and a shift in spine stability through a change in the expression profile of adhesion molecules (e.g. integrins or N-cadherin) (Bozdagi et al., 2010; McGeachie et al., 2011; Koleske, 2013) or through changes in the organization of the astrocytic processes around specific synapses (Bernardinelli et al., 2014b).

-Adult neurogenesis represents the addition of new, functional neurons derived from neural stem cells to the adult brain. It takes place in very specific brain regions, namely the SGZ of the DG and the subventricular zone of the lateral ventricle (Kuhn et al., 1996; Eriksson et al., 1998; Toda and Gage, 2018). Hippocampal adult neurogenesis has been involved in the regulation of multiple processes, such as pattern separation (Aimone et al., 2014), control of the emotional status (Snyder et al., 2011) and importantly, learning and memory. Considering that the hippocampus is strongly associated with learning and memory, it is not surprising that adult neurogenesis, a process continuously adding new neurons to the hippocampal circuitry (Spalding et al., 2013), plays a role in cognition. Nowadays, accumulating evidence has shed light on the details, showing that neurogenesis regulates hippocampal trace memories (Shors

et al., 2001), plasticity of learning strategies (Garthe et al., 2009), contextual fear memory (Saxe et al., 2006) and even associative fear memory relocation from the hippocampus to other brain regions (Kitamura et al., 2009), albeit with some inconsistencies among the numerous studies, probably due to the differences in methodologies and species used.

1.6 Protein Kinase D

The Protein Kinase D (PKD) family of serine/threonine kinases belongs to the CaMKII superfamily, and consists of three isoforms in mammals, PKD1-3, all of which are expressed in neuronal cells from early embryonic age (Oster et al., 2006; Ellwanger et al., 2008). The members of this family are DAG effectors and are activated downstream of multiple stimuli e.g. growth factors, hormones or neurotransmitters. They have been described to perform multiple roles in mammalian cells, ranging from regulation of vesicle fission at the trans-Golgi network (TGN) and intracellular trafficking to control of carcinogenesis through modulation of proliferation, epithelial-to-mesenchymal transition and apoptosis (Li and Wang, 2014).

1.6.1 Structure

PKD isoforms are evolutionarily conserved, with a high sequence homology. In spite of this, they also present a certain degree of structural and functional diversity (Ellwanger and Hausser, 2013). All members of the family contain a regulatory NTD comprising two cysteine-rich Zn finger-like domains called C1a and C1b, a region with a high number of negatively charged AAs, a pleckstrin homology (PH) domain and a C-terminal kinase domain. In addition to these “classical domains”, the existence of a highly conserved regulatory domain in the NTD, the so-called ubiquitin-like domain (ULD, Figure 4), mostly composed of hydrophobic residues, was recently described (Li and Wang, 2014; Aicart-Ramos et al., 2016; Adhiraj et al., 2017; Elsner et al., 2019).

Regulatory domains at the NTD of PKD are involved in the maintenance of the basal cytosolic autoinhibited state of the protein. In addition, the C1 domains anchor PKD to subcellular membranes upon binding to DAG or phorbol esters, being thus involved in the localization of PKD to compartments such as the TGN membrane, the PM and the mitochondria (Matthews et al., 2000; Liljedahl et al., 2001; Storz et al., 2005). PKD is further regulated by phosphorylation and protein interaction partners. While the newly discovered ULD domain mediates the dimerization of PKD subunits upon DAG binding via a hydrophobic surface, the PH region plays an autoregulatory role, in addition to controlling nuclear export and protein-

protein interactions with PKCη, PKCε or the trimeric G protein subunit Gβγ, for instance. Lastly, the CTD presents the kinase domain with the activation loop (S744/748 in murine PKD1) and, only in PKD1/2, the autophosphorylation site (S916 in murine PKD1) and the PDZ binding domain, which is capable of regulating protein-protein interactions with PKD interaction partners including NHERF-1 and Kidins220 (Jamora et al., 1999; Matthews et al., 1999; Waldron et al., 1999; Sánchez-Ruiloba et al., 2006; Kunkel et al., 2009; Adhiraj et al., 2017; Elsner et al., 2019). The existence of these regulatory domains suggests that PKD structure, subcellular location and catalytic functions are closely connected.

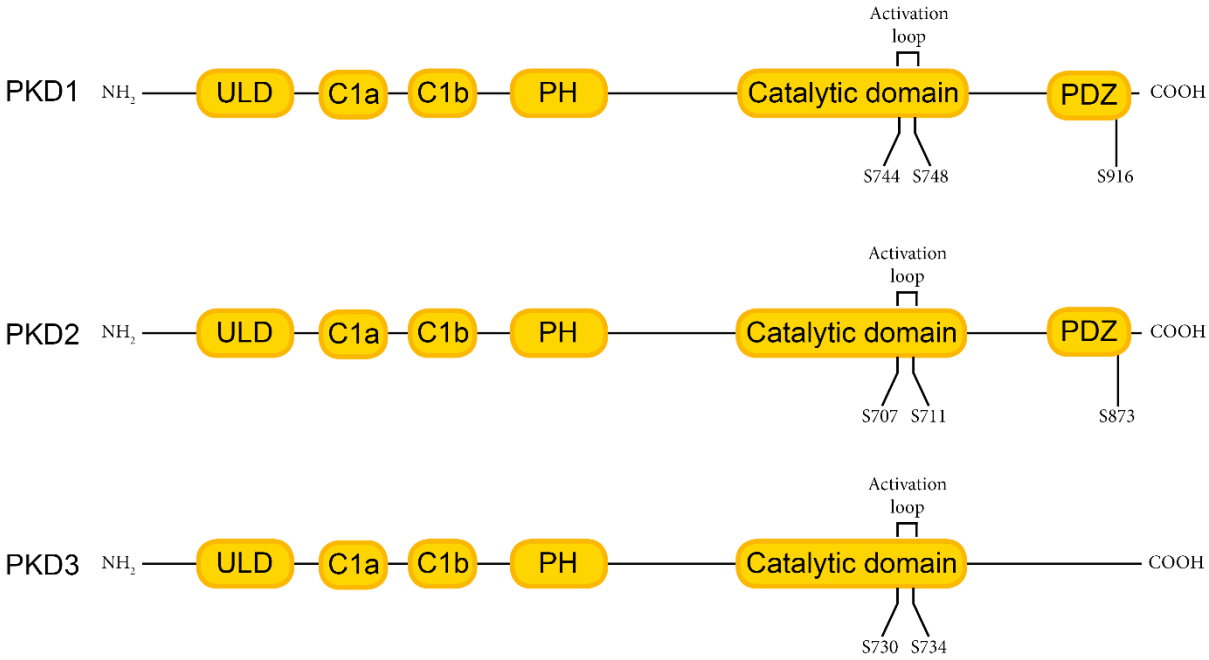

Figure 4 Structure of the PKD family of serine/threonine kinases. For details please refer to text. ULD: ubiquitin-like domain; PH: plekstrin homology domain; PDZ: postsynaptic density-95/discs large/zonula occludens-1 binding motif. Adapted from (Ellwanger and Hausser, 2013)

1.6.2 Activation mechanisms and localization

Cells on a resting state present a certain level of PKD activity, which can be upregulated through receptor tyrosine kinases (RTKs) or GPCRs by a myriad of physiological factors, among which are bioactive peptides, lipids, growth factors, the tumour necrosis factor, hormones, reactive oxygen species (ROS) and neurotransmitters (Adhiraj et al., 2017; Kolczynska et al., 2020; Reinhardt et al., 2020). The body of work generated about this topic over the last two decades has proposed two main activation pathways. According to the classical hypothesis, initiation of transduction cascades by GPCRs or RTKs mediates PLC activation, which in turn hydrolyses the phospholipid phosphatidylinositol 4,5-biphosphate

giving rise to DAG and inositol 1,4,5-triphosphate. DAG induces the recruitment and activation of PKC and PKD, and additionally, PKD is phosphorylated at the activation loop serines by the novel PKC members PKCε or PKCη (Matthews et al., 2000; Vertommen et al., 2000; Hausser et al., 2002; Cowell et al., 2009; Olayioye and Hausser, 2012). On the contrary, the model proposed by the laboratory of Thomas Leonard postulates that the inhibited, cytosolic and monomeric pool of PKD is recruited to membranes upon DAG production. This relieves their autoinhibition and promotes dimerization through their ULD domains due to the increase in the local concentration of PKD. Subsequently, their kinase domains dimerize as well, ultimately leading to trans-autophosphorylation of their activation loops, followed by dissociation of the monomers and further transduction of the signal through their downstream substrates (Aicart-Ramos et al., 2016; Elsner et al., 2019; Reinhardt et al., 2020). An intriguing idea brought forth by this second model is that PKD phosphorylation by upstream kinases (namely PKC) may not be necessary, a hypothesis that has yet to be clarified.

The main location of PKD in resting cells is the cytoplasm, with a secondary pool residing at the TGN. PKD transport to other subcellular locations depends on the activated transduction pathway and DAG membrane availability, as well as on the specific interactions established with different lipids and proteins, a process also involving its regulatory domains. The C1a domain is important for Golgi localization, while C1b is involved in PKD translocation to the plasma or mitochondrial membrane and nuclear import. Finally, the PH domain regulates its nuclear export (Maeda et al., 2001; Rey et al., 2001; Hausser et al., 2002; Storz et al., 2005). If, in response to the aforementioned stimuli, there is an increase in DAG concentration at the plasma or mitochondrial membranes, PKD traffics from the cytosol to these compartments, then leaves the membrane again and is able to enter the nucleus (Matthews et al., 2000; Cowell et al., 2009; Li and Wang, 2014; Aicart-Ramos et al., 2016; Kolczynska et al., 2020).

1.6.3 PKD functions

Upon activation, PKD recognizes the consensus sequence LXRXXS/T (where X represents any AA), and phosphorylates its downstream substrates. Due to its presence in numerous subcellular locations, PKD acts as a regulatory element of many cellular functions: induction of cell proliferation (Sinnett-Smith et al., 2009), cell motility and migration suppression through the modulation of multiple actin cytoskeleton regulators (Jaggi et al., 2005; Eiseler et al., 2009; Peterburs et al., 2009; Du et al., 2010; Eiseler et al., 2010; Ziegler et al., 2011; Ellwanger and Hausser, 2013; Olayioye et al., 2013), pro-survival signalling induced by oxidative stress (Storz et al., 2005; Cowell et al., 2009), angiogenesis (Wong and Jin, 2005; Hao et al., 2009) proliferation and signal transduction in T-cells (Navarro et al., 2014) or regulation of

transcription factors such as MEF2 and RUNX2 (Jensen et al., 2009; Ellwanger and Hausser, 2013; Aicart-Ramos et al., 2016; Adhiraj et al., 2017; Xu and Pozzo-Miller, 2017). Many of these processes are hallmarks of tumour progression, and thus it is not surprising that PKD has been classically described as a regulator of tumour progression. However, the role of PKD in oncogenic processes is not clear-cut, as depending on the tissue and the isoform under study, PKD has been described either as a tumour suppressor or as a neoplastic driver (Adhiraj et al., 2017).

A key role of PKD is the regulation of membrane trafficking. Here, PKD has been shown to control constitutive vesicle fission from the TGN and to modulate endocytic trafficking. Upon DAG generation at the TGN membrane, PKD1/2 have been observed to promote the production of phosphatidylinositol 4-phosphate by phosphorylation of the lipid kinase phosphatidylinositol 4-kinase IIIβ at S294. This stimulates recruitment of lipid transfer proteins such as the ceramide transport protein CERT, a promoter of ceramide transfer from the ER to the TGN, and the oxysterol-binding protein 1, involved in the synthesis of sphingomyelin (SM); which in turn leads to the generation of SM and DAG from ceramide and phosphatidylcholine. Another key regulator of vesicle fission is Arfaptin-1, a BAR (Bin/Amphiphysin/Rvs)-domain-containing protein which can bind PI4P-containing liposomes and TGN membranes and shield the necks of forming vesicles from being severed through the inhibition of the ADP ribosylation factor (a component of the vesicle scission machinery). Importantly, PKD-mediated phosphorylation of Arfaptin-1 at S132 prevents this inhibition, thus enabling vesicle fission. In line with this, impaired interaction of PKD and Arfaptin-1 leads to secretion block, as shown by the loss of glucose-stimulated insulin secretion in pancreatic β cells expressing nonphosphorylatable Arfaptin-1 (Liljedahl et al., 2001; Hausser et al., 2005; Hanada et al., 2009; Malhotra and Campelo, 2011; Ellwanger and Hausser, 2013). Lastly, in breast cancer cells PKD has been described to regulate endocytic trafficking through phosphorylation of its effector protein Rabaptin-5 on S407. Rabaptin-5 is a scaffold protein with an amino terminal Rab4-binding domain and a carboxy terminal Rab5-binding domain (Stenmark et al., 1995; Vitale et al., 1998). In addition, Rabaptin-5 can bind Rabex-5, a Rab5 guanosine nucleotide exchange factor (Rab5GEF) (Horiuchi et al., 1997; Zhang et al., 2014). So far, PKD has been described to promote recycling of vesicle cargo back to the membrane through the fast recycling loop via interaction with Rab4 (Woods et al., 2004; Christoforides et al., 2012), but the interaction of Rabaptin-5 and Rab5 suggests that PKD could also play a role in endocytosis regulation.

1.6.4 The role of PKD in neuronal cells

In neuronal cells, PKD is expressed since the early stages of embryonic development, albeit with variations among the different isoforms: PKD1 mRNA expression could be observed from day E8.5 in most brain regions, while PKD2 was detected from day E14.5 onwards and PKD3 briefly between E9.5-10.5 in the mid- and forebrain before being widely expressed from day E14.5 on (Oster et al., 2006). Expression of the PKD3 protein was also detected in the embryonic brain from day E12.5 on (Ellwanger et al., 2008). In line with its activation in other cell types, PKD translocates to the PM upon phorbol ester stimulation, concretely to lipidic rafts, where it phosphorylates its neuronal specific substrate Kiddins220 by a mechanism involving PKCε and the small tyrosine kinase Src (Cabrera-Poch et al., 2004).

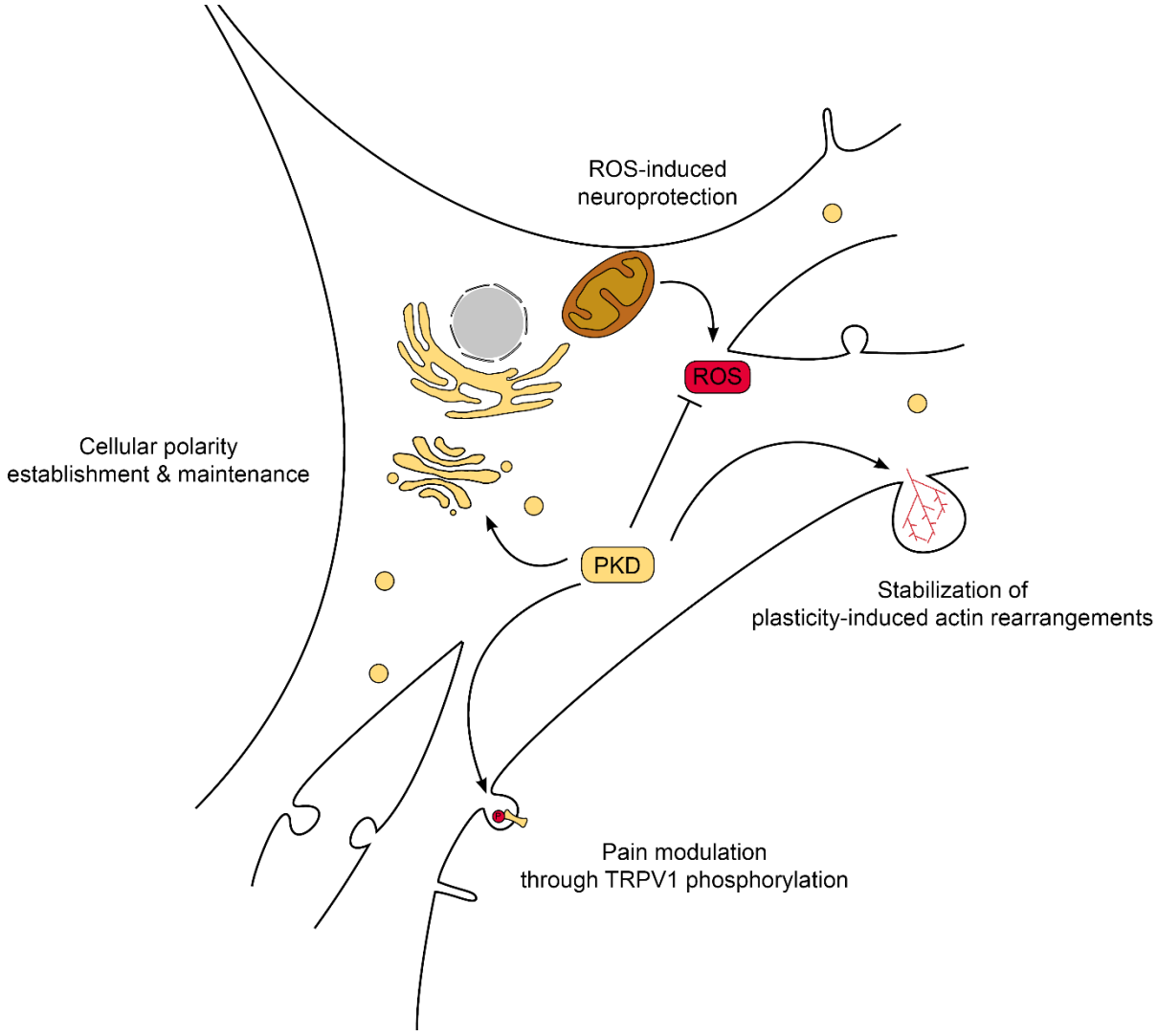

Figure 5 PKD functions in neuronal cells. For details please refer to text. ROS: reactive oxygen species.

Multiple publications describe PKD as a regulator of key neuronal processes. For instance, in rat hippocampal neuronal cells nuclear PKD was reported to phosphorylate histone deacetylase 5 (HDAC5) at two critical sites for its nuclear export (S259 and S498) in response to treatment with ketamine, a non-competitive NMDAR antagonist, or with oleanolic acid or its derivates, a naturally occurring triterpenoid compound typically found in food and plants. This promoted the nuclear export of HDAC5, thus releasing the transcriptional activity of MEF2 and the upregulation of its downstream targets, including *Bdnf*, *Klf6* and *Nr4a1* (Choi et al., 2015; Volmar and Wahlestedt, 2015; Jo et al., 2017).

Moreover, PKD acts as a promoter of neuroprotection during the early stages of oxidative stress and cerebral ischemia through the phosphorylation of the Heat Shock Protein 27 (HSP27), which in turn inhibits the Apoptosis Signal-regulating Kinase 1 (ASK1). Consequently, downregulation of PKD activity via knockdown or treatment with its specific inhibitor CID755673 abrogated HSP27 binding to ASK1 and its neuroprotective effects (Stetler RA, 2012). Similarly, in a cellular model of PD dopaminergic neuron degeneration, cell exposure to hydrogen peroxide or to the parkinsonian specific toxicant 6-OHDA induced phosphorylation of PKD on its activation loop through a mechanism involving the catalytic fragment of PKCδ, which promoted a decrease in oxidative stress-mediated apoptosis. In line with this, inhibition of PKD activity via RNAi knockdown or overexpression of the phosphodeficient mutant PKD S916A led to an increase in apoptosis, and conversely, upregulation of PKD activity via overexpression of the full length protein or of a constitutively active mutant decreased apoptosis in response to oxidative stress (Asaithambi et al., 2011; Asaithambi et al., 2014). Curiously, and in contrast to non-neuronal cells (Storz et al., 2005), this role of PKD is not mediated through NFκB, as their nuclear translocation in response to PKD activation could not be observed. Instead Liliom *et al* suggested that this neuroprotective role could be linked to a mitochondrial role performed by PKD, as the authors reported on the presence of PKD at the mitochondrial membrane (Liliom et al., 2017).

PKD was also described as a regulator of pain modulation in a model of inflammatory pain using dorsal root ganglia (DRG) cells. Both the Transient Receptor Potential V1 (TRPV1) and PKD were observed to translocate to the PM following treatment with Complete Freund's Adjuvant (CFA), where they interact after PKD transphosphorylation (as proven via co-immunoprecipitation). Moreover, upregulation of PKD activity via PKD1 overexpression was described to increase the amount of TRPV1's and enhance heat hypersensitivity in DRG cells, whereas PKD downregulation through intrathecal injection of PKD1 antisense or overexpression of kinase-dead PKD1 (kdPKD1) alleviated CFA-induced thermal hypersensitivity and decreased TRPV1's amount in DRG cells (Zhu et al., 2008).

Several studies, in line with its role in non-neuronal cells, have proposed PKD as a key regulator of the Golgi apparatus and secretory trafficking in neuronal cells. More specifically, Czöndör *et al* demonstrated that PKD is involved in the maintenance of dendritic arborization and Golgi structure in embryonic hippocampal neuronal cells. Using a reporter of PKD activity, the kinase was described to be basally active at the neuronal somatodendritic compartment, and specifically at the TGN. Furthermore, overexpression of EGFP-tagged wtPKD1 and constitutively-active PKD1 (caPKD1) promoted an increase in the complexity of the neuronal dendritic tree without interfering with the TGN morphology, in contrast to the Golgi fragmentation and the reduction in the complexity of the neuronal dendritic tree caused by overexpression of kdPKD, an observation also made in mice with neuron-specific, inducible expression of kdPKD1 (Czöndör et al., 2009). Similarly, PKD has been presented as a promoter of early dendritic development and neuronal polarity through the regulation of TGN-derived vesicle directionality, based on the observations that kdPKD expression led to dendritic growth inhibition in hippocampal pyramidal neurons (Horton et al., 2005); and that downregulation of PKD activity using the specific inhibitor Gö6976 led to symmetric post-Golgi membrane trafficking, and thus to the equal growth of all dendrites (Yin et al., 2008). In contrast to these studies, the Cáceres group reported that while PKD1 is not crucially involved in the maintenance of Golgi morphology or the exit of either axonal- or dendritic-bound vesicles from the TGN, it modulates the correct packaging of dendritic membrane proteins at the Golgi apparatus. This is based on their observations after RNAi- based PKD knockdown or expression of kdPKD, which led to axonal expression of the typically dendritic transferrin receptor (TfR) and low density receptor-related protein (LRP) but did not cause Golgi tubulation (Bisbal et al., 2008). Thus, while a role of PKD in the neuronal Golgi complex and in the regulation of the TGN-derived vesicles is apparent, further studies are required to fully understand the details of its regulation.

Lastly, a few studies support a role for PKD as a regulator of synaptic plasticity. Initially, Krueger *et al* reported that stimulation of mGluR5 in hippocampal neuronal cultures and hippocampal slices using its specific DHPG agonist results in the phosphorylation of PKD on 916 serine in a mechanism dependent on PLC and PKC (Krueger et al., 2010). Subsequently, this novel role of PKD was further described by the Yu group, which observed that PKD activation downstream of DHPG promotes the phosphorylation of the surface expressed NMDARs on their C-termini, thus downregulating their activity. Moreover, the block of PKD activity via knockdown or infusion of the PKD inhibitor CID755673 into the bilateral hippocampal CA1 prevented the decrease in the NMDAR-mediated miniature excitatory post-synaptic currents while leaving those mediated by AMPARs unaffected (Fang et al., 2015; Wang et al., 2018). These studies thus present PKD as a regulator of mGluR-LTD. Moreover,

PKD could be involved in the downregulation of synaptic transmission through its downstream substrate Ras and Rab interactor 1 (RIN1), a Ras effector protein highly expressed in mature neurons which acts as a negative regulator of memory stabilisation during amygdala-related fear learning and experience-mediated fear extinction through the destabilisation of synaptic connections and the regulation of AMPAR and EphA4 endocytosis (Dhaka et al., 2003; Deininger et al., 2008; Bliss et al., 2010; Szíber et al., 2017). RIN1 has the ability to activate Abl kinases, which in turn control actin cytoskeletal remodelling (a key mediator of the morphological changes that occur at synapses during synaptic plasticity) (Hu et al., 2005). Importantly, this function of RIN1 is dependent on its phosphorylation by PKD at S292, an observation that suggests that PKD could regulate the activity-mediated changes that the actin cytoskeleton undergoes through the phosphorylation of RIN1 (Ziegler et al., 2011; Szíber et al., 2017). Intriguingly, PKD was described to be important not only for LTD expression, but also for the morphological changes that occur at dendritic spines during LTP. Impaired PKD activity via overexpression of kdPKD or via treatment with the specific PKD inhibitor kbNB 142-70 attenuates such changes and has deleterious effects on spatial memory formation. These observations can be attributed to the PKD-mediated modulation of the actin-binding protein cofilin through its downstream substrate slingshot 1 (SSH1). Cofilin has been described as a central regulator of actin filament turnover through its ability to sever F-actin filaments and generate free barbed ends. Interestingly, cofilin activity depends on its phosphorylation state: the LIM kinase family and the related testicular protein promote phosphorylation of cofilin at S3, leading to its inactivation, whereas the phosphatases chronophin and SSH1 dephosphorylate cofilin at S3, consequently activating it. Additionally, SSH1 dephosphorylates and subsequently inactivates the LIM kinases 1 and 2, hence further promoting cofilin activation. PKD, in turn, mediates the inactivation of SSH1 through its phosphorylation at S937 and S938, which promotes SSH1 sequestering in the cytoplasm through binding to 14-3-3 proteins, and at S402, thus preventing SSH1 from binding and dephosphorylating cofilin and ultimately leading to cofilin inactivation and stabilisation of the enlarged dendritic spines following LTP (Olayioye et al., 2013; Bencsik et al., 2015).

In spite of the role PKD plays as a regulator of endocytic trafficking in both neuronal and non-neuronal cells and of synaptic plasticity, it has not yet been described to be involved in AMPAR trafficking, the main mechanism behind learning, memory and synaptic plasticity.

1.7 Aims of the thesis

The AMPAR family of iGluRs mediates the majority of excitatory neurotransmission in the mammalian brain by means of allowing ion trafficking and promoting local depolarization, critical functions for normal CNS physiology (Ashby et al., 2008). Several intracellular pools of AMPARs can be found in neuronal cells, namely, the synaptic, extrasynaptic and endosomal populations. To allow for regulation of the strength of synaptic transmission, it is crucial that these different pools are continuously interconnected through endo- and exocytic processes, which in turn must be regulated in a dynamic manner (Ehlers, 2000; Henley and Wilkinson, 2013). Despite the body of work gathered during the last decades, the molecular mechanisms underlying these trafficking processes and their regulation are not fully understood yet.

The PKD family of serine/threonine kinases constitutes an important regulatory hub for many key cell processes, such as proliferation, cell motility and migration, transcription factor regulation and, importantly, regulation of vesicle fission from the TGN and endocytic trafficking (Ellwanger and Hausser, 2013; Adhiraj et al., 2017). In neuronal cells, all PKD isoforms are expressed from an early embryonic age, and they have also been described to regulate key specific neuronal processes, i.e., regulation of the establishment and maintenance of neuronal polarity or neuroprotection against oxidative damage (Czöndör et al., 2009; Asaithambi et al., 2011). Thrillingly, a new role of PKD in the regulation of learning and memory was previously described (Bencsik et al., 2015). In their work, the authors described the involvement of PKD in activity-induced plasticity within dendritic spines by stabilizing the newly formed actin filaments upon dendritic spine growth. Strikingly, a role of PKD in the regulation of AMPAR trafficking, the main mediator of synaptic plasticity, learning and memory, has not been described yet.

In order to elucidate whether PKD could also regulate synaptic plasticity by acting as a regulator of AMPAR trafficking, my work focused on addressing three main points:

-First, I characterized PKD as a basal regulator of AMPAR endocytosis in neuronal cells through the use of biochemical and immunofluorescence techniques with primary hippocampal neurons.

-Subsequently, I further described PKD as a regulator of activity-mediated AMPAR endocytosis on hippocampal neurons, employing for this purpose biotinylation of surface proteins upon agonist- or cLTD-mediated receptor endocytosis.

-Lastly, I shed light on the molecular mechanism by analysing the effects of perturbed PKD signalling on Rab5 activity and AMPAR surface expression.

2. Material and methods

2.1 Materials

2.1.1 Animal strains

With the aim of obtaining embryonic primary hippocampal neurons for this work, the CD1 mouse strain was selected due to their multipurpose suitability. The mice were obtained from the Charles Rivers company and kept in the animal facility of the Institute of Cell Biology and Immunology (IZI) of the University of Stuttgart, or of the Biological Institute of the Eötvös Loránd University, at 22 ± 1°C with 12 h light and dark cycles and *ad libitum* access to water and food. The animals were maintained and handled in accordance with the Guidelines for Accommodation and Care of Animals, according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

2.1.2 HEK293T cell line

While most of the experiments in this study were performed on primary hippocampal embryonic neurons, HEK293T cells were also employed. Derived from a stable clone of the human embryonic kidney 293 cell line, these cells were selected because of their suitability for cell culture, transient transfection and protein production.

2.1.3 Bacterial strains

Competent *Escherichia coli* (*E. coli*) bacteria of the DH5α strain were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

2.1.4 Media, supplements and consumables for cell culturing

Table 1. Table of media, supplements and consumables for cell culturing

Medium / supplement / consumable	Supplier / composition
Amphotericin B	Thermo Fisher Scientific, Waltham, MA, USA
B27™ supplement (50X), serum-free	Thermo Fisher Scientific, Waltham, MA, USA
Brainphys™ neuronal medium	Stemcell Technologies SARL, Grenoble, France
Corning® non-treated culture dishes (35/10 mm)	Merck, Darmstadt, Germany

Medium / supplement / consumable	Supplier / composition
Greiner CELLSTAR® 6-well culture plate	Greiner Bio-One GmbH, Kremsmünster, Austria
Greiner CELLSTAR® 24-well culture plate	Greiner Bio-One GmbH, Kremsmünster, Austria
CELLview™ dish with glass bottom	Greiner Bio-One GmbH, Kremsmünster, Austria
Cytosin-arabinofuranoside	Thermo Fisher Scientific, Waltham, MA, USA
Deoxyribonuclease I (DNAse I) from bovine pancreas	Merck, Darmstadt, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	Merck, Darmstadt, Germany
Foetal Bovine Serum Brazil One (FBS)	Pan Biotech, Aidenbach, Germany
Filtering sterile mesh	EmTek, Budapest, Hungary
Glass round coverslips Ø12 mm	Carl Roth, Karlsruhe, Germany
Gentamycin	Merck, Darmstadt, Germany
GlutaMAX™ (100x)	Merck, Darmstadt, Germany
Laminin	Merck, Darmstadt, Germany
NeuroBasal™ medium	Thermo Fisher Scientific, Waltham, MA, USA
NeuroCult™ SM1 Neuronal Supplement	Stemcell Technologies SARL, Grenoble, France
Poly-L-lysine-hydrobromide mol. weight ≥ 300000	Merck, Darmstadt, Germany
RPMI 1640 medium	Thermo Fisher Scientific, Waltham, MA, USA
Supplemented NeuroBasal™ medium (supplemented neurobasal)	NeuroBasal™ medium, 5% FBS, B27™ supplement, 0.5 M GlutaMAX™, 2.5 µg/ml amphotericin B and 40 µg/ml gentamycin
Supplemented Brainphys™ neuronal medium (supplemented brainphys)	Brainphys™ neuronal medium supplemented with SM1, 2.5 µg/ml amphotericin B and 40 µg/ml gentamycin
Trypsin-EDTA (0.5%)	Thermo Fisher Scientific, Waltham, MA, USA

2.1.5 Solutions and buffers

Table 2. Table of buffers and solutions

Cell lysis, SDS-PAGE and Western Blotting	
Buffer / solution	**Composition / supplier**
DPBS, calcium, magnesium (PBSCM)	Thermo Fisher Scientific, Waltham, MA, USA
Glutathione cleavage buffer	50 µM L-Glutathione, 75 µM NaCl, 10 mM EDTA, 1% BSA (v/v) in ddH_2O, pH 8.6
HEK293T homogenization lysis buffer	300 mM NaCl, 10 mM $MgCl_2$, 20mM HEPES, 10% glycerol (v/v), 1mM DTT, 0.1% NP-40 (v/v) in ddH_2O
HEK293T homogenization washing buffer	300 mM NaCl, 10 mM $MgCl_2$, 20mM HEPES, 10% glycerol (v/v), 1mM DTT in ddH_2O
Laemmli protein loading buffer (5x)	312.5 mM Tris pH 6.8, 25% β-mercaptoethanol (v/v), 25% glycerine (v/v), 10% SDS (w/v) and 0.05% (w/v) bromophenol blue in ddH_2O

Buffer / solution	Composition / Supplier
Membrane blocking solution	0.5% Western blocking reagent (v/v, Roche), 0.05% Tween 20 (v/v) and 0.1% NaN_3 (v/v) in PBS
NuPAGE™ Antioxidant	ThermoFisher Scientific, Waltham, MA, USA
NuPAGE™ MES SDS Running Buffer (20x)	ThermoFisher Scientific, Waltham, MA, USA
PBS-Tween-20	0.05% (v/v) Tween-20 in PBS
Phosphate Buffered Saline (PBS)	140 mM NaCl, 2.7 mM KCl, 8mM Na_2HPO_4, 1.5 mM KH_2PO_4 in ddH_2O, pH 7.4
Ponceau S solution	0.1% (w/v) Ponceau S in 5% (w/v) acetic acid
TDN Buffer	50 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT in ddH_2O
TED Buffer	50 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT in ddH_2O
TEDN buffer	50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT in ddH_2O
Triton X-100 lysis buffer	1% Triton X-100 (v/v), 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA in ddH_2O
Immunofluorescence	
Buffer / solution	**Composition / Supplier**
Blocking buffer	5% FBS (v/v) + 0.1% NaN_3 (v/v) in PBS
Live Cell Imaging buffer (LCI)	142 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM HEPES in ddH_2O, pH 7.4
Paraformaldehyde fixing solution	4% paraformaldehyde (v/v) in PBS
Permeabilization buffer	0.1% Triton X-100 (v/v) + 0.1% NaN_3 (v/v) in PBS
Other buffers	
Buffer / solution	**Composition / Supplier**
Lysogeny broth medium (LB)	10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl in ddH_2O
TCM Buffer	10 mM Tris pH 8.0, 10 mM $CaCl_2$, 10 mM $MgCl_2$ in ddH_2O

2.1.6 General chemicals, reagents and consumables

Table 3. Table of general chemicals, reagents and consumables

Chemical / reagent / consumable	Supplier
Acetic acid ($C_2H_4O_2$)	Carl Roth, Karlsruhe, Germany
Ampicillin	Thermo Fisher Scientific, Waltham, MA, USA
β-Mercaptoethanol	Merck, Darmstadt, Germany
Bromophenol blue Na-salt	Serva, Heidelberg, Germany
Bovine Serum Albumin (BSA)	Thermo Fisher Scientific, Waltham, MA, USA
cOmplete™ Protease Inhibitor Cocktail Tablets	Roche, Rotkreuz, Switzerland
Calcium chloride ($CaCl_2$)	Merck, Darmstadt, Germany
CRT 0066101	Tocris Bioscience, Bristol, United Kingdom
D-APV	Tocris Bioscience, Bristol, United Kingdom

Chemical / reagent / consumable	Supplier
DEPC-treated water	Thermo Fisher Scientific, Waltham, MA, USA
Dimethyl sulfoxide (DMSO)	Carl Roth, Karlsruhe, Germany
Disodium phosphate (Na_2HPO_4)	Carl Roth, Karlsruhe, Germany
Dithiothreitol (DTT)	Carl Roth, Karlsruhe, Germany
Ethanol ≥ 99.8%	Carl Roth, Karlsruhe, Germany
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	Carl Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe, Germany
EZ-Link™ Sulfo-NHS-SS-Biotin	Thermo Fisher Scientific, Waltham, MA, USA
GeneRuler™ 1kb DNA ladder	Thermo Fisher Scientific, Waltham, MA, USA
Glucose	Carl Roth, Karlsruhe, Germany
Glutathione Sepharose™ 4B	GE Healthcare, Chicago, IL, USA
Glycerin	Carl Roth, Karlsruhe, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Carl Roth, Karlsruhe, Germany
HEPES	Carl Roth, Karlsruhe, Germany
iBlot™ Transfer Stack, nitrocellulose, mini and regular	Thermo Fisher Scientific, Waltham, MA, USA
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Merck, Darmstadt, Germany
Leupeptin	Tocris Bioscience, Bristol, United Kingdom
L-glutathione	Merck, Darmstadt, Germany
Lipofectamine™ 2000	Thermo Fisher Scientific, Waltham, MA, USA
Magnesium chloride ($MgCl_2$)	Carl Roth, Karlsruhe, Germany
Magnesium sulfate ($MgSO_4$)	Carl Roth, Karlsruhe, Germany
Nitric Acid (HNO_3)	Merck, Darmstadt, Germany
NMDA	Merck, Darmstadt, Germany
Nonidet™ P40 (NP-40)	Merck, Darmstadt, Germany
NuPAGE™ 4-12%, Bis-Tris Midi Protein Gel	Thermo Fisher Scientific, Waltham, MA, USA
NuPAGE™ 4-12%, Bis-Tris Mini Protein Gel	Thermo Fisher Scientific, Waltham, MA, USA
Opti-MEM™	Thermo Fisher Scientific, Waltham, MA, USA
PageRuler™ prestained protein ladder	Thermo Fisher Scientific, Waltham, MA, USA
Paraformaldehyde	Carl Roth, Karlsruhe, Germany
PhosSTOP™	Roche, Rotkreuz, Switzerland
Phorbol 12,13-dibutyrate (PdbU)	Tocris Bioscience, Bristol, United Kingdom
Pierce™ NeutrAvidin™ Agarose	Thermo Fisher Scientific, Waltham, MA, USA
Ponceau S	Merck, Darmstadt, Germany
Potassium Chloride (KCl)	Carl Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth, Karlsruhe, Germany
ProLong™ Gold Antifade Mountant	Thermo Fisher Scientific, Waltham, MA, USA
Protein G Agarose beads	Seracare, Milford, MA, USA
(S)-AMPA	Hello Bio, Bristol, United Kingdom
Sodium Azide (NaN_3)	Merck, Darmstadt, Germany
Sodium Chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium dihydrogen phosphate (Na_2HPO_4)	Carl Roth, Karlsruhe, Germany
Sodium dodecyl sulphate	Carl Roth, Karlsruhe, Germany
TransIT®-293 transfection reagent	Mirus Bio, Madison, USA
Tris-hydroxymethyl-aminomethane (Tris)	Carl Roth, Karlsruhe, Germany
Triton X-100	Carl Roth, Karlsruhe, Germany
Tween-20	Carl Roth, Karlsruhe, Germany
Wash-N-Dry Coverslip Rack	Diversified Biotech INC., Boston, MA, USA
Western blocking reagent	Roche, Rotkreuz, Switzerland

2.1.7 Antibodies and fluorescent dyes

Table 4. Table of antibodies and fluorescent dyes

Immunoblotting			
Primary antibody	**Species**	**Dilution**	**Supplier**
Anti-actin	Mouse	1:1000	Merck, Darmstadt, Germany
Anti-GFP	Mouse	1:2000	Roche, Rotkreuz, Switzerland
Anti-N-terminal GluA1	Mouse	1:1000	Merck, Darmstadt, Germany
Anti-PKD1	Rabbit	1:1000	Cell Signalling, Cambridge, UK
Anti-PKD Substrate	Rabbit	1:1000	Cell Signalling, Cambridge, UK
Anti-pS831 GluA1	Rabbit	1:1000	Cell Signalling, Cambridge, UK
Anti-pS845 GluA1	Rabbit	1:1000	Cell Signalling, Cambridge, UK
Anti-pS916 PKD	Rabbit	1:1000	Cell Signalling, Cambridge, UK
Secondary antibody	**Species**	**Dilution**	**Supplier**
HRP anti-mouse IgG	Goat	1:10000	DIANOVA, Hamburg, Germany
HRP anti-rabbit IgG	Goat	1:10000	DIANOVA, Hamburg, Germany
Immunofluorescence			
Primary antibody	**Species**	**Dilution**	**Supplier**
Anti-EEA1	Rabbit	1:100	Cell Signalling, Cambridge, UK
Anti-N-terminal GluA1	Mouse	1:500	Merck, Darmstadt, Germany
Anti-Shank2	Guinea Pig	1:2000	Synaptic systems, Göttingen, Germany
Secondary antibody	**Species**	**Dilution**	**Supplier**
Alexa Fluor® 488/546/633 anti-mouse	Goat	1:500	Thermo Fisher Scientific, Waltham, MA, USA
Alexa Fluor® 488/546/633 anti-rabbit	Goat	1:500	Thermo Fisher Scientific, Waltham, MA, USA

2.1.8 Kits

Table 5. Table of kits

Kit	**Supplier**
SuperSignal® west pico PLUS & Dura chemiluminescent substrates	Thermo Fisher Scientific, Waltham, MA, USA
NucleoBond® XtraMidi	Macherey-Nagel, Düren, Germany
*Trans*IT-HeLaMONSTER® Transfection Kit	Mirus Bio, Madison, USA

2.1.9 Plasmids

Table 6. Table of plasmids

Plasmid	Source
pEGFP-N1-PKD1 S738/742E	IZI, University of Stuttgart
pEGFP-C2-Vector	Clontech Europe
pEGFP-C3-Rabaptin 5 wt	Alex Toker, Beth Israel Deaconess Center, Harvard Medical School, Boston, MA, USA
pEGFP-C3-Rabaptin 5 S407A	Alex Toker, Beth Israel Deaconess Center, Harvard Medical School, Boston, MA, USA
GFP-Rab5 wild type	Lucas Pelkmans, University of Zurich, Switzerland
GFP-Rabex5	Juan Bonifacino, NIH, Bethesda, MD, USA
pcDNA3-mRuby2	Michael Lin, Stanford University, CA, USA Addgene (#40260)
pCl-SEP-GluR1	Robert Malinow, UCSD, CA, USA Addgene (#24000)
pGEX-4 T-2/Rabaptin-5:R5BD	Guangpu Li, Fujian Agriculture & Forest University, China

2.1.10 Equipment

Table 7. Table of equipment

Name	Manufacturer
Amersham™ Imager 600	GE Healthcare, Chicago, IL, USA
iBlot® Dry Blotting System	Thermo Fisher Scientific, Waltham, MA, USA
LSM 710 Confocal Laser Scanning Microscope	Carl Zeiss, Oberkochen, Germany
UGA-42 Firefly Photomanipulation System	Rapp OptoElectronic, , Germany
XCell4 SureLock™ Midi/Mini-Cell electrophoresis system	Thermo Fisher Scientific, Waltham, MA, USA
Zeiss Axio Observer Spinning Disk	Carl Zeiss, Oberkochen, Germany

2.1.11 Software

Table 8. Table of software

Software	Developer company
Adobe InDesign CC 2019	Adobe Systems Incorporated, San Jose, CA, USA
Endnote X7.8	Thomson Reuters, Albuquerque, NM, USA
GraphPad Prism 8	GraphPad software, La Jolla, CA, USA
Image J 1.51n	Wayne Rasband, National Institute of Health, Bethesda, MD, USA
ImageStudio Lite	LI-COR Biosciences, Lincoln, NE, USA
Microsoft Office	Microsoft, Redmond, WA, USA
ZEN 3.0 (Blue edition)	Carl Zeiss, Oberkochen, Germany

2.2 Methods

2.2.1 Bacterial transformation

Competent *E. coli* were transformed via the heat shock method with recombinant plasmid DNA. Initially bacteria were left to thaw on ice, and 50 µl of TCM buffer, 10 µl of plasmid DNA and 10 µl of ddH_2O were added to 125 µl of recombinant bacteria. After incubating for 30 min on ice, the mix was submerged on water at 42°C for 2 minutes. Subsequently, 800 µl of Lysogeny Broth medium (10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl in ddH_2O) were added and the mixture was left for 1 h at 37°C. Then, bacteria were centrifuged for 30 s at 16100 Gs and most of the supernatant was discarded by inversion. Finally, bacteria were resuspended on the remaining supernatant volume, plated on an agar plate with the corresponding antibiotic, and incubated overnight at 37°C.

2.2.2 Isolation of plasmid DNA

In order to amplify recombinant plasmid DNA, a single colony of bacteria was inoculated in 5 ml of LB medium with 100 mg/ml ampicillin and left to incubate overnight at 37°C with shaking. The next day, 20 µl of that bacterial solution were used to inoculate 175 ml of LB with ampicillin and again left overnight at 37°C with shaking to further expand the culture. Afterwards, in accordance with the instructions provided by the NucleoBond® XtraMidi kit, the bacterial suspension was centrifuged at 6000 Gs for 10 min at 4°C, the supernatant was discarded and the bacterial pellet resuspended in 8 ml of “RES Resuspension Buffer” + RNase A. Subsequently, 8 ml of “LYS Lysis Buffer” were added to the suspension, which was then inverted 5 times and incubated at room temperature (RT) for 5 min. 8 ml of “NEU Neutralization Buffer” were then added, and the suspension was then gently inverted, clarified and loaded to the previously equilibrated NucleoBond® Xtra Columns. The first column wash was performed with 5ml of “EQU Equilibration Buffer”, after which the filter was removed and the column was washed for a second time using 8 ml of the “WASH Buffer Wash”. Then, 5 ml of “ELU Elution Buffer” were used to collect the eluate, 3.5 ml of isopropanol were added to precipitate the plasmid DNA and the solution was centrifuged at 15000 Gs for 30 min at 4°C. After discarding the supernatant, 2 ml of 70% ethanol were added and the solution was again centrifuged at 15000 Gs for 30 min at 4°C in order to ensure pellet stability. Lastly, the supernatant was carefully removed and the pellet was allow to dry at RT, after which it was resuspended in DEPC-treated water.

2.2.3 Preparation of GST-Rab5BD beads

In order to be able to specifically pull down active Rab5 from cell lysates, a single colony of bacteria transformed with the "pGEX-4 T-2/Rabaptin-5:R5BD" plasmid (expressing the active Rab5 binding domain of the Rabaptin-5 protein) was inoculated into 20 ml of LB media with 100 mg/ml ampicillin and incubated overnight at 37°C with shaking. The next day, 10 ml of the inoculated culture were added to 500 ml of LB with ampicillin. Bacteria were left to grow through the day and their OD_{600} value was measured periodically. Once it was within the 0.8-1 range, 0.3 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to the bacterial culture in order to induce production of the GST-Rab5BD protein, and it was left to incubate overnight at RT with shaking. The day after, the culture was centrifuged at 1380 Gs for 10 min at 4°C, the supernatant was removed and the bacterial pellet was frozen by placing it on dry ice for 30 min. While always keeping the bacterial extracts on ice, the pellet was resuspended in 20 ml of TED buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT in ddH_2O) supplemented with protease and phosphatase inhibitors, 0.1 mg/ml of lysozyme were added and the solution was incubated for 30 min with gentle shaking. Subsequently, 5 mM $MgCl_2$ and 20 ng/µl DNase I (Merck) were added, the solution was left again to incubate for 30 min at 4°C with gentle shaking and ultracentrifuged for 40 min at 30000 Gs and 4°C. During that time, the Glutathione Sepharose™ 4B beads (GE Healthcare) were prepared: 2ml of a 30% bead slurry stock were centrifuged for 5 min at 500 Gs, the supernatant was removed, the beads were washed and subsequently centrifuged with 5ml of TEDN buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT in ddH_2O) three times and finally resuspended in 500 µl of TEDN buffer to obtain a 50% bead slurry. After the centrifugation, the supernatant was collected, 1 ml of 50% glutathione bead slurry was added to it and it was left to incubate for 1 h at 4°C on a rocking platform in order to allow for binding of the Rab5BD to the beads. The beads were then pelleted via centrifugation at 500 Gs for 5 min at 4°C, the supernatant was discarded, and beads were washed three times with 4 ml of TEDN buffer supplemented with protease and phosphatase inhibitors (Roche). Finally, the beads were washed once with 4 ml of TDN buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT in ddH_2O) and subsequently resuspended in 500 µl of that same buffer, yielding 1 ml of 50% slurry of GST-Rab5BD beads.

2.2.4 Coverslip acid washing

In order to remove all impurities from the surface of coverslips, thus improving the health of the neuronal cultures, coverslips were consistently washed in a 65% solution of nitric acid (Merck) for 18 hours, using the Wash-N-Dry coverslip racks (Diversified Biotech Inc.) to ensure thorough cleaning. Afterwards, coverslips were rinsed in ddH_2O for 30 min six times before

being submerged in 100% ethanol (Carl Roth) for 10 min. Lastly, they were placed on a glass petri dish covered by aluminium foil and dry heat sterilized for 8 hours at 240 °C.

2.2.5 Poly-L-lysine and laminin surface coating

All plates and coverslips meant to be used for neuronal culture needed to be pre-treated in order to boost their cell adhesion capabilities and be suitable for neurons. To this aim, both plastic and glass surfaces [Corning® non-treated culture dishes (35/10 mm) (Merck), Greiner CELLSTAR® 24-well culture plate (Greiner) and CELLview™ dish with glass bottom (Greiner), glass round coverslips Ø12 mm (Carl Roth)] were coated with 4 µg/ml of poly-L-lysine (PLL, Merck) dissolved in ddH_2O, a positively charged amino acid that facilitates cell attachment by enhancing electrostatic interactions with the negatively charged ions of the cell surface. In sterile conditions, the bottom of each dish or well was covered with PLL solution and incubated for 30 min in the dark. Then, the solution was aspirated and the dishes or wells were left to dry for 90 min again in the dark; after which the last two steps were repeated. These plates were kept in the dark at 4°C for up to two weeks.

Glass surfaces needed an additional layer of coating with 20 µg/ml laminin dissolved in PBS (140 mM NaCl, 2.7 mM KCl, 8mM Na_2HPO_4, 1.5 mM KH_2PO_4 in ddH_2O, pH 7.4) to be able to sustain neuronal cultures. As a key component of the basal lamina, laminin as a cell culture substratum supports growth and attachment of many different cell types, including neurons. In sterile conditions, the surface of glass coverslips or dishes was covered with the laminin solution, and they were left to incubate overnight in the dark in a humidified chamber at 37°C and 5% CO_2. For glass coverslips, special care was taken that air bubbles did not remain between the bottom of the plate and the coverslip, as these could cause the coverslip to float and prevent adequate coating.

Finally, all culture surfaces were added supplemented NeuroBasal™ medium [supplemented neurobasal; Neurobasal™ (Thermo Fisher Scientific), 5% Fetal Bovine Serum (FBS, PAN Biotech), B27™ supplement (Thermo Fisher Scientific), 0.5 M GlutaMAX™ (Merck), 250 ng/ml amphotericin B (ThermoFisher Scientific) and 40 ng/ml gentamycin (Merck)] and left in a humidified chamber at 37°C and 5% CO_2 during at least a few hours before cells were plated on them.

2.2.6 Dissection and culturing of embryonic hippocampal neurons

Co-cultures of primary embryonic hippocampal neurons and primary glial cells were obtained from embryos on day E18. Dissections were carried out on DMEM (Merck) supplemented with 250 ng/ml amphotericin B (Thermo Fisher Scientific) and 40 ng/ml gentamycin (Merck) following a published protocol (Czöndör et al., 2009). First, the brain was aseptically removed from the skull, and the hippocampi were dissected. After carefully removing the meninges, the hippocampi were cut into small pieces and incubated in PBS containing 0.05% trypsin-EDTA (Thermo Fisher Scientific) and 0.05% DNase I for 15 min at 37°C. Subsequently, tissue was centrifuged briefly, supernatant was discarded and cells were resuspended in supplemented neurobasal medium before being filtered through a 42-µm pore size sterile mesh (EmTek). Cell number was determined via trypan blue exclusion, and cells were seeded in supplemented neurobasal medium. For immunofluorescence and microscopy applications, neurons were seeded on glass coverslips or CELLview™ dishes at a density of 1.14 x 10^5 per coverslip or compartment, whereas for protein extraction and western blotting cells were seeded on plastic 35 mm cell culture dishes at a density of 3.94 x 10^5 per dish. Cells were carefully followed on a daily basis, and 10 µM of cytosin-arabinofuranoside (Thermo Fisher Scientific) were added to the culture as the glial cells were reaching 70% confluence, to prevent them from disturbing neuronal growth. Additionally, on days in vitro (DIV) 5, 8 and 11, cells underwent a half medium change for supplemented Brainphys™ neuronal medium [supplemented brainphys; Brainphys™ neuronal medium supplemented with NeuroCult™ SM1 Neuronal Supplement, 250 ng/ml amphotericin B and 40 ng/ml gentamycin]. Cells were cultivated overall for 13 days under sterile conditions in a humidified chamber at 37°C and 5% CO_2, thus reaching morphological and functional maturity. Chemically-induced Long Term Depression (cLTD) was induced by treating the neuronal cultures with 50 µM NMDA (Sigma, #M3262) for 5 min in conditioned medium. Then, cells were changed to a NMDA-free medium, and incubated for 15 min before being further processed.

2.2.7 Culturing of HEK293T cells

HEK293T cells were cultivated in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS under sterile conditions, in a humidified chamber at 37°C and 5% CO_2. When cells reached approximately 80% confluence, they were passaged 1:5-1:10 using 1x trypsin/EDTA in PBS.

2.2.8 Transient cell transfection

Embryonic hippocampal neuronal cultures were transiently transfected on DIV 12 using Lipofectamine™ 2000, according to the manufacturer's instructions. Initially, the transfection mixes were prepared by placing 50 µl of Brainphys™ neuronal medium times the number of coverslips or Cellview™ chambers to be transfected into 2 tubes. To those tubes, either plasmid DNA or Lipofectamine 2000 were added in a 1 µg : 2 µl ratio. For single transfections, 0.5 µg plasmid DNA were used, for double transfections, 0.8 µg in total. After 5 min of incubation, both tubes were mixed and subsequently left to rest for 20 min at RT, to allow for liposome formation. In the meantime, the conditioned medium of the culture was removed and stored, and cells were changed to Brainphys™ neuronal medium. Afterwards, the liposome mix was added in a dropwise manner to the cells, and the culture was incubated for 4 hours in a humidified chamber at 37°C and 5% CO_2. Lastly, the transfection medium was removed from the culture, cells were returned to the conditioned medium and the culture was incubated overnight.

For transient transfection of HEK293T cells, the *Trans*IT™-293 Transfection Reagent was employed according to the manufacturer's instructions. Briefly, the reagent was allowed to reach RT and gently vortexed. For every well, 2.5 µg of plasmid DNA and 7.5 µl of the reagent were added to 250 µl of OPTI-MEM and gently mixed, after which the transfection solution was incubated for 20 min at RT. Afterwards, the mix was added drop by drop to the cells, which were incubated overnight at 37°C.

2.2.9 Chemical treatments, cell surface staining and biotinylation

In order to block the activity of all PKD isoforms, the pan-inhibitor CRT 0066101 (CRT, Tocris #4975) (Harikumar et al., 2010) was diluted in dimethyl sulfoxide (DMSO) and added to the culture at a final concentration of 2 µM. Conversely, to promote activation of PKD, the phorbol ester phorbol 12,13-dibutyrate (PdbU, Tocris #4153) was used at a concentration of 1 µM.

For surface biotinylation of endogenous GluA1, DIV 13 neurons were placed on ice, their medium was removed and they were washed twice with Ca^{2+}- and Mg^{2+}-containing PBS (PBSCM, Thermo Fisher). Subsequently, they were incubated with 1.5 mg/ml of EZ-Link™ Sulfo-NHS-SS-Biotin (biotin, Thermo Fisher Scientific) diluted in PBSCM for 15 minutes and washed twice again with 20 mM glycine (Carl Roth) in order to bind free biotin, before being lysed.

To visualize surface GluA1 a previously described surface staining protocol was followed (Szíber et al., 2017). Cells were initially incubated in conditioned medium with an N-terminal specific GluA1 antibody (1:100, Merck) for 10 min in a humidified chamber at 37°C and 5% CO_2. Subsequently, cells were washed twice with PBS and fixed with paraformaldehyde fixing solution [4% paraformaldehyde (v/v, Carl Roth) in PBS] for 10 min at RT. Without permeabilization, cells were blocked for 1 h with blocking buffer [5% FBS (v/v) + 0.1% sodium azide (NaN_3, v/v, Merck) in PBS] and subsequently incubated with a secondary Alexa Fluor™ anti-mouse antibody (Thermo Fisher Scientific). Afterwards, cells were washed three times with PBS, fixed again with paraformaldehyde fixing solution for 10 min and further processed for Shank2 immunostaining (please see immunostaining staining section).

To analyse AMPAR endocytosis in response to agonist binding, biotinylation assays of receptor internalization were performed as previously described (Ehlers, 2000). Initially, lysosomal protein degradation was inhibited by incubating the culture with 100 µg/ml Leupeptin (Tocris) for 1h in a humidified chamber at 37°C and 5% CO_2. Subsequently, 1.5 mg/ml biotin were added to the culture, which was incubated for 1 h at 4°C. After the incubation period, cells were transferred back to a biotin-free medium and treated with 100 µM of S-AMPA (Hello Bio) and 50 µM D-APV (Tocris) for 2 min. Cells were then returned for 10 min to a S-AMPA- and D-APV-free medium to allow for AMPAR endocytosis, placed on ice, washed twice with cold PBSCM and remaining surface biotin was cleaved by incubation with glutathione cleavage buffer [50 µM L-Glutathione (Sigma), 75 µM NaCl, 10 mM EDTA (Carl Roth), 1% BSA (v/v, Thermo Fisher Scientific) in ddH_2O, pH 8.6] for 15 min. Lastly, cells were washed twice with PBSCM and further processed for protein extraction (please see protein extraction section).

2.2.10 Protein extraction, biotin pulldown and Western Blotting

Neuronal cells were lysed in cold Triton X-100 lysis buffer [1% Triton X-100 (v/v, Carl Roth), 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA (Carl Roth) in ddH_2O] supplemented with protease and phosphatase inhibitors with the help of cell scrapers, and subsequently centrifuged at 13000 Gs and 4°C for 10 min. To pull down biotinylated proteins, avidin-coated Pierce™ NeutrAvidin™ Agarose beads (avidin beads, Thermo Fisher Scientific) were first washed with 1 ml of empty lysis buffer and centrifuged at 2500 Gs for 2 min three times, and subsequently resuspended in empty lysis buffer to have a 50% bead slurry. To equal volumes of cell lysate, 40 µl of avidin beads and supplemented Triton X-100 lysis buffer were added up to a volume of 1 ml, and the mixture was incubated on a rocking platform for 1.5 h at 4°C. Subsequently, complexes were washed with 1 ml of supplemented Triton X-100

lysis buffer and centrifuged at 2500 Gs and 4°C for 2 min four times. To immunoprecipitate Rabaptin-5, Protein G Agarose beads (Seracare) were first washed with 1 ml of cold 10 mM Tris 7.4 pH and centrifugated at 2500 Gs and 4°C for 2 min three times, and subsequently resuspended in cold 10 mM Tris 7.4 pH to have a 50% bead slurry. 2 µg of anti-Rabaptin-5 antibody (Table 4) and supplemented Triton X-100 lysis buffer were added to equal volumes of lysate up to a volume of 1 ml, and the mixture was incubated on a rocking platform for 2 hours at 4°C. Subsequently, 30 µl of washed Protein G Agarose beads were added to each sample, which were further incubated for an additional hour. Then, complexes were washed with 1 ml of supplemented Triton X-100 lysis buffer and centrifuged at 17500Gs and 4°C for 1 min four times.

HEK293T cells were harvested in cold HEK293T homogenization lysis buffer [300 mM NaCl, 10 mM $MgCl_2$, 20mM HEPES (Carl Roth), 10% glycerol (v/v, Carl Roth), 1mM DTT (Carl Roth), 0.1% NP-40 (v/v, Merck) in ddH_2O] supplemented with protease and phosphatase inhibitors, shortly vortexed and subsequently centrifuged at 9300 Gs for 3 min at 4°C. In order to determine the activation level of Rab5, firstly GST-Rab5BD beads (Qi et al., 2015) were washed with 1 ml of cold PBS, centrifuged at 2500 Gs for 2 min at 4°C and resuspended in PBS to obtain a 50% bead slurry. Then, equal amounts of lysate were incubated on a rocking platform with 20 µl of GST-Rab5BD beads and lysis buffer up to a volume of 1 ml for 1 h at 4°C. Then, beads were centrifuged at 400 Gs for 1 min, washed with 1ml of HEK293T homogenization washing buffer [300 mM NaCl, 10 mM $MgCl_2$, 20mM HEPES, 10% glycerol (v/v), 1mM DTT in ddH_2O] supplemented with protease an phosphatase inhibitors after the supernatant was discarded and finally resuspended in a small volume of washing buffer.

After extraction, proteins were denatured with 1x sodium dodecyl sulphate (Carl Roth) for 5 min at 95°C, spined down and equal amounts of lysate were run on NuPAGE™ 4 to 12%, Bis-Tris Midi Protein Gel (Thermo Fisher Scientific). Subsequently, membranes were blocked with membrane blocking solution [0.5% Western blocking reagent (v/v, Roche), 0.05% Tween-20 (v/v, Carl Roth) and 0.1% NaN_3 (v/v) in PBS] for 30 min at RT and incubated with a primary antibody (Table 4) diluted in blocking buffer at 4°C overnight. The next day, membranes were washed multiple times with PBS-Tween-20 [0.05% (v/v) Tween-20 in PBS] and incubated with a HRP-conjugated secondary antibody (Table 4, Dianova) diluted in membrane blocking solution for 90 min at RT. Lastly, membranes were washed again multiple times with PBS-Tween-20 and visualized using the SuperSignal™ west pico PLUS & Dura chemiluminescent substrates (Thermo Fisher Scientific).

Proteins were visualized using the Amersham™ Imager 600, an enhanced chemiluminescence detection system (GE Healthcare). Quantitative Western Blotting

chemiluminescence was detected at a depth of 16-bit in the linear detection range of the device, equipped with a 3.2-megapixel super-honeycomb CCD camera fitted with a large aperture f/0.85 FUJINON lens. Special care was taken not to overexpose in order to guarantee accurate quantifications. Densitometry was performed using Image Studio Lite 5.2 (Li-COR Biosciences, Bad Homburg, Germany). For each protein, the integrated density of the signal was measured, corrected for background signals and adjusted to loading controls.

2.2.11 Immunofluorescence staining

Hippocampal neuronal cultures were fixed on DIV 13 with paraformaldehyde fixing solution for 10 min. After a washing step with PBS, cells were permeabilized with permeabilization buffer [0.1% Triton X-100 (v/v) + 0.1% NaN_3 (v/v) in PBS] for 5 min. Cells were again washed with PBS and blocked with blocking buffer for 1 h at RT. Subsequently, cells were incubated with the corresponding primary antibody (Table 4) diluted in blocking buffer for 1.5 h at RT. Then, cells were washed with PBS and incubated with Alexa Fluor™ labelled antibodies (Table 4) diluted in blocking buffer for 1 h at RT. Finally, samples were mounted using ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific).

2.2.12 Microscopy imaging

In order to investigate AMPAR surface trafficking dynamics, fluorescence recovery after photobleaching (FRAP) of surface GFP was carried out in neurons transiently transfected with the super ecliptic pHluorin-tagged GluA1 (pCl-SEP-GluR1, Addgene #24000) and mRuby2 (pcDNA3-mRuby2, Addgene # 40260) proteins (5:3 plasmid ratio). Initially the culture medium was changed to pre-heated live cell imaging buffer [LCI buffer, 142 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5 mM glucose (Carl Roth) and 25 mM HEPES in ddH_2O, pH 7.4] and the diffusion of GluA1-containing AMPARs was analysed at 37°C and 5% CO2 on a Zeiss Axio Observer Spinning Disc Microscope, using an alpha Plan-Apochromat 100x/1.46 Oil DIC objective and a Photometrix Evolve 512 EMCCD camera for image acquisition. Photobleaching was executed with a UGA-42 firefly photomanipulation system equipped with a 100 mW 473 nm laser (Rapp OptoElectronic, Germany). After treatment with CRT or DMSO, selected dendritic spines were bleached with a high-intensity laser light (473 nm line, 10% laser power). Fluorescence intensity in the bleached areas was measured over time both before and after the bleaching event, taking images every 30 seconds for approx. 25 min. Intensity values were corrected with the background intensity values and normalized to the unbleached region. Fitting of the curves was performed with a

one-phase exponential equation *Y=Y0+(Plateau-Y0)*(1-exp(-K*x))*, (Prism 8, GraphPad Software).

All other samples were imaged using a confocal laser scanning microscope (LSM 710, Carl Zeiss) equipped with either a Plan Apochromat 63x/1.40 DIC M27 or an alpha Plan-Apochromat 100x/1.46 Oil DIC objective (Carl Zeiss), using sequential excitation with an 488 nm Argon laser, an 561 DPSS laser or an 633 nm HeNe laser. Image acquisition for the quantitative measurement of GluA1 intensity was done as follows: z-stacks of 0.32 µm intervals were acquired throughout the selected neuronal branches of at least 20 µm in length. Image processing and analysis was were performed with either ZEN blue (Carl Zeiss) or with ImageJ. Regions of interests were selected manually according to clear Shank2 immunopositivity at the plasma membrane along the shaft or within dendritic spines. Mean pixel intensity values of the GluA1 channel within the selected regions of interests were measured, background corrected and normalized to the staining intensity in the control condition. In order to analyze the co-localization between GluA1 and EEA1 signals, single images of selected neuronal branches were acquired. In all experiments, laser power was set so that there would be no saturation and maintained constant throughout the analyses of different samples from the same experiment. Image processing and analysis was performed with ZEN blue (Carl Zeiss) and ImageJ. The Mander's overlap coefficient of GluA1 with EEA1 was calculated and used as a measure of co-localization.

2.2.13 Statistical analysis

Results are shown as mean ± SEM, mean ± 95% CI or as Tukey's box-and-whiskers plots; as specified for each graph in its figure legend. When normalizing data, each data set was normalized to the control group, whose average was set to 1. For statistical evaluation, the normal distribution of the data was routinely tested using the Shapiro-Wilk normality test. For the comparison of two groups, an unpaired Student´s t test, a multiple Student´s t test (if data passed the normality test) or a Mann-Whitney test (if data failed the normality test) was employed; whereas to compare three or more groups, a one-way ANOVA (if data passed the normality test) or a Kruskal-Wallis test (if data failed the normality test) was used, followed by a post-hoc test for multiple comparisons. Statistical analysis was performed using GraphPad Prism 8 software. P values: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

3. Results

3.1 PKD regulates basal AMPAR trafficking in hippocampal neurons

3.1.1 Short-term treatment with CRT0066101 effectively blocks PKD activation *in vitro* in hippocampal neurons

In order to study a potential role of PKD in the regulation of fast local changes in AMPAR trafficking, embryonic hippocampal neuronal cultures were subjected to an acute, short-term PKD inhibition (10 minutes) using CRT0066101, a selective pan-inhibitor of all PKD isoforms [CRT, (Harikumar et al., 2010)], or DMSO, its vehicle. The rationale behind this protocol is that, in opposition to PKD knockdown or expression of a dominant negative protein mutant, CRT treatment allows for the immediate inhibition of the kinase, thus preventing a potential masking of the results by a defect in exocytic trafficking caused by the role of PKD at the TGN.

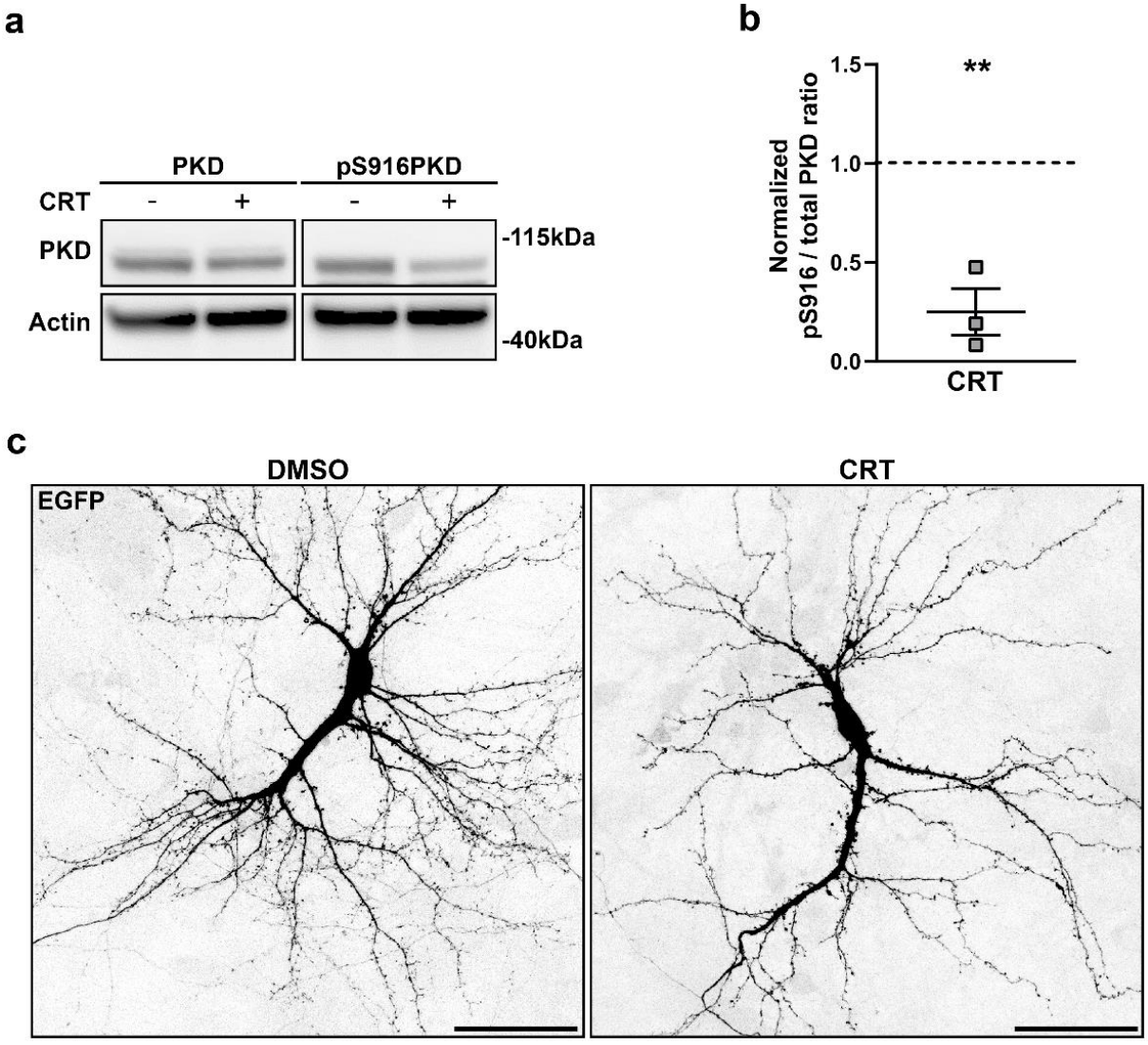

Figure 6 Short-term CRT0066101 treatment effectively inhibits PKD in cultured hippocampal mouse neurons with no impact on cell health. (a) Representative Western Blots obtained from hippocampal neuronal cultures treated with DMSO (-) or CRT (+) for 10 minutes, displaying total PKD and pS916PKD levels. Actin served as a loading control. **(b)** Quantification of data shown in (a) using densitometry analysis. Data from CRT-treated cultures are presented as mean line density ± SEM normalised to the control cultures (represented by the spotted line), each dot indicates one independent experiment. Statistical analysis was performed by unpaired two-tailed t-test. **(c)** Representative inverted single-channel pictures of neurons expressing EGFP. Cells were treated with DMSO or CRT for 10 minutes and fixed. **p<0.01. Scale bar 50 µm.

To prove that this short-term treatment with CRT effectively inhibits PKD activity, I analysed autophosphorylation of PKD at serine 916 (pS916PKD) as an indicator of kinase activity (Matthews et al., 1999) along with the total levels of PKD (Figure 6a). In order to investigate whether short-term inhibition of PKD has any effect on neuronal morphology, hippocampal neuronal cultures overexpressing EGFP were treated with CRT or DMSO for 10 minutes before being fixed (figure 6c). As shown by the images, CRT-treated neurons retain a fully developed morphology and show abundant dendrites and dendritic spines that are no different from those of control cells.

Hence, my data convincingly demonstrate that short-term treatment with CRT effectively inhibits PKD activity in hippocampal neurons with no impact to their health.

3.1.2 Acute inhibition of PKD increases AMPAR surface amount

Once the efficacy of the PKD inhibitor in these conditions had been confirmed, I set out to determine whether PKD activity is involved in the regulation of surface AMPAR levels. To do so, hippocampal neuronal cultures were treated with CRT or DMSO for 10 minutes and placed on ice, where surface proteins were tagged using sulfo-NHS-SS-biotin. Afterwards, cells were lysed, biotinylated proteins were precipitated, and the total expression and surface levels of the AMPAR subunit GluA1 were quantified via Western Blot analysis (Figure 7a). The logic behind the choice of GluA1 as the AMPAR subunit to study is two-fold: on the one hand, it is part of the most common AMPAR tetramer, GluA1/2; and on the other hand, GluA1-containing AMPARs are those trafficked and regulated in response to activity-mediated events, a major point of interest in my research. The results clearly demonstrated that PKD inhibition leads to a significant increase in the relative surface amount of GluA1 (172 ± 21% SEM compared to the control, Figure 7b), thus proving that even a short-term inhibition of PKD activity affects the surface expression of GluA1-containing AMPAR.

3.1.3 Acute inhibition of PKD increases AMPARs localisation to synaptic membranes

Under basal conditions, AMPARs have a high turnover within spines (Passafaro et al., 2001; Shi et al., 2001), and are internalized in their vicinity (Rosendale et al., 2017). To investigate whether short-term PKD inhibition affects the amount of GluA1 within synaptic membranes, hippocampal neuronal cultures were treated with CRT or DMSO, and subsequently incubated with an antibody detecting the extracellular N-terminal domain of GluA. Cells were then fixed and incubated with a fluorescently labelled secondary antibody, followed by fixation, permeabilization and immunostaining for Shank2, a marker of the PSD (Naisbitt et al., 1999) (Figure 8a). Notably, the analysis revealed that CRT treatment significantly increases GluA1 intensity within surface Shank2-positive areas compared to the control (Figure 8b), thus advocating for PKD as a regulator of GluA1-containing AMPAR synaptic levels.

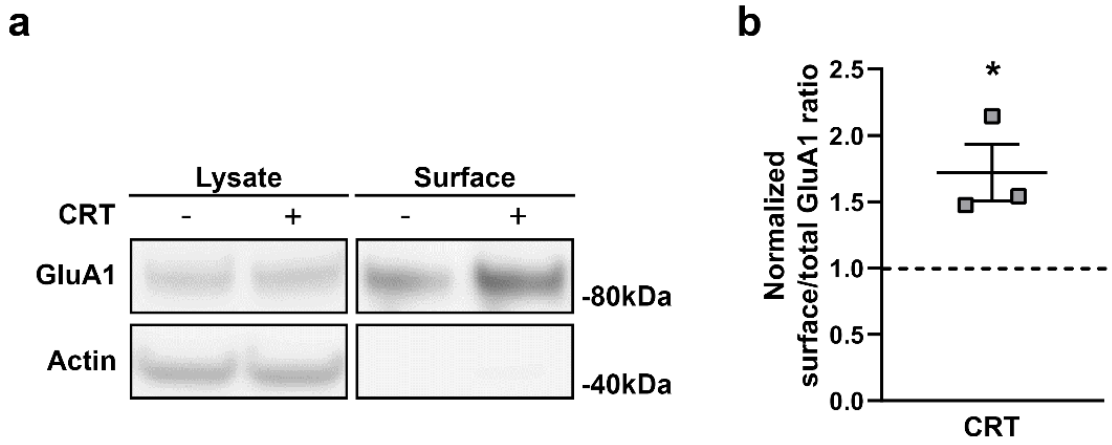

Figure 7 Short-term PKD inhibition promotes an increase in surface AMPAR levels. (a) Representative Western Blots obtained from hippocampal neuronal cultures treated with DMSO (-) or CRT (+) for 10 minutes, displaying total and surface GluA1 levels. Actin served as a loading control and is absent from the precipitated samples. **(b)** Quantification of data shown in (a) using densitometry analysis. Data from CRT-treated cultures are presented as mean line density ± SEM normalised to the control cultures (represented by the spotted line), each dot indicates one independent experiment. Statistical analysis was performed by unpaired two-tailed t-test. *p<0.05.

3.1.4 Transient expression of constitutively active PKD promotes a decrease in the synaptic amount of AMPARs

In order to corroborate this, I decided to transfect hippocampal neuronal cultures with the pEGFP-N1-PKD1 S738/742E plasmid, which encodes a constitutively active S738/742E mutant form of PKD1 (caPKD-EGFP), and then quantify the changes in GluA1 synaptic amount. During the realisation of this experiment I was assisted by Attila Ignácz, who imaged and analysed some of the samples included in the analysis. Hippocampal neurons transiently

expressing caPKD (pEGFP-N1-PKD1 S738/742E, IZI, University of Stuttgart) or EGFP (pEGFP-C2-Vector, Clontech Europe) were incubated with the N-terminal GluA1 antibody and subsequently fixed and stained without permeabilization for the surface-bound GluA1 antibody, before being fixed again, permeabilized and stained for Shank2 (Figure 9a).

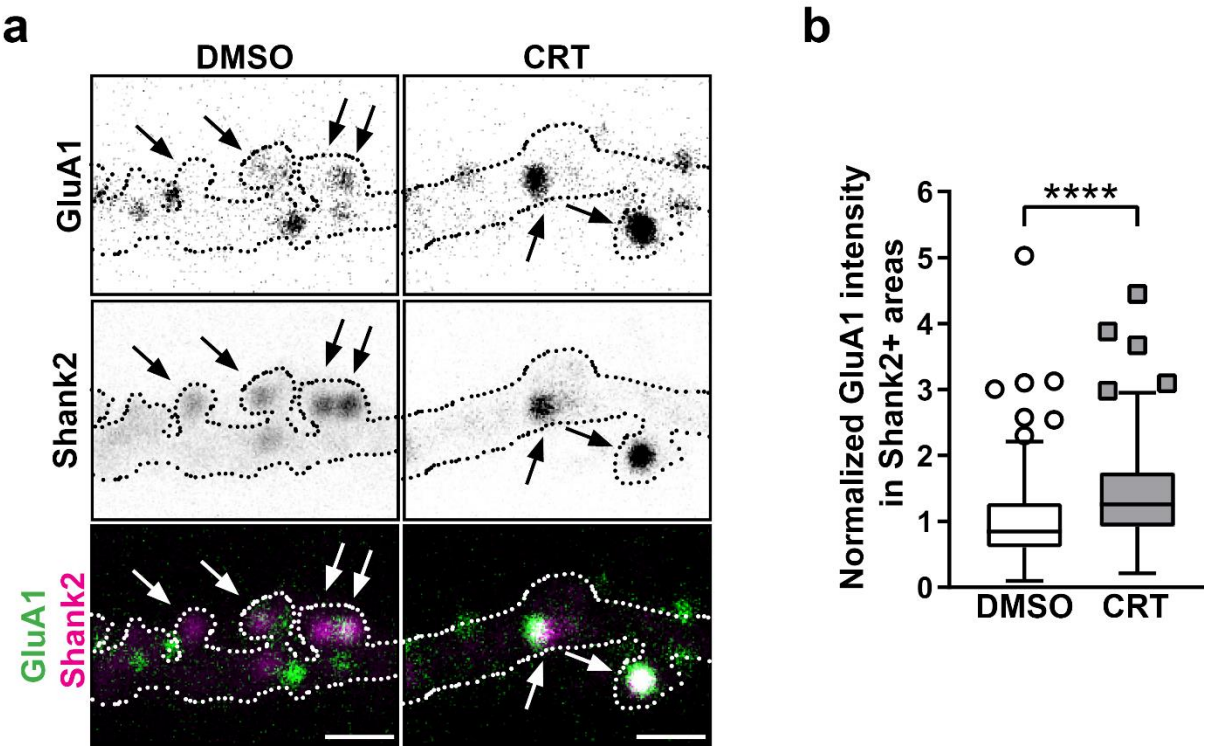

Figure 8 Short-term PKD inhibition increases synaptic GluA1-containing AMPAR levels. (a) Representative inverted single-channel and coloured merged pictures of neuronal dendritic branches stained for the extracellular N-terminal domain of GluA1 and the PSD marker Shank2. Arrows point to Shank2-positive areas included in the analysis. **(b)** Quantification of the images shown in (a). Data were normalised for the DMSO control. The boxplot shows the results of three independent experiments. Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. The number of investigated sample points is N=171 and 273. The significance of differences was analysed by a Mann-Whitney test (two-tailed). ****p<0.0001. Scale bar 2 µm.

Strikingly, the analysis revealed that expression of caPKD led to a significant decrease in the amount of GluA1 within Shank2 positive areas (Figure 9b), in line with the previously obtained results.

In conclusion, the obtained data strongly advocate for PKD as a regulator of surface and synaptic GluA1-containing AMPAR amounts in hippocampal neurons under basal conditions.

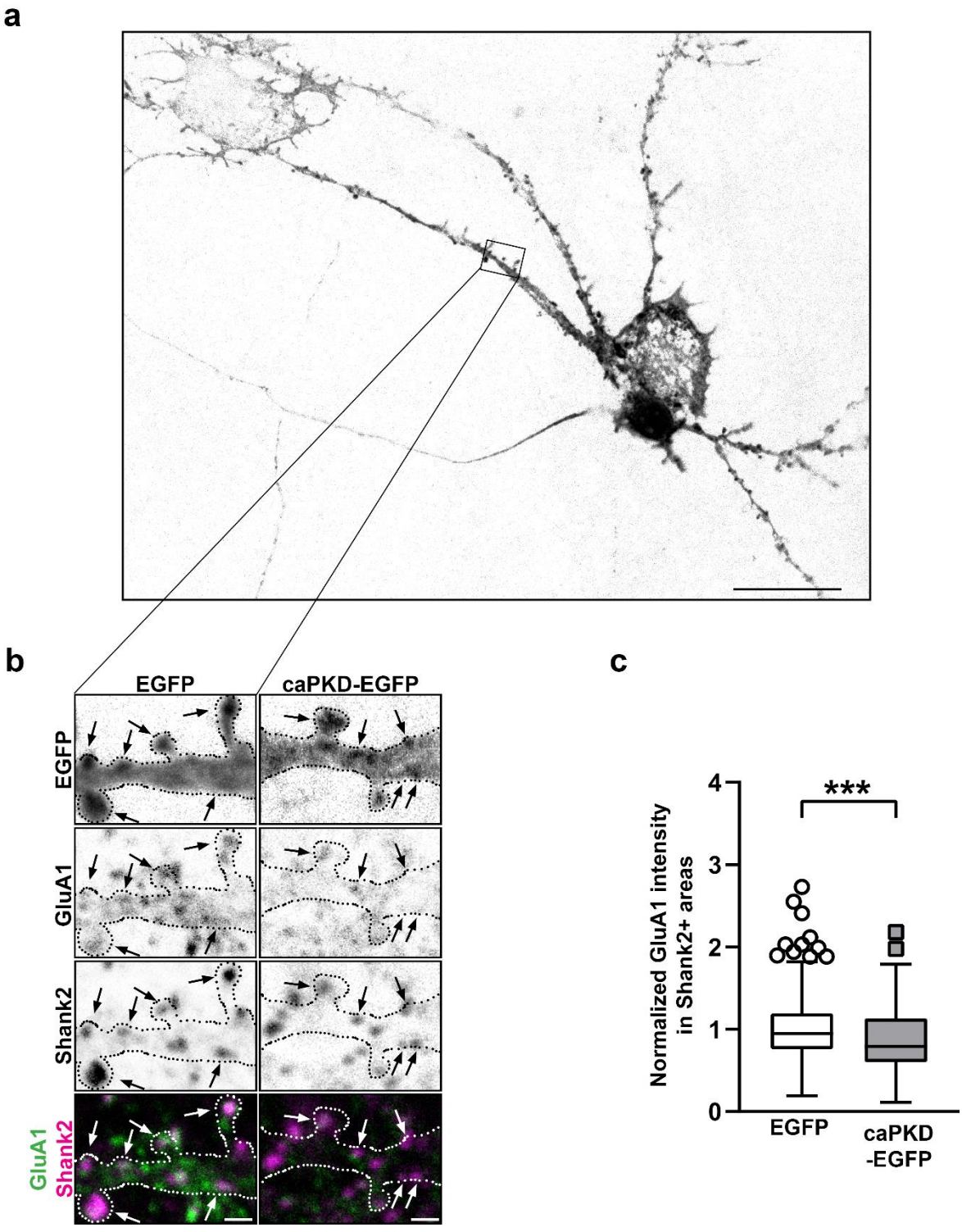

Figure 9 Expression of constitutively active PKD promotes a decrease in synaptic GluA1-containing AMPAR levels. (a) Representative inverted single-channel picture of an EGFP-expressing neuron. Scale bar 20 µm. **(b)** Representative inverted single-channel and coloured merged pictures of neuronal dendrites expressing EGFP or constitutively active PKD-EGFP (caPKD-EGFP), stained for the extracellular N-terminal domain of GluA1 and the PSD marker Shank2. Arrows point to Shank2-positive areas included in the analysis. The EGFP signal is not included in the coloured merge. **(c)** Quantification of pictures shown in (b). Data were normalised to the EGFP control. The boxplot shows the results of three independent experiments. Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. The number of investigated sample points is N=220 and 223. The significance of differences was analysed by a Mann-Whitney test (two-tailed). ***p<0.001. Scale bar 2 µm.

3.2 PKD specifically regulates AMPAR endocytosis

3.2.1 AMPAR surface trafficking is slowed down upon short-term PKD inhibition

While the results presented so far convincingly demonstrate that PKD plays a role in the surface expression of AMPARs, they were insufficient to clarify the mechanism behind it. Two different hypotheses could explain the obtained data: i) PKD activity leads to the inhibition of AMPAR recycling from the endosomal system or ii) PKD activity promotes endocytosis of AMPARs. In order to clarify this, I first decided to examine whether perturbed PKD activity affects the trafficking dynamics of surface AMPARs. This was achieved by measuring fluorescence recovery after photobleaching (FRAP) of Super Ecliptic pHluorin-tagged GluA1 (SEP-GluA1). SEP is a pH-sensitive version of GFP that only emits fluorescence at pH>6 (Miesenböck et al., 1998), a trait that turns it into an ideal tool to investigate specific subpopulations of a SEP-tagged protein cycling between the acidic endosomal compartment (Hu et al., 2015) and the neutral pH of the cellular surface, as is the case for AMPARs (Sankaranarayanan et al., 2000; Kopec et al., 2006). Hippocampal neuronal cultures co-expressing mRuby2 (pcDNA3-mRuby2, Addgene #40260), to visualize neuronal morphology, and SEP-GluA1 (pCl-SEP-GluR1, Addgene #24000) were treated with CRT or DMSO for 10 minutes before undergoing bleaching of the fluorescent GluA1 signal within the dendritic spines of the imaged dendritic branches. Fluorescence recovery was then measured for 10 additional minutes (Figure 10a, b). In order to calculate the half-time of fluorescence recovery as well as the mobile fraction of GluA1, the recovery curve was fitted with a one-phase exponential equation (Figure 10c). In DMSO-treated neurons, photobleaching of the SEP-GluA1 spine signal to background levels was followed by a rapid recovery (t½ = 14.52 seconds with 95% Cl of + 15.08 CI, Figure 10d) plateauing at 61.16% with a 95% CI of ± 3.4% of the original signal after 183 seconds (Figure 10e). On the contrary, CRT-treated neurons presented a significantly slower fluorescent signal recovery after photobleaching (t½ = 114.47 seconds with 95% Cl of + 77.67 and - 38.26 CI, Figure 10d), albeit to the same level as the control cells (Figure 10e). Consequently, while the mobile GluA1 fraction was not affected by the inhibition of PKD activity, these data clearly demonstrate that inhibition of PKD activity leads to a slowing down of surface GluA1 trafficking dynamics. Furthermore, since the inhibition of PKD activity promotes a slower recovery of the fluorescent signal in the bleached areas, I hypothesized that PKD is a regulator of AMPAR endocytosis: impaired internalization of bleached SEP-GluA1 molecules means that the rate at which slots open in the PSD is reduced (Shi et al., 2001; Malinow and Malenka, 2002; Lisman and Raghavachari, 2006; Opazo et al., 2012), and consequently, the speed at which newly exocytosed, non-bleached SEP-GluA1 molecules can enter the postsynaptic membrane.

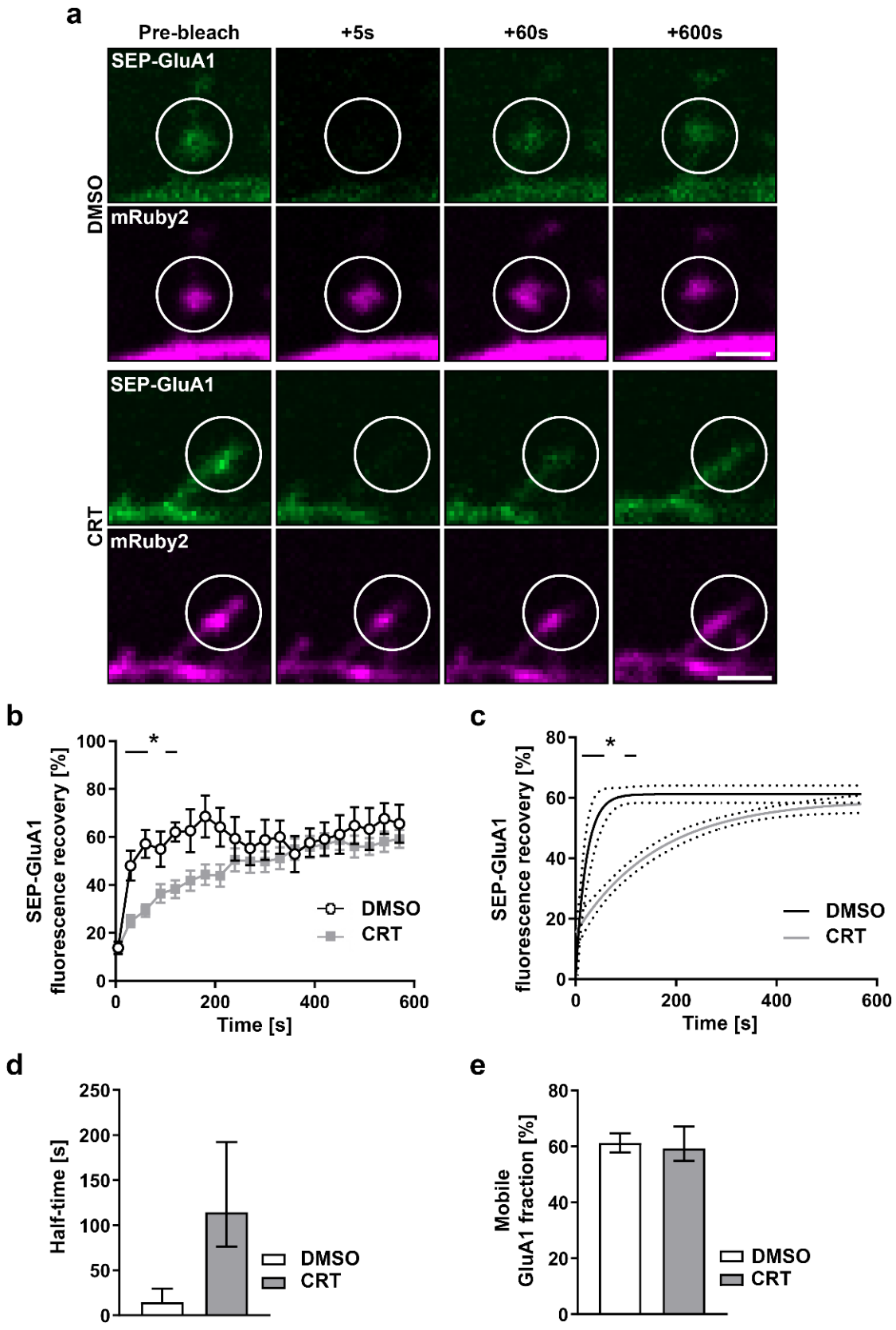

Figure 10 Short-term PKD inhibition slows down surface GluA1-containing AMPAR trafficking. (a) Representative pictures of neuronal dendritic spines expressing Super Ecliptic pHluorin-tagged GluA1 (SEP-GluA1) and mRuby2. Cells were treated with DMSO as a control or CRT for 10 minutes prior to the bleaching event. Images show dendritic spines before bleaching, as well as 5, 60 and 600 seconds afterwards. **(b)**

Fluorescence recovery curve and **(c)** one-phase association curve fitting of the SEP-GluA1 intensity after bleaching. Data shown as mean ± SEM (b) or with 95% CI (c); n=14 and 27 analysed dendritic spines from 12 and 16 neurons, for DMSO and CRT-treated conditions, respectively, coming from 3-4 independent cultures. The significance of differences was analysed by a multiple t-test followed by Holm-Sidak's multiple comparisons test. **(d)** Recovery half-time of the SEP-GluA1 signal and **(e)** mobile GluA1 fraction. Data shown as mean ± 95% CI. *p<0.05. Scale bar 2 µm.

3.2.2 PKD activity promotes AMPAR endocytosis

To corroborate this hypothesis, I decided to investigate next whether PKD is involved in AMPAR endocytosis. It is well established that constitutive endocytosis at perisynaptic zones regulates the number of AMPARs at the postsynaptic level (Lüscher et al., 1999; Ehlers, 2000; Man et al., 2000; Rosendale et al., 2017). Furthermore, under basal conditions, AMPARs undergo a robust time-dependent endocytosis (Ehlers, 2000).

Thus, I firstly investigated whether PKD activity affects basal trafficking of AMPARs to EEs. Hippocampal neuronal cultures expressing caPKD were treated with CRT or DMSO for 10 minutes, fixed and stained for EEA1, a marker of EEs (Mu et al., 1995; Selak et al., 2006), and for GluA1. EGFP-expressing neurons served as the control. EEA1 and GluA1 were present on dot-like structures distributed all over the dendritic branch (Figure 11a). This is in line with earlier reports showing that EEA1 participates in the endocytosis of AMPARs and is highly expressed in the postsynaptic compartment of hippocampal synapses (Selak et al., 2000; Selak et al., 2006). Importantly, no differences in the size of the EEA1+ vesicles were observed among the different conditions (data not shown). To assess whether the amount of GluA1 in EEs is influenced by PKD activity, I quantified the co-occurrence of GluA1 and EEA1 using Mander´s coefficient. The obtained data demonstrate that expression of caPKD led to a significant increase in the amount of GluA1 at EEs compared to the EGFP control. Interestingly, when cells were treated with CRT prior to fixation, GluA-EEA1 co-occurrence remained at control levels (Figure 11b), further supporting a role for PKD as a promoter of basal AMPAR endocytosis in hippocampal neurons.

3.2.3 Acute inhibition of PKD promotes an increase in GluA1 S831 phosphorylation levels

Additionally, I studied the phosphorylation of GluA1 at S831 and S845 in dependence on PKD activity. These two phosphorylation sites are among the best understood posttranslational modifications of AMPARs. S831 phosphorylation is mediated by CaMKII and PKC, and promotes targeting of GluA1 to the PSD in addition to increasing single-channel conductance,

whereas S845 phosphorylation is mediated by PKA, and leads to increased single-channel open probability and to targeting or retention of GluA1 at the cell surface (Banke et al., 2000; Man et al., 2007; Diering and Huganir, 2018). Cells were treated with CRT or DMSO for 10 minutes, lysed, and both the total and phosphorylated levels of GluA1 were measured via Western Blot (Figure 12a and c).

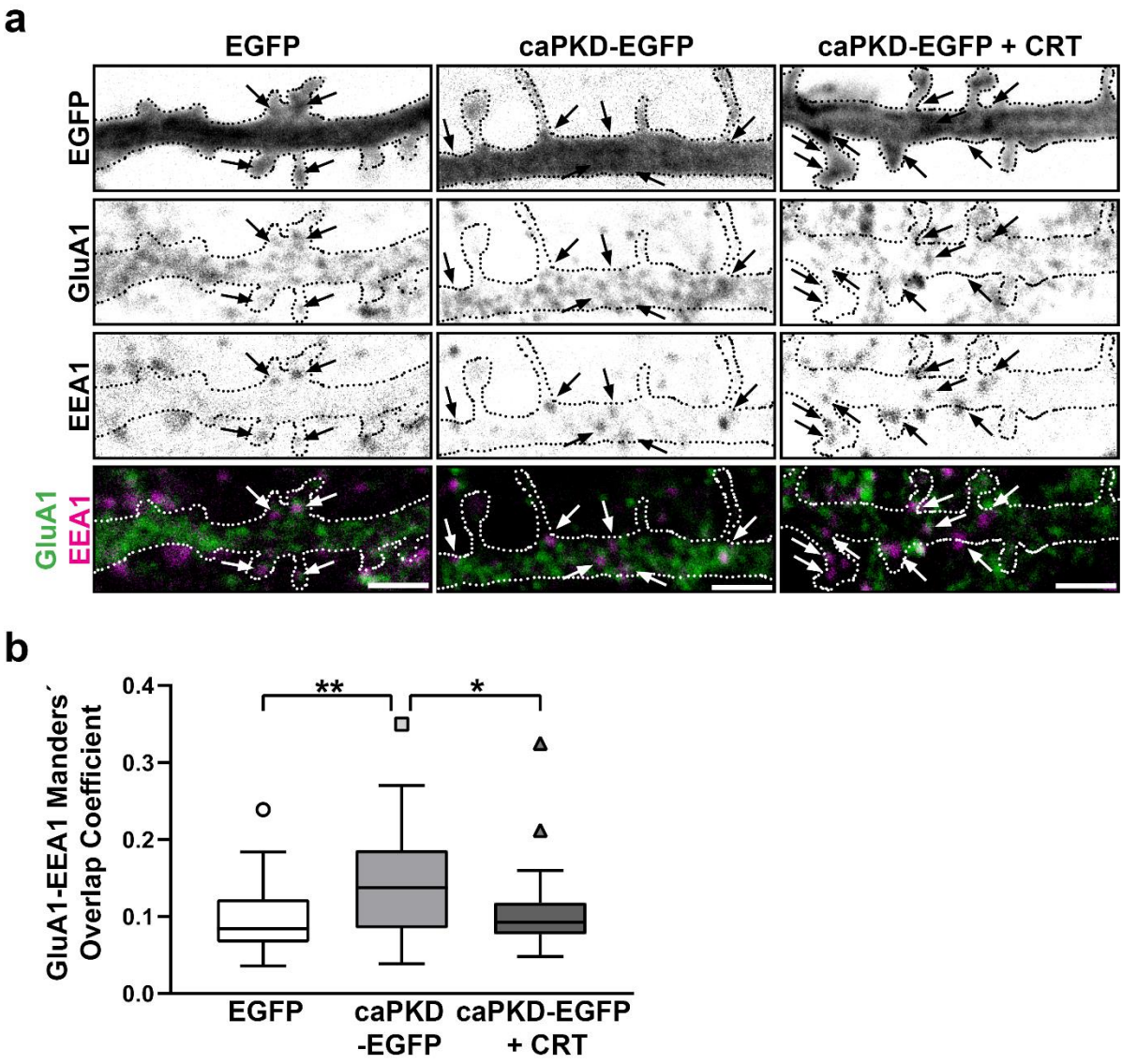

Figure 11 PKD activity promotes endocytic trafficking of surface GluA1-containing AMPARs to early endosomes. (a) Representative inverted single-channel and coloured merged pictures of neuronal dendritic branches expressing EGFP or constitutively active PKD-EGFP (caPKD-EGFP). Cells were treated with DMSO (-) or CRT for 10 minutes, fixed and stained for the early endosome marker EEA1 and for GluA1. Arrows point to the EEA1-positive early endosomes included in the analysis. The EGFP signal is not included in the coloured merged image. **(b)** Quantification of the pictures shown in (a). Manders´ overlap coefficient for EEA1 and GluA1. The boxplot shows the results of three independent experiments. Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; N = 28 sample points each. The significance of differences was analysed by a Kruskal-Wallis test followed by Dunn's multiple comparisons test. *p<0.05, **p<0.01. Scale bar 2 µm.

Remarkably, while short-term inhibition of PKD did not modify basal pS845 GluA1 levels compared to the control (Figure 12b), the data show a significant increase in basal pS831 GluA1 in CRT-treated cells when compared to the control (Figure 12d). These observations are in line with the previous results showing enhanced GluA1 levels at the PSD and slowed down GluA1 cell surface trafficking upon PKD inhibition. As S831-phosphorylated GluA1 subunits are enriched at the PSD (Diering et al., 2016), it further suggests that the occurrence of GluA1-containing AMPARs at the PSD is enriched in PKD-inhibited cells.

Collectively, the data show that the basal endocytic trafficking of GluA1-containing AMPARs requires PKD activity.

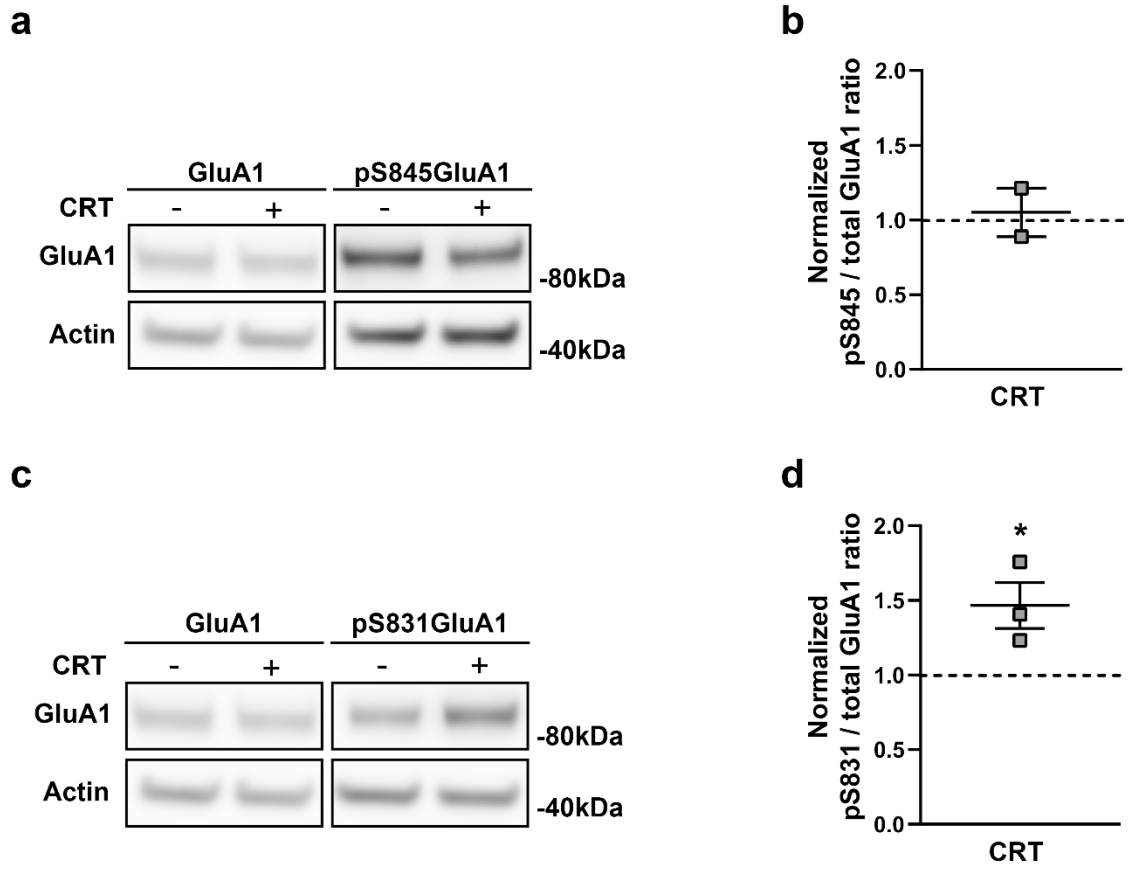

Figure 12 PKD inhibition leads to an increase in pS831GluA1. (a) Representative Western Blots obtained from hippocampal neuronal cultures treated with DMSO (-) or CRT (+) for 10 minutes, displaying total and pS845 GluA1 levels. Actin served as a loading control. **(b)** Quantification of data shown in (a) using densitometry analysis. Data from CRT-treated cultures is presented as mean line density ± SEM normalized to the control (represented by the spotted line), each dot indicates one independent experiment. Statistical analysis was performed by unpaired two-tailed t-test. **(c)** Representative Western Blots obtained from hippocampal neuronal cultures treated with DMSO (-) or CRT (+) for 10 minutes, displaying total and pS831 GluA1 levels. Actin served as a loading control. **(d)** Quantification of data shown in (c) using densitometry analysis. Data from CRT-treated cultures is presented as mean line density ± SEM normalized to the control (represented by the spotted line), each dot indicates one independent experiment. The dotted line indicates the control level. Statistical analysis was performed by unpaired two-tailed t-test. *p<0.05.

3.3 PKD regulates activity-mediated AMPAR endocytosis

3.3.1 PKD activity is necessary for agonist-induced AMPAR endocytosis

Given that PKD activity promotes basal endocytic AMPAR trafficking, I next investigated whether the kinase also plays a role in agonist-induced AMPAR endocytosis from synapses. To do so, I employed the biotinylation assay of receptor endocytosis using sulfo-NHS-SS-biotin (Ehlers, 2000). Briefly, biotinylated hippocampal cultures were treated with CRT or DMSO, followed by 2 minutes of 100 µM AMPA-evoked stimulation of AMPARs while simultaneously blocking NMDA receptors with 50 µM D-AP5 (Figure 13a). Cells were incubated additionally for 10 minutes to allow for endocytosis before the remaining cell surface biotin was cleaved and cells were processed for analysis. In this way, GluA1 subunits remain biotinylated only within endosomes. In line with previous results, AMPA treatment induced GluA1 endocytosis as indicated by increased levels of biotinylated GluA1 in comparison to total GluA1 (126 ± 8% SEM compared to the control, Figure 13b and c). Strikingly, ligand-induced endocytosis of AMPARs was completely rescued when cells were pre-treated with CRT (72 ± 13% SEM compared to the control, Figure 13b and c). Notably, AMPA treatment also enhanced autophosphorylation of PKD, indicating that the kinase is a downstream target of AMPAR signalling. The AMPA-induced increase in kinase activity was completely blocked by pre-treatment of cells with CRT (Figure 13d and e).

3.3.2 PKD activity mediates AMPAR endocytosis in response to NMDAR-cLTD

Treatment of hippocampal neuronal cultures with NMDA promotes activity-dependent endocytosis of AMPARs thus evoking NMDAR-cLTD (designated as cLTD) (Lee et al., 1998; Beattie et al., 2000; Lee et al., 2002; Lin and Huganir, 2007; Collingridge et al., 2010).

To investigate whether PKD activity is specifically required for cLTD induction, I treated cells with CRT or DMSO for 10 minutes, and subsequently with 50 µM NMDA for an additional 5 minutes. Cells were then changed to an NMDA-free medium and incubated for 15 minutes to allow for receptor endocytosis before undergoing surface protein biotinylation using sulfo-NHS-SS-biotin (Figure 14a). After cell lysis, biotinylated proteins were precipitated and the total expression and surface levels of GluA1 were detected through Western Blot analysis (Figure 14b). Remarkably, cLTD treatment promoted a significant decrease in surface GluA1 (78 ± 1% SEM compared to the control), which could be rescued with CRT (124 ± 10% SEM compared to the control, Figure 14c). The data also show an enhanced PKD phosphorylation upon cLTD treatment indicating that PKD is activated downstream of NMDARs. In line with

the previous data (Figure 6), CRT treatment completely prevented NMDA-induced PKD activation (Figure 14d and e).

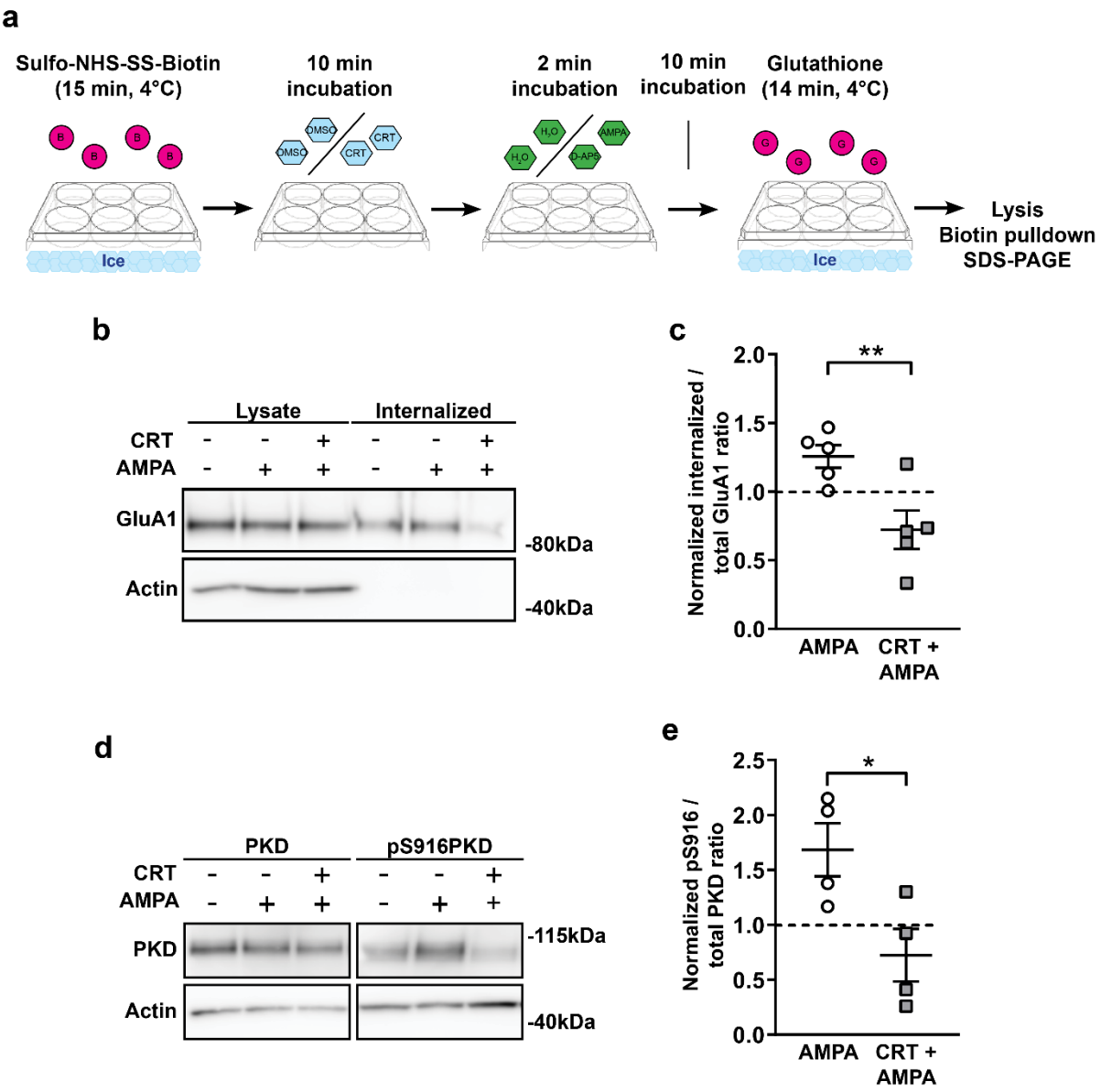

Figure 13 PKD activity is required for agonist-induced endocytosis of GluA1-containing AMPARs. (a) Scheme displaying the surface biotinylation assay for receptor internalization. After surface biotinylation, hippocampal neuronal cultures were treated with DMSO or CRT, followed by AMPA and D-AP5 application. Afterwards, cultures were incubated for 10 minutes to allow for AMPAR internalization, subsequently treated with glutathione to remove remaining surface biotin and further processed. Thereby, biotinylated GluA1 signal is detected only in endocytosed AMPARs, evoked by combined AMPA and D-AP5 treatment. **(b)** Representative Western Blots displaying total and internalized GluA1 levels. Actin served as a loading control and is absent from the precipitated samples. **(c)** Quantification of data shown in (b) using densitometry analysis. Data of the treated samples (AMPA and CRT plus AMPA) are presented as mean line density ± SEM normalized to the control (represented by the dotted line), each dot indicates one independent experiment. Statistical comparison was done by one-way ANOVA followed by Sidak's multiple comparisons test. **(d)** Representative Western Blots displaying total PKD and pS916PKD levels. Actin served as a loading control. **(e)** Quantification of data shown in (d) using densitometry analysis. Data of the treated samples (AMPA and CRT plus AMPA) are presented as mean line density ± SEM normalized to the control (represented by the dotted line), each dot indicates one independent experiment. Statistical comparison was done by one-way ANOVA followed by Sidak's multiple comparisons test. *p<0.05, **p<0.01.

GluA1 dephosphorylation at S845 is a prerequisite for the cLTD-induced endocytosis of AMPARs (Lee et al., 1998; Lee et al., 2003; Diering and Huganir, 2018). In line with the literature, cLTD treatment promoted a significant decrease in the level of pS845 GluA1 compared to the control. Notably, the NMDA-induced dephosphorylation of S845 was not blocked with CRT treatment (Figure 14f and g), suggesting that PKD acts further downstream.

Together, my data demonstrate that PKD activity is required for the endocytosis of GluA1-containing AMPARs during basal and activity-dependent trafficking.

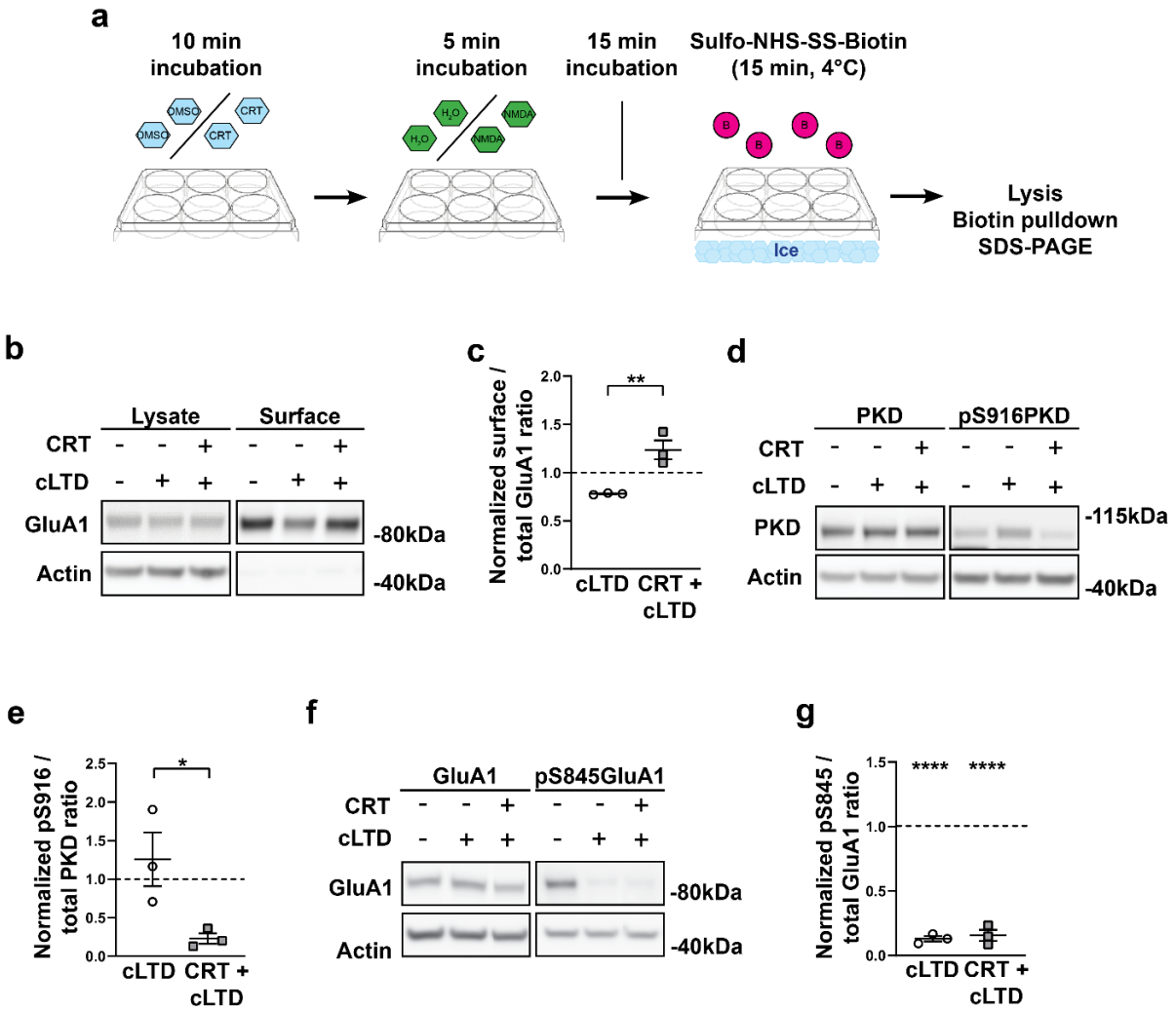

Figure 14 PKD activity is required for cLTD-induced AMPAR endocytosis. (a) Scheme displaying the cell surface biotinylation assay to detect the remaining surface GluA1 receptors after cLTD treatment. Hippocampal neuronal cultures were treated with DMSO or CRT followed by NMDA application. After the cLTD treatment, cultures were incubated for 15 minutes to allow for AMPAR internalization, subsequently surface biotinylated using sulfo-NHS-SS-biotin and further processed. **(b)** Representative Western Blots displaying total and surface GluA1 levels. Actin served as a loading control and is absent from the precipitated samples. **(c)** Quantification of data shown in (b) using densitometry analysis. Data of the treated samples (cLTD and CRT plus cLTD) are presented as mean line density ± SEM normalized to the control (represented by the dotted line), each dot indicates one independent experiment. Statistical comparison was done by one-way ANOVA followed by Sidak's multiple comparisons test. **(d)** Representative Western Blots displaying total PKD and pS916PKD levels. Actin served as a loading control. **(e)** Quantification of data shown in (d) using densitometry analysis. Data of the treated samples

(cLTD and CRT plus cLTD) are presented as mean line density ± SEM normalized to the control (represented by the dotted line), each dot indicates one independent experiment. Statistical comparison was done by one-way ANOVA followed by Sidak's multiple comparisons test. **(f)** Representative Western Blots displaying total GluA1 and pS845GluA1 levels. Actin served as a loading control. **(g)** Quantification of data shown in (f) using densitometry analysis. Data of the treated samples (cLTD and CRT plus cLTD) are presented as mean line density ± SEM normalized to the control (represented by the spotted line), each dot indicates one independent experiment. Statistical comparison was done by one-way ANOVA followed by Sidak's multiple comparisons test. *p<0.05, **p<0.01, ****p<0.0001.

3.4 PKD phosphorylation of Rabaptin-5 and subsequent Rab5 activation: a regulatory mechanism for AMPAR endocytosis?

3.4.1 PKD-mediated phosphorylation of Rabaptin-5 promotes Rab5 activation in HEK293T cells

Once the role of PKD in basal and activity-mediated AMPAR endocytosis had been described, I decided to characterize the molecular pathway by which the kinase exerts its regulation. The small GTPase Rab5 has been described to control the fusion kinetics of plasma-derived endocytic vesicles with EEs in both basal and activity-mediated trafficking of AMPARs (Gorvel et al., 1991; Bucci et al., 1992; Hausser and Schlett, 2017; Szíber et al., 2017), and thus I decided to investigate whether PKD activity is required for proper Rab5 activation.

To this end, I first attempted to measure changes in fluorescence resonance energy transfer (FRET) efficiency in primary hippocampal neurons transfected with the Raichu-Rab5 FRET biosensor (Kitano et al., 2008). FRET is a distance-dependent physical process by which energy is transferred non-radiatively from an excited molecular donor fluorophore to an acceptor fluorophore by means of intermolecular long-range dipole-dipole coupling, which can provide an accurate measurement of molecular proximity at angstrom distances. The fraction of energy absorbed by the donor molecules that is in this way transferred to acceptor molecules represents the FRET efficiency (Sekar and Periasamy, 2003; Kedziora and Jalink, 2015). The Rab5-Raichu probe comprises the modified yellow fluorescent protein called Venus, the amino-terminal Rab5-binding domain of EEA1, the modified cyan fluorescent protein SECFP and Rab5; and is designed so that an increase in Rab5-GTP results in elevated FRET efficiency (Kitano et al., 2008). Hippocampal neuronal cultures were transfected with the Raichu-Rab5 probe overnight. Then, cells were changed into a buffer resembling a cellular extracellular solution, treated with DMSO or CRT for 10 minutes, and then either cLTD-induced or added empty buffer as a control. According to my hypothesis, PKD activity is necessary for Rab5 activation, which in turn leads to AMPAR endocytosis, and thus I expected a decrease in FRET efficiency in CRT-treated cells compared to control cells. Likewise, in cLTD-induced cells, FRET efficiency should be higher than in the control, and pre-treatment

with CRT should completely block the effect. Unfortunately, this combination of technique and treatments repeatedly led to a very high neuronal death rate (data not shown), thus making it impossible to accurately gather data.

In order to surmount this obstacle, I decided to look for downstream substrates of PKD which could be involved in Rab5 regulation. The scaffolding protein Rabaptin-5 is a PKD substrate implicated in the regulation of integrin receptor trafficking in cancer cells (Christoforides et al., 2012). Interestingly, Rabaptin-5 forms a complex with Rabex-5, a Rab5-GEF, and Rab5, thereby promoting Rab5 activation during endocytosis (Horiuchi et al., 1997; Zhang et al., 2014). In the light of these studies, I investigated whether PKD-mediated phosphorylation of Rabaptin-5 is required for Rab5 activity. To this end, I initially performed an effector pulldown assay using HEK293T cells transiently transfected with plasmids encoding EGFP-Rab5 (Rab5, GFP-Rab5 wild type) in combination with either EGFP as a control or EGFP-Rabex-5 (Rabex-5, GFP-Rabex5) together with EGFP-Rabaptin-5 wild type (Rabaptin-5 wt, pEGFP-C3-Rabaptin 5 wt) or EGFP-Rabaptin-5 S407A (Rabaptin-5 S/A, pEGFP-C3-Rabaptin 5 S407A Figure 15a). In the latter case, the PKD-specific phosphorylation site of Rabaptin-5 has been exchanged for an alanine, preventing upstream phosphorylation. In line with other reports (Stenmark et al., 1995; Horiuchi et al., 1997; Zhang et al., 2014), I detected an increased level of active Rab5 in cells expressing Rabaptin-5 wt compared to the control cells. By contrast, the phosphodeficient Rabaptin-5 S407A mutant was not able to promote Rab5 activity when compared to control cells (Figure 15b).

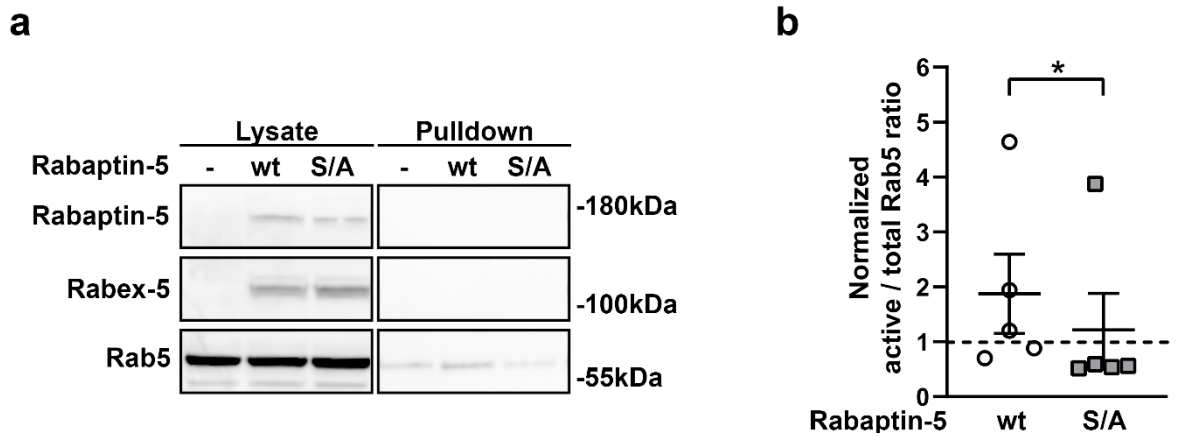

Figure 15 PKD regulates Rab5 activity in HEK293T cells through Rabaptin-5 phosphorylation. (a) Representative Western Blot obtained from HEK293T cells expressing Rab5 in combination with either EGFP (-), Rabex-5 and Rabaptin-5 wt (wt) or Rabex-5 and Rabaptin-5 S407A (S/A); displaying total and active Rab5 levels. Active Rab5 was precipitated from the cell lysates using GST-tagged Rabaptin-5:Rab5 binding domain bound to glutathione sepharose beads. **(b)** Quantification of data shown in (a) using densitometry analysis. Data of the wt and S/A samples are presented as mean line density ± SEM normalised to the control (represented by the spotted line), each dot indicates one independent experiment. Statistical comparison was done by unpaired two-tailed t-test. *p<0.05.

Next, I set out to investigate whether PKD activity also promotes phosphorylation of Rabaptin-5 S407 in neuronal cells. To achieve this, I employed a polyclonal antibody specific for the phosphorylated substrate motif of PKD (PKD pMOTIF) which had already been described to detect phosphorylated Rabaptin-5 (Christoforides et al., 2012). Initially, the specificity of the antibody was tested by Viktória Szentgyörgyi and Gisela Link, who treated HEK293T cells overexpressing either Rabaptin-5 wt or Rabaptin-5 S407A for 15 minutes with 1 µM phorbol 12,13-dibutyrate (PdbU), a potent PKD activator, or its vehicle DMSO. After cell lysis, the levels of total and phosphorylated Rabaptin-5 S407 were analysed via Western Blot. Whilst very faint, the signal for the phosphorylated Rabaptin-5 is visible in those cells overexpressing Rabaptin-5 wt and becomes stronger upon PdbU treatment, while it is not present in those overexpressing the Rabaptin-5 S407A mutant, regardless of PdbU addition (Figure 16a). Subsequently, I induced cLTD on neuronal hippocampal cultures that had been previously treated with CRT or DMSO and lysed the cells. A fraction of that lysate was kept to analyse the total level of Rabaptin-5 and the rest was used to enrich for Rabaptin-5 via immunoprecipitation. Later, the total Rabaptin-5 and the phosphorylated levels of Rabaptin-5 S407 were measured using the total and immunoprecipitated lysates, respectively, via Western Blot analysis. Disappointingly, a specific signal for phosphorylated Rabaptin-5 could not be detected in neuronal cells (Figure 16b). This, combined with the fact that the signal obtained in HEK293T cells overexpressing Rabaptin-5 was already very weak, points to neuronal expression of Rabaptin-5 being too low for the PKD pMOTIF antibody to be able to recognise the phosphorylated protein fraction, even after enrichment via immunoprecipitation.

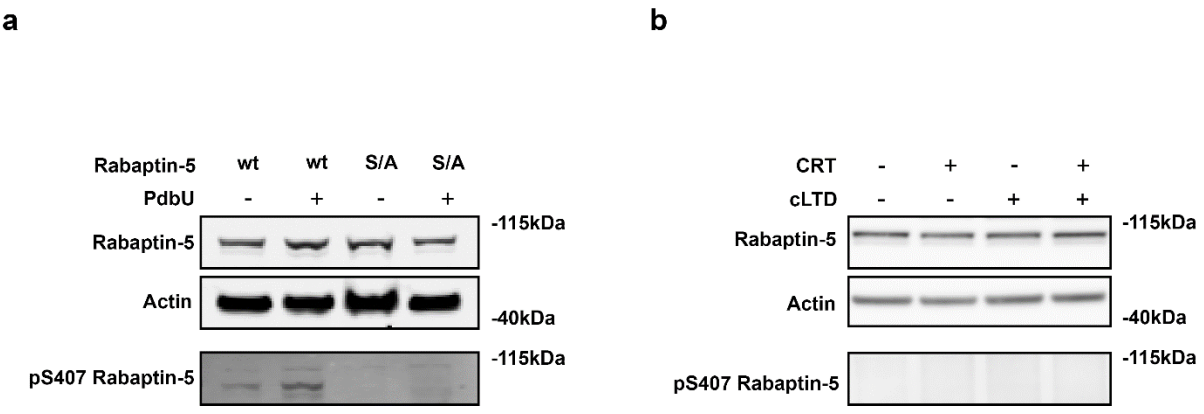

Figure 16 PKD activation promotes an increase in Rabaptin-5 S407 phosphorylation in HEK293T cells. (a) Representative Western Blot obtained from HEK293T cells expressing Rabaptin-5 wt (wt) or Rabaptin-5 S407A (S/A) treated with 1µM PdbU or DMSO for 15 minutes, displaying total and phosphorylated Rababaptin-5 levels. Actin served as a loading control. Experiment was conducted by Gisela Link and Viktoria Szentgyörgyi. **(b)** Representative Western Blot obtained from cLTD-induced or control primary hippocampal neurons following treatment with CRT or DMSO for 10 minutes, displaying total and phosphorylated Rababaptin-5 levels. Actin served as a loading control.

All together, these results demonstrate that PKD-mediated phosphorylation of Rabaptin-5 at S407 promotes activation of Rab5 in HEK293T cells.

3.4.2 Phosphorylation of Rabaptin-5 on its S407 is required for NMDAR-cLTD endocytosis

While direct proof that PKD phosphorylates Rabaptin-5 on S407 in neurons could not be found, I decided to explore next whether phosphorylation of Rabaptin-5 on S407 (presumably by PKD) is required to promote synaptic removal of AMPARs upon NMDA-cLTD. For this, I incubated neuronal hippocampal cultures transfected with plasmids encoding EGFP, Rabaptin-5 wt or Rabaptin-5 S407A with an antibody that detects the extracellular N-terminal domain of GluA1 after the induction of cLTD. Subsequently, cells were fixed and incubated with a fluorescently labelled secondary antibody, followed by fixation, permeabilization and immunostaining for Shank2 (Figure 17a). When analysing GluA1 intensity in Shank2-positive areas of untreated cells, I observed a significant increase in this value in the Rabaptin-5 S407A expressing cells in comparison with the EGFP control cells. This, however, was not the case for the untreated Rabaptin-5 wt expressing condition, and clearly indicates that expression of the phosphodeficient mutant of Rabaptin-5 increases synaptic GluA1 surface levels under basal conditions. Most importantly, cLTD treatment induced a significant decrease in GluA1 intensity within the synaptic areas both upon EGFP and Rabaptin-5 wt overexpression but not in Rabaptin-5 S407A expressing neurons (Figure 17b).

The data thus provide evidence that phosphorylation of Rabaptin-5 at S407 is necessary for GluA1-containing AMPAR endocytosis in response to NMDAR-cLTD, a mechanism likely to be regulated by PKD in neurons as well.

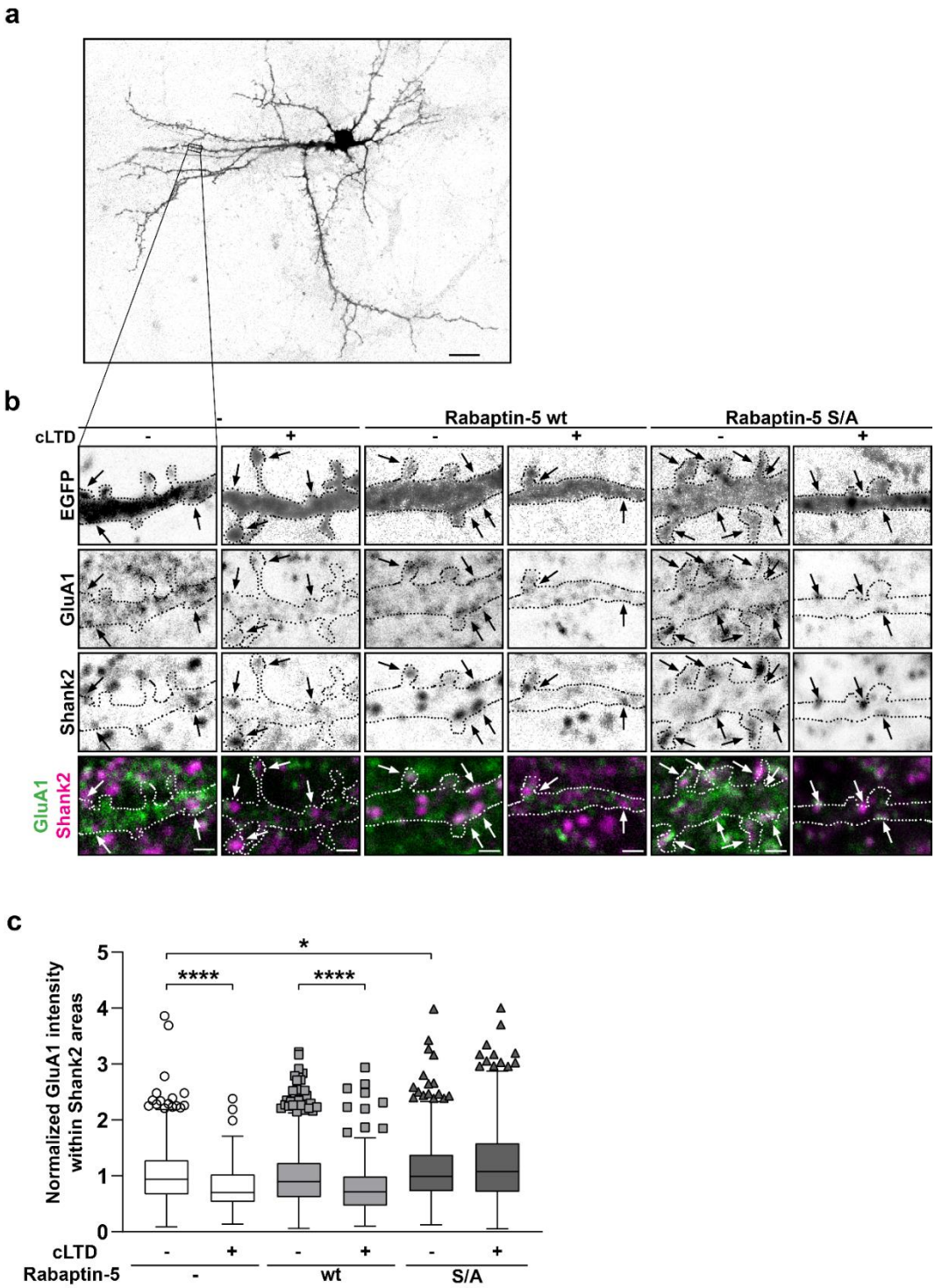

Figure 17 PKD-mediated phosphorylation of Rabaptin-5 is required for cLTD-induced AMPAR endocytosis. **(a)** Representative inverted single-channel picture of an EGFP-expressing neuron. Scale bar 20 µm. **(b)** Representative inverted single-channel and coloured merged pictures of neuronal dendritic branches expressing EGFP, EGFP-tagged Rabaptin-5 wild-type (EGFP-Rabaptin-5 wt) or EGFP-tagged Rabaptin-5 S407A (EGFP-Rabaptin5 S/A), treated with sterile water (-) or NMDA. Cells were then stained for the extracellular domain of surface GluA1 and the PSD marker Shank2. Arrows point to Shank2-positive PSD areas included in the analysis. The EGFP signal is not included in the coloured merged image. **(b)** Quantification of the images shown in (a). The boxplot shows the results of three independent experiments. Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. N=769, 385, 835, 323, 661 and 331 sample points. The significance of differences was analysed by a Kruskal-Wallis test followed by Dunn's multiple comparisons test. *p < 0.05, **p < 0.01, ****p < 0.0001. Scale bar 2 µm.

4. Discussion

As key players in the depolarization of the neuronal PM, AMPARs are central to maintain the physiology of the neuronal cell. In particular, AMPAR trafficking and the mechanisms regulating it have attracted a lot of attention from the scientific community, due to their direct impact on synaptic plasticity, learning and memory. Despite this, understanding of these mechanisms is still incomplete.

Since its discovery in 1994, the role of PKD as a regulator of multiple cellular processes has been intensively studied (Valverde et al., 1994). In addition to its modulation of general cell physiology, PKD presents specific functions in neuronal cells encompassing pain modulation (Zhu et al., 2008), regulation of the establishment and maintenance of neuronal polarity (Yin et al., 2008; Czöndör et al., 2009) or ROS-induced neuroprotection (Cabrera-Poch et al., 2004; Asaithambi et al., 2011; Asaithambi et al., 2014; Liliom et al., 2017). Excitingly, PKD has been recently described as a regulator of learning and memory through modulation of both LTP and LTD (Krueger et al., 2010; Bencsik et al., 2015; Wang et al., 2018), indicating that there still are undescribed PKD functions.

In this context, I wanted to investigate whether PKD activity regulates synaptic plasticity, learning and memory by controlling AMPAR trafficking. First, I demonstrated that up- and downregulation of PKD activity promote a decrease or an increase in the surface and synaptic amount of AMPARs, respectively. Subsequently, I showed that short-term inhibition of PKD affects AMPAR surface trafficking dynamics, and that PKD activity promotes endocytosis of AMPARs to EEs. Interestingly, PKD does not only act as a regulator of basal AMPAR endocytosis, but also of activity-mediated endocytosis, as I proved that PKD activity is necessary for the internalization of the receptor in response to activation of both AMPARs and NMDARs. Finally, I studied the effects of perturbed PKD signalling on Rab5 activity and AMPAR surface expression and propose that phosphorylation of the Rab5 interactor and downstream substrate of PKD Rabaptin-5 on S407, presumably by PKD, is necessary for both basal and activity-mediated endocytosis of AMPARs.

4.1 PKD activity regulates basal AMPAR endocytosis

In both non-neuronal and neuronal cells several pools of PKD are present at different subcellular localizations, which enables each of them interaction with a subset of locally available substrates thus ultimately determining the cellular response effected by PKD. Briefly, PKD is mostly present at the cytoplasm in resting cells, with a small pool present at the Golgi. However, an increase in the amount of DAG at the plasma or mitochondrial membranes in

response to specific stimuli can promote the translocation of PKD from the cytosol. Then, upon DAG- and PKC-mediated activation, PKD dissociates from the membrane and returns to the cytoplasm, being then able to enter the nucleus or to localize to the Golgi (Maeda et al., 2001; Rey et al., 2001; Hausser et al., 2002; Rozengurt et al., 2005; Li and Wang, 2014). As an example of this, nuclear PKD in neuronal cells is able to upregulate transcription of MEF2 downstream targets through phosphorylation and nuclear export of HDAC5 (Choi et al., 2015; Volmar and Wahlestedt, 2015; Jo et al., 2017), whereas at the mitochondria it acts as a neuroprotective agent through phosphorylation of HSP27 and through diminishing oxidative stress-mediated apoptosis in a NFκB-independent mechanism (Cabrera-Poch et al., 2004; Asaithambi et al., 2011; Asaithambi et al., 2014; Liliom et al., 2017) and at the PM as a modulator of inflammatory pain through its interaction with the TRPV1 receptor (Zhu et al., 2008).

While this work is, to the best of my knowledge, the first one to present PKD as a promoter of AMPAR endocytosis, my data is in line with that of other groups describing a role for PKD as a regulator of the secretory and endosomal trafficking of PM-targeted proteins in both non-neuronal and neuronal cells. In NIH 3T3 fibroblasts, PKD was described to strongly associate with αγβ3 integrin within endosomes upon primaquine treatment, a weak base which can accumulate at endosomes and neutralise their pH, thus inhibiting the recycling of endocytosed integral membrane proteins. Moreover, impairing PKD activity or preventing its association with αγβ3 integrin impaired PDGF-dependent short-loop recycling of αγβ3 integrin from EEs to the PM (van Weert et al., 2000; Woods et al., 2004). In hippocampal neuronal cells and in organotypic brain slices, the role of PKD as a regulator of secretory trafficking from the TGN has been described to be required for normal neuronal morphogenesis, specifically for dendrite growth, initiation and maintenance (Horton et al., 2005). Later, also in hippocampal neurons, PKD activity at the TGN was proposed to be essential for the integrity of the Golgi complex, the maintenance of the dendritic tree and the establishment and preservation of neuronal polarity (Yin et al., 2008; Czöndör et al., 2009). In agreement with this, the generation of Golgi outposts, usually localised at the main dendrite and contributing to dendritic growth and branch dynamics through the regulation of polarised secretory trafficking, was described by the Cáceres group to be dependent on PKD activation at the Golgi downstream of RhoA and Rock. PKD phosphorylates SSH1, thus leading to cofilin inhibition and to the fission of somatic Golgi derived tubules (Horton et al., 2005; Ye et al., 2007; Quassollo et al., 2015). Excitingly, using multi-electrode arrays, we and our collaborators recently demonstrated that acute chemical inhibition of PKD affects neuronal functionality, as firing activity and burstiness were increased in primary hippocampal cultures following impaired AMPAR endocytosis (Oueslati Morales et al., 2020).

Intriguingly, the Cáceres group also described that impairment of PKD activity through expression of kdPKD promotes an increased endocytosis of the plasma membrane proteins TfR and LRP in hippocampal neuronal cultures (Bisbal et al., 2008). While this data may initially appear contradictory to my own, it should be first taken into consideration that the two protocols employed to impair PKD activity are greatly different: on the one hand, the Cáceres group promoted overexpression of kdPKD for a day; on the other hand, this study employed short-term chemical inhibition of PKD activity. Furthermore, the effect observed by the Caceres group was dependent on the Golgi localization of kdPKD, because it was not detected after expression of a mutant devoid of Golgi binding (Bisbal et al., 2008). In contrast, basal and activity-dependent AMPAR endocytosis is a fast event that is likely to be regulated by a dendritic spine-localized PKD pool. In support of this, PKD has been localized at the postsynaptic region and periphery of dendritic spines (Bencsik et al., 2015). More importantly, it has been demonstrated that kdPKD expression causes a mis-sorting of TfR and LRP into axonal vesicles (Bisbal et al., 2008). Mis-sorting of basolateral cargo to the apical membrane has been also observed in polarized epithelial cells expressing kdPKD (Yeaman et al., 2004). Axonal proteins are regularly trafficked to both dendrites and axons and then rapidly endocytosed from dendritic branches (Sampo et al., 2003; Wisco et al., 2003). The increased endocytosis of TfR and LRP at the dendritic membrane could thus be an indirect effect of the mis-sorting of this cargo at the level of the Golgi complex. In sum, due to the many roles played by PKD in neuronal physiology, long-term inhibition of PKD activity is likely to affect, either directly or indirectly, several aspects of neuronal function, as is the case of the number and complexity of neurites (Czöndör et al., 2009). In contrast, the data obtained in this study point to PKD activity being directly implicated in the endocytosis of AMPARs, and the approach employed, due to the short-term nature of the inhibition, is much better suited for the study of fast changes in protein surface amount and less likely to promote additional effects (as shown in Figure 1c). Despite these differences, the work of Bisbal *et al* raises the question of what consequences a long-term inhibition of PKD activity has on AMPAR surface amount and trafficking. While the data presented in this work show that short-term block of PKD activity promotes a strong and fast increase in the amount of AMPAR present at the PM and at post-synaptic sites, it is likely that long-term inhibition of PKD activity would impair its role as a regulator of vesicle fission and cargo sorting at the TGN (Liljedahl et al., 2001; Hausser et al., 2005) and as a modulator of endosomal protein recycling (Woods et al., 2004). When these works are put together, it becomes apparent that PKD could be influencing the *de novo* exocytosis, the endocytosis and the recycling of AMPARs. It is thus tempting to speculate that the combined effects of the impairment of *de novo* exocytosis and recycling following long-term inhibition of PKD could compensate the impairment of endocytosis on the surface amount of AMPARs, thus promoting a net decrease in the amount of AMPARs at the PM and PSDs.

However, experiments directed at studying AMPAR surface levels and trafficking following long-term chemical inhibition of PKD or expression of kdPKD need to be performed in order to obtain the answer to this question.

The fact that PKD could be involved in the regulation of all steps of the trafficking of a specific protein, such as the AMPARs, could also give some additional context to my observations of the increase in the recovery half-time of the bleached GluA1-SEP signal within dendritic spines following acute inhibition of PKD activity. While this data suggests that PKD acts as a regulator of AMPAR surface dynamics through the regulation of AMPAR endocytosis, the work of Woods *et al* also suggests that PKD may do so through the regulation of the recycling of the receptor (Woods et al., 2004). Interestingly, the prominent “PSD slot” hypothesis offers a way to reconcile these two processes based on the highly dynamic trafficking AMPARs present, as well as on the continuous exocytosis, endocytosis and lateral diffusion among its multiple cellular pools: the PSD contains “slots” that may be occupied by AMPARs supplied by lateral diffusion from the perisynaptic region, a pool that in turn is replenished by exocytosis of the AMPARs present in the endosomal pool. Eventually, AMPARs diffuse out of the PSD and are endocytosed at the perisynaptic region, returning to the endosomal compartment and closing the circle (Ashby et al., 2006; Makino and Malinow, 2009; Opazo et al., 2012; Henley and Wilkinson, 2013; Diering and Huganir, 2018). Towards this end, a biotinylation assay of AMPAR endocytosis and recycling could be performed on primary hippocampal neurons to find out whether the effect of PKD activity on AMPAR surface trafficking is due solely to regulation of its endocytosis or of its recycling as well (Ehlers, 2000). Additionally, the functional consequences of acute PKD inhibition could be further explored by performing acute PKD inhibition in mouse-derived organotypic brain slices followed by the study of neuronal excitability and burstiness of Schaffer collaterals.

The use of small-molecule inhibitors, such as CRT, presents numerous advantages in addition to precise temporal control of the inhibition. These include high penetration across the whole cell population, ease of use, portability among multiple systems and control of the extent of the inhibition through modulation of the concentration used (via titration experiments). However, its use is not without disadvantages either, namely, off-target effects caused by inhibition of proteins with a similar conformation to the specific target (an issue especially acute for kinase inhibitors) or the challenge that constitutes their development for proteins that do not have a well-defined substrate (proteins other than kinases, phosphatases or receptors) and instead perform their role through protein-protein interactions (as is the case for scaffolding proteins or transcription factors) (Weiss et al., 2007; Gurevich and Gurevich, 2015). Thus, while CRT has been described as a specific PKD inhibitor (Harikumar et al., 2010), it would be interesting to inhibit PKD activity through an alternative method that allows for

corroboration and expansion of the results described here while also overcoming the limitations of small-molecule inhibitors.

However, most of the commonly used techniques to perform knock-down of a specific protein (such as RNAi, CRISPR/Cas9 or expression of a dominant negative mutant of the protein of interest) are not suitable for acute inhibition, as they involve complex protocols and require time to downregulate protein activity. Excitingly, small-molecule-induced selective protein degradation is a relatively novel field which constitutes an attractive approach to elucidate the function of a protein or study a promising therapeutical target in many disorders. While numerous systems to achieve this have been described [for more information, please see (Burslem and Crews, 2017)], I will focus here on the current prevailing approach of the proteolysis-targeting chimera (PROTAC), as well as on other promising alternatives for the near future. PROTAC constitutes a relatively novel post-translational protein degradation system that allows for the hijacking of the ubiquitin-proteasome system, promoting the degradation of specific target proteins. Briefly, an E3 ligase ligand is bound through a variable chemical linker to a targeting element of the protein of interest, promoting the ectopic ubiquitination of the target and its degradation at the proteasome. Encouragingly, PROTACs function against a wide number of protein classes in multiple subcellular localizations, including the cytosol, the nucleus and the plasma membrane; with some specific E3 ligands, due to their restricted localization, allowing for degradation only in specific compartments. Moreover, they constitute promising therapeutic drugs for CNS disorders, as they can be designed to penetrate the blood-brain barrier (Arvinas, 2019; Zhang et al., 2019). Thus, PROTAC technology works in an analogous manner to RNAi, albeit targeting the protein directly rather than the mRNA molecule, and constitutes a very powerful tool, as it combines the advantages of small-molecule inhibitors with those of the gene silencing and editing techniques. Moreover, the development of the HaloPROTAC and dTAG systems removes the need to develop a specific PROTAC for the protein of interest, as any protein covalently bound to either the HaloTag or the F36V FKBP protein can be specifically targeted and degraded (Burslem and Crews, 2020; Ding et al., 2020). Based on this, mouse embryos could be genetically engineered via CRISPR/Cas9 to express a transgenic version of PKD that is covalently bound to one of the aforementioned tags. Once the new transgenic line has been sufficiently expanded, primary hippocampal neurons could be harvested from it, and the effect of acute PKD inhibition in AMPAR trafficking (or any other physiological process) could be analysed upon addition of the corresponding PROTAC. Moreover, by genetically engineering a single isoform of PKD, potential isoform-specific functions could also be investigated.

Despite the many advantages offered by this booming field, it is worth keeping in mind that it is not without its limitations: PROTACs tend to present low solubility and poor cell permeability,

they are restricted by the small pool of E3 ligases currently used and are not very effective at degrading extracellular proteins, protein aggregates or proteins with expanded repeat sequences, a common trait of neurodegenerative disorders (Ding et al., 2020) (Burslem and Crews, 2020). To overcome these limitations, novel concepts have recently emerged that attempt to harness the cellular lysosomal system, namely the lysosome-targeting chimera (LYTAC), the autophagy-targeting chimera (AUTAC) and the autophagosome-tethering compound (ATTEC). In short, LYTAC molecules consists on a specific antibody targeting the protein of interest and a 20- or 90-mer of mannose-6-phosphatase covalently bound to the antibody, which promotes binding to the cation independent mannose-6-phosphatase receptor and subsequently degradation via the lysosome mechanism for glycosylated proteins. While the LYTAC technology enables targeting of both extracellular and membrane associated proteins and takes advantage of a ubiquitously expressed degradation pathway, LYTAC molecules are so large that they are not considered small-molecule drugs, and they are unable to promote degradation of cytosolic proteins (Banik et al., 2020). In contrast to the K48 polyubiquitination of PROTACs, AUTACs promote S-guanylation-mediated K63 polyubiquitination, leading to the degradation of the protein of interest via the autophagy pathway (Takahashi et al., 2019). Lastly, ATTECs take this concept a step forward by directly tethering the protein of interest to the key autophagosome protein LC3. Excitingly, ATTECs have been observed to cross the blood-brain barrier and have shown promising results in studies of HD and type III spinocerebellar ataxia. However, the AUTAC and ATTEC mechanisms require further study, as perturbation of global autophagy could lead to very deleterious consequences (Li et al., 2019; Ding et al., 2020; Li et al., 2020).

An additional open question left by my data is whether PKD regulates the endocytosis of all AMPAR tetramers, regardless of their subunit composition, or of only those containing the GluA1 subunit. AMPARs in the CNS are typically composed of either GluA1/2 or GluA2/3 dimers (Wenthold et al., 1996; Lu et al., 2009), with a small percentage being GluA1 homotetramers or GluA1/3 heterotetramers. The trafficking of these tetramers is regulated differently, and while basal PM insertion of GluA1-containing AMPARs is low in basal conditions and becomes enhanced during activity-mediated events (such as NMDAR activation), the GluA2 subunit largely determines constitutive AMPAR endocytosis and recycling, with GluA2/3 AMPARs undergoing fast constitutive cycling between the PSD and the endosomal compartment (Passafaro et al., 2001; Shi et al., 2001). GluA1/1 constitutes a special case, because their lack of a GluA2 subunit makes the channel permeable to calcium. It is currently suggested that GluA1/1 are mainly delivered to synapses during the first moments of potentiating synaptic plasticity events, possibly from recycling endosomes, and rapidly reinternalized and substituted with calcium impermeable AMPARs (Hanley, 2014;

Moretto and Passafaro, 2018; Parkinson and Hanley, 2018). Notwithstanding these differences, the basic principles that underlie the trafficking of GluA1- and GluA2-containing subunits (e.g. location of the protein pools, molecular interactors, regulation via PTMs) are the same. Moreover, the basal trafficking of the GluA1/2 tetramer is determined by the GluA2 subunit, and I was able to demonstrate that PKD activity regulates basal endocytosis of GluA1-containing AMPARs. Therefore, this suggests that PKD activity regulates trafficking of all AMPAR heteromers. Nevertheless, to experimentally test this hypothesis, the data shown in this work would need to be complemented by similar biochemical and microscopy studies analysing GluA2 trafficking.

4.2 PKD activity is necessary for activity-mediated AMPAR endocytosis

PTMs play a hugely relevant role in synaptic plasticity, with phosphorylation being to date the best described among them. It is therefore not surprising that several kinases have been implicated in the induction, expression and modulation of synaptic plasticity through their interactions with key players in this process, as is the case for PSD95, NMDARs and, importantly, AMPARs (Kim et al., 2007b; Lussier et al., 2015; Diering and Huganir, 2018). Briefly, in the context of LTP, phosphorylation of GluA1 S811 by PKC promotes AMPAR exocytosis from endosomes, while phosphorylation of GluA1 S831 is mediated by PKC in combination with CaMKII and promotes an increase in single channel conductance and trafficking of AMPARs to the PSD (Roche et al., 1996; Barria et al., 1997; Derkach et al., 1999; Lin et al., 2009; Ren et al., 2013). The increase in single-channel open probability and PM targeting of AMPARs following GluA1 S845 phosphorylation by PKA has similarly been well characterised (Banke et al., 2000; Ehlers, 2000; Man et al., 2007). These kinases and phosphorylation sites also modulate LTD and synaptic downscaling: CaMKII phosphorylation of GluA1 S567 and PKC phosphorylation of GluA2 S880 in response to LTD-inducing stimuli promote AMPAR removal from the PSD and modulate its membrane trafficking, while dephosphorylation of GluA1 S831 increases the rate at which AMPARs can dissociate from the PSD scaffold, and dephosphorylation of GluA1 S845 is required for the removal of CP-AMPARs from the PSD during LTD (Lu et al., 2010; Coultrap et al., 2014; Sanderson et al., 2016; Diering and Huganir, 2018). A further role regulated by the GluA1 S831 and S845 sites was proposed in a study using knock-in mice with prevented phosphorylation of GluA1 S831 and S845. These animals presented reduced object and spatial working memory capacity, as well as reduced spatial long-term memory, which points to a role of these PTM sites in the regulation of the information load (Olivito et al., 2016; Diering and Huganir, 2018).

In accordance with these studies, in this work I observed that acute PKD inhibition promotes an increase in the phosphorylation level of GluA1 S831, suggesting that PKD activity is involved in the dephosphorylation of this site. Intriguingly, the mechanisms by which the GluA1 S831 site is dephosphorylated have not been clarified yet. By contrast, the dephosphorylation of GluA1 S845 is much better described: during LTD, dephosphorylation of GluA1 on S845 is mediated by calcineurin, while during homeostatic scaling down decreased GluA1 phosphorylation on S845 is mediated by a decrease in the targeting of its corresponding kinase, PKA, through uncoupling with the AKAP5 scaffold (Diering et al., 2014; Sanderson et al., 2016). In the light of this data, it can be hypothesized that PKD activity regulates the dephosphorylation of GluA1 on S831 through phosphatase activation [PKD is a well-known regulator of phosphatases such as SSH1 or SH2-containing protein tyrosine phosphatase-1 (Olayioye et al., 2013; Ishikawa et al., 2016)] or via promoting the uncoupling of PKC and CaMKII with the PSD. To elucidate this latter point, the co-localisation of these kinases with the PSD could be analysed via fluorescent microscopy following acute inhibition of PKD activity. However, the detailed study of protein localisation within dendritic spines requires a level of resolution greater than the one provided by conventional confocal microscopy, therefore, super-resolution microscopy studies must be conducted in order to answer this question. Alternatively, it can be hypothesized that PKD activity could indirectly impair PKC/CaMKII-mediated phosphorylation of GluA1, and thus we observe an increase in pS831 GluA1 following PKD inhibition. Finally, it must be considered that the increase in GluA1 phosphorylation may not be a consequence of impaired PKD activity, but rather of the impaired endocytosis *per se*, as this would increase its prevalence at the membrane and consequently also its phosphorylation level.

Importantly, PKD activity has not only been related to the downregulation of synaptic transmission and plasticity through the regulation of the PTMs of AMPARs. mGluR-LTD is a form of synaptic plasticity induced at excitatory synapses which promotes a reduction in synaptic strength and is relevant for learning and memory (Collingridge et al., 2010). Interestingly, PKD activity was described to be involved in this process, as in hippocampal neuronal cultures and brain slices derived from rat, activation of mGluR5 using the group I specific agonist DHPG resulted in increased phosphorylation of PKD on its autocatalytic site in a mechanism dependent on PLC and PKC (Krueger et al., 2010). Later, this PKD function was further described by the Yu group, as they observed that PKD activation downstream of DHPG promotes the phosphorylation of the surface remaining NMDARs on their C-termini, thus downregulating their activity (Fang et al., 2015; Wang et al., 2018). Additionally, the involvement of PKD in the endocytosis of AMPARs upon NMDAR-cLTD induction could be demonstrated in a functional manner, as MEA studies performed in primary hippocampal

neurons showed that inhibition of PKD activity partially impaired the reduction in network activity promoted by NMDA treatment (Oueslati Morales et al., 2020). Moreover, Quassollo *et al* demonstrated that PKD activity is necessary for the generation of Golgi outposts, important platforms that regulate the delivery of receptors to synapses (Quassollo et al., 2015), and in a recent study, the Chen group performed behavioural studies on *Fld* mice (a strain characterised by a deficiency of the Lpin1 gene, a generator of DAG and upstream activator of PKD), showing that PKD acts as a regulator of synaptic plasticity through the activation of the ERK and CREB pathways, as these mice present cognitive impairment in hippocampal-based tasks and a reduced synapse number (Shang et al., 2020).

The downregulation of synaptic transmission and plasticity could also be modulated by PKD in an indirect manner, as some of its downstream substrates have been implicated in the control of AMPAR endocytosis. PKD-mediated phosphorylation of RIN1, a negative regulator of memory stabilisation during amygdala-related fear learning and experience-mediated fear extinction, has been described to promote activation of the Abl kinases. As they, in turn, control actin cytoskeletal remodelling (a key component mediating the morphological changes necessary for the expression of synaptic plasticity), PKD was suggested to regulate the structural changes that occur during synaptic plasticity through RIN1 phosphorylation (Dhaka et al., 2003; Hu et al., 2005; Bliss et al., 2010; Ziegler et al., 2011; Szíber et al., 2017). Another relevant substrate is Kidins220/ARMS, a membrane scaffold protein involved in the modulation of multiple aspects of the neuronal physiology. Kidins220 presents multiple phosphorylation sites, such as the PKD site S919, and the PTM state of this protein determines its interactions with specific target proteins. Excitingly, it was shown that Kidins220 overexpression or knockdown in rat organotypic brain slices caused reverse changes in GluA1 expression and in the amplitude of AMPAR-mediated excitatory post-synaptic currents. Furthermore, basal synaptic transmission of hippocampal slices prepared from 1 month old ARMS +/- mice was slightly increased, which pointed to the association of Kidins220 with GluA1, and to the regulation of GluA1 PTM state and localisation (Cabrera-Poch et al., 2004; Arévalo et al., 2010; Scholz-Starke and Cesca, 2016).

Strikingly, PKD has not only been described as a regulator of AMPAR endocytosis and LTD, but also synaptic potentiation in the form of LTP, as Bencsik *et al* demonstrated that PKD activity is necessary for the actin stabilisation and enlargement of dendritic spines through the inactivation of the cofilin phosphatase SSH1 (Eiseler et al., 2009; Olayioye et al., 2013; Bencsik et al., 2015). In this same line, it has been suggested that the interaction between Kidins220 and GluA1 could regulate LTP, as this process was upregulated in hippocampal Schaffer collaterals – CA1 synapses in young ARMS +/- mice. Furthermore, hippocampal slices from ARMS+/- mice and Kiddins220-depleted hippocampal neurons showed an

increase in the phosphorylation of two critical serines for LTP induction, S831 and S845 (Lee et al., 2003; Arévalo et al., 2010; Lee et al., 2010). Lastly, Kidins220 (and therefore PKD) could also potentially modulate LTP through its interaction with the NMDAR subunits NR1, NR2A and NR2B, as NMDARs are critically involved in LTP induction (López-Menéndez et al., 2009; Scholz-Starke and Cesca, 2016).

While it may seem initially puzzling that the same kinase regulates these antagonistic processes, some factors could explain how PKD acts as a general regulator of synaptic plasticity by modulating both LTP and LTD. On the one hand, the current understanding of the pathways linking PKD to the regulation of synaptic plasticity is incomplete, as a direct link between the Kidins220-GluA1 complex and LTP has not been described yet, similarly to the actual consequences of the interaction between Kidins220 and NMDARs. On the other hand, it must be considered that PKD is activated downstream of a myriad of different stimuli, which determine the final subcellular localization of PKD, the downstream substrates it can interact with and, ultimately, the role it plays. As LTP and LTD are not induced by the same stimuli, it seems unlikely that the role performed by PKD in these processes is the same. Despite these remarks, it is clear that further studies in this area must be performed in order to understand the specific molecular cascades through which PKD modulates both processes.

4.3 Phosphorylation of Rabaptin-5 on S407 is necessary for Rab5 activation and AMPAR endocytosis

As a promoter of the fusion between endocytic vesicles and early endosomes, Rab5 has been previously described as a critical regulator of both constitutive and activity-dependent AMPAR endocytosis (Brown et al., 2005; Glebov et al., 2014), thus playing an essential role in the regulation of synaptic transmission and in the expression of LTD. Consequently, loss or inhibition of the Rab5 effector protein EEA1 resulted in an increase in the surface amount of GluA1-containing AMPARs (Selak et al., 2006; Xu and Pozzo-Miller, 2017).

Rab5 activation *in vivo* has been described to take place via binding to Rabex-5 and Rabaptin-5 (Stenmark et al., 1995; Horiuchi et al., 1997; Zhang et al., 2014). Rabex-5 acts as a GEF protein for multiple Rabs, including Rab5, Rab17 and Rab21 (Delprato and Lambright, 2007; Mori et al., 2013), while Rabaptin-5 constitutes a scaffolding protein conformed by four coiled-coil domains, serving as a molecular bridge between the multiple trafficking events that take place within the cell thanks to its ability to bind multiple interaction partners. The binding to GTP-bound Rab5, recruitment to early endosomes and participation in protein endocytosis is mediated by its CTD, while its middle region allows interaction with the GGA (Golgi-localized,

gamma-ear containing, ADP-ribosylation factor binding) proteins, that bind ARF and act as effectors in TGN-modulated tethering and fusion processes (Mattera et al., 2003; Zhu et al., 2004)]. Lastly, through its NTD, Rabaptin-5 is able to interact with Rab8 and with Rab4, suggesting that it could be recruited to secretory vesicles and REs, respectively (Vitale et al., 1998; Brown et al., 2007; Omori et al., 2008; Hausser and Schlett, 2017). Intriguingly, as Rabaptin-5 interacts with both Rabs and Arfs, it has been speculated that it could promote tethering and fusion of early endosomes with TGN-derived vesicles (Zhang et al., 2014).

Rab4 is typically localized at REs, where it regulates cargo recycling (Van der Slujis et al., 1992; Wandinger-Ness and Zerial, 2014), but, remarkably, it was also observed at EEs (Ehlers, 2000; Sönnichsen et al., 2000). In neuronal cells, Rab4 regulates dendritic spine maintenance and constitutive AMPAR recycling from sorting endosomes (Ehlers, 2000; Brown et al., 2007; Gu et al., 2016). Interestingly, Rab4 and Rabex-5 are able to recruit Rabaptin-5 to EEs, which in turn leads to Rab5 activation (Kälin et al., 2015). In the light of this evidence, I speculate that activation of Rab5 by Rabaptin-5 can take place through two different mechanisms: 1) Rabaptin-5 directly binds Rabex-5 and Rab5 or 2) Rabaptin-5 interacts firstly with Rab4 and Rabex-5 and subsequently with Rab5; with both pathways leading to the induction of endocytosis.

Importantly, PKD has been described as an upstream activator of Rabaptin-5 through phosphorylation of S407 in breast cancer cells, where this interaction was seen to regulate integrin recycling from EEs in a Rab4-dependent manner (Woods et al., 2004; Christoforides et al., 2012). This first evidence that PKD could be implicated in the endosomal trafficking of PM proteins through its substrate Rabaptin-5 is not only in line with my own observations in this work, where I show that i) phosphorylation of Rabaptin-5 on S407, the PKD phosphorylation site, regulates both the basal and activity-mediated level of AMPARs on the PSD, and ii) expression of caPKD leads to an increased co-occurrence between EEA1 and GluA1 at EEs; but also with other work supporting a role for Rabaptin-5 in ion channel endocytosis and cognitive development. Ninkovic *et al* demonstrated that a slight increase in the surface amount of Kv10.1, a potassium channel involved in the control of neuronal excitability, can be observed in HEK293T following complete Rabaptin-5 knockdown (Ninkovic et al., 2012); and the Matalon group proposed, in a study performed on a phenylketonuria mouse model, that the cognitive declined associated with this metabolic disease is partially due to decreased expression levels of Rabaptin-5 (Surendran et al., 2005). In view of this evidence, I attempted to obtain direct proof that PKD promotes Rab5 activation in neuronal cells through two different approaches, first via FRET measurement of the Rab5-Raichu probe following treatment with CRT and/or NMDA, and later through the quantification of the phosphorylated Rabapin-5 fraction in response to PKD inhibition and/or cLTD induction using

a PKD pMOTIF antibody (Christoforides et al., 2012). However, evidence that Rabaptin-5 is a PKD substrate in neuronal cells is still missing. In order to address this, the development of a phosphospecific antibody for Rabaptin-5 S407, with a higher specificity for the site than the PKD pMOTIF has, would prove very useful. Moreover, the level of active Rab5 in hippocampal neuronal cultures treated with CRT and/or NMDA could be analysed via immunofluorescence. Additionally, changes in the level of Rab5 activity could also be analysed via FLIM-FRET in neurons expressing the Rab5-Raichu probe following activity-mediated AMPAR endocytosis and/or PKD inhibition, as this technique presents several benefits when compared with classical FRET, including reduced phototoxicity (Ishikawa-Ankerhold et al., 2012).

Another open question relates to the precise localisation of PKD and Rabaptin-5 within dendritic spines. While a general knowledge of their intracellular localisation has already been achieved, the specific localisation of their interaction and regulation of endosomal trafficking is not known yet. Rabaptin-5 association with Rab5 and Rab4 at EEs and REs, respectively, could follow its phosphorylation by PKD at the PM. Another possibility would be that PKD activates Rabaptin-5 locally, associated at the vesicular membrane or it could take place within the cytoplasm of the spine. Furthermore, due to the ability Rabaptin-5 presents to interact with Rab8 through its NTD, and the fact that in HeLa cells PKD has been described as a promoter of Rab8 recruitment to Golgi membranes (Omori et al., 2008; Eisler et al., 2018), the possibility that PKD and Rabaptin-5 associate with Rab8 to direct the trafficking of secretory vesicles must also be addressed. To study the localisation of these proteins within dendritic spines, super-resolution microscopy studies must be carried out in neuronal cells overexpressing fluorescent PKD and Rabaptin-5 following the immunostaining of relevant markers of the endocytic system, such as Rab5, Rab4 and Rab8. In order to avoid the massive expression caused by the CMV promoter, new plasmids expressing PKD and Rabaptin-5 must be cloned using a specific neuronal promoter with a lower level of activity, such as synapsin (Chin et al., 1994). After the analysis of the localisation of the exogenous proteins, the study of the localisation of endogenous Rabaptin-5 and PKD could follow.

Finally, it is worth considering whether PKD acts as a specific promoter of AMPAR endocytosis, or by contrast, whether it is a general regulator of surface protein endocytosis in neuronal cells. Since Rab5 acts a general promoter of the fusion between plasma-derived endocytic vesicles and early endosomes in both neuronal and non-neuronal cells (Bucci et al., 1992; de Hoop et al., 1994; Hausser and Schlett, 2017), the PKD-Rabaptin-5-Rab5 axis could be involved in the trafficking of many surface proteins from the membrane to EEs. Interestingly, the Iglesias group showed that, in contrast to Kidins220, the cellular distribution of the neurotrophin receptors Trks and p75 remained unaltered in primary neurons expressing kdPKD (Sánchez-Ruiloba et al., 2006), thus pointing to the fact that the role of PKD as a

mediator of endocytosis is selective rather than general. Despite this, experiments addressing whether PKD activity regulates the surface levels of other proteins involved in synaptic transmission and plasticity, such as the NMDAR, must be performed to better understand this newly described function.

Based on the obtained data, I propose the following model on how PKD activity regulates the endocytosis of AMPARs: under basal conditions, endogenous PKD is active and promotes phosphorylation of Rabaptin-5 at S407, which leads to its interaction with Rab4 on early endosomes and the activation of Rab5 through Rabex-5. As a result, AMPARs are internalized from the synaptic membrane through Rab5-dependent endocytosis. Activity-mediated events activate AMPA and NMDA receptors, which promote an increase in the activity level of PKD thus leading to elevated internalization of AMPARs from the surface (Figure 18).

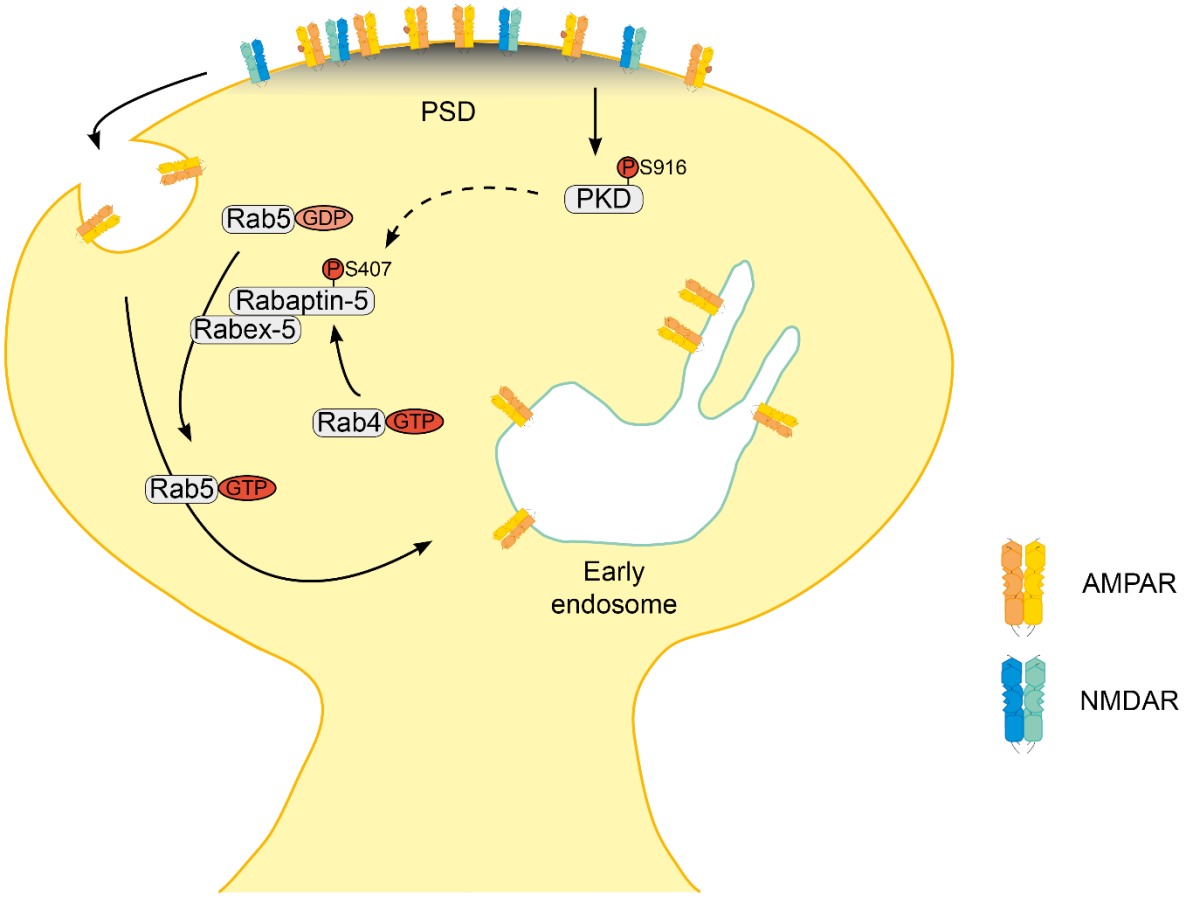

Figure 18 Proposed model of AMPAR endocytosis in response to PKD, Rabaptin-5 and Rab5 signalling. For details please refer to text. PSD: post-synaptic density.

4.4 AMPARs in neuronal diseases – a promising therapeutic target

Many of the neurological disorders that currently afflict human beings are caused by impaired synaptic function at the CNS. Impaired glutamate homeostasis and the typically associated neuronal death by excitotoxicity have important neuropathological consequences and have been associated to neurological diseases such as AD, HD, PD or epilepsy, among others (Iovino et al., 2020).

Similarly, as the main mediators of fast excitatory neurotransmission and synaptic plasticity, dysregulation of the normal AMPAR physiology plays a significant role in the development of these diseases. Indeed, animal models of AD, HD and PD all show deficits in synaptic transmission and plasticity, which carry as a result problems in learning, memory and motor function (Usdin et al., 1999; Picconi et al., 2005; Kolodziejczyk et al., 2014). The role of AMPARs in AD has been the best reported so far: the observed cognitive decline has been correlated with a decrease in AMPAR-mediated synaptic transmission in animal AD models and with synaptic loss in both animal models and human patients (DeKosky and Scheff, 1990; DeKosky et al., 1996; Penzes and VanLeeuwen, 2011). Moreover, the presence of Aβ plaques has been reported to promote AMPAR endocytosis, thus impairing basal and activity-mediated trafficking and also leading to synaptic loss (Hsieh et al., 2006; Ganesh et al., 2007). Interestingly, this increased endocytosis of AMPARs seems to involve the endosomal pathway, as hyperactivation of Rab5 and enlarged Rab5-positive early endosomes have been observed in mouse models and human AD patients (Cataldo et al., 2000; Ginsberg et al., 2010a; Ginsberg et al., 2010b; Ginsberg et al., 2011). Studies on HD have focused mostly on NMDARs due to their involvement in the death of striatal medium spiny neurons, a landmark trait of HD (Fan and Raymond, 2007). However, a decrease in the amount of AMPAR located at the PSD has been observed in both mouse models and post-mortem HD human brains, potentially via a kinesin-dependent mechanism that would impair microtubule-associated trafficking (Mandal et al., 2011; Fourie et al., 2014). Interestingly, a decrease in the levels of AMPAR in the striatum was similarly observed in post-mortem brains of PD patients, together with elevated levels of AMPAR expression and dephosphorylation in animal models of PD (Silverdale et al., 2010; Ba et al., 2011; Lee et al., 2016).

When all these lines of evidence are put together, it becomes apparent that AMPARs are promising therapeutical targets. Thus, effort was made to develop drugs capable of modulating AMPAR activity. AMPAkines, pharmacological agents with the ability to modulate AMPARs, initially gathered abundant interest due to their potential for treating cognitive decline via enhancement of excitatory glutamatergic neurotransmission (Ornstein et al., 2000; Baumbarger et al., 2001). While the interest in these compounds as a treatment against AD

has mostly waned due to the lack of positive results in phase III clinical trials (Chang et al., 2012), CX929 is still being investigated as a potential treatment against HD, promoting, among other effects, a rescue of synaptic plasticity and memory in a mouse model of HD (Simmons et al., 2009; Simmons et al., 2011). The viability of AMPAR antagonists as a treatment against PD has been explored, producing mixed results: on the one hand, they were shown to elevate the antiparkinsonian effects of levodopa and the resistance in levodopa-induced dyskinesia (Kobylecki et al., 2010); on the other hand, perampanel, a non-competitive AMPAR antagonist, did not improve levodopa-induced dyskinesias when given along levodopa to patients (Lees et al., 2012; Lee et al., 2016).

Epilepsy is characterized by excessive or hypersynchronous neural network activity that causes an imbalance between excitatory and inhibitory neurotransmission. Current drug treatments attempting to restore this disequilibrium are not always effective and present substantial side effects (Chaves and Sander, 2005; Thomas et al., 2006), therefore, other options such as AMPAR antagonists are being explored in both animal models (De Sarro et al., 2005) and clinical research in humans (Citraro et al., 2014; Hanada, 2014a). While research on competitive AMPAR agonists has been proven unsuccessful due to severe side effects and problems regarding their tolerability and pharmacokinetic profile, perampanel has been approved for treatment of epilepsy (Hanada et al., 2011; Hanada, 2014b; Lee et al., 2016).

Lastly, there are several lines of evidence indicating an altered AMPAR physiology in ASD, namely, the deletion of the gene encoding GluA2 (Ramanathan et al., 2004) or the observed decrease in AMPAR density in the cerebellum of post-mortem studied brains from ASD patients (Purcell et al., 2001). Moreover, in fragile X syndrome, mGluR5 signalling is upregulated, causing increased AMPAR internalization and deficits in synaptic plasticity (Nakamoto et al., 2007). To alleviate these symptoms, modulators of general excitatory neurotransmission and specific regulators of AMPARs have been studied during the last fifteen years. While these studies yielded some positive results (Doyle and McDougle, 2012; Silverman et al., 2013), the need for an effective way to modulate AMPAR physiology in ASD remains (Lee et al., 2016).

Excitingly, while not much research has been done yet to study the potential involvement of PKD in neurological diseases, two studies suggest that this kinase could be involved in the pathophysiology of, at least, PD and ASD. In an *in vitro* model of PD, PKD was activated during the early stages of oxidative damage and protected neurons from excitotoxicity and death (Asaithambi et al., 2014); whereas for ASD, spontaneous PKD mutations causing loss

of protein function have been reported in sporadic cases, pointing to them possibly constituting a risk factor for the disease (Matsumura et al., 2019).

In addition to its role as a neurotransmitter, glutamate has been described as a regulator of tumour development, by acting as a growth factor and signal mediator in numerous neuronal and non-neuronal tumours (Stepulak et al., 2014). Multiple studies have demonstrated that glutamate is secreted in a myriad of cancer types, both neuronal and non-neuronal (Takano et al., 2001; Seidlitz et al., 2009). Likewise, the expression of glutamate receptor subunits is not restricted to neoplastic cells with origin at the CNS, but they have been observed in numerous non-neural cancers as well (Stepulak et al., 2009). AMPARs, in particular, have been associated with tumour malignancy in several cancer subtypes, including glioblastoma multiforme (GBM) and pancreatic cancer (de Groot et al., 2008; Herner et al., 2011). Indeed, in GBM AMPARs are abundantly expressed, where they play the key role of enhancing the malignant phenotype of tumorous cells by mediating glutamate proliferative signals (Sontheimer, 2008; de Groot and Sontheimer, 2011). When these results are put together with the role of PKD as a tumour regulator, the possibility that AMPARs or PKD could be targeted as therapeutic targets for the treatment of different cancer subtypes becomes apparent. Remarkably, a study performed with human GBM cell lines recently showed that combinatorial inhibition of PKD and the AMPAR subunits GluA1/2 by treatment with CRT and talampanel, a non-competitive AMPAR antagonist, was able to decrease general cell viability, while specifically diminishing the number of cancer stem cells as well as their self-renewal efficiency (Föller, 2019).

These few studies present PKD as a potential therapeutical target for the treatment of neurological disorders, however, further research must be conducted to ascertain whether PKD regulation of AMPAR trafficking could be modulated in order to alleviate AMPAR mislocalization and the defects on synaptic plasticity. While a therapy based on the modulation of PKD activity may be a challenging task, due the ubiquitous expression of the kinase and the many processes it is involved in, a deeper understanding of the mechanisms regulating AMPAR trafficking alone would be invaluable for the development of future therapies.

5. Outlook

PKDs are expressed in neuronal cells from a very early embryonic stage, where they have been described to regulate multiple functions. Among other things, our lab was able to prove that PKD plays a role in the regulation of LTP, learning and memory through stabilisation of the actin cytoskeleton following plasticity-induced phenomena. Building upon those results, I further describe PKD as a modulator of synaptic plasticity, learning and memory based on its regulation of AMPAR endocytosis. Furthermore, I propose that PKD does so via phosphorylation of its substrate Rabaptin-5 on serine 407, promoting its interaction with Rab5 and Rabex-5 and ultimately leading to AMPAR endocytosis. These results open the door to further investigation into the role of PKD as: 1) a regulator of AMPAR trafficking and synaptic plasticity and 2) a regulator of general protein endocytosis. Considering that my results on AMPAR trafficking were obtained by studying the GluA1 subunit alone, biochemical and microscopy studies analysing the surface amount and trafficking dynamics of GluA2-containing AMPARs need to be performed before PKD can be established as a promoter of endocytosis of all AMPAR tetramer variants. Furthermore, patch-clamp experiments on dissociated hippocampal neurons and on organotypic slices must be performed to analyse the functional consequences of PKD inhibition at a single cell level and, importantly, direct proof that PKD phosphorylates Rabaptin-5 in neuronal cells needs to be obtained. Finally, a potential role of PKD as a general regulator of protein endocytosis should be addressed. For this, the surface amount and trafficking dynamics of other neuronal PM proteins, such as NMDARs or TfR, could be analysed following inhibition of PKD activity. The information gained from these experiments, when added to that obtained in my work, will grant not only a better understanding of the role of PKD in neuronal cells, but also greater comprehension of AMPAR trafficking and synaptic plasticity.

6. References

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7. Publications

This work was published in part in the following publication:

2020 Oueslati Morales CO, Ignácz A, Bencsik N, Rátkai AE, Lieb WS, Eisler SA, Schlett K et Hausser A

PKD promotes activity-dependent AMPA receptor endocytosis in hippocampal neurons

Published in bioRxiv under:
https://www.biorxiv.org/content/10.1101/2020.04.09.033514v2

doi: https://doi.org/10.1101/2020.04.09.033514

8. Acknowledgements

My time as a PhD student, both in Stuttgart and in Budapest, has taught me plenty of lessons both in the professional and personal areas, allowed me to meet people who would change my life forever, and forged me into the person I am today. I am incredibly grateful for this experience, which would not have been possible without the help and support of many people. I would like to highlight a few of them:

Angelika Haußer: first of all, I would like to thank you. I am very grateful for the opportunity you gave me, for being the main driver of my professional development, for the continued help and support both when things worked and when they did not, for the enriching scientific discussions, for those moments when we just shared some small talk and some laughs… In short, thanks for being an amazing supervisor.

Katalin Schlett: my PhD would have been only half as special if it was not for you and your involvement in this project. Thank you for the opportunity to stay a few precious weeks every year in your laboratory, for teaching me how to have a critical eye for neuronal health and imaging, for fostering my development and allowing the exchange of ideas and techniques between me and the members of your lab… A heartfelt thank you.

Sebastian Lieb: in these few years we have spent together in the laboratory, you have become not only my dear friend, but also a bit the older brother I never had. Thank you, for all the support and the scientific discussions, for helping me develop my presenting skills, for all those laughs over a coffee or a Döner and, specially, for transmitting me some of your incredible drive and your “never give up” mentality.

Gisela Link: what would become of the lab without you! Every time you went on (hard-earned) holidays, it descended progressively into chaos :) Thank you for all your help, for teaching me so many things, and for those relaxed chats in German and Spanish over a cup of coffee.

Stefanie Föller: I am grateful to have had the chance to be your supervisor. Working together was a great experience which definitely turned me into a better scientist. Thank you!

Schlett group: what can I say about you guys! You were a big part in why I was always happy to go to Budapest. Attila I. Hanna, Zsófia, Anikó, Attila S, Krisztián, Norbert… It was a pleasure working together.

Biswajit Podder: after a rocking start sorting out your origins and the correct pronunciation of your name (Johnny), I discovered that you are the kindest person I have ever met. I am grateful for all the time we spent together, for the scientific discussions, for the funny dancing, for our

“loving” pictures, and for the heart-to-hearts over a coffee. I am very lucky to count you as my friend.

Monilola Olayioye: thank you for always providing helpful feedback and novel viewpoints during the scientific discussions of my project. Both the project and I improved thanks to you.

The MOlab: my PhD would not have been the same without having you guys next door. It did not matter whether I needed a helpful hand, a good scientific discussion over how to troubleshoot a certain issue or a good time over some coffee & chocolate or the good Mensa food, you were always there. David, Yannick, Ismael, Cristiana, Florian and the rest of you…thanks.

Stephan Eisler and Melanie Noack: a sincere thank you to the both of you for your technical help and advice about how to keep alive and properly image my not-so-resilient transfected neurons… It would have been madness without you.

To many other IZIaners: so many other people made my experience a better one… Josip, Vesna, Raluca, Gabi, Julie, Hannah, Katrin, Alex, Chiara, Elena, Kai, Sylke, Filipa, Helena… Thank you for the scientific advice and the leisure time we spent together.

To my friends: getting to know so many of you during these years was already an invaluable experience. Péter, Álvaro, Rubén, Marta, Bálint, Marc, Andoni, Chris, Dorina, Eszter, … Thanks for the good time and the emotional support that helped me get here. Even though half of you continued mistaking mice and rats ;)

A mi familia: si he llegado hasta aquí y soy quien soy es gracias a que vosotros cuidasteis de mí y me apoyasteis durante toda mi vida. Gracias por convencerme de que puedo conseguir cualquier cosa que me proponga. Gracias por vuestro apoyo durante todos estos años. Gracias por los buenos momentos. Mamá, Papá, Ismael… os echo de menos.

Viktória Szentgyörgyi: last, but definitely not least, I am grateful to you. Thank you for your continued support, your kindness, your patience and your understanding, specially during this last difficult year, because they allowed me to complete this period of my life. Getting to know you was the very large cherry on the cake of my doctorate.