

Functional redundancy of protein kinase D1 and protein kinase D2 in neuronal polarity



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ABSTRACT

Mammalian protein kinase D (PKD) isoforms have been proposed to regulate diverse biological processes, including the establishment and maintenance of neuronal polarity. To investigate the function of PKD in neuronal polarization *in vivo*, we generated PKD knockout (KO) mice. Here, we show that the brain, particularly the hippocampus, of both PKD1 KO and PKD2 KO mice was similar to that of control animals. Neurite length in cultured PKD1 KO and PKD2 KO hippocampal neurons was similar to that of wild-type neurons. However, hippocampal neurons deficient in both PKD1 and PKD2 genes showed a reduction in axonal elongation and an increase in the percentage of neurons with multiple axons relative to control neurons. These results reveal that whereas PKD1 and PKD2 are essential for neuronal polarity, there exists a functional redundancy between the two proteins.

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1. Introduction

Neuronal polarity is established early in development as neurons differentiate and extend processes (Craig and Banker, 1994; Horton and Ehlers, 2003; Arimura and Kaibuchi, 2007; Li and Wang, 2014). However, the molecular mechanisms that underlie the development and specification of axons and dendrites—a process known as neuronal polarization—are still unclear. The Golgi apparatus plays a crucial role in neuronal polarization (Arimura and Kaibuchi, 2007), as several of its constitutive molecules have been known to be involved in this process. Belonging to this group of proteins, protein kinase Ds (PKDs) localize on the Golgi and are known to be involved in both the establishment and maintenance of neuronal polarity (Yin et al., 2008; Bisbal et al., 2008).

PKD is a member of the serine/threonine protein kinase family. The mammalian PKD comprises three different but closely related

isoforms: PKD1, PKD2, and PKD3. All have a highly conserved N-terminal regulatory domain consisting of two cysteine-rich DAG (diacylglycerol)-binding domains and an auto-inhibitory PH (pleckstrin homology) domain. Half of the C-terminus comprises the catalytic kinase domain, which consists of an activation loop (Baron and Malhotra, 2002; Fu and Rubin, 2011).

Previous reports have shown that PKDs are expressed in the brain, where they are involved in many cellular processes, including regulation of membrane trafficking in dendrites and establishment and maintenance of neuronal polarity (Bisbal et al., 2008; Yin et al., 2008).

Here we examined the brains of previously generated PKD1 knockout (KO) and PKD2 KO mice. The brains—specifically, the hippocampi—of both KO strains were similar to control animals in the analyses we performed. Neurite lengths of cultured PKD1 KO and PKD2 KO hippocampal neurons were similar to those of wild-type neurons. However, when we generated hippocampal neurons deficient in both PKD1 and PKD2 genes using Cre recombinase, we found that their axonal elongation was inhibited and the percentage of neurons with multiple axons was increased relative to control neurons. Taken together, our results suggest that PKD1 and PKD2 are essential for neuronal polarity, though there exists a functional redundancy between them.

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2. Materials and methods

2.1. Animals

All experimental protocols were conducted according to the guidelines of Animal Care and Experimentation Committee of Gunma University and Osaka University. Animals were bred at the Institute of Animal Experience Research of Gunma University and Osaka University. Animals were housed under 12:12-h light–dark cycle with controlled humidity and temperature and free access to food pellets and tap water. PKD1 KO (PKD1^{geo/geo}), PKD2 KO (PKD2^{geo/geo}) and PKD1^{flox/flox}; PKD2^{flox/flox} mice were generated as reported previously (Atik et al., 2014).

2.2. Histological and western blot analysis

Eight- to nine-week-old mice were used for histology and immunofluorescence microscopy. They were fixed by perfusion with 3% (w/v) paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) and processed as previously described (Sato et al., 2011; Atik et al., 2014). We performed hematoxylin–eosin (HE) staining and immunofluorescence staining according to previous methods (Sato et al., 2011; Atik et al., 2014). A laser scanning confocal microscope (FV-1000D, Olympus, Tokyo, Japan) was used to analyze the samples. The images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Western blot analysis was performed as previously described (Sato et al., 2011; Atik et al., 2014) using 25 µg of protein loaded per lane. The following primary antibodies and a reagent were used; anti-PKD1 (Sigma, St. Louis, MO, USA), anti-PKD1 (C20: Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PKD3 (Cell Signaling Technology, Danvers, MA, USA), anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tau (Tau1: Chemicon, Temecula, CA, USA), anti-GS28 (BD Biosciences, San Jose, CA, USA), anti-MAP1A (Harada et al., 1994), anti-GFP (Atik et al., 2014), and rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

2.3. Primary culture of hippocampal neurons

Hippocampal neurons were obtained by dissecting PKD1^{+/+} or PKD1^{geo/geo} embryos generated from PKD1^{geo/+} intercrosses. PKD2^{+/+} or PKD2^{geo/geo} embryos were obtained by PKD2^{geo/+} intercrosses. PKD1^{flox/+} and PKD2^{flox/+} were obtained by mating PKD1^{geo/+} mice and PKD2^{geo/+} mice with FLPe transgenic mice (Jackson Laboratories, Bar Harbor, ME, USA), respectively.

Neurons were dissociated and cultured as previously described (Sato et al., 2011). In brief, the hippocampi of embryonic day 17.5 (E17.5) mice were cut into pieces and incubated for 20 min at 37 °C in PBS containing 1% BSA (Sigma, St. Louis, MO, USA), DNase (Sigma, St. Louis, MO, USA), papain (Sigma, St. Louis, MO, USA), and glucose (Wako, Osaka, Japan). The supernatants were discarded, and the same solution without papain was added to the pellets. The resulting tissue fragments were gently triturated with a pipette and incubated for 15 min at 37 °C. After centrifugation, dissociated cells were plated at a density of 1×10^5 per ml on a 35-mm dish that had been pretreated with poly-D-lysine and laminin (BD Biosciences, San Diego, CA, USA). The cells were cultured in Neurobasal medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with B27 and L-alanyl-L-glutamine (Wako, Osaka, Japan) at 37 °C in a humidified 5% CO₂/95% air atmosphere.

Expression of Cre recombinase was achieved by infection with a recombinant adenovirus encoding Cre recombinase (Ad-Cre) (Takara, Otsu, Shiga, Japan). PKD1^{flox/flox}; PKD2^{flox/flox} hippocampal neurons were infected with Ad-Cre 3 h after plating to deplete PKD1 and PKD2. We infected a recombinant adenovirus encoding

LacZ (Ad-LacZ) to PKD1^{flox/flox}; PKD2^{flox/flox} hippocampal neurons and used them as control neurons. After incubation with Ad-Cre or Ad-LacZ for 48 h, the culture medium was changed to the virus-free medium. Neurons were either lysed for western blot analysis or fixed and stained for measurement of axonal length at 48 and 72 h after addition of adenovirus.

Transfection of PKD1-EGFP or PKD2-EGFP cDNA and PKD1 or PKD2 siRNA were achieved by electroporation using mouse neuron nucleofector kit (Lonza, Cologne, Germany) according to the manufacturer's instructions.

2.4. RNA interference

The sequences of PKD1, PKD2 and control short interfering RNA (siRNA) were the same as previously described (Yin et al., 2008). For knockdown experiments, siRNAs targeting PKD1 and PKD2 (Sigma, St. Louis, MO, USA) were electroporated into 5×10^6 hippocampal neuron cells.

2.5. Measurement of axonal length and analysis of neuronal morphology

Hippocampal neurons were fixed at 24, 48, and 72 h after plating with 3.7% formaldehyde in PBS for 1 h at 37 °C, washed with PBS, and permeabilized as previously described (Mori et al., 2007). They were then stained with the aforementioned antibodies. To measure the length of axons, areas of 600 µm × 600 µm from culture dishes were imaged at 20× magnification using a confocal microscope. The lengths of axons in randomized areas were measured using ImageJ (NIH, Bethesda, MD, USA). A neurite was considered to be an axon if either the length of the neurite was both more than twice as long as any other process and more than twice the diameter of the cell body (Yin et al., 2008) or if it stained positively for the axonal marker Tau1. Stages 1, 2, and 3 were determined as previously described (Tahirovic and Bradke, 2009). The data represent the averages ± SD (the number each of control, PKD1 KO, PKD2 KO, and PKD1,2 double knockout (DKO) neurons was over 300 for each. These neurons were taken from 3 independent hippocampi).

2.6. Plasmids

A full-length cDNA of PKD1 or PKD2 were amplified by PCR and cloned into pCDNA5/FRT/to EGFP (Addgene, Cambridge MA, USA) to create a PKD1- and PKD2-EGFP tagged plasmids.

2.7. Statistical analysis

Significance was determined by Student's *t*-tests or one-way analysis of variance (ANOVA) followed by a multiple comparison test when samples are more than 2 groups. Results are presented as mean ± SD and statistically significant differences are defined as a *p* value less than 0.05.

3. Results

3.1. Expression of PKD1 and PKD2 in brain and hippocampal neurons

Our previous work showed that PKD1 and PKD2 were expressed in various tissues, including the brain (Atik et al., 2014). As in other tissues shown (Atik et al., 2014), PKD1 and PKD2 were absent in the brains of PKD1^{geo/geo} (PKD1 KO) mice and PKD2^{geo/geo} (PKD2 KO) mice, respectively (Fig. 1A). PKD1 was much more abundantly expressed than PKD2 both in the brain and hippocampal neurons

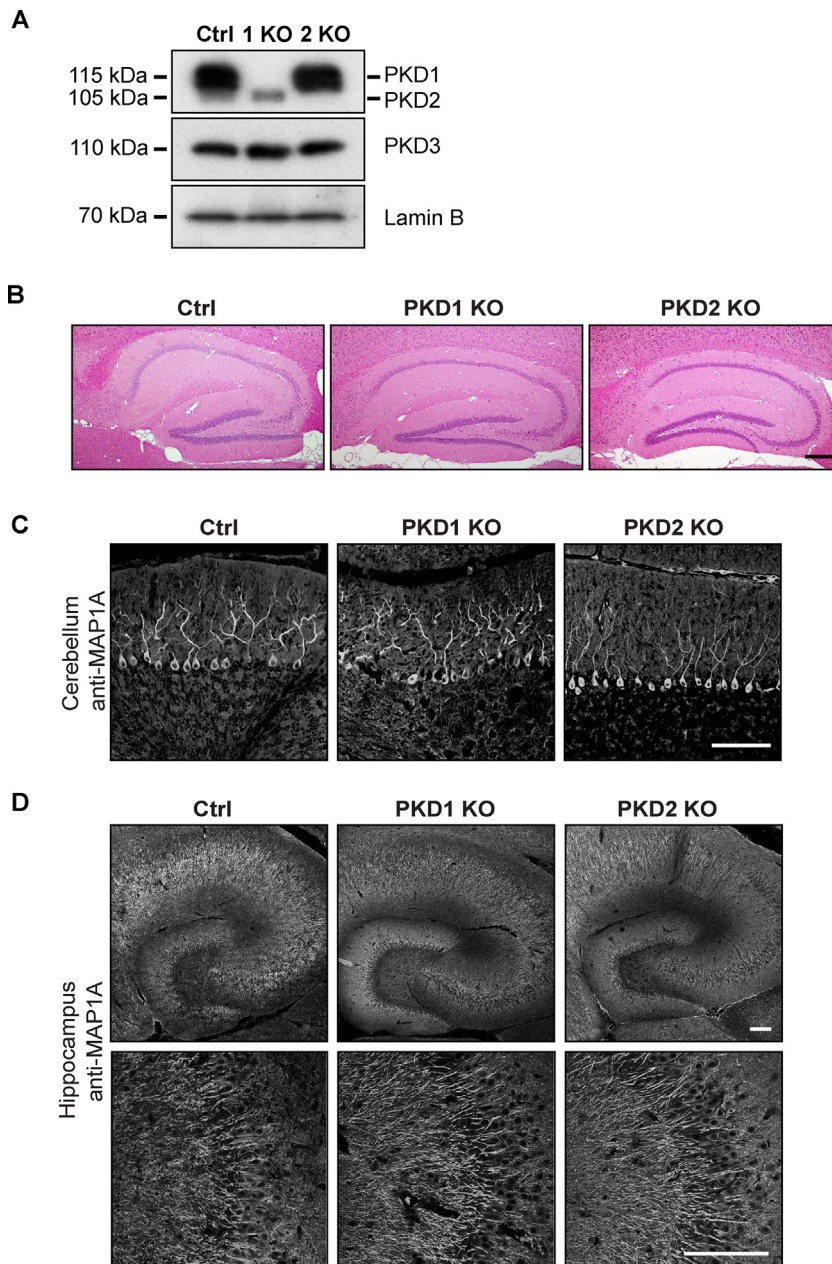


Fig. 1. PKDs protein expression and morphological analysis of the central nervous system of PKD1 KO and PKD2 KO mice. (A) Western blot analysis of PKD1 and PKD2 in the brain using anti-PKD1 antibody (Sigma) that detects both isoforms. In PKD1 KO sample ('1KO') and PKD2 KO sample ('2KO'), PKD1 and PKD2 proteins were not detected, respectively (top panel). There is no difference in the expression of PKD3 among PKD1, PKD2, and control ('Ctrl') samples (middle panel). Lamin B was used as a loading control (bottom panel). (B) HE-stained paraffin sections of hippocampus from control, PKD1 KO, and PKD2 KO mice. Bar, 200 μ m. (C and D) Immunofluorescence micrographs of the cerebellum (C) and the hippocampus (D). Whole hippocampus (upper panels) and the CA3 region of the hippocampus (lower panels) were stained with anti-MAP1A antibody. Bars, 100 μ m.

(Fig. 1A and 4B). We did not observe a difference in the amount of PKD3 in the brains of PKD1 KO, PKD2 KO, and control mice (Fig. 1A).

3.2. Architecture of the brain and neuronal polarity are not disturbed in either PKD1 KO or PKD2 KO mice

Previous studies reported that neurite outgrowth was impaired in cultured hippocampal neurons in which PKD1 or PKD2 was knocked down either by RNAi or PKD-specific inhibitors (Bisbal et al., 2008; Yin et al., 2008).

To assess structural deficits in the central nervous system of KO mice, we examined tissue sections of the hippocampus using light microscopy (Fig. 1B). The gross structures of the hippocampus

of PKD1 KO or PKD2 KO mice were indistinguishable from those of control mice as visualized by HE staining. Furthermore, when we stained frozen sections of the cerebellum and hippocampus for MAP1A, dendrites of cerebellar Purkinje and hippocampal pyramidal cells were similar among PKD1 KO, PKD2 KO, and control mice (Fig. 1C and D).

To further examine the respective roles of PKD1 and PKD2 in axonal outgrowth, we cultured control and KO hippocampal neurons and measured axonal length at 24, 48, and 72 h after plating (Figs. 2 and 3). For quantitative analysis, we first measured the fraction of neurons at stage 3 of development, which corresponds to the stage of axonal elongation (Dotti et al., 1988; Bisbal et al., 2008; Tahirovic and Bradke, 2009; Sato et al., 2011). The proportion of

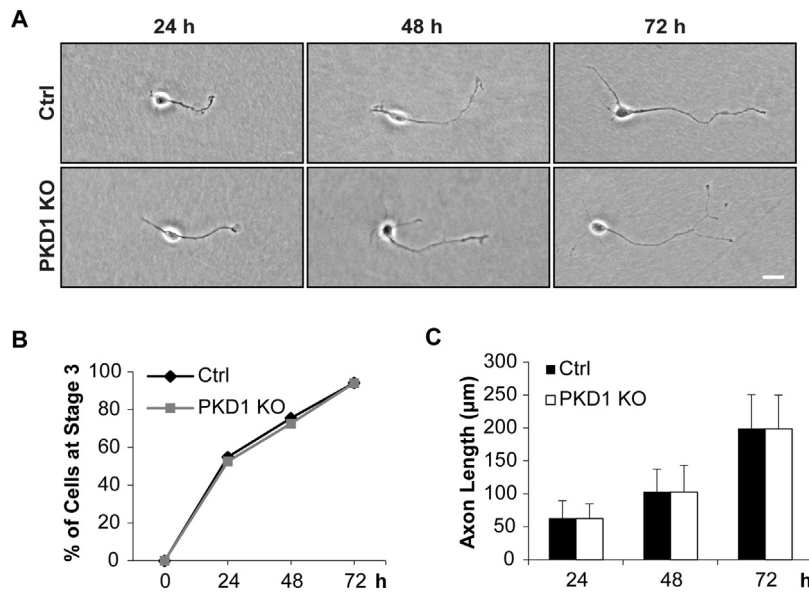


Fig. 2. Neuronal architecture and axonal elongation in PKD1 KO mice. (A) Micrographs of cultured hippocampal neurons at 24, 48, and 72 h after plating to show axonal elongation both in control (Ctrl) and PKD1 KO neurons. (B) The percentage of cultured hippocampal neurons at stage 3 derived from control and PKD1 KO mice. (C) Measurements of axonal lengths of cultured hippocampal neurons at 24, 48 and 72 h after plating, taken from control and PKD1 KO mice. The data represent the averages \pm SD. The data were collected from more than 300 neurons derived from three independent embryonic hippocampi. Bar, 20 μ m.

PKD1 KO or PKD2 KO neurons at stage 3 increased similarly to that of control neurons at all time points examined (Figs. 2B and 3B). To analyze the axonal elongation more precisely, we measured the lengths of axons from stage 3 neurons at 24, 48, and 72 h after plating. We identified that axonal length in both PKD1 KO and PKD2 KO neurons was not significantly different from that in control neurons (Figs. 2C and 3C). In the previous study, the percentage of neurons with multiple axons was increased in both PKD1 knockdown and PKD2 knockdown neurons (Yin et al., 2008). However, we found the number of neurons with multiple axons in PKD1 KO and PKD2 KO similar to that of control neurons (data not shown).

These results suggest that neither neuronal polarization nor axonal elongation is affected in either PKD1 KO or PKD2 KO neurons.

3.3. Depletion of both PKD1 and PKD2 in combination affects neuronal polarity and axonal elongation in hippocampal neurons

Due to the functional redundancy amongst PKD isoforms, no difference in neuronal polarity was detected in either PKD1 KO or PKD2 KO neurons. However, as our previous paper showed that PKD1,2 DKO (PKD1^{geo/geo}; PKD2^{geo/geo}) mice died between

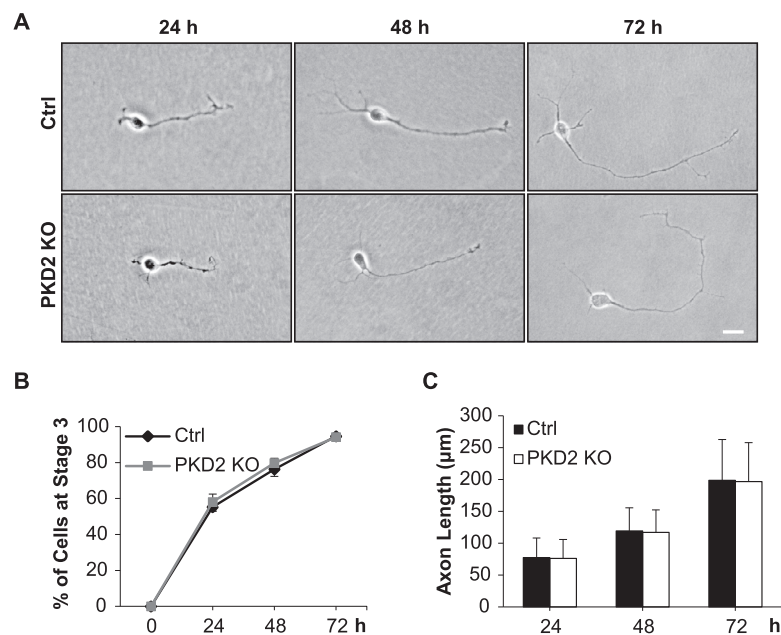


Fig. 3. Neuronal architecture and axonal elongation in PKD2 KO mice. (A) Micrographs of cultured hippocampal neurons at 24, 48, and 72 h after plating to show axonal elongation both in control (Ctrl) and PKD2 KO neurons. (B) The percentage of cultured hippocampal neurons at stage 3 derived from control and PKD2 KO mice. (C) Measurements of axonal lengths of cultured hippocampal neurons at 24, 48 and 72 h after plating, taken from control and PKD2 KO mice. The data represent the averages \pm SD. The data were collected from more than 300 neurons derived from three independent embryonic hippocampi. Bar, 20 μ m.

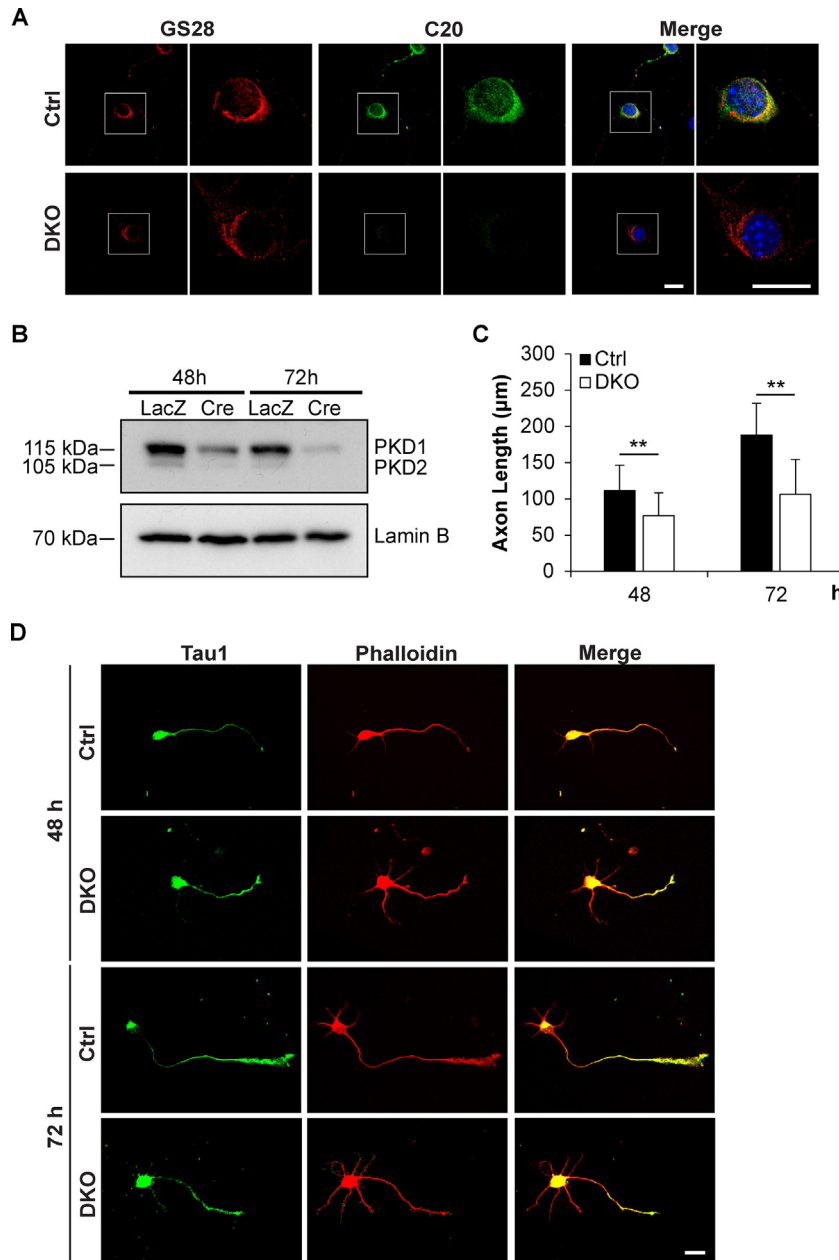


Fig. 4. Depletion of PKD1 and PKD2 in hippocampal neurons resulted in defects in axonal elongation. (A) PKD1^{fllox/fllox}; PKD2^{fllox/fllox} hippocampal neurons were infected with Ad-Cre to generate PKD1,2 DKO (DKO) neurons. Infection with Ad-LacZ served as a control (Ctrl). Neurons were then fixed and stained 72 h after adenovirus infection. The Golgi, nucleus, and PKDs were stained with anti-GS28 antibody (red), DAPI (blue), and anti-PKD1 antibody (C20) which could detect both PKD1 and PKD2 (green), respectively. The inset is a picture at higher magnification. (B) Lysates from neurons 48 and 72 h after the addition of adenovirus were analyzed via western blot with an anti-PKD1 antibody (Sigma) that detected both PKD1 and PKD2. (C) Measurements of axonal lengths in cultured hippocampal neurons 48 and 72 h after the addition of adenovirus in control and PKD1,2 DKO neurons. The data represent the averages \pm SD. ** $p < 0.001$. The data were collected from more than 300 neurons derived from three independent embryonic hippocampi. (D) Immunofluorescence of cultured hippocampal neurons 48 and 72 h after the addition of adenovirus, showing axonal elongation in control (Ctrl) and PKD1,2 DKO neurons. Rhodamine-phalloidin (red) and anti-tau (Tau1) (green) were used as neurite and axonal markers, respectively. Bars, 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

8.5 dpc and 10.5 dpc, we were not able to derive neurons from DKO mice (Atik et al., 2014). Thus, to examine the function of PKD1 and PKD2 in neuronal polarity, we generated PKD1 and PKD2 double floxed (PKD1^{fllox/fllox}; PKD2^{fllox/fllox}) mice, collected their hippocampal neurons, and treated them with Ad-Cre to obtain DKO neurons. After treatment with adenovirus to drive the expression of Cre, expression of both PKD1 and PKD2 were dramatically decreased by immunofluorescence and WB analysis (Fig. 4A and B). At 48 and 72 h after the addition of adenovirus, we stained the axons of hippocampal neurons and measured their length. We found that the axonal length of DKO neurons was

significantly shorter than that of Ad-LacZ treated control neurons (48 h: control, $111.6 \pm 34.7 \mu\text{m}$; DKO, $76.9 \pm 31.7 \mu\text{m}$ and 72 h: control, $188.2 \pm 43.7 \mu\text{m}$; DKO, $106.3 \pm 47.8 \mu\text{m}$; three independent experiments; $p < 0.001$) (Fig. 4C and D). The percentage of stages 1 and 2 was similar between DKO and control neurons, however the percentage of stage 3 was significantly reduced in DKO neurons compared to control neurons (48 h: control, $64.3 \pm 6.2\%$; DKO, $42.2 \pm 8.9\%$ and 72 h: control, $84.1 \pm 2.1\%$; DKO, $52.1 \pm 5.6\%$; three independent experiments; $p < 0.05$) (Fig. 5A and B).

Interestingly, the percentage of DKO neurons with multiple axons was increased relative to control neurons (48 h: control,

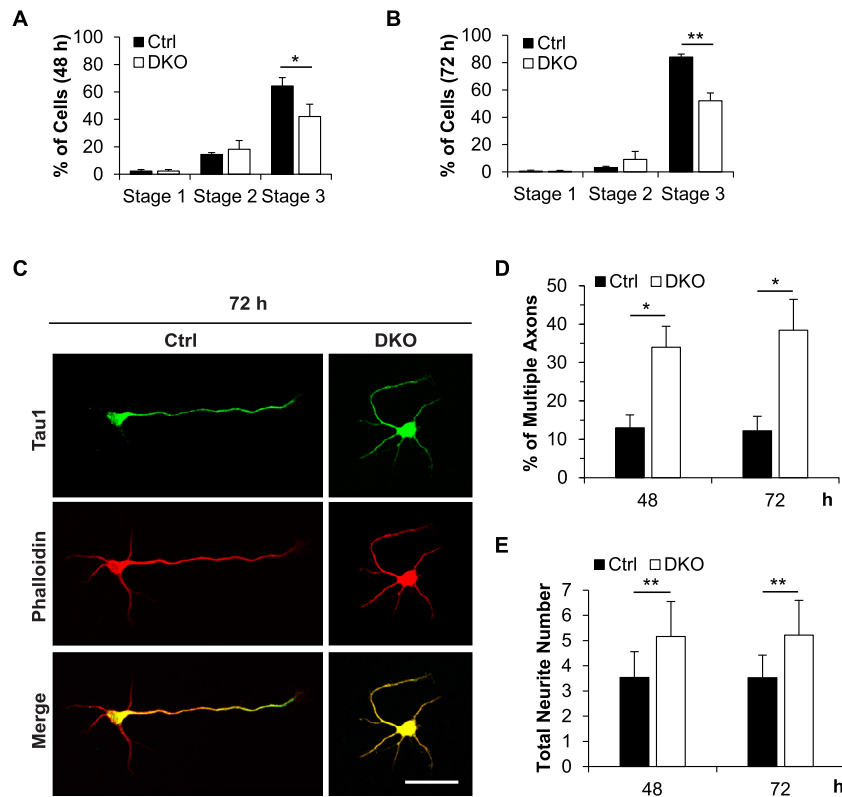


Fig. 5. Depletion of PKD1 and PKD2 in hippocampal neurons induced formation of multiple axons. (A and B) Quantification of the number of stages 1, 2, or 3 neurons 48 (A) and 72 h (B) after adenovirus infection in control and PKD1,2 DKO neurons. (C) A PKD1,2 DKO neuron developed multiple axons, stained with rhodamine-phalloidin (red) and anti-tau (green). (D and E) Percentage of neurons with multiple axons (D) and total number of neurites (E) in cultured hippocampal neurons 48 and 72 h after the addition of adenovirus in control and PKD1,2 DKO neurons. The data represent the averages \pm SD. * $p < 0.05$, ** $p < 0.001$. The data were collected from more than 300 neurons derived from three independent embryonic hippocampi. Bar, 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

13.0 \pm 3.3%; DKO, 34.0 \pm 5.5% and 72 h: control, 12.2 \pm 3.7%; DKO, 38.4 \pm 8.0%; three independent experiments; $p < 0.05$) (Fig. 5C and D). In addition, the total number of neurites per neuron was increased in DKO neurons relative to control neurons (48 h: control, 3.5 \pm 1.0; DKO, 5.2 \pm 1.4 and 72 h: control, 3.5 \pm 0.9; DKO, 5.2 \pm 1.4; three independent experiments; $p < 0.001$) (Fig. 5E).

Next, we used PKD1 and PKD2 siRNA to generate PKD1 and PKD2 double knockdown (DKD) for confirming the results of DKO neurons. At 48 and 72 h after electroporation of control or PKD1 together with PKD2 siRNA, the hippocampal neurons were stained and observed. Indeed, we found similar abnormalities between DKO and DKD. The percentage of neurons at stages 1 and 2 was similar between DKD and control neurons (Fig. 6A and B). Nevertheless, percentage of stage 3 was significantly reduced in DKD neurons compared to control neurons (48 h: control, 61.6 \pm 2.8%; DKD, 45.6 \pm 5.1% and 72 h: control, 69.0 \pm 5.7%; DKD, 57.8 \pm 2.5%; three independent experiments; $p < 0.05$) (Fig. 6A and B). The percentage of DKD neurons which have multiple axons was increased to that of control neurons (48 h: control, 15.0 \pm 5.8%; DKD, 28.7 \pm 6.5% and 72 h: control, 21.3 \pm 4.1%; DKD, 32.6 \pm 3.6%; three independent experiments; $p < 0.05$) (Fig. 6A and B).

Axonal length of DKD neurons was significantly reduced relative to that of control neurons (48 h: control, 95.2 \pm 40.3 μ m; DKD, 76.4 \pm 31.2 μ m and 72 h: control, 169.5 \pm 65.4 μ m; DKD, 115.9 \pm 41.8 μ m; three independent experiments; $p < 0.001$) (Fig. 6C and E). We also found that the total number of neurites per neuron was increased in DKD neurons relative to that of control (48 h: control, 3.0 \pm 1.0; DKD, 4.2 \pm 1.2 and 72 h: control, 3.0 \pm 0.9; DKD, 4.5 \pm 1.3; three independent experiments; $p < 0.001$) (Fig. 6D).

Our findings suggest that PKD1 and PKD2 compensate for one another and are necessary for neuronal polarization.

3.4. Expression of PKD1-EGFP or PKD2-EGFP can rescue the defects in axonal elongation and neuronal polarization in DKO hippocampal neurons

The above data showed that the defects of neuronal polarity and axonal elongation appeared when we depleted both PKD1 and PKD2 in combination but not in PKD1 KO or PKD2 KO alone. Next, we tested whether we can rescue the defects in DKO neurons by PKD1 or PKD2 cDNA.

Expression of PKD1-EGFP or PKD2-EGFP into PKD1 and PKD2 double floxed (PKD1^{lox/lox}; PKD2^{lox/lox}) hippocampal neurons were achieved by electroporation prior to Ad-Cre infection (respectively referred to DKO + PKD1 or DKO + PKD2). Neurons were plated after electroporation procedure, then treated with Ad-Cre or Ad-LacZ. Again, at 48 and 72 h after adenovirus infection, we stained the hippocampal neurons and observed their developmental stages, axonal length, and the number of neurites and axons per neuron (Fig. 7A).

The percentage of neurons at stage 3 was significantly increased in DKO + PKD1 or DKO + PKD2 neurons compared to DKO neurons (48 h: DKO, 48.9 \pm 5.9%; DKO + PKD1, 60.3 \pm 5.1%; DKO + PKD2, 63.5 \pm 5.2%; control, 63.3 \pm 1.7%, and 72 h: DKO, 56.2 \pm 4.9%; DKO + PKD1, 70.0 \pm 6.6%; DKO + PKD2, 69.1 \pm 3.7%; control, 68.1 \pm 5.3%; three independent experiments; $p < 0.05$) (Fig. 7B and C). The percentage of DKO + PKD1 or DKO + PKD2 neurons with multiple axons was decreased relative to DKO neurons (48 h: DKO, 26.6 \pm 6.6%; DKO + PKD1, 14.8 \pm 3.0%; DKO + PKD2, 12.6 \pm 3.8%;

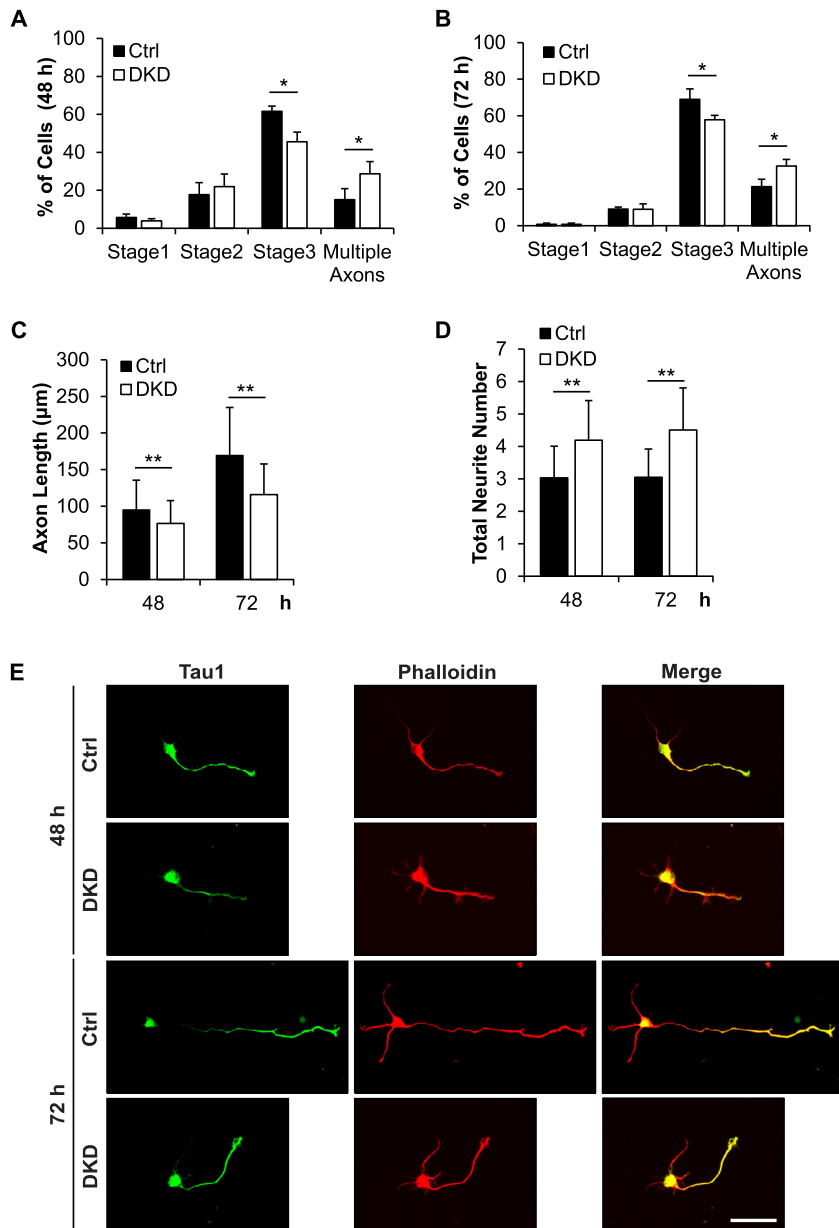


Fig. 6. Knockdown of PKD1 and PKD2 in hippocampal neurons using siRNA showed similar phenotypes to PKD1,2 DKO hippocampal neurons. (A and B) Hippocampal neurons were transfected with control siRNA (Ctrl) or PKD1 and PKD2 siRNA as PKD1,2 double knockdown (DKD) neurons. The percentage of the number of neurons at stages 1, 2, 3, and the one of neurons with multiple axons 48 (A) and 72 h (B) after transfection in control and PKD1,2 DKD neurons. (C) Measurement of axonal lengths in cultured hippocampal neurons 48 and 72 h after siRNA treatment. (D) The average of total neurite number per neuron of cultured hippocampal neurons at 48 and 72 h after siRNA treatment. (E) Immunofluorescence of control and PKD1,2 DKD neurons at 48 and 72 h after the transfection. Rhodamine-phalloidin (red) and anti-tau (Tau1) (green) were used as neurite and axonal markers, respectively. The data represent the averages \pm SD. * $p < 0.05$, ** $p < 0.001$. The data were collected from more than 300 neurons derived from three independent embryonic hippocampi. Bar, 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

control, $9.8 \pm 3.7\%$, and 72 h: DKO, $23.3 \pm 0.8\%$; DKO+PKD1, $10.7 \pm 4.5\%$; DKO+PKD2, $14.0 \pm 2.4\%$; control, $12.4 \pm 3.3\%$; three independent experiments; $p < 0.05$) (Fig. 7B and C).

The expression of PKD1 or PKD2 in DKO neuron also rescued the defects in axonal elongation (48 h: DKO, $78.8 \pm 27.7 \mu$ m; DKO+PKD1, $93.1 \pm 33.4 \mu$ m; DKO+PKD2, $90.6 \pm 37.9 \mu$ m and 72 h: DKO, $117.4 \pm 39.6 \mu$ m; DKO+PKD1, $151.4 \pm 60.2 \mu$ m; DKO+PKD2, $137.2 \pm 54.8 \mu$ m; three independent experiments; $p < 0.001$). However, axons of DKO+PKD1 or DKO+PKD2 were still shorter than that of control neurons (48 h: control, $107.0 \pm 42.5 \mu$ m and 72 h: control, $172.2 \pm 50.9 \mu$ m; three independent experiments; $p < 0.001$) (Fig. 7D). Finally, we found that the total

number of neurites per neuron was decreased in DKO+PKD1 or DKO+PKD2 neurons compared to DKO neurons (48 h: DKO, 4.2 ± 1.6 ; DKO+PKD1, 3.3 ± 1.0 ; DKO+PKD2, 3.2 ± 0.9 ; control, 3.3 ± 1.0 and 72 h: DKO, 4.3 ± 1.3 ; DKO+PKD1, 3.2 ± 0.9 ; DKO+PKD2, 3.3 ± 0.9 ; control 3.1 ± 1.2 ; three independent experiments; $p < 0.001$) (Fig. 7E).

Taken together, we suggest that PKD1 or PKD2 expression can rescue the defects in the axonal elongation and neuronal polarization in DKO hippocampal neurons. These observations are consistent with the idea that PKD1 and PKD2 compensate for one another and are necessary for neuronal polarization process.

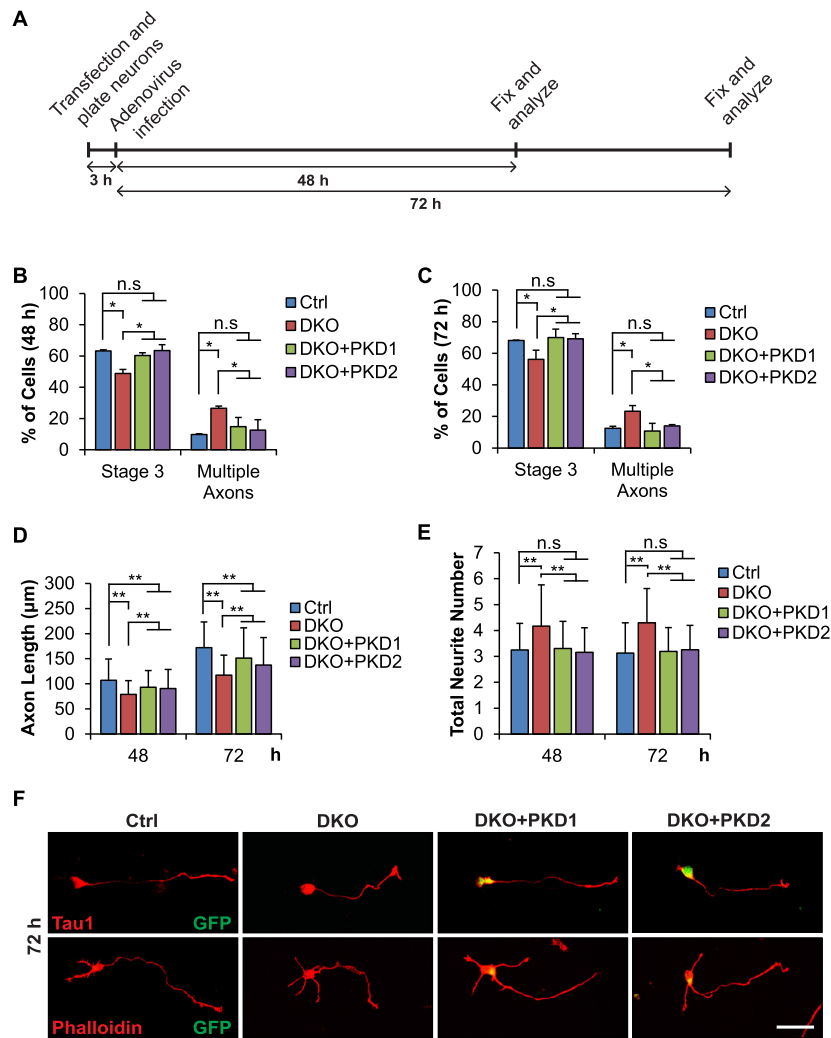


Fig. 7. Expression of PKD1-EGFP or PKD2-EGFP can rescue the defects in axonal elongation and neuronal polarization in PKD1,2 DKO hippocampal neurons. (A) Schematic representation of the rescue experiment. (B and C) Proportion of neurons at stage 3 or neurons with multiple axons at 48 (B) and 72 h (C) after Ad-Cre treatment and PKD1-EGFP transfection (DKO + PKD1), Ad-Cre treatment and PKD2-EGFP (DKO + PKD2) transfection, Ad-Cre treatment only (DKO), and Ad-LacZ infection (Ctrl). (D and E) Measurements of axonal lengths (D) and quantification of total neurite number per neuron (E) in control, DKO, DKO + PKD1, and DKO + PKD2 neurons at 48 and 72 h after adenovirus infection. (F) Control, DKO, DKO + PKD1, and DKO + PKD2 neurons at 72 h after adenovirus infection. Anti-tau (Tau1) (red) in the upper panels, rhodamine-phalloidin (red) in the lower panels, and GFP (green) in both panels were used as markers for axons, neurites, and PKD1 or PKD2 transfected cells, respectively. The data represent the averages \pm SD. * $p < 0.05$, ** $p < 0.001$, one-way analysis of variance (ANOVA). The data were collected from more than 300 neurons derived from three independent embryonic hippocampi. Bar, 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

Neuronal cells become polarized and establish two structurally and functionally distinct compartments known as axons and dendrites. PKD1 and PKD2 have been reported to be involved in this process (Bisbal et al., 2008; Yin et al., 2008). This previous work suggested that neuronal polarity was disturbed when PKD1 or PKD2 was knocked down in hippocampal neurons *via* siRNA. The authors proposed that PKD1 and PKD2 may need to be dimerized to regulate polarized membrane trafficking in neurons (Yin et al., 2008). However, we showed that our individual knockout mice of PKD1 and PKD2 exhibited no gross abnormalities in the hippocampus or cerebellum (Fig. 1). In addition, axonal outgrowth of cultured PKD1 KO and PKD2 KO hippocampal neurons was similar to that of normal neurons (Figs. 2 and 3). These data enable us to postulate that PKD1 and PKD2 have functional redundancy in establishing neuronal polarity.

We examined the function of PKD1 and PKD2 in neuronal polarity by depleting both PKD1 and PKD2 and showed that there were defects in neuronal polarity and axonal elongation (Figs. 4–6).

Axonal elongation defects could be rescued by expressing either PKD1 or PKD2 (Fig. 7).

Our findings of unaltered polarity in PKD1 KO neurons vary from previous work (Yin et al., 2008). This discrepancy may be caused by the difference between acute siRNA-mediated depletion of PKD1 or PKD2 and chronic depletion induced by gene targeting. Alternatively, we have not excluded the possibility that the defects in PKD1 knockdown neurons resulted from off-target effects. In the case of PKD2, as the amount of PKD2 was very low in hippocampal neurons (Bisbal et al., 2008), the authors concluded that the role of PKD2 in neuronal polarity did not seem to be significant. Based on our current findings, the role of PKD1 and PKD2 in neuronal polarization should be re-examined.

To further elucidate the molecular mechanism by which PKD regulates neuronal polarity, it is important to determine what molecules or substrates of PKD are involved in axon formation. It has been reported that Kidins220/ARMS (kinase D-interacting substrate of 220 kDa/ankyrin repeat-rich membrane spanning) is a substrate of PKD1 and PKD2 and is involved in regulating neuronal polarity (Iglesias et al., 2000; Higuero et al., 2010). Kidins220/ARMS

is an integral membrane protein associated with lipid rafts, concentrated at the tip of extending neurites, and abundant in the developing nervous system and adult brain (Iglesias et al., 2000; Sánchez-Ruiloba et al., 2006; Higuero et al., 2010). A previous report (Sánchez-Ruiloba et al., 2006) indicated the importance of PKD1 and PKD2 in the localization of Kidins220 by the following reasons. First, transportation of Kidins220 from the TGN to the plasma membrane was shown to require the autophosphorylation of PKD at Ser916. Second, expression of kinase-inactive PKD in neuronal cells alters the localization of Kidins220/ARMS from the plasma membrane to intracellular vesicle clusters. Furthermore, a more recent study (Higuero et al., 2010) reported that shRNA-mediated knock-down of Kidins220 in hippocampal neurons induced the formation of multiple axon-like processes, similar to our reported phenotype in PKD1 and PKD2 DKO neurons. Taken together, in the absence of PKD1 and PKD2, Kidins220/ARMS transport to the plasma membrane may be disturbed, which may then lead to neuronal polarity defects. As there are a variety of other substrates of PKDs (Par1, etc.), we should further assess which substrates are the most important for the role of PKDs in the establishment of neuronal polarity. At the same time, to clarify the significance of PKDs in neuronal polarity *in vivo*, analysis of neuron-specific DKO mice should be performed as well.

5. Conclusion

We report here that the morphology in various brain regions in PKD1 KO and PKD2 KO mice was similar to that of control mice. Axonal length in PKD1 KO or PKD2 KO hippocampal neurons was similar to that in wild-type neurons. However, when we deleted both PKD1 and PKD2 in hippocampal neurons, axonal length was diminished, the number of neurons with multiple axons was increased, and the number of neurites per neuron was increased. This supports the importance of PKD1 and PKD2 in the establishment of axonal elongation and suggests functional redundancy between PKD1 and PKD2 with respect to neuronal polarity *in vivo* and *in vitro*.

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References

- Arimura, N., Kaibuchi, K., 2007. Neuronal polarity: from extracellular signals to intracellular mechanisms. *Nat. Rev. Neurosci.* 8, 194–205.
- Atik, N., Kunii, M., Avriyanti, E., Furumoto, N., Inami, K., Yoshimura, S., Harada, R., Harada, A., 2014. The role of PKD in cell polarity, biosynthetic pathways, and organelle/F-actin distribution. *Cell Struct. Funct.* 39, 61–77.
- Baron, C.L., Malhotra, V., 2002. Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. *Science* 295, 325–328.
- Bisbal, M., Conde, C., Donoso, M., Bollati, F., Sesma, J., Quiroga, S., Díaz Añel, A., Malhotra, V., Marzolo, M.P., Cáceres, A., 2008. Protein kinase D regulates trafficking of dendritic membrane proteins in developing neurons. *J. Neurosci.* 28, 9297–9308.
- Craig, A.M., Banker, G., 1994. Neuronal polarity. *Annu. Rev. Neurosci.* 17, 267–310.
- Dotti, C.G., Sullivan, C.A., Banker, G.A., 1988. The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* 8, 1454–1468.
- Fu, Y., Rubin, C.S., 2011. Protein kinase D: coupling extracellular stimuli to the regulation of cell physiology. *EMBO Rep.* 12, 785–796.
- Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Takei, Y., Noda, T., Hirokawa, N., 1994. Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature* 369, 488–491.
- Higuero, A.M., Sánchez-Ruiloba, L., Doglio, L.E., Portillo, F., Abad-Rodríguez, J., Dotti, C.G., Iglesias, T., 2010. Kidins220/ARMS modulates the activity of microtubule-regulating proteins and controls neuronal polarity and development. *J. Biol. Chem.* 285, 1343–1357.
- Horton, A.C., Ehlers, M.D., 2003. Neuronal polarity and trafficking. *Neuron* 40, 277–295.
- Iglesias, T., Cabrera-Poch, N., Mitchell, M.P., Naven, T.J., Rozengurt, E., Schiavo, G., 2000. Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. *J. Biol. Chem.* 275, 40048–40056.
- Li, G., Wang, Y., 2014. Protein kinase D: a new player among the signaling proteins regulate functions in the nervous system. *Neurosci. Bull.* 30, 497–504.
- Mori, T., Wada, T., Suzuki, T., Kubota, Y., Inagaki, N., 2007. Singar 1, a novel RUN domain-containing protein, suppresses formation of surplus axons for neuronal polarity. *J. Biol. Chem.* 282, 19884–19893.
- Sánchez-Ruiloba, L., Cabrera-Poch, N., Rodríguez-Martínez, M., López-Menéndez, C., Jean-Mairet, R.M., Higuero, A.M., 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.
- Sato, M., Yoshimura, S., Hirai, R., Goto, A., Kunii, M., Atik, N., Sato, T., Sato, K., Harada, R., Shimada, J., Hatabu, T., Yorifuji, H., Harada, A., 2011. The role of VAMP7/TI-VAMP in cell polarity and lysosomal exocytosis *in vivo*. *Traffic* 12, 1383–1393.
- Tahirovic, S., Bradke, F., 2009. Neuronal polarity. *Cold Spring Harb. Perspect. Biol.* 1, a001644.
- Yin, D.M., Huang, Y.H., Zhu, Y.B., 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.