Supplementary Material

Pathophysiological characteristics associated with epileptogenesis in human hippocampal sclerosis

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Supplementary Methods

Slice preparations and maintenance

All of the MTLE patients underwent anteromedial temporal lobectomy with en bloc resection of the hippocampus. About 3-4 cm of hippocampal tissue was resected longitudinally, and coronal brain blocks about 5 mm thick were sectioned at 1 cm from the head of the hippocampus in the operating room. As described previously (Kitaura et al., 2011; Kitaura and Kakita, 2013), the sectioned hippocampal specimens were immediately immersed in ice-cold transport and cutting solution containing (in mM): KCl, 2.5; NaH_2PO_4, 1.2; $MgSO_4$, 10; $CaCl_2$, 0.5; $NaHCO_3$, 25; glucose, 25; and sucrose 105. The tissue was transported to our laboratory bubbled with 95% O_2 and 5% CO_2 within 30 min after removal. Three coronal slices 500 μm thick were prepared from each specimen per patient with the aid of a microslicer (Linearslicer, Dosaka, Japan), unless the condition of specimens prevented this, and these were transferred to an incubation chamber containing oxygenated artificial cerebrospinal fluid (ACSF) at 30 ^oC for more than 1 h until recordings were taken (Fig. S3). We used one slice per patient for imaging studies or field potential recordings, unless otherwise stated. The composition of the ACSF (in mM) was: NaCl, 124; KCl, 3; NaH_2PO_4, 1.24; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; and glucose, 10.

Histological procedures

For Timm staining, another fresh specimen was immersed in 0.2% Na_2S solution at 37 ^oC 10 min, and then fixed with 10% buffered formalin for 48 h. The tissues were rinsed with phosphate-buffered saline and embedded in OCT compound after immersion in 30% sucrose. Cryostat frozen sections 15 μm thick were mounted on slides coated with gelatin and visualized with developing solution (1.5 g citric acid, 1.2 g sodium citrate, 1.0 g hydroquinone, 10 g gum Arabic and 0.1 g silver nitrate dissolved in 60 ml of distilled water) in darkness for 10 min. All sections were counterstained with

hematoxylin, dehydrated and coverslipped.

For the quantitative analysis of granule cell layer width (Fig. 4E), we measured the width of the granule cell layer on a single Klüver-Barrera-stained section of each case. With the aid of a light microscope, we identified individual granule cells on the basis of their morphology, i.e. small, round, and dark nuclei with scant cytoplasm. The perpendicular distance to the granule cell layer, from the border towards the hilus dentatus to the outer border of the most distal granule cell, was measured at three points for each case, and then averaged. For quantitative analysis of immunofluorescence area, each of Kir 4.1 and GFAP fluorescence images (512x512 pixels, 0.311 μm/pixel) were converted into 16-bit grayscale using ImageJ software (ImageJ.net). We then quantified the immunostained area of the structures using AQUACOSMOS (Hamamatsu Photonics, Hamamatsu, Japan), as described previously (Tada et al., 2016). Areas of less than 20 pixels were discarded as a noise.

References

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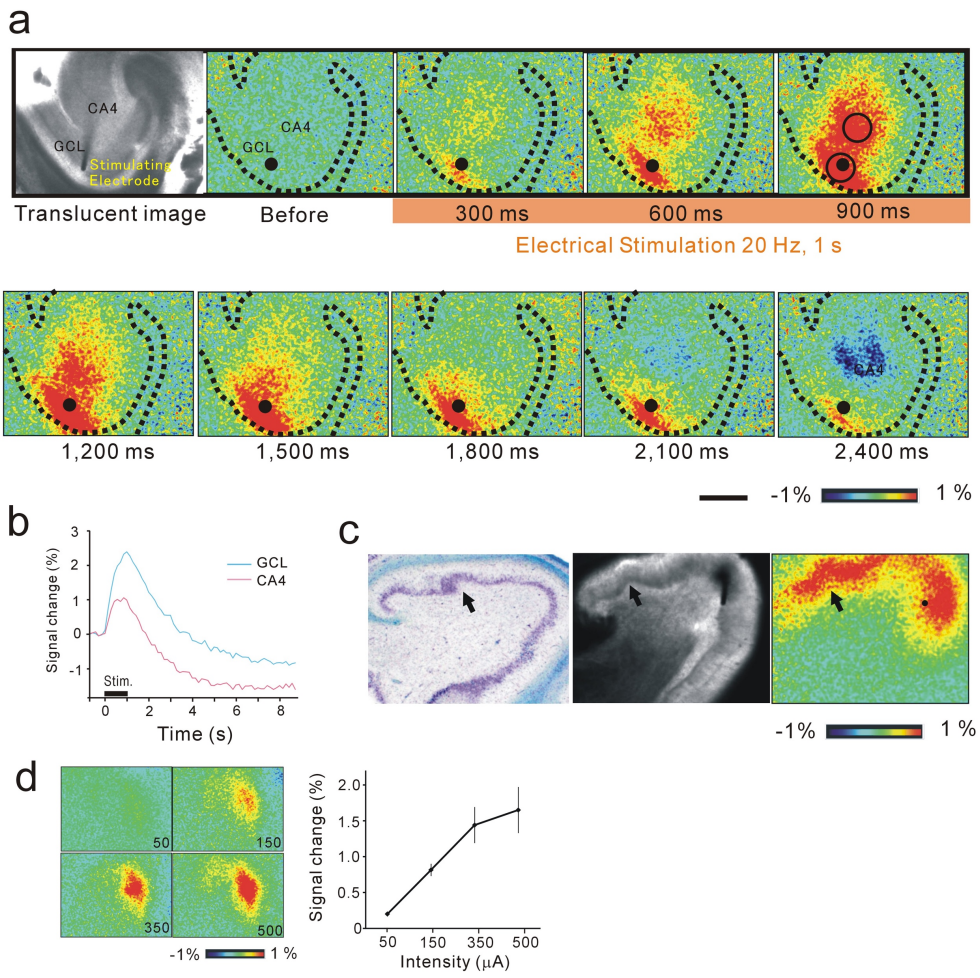
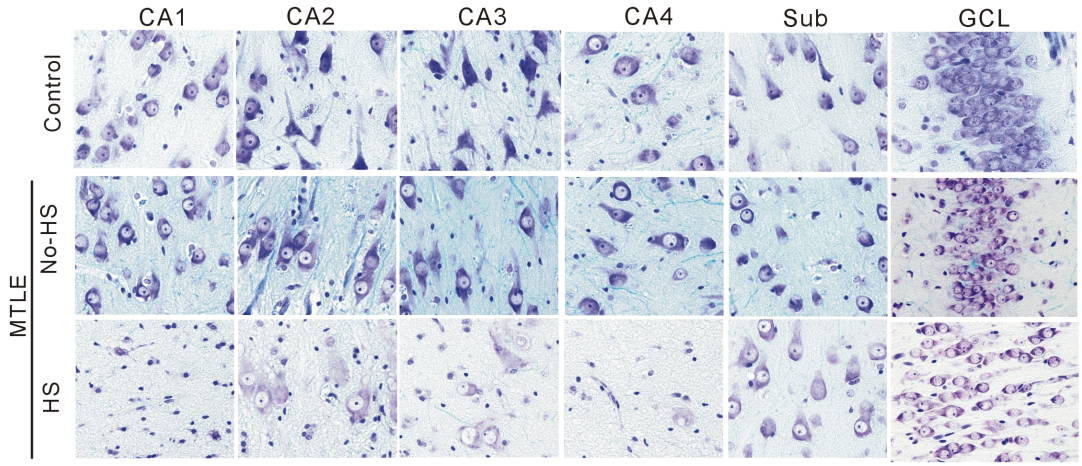
Tada M, Konno T, Tada M, Tezuka T, Miura T, Mezaki N, et al. Characteristic microglial features in patients with hereditary diffuse leukoencephalopathy with spheroids. Ann Neurol. 2016; 80: 554-565.



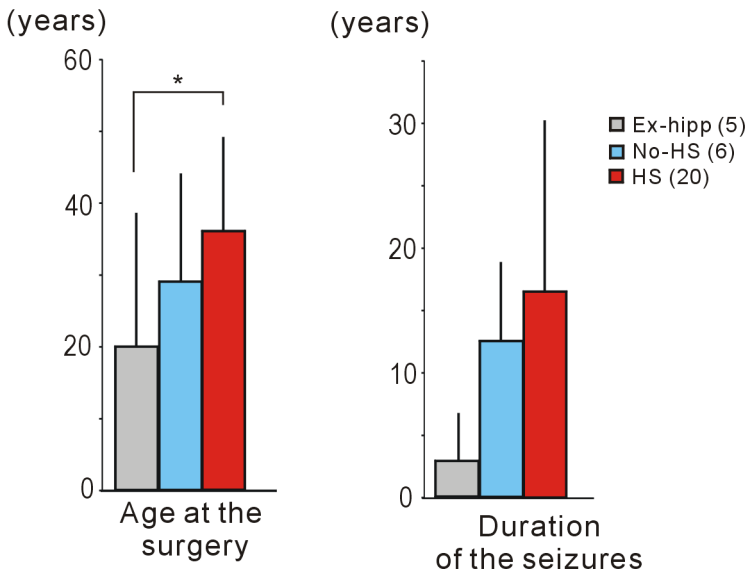
Supplementary Figure 1. Sufficiency of temporal and spatial resolution of flavoprotein fluorescence imaging.

(a) Representative sequential flavoprotein fluorescence images after stimulation of the granule cell layer (GCL) in the No-HS group. *Black circles* indicate the stimulation points. Scale Bar: 1 mm. (**b)** Time course of fluorescence signal changes in the GCL and CA4. The recording locations are indicated by two ROIs (*circles*) depicted in the "900 ms" panel in **a**. (**c)** A morphological picture of the GCL, and clear reproduction of the picture by flavoprotein fluorescence imaging. *Arrows* indicate an identical point in the GCL. *Left*: Klüver-Barrera staining, *middle*: translucent image of the slice, and *right*: flavoprotein fluorescence image at 1 s after stimulation onset. (**d)** GCL stimulus-response curve in the same case.



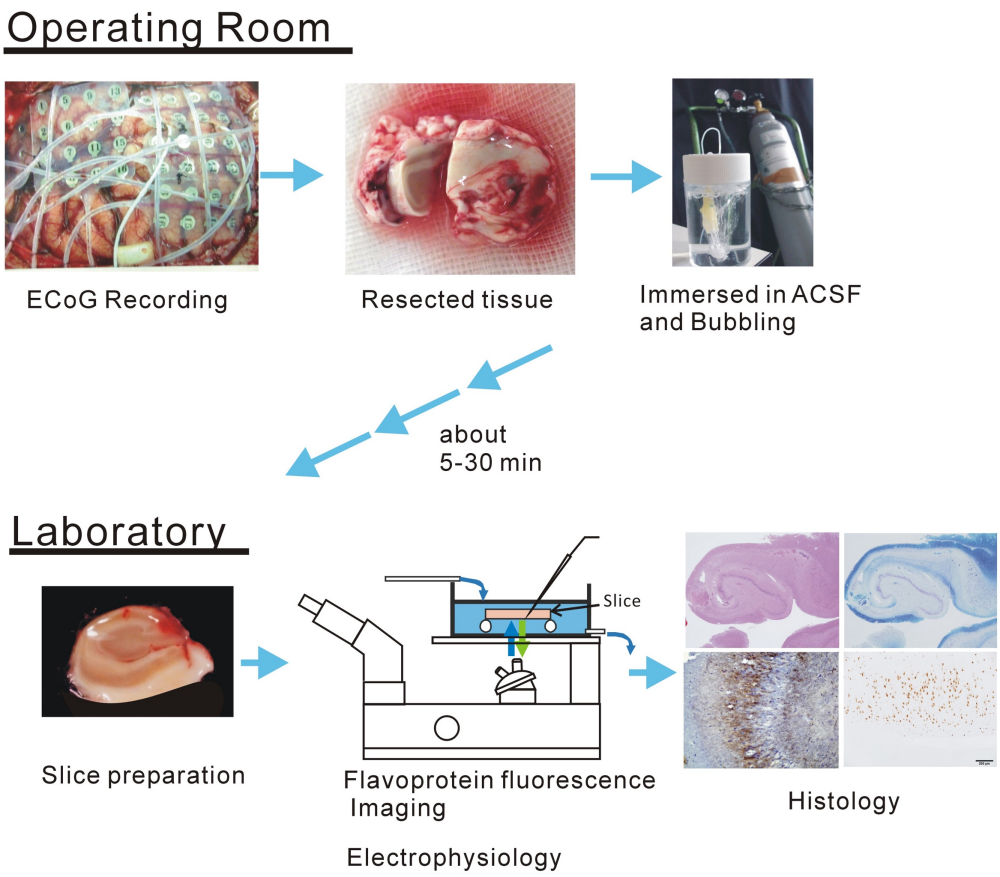
Supplementary Figure 2. Histological findings and classification.

Representative photomicrographs of the hippocampal CA1, CA2, CA3 and CA4 subfields, subiculum (Sub) and granule cell layer (GCL) in the Control, No-HS and HS groups. In the HS group, severe neuronal loss is evident in the CA1, 3, and 4 subfields, whereas no neuronal loss is evident in either the Control or the No-HS group. Klüver-Barrera staining. Bar = 20 μm.



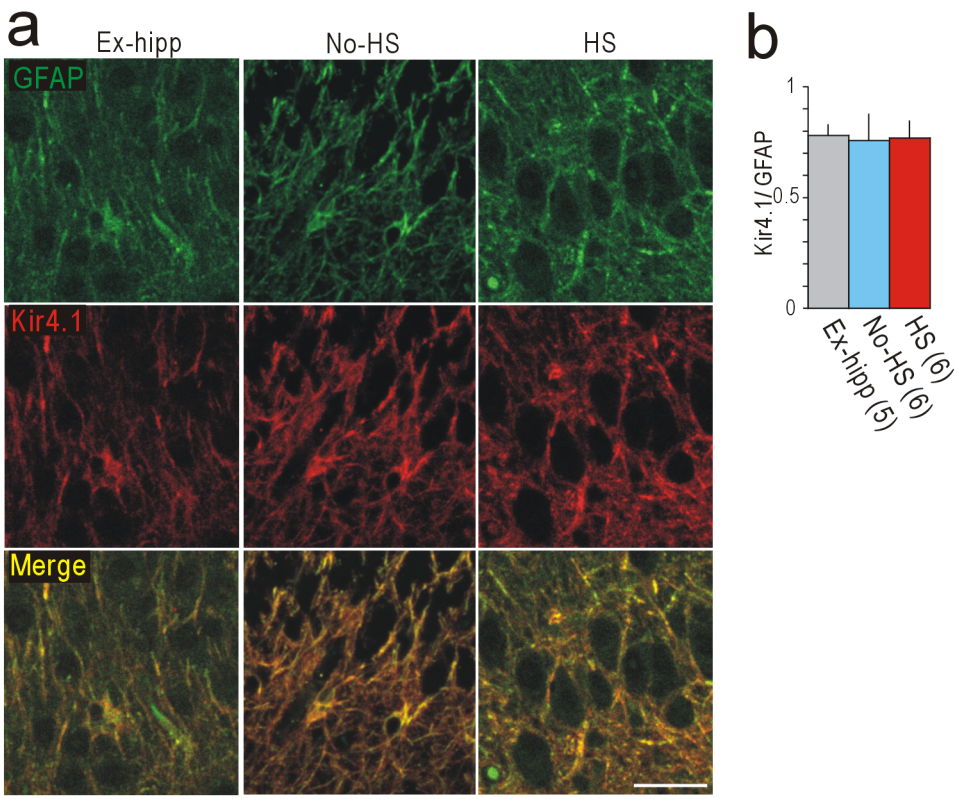
Supplementary Figure 3. Patient age at surgery and duration of seizures.

HS patients tended to be older at surgery and to show a longer seizure duration than patients in the control and No-HS groups, but not to a significant degree (one-way ANOVA with Tukey-Kramer test, median ± S.D.).



Supplementary Figure 4. Methodology of the experiments.

After the ECoG recordings, the resected hippocampal tissue was cut into 5-mm-thick coronal brain blocks, which were immediately immersed in ice-cold ACSF in the operating room. These tissue blocks bubbled with 95% O_2 and 5% CO_2 were then transported to the laboratory, and coronal slices 500 μm thick were then prepared. Each slice was transferred to a recording chamber and subjected to flavoprotein fluorescence imaging or LFP recording. The brain block corresponding to the mirror surface of each slice was used for histopathological examination (see materials and methods).



Supplementary Figure 5. **(a)** Double immunofluorescence images in GCL showing a similar expression pattern for both Kir 4.1 (*red*) and GFAP (*green*). Scale bar: 25 μm. **(b)** Quantitative analysis of stained area of immunofluorescence were shown. Error bars: SEM.