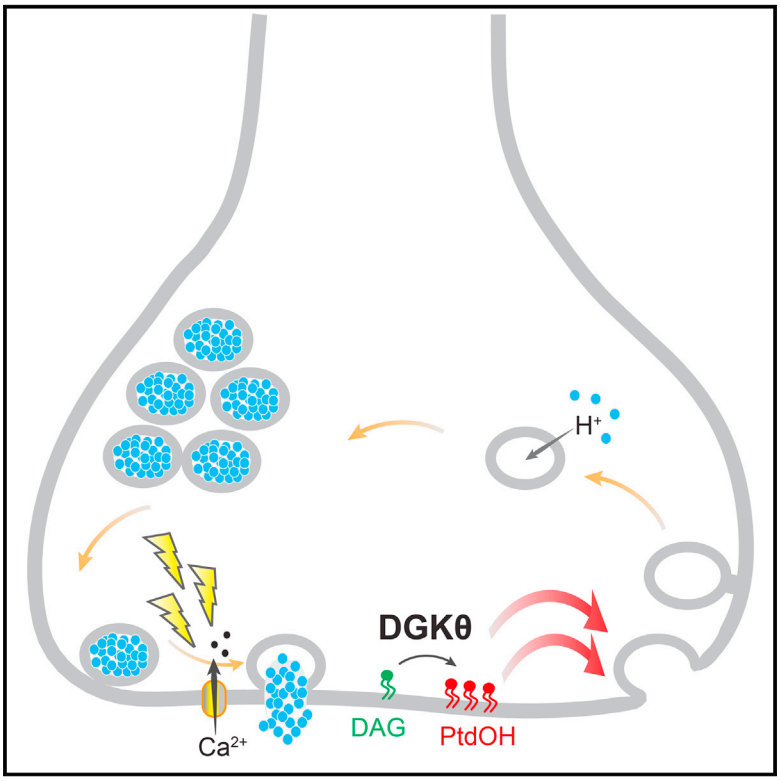
Report

Cell Reports

DGKθ Catalytic Activity Is Required for Efficient Recycling of Presynaptic Vesicles at Excitatory Synapses

Graphical Abstract



Authors

Hana L. Goldschmidt, Becky Tu-Sekine, Lenora Volk, Victor Anggono, Richard L. Huganir, Daniel M. Raben

Correspondence

rhuganir@jhmi.edu (R.L.H.), draben@jhmi.edu (D.M.R.)

In Brief

Goldschmidt et al. identified a cellular role for the lipid kinase DGKθ in the mammalian brain. DGKθ was found to localize to excitatory synapses where its activity is required cell autonomously to promote efficient retrieval of synaptic vesicles following sustained neuronal activity.

Highlights

- DGKθ functions at excitatory synapses of the mammalian CNS
- Loss of DGKθ slows presynaptic vesicle retrieval following neuronal stimulation
- DGKθ catalytic activity promotes efficient synaptic vesicle recycling in neurons

Goldschmidt et al., 2016, Cell Reports *14*, 200–207
January 12, 2016
http://dx.doi.org/10.1016/j.celrep.2015.12.022

CellPress

OPEN ACCESS
CellPress

Cell Reports
Report

DGKθ Catalytic Activity Is Required for Efficient Recycling of Presynaptic Vesicles at Excitatory Synapses

Hana L. Goldschmidt,[1] Becky Tu-Sekine,[1] Lenora Volk,[2] Victor Anggono,[3] Richard L. Huganir,[2,*] and Daniel M. Raben[1,*]
[1]Department of Biological Chemistry, Johns Hopkins University School of Medicine, Hunterian 503, 725 N. Wolfe Street, Baltimore, MD 21205, USA
[2]Department of Neuroscience, Johns Hopkins University School of Medicine, Hunterian 1001, 725 N. Wolfe Street, Baltimore, MD 21205, USA
[3]Clem Jones Centre for Ageing Dementia Research, Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia
*Correspondence: rhuganir@jhmi.edu (R.L.H.), draben@jhmi.edu (D.M.R.)
http://dx.doi.org/10.1016/j.celrep.2015.12.022

SUMMARY

Synaptic transmission relies on coordinated coupling of synaptic vesicle (SV) exocytosis and endocytosis. While much attention has focused on characterizing proteins involved in SV recycling, the roles of membrane lipids and their metabolism remain poorly understood. Diacylglycerol, a major signaling lipid produced at synapses during synaptic transmission, is regulated by diacylglycerol kinase (DGK). Here, we report a role for DGKθ in the mammalian CNS in facilitating recycling of presynaptic vesicles at excitatory synapses. Using synaptophysin- and vGlut1-pHluorin optical reporters, we found that acute and chronic deletion of DGKθ attenuated the recovery of SVs following neuronal stimulation. Rescue of recycling kinetics required DGKθ kinase activity. Our data establish a role for DGK catalytic activity at the presynaptic nerve terminal in SV recycling. Altogether, these data suggest that DGKθ supports synaptic transmission during periods of elevated neuronal activity.

INTRODUCTION

Efficient communication between neurons is essential for proper brain function. This process is triggered by Ca^{2+}-influx into presynaptic nerve terminals, resulting in fusion of synaptic vesicles (SVs) with the plasma membrane (exocytosis) and release of neurotransmitters into the synaptic cleft. A typical nerve terminal contains a relatively small number of vesicles, enough to maintain about 5–10 s of neurotransmission. Thus after exocytosis, SVs must be retrieved and recycled by endocytosis in order to maintain synaptic transmission (Südhof, 2004). This becomes particularly critical during periods of elevated neuronal activity, where multiple SVs undergo exocytosis over a short period of time (Cheung et al., 2010). SV recycling is therefore essential for neuronal function, and its dysregulation may contribute to several neurological and psychiatric disorders (Kavalali, 2006).

Despite its being a well-studied cellular process, the mechanisms that mediate the steps of the SV cycle, particularly those involved in endocytosis, remain a matter of debate. To date, four mechanisms of SV endocytosis have been described: (1) clathrin-mediated endocytosis (CME), (2) activity-dependent bulk endocytosis (ADBE) (Cheung et al., 2010), (3) kiss-and-run (Südhof, 2004), and (4) ultra-fast-endocytosis (Watanabe et al., 2013). These pathways are differentially utilized depending on the strength and duration of neuronal activity, as well as differ in their molecular machinery, speed, and capacity for membrane retrieval (Clayton and Cousin, 2009; Kononenko and Haucke, 2015; Südhof, 2004; Watanabe et al., 2013; Wu et al., 2014).

Numerous proteins regulate SV endocytosis in mammalian central neurons (Haucke et al., 2011). Equally important, the lipid composition of the presynaptic membrane plays an active role in this process. Of the membrane lipids studied so far, phosphoinositides have the most well established role in SV endocytosis (Puchkov and Haucke, 2013; Rohrbough and Broadie, 2005). Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) modulates SV recycling by recruiting and activating key molecules, such as synaptotagmin I (Chapman, 2008), clathrin adaptor protein AP2 and dynamin-1 (Burger et al., 2000; Di Paolo et al., 2004) to the presynaptic membrane. Genetic deletions of the lipid kinase (phosphatidylinositol phosphate kinase type Iγ, PIPK1γ) (Di Paolo et al., 2004), or the lipid phosphatase (synaptojanin 1) (Cremona et al., 1999; Mani et al., 2007) that mediate the generation and metabolism of PtdIns(4,5)P_2, respectively, result in multiple synaptic defects, including impaired SV recycling. PtdIns(4,5)P_2 is also a substrate for phospholipase C, which produces the signaling lipid, diacylglycerol (DAG).

DAG has been implicated in synaptic function and may play at least three roles in the SV cycle (Tu-Sekine and Raben, 2011). First, DAG enhances the activity of Munc13-1, which mediates the priming of SVs, a crucial step in SV exocytosis during spontaneous and evoked synaptic transmission (Augustin et al., 1999; Bauer et al., 2007). Second, DAG activates protein kinase C (PKC), which phosphorylates and thereby regulates the activities of presynaptic SNARE complex proteins, including Munc-18 and SNAP-25 (Di Paolo et al., 2004; Rhee et al., 2002). Finally, termination of DAG signaling through its phosphorylation by DAG kinases (DGKs) results in the production of phosphatidic acid (PtdOH), an acidic phospholipid which can serve as a signaling

molecule itself as well as a precursor for the generation of PtdIns(4,5)P_2 (Antonescu et al., 2010; Luo et al., 2004).

Despite the importance of DAG and PtdOH in SV recycling, not much is known regarding the roles of DGKs in regulating SV recycling and presynaptic function. Understanding these functions is complicated by the fact there are ten mammalian DGK isoforms (α, β, γ, δ, ε, ζ, η, θ, ι, κ), all of which possess the same catalytic activity, are expressed in the CNS, and nine of the ten isoforms are found in neurons (Mérida et al., 2008; Tu-Sekine and Raben, 2011). Several functional studies have implicated individual DGK isoforms (β, ζ, ε, η) in modulating spine dynamics, neuronal plasticity, and neurological disorders (Kakefuda et al., 2010; Kim et al., 2010; Musto and Bazan, 2006; Shirai et al., 2010). However, the roles of DGK isoforms localized to the presynaptic terminal are less well understood. Indeed, DGKι is the only isoform that has been implicated in presynaptic release (Yang et al., 2011). The notion that this family of enzymes might play a role in regulating SV recycling is supported by studies in *C. elegans* that found that DGK-1 activity suppressed acetylcholine release from the neuromuscular junction (McMullan et al., 2006; Nurrish et al., 1999). The ortholog of DGK-1 in mammals is DGKθ, the only type V DGK isoform and whose function has never been described in the nervous system.

Here, we report that DGKθ plays an important role in modulating SV recycling in mammalian central neurons. Both shRNA-mediated knockdown of DGKθ and neurons derived from DGKθ knockout (KO) mice exhibit a decreased rate of SV endocytosis compared to wild-type neurons. Importantly, this defect can only be rescued by ectopic expression of enzymatically active DGKθ but not a kinase-dead mutant of the enzyme. Our data establish a role for DGKθ kinase activity in the regulation of SV recycling and suggest that DGKθ supports synaptic transmission during periods of sustained neuronal activity.

RESULTS

DGKθ Localizes to Excitatory Synapses in the Mouse Forebrain

DGKθ expression has been detected in multiple regions of the late embryonic (Ueda et al., 2013) and adult mouse brain (Houssa et al., 1997; Tu-Sekine and Raben, 2011), albeit at low cellular and temporal resolution. In order to study the role of DGKθ in brain function, we first examined DGKθ expression across multiple brain regions in adult mice. Western blot analysis using an isoform-specific antibody that recognizes the C-terminal region of DGKθ revealed wide-spread expression of DGKθ protein in all brain regions examined, including the cortex and hippocampus (Figure 1A), which is consistent with the previously reported mRNA expression pattern (Houssa et al., 1997). DGKθ was detected in the forebrain but not in cultured glial cells prepared from the same brain region (Figure 1B), indicating a specific expression of DGKθ in neuronal cells. The expression of DGKθ was upregulated during development, which peaked at postnatal day 14 (P14), coincided with the expression of synaptophysin, an integral SV protein with an established function at synapses (Figure 1C). A similar increase in DGKθ protein during synapse formation and development was also observed in cultured neurons (DIV 7–14, data not shown). Subcellular fractionation analysis of an adult mouse brain showed a general distribution of DGKθ in the cytosolic, microsomal, and synaptosomal fractions (Figure 1D). DGKθ was also detected in the post-synaptic density fraction, albeit at a lower level, suggesting its presence at both pre- and postsynaptic sites.

To verify the biochemical results we examined endogenous DGKθ localization by immunofluorescence microscopy. Consistent with fractionation experiments, DGKθ was detected throughout the neuron, including the soma, MAP2-positive dendrites, and MAP2-negative axons (Figures 1E and 1F). Strikingly, we found that DGKθ had a punctate distribution along dendrites that significantly overlapped with the excitatory presynaptic protein vGlut1 (Figures 1E and 1F). Quantification of the overlap between these two signals showed that 62.6% ± 1.4% of DGKθ overlapped with vGlut1 and 56.6% ± 1.5% of vGlut1 overlapped with DGKθ per micrometer of dendrite (Figure 1G). In contrast, only 10.0% ± 0.7% of DGKθ overlapped with the inhibitory presynaptic protein VGAT per micrometer of dendrite (Figures 1F and 1G), suggesting that DGKθ preferentially localizes to excitatory synapses.

Knockdown of DGKθ Slows the Rate of Synaptic Vesicle Recycling

DGKθ localization to excitatory synapses and the onset of its expression during synaptogenesis suggested a potential role for this enzyme in excitatory synaptic transmission. Given the role of the DGKθ ortholog in *C. elegans* (McMullan et al., 2006; Nurrish et al., 1999), we predicted that DGKθ might possess a similar synaptic function at the presynaptic terminal. To test this, we generated a short-hairpin RNA construct directed against endogenous DGKθ mRNA (Figure 2A) to suppress DGKθ protein expression. Western blot analysis showed that lentiviral-mediated expression of DGKθ-shRNA in cortical neurons resulted in >90% knockdown of DGKθ protein compared to control shRNA-infected cells (Figure 2B).

To determine the effect of reduced DGKθ protein levels on presynaptic function, we used the pH-sensitive optical reporter synaptophysin-pHluorin (sypHy; Granseth et al., 2006) to monitor SV recycling dynamics. Cortical neurons were co-transfected with sypHy and DGKθ-shRNA or a control shRNA and allowed to express for 48 hr prior to measuring SV recycling kinetics. Knockdown of DGKθ significantly slowed the rate of SV endocytosis following KCl-induced neuronal depolarization compared to control neurons (Figures 2C and 2D; τ = 59.2 ± 4.2 s and 32.4 ± 1.9 s, respectively). Moreover, expression of shRNA-resistant human DGKθ (^{myc}DGKθ) was sufficient to rescue the observed defect in endocytosis in neurons expressing DGKθ-shRNA, thus verifying the specificity of DGKθ-shRNA in our study (Figure 2D; τ = 34.9 ± 4 s). Importantly, similar results were obtained when neurons were stimulated with a train of 300 action potentials (APs, 10Hz), supporting the role of DGKθ in regulating SV recycling in central neurons (Figures 2E, 2F, and S1A). To confirm that the effect of DGKθ knockdown was not due to a specific defect in the sorting of a particular SV protein (synaptophysin in this case), we measured SV recycling kinetics using a different pHluorin reporter, vGlut1-pHluorin

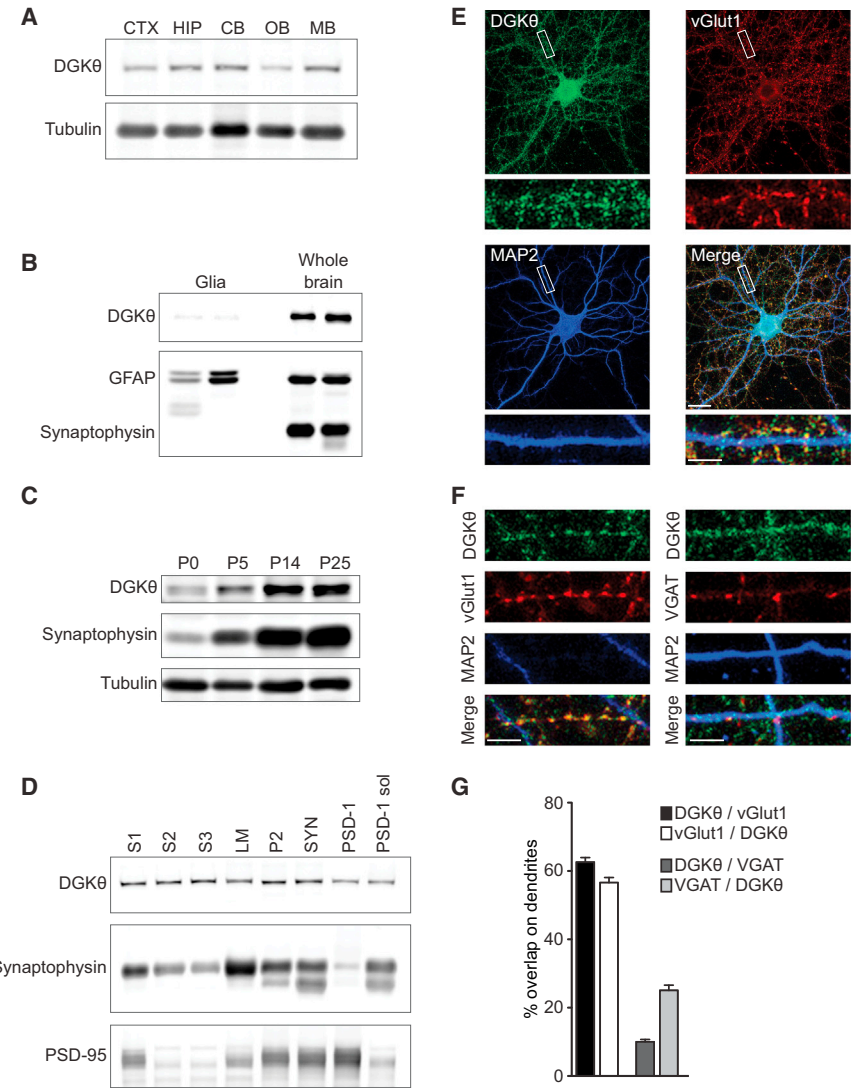


Figure 1. DGKθ Is an Excitatory Synaptic Protein

(A) Protein extracts prepared from adult mouse cortex (CTX), hippocampus (HIP), cerebellum (CB), olfactory bulb (OB), and midbrain (MB) were assayed for DGKθ protein expression by western blot using specific antibodies against DGKθ and tubulin (loading control).

(B) Whole-cell extracts from primary glial cultures and rat forebrain were blotted with specific antibodies against DGKθ, GFAP (glial marker), and synaptophysin (neuronal marker).

(C) Whole-brain lysates from mice between postnatal day 0 (P0) and 25 (P25) were assayed for DGKθ protein expression by western blot using antibodies against DGKθ, synaptophysin, and tubulin.

(D) Biochemical fractionation of adult mouse brain reveals a wide distribution of DGKθ in various subcellular compartments. Synaptophysin and PSD-95 (pre- and postsynaptic protein markers, respectively) were used as controls for successful isolation of synaptic fractions. Post-nuclear supernatant (S1), cytosol after P2 precipitation (S2), cytosol after LM precipitation (S3), light membranes (LM), crude synaptosomes/membranes (P2), synaptosomes (SYN), postsynaptic density (PSD-I), remaining soluble fraction after PSD-I precipitation (PSD-I sol). 20 μg of protein was loaded for each fraction.

(E and F) Cultured hippocampal neurons (DIV 28) were immunostained with specific antibodies against DGKθ, vGlut1 (excitatory presynaptic protein) or VGAT (inhibitory synaptic marker) and MAP2 (dendritic protein). Scale bars represent 20 μm and 5 μm (cropped region).

(G) Quantification of the average overlap between DGKθ/vGlut1 and DGKθ/VGAT staining on MAP2-positive secondary dendrites. Data represent mean ± SEM (n = 3 independent coverslips).

(vGlut1-pH, (Balaji and Ryan, 2007)). Consistent with the sypHy data, the kinetics of vGlut1-pH recycling following a train of 300 APs (10Hz) were also significantly slower in DGKθ-shRNA expressing neurons compared to controls (Figure S1B). Together, these data indicate that DGKθ is involved in the general retrieval mechanism of SVs, rather than regulating the sorting of a specific SV protein.

Because the strength of the stimulus can activate distinct recycling mechanisms (Clayton and Cousin, 2009; Kononenko and Haucke, 2015), we speculated that elevated neuronal activity may exaggerate the defect in SV recycling observed in DGKθ-knockdown neurons. As shown in Figures S1B and S1C, the delay in endocytosis kinetics of sypHy or vGlut1-pH in DGKθ knockdown neurons was larger when stimulated with trains of 600 APs (50 Hz) compared to 300 APs (10 Hz). Furthermore, a relatively smaller, but significant, delay in the rate of endocytosis was also observed when neurons were stimulated with a train of 40 APs (20Hz), which is known to mobilize primarily the readily releasable pool of SVs (Burrone et al., 2006) (Figure S1D). Taken together, these data demonstrate that DGKθ is crucial for maintaining efficient recycling of SVs via distinct pathways of membrane retrieval in a use-dependent manner.

DGKθ KO Mice Have Reduced DGK Activity in the Brain and Exhibited an Impairment in SV Recycling Efficiency

Next, we investigated the role of DGKθ with the use of a DGKθ homozygous KO mouse (see methods, Figure 3A). The genotypes and the levels of DGKθ protein expression in wild-type (WT), heterozygous (Het) and KO mice were confirmed by PCR and western blotting analyses, respectively (Figures 3A and 3B). DGKθ KO mice appeared overtly healthy and did not display any obvious gross morphological differences in body size, mating, and lifespan compared to WT mice (data not shown). In addition, we did not observe any compensatory increase in other neuronal DGK isoforms, all of which are capable of catalyzing the production of PtdOH, in brain extracts from DGKθ KO mice

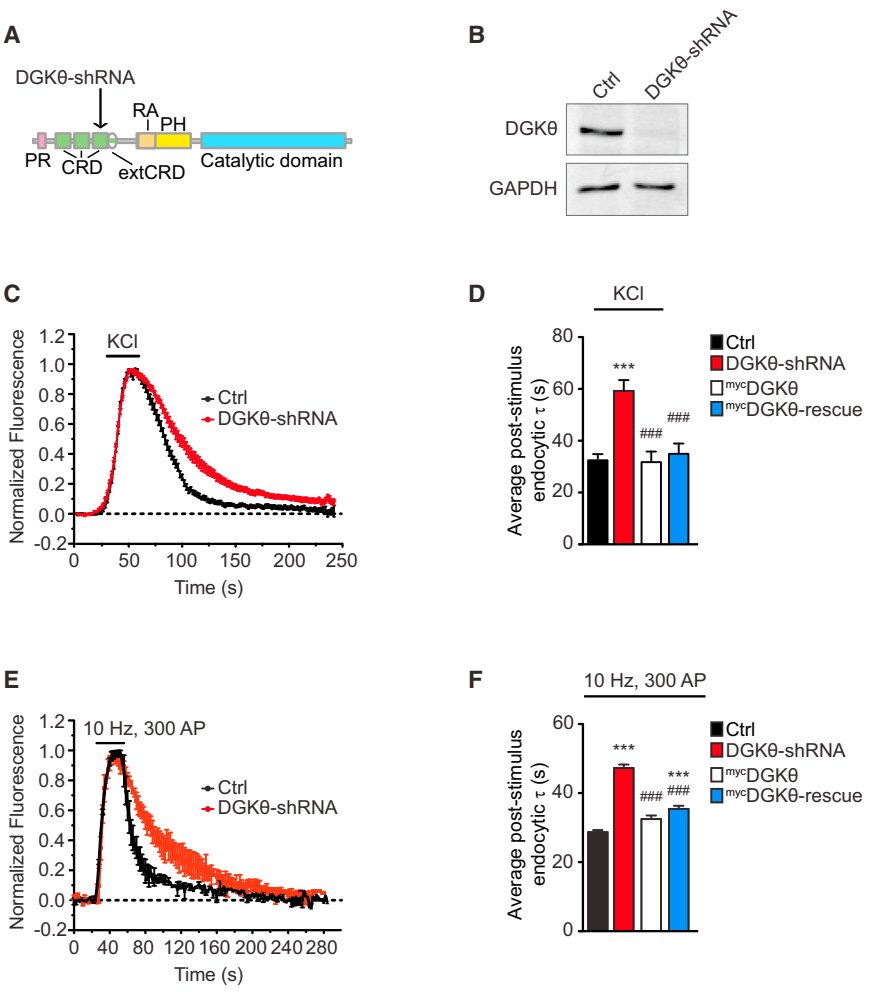


Figure 2. DGKθ Regulates the Kinetics of SV Recycling in Cortical Neurons

(A) Schematic of the domain structure of DGKθ and the relative location of the DGKθ-shRNA target.

(B) Lysates from cultured cortical neurons infected with either a control (Ctrl) or DGKθ-shRNA lentivirus were subjected to western blot analysis with antibodies against DGKθ and GAPDH (loading control).

(C) Normalized average traces from neurons expressing sypHy with either control (black) or DGKθ-shRNA (red) in response to 60 s stimulation with high K^+ buffer.

(D) Comparison of average τ values between control and DGKθ-shRNA from (C), ^{myc}DGKθ (+ctrl shRNA, gray), and ^{myc}DGKθ-rescue (green) neurons. The decay phases of the traces were fitted with single exponential functions and τ values were calculated from the fits.

(E) Normalized average traces from neurons expressing sypHy with control or DGKθ-shRNA in response to a train of 300 APs (10 Hz).

(F) Comparison of average τ values between control and DGKθ-shRNA from (E), ^{myc}DGKθ and ^{myc}DGKθ-rescue neurons in response to the 10 Hz stimulus.

For all experiments shown in Figure 2, data represent mean ± SEM from ≥100 boutons; ***p < 0.001 against Ctrl; ### p < 0.001 against DGKθ-shRNA, a one-way ANOVA with Tukey's post hoc test.

(Figure 3C). Surprisingly, we detected a significant decrease in the total DGK activity measured in protein extracts from adult KO forebrain compared to WT (Figure 3D; Tu-Sekine and Raben, 2012). These data highlight an important role for DGKθ in the production of PtdOH in the brain.

To confirm the role of DGKθ in regulating the kinetics of SV recycling, we measured the efficiency of sypHy retrieval in neurons derived from WT and DGKθ KO mice. Consistent with our previous results, the rate of endocytosis was significantly slower in KO neurons following a train of 600 APs compared to WT controls (Figures 4A and 4B; τWT = 27.9 ± 0.8 s versus τKO = 60.2 ± 1.1 s). The endocytic defect in KO neurons was accompanied by a significant increase in sypHy fluorescence intensity 200 s post-stimulation (Figure S2B), indicating the accumulation of uninternalized SV proteins on the cell surface. We also ruled out the role of DGKθ in regulating SV exocytosis in neurons (Figures S2C–S2E). Importantly, expression of ^{myc}DGKθ completely rescued the endocytic defect in KO neurons (Figure 4B; τ^{myc}DGKθ = 37.0 ± 1.1 s).

Due to the significant reduction of total DGK activity in DGKθ KO brain tissue, we predicted that the catalytic activity of DGKθ might be essential for promoting efficient recycling of SVs. To test this hypothesis, we transiently transfected KO neurons with ^{myc}DGKθ harboring the G648A point mutation that renders the enzyme catalytically inactive (Los et al., 2004) . Consistent with our hypothesis, expression of the ^{myc}DGKθ kinase-dead mutant (^{myc}DGKθ-kd) failed to rescue the delay in SV recycling kinetics in KO neurons (Figure 4B; τ^{myc}DGKθ-kd = 62.1 ± 1.5 s). This was not due to the low expression or mis-targeting of the mutant as ^{myc}DGKθ-kd colocalized with sypHy along neuronal processes (Figure 4C). Taken together, these data demonstrate that DGKθ catalytic activity is necessary for efficient recycling of SVs following neuronal activity.

Finally, we evaluated the effects of frequency and duration of stimuli on the rate of endocytosis. Whereas WT neurons displayed comparable endocytic time constants across various neuronal stimuli, DGKθ KO neurons displayed a significant augmentation of the SV recycling defect with increases in the strength and frequency of neuronal stimulation (Figures 4D–4F). Thus, we conclude that during periods of sustained neuronal activity, when more APs are fired, DGKθ plays a more critical role in promoting efficient retrieval of SVs.

DISCUSSION

Despite speculations regarding the roles of mammalian DGKs in synaptic transmission, the function of DGKθ in the brain has

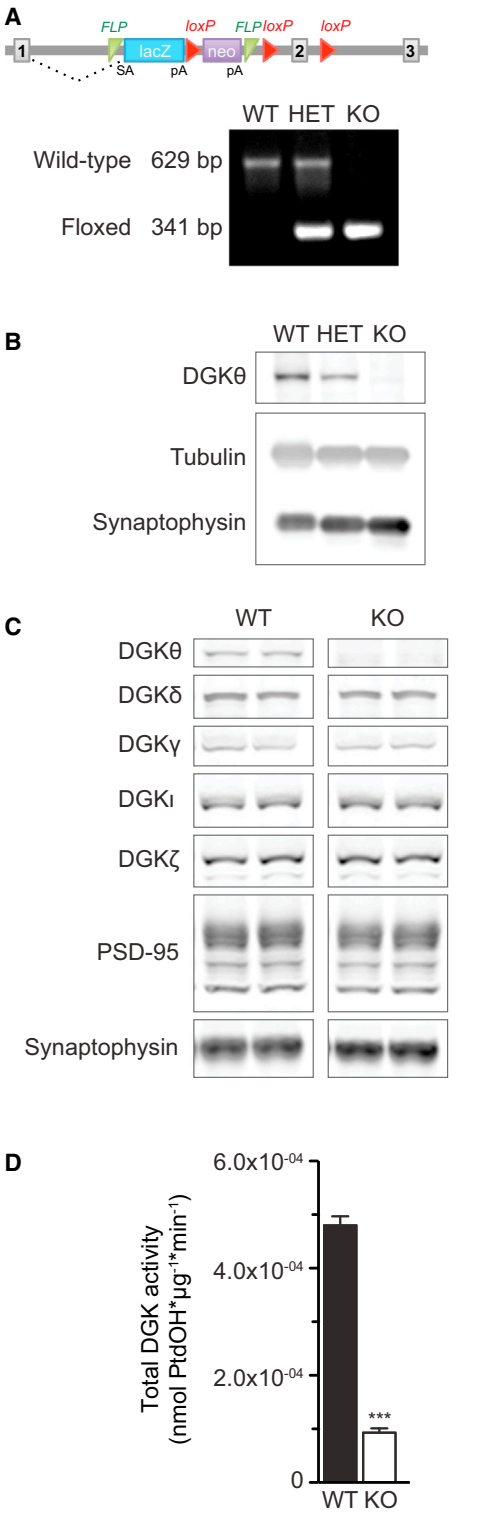


Figure 3. Total DGK Activity Is Reduced in DGKθ KO Mice

(A) Top: schematic of DGKθ KO allele (Dgkq$^{tm1a(KOMP)Wtsi}$). Exon 1 is spliced to the artificial splice acceptor (SA) in front of lacZ instead of another exon in the DGKθ gene. The poly-adenylation site (pA) terminates transcription after lacZ, preventing the transcription of the DGKθ RNA. Bottom: typical result of PCR for genotyping. Bands at 629bp and 341bp are indicative of DGKθ WT and KO alleles, respectively.

(B) Western blot analysis of protein extracts prepared from DGKθ WT, HET, and KO mouse brain tissue confirms the loss of DGKθ protein in KO lysates. Tubulin and synaptophysin blot showed equal loading of protein lysates.

(C) Western blot analysis of brain tissue isolated from two pairs of WT and KO mice run on the same gel. Samples were immunoblotted with antibodies against DGKθ, -γ, -ι, -ζ, representing four classes of DGKs. Synaptophysin and PSD-95 blots showed equal loading of protein lysates.

(D) Average total DGK activity measured in vitro in 5 µg of S1 fractions from five pairs of age-matched WT and KO forebrain tissues. Averages include three technical replicates per sample. Error bars represent SEM. Student's t test, ***$p < 0.0001$ against WT.

remained unknown. In this study, we tested the hypothesis that DGKθ modulates neurotransmitter release at central synapses. We found that DGKθ protein expression is elevated during synaptogenesis and localizes specifically to excitatory synapses. Both acute and chronic loss of DGKθ slowed SV retrieval following neuronal stimulation. The endocytic defect could be rescued by ectopic expression of WT, but not catalytically inactive DGKθ, thus implicating DGKθ enzymatic activity in promoting the efficient recycling of SVs following neuronal activity.

A potential consequence of reduced synaptic DGK activity observed in DGKθ KO mice could be elevated levels of DAG in the plasma membrane. Since functional analogs of DAG are known to potentiate synaptic transmission (Rhee et al., 2002), we hypothesized that the slowed recycling kinetics measured in DGKθ KO neurons could be the result of augmented SV exocytosis. However, the rate of SV exocytosis reported by sypHy, assayed in the presence of the vesicular ATPase inhibitor, bafilomycin A1, was essentially identical in WT and KO neurons (Figure S2). These findings suggest that the SV recycling defect observed in DGKθ KO neurons is not secondary to altered exocytosis, and argues that DGKθ directly regulates the rate of SV endocytosis.

If DGKθ is regulating a distinct pool of DAG, not relevant for SV exocytosis, it raises the intriguing possibility that it is the PtdOH produced by DGKθ, rather than its consumption of DAG, that is crucial for maintaining efficient SV recycling. This notion is corroborated by the fact that DGKθ is responsible for generating a significant amount of PtdOH and, potentially PtdIns, in the brain. Interestingly, the role of DGKθ becomes much more prominent during intense neuronal stimulation, presumably due to an increase demand of PtdOH production at synapses. Consistent with this notion, previous studies in non-neuronal cells have shown that PtdOH production by DGKs as well as phospholipase D is important for clathrin-mediated endocytosis (Antonescu et al., 2010; Kawasaki et al., 2008; Los et al., 2004). Although our data implicate a role for DGK-mediated PtdOH production in SV endocytosis at central synapses, the involvement of a PLD cannot be ruled out. A deeper understanding of the distinct mechanisms used by individual lipid-metabolizing enzymes within the presynaptic terminal and how these pathways are integrated to ensure lipid homeostasis and efficient neuronal function will be critical for understanding the molecular basis of synaptic transmission as well as neurological diseases.

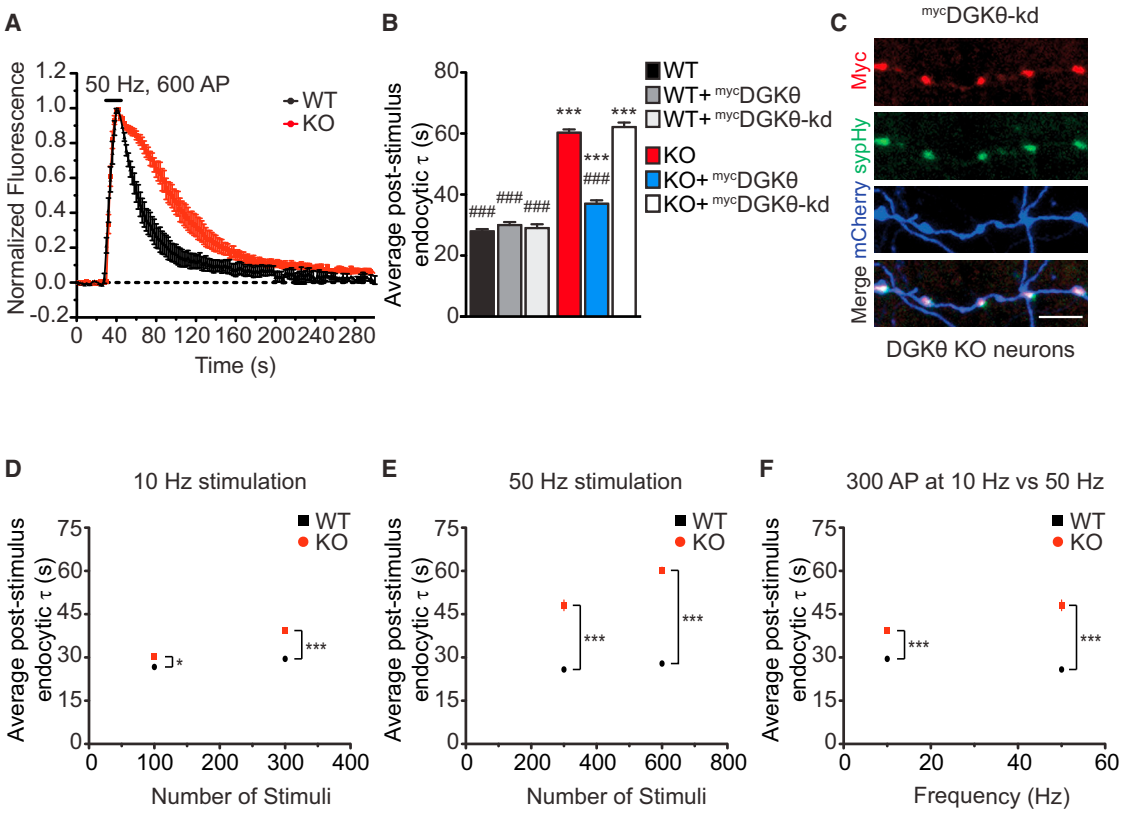


Figure 4. DGKθ Enzymatic Activity Is Required for Efficient SV Recycling

(A) Normalized average traces from WT (black) and KO (red) neurons expressing sypHy in response to a train of 600 APs (50 Hz).

(B) Comparison of average τ_{endo} values between WT and KO neurons expressing empty vector from (A), ^{myc}DGKθ, or kinase-dead DGKθ (^{myc}DGKθ-kd) in response the 50 Hz stimulus.

(C) DGKθ KO neurons (DIV20) expressing sypHy, mCherry, ^{myc}DGKθ-kd were stained with anti-myc (red), anti-GFP (green), and anti-mCherry (blue) antibodies. Merge image (bottom) shows ^{myc}DGKθ-kd colocalizes with sypHy reporter. Scale bar represents 5 μm.

(D–F) Defect in SV recycling is exaggerated with increasing number of stimuli (D and E) and higher frequency stimulation (F). (D) Average τ values measured in WT and KO neurons following trains of 100 or 300 APs delivered at 10 Hz. (E) Average τ values measured in WT and KO neurons following trains of 300 or 600 APs delivered at 50 Hz. (F) Comparison of the average τ values from (D) and (E) following a train of 300 APs delivered at 10 Hz and 50 Hz.

For all experiments shown in Figure 4, data represent ± SEM from ≥200 boutons per condition; ***p < 0.001, against WT, ### p < 0.001 against KO, ANOVA with Tukey's post hoc test.

EXPERIMENTAL PROCEDURES

Animals

The DGKθ KO mouse (Dgkq$^{tm1a(KOMP)Wtsi}$) was obtained from the KOMP Repository. Sprague Dawley rats were used to generate embryos for neuronal cultures. All animals were treated in accordance with the Johns Hopkins University Animal Care and Use Committee guidelines.

Neuronal Culture and Transfection

Cortical neurons from E18 rat or E17 mouse pups were plated onto poly-L-lysine coated dishes or 18 mm coverslips in Neurobasal growth medium supplemented with 2%B27, 2 mM Glutamax, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5% Horse serum. FDU was added at days in vitro (DIV) 4 and neurons were maintained in glial-conditioned growth medium (1% serum) and fed twice a week. Neurons were transfected at DIV 11-12 using lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. SypHy and vGlut1-pH live-cell imaging was performed DIV 14–17.

Live-Cell Imaging

Coverslips containing neurons were mounted into a custom-built perfusion chamber and held at 37°C on the heated microscope stage. Cells were continuously perfused at with pre-warmed ACSF (in millimoles: 122.5, NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 30 $_D$-glucose, 25 HEPES [pH 7.4]) and imaged at 0.5 Hz through a 40× (1.6 NA) oil objective using a Zeiss spinning-disk confocal microscope. SypHy and vGlut1-pH fluorescence was imaged at 488 nm excitation and collected through a 505–550 nm filter, whereas mCherry signal was imaged at 561 nm excitation and 575–615 nm emission. For KCl stimulation (ACSF with 50 mM KCl, 75 mM NaCl), 1 μM tetrodotoxin (TTX) was added to all buffers. For field stimulation, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM D,L-2 amino-5-phosphonovaleric acid (AP5) were added to the ACSF instead of TTX. APs were delivered using platinum wires embedded in the imaging chamber at 100 mA and 1 ms pulse width. Quantitative imaging analyses were performed with ImageJ using the time-series plugin (Granseth et al., 2006), and the data were fitted using Prism 5 software (GraphPad Software). See Supplemental Experimental Procedures for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.022.

AUTHOR CONTRIBUTIONS

Conceptualization, H.L.G, B.T. and D.M.R, Methodology, H.L.G, V.A., R.L.H. and D.M.R., Investigation, H.L.G, V.A., B.T. and L.V., Writing - Original Draft, H.L.G, R.L.H. and D.M.R., Writing - Review & Editing draft, H.L.G, V.A., B.T., L.V., R.L.H. and D.M.R., Funding Acquisition, H.L.G, V.A., R.L.H. and D.M.R., Supervision, R.L.H. and D.M.R.

ACKNOWLEDGMENTS

The authors thank Brian Loeper and Rebecca White for assistance in generating DGKθ KO mice; Seth Margolis, Michael Wolfgang, and members of the R.L.H. lab for critical discussions. This work was supported by RO1NS077923 (to D.M.R.), T32GM007445 (to H.L.G., BCMB Training Grant), RO1NS036715 (to R.L.H.), and the John T. Reid Charitable Trusts (to V.A.).

Received: January 14, 2015
Revised: October 27, 2015
Accepted: November 25, 2015
Date: December 31, 2015

REFERENCES

Antonescu, C.N., Danuser, G., and Schmid, S.L. (2010). Phosphatidic acid plays a regulatory role in clathrin-mediated endocytosis. Mol. Biol. Cell *21*, 2944–2952.

Augustin, I., Rosenmund, C., Südhof, T.C., and Brose, N. (1999). Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature *400*, 457–461.

Balaji, J., and Ryan, T.A. (2007). Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. Proc. Natl. Acad. Sci. USA *104*, 20576–20581.

Bauer, C.S., Woolley, R.J., Teschemacher, A.G., and Seward, E.P. (2007). Potentiation of exocytosis by phospholipase C-coupled G-protein-coupled receptors requires the priming protein Munc13-1. J. Neurosci. *27*, 212–219.

Burger, K.N., Demel, R.A., Schmid, S.L., and de Kruijff, B. (2000). Dynamin is membrane-active: lipid insertion is induced by phosphoinositides and phosphatidic acid. Biochemistry *39*, 12485–12493.

Burrone, J., Li, Z., and Murthy, V.N. (2006). Studying vesicle cycling in presynaptic terminals using the genetically encoded probe synaptopHluorin. Nat. Protoc. *1*, 2970–2978.

Chapman, E.R. (2008). How does synaptotagmin trigger neurotransmitter release? Annu. Rev. Biochem. *77*, 615–641.

Cheung, G., Jupp, O.J., and Cousin, M.A. (2010). Activity-dependent bulk endocytosis and clathrin-dependent endocytosis replenish specific synaptic vesicle pools in central nerve terminals. J. Neurosci. *30*, 8151–8161.

Clayton, E.L., and Cousin, M.A. (2009). The molecular physiology of activity-dependent bulk endocytosis of synaptic vesicles. J. Neurochem. *111*, 901–914.

Cremona, O., Di Paolo, G., Wenk, M.R., Lüthi, A., Kim, W.T., Takei, K., Daniell, L., Nemoto, Y., Shears, S.B., Flavell, R.A., et al. (1999). Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell *99*, 179–188.

Di Paolo, G., Moskowitz, H.S., Gipson, K., Wenk, M.R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R.M., Ryan, T.A., and De Camilli, P. (2004). Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature *431*, 415–422.

Granseth, B., Odermatt, B., Royle, S.J., and Lagnado, L. (2006). Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron *51*, 773–786.

Haucke, V., Neher, E., and Sigrist, S.J. (2011). Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. Nat. Rev. Neurosci. *12*, 127–138.

Houssa, B., Schaap, D., van der Wal, J., Goto, K., Kondo, H., Yamakawa, A., Shibata, M., Takenawa, T., and van Blitterswijk, W.J. (1997). Cloning of a novel human diacylglycerol kinase (DGKtheta) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping Ras-associating domain. J. Biol. Chem. *272*, 10422–10428.

Kakefuda, K., Oyagi, A., Ishisaka, M., Tsuruma, K., Shimazawa, M., Yokota, K., Shirai, Y., Horie, K., Saito, N., Takeda, J., and Hara, H. (2010). Diacylglycerol kinase β knockout mice exhibit lithium-sensitive behavioral abnormalities. PLoS ONE *5*, e13447.

Kavalali, E.T. (2006). Synaptic vesicle reuse and its implications. Neuroscientist *12*, 57–66.

Kawasaki, T., Kobayashi, T., Ueyama, T., Shirai, Y., and Saito, N. (2008). Regulation of clathrin-dependent endocytosis by diacylglycerol kinase delta: importance of kinase activity and binding to AP2alpha. Biochem. J. *409*, 471–479.

Kim, K., Yang, J., and Kim, E. (2010). Diacylglycerol kinases in the regulation of dendritic spines. J. Neurochem. *112*, 577–587.

Kononenko, N.L., and Haucke, V. (2015). Molecular mechanisms of presynaptic membrane retrieval and synaptic vesicle reformation. Neuron *85*, 484–496.

Los, A.P., van Baal, J., de Widt, J., Divecha, N., and van Blitterswijk, W.J. (2004). Structure-activity relationship of diacylglycerol kinase theta. Biochim. Biophys. Acta *1636*, 169–174.

Luo, B., Prescott, S.M., and Topham, M.K. (2004). Diacylglycerol kinase zeta regulates phosphatidylinositol 4-phosphate 5-kinase Ialpha by a novel mechanism. Cell. Signal. *16*, 891–897.

Mani, M., Lee, S.Y., Lucast, L., Cremona, O., Di Paolo, G., De Camilli, P., and Ryan, T.A. (2007). The dual phosphatase activity of synaptojanin1 is required for both efficient synaptic vesicle endocytosis and reavailability at nerve terminals. Neuron *56*, 1004–1018.

McMullan, R., Hiley, E., Morrison, P., and Nurrish, S.J. (2006). Rho is a presynaptic activator of neurotransmitter release at pre-existing synapses in C. elegans. Genes Dev. *20*, 65–76.

Mérida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. Biochem. J. *409*, 1–18.

Musto, A., and Bazan, N.G. (2006). Diacylglycerol kinase epsilon modulates rapid kindling epileptogenesis. Epilepsia *47*, 267–276.

Nurrish, S., Ségalat, L., and Kaplan, J.M. (1999). Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. Neuron *24*, 231–242.

Puchkov, D., and Haucke, V. (2013). Greasing the synaptic vesicle cycle by membrane lipids. Trends Cell Biol. *23*, 493–503.

Rhee, J.S., Betz, A., Pyott, S., Reim, K., Varoqueaux, F., Augustin, I., Hesse, D., Südhof, T.C., Takahashi, M., Rosenmund, C., and Brose, N. (2002). Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell *108*, 121–133.

Rohrbough, J., and Broadie, K. (2005). Lipid regulation of the synaptic vesicle cycle. Nat. Rev. Neurosci. *6*, 139–150.

Shirai, Y., Kouzuki, T., Kakefuda, K., Moriguchi, S., Oyagi, A., Horie, K., Morita, S.-Y., Shimazawa, M., Fukunaga, K., Takeda, J., et al. (2010). Essential role of neuron-enriched diacylglycerol kinase (DGK), DGKbeta in neurite spine formation, contributing to cognitive function. PLoS ONE *5*, e11602.

Südhof, T.C. (2004). The synaptic vesicle cycle. Annu. Rev. Neurosci. *27*, 509–547.

Tu-Sekine, B., and Raben, D.M. (2011). Regulation and roles of neuronal diacylglycerol kinases: a lipid perspective. Crit. Rev. Biochem. Mol. Biol. *46*, 353–364.

Tu-Sekine, B., and Raben, D.M. (2012). Dual regulation of diacylglycerol kinase (DGK)-θ: polybasic proteins promote activation by phospholipids and increase substrate affinity. J. Biol. Chem. *287*, 41619–41627.

Ueda, S., Tu-Sekine, B., Yamanoue, M., Raben, D.M., and Shirai, Y. (2013). The expression of diacylglycerol kinase theta during the organogenesis of mouse embryos. BMC Dev. Biol. *13*, 35.

Watanabe, S., Rost, B.R., Camacho-Pérez, M., Davis, M.W., Söhl-Kielczynski, B., Rosenmund, C., and Jorgensen, E.M. (2013). Ultrafast endocytosis at mouse hippocampal synapses. Nature *504*, 242–247.

Wu, Y., O'Toole, E.T., Girard, M., Ritter, B., Messa, M., Liu, X., McPherson, P.S., Ferguson, S.M., and De Camilli, P. (2014). A dynamin 1-, dynamin 3- and clathrin-independent pathway of synaptic vesicle recycling mediated by bulk endocytosis. eLife *3*, e01621.

Yang, J., Seo, J., Nair, R., Han, S., Jang, S., Kim, K., Han, K., Paik, S.K., Choi, J., Lee, S., et al. (2011). DGKι regulates presynaptic release during mGluR-dependent LTD. EMBO J. *30*, 165–180.