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Dopamine and the phosphorylated dopamine transporter are increased in the diacylglycerol kinase η-knockout mouse brain

Maho Asami, Yuji Suzuki and Fumio Sakane

Department of Chemistry, Graduate School of Science, Chiba University, Japan

Correspondence
F. Sakane, Department of Chemistry, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
Tel: +81 43 290 3695
E-mail: sakane@faculty.chiba-u.jp

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The molecular mechanisms generating the mania-like abnormal behaviors caused by diacylglycerol (DG) kinase (DGK) η deficiency remain unclear. Here, we found that DGKη knockout markedly increased dopamine (DA) levels in the midbrain (DA-producing region, 2.8-fold) and cerebral cortex (DA projection region, 1.2-fold). Moreover, DGKη deficiency significantly augmented phosphorylated DA transporter (DAT) levels (1.4-fold increase), which induce DA efflux to the synaptic cleft, in the cerebral cortex. Moreover, phosphorylation levels of protein kinase C-β, which is activated by DG and involved in DAT phosphorylation, were also increased. DAT expressed in Neuro-2a cells recruited DGKη to the plasma membrane and colocalized with it. These results strongly suggest that dopaminergic hyperfunction caused by DGKη deficiency in the brain leads to mania-like behaviors.

Keywords: bipolar disorder; diacylglycerol kinase; dopamine; Parkinson's disease; protein kinase C; schizophrenia

Bipolar disorder (BPD) is a mental disorder characterized by unusual conversion between the heights of mania and the depths of depression [1]. Current reports indicate that the lifetime prevalence of BPD may be close to 5% [1]. Although BPD has been gradually recognized as a major health problem, the underlying neurobiology of BPD remains largely unknown.

Several genome-wide association studies (GWASs) have repeatedly demonstrated that single nucleotide polymorphisms (SNPs) of *DGKH* [diacylglycerol kinase (DGK) η gene] are associated with the etiology of BPD (https://www.gwascentral.org) [2–5]. In addition, *DGKH* is located within the BPD linkage region on 13q14 [6,7]. Intriguingly, the mRNA levels of DGKη were markedly augmented in the brains of patients with BPD (many of whom are likely in a depressive or normal state because the manic state is often brief) [8].

DGK, which comprises 10 isozymes, phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA) [9–12]. DGK isozymes, which are distributed in specific tissues and cells [9–12], phosphorylate different DG molecular species [13,14] and regulate a variety of physiological and pathological events [3,15–18]. Among them, DGKη (type II isozyme [19]) is most strongly expressed in the brain [20,21], and it was intensively detected in layers II–VI of the cerebral cortex, in the dentate gyrus region of the hippocampus, and in Purkinje cells in the cerebellum of 1- to 32-week-old mice [21]. The pleckstrin homology domain of DGKη interacts with phosphatidylinositol 4,5-bisphosphate [22], which is a main effector molecule of phosphatidylinositol turnover [23]. Moreover, DGKη interacts with and activates C-Raf in the Ras–B-Raf–C-Raf–MEK–ERK signaling cascade [24]. DGKη

Abbreviations
BPD, bipolar disorder; DA, dopamine; DAT, dopamine transporter; DGK, diacylglycerol kinase; KO, knockout; MAO, monoamine oxidase; PA, phosphatidic acid; PKC, protein kinase C; PUFA, polyunsaturated fatty acid; SERT, serotonin transporter; TH, tyrosine hydroxylase; WT, wild-type.

regulates the proliferation of myoblasts by controlling the expression of mammalian target of rapamycin [25].

Recently, we generated DGKη-knockout (KO) mice and performed various behavioral tests [26]. It is noteworthy that DGKη-KO mice exhibited an overall behavioral profile that is analogous to the BPD manic state, such as hyperactivity, decreased anxiety, and lesser depressive states. Moreover, these phenotypes were markedly inhibited by the administration of lithium, a therapeutic drug for mania [26]. These results strongly implied the existence of an association between DGKη and BPD. Moreover, microarray analyses using DGKη-KO mouse brains showed that the mRNA levels of growth hormone and prolactin, which are increased in BPD patients and model animals [27,28], were drastically increased [29].

However, the molecular mechanisms causing the mania-like abnormal behaviors of DGKη-KO mice remain unclear. Because dopamine (DA) D2 receptor antagonists (atypical antipsychotics) are utilized for mania treatment, the DA hypothesis has been a key theory of the pathophysiology of the manic phase of BPD [30]. Therefore, the purpose of the present work was to clarify the relationship between DGKη and the DA nervous system. Intriguingly, in the brain, DGKη deficiency increased DA levels and enhanced DA transporter (DAT) phosphorylation, which induced DAT-mediated DA efflux instead of DA uptake and, thus, increased the synaptic level of DA [31]. Therefore, it is suggested that dopaminergic hyperfunction caused by DGKη deficiency in the brain leads to the mania-like behaviors.

Materials and methods

Mouse

The animals (male, 12 weeks old, ~ 25 g) were housed at 24 ± 2 °C under a 12-h light–dark cycle (lights on from 7:00 to 19:00) with *ad libitum* access to food and water. DGKη-KO mice (*dgkh*$^{-/-}$, Accession No. CDB0606K) were generated as previously described [26]. In brief, part of the catalytic domain encoded by exons 5 and 6 of the DGKη gene in mice was deleted by homologous recombination. We confirmed that the DGKη protein was not detectable in DGKη-KO mice [26]. Wild-type (WT, C57BL/6) male littermates were used as a control group for DGKη-KO mice. WT and KO samples were prepared and treated pairwise. This study received approval from the Animal Experiment Committee of Chiba University (Permission Numbers: 30-185, 1-51, and 2-227). All procedures relating to animal care and treatment were conducted in compliance with the National Institutes of Health: Guide for the Care and Use of Laboratory Animals.

DA enzyme-linked immunosorbent assay (ELISA)

The mouse cerebral cortex (12-week-old male) was homogenized on ice with lysis buffer [50 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM $MgCl_2$, cOmplete™ EDTA-free protease inhibitor (1 tablet/50 Ml; Roche Applied Science, Mannheim, Germany)] and 1 mM phenylmethylsulfonyl fluoride. The total protein content of each sample was determined by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, DA was quantified using a DA ELISA Kit (Abnova, Taipei, Taiwan) according to the instructions from the manufacturer. The sample volume used was 100 µL. A normalization for protein level was made between samples.

Western blotting

Western blotting was performed as described previously [32]. The mouse midbrain and cerebral cortex were homogenized in ice-cold lysis buffer [50 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, cOmplete™ EDTA-free protease inhibitor (1 tablet/50 mL), and 1 mM phenylmethylsulfonyl fluoride and Phosphatase Inhibitor Cocktail II (1×; Sigma-Aldrich, St. Louis, MO, USA)]. The homogenates were separated by SDS/polyacrylamide gel electrophoresis. The separated proteins were transferred to a PVDF membrane (Pall Corporation, Tokyo, Japan) and blocked with 5% (W/W) skim milk. The membrane was incubated with an anti-DAT antibody (sc-14002; Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-phosphorylated-DAT (Thr-53) antibody (p435-53; PhosphoSolutions, Littleton, CO, USA), anti-protein kinase C (PKC) βI/II antibody (3223SA; Gibco BRL Products, Gaithersburg, MD, USA), anti-phosphorylated-PKCβI/II (Thr-500) antibody (ab5817; Abcam, Cambridge, UK), or anti-β-actin (A5441; Sigma-Aldrich) in 5% (W/W) skim milk for 1 h. The immunoreactive bands were then visualized using a peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and the Enhanced Chemiluminescence Western Blotting Detection System (GE Healthcare, Chicago, IL, USA).

Cell culture and transfection

Neuroblastoma Neuro-2a cells (dopaminergic cells) were grown in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Tokyo, Japan) supplemented with 10% FBS (Corning, Corning, NY, USA), 100 units·mL^{-1} penicillin, and 100 µg·mL^{-1} streptomycin (Wako Pure Chemical Industries). The cells were maintained at 37 °C in an atmosphere containing 5% CO_2.

Neuro-2a cells were seeded in a poly-L-lysine-coated 12-well plate with coverslips at a density of 1 × 10^4 cells/well. pEGFP-human DGKη1 and pDsRed monomer-human DAT were transiently transfected using Lipofectamine 2000

Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The transfected cells were then allowed to grow in DMEM containing 10% FBS for 24 h.

Confocal laser scanning microscopy

The cells were fixed in 4% paraformaldehyde. The coverslips were mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were acquired using an Olympus FV1000-D (IX81) confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with UPLSAPO 60× 1.35 NA oil at room temperature. EGFP fluorescence was excited at 488 nm, and DsRed fluorescence was excited at 543 nm. Images were acquired using FV-10 ASW software (Olympus).

Analysis of protein accumulation at the plasma membrane

To quantify the amount of DsRed monomer-tagged DAT and EGFP-tagged DGKη accumulated at the plasma membrane compared with cytosol, we used an IMAGEJ plugin [33] that measures average intensity value of the image in a small circular area; we monitored the background, the cytosol of COS-7 cells, and the plasma membrane when the fluorescent protein was expressed (we verified that the fluorescence intensities were almost equal among cells that were analyzed) [34]. The average pixel value was computed for each measurement (background, cytosol, and plasma membrane). From these observed values, we separated the contribution of each component (background, cytosol, and plasma membrane). Finally, we computed the ratio between fluorescence at the plasma membrane and fluorescence in the cytosol. Ratio values are represented as dot plots, with each dot representing an individual cell.

Statistical analysis

Data are represented as the means ± SEM. Statistical comparisons were performed using a two-tailed *t*-test (a paired-samples *t*-test) for the comparison of two groups or an ANOVA–Kruskal–Wallis followed by Dunn's multiple comparison test for multiple comparisons using PRISM 8 (GraphPad Software, San Diego, CA, USA) to determine any significant differences. $P < 0.05$ was considered significant.

Results

DA levels are increased in the DGKη-KO mouse brain

To elucidate the relationship between DGKη and the DA nervous system, we first investigated whether DGKη deficiency affects the amount of DA in the brain. We used 12-week-old male mice. DA is produced in the midbrain and is then carried to the DA projection region (the cerebral cortex) [35]. Therefore, we determined DA levels in the midbrain (DA-producing region) and cerebral cortex (DA projection region) of WT and DGKη-KO mice. As shown in Fig. 1A, DGKη deficiency drastically increased DA levels in the midbrain (approximately 2.8-fold) compared with the control. Moreover, DA in the cerebral cortex of DGKη-KO mice was also moderately but significantly augmented (approximately 1.2-fold) (Fig. 1B). These results indicate that DA levels are increased not only in the DA-producing region but also in the DA projection (DA-carried) region in the absence of DGKη.

Phosphorylation of DAT is enhanced in the DGKη-KO mouse brain

DAT usually takes up DA from the synaptic cleft in the DA projection region. However, amphetamine-dependent phosphorylation of DAT at Thr-53 reversely induces DAT-mediated DA efflux, but not DA uptake, and consequently increases the synaptic level of DA [31]. Therefore, we next examined whether DGKη deficiency affects the phosphorylation levels of DAT (Thr-53) in the cerebral cortex of WT and DGKη-deficient mice. DAT protein levels were not changed in the DGKη-KO cerebral cortex (Fig. 2A,B). However, the amounts of phosphorylated DAT (p-DAT) were significantly increased (p-DAT/β-actin and p-DAT/DAT: approximately 1.4- and 1.3-fold, respectively) in the DGKη-deficient cerebral cortex compared with the WT cerebral cortex (Fig. 2A,C,D). These results indicate that the absence of DGKη augments the phosphorylation of DAT (Thr-53), which provokes DA efflux [31].

Phosphorylation levels of PKCβ are augmented in the DGKη-KO mouse brain

PKCβ was reported to selectively enhance the phosphorylation of DAT at Thr-53 and to trigger its DA efflux to the synaptic cleft, but not DA uptake, in an amphetamine-dependent manner [36,37]. Thus, we next determined whether the levels of PKCβ phosphorylation at Thr-500, which activates PKC activity [38], are changed in the DGKη-KO mouse brain (cerebral cortex). The protein levels of PKCβ were not changed (Fig. 3A,B). However, the levels of phosphorylated PKCβ (p-PKCβ) were significantly increased (p-PKCβ/β-actin and p-PKCβ/

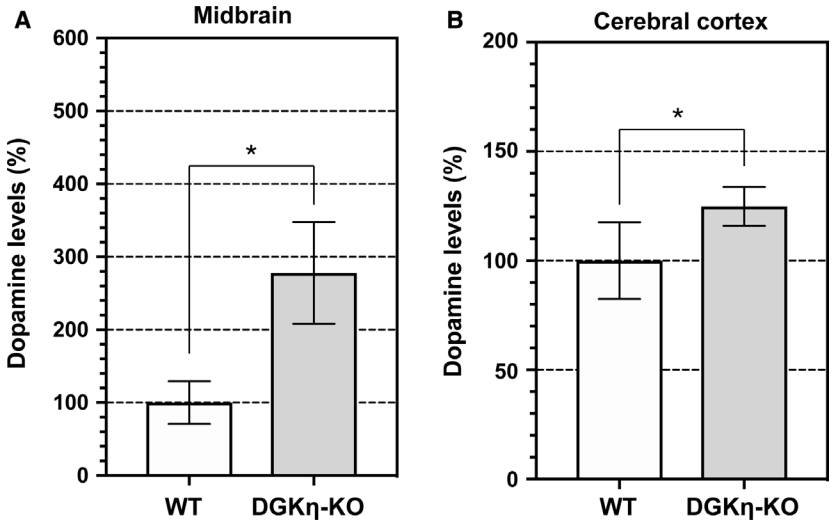
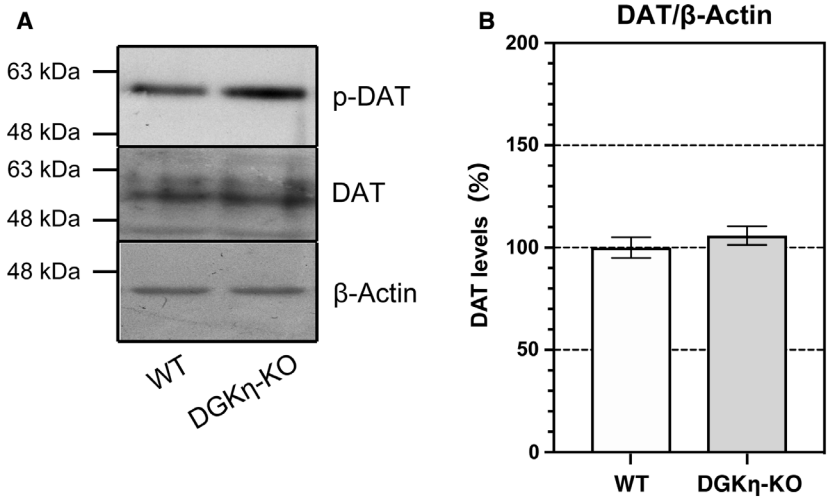


Fig. 1. DA levels in the DGKη-KO mouse midbrain and cerebral cortex. (A) The amounts of dopamine in the midbrains of 12-week-old male control ($n = 6$) and DGKη-KO ($n = 6$) mice were measured using a dopamine ELISA Kit. Data are shown as the means ± SEM. $*P < 0.05$ vs. control mice. (B) The amounts of dopamine in the cerebral cortex of 12-week-old male control ($n = 8$) and DGKη-KO ($n = 8$) mice were measured using a dopamine ELISA Kit. Data are shown as the means ± SEM. $*P < 0.05$ vs. control mice.



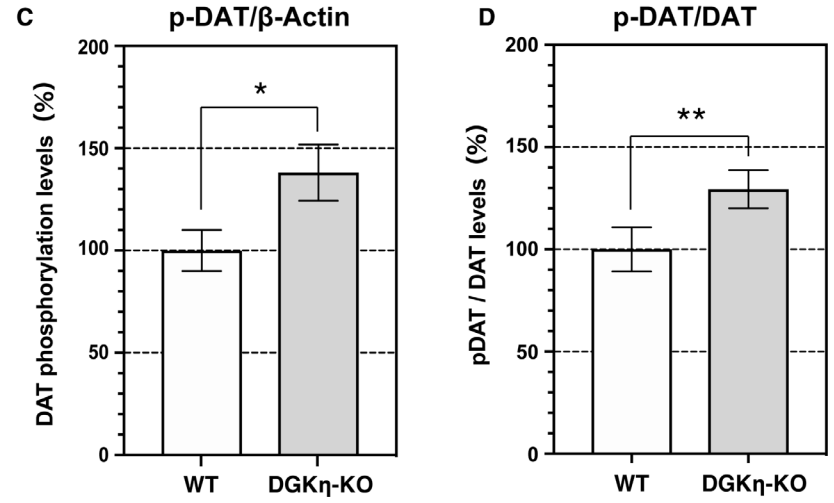


Fig. 2. Phosphorylation levels in the DAT in the cerebral cortex of DGKη-KO mice. (A) Protein samples (20 μg) from the cerebral cortex of 12-week-old male control and DGKη-KO mice were probed with anti-DAT and anti-phosphorylated DAT (p-DAT, Thr-53) antibodies. (B–D) Quantitative analysis of western blotting of DAT (B), p-DAT (C), and p-DAT/DAT (D). DAT and p-DAT immunoblots were scanned, and protein levels were quantified using IMAGEJ software. DAT and p-DAT levels were normalized relative to the β-actin levels. DAT, p-DAT, and p-DAT/DAT levels in the control mouse were set to 100%. Data are shown as the means ± SEM of eight independent experiments. $*P < 0.05$ vs. control mice; $**P < 0.01$.

PKCβ: approximately 1.2- and 1.3-fold, respectively) (Fig. 3A,C,D). These results indicate that DGKη deficiency enhances phosphorylation (activation) of PKCβ, which induces the phosphorylation of DAT at Thr-53 and provokes its DA efflux [36,37].

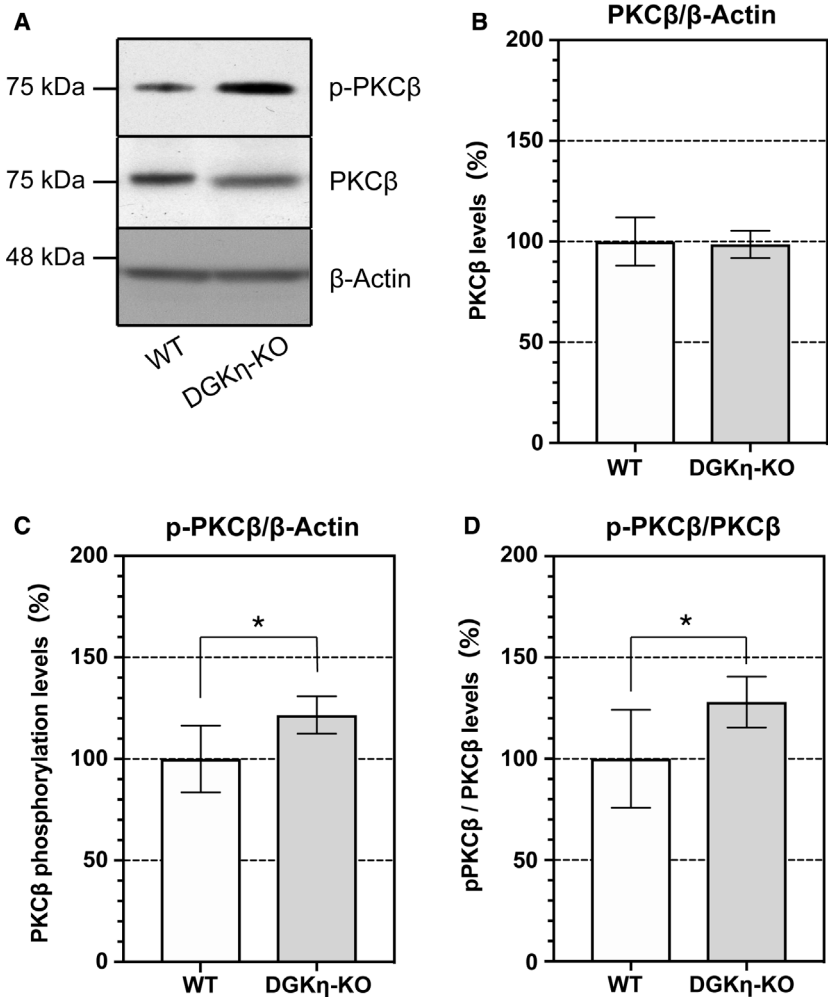


Fig. 3. Phosphorylation levels of PKCβ in the DGKη-KO mouse cerebral cortex. (A) Protein samples (50 μg) from the cerebral cortex of 12-week-old male control and DGKη-KO mice were probed with anti-PKCβ and anti-phosphorylated PKCβ (p-PKCβ, Thr-500) antibodies. (B–D) Quantitative analysis of western blotting (PKCβ (B), p-DAT (C), and p-DAT/DAT (D)). The graphs show the relative amounts of PKCβ (B), p-PKCβ (C), and p-PKCβ/PKCβ. PKCβ and p-PKCβ immunoblots were scanned, and protein levels were quantified using IMAGEJ software. PKCβ and p-PKCβ levels were normalized relative to the β-actin levels. PKCβ, p-PKCβ, and p-PKCβ/PKCβ levels in the control mice were set to 100%. Data are shown as the means ± SEM of eight independent experiments. $*P < 0.05$ vs. control mice.

DGKη is colocalized with DAT in Neuro-2a cells

To investigate whether DGKη is associated with DAT in cells, we next examined whether DGKη and DAT are colocalized in Neuro-2a neuroblastoma cells (dopaminergic cells). DsRed monomer-tagged DAT, which is a 12-transmembrane protein, was primarily located at the plasma membrane (plasma membrane/cytosol ratio: 4–5) in Neuro-2a cells (Fig. 4A,B). On the other hand, EGFP-tagged DGKη was broadly distributed in the cytoplasm (plasma membrane/cytosol ratio: approximately 0.8) in the absence of DsRed monomer-DAT (Fig. 4A,C). However, when DsRed monomer-DAT was coexpressed, EGFP-DGKη was markedly translocated to the plasma membrane (plasma membrane/cytosol ratio: approximately 1.8) and substantially colocalized with DsRed monomer-DAT (Fig. 4A,C). These results indicate that DAT recruits DGKη to the plasma membrane and colocalizes the enzyme, suggesting that DGKη directly or indirectly interacts with DAT.

Discussion

We previously reported that DGKη-KO mice display lithium (mania/BPD remedy)-sensitive mania-like behaviors. However, the molecular mechanisms causing the mania-like abnormal behaviors of DGKη-KO mice are not clear. In the present study, we demonstrated that DGKη-KO increased DA in the midbrain (DA-producing region) and cerebral cortex (DA projection region) (Fig. 1 and Fig. S1). Moreover, DGKη deficiency enhanced DAT phosphorylation at Thr-53 (Fig. 2 and Fig. S1), which reversely changes the transport direction (to efflux) of DA instead of uptake from the synaptic cleft in an amphetamine-dependent manner [31]. Therefore, it is likely that the hyperactivity of the DA nervous system at least in part contributes to mania-like behaviors caused by DGKη deficiency (Fig. S1).

Mice with brain-specific KO of DGKδ (type II isozyme), which is similar to DGKη [19], showed

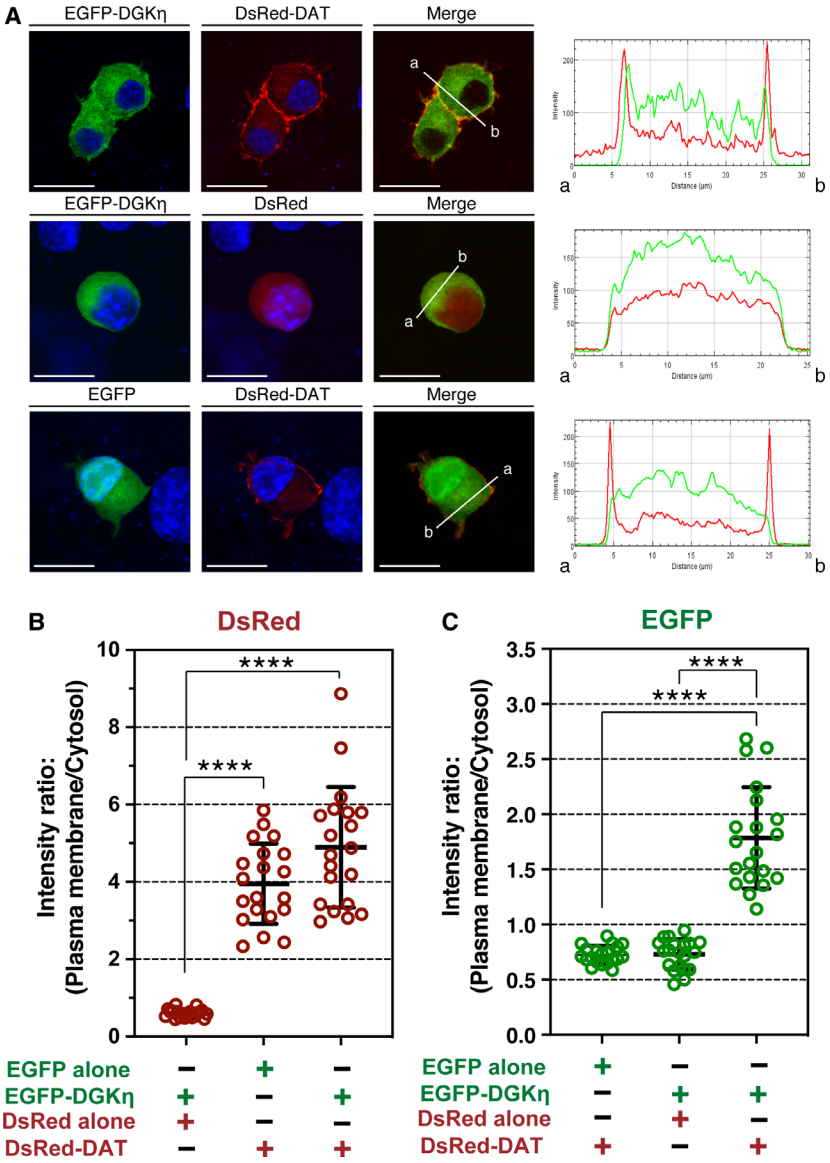


Fig. 4. Subcellular localization of EGFP-DGKη and DsRed monomer-DAT in Neuro-2a cells. (A) Neuro-2a cells were cotransfected with plasmids encoding EGFP-tagged DGKη1 or EGFP alone and DsRed monomer-tagged DAT or DsRed monomer alone as indicated. Cells were examined using an inverted confocal laser scanning microscope (FV1000-D; Olympus). Bar = 20 μm. (B) Quantitative image analysis of DsRed monomer alone (n = 20), DsRed monomer-DAT alone (n = 20), and DsRed monomer-DAT cotransfected with EGFP-DGKη (n = 20). (C) Quantitative image analysis of EGFP alone (n = 20), EGFP-DGKη alone (n = 20), and EGFP-DGKη cotransfected with DsRed monomer-DAT (n = 20). (B), (C) Each dot shows the plasma membrane : cytosol intensity ratio. Data are shown as the means ± SEM. ****P < 0.001, ANOVA–Kruskal–Wallis followed by Dunn's multiple comparison tests.

serotonin reuptake [serotonin transporter (SERT)] inhibitor-sensitive obsessive–compulsive disorder-like behaviors [39]. Moreover, we demonstrated that DGKδ-KO increased SERT protein levels in the cerebral cortex [40] and that DGKδ induced SERT degradation through the Praja-1 E3 ubiquitin–protein ligase–ubiquitin–proteasome system in a DGK activity [18:0/22:6-PA (X:Y = the total number of carbon atoms:the total number of double bonds in the fatty acyl moiety of the glycerol backbone)]-dependent manner [41,42]. Intriguingly, two important neurotransmitter transporters (DAT and SERT) were critically regulated by closely related type II DGK isozymes (η and δ) in different ways (phosphorylation by DG-dependent PKCβ and ubiquitination by 18:0/22:6-PA-dependent Praja-1). DGKβ-KO mice also showed mania-like behaviors [43]. However, DA abnormalities in DGKβ-deficient mice have not been reported. Therefore, it is suggested that DGKβ deficiency caused mania-like phenotypes through mechanisms different from those of DGKη-KO.

PKCβ was demonstrated to directly or indirectly phosphorylate DAT and enhance its DA efflux instead of DA uptake in an amphetamine-dependent manner [36,37]. In the DGKη-KO mouse brain (cerebral cortex), the Thr-500 phosphorylation (activation [38]) levels of PKCβ were significantly increased (Fig. 3 and Fig. S1). PKCβ, a conventional PKC, is activated by DG [44]. In the DGKη-KO mouse cerebral cortex, the amounts of polyunsaturated fatty acid (PUFA)-

containing PA species (reaction products of DGK), such as 18:1/18:2-PA, 18:1/20:2-PA, 18:0/22:5-PA, 20:0/20:4-PA, and 18:1/22:2-PA, were decreased [29]. Therefore, although accumulation of DG in the DGKη-KO cerebral cortex was not detected because of the high DG background, it is likely that PUFA-containing DG species were augmented and activated PKCβ. PKCβ did not exhibit DG species selectivity and thus was equally activated by 16:0/16:0-DG, 16:0/18:1-DG, 18:1/18:1-DG, 18:0/20:4-DG, and 18:0/22:6-DG [45]. Therefore, PKCβ would be activated by PUFA-containing DG species, 18:1/18:2-DG, 18:1/20:2-DG, 18:0/22:5-DG, 20:0/20:4-DG, and 18:1/22:2-DG. Because there are many PA-binding proteins [13], 18:1/18:2-PA, 18:1/20:2-PA, 18:0/22:5-PA, 20:0/20:4-PA, and 18:1/22:2-PA may affect DAT activity and DA levels through certain PA-binding proteins.

DAT recruited DGKη to the plasma membrane and colocalized with DGKη (Fig. 4 and Fig. S1), suggesting that DGKη physically interacts with DAT. Although we performed coimmunoprecipitation analyses, coimmunoprecipitation of DGKη and DAT was not detectable. To solubilize DAT, which is a 12-transmembrane protein, for coimmunoprecipitation analyses, a high concentration of detergent (1% Nonidet P-40) was needed. Therefore, the detergent at high concentrations may destroy the interaction between DGKη and DAT.

DA increased in the DGKη-KO mouse midbrain and cerebral cortex (Fig. 1 and Fig. S1). DA is synthesized by tyrosine hydroxylase (TH) in the midbrain and is degraded by monoamine oxidase-B (MAO-B) in the cerebral cortex [46]. However, the level of TH was not increased in the midbrain, and the amount of MAO-B was not decreased in the cerebral cortex (M. Asami & F. Sakane, unpublished work). Thus, it is possible that activation of TH and inactivation of MAO-B, instead of amount changes, occur in the DGKη-KO brain. Other enzymes, such as dihydroxyphenylalanine decarboxylase, may be changed. Enhanced DA efflux of DAT may secondarily increase DA. When DA efflux of DAT is augmented, intracellular (presynapse) DA is expected to be decreased. However, intracellular (presynapse) and extracellular (synaptic cleft) distribution of DA is unknown at present. Thus, it is unclear whether intracellular (presynapse) DA in the DGKη-KO mouse brain is decreased or not. In any cases, total DA levels were increased and DA efflux (phosphorylated DAT) was augmented in the DGKη-KO mouse brain (Figs 1 and 2; Fig. S1), suggesting that DA levels in the synaptic cleft were increased, and consequently, a hyperactivity of the DA nervous system occurs. However, further analyses are needed to address these issues.

GWASs have suggested that *DGKH* (DGKη gene) is associated with the pathogenesis of BPD [2–5]. *DGKH* is located within the BPD linkage region [6,7]. The mRNA levels of DGKη were markedly augmented in patients with BPD [8]. DGKη-KO mice showed mania-like behaviors [26]. Prolactin and growth hormone levels, which were reported to be higher in BPD patients than in controls [27,28], were increased in DGKη-KO mouse brains [29]. In the present study, we demonstrated that DGKη deficiency caused hyperactivity of the dopaminergic system. Therefore, the molecular mechanisms by which DGKη-deficient mice exhibit mania-like behaviors became clearer, and DGKη-KO mice were more likely to be a good model for BPD. Moreover, our current results support the DA hypothesis of BPD, which has been a key theory of the pathogenesis of the manic phase of BPD for over four decades [30]. Hypoactivity of the dopamine system is thought to be related to many psychiatric disorders [30,47–49], such as the depression phase of BPD, major depression, negative symptoms of schizophrenia, and Parkinson's disease. Schizophrenia and Parkinson's disease are associated with SNPs of *DGKH* by GWASs, in addition to BPD (https://www.gwascentral.org). Therefore, further study on the functions of DGKη in the brain may contribute to exploring the mechanisms of pathogenesis of these neurological disorders. Moreover, DGKη-specific inhibitors, if developed, are likely to be good therapeutics for these diseases.

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Author contributions

MA primarily performed the experiments and analyzed the data. YS performed the experiments and analyzed the data. FS and MA wrote the manuscript. FS supervised the project. All authors reviewed and approved the final manuscript.

Data accessibility

The data that support the findings of this study are available from the corresponding author (sakane@faculty.chiba-u.jp) upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Schematic representation of DA and DAT regulation by DGKη.