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# 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.



## 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 $1 \times 10^5$ 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% $CO_2$/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 $5 \times 10^6$ 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



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