Comprehensive Supplemental Information Document

DGKθ catalytic activity is required for efficient recycling of presynaptic vesicles at excitatory synapses

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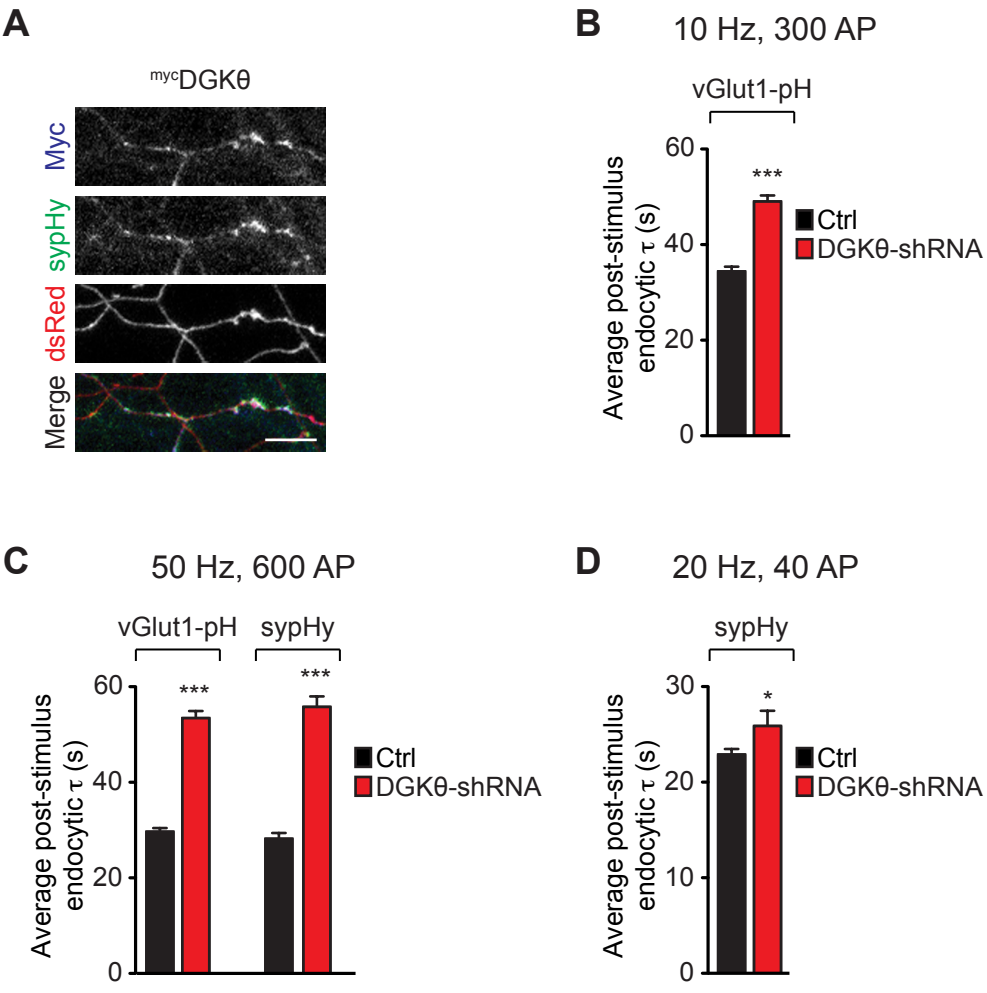

Figure S1 (related to Figure 2)

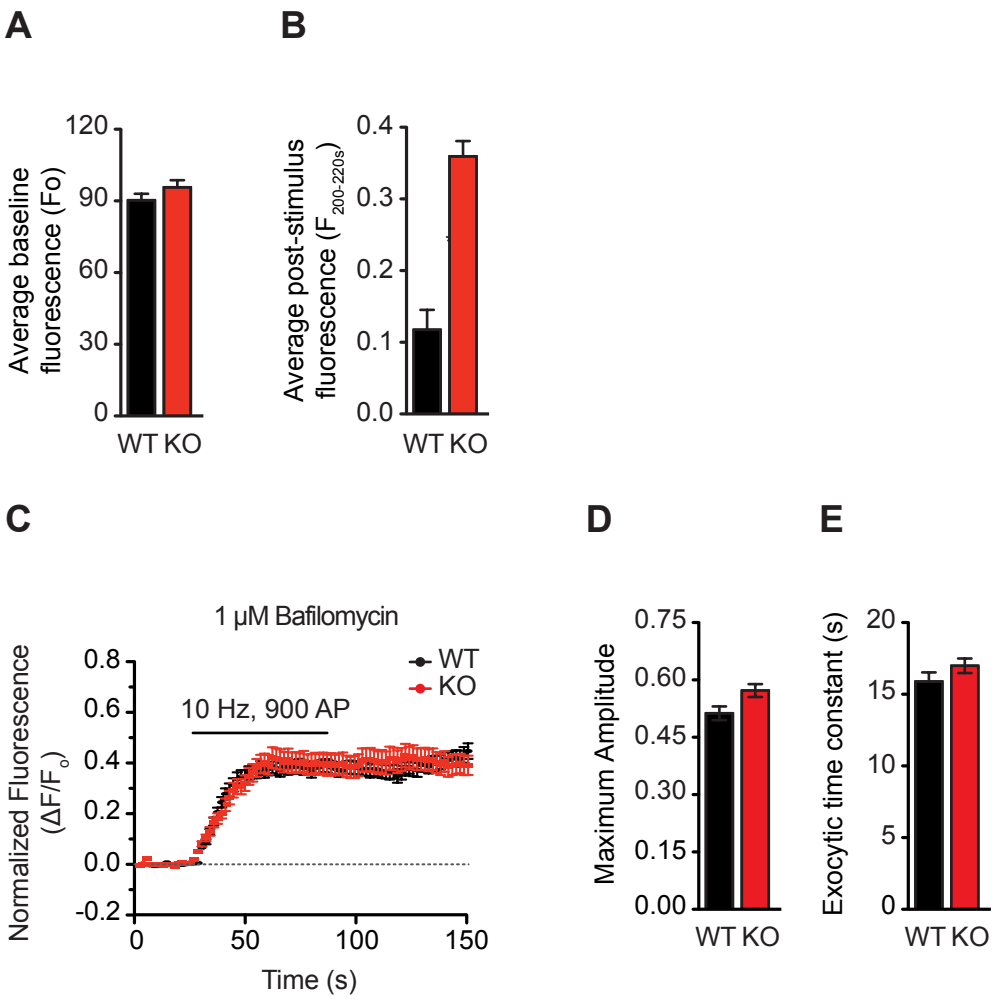

Figure S2 (related to Figure 4)

Supplementary Figure Legends

Figure S1, Related to Figure 2. SV recycling kinetics measured with vGlut1-pHluorin are slower in DGKθ-knockdown neurons**.**

(A) Cultured cortical neurons transfected with ^{myc}DGKθ, sypHy, and mCherry were and immunostained with specific antibodies against Myc (^{myc}DGKθ, blue), GFP (sypHy, green) and dsRED (mCherry). The overlap between the Myc and GFP signals along neuronal processes indicates ^{myc}DGKθ rescues SV recycling kinetics (**Figure 2**) in DGKθ-shRNA expressing neurons neurons at synaptic sites. Scale bar, 5μm.

(B) Comparison of average post-stimulus endocytic time constants (τ) values measured using the vGlut1-pHluorin reporter (vGlut1-pH) between control (Ctrl, black) and DGKθ-shRNA (red) in response to a train of 300 APs (10Hz). Data represent mean ± SEM from >300 boutons for each condition. Student's t test, *** P<0.001 against Ctrl.

(C) Comparison of average τ values measured using either vGlut1-pH or sypHy between control (Ctrl, black) and DGKθ-shRNA (red) in response to a train of 600 APs (50Hz). Data represent mean ± SEM from >300 boutons (vGlut1-pH) and >100 boutons (sypHy) for each condition; *** P<0.001 against Ctrl with vGlut1-pH.

P<0.001 against Ctrl with sypHy, a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test.

(D) Comparison of average τ values measured using sypHy between control (Ctrl, black) and DGKθ-shRNA (red) in response to a train of 40 APs (20Hz). Data represent mean ± SEM from >300 boutons for each condition. Student's t test, * P = 0.04 against Ctrl.

Figure S2, Related to Figure 4. DGKθ regulates the retrieval of SV proteins following elevated neuronal activity.

(A) Comparison of the average sypHy fluorescence intensity values during the 20 s prior to stimulation (Fo) measured in WT (black) and KO (red) neurons. Fo values were corrected for differences in background fluorescence by subtracting the average fluorescence values from 3 ROIs selected in regions of the imaging area that were not on neuronal processes (indicated by mCherry fluorescence). There was no significant (ns) difference in the average Fo between WT and KO neurons (Student's t test). Data represent mean ± SEM from >500 boutons per genotype.

(B) Average sypHy fluorescence intensity is significantly higher in DGKθ KO neurons >2 minutes following termination of a train of 600APs (50Hz). Values were first normalized to Fo and the average fluorescence intensities were calculated from 10 frames (20 s) 160 s following the end of the 50 Hz stimulus ($F_{200\text{-}220s}$). Data represent mean ± SEM from >160 boutons per genotype; Student's t test.

(C) DGKθ KO neurons show no significant difference in the kinetics of SV exocytosis compared to WT. Average traces of sypHy fluorescence in WT and KO neurons in response to a train of 900 APs (10Hz) delivered in the presence of bafilomycin A1 (baf). Values were normalized to the average baseline before stimulation (Fo, as in described in (**A**)). Representative traces from 1 coverslip of each genotype are shown; data represent mean from ± SEM from >100 boutons per genotype.

(D) Comparison of the average maximum sypHy amplitudes between WT and KO neurons in response to a train of 900 APs (10Hz) delivered in the presence of bafilomycin. Values were calculated from ΔF/F curves (example traces shown in **(C)**). There was no significant difference between sypHy amplitudes measured in WT and KO neurons. Data represent mean ± SEM from ≥300 boutons per genotype, Student's t test.

(E) Exocytic rate constants for WT and KO neurons were determined by first normalizing ΔF/F traces for each ROI to the maximum sypHy amplitude (0-1 scale). Tau values were calculated by fitting the rise-portion of the response during stimulation to a one-phase association curve. Tau values measured in WT and KO neurons were not significantly different. Data represent mean ± SEM from ≥300 boutons per genotype, Student's t test.

Detailed Supplementary Experimental Procedures

Reagents

Imaging reagents: TTX, DL-AP5, and CNQX (Tocris), were resuspended with water to make 1000x stock solutions (1 mM, 50 mM, 10 mM, respectively), aliquots were stored at -20°C until use. Bafilomycin A1 (Calbiochem) was resuspended in DMSO to make 1mM stock solution, aliquots were stored at -20°C until use. Antibodies: mouse anti-DGKθ, DGKι, DGKγ from BD biosciences, chicken anti-MAP2 (Novus), mouse anti-PSD-95 (NeuroMab), guinea pig anti-vGlut1 (Synaptic systems), mouse β-Tubulin (Sigma), rabbit anti-GluR1 (RLH lab), rabbit anti-dsRed (Clontech), chicken anti-GFP (Abcam). IRDye-conjugated secondary antibodies were used for infrared imaging (Odyssey; LICOR).

DNA constructs (Molecular Biology)

shRNA-knockdown and rescue plasmids. Complementary primers containing DGKθ or control shRNA (Ctrl) sequences were annealed and cloned into *Sac*I and *Xho*I sites of pSuper vector and driven by H1 RNA polymerase III promoter. Ctrl shRNA sequence was specific for luciferase. shRNA targeting sequences: DGKθ-shRNA (Baldanzi et al., 2010), 5'- GTGTACATTTGGACGTCTA -3'; luciferase shRNA, 5'-CGCTGAGTACTTCGAAATGTC -3'. For rescue experiments, human DGKθ or DGKθ-kinase dead (DGKθ-kd, point-mutation G648A in kinase domain (Los et al., 2004)) was subcloned into pRK5-Myc between *Sal*I and *Not*I restriction sites and driven by CMV promoter (^{Myc}DGKθ and ^{Myc}DGKθ-kd, respectively). vGlut1-pHluorin was subcloned into pRK5 from a lentiviral vector (Beijing University). All constructs were verified by restriction enzyme digest and DNA sequencing. Other DNA plasmids used: synaptophysin-pHluorin (CMV::SypHy A4) was obtained from Addgene (plasmid 24478).

Lentivirus

Control and DGKθ-shRNA were subcloned into FuGW lentiviral vector and lentivirus was produced as described previously(Anggono et al., 2011). Briefly, HEK 293 cells were transfected with FuGW, Δ8.9, VSV-G, using lipofectamine 2000. Cells were treated with sodium butyrate 18hrs post-transfection, Virus was collected 8hrs, and 24hrs post treatment. Virus was concentrated, and resuspended in NB, aliquots were frozen and stored at -80C. For DGKθ knockdown experiments, rat cortical neurons were infected with control or DGKθ-shRNA lentivirus DIV 8, and lysates were assayed by western blot DIV 14-16.

Immunocytochemistry

Neurons were rinsed with PBS and fixed for 15 minutes at room temperature in PBS containing 4% paraformaldehyde (PFA) and 4% sucrose. Cells were washed 3 times and permeabilized with 0.25%TX-100 in PBS for 10 minutes. Neurons were then blocked with 10% BSA serum in PBS for 1hr at 37^{o}C and incubated in primary antibodies in PBS containing 3% BSA at room temperature. Neurons were washed 5 times before they were incubated with fluorescently labeled secondary antibodies (goat conjugated Alexafluor 647, 568, or 488). Following 5 final washes with PBS, coverslips and mounted onto glass slides using Fluoromount-G (Southern Biotech). Images were obtained using a 510-laser scanning confocal microscope (Zeiss).

DGK activity assay

Assay was performed as described previously (Tu-Sekine and Raben, 2012). Large unilamellar vesicles were prepared on the day of assay as follows: stock lipids were

combined, dried under nitrogen and stored under vacuum at 4° C for 2-20 hours to remove residual $CHCl_3$. Lipid films were rehydrated in DGK assay buffer (55 mM Hepes, 100 mM NaCl) for 30 min at 40°C with occasional vortexing and sonication (30 seconds, Branson Sonicator). Vesicles were formed at 37-40C by extrusion through a 0.1μm polycarbonate membrane using an Avanti mini extruder per manufacturer instructions. Lipid composition was POPC:POPE:POPS:DOG at mole fractions of 26:51:15:8, respectively. 5μg of S1 fractions were added to the vesicles and reactions were incubated 30 min at 37C without agitation. The final assay contained: 1.2 mM total lipid in 50 mM Hepes pH 7.5, 1 mM DTT, 1.5 mM $MgCl_2$, 1 mM [γ^{32}P]ATP (specific activity = $2.5x10^5$ cpm/nmol ATP). Reactions were terminated by addition of chloroform/methanol/1M NaCl (1:2:0.8) (v:v), and phases separated by addition of 1 ml each of $CHCl_3$ and 1M NaCl. The organic phase was washed with 2 ml of 1M NaCl, dried under nitrogen gas, resuspended in $CHCl_3$:MeOH (95:5) and spotted onto a silica gel 60 TLC plate. Phosphatidic acid (PtdOH) was separated from other lipids with chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1) (v:v). The amount of [γ^{32}P]PtdOH was measured by liquid scintillation spectrophotometry in a Wallac 1410 liquid scintillation counter. DGK activity was quantified as nmol PtdOH min^{-1} μg^{-1} of protein. Assay conditions used in these experiments are routinely used to measure DGK activity of purified forms of DGKθ, DGKζ, and DGKδ (personal communication from Becky Tu-Sekine). By excluding calcium from the DGK assay buffer, we minimized activity measured in cell fraction from the type I DGK isoforms (-α, -β, and -γ).

PSD preparation

Forebrain or whole brain was isolated from adult mouse and frozen on dry ice. Brain was thawed in homogenization buffer (320 mM sucrose, 5 mM sodium pyrophosphate, 1 mM

EDTA, 10 mM HEPES pH 7.4, 200 nM okadaic acid, protease inhibitor cocktail (Roche)) and homogenized 30 times in glass homogenizer. The homogenate was centrifuged at 800xg for 10 minutes at 4^{o}C to yield P1 and S1. S1 was further centrifuged at 10,000xg for 20 minutes at 4^{o}C to yield P2 and S2. P2 was layered on top of a sucrose gradient composed of 0.8, 1, 1.2 mM sucrose buffers, respectively, containing protease and phosphatase inhibitors. The gradient was centrifuged 82,500 xg for 2hrs at 4^{o}C . The layer containing synaptosomes (between 1 and 1.2mM sucrose) was isolated, diluted with 10mM Hepes buffer, pH 7.4, and synaptosomes were pelleted by centrifugation at 150,000 x g for 30 minutes at 4^{o}C. Synaptosomal pellet (SYN) was resuspended in 50mM HEPES pH 7.4, mixed with an equal volume of 1% triton X-100, and incubated with agitation at 4^{o}C for 15 minutes. The PSD was generated by centrifugation at 32,000xg for 20 minutes at 4^{o}C. The final PSD pellet was resuspended in 50mM HEPES pH 7.4 followed by protein quantification and western blot.

Live cell imaging and analysis

Neurons were transfected DIV 11-12 using lipofectamine 2000 (Invitrogen) and sypHy live-cell imaging was performed DIV 14-20. Concentration of DNAs used are as follows: 1 µg sypHy or vGlut1-pH, 0.4 µg mCherry, 0.5 µg empty vector (pRK5-Myc) or ^{Myc}DGKθ, or ^{Myc}DGKθ-kd; for shRNA-knockdown 1 µg of control or DGKθ-specific shRNA (shLuc and sh#1, respectively) was also included.

Coverslips containing neurons were mounted into a custom-built perfusion and stimulation chamber which held at 37°C on the heated microscope stage. Healthy, transfected neurons were identified by mCherry expression. During image acquisition, cells were continuously perfused at 0.5-1.0 mL/min with pre-warmed ACSF. In all experiments, images were acquired for 30 s prior to stimulation to establish a stable baseline.

Images were analyzed in ImageJ (http://rsb.info.nih.gov/ij/) using the time-series plugin (http://rsb.info.nih.gov/ij/plugins/time-series.html). τ values were calculated from fluorescence intensity changes individual boutons (≥3 coverslips per genotype, from ≥3 batches of neurons). Values were normalized to the initial baseline, $\Delta F/Fo$, (Fo = average F values 20 s prior to stimulation) and then normalized to their maximum amplitude (ΔF). τ values were calculated by fitting the decay phase for each ΔF trace (corresponding to 1 bouton) to a single exponential function in Prism 5.

Supplemental References

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