Supplementary Information for

Global Enhancement of Cortical Excitability Following Co-Activation of Large Neuronal Populations

Authors

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Materials and Methods

Figures S1 to S8

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Materials and Methods

Animals

Adult C57 mice purchased from Slac Laboratory Animals Inc. were used. All procedures were performed following protocols approved by the Animal Care and Use Committee of Shanghai Institutes for Biological Sciences, Chinese Academic of Sciences. Mice were group-housed in disposable plastic cages with standard bedding in a room with a normal 12-hr light / 12-hr dark cycle. Each animal used only once for an experiment.

Surgery

Experiments were performed on adult C57 male mice (2-6 months old, 20-35 g). For acute experiments, the mouse was anesthetized with intraperitoneal (i.p.) injection of chlorprothixene (5mg/kg), followed by urethane (1.4-1.5g per kilogram body weight). Mice were restrained in a stereotaxic apparatus, with body temperature kept at 37.5℃ using a heating pad. After asepsis, the skin was incised to expose the skull and the overlying connective tissue was removed. A 3 x 3 mm^2 craniotomy was made above the right V1 or the M2. In some experiments, two separated 2 x 3 mm^2 craniotomy was made above the right V1 and S1. In cross-hemisphere experiments, two 2 x 3 mm^2 craniotomy was made above the V1 of each hemisphere or above the ipsilateral V1 and contralateral S1. After the craniotomy, the underlying dura was removed to allow the insertion of the multi-electrode array manually or by a pneumatic insertion device (Cyberkinetics). The cortical surface was constantly superfused with warmed (34℃) artificial cerebral spinal fluid (aCSF) containing (in mM) 119 NaCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 22 glucose. The insertion depths were ~300 μm. For recording experiments on awake mice, the surgery was performed under ketamine (i.p., 100 mg kg^{-1}) and xylazine (i.p., 10 mg kg^{-1}) anesthesia, in which the mice were fitted with a dental cement head-mount with four mounting screws and the mice were held in the head-fixation and body restraining device. The recording was performed 2 hours after surgery, and the experiment was stopped if the mice showed physiological signs of stress.

Viral transfection

For virus injection, mice were anesthetized with sodium pentobarbital (7 mg/kg) and positioned

in a stereotaxic frame (Reward Co.). Body temperature was maintained at 37 °C using a heating pad. Viruses were injected using a glass micropipette with a tip diameter of 15–20 μm, through a small skull opening (<0.5 mm^2), with a micro-injector (QSI or Micro 4). Three injection points were distributed in a triangle with equal distance within V1, M2 or S1. Stereotaxic coordinates for V1: from 0 to 2.5 mm anterior of the lambda; from 2 to 4 mm lateral of the midline, and 0.3~0.5 mm vertical from the cortical surface. Stereotaxic coordinates for M2: -0.5 mm to 2.5 mm anterior of the bregma, 1.8 mm to 4 mm lateral from the midline, and 0.3~0.5 mm vertical from the cortical surface. In the experiment of long-distance co-activation, three injection positions were located within ipsilateral M1, S1, and V1, distributed 1 to 4 mm posterior to the bregma with equal distance and 2 mm lateral from the midline, and at a depth of 0.3 mm from the cortical surface. For AAV- CaMKIIα-ChR2-2a-mCherry, we injected 1 μl (10^{12} virus particles per ml) at each injection site in V1, S1 or M1/M2. In the experiment of midline/intralaminar thalamus inhibition, three positions in V1 inject AAV- CaMKIIα-ChR2-2a-mCherry, and one additional injection position (0.9 mm or 2 mm posterior to bregma, 0.2-0.3 mm lateral from midline, and at a depth of 2.9 mm from the cortical surface) inject AAV:HA-hM4D(Gi)-IRES-mCitrine (1 μl,10^{12} virus particles per ml). All the injected mice were waiting for more than 4 weeks for maximal expression before using for *in vivo* recording.

Multi-unit recording

The electrode array was custom made with Ni-Cr alloy wire (California Fine Wire Company, CFW 100188), consisting of 8 channels, with 150, 280 or 500 μm between neighboring channels and the impedance of 300k Ohm. Signals were recorded with the Cerebus system (Cyberkinetics) and TDT System-3 neurophysiology workstation (with an RA16 preamplifier + RZ5 workstation) controlled by OpenEx software (TDT). All data analysis was performed in Matlab (Mathworks). Spiking signals were band-pass filtered at 250 - 7,500 Hz and sampled with a rate of 30 kHz. Recorded spikes represent multi-unit activities, and the threshold for spike detection was set 3 times (in the Cyberkinetics system) or 1.5 times (in TDT system) the noise level. Recordings were made mainly in layer 2/3 of cortical neurons. PSTH for spiking activities was constructed using 2-ms bins.

***In vivo* whole-cell recording**

Whole-cell recordings were made in V1 of anesthetized mice. The whole-cell current-clamp recording was made with Multi-clamp 700A/B amplifier (Molecular Devices). Data were filtered at 2 kHz, digitized at 1-5 kHz, stored on a computer, and analyzed offline using Matlab (Math works). Borosilicate glass patch pipettes (resistance 7-10 MΩ) were filled with a solution containing (in mM) 140 K-gluconate, 5 KCl, 0.2 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3 Na_2-GTP, 10 Na-$_2$-phosphocreatine, and 10 HEPES (pH 7.3, 290-300 mOsm) for the recording of excitatory postsynaptic potentials. Only neurons recorded in superficial layers were analyzed (~300 μm from the pia surface).

Estimate of light intensity for test and co-activation stimuli

The intensity of the test light spot applied at the center of each RA was determined by the following procedure. We varied the light level of the test spot in a stepwise manner over a three-fold range (frequency, 0.25 Hz; duration, 16.7 ms), and chose the light intensity for each RA that evoked spiking of the multi-units recorded at all chosen RAs. As illustrated in Fig. S1A for one specific RA ("1" in Fig. 1D), when the intensity of test light at RA1 was increased, the total number of evoked spikes increased and the onset of spiking decreased at all four RAs. We chose the intensity (labeled by the green color) at which the test stimulation at the center of RA1 evoked multi-unit firing at all four electrodes, as indicated by the PSTH (bottom panel). Fig. S1B summarizes the results from all mice showing the relationship between the light intensities of test spots and the onset time (or the evoked firing rate) at all RAs chose for all experiments in this study.

Parameters for test and co-activation stimuli

The duration of test stimuli was set to frame (1/60 s, 16.7 ms), for the reason that in our DMD-based system, the maximal refresh rate was 60 Hz. The previous work in our lab (1) showed that 2-second interval was appropriate for reliable testing of cortical neuronal responses, and this interval was used in our standard tests. The stimulus pattern of co-activation conditioning was in a modified theta-burst pattern (five 30-Hz burst stimulation instead of four 100-Hz stimuli (as in the standard TBS)). The interval between bursts is 1 second.

Drug infusion

All drug infusion experiments were performed on anesthetized mice. For the application of APV or CNQX, 50 μl of ACSF containing APV (Tocris, 0.5 μM) or CNQX (Sigma-Aldrich, 0.05 or 0.5 μM) was loaded onto the cortical surface. We found that although APV or CNQX application caused a decrease in cortical firing rate, the rate reached a steady-state within 20 min after APV application and 15 min after CNQX application (Fig. S3C) and remained stable during the optogenetic activation experiment.

Data analysis

The evoked firing rate was calculated by the average spike rate within the 30-ms window after the stimulus subtracted by that within the 30-ms window before the stimulus. The after/before ratio was calculated by the evoked firing rate after co-activation divided by that before co-activation. The EPSPs were truncated by median filtering with 5-ms bin. All data analysis was performed by Matlab (Mathworks). Data are presented as mean ± SEM. unless stated otherwise. The statistical significance was tested with the two-tail *t* test and Kolmogorov-Smirnov test. One-way ANOVA and Bonferroni posthoc test following one-way ANOVA was used for testing significance between more than two groups.

Estimate of the number of neurons activated by optogenetic stimulation

To examine the number of ChR2-expressing neurons in the visual cortex, the mouse was fixed by perfusion with 4% paraformaldehyde after *in vivo* recording. The dissected brain was removed and fixed with paraformaldehyde at 4℃ for 1 day, mounted in agar, and cut into 50 μm coronal sections on a Leica VT1200 vibratome. Fluorescence images of cortical sections were acquired using an Olympus FV10I confocal microscope with a 10X objective lens, using z-stacks at 5 μm steps. The neuron number in the acquired images was counted using NIH Image J software. In 4 coronal sections of a typical mouse brain that was used in the optogenetic stimulation experiment, we counted the total number of labeled neurons within 50 x 100 x 100 (depth) μm cortical columns. The result was summarized in Fig. S7A, showing the distribution of ChR2-expressing neurons at different cortical depths.

To determine the optogenetically stimulated neuronal population, we next estimated the distribution of the test light in the cortex. A typical mouse brain was rapidly dissected, mounted in agar, and cut along a coronal plane through the visual cortex. The brain block was then placed on the experimental platform in the same manner as that in the co-activation experiments. A spot of laser light with the same size as that was used in the test stimulation was applied to the surface of the visual cortex adjacent to the edge of the cut surface of the brain block. The distribution of laser light on the cut surface was imaged using a Nikon D90 camera (Fig. S7B). For the example mouse used above in estimating the number of the labeled neurons, we measured the border of the cortical area illuminated by higher than 50% of total light intensity (see Fig. S7B), and then used the function below to estimate the number of active neurons within the laser illuminated area.

$$\text{Number} = \sum_{d=0}^{D} \rho(d) * \pi * R(d)^2$$

Where "D" is the maximal depth below the cortical surface within the border, ρ(d) is the density function of ChR2-expressing neurons at different depth (d), R(d) is the function of the radius of the border at different depth. Based on data from the example mouse described in Fig. S7A and B, the estimated total number of ChR2-expressing neurons activated by the standard test light stimulus was ~6000 neurons in the example shown in Fig. S7A.

Immunostaining and confocal imaging

Mice were fixed by transcardial perfusion with 4% paraformaldehyde after *in vivo* recording. Dissected brains were removed and fixed with paraformaldehyde at 4℃, cryoprotected with 30% sucrose, and then cut into 40 μm coronal sections on a Leica HM525 cryostat. Cortical sections were incubated with rabbit polyclonal antibody against CaMKII (1:1000, Sigma-Aldrich) or against GABA (1:1000, Millipore) overnight at 4℃. Secondary antibodies, Alexa Fluor 488 donkey against rabbit IgG antibody (1:1000 Invitrogen) were used. The nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole, Sigma-Aldrich). Fluorescence images of V1 sections were acquired using a Nikon A1 confocal microscope with a 60X oil-emersion objective lens, using z-stacks at 0.3 μm steps. Fluorescence images of midline/intralaminar thalamus sections were acquired using Olympus FV3000 confocal microscope with a 20X objective lens. Quantification of the number

was processed using NIH Image J software and custom write Matlab script.

Supplementary Figure Legends

Fig. S1.

Spiking Responses Evoked by Test Light of Different Intensities.

(A) Peri-stimulus spiking responses evoked by test stimuli (frequency, 0.5 Hz; duration, 16.7 ms) applied to RA1 at 8 different intensity levels (color-coded) in a stepwise manner over a five-fold range. Top panel, spiking at all 4 RAs in response to test stimuli applied to RA1. Vertical dashed lines, test stimulus onset. Blue bar, duration of the light stimulus. Bottom panel, peri-event histograms of the test-evoked spiking rate (average in 5-ms bins) at the light intensity level 6 (marked by horizontal dashed lines). Note that, at this light level, the spike activities were clearly observed in both test light-stimulated RA ("self") and neighboring RAs ("hetero"), a criterion used for determining the light intensity used in each experiment.

(B) Dependence of the onset time of spiking and average spiking rates (within 30-ms window after stimulus onset) on the light intensity, at self-activated (red) and hetero-activated (blue) RAs.

Fig. S2.

Activation of V1 Neurons in Random Sequences did not Change V1 Neuronal Excitability.

(A) Typical experimental protocol. Test stimuli (light pulse of 16.7ms, 50 pulses at 2-s interval) were applied before and after co-activation: Conditioning stimuli were 50 bursts at 1-s intervals (5 pulses/burst at 15 Hz), with pulses applied in random sequence to the 4 RAs (marked by different colors), as illustrated for 10 trials.

(B) An example of a peri-stimulus spike raster plot for evoked responses at four RAs evoked by test stimuli applied to RA3 over 50 trials, before (left), during (middle), and after (right) random sequence activation. Red dashed line, test stimulus onset. Blue bar, test stimulus (duration, 16.7 ms). Shaded blue, random sequence activation (duration, 334 ms).

(C) Data shown in B, plotted as peri-stimulus histograms, for firing at all four RAs evoked by test stimulation at RA3, before (blue) and after (red) random sequence activation.

(D) Pair-wise comparison of after/before ratio of spiking response at all RAs, for responses evoked by stimuli applied to the RA itself ("self") and to those applied to other RAs ("hetero"), before (blue) and after (red) random sequence co-activation. Each data point represents the average ratio for all tested RAs observed in one mouse ($p>0.5$ for both data sets, n = 5 mice, paired *t* test).

Fig. S3.

Co-activation Did Not Induce Detectable Change in Spontaneous Firing and Dependence of the Enhancement Effect on NMDA Receptor Activation.

(A) Spontaneous firing rate (30 ms before the test stimulus onset) at different times before and after co-activation (vertical dashed line), in the co-activated RAs and RAs not co-activated. Data represented mean ± SEM. (n =13 mice).

(B) Pair-wise comparison of the spontaneous firing rate (over the 30-ms period before the test stimulus onset), averaged over all trials during a 10-min test period, before (red) and after (blue) co-activation. The same data set as in A. No significant difference was observed ($p>0.19$ for both data sets, paired *t* test).

(C) Changes in the firing rate with time after cortical perfusion of APV or CNQX. The mean firing rate decreased to a stable level of about 40.8 ± 4.4 spikes/s (SEM, n = 24 mice) at about 10 min after APV (0.5 μM) and 38.9 ± 7.9 spikes/s (SEM, n = 4 mice) at 20 min after CNQX (0.05 μM) perfusion, respectively. The drug was applied at time 0. Data points represent mean firing rates within 1-min bins, and error bars depict SEM.

(D) Co-activation-induced enhancement of test stimulus-evoked spiking was abolished after the APV application. Data from one example experiment on co-activation of V1 neurons. Top two panels: Peri-stimulus raster plots of spiking activity in all four RAs (shown only for RA1 and RA2) in response to test stimuli applied to RA2 over 50 trials, before, during, and after co-activation of four RAs, with the mouse V1 under constant perfusion with ACSF throughout the experiment. Red dashed line, light stimulus onset. Blue bar, light stimulation. Note the increased spiking after co-activation at all RAs in

response to RA2 stimulation. Middle two panels: The perfusion solution was subsequently changed to ACSF plus APV (500 μM). Further co-activation experiment using the same protocol as above resulted in no significant enhancement of test stimulus-evoked firing at all four RAs. Bottom two panels: Further perfusion of V1 with ACSF alone afterward resulted in a significant enhancement in all RAs following the third round of co-activation.

(E) Data from D were illustrated with peri-stimulus histograms, showing the enhancement of test stimulus-evoked spiking following co-activation before and after inhibition of NMDA receptors with APV.

Fig. S4.

Stability of Response Area (RA) of a Single Neuron Recorded by In Vivo Whole-cell Recording.

Depolarizations recorded at different times (20, 30, 40 ms) after the stimulus onset, coded in grayscales (shown by scale bar below), recorded at two-time points (t = 0 hr and t = 2 hr).

Fig. S5.

Stability of Input Resistance and time constant of local co-activated Neuron and distant non-co-activated neuron.

(A) Summary of input resistance of the neurons co-activated (local) and not co-activated (distant) before and after co-activation (n = 5 neurons).

(B) Summary of time constant of the neurons co-activated (local) and not co-activated (distant) before and after co-activation (n = 5 neurons).

Fig. S6.

Cooperativity and Cross-hemispheric Enhancement Effect.

(A) Enhancement of evoked responses following co-activation of neurons in distant cortical regions in an anesthetized mouse. Left, a schematic diagram showing co-activating areas. Dashed circles, RAs for the multi-electrodes in two cortical

regions. Solid circles, tested RAs; white squares, testing light spots in this example experiment; blue squares, co-activated areas; scale bar, 300 μm. Right, examples of peri-stimulus raster plots and PSTH of spike trains in 7 RAs in each cortical region (V1 and S1), in response to 50 trials of test stimuli (white squares) applied to 2 example RAs (white circles), before and after co-activation. Red and gray dashed lines, stimulus onset; blue bar, duration of test stimulation.

(B-G) Two example experiments showing enhancement of evoked responses following co-activation of neurons in distant or cross-hemispheric cortical regions in an anesthetized mouse (A-C) and an un-anesthetized mouse (D-F). (A) and (D): schematic diagrams showing co-activated areas. Dashed circles, RAs for multi-unit electrodes in two cortical regions. Solid circles, tested RAs; white squares, testing light spots; blue squares, co-activated areas. Scale bar, 300 m. (B) and (E): Examples of peri-stimulus raster plots of spike trains in five RAs in each cortical region (V1 and S1) or hemisphere (left V1 and right V1), in response to 50 trials of four RAs co-activation stimulation. Red dashed line, light stimulus onset; blue bar, duration of test light stimulation. (C) and (F): Peri-stimulus raster plots and PSTH of spike trains in five RAs in each cortical region (V1 and S1) or hemisphere (left V1 and right V1), in response to 50 trials of test stimuli (white squares) applied to two example RAs (white circles), before and after co-activation. Red dashed line, light stimulus onset; blue bar, duration of test light stimulation.

(H) Summary of experiments involving test RAs located at contralateral V1 from the co-activated V1 in awaked mice (n = 3), for co-activation of four RAs. Significant enhancement was observed not only in the co-activated hemisphere but also in the non-co-activated hemisphere. Data points represent mean ± SEM (* $p<0.05$ ** $p<0.01$; paired *t* test).

Fig. S7.

Distribution of Light-activated Neuronal Populations.

(A) Distribution of ChR2-expressing neurons at different cortical depths. Left: a coronal cortical section (thickness: 50 μm) from a virus-infected mouse, showing

mCherry-labeled neurons. Right: Estimated number of ChR2-expressing neurons within a cortical volume of 100 x 100 x 50 μm at different cortical depths.

(B) Map of the cortical region activated by the test light, with the relative intensity coded by colors in log scale. White dashed circle marked the area illuminated by light with 50% of maximal light intensity, which is known to directly stably activate neurons, as detected by multi-unit recording (see Fig. S1). Each RA with the light intensity sufficient to stably activate labeled neurons was ~500 μm, and co-activation that reliably induced enhancement effect thus corresponded to a cortical volume with a diameter of ~1 mm. Scale, 1mm.

Fig. S8.

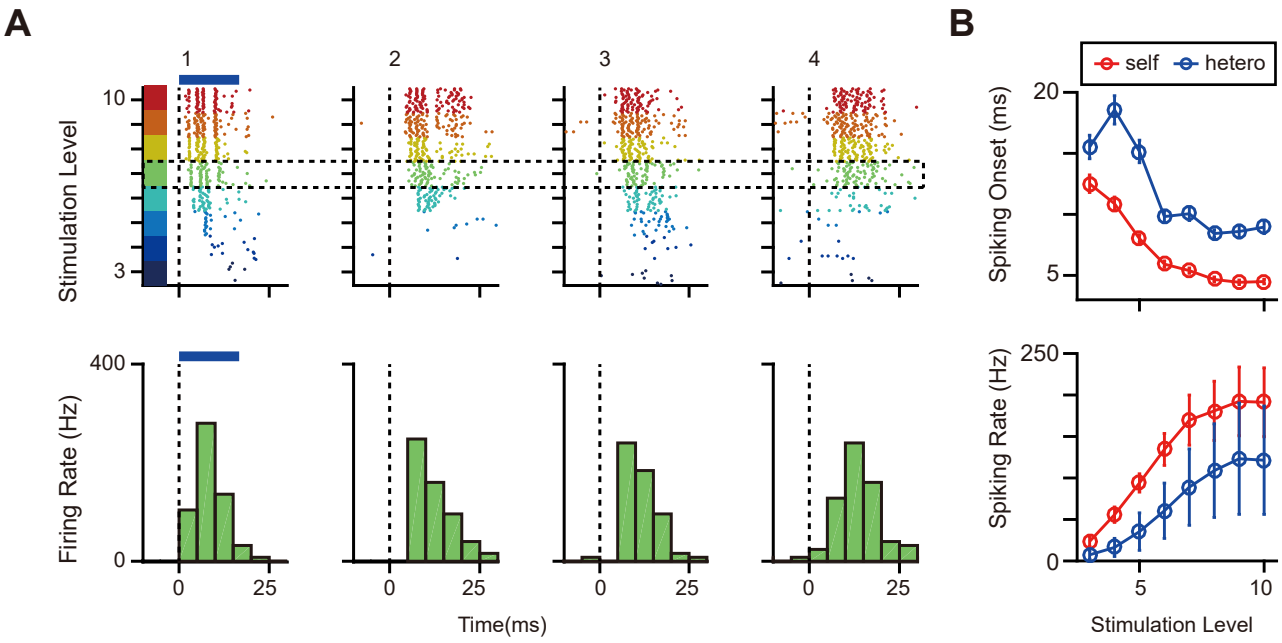
Activation of the midline/intralaminar Thalamus during Co-activation.

(A) Neuronal spiking observed in cortical and subcortical regions during cortical co-activation. Schematic diagrams (left) depict one cortical and four sub-cortical recording sites (marked by black stars) in an example experiment. Red lines, recording electrodes. Peri-stimulus raster plots (right) at each recording site in response to 50 optogenetic stimuli, using two different sizes of light spot (blue squares) applied to V1. Red dashed line, light stimulus onset. Blue bar, duration of the light stimulus.

(B) Immunostaining of c-Fos (green) observed in brain sections. The dashed line marked different brain regions based on stereotaxic coordinates in mouse brain atlas (Paxinos and Franklin, 2003). Top: example coronal section of the cortex (CTX); bottom: 1-4 corresponding to the coronal sections at bregma -0.82 mm, -1.06 mm, -1.34 mm and -1.82 mm in the mouse brain atlas, respectively, Scale, 200 μm. CL, centrolateral thalamic nucleus; CM, central medial thalamic nucleus; IAD, interanterodorsal thalamic nucleus; IMD, intermediodorsal thalamic nucleus; LHb, lateral habenular nucleus; MD, mediodorsal thalamic nucleus; PC, paracentral thalamic nucleus; PV, paraventricular thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior part.

Reference

1. S.J. Xu, W.C. Jiang, M.M. Poo, Y. Dan, Activity recall in a visual cortical ensemble. *Nat Neurosci*. 15, 449-55 (2012).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3
4
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Firing Rate (Hz)
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25
Time(ms)
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hetero
20
5
Spiking Onset (ms)
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Spiking Rate (Hz)
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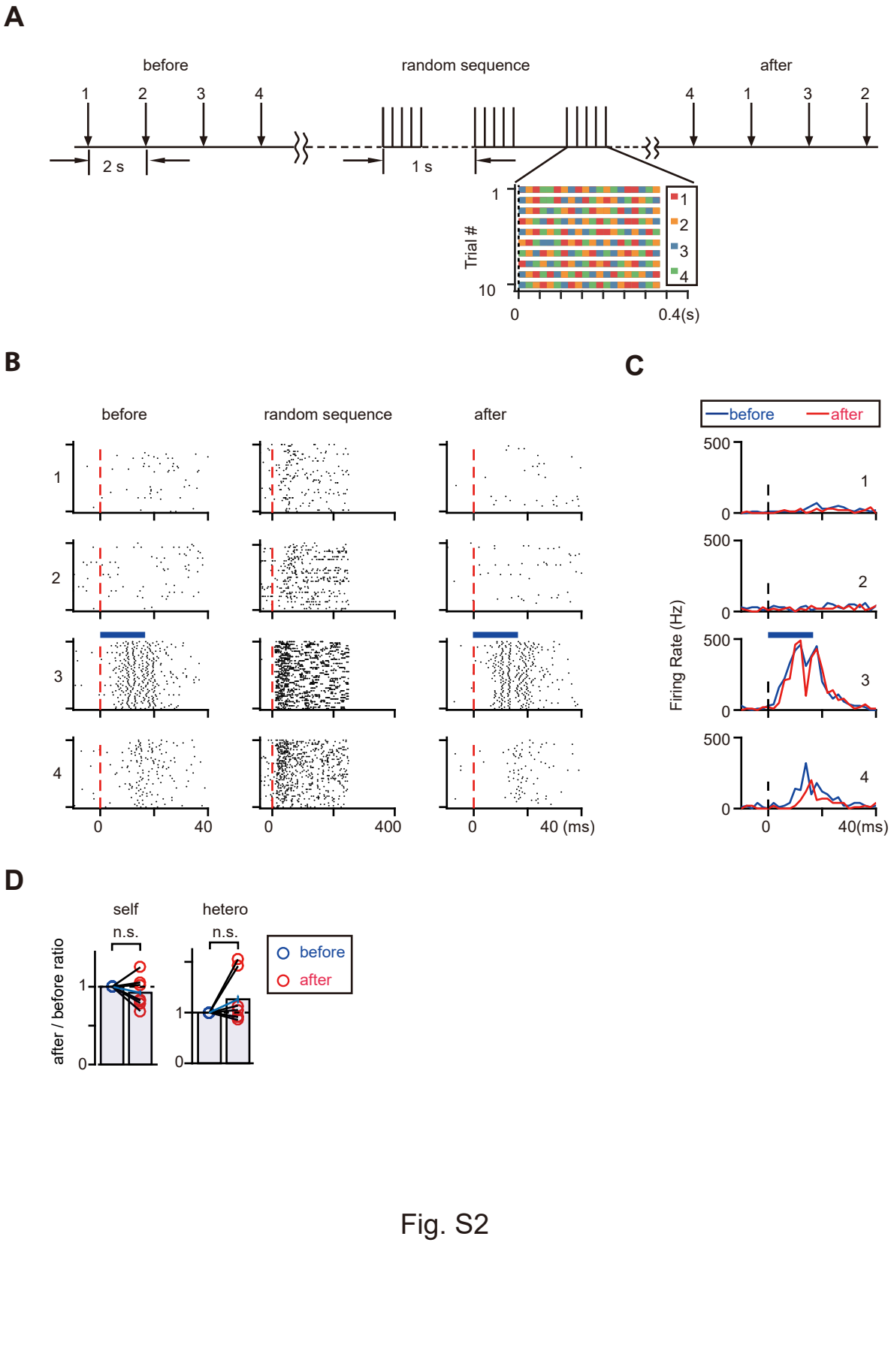
Stimulation Level

Fig. S1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before
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after
40
400
40 (ms)
C
before
after
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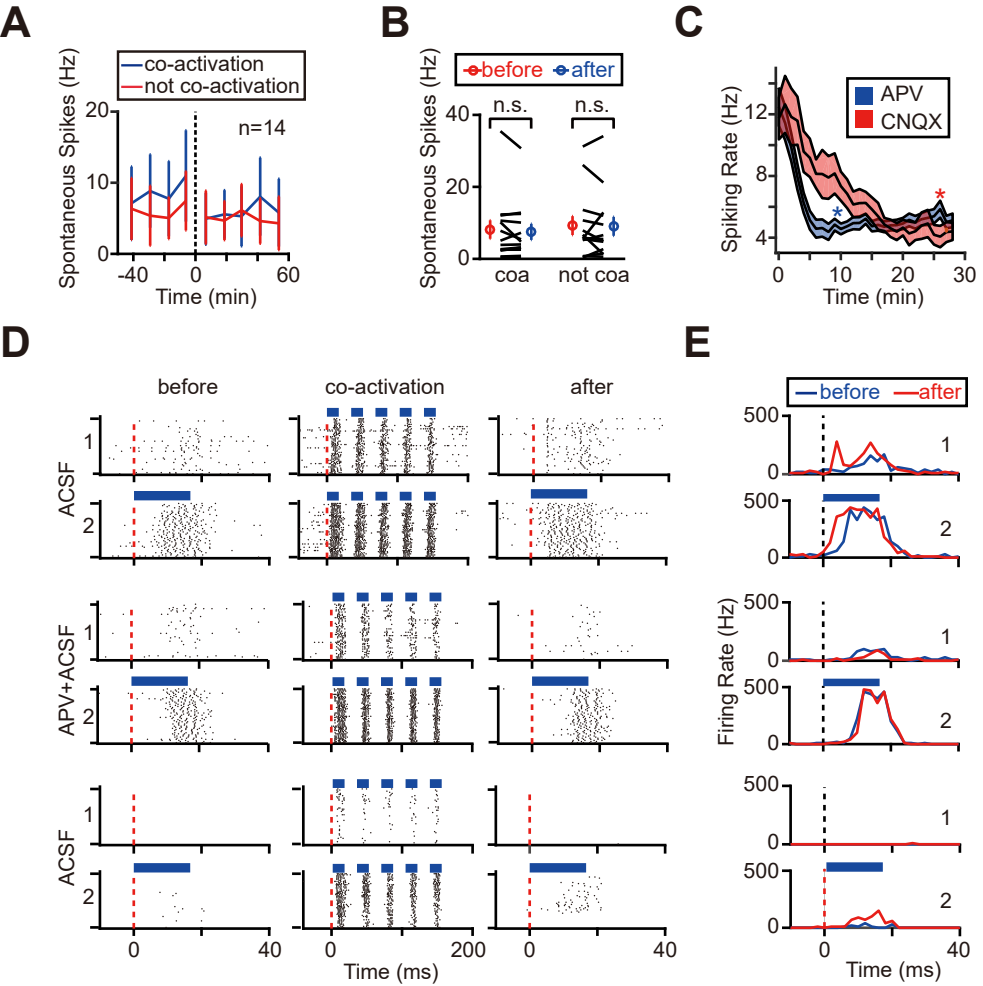
n.s.
after / before ratio
before
after

Fig. S2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before
after
n.s.
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coa
not coa
C
APV
CNQX
Spiking Rate (Hz)
Time (min)
D
before
co-activation
after
ACSF
APV+ACSF
ACSF
Time (ms)
E
before
after
Firing Rate (Hz)
Time (ms)

Fig. S3

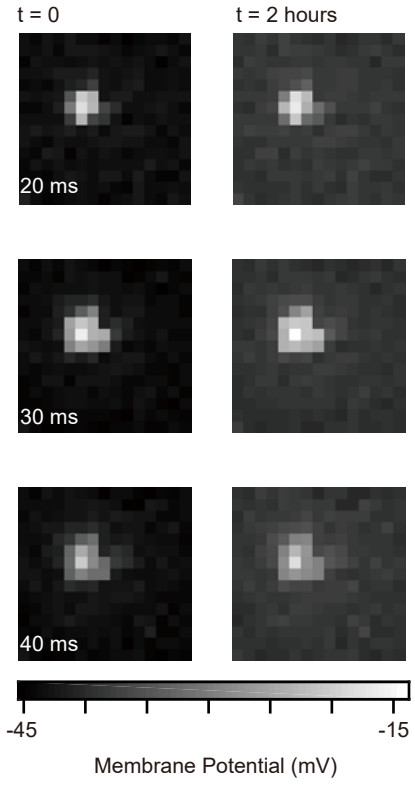
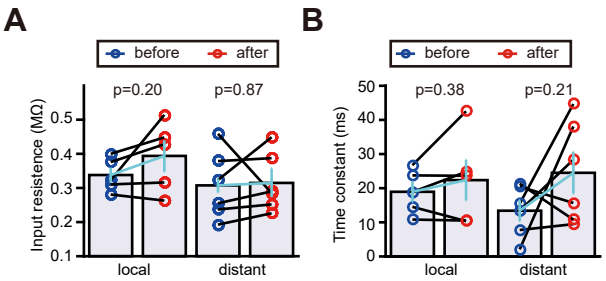


Fig. S4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p=0.21
Time constant (ms)
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40
30
20
10
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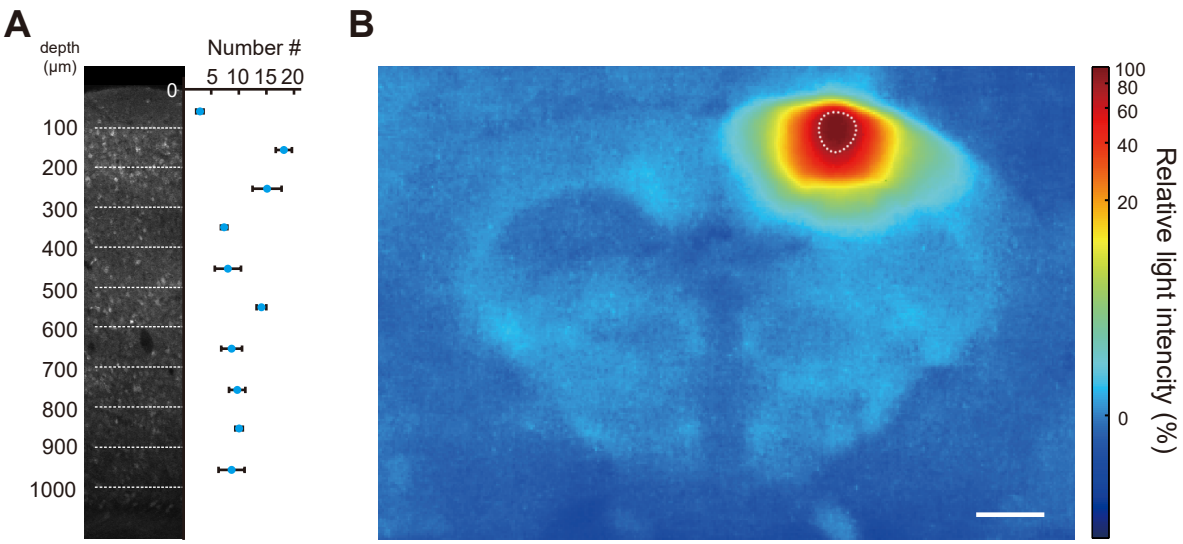
local
distant

Figure S5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800
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1000
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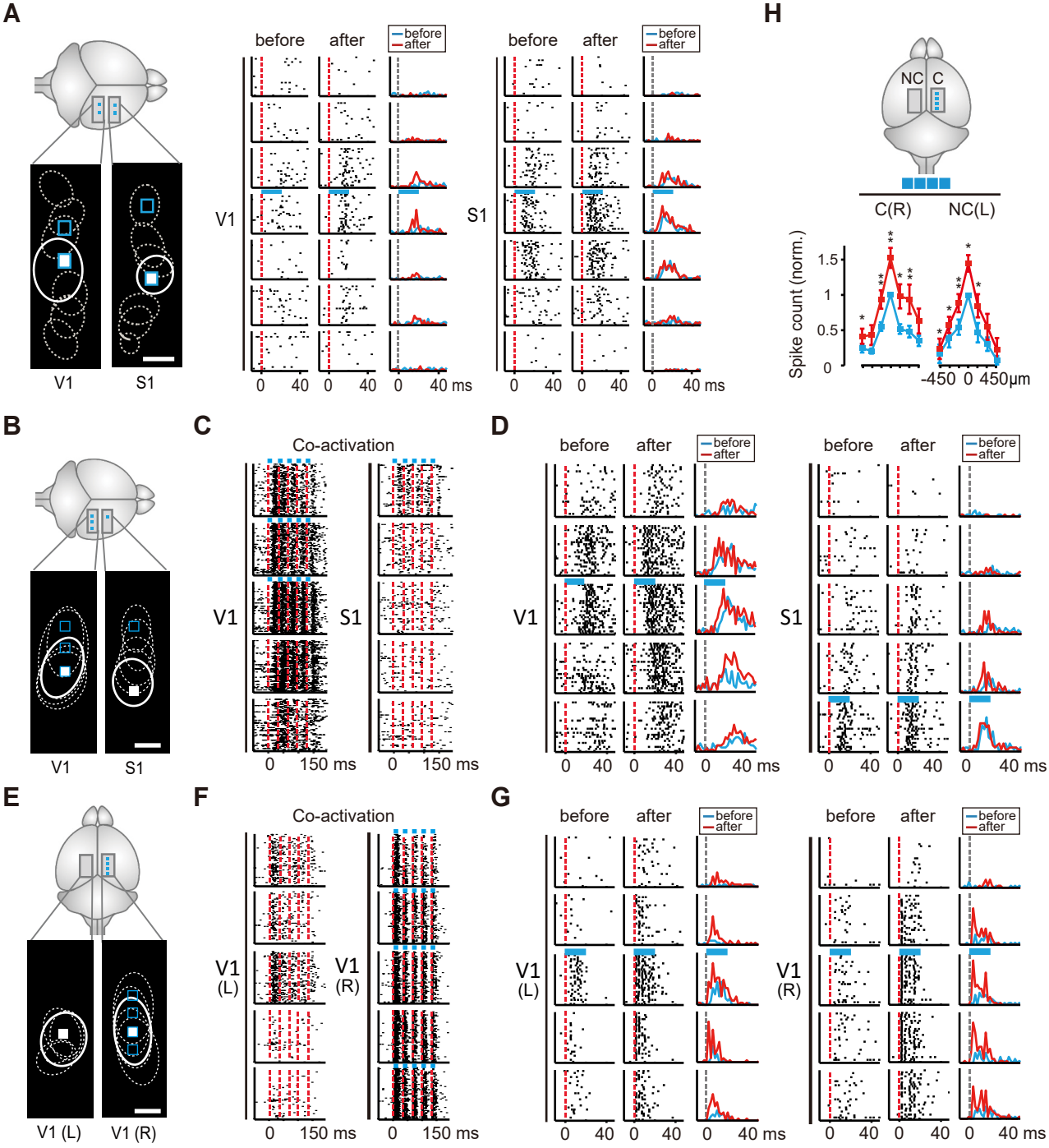
80
60
40
20
0
Relative light intencity (%)

Fig. S6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V1
S1
before
after
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40 ms
B
C
Co-activation
150 ms
D
E
V1 (L)
V1 (R)
F
G
H
NC
C
C(R)
NC(L)
Spike count (norm.)
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1
0.5
0
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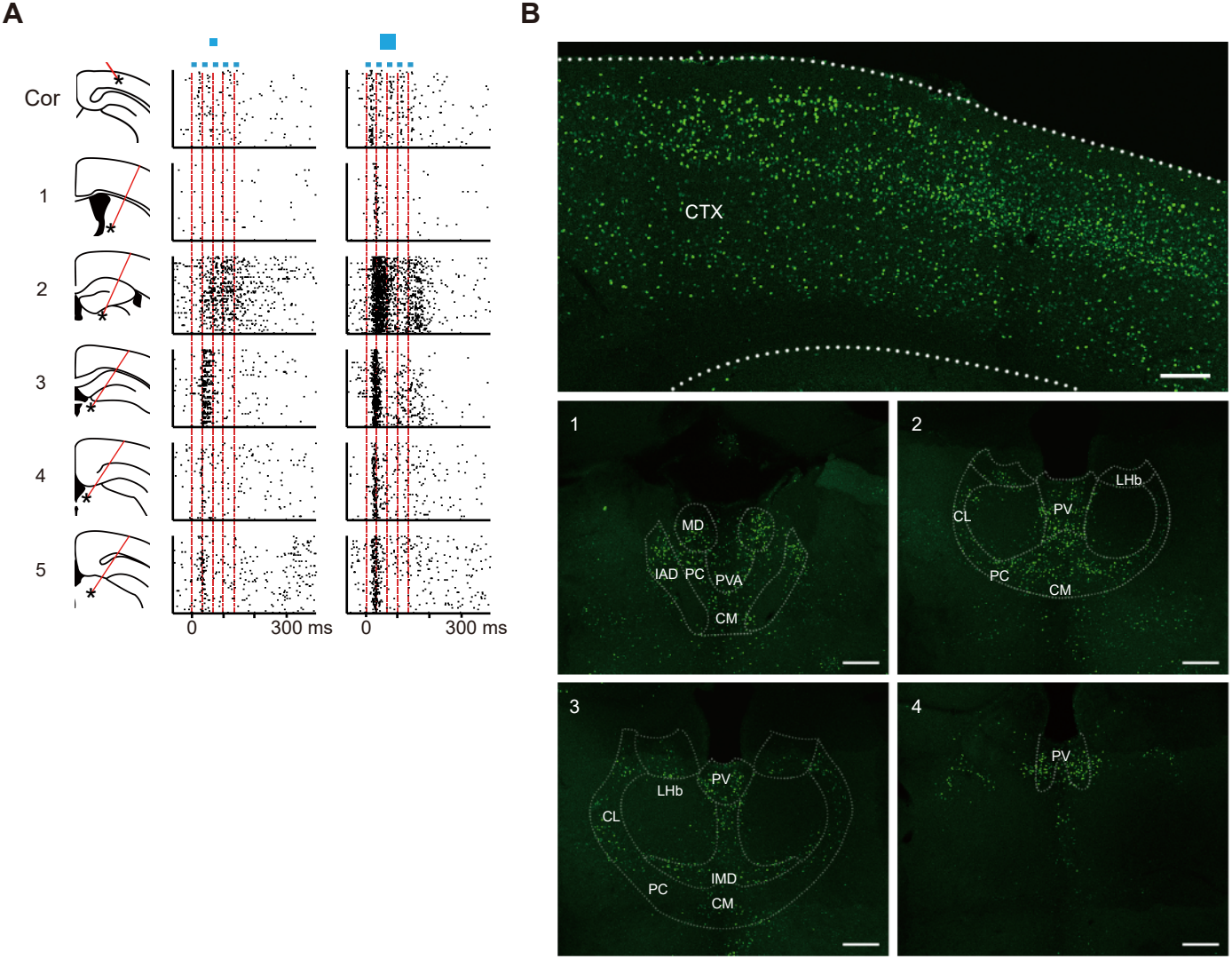
Fig. S7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4
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300 ms
0
300 ms
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CTX
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MD
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PVA
CM
2
LHb
CL
PV
PC
CM
3
PV
LHb
CL
IMD
PC
CM
4
PV

Fig. S8