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ORIGINAL ARTICLE

Deficiency of diacylglycerol kinase η induces lithium-sensitive mania-like behavior

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Abstract

The η isozyme of diacylglycerol kinase (DGK) is highly expressed in the hippocampus and Purkinje cells in the central nervous system. Recently, several genome-wide association studies have implicated *DGK*η in the etiology of bipolar disorder (BPD). However, it is still unknown whether DGKη is indeed related to BPD. In this study, we generated DGKη-knockout (KO) mice and performed behavioral tests such as the open field test, the elevated plus maze test and tail suspension test using the KO mice to investigate the effects of DGKη deficits on psychomotor behavior. Intriguingly, DGKη-KO mice displayed an overall behavioral profile that is similar to human mania, including hyperactivity, less anxiety and less depression-like behavior. In addition, these phenotypes were significantly attenuated by the administration of a BPD (mania) remedy, namely, lithium. Moreover, DGKη-KO mice showed impairment in glycogen synthase kinase (GSK) 3β signaling, which is closely related to BPD. These findings clearly support the linkage between BPD and DGKη that is implicated by genome-wide association studies. Moreover, this study provides DGKη-KO mice as a previously unrecognized model that reflects several features of human BPD with manic episodes and revealed an important role for DGKη in regulating behavior and mood through, at least in part, GSK3β signaling.

Keywords: bipolar disorder, depression, diacylglycerol, diacylglycerol kinase, lithium, mania, phosphatidic acid, signal transduction.

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Bipolar disorder (BPD) is a mental disorder characterized by unusual shifts in mood from the heights of mania to the depths of depression (Martinowich *et al.* 2009). The lifetime prevalence of BPD was thought to be approximately 1%, but current reports indicate that this figure may be closer to 5% (Martinowich *et al.* 2009). Because of the elevated morbidity and mortality suffered by individuals with the disorder, BPD has been increasingly recognized as a major health problem. Despite advances in its diagnosis and recognition, the underlying neurobiology of BPD remains largely unknown.

Recently, several genome-wide association studies (GWASs) conducted by Baum *et al.* (2008), Weber *et al.* (2011) and Zeng *et al.* (2011) have successively implicated *DGK*η (diacylglycerol kinase (DGK) η gene) in the etiology of BPD. Moreover, Moya *et al.* (2010) also found that in patients with BPD, DGKη mRNA levels are significantly increased. Therefore, *DGK*η is attracting much attention as a BPD-related candidate gene. However, it is still unknown whether *DGK*η is indeed related to BPD because DGKη gene-deficient animals have not yet been established.

DGK phosphorylates diacylglycerol to generate phosphatidic acid (Goto *et al.* 2006; Sakane *et al.* 2007; Merida *et al.* 2008; Topham and Epand 2009). Ten mammalian DGK isozymes (α–κ), which commonly contain two or three

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Abbreviations used: ADHD, attention deficit hyperactivity disorder; BPD, bipolar disorder; DGK, diacylglycerol kinase; GSK, glycogen synthase kinase; GWAS, genome-wide association study; KO, knockout.

characteristic cysteine-rich, zinc finger-like C1 domains and a catalytic region, are subdivided into five groups according to their structural features. DGKη belongs to type II DGK (Sakai and Sakane 2012). Type II DGKs commonly have a pleckstrin homology domain at their N-termini and a catalytic domain that is divided into two subdomains. We revealed that DGKη positively regulates the epidermal growth factor receptor pathway through the activation of C-Raf in HeLa cervical cancer cells (Yasuda *et al.* 2009). Intriguingly, this DGK isozyme is most abundantly expressed in the brain (Klauck *et al.* 1996; Usuki *et al.* 2015). The expression of DGKη increased between 1 and 4 weeks, which is coincident with synapse formation of the brain (Usuki *et al.* 2015). Moreover, a substantial amount of DGKη was detected in layers II–VI of the cerebral cortex; in the CA1, CA2, and dentate gyrus regions of the hippocampus; in the mitral cell and glomerular layer of the olfactory bulb; and in the Purkinje cells in the cerebellum of one- to 32-week-old mice (Usuki *et al.* 2015). However, the function of DGKη in the brain remains unclear.

The purpose of our present work, based on the results of GWASs (Baum *et al.* 2008; Weber *et al.* 2011; Zeng *et al.* 2011), was to investigate the involvement of DGKη in BPD to gain insight into the neurogenic mechanisms of BPD. For this purpose, we have generated DGKη-knockout (KO) mice and used these mice to perform behavioral and pharmacological tests.

Materials and methods

Mice

This study received approval from the Animal Experiment Committee of Chiba University (permission number: 2012–95, 2013–149, 2013–251, 2014–096, and 2014–370) and the Institutional Animal Care and Use Committee of the RIKEN Kobe Branch (Permit Number: AH13-03-65). All procedures relating to animal care and treatment conformed to the animal care guidelines of the committees. All efforts were made to minimize both the suffering and the number of animals used. The animals (male, 10–14 weeks old) 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 were originated from C57BL/6 and CBA background (RIKEN, Kobe, Japan), and then backcrossed with C57BL/6 mice (Japan SLC, Hamamatsu, Japan) for at least five generations to eliminate any background effects on the observed phenotypes. In all experiments, we used wild-type (WT) littermates as a control group for DGKη-KO mice. Behavioral experiments were performed between 10:00 and 18:00.

Anti-DGKη antibody

For preparation of anti-human DGKη antibody, an oligopeptide corresponding to amino acids 600–620 of human DGKη (IGKPSSQKAVKPREIMLRANS) was used as the antigen. Rabbits were immunized by multiple subcutaneous injections of 100 μg of the peptide emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Injections were repeated at 4-week intervals, and the serum was obtained after the fifth injection. The IgG was purified by column chromatography on the antigen peptide-conjugated Hi-Trap NHS-activated Sepharose HP (GE Healthcare Bio-Sciences; Piscataway, NJ, USA).

Western blotting

Western blotting of the brain was performed using the anti-DGKη antibody (see above), the anti-glycogen synthase kinase (GSK) 3β (H-76, Santa Cruz Biotechnology, Santa Cruz, CA) and the anti-phospho-GSK3β (Ser9, Cell Signaling Technology, Danvers, MA, USA) as previously described (Usuki *et al.* 2015).

PCR of the DGK gene and RT-PCR amplification of the DGK mRNA

As indicated in Supplemental Fig. 1, genomic DNA was subjected to PCR using the following primers: A) 5′-TTCGTCGCTTTAAA CAGTTGC-3′, B) 5′-CGAAGAGGTTCACTAGTTCTAGAGC-3′, and C) 5′-GTCAATCACCAGCACCATTC-3′. The PCR conditions were 94°C for 3 min; 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and 72°C for 5 min.

RT-PCR was performed as previously described (Usuki *et al.* 2015). Brains were homogenized in QIAzol lysis reagent, and total RNA was isolated with the RNeasy Lipid Tissue Midi Kit (Qiagen, Venlo, Netherlands). cDNA synthesis was performed with the Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics), using 0.5 μg of total RNA and random hexamer primers. PCR amplification was performed using Ex Taq DNA polymerase (Takara Bio, Kusatsu, Japan). The DGKη primer sequences were as follows: forward primer D (nucleotide positions 65–88), 5′-GCAGA GAACCCTATGAGGTGGCCC-3′ and reverse primer E (nucleotide positions 473–496) 5′-GGCCAAGGGGACAGACGGGGATGG-3′. The PCR conditions were 94°C for 5 min; 45 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s; and 72°C for 5 min.

Open field test

Each mouse (WT: n = 21, DGKη-KO: n = 28) was placed in the periphery of the open field apparatus (length 60 × width 60 × height 40 cm). The time spent in the center area (length 30 × width 30 cm), the number of entries into the center area and the number of behavioral switches were analyzed for 25 min for each test session in a blind manner by a single observer. The total distance moved in the arena was quantified using Image J software (NIH, Bethesda, MD, USA).

Elevated plus maze test

The elevated plus maze test apparatus consisted of two open arms (length 30 × width 5 cm) and two closed arms of the same size, along with a wall (height 15 cm) and central platform (length 5 × width 5 cm). These arms and central platform were elevated 50 cm above the floor. Each mouse (WT: n = 15, DGKη-KO: n = 18) was placed in the central platform facing one of the open arms. The number of entries into the open arms and the time spent in the open arms of the apparatus were scored.

Tail suspension test

For the tail suspension test, a mouse (WT: n = 18, DGKη-KO: n = 20) was tail-suspended with an adhesive tape 50 cm above the floor, and their behavior was recorded for 7 min.

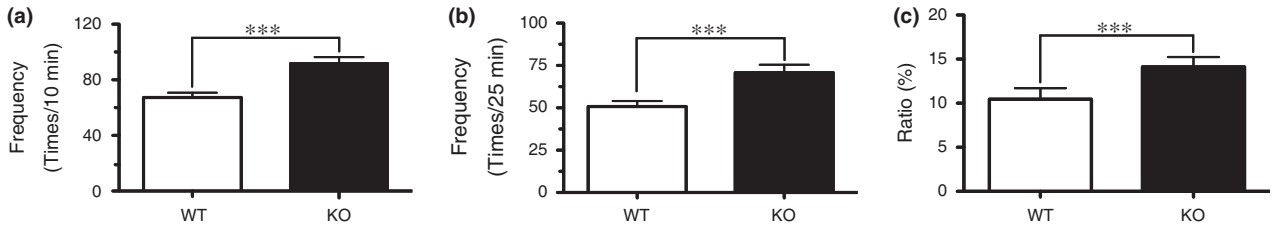

Fig. 1 Open field test of WT and diacylglycerol kinase (DGK)η-KO mice. WT and DGKη-KO mice were placed in the open field apparatus to measure their frequency of behavioral switching for 10 min (a), their frequency of entering the center area for 25 min (b) and the ratio of staying time in the center area for 25 min (c). The values are presented as the mean ± SEM (WT: $n = 21$, KO: $n = 28$). ***$p < 0.005$ versus WT mice.

Barnes maze test

Barnes maze consisted of a round table (90 cm in height) with 20 holes evenly distributed around the edge. An escape box was placed underneath one of the holes. Three Visual cues with different colors and shapes were placed around the maze. The maze was rotated daily, with the spatial location of the target unchanged with respect to the distal visual cues, to prevent a bias based on an olfactory cue or proximal cues within the maze. As a mildly aversive stimulus, two table lights located above the center of the maze top that produced approximately brightness. On the first test day, each mouse (WT: $n = 10$, DGKη-KO: $n = 8$) was gently guided to the escape box, which was then covered for 3 min. After these 3 min, the mouse was kept in the start box for 10 s in the center of the platform, and the following was recorded by a video camera. The mouse was allowed to walk around the maze for 3 min to find and enter the escape box. The escape box was placed underneath the target hole. If the mouse located the escape box, it was allowed 60 s inside before being returned to its home cage. If the mouse failed to enter the escape box within 3 min, it was also gently guided to the box and remained in the box for 60 s prior to being returned to its home cage. For four successive days these trials were conducted at three per day with 25 min intervals between trials. The number of nose pokes into the holes (frequency) and time spent to find the target hole (latency) were measured during the experiment as outcome factors.

Drug treatment

LiCl was mixed into the drinking water at 600 mg/L and administered for 10 days (Dehpour *et al.* 2002; Roybal *et al.* 2007; Kakefuda *et al.* 2010).

Statistical analysis

Data are presented as the means ± SEM. Pairwise comparisons were made by Student's *t*-test (Figs 1–3 and 6). Other datasets were analyzed by one-way ANOVA followed by a Tukey's *post hoc* test (Figs 4 and 5). A p-value of 0.05 or less was considered statistically significant.

Results

Generation of DGKη-deficient mice by homologous recombination

Given the links between *DGK*η and BPD, we determined whether mice carrying a mutation in the DGKη gene exhibit mood-related behavioral abnormalities. By homologous recombination, we deleted part of the catalytic domain encoded by exons 5 and 6 of the DGKη gene in mice (Fig. S1A). The knockout allele followed Mendelian distribution of inheritance. The mice lacking *DGK*η (*dgkh*$^{-/-}$) (Accession No. CDB0606K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) had a normal gender ratio, were grossly normal, and did not express truncated or full-length DGKη mRNA (Fig. S1B). Moreover, we confirmed that the DGKη protein was not detected in DGKη-KO mice (Fig. S1C). We verified that deleting DGKη did not significantly affect protein expression of other DGK isozymes (α, β, γ, δ, κ, ε, ζ, ι, and θ) in brain tissue (Fig. S2).

DGKη-KO mice exhibit hyperactivity

BPD patients in the manic state often display hyperactivity, low levels of anxiety and reduced depression. Therefore, we determined whether the DGKη-KO mice displayed these three phenotypes. First, DGKη-KO and WT mice were subjected to an open field test, and their activity and stereotyped behavior were compared. The traveled distance of the DGKη-KO mice was not significantly different compared to that of WT mice (data not shown). However, we next took note of the frequency of behavioral switching, that is, transition to walking, standing, grooming, scratching, and jumping. Intriguingly, we found that the frequency of behavioral switching in DGKη-KO mice was significantly (approximately 1.4-fold, $p = 0.000067$) greater than that of WT mice (Fig. 1a). The results show that the DGKη-KO mice quickly changed their behavior, indicating that they were in a hyperactive state.

DGKη-KO mice exhibit less anxiety

In the open field test, we also assessed the anxiety level of DGKη-KO mice by measuring their frequency of entering into the center area of an open field apparatus (anxiety-provoking area for mice). In this assessment, DGKη-KO mice more frequently (approximately 1.4-fold, $p = 0.0010$) entered into the center area of the apparatus than WT mice (Fig. 1b). Moreover, the time spent in the center area for the DGKη-KO mice was longer (approximately 1.3-fold, $p = 0.00094$) than that of the WT mice as well (Fig. 1c).

To further assess the anxiety levels of the DGKη-KO mice, we conducted another anxiety-measuring behavioral test: an elevated plus maze test. In this test, the number of entries into the open arms for the DGKη-KO mice was markedly increased (approximately 1.7-fold, $p = 0.000092$) compared with the entries for the WT mice (Fig. 2a). In addition, the time spent in the open arms for the DGKη-KO mice was also greater (approximately 3-fold, $p = 0.0061$) than that in the WT mice (Fig. 2b). These results indicate that the DGKη-KO mice showed less anxiety.

DGKη-KO mice decrease immobility in the tail suspension test

The mouse model for mood disorders generally exhibits changes in their depressive states, *i.e.,* the mouse model of mania exhibits lower depressive states (Roybal *et al.* 2007). Thus, we evaluated the change in DGKη-KO mice using the tail suspension test. In this test, DGKη-KO mice showed a reduced state of despair (approximately 0.7-fold, $p = 0.0000038$), as assessed by immobility time (Fig. 3).

DGKη-KO mice exhibited moderate impaired learning and memory performance in the Barnes maze test

Cognitive impairment is implicated in mania episode in BPD (Daglas *et al.* 2015). We performed the Barnes maze test to determine spatial learning and memory performance. As shown in Fig. 4, DGKη-KO mice exhibited moderate impaired learning and memory performance, with increased latency to reach the target hole (Fig. 4b, $p = 0.00029$ (Day 1)) and increased errors (Fig. 4a, $p = 0.045$ (Day 4)).

The manic-like behavior of DGKη-KO mice can be reversed with LiCl treatment

Lithium is a commonly prescribed mood stabilizer that is particularly effective in treating mania (Shastry 1997). To determine whether the behavioral abnormalities of the DGKη-KO mice can be reduced by a low-dose lithium treatment, we administered LiCl in drinking water at 600 mg/liter for 10 days as described previously (Dehpour *et al.* 2002; Roybal *et al.* 2007; Kakefuda *et al.* 2010). This paradigm produces a stable serum Li^+ concentration of approximately 0.4 mmol/L, which is at the low end of the therapeutic range for human patients (Roybal *et al.* 2007). To investigate the effect of lithium on the hyperactivity of DGKη-KO mice, the frequency of behavioral switching in the open field test was measured. As shown in Fig. 5a, the frequency of behavioral switching of the DGKη-KO mice was reduced by a low-dose treatment of lithium compared with the vehicle-treated DGKη-KO mice ($p = 0.00017$) and returned to basal levels (the values of WT mice). The behavior of the WT mice was not significantly affected by this concentration of LiCl. The results indicate that the hyperactivity of the DGKη-KO mice is lithium-sensitive.

Next, the effect of low-dose LiCl on the frequency of entering into the center area and the time spent in the center area of an open field apparatus was determined. Both of these measures were significantly decreased in lithium-treated DGKη-KO mice when compared with the vehicle-treated DGKη-KO mice ($p = 0.0000064$ and $p = 0.025$,

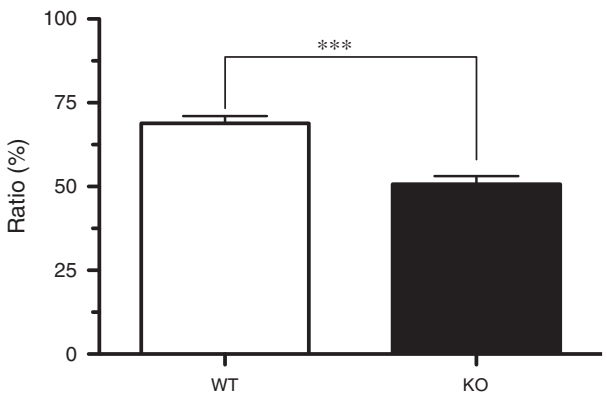

Fig. 3 Tail suspension test of WT and diacylglycerol kinase (DGK)η-KO mice. WT and DGKη-KO mice were tail-suspended with adhesive tape 50 cm above the floor over a period of 7 min; the immobility time was measured in only the last 6 min. The values are presented as the mean ± SEM (WT: $n = 18$, KO: $n = 20$). ***$p < 0.005$ versus WT mice.

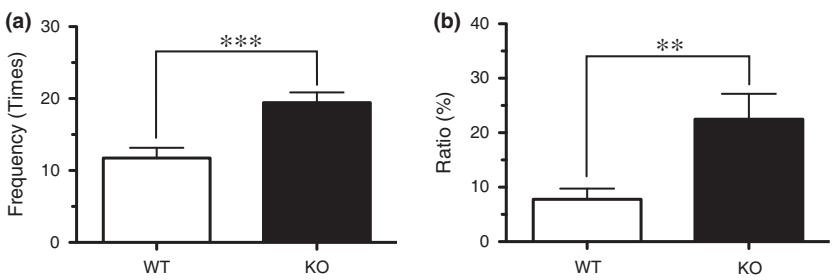

Fig. 2 Elevated plus maze test of WT and diacylglycerol kinase (DGK)η-KO mice. WT and DGKη-KO mice were placed in the elevated plus maze apparatus for 10 min to measure their frequency of entering the open arms (a) and the ratio of staying time in the open arms (b). The values are presented as the mean ± SEM (WT: $n = 15$, KO: $n = 18$). **$p < 0.01$, ***$p < 0.005$ versus WT mice.

respectively) and returned to basal levels (Fig. 5b and c). We next analyzed the effects of LiCl on DGKη-KO mice in the elevated plus maze test. Lithium treatment attenuated the frequency of entering the open arms (Fig. 6d, $p = 0.00014$) and the time spent in open arms (Fig. 5e, $p = 0.0011$). The behavior of the WT mice was not significantly affected by LiCl in the open field and elevated plus maze tests. These results indicate that the lowered anxiety of the DGKη-KO mice is also lithium-susceptible.

Finally, we determined the effects of LiCl on DGKη-KO mice in the tail suspension test. As shown in Fig. 5f, lithium treatment reversed the reduction in the despair state in the tail suspension test ($p = 0.0015$). Lithium treatment did not markedly affect the behavior of the WT mice. Taken together, these results indicate that all of the mania-like phenotypes of the DGKη-KO mice are sensitive to lithium.

Phosphorylation levels of GSK3β are decreased in the cerebral cortex and the cerebellum of DGKη-KO mice

GSK3β is the downstream factor of diacylglycerol-dependent protein kinase C (Baum *et al.* 2008; Kaidanovich-Beilin and Woodgett 2011) and of the MEK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) pathway (Saito *et al.* 1994; Kaidanovich-Beilin and Woodgett 2011), which is activated by DGKη (Yasuda *et al.* 2009), and one of target proteins of lithium (Jope 2003; Zhang *et al.* 2003; Beaulieu *et al.* 2004). Using western blot analysis, we evaluated the phosphorylation level of GSK3β. As shown in Fig. 6, the phosphorylation levels of GSK3β (Ser9) were significantly decreased in the cerebral cortex ($p = 0.019$) and the cerebellum ($p = 0.0036$) of DGKη-KO mice, despite the normal levels of total GSK3β proteins. These results indicate that DGKη-KO mice showed impairment in GSK3β signaling, which is closely related to BPD pathogenesis (Kaidanovich-Beilin and Woodgett 2011).

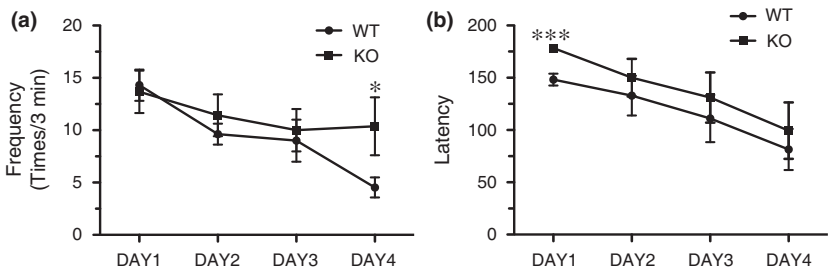

Fig. 4 Barnes maze test of WT and diacylglycerol kinase (DGK)η-KO mice. The frequencies of errors (a) and latency to target (b) were measured. The values are presented as the mean ± SEM (WT: $n = 10$, KO: $n = 8$). $^*p < 0.05$, $^{***}p < 0.005$ versus WT mice.

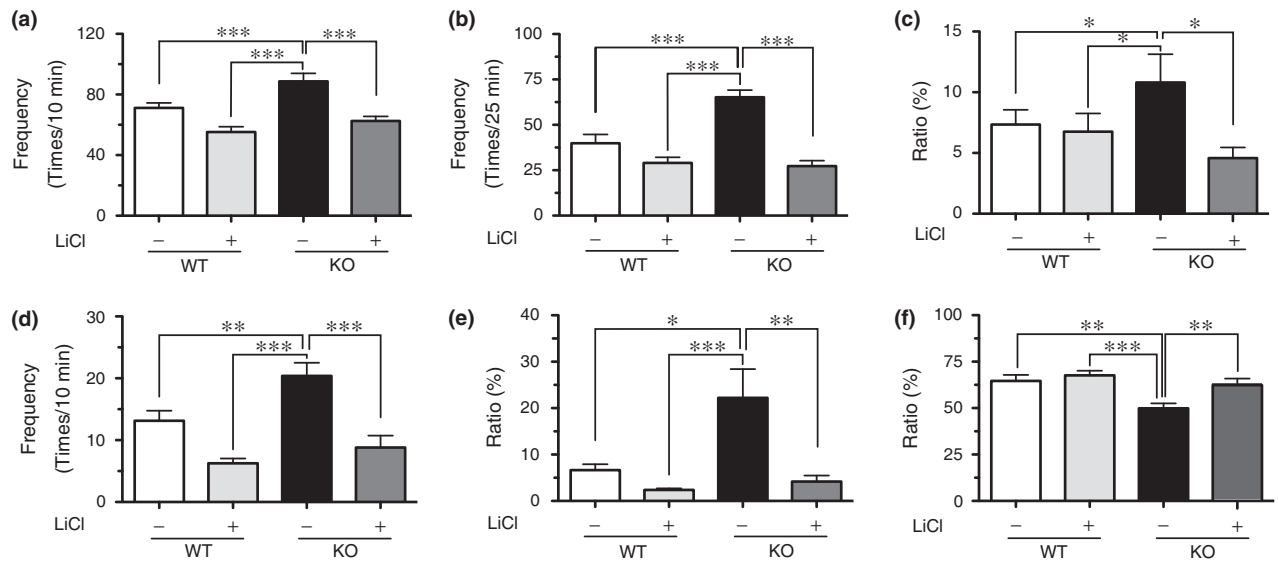

Fig. 5 Assessment of the lithium effect on diacylglycerol kinase (DGK) η-KO mice in the open field test, the elevated-plus maze test and the tail suspension test. Before (–) and after (+) 10-days of LiCl treatment, WT and DGKη-KO mice were subjected to the open field test (a–c), the elevated plus maze test (d and e) and the tail suspension test (f). In the open field test, the frequency of behavioral switching (a), the frequency of entering the center area (b) and the ratio of staying time in the center area (c) were measured. In the elevated-plus maze test, the frequency of entering open arms (d) and the ratio of staying time in open arms (e) were assessed. The values are presented as the mean ± SEM (WT: $n = 8$, KO: $n = 10$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$: KO mice with LiCl treatment versus KO mice without LiCl treatment.

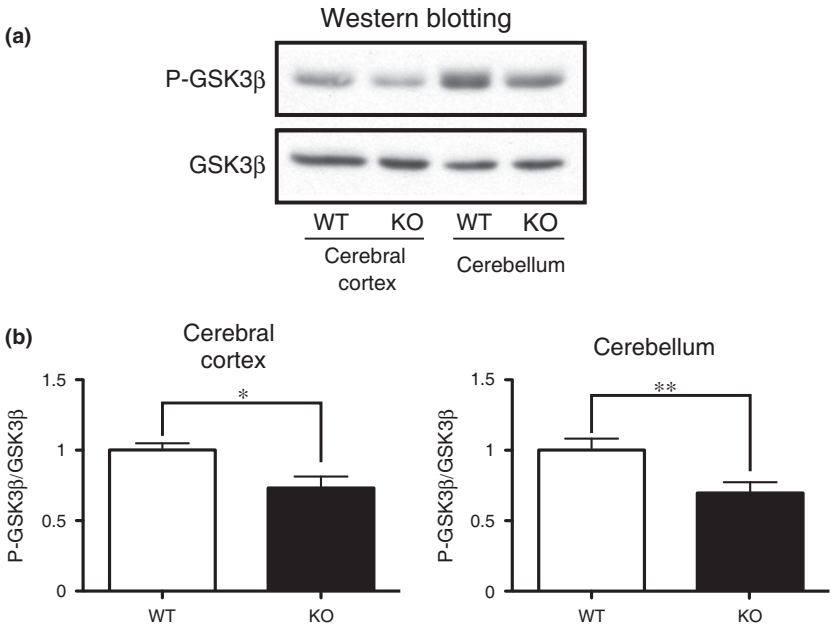

Fig. 6 Western blot analysis of phosphorylated GSK3β. (a) Western blotting of lysates from WT or KO mouse cerebral cortexes and cerebellums was performed to detect expression of total and phosphorylated GSK3β (Ser9). (b) Quantitative analysis of western blotting. The values of WT mice were set to 1. The values are presented as the mean ± SEM (WT: $n = 3$, KO: $n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus WT mice.

Discussion

Recently, GWASs implicated the DGKη gene in the etiology of BPD (Baum *et al.* 2008; Weber *et al.* 2011; Zeng *et al.* 2011). Moreover, DGKη mRNA levels are increased in patients with BPD (Moya *et al.* 2010). To test the linkages between *DGK*η and BPD, DGKη-KO mice are required. However, the generation of DGKη-KO mice has not been accomplished until now. In this study, we succeeded in generating DGKη-KO mice (Fig. S1) and performed a comprehensive behavioral analysis of the mice (Figs 1–5) to investigate the role of DGKη in higher brain functions, and the relationship between *DGK*η and BPD.

DGKη-KO mice exhibited increased open field activity (Fig. 1a), increased open field center time/frequency (Fig. 1b and c), increased open arm time/frequency in elevated plus maze (Fig. 2), increased antidepressant-like behavior (Fig. 3). Moreover, these phenotypes were sensitive to a BPD remedy, namely, lithium (Fig. 5). Furthermore, moderate cognitive impairment, which is implicated in mania episodes in BPD (Daglas *et al.* 2015), was detected in DGKη-KO mice (Fig. 4). The behavioral profile (hyperactivity, lower anxiety, lower depressive states, and cognitive impairment) of DGKη-KO mice is similar in several behavioral dimensions to BPD patients in the manic state (Martinowich *et al.* 2009) (Figs 1–4), including the disappearance of their phenotypes by treatment with lithium (Fig. 5). These lithium sensitive-phenotypes were commonly observed in representative BPD model mice such as Neurocan-KO (Miro *et al.* 2012), Clock-KO (Roybal *et al.* 2007), glutamate receptor 6-KO (Shaltiel *et al.* 2008), DGKβ-KO (Kakefuda *et al.* 2010; Shirai *et al.* 2010), and GSK3β-transgenic (Spittaels *et al.* 2000; Prickaerts *et al.* 2006) mice. Therefore, these findings strongly suggest that DGKη is one of the key enzymes involved in BPD pathogenesis and support the GWAS results. The lack of availability of suitable animal models of mania has been one of the greatest impediments in the field. Our results indicate that the DGKη-KO mice would represent a bona fide model of human BPD with mania. Therefore, it is likely that these mice are particularly useful for studying the pathophysiology of mania.

*DGK*η has also been found to be associated with attention deficit hyperactivity disorder (ADHD) by GWAS (Weber *et al.* 2011). Moreover, mania-like behavior is similar to ADHD symptoms. Therefore, DGKη-KO mice could also represent a model for ADHD. Although the focus of this study is on the role of *DGK*η in BPD, there may be a possible link between *DGK*η and ADHD in addition to BPD.

In addition to the common tests among the five representative BPD model mice described above, Neurocan-KO mice showed increased preference for saccharine, increased marble burying and increased acoustic startle (Miro *et al.* 2012). Clock-KO mice exhibited increased preference for sucrose and decreased hyponeophagia (Roybal *et al.* 2007). Glutamate receptor 6-KO mice showed increased aggression (Shaltiel *et al.* 2008). GSK3β-transgenic mice exhibited increased acoustic startle (Spittaels *et al.* 2000; Prickaerts *et al.* 2006). We performed marble burying, sucrose preference and cotton bud biting (aggression) tests. However, we have not detected significant increases in these tests so far (data not shown). Nevertheless, it is reasonable that the phenotypes of DGKη-KO mice may be moderately different from other mania model mice, because BPD and mania are multigenic disorders that result from a combination of different genetic profiles.

DGKη mRNA is expressed much more in the dentate gyrus than in the CA region of the hippocampus (Usuki *et al.* 2015). Dysgeneration of the dentate gyrus is known to be a cause of BPD (Walton *et al.* 2012; Hagihara *et al.* 2013). Therefore, it is possible that a high expression of DGKη in the dentate gyrus is involved in the pathogenesis of BPD. DGKη is also highly expressed in the Purkinje cells of the cerebellum (Usuki *et al.* 2015). Purkinje neurons are also associated with BPD (Maloku *et al.* 2010). Therefore, DGKη expression in Purkinje cells may play a role in the pathogenesis of BPD as well. Dysregulation of G-protein-coupled receptor activity is involved in the pathology of many psychiatric disorders, including BPD (Catapano and Manji 2007). Rittiner *et al.* (2014) reported that DGKη enhances G-protein-coupled receptor signaling by reducing protein kinase C activation. Therefore, DGKη may regulate pathogenesis of BPD through G-protein-coupled receptor.

Although GWAS has not implicated *DGK*β in the etiology of BPD so far, DGKβ-KO mice also exhibited mania-like phenotypes (Kakefuda *et al.* 2010; Shirai *et al.* 2010). DGKβ is widely distributed in the brain, particularly in the olfactory bulb, cerebral cortex, striatum, and hippocampus (Goto and Kondo 1993). Therefore, the expression pattern of DGKβ is different from that of DGKη (Usuki *et al.* 2015) as described above. DGKβ is stably distributed at the plasma membrane in cells (Goto and Kondo 1993; Shirai *et al.* 2010), whereas DGKη is translocated to the non-ionic detergent-resistant membrane in punctate vesicles from the cytoplasm in response to stress stimuli (Murakami *et al.* 2003; Matsutomo *et al.* 2013). Although further studies are required, these differences suggest that DGKη and DGKβ regulate BPD (mania) pathogenesis through distinct mechanisms.

Phosphatidylinositol turnover, which generates DG, has been hypothesized to play an important role in the mechanism of action of LiCl (Martinowich *et al.* 2009). DGK is one of the components of the phosphatidylinositol turnover (Goto *et al.* 2006; Sakane *et al.* 2007; Merida *et al.* 2008; Topham and Epand 2009). Moreover, we recently found that the pleckstrin domain of DGKη is strongly bound to phosphatidylinositol 4,5-bisphosphate, a product of the phosphatidylinositol turnover (Kume *et al.* 2016). We also revealed that DGKη is a unique enzyme with high affinity for DG (Komenoi *et al.* 2015). In addition, DGKη is a positive regulator of the epidermal growth factor receptor pathway (Yasuda *et al.* 2009), which drives phosphatidylinositol turnover and is implicated in BPD (Sklar *et al.* 2008). It will be interesting to determine what role DGKη plays in the phosphatidylinositol turnover-related, lithium-sensitive molecular mechanisms of BPD pathogenesis.

Thus far, there have been no indications that DGKη-KO mice cycle between mania and depression. However, future studies are needed to determine whether depression-like symptoms occur in these mice after periods of stress. Interestingly, in patients with BPD, DGKη mRNA levels were increased (Moya *et al.* 2010). Because the depression/normal state is considerably longer than the mania state in BPD patients, many of these patients were in the depression state. If this is the case, in consideration of the manic-like phenotypes of the DGKη-deficient mice (Figs 1–5), these results suggest that abnormally low and high expression levels of DGKη cause manic and depressive states of BPD, respectively. Intriguingly, Klauck *et al.* (1996) and we (Murakami *et al.* 2003) reported that a glucocorticoid treatment markedly increased the expression levels of DGKη. In addition, excess glucocorticoid is associated with human depression and anxiety disorders (Holsboer 2000; Korte 2001). It will be interesting to perform experiments using the DGKη-transgenic mice and mice with high glucocorticoid levels to verify this hypothesis.

All of the SNPs in *DGK*η (Baum *et al.* 2008; Weber *et al.* 2011; Zeng *et al.* 2011) that are implicated in the etiology of BPD by GWASs are located in introns. For example, the SNP rs1170191, which is identified in three independent reports (Baum *et al.* 2008; Weber *et al.* 2011; Zeng *et al.* 2011), is located in the first intron of *DGK*η. Therefore, it is likely that the SNPs lead to dysregulation of the expression of DGKη, which probably causes BPD as described above.

GSK3β lies downstream of diacylglycerol-dependent protein kinase C (Baum *et al.* 2008; Kaidanovich-Beilin and Woodgett 2011) and of the MEK/ERK pathway (Saito *et al.* 1994; Kaidanovich-Beilin and Woodgett 2011), and one of target proteins of lithium (Jope 2003; Zhang *et al.* 2003; Beaulieu *et al.* 2004). Transgenic mice over-expressing GSK3β display mania-like behavior (Prickaerts *et al.* 2006). Moreover, the decreases in levels of phosphorylated GSK3β were also observed in the brains of BPD (mania) model mice such as dopamine transporter-KO (Beaulieu *et al.* 2004) and DGKβ-KO mice (Kakefuda *et al.* 2010). Therefore, to analyze the mechanisms of mania-like behavior generation in DGKη-KO mice, we assessed the phosphorylation (inactivation) level of GSK3β in the cerebral cortex and the cerebellum. We confirmed that the phosphorylation levels of GSK3β (Ser9) were significantly decreased in the cerebral cortex and the cerebellum of DGKη-KO mice (Fig. 6). The results strongly suggest that DGKη regulates behavior and mood through, at least in part, GSK3β signaling and further support the linkage between DGKη and BPD. GSK3β was reported to be phosphorylated and inactivated by the Raf/MEK/ERK pathway (Saito *et al.* 1994; Kaidanovich-Beilin and Woodgett 2011). Interestingly, the Raf/MEK/ERK pathway is activated by DGKη (Yasuda *et al.* 2009). Therefore, it is possible that DGKη modifies GSK3β activity through the regulation of the Raf/MEK/ERK pathway.

Taken together, it is strongly suggested that DGKη is one of the key enzymes of the etiology of BPD and that the DGKη-KO mice would represent a bona fide model of human mania. Our findings facilitate the design and

development of novel therapeutic strategies for BPD. For example, DGKη-specific inhibitors may be valuable as novel drugs for depressive episodes of BPD. However, the molecular mechanism underlying the behavioral effects of DGKη deficiency remains to be elucidated. Further studies will be necessary to explore this issue.

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Generation of DGKη-deficient mice by homologous recombination.

Figure S2. Expression of DGK isozymes in WT and DGKη-KO mouse brains.

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