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# Behavioral and pharmacological phenotypes of brain-specific diacylglycerol kinase δ-knockout mice

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ABSTRACT

Diacylglycerol kinase (DGK) is a lipid-metabolizing enzyme that phosphorylates diacylglycerol to produce phosphatidic acid. Previously, we reported that the δ isozyme of DGK was abundantly expressed in the mouse brain. However, the functions of DGKδ in the brain are still unclear. Because conventional DGKδ-knockout (KO) mice die within 24 h after birth, we have generated brain-specific conditional DGKδ-KO mice to circumvent the lethality. In the novel object recognition test, the number of contacts in the DGKδ-KO mice to novel and familiar objects was greatly increased compared to the control mice, indicating that the DGKδ-KO mice showed irrational contacts with objects such as compulsive checking. In the marble burying test, which is used for analyzing obsessive-compulsive disorder (OCD)-like phenotypes, the DGKδ-KO mice buried more marbles than the control mice. Additionally, these phenotypes were significantly alleviated by the administration of an OCD remedy, fluoxetine. These results indicate that the DGKδ-KO mice showed OCD-like behaviors. Moreover, the number of long axon/neurites increased in both DGKδ-KO primary cortical neurons and DGKδ-knockdown neuroblastoma Neuro-2a cells compared to control cells. Conversely, overexpression of DGKδ decreased the number of long axon/neurites of Neuro-2a cells. Taken together, these results strongly suggest that a deficiency of DGKδ induces OCD-like behavior through enhancing axon/neurite outgrowth.



## 1. Introduction

Diacylglycerol kinase (DGK) is a lipid-metabolizing enzyme that phosphorylates diacylglycerol to produce phosphatidic acid. Diacylglycerol and phosphatidic acid act as lipid second messengers in a wide variety of biological processes in mammalian cells (English, 1996; Exton, 1994; Hodgkin et al., 1998). Thus, DGK plays a pivotal role in various intracellular signaling pathways by regulating diacylglycerol and phosphatidic acid concentrations.

DGK represents a large enzyme family (Goto et al., 2006; Merida et al., 2008; Sakane et al., 2007; Topham and Epand, 2009). Ten DGK isozymes (α, β, γ, δ, η, κ, ε, ζ, ι, and θ) have been identified and classified into five subtypes based on their structural features. The type II DGK (Sakai and Sakane, 2012) comprises δ (Sakane et al., 1996), η (Klauck et al., 1996), and κ (Imai et al., 2005). Moreover, alternative splicing products of DGKδ (δ1 and δ2) (Sakane et al., 2002) and DGKη (η1 and η2) (Murakami et al., 2003) have been identified. All of the type II DGK isoforms possess a pleckstrin homology domain at their N-termini and a separate catalytic region, and DGKs δ1, δ2, and η2, but not DGKη1, contain a sterile α-motif domain at their C-termini. DGKδ2 specifically contains the Pro-rich 52 residues extending from the N-terminus (Sakane et al., 2002).

A tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate, induces the phosphorylation, oligomer-monomer conversion and translocation to the plasma membrane of DGKδ1 (Imai et al., 2002; Imai et al., 2004; Sakane et al., 2002). DGKδ1 translocates from the cytoplasm to the plasma membrane via the pH and C1 domains in response to high glucose levels (Takeuchi et al., 2012). DGKδ forms oligomeric (at least tetrameric) structures *in vitro* and *in vivo* and that the SAM domain plays a critical role in oligomer formation (Harada et al., 2008; Imai et al., 2002; Knight et al., 2010; Sakane et al., 2002).

Based on the analysis of DGKδ-knockout (KO) mice, it has recently been reported that DGKδ regulates the epidermal growth factor receptor pathway in epithelial cells of the lungs and skin

*Abbreviations:* DGK, diacylglycerol kinase; KO, knockout; OCD, obsessive-compulsive disorder; PTZ, pentylenetetrazol; SSRI, selective serotonin reuptake inhibitor

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(Crotty et al., 2006) and insulin receptor signaling in skeletal muscle (Chibalin et al., 2008; Miele et al., 2007) by modulating PKC activity.

In addition to being expressed in skeletal muscle cells (Chibalin et al., 2008; Miele et al., 2007; Sakai et al., 2014; Sakane et al., 1996), DGKδ was abundantly expressed in mouse brains (Usuki et al., 2015). DGKδ2, but not DGKδ1, was highly expressed in layers II–VI of the cerebral cortex, hippocampus, dentate gyrus, the mitral cell, granule cell and glomerular layers of the olfactory bulb and the granule cell layer of the cerebellum in one- to 32-week-old mice (Usuki et al., 2015). DGKδ2 was expressed just after birth, and its expression levels dramatically increased from one to four weeks. Moreover, DGKδ has been reported to be related to neurological disorders (Leach et al., 2007). A female patient with a *de novo* balanced translocation, 46,X,t(X;2)(p11.2;q37)dn, exhibited seizures, capillary abnormality, developmental delay, infantile hypotonia and obesity (Leach et al., 2007). However, the functions of DGKδ in neurological disorders are still unclear.

Because conventional KO mice die within 24 h of birth, it is difficult to analyze higher brain functions using them. Therefore, in this study, we generated brain-specific conditional DGKδ-KO mice and used these mice to perform behavioral and pharmacological tests.

## 2. Results

### 2.1. Generation of brain-specific DGKδ-deficient mice

To investigate a potential role for DGKδ in the brain *in vivo*, we generated a brain-specific DGKδ conditional allele targeting exon 9 (Suppl. Fig. 1A), circumventing the immediate postnatal lethality associated with the germline deletion of DGKδ in mice (Crotty et al., 2006). PCR screening of tail-derived genomic DNA showed that *LoxP* sites were placed flanking exon 9 of *DGK*δ (Suppl. Fig. 1B). Moreover, we confirmed that the DGKδ2 protein, which was expressed in the control mice ($cre^{-/-}$: $loxP^{+/+}$), was not detected in the brain of the conditional DGKδ-KO mice ($cre^{+/-}$: $loxP^{+/+}$) (Suppl. Fig. 1C). Conversely, the DGKδ2 expression level in the testis, in which the protein was strongly expressed in wild-type mice (Shionoya et al., 2015; Usuki et al., 2015), was almost the same as that of the control littermates. We verified that deleting DGKδ did not significantly affect protein expression of other DGK isozymes (α, β, γ, η, κ, ε, ζ, ι and θ) in the brain (Suppl. Fig. 2).

The brain-specific DGKδ-KO mice grew normally and were reproductively active. A previous report (Leach et al., 2007) demonstrated that a female patient lacking the DGKδ gene exhibited seizures. A potential seizure phenotype in mutant mice was investigated by a seizure-susceptibility study using a subthreshold dose of pentylenetetrazol (PTZ). However, no significant difference in susceptibility to PTZ-induced seizures was detected (Suppl. Fig. 3). Moreover, we performed the open field test (for activity and anxiety abnormalities) (Suppl. Fig. 4), the elevated plus-maze test (anxiety abnormality) (Suppl. Fig. 5), the sociability test (Suppl. Fig. 6), the Barnes maze test (cognitive defects) (Suppl. Fig. 7), the cotton bud biting test (aggressiveness) (Suppl. Fig. 8), and the tail suspension test (anti-depressive activity) (Suppl. Fig. 9). However, substantial differences between the DGKδ-KO and control mice were not observed in any tests (Table 1).

### 2.2. DGKδ-KO mice exhibit compulsive checking of objects

We next performed the novel object recognition test. As shown in Fig. 1A, the DGKδ-KO mice more frequently returned to the novel and familiar objects, and the numbers of contacts of the DGKδ-KO mice to both objects were significantly increased (approximately 6- and 7-fold increases, respectively) compared to the control mice. Moreover, the time of contact in the DGKδ-KO mice to the novel and familiar objects was also greatly augmented (approximately 11- and 12-fold increases, respectively) (Fig. 1B). These results indicate that the DGKδ-KO mice showed irrational contacts to objects. Because ritual-like compulsive checking of objects in OCD model mice were reported (Joel, 2006; Szechtman et al., 2001), these results suggested that the DGKδ-KO mice showed OCD-like behaviors. An increase of novel preference of the DGKδ-KO mice was not detected because the time spent on the novel object/the time spent on both objects and the number of contacts to the novel object/the number of contacts to both objects failed to be significantly different between the DGKδ-KO and the control mice (Fig. 1C and D).

### 2.3. Brain-specific DGKδ-KO mice buried more marbles than control in marble burying test

Because the marble burying test is related to compulsive behaviors and is used to analyze an OCD-like phenotype (Broekkamp et al., 1986; Joel, 2006), we carried out this test. In the marble burying test, the DGKδ-KO mice buried markedly more marbles (more than 2-fold increase) than the control mice (Fig. 2). The results further suggest that the DGKδ-KO mice showed OCD-like behaviors.

### 2.4. Fluoxetine treatment alleviates OCD-like behaviors in brain-specific DGKδ-KO mice

A selective serotonin reuptake inhibitor (SSRI), fluoxetine, is utilized as an OCD remedy (Joel, 2006; Yadin et al., 1991). Therefore, we analyzed the effects of the SSRI on abnormal behaviors in the novel object recognition test and the marble burying test. Increased number and time of contact to a novel object in the DGKδ-KO mice were significantly alleviated to basal levels by the administration of fluoxetine (Fig. 3A and B). Moreover, the enhanced marble burying in DGKδ-KO mice was also significantly decreased to basal levels d by the administration of the SSRI (Fig. 3C). Taken together, these results further suggest that DGKδ-KO mice exhibited OCD-like behaviors (Table 1).

### 2.5. A defect in DGKδ increases axon/neurite outgrowth in primary cultured cortical neurons and Neuro-2a neuroblastoma cells

To investigate the effects of the brain-specific DGKδ-deficiency on neuronal cell functions, primary cultured cortical neurons were prepared from the DGKδ-KO mice. We performed quantification of cells that had 0, 1, 2, and more than 3 axon/neurites in control and DGKδ-KO cortical neurons. As shown in Fig. 4, the percentage of cells that had more than 3 axon/neurites significantly increased (approximately 2.5-fold) in DGKδ-KO neurons, compared to the control cells.

To verify the effects of DGKδ-deficiency on the extension of axon/neurites in primary cultured neuronal cells, we transfected DGKδ-specific siRNA into Neuro-2a neuroblastoma cells and observed axon/neurite extension. We confirmed that the expression levels of the DGKδ protein were markedly reduced (Fig. 5A). We counted the numbers of cells that had 0, 1, 2, and more than 3 axon/neurites in control and DGKδ-knockdown Neuro-2a cells. As observed in DGKδ-deficient neuronal cells (Fig. 4), the percentage of cells that had more than 3 axon/neurites substantially increased (approximately 2.5-fold) in DGKδ-silenced cells compared to control cells (Fig. 5B and C).

Next, we overexpressed DGKδ in Neuro-2a cells. We performed quantification of cells that had 0, 1, 2, and more than 3 axon/neurites in AcGFP alone and AcGFP-DGKδ-overexpressed Neuro-2a cells as

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