

Supplementary Information

The Supplementary Information file contains Supplementary Fig. 1 (Cortical organoids contain multi-layer progenitor zones and six cortical layers); Supplementary Fig. 2 (Global transcriptome analysis and electrophysiological properties of developing cortical organoids); Supplementary Fig. 3 (Modeling JEV infection using cortical organoids); Supplementary Fig. 4 (JEV can target hESC-derived cortical NPCs and immature neurons); Supplementary Fig. 5 (Gene expression of *ISGs*, *TLRs* and *IRFs* in brain organoids upon JEV infection).

Supplementary Figure Legends

Supplementary Fig. 1. Cortical organoids contain multi-layer progenitor zones and six cortical layers. **a** Immunohistochemistry of SOX2 (green), β -CATENIN (red) and NESTIN (blue) at day 35 cortical organoid, demonstrate the presence of a neuroepithelium-like structures near a lumen (white dotted line). Scale bar: 50 μ m. **b** Immunostaining for PH3⁺ (green) PAX6⁺ (red) proliferating cells in a Hoechst-stained (HO, gray) cortical organoid. Scale bar: 50 μ m. **c** Intermediate progenitor cells marker TBR2 (red) are present in a SVZ-like region beyond the PAX6⁺ (green) VZ-like region. Scale bar: 20 μ m. **d** Q-CTS-hESC-1-derived cortical organoids contained SOX2⁺ (green) PAX6⁺ (red) and TBR2⁺ (blue) multi-layer progenitor zones. Scale bar: 50 μ m. **e** Cortical plate marker CTIP2 (green) at day 70 organoids. Scale bar: 100 μ m. **f** Schematic representation of marker expression for all six cortical layers. **g** Immunostaining for deep cortical layers marker CUX1 (green), TBR1 (red), and Cajal-Retzius neuron cell marker REELIN (blue) at day 120 cortical organoid. Scale bar: 50 μ m. **h** Immunohistochemical analyses demonstrate the presence of early-born CTIP2⁺ (green) layer, late-born SATB2⁺ (red) neurons and BRN2⁺ (blue) neurons at day 120 cortical organoid. Scale bar: 50 μ m.

Supplementary Fig. 2. Global transcriptome analysis and electrophysiological properties of developing cortical organoids. **a-b** Heatmap of Pearson's correlation analysis of RNA-seq datasets at day 90 and 190 cortical organoids, with Roost et al. published datasets from 21 different human fetal organs (**a**) and Allen Brain Atlas's published datasets from 3 different cortical subregions (**b**). **c** An example of cells under the patch-clamp in a day 300 cortical organoid. **d** Representative traces of membrane potential responding to step depolarization by current injection steps from -10 pA to +30 pA in 10-pA increments. Membrane potential was current-clamped at around -70 mV. **e** Representative traces of whole-cell currents in voltage-clamp mode; cells were held at -70 mV; step depolarization from -80mV to +60 mV at 10-mV intervals was delivered. The inset shows Na⁺ currents. **f** Spontaneous action potentials recorded from cell of cortical organoid slice. No current injection was applied. **g-i** Quantification of resting membrane potential (RMP, **g**), membrane capacitance (*C_m*, **h**) and membrane resistance (*R_{in}*, **i**). n=7 cortical organoid neurons.

Supplementary Fig. 3. Modeling JEV infection using cortical organoids. **a-b** Immunostaining images of SOX2 (green), JN1 (red) and caspase-3 (blue) in Hoechst-stained (HO, gray) cortical organoids exposed to JEV (SA14, 10⁵PFU) or mock treated at day 9 (**a**) and day 55 (**b**). While arrows point to detached cells. Scale bars: 50µm.

Supplementary Fig. 4. JEV can targets hESC-derived cortical NPCs and immature neurons. **a-b** Cortical neural progenitor cells exposed to JEV (SA14, 10⁵ PFU) or mock treated for 24 hrs and analyzed 24 hrs later. Immunostaining images (**a**) and JEV titers (**b**) were shown. Scale bar: 100µm. **c-d** Cortical immature neurons exposed to JEV (SA14, 10⁵ PFU) or mock treated for 24 hrs and analyzed 2 days later. Immunostaining images (**c**) and JEV titers (**d**) were shown. Scale bar: 100µm.

Supplementary Fig. 5. Gene expression of ISGs, TLRs and IRFs in brain organoids upon JEV infection. **a–j** Gene expression of *IFITM3* (**a**), *ISG54* (**b**) *OAS1* (**c**), *MDA5* (**d**), *TLR2* (**e**), *TLR3* (**f**), *TLR7* (**g**), *IRF3* (**h**), *IRF7*(**i**) and *STAT2* (**j**) was quantified by qRT-PCR. **k** Western blots data showing the alterations of p-STAT2 in protein levels. Brain cortical organoids at day 24 and day 100 were inoculated with JEV (SA14, 10⁵ PFU) or mock treated for 24 hours and analyzed by western blot with indicated antibodies on 2 days post infection.