

**THE ROLE OF PROTEIN KINASE D IN NEURONAL TRANSPORT
PROCESSES AND IN DENDRITIC DEVELOPMENT**

PhD thesis

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ABBREVIATIONS

AIS	axon initial segment
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF	brain-derived neurotrophic factor
BFA	brefeldin-A
CaMK	Ca ²⁺ /calmodulin-dependent protein kinase
CNS	central nervous system
CREB	cAMP-response element-binding protein
CRMP-2	collapsin response mediator protein 2
CYS1/2	cystein-rich zinc-finger domain 1 and 2
DAG	diacylglycerol
diC8	1,2-dioctanoylglycerol
DIV	days in vitro
Dox	doxycycline
ER	endoplasmic reticulum
ERES	endoplasmic reticulum exit site
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
ERK	extracellular signal regulated kinase
FC	fast component of axonal transport
GABA	γ -amino-butiric acid
G_{$\beta\gamma$}	$\beta\gamma$ subunit of the heterotrimeric G protein
GI	Golgi apparatus enrichment index
G-PKDrep	Golgi-targeted PKD activity reporter
G-PKDrep S/A	Golgi-targeted PKD activity reporter containing a serine to alanine mutation
GPCR	G-protein coupled receptor
GRIP	glutamate receptor-interacting protein
GSK-3β	glycogen synthase kinase-3 β
HDAC	histon deacetylase
JNK	c-Jun N-terminal kinase
KHC	kinesin heavy chain
Kidins220	kinase D-interacting substrate of 220 kDa
KIF	kinesin family protein
KLC	kinesin light chain
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
NgCAM	neuron-glia cell adhesion molecule

NGF	nerve growth factor
NMDA	N-methyl D-aspartate
NR1	NMDA receptor subunit 1
PDBu	phorbol 12,13-dibutyrate
PH	pleckstrin homology domain
PI3-kinase	Phosphoinositide-3-kinase
PI4KIIIβ	Phosphatidylinositol 4-kinase III β
PKC	protein kinase C
PKD	protein kinase D
PKDrep	non-targeted PKD activity reporter
PKDrep S/A	non-targeted PKD activity reporter containing a serine to alanine mutation
PLC	phospholipase C
PSD	post synaptic density
PSD-95	postsynaptic density protein 95
RE	recycling endosome
RIN1	Ras and Rab interactor protein 1
SAP	synapse associated protein
SER	smooth endoplasmic reticulum
SCb	slow component-b type of axonal transport
SNARE	soluble N-ethyl maleimide sensitive factor adaptor receptor
TGN	trans-Golgi network
Trk	tyrosine kinase receptor
VAMP4	Vesicle Associated Membrane Protein 4
VZ	ventricular zone

INTRODUCTION

3.1. Development of the highly polarised structure of mature neurons

3.1.1. Development of polarised neurons in the mammalian central nervous system

Neurons are generated in the inner part of the neuroepithelium, which surrounds the ventricles following the formation of the neuronal tube in the vertebrate nervous system (Zhong and Chia, 2008). Neuronal precursors and postmitotic neurons leave the site of origin and migrate towards their final destination, where they differentiate into distinct cell types establishing a complicated neuronal network with functioning connections (Luskin, 1994; Nadarajah and Parnavelas, 2002).

Investigating the complex regulation and mechanism of brain development has been one of the central enquiries of neuroscientists in the last decades. Most of our knowledge in term of neurogenesis and neuronal migration arises from studies of the neocortex and the cerebellum, structures which constitute of well-determined cell-types organised in a laminar structure. It has been shown that different neuronal populations use different modes of migration and have to cover distances in a wide range. In general, two types of migration have been identified in the forebrain and other regions of the central nervous system (CNS): radial migration is directed from the progenitor zone towards the surface, whereas tangential migration takes place orthogonal to the direction of radial migration.

Radial migration has been widely investigated particularly in the developing cortex. Cortical projection neurons are born in the proliferative ventricular zone (VZ) of the dorsal forebrain and establish cortical laminar organisation upon migrating towards the pial surface basically in two distinct modes: somal translocation and glia-guided migration (Nadarajah, 2003; Kriegstein and Noctor, 2004; see Figure 1). At early stages of development, when the cerebral wall is relatively thin, postmitotic neurons in the VZ undergo somal translocation in order to form the preplate (PP) at the surface of the cerebral vesicles (Figure 1B). Neurons possess a long, radially oriented leading process that terminates at the pial surface, and a short, transient trailing process. The leading process remains attached to the surface and becomes progressively shorter as the soma advances toward the pial surface, displaying continuous translocation. During the later stages of cortical formation, when the cerebral wall is considerably thicker, cortical cells establish the laminar organisation upon migrating along glial fibers (Figure 1C). The migratory behaviour of glia-guided neurons show fundamental distinctions compared to neurons

undergoing somal translocation, as they have a free motile leading process that maintains a relatively constant length during migration and show saltatory pattern of locomotion.

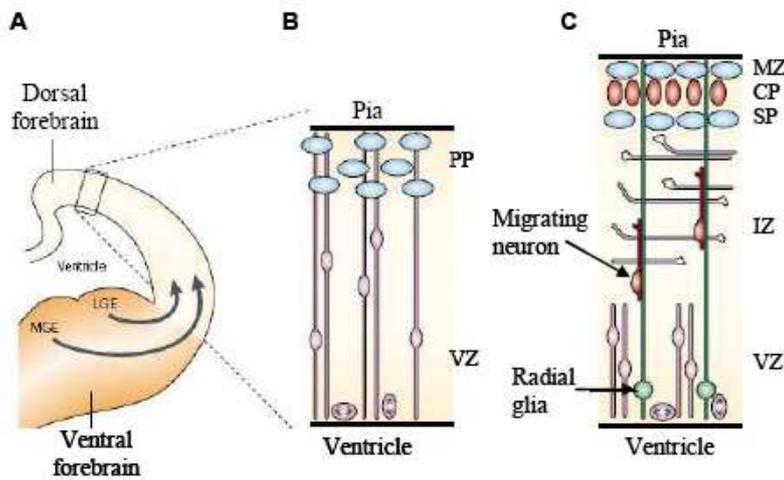


Figure 1. Schematic diagram of cortical development. A: Section through the developing rodent forebrain. Arrows indicate tangential migratory routes of cortical interneurons arising from the lateral and medial ganglionic eminence (LGE and MGE, respectively). B-C: Different stages of the developing dorsal forebrain indicated by the boxed area on A. During early stages (B), postmitotic neurons in the ventricular zone (VZ) undergo somal translocation and form the

preplate (PP). During later stages (C), cortical neurons migrate along glial fibers through the intermediate zone (IZ) to form the cortical plate (CP). At the same time, PP gets splitted into the marginal zone (MZ) and the subplate (SP). Modified from Nadarajah and Parnavelas, 2002.

Unlike pyramidal neurons, cortical interneurons that originate in the ventral telencephalon need to migrate tangentially over hundreds of micrometers to reach their final destination (Metin et al., 2006; Figure 1A). During tangential migration, cells can use different types of substrate for migration, however, in case of cortical GABAergic interneurons, no special substrate has been found so far. On the other hand, interneurons of the olfactory bulb are known to use each other to promote their migration in the rostral migratory system (so-called chain migration), while neurons expressing gonadotropin-releasing hormone migrate along growing axons (Marin and Rubenstein, 2003). It has been also shown that migrating interneurons turn by extending new processes in new directions rather than re-orienting the trajectory of their leading growth cone, resulting in diverse morphology of branched leading processes.

Although the morphology of different types of migrating neurons varies largely during the development of the CNS, increasing amount of evidence suggests that migrating neurons rely on a common set of signalling mechanisms (Marin et al., 2006). In general, the movement of neurons can be described in three steps: I) extension of the leading process that explores the immediate environment for different guiding cues, II) nucleokinesis, when the nucleus moves into the leading process and III) the retraction of the trailing process. These processes are fundamentally based on cytoskeletal dynamics involving a very complex interplay of different environmental signals and specific intracellular events. In vivo, directional guidance of migrating cells is attained by spatially

and temporally restricted expression of chemoattractive and chemorepulsive factors, including extracellular matrix molecules, cell adhesion molecules or soluble and membrane-bound factors like neurotrophins, growth factors and neurotransmitters (Sobeih and Corfas, 2002). On the other hand, intrinsic expression pattern of neurotransmitter receptors, ion-channels, and other proteins involved in intracellular signalling pathways and cytoskeletal regulation are also indispensable for the controlled regulation of migratory processes (Komuro and Rakic, 1998; Feng and Walsh, 2001).

As demonstrated above, CNS neurons undergo polarisation already during their migration by establishing neurites of different morphology depending on migratory behaviour (Solecki et al., 2006). Radially migrating neurons are typically described as bipolar cells with opposing leading and trailing processes, while tangentially migrating neurons appear to have a much more diverse morphology with distinctly branched leading process in various lengths. Investigations on the development of cortical projection neurons and cerebellar granule cells indicate that the future axon evolves from the trailing process of the migrating neuron, while dendrites develop mainly from the leading process (Barnes et al., 2008). However, this was not proven in other types of neuronal cells.

In parallel with axonal outgrowth and target finding, pre- and postsynaptic specialisation occurs including the orchestration of signalling cascades, cytoskeletal rearrangement and polarised protein trafficking. Mature neurons, consequently, are extremely polarised cells with functionally and structurally different axonal and dendritic compartments - this feature is an absolute prerequisite for the proper transfer and processing of synaptic information (Mattson, 1999; Gundelfinger and tom Dieck, 2000).

3.1.2. The well-described stages of neuronal development in primary hippocampal cultures

Rodent hippocampal or cortical dissociated neuronal cultures have been successfully used to explore the cellular and molecular mechanisms underlying neuronal polarisation. As it was shown originally by Dotti et al. (1988), hippocampal neurons plated on two-dimensional substrate undergo a defined sequence of morphological events leading from a non-polarised to a polarised state within 1 week. Tracking of individual living neurons throughout the course of their development in low-density embryonal hippocampal cultures has led to the description of a characteristic sequence of events during *in vitro* hippocampal neuronal development (Figure 2).

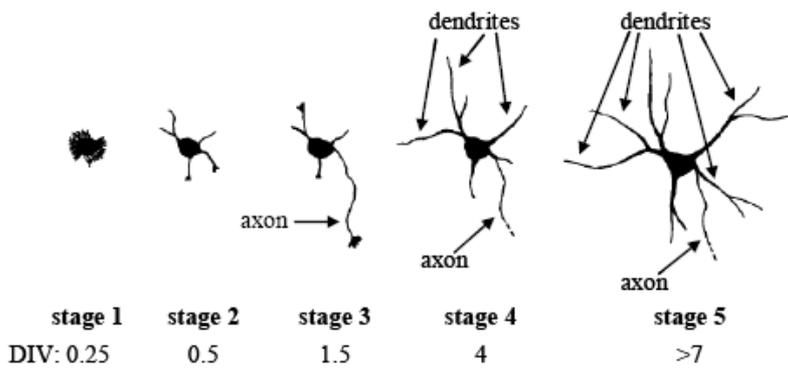


Figure 2. Developmental stages of hippocampal neurons growing in culture. Stages indicate the following processes: stage 1: lamellipodia formation, stage 2: development of minor processes, stage 3: axonal outgrowth, stage 4: dendritic outgrowth and stage 5: further maturation. Timepoints when cells approximately enters each of the stages are indicated in days in vitro (DIV). Modified from Dotti et al., 1988.

After plating, neurons have a spherical form and need 1-2 hours to attach to the culture surface. Substrate-attached neurons first develop highly motile lamellipodia around the circumference of the cell body (*stage 1*). Within half a day, lamellipodia break up into discrete patches along the cell periphery marking the sites where neurites will emerge and soon multiple and morphologically identical minor processes with flattened growth cones appear (*stage 2*). Once established, minor processes persist for days and are quite stable, extending and retracting only in short distances. 1-2 days after plating, one of the minor processes begins to grow 5-10 times faster than the other processes and becomes the axon (*stage 3*). This step results in the establishment of the polarised morphology of the cultivated neuron. Within the next days, the axon rapidly elongates and forms axonal collaterals. Dendritic growth begins around 4 days after plating, from the residual minor processes (*stage 4*) and at a much slower rate compared to axonal growth.

From the second week of plating, axonal and dendritic arbours undergo further maturation and develop functional synaptic connections (*stage 5*). In contrast to the well-defined morphological changes observed during earlier stages (stage 1-4), the distinction between stage 4 and 5 is less evident and full maturation *in vitro* can last for several weeks after plating. While initial polarisation and neurite outgrowth can happen cell-autonomously and can be regarded as the outcome of mainly endogenous regulatory processes, stage 4-5 maturation normally requires cell-to-cell interactions. All cultured hippocampal pyramidal neurons develop in the above sequence, albeit the onset of the individual stages can vary within one culture.

Most of our knowledge in terms of molecules regulating the establishment of axon-dendrite polarity during mammalian CNS development arises from *in vitro* studies. However, results from *in vitro* studies must be always handled cautiously as *in vivo*

investigations have provided both consonant and contradictory results when compared to cell culture studies (Barnes et al., 2008).

3.1.3. Molecular mechanisms underlying axonal and dendritic growth

3.1.3.1. The regulation of axon formation and extension

The break of the initial morphological symmetry happens when one out of the multiple neurites begins to grow rapidly (Figure 3). Several investigations have addressed the question how the future axon is selected from the several, morphologically identical minor processes of a developing neuron. Careful investigations proved that when one neurite has acquired axonal properties, the remaining processes are prevented from becoming axons (Bradke and Dotti, 2000). Additionally, when the original axon is cut close to the cell body, a future dendrite can change its fate and develop into an axon as it was shown both in hippocampal and cortical neuronal cultures (Dotti and Banker, 1987; Hayashi et al., 2002). Therefore, all neurites of a stage 2 neuron have the initial potential to become the axon.

Recent examinations revealed that certain level of polarisation occurs already in morphologically non-polarised neurons. The centrosome, the Golgi apparatus, and endosomes were shown to cluster together at one pole of the non-polarised stage 1 neuron, close to the area where the first neurite will form (de Anda et al., 2005). In stage 2 neurons, when minor processes are established, the actin network inside the future axonal growth cone becomes highly dynamic prior to axonal elongation. Additionally, membranous organelles and TGN-derived vesicles were shown to be enriched in the future axon, already before morphological polarisation (Bradke and Dotti, 1997, 1999; Figure 3A, B).

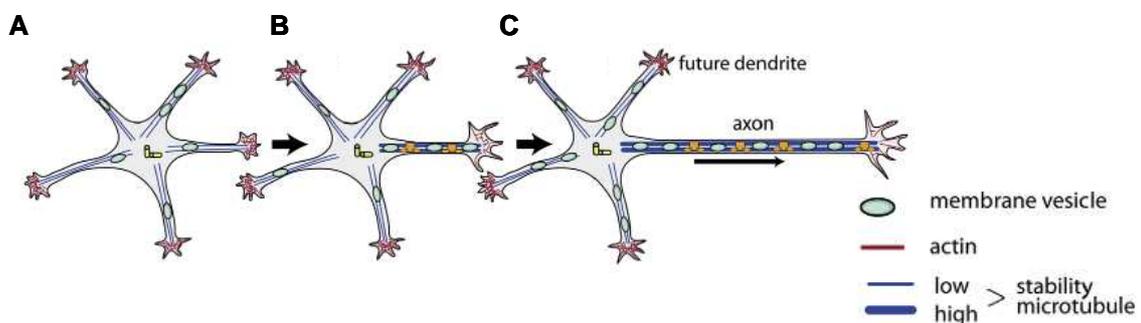


Figure 3. Initiation and rapid elongation of the axon during neuronal development. A: Initially, stage 2 neurons possess uniform neurites. B: Stabilisation of microtubules, enhanced actin dynamics and increased membrane flow occur in the future axon before morphological polarisation. C: Axonal elongation in stage 3 neurons is supported by enhanced microtubule stabilisation and continuous membrane supply restricted to the growing axon. Modified from Witte and Bradke, 2008.

In order to ensure the required large increase in surface area and volume, cytoskeletal rearrangements and polarised membrane traffic are crucial processes underlying elongation of the future axon (Figure 3C). The selective growth of one neurite besides the others requires basic **changes in the cytoskeleton**. Increased microtubule stability and destabilisation of actin filaments in the growth cone were shown to occur already before axon formation and are known to characteristically label the axon of morphologically polarised neurons (Witte and Bradke, 2008). Enhanced stability of microtubules is needed for the selective protrusion of microtubule bundles into the selected neurite, while increased actin dynamics in the growth cone allows the microtubule to penetrate into the leading tip. The importance of cytoskeletal rearrangements in neuronal polarisation and axon elongation was highlighted upon initiating abnormal neurite growth by treating developing neurons with actin destabilising (Da Silva et al., 2003) or microtubule stabilising agents (Witte et al., 2008).

Increased flow rates of membrane constituents contribute also significantly to the rapid growth of the future axon. The involvement of polarised membrane traffic in axonal extension was indicated by treating hippocampal neurons with brefeldin-A (BFA), which is known to disrupt Golgi-mediated transport. BFA treatment prevented the polarisation of non-polarised neurons and led to the shrinkage of the axon in stage 3 neurons (Jareb and Banker, 1997; de Anda et al., 2005). Additionally, several transmembrane and membrane-associated proteins were shown to be enriched in growing axons including axonal proteins (Fletcher et al., 1991) as well as dendritic components (Killisch et al., 1991; Craig et al., 1993; Bradke and Dotti, 1997). Based on recent works, it is a feasible hypothesis that the enrichment of proteins in the growing axon can be a consequence of non-specific bulk flow, as distinct organelles like mitochondria, peroxisomes, ribosomes and trans-Golgi derived vesicles were also shown to be delivered to the axon (Bradke and Dotti, 2000). These data suggest that an important aspect of neuronal polarisation is the reorganisation of non-specific intracellular transport machinery from multidirectional to unidirectional way to ensure the delivery of essential molecules to the future axon. It remains yet to be clarified to what extent certain axon-enriched proteins exert specific functions during the establishment of the axon.

Changes in cytoskeletal dynamics and polarised cytoplasmic flow are complementary processes and interact with each other to a great extent during polarised neurite outgrowth. Stabilised microtubules serve as specific guides for directed vesicle transport and contribute to the enrichment of molecules regulating microtubule or actin dynamics in the

rapidly elongated axon. At the same time, minor processes become depleted from these molecules.

In the last decades, several attempts have been made to define **intracellular regulatory molecules** playing key roles in the development of neuronal polarity (Arimura and Kaibuchi, 2005; Yoshimura et al., 2006a). Several molecules that regulate the dynamics of cytoskeletal components have been identified to integrate various signals controlling axon specification and elongation. Phosphoinositide-3-kinase (PI3-kinase) and its lipid product, phosphatidylinositol (3,4,5)-trisphosphate (PIP3) are key mediators affecting different regulators of actin and microtubule dynamics during neuronal polarisation (Cosker and Eickholt, 2007; see Figure 4 for summary). PI3-kinase is controlled by diverse extracellular growth factors and their appropriate receptors either directly or indirectly via the inhibitory lipid phosphatases PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHIP (Src homology domain 2-containing inositol phosphatase). The significance of PI3-kinase in axonal specification was shown by the findings that its activity highly localises to the tip of the newly specified axon of stage 3 hippocampal neurons. Additionally, inhibiting PI3-kinase activity leads to the arrest of axonal elaboration in non-polarised neurons (Shi et al., 2003).

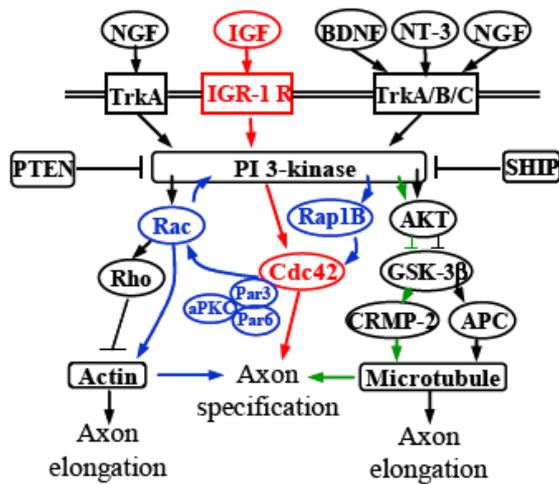


Figure 4. Control of axon specification and elongation by PI 3-kinase mediated signalling pathways. Diverse extracellular factors (e.g. neurotrophin-3 (NT-3), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF) can activate PI3-kinase via interacting with Trk receptors or IGF-1 receptor. Activated PI3-kinase promote axonal elongation by acting on actin cytoskeleton via Rac activation and Rho inhibition or by regulating microtubule dynamics via Akt/GSK-3 β /CRMP-2 pathway (black). The specific activity of PI3-kinase, GSK-3 β and CRMP-2 in the growing axon contributes largely to axon specification (green) as well as the positive feedback-loop comprising Rap 1B, Cdc42, Par3-Par6-aPKC and Rac (blue). PI3-kinase activity is negatively regulated by the lipid phosphatases PTEN and SHIP. Modified from Cosker and Eickholt, 2007.

PI3-kinase was shown to regulate cytoskeletal rearrangements via two major signalling cascades: a positive feed-back loop composed of Rho GTPases and the Par3/Par6/aPKC (atypical protein kinase C) as well as the Akt/glycogen synthase kinase-3 β (GSK-3 β)/CRMP-2 pathway. Rho GTPases are known as central regulators of actin cytoskeleton (Govek et al., 2005). Rac1 and Cdc42 activity was shown to be needed for

proper axonal maturation, acting on the actin depolymerising factor cofilin. In contrast, RhoA was defined as negative regulator of neurite growth by inducing actin polymerisation via profilin (Fukata et al., 2002). Consequently, several downstream targets as well as upstream regulators of Rho GTPases have been demonstrated to affect neuronal polarisation, like the guanine-nucleotide exchange factor Tiam-1, enabled vasodilator-stimulated phosphoprotein (Ena), or the neuronal Wiskott-Aldrich syndrome protein (N-WASP) (Kunda et al., 2001; Nishimura et al., 2005; Marin et al., 2006). The polarised localisation of Par3 and Par6 as a consequence of polarised activity of PI3-kinase in the growing axon was also shown to be crucial for axon specification (Shi et al., 2003). It has been also shown that PI3-kinase and Cdc42 control the position of the centrosome indicating that the special localisation of the centrosome at the future axon may not be the cause for axon formation, but rather a result of axon-inducing cues (Arimura and Kaibuchi, 2007).

PI3-kinase was also shown to act on microtubule-binding proteins via regulating the activity of GSK3 β (Yoshimura et al., 2006b; Cosker and Eickholt, 2007). It has been shown that PI3-kinase activity leads to the inactivation of GSK3 β resulting in the promotion of microtubule polymerisation and stabilisation via dephosphorylation of collapsin response mediator protein 2 (CRMP-2) and adenomatous polyposis coli protein (APC). The selective enrichment of both APC and CRMP-2 at axonal growth cone further supports the importance of these molecules during early neuronal development (Jan and Jan, 2003; Votin et al., 2005). The GSK3 β upstream signalling pathway is under dispute (Gartner et al., 2006) but many publications indicate that GSK3 β is regulated via Akt/PKB (Yoshimura et al., 2006b; Cosker and Eickholt, 2007).

In contrast to the pathway described above, active GSK3 β can also induce microtubule instability via phosphorylation of the microtubule associated protein 1B (MAP1B). These data suggest that efficient axonal growth requires the orchestration of spatially restricted activation and inactivation of signalling molecules. Phosphorylation-state-specific gradient of Tau and MAP1B in the axon was also detected indicating that these gradients also contribute to the microtubule changes necessary for axon elongation (Mandell and Banker, 1996; Trivedi et al., 2005).

The distribution of microtubule-based motor proteins during the establishment of neuronal polarisation revealed specific accumulation of the Kinesin-1 family member KIF5 in the axonal growth cone already at the initiation of axonal specification, while other

kinesins were shown to be distributed non-specifically in all developing neurites (Jacobson et al., 2006). The selective translocation of KIF5 in early stages of axonal development can play a significant role in targeting Kinesin-1 cargo proteins, like CRMP-2 to the developing axon, resulting in a positive-feedback loop that contributes to further differentiation of the axon.

Besides the effects of cytoskeletal regulating proteins, neurite extension and growth cone dynamics show complex responses that depend also on the localisation, concentration and temporal dynamics of intracellular Ca^{2+} signal, implying a regulatory role in neuronal polarisation for Ca^{2+} -dependent proteins, as well. Among several other molecules, Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK) and Ca^{2+} /calmodulin-dependent protein kinase I (CaMKI) were shown to be required for normal axonal outgrowth and growth cone morphology in cultured hippocampal and cerebellar granule cells (Wayman et al., 2004).

Neuronal development and maturation is inevitably regulated by multiple **extracellular cues**, too. Neurotrophins, like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) have been demonstrated to promote axonal growth as well as to determine the direction of axon extension acting via high-affinity tyrosine kinase receptors (Trks) or the low-affinity receptor called p75 (Gallo and Letourneau, 2004). Semaphorins, ephrins, slits, and netrins were also shown to be involved in axonal guidance and elongation (Hur and Kim, 2007). Besides secreted or membrane-bound factors, extracellular matrix molecules, like laminin or neuron-glia cell adhesion molecule (NgCAM), also support axon formation (Esch et al., 1999). These diverse external factors were shown to affect axonal growth through receptors coupled to the aforementioned intracellular pathways.

3.1.3.2. The development of dendrites

Compared to data on initial axonal outgrowth, much less is known about mechanisms underlying dendritic development and branching. Dendritogenesis is often used in the literature to refer both to the elongation of dendrites in stage 4, as well as to the later occurring maturation of the dendritic arbour in stage 5 neurons. These two periods of dendritic development, however, should be distinguished and addressed separately for better understanding. Initial dendritic outgrowth is discussed in this chapter, while spine formation and the refinement of already developed dendritic tree is presented in chapter 3.1.4.2.

Axons and dendrites are fundamentally similar in their ability to elongate and branch. The observed distinctions in the time of initiation, the growing rate and the involved regulatory molecules, however, indicate at least partially different regulation of axonal versus dendritic development (Jan and Jan, 2003; Georges et al., 2008). Regulatory differences are probably due to the well-described distinctions between axons and dendrites including distinct microtubule orientation or spacing and the selective intracellular localisation of proteins required for specific function.

Distinct role of **cytoskeletal dynamics** in dendritic extension was shown by treating neurons with taxol, a drug known to stabilise microtubule. Increased microtubule stability led to the turn of dendrites into axons (Gomis-Ruth et al., 2008). While axons were shown to use stable, uniaxially oriented microtubules in order to ensure rapid elongation, dendritic growth seems to be less influenced by the stabilisation of microtubules (Georges et al., 2008). Microtubules are known to be oriented biaxially in the dendrites and are associated with a different set of MAPs resulting in a frame of less densely packed microtubules. While axonal elongation is ensured mainly by stable microtubule bundles, dendrites rely more on cell-substrate adhesive forces to possibly compensate lower microtubule concentration.

The kinesin-related motor protein CHO1/MKLP1 was shown to be responsible for the specific transportation of minus-end distal microtubules into the dendrites hence playing a significant role in the development of non-uniformly oriented microtubules within the dendritic arbour (Sharp et al., 1997). The suppression of CHO1/MKLP1 during early development of sympathetic neurons has been shown to inhibit dendrite formation without affecting axonal growth.

Similarly to axonal growth, intracellular molecules regulating microtubule and actin dynamics also play a central role in dendritic growth and branching. The members of the actin regulator Rho family GTPases, like Rac1 and Cdc42, are involved in all aspects of early dendrite development including initiation, growth and branching (Threadgill et al., 1997; Koh, 2006). Members of the Ras subgroup of small GTPases, like Rit also play an important role during early neuronal polarisation processes. Depending on its activation status, Rit can affect dendritic and axonal growth adversely (Lein et al., 2007). Downstream effectors of small RhoGTPases, like p21-activating kinase-1 (Pak1) in case of Rac1/Cdc42, were also shown to influence the number of basal dendrites as well as the extent of primary branching of apical dendrites in cortical pyramidal neurons (Hayashi et al., 2007). Upstream regulators of small GTPases, like the lipid-raft associated CaMKI γ ,

also influence early dendritogenesis. This is supported by recent data about Rac-dependent cytoskeletal reorganisation in developing dendrites (Takemoto-Kimura et al., 2007).

Polarised surface-directed transport of membrane materials is also required during the development of dendrite arbour (Tang, 2008). Recent findings have revealed that the growth of neuronal dendrites depends much more on the secretory pathway compared to axons (Ye et al., 2007). The special role of membrane traffic is indicated by the specific presence of discrete, satellite secretory apparatus within growing dendrites, including endoplasmic reticulum (ER), ER exit sites and Golgi structures referred as Golgi outposts. Polarised orientation of the somatic Golgi towards the longest dendrite or the appearance of Golgi outposts were described already at early stages in cultured hippocampal neurons (Horton and Ehlers, 2003a). The significance of secretory trafficking in dendritic growth was supported by the decrease of total dendritic length in stage 4 neurons upon disassembling the Golgi apparatus by BFA treatment or upon blocking late secretory trafficking by expression of kinase-dead mutant of protein kinase D (PKD) (Horton et al., 2005). Furthermore, reducing the level of Sar1, a protein involved in the trafficking from ER to Golgi, resulted in a dramatic decrease in total length of developing dendrites in hippocampal neurons. The direct role of Golgi outposts in dendritic growth has been additionally indicated by markedly reduced dendritic dynamics after the elimination of Golgi outposts by intense laser illumination (Ye et al., 2007).

Up to date, several **extracellular signalling proteins** have been identified to influence dendrite patterning. For example, BDNF, neurotrophin-3 (NT-3) and NGF were shown to act as extrinsic factors regulating dendritic morphology of pyramidal cells (Snider, 1988; McAllister et al., 1997). Furthermore, the extracellular matrix glycoprotein reelin and agrin have been also implicated to play a role in neurite development, showing effects on both actin and microtubule dynamics (Georges et al., 2008). Several members of the bone morphogenetic protein (BMP) family as well as the signalling molecule Notch and other diverse cell adhesion molecules were also shown to influence dendritic growth (McAllister, 2000). More detailed discussion of all the possible regulatory molecules, however, would exceed the scope of this introduction.

3.1.4. Pre- and postsynaptic specialisation in neurons

3.1.4.1. Stabilisation of axonal connections

During neuronal development, axonal branching and direction of growth is controlled by diverse environmental cues leading to the establishment of synaptic contacts with the

appropriate target. In the last decades, external cues involved in axonal pathfinding have been extensively investigated and revealed a high number of extracellular factors acting through distinct receptor classes and coupled to different intracellular cascades (see in detail: (Cooper, 2002; Wen and Zheng, 2006).

During the development of synaptic connections, axons undergo highly dynamic rearrangements including selective stabilisation of some branches and synapses while elimination of others. Growing axons were shown to make many immature synaptic contacts via continuously formed exploratory side branches. Several investigations regarding development of different brain areas indicated that elimination or stabilisation of axonal side branches and transient contacts are mainly regulated by activity-dependent mechanisms (Ernst et al., 1998; Yu et al., 2004; Hua et al., 2005).

Axonal contacts made with appropriate target sites initiate the formation of the presynaptic area which includes *i*) the termination of axonal growth, *ii*) the establishment of the presynaptic active zone, a specialised cytoskeletal structure and *iii*) the clustering of synaptic vesicles (Zhen and Jin, 2004). Active zone components are transported via vesicular trafficking, but separately and earlier than synaptic vesicles to the presynaptic terminal. To date, mechanisms underlying fast accumulation of presynaptic components upon generation of contact sites are not completely clarified. The involvement of adhesion molecules in these processes is highly feasible, as adhesion molecules play significant role in diverse aspects of synaptogenesis including target recognition, synapse specification, stabilisation and function (Yamagata et al., 2003). Adhesion molecules can anchor presynaptic organelles as indicated by a direct association between TGN-derived structures and neural cell adhesion molecule (NCAM) clusters. Furthermore, NCAM was shown to form a complex with synaptic organelles and to be transported along neurites prior to contact formation. These observations raise the possibility that organelles containing presynaptic molecules accumulate at contact sites via NCAM-mediated interactions (Sytnyk et al., 2004).

3.1.4.2. Formation of extended dendritic arborisation and postsynaptic maturation

The spatial pattern of dendritic arborisation contributes significantly to neuronal function by determining the number and type of inputs received from distinct connections. The formation of dendrite arborisation has been investigated extensively, however, the mechanisms underlying the development of such a complex and diverse structure need further clarifications.

The CNS contains several types of neurons possessing highly distinct dendrite morphology. Genetic specification of dendrite morphology was indicated by the development of distinct dendritic patterns characteristic for different cell-types upon minimal external effects under *in vitro* conditions (Kriegstein and Dichter, 1983; Horton et al., 2006). However, regulation by environmental signals including secreted molecules from distant targets, interactions with neighbouring cells, and contact with presynaptic cells play a significant role in the formation of the dendritic arbour (McAllister et al., 1995; Kapfhammer, 2004).

Dominant processes during later stages of dendritic development involve the dynamic formation of branches and dendritic spines, as well as fine rearrangements of existing dendritic branches. Several molecules including small GTPases as well as their downstream effectors and upstream regulators are involved also in the maturation processes of the dendritic arbour (Nakayama et al., 2000; Koh, 2006; Hayashi et al., 2007; Tolia et al., 2007). Molecules regulating cytoskeletal dynamics independent of small GTPase pathways can influence dendritic development (Jones et al., 2004; Tang, 2008). MAP2, for example, is involved in the regulation of dendritic arborisation (Harada et al., 2002), and acts depending on its phosphorylation state (Tang, 2008). Scaffolding proteins of the post-synaptic density (PSD), like postsynaptic density protein 95 (PSD-95), cypin, GRIP and Shank also play a role in dendritic branching and arborisation by providing a link between actin cytoskeleton and microtubules (Charych et al., 2006; Vessey and Karra, 2007).

The importance of polarised membrane traffic was indicated by the somatic Golgi apparatus oriented towards the longer and more complex dendrites in stage 5 neurons as well as the localisation of Golgi outposts at dendritic branchpoints. It is an intriguing idea that local Golgi apparatus serves as potential source of membrane components at regions of intensive growth (Horton et al., 2005).

The complexity of received inputs increases continuously with the ongoing development of the nervous system. Activity-dependent mechanisms were shown to be decisive during synaptogenesis indicating their relevance in shaping developing dendritic arborisation (McAllister, 2000; Wong and Ghosh, 2002). Signalling events involved in activity-dependent control of dendritic growth and patterning were shown to be regulated mainly by calcium-dependent processes, e.g. via changing the transcription of target genes or regulating local alterations in dendritic structures via the cytoskeleton (Scott and Luo, 2001).

Synaptogenesis is one of the central processes occurring during neuronal maturation and involves the extensive formation of dendritic filopodia and spines (Jontes and Smith, 2000). Dendritic filopodia are long, thin and highly motile protrusions possessing multiple functions during dendritic arborisation (Evers et al., 2006). Filopodia are key players in environmental exploration and were shown to be involved in steering dendritic growth, guiding growing axon terminals towards the dendrite, formation of new dendrites and spinogenesis. Dendritic spines, on the other hand, are short, bulbous structures localised along the dendrites of several neuronal types and are mainly involved in the establishment of excitatory synapses. Importantly, dendritic spines assure the occurrence of independent chemical changes within individual synapses. Regulation of spine formation and elimination, as well as the control of spine number, shape and size are the central components of activity dependent modulation of synaptic strength (Tada and Sheng, 2006). The detailed discussion of spine morphogenesis and molecular mechanisms underlying long-term potentiation and depression, however, is beyond the scope of the present work and readers are referred to more specialised reviews (Nimchinsky et al., 2002; Carlisle and Kennedy, 2005).

3.2. The maintenance of polarised neuronal surface and specialised intracellular composition

During neuronal development, the axon and the somatodendritic compartments become highly specialised for either transmitting or receiving and integrating cellular signals, respectively. To preserve molecular and functional polarity, plasma membrane domains constituting fundamentally different molecular components must be established and continuously supported throughout the lifespan of the neuron. As part of the orchestrated intracellular regulatory mechanisms, secretory and endosomal pathways provide continuous membrane supply and selective targeting of proteins. Additionally, free diffusion of selectively localised proteins can be inhibited by physical barriers within the membrane or by binding to the submembraneous cytoskeleton.

3.2.1. Selective localisation of membrane components: restricted diffusion and tethering

In order to maintain polarised localisation of membrane-attached proteins, lateral diffusion between specific subcellular domains has to be inhibited. Immunocytochemical analyses of axonal and somatodendritic membrane protein distribution in cultured hippocampal neurons indicated that axon initial segment (AIS) comprises a boundary

between the two main subcellular domains. Measuring lateral mobility of diverse axonal and somatodendritic membrane proteins revealed a markedly reduced mobility in the initial segment of the axon suggesting that AIS represents a general diffusion barrier in neurons (Winckler et al., 1999). Structural analysis of the initial segment fence revealed the presence of a specialised, detergent-insoluble complex of cytoskeletal proteins, consisting of the brain-specific isoform of ankyrin G, F-actin and spectrin (Jenkins and Bennett, 2001). The importance of ankyrin G in neuronal polarisation was demonstrated by the subsequent disassembly of the AIS and the redistribution of somatodendritic proteins into the axon upon ankyrin G silencing (Hedstrom et al., 2008). Besides cytoskeletal elements, membrane proteins required for specialised functions, such as axonal signal propagation, accumulate at the AIS and get immobilised via binding to ankyrin G. The densely packed membrane proteins anchored to the underlying cytoskeletal complex thus form an effective physical barrier to inhibit the penetration of other membrane proteins. In contrast to membrane proteins, experiments with fluorescent lipid analogues indicated that the molecular fence of AIS does not impede the diffusion of membrane lipids (Winckler and Poo, 1996).

The free diffusion of membrane proteins is also hampered upon stabilisation at certain membrane domains and via binding to submembraneous cytoskeletal structures. Lipid rafts are cholesterol and sphingolipid enriched microdomains within the plasma membrane of both axons and dendrites (Tsui-Pierchala et al., 2002). The influence of lipid rafts on axonal protein sorting was indicated by disturbing cholesterol and sphingolipid synthesis in mature hippocampal neurons, which resulted in the missorting of the axonal cell adhesion molecule Thy-1 to dendrites in almost half of the investigated cells (Ledesma et al., 1998). Lipid rafts are also present throughout the somatodendritic part of neurons and play important role in synaptic stabilisation (Hering et al., 2003). Disruption of lipid rafts by inhibiting sphingolipid and cholesterol synthesis in mature hippocampal neurons markedly decreased the density of postsynaptic protein markers PSD-95, Shank and NMDA (N-methyl D-aspartate) receptor subunit 1 (NR1) while the size of remaining clusters was elevated. These observations indicate a special role for lipid rafts in tethering certain membrane proteins within the plasma membrane.

The direct or indirect binding of membrane proteins to the submembraneous cytoskeleton leads also to the restricted localisation of proteins to certain parts of the plasma membrane. In case of the presynaptic and postsynaptic regions, cytoplasmic and membrane proteins required for synaptic function are organised in electron-dense

structures which are localised close to the synaptic membrane. These structures are composed of several hundred proteins which are organised mainly by diverse multidomain scaffolding proteins equipped with a huge set of protein-protein interaction domains. Clustering of neurotransmitter receptors, ion-channels and cell adhesion molecules at the postsynaptic membrane is provided by scaffolding proteins including PSD-95 and other synapse associated proteins (SAPs). These scaffolding proteins are bound directly or indirectly to the intracellular tail of membrane-bound receptors and channels, as well as to other scaffolding proteins linked to the actin-based cytoskeleton (Boeckers, 2006). The role of the actin cytoskeleton in neurotransmitter receptor anchoring was shown by depolymerisation of the actin cytoskeleton in cultured hippocampal neurons, which induced the dispersion of clustered NMDA receptors and α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors away from synaptic sites (Allison et al., 1998). The presynaptic scaffolding proteins Munc13 and Bassoon/Piccolo also participate in determining the spatial restriction of neurotransmitter release sites by interacting with proteins of the plasma membrane, synaptic vesicles, the cytosol and the cortical cytoskeleton (Dresbach et al., 2001). The actin cytoskeleton plays a significant role in clustering synaptic vesicles and regulating vesicle docking and release, as well (Doussau and Augustine, 2000).

3.2.2. Continuous membrane supply and selective targeting of proteins to axons and dendrites

Membrane dynamics is indispensable for neuronal function and is controlled by orchestrating secretory and endocytotic pathways. Targeted delivery of proteins to specialised subcellular domains involves diverse sorting mechanisms. In a subset of proteins destined to different surface domains, segregation occurs at the level of the TGN by forming separate classes of post-Golgi carriers. Preferential delivery of post-Golgi carriers to axons or dendrites is ensured via the selective binding of the cargo to different transport complexes including motor proteins moving along differently orientated microtubules. Sorting of certain membrane components, however, can also happen directly at the plasma membrane by *i*) selective fusion determined by specified fusion sites, *ii*) selective retention of appropriate proteins and *iii*) rapid internalisation of mistargeted membrane proteins.

3.2.2.1. Intracellular distribution of the secretory pathway components and their role in polarised membrane trafficking

Continuous supply of membrane material to the surface is provided by the biosynthetic transport of proteins and lipids along the exocytotic pathway. As in non-neuronal cells, neuronal membrane proteins and lipids are synthesised and subjected to certain posttranslational modification within the ER and get accumulated at the ER exit sites (ERES). The cargo leaves the ER in carriers coated with coat protein II (COPII) and merge with the ER-Golgi intermediate compartments (ERGICs). Afterwards, secretory cargo passes through the cis, medial and trans compartments of the Golgi apparatus and goes through further modifications. At the TGN, secretory cargo gets sorted in diverse vesicles in order to be transported either to the plasma membrane or to endosomal compartments (Hanus and Ehlers, 2008).

A unique feature observed in neurons is the selective intracellular localisation of the secretory compartments, thus providing increased amount of membrane material specifically at required compartments. The secretory pathway components including smooth and rough ER (SER and RER), ERGICs, ERES and the Golgi complex, extend throughout the soma and the dendrites but are excluded from the axons (Gardiol et al., 1999; Tang, 2008). Additionally, dendrites comprise discrete Golgi structures termed *Golgi outposts*, initially defined by their immunoreactivity for the Golgi matrix protein GM130 and their characteristic mini-Golgi stack ultrastructure (Horton and Ehlers, 2003a). The selective localisation of Golgi outposts to longer and more complex dendrites observed in hippocampal neurons indicated a role for Golgi outposts in asymmetric dendritic growth. Indeed, Golgi outposts are involved in the transport of both integral membrane proteins and secreted factors. Therefore, in certain dendrites their presence enables post-ER trafficking independently from the distant somatic Golgi complex.

Additionally, a satellite secretory pathway comprising RER, ERGIC, Golgi and TGN markers in dendritic spines is responsible for producing proteins required for local synapse-specific modifications (Pierce et al., 2001). In a subset of dendritic spines, ER and Golgi markers are localised to a special structure termed *spine apparatus*, which is composed of SER derived stacks interdigitated by a densely stained material. Although the precise compartmental identity and function of spine apparatus is still elusive, recent investigations suggest a role in synaptic plasticity processes including local regulation of calcium kinetics and post-translational modification of locally synthesised proteins (Jedlicka et al., 2008).

The sorting of proteins at the TGN is based on certain amino acid motifs serving as intrinsic sorting signals, which direct proteins to distinct vesicles. Investigations in neurons revealed the presence of characteristic sorting motifs in a subset of membrane proteins with preferential axonal or dendritic localisation. The exclusive somatodendritic targeting was shown to rely mainly on tyrosine- and dileucine-based motifs in the cytoplasmic domain of the proteins, known to serve as basolateral transport signals in polarised epithelial cells, as well (Horton and Ehlers, 2003b). In contrast, axonal sorting signals are just rarely found and exert disparate signals rather than stereotypical motifs uncovered for dendritic targeting (Sampo et al., 2003; Rivera et al., 2005). As axonal proteins are frequently detected in both axons and dendrites, the polarised localisation of axonal proteins is rather a consequence of sorting mechanisms occurring at later points of the secretory pathway.

3.2.2.2. Intracellular transport mechanisms and their significance in the directed delivery of axonal and dendritic molecules

Post-Golgi carriers as well as the required organelles and cytoplasmic proteins are delivered to their destination upon binding to specialised cytoskeleton-associated motor proteins which move along microtubules or microfilament tracks. Long distance organelle transport happens along longitudinally oriented microtubules and is carried out by members of the kinesin (KIF) and dynein motor protein superfamily. KIFs are coded by a large molecular gene family composed mainly of plus-end-directed motors and were shown to be the main long-distance transporters towards the periphery in neurons (Hirokawa and Takemura, 2005). KIFs constitute of the kinesin heavy chains (KHCs), which contain the motor domain, associated with the kinesin light chains (KLC) and display individual cargo specificity through binding cargos via diverse scaffold proteins (Setou et al., 2002). Cytoplasmic dynein is a minus-end directed motor and plays a significant role in transporting organelles, vesicles and signalling complexes from axonal and dendritic terminals towards the cell body, indicating its important role in retrograde signalling and protein degradation (Levy and Holzbaur, 2006). Cytoplasmic dynein is a multimeric complex composed of two heavy chains and variable number of associated intermediate and light chains. Although several of the dynein chains can associate with different cargos, the interaction of cytoplasmic dynein with their cargos was also shown to be mediated by dynactin, a multisubunit complex required for dynein function (Gunawardena and Goldstein, 2004).

Actin filaments, enriched mainly at the cell membrane, serve as tracks for motors of the myosin superfamily and provide short-range, dispersive distribution of vesicles and organelles towards the cell periphery (Bridgman, 2004). Members of the myosin classes I, V and VI play significant roles in neuronal transport processes mainly at regions devoid from microtubules, including areas adjacent to plasma membrane, presynaptic terminals and dendritic spines.

Microtubule based long-range transport was investigated predominantly in axons containing highly structured, uniformly oriented microtubules with the plus ends oriented towards the axon terminal. As axons lack local protein synthesis, synaptic precursors, ion channels, adhesion molecules or mitochondria often have to cover very long distances from the soma to reach their final localisation at synapses or at other distal destinations. Axonal proteins, lipids and organelles are delivered either by association to membranous vesicles or as multiprotein complexes, but in both cases, are connected to motor proteins (Brown, 2003). The anterograde transport of axonal cargos is usually based on the kinesin mediated transport, with distinct carriers moving at different rates. Golgi-derived vesicles are transported in the *fast component (FC) of axonal transport*, which is characterised by a constant and unidirectional movement at maximal rates of $\sim 2\text{-}5 \mu\text{m}/\text{sec}$. Most of the synaptic proteins are delivered in precursor-form by the FC axonal transport and have been shown to associate with distinct subclasses of membrane organelles and distinct kinesin subtypes (Okada et al., 1995). In contrast, cytoskeletal polymers and cytosolic protein complexes are transported in the axons at a slower rate, by the *slow component (SC) type of axonal transport*. SC transport in cultured neurons can be subdivided further: slow component a (SCa) and b (SCb) transport is characterised with an average rate of $0.4\text{-}0.7 \mu\text{m}/\text{sec}$ or $\sim 1 \mu\text{m}/\text{sec}$, respectively (Roy et al., 2007). SCa transport delivers diverse cytoskeletal elements, while SCb transport is involved in the targeting of several cytosolic axonal proteins needed for synaptic vesicle release. SC axonal transport is bidirectional and often halts for longer pauses between periods of rapid movement. The low average speed defined upon investigating SC axonal transport can thus be explained by the observed 'stop and go' movement and suggests the involvement of the same fast motors as in FC transport (Shah and Cleveland, 2002).

In contrast to axons, long-range transport in dendrites has been less extensively studied and its mechanisms appear to be less clear. The distinctions between axonal and dendritic transport processes depend on the fact that dendrites contain microtubules with mixed polarity and proteins of the postsynaptic density, neurotransmitter receptors, ion

channels, or specific mRNAs have to be transported over much shorter distances and in a more complex structure compared to axonal molecules. A subset of dendritic proteins like NMDA and AMPA receptor subunits, mitochondria or RNA-containing granules are also transported via binding to different kinesin motors but in a less characterised way as in the axons (Hirokawa and Takemura, 2005). Furthermore, recruitment of postsynaptic proteins to nascent synapses in dendrites and in dendritic spines occurs in a gradual manner, without the involvement of discrete transport particles (Bresler et al., 2004). The presence of minus-end-distal microtubules in the dendrites also indicates a special role for the minus-end motor dynein in the selective targeting of dendritic proteins and diverse organelles (Zheng et al., 2008).

The distinct microtubule arrangement between axons and dendrites as well as the selective binding of motor proteins to different organelles and cargo proteins provide a pivotal role for motor proteins in the selective targeting of neuronal membrane proteins, too (Hirokawa and Takemura, 2005). Certain KIFs mediate selective transport by specific localisation to either dendrites or axons (Okada et al., 1995; Marszalek et al., 1999; Guillaud et al., 2003). However, most of the motor proteins are involved in both axonal and dendritic cargo delivery and provide specific transport via binding to different adaptor complexes. In case of KIF5, the delivery of proteins to the axon or to dendrites depends on the interaction with the cargo via adaptor proteins bound either to KLC or KHC, respectively (Setou et al., 2002). The role of adaptor proteins in regulating the direction of kinesin-mediated traffic was also indicated, as the expression of the kinesin-binding domains of axon or dendrite-specific adaptor proteins resulted in the redistribution of KIF5 to the axonal or somatodendritic compartment of neurons, respectively (Setou et al., 2002).

3.2.2.3. Sorting mechanisms at the plasma membrane: selective fusion and selective retention

Several live cell recordings revealed that certain axonal proteins are transported to both axons and dendrites, therefore, selective axonal enrichment can be also a consequence of selective fusion or retention at the axonal membranes (Burack et al., 2000; Sampo et al., 2003).

Selective membrane fusion of the targeted vesicles can lead to polarised enrichment of non-specifically delivered membrane proteins (Sampo et al., 2003). The localisation and specificity of membrane fusion can be determined by diverse membrane-associated protein complexes like the SNARE (soluble N-ethyl maleimide sensitive factor adaptor receptor)

complex. SNAREs are key components of the vesicle fusion apparatus and are involved in constitutive membrane supply in neurons (Horton and Ehlers, 2003b). As SNARE mediated fusion is based on specific binding of diverse SNARE proteins localised in the plasma membrane and on the vesicle, the restricted localisation of specific SNAREs could determine distinct fusion events in different subcellular components. This idea was supported by the cleavage of certain SNARE proteins resulting in reduced axonal growth without affecting dendritic growth (Osen-Sand et al., 1996). Lipid rafts with specific lipid and protein compositions can be also involved in polarised membrane protein distribution in neurons (Hering et al., 2003). As an example, the axon specific localisation of the GPI-anchored cell adhesion molecule Thy-1 was shown to develop in parallel with the establishment of lipid rafts in cultured hippocampal neurons (Ledesma et al., 1999).

A subset of membrane proteins incorporate in the plasma membrane nonspecifically, while polarised distribution is achieved via rapid internalisation from the inappropriate membrane compartments due to selective endosomal pathways. Newly internalised endocytic vesicles fuse with each other or pre-existing endosomes to form early endosomes (EE) which enter either the late endosomal (LE) pathway for degradation or are transferred back to the cell surface either directly or through recycling endosomes (RE). The endosomal components localise throughout the soma and all processes in neurons and play significant role in the recycling of neurotransmitter containing vesicles, neurotransmitter receptors and in plasticity induced synaptic alterations (Buckley et al., 2000), (Bonanomi et al., 2006). Endosomes are also involved in signalling pathways by internalising signalling receptors or cell adhesion molecules upon stimulation (Kamiguchi and Lemmon, 2000; Saxena et al., 2005) and by providing membrane for activity-dependent spine growth and remodelling (Park et al., 2006). The importance of endocytic pathway in postsynaptic events was further supported by the finding that the majority of endosomal structures are situated within or at the base of dendritic spines (Cooney et al., 2002).

The importance of the endosomal system in polarised localisation of membrane proteins was elegantly demonstrated by examining the localisation and transport of the axonal protein VAMP2 (Sampo et al., 2003). Inactivating the endocytotic signal of VAMP2 led to a uniform distribution of the mutant construct throughout the surface of both axons and dendrites indicating the importance of selective dendritic endocytosis for proper axonal enrichment. Selective endocytosis was also indicated in the polarised localisation of the sodium channel Nav1.2 by using surface labelling experiments in living cells. The internalisation of a chimera protein containing the axonal targeting sequence of

Nav1.2 was clearly detected in the somatodendritic compartment of cultured hippocampal neurons and the internalised vesicles were positive for markers of early and recycling endosomes (Garrido et al., 2001). The L1/NgCAM axonal protein is also transported to both axons and dendrites but is finally enriched in axonal plasma membrane via the indirect transcytotic pathway (Wisco et al., 2003). L1/NgCAM is first inserted into the somatodendritic domain, then gets selectively internalised into the somatodendritic endosomes. The final sorting to the axon is further directed by the presence of axonal sorting signals, indicating the sequential activation of different targeting pathways (Yap et al., 2008).

3.3. Structure and function of protein kinase D

3.3.1. Classification and structural characterisation of PKD

The PKD family has been recently described as a separate family among the serine/threonine protein kinases and comprises 3 isoforms in mammals: PKD1, PKD2 and PKD3 (Van Lint et al., 2002; Figure 5). Originally, PKD was identified as a member of the protein kinase C (PKC) family (the initial name for the human isotype of PKD1 is PKC μ while PKD3 was originally named as PKC ν) (Hayashi et al., 1999). Based on the low homology between the catalytic domains of PKCs and PKDs and the distinct substrate and inhibitor specificity, PKC μ /PKD1 and PKC ν /PKD3 have been classified into a new serine/threonine kinase family together with the more recently described PKD2 (Sturany et al., 2001).

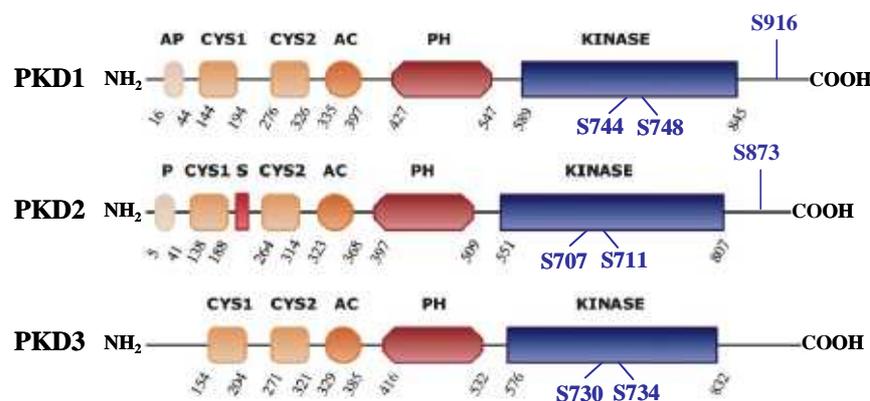


Figure 5. The multidomain structure of PKD isoforms. Amino acid numbering reflects mouse PKDs. Serine (S) phosphorylation sites within the activation loop and autophosphorylation sites at the C-terminal are indicated in blue. AP: alanine- and proline-rich domain; P: proline-rich domain; CYS: cysteine-rich Zn finger domain; S: serine-rich domain; AC: negatively charged amino acids; PH: pleckstrin homology domain; KINASE: kinase catalytic domain. Modified from Rykx et al., 2003.

The overall molecular architecture of PKD1, 2 and 3 shows high degree of sequence homology between the three isoforms (Ryck et al., 2003). The regulatory domain at the N-terminal contains two cysteine-rich domains (CYS1 and CYS2) separated by a long linker region and followed by a stretch of negatively charged amino acids (AC) and a pleckstrin homology domain (PH). Individual differences between the isoforms consist of an apolar region rich in alanine and proline (AP) or just proline (P) at the N-terminal of PKD1 and PKD2, respectively. PKD2 additionally contains a serine-rich stretch (S) within the CYS linker region. The C-terminus contains the conservative serine/threonine kinase catalytic domain, which possesses high sequence homology with the myosin light chain kinase. Based on structural and substrate similarities, PKDs are now classified within the CaMK superfamily (Avkiran et al., 2008).

The individual motifs of the three isoforms can confer isoform-specific functions, although in many cases, PKD1-3 display redundant functions. The most extensively studied isoform is PKD1 - consequently, PKD2 and PKD3 mediated cellular events are less clear and the selective localisation or functional roles of these isoforms need still to be described.

3.3.2. Activity dependent subcellular localisation of PKD

In resting cells, the main fraction of PKD is located in the cytoplasm, with a lesser extent at intracellular compartments including the nucleus, Golgi apparatus and the mitochondria (Rozenfurt et al., 2005).

Upon stimuli induced activation, PKD undergoes rapid translocation to various subcellular compartments and executes its action on diverse intracellular processes (Figure 6). In response to receptor activation, cytoplasmic PKD gets transiently recruited to the plasma membrane, followed by the redistribution into the cytoplasm in an activated state (Rey et al., 2001a). In unstimulated cells, PKD shuttles continuously between the cytosol and the nucleus but gets transiently accumulated in the nucleus after stimulation induced activation at the plasma membrane (Rey et al., 2001b). The dynamic translocation of PKD upon activation enables PKD to transmit signals between different subcellular compartments.

The localisation of PKD to distinct parts of the cell requires interactions through different domains. Plasma membrane localisation was shown to be based on the CYS2 domain via binding to diacylglycerol (DAG), produced in the inner leaflet of the plasma membrane (Matthews et al., 1999).

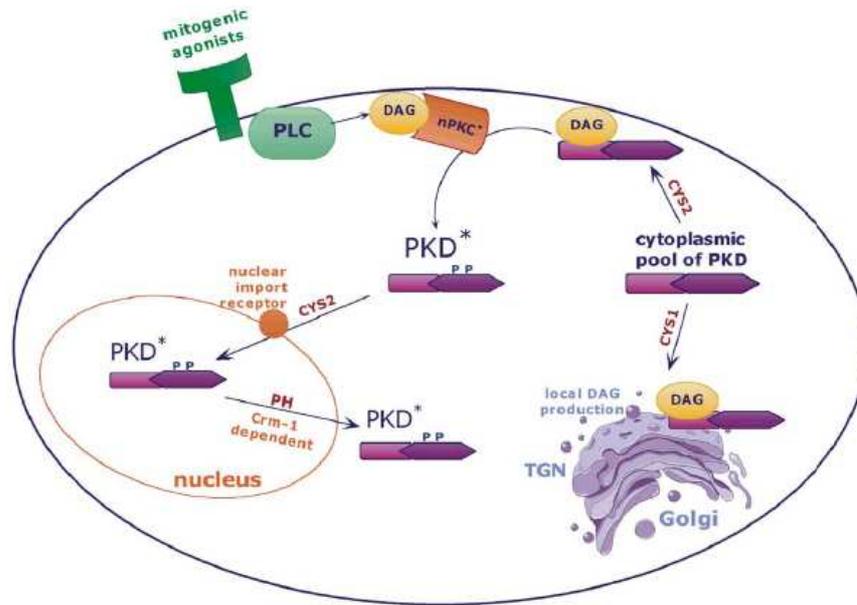


Figure 6. Stimulation induced translocation of PKD to diverse subcellular compartments. Upon extracellular stimulation of the cell, PKD translocates from the cytoplasm to the plasma membrane and gets activated by nPKC mediated transphosphorylation. Afterwards, activated PKD relocates to the cytosol and accumulates transiently within the nucleus. The DAG dependent localisation of PKD to the plasma membrane is mediated through its second cystein-rich Zn finger domain (CYS2), which is also required for its import into the nucleus. Export from the nucleus is mediated by the PH domain. PKDs recruitment to the TGN requires CYS1 mediated binding upon local DAG production. PKD*: activated PKD; nPKC: novel PKC; PLC: phospholipase C; CYS: cystein-rich Zn finger domain; PH: pleckstrin homology domain; DAG: diacylglycerol (Rykx et al., 2003).

CYS2 is also required for the nuclear import of PKD, while the export from the nucleus was shown to be regulated by the PH domain and the Crm-1 dependent nuclear export pathway (Rey et al., 2001b). The recruitment of PKD to the Golgi apparatus is mediated by the CYS1 and seems to depend on local DAG production but is independent of its kinase activity (Maeda et al., 2001).

The reversible translocation from the cytosol to the plasma membrane upon receptor activation was also shown in case of PKD2 and PKD3, however, in contrast to PKD1 and 3, which show accumulation in the nucleus, PKD2 remained predominantly in the cytoplasm (Rey et al., 2003).

3.3.3. Intracellular mechanisms involved in the regulation of PKD activity

In non-stimulated cells, PKD displays low level of kinase activity due to the inhibitory effect of the regulatory domain. Diverse mutations in the PH domain or the deletion of both CYS domains were shown to fully activate PKD, indicating that PKD activity is at least partly regulated intramolecularly (Iglesias and Rozengurt, 1998, 1999).

PKD can get activated through multiple mechanisms dependent on the type of external stimuli and the subcellular localisation. The most extensively studied mechanism

of PKD activation involves DAG dependent recruitment to the plasma membrane and PKC-mediated phosphorylation (Wang, 2006). Various external stimuli including growth factors, neuropeptides, cytokines acting through G-protein coupled receptor (GPCR) or tyrosine kinase receptor activation, and pharmacological agents like phorbol esters were shown to provoke phospholipase C (PLC)-mediated DAG production and successive PKD activation (Zugaza et al., 1997; Van Lint et al., 1998; Yuan et al., 2000). Production of DAG induces the CYS2-mediated recruitment of PKD from the cytosol to the plasma membrane and simultaneously activates PKCs. Novel PKCs (PKC ϵ , PKC δ and PKC η) then activate PKD by transphosphorylating two serine (S) residues (S744 and S748 for mouse PKD1 and S738 and S742 for human PKD1) within the activation loop of the catalytic domain (Waldron and Rozengurt, 2003), which leads to the release of PH domain mediated autoinhibition. Members of the novel PKCs involved in PKD activation show isoform specificity depending on cell context, type and stimuli (Brandlin et al., 2002; Rey et al., 2004; Berna et al., 2007). Additionally, in some cases, the α -subtype of classical PKC also contributes to activation loop phosphorylation of PKD (Wong and Jin, 2005). Recent studies revealed a PKC-independent mechanism of S748 phosphorylation, as well, achieved by autophosphorylation during late phase of GPCR agonist induced PKD activation (Jacamo et al., 2008). Disturbing PKC-mediated phosphorylation of PKD did not affect its recruitment to the plasma membrane but strikingly retarded its translocation to the cytosol indicating that PKC-mediated activation is required only for the rapid reverse translocation of PKD whereas the recruitment occurs independently of PKD activity (Rey et al., 2001a). Similarly to activation at the plasma membrane, CYS1-mediated recruitment of PKD to the Golgi apparatus depends on local DAG production and was shown to be activated upon G $_{\beta\gamma}$ ($\beta\gamma$ subunit of the heterotrimeric G protein) induced transphosphorylation by recruited and activated PKC η (Ghanekar and Lowe, 2005). However, in contrast to plasma membrane recruitment, PKD remains associated with the Golgi apparatus and phosphorylates effector molecules localised there.

Besides the two serines within the activation loop, PKDs are further phosphorylated on additional serine sites during *in vivo* activation. The autophosphorylation site located at the C-terminal (S916 in mouse PKD1 and S910 in human PKD1) is thought to regulate conformational changes and to interact with PDZ proteins (Sanchez-Ruiloba et al., 2006). The phosphorylation event of S916 is often used to determine the activation state of PKD. Two other putative phosphorylation sites have been also described within the regulatory

domain: S203, another autophosphorylation site mediating the interaction of PKD with the regulator protein 14-3-3 and S255, which is a transphosphorylation site phosphorylated by downstream targets of PKC pathway (Vertommen et al., 2000).

PKD2 and PKD3 also get activated through the DAG-PLC-PKC-dependent pathway showing transphosphorylation of analogue serine sites within the activation loop as described earlier for PKD1 (Sturany et al., 2002; Matthews et al., 2003; see also Figure 5). Compared to PKD1 and PKD2, PKD3 does not have an autophosphorylation site at its C-terminal (Sanchez-Ruiloba et al., 2006).

Besides phosphorylation of serine residues, tyrosine (Tyr) phosphorylation was also shown to regulate PKD activation. Oxidative stress induced Src activation causes Tyr phosphorylation within the PH domain leading to the binding of PKC δ and the consecutive activation of PKD via transphosphorylation of the activation loop (Doppler and Storz, 2007). Moreover, PKD can be also activated independently of PKC-mediated phosphorylation. Upon apoptosis induced by genotoxic agents, PKD was shown to be activated by caspase-mediated cleavage leading to the release of the catalytic domain and the consequent activation of PKD (Endo et al., 2000). PKD is also activated by bone morphogenetic protein 2 and endothelin-I independent of PKC-mediated phosphorylation, but the underlying mechanisms remain to be elucidated (Lemonnier et al., 2004; Harrison et al., 2006).

3.3.4. Multiple cellular functions of PKD

According to the various subcellular signalling pathways involved in PKD activation, PKD has been indicated to play a role in diverse cellular processes including cell growth, proliferation, migration, invasion, differentiation, signal transduction, and membrane transport. Besides regulating common cellular processes, there is growing evidence for the involvement of PKD in relevant organ specific functions exerted in the heart, in the vascular system, in the immune system, and in the nervous system. However, as the elucidation of PKD-mediated regulatory pathways and the identification of direct substrates has been started only recently, several details are still not clarified and need further research.

3.3.4.1. Cytoplasmic PKD influences diverse intracellular signalling pathways

After the recruitment to and activation of PKD at the plasma membrane induced by external stimuli, PKD returns to the cytosol and exerts its effect by modulating major

signalling pathways. PKD is involved in the regulation of cell proliferation and differentiation by modulating mitogen-activated protein kinase (MAPK) cascades in response to growth factor or phorbol ester stimulation (Bagowski et al., 1999; Celil and Campbell, 2005; Wong and Jin, 2005). Activated PKD upregulates the extracellular signal regulated kinase (ERK) pathway via Ras and Rab interactor protein 1 (RIN1), an identified substrate of PKD. The phosphorylation of RIN1 by PKD increases its association with 14-3-3 protein and thus blocks the interaction of RIN1 with Ras. Consequently, free Ras can activate ERK through the proximal kinases of ERK kinase cascade (Wang et al., 2002). In contrast to the ERK pathway, activation of PKD can lead to the downregulation of another MAPK cascade involving the c-Jun N-terminal kinase (JNK). Activated PKD interacts directly with JNK or phosphorylate c-Jun at sites distinct from those phosphorylated by JNK thus influencing the interaction of c-Jun and JNK (Hurd et al., 2002). This mechanism can explain how PKD is involved in platelet derived growth factor (PDGF)-induced suppression of epidermal growth factor (EGF)-mediated JNK activation (Bagowski et al., 1999).

PKD-mediated regulation of ERK and JNK pathways plays a role in pro- and antiapoptotic responses induced by asbestos (Buder-Hoffmann et al., 2009). The involvement of PKD in processes regarding cell survival and death was further indicated by showing PKD activation during oxidative stress induced responses. PKD gets activated upon H₂O₂ induced oxidative stress via distinct pathways involving PKC δ -mediated phosphorylation and exerts effects in both apoptosis induction and prevention (Zhang et al., 2005; Song et al., 2006; Doppler and Storz, 2007).

3.3.4.2. Nuclear PKD modulates gene transcription

PKD localised in the nucleus can regulate gene expression indirectly, via interaction with the class II histon deacetylases (HDACs) (Harrison et al., 2006). The direct phosphorylation by PKD induces the interaction of HDACs with 14-3-3 proteins, which triggers the nuclear export of HDACs. Consequently, HDAC-mediated transcriptional repression is released. Gene expression induced by PKD-mediated nuclear export of HDACs has been shown to play a role in diverse physiological and pathological processes including vascular endothelial growth factor (VEGF)-mediated angiogenesis or stress induced cardiac hypertrophy (Harrison et al., 2006; Wang et al., 2008).

Recently, the transcription factor cAMP-response element-binding protein (CREB) was also identified as direct PKD substrate: phosphorylation of CREB upon PKD

activation induces the recruitment of co-activators and leads to the expression of CREB-responsive genes, like the early response gene Nur77. As CREB-responsive genes are widely involved in proliferative responses, this work has described an additional way for PKD to regulate cell proliferation, differentiation, apoptosis and secretory pathways (Johannessen et al., 2007).

3.3.4.3. PKD associated to the Golgi apparatus regulates protein transport and membrane trafficking

Presumably the best characterised functions of PKD are the regulation of vesicle fission at the Golgi apparatus and the involvement in the regulation of plasma membrane directed protein transport. Based on the findings that the marine sponge metabolite ilimaquinone (IQ)-induced Golgi fragmentation involves the activation of PKD by direct interaction with $G_{\beta\gamma}$, PKD was described as a central regulator of Golgi organisation (Jamora et al., 1999). Further investigations using a kinase inactive form of PKD indicated a role of Golgi-localised PKD in the formation and fission of transport vesicles from the TGN, since the expression of kinase inactive PKD induced the tubulation of TGN and was shown to inhibit protein transport from the TGN to the plasma membrane (Liljedahl et al., 2001). The mechanism underlying PKD-mediated vesicle fission has been extensively studied in the last decade leading to a more detailed but still not completely clarified picture of PKD's role in transport carrier formation and plasma membrane directed protein transport. In summary, according to the model proposed by Diaz-Anel (Diaz Anel, 2007), $G_{\beta\gamma}$ is generated by activation of GPCR at the plasma membrane by a still undefined signal and is subsequently translocated to the TGN. TGN located $G_{\beta\gamma}$ activates PLC β 3 leading to DAG production, which recruits PKD to the Golgi and activates PKC η simultaneously. Activated PKC η interacts with the PH domain of PKD and activates PKD by direct phosphorylation of the activation loop serines. Phosphatidylinositol 4-kinase III β (PI4KIII β) is involved in vesicle fission from the TGN and was recently identified as one of the direct substrates of activated PKD (Hausser et al., 2005). Therefore, PKD can regulate vesicle fission via controlling lipid kinases, which play an important role in regulating the generation of diverse lipids leading to membrane deformation and the subsequent vesicle budding upon transport carrier formation (Bankaitis, 2002; Bard and Malhotra, 2006). The downstream PKD effectors and mechanisms involved in vesicle budding, however, are far from well understood. The linkage of PKD and lipid homeostasis was further supported by identifying the lipid transfer protein (CERT) as a

novel substrate of PKD (Fugmann et al., 2007). The regulation of CERT affinity towards its lipid targets by PKD-mediated phosphorylation was indicated to play a critical role in membrane directed cargo transport.

PKD2 and PKD3 isoforms can also exert a role in TGN-derived vesicle formation as the siRNA mediated knockdown of either PKD2 or PKD3 inhibits Golgi-to-cell surface transport (Bossard et al., 2007). Furthermore, as the depletion of a single isoform was shown to affect surface directed transport markedly, PKD2 and PKD3 were proposed to regulate the fission of exocytic vesicles as dimers.

Besides the investigations in non-polarised cells described above, the role of PKD in surface directed transport has been also examined in polarised epithelial cells. PKD is specifically involved in the basolateral directed protein transport indicated by the missorting of basolateral cargo to the apical membrane upon the expression of kinase inactive PKD1 or PKD2 (Yeaman et al., 2004). The transport of proteins carrying apical sorting signal, on the other hand, was unaffected. PKD3 was not involved in the selective protein transport, at least in the investigated epithelial cells.

3.3.5. The function of PKD in neurons

Although all three PKD isoforms are expressed in the nervous system of rodents already at early embryonic stages, little is known about the functional relevance of PKD in neurons (Oster et al., 2006).

A special role of PKD in neurons was indicated by the identification of the first physiological PKD substrate termed kinase D-interacting substrate of 220 kDa (Kidins220 or alternatively Ankyrin-rich membrane spanning/ARMS), which is an integral membrane protein predominantly expressed in the brain and in neuroendocrine cells (Iglesias et al., 2000). Further studies indicated that PKD1 and PKD2 are involved in the surface targeted trafficking of Kidins220 and suggested a significant role for the autophosphorylation site located in the PDZ binding domain of PKD1 and PKD2 in regulating Kidins220 transport (Sanchez-Ruiloba et al., 2006). Recent investigations revealed a role for Kidins220 in neurotrophic signalling-mediated neuronal differentiation or survival and in neurotensin secretion, as well (Cortes et al., 2007; Li et al., 2008; Sniderhan et al., 2008).

RIN1, another known substrate of PKD, is predominantly expressed in the nervous system with high expression levels in forebrain structures (Wang et al., 2002; Bliss et al., 2006). Investigations using RIN1 null mice indicated a role for RIN1 in negative regulation of neuronal plasticity and memory formation in the amygdala (Dhaka et al., 2003). An

underlying mechanism was proposed by showing the involvement of RIN1 in ephrinA4 (EphA4) endocytosis suggesting a role for RIN1 in regulating synaptic plasticity by modulating Eph mediated intracellular events (Deininger et al., 2008).

A role for PKD during neuronal development has been also indicated, as the expression of kinase inactive PKD mutant was shown to decrease dendritic outgrowth and complexity both *in vivo* and *in vitro* (Horton et al., 2005). Furthermore, recent investigations implicated the involvement of PKD in the establishment of neuronal polarity, as decreased PKD activity led to multiple axon formation in developing hippocampal neurons (Yin et al., 2008). Additionally, recent study revealed a role for PKD in polarised membrane trafficking of mature neurons (Bisbal et al., 2008).

MAIN GOALS

With the aid of transfecting mouse embryonal hippocampal neuronal cultures with various, fluorescently labelled constructs, our aim was:

1. To investigate the localisation of endogenous PKD activity during neuronal development and in mature neurons by expressing a PKD activity reporter in primary hippocampal neuronal cultures.
2. To unravel the intracellular localisation and possible interactions of fluorescently labelled wild type and mutant PKD proteins in mature hippocampal neurons by colocalisation studies.
3. To analyse PKD-mediated effects on neuronal Golgi structure and dendritic arborisation in mature neurons upon expression of kinase inactive and constitutive active PKD proteins.
4. To investigate the axonal transport of PKD, as well as its possible effect on the transport of diverse axonal proteins with the aid of fast live cell imaging recordings.

MATERIALS AND METHODS

Animals

CD1, CaMKII α rtTA2 (Michalon et al., 2005) or kdPKD1-EGFP transgenic mice (Czondor et al., 2009) were housed in the Animal Facility of the Biological Institute, Eotvos Lorand University or the Institute of Cell Biology and Immunology, University of Stuttgart at 22 \pm 1 °C with 12h light/dark cycles and with ad libitum access to food and water. All procedures were performed under the supervision of Local Animal Care Committee, in agreement with the European Union and Hungarian legislation (Budapest) or approved by the Regierungspräsidium Stuttgart. All experiments were complied with local guidelines and regulations for the use of experimental animals (35-9185.81/0209, 35-9185.81/0247 and #878/003/2004 for the experiments carried out in Stuttgart or in Budapest, respectively).

Expression constructs

pEGFP-N1, pmRFP-N1 and pmCherry-N1 vectors were from Clontech and A. Jeromin, Allen Institute for Brain Science, Seattle, USA, respectively. Fluorescently tagged plasmids are summarised in Table 1. Constructs marked with * were generated in the laboratory of A. Hausser, Institute of Cell Biology and Immunology, Stuttgart, Germany. Plasmids marked with § were kind gifts of A. Jeromin. The NR1-GFP plasmid was from K. McAllister, Center for Neuroscience, California, USA; the pMH4-SYN-P65-I-EGFP plasmid from A. Illiev, European Neuroscience Institute, Göttingen; the pEGFP-N1- α -Synuclein construct from Robert Edwards, University of California, San Francisco; the pEGFP-Synapsin Ia from George Augustine, Duke University Medical Center, Durham, NC and the pEGFP-C1 MyosinV brain construct was generated in the laboratory of L. Nyitray, Eötvös Loránd University, Budapest.

Cell cultures and transfection

Primary cultures of embryonal hippocampal cells were prepared from CD1 mice on embryonic day 18. After aseptically removing hippocampus from the skull, tissue was freed from meninges and incubated in 0.05% trypsin-EDTA (Gibco, Hungary) solution with 0.05% DNaseI in PBS for 15 min at 37°C. After a brief centrifugation, cells were

Abbreviation	Plasmids	Description	Reference
<i>PKD constructs</i>			
wtPKD1	pEGFP-N1-pkcμ * pmRFP-N1-PKD1 * pCherry-N1-PKD1 *	wild type human PKD1	Hausser et al., 2002
kdPKD1	pEGFP-N1-PKCμK612W * pmRFP-N1-PKD1 K612W * pCherry-N1-PKD1 K612W *	kinase inactive / kinase dead human PKD1	
caPKD1	pEGFP-N1-PKD1 S738/742E * pmCherry-N1-PKD1 S738/742E *	constitutive active human PKD1	Storz et al., 2004
G-PKDrep	pEGFP-L-pS294-L-p230-98aa *	Golgi targeted PKD activity reporter	Fuchs et al., 2009
G-PKDrep S/A	pEGFP-L-A294-L-p230-98aa *	G-PKDrep, containing a serine to alanine mutation in the PKD target sequence	
PKDrep	pEGFP-C1-L-pS294-L *	non-targeted PKD activity reporter	Czondor et al., 2009
PKDrep S/A	pEGFP-C1-L-pS294A-L *	PKDrep, containing a serine to alanine mutation in the PKD target sequence	
<i>Constructs of dendritic proteins</i>			
PSD-95	pEGFP-N1-PSD 95 §	scaffolding protein of the postsynaptic density	Bresler et al., 2001
Kv4.2	pEGFP-N1-Kv 4.2 §	K ⁺ channel localised to the somatodendritic region and contributes largely to dendritic excitability	Chu et al., 2006
NR1	NR1-GFP	indispensable subunit of NMDA receptors	Bresler et al., 2004
SAP97	pmCherry-C1-SAP 97 *	scaffold protein involved in transport and localisation of neurotransmitter receptors and ion-channels	Gardoni et al., 2007
<i>Constructs of axonal proteins</i>			
synaptotagmin	pMH4-SYN-P65-I-EGFP	presynaptic protein involved in vesicle release	Stagi et al., 2005
synaptophysin	p38mCherry pcDNA3 §	presynaptic protein localised in the membrane of synaptic vesicles	Roy et al., 2007
synuclein	pEGFP-N1- α-Synuclein	soluble protein localised mainly in presynaptic terminals with unknown function	
synapsin	pEGFP-Synapsin Ia	presynaptic protein involved in vesicle release	
myosin Vb	pEGFP-C1 MyosinV brain	actin based motorprotein involved in recycling processes of the plasma membrane	Lapierre et al., 2001

Table 1. Fluorescently labelled constructs used in transfection studies.

trituated in NeuroBasal (NB) supplemented with B27 (Gibco) containing 5% Fetal Calf Serum (FCS; Sigma, Hungary), 0.5 mM glutamax (Gibco), 40 µg/ml gentamicin (Hungaropharma) and 2.5 µg/ml amphotericin B and filtered through a sterile polyester mesh with 42 µm pore size (EmTek Ltd, Hungary). Cell number was determined by trypan blue exclusion, and cells were seeded in NB culture medium onto poly-L-lysine (Sigma, Hungary) – 1 µg/cm² laminin (Sigma, Hungary) coated glass coverslips (13 mm diameter) in 24-well plates at 6x10⁴ cells/cm² cell density for microscopic observations. For live cell imaging experiments, 4x10⁵ cells were plated onto PLL-laminin coated glass-bottom 35 mm petri dishes (Greiner, Germany). Cytosin-arabinofuranoside (CAR, 10 µM; Sigma, Hungary) was added to the cultures 48 h after plating, then one third part of the culture medium was changed to NeuroBasal supplemented with B27 without FCS on the fourth day of cultivation. One third of the medium was replaced every 3-4 days afterwards. Cells were cultivated for 10 days at 37°C in 5% CO₂/95% air atmosphere. Immunocytochemical stainings revealed relatively low amount (less than 5%) of interneurons containing GABA and high level of vesicular glutamate transporter-1 (vGluT1) in our cultures (data not shown).

9 days after plating, transfection of hippocampal cells was carried out using Lipofectamine2000 (Invitrogen, Csertex, Hungary) according to the manufacturer's instructions. In 24-well plates, 0.3-0.5 µg plasmid DNA was mixed with Lipofectamine in a 1µg:2 µl ratio. Medium was changed 5-8 hours after the transfection to the original cultivation medium, and cells were analysed at the indicated time points after transfection. Phorbol ester treatment of cultures was carried out either by phorbol 12,13-dibutyrate (PDBu; 1 µM; 2mM stock solution dissolved in DMSO; Sigma, Germany) or by the cell permeable DAG analogue, sn-1,2-dioctanoylglycerol (diC8; 25 µM; 25mM stock solution dissolved in DMSO; Sigma, Germany) treatment in the culture medium.

In case of doxycycline (Dox) induced kdPKD1-EGFP expression, primary E18 embryonal hippocampal cultures were prepared from pregnant females as described above, after heterozygous kdPKD1-EGFP mice were crossed with heterozygous mice carrying the tetracycline-controlled transactivator rtTA2. In this way, approximately 25% of the embryos were double transgenic which was verified by PCR analyses (data not shown). As rtTA2 is under the control of CaMKIIα promoter, the transactivator shows forebrain and hippocampus-specific expression pattern providing thus neuron-specific transgene-expression upon Dox mediated induction (Michalon et al., 2005). To induce kdPKD1-

EGFP expression, pregnant females were fed with Dox (Fagron GmbH Co. KG, Barsbüttel, Germany) containing food at 6mg/g dose between E13 to E18, while 1 µg/ml Dox was present in the culture medium during in vitro cultivation, as well.

Immunostaining

Hippocampal cultures were fixed on the tenth day after plating (DIV10) for 20 min with cold 4% paraformaldehyde - 4% sucrose in PBS. After rinsing, cells were permeabilised with 0.1% Triton X-100 for 5 minutes. Non-specific antibody binding was blocked by incubation with 2% BSA - 0.1% Na azide in PBS (PBS-BSA) for 1 hour at room temperature. Primary antibodies like anti-GM130 (mouse IgG1, 1:250, BD Transduction Laboratories), anti-VAMP4 (rabbit, 1:300, Sigma), anti-pS294 (rabbit, 1:750; Hausser et al., 2005), or anti-PKD1 (JP2; monoclonal IgG, 1:500; Johannes et al., 1995) were diluted in PBS-BSA and used at 4 °C overnight. Anti-mouse IgGs conjugated with Alexa 633 (1:500, Molecular Probes) or anti-rabbit-Alexa 546 (1:1000, Molecular Probes) or F_(ab)2 fragments of anti-mouse IgG conjugated with Alexa 488 (for visualising JP2 signal; 1:500, Molecular Probes) were employed for 1.5 hour at room temperature. Cultures were mounted with Mowiol 4.88 (Polysciences, Germany) supplemented with bis-benzimide. In all cases, control samples were processed similarly, except that the first layer antibodies were omitted.

Microscopy

Microscopic analyses were carried out only from those transfected neurons, which had a clear distinction between dendrites and the axon, a complete dendritic tree with dendritic spines and an intact nucleus. Pictures were taken with Zeiss HS CellObserver inverse microscope equipped with AxioCam HR 12 bit camera, the Apotome system and with Plan Neofluar 10x/0.3 M27, Plan Apochromat 20x/0.8 M27 or Plan Apochromat 63x/1.4 oil DIC M27 objective lenses. 3D reconstructions of the Golgi structure were made out of 28 to 33 z-stacks using the Inside 4D tool of the AxioVision 4.6 software. In this case, adjacent z-stacks were recorded by 0.15 µm steps. Confocal microscopy was performed with a Leica (TCS SP2) confocal scanning microscope at Airy pinhole using 488, 543, and 633 nm excitation and a 100x/1.4 HCX PL APO objective lens. Wide-field fluorescent pictures were taken by a BX51 Olympus microscope equipped with a

FluoViewII camera and the AnalysisPro software, using either 60x/1.4 or 100x/1.2 oil Plan Apochromat phase contrast objectives.

Live cell imaging observations were carried out with the Zeiss HS CellObserver and with a 488 nm blue led illumination by the Colibri system. In case of fast live cell recordings using dual colour labelling, the fast switch between the green and red channels was ensured by the Lambda DG-4 illumination system (Sutter Instrument) containing galvanometers utilizing interference filters for fast wavelength selection and a dual emission filter. These equipments enable taking pictures every 300-500 milliseconds. Neurons were observed by the Plan Apochromat 63x/1.4 oil DIC M27 objective lens and with the AxioCam HR camera with 200 msec frame rate and 2x2 binning. During imaging, cultures were kept in imaging buffer (142 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM NaH₂PO₄, 25 mM HEPES, 5 mM glucose, 0.8 mM MgCl₂, pH 7.4) and at 37 °C. In case of PDBu treatment, 1 μM PDBu was applied to the observed petri dish directly on the stage of the microscope.

Fluorescence recovery after photobleaching (FRAP) experiments were carried out with the Leica TCS SP2 confocal scanning microscope using 488-nm laser illumination and a 63x/1.4 HCX PlanAPO oil immersion objective. During the experiments, living cells were kept in the imaging buffer described above, at 37 °C. Initially, basic fluorescence signal within the defined dendritic region was recorded by 5 scans in every 15 seconds. Selective bleaching was performed by 5 consecutive scans at full laser power. Recovery of the fluorescence signal within the same region was followed immediately after bleaching, either in every 3 seconds up to 3 minutes or in every 30 seconds for 10 minutes.

Quantitative analyses of microscopy data

Golgi apparatus enrichment index (GI) was calculated to quantify the intracellular distribution of GFP-tagged PKD1 constructs in DIV10 neurons. 12 bit fluorescent images were recorded below saturation level with Plan Apochromat 63x/1.4 oil DIC M27 objective lens, using the Zeiss Apotome system. Average fluorescent intensity was calculated from 5x1 μm² areas located over the cytoplasm or over the VAMP4-stained TGN structures followed by subtracting background fluorescent intensity. Average TGN intensity values were compared to the corresponding cytoplasmic intensity values, resulting in GI for each cell. Average GI was calculated from 13 EGFP, 13 wtPKD1-EGFP, 26 kdPKD1-EGFP and 20 caPKD1-EGFP transfected neurons.

Golgi morphology was analysed in 3 independent cultures, transfected with the indicated constructs in parallel. In total, Apotome recordings were made from 34 EGFP, 46 wtPKD1-EGFP, 37 kdPKD1-EGFP and 53 caPKD1-EGFP transfected neurons, using the Plan Apochromat 63x/1.4 oil DIC M27 objective lens. Only those cells were taken into account where VAMP4 immunostaining of the Golgi complex was clearly detectable. Neuronal Golgi morphology was regarded as thread-like when cis- and trans-Golgi structures appeared as reticular networks with mostly continuous filaments. Dispersed Golgi structure had much shorter (if any) reticular structures and many small fragments, all labelled with VAMP4 and GM130, as well.

The same criteria were used for determining Golgi morphology of kdPKD1-EGFP expressing neurons at different posttransfectional time-points. Golgi morphology was analysed 12, 18 and 24 hours after transfection in 54, 72 and 50 transfected neurons from 3 parallel cultures, respectively. Changes in the average expression levels of the same kdPKD1-EGFP transfected neurons were analysed using 8-bit wide-field fluorescent images recorded by the BX51 Olympus microscope with identical exposure and gamma settings. Average intensity values were calculated by determining average fluorescent intensity in ROIs drawn manually around the soma of the transfected neurons.

Dendritic tree morphology of 27 EGFP, 23 PKD1-EGFP, 28 kdPKD1-EGFP and 27 caPKD1-EGFP transfected cells was analysed by a modified Sholl analysis. A template consisting of circles with consecutively increasing diameter (with 40 μm steps) was placed on the inverted pictures of the transfected neurons and the number of intersections was determined at every level. Average number of primary dendrites was calculated in 5 EGFP, 10 wtPKD1-EGFP and 10 kdPKD1-EGFP transfected cells. Average thickness of primary dendrites was measured at the most proximal part of the primary dendrites in the same cells. The correlation between Golgi morphology and the extent of dendritic arborisation was investigated by simultaneously analysing 37 kdPKD1-EGFP transfected cells' dendritic arborisation and Golgi complex.

During quantitative evaluation of the microscopy data, only cells with intact nuclei (visualised by DAPI staining) were taken for analyses. For statistical evaluation, Student t-test was used in all cases ($p < 0.05$).

FRAP analyses of wtPKD1-EGFP ($n=6$) and kdPKD1-EGFP ($n=4$) transfected neurons were demonstrated by showing average fluorescence signal values at each time point of the measurement calculated from the recordings made under identical settings and normalised to the average fluorescence values measured before bleaching.

RESULTS

6.1. PKD activity is required for the maintenance of neuronal Golgi structure

6.1.1. PKD is enriched at the neuronal trans-Golgi network

We have visualised neuronal Golgi apparatus in cultured mouse hippocampal neurons using GM130 and VAMP4 (Vesicle Associated Membrane Protein 4) immunostaining. GM130 has been widely used both in non-neuronal and neuronal cells to label cis-Golgi (Nakamura et al., 1995; Horton et al., 2005) while VAMP4, a member of the SNARE complex, is known to be highly enriched at the TGN and regulate the traffic and sorting of recycling endosomes (Steggmaier et al., 1999; Tran et al., 2007). Both GM130 and VAMP4 highlighted filamentous, perinuclear staining in cultivated neurons (Figure 7). Cis- and trans-Golgi compartments were located side-by side forming a reticular structure in the soma. Filamentous Golgi structures often entered one or two dendrites, as well.

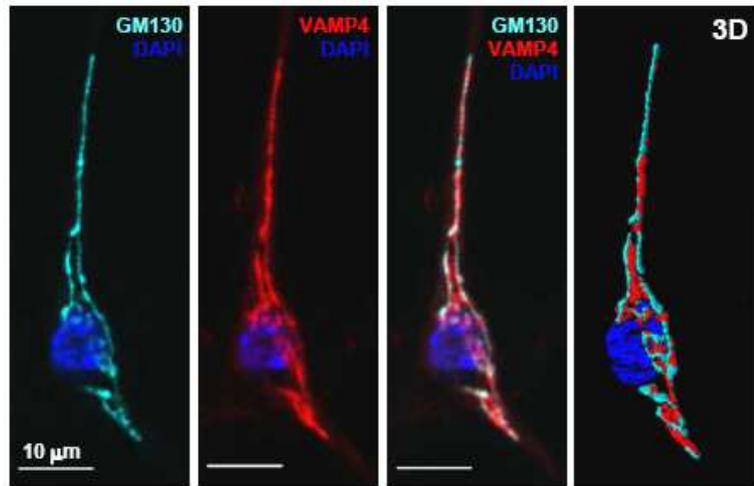


Figure 7. Golgi structure in non-transfected hippocampal neurons. GM130-positive cis- and VAMP4-positive trans-Golgi compartments are located side-by side forming a reticular structure in the soma and enter into one or two dendrites, as well. Pictures on the left show individual z-stacks, while the right-most picture is a 3D reconstruction made from 29 z-stacks. Nucleus is stained by DAPI.

The intracellular localisation of PKD was followed by introducing EGFP-tagged human wild-type PKD1 (wtPKD1-EGFP) into hippocampal neurons already possessing elaborate axonal arborisation and well developed dendrites (9 days after plating; DIV9). Cultures were fixed 22-24 hours after transfection and were processed for GM130 and VAMP4 immunocytochemistry (Figure 8). Besides a rather homogenous cytoplasmic distribution, wtPKD1-EGFP was enriched at the Golgi apparatus (Figure 8B). The extent of wtPKD1-EGFP accumulation at the Golgi complex was calculated by defining a Golgi apparatus enrichment index (GI) as described in Materials and Methods. Briefly, background intensity values, average fluorescence intensity over the cytoplasmic region or over the VAMP4-positive TGN were calculated for each cell. GI was determined by

comparing average TGN intensity to the corresponding cytoplasmic intensity values after the subtraction of background fluorescent intensity from individual cytoplasmic or TGN intensity values. While average cytoplasmic intensity values did not differ significantly between EGFP and wtPKD1-EGFP transfected neurons, wtPKD1-EGFP signal was significantly enriched at the TGN (Table 2). On closer examination, wtPKD1-EGFP was localised side-by-side with GM130 (Figure 8C, see enlargement) but showed partially overlapping localisation with VAMP4-stained structures (Figure 8D, see enlargement).

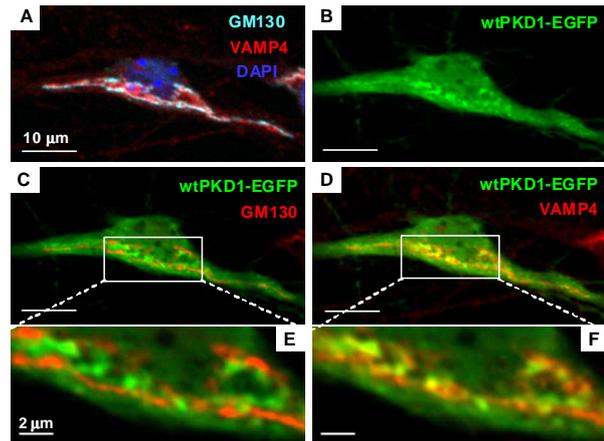


Figure 8. Localisation of wtPKD1-EGFP in transfected hippocampal neurons. wtPKD1-EGFP shows side-by-side localisation with GM130 (C) and strongly colocalises with VAMP4-stained structures (D). E, F: Enlarged perinuclear regions from C and D, respectively. For the sake of better comparison, GM130 and VAMP4 are similarly displayed in red color. Nucleus is stained by DAPI.

transfection	number of analysed cells	Golgi apparatus enrichment index (GI)	average fluorescence intensity in the cytoplasm	average fluorescence intensity at the TGN	average background fluorescence intensity
EGFP	13	1.27 ± 0.05	213.58 ± 18.19	267.65 ± 19.57	2.83 ± 0.50
wtPKD1-EGFP	13	1.91 ± 0.10*	253.05 ± 28.49	463.01 ± 44.30*	2.12 ± 0.26
caPKD1-EGFP	20	1.89 ± 0.08*	246.36 ± 19.38	445.63 ± 36.47*	3.29 ± 0.41
kdPKD1-EGFP	16	8.24 ± 0.83**	120.39 ± 17.36**	692.81 ± 41.63**	4.12 ± 0.42

Table 2. PKD1 is enriched at the TGN of the transfected neurons. GI was determined by comparing average EGFP intensity at the TGN to the corresponding cytoplasmic EGFP intensity values after the subtraction of background fluorescent intensity from individual cytoplasmic or TGN intensity values. In comparison to values obtained from EGFP transfected neurons, wtPKD1-EGFP and caPKD1-EGFP were significantly enriched at the TGN while kdPKD1-EGFP signal was reduced in the cytoplasm but highly trapped at the TGN. Data are given as mean ± s.e.m. Asterisks indicate significant changes compared to the corresponding EGFP values (*: p<0.05; **: p<0.001). Significance of differences was calculated by an unpaired Students t-test.

Phorbol ester treatment is known to cause plasma membrane translocation and activation of PKD (Zugaza et al., 1996; Matthews et al., 1999; Rey et al., 2004). In live cell

imaging of hippocampal neurons, 1 μ M PDBu treatment led to a rapid recruitment of wtPKD1-EGFP to the plasma membrane, followed by the intracellular accumulation of the fluorescently tagged protein (Figure 9A). In addition, immunocytochemical staining with VAMP4 confirmed that 15 minutes of PDBu treatment enhanced the translocation of wtPKD1-EGFP to the TGN as well as to the nuclear membrane and to other intracellular structures (Figure 9B). These results indicate that fluorescently labelled PKD1 behaved similarly in transfected neurons as endogenous PKD in non-neuronal cells.

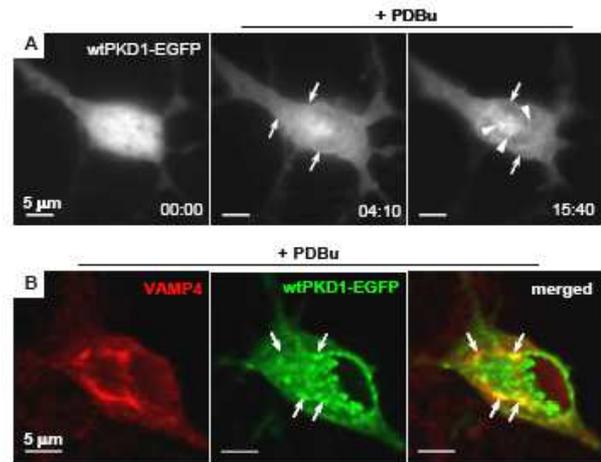


Figure 9. PDBu treatment leads to the translocation of wtPKD1-EGFP. (A) PDBu treatment induces the recruitment of wtPKD1-EGFP to the plasma membrane (see arrows) and to internal structures (see arrowheads) in live cell imaging recordings (elapsed time is indicated as min:sec). (B) wtPKD1-EGFP is localised to the TGN (indicated by the arrows) after 15 minutes of 1 mM PDBu treatment. TGN is immunostained with anti-VAMP4.

6.1.2. Endogenous PKD is active at the neuronal trans-Golgi network

The consensus PKD target site has been mapped and antibodies raised against the phosphorylated serine of the consensus PKD target site have been successfully used to demonstrate PKD-mediated phosphorylation of various substrate molecules (Doppler et al., 2005; Hausser et al., 2005). PI4KIII β is a known target of PKD and gets selectively phosphorylated by PKD on S294 (Hausser et al., 2005). In order to visualise endogenous PKD activity at the Golgi complex, a Golgi-targeted EGFP-tagged PKD activity reporter construct (Figure 10A; G-PKDrep) was created in Stuttgart using the PKD specific substrate sequence of PI4KIII β fused to the GRIP domain of p230, a known trans-Golgi localised protein (Fuchs et al., 2009). PKD-mediated phosphorylation of the transfected construct can be detected by an antibody specific for phosphorylated S294 (anti-pS294; (Hausser et al., 2005). Specificity of the immunostaining was proven by expressing a reporter containing a serine to alanine mutation inside the PKD target sequence (Figure 10B; G-PKDrep S/A).

The GRIP domain of p230 led to a selective enrichment of both Golgi-targeted PKD reporter constructs at the Golgi complex, the latter visualised by GM130 staining (Figure 10). As expected, pS294 antibody staining was detected only in those cells which were transfected with the PKD reporter containing the original serine site. GM130

immunostaining revealed a side-by-side localisation with the pS294 signal which is in accordance with the TGN-directed localisation of the PKD activity reporter. Importantly, Golgi-targeted reporter was highly phosphorylated at the TGN in transfected neurons, even without phorbol ester treatment.

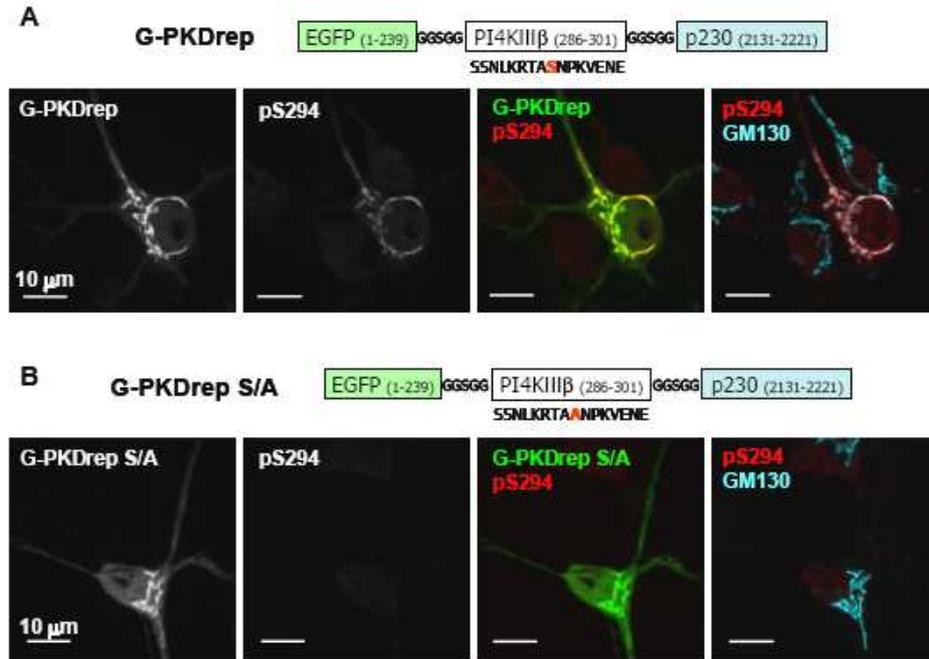


Figure 10. Endogenous PKD activity is detected at the neuronal Golgi complex. (A) Phosphorylation of the G-PKDrep construct (depicted by the cartoon) is detected by immunostaining with a pS294-specific antibody. pS294 signal strongly colocalises with the EGFP-tagged G-PKDrep signal and shows side-by-side localisation with the GM130-stained cis-Golgi, as well. (B) No pS294 signal is detected in the neurons which express a G-PKDrep control construct with a serine to alanine mutation (see cartoon). Regardless to the mutation, the control construct is also localised to the neuronal Golgi complex.

6.1.3. Kinase inactive PKD1 mutant strongly accumulates at the trans-Golgi network

6.1.3.1. Kinase inactive mutation of PKD1 leads to restricted intracellular localisation

In order to investigate the effects of different PKD mutations on neuronal Golgi complex morphology, DIV9 hippocampal neurons were transfected with EGFP-tagged wild-type, constitutive active (caPKD1-EGFP; PKD1^{S738/742E}-EGFP) and dominant negative kinase inactive (kdPKD1-EGFP; PKD1^{K612W}-EGFP) forms of human PKD1. 24 hours after transfection, caPKD1-EGFP showed similar distribution pattern to wtPKD1-EGFP (see Figure 11 versus Figure 8B), being evenly distributed in the cytoplasm and enriched around the neuronal Golgi complex. Quantitative analyses of the enrichment confirmed our observations, as GI was significantly enhanced in caPKD1-EGFP transfected neurons compared to EGFP expressing cells (Table 2). Importantly, expression

of caPKD1-EGFP did not disturb the thread-like structure of the neuronal Golgi complex, either (Figure 11).

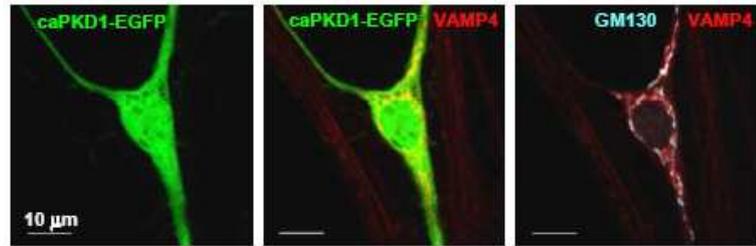


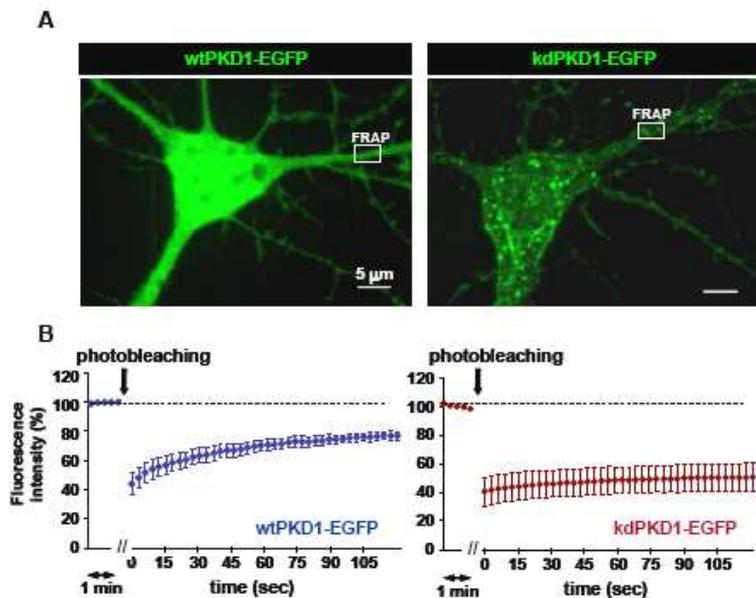
Figure 11. Morphology of the Golgi apparatus in hippocampal neurons transfected with the constitutive active mutant PKD1 construct (caPKD1-EGFP). caPKD1-EGFP is distributed in the cytoplasm with clear enrichment at the Golgi structure and does not disturb thread-like neuronal Golgi structure.

The expression of kinase inactive PKD1 mutant, on the other hand,

resulted in a completely different distribution pattern in comparison to wild-type or constitutive active PKD1 expression (Figure 12A). Besides relatively low cytoplasmic expression (see Table 2), kdPKD1-EGFP was concentrated in discrete spots in the perinuclear region and in the dendrites.

To compare the intracellular mobility of wild-type and kinase inactive, EGFP tagged PKD1 construct in the dendritic part of the transfected neurons, fluorescence recovery after photobleaching (FRAP) experiments were carried out with a Leica SP2 confocal microscope (Figure 12B). Bleaching was achieved by illuminating the defined dendritic region 5 times with maximum laser intensity. Successive scanning with high laser power led in general to a 55-70 % loss in fluorescence intensity. Recovery of the fluorescence signal within the same region was followed either in every 3 seconds (up to 3 minutes; Figure 12B) or in every 30 seconds for 10 minutes.

Figure 12. Intracellular (re)distribution of wtPKD1-EGFP and the kinase inactive mutant (kdPKD1-EGFP) differs in transfected neurons. (A) wtPKD1-EGFP (left panel) is homogenously distributed in the cytoplasm. kdPKD1-EGFP (right panel) shows local accumulation in the perinuclear region and in the dendrites with relatively low level of cytoplasmic expression. The pictures are projections of 7 z-stacks and 35 z-stacks, respectively. (B) FRAP analyses in wtPKD1-EGFP (left graph) and kdPKD1-EGFP (right graph) transfected neurons. Normalised average fluorescence intensity values in dendritic regions (see boxed areas on A) are presented before and after photobleaching. Data are indicated as mean \pm s.e.m.

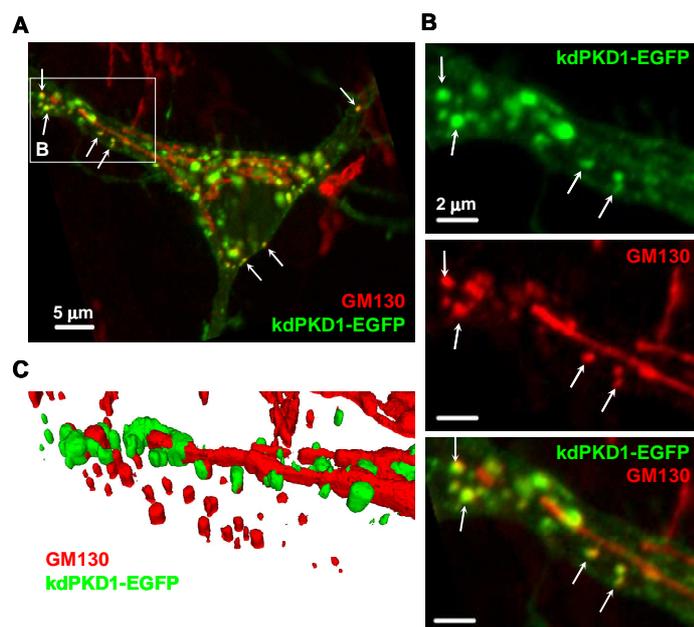


The recovery of fluorescence intensity in wtPKD1-EGFP and kdPKD1-EGFP transfected neurons showed different kinetics already within 3 minutes: relative fluorescence intensity of wtPKD1-EGFP rose from $44,11 \pm 7,67$ % (immediately after photobleaching) to $76,95 \pm 2,48$ % of the prebleaching values, while the increase in fluorescence intensity was much lower in case of kdPKD1-EGFP transfected neurons (from $40,40 \pm 10,11$ % to $50,48 \pm 9,91$ % within 3 minutes). The same difference was observed upon longer acquisition time, as well: fluorescence intensity recovered more slowly and to a much lesser extent in case of kdPKD1-EGFP compared to wtPKD1-EGFP construct (kdPKD1-EGFP: from $44,13$ % to $71,34$ %; wtPKD1-EGFP: from $32,90 \pm 2,03$ % to $87,93 \pm 1,40$ % within 10 minutes). During the time period of our measurements, fluorescence recovery did not reach initial fluorescent intensity values in either of the cases. The observed differences between initial and final fluorescence intensity values most likely represent the immobile fraction of expressed PKD1 constructs.

6.1.3.2. Kinase inactive PKD1 mutant is enriched at TGN structures

To investigate the interaction between kdPKD1-EGFP and the Golgi apparatus, GM130 immunostaining was carried out on kdPKD1-EGFP transfected neurons (Figure 13). In the somatic area, kdPKD1-EGFP signal was clearly localised to GM130 positive, partially fragmented Golgi structures (Figure 13A and B). 3D reconstruction additionally confirmed that kdPKD1-EGFP was mainly localised to the terminal parts of the Golgi filaments and to small Golgi fragments (Figure 13C). Quantitative analyses unravelled an eight-fold increase of GI in contrast to EGFP transfected neurons (Table2).

Figure 13. kdPKD1-EGFP is enriched at the Golgi apparatus in transfected hippocampal neurons. (A) Single z-stack from the somatic area of a kdPKD1-EGFP transfected neuron, where Golgi apparatus is highlighted with GM130 immunostaining. Small arrows indicate kdPKD1-EGFP patches overlapping with GM130 positive Golgi fragments. (B) Enlarged area from A, indicating strong colocalisation between kdPKD1-EGFP signal and the Golgi apparatus. GM130-immunopositive fragments are indicated by small arrows. (C) 3D reconstruction of the same area. Terminal regions of filamentous Golgi apparatus as well as Golgi fragments are surrounded by kdPKD1-EGFP signal.



The localisation of kdPKD1-EGFP to the Golgi apparatus was also obvious in cultures arising from kdPKD1-EGFP transgenic mice, where the expression of human kdPKD1-EGFP was under the control of the tetracycline-responsive (TetO) hCMV promoter. Heterozygous kdPKD1-EGFP mice were crossed with heterozygous mice carrying rtTA2 under the control of CaMKII α promoter, known to provide a forebrain and hippocampus-specific expression pattern of the transactivator (Michalon et al., 2005). Primary E18 embryonal hippocampal cultures were prepared from pregnant females which were fed with doxycycline (Dox) containing food between E13 to E18. 1 μ g/ml Dox was present in the culture medium, as well.

In comparison to the Lipofectamine-mediated transfection, the level of Dox-induced kdPKD1-EGFP expression was lower and developed gradually (data not shown). In accordance with data from transfected neurons, kdPKD1-EGFP was side-by-side localised to the GM130 positive Golgi structures in DIV10 neurons (Figure 14). These results support that the accumulation of kinase inactive PKD at the Golgi apparatus is independent from the way of expression.

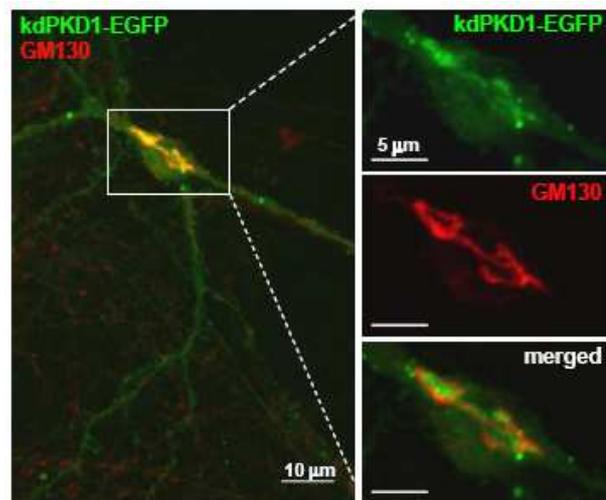


Figure 14. Doxycycline induced kdPKD1-EGFP expression in a CaMKII α rtTA2 x kdPKD1-EGFP double transgenic DIV10 neuron. kdPKD1-EGFP is weakly expressed in the cytoplasm but accumulates in the soma and in the proximal part of the dendrites. kdPKD1-EGFP signal is clearly localised side-by-side to the GM130 positive Golgi structure (see also the enlargements).

6.1.4. Expression of kinase inactive PKD1 mutant leads to the dispersion of the Golgi complex

In a large portion of kdPKD1-EGFP transfected neurons, VAMP4 and GM130 immunostaining revealed that the Golgi complex was disrupted into several small fragments containing both cis- and trans-Golgi elements (Figure 15A). In many cases, a sandwich-like arrangement between GM130 and VAMP4 positive structures and kdPKD1-EGFP was observed, with VAMP4-labeled trans-Golgi parts being in the middle (Figure 15B). Quantitative analysis of Golgi morphology from 3 independent cultures in 34 EGFP, 46 wtPKD1-EGFP, 37 kdPKD1-EGFP and 53 caPKD1-EGFP transfected neurons additionally confirmed the above findings (Figure 15C): the ratio of transfected

neurons possessing dispersed Golgi complex was significantly increased upon kdPKD1-EGFP expression but did not differ between the vector transfected neurons and wtPKD1-EGFP or caPKD1-EGFP expressing neurons.

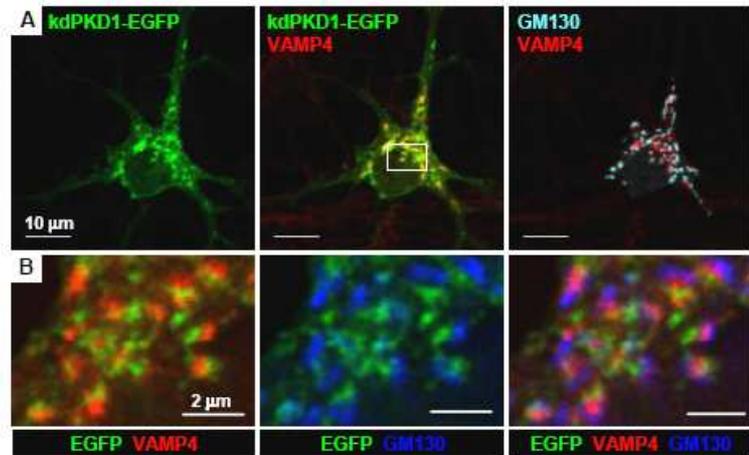
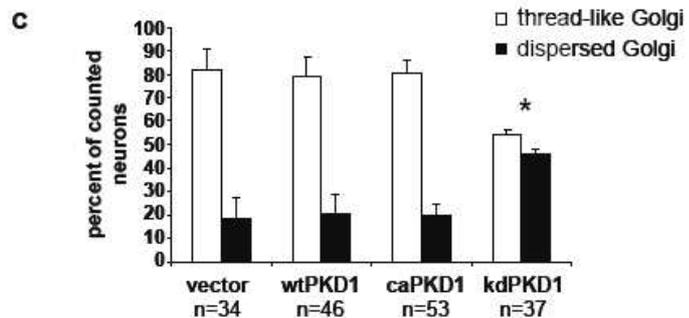


Figure 15. kdPKD1-EGFP expression leads to the dispersion of the Golgi complex. (A) kdPKD1-EGFP forms patches in the perinuclear region and in the dendrites and leads to the dispersal of the Golgi complex. (B) Confocal images of the boxed area on A. kdPKD1-EGFP is localised mainly to the VAMP4-positive, trans-Golgi side of the dispersed neuronal Golgi. (C) Quantitative evaluation of neuronal Golgi morphology in EGFP, wtPKD1-EGFP, kdPKD1-EGFP and caPKD1-EGFP transfected neurons. Data are shown as averages and s.e.m. *: $p < 0.05$.



To analyse the time-course of Golgi dispersal, Golgi morphology of kdPKD1-EGFP expressing neurons as well as the percentage of transfected neurons possessing disrupted Golgi complex was examined 12, 18 or 24 hours after transfection (Table 3). The level of expression was followed by determining the average fluorescent intensity over the whole soma region. According to our results, strong correlation between Golgi structure dispersal and the expression level of kdPKD1-EGFP was found in all time points, since neurons with disrupted Golgi complex had higher kdPKD1-EGFP expression levels compared to those cells where Golgi apparatus was preserved at least partially in its thread-like morphology. This difference was significant at 18 and 24 hours after transfection. As expected, kdPKD1-EGFP average expression level increased in parallel with post-transfection time and the ratio of transfected neurons with dispersed Golgi apparatus increased significantly between 12, 18 and 24 hours after transfection. These data indicate that dominant negative effects of kdPKD1-EGFP develop gradually and emphasise the importance of PKD activity in the maintenance of the normal Golgi apparatus structure in neurons.

kdPKD1-EGFP transfected neurons		12h		18h		24h	
		%	average fluorescent intensity	%	average fluorescent intensity	%	average fluorescent intensity
Golgi complex morphology	thread-like	84.29 ± 1.10	89.31 ± 4.48	80.19 ± 19.81	101.99 ± 3.99	64.39 ± 0.24	110.84 ± 7.44
	dispersed	15.71 ± 1.10	118.34 ± 16.7	19.81 ± 0.27 [§]	146.69 ± 5.96*	35.61 ± 0.24 [§]	173.61 ± 10.03*
average expression (fluorescent intensity over the whole soma region)		93.07 ± 4.59		110.68 ± 3.99		132.18 ± 7.28	
number of analysed cells		54		72		50	

Table 3. The effect of kdPKD1-EGFP expression on Golgi complex morphology increases over time. The percentage of transfected neurons possessing disrupted Golgi complex increases significantly between 12, 18 and 24 hours after transfection (see § for significant changes; $p < 0.05$). Neurons with disrupted Golgi complex have significantly higher kdPKD1-EGFP expression levels compared to those cells where Golgi apparatus is preserved at least partially in its tread-like morphology (*: $p < 0.05$). Data are given as mean \pm s.e.m from 3 parallel cultures.

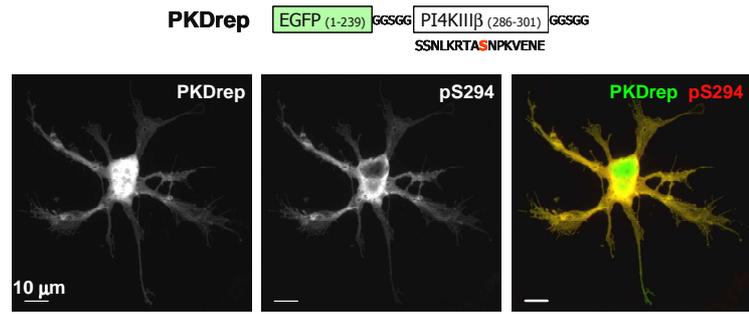
6.2. PKD activity is restricted to the somatodendritic compartment of mature neurons and is required for the maintenance of dendritic arborisation

6.2.1. PKD is ubiquitously active in non-polarised neurons and disappears from developing axons

To reveal temporal and spatial endogenous PKD activity during development, DIV1-DIV3 hippocampal neurons (stage2/3) were transfected with the non-targeted versions of PKD activity reporter¹ (PKDrep or PKDrep S/A). Both the control, alanine containing construct (PKDrep S/A; data not shown) and PKDrep were evenly distributed in the cytoplasm of the transfected neurons. In non-polarised stage 2 neurons, PKDrep phosphorylation was evident in all neurites and in the soma (Figure 16).

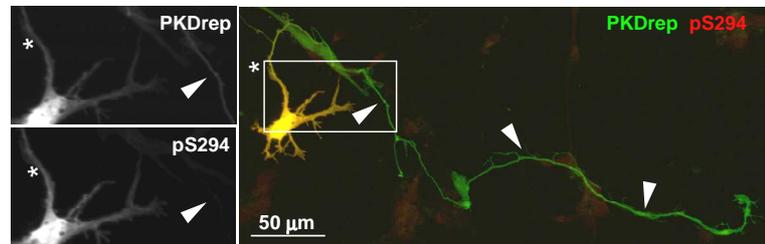
¹ Similarly to the Golgi-targeted PKD activity reporter, described in detail by Fuchs et al. (2009), PKDrep was shown to be a sensitive and specific tool to follow dynamics of PKD activation (Czöndör et al., 2009). Upon the cotransfection of HEK293T cells with the reporter and different PKD1 constructs, mutant PKD1 lacking the inhibitory PH domain led to increased phosphorylation of the PKD target site compared to wtPKD1, while alanine mutation of PKDrep completely abolished pS294 reactivity. Rapid and reversible phosphorylation of PKDrep was shown after FCS treatment of serum-starved HEK293T cells. Application of diC8 (1,2-dioctanoylglycerol; 25 μ M), a cell permeable DAG analogue, induced rapid changes in the localisation and intensity of pS294 signal in hippocampal neurons, as well (see also Figure 21). Thus, alike a natural substrate, phosphorylation of the reporter allows the dynamic monitoring of endogenous PKD activity.

Figure 16. Endogenous PKD activity is ubiquitously distributed in non-polarised, stage 2 neurons. Phosphorylation of the evenly distributed, non-targeted PKDrep construct (see cartoon and the left panel) is visualised by the overlapping pS294 immunostaining (middle panel) in the soma and in the developing neurites, as well.



On the other hand, phosphorylation of PKDrep was gradually diminished in parallel with axonal maturation. In stage 3 neurons, which possess already elongated axons, endogenous PKD activity in the axon was detected only in the proximal segment (Figure 17; see asterisk). pS294 signal was also present in the soma and in the developing dendrites.

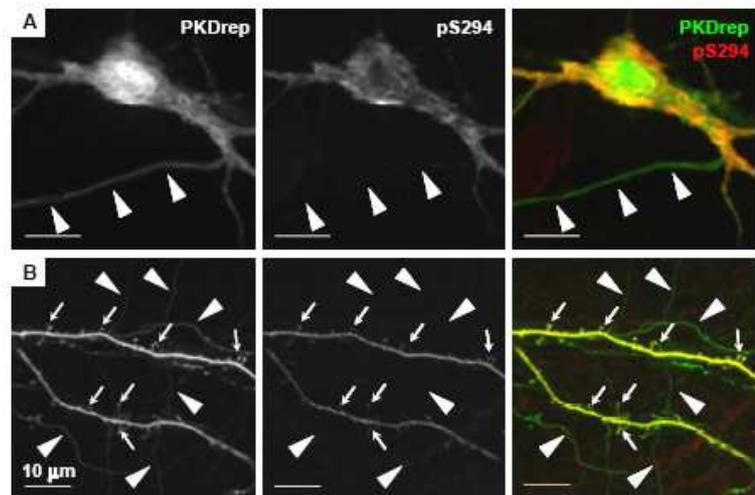
Figure 17. PKDrep phosphorylation is excluded from the developing axon. PKDrep is evenly distributed in the transfected neuron, while overlapping pS294 signal is detectable only in the soma, in the dendrites and in the proximal axonal segment (see asterisk and orange colour in the merged image). pS294 signal is excluded from the distal axon (indicated by arrowheads; only the green colour of PKDrep is visible) of a stage 3 neuron.



6.2.2. In mature neurons, endogenous PKD activity is spatially restricted inside the somatodendritic compartments

In mature neurons (DIV10), pS294 signal was completely excluded from the axons, both from the axonal initial segment (Figure 18A) or from the axon collaterals (Figure 18B).

Figure 18. In DIV10 neurons, PKDrep phosphorylation is absent from the axons. PKDrep construct (left panels) is distributed evenly in the somatodendritic compartments as well as in the axons, but pS294 immunostaining (middle panels) is absent from the initial axonal segment (A; arrowheads) and from the axon collaterals (B; arrowheads). Strong pS294 signal is evident in the soma (A), in the dendrites and in the dendritic spines, as well (B; small arrows).



At the same time, endogenous PKD activity was evident in the soma and in the main dendrites (Figure 18A), as well as in the smaller dendritic branches and dendritic spine heads (see small arrows on Figure 18B).

caPKD1-mCherry distributed evenly in the cytoplasm, including proximal (Figure 19) and distal axonal segments of mature neurons (data not shown). This spatial distribution was very similar to the localisation of the PKDrep construct. Cotransfection of PKDrep and caPKD1-mCherry led to clear phosphorylation of PKDrep in the axons (see arrowheads on

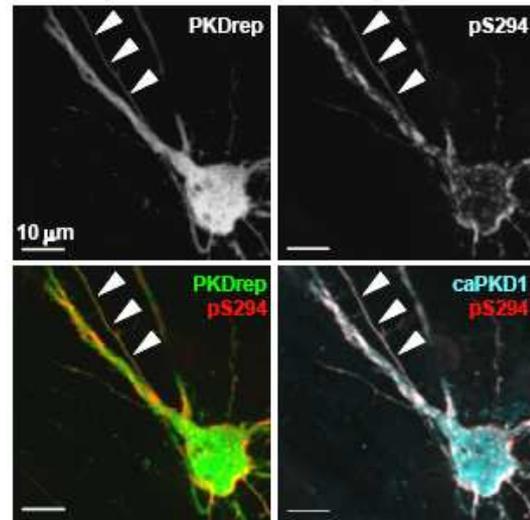


Figure 19. Cotransfection of constitutive active PKD1-mCherry (caPKD1) and PKDrep constructs leads to the appearance of pS294 signal in the axon (indicated by arrowheads). pS294 immunoreactivity also reveals patch-like, spatially restricted PKD activity in the soma and in the main dendrites of cotransfected mature neurons.

Figure 19). Additionally, spatially restricted pS294 signal became evident in the soma and in the main dendrites, as well.

Localised endogenous PKD activity in mature neurons was further supported by the phosphorylation pattern of the non-targeted PKD reporter. In the soma of transfected neurons, pS294 signal was elevated in regions near the plasma membrane (Figure 18A and 20B). Endogenous PKD activity was also evident in large segments of the main dendrites but was absent from certain regions (Figure 20C). Spatially localised PKD activity pattern was even more obvious upon stimulating transfected neurons with diC8, a membrane-permeable DAG analogue (Figure 21). Within 2 minutes of diC8 application, discrete, plasma membrane localised pS294 signal with increased intensity appeared in the soma and in the main dendrites, resembling patch-like localisation of pS294 signal upon coexpression of PKDrep and caPKD1-mCherry (see Figure 19).

6.2.3. PKD activity regulates dendritic arborisation of mature neurons

In order to investigate the possible role of PKD in the dendritic tree, dendritic morphology and rearrangement in DIV10 hippocampal neurons expressing various PKD1 constructs were quantitatively analysed 22-24 hours after transfection.

Figure 20. PKD activity is spatially restricted to certain somatodendritic regions of DIV10 hippocampal neurons. (A) pS294 immunostaining reveals PKDrep phosphorylation in the soma and in most of the dendritic tree while pS294 signal is excluded from the axon (only the green colour of PKDrep is visible as indicated by arrowheads). (B) Enlargement of the corresponding boxed area on A. In the soma, evenly distributed PKDrep is highly phosphorylated close to the plasma membrane. (C) Enlargement of the corresponding boxed area on A. pS294 staining shows different levels in local PKD activity in the main dendrites (see small arrows for low pS294 signal). All images are single z-stacks.

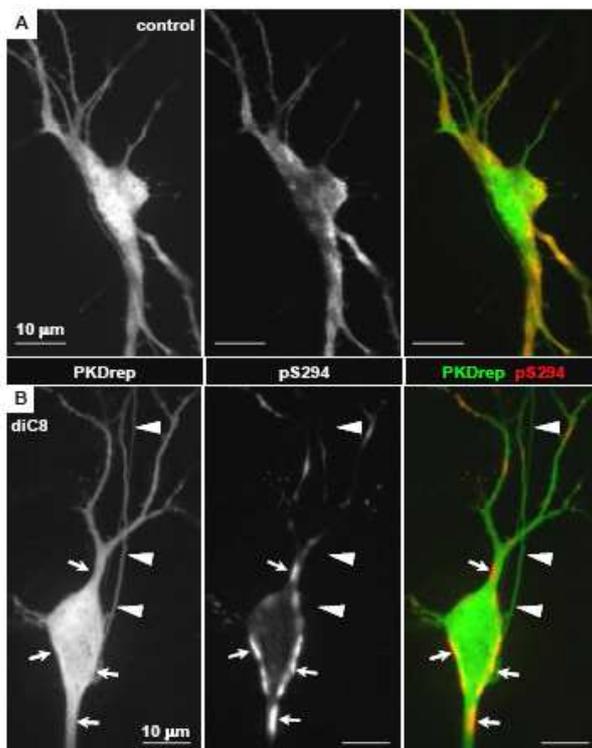
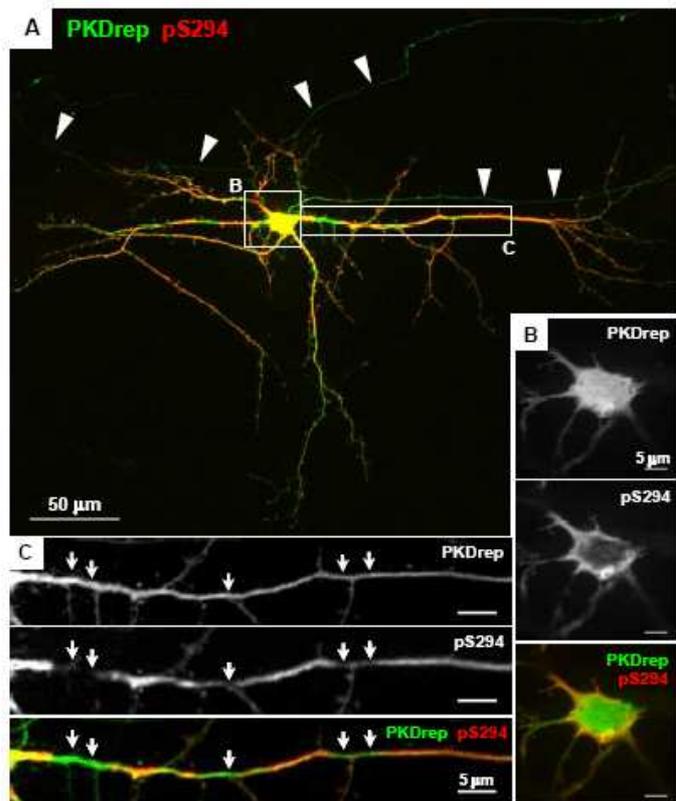


Figure 21. 1,2-dioctanoylglycerol (diC8, 25 mM) treatment rapidly increases PKDrep phosphorylation at the plasma membrane in DIV10 hippocampal neurons. (A) Strong pS294 signal is detected in spatially restricted areas in the cytoplasm of the soma and the dendrites in non-treated neurons. (B) Elevated, plasma membrane localised pS294 staining is detected in the soma and dendrites already upon 2 minutes of diC8 treatment (small arrows). diC8 treatment does not increase axonal pS294 signal (arrowheads). Images were recorded under identical settings and show single z-stacks.

To investigate the extent of dendritic branching, modified Sholl analysis was carried out in the transfected neurons by determining dendritic intersection numbers around the soma at 20 μm steps (Figure 22A shows the outline of representative neurons). Average

number of intersections was calculated as a function of distance from the soma (Figure 22B).

Control, vector-only transfected neurons had the highest number of intersections in the close (within 40 μm) vicinity of the soma, with dendrites extending up to 300 μm distance from the soma. The extent of arborisation gradually decreased in parallel with the increasing distance. The presence of kdPKD1-EGFP led to a significant decrease in the intersection numbers at almost all levels (indicated by asterisks on Figure 22B) and dendrites did not extend further than 200 μm distance. Introducing caPKD1-EGFP into the neurons, on the other hand, led to a significant increase in dendritic arborisation. Not only did the distribution of the highest intersection numbers shift towards outer regions around the soma (60 - 80 μm), but the average number of cross-points was also significantly elevated (see + signs on Figure 22B). Expression of wtPKD1-EGFP led to similar changes, but to a lesser extent.

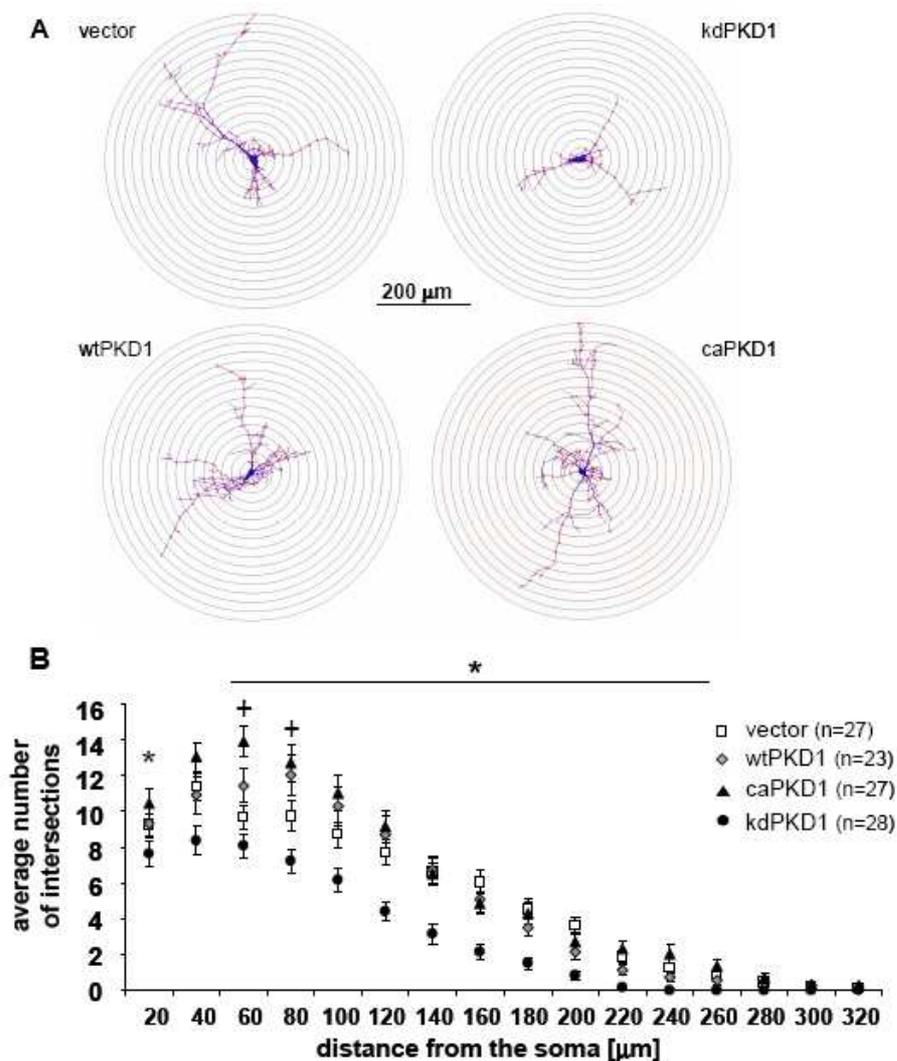


Figure 22. Sholl analysis of transfected hippocampal neurons. (A) Representative Sholl analysis images of neurons transfected with different PKD1 constructs. Dendritic intersections are marked by red dots. (B) Average intersection numbers at certain distance from the soma in transfected neurons. Data are presented as averages and s.e.m. * and +: p<0.05 for kdPKD1-EGFP (*) or for caPKD1-EGFP (+) expressing neurons.

The average number and the average thickness of primary dendrites were also determined in EGFP, wtPKD1-EGFP or kdPKD1-EGFP expressing neurons (Figure 23). Compared to EGFP expressing neurons, the average number of primary dendrites was slightly decreased while the thickness of primary dendrites was increased in case of kdPKD1-EGFP transfected neurons. Albeit the observed alterations were not significant, they supported previous findings that inactivation of PKD leads to rapid dendritic rearrangements.

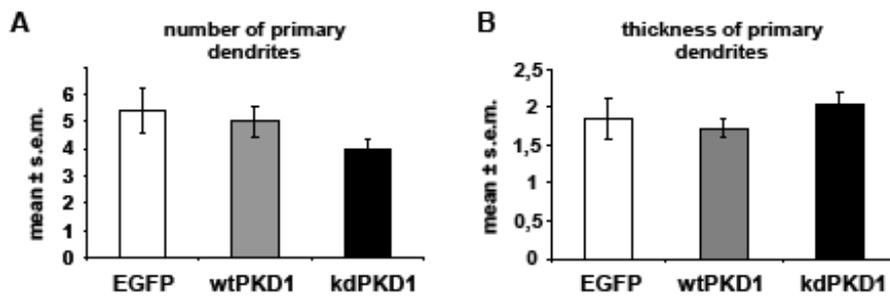


Figure 23. Average number (A) and average thickness (B) of primary dendrites in EGFP (n=5), wtPKD1-EGFP (n=10) or kdPKD1-EGFP (n=10) transfected DIV10 neurons.

To reveal potential correlation between dendritic arborisation and Golgi complex morphology, high magnification pictures were recorded from the soma region of EGFP and kdPKD1-EGFP transfected neurons, followed by evaluating dendritic arborisation of the same cells. In neurons transfected with EGFP, no direct correlation was found between the extent of dendritic arborisation and the appearance of dispersed Golgi complex (average number of dendritic intersections and s.e.m. values were 76.45 ± 6.92 and 85.63 ± 7.11 for neurons having normal, thread-like or dispersed Golgi complex, respectively; 29 cells investigated). In case of kdPKD1-EGFP expressing neurons, on the other hand, strong correlation was found (Figure 24; 37 cells investigated). Neurons, which possessed highly dispersed Golgi apparatus, had an even more severely reduced dendritic arborisation compared to those cells where Golgi complex was at least partially preserved in a filamentous, thread-like state. Similar findings were observed already during shorter (12 and 18h) posttransfection times, when kdPKD1-EGFP transfected neurons possessing dispersed Golgi apparatus had severely reduced dendritic tree, as well (data not shown). Importantly, the ratio of these cells increased with time (see Table 3).

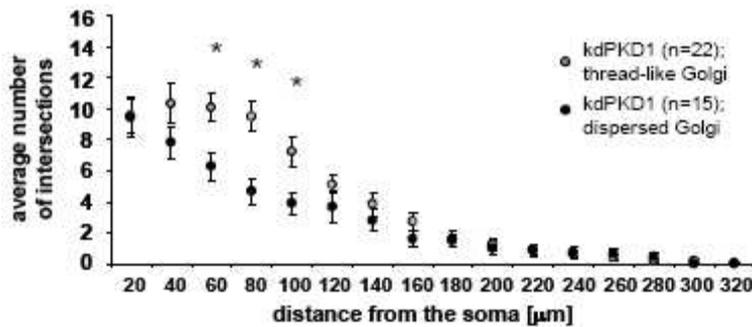


Figure 24. The extent of Golgi complex dispersal correlates with more reduced dendritic arborisation in kdPKD1-transfected neurons. Data are presented as averages and s.e.m. *: $p < 0.05$

6.2.4. PKD1 mediated effects on the intracellular distribution of dendritic proteins

To examine the possible role of PKD1 regulating the intracellular transport and/or localisation of different dendritic proteins, neurons were cotransfected with PKD1 constructs and various fluorescently labelled proteins known to be localised specifically in dendrites. It is known that proteins needed for postsynaptic processes are clustered into the postsynaptic density (PSD) of the neurons (Boeckers, 2006). One important component of the PSD is PSD-95, which serves as a scaffold protein within the postsynaptic machinery (Dosemeci et al., 2007). In the control experiments, when PSD-95-EGFP was cotransfected with the mRFP vector, PSD-95-EGFP was localised to discrete spots in the main dendrites and in the thinner dendritic branches (data not shown). The spot-like distribution of PSD-95-EGFP was also observed upon cotransfection with wtPKD1-RFP or kdPKD1-RFP indicating that overexpressed PKD1 has no apparent effects on the distribution of PSD-95 (Figure 25A). Furthermore, wtPKD1-RFP was not accumulated at bright PSD-95-EGFP spots, however, some of the kdPKD1-RFP signal overlapped with the spatially restricted PSD95-EGFP dots.

Besides scaffold proteins, neurotransmitter receptors like N-methyl D-aspartate (NMDA) receptors in case of glutamatergic synapses are also crucial for the postsynaptic response (Cull-Candy and Leszkiewicz, 2004). EGFP-tagged NMDA receptor subunit 1 (NR1-EGFP) was expressed in neurons. NR1-EGFP signal was located to bigger patches in the cytoplasm of thicker dendrites, presumably reflecting enrichment at membranous organelles like ER or endosomes, or to some larger spots close to the surface of thinner dendrites. This intracellular distribution of NR1-EGFP was observed both upon cotransfection with mRFP (data not shown) or with wtPKD1-RFP (Figure 25B) and NR1-EGFP formed patches were partially overlapped with wtPKD1-RFP signals. Cotransfection

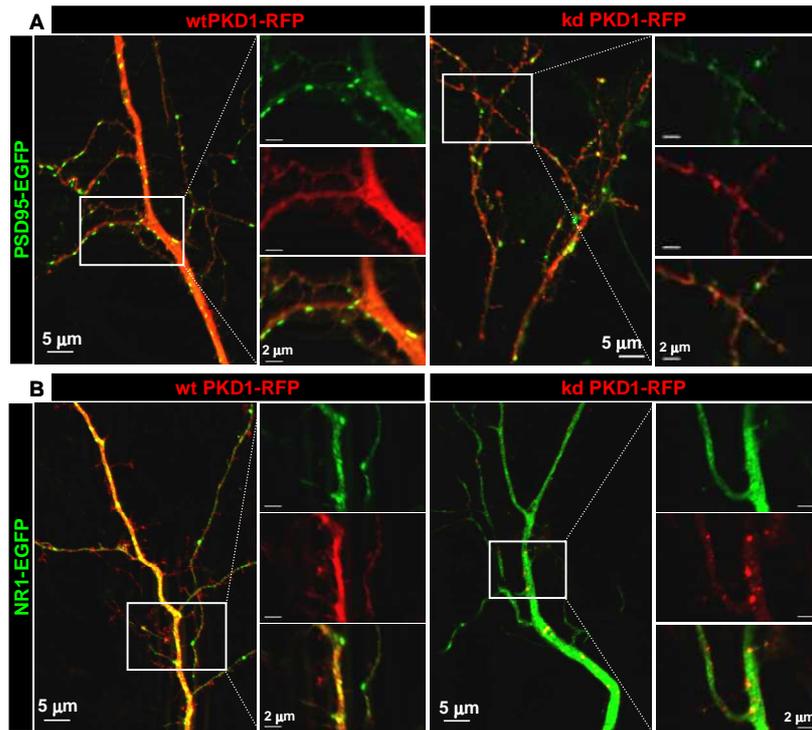


Figure 25. Intracellular distribution of the dendritic proteins PSD95-EGFP (A) and NR1-EGFP (B) in hippocampal neurons cotransfected with wtPKD1-RFP (left panels) or kdPKD1-RFP (right panels). Images on the left are projections of 12, 14, 9 and 16 z-stacks, respectively, while enlarged pictures on the right show single z-stacks from single fluorescent signals or the merged images.

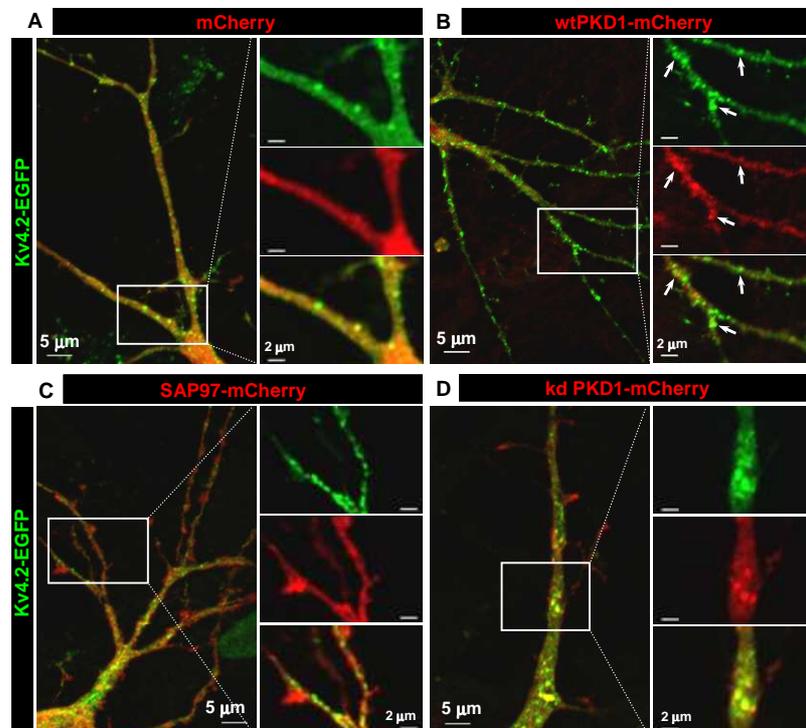


Figure 26. Intracellular distribution of Kv4.2-EGFP in hippocampal neurons cotransfected with mCherry (A), wtPKD1-mCherry (B), SAP97-mCherry (C) and kdPKD1-mCherry (D). Images on the left are projections of 17, 12, 16 and 11 z-stacks, respectively, while enlarged pictures on the right show single z-stacks from single fluorescent signals or the merged images. Arrows indicate colocalisation of Kv4.2-EGFP and wtPKD1-mCherry signals.

with kdPKD1-RFP did not cause apparent changes in the expression pattern of NR1-EGFP, and no colocalisation was found between locally enriched NR1-EGFP and kdPKD1-RFP signals.

PKD-mediated effects were also investigated by cotransfecting neurons with various PKD1 constructs and EGFP labelled voltage-gated K⁺ channel Kv4.2, one of the major regulatory proteins of dendritic excitability (Tkatch et al., 2000). In DIV10 neurons transfected with the mCherry vector only, Kv4.2-EGFP signal was ubiquitously distributed in the cytoplasm with some local enrichments at the surface of the dendrites (Figure 26A). When Kv4.2-EGFP was coexpressed with wtPKD1-mCherry, on the other hand, enrichment of Kv4.2-EGFP was more obvious and showed clear, dot-like structures, especially at distal dendritic regions (Figure 26B). Importantly, the distribution of wtPKD1-mCherry was very similar and often overlapped with Kv4.2-EGFP (see small arrows on Figure 26B). The dot-like distribution resembled the expression pattern of Kv4.2-EGFP when cotransfected with synapse associated protein 97 (SAP-97), a protein known to play important role in the transport and localisation of these potassium channels (Gardoni et al., 2007; Figure 26C). The similarity in the effects of wtPKD1 and SAP97 overexpression on the intracellular distribution of Kv4.2-EGFP indicates that PKD1 can - directly or indirectly - influence the intracellular localisation of Kv4.2. This possibility was further supported by the altered intracellular distribution of Kv4.2-EGFP in the presence of kdPKD1-mCherry (Figure 26D): Kv4.2-EGFP signal was frequently localised in the inner part of the dendrites and was mainly retained in the main dendritic shafts compared to cotransfection with wtPKD1 or SAP97. Additionally, interaction of Kv4.2-EGFP and kdPKD1-mCherry was also indicated by detecting partial colocalisation between the two expressed proteins.

6.3. Investigating axonal localisation and activity of PKD

6.3.1. Endogenous PKD is present in the axon of mature neurons

Our previous examinations in PKDrep expressing neurons revealed the absence of endogenous PKD activity in mature axons (see Figure 18). In order to investigate whether the lack of endogenous PKD activity is explained by the absence of PKD1 protein in the axon, immunostaining with a monoclonal PKD1 antibody was carried out. To prove the specificity of the detection, cultures transfected with wtPKD1-mCherry or mCherry were also used. According to the results, anti-PKD1 signal was detected in the axons

(Figure 27A). Staining intensity in the axons did not differ between non-transfected cells or neurons transfected with mCherry (Figure 27A), while overexpression of wtPKD1-mCherry specifically led to increased signal intensity in the transfected axons (Figure 27B). Omitting the first layer antibody completely eliminated anti-PKD1 immunostaining, confirming the specificity of our immunostaining (Figure 27C).

These data and the results from PKDrep expressing neurons clearly indicate that endogenous PKD1 is present but its kinase activity is inhibited in the axons of mature neurons. It is important to note that PKD activity in the axons can not be induced even by elevated DAG signalling as axonal pS294 signal was not increased even after stimulation with diC8, a cell permeable DAG analogue treatment (see Figure 21).

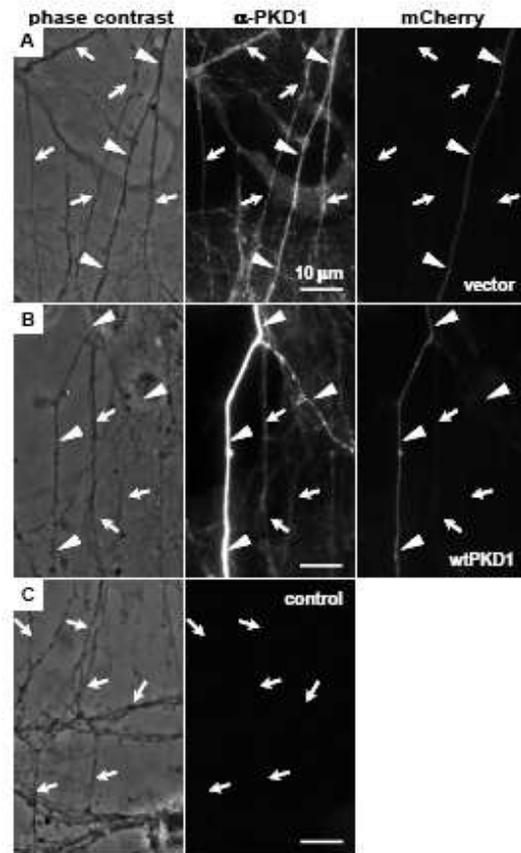


Figure 27. Endogenous PKD1 is present in the axons. (A) In cultures transfected with the mCherry vector, staining intensity is similar between transfected (arrowheads) and non-transfected (small arrows) axons. (B) Overexpression of wtPKD1 leads to highly increased signal intensity in the wtPKD1-mCherry transfected axon. (C) The specificity of the immunostaining is proved by the lack of immunostaining when the first layer antibody was omitted.

6.3.2. wtPKD1-mCherry is partially cotransported with the Slow Component b proteins *synapsin-EGFP* and *myosin-EGFP*

A special microscopic system (Zeiss, CellObserver) providing proper conditions for investigating living cells and capable for fast fluorescent recordings was used to examine the axonal transport of various PKD constructs. Pictures from different axonal regions of transfected neurons were taken in every 300-500 milliseconds up to 5 minutes. The axonal transport of wtPKD1-EGFP was characterised by analysing the direction and the speed of fluorescently labelled structures revealing mostly unidirectional movement of wtPKD1-EGFP associated structures in both directions in the axons (see Figure 28A for designated pictures of a live cell recording). The movement of labelled structures was often

interrupted by prolonged pauses resulting in an average speed distribution between 0.1 to 1 $\mu\text{m}/\text{sec}$ (Figure 28B). The maximum speed of transport was around 2 $\mu\text{m}/\text{sec}$ (data not shown). In case of kdPKD1-EGFP transfected neurons, on the other hand, kdPKD1-EGFP labelled structures were rarely found in the axons and were often immobile (90% of the structures associated with kinase inactive PKD1-EGFP were slower than 0.1 $\mu\text{m}/\text{sec}$; Figure 28B).

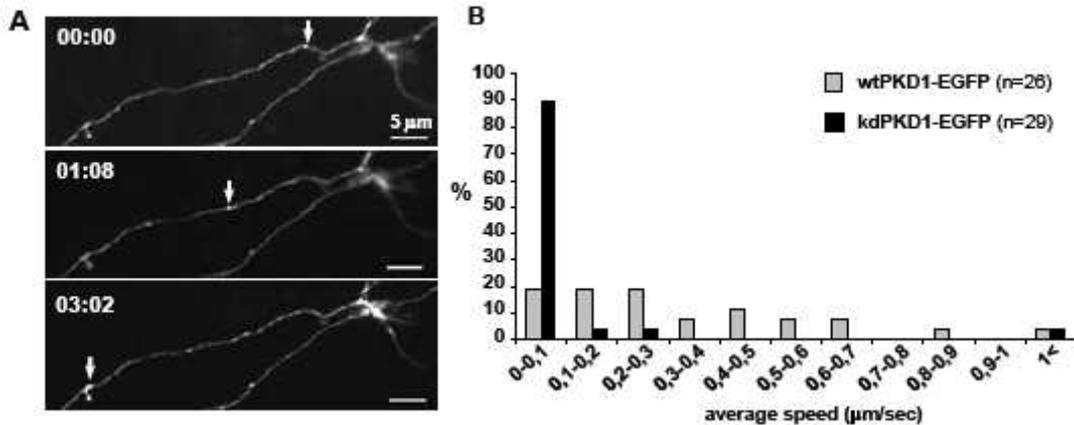


Figure 28. Transport of wtPKD1-EGFP in the axon. (A) Unidirectional transport of a wtPKD1-EGFP associated vesicle-like structure (small arrow) in the axon of a transfected neuron, depicted by three images from a live cell recording. Elapsed time is indicated as min:sec. (B) Relative frequency of average speed categories, characteristic for wtPKD1-EGFP and kdPKD1-EGFP associated vesicle movements.

In order to directly investigate PKD1's axonal transport characteristics and potential interactions with known axonal proteins, fast dual colour live cell imaging was used. During the live cell imaging experiments, EGFP-labelled synaptotagmin and synaptophysin constructs were used as indicators of the fast component (FC) axonal transport (Okada et al., 1995), while slow component b (SCb) type transport was highlighted by the translocation of fluorescently tagged myosin Vb, synuclein or synapsin I (Roy et al., 2007) constructs. To reveal any PKD-mediated effects on axonal transport processes, EGFP-labelled axonal constructs were cotransfected with mCherry-labelled wild-type and mutant PKD1 molecules.

In neurons cotransfected with synaptotagmin-EGFP and synaptophysin-mCherry, fast axonal transport of both marker proteins was clearly detectable: fluorescently labelled vesicles showed continuous, unidirectional transport (both in the anterograde and retrograde direction) with ~ 2 $\mu\text{m}/\text{sec}$ average speed (data not shown). When wt or kdPKD1 constructs were co-expressed with either synaptotagmin or synaptophysin, EGFP and mCherry tagged proteins were transported separately. On the other hand, colocalisation was evident in stationary structures, located either at axonal branch points or at the

terminal axonal tips (Figure 29A). The investigated characteristics of fast axonal transport (e.g. average speed or the direction) were not altered upon the cotransfection of wt or kdPKD1, either.

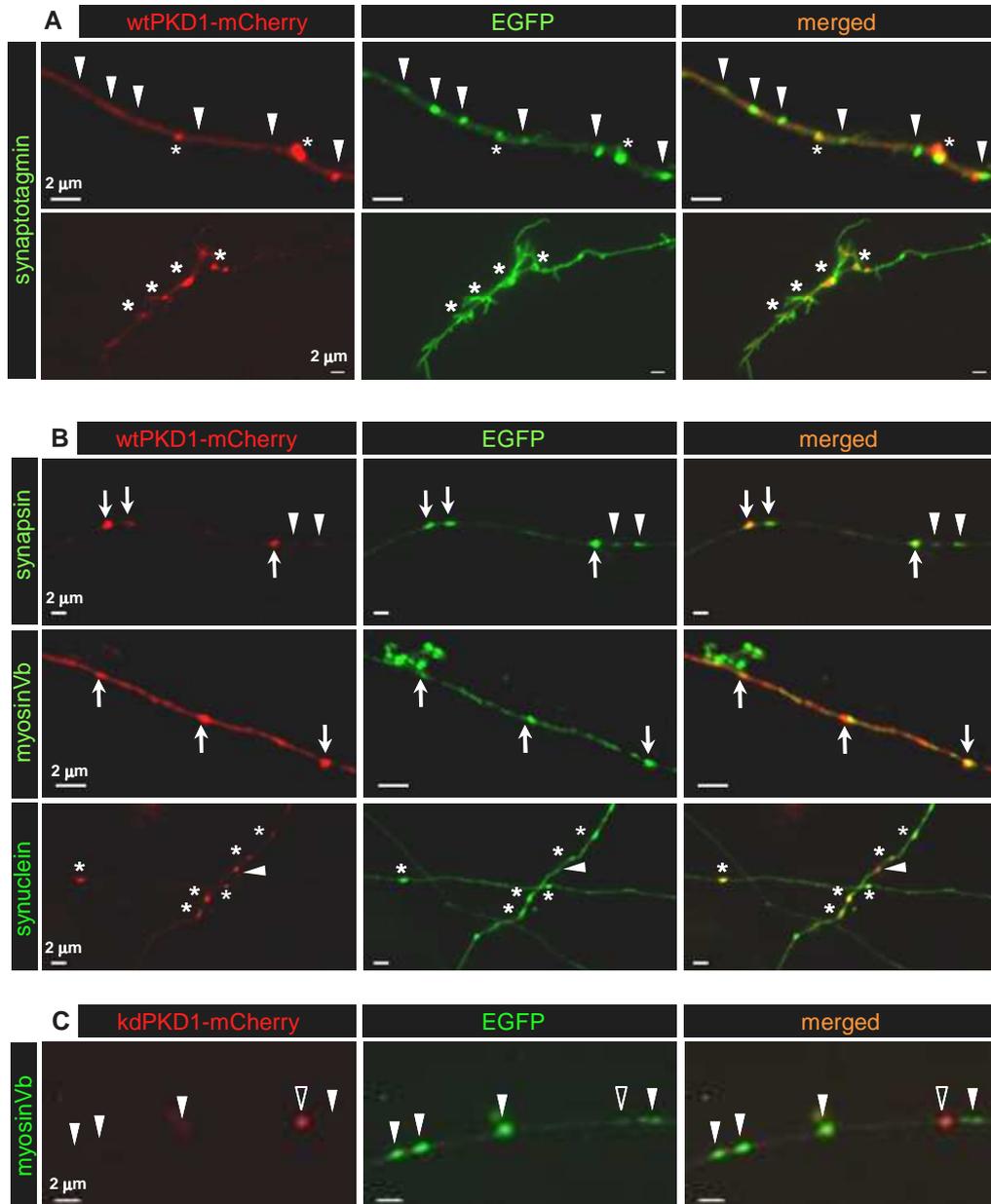


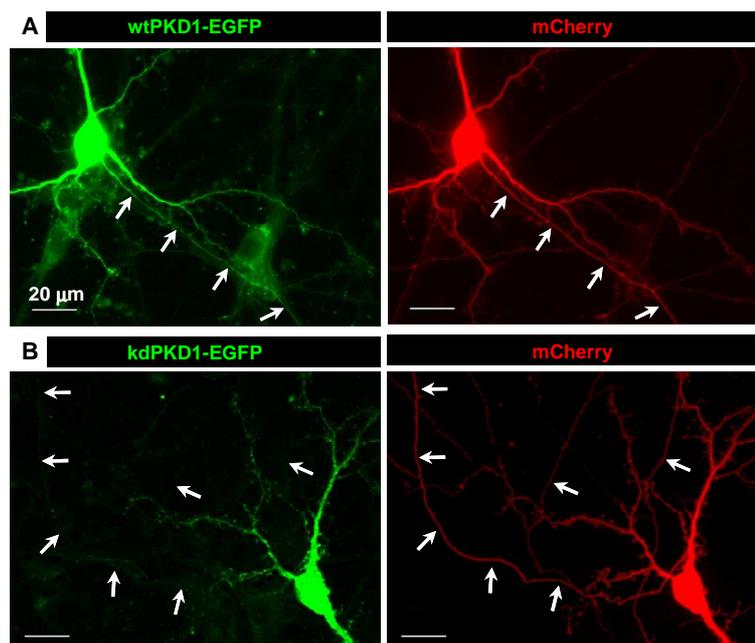
Figure 29. Intracellular transport of wtPKD1-mCherry in hippocampal neurons cotransfected with markers of different types of axonal transport (A) The transport of FC protein synaptotagmin-EGFP is independent from wtPKD1-mCherry (see arrowheads to label transported synaptotagmin-EGFP vesicles). Colocalisation between the two constructs is detected only in stationary structures (see asterisks), which are frequently located to the branching points at the terminal part of the axon. (B) Colocalisation and cotransport of wtPKD1-mCherry with known SCb proteins like synapsin-EGFP (upper panel), myosinVb-EGFP (middle panel) and synuclein-EGFP (lower panel). (C) kdPKD1-mCherry associated vesicle-like structure was transported independently from myosin Vb-EGFP within the axon (see empty arrowhead). Dual-labelled, mobile structures are indicated by small arrows, asterisks mark colocalised but immobile structures, while arrowheads point to single labelled vesicles.

In case of the SCb transport, on the other hand, wtPKD1-mCherry was often cotransported with synapsin-EGFP or myosin Vb-EGFP (see small arrows on Figure 29B), although the majority of the EGFP-labelled structures were not associated with wtPKD1. On the other hand, randomly moving or stationary kdPKD1-mCherry signals in the axons were never associated with any of the transported SCb marker proteins. Moreover, transport characteristics or the localisation of SCb markers were not affected by the coexpression of kdPKD1 in either of the cases (data not shown). Stationary deposits of wtPKD1-mCherry were often detected in the axons and colocalised frequently with immobile synuclein or synapsin I signals, but cotransport of wtPKD1 and synuclein molecules was not observed. In accordance with our previous findings, kdPKD1-mCherry associated structures were rarely found in the axon and in these cases, kdPKD1 was mainly stationary or was moving independently from SCb markers.

6.3.3. Kinase activity of PKD is needed for the axonal transport of PKD

We have already shown that intracellular localisation of kdPKD1-EGFP differs from the distribution of wtPKD1-EGFP in transfected neurons (see Figure 12). While wtPKD1-EGFP was ubiquitously distributed throughout the whole neuron, kdPKD1-EGFP was hardly detected in the axon and was mainly restricted to certain intracellular compartments in the soma or in the dendrites. In order to examine the presence of the different PKD1 constructs within the axon, neurons were cotransfected with wtPKD1-EGFP or kdPKD1-EGFP together with the mCherry vector (Figure 30).

Figure 30. kdPKD1-EGFP is excluded from the axon of transfected neurons. (A) wtPKD1-EGFP is ubiquitously distributed throughout the transfected neuron and colocalises with mCherry in the soma, in the dendrites and in the axon as well. (B) kdPKD1-EGFP, on the other hand, is excluded from the axon. In cotransfected neurons, only mCherry signal is detected in the axon. Small arrows indicate the axon of the corresponding neuron. Images are projections of 5 (wtPKD1-EGFP) or 3 (kdPKD1-EGFP) z-stacks.



In case of wtPKD1-EGFP, the distribution of the two constructs was similar and both signals were evident in the cytoplasm of cotransfected axons. In transfected neurons, kdPKD1-EGFP signal was evident in the soma and in the dendritic tree. On the other hand, cotransfected axons (especially the distal regions and the axon collaterals) lost cytoplasmic kdPKD signal and within 24 hours posttransfection time, contained almost exclusively the mCherry signal (Figure 30B). In accordance with live cell imaging observations, some kdPKD1-EGFP associated vesicle-like structures were also present in the cotransfected axons. These data indicate that the kinase inactive mutation of PKD1 interferes with its proper axonal transport.

Summary of the experimental results:

1. In mature neurons, endogenous PKD activity was detectable at the Golgi apparatus. Polarised PKD activity developed in parallel with neuronal maturation, as PKD activity gradually diminished from the axon simultaneously with axonal development. In mature neurons, PKD-mediated phosphorylation of the activity reporter construct was restricted to the somatodendritic compartment while being absent from the axon.
2. wtPKD1-EGFP and caPKD1-EGFP were ubiquitously distributed in the cytoplasm of transfected neurons and were slightly enriched at the Golgi compartment. In contrast, kdPKD1-EGFP showed relatively low cytoplasmic expression and was rather concentrated in discrete spots in the perinuclear region and in the dendrites of transfected neurons. The enrichment of kdPKD1-EGFP at Golgi structures was elevated compared to wild-type or constitutive active PKD expressing neurons.
3. Expression of kdPKD1-EGFP led to the gradual dispersal of the neuronal Golgi complex. In contrast, wtPKD1-EGFP and caPKD1-EGFP did not disturb the filamentous structure of the neuronal Golgi apparatus.
4. Inhibition of PKD activity led to the severe reduction, while constitutively active PKD1 increased the extent of dendritic tree of transfected neurons. kdPKD1-EGFP mediated shrinkage of dendritic arborisation was in a good time-correlation with the dispersal of Golgi complex.
5. Cotransfection experiments between fluorescently labelled PKD variants and dendritic marker proteins indicated that PKD activity can influence the transport and/or the localisation of certain dendritic proteins such as Kv4.2 ion channels.
6. Wild-type PKD1-EGFP was transported in the axon bidirectionally and in a stop-and-go manner. wtPKD1-EGFP was partially cotransported with synapsin-EGFP or myosin Vb-EGFP signals, which are known markers of SCb type of axonal transport. In contrast to wtPKD1-EGFP, cytoplasmic kdPKD1-EGFP signal gradually disappeared from the transfected axons. kdPKD1-EGFP associated structures with random and short-term movements were occasionally detected in the axons and did not colocalise with axonal transport markers.

DISCUSSION

Based on studies in non-neuronal cells, PKD was shown to play a significant role in surface targeted membrane trafficking (Liljedahl et al., 2001). Furthermore, investigations in polarised epithelial cells revealed that PKD is selectively involved in the basolateral membrane protein transport (Yeaman et al., 2004). The maintenance of extremely polarised structure and function in neurons requires constant membrane support and directed protein transport indicating the involvement of a highly organised machinery in surface directed delivery. The involved mechanisms and their regulation, however, are far from well understood and the possible contribution of PKD-mediated effects needs further clarification. In line with recent publications (Horton et al., 2005; Bisbal et al., 2008), the current work provides additional evidence on the Golgi-localised action of PKD and on its involvement in the maintenance of the dendritic arbour.

PKD can influence early neuronal development and polarisation

Polarised activity of PKD developed gradually during neuronal maturation, as phosphorylation of PKDrep was detected in every compartment of young, non-polarised neurons but diminished from the axon in parallel with neuronal development. The ubiquitous distribution of endogenous PKD activity in non-polarised neurons already indicates a role of PKD during early neuronal development. In addition, Western blot experiments detecting autophosphorylation (thus, the activation) of endogenous PKD revealed high PKD activity in neurons already from early differentiation stages (Czondor et al., 2009). The involvement of PKD in neuronal development was also suggested by a recent study showing that PKD can directly regulate Par-1 protein activity, which is one of the pivotal proteins involved in regulating cell polarity (Watkins et al., 2008). Furthermore, PKD was reported to be required for the polarisation of cultured hippocampal neurons via regulating polarised membrane trafficking towards the future axon (Yin et al., 2008). Based on these studies, PKD contributes to neuronal polarisation processes during early developmental stages, however, there are some contradictory results which need further clarification.

The multiple axon formation upon decreased PKD activity described by Yin et al. (2008) is in contrast to other published data describing that PKD was involved specifically in dendritic growth and that impaired PKD activity did not show any effect on axonal development (Horton et al., 2005). That PKD is rather involved in dendritic formation is

also indicated by our investigations showing diminished PKD activity in parallel with axon formation. Furthermore, PKD is involved in Par-1 mediated activation of the polarity complex Par-3/Par-6/aPKC, as PKD-mediated phosphorylation of Par-1 leads to the sequestration of Par-1 by 14-3-3 proteins and to the consequent release of Par-1 mediated inhibition on Par-3/Par-6/aPKC (Watkins et al., 2008). Accordingly, PKD can exert an effect on axonal development, but in this case, kdPKD expression would rather lead to the inhibition of Par-3/Par-6/aPKC activity and axonal polarisation/commitment. Thus, further work is needed to clarify the involvement of PKD during neuronal polarisation.

Last but not least, in our own experiments, downregulation of PKD activity resulted in decreased axonal length while constitutive active PKD induced neurite elongation in stage 3 neurons (unpublished data). These observations indicate that besides influencing early neuronal polarisation, PKD can contribute to developmental processes by providing the necessary membrane supply for neurite elongation or via controlling the required cytoskeletal changes. However, further experiments are needed in order to explore the versatile function of PKD during neuronal development.

PKD-mediated axonal effects

In contrast to non-polarised neurons, PKD activity showed polarised localisation in mature neurons being present in the somatodendritic regions and excluded from the axons. Treatment with diC8, a cell permeable DAG analogue, or expression of caPKD1 led to highly elevated pS294 immunostaining at the plasma membrane of the soma and in the dendrites, reflecting local recruitment of activated PKD. In contrast, diC8 treatment did not increase axonal pS294 signal despite that endogenous PKD1 was clearly detectable within the axons. Axonal PKD activity became detectable only upon overexpression of caPKD1, which was evenly distributed throughout the neurons.

Despite the lack of endogenous PKD activity in axons, PKD can be involved in axonal processes. On one hand, immunocytochemical stainings revealed the presence of endogenous PKD in the axon. Additionally, live cell imaging experiments have shown that overexpressed wild type PKD is clearly transported within the axons. The average speed of wtPKD1-EGFP associated structures as well as the stop-and-go way of transport resembled mostly the SCb axonal transport. Although we can not exclude the possibility of some experimental artefacts due to overexpression, recordings showing cotransport of wtPKD1

with known SCb marker proteins further support our assumption that PKD is transported in the SCb type of axonal transport.

In contrast to wtPKD1 or caPKD1 constructs, kdPKD1-EGFP was excluded from the axon in parallel with increasing posttransfection time, indicating that the axonal transport of PKD depends on its kinase activity. As endogenous PKD is inactive in the axon, kinase activity can be needed for connecting PKD with motor proteins either directly or via scaffolding/adaptor proteins. Accordingly, live cell imaging recordings revealed that while wtPKD1 was cotransported with some axonal proteins, kdPKD1 associated structures were just occasionally detected in the axon and did neither colocalise with transported axonal proteins nor possessed movements characteristic for wtPKD1. These results further emphasize the requirement of PKD activity for its axonal transport and that PKD activity is needed for its interaction with the axonal transport machinery.

One possible mechanism for connecting PKD activity to the axonal transport machinery implicates the involvement of autophosphorylation events. The autophosphorylation site (S916) at the C-terminus of PKD is localised within a PDZ-binding domain which is supposedly required for interaction with PDZ proteins (Sanchez-Ruiloba et al., 2006). As several PDZ proteins are involved in transport processes by coupling to kinesins as well as to transport carriers (Kim and Sheng, 2004), it is a feasible idea that wtPKD is transported along microtubules by binding to kinesins via PDZ domain containing adaptor proteins. Accordingly, the interaction with PDZ proteins can be impaired in the lack of autophosphorylation upon inhibiting PKD activity.

Recent data have proved that the S916 site can be also transphosphorylated (Sanchez-Ruiloba et al., 2006). Consequently, besides the inhibition of autophosphorylation, the development of dominant-negative kdPKD effect can inhibit transphosphorylation of kdPKD proteins, mediated by endogenous PKDs. This is in accordance with the fact that the exclusion of cytoplasmic kdPKD1 from the axon developed gradually during posttransfectional time. Therefore, it is a feasible idea that the transport of PKD can also depend on transphosphorylation events being abolished in parallel with the evolvement of the dominant negative effect of kdPKD1 (Johannes et al., 1998; Sanchez-Ruiloba et al., 2006).

Taken together, the regulation of PKD transport can be based on phosphorylation dependent interactions with motor proteins or adaptor proteins. At first sight, the cotransport of wtPKD1 and myosin constructs, as well as the lack of interaction upon kdPKD1 expression is in accordance with this idea. Myosin is responsible for actin based

transport and is delivered to actin containing parts of the cell by being transported along microtubules in association with transport carriers coupled to kinesins. Thus, it is highly likely that the observed cotransport of wtPKD1 and myosin reflects the delivery of the two constructs in the same transport carriers. The cotransport of wtPKD1 with myosin and other SCb marker proteins indicate that PKD1 is delivered in the axon in association with multiprotein complexes and not to vesicles (Roy et al., 2007). However, further experiments are required to clarify the structural background and the underlying mechanisms of PKD transport.

Based on our observations, we propose that PKD-mediated (auto)phosphorylation is required for its interaction with the transport machinery. On the other hand, PKD's activity is selectively inhibited in the axons, as it was shown with the aid of the PKD activity reporter. The lack of axonal pS294 staining can be a consequence of the inactivation of PKD via sequestration by 14-3-3 adapter proteins (Hausser et al., 1999; Taya et al., 2007) or by selective dephosphorylation of PKD and/or the reporter within the axons (Uetani et al., 2006). Thus, PKD could exert its effect only in restricted axonal regions. As one possibility, PKD can be delivered to presynaptic areas regulating synaptic processes, which would be in line with the findings that SCb transport is responsible for the delivery of several hundreds of cytoplasmic proteins involved in presynaptic function (Brown, 2003). The colocalisation of wtPKD1 with the presynaptic proteins synapsin and synuclein in stationary deposits localised at axon terminals or at axonal branching points further indicates a role for PKD in presynaptic functions.

Recent publications have shown that kdPKD1 expression had no effects on axonal elongation (Horton et al., 2005) or on the localisation of axonal membrane proteins (Bisbal et al., 2008). However, our results show that the axonal transport of kdPKD1 is impaired, therefore, the lack of any axonal consequences upon kdPKD1 expression is not surprising. Thus, further studies about the axonal effect of increased PKD activity are needed to reveal PKD's function in axonal processes.

PKD is involved in the maintenance of neuronal Golgi organisation

In our mature hippocampal cultures, PKD1 constructs were enriched at the TGN and Golgi targeted PKD reporter revealed high endogenous PKD activity at the neuronal Golgi complex. These observations already suggest a role for PKD in neuronal Golgi function, similarly to non-neuronal cells (Liljedahl et al., 2001; Diaz Anel and Malhotra, 2005;

Ghanekar and Lowe, 2005). Upon introducing a kinase inactive PKD mutant carrying a point mutation within the ATP-binding motif of the kinase domain (Johannes et al., 1998) into the neurons, accumulation at the Golgi apparatus was further increased. The enhanced enrichment of kinase inactive PKD at the Golgi compartment is in accordance with former observations made in HeLa cells, showing that the point mutation inhibits kinase activity but not binding to the Golgi compartment (Hausser et al., 2002). Moreover, the detachment of kinase inactive PKD mutant appeared to be impaired resulting in an accumulation at the TGN (Maeda et al., 2001). These observations suggest that autophosphorylation or substrate phosphorylation events are likely required for dissociation from membrane structures. In our case, accumulation of kdPKD1-GFP to VAMP4 positive TGN structures was observed both in transfected neurons as well as upon Dox-induced PKD mutant expression in neurons arising from CaMKII α rtTA2 x kdPKD1-EGFP double transgenic mice indicating an independent effect from the way of expression.

Importantly, kdPKD1 expression led to the dispersal of the neuronal Golgi apparatus into smaller fragments, still possessing cis- and trans-Golgi elements. Prolonged or elevated kdPKD1 expression level led to a gradual increase in the ratio of cells with impaired Golgi complex morphology. These data indicate that a certain level of kdPKD1 expression is needed to affect Golgi complex morphology, likely due to the development of the dominant negative effect towards endogenous PKD. A correlation between the expression level of kdPKD1 and Golgi morphology was further supported by the analysis carried out in CaMKII α rtTA2 x kdPKD1-EGFP cultured hippocampal neurons possessing lower level of kdPKD1 expression upon Dox mediated induction, where double transgenic neurons showed only subtle alterations in Golgi morphology. Differences in the expression level of kdPKD1, the time point of analyses as well as the distinct evaluation methods can also explain the differences between our results and that of Bisbal and colleagues published recently (Bisbal et al., 2008), where no morphological changes in the Golgi apparatus was observed upon interference with neuronal PKD activity during 12 to 18h after transfection.

Fragmentation of the Golgi apparatus is at variance with the well-described tubulation of the TGN in kdPKD expressing non-neuronal cells, which is widely accepted as a consequence of impaired vesicle fission (Bard and Malhotra, 2006; De Matteis and Luini, 2008). Accordingly, the vesiculation of the Golgi apparatus would be rather explained by the overactivation of the fission machinery, as it was indicated by

investigating the involvement of PKD in $G_{\beta\gamma}$ induced dispersion of Golgi organisation (Jamora et al., 1999). A seemingly opposite effect of kdPKD on Golgi morphology might be explained by the basic differences concerning Golgi organisation and function described in neurons compared to non-neuronal cells (Horton and Ehlers, 2004). Neurons specifically exhibit filamentous and well extended perinuclear Golgi complex together with uniquely polarised and discontinuous Golgi elements (so-called Golgi outposts) when compared to non-neuronal cells. Additionally, as neurons are non-dividing cells, neuronal Golgi is not subject to cyclic fragmentation and reassembly as in case of dividing cells. These differences already indicate distinctions in the processes involved in Golgi organisation in neurons and non-neuronal cells. Therefore, despite differences in phenotypical changes upon kdPKD expression, our results are consistent with observations from earlier studies carried out in non-neuronal cells in the following aspects: *i*) PKD activity is required for the maintenance of the cell type specific Golgi complex architecture and *ii*) interfering with PKD activity rapidly changes Golgi complex integrity.

The morphology of the Golgi complex is a result of highly dynamic processes including continuous membrane shuttle into and out of the Golgi complex and depends to a large extent on the integrity of the surrounding cytoskeletal elements. Whereas microtubules are thought to regulate mainly the positioning of the Golgi apparatus, actin cytoskeleton contributes to the maintenance of the Golgi structure (Lippincott-Schwartz, 1998; Egea et al., 2006). It is known that PKD activity regulates cytoskeletal organisation, cell migration and invasion in non-neuronal cells (Bowden et al., 1999; Qiang et al., 2004; De Kimpe et al., 2009; Eiseler et al., 2009; Scholz et al., 2009). Interaction between PKD and the actin cytoskeleton was further supported by recent studies identifying the actin regulatory proteins Slingshot and cortactin as direct substrates of PKD (De Kimpe et al., 2009; Eiseler et al., 2009). Furthermore, influence of PKD on nocodazole-mediated disruption of the Golgi complex was also recently published (Fuchs et al., 2009). Therefore, besides interfering with the secretory function of the Golgi apparatus, PKD can affect Golgi organisation also via acting on the cytoskeletal system.

Taken together, the observed disruption of the Golgi complex upon kdPKD1 expression might evolve due to the disturbed balance of Golgi membrane dynamics or as a consequence of altered cytoskeletal organisation. Importantly, Golgi fragmentation is one of the significant processes in human neurodegenerative diseases, occurring at early stages prior to apoptosis. Experimentally induced Golgi fragmentation can trigger apoptosis, but can evoke cell death by other mechanisms, as well (Gonatas et al., 2006). Thus, kdPKD1-

EGFP mediated Golgi fragmentation could be also interpreted as an early indicator of cell death, a process occurring in a larger time-scale compared to the duration of our observations.

Enhancement of PKD activity either by overexpressing wild type PKD or constitutive active PKD did not lead to any detectable changes in Golgi morphology using light microscopy. Consequently, the amount of available PKD substrates might be a limiting factor for considerable changes of Golgi structure, however, we can not rule out ultrastructural alterations in Golgi morphology detectable only by high resolution (preferably electron microscopic) analysis.

PKD regulates dendritic arborisation of mature neurons

Besides the effect on Golgi organisation, kdPKD1 expression led to dramatic shrinkage of the dendritic tree within 24 hours in mature neurons. As maintenance of the huge and extremely polarised surface of neurons is mainly provided by components of the secretory and endocytotic system, reduced dendritic arborisation can be a consequence of impaired secretory function(s) and decreased membrane supply occurring upon kdPKD1 induced Golgi disruption. This idea was supported by a strong correlation between the onset of Golgi apparatus disruption and the extent of impaired dendritic arborisation in kdPKD1 transfected neurons. In line with our results, recent investigations showed that the disturbance of the ER-to-Golgi transport or the reversible disassemble of the Golgi apparatus leads to reduced dendritic arbour in mature neurons (Horton et al., 2005; Ye et al., 2007). Golgi outposts in the longer and more complex dendrites further emphasize the role of secretory pathway in providing dendritic surface material.

According to recent data, impairment of PKD activity results in the missorting of dendritic proteins at the Golgi compartment (Bisbal et al., 2008). The authors proposed that Golgi localised PKD is required for the sorting of dendritic proteins into the appropriate vesicles and that the halt of surface maintenance upon kdPKD1 expression would be partly a consequence of impaired delivery of dendritic membrane proteins regulating outgrowth. However, kdPKD1 was shown to exert an effect also on endosomal dynamics as kinase inactive PKD resulted in increased endocytosis of dendritic membrane proteins. PKD-mediated regulation of the endosomal pathway is further supported by the recent observations that RIN1, a characterised substrate of PKD1, directly regulates the endocytosis of EphA4 receptor and consequently, synaptic plasticity (Deininger et al.,

2008). PKD-mediated phosphorylation promotes the interaction of RIN1 with 14-3-3 proteins with consequent sequestration and inactivation (Wang et al., 2002). As RIN1 is known to stimulate endocytosis via activating Rab5, an important component of the endocytotic machinery (Tall et al., 2001), kinase inactive PKD would lead to enhanced endocytosis, which is in accordance with the observations of Bisbal et al. (2008). However, the relationship between PKD and the endosomal system is far from well understood.

The observed changes in the intracellular localisation of the Kv4.2 ion channel subunit upon altered PKD activity additionally indicate a role of PKD in the organisation of dendritic surface structure. Our initial observations presented here and further ongoing analysis already suggest that the transport and/or plasma membrane localisation of Kv4.2 can be influenced by PKD activity (Krenkler J, 2009). Accumulation of Kv4.2-EGFP patches in the membrane seemed to be increased by elevated PKD activity, similarly to the effects of SAP97 overexpression. As SAP97 is involved in the transport of Kv4.2 depending on CaMKII mediated phosphorylation events, it is a feasible idea that PKD might be involved in the dendritic localisation of Kv4.2 via phosphorylating proteins involved in the transport and localisation of Kv4.2 (Gardoni et al., 2007). SAP97 contains several consensus PKD phosphorylation sites² which further supports the potential connection between the two molecules. However, further studies are required to confirm the results of our initial microscopic analysis and to clarify the underlying mechanisms involved in PKD mediated localisation of Kv4.2. The potential difference in the influence of PKD activity on the localisation of other investigated dendritic membrane proteins (like NR1 and PSD-95) also requires further studies, including quantitative analysis and live-cell observations.

Cytoskeletal rearrangements fundamentally influence the formation and maintenance of dendritic structures, including elongation and branching of dendrites or spine formation (Gauthier-Campbell et al., 2004; Kim et al., 2006; Hayashi et al., 2007; Tada et al., 2007; Zhang and Macara, 2008). Thus, direct or indirect cytoskeletal effects of PKD can also participate in the observed dendritic rearrangements in transfected neurons, especially when taking into account that high level of PKD activity was observed not only around the neuronal Golgi, but also in the cytoplasm of dendrites and even in the dendritic spines.

² <http://scansite.mit.edu>, using the consensus I/LXRXXS/T PKD target motif for search

Potential role of PKD in local signalling, synapse formation and plasticity

Based on the findings that endogenous PKD is active within the dendrites we propose that PKD can influence diverse dendritic mechanisms. As PKDrep phosphorylation was spatially restricted to certain dendritic regions, PKD might exert local effects within the dendrites. Accordingly, expressing caPKD1 or activating PKD by diC8 treatment resulted in elevated pS294 signal concentrated to discrete regions of the plasma membrane in the soma and in the dendrites, as well. These observations indicate the involvement of PKD in local signalling of dendritic membrane segments, which is in line with previous findings showing that PKD is associated to lipid rafts upon activation (Cabrera-Poch et al., 2004). Thus, PKD can be involved in local signal transduction pathways by interacting with lipid raft associated GPCRs, Trks and downstream molecules - all known to influence dendritic maintenance or arborisation.

High endogenous PKD activity was also detected in dendritic spines. The involvement of PKD in regulating postsynaptic processes is highly likely as neuronal synapses are significant sites of DAG production (Kim et al., 2009). As an example, the importance of DAG signalling in postsynaptic function was indicated by showing that DAG kinases play a crucial role in the maintenance of dendritic spines (Kim et al., 2009). Furthermore, spine morphology depends also to a great extent on processes which can be influenced by PKD, like actin dynamics (Hotulainen et al., 2009) or the endosomal system (Park et al., 2006). As the dynamic regulation of spine morphology is crucial for synapse plasticity, PKD can be also involved in the regulation of synaptic dynamics. Accordingly, the involvement of the PKD substrate RIN1 in the regulation of aversive memory and long term potentiation in the amygdala has been already shown (Dhaka et al., 2003). Furthermore, PKD might influence synaptic plasticity via regulating MEF2-dependent gene transcription by phosphorylating HDACs, as MEF2C was indicated to play a role in hippocampal-dependent learning and memory (Barbosa et al., 2008).

Taken together, in order to unravel the role of PKD in synaptic plasticity, further studies are required including investigating the effect of induced kdPKD1-EGFP expression on the memory of double transgenic mice.

SUMMARY

The protein kinase D (PKD) family has recently become a separate family among the serine-threonine kinases. Based on experiments in non-polarised cells, PKD is known to participate in various cellular processes including vesicular transport, cell proliferation, survival, migration and immune responses. A portion of PKD is localised to the Golgi membrane and was shown to regulate the formation of plasma membrane targeted transport carriers. However, just few aspects of PKD functions are known in the extremely polarised neuronal cells.

In the present work, PKD function was investigated in mature neurons using mouse embryonal hippocampal neuronal cultures. Expression of a GFP-tagged PKD activity reporter revealed high endogenous PKD activity at the Golgi complex and in the dendrites of mature neurons, while PKD activity was excluded from the axon in parallel with axonal maturation. Expression of wild type PKD1 and constitutive active PKD1 (caPKD1) in neurons revealed that both proteins were slightly enriched at the trans-Golgi network (TGN) and did not interfere with its thread-like morphology. By contrast, expression of dominant-negative kinase inactive PKD1 (kdPKD1) led to the disruption of the neuronal Golgi complex, with kdPKD1 strongly localised to the TGN fragments. As a prominent consequence of kdPKD1 expression, the dendritic tree of transfected neurons was reduced in correlation with the state of Golgi structure, while caPKD1 increased dendritic arborisation. Taken together, these findings indicate a role for PKD in the maintenance of dendritic arborisation partly via its function localised to the Golgi compartment. PKD was also indicated to influence the transport and/or the localisation of Kv4.2 ion channels, which further suggests a role for PKD in regulating dendritic membrane structure.

Despite the lack of endogenous PKD activity in the axon of mature neurons, PKD was shown to be present in the axon upon detection with a specific antibody. Furthermore, live-cell imaging recordings revealed that wtPKD1-EGFP is cotransported with some axonal proteins, presumably by the SCb type of axonal transport. As kdPKD1-EGFP was excluded from the axon gradually with posttransfectional time, transport of PKD is likely to be dependent on its kinase activity.

Based on our results, PKD is involved in the maintenance of Golgi structure and dendritic arborisation in mature neurons, however, the presence and transport of PKD in the axon suggests also a role for PKD in axonal processes which remain to be discovered.

ÖSSZEFOGLALÁS

A protein kináz D (PKD) a szerin-treonin kinázok egy nemrég elkülönített, önálló családja. Az eddigi, nem-neurális sejteken végzett vizsgálatok alapján a PKD rendkívül szerteágazó hatással bír: szerepét különböző immunrendszeri folyamatokban, sejtosztódás, sejt vándorlás és programozott sejthalál alatt is kimutatták. A PKD idegsejtekben betöltött szerepéről azonban nagyon kevés információ áll rendelkezésünkre.

Doktori munkám során érett embrionális hippokampális idegsejttenyészeteken vizsgáltam a PKD idegsejtekben betöltött szerepét. Az érett idegsejtek Golgi készülékénél és a szomatodendritikus területeken GFP-vel jelölt, PKD aktivitást detektáló konstrukció expressziójának segítségével magas endogén PKD aktivitást detektáltunk. A PKD endogén aktivitása azonban az idegsejtek fejlődésével párhuzamosan eltűnt az axonból.

Az idegsejtekben termeltetett, fluoreszcensen jelzett vad típusú és konstitutívan aktív PKD (caPKD) kisebb mértékű felhalmozódást mutatott az idegsejtekre jellemző fonalas struktúrájú Golgi készüléknél. Ezzel szemben a kináz inaktív PKD (kdPKD) túltermeltetése a Golgi készüléknél történő felhalmozódás mértékét megnövelte és a Golgi készülék fragmentálódásához vezetett. Emellett a kdPKD túltermeltetése a Golgi készülék épségének függvényében jelentős mértékű dendritfa redukálódáshoz vezetett. Mindezek alapján arra következtetünk, hogy érett idegsejtekben a PKD a dendritfa fenntartásában jelentős szerepet játszik. Ezt támasztja alá az is, hogy caPKD túltermeltetése során a dendritfa növekedése volt megfigyelhető. A PKD a Kv4.2 ioncsatorna szállítását és/vagy eloszlását is befolyásolta, ami szintén a PKD-nak a dendritikus membránszerkezet kialakításában betöltött szerepét jelzi.

Annak ellenére, hogy az érett idegsejtek axonjában endogén PKD aktivitás nem volt kimutatható, a PKD fehérje az axonban detektálható volt. Élő sejtek mikroszkópos vizsgálatokor emellett a fluoreszcensen jelzett PKD konstrukciók axonális szállítása is megfigyelhető volt. A transzport sebessége, valamint más axonális fehérjékkel történő közös szállítás alapján valószínű, hogy a PKD szállítása SCb transzporttal történik. A PKD kináz aktivitása az axonális szállításához elengedhetetlen, mivel a kdPKD1-EGFP jelenléte az axonban a transzfekeció utáni idő elteltével fokozatosan csökkent.

Eredményeink alapján érett idegsejtekben a PKD szerepet játszik a Golgi struktúrájának és a dendritfa arborizációjának fenntartásában. A PKD jelenléte és szállítása az axonban azonban arra utal, hogy a PKD axonális folyamatokban is szerepet játszik, de ennek feltárásához még további vizsgálatokra van szükség.

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REFERENCES

- Allison DW, Gelfand VI, Spector I, Craig AM (1998) Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. *J. Neurosci.* **18**:2423-2436.
- Arimura N, Kaibuchi K (2005) Key regulators in neuronal polarity. *Neuron* **48**:881-884.
- Arimura N, Kaibuchi K (2007) Neuronal polarity: from extracellular signals to intracellular mechanisms. *Nat. Rev. Neurosci.* **8**:194-205.
- Avkiran M, Rowland AJ, Cuello F, Haworth RS (2008) Protein kinase d in the cardiovascular system: emerging roles in health and disease. *Circ. Res.* **102**:157-163.
- Bagowski CP, Stein-Gerlach M, Choidas A, Ullrich A (1999) Cell-type specific phosphorylation of threonines T654 and T669 by PKD defines the signal capacity of the EGF receptor. *EMBO J.* **18**:5567-5576.
- Bankaitis VA (2002) Cell biology. Slick recruitment to the Golgi. *Science* **295**:290-291.
- Barbosa AC, Kim MS, Ertunc M, Adachi M, Nelson ED, McAnally J, Richardson JA, Kavalali ET, Monteggia LM, Bassel-Duby R, Olson EN (2008) MEF2C, a transcription factor that facilitates learning and memory by negative regulation of synapse numbers and function. *Proc. Natl. Acad. Sci. U. S. A.* **105**:9391-9396.
- Bard F, Malhotra V (2006) The formation of TGN-to-plasma-membrane transport carriers. *Annu. Rev. Cell Dev. Biol.* **22**:439-455.
- Barnes AP, Solecki D, Polleux F (2008) New insights into the molecular mechanisms specifying neuronal polarity in vivo. *Curr. Opin. Neurobiol.* **18**:44-52.
- Berna MJ, Hoffmann KM, Tapia JA, Thill M, Pace A, Mantey SA, Jensen RT (2007) CCK causes PKD1 activation in pancreatic acini by signaling through PKC-delta and PKC-independent pathways. *Biochim. Biophys. Acta* **1773**:483-501.
- Bisbal M, Conde C, Donoso M, Bollati F, Sesma J, Quiroga S, Diaz Anel A, Malhotra V, Marzolo MP, Caceres A (2008) Protein kinase d regulates trafficking of dendritic membrane proteins in developing neurons. *J. Neurosci.* **28**:9297-9308.
- Bliss JM, Venkatesh B, Colicelli J (2006) The RIN family of Ras effectors. *Methods Enzymol.* **407**:335-344.
- Boeckers TM (2006) The postsynaptic density. *Cell Tissue Res.* **326**:409-422.
- Bonanomi D, Benfenati F, Valtorta F (2006) Protein sorting in the synaptic vesicle life cycle. *Prog. Neurobiol.* **80**:177-217.
- Bossard C, Bresson D, Polishchuk RS, Malhotra V (2007) Dimeric PKD regulates membrane fission to form transport carriers at the TGN. *J. Cell Biol.* **179**:1123-1131.
- Bowden ET, Barth M, Thomas D, Glazer RI, Mueller SC (1999) An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation. *Oncogene* **18**:4440-4449.
- Bradke F, Dotti CG (1997) Neuronal polarity: vectorial cytoplasmic flow precedes axon formation. *Neuron* **19**:1175-1186.
- Bradke F, Dotti CG (1999) The role of local actin instability in axon formation. *Science* **283**:1931-1934.

- Bradke F, Dotti CG (2000) Establishment of neuronal polarity: lessons from cultured hippocampal neurons. *Curr. Opin. Neurobiol.* **10**:574-581.
- Brandlin I, Hubner S, Eiseler T, Martinez-Moya M, Horschinek A, Hausser A, Link G, Rupp S, Storz P, Pfizenmaier K, Johannes FJ (2002) Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways. *J. Biol. Chem.* **277**:6490-6496.
- Bresler T, Ramati Y, Zamorano PL, Zhai R, Garner CC, Ziv NE (2001) The dynamics of SAP90/PSD-95 recruitment to new synaptic junctions. *Mol. Cell. Neurosci.* **18**:149-167.
- Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M, Garner CC, Rosenblum K, Gundelfinger ED, Ziv NE (2004) Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. *J. Neurosci.* **24**:1507-1520.
- Bridgman PC (2004) Myosin-dependent transport in neurons. *J. Neurobiol.* **58**:164-174.
- Brown A (2003) Axonal transport of membranous and nonmembranous cargoes: a unified perspective. *J. Cell Biol.* **160**:817-821.
- Buckley KM, Melikian HE, Provoda CJ, Waring MT (2000) Regulation of neuronal function by protein trafficking: a role for the endosomal pathway. *J Physiol* **525 Pt 1**:11-19.
- Buder-Hoffmann SA, Shukla A, Barrett TF, MacPherson MB, Lounsbury KM, Mossman BT (2009) A protein kinase Cdelta-dependent protein kinase D pathway modulates ERK1/2 and JNK1/2 phosphorylation and Bim-associated apoptosis by asbestos. *Am. J. Pathol.* **174**:449-459.
- Burack MA, Silverman MA, Banker G (2000) The role of selective transport in neuronal protein sorting. *Neuron* **26**:465-472.
- Cabrera-Poch N, Sanchez-Ruiloba L, Rodriguez-Martinez M, Iglesias T (2004) Lipid raft disruption triggers protein kinase C and Src-dependent protein kinase D activation and Kidins220 phosphorylation in neuronal cells. *J. Biol. Chem.* **279**:28592-28602.
- Carlisle HJ, Kennedy MB (2005) Spine architecture and synaptic plasticity. *Trends Neurosci.* **28**:182-187.
- Celil AB, Campbell PG (2005) BMP-2 and insulin-like growth factor-I mediate Osterix (Osx) expression in human mesenchymal stem cells via the MAPK and protein kinase D signaling pathways. *J. Biol. Chem.* **280**:31353-31359.
- Charych EI, Akum BF, Goldberg JS, Jornsten RJ, Rongo C, Zheng JQ, Firestein BL (2006) Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95. *J. Neurosci.* **26**:10164-10176.
- Chu PJ, Rivera JF, Arnold DB (2006) A role for Kif17 in transport of Kv4.2. *J. Biol. Chem.* **281**:365-373.
- Cooney JR, Hurlburt JL, Selig DK, Harris KM, Fiala JC (2002) Endosomal compartments serve multiple hippocampal dendritic spines from a widespread rather than a local store of recycling membrane. *J. Neurosci.* **22**:2215-2224.
- Cooper HM (2002) Axon guidance receptors direct growth cone pathfinding: rivalry at the leading edge. *Int. J. Dev. Biol.* **46**:621-631.

- Cortes RY, Arevalo JC, Magby JP, Chao MV, Plummer MR (2007) Developmental and activity-dependent regulation of ARMS/Kidins220 in cultured rat hippocampal neurons. *Dev Neurobiol* **67**:1687-1698.
- Cosker KE, Eickholt BJ (2007) Phosphoinositide 3-kinase signalling events controlling axonal morphogenesis. *Biochem. Soc. Trans.* **35**:207-210.
- Craig AM, Blackstone CD, Huganir RL, Banker G (1993) The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. *Neuron* **10**:1055-1068.
- Cull-Candy SG, Leszkiewicz DN (2004) Role of distinct NMDA receptor subtypes at central synapses. *Sci. STKE* **2004**:re16.
- Czondor K, Ellwanger K, Fuchs YF, Lutz S, Gulyas M, Mansuy IM, Hausser A, Pfizenmaier K, Schlett K (2009) Protein kinase d controls the integrity of Golgi apparatus and the maintenance of dendritic arborization in hippocampal neurons. *Mol. Biol. Cell* **20**:2108-2120.
- Da Silva JS, Medina M, Zuliani C, Di Nardo A, Witke W, Dotti CG (2003) RhoA/ROCK regulation of neuriteogenesis via profilin IIa-mediated control of actin stability. *J. Cell Biol.* **162**:1267-1279.
- de Anda FC, Pollarolo G, Da Silva JS, Camoletto PG, Feiguin F, Dotti CG (2005) Centrosome localization determines neuronal polarity. *Nature* **436**:704-708.
- De Kimpe L, Janssens K, Derua R, Armacki M, Goicoechea S, Otey C, Waelkens E, Vandoninck S, Vandenhede JR, Seufferlein T, Van Lint J (2009) Characterization of cortactin as an in vivo protein kinase D substrate: interdependence of sites and potentiation by Src. *Cell. Signal.* **21**:253-263.
- De Matteis MA, Luini A (2008) Exiting the Golgi complex. *Nat. Rev. Mol. Cell Biol.* **9**:273-284.
- Deininger K, Eder M, Kramer ER, Zieglgansberger W, Dodt HU, Dornmair K, Colicelli J, Klein R (2008) The Rab5 guanylate exchange factor Rin1 regulates endocytosis of the EphA4 receptor in mature excitatory neurons. *Proc. Natl. Acad. Sci. U. S. A.* **105**:12539-12544.
- Dhaka A, Costa RM, Hu H, Irvin DK, Patel A, Kornblum HI, Silva AJ, O'Dell TJ, Colicelli J (2003) The RAS effector RIN1 modulates the formation of aversive memories. *J. Neurosci.* **23**:748-757.
- Diaz Anel AM (2007) Phospholipase C beta3 is a key component in the Gbetagamma/PKCeta/PKD-mediated regulation of trans-Golgi network to plasma membrane transport. *Biochem. J.* **406**:157-165.
- Diaz Anel AM, Malhotra V (2005) PKCeta is required for beta1gamma2/beta3gamma2- and PKD-mediated transport to the cell surface and the organization of the Golgi apparatus. *J. Cell Biol.* **169**:83-91.
- Doppler H, Storz P (2007) A novel tyrosine phosphorylation site in protein kinase D contributes to oxidative stress-mediated activation. *J. Biol. Chem.* **282**:31873-31881.
- Doppler H, Storz P, Li J, Comb MJ, Toker A (2005) A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. *J. Biol. Chem.* **280**:15013-15019.

- Dosemeci A, Makusky AJ, Jankowska-Stephens E, Yang X, Slotta DJ, Markey SP (2007) Composition of the synaptic PSD-95 complex. *Mol. Cell. Proteomics* **6**:1749-1760.
- Dotti CG, Banker GA (1987) Experimentally induced alteration in the polarity of developing neurons. *Nature* **330**:254-256.
- Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* **8**:1454-1468.
- Doussau F, Augustine GJ (2000) The actin cytoskeleton and neurotransmitter release: an overview. *Biochimie* **82**:353-363.
- Dresbach T, Qualmann B, Kessels MM, Garner CC, Gundelfinger ED (2001) The presynaptic cytomatrix of brain synapses. *Cell. Mol. Life Sci.* **58**:94-116.
- Egea G, Lazaro-Diequez F, Vilella M (2006) Actin dynamics at the Golgi complex in mammalian cells. *Curr. Opin. Cell Biol.* **18**:168-178.
- Eiseler T, Doppler H, Yan IK, Kitatani K, Mizuno K, Storz P (2009) Protein kinase D1 regulates cofilin-mediated F-actin reorganization and cell motility through slingshot. *Nat. Cell Biol.*
- Endo K, Oki E, Biedermann V, Kojima H, Yoshida K, Johannes FJ, Kufe D, Datta R (2000) Proteolytic cleavage and activation of protein kinase C [micro] by caspase-3 in the apoptotic response of cells to 1-beta -D-arabinofuranosylcytosine and other genotoxic agents. *J. Biol. Chem.* **275**:18476-18481.
- Ernst AF, Journey WM, McLoon SC (1998) Mechanisms involved in development of retinotectal connections: roles of Eph receptor tyrosine kinases, NMDA receptors and nitric oxide. *Prog. Brain Res.* **118**:115-131.
- Esch T, Lemmon V, Banker G (1999) Local presentation of substrate molecules directs axon specification by cultured hippocampal neurons. *J. Neurosci.* **19**:6417-6426.
- Evers JF, Muench D, Duch C (2006) Developmental relocation of presynaptic terminals along distinct types of dendritic filopodia. *Dev. Biol.* **297**:214-227.
- Feng Y, Walsh CA (2001) Protein-protein interactions, cytoskeletal regulation and neuronal migration. *Nat. Rev. Neurosci.* **2**:408-416.
- Fletcher TL, Cameron P, De Camilli P, Banker G (1991) The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J. Neurosci.* **11**:1617-1626.
- Fuchs YF, Eisler SA, Link G, Schlicker O, Bunt G, Pfizenmaier K, Hausser A (2009) A Golgi PKD activity reporter reveals a crucial role of PKD in nocodazole-induced Golgi dispersal. *Traffic in press*
- Fugmann T, Hausser A, Schoffler P, Schmid S, Pfizenmaier K, Olayioye MA (2007) Regulation of secretory transport by protein kinase D-mediated phosphorylation of the ceramide transfer protein. *J. Cell Biol.* **178**:15-22.
- Fukata Y, Kimura T, Kaibuchi K (2002) Axon specification in hippocampal neurons. *Neurosci. Res.* **43**:305-315.
- Gallo G, Letourneau PC (2004) Regulation of growth cone actin filaments by guidance cues. *J. Neurobiol.* **58**:92-102.
- Gardiol A, Racca C, Triller A (1999) Dendritic and postsynaptic protein synthetic machinery. *J. Neurosci.* **19**:168-179.

- Gardoni F, Mauceri D, Marcello E, Sala C, Di Luca M, Jeromin A (2007) SAP97 directs the localization of Kv4.2 to spines in hippocampal neurons: regulation by CaMKII. *J. Biol. Chem.* **282**:28691-28699.
- Garrido JJ, Fernandes F, Giraud P, Mouret I, Pasqualini E, Fache MP, Jullien F, Dargent B (2001) Identification of an axonal determinant in the C-terminus of the sodium channel Na(v)1.2. *EMBO J.* **20**:5950-5961.
- Gartner A, Huang X, Hall A (2006) Neuronal polarity is regulated by glycogen synthase kinase-3 (GSK-3beta) independently of Akt/PKB serine phosphorylation. *J. Cell Sci.* **119**:3927-3934.
- Gauthier-Campbell C, Brecht DS, Murphy TH, El-Husseini Ael D (2004) Regulation of dendritic branching and filopodia formation in hippocampal neurons by specific acylated protein motifs. *Mol. Biol. Cell* **15**:2205-2217.
- Georges PC, Hadzimechalis NM, Sweet ES, Firestein BL (2008) The yin-yang of dendrite morphology: unity of actin and microtubules. *Mol. Neurobiol.* **38**:270-284.
- Ghanekar Y, Lowe M (2005) Protein kinase D: activation for Golgi carrier formation. *Trends Cell Biol.* **15**:511-514.
- Gomis-Ruth S, Wierenga CJ, Bradke F (2008) Plasticity of polarization: changing dendrites into axons in neurons integrated in neuronal circuits. *Curr. Biol.* **18**:992-1000.
- Gonatas NK, Stieber A, Gonatas JO (2006) Fragmentation of the Golgi apparatus in neurodegenerative diseases and cell death. *J. Neurol. Sci.* **246**:21-30.
- Govek EE, Newey SE, Van Aelst L (2005) The role of the Rho GTPases in neuronal development. *Genes Dev.* **19**:1-49.
- Guillaud L, Setou M, Hirokawa N (2003) KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons. *J. Neurosci.* **23**:131-140.
- Gunawardena S, Goldstein LS (2004) Cargo-carrying motor vehicles on the neuronal highway: transport pathways and neurodegenerative disease. *J. Neurobiol.* **58**:258-271.
- Gundelfinger ED, tom Dieck S (2000) Molecular organization of excitatory chemical synapses in the mammalian brain. *Naturwissenschaften* **87**:513-523.
- Hanus C, Ehlers MD (2008) Secretory outposts for the local processing of membrane cargo in neuronal dendrites. *Traffic* **9**:1437-1445.
- Harada A, Teng J, Takei Y, Oguchi K, Hirokawa N (2002) MAP2 is required for dendrite elongation, PKA anchoring in dendrites, and proper PKA signal transduction. *J. Cell Biol.* **158**:541-549.
- Harrison BC, Kim MS, van Rooij E, Plato CF, Papst PJ, Vega RB, McAnally JA, Richardson JA, Bassel-Duby R, Olson EN, McKinsey TA (2006) Regulation of cardiac stress signaling by protein kinase d1. *Mol. Cell. Biol.* **26**:3875-3888.
- Hausser A, Storz P, Martens S, Link G, Toker A, Pfizenmaier K (2005) Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. *Nat. Cell Biol.* **7**:880-886.
- Hausser A, Link G, Bamberg L, Burzlaff A, Lutz S, Pfizenmaier K, Johannes FJ (2002) Structural requirements for localization and activation of protein kinase C mu (PKC mu) at the Golgi compartment. *J. Cell Biol.* **156**:65-74.

- Hausser A, Storz P, Link G, Stoll H, Liu YC, Altman A, Pfizenmaier K, Johannes FJ (1999) Protein kinase C mu is negatively regulated by 14-3-3 signal transduction proteins. *J. Biol. Chem.* **274**:9258-9264.
- Hayashi A, Seki N, Hattori A, Kozuma S, Saito T (1999) PKC ν , a new member of the protein kinase C family, composes a fourth subfamily with PKC μ . *Biochim. Biophys. Acta* **1450**:99-106.
- Hayashi K, Kawai-Hirai R, Ishikawa K, Takata K (2002) Reversal of neuronal polarity characterized by conversion of dendrites into axons in neonatal rat cortical neurons in vitro. *Neuroscience* **110**:7-17.
- Hayashi K, Ohshima T, Hashimoto M, Mikoshiba K (2007) Pak1 regulates dendritic branching and spine formation. *Dev Neurobiol* **67**:655-669.
- Hedstrom KL, Ogawa Y, Rasband MN (2008) AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. *J. Cell Biol.* **183**:635-640.
- Hering H, Lin CC, Sheng M (2003) Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *J. Neurosci.* **23**:3262-3271.
- Hirokawa N, Takemura R (2005) Molecular motors and mechanisms of directional transport in neurons. *Nat. Rev. Neurosci.* **6**:201-214.
- Horton AC, Ehlers MD (2003a) Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J. Neurosci.* **23**:6188-6199.
- Horton AC, Ehlers MD (2003b) Neuronal polarity and trafficking. *Neuron* **40**:277-295.
- Horton AC, Ehlers MD (2004) Secretory trafficking in neuronal dendrites. *Nat. Cell Biol.* **6**:585-591.
- Horton AC, Yi JJ, Ehlers MD (2006) Cell type-specific dendritic polarity in the absence of spatially organized external cues. *Brain Cell Biol.* **35**:29-38.
- Horton AC, Racz B, Monson EE, Lin AL, Weinberg RJ, Ehlers MD (2005) Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis. *Neuron* **48**:757-771.
- Hotulainen P, Llano O, Smirnov S, Tanhuanpaa K, Faix J, Rivera C, Lappalainen P (2009) Defining mechanisms of actin polymerization and depolymerization during dendritic spine morphogenesis. *J. Cell Biol.* **185**:323-339.
- Hua JY, Smear MC, Baier H, Smith SJ (2005) Regulation of axon growth in vivo by activity-based competition. *Nature* **434**:1022-1026.
- Hur EM, Kim KT (2007) A role of local signalling in the establishment and maintenance of the asymmetrical architecture of a neuron. *J. Neurochem.* **101**:600-610.
- Hurd C, Waldron RT, Rozengurt E (2002) Protein kinase D complexes with C-Jun N-terminal kinase via activation loop phosphorylation and phosphorylates the C-Jun N-terminus. *Oncogene* **21**:2154-2160.
- Iglesias T, Rozengurt E (1998) Protein kinase D activation by mutations within its pleckstrin homology domain. *J. Biol. Chem.* **273**:410-416.
- Iglesias T, Rozengurt E (1999) Protein kinase D activation by deletion of its cysteine-rich motifs. *FEBS Lett.* **454**:53-56.

- Iglesias T, Cabrera-Poch N, Mitchell MP, Naven TJ, Rozengurt E, Schiavo G (2000) Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. *J. Biol. Chem.* **275**:40048-40056.
- Jacamo R, Sinnott-Smith J, Rey O, Waldron RT, Rozengurt E (2008) Sequential protein kinase C (PKC)-dependent and PKC-independent protein kinase D catalytic activation via Gq-coupled receptors: differential regulation of activation loop Ser(744) and Ser(748) phosphorylation. *J. Biol. Chem.* **283**:12877-12887.
- Jacobson C, Schnapp B, Banker GA (2006) A change in the selective translocation of the Kinesin-1 motor domain marks the initial specification of the axon. *Neuron* **49**:797-804.
- Jamora C, Yamanouye N, Van Lint J, Laudenslager J, Vandenheede JR, Faulkner DJ, Malhotra V (1999) Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. *Cell* **98**:59-68.
- Jan YN, Jan LY (2003) The control of dendrite development. *Neuron* **40**:229-242.
- Jareb M, Banker G (1997) Inhibition of axonal growth by brefeldin A in hippocampal neurons in culture. *J. Neurosci.* **17**:8955-8963.
- Jedlicka P, Vlachos A, Schwarzacher SW, Deller T (2008) A role for the spine apparatus in LTP and spatial learning. *Behav. Brain Res.* **192**:12-19.
- Jenkins SM, Bennett V (2001) Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at Purkinje neuron initial segments. *J. Cell Biol.* **155**:739-746.
- Johannes FJ, Prestle J, Dieterich S, Oberhagemann P, Link G, Pfizenmaier K (1995) Characterization of activators and inhibitors of protein kinase C mu. *Eur. J. Biochem.* **227**:303-307.
- Johannes FJ, Horn J, Link G, Haas E, Siemienski K, Wajant H, Pfizenmaier K (1998) Protein kinase Cmu downregulation of tumor-necrosis-factor-induced apoptosis correlates with enhanced expression of nuclear-factor-kappaB-dependent protective genes. *Eur. J. Biochem.* **257**:47-54.
- Johannessen M, Delghandi MP, Rykx A, Dragset M, Vandenheede JR, Van Lint J, Moens U (2007) Protein kinase D induces transcription through direct phosphorylation of the cAMP-response element-binding protein. *J. Biol. Chem.* **282**:14777-14787.
- Jones SB, Lu HY, Lu Q (2004) Abl tyrosine kinase promotes dendrogenesis by inducing actin cytoskeletal rearrangements in cooperation with Rho family small GTPases in hippocampal neurons. *J. Neurosci.* **24**:8510-8521.
- Jontes JD, Smith SJ (2000) Filopodia, spines, and the generation of synaptic diversity. *Neuron* **27**:11-14.
- Kamiguchi H, Lemmon V (2000) Recycling of the cell adhesion molecule L1 in axonal growth cones. *J. Neurosci.* **20**:3676-3686.
- Kapfhammer JP (2004) Cellular and molecular control of dendritic growth and development of cerebellar Purkinje cells. *Prog. Histochem. Cytochem.* **39**:131-182.
- Killisch I, Dotti CG, Laurie DJ, Luddens H, Seeburg PH (1991) Expression patterns of GABAA receptor subtypes in developing hippocampal neurons. *Neuron* **7**:927-936.
- Kim E, Sheng M (2004) PDZ domain proteins of synapses. *Nat. Rev. Neurosci.* **5**:771-781.

- Kim K, Yang J, Zhong XP, Kim MH, Kim YS, Lee HW, Han S, Choi J, Han K, Seo J, Prescott SM, Topham MK, Bae YC, Koretzky G, Choi SY, Kim E (2009) Synaptic removal of diacylglycerol by DGK ζ and PSD-95 regulates dendritic spine maintenance. *EMBO J.* **28**:1170-1179.
- Kim Y, Sung JY, Ceglia I, Lee KW, Ahn JH, Halford JM, Kim AM, Kwak SP, Park JB, Ho Ryu S, Schenck A, Bardoni B, Scott JD, Nairn AC, Greengard P (2006) Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. *Nature* **442**:814-817.
- Koh CG (2006) Rho GTPases and their regulators in neuronal functions and development. *NeuroSignals* **15**:228-237.
- Komuro H, Rakic P (1998) Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca²⁺ fluctuations. *J. Neurobiol.* **37**:110-130.
- Krenkler J HA, Schlett K (2009) Protein kinase D (PKD) mediated effects on Kv4.2 localization in hippocampal neurons. In: 12th Meeting of the Hungarian Neuroscience Society. Budapest.
- Kriegstein AR, Dichter MA (1983) Morphological classification of rat cortical neurons in cell culture. *J. Neurosci.* **3**:1634-1647.
- Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. *Trends Neurosci.* **27**:392-399.
- Kunda P, Paglini G, Quiroga S, Kosik K, Caceres A (2001) Evidence for the involvement of Tiam1 in axon formation. *J. Neurosci.* **21**:2361-2372.
- Lapierre LA, Kumar R, Hales CM, Navarre J, Bhartur SG, Burnette JO, Provance DW, Jr., Mercer JA, Bahler M, Goldenring JR (2001) Myosin vb is associated with plasma membrane recycling systems. *Mol. Biol. Cell* **12**:1843-1857.
- Ledesma MD, Simons K, Dotti CG (1998) Neuronal polarity: essential role of protein-lipid complexes in axonal sorting. *Proc. Natl. Acad. Sci. U. S. A.* **95**:3966-3971.
- Ledesma MD, Brugger B, Bunning C, Wieland FT, Dotti CG (1999) Maturation of the axonal plasma membrane requires upregulation of sphingomyelin synthesis and formation of protein-lipid complexes. *EMBO J.* **18**:1761-1771.
- Lein PJ, Guo X, Shi GX, Moholt-Siebert M, Bruun D, Andres DA (2007) The novel GTPase Rit differentially regulates axonal and dendritic growth. *J. Neurosci.* **27**:4725-4736.
- Lemonnier J, Ghayor C, Guicheux J, Caverzasio J (2004) Protein kinase C-independent activation of protein kinase D is involved in BMP-2-induced activation of stress mitogen-activated protein kinases JNK and p38 and osteoblastic cell differentiation. *J. Biol. Chem.* **279**:259-264.
- Levy JR, Holzbaur EL (2006) Cytoplasmic dynein/dynactin function and dysfunction in motor neurons. *Int. J. Dev. Neurosci.* **24**:103-111.
- Li J, Chen LA, Townsend CM, Jr., Evers BM (2008) PKD1, PKD2, and their substrate Kidins220 regulate neurotensin secretion in the BON human endocrine cell line. *J. Biol. Chem.* **283**:2614-2621.

- Liljedahl M, Maeda Y, Colanzi A, Ayala I, Van Lint J, Malhotra V (2001) Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. *Cell* **104**:409-420.
- Lippincott-Schwartz J (1998) Cytoskeletal proteins and Golgi dynamics. *Curr. Opin. Cell Biol.* **10**:52-59.
- Luskin MB (1994) Neuronal cell lineage in the vertebrate central nervous system. *FASEB J.* **8**:722-730.
- Maeda Y, Beznoussenko GV, Van Lint J, Mironov AA, Malhotra V (2001) Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain. *EMBO J.* **20**:5982-5990.
- Mandell JW, Banker GA (1996) A spatial gradient of tau protein phosphorylation in nascent axons. *J. Neurosci.* **16**:5727-5740.
- Marin O, Rubenstein JL (2003) Cell migration in the forebrain. *Annu. Rev. Neurosci.* **26**:441-483.
- Marin O, Valdeolmillos M, Moya F (2006) Neurons in motion: same principles for different shapes? *Trends Neurosci.* **29**:655-661.
- Marszalek JR, Weiner JA, Farlow SJ, Chun J, Goldstein LS (1999) Novel dendritic kinesin sorting identified by different process targeting of two related kinesins: KIF21A and KIF21B. *J. Cell Biol.* **145**:469-479.
- Matthews S, Iglesias T, Cantrell D, Rozengurt E (1999) Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation. *FEBS Lett.* **457**:515-521.
- Matthews SA, Dayalu R, Thompson LJ, Scharenberg AM (2003) Regulation of protein kinase C η by the B-cell antigen receptor. *J. Biol. Chem.* **278**:9086-9091.
- Mattson MP (1999) Establishment and plasticity of neuronal polarity. *J. Neurosci. Res.* **57**:577-589.
- McAllister AK (2000) Cellular and molecular mechanisms of dendrite growth. *Cereb. Cortex* **10**:963-973.
- McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* **15**:791-803.
- McAllister AK, Katz LC, Lo DC (1997) Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron* **18**:767-778.
- Metin C, Baudoin JP, Rakic S, Parnavelas JG (2006) Cell and molecular mechanisms involved in the migration of cortical interneurons. *Eur. J. Neurosci.* **23**:894-900.
- Michalon A, Koshibu K, Baumgartel K, Spirig DH, Mansuy IM (2005) Inducible and neuron-specific gene expression in the adult mouse brain with the rtTA2S-M2 system. *Genesis* **43**:205-212.
- Nadarajah B (2003) Radial glia and somal translocation of radial neurons in the developing cerebral cortex. *Glia* **43**:33-36.
- Nadarajah B, Parnavelas JG (2002) Modes of neuronal migration in the developing cerebral cortex. *Nat. Rev. Neurosci.* **3**:423-432.

- Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, Slusarewicz P, Kreis TE, Warren G (1995) Characterization of a cis-Golgi matrix protein, GM130. *J. Cell Biol.* **131**:1715-1726.
- Nakayama AY, Harms MB, Luo L (2000) Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J. Neurosci.* **20**:5329-5338.
- Nimchinsky EA, Sabatini BL, Svoboda K (2002) Structure and function of dendritic spines. *Annu. Rev. Physiol.* **64**:313-353.
- Nishimura T, Yamaguchi T, Kato K, Yoshizawa M, Nabeshima Y, Ohno S, Hoshino M, Kaibuchi K (2005) PAR-6-PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1. *Nat. Cell Biol.* **7**:270-277.
- Okada Y, Yamazaki H, Sekine-Aizawa Y, Hirokawa N (1995) The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell* **81**:769-780.
- Osen-Sand A, Staple JK, Naldi E, Schiavo G, Rossetto O, Petitpierre S, Malgaroli A, Montecucco C, Catsicas S (1996) Common and distinct fusion proteins in axonal growth and transmitter release. *J. Comp. Neurol.* **367**:222-234.
- Oster H, Abraham D, Leitges M (2006) Expression of the protein kinase D (PKD) family during mouse embryogenesis. *Gene Expr. Patterns* **6**:400-408.
- Park M, Salgado JM, Ostroff L, Helton TD, Robinson CG, Harris KM, Ehlers MD (2006) Plasticity-induced growth of dendritic spines by exocytic trafficking from recycling endosomes. *Neuron* **52**:817-830.
- Pierce JP, Mayer T, McCarthy JB (2001) Evidence for a satellite secretory pathway in neuronal dendritic spines. *Curr. Biol.* **11**:351-355.
- Qiang YW, Yao L, Tosato G, Rudikoff S (2004) Insulin-like growth factor I induces migration and invasion of human multiple myeloma cells. *Blood* **103**:301-308.
- Rey O, Yuan J, Rozengurt E (2003) Intracellular redistribution of protein kinase D2 in response to G-protein-coupled receptor agonists. *Biochem. Biophys. Res. Commun.* **302**:817-824.
- Rey O, Young SH, Cantrell D, Rozengurt E (2001a) Rapid protein kinase D translocation in response to G protein-coupled receptor activation. Dependence on protein kinase C. *J. Biol. Chem.* **276**:32616-32626.
- Rey O, Sinnott-Smith J, Zhukova E, Rozengurt E (2001b) Regulated nucleocytoplasmic transport of protein kinase D in response to G protein-coupled receptor activation. *J. Biol. Chem.* **276**:49228-49235.
- Rey O, Reeve JR, Jr., Zhukova E, Sinnott-Smith J, Rozengurt E (2004) G protein-coupled receptor-mediated phosphorylation of the activation loop of protein kinase D: dependence on plasma membrane translocation and protein kinase Cepsilon. *J. Biol. Chem.* **279**:34361-34372.
- Rivera JF, Chu PJ, Arnold DB (2005) The T1 domain of Kv1.3 mediates intracellular targeting to axons. *Eur. J. Neurosci.* **22**:1853-1862.
- Roy S, Winton MJ, Black MM, Trojanowski JQ, Lee VM (2007) Rapid and intermittent cotransport of slow component-b proteins. *J. Neurosci.* **27**:3131-3138.

- Rozengurt E, Rey O, Waldron RT (2005) Protein kinase D signaling. *J. Biol. Chem.* **280**:13205-13208.
- Rykx A, De Kimpe L, Mikhalap S, Vantus T, Seufferlein T, Vandenhede JR, Van Lint J (2003) Protein kinase D: a family affair. *FEBS Lett.* **546**:81-86.
- Sampo B, Kaech S, Kunz S, Banker G (2003) Two distinct mechanisms target membrane proteins to the axonal surface. *Neuron* **37**:611-624.
- Sanchez-Ruiloba L, Cabrera-Poch N, Rodriguez-Martinez M, Lopez-Menendez C, Jean-Mairet RM, Higuero AM, Iglesias T (2006) Protein kinase D intracellular localization and activity control kinase D-interacting substrate of 220-kDa traffic through a postsynaptic density-95/discs large/zonula occludens-1-binding motif. *J. Biol. Chem.* **281**:18888-18900.
- Saxena S, Howe CL, Cosgaya JM, Steiner P, Hirling H, Chan JR, Weis J, Kruttgen A (2005) Differential endocytic sorting of p75NTR and TrkA in response to NGF: a role for late endosomes in TrkA trafficking. *Mol. Cell. Neurosci.* **28**:571-587.
- Scholz RP, Regner J, Theil A, Erlmann P, Holeiter G, Jahne R, Schmid S, Hausser A, Olayioye MA (2009) DLC1 interacts with 14-3-3 proteins to inhibit RhoGAP activity and block nucleocytoplasmic shuttling. *J. Cell Sci.* **122**:92-102.
- Scott EK, Luo L (2001) How do dendrites take their shape? *Nat. Neurosci.* **4**:359-365.
- Setou M, Seog DH, Tanaka Y, Kanai Y, Takei Y, Kawagishi M, Hirokawa N (2002) Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. *Nature* **417**:83-87.
- Shah JV, Cleveland DW (2002) Slow axonal transport: fast motors in the slow lane. *Curr. Opin. Cell Biol.* **14**:58-62.
- Sharp DJ, Yu W, Ferhat L, Kuriyama R, Rueger DC, Baas PW (1997) Identification of a microtubule-associated motor protein essential for dendritic differentiation. *J. Cell Biol.* **138**:833-843.
- Shi SH, Jan LY, Jan YN (2003) Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* **112**:63-75.
- Snider WD (1988) Nerve growth factor enhances dendritic arborization of sympathetic ganglion cells in developing mammals. *J. Neurosci.* **8**:2628-2634.
- Sniderhan LF, Stout A, Lu Y, Chao MV, Maggirwar SB (2008) Ankyrin-rich membrane spanning protein plays a critical role in nuclear factor-kappa B signaling. *Mol. Cell. Neurosci.* **38**:404-416.
- Sobeih MM, Corfas G (2002) Extracellular factors that regulate neuronal migration in the central nervous system. *Int. J. Dev. Neurosci.* **20**:349-357.
- Solecki DJ, Govek EE, Tomoda T, Hatten ME (2006) Neuronal polarity in CNS development. *Genes Dev.* **20**:2639-2647.
- Song J, Li J, Lulla A, Evers BM, Chung DH (2006) Protein kinase D protects against oxidative stress-induced intestinal epithelial cell injury via Rho/ROK/PKC-delta pathway activation. *Am. J. Physiol. Cell Physiol.* **290**:C1469-1476.
- Stagi M, Dittrich PS, Frank N, Iliev AI, Schwille P, Neumann H (2005) Breakdown of axonal synaptic vesicle precursor transport by microglial nitric oxide. *J. Neurosci.* **25**:352-362.

- Steedmaier M, Klumperman J, Foletti DL, Yoo JS, Scheller RH (1999) Vesicle-associated membrane protein 4 is implicated in trans-Golgi network vesicle trafficking. *Mol. Biol. Cell* **10**:1957-1972.
- Storz P, Doppler H, Toker A (2004) Protein kinase Cdelta selectively regulates protein kinase D-dependent activation of NF-kappaB in oxidative stress signaling. *Mol. Cell. Biol.* **24**:2614-2626.
- Sturany S, Van Lint J, Gilchrist A, Vandenheede JR, Adler G, Seufferlein T (2002) Mechanism of activation of protein kinase D2(PKD2) by the CCK(B)/gastrin receptor. *J. Biol. Chem.* **277**:29431-29436.
- Sturany S, Van Lint J, Muller F, Wilda M, Hameister H, Hocker M, Brey A, Gern U, Vandenheede J, Gress T, Adler G, Seufferlein T (2001) Molecular cloning and characterization of the human protein kinase D2. A novel member of the protein kinase D family of serine threonine kinases. *J. Biol. Chem.* **276**:3310-3318.
- Sytnyk V, Leshchyns'ka I, Dityatev A, Schachner M (2004) Trans-Golgi network delivery of synaptic proteins in synaptogenesis. *J. Cell Sci.* **117**:381-388.
- Tada T, Sheng M (2006) Molecular mechanisms of dendritic spine morphogenesis. *Curr. Opin. Neurobiol.* **16**:95-101.
- Tada T, Simonetta A, Batteredon M, Kinoshita M, Edbauer D, Sheng M (2007) Role of Septin cytoskeleton in spine morphogenesis and dendrite development in neurons. *Curr. Biol.* **17**:1752-1758.
- Takemoto-Kimura S, Ageta-Ishihara N, Nonaka M, Adachi-Morishima A, Mano T, Okamura M, Fujii H, Fuse T, Hoshino M, Suzuki S, Kojima M, Mishina M, Okuno H, Bito H (2007) Regulation of dendritogenesis via a lipid-raft-associated Ca²⁺/calmodulin-dependent protein kinase CLICK-III/CaMKIgamma. *Neuron* **54**:755-770.
- Tall GG, Barbieri MA, Stahl PD, Horazdovsky BF (2001) Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. *Dev. Cell* **1**:73-82.
- Tang BL (2008) Emerging aspects of membrane traffic in neuronal dendrite growth. *Biochim. Biophys. Acta* **1783**:169-176.
- Taya S, Shinoda T, Tsuboi D, Asaki J, Nagai K, Hikita T, Kuroda S, Kuroda K, Shimizu M, Hirotsune S, Iwamatsu A, Kaibuchi K (2007) DISC1 regulates the transport of the NUDEL/LIS1/14-3-3epsilon complex through kinesin-1. *J. Neurosci.* **27**:15-26.
- Threadgill R, Bobb K, Ghosh A (1997) Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. *Neuron* **19**:625-634.
- Tkatch T, Baranauskas G, Surmeier DJ (2000) Kv4.2 mRNA abundance and A-type K(+) current amplitude are linearly related in basal ganglia and basal forebrain neurons. *J. Neurosci.* **20**:579-588.
- Tolias KF, Bikoff JB, Kane CG, Tolias CS, Hu L, Greenberg ME (2007) The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development. *Proc. Natl. Acad. Sci. U. S. A.* **104**:7265-7270.
- Tran TH, Zeng Q, Hong W (2007) VAMP4 cycles from the cell surface to the trans-Golgi network via sorting and recycling endosomes. *J. Cell Sci.* **120**:1028-1041.

- Trivedi N, Marsh P, Goold RG, Wood-Kaczmar A, Gordon-Weeks PR (2005) Glycogen synthase kinase-3 β phosphorylation of MAP1B at Ser1260 and Thr1265 is spatially restricted to growing axons. *J. Cell Sci.* **118**:993-1005.
- Tsui-Pierchala BA, Encinas M, Milbrandt J, Johnson EM, Jr. (2002) Lipid rafts in neuronal signaling and function. *Trends Neurosci.* **25**:412-417.
- Uetani N, Chagnon MJ, Kennedy TE, Iwakura Y, Tremblay ML (2006) Mammalian motoneuron axon targeting requires receptor protein tyrosine phosphatases sigma and delta. *J. Neurosci.* **26**:5872-5880.
- Van Lint J, Ni Y, Valius M, Merlevede W, Vandenhede JR (1998) Platelet-derived growth factor stimulates protein kinase D through the activation of phospholipase C γ and protein kinase C. *J. Biol. Chem.* **273**:7038-7043.
- Van Lint J, Rykx A, Maeda Y, Vantus T, Sturany S, Malhotra V, Vandenhede JR, Seufferlein T (2002) Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol.* **12**:193-200.
- Vertommen D, Rider M, Ni Y, Waelkens E, Merlevede W, Vandenhede JR, Van Lint J (2000) Regulation of protein kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis. *J. Biol. Chem.* **275**:19567-19576.
- Vessey JP, Karra D (2007) More than just synaptic building blocks: scaffolding proteins of the post-synaptic density regulate dendritic patterning. *J. Neurochem.* **102**:324-332.
- Votin V, Nelson WJ, Barth AI (2005) Neurite outgrowth involves adenomatous polyposis coli protein and beta-catenin. *J. Cell Sci.* **118**:5699-5708.
- Waldron RT, Rozengurt E (2003) Protein kinase C phosphorylates protein kinase D activation loop Ser744 and Ser748 and releases autoinhibition by the pleckstrin homology domain. *J. Biol. Chem.* **278**:154-163.
- Wang QJ (2006) PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol. Sci.* **27**:317-323.
- Wang S, Li X, Parra M, Verdin E, Bassel-Duby R, Olson EN (2008) Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7. *Proc. Natl. Acad. Sci. U. S. A.* **105**:7738-7743.
- Wang Y, Waldron RT, Dhaka A, Patel A, Riley MM, Rozengurt E, Colicelli J (2002) The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. *Mol. Cell. Biol.* **22**:916-926.
- Watkins JL, Lewandowski KT, Meek SE, Storz P, Toker A, Piwnicka-Worms H (2008) Phosphorylation of the Par-1 polarity kinase by protein kinase D regulates 14-3-3 binding and membrane association. *Proc. Natl. Acad. Sci. U. S. A.* **105**:18378-18383.
- Wayman GA, Kaech S, Grant WF, Davare M, Impey S, Tokumitsu H, Nozaki N, Banker G, Soderling TR (2004) Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I. *J. Neurosci.* **24**:3786-3794.
- Wen Z, Zheng JQ (2006) Directional guidance of nerve growth cones. *Curr. Opin. Neurobiol.* **16**:52-58.
- Winckler B, Poo MM (1996) No diffusion barrier at axon hillock. *Nature* **379**:213.

- Winckler B, Forscher P, Mellman I (1999) A diffusion barrier maintains distribution of membrane proteins in polarized neurons. *Nature* **397**:698-701.
- Wisco D, Anderson ED, Chang MC, Norden C, Boiko T, Folsch H, Winckler B (2003) Uncovering multiple axonal targeting pathways in hippocampal neurons. *J. Cell Biol.* **162**:1317-1328.
- Witte H, Bradke F (2008) The role of the cytoskeleton during neuronal polarization. *Curr. Opin. Neurobiol.* **18**:479-487.
- Witte H, Neukirchen D, Bradke F (2008) Microtubule stabilization specifies initial neuronal polarization. *J. Cell Biol.* **180**:619-632.
- Wong C, Jin ZG (2005) Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. *J. Biol. Chem.* **280**:33262-33269.
- Wong RO, Ghosh A (2002) Activity-dependent regulation of dendritic growth and patterning. *Nat. Rev. Neurosci.* **3**:803-812.
- Yamagata M, Sanes JR, Weiner JA (2003) Synaptic adhesion molecules. *Curr. Opin. Cell Biol.* **15**:621-632.
- Yap CC, Nokes RL, Wisco D, Anderson E, Folsch H, Winckler B (2008) Pathway selection to the axon depends on multiple targeting signals in NgCAM. *J. Cell Sci.* **121**:1514-1525.
- Ye B, Zhang Y, Song W, Younger SH, Jan LY, Jan YN (2007) Growing dendrites and axons differ in their reliance on the secretory pathway. *Cell* **130**:717-729.
- Yeaman C, Ayala MI, Wright JR, Bard F, Bossard C, Ang A, Maeda Y, Seufferlein T, Mellman I, Nelson WJ, Malhotra V (2004) Protein kinase D regulates basolateral membrane protein exit from trans-Golgi network. *Nat. Cell Biol.* **6**:106-112.
- Yin DM, Huang YH, Zhu YB, Wang Y (2008) Both the establishment and maintenance of neuronal polarity require the activity of protein kinase D in the Golgi apparatus. *J. Neurosci.* **28**:8832-8843.
- Yoshimura T, Arimura N, Kaibuchi K (2006a) Signaling networks in neuronal polarization. *J. Neurosci.* **26**:10626-10630.
- Yoshimura T, Arimura N, Kawano Y, Kawabata S, Wang S, Kaibuchi K (2006b) Ras regulates neuronal polarity via the PI3-kinase/Akt/GSK-3beta/CRMP-2 pathway. *Biochem. Biophys. Res. Commun.* **340**:62-68.
- Yu CR, Power J, Barnea G, O'Donnell S, Brown HE, Osborne J, Axel R, Gogos JA (2004) Spontaneous neural activity is required for the establishment and maintenance of the olfactory sensory map. *Neuron* **42**:553-566.
- Yuan J, Slice L, Walsh JH, Rozengurt E (2000) Activation of protein kinase D by signaling through the alpha subunit of the heterotrimeric G protein G(q). *J. Biol. Chem.* **275**:2157-2164.
- Zhang H, Macara IG (2008) The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. *Dev. Cell* **14**:216-226.
- Zhang W, Zheng S, Storz P, Min W (2005) Protein kinase D specifically mediates apoptosis signal-regulating kinase 1-JNK signaling induced by H₂O₂ but not tumor necrosis factor. *J. Biol. Chem.* **280**:19036-19044.

- Zhen M, Jin Y (2004) Presynaptic terminal differentiation: transport and assembly. *Curr. Opin. Neurobiol.* **14**:280-287.
- Zheng Y, Wildonger J, Ye B, Zhang Y, Kita A, Younger SH, Zimmerman S, Jan LY, Jan YN (2008) Dynein is required for polarized dendritic transport and uniform microtubule orientation in axons. *Nat. Cell Biol.* **10**:1172-1180.
- Zhong W, Chia W (2008) Neurogenesis and asymmetric cell division. *Curr. Opin. Neurobiol.* **18**:4-11.
- Zugaza JL, Sinnott-Smith J, Van Lint J, Rozengurt E (1996) Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J.* **15**:6220-6230.
- Zugaza JL, Waldron RT, Sinnott-Smith J, Rozengurt E (1997) Bombesin, vasopressin, endothelin, bradykinin, and platelet-derived growth factor rapidly activate protein kinase D through a protein kinase C-dependent signal transduction pathway. *J. Biol. Chem.* **272**:23952-23960.

AUTHOR'S PUBLICATIONS

Publication based on the presented work:

Czöndör K., Ellwanger K, Fuchs YF, Lutz S, Gulyás M, Mansuy IM, Hausser A, Pfizenmaier K, Schlett K. (2009) Protein kinase D controls the integrity of Golgi apparatus and the maintenance of dendritic arborization in hippocampal neurons, Mol Biol Cell. Apr;20(7):2108-20. if: 6.028

see attached reprint

Publications independent from the presented work:

Tárnok K., Czirók A., **Czöndör K.**, Schlett K. (2005) Cerebellar granule cells show age-dependent migratory differences in vitro, J Neurobiology, Nov;65(2):135-45. if: 4.17

Tárnok K., **Czöndör K.**, Jelitai M., Czirók A., Schlett K. (2008) NMDA receptor NR2B subunit over-expression increases cerebellar granule cell migratory activity, J Neurochem. Feb;104(3):818-29 if: 4.451