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Supplemental Information

A MicroRNA-Based Gene-Targeting Tool for Virally Labeling Interneurons in the Rodent Cortex

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Supplemental Tables and Figures

SUMMARY OF INTERNEURON LABELLING			
Virus Type	Animal and Injection site	GABA Colocalization[e,f]	
		mAGNET	Control
LV	C57BL/6 wt mouse[a] (cortex 1 & 2)[d]	91 ± 3% *** (339)	12 ± 3% (384)
	Sprague-Dawley wt rat[a] (cortex 1 & 2) [d]	74 ± 3% *** (337)	16 ± 3% (408)
AAV	C57BL/6 wt mouse[a] (cortex 1)	98 ± 2% *** (184)	23 ± 2% (195)
	FVP/NJ-Tg(Ube3a)1Mpan/J mouse[b] (cortex 1)	96 ± 2% (134)	NA
	FVB/NJ wt mouse[c] (cortex 1)	95 ± 3% (177)	NA
	Sprague-Dawley wt rat[a] (cortex 1 & 2) [d]	82 ± 3% *** (348)	21 ± 4% (411)
	Sprague-Dawley wt rat[a] (hippocampus)	80 ± 5% *** (176)	8 ± 2% (174)

Table S1. Summary of interneuron labelling. Related to Figures 1, 2 and 3.

[a] n = 3 animals each for GABA mAGNET and Control virus
[b] n = 2 animals, GABA mAGNET virus only
[c] n = 3 animals, GABA mAGNET virus only
[d] data are pooled across both cortical injection sites
[e] numbers in parentheses indicate the number of EGFP+ neurons analyzed (at least 50 per animal per injection site, see Methods)
[f] *** $p<0.005$ Pearson's chi squared test (see Supplemental Experimental Procedures).

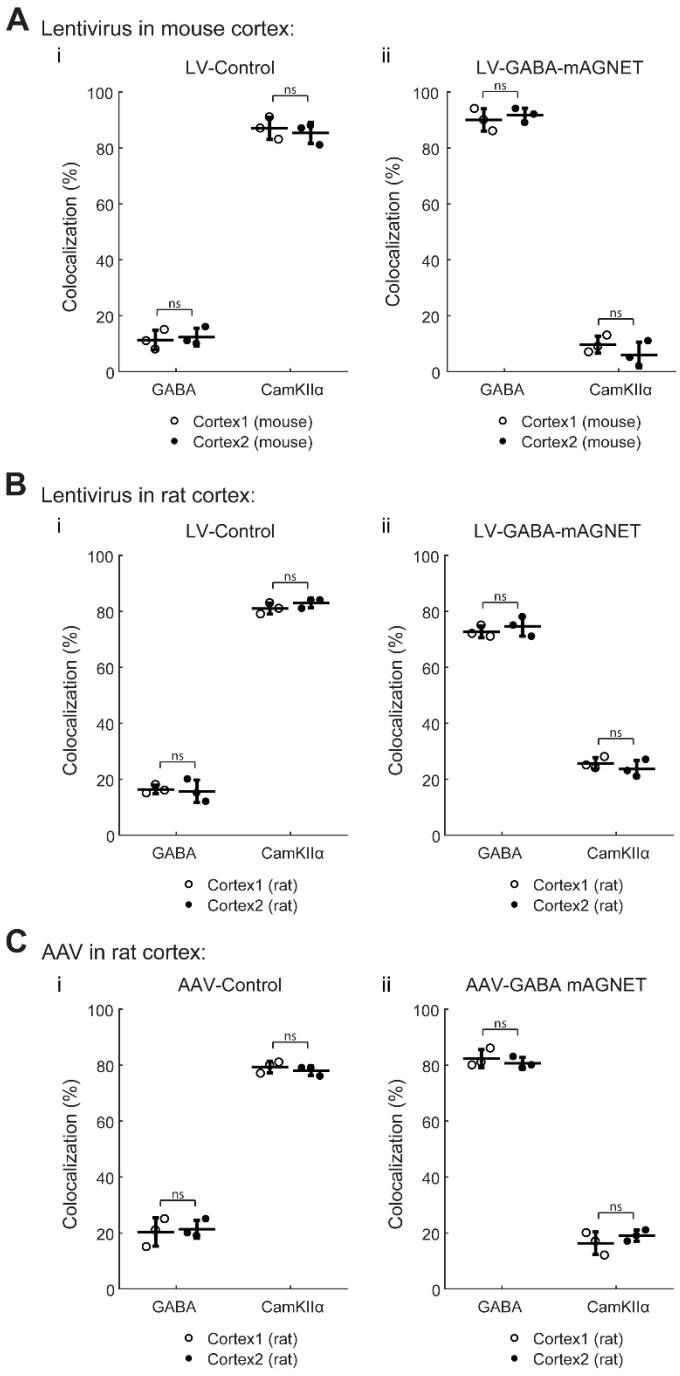

Figure S1. Consistency in interneuron targeting across cortical injection sites. Related to Figures 1, 2 and 3. (A) – (C) Comparison of EGFP colocalization (%) with immunohistology markers for inhibitory neurons (GABA) or excitatory neurons (CamKIIα) - reveals no significant differences (ns, Pearson's chi squared test) between the two motor cortex sites injected for (A) lentiviruses in the mouse cortex, (B) lentiviruses in the rat cortex, or (C) AAVs in the rat cortex. Note that AAVs were only injected at one site in the mouse cortex, so there is no comparison data across cortical sites. Scatter plots show individual animals (circles) and mean (horizontal bar) ± SD.

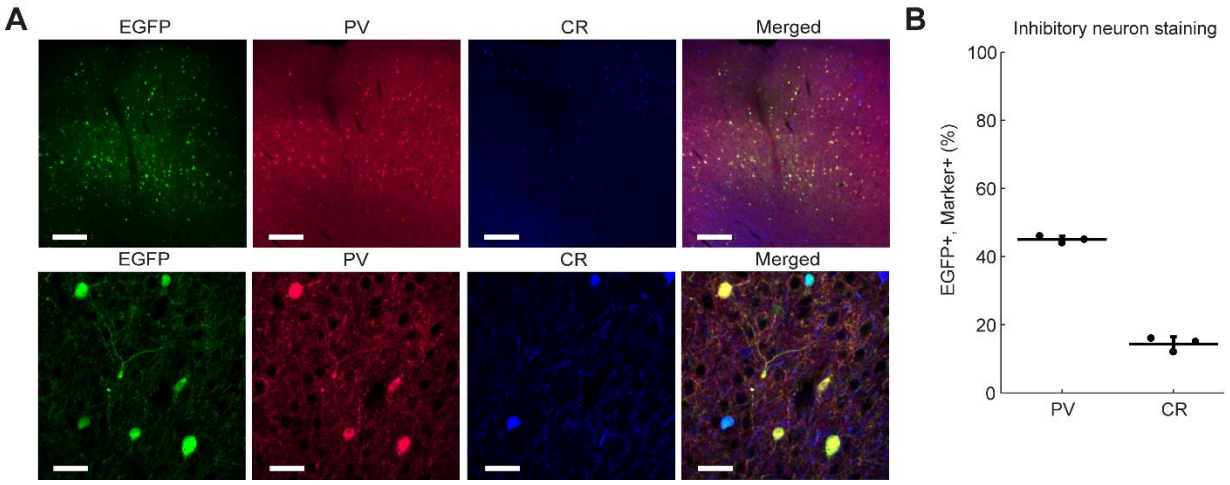

Figure S2. AAV-GABA-mAGNET labelling of interneuron subtypes by cortical layer. Related to Figure 2.
(A) Representative confocal images of EGFP fluorescence from cortical cells transduced with AAV GABA mAGNET, immunofluorescence of non-overlapping parvalbumin (PV) and calretinin (CR) cell markers, and colocalization at low (top) and high (bottom) magnification. Top scale bars are 210 µm. Bottom scale bars are 35 µm.
(B) Colocalization of EGFP (%) with PV and CR marker immune stains in the wild type mouse cortex (n = 3 mice). Scatter plot shows individual animals (circles) and mean (horizontal bar) ± SD. Colocalization of EGFP (%) with immunohistology markers for sub-types of inhibitory neurons – parvalbumin (PV) and calretinin (CR) – follows the expected distribution of these cells in mouse cortex.

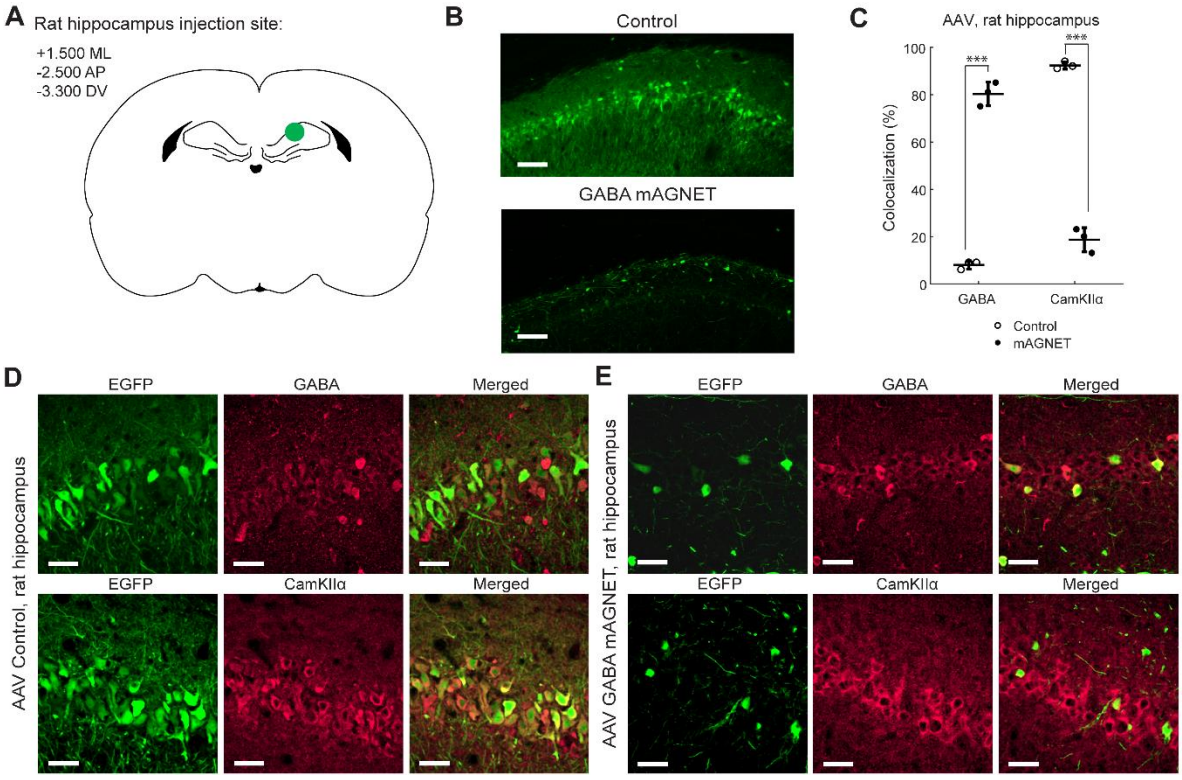

Figure S3. AAV-GABA-mAGNET targeting in the wild type rat hippocampus. Related to Figure 3.

(A) Schematic of stereotaxic virus injection sites in rat hippocampus.

(B) Confocal microscopy images showing comparison of AAV-Control and AAV-mAGNET-transduction in rat hippocampus. Scale bars are 250 µm.

(C) Colocalization of EGFP with inhibitory (GABA) and excitatory (CamKIIα) neuron marker immune stains in the rat hippocampus (n = 3 rats each). Scatter plot shows individual animals (circles) and mean (horizontal bar) ± SD. ***: $p<0.005$ Pearson's chi squared test (see Methods).

(D) Representative confocal images of EGFP fluorescence from cortical cells transduced with AAV-Control, immunofluorescence of inhibitory (GABA) and excitatory (CamKIIα) cell markers, and colocalization. Scale bars are 35 µm.

(E) Same as (D), for cells transduced with AAV-GABA-mAGNET.

Supplemental Experimental Procedures

Viral vector construction. Standard molecular cloning procedures (PCR and restriction enzyme digestion) were used to construct genetic classifiers in viral vectors unless otherwise stated. LV GABA mAGNET and LV Control vectors were made by replacing the 1.2 kb Ef-1α promoter in our previously published 8X2C mAGNET (Addgene plasmid #67955) with human Synapsin 1 promoter (Addgene plasmid #86537) via EcoRI and BamHI restriction sites. The expression cassettes (including promoter, transgene and miRNA sites for mAGNET) from the resulting LV GABA mAGNET and LV Control vectors were amplified via PCR and inserted between the ITRs of an AAV-2 genomic vector obtained from Roger Tsien (Addgene plasmid #50954) via BglII and HindIII restriction sites. To produce the AAV CamKIIα mRuby2 vector, the CamKIIα promoter obtained from Ed Boyden (Addgene plasmid #64545) was cloned into the AAV-2 vector simultaneously with an mRuby2 reporter obtained from Michael Davidson (Addgene plasmid #54771) via EcoRI, AscI and Sbf1 sites. To generate Jaws GABA mAGNET, the Jaws transgene fused to an ER2 tag was obtained from Ed Boyden (Addgene plasmid #65013) and fused to mRuby2, replacing EGFP in the AAV GABA mAGNET and AAV Control vectors via a Gibson reaction. Finally, the CamKIIα ChR2 vector was produced by replacing the mRuby2 transgene in the AAV CamKIIα mRuby2 vector with ChR2-EGFP obtained from Ed Boyden (Addgene plasmid #58881) via AscI and SpeI sites. For sequences, see Supplementary Table 1. All new viral vectors produced here will be deposited to GenBank and Addgene.

Lentivirus preparation. Replication-incompetent lentivirus was packaged via triple transfections of the LV-hSyn-EGFP or LV-hSyn-EGFP-8X2C lentiviral transfer plasmid, plasmid pMD2.G encoding for VSV-G pseudotyping coat protein (Addgene plasmid #12259), and pDelta 8.74 (Addgene plasmid #5682) helper packaging plasmid, into HEK293FT cells (LifeTechnologies, R700-07) using TransIT-293 transfection reagent (Mirus Bio LLC, MIR 2700),

and then purified through ultracentrifugation. Viral transduction was first tested in primary rat neuron cultures before *in vivo* injections were performed, and viral titer was estimated to be ~1E9 transducing units/mL based on transduction efficiency of serial dilutions (Han, Qian et al. 2009).

Adeno-associated virus (AAV) preparation. Replication-incompetent AAV was packaged via triple transfections of the AAV-2 transfer vector (rAAV2.9-hSyn-EGFP-8XC, rAAV2.9-hSyn-EGFP, rAAV2.9-CamKIIα-mRuby2, rAAV2.9-hSyn-Jaws_mRuby2-8X2C, or rAAV2.9-CamKIIα-ChR2_EGFP), cap-9 plasmid encoding the Rep and Cap proteins (serotype 9), and pXX680 helper packaging plasmid, into HEK293FT cells (LifeTechnologies, R700-07) using TransIT-293 transfection reagent (Mirus Bio LLC, MIR 2700). Two days post-transfection, HEK cells were washed and lysed via three freeze-thaw cycles followed by sonication, and virus was purified from the lysate via PEG precipitation (System Biosciences, LV810A-1). Viral titer was determined via rt-PCR (Takara, AAVpro Titration Kit Ver.2) to be ~2E12 vg/mL. Viral transduction was first tested in primary rat neuron cultures before *in vivo* injections were performed.

Stereotaxic virus injection surgeries. All procedures were done in accordance with the National Institutes of Health Guide for Laboratory Animals and were approved by the Boston University Institutional Animal Care and Use and Biosafety Committees. Mice used for Figure 1, and 2A-E were adult female C57bL/6 wild type mice (Taconic Biosciences) eight weeks old at time of injection. We injected lentivirus at two locations in the mouse neocortex: cortex1 (+1.000 mm ML, +1.800 mm AP, -1.250 mm DV) and cortex2 (+1.000 mm ML, -1.500 mm AP, -0.900 mm DV). Approximately 1.0 µL (1E6 infectious particles) of lentivirus was injected per site. Three mice were injected per condition (n = 6 mice total). We injected the AAV GABA mAGNET or AAV Control only at the cortex 1 coordinates in the mouse cortex. Approximately 0.5 µL of virus was injected for each AAV (1E9 viral particles). Three mice were used per virus (n = 6 mice total).

Mice used in Figure 2F-I were adult female FVP/NJ-Tg(Ube3a)1Mpan/J autism mice (The Jackson Laboratory stock #019730) or adult female FVP/NJ wild type littermates (The Jackson Laboratory stock #001800), twelve weeks old at time of injection. To obtain Ube3a 2X Tg animals, heterozygous (1X Tg) males were mated with heterozygous (1X Tg) females, and the offspring homozygous (2X Tg) animals were used for experiments. We injected ~1.0 µL of AAV GABA mAGNET premixed with AAV CamKIIα mRuby2 (1E9 viral particles per virus) at the cortex 1 coordinates. Two Ube3a 2X Tg autism mice and three wild type littermates were used (n = 5 mice total).

All rats used were adult male Sprague-Dawley wild type rats, eight weeks old at the time of injection. We injected lentivirus at two locations in the rat brain: cortex 1 (+1.700 mm ML, +2.800 mm AP, -1.600 mm DV) and cortex 2 (+1.500 mm ML, -2.500 mm AP, -1.600 mm DV), analogous to the corresponding cortical injection sites in the mice. ~1.0 µL of lentivirus was injected per site. Three rats were injected per lentivirus (n = 6 rats total). ~0.5 µL AAV GABA mAGNET or AAV Control was injected at both cortical sites in the rats and additionally at a site in the hippocampus (+1.500 mm ML, -2.500 mm AP, -3.300 mm DV). Three rats each were used for each AAV virus (n = 6 rats total). All injection surgeries were performed using sterile surgical techniques under isoflurane anesthesia, and were accomplished using a standard rodent stereotaxic instrument and an automated microinjection syringe pump (World Precision Instruments, UMP3-1).

Immunohistology. At two weeks (LV) or three weeks (AAV) post-injection, animals were sacrificed and perfused with 4% PFA. Brains were post-fixed in 4% PFA for three hours, cryoprotected in a 30% sucrose, then flash froze in OCT. Frozen brains were sectioned (coronal) at 50 µm on a cryostat and slices were stored in PBS+0.05% sodium azide. Brian slices were stained with antibodies against GABA (Sigma, A2052, 1:1000) or CamKIIα (Santa Cruz Biotech, sc-13082, 1:50), followed by Alexa Fluor 568 goat anti-rabbit secondary antibody (Invitrogen A21071, 1:1000) and ToPRO-3 nuclear dye (Life Technologies, 1:5000), or just Alexa Fluor 633

goat anti-rabbit secondary antibody (Invitrogen A11011, 1:1000). The same GABA and CamKIIα primary antibodies (as well as Alexa Fluor secondary antibodies) were used for mice and rat brain slices, as they were shown by the manufacturer to be reactive in both species. For parvalbumin (PV) and calretinin (CR) staining, brain slices were co-stained with a mix of PV (SWANT GP72 1:2000) and CR antibodies (SWANT 7697 1:1000), and then a mix of secondary antibodies: Alexa Fluor 568 goat anti-guinea pig (Invitrogen A11075 1:1000) and Alexa Fluor 633 goat anti-rabbit secondary antibody (Invitrogen A11011, 1:1000).

Confocal imaging and quantification. Confocal imaging was performed on an Olympus FV1000 scanning confocal microscope, using a 10X lens or 60X water immersion lens. Z-stacks were taken by imaging at 2 µm intervals throughout the slices. Confocal image stacks were taken at least 150 µm away from the center of the injection site for LV, and at least 250 µm away from the center of the injection site for AAV. Consistent laser settings were used for all imaging sessions: 488 nm laser 3.5%, 350 V; 543 nm laser 13%, 600 V; 633 nm laser 5%, 600V. EGFP+ cell bodies were identified by comparison with ToPRO-3 nuclear staining, then each identified EGFP+ cell was categorized as immunopositive or immunonegative for the antibody stain. Note that staining for each immune marker (ex. GABA, CamKIIα) was performed on separate cortical brain slices unless otherwise stated. Immunopositive cell counts were pooled across slices stained for the same marker for each animal, and normalized to the total EGFP+ cell count across those slices. In this way, the proportion (%) of EGFP+ (or labelled) cells that colocalize with each immunomarker was calculated independently. Conversely, we determined that the cortical interneuron transduction efficiency of our AAVs at the delivered dose was 70 ± 3% (n = 246 cells), based on the percentage of GABA+ cells expressing the reporter, consistent with the general observation of the overall transduction efficiency of AAVs. For each animal, we analyzed at least 1-4 non-overlapping confocal stacks from each of 2-6 slices per injection site. A similar number of cells (at least 50 EGFP+ cells per animal, see Statistical analysis) at similar distances from the

injection site (~150 – 250 µm for LV and ~250 – 350 µm for AAV) were analyzed for each animal. All hippocampal images were taken in the CA1 area.

Statistical analysis. To determine the minimal sample size (number of animals) needed to ensure adequate power to detect an inhibitory neuron targeting effect, we performed a power analysis with the following parameters: mean control group estimated value = 0.2, mean experimental group estimated value = 0.8, expected standard deviation = 0.05, alpha = 0.05, power = 0.8, 2 sided test. Randomization and investigator blinding were not considerations of this study design.

A chi squared test of independence with one degree of freedom (as cortical neurons examined are either excitatory or inhibitory) requires an expected frequency of at least 10 to produce reliable approximations, and because inhibitory cells make up approximately 20% of neurons in the cortex, 50 neurons per mouse in each test condition was determined to be the minimum sample size. The Pearson's chi squared test statistic with one degree of freedom was calculated for interneuron targeting in each condition by taking the difference between the observed number of counts for inhibitory cells compared to the expected distribution of 20% inhibitory cells, to determine the statistical significance of the targeting effect.

Optogenetics and electrophysiology. All mice used were adult female C57bL/6 wild type mice. At eight weeks old, mAGNET mice (n = 6) were injected with ~1.0 µL an equal mixture (1E9 viral particles each) of AAV Jaws GABA mAGNET and AAV CamKIIα ChR2 at cortex 1 coordinates (+1.000 mm ML, +1.800 mm AP, -1.250 mm DV). During the same surgery, a metal pin was implanted in the opposite hemisphere serving as recording ground, and a head plate was secured to the animal's skull via dental cement. Sham animals (n = 5) underwent surgery for metal ground pin and head plate implantation, but did not receive any viral injection.

After 6-7 weeks post-surgery, recordings were performed with a borosilicate glass electrode (G100F-4, Warner Instruments; 1-5 MOhm and filled with saline) to avoid laser light induced artifact on metal electrodes, while mice were awake and head-fixed. Signals were amplified with a Multiclamp 700B (Molecular Device), digitized with a Digidata 1440A, and recorded with the pClamp software (Molecular Device) at 20 kHz. Optogenetic light illumination for ChR2 and Jaws were achieved with a 473 nm blue laser (Shanghai Laser) and a 635 nm red laser (Shanghai Laser), respectively. To deliver light, two optical fibers (200 μm in diameter, ThorLabs) were coupled with the glass electrode. The tips of the optical fibers were positioned about 500 μm above the tip of the glass electrode, and the power at the optic fiber tips was adjusted to 10 mW for both blue and red light. The fiber-electrode bundle was insert into the viral injection site (+1.000 mm ML, +1.800 mm AP, -0.400 DV). Illumination patterns consisted of three conditions: red laser alone to silence inhibitory neurons (R), blue laser alone to activate excitatory neurons (B), and both lasers (R+B). For each condition, we tested two illumination patterns (40 Hz pulse with 50% duty cycle, or a single constant pulse of 200 ms in duration). The illumination patterns were generated by a function generator (33210A Agilent), which was triggered by pClamp to maintain precise timing of the illumination patterns. Each trial contained three illumination periods (red laser alone, blue laser alone, and red + blue lasers) with 10 seconds intervals between each illumination and 5 second window before the first illumination period and after the last illumination period. The illumination order was randomized across mice, and each mouse underwent 10 trials of each illumination pattern.

Electrophysiology data analysis. LFP data analysis was performed in Matlab with Chronux (http://chronux.org/). Spectrograms were generated with mtspecgramc (tappers=[3 5], window size=500 ms, step size=5 ms) and normalized as decibel (dB) using: $power_{normalized} = 10 \times \log \frac{power}{power_{max}}$

where $power_{max}$ is the maximum value of the LFP power during each trial. The power pre- and post-stimulation for each trial was calculated with mtspectrumc (tappers=[3 5]) from LFP signals during the 1- second windows before and after illumination onset, respectively. To remove the variabilities between individuals, we further normalize the power for each illumination as follows:

$$power_{normalized} = 10 \times \log \frac{power}{power_{baseline}}$$

where $power_{baseline}$ is the averaged power during the 1-second window prior to the pre-illumination window. The powers were first averaged across trials to obtain the representative power for each animal, and then averaged across animals.