Modeling Intrahippocampal Effects of Anterior Hippocampal Hyperactivity Relevant to Schizophrenia Using Chemogenetic Excitation of Long Axis-Projecting Mossy Cells in the Mouse Dentate Gyrus

Supplemental Information

SUPPLEMENTAL METHODS AND MATERIALS

Adeno-associated viruses

The following adeno-associated virus particles (AAVs) were purchased from Addgene (Watertown, MA). Citations for published constructs are provided in the main manuscript.

1. AAV.pgk.Cre was a gift from Patrick Aebischer (Addgene viral prep # 24593-AAVrg; http://n2t.net/addgene:24593; RRID:Addgene_24593). Viral titer = 9.3 x 10^{12} genome copies (GC)/mL.
2. pAAV.Syn.Flex.GCaMP6f.WPRE.SV40 was a gift from Douglas Kim & GENIE Project (Addgene viral prep # 100833-AAV1; http://n2t.net/addgene:100833; RRID:Addgene_100833). Viral titer = 1.9 x 10^{13} GC/mL.
3. pAAV-hSyn-DIO-mCherry was a gift from Bryan Roth (Addgene viral prep # 50459-AAV8; http://n2t.net/addgene:50459; RRID:Addgene_50459). Viral titer = 2.6 x 10^{13} GC/mL.
4. pAAV-hSyn-DIO-hM3D(Gq)-mCherry was a gift from Bryan Roth (Addgene viral prep # 44361-AAV8; http://n2t.net/addgene:44361; RRID:Addgene_44361). Viral titer = 2.0 x 10^{13} GC/mL.
5. pENN.AAV.hSyn.Cre.WPRE.hGH was a gift from James M. Wilson (Addgene viral prep # 105553-AAV1; http://n2t.net/addgene:105553 ; RRID:Addgene_105553). Viral titer = 1.9 x 10^{13} GC/mL.
6. pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA was a gift from Karl Deisseroth (Addgene viral prep # 20297-AAV8; http://n2t.net/addgene:20297 ; RRID:Addgene_20297). Viral titer = 1.9 x 10^{13} GC/mL.

Stereotaxic surgery and viral infusion

Male CD-1 mice were anesthetized using inhaled isoflurane (Piramal Critical Care, Telangana, India) administered by a tabletop anesthesia machine (VetEquip, Livermore, CA) with thermal support provided by Deltaphase isothermal pads (Braintree Scientific, Braintree, MA) and mounted in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Adeno-associated virus (0.2 – 0.4 μL, depending on target region) was infused at a rate of 50 nL/min using a 2 μL syringe (Hamilton, Reno, NV) to the following structures:

1) Dorsal dentate gyrus (DG) inner molecular layer: Anterior/posterior (AP): -1.94 mm, medial/lateral (ML): 1.20 mm left; dorsal/ventral (DV): -2.50 mm.

2) Ventral DG hilus: AP: -3.40 mm, ML: ±3.00 mm, DV: -3.50 mm.

The syringe was left in place for an additional 3 min to reduce backflow. Fiber photometry cannulas were targeted to the same coordinates as ventral DG hilus and cemented in place with C&B-Metabond (Parkell, Edgewood, NY). The skin was closed with VetBond (3M, Saint Paul, MN). Mice were housed for at least three weeks prior to experimentation to allow for viral expression. Targeting was confirmed by fluorescence microscopy. Mice with either unilateral or bilateral ventral mossy cell expression were included in behavioral analyses due to extensive bilateral projections.

Fluorescent immunostaining, microscopy, and cell counting

Mice were terminally anesthetized with pentobarbital sodium (Vortech Pharmaceuticals, Dearborn, MI) then perfused intracardially with ice-cold phosphate buffered saline (PBS, 0.1 M, pH 7.3) followed by ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed for 48 h in 4% PFA at 4 °C, then stored in PBS until sectioning. 40 μm sections were cut on a vibrating microtome (Leica, Buffalo Grove, IL). For immunostaining, tissue sections were permeabilized and blocked in 0.3% Triton X-100 solution and 3% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA) in PBS (blocking buffer) for 2 hours at room temperature followed by incubation in primary antibody. Primary antibodies used were mouse anti-calretinin (MAB1568, Millipore, Burlington, MA, diluted in blocking

buffer) and rabbit anti-cFos (226 003, Synaptic Systems, Goettingen, Germany, diluted in 0.1% Triton X-100 and 1% NDS in PBS), both diluted 1:1000 with overnight incubation at 4 ℃. Sections were rinsed in PBS, then incubated in secondary antibody (Alexa Fluor 488 Donkey Anti-Mouse, Alexa Fluor 488 Donkey Anti-Rabbit, or Alexa Fluor 647 Donkey Anti-Rabbit) diluted 1:1000 in blocking buffer for 2 hours at room temperature. Sections were rinsed in PBS, then incubated in 4′,6-diamidino-2-phenylindole (DAPI, Millipore) diluted 1:5000 in PBS at room temperature for 5 mins. Sections were again washed in PBS, then mounted on slides using Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Widefield images were acquired with an AF6000 LX fluorescent microscopy system (Leica) equipped with an HCX PL FLUOTAR 10.0x objective (NA = 0.30), while confocal images were acquired with an LSM 710 META Inverted microscope (Zeiss, White Plains, NY) equipped with a 20x Plan-Apochromat objective (NA = 0.8). Acquisition software was Leica LAS AF for widefield images and Zeiss Zen for confocal images. To quantify the overlap between GCaMP6f and calretinin, two adjacent coronal sections were imaged as Z stacks and collapsed using maximum projection. The number of hilar GCaMP6f+, calretinin+, and GCaMP6f+calretinin+ double labeled neurons were counted, enabling calculation of GCaMP6f+calretinin+/GCaMP6f+ and GCaMP6f+calretinin+/calretinin+. These values were averaged across two sections to obtain a single value for each mouse, and these single values were averaged across mice. For off-target GCaMP6f quantification, the same approach was used and the number of GCaMP6f+ neurons was divided by the area of the dentate gyrus granule cell layer as delineated by DAPI staining or area CA3. Quantification of hilar cFos+mCherry+/mCherry+ neurons to calculate fraction of hilar cells activated by DREADD strategy was performed in a similar fashion, as was quantification of cFos+ neurons per unit area in dentate gyrus granule cells of the ventral hippocampus. To quantify cFos+ neurons in the dorsal hippocampus, the same technique was used except cFos+ neurons were counted in bilateral hippocampus for each slice and normalized to granule cell layer area, averaged to get a single value per slice, and then averaged with a second slice so that each mouse contributed a single data point consisting typically of 4 hippocampi.

Electrophysiology

Validation of DREADD activation

Mice were rapidly decapitated under deep isoflurane anesthesia and 300-µm horizontal sections containing the ventral DG were prepared in *N*-methyl-D-glucamine cutting solution (in mM): 93 *N*-methyl-D-glucamine, 20 HEPES, 2.5 KCl, 0.5 $CaCl_2$, 10 $MgCl_2$, 1.2 NaH_2PO_4, 25 glucose, 5 Na-ascorbate, and 3 Na-pyruvate. Slices recovered in 30° cutting solution for 10 minutes and then in 23° artificial cerebrospinal fluid for at least 60 minutes prior to recording. All solutions were continuously bubbled with 95/5% O_2/CO_2. Slices were transferred to a 30° recording chamber perfused at 2 mL/min with artificial cerebrospinal fluid (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 MgCl2, 1 NaH_2PO_4, 11 glucose, and 26 $NaHCO_3$. Mossy cells were identified within the hilus, expressed red fluorescent protein, and were patched with borosilicate glass pipettes (4-6 MΩ). Whole cell access was obtained, and cells were dialyzed for 5 minutes with internal solution (in mM): 125 K-gluconate, 4 NaCl, 10 HEPES, 4 MgATP, 0.3 NaGTP, 10 Tris-phosphocreatine. Mossy cells were voltage-clamped at -75 mV and 10 µM CNO was perfused in the bath for 5 minutes. Recordings were acquired with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 10 kHz. The mean of the holding current was quantified before and after CNO perfusion using pClamp 10.4 software (Axon Instruments, Union City, CA).

Optical activation of ventral mossy cell terminals in dorsal dentate gyrus

Brain slices were prepared as previously described (1, 2). Mice were deeply anesthetized and transcardially perfused with an N-methyl-D-glucamine (NMDG)-based ice-cold solution (components below) and decapitated. Coronal brain slices (300-320 µm thickness) containing dorsal hippocampus were prepared with a vibratome (Leica VT 1200S, Leica Biosystems Inc) in an NMDG dissection solution (mM: NMDG 92, KCl 2.5, $CaCl_2$ 0.5, NaH_2PO_4 1.25, HEPES 20, $MgSO_4$ 10, $NaHCO_3$ 30, glucose 25, Thiourea 2, Na-ascorbate 5, Na-pyruvate 3, pH 7.3-7.4 and O_2 95%+5%CO_2 bubbled) (3) and slices were later incubated in a chamber at 35-36°C for 40 min with continuously oxygenated ACSF (see below for

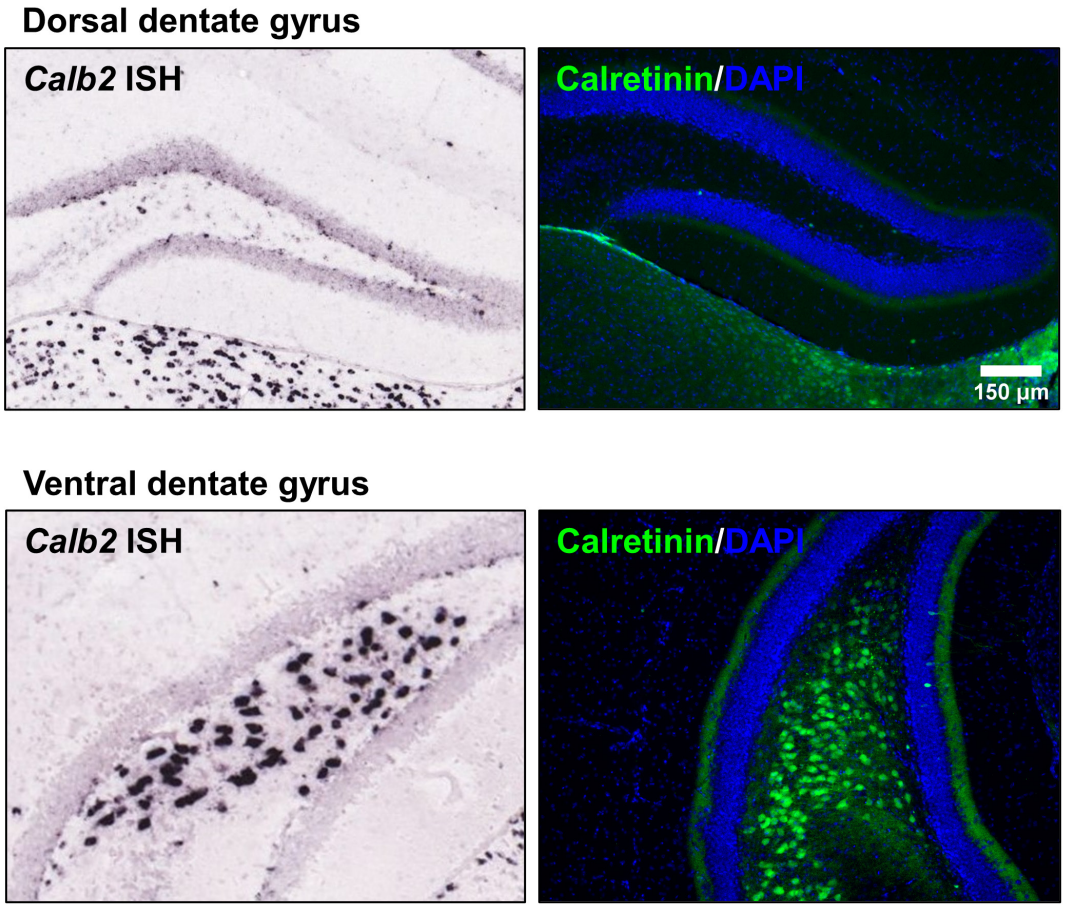
components). Slices remained at room temperature for at least 1 hour before electrophysiological recordings at room temperature. Whole-cell patch-clamp recordings were made from dorsal hippocampal dentate gyrus granule neurons by using a Nikon infrared/DIC microscope (Eclipse FN1, Nikon Corp. Inc., Melville NY), and slices were continuously superfused (flow speed 1-1.5 ml/min) with an ACSF solution (containing [in mM]: 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 10 D-glucose, pH 7.4) bubbled with 95% O_2/5% CO_2. Filled electrodes had resistances of 2~5 MΩ with one internal solution (consisted of [in mM]: 120 K-gluconate, 11 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 0.6 EGTA, 10 HEPES, 2 Na-ATP, 0.6 Na-GTP, 10 K-creatine-phosphate, pH 7.3 (4, 5)) for spontaneous (s) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated sEPSCs, recorded at a holding potential -55.8mV (Cl^- reversal potential). To record action potentials (APs), current-clamp mode was used with the same internal solution for sEPSC recordings. Access resistance (Ra, voltage-clamp mode) was continuously monitored during recordings and recordings with Ra larger than 25 MΩ or 20% change were discarded. The blue laser light (5 ms pulse duration, 20 Hz, 1 s duration) was delivered through a fiberoptic cable within brain slices for stimulating axon terminals containing ChR2 within dentate gyrus, controlled by a DPSS laser (MBL-III-473 (100mW, Tetralayers Co., Inc)) and the timing of laser delivery was controlled by Clampex 10 software (Molecular Devices Inc., Union City, CA). Data were collected using one multiClamp 700B amplifier and Clampex 10 software (Molecular Devices Inc., Union City, CA) and filtered at 2 kHz, and digitized at 20 kHz using a Digidata 1440A (Molecular Devices Inc., Union City, CA).

Fiber photometry

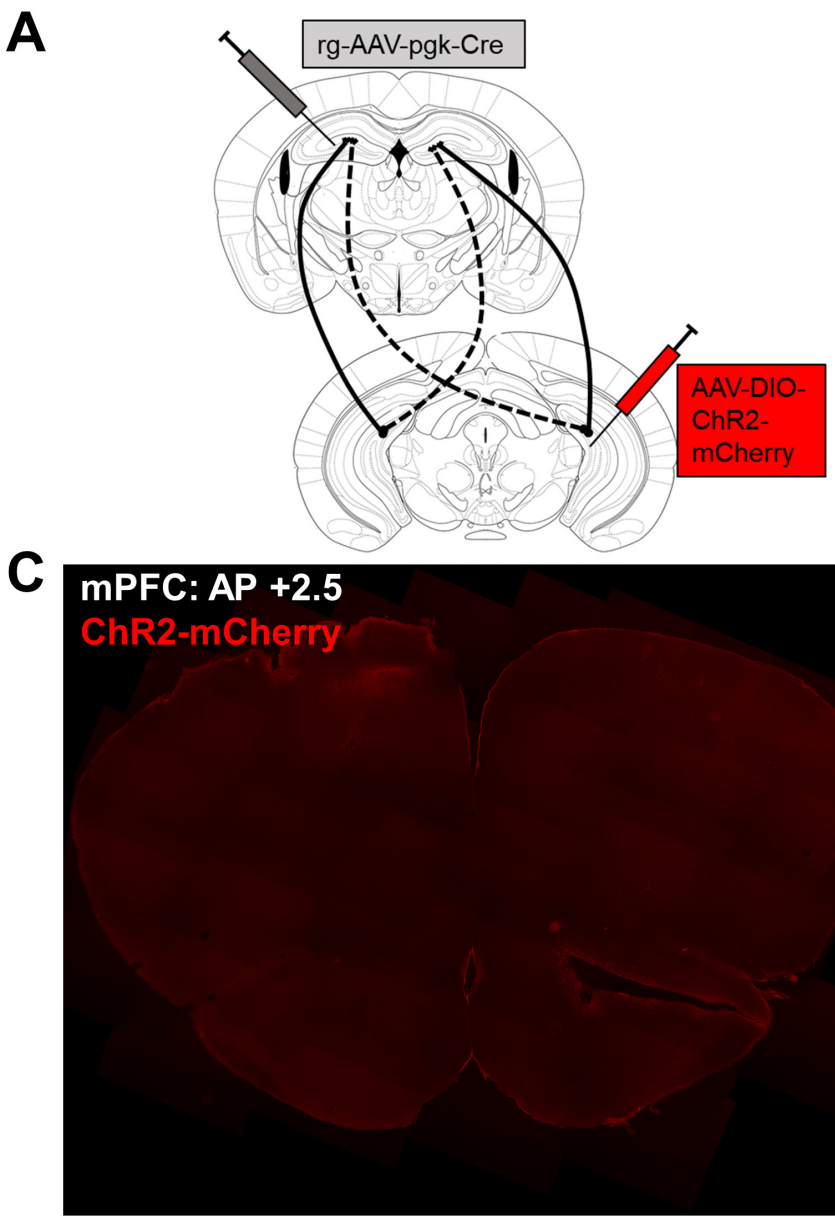
Fiber photometry cannula (Doric Lenses, Quebec, Canada) implanted male CD-1 mice expressing GCaMP6f in vMCs underwent *in vivo* calcium recording while mice ambulated their home cage with cage lid removed in a novel recording room. Photometry was performed using the Doric Lenses system controlled by Doric Neuroscience Studio (DNS) version 5.3, consisting of 405 nm and 465 nm light-emitting diodes (LEDs) run by an LED driver and routed through a 4-port fluorescent minicube.

GCaMP6f output excited by 405 nm (calcium-independent signal) and 465 nm (calcium-dependent signal) LEDs were measured by a Newport Visible Femtowatt Photoreceiver and separated using lock-in demodulation. Behavior was time-locked with photometry signal by USB 3.0 color camera (Doric Lenses) input to the photometry console. Signal processing was performed by DNS photometry analyzer. $\Delta F/F_0$ was calculated for the 405 nm and 465 nm channels independently using a least mean square fit of the whole trace, and then $\Delta F/F_0$ (405 nm) subtracted from $\Delta F/F_0$ (465 nm) to yield a corrected trace. This trace was subsequently lowpass filtered at 2 Hz (6) to yield the final bulk calcium signal and Z-normalized. Videos were annotated manually to mark time of exploratory rearing (centered around initially point of rearing apogee) and the time of transition when mice started or stopped horizontal XY plane movement ("stop to start" and "start to stop", respectively). From the averaged $\Delta F/F_0$ Z-score curves for each mouse, area under the curve was calculated for the 2 seconds before (-2 s to 0 s) and after (0 s to +2 s) the indicated behavioral event and analyzed by two-way repeated measures ANOVA. Data analysis was performed using custom written scripts in Matlab R2019a (MathWorks, Natick, MA). Only mice with histologically verified cannula and GCaMP6f targeting were analyzed.

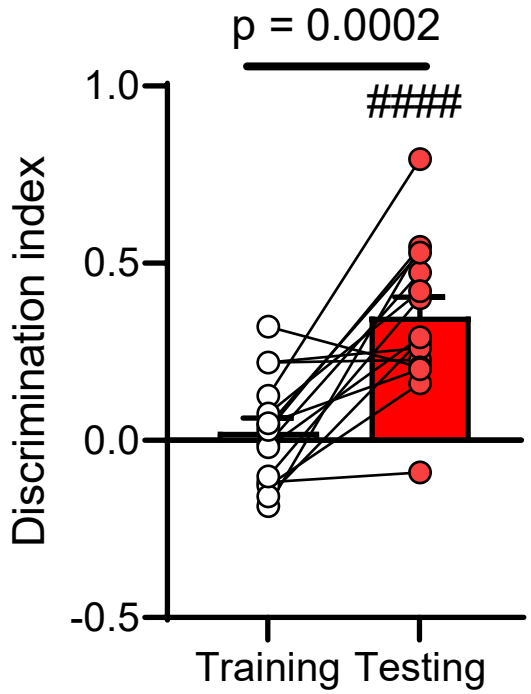
SUPPLEMENTAL FIGURES



Supplemental Figure S1. Calretinin expression in the dorsal and ventral hippocampal hilus. In situ hybridization (ISH) for the gene encoding calretinin, *Calb2* (left), and immunofluorescent images for calretinin protein (right) confirmed previous studies that ventral mossy cells (bottom) but not dorsal mossy cells (top) express calretinin mRNA and protein. In situ hybridization is from the Allen Mouse Brain Atlas (7, 8).


A
rg-AAV-pgk-Cre
AAV-DIO-ChR2-mCherry
B
Dorsal DG
ChR2-mCherry
DAPI
100 µm
Ventral DG
ChR2-mCherry
DAPI
100 µm
C
mPFC: AP +2.5
ChR2-mCherry

Supplemental Figure S2. No evidence of ventral mossy cell projection to medial prefrontal cortex (mPFC). A Channelrhodopsin-mCherry (AAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA) was expressed in ventral mossy cells projecting to the dorsal dentate gyrus (DG) using an intersectional targeting technique. **B** ChR2-mCherry projections were clearly visible in the inner molecular layer of the dorsal DG (top) originating from mossy cells in the ventral DG hilus (bottom). **C** Stitched image from the same mouse shown in (**B**) fails to reveal any significant projection to the medial prefrontal cortex. Experiment is representative of 9 total mice expressing either ChR2-mCherry or mCherry in ventral mossy cells that do not demonstrate projections to the mPFC.



Supplemental Figure S3. CD-1 wildtype mice show intact object location memory 24 hrs after training session as evidenced by a significant increase in discrimination index (DI) between training and testing sessions (N = 14, t(14) = 4.94, p = 0.0002). ####$p < 10^{-4}$ by one-sample t test versus DI = 0 (t(14) = 6.37).

SUPPLEMENTAL REFERENCES

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