Supplementary Material

Differential contribution of excitatory and inhibitory neurons in shaping neurovascular coupling in different epileptic neural states

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Quantification of vessel diameters and GCaMP6f signals derived from two-photon imaging data

We used a 2D Gaussian filter with a 1.73 μm full width at half maximum (FWHM) to enhance the signal-to-noise ratio (SNR) of the vessel imaging data. The filtered data were then processed to correct the movement artifacts using the "StackReg" plugin with a rigid-body transformation in Fiji.[1] Following this alignment, we selected three to five points on different branches of pial arterioles and venules within 700 μm from the seizure focus (187 arterioles and 163 venules from 53 seizures in 8 mice). The identification of the pial arterioles and venules was based on morphology.[2] Lines perpendicular to the vascular lumens were then made on the selected points, generating a stack of lines measured over time. To calculate the changes in vessel diameter, these stacked measurements were then processed using the "Diameter" plugin in Fiji,[3] which applies a modified algorithm from the full width at half-maximum (FWHM). Manual measurements from eye inspection evaluated and confirmed the quantified diameters.

To quantify GCaMP6f fluorescence intensity change, we first used Fiji (ImageJ) to apply a Gaussian filter with a 1.73 μm FWHM kernel to all image stacks in order to improve the signal to noise ratio. After Gaussian filtering, all image stacks were concatenated for each individual animal and realigned using the "Image stabilizer" plug-in in Fiji to compensate for potential in-plane movements. Our field of view (FOV) was 885.76 μm x 885.76 μm, and FOV generally includes up to 750 μm from the injection focus.

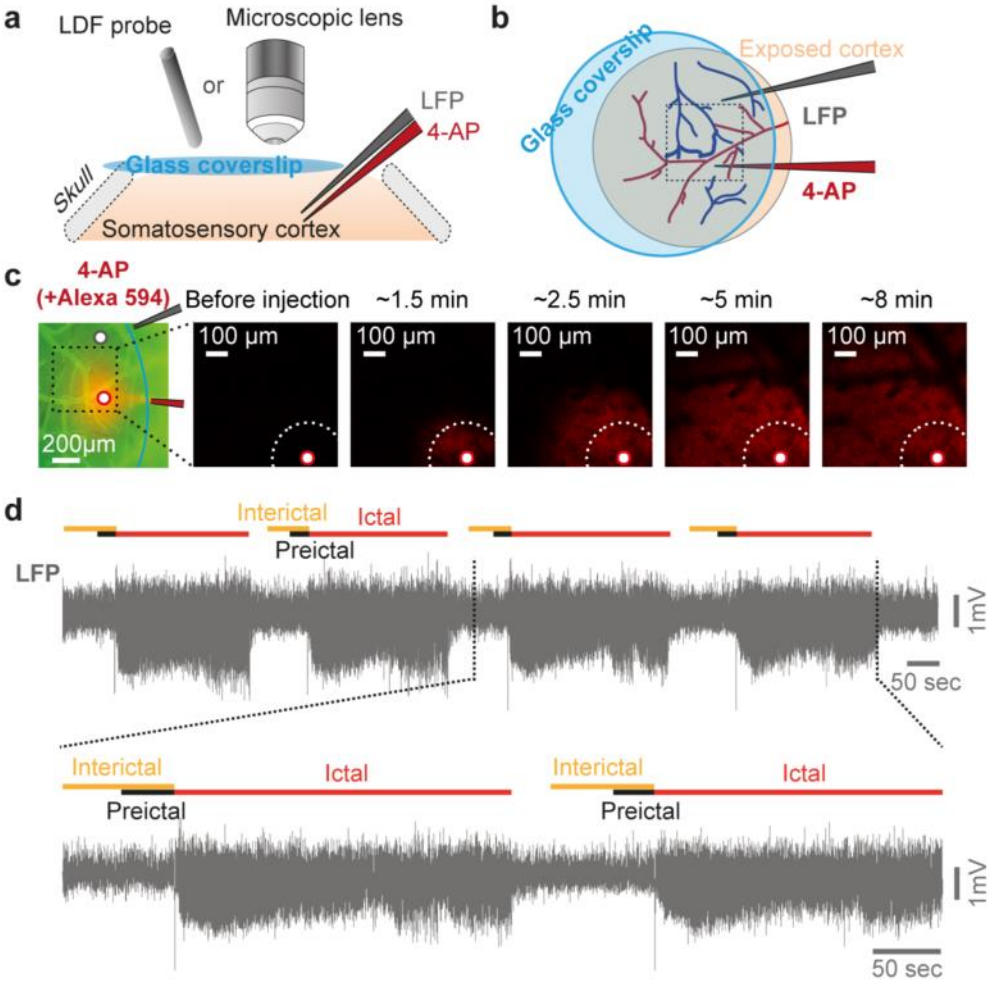
The 200 μm-annuli range from the injection focus was excluded to avoid possible damage to neuronal cells, resulting from the insertion of a glass pipette. The signal intensity was averaged within the area of 200–700 μm from the 4-AP injection site to quantify changes in excitatory and inhibitory neuronal calcium activities. The intensity of pixels within 200-700 μm of the

seizure focus was averaged in each different epileptic state: pre-injection, preictal and ictal period, using the same time criteria used for the vessel diameter analysis. To obtain cell contours of individual neurons (neuron ROIs), we generated a standard deviation (SD) map using from the 1-min spatiotemporal data following seizure onset. The temporal SD map was used to identify a cell soma where the calcium changes mainly occurred.

To accurately isolate the cell soma regions (neuron ROIs) from the background signal, we created a ~20 μm FWHM kernel Gaussian filtered image and subtracted it from the raw SD map. A local threshold with "Otsu" algorithm was conducted to cluster the cell soma. After this transformation, the cell soma clusters were segmented as a single cell shape using a "watershed" algorithm to divide the boundaries of overlapping cells. We also confirmed the division visually and excluded clusters with diameters less than 5 μm to eliminate false-positive signals. The fluorescence time course of each cell was measured by averaging all pixels within each neuron ROI. We also excluded the clusters whose intensity did not increase more than 2 SD over the preictal level. We then calculated the coordinates of the center of mass of the defined cell cluster. The distance from the center of the 4AP injection to the cell cluster center of mass was measured and assigned to the cell cluster. Based on the coordinates of the cell cluster, the calcium transients of the isolated cells were acquired in each seizure trial. To calculate neuronal synchrony, we calculated correlation coefficients between a pair of two neuron ROIs' calcium transients (window:1s, step: 1s) for all pairs of neuron ROIs identified in each seizure. In each seizure trial, the correlation values were averaged for the pairs that showed statistical significance at 95% confidence level.

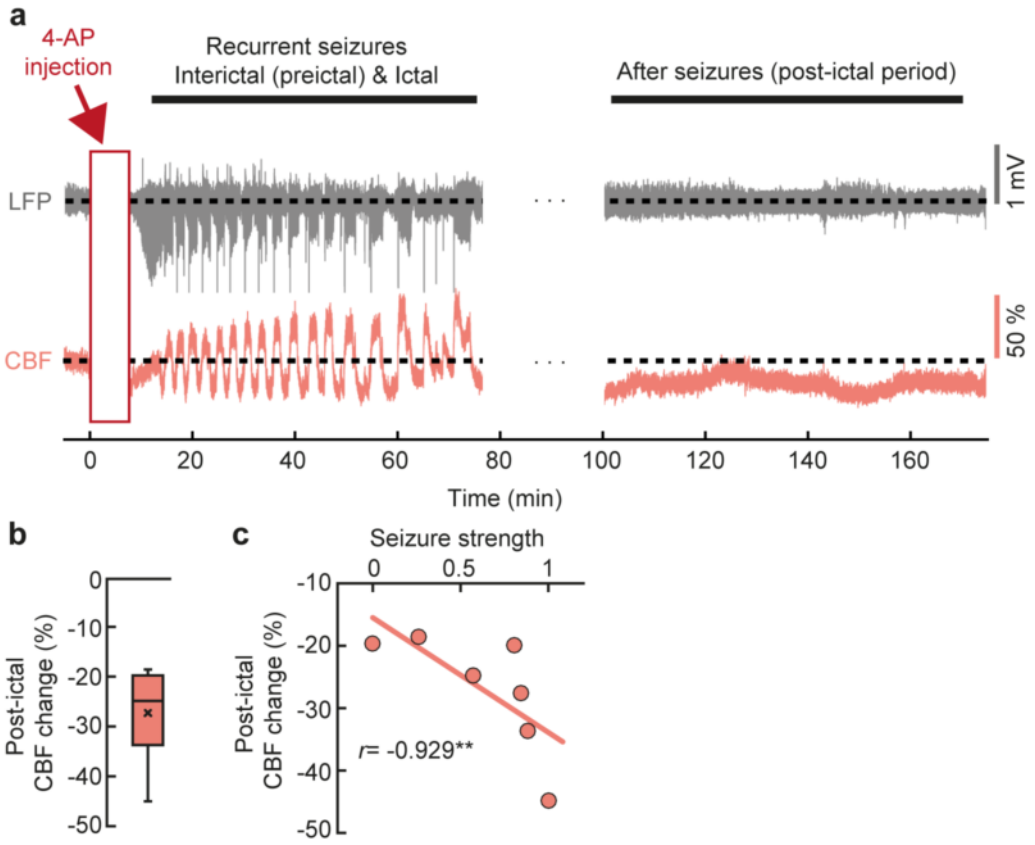
Immunostaining

Anesthetized mice (Zoletil, 30 mg/kg, i.p., Virbac, France) were perfused with saline and 4% paraformaldehyde (PFA), and their brains were extracted. The brains were fixed in 4% PFA at 4°C for 24 hours and were immersed in 30% sucrose with 0.1% sodium-azide solution for 3 days. The brains were then frozen and coronally sectioned to a thickness of 40-μm by cryostat. The primary antibodies used were mouse anti-green fluorescent protein (GFP, Abcam, USA, 1:200), rabbit anti-neuronal nuclei (NeuN, Abcam, 1:400), rabbit anti-parvalbumin (PV, Abcam, 1:200), and rabbit anti-somatostatin (SOM, Peninsula, USA, 1:1,000). The secondary antibodies used were anti-mouse Alexa 488 (Molecular probes, USA, 1:200) and anti-rabbit Alexa 568 (Molecular probes, 1:350) antibodies. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, 1:10,000). The stained brain slices were imaged using confocal laser microscopy (TCS SP8, Leica).

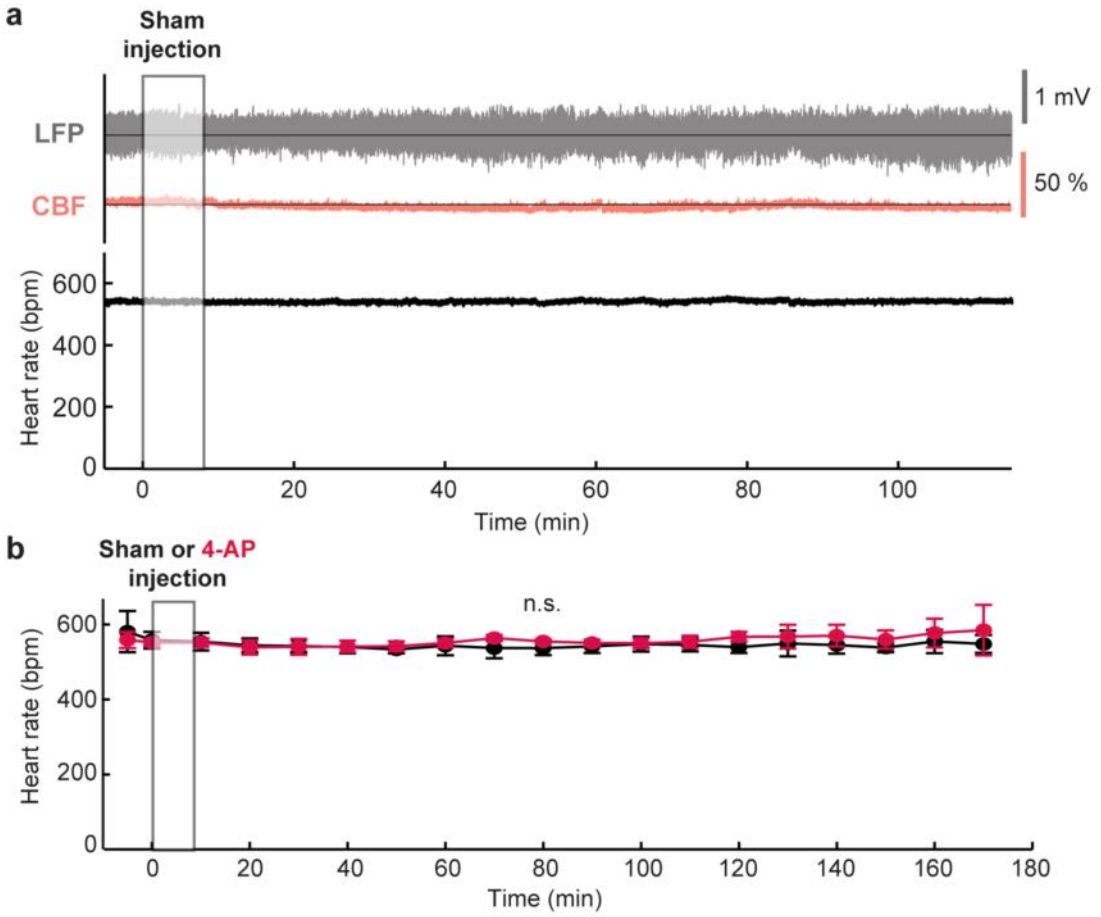


Supplementary Figure 1. Experimental set-ups for LDF recording or two-photon imaging with concurrent recording of LFP signals and induction of recurrent seizures (a, b) Schematic drawing of the top and side views of the cranial window, partially covered with a glass coverslip. A glass pipette and an LFP recording probe was intracortically inserted into layer 2/3 of somatosensory cortex through the partially exposed cranial window. (c) The seizure focus (the tip of the glass pipette) and the temporal spread of the solution are visualized by a mixture of Alexa594 (10 μM) with 4-AP (15 mM) to confirm 4-AP injection. (d) An example of LFP signals of consecutive seizures induced by the 4-AP injection, showing different

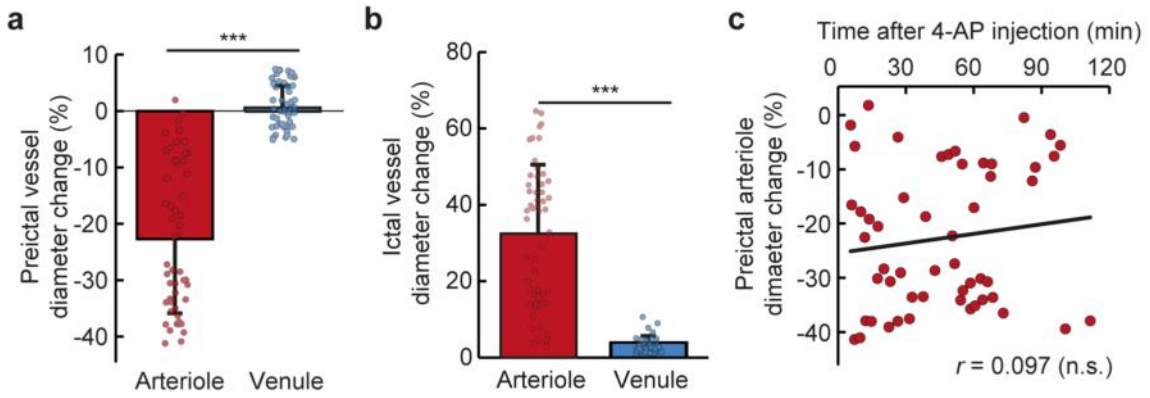
epileptic states: interictal (the period between consecutive seizures), preictal (up to 30 s period preceding each ictal onset) and ictal events.



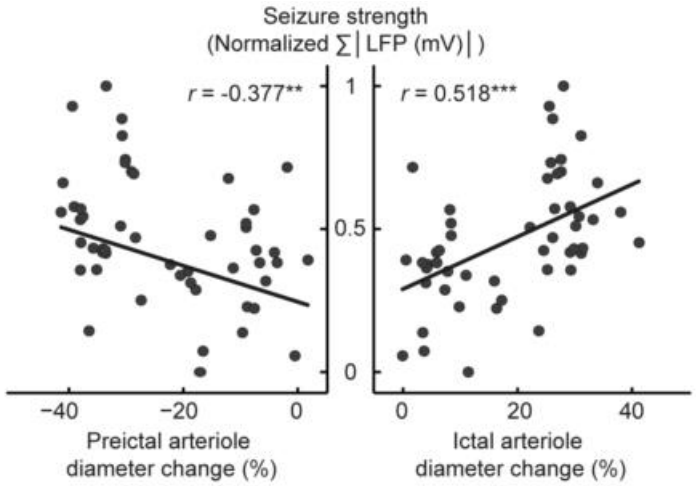
Supplementary Figure 2. CBF changes after recurrent seizures. (a) Representative examples showing the dynamics of LFP and CBF before, during and after recurrent seizures induced by 4-AP. (b) Post-ictal CBF changes (n=7, -26.97 ± 9.50%, mean ± SD). CBF levels during 20-80 mins after last seizure were averaged and normalized according to pre-injection CBF levels. (c) Relationship between post-ictal CBF changes and ictal strength of recurrent seizures prior to the post-ictal period (Spearman's r=-0.929, p=0.007**, R^2=0.509). Absolute values of LFP amplitudes (>3 SD above the pre-injection LFP signals) during recurrent seizures were combined to estimate ictal strength.



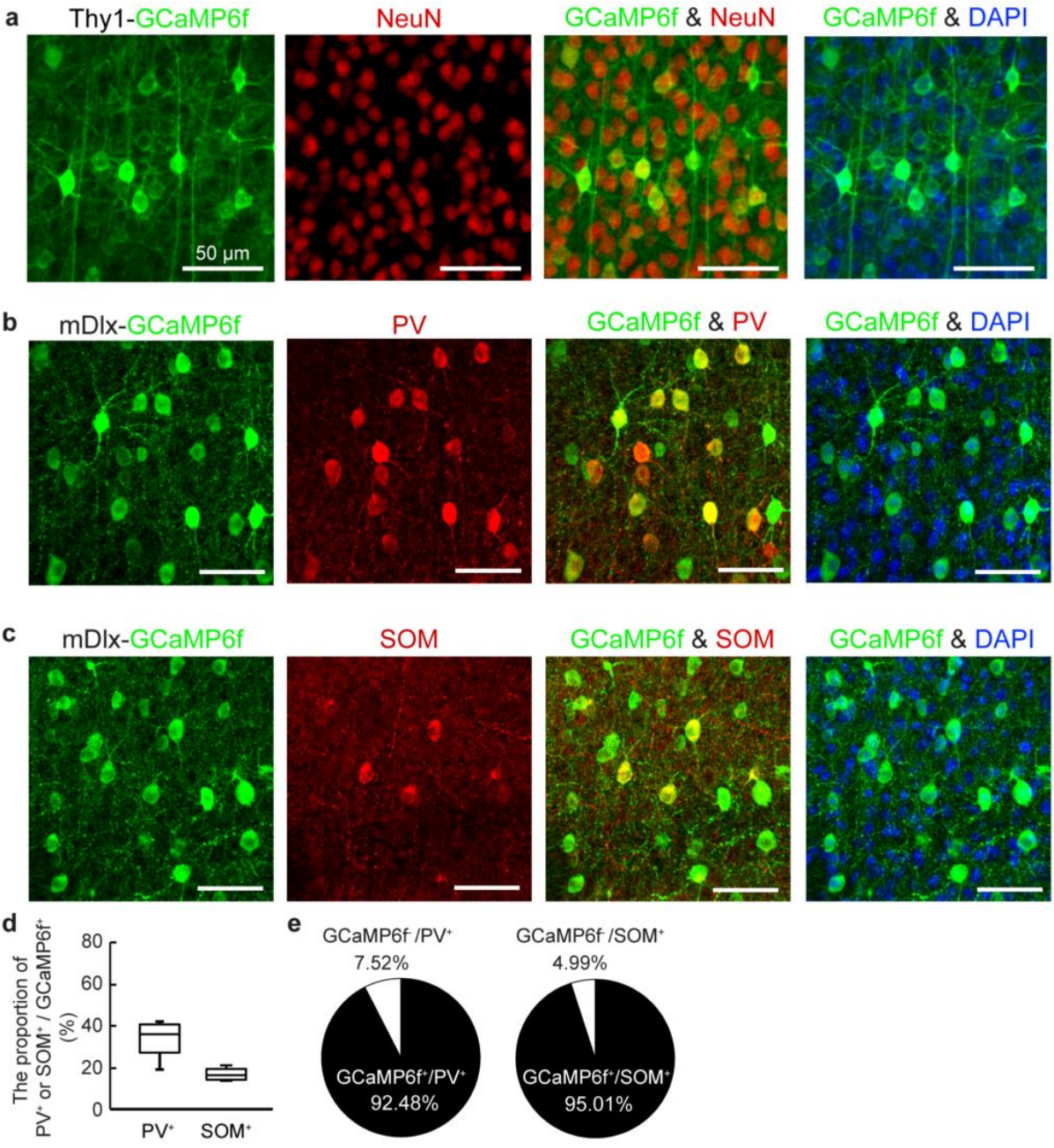
Supplementary Figure 3. Time-course traces of LFP signals, CBF changes and heart rate in sham control, and heart rate over time in sham or 4-AP injected mice. (a) Examples of LFP, CBF and heart rate over time in a sham control mouse for ~2 hr, showing no epileptiform activity by using saline injection alone (sham control) without any apparent change in systemic physiological conditions. (b) Averages of the heart rate measured for ~3 hr in mice treated with sham (n=3, 546.26 ± 20.20 bpm, mean ± SD) and 4-AP injection (n=4, 556.51 ± 10.40 bpm, mean ± SD) (n.s. indicates no statistical significance between the two groups at each time point, tested by Mann-Whitney *U* test) under urethane (1.25 g/kg, i.p.) anesthesia.



Supplementary Figure 4. Changes in the diameter of preictal and ictal pial vessels (arterioles and venules). (a) Changes in oreictal arteriole (-22.71 ± 13.14 %, mean ± SD) and venule (0.49 ± 3.98 %, mean ± SD) diameter (total number of seizures=53, n=8, ***p<0.001 by Mann-Whitney *U* test). (b) Maximal diameter changes in arteriole (32.32 ± 18.25 %, mean ± SD) and venule (3.67 ± 1.98 %, mean ± SD) during the ictal period (total number of seizures=53, n=8, ****p*<0.001 by Mann-Whitney *U* test). (c) Changes in preictal arteriole diameter over time after the 4-AP injection. Preictal arteriole constriction was consistently observed with no significant linear relation with time (total number of seizures=53, n=8, Spearman's *r*=0.097, *p*=0.488; n.s. indicates non- significance).

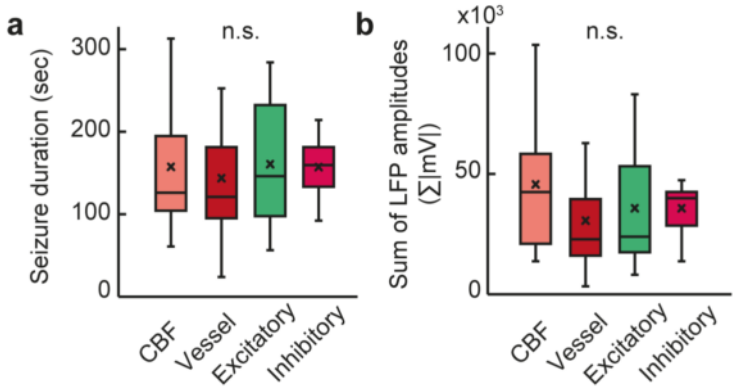


Supplementary Figure 5. Relationship between changes in preictal and ictal arteriole diameter and ictal LFP amplitudes. The ictal LFP amplitudes were calculated by summing the absolute LFP amplitudes during the period between each seizure onset and offset. The cumulative values were then normalized (total number of seizures=53, n=8, left: Spearman's r=-0.377, **p=0.007, R^2=0.145; right: Spearman's r=0.518, ***p<0.001, R^2=0.242).

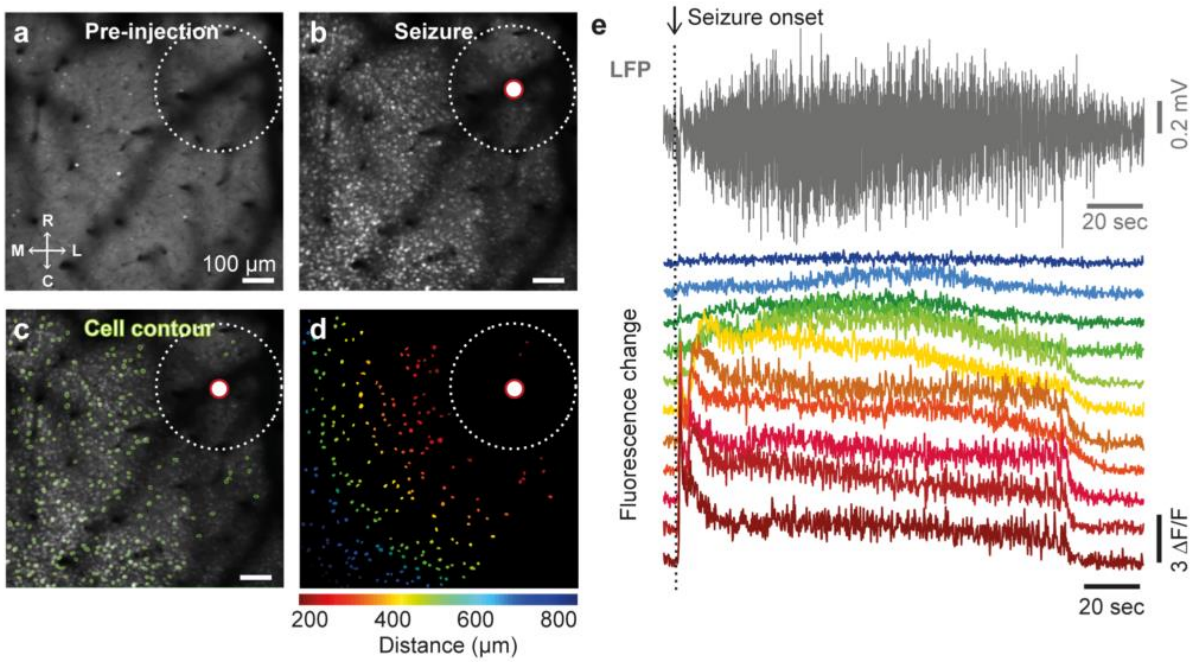


Supplementary Figure 6. GCaMP6f expression in layer 2/3 of somatosensory cortex in transgenic mice (Thy1-GCaMP6f-GP5.17DKim/J) and in C57BL/6 mice that receiving intracortical viral injections (AAV9-mDlx-GCaMP6f-Fishell-2). (a) Transgenic expression of GCaMP6f under Thy1 promoter in mouse somatosensory cortex. GCaMP6f^{+} cells were overlaid with NeuN^{+} cells. Cell nuclei were stained with DAPI. (b-c) Viral expression of

GCaMP6f under the mDlx promoter in mouse somatosensory cortex. Representative examples of colocalization between GCaMP6f$^+$ cells and PV$^+$ or SOM$^+$ cells. (d) Box-whisker diagram showing the proportions of PV$^+$ and SOM$^+$ in GCaMP6f$^+$ cells (PV$^+$: 33.57 ± 9.37 %, SOM$^+$: 17.09 ± 3.08 %, n=6, mean ± SD) in layer 2/3 of somatosensory cortex. (e) The proportions of GCaMP6f$^+$ cells in PV$^+$ (92.48 ± 9.80 %, n = 6, mean ± SD) and SOM$^+$ (95.01 ± 4.13 %, n=6, mean ± SD) cells indicate that the mDlx promoter effectively expressed GCaMP6f in GABAergic inhibitory neurons.



Supplementary Figure 7. (a) Duration of the seizures measured in the four sets of experiments (CBF: total number of seizures=42, n=5; Vessel: total number of seizures=53, n=8; Excitatory, total number of seizures=16, n=5; Inhibitory, total number of seizures=19, n=4). (b) Sum of LFP amplitudes between seizure onset and offset in the experiments. Boxes represent 25^{th}-75^{th} percentiles, and the horizontal lines inside the boxes indicate the medians.



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148 **Supplementary Figure 8. Isolation of neuron ROIs.** (a-b) Representative GCaMP6f
149 fluorescence images of neuronal activity before 4-AP injection (pre-injection period) and the
150 full ictal event. (c) Contour plot of the registered soma ROIs is overlaid on the ictal image
151 shown in (b). (d) Image of the colored neuron ROIs according to their distance from the
152 injection focus (red-lined white dot). (e) Example of a seizure event with LFPs and its
153 corresponding calcium transient in 11 representative cells within a 200-700 µm range from the
154 seizure focus.

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Supplementary Table 1.

Coefficients of correlation (Pearsons' *r*) between preictal LFP powers at different neural frequency bands and the preictal excitatory level shown in Figure 5(g).

	δ	**θ**	**α**	**β**	**γ**
r	0.368	-0.447	0.368	-0.596	-0.611
***p* value**	0.160	0.083	0.161	0.015*	0.012*
R^2	0.136	0.199	0.135	0.355	0.374

Supplementary Table 2.

Coefficients of correlation (Pearson's *r*) between the preictal LFP power at different neural frequency bands and the preictal inhibitory level shown in Figure 5(g).

	δ	**θ**	**α**	**β**	**γ**
r	-0.268	0.349	0.535	0.350	0.495
***p* value**	0.268	0.143	0.018*	0.142	0.031*
R^2	0.072	0.122	0.286	0.122	0.245

Supplementary Movie 1. Examples of spatiotemporal dynamics of preictal and ictal activities of excitatory (left, green) and inhibitory (right, red) neurons.

$\Delta F/F = (F-F_0) / F_0$, where F_0 represents an average of preictal fluorescence intensity and F denotes fluorescence intensity over time. The two gray traces below are LFP signals, which were measured concurrently with excitatory and inhibitory activities, respectively.

References

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