

IL-6–dependent neuroimmune signalling is a key contributor to neuronal hyperexcitability and seizures in HSV encephalitis

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Article

Keywords:

Posted Date: October 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11232670/v1>

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Additional Declarations: **Yes** there is potential Competing Interest. T.S. has been a member of Encephalitis International since 1998 and President of Encephalitis International since 2019 (unpaid). T.S. is the Director of The Pandemic Institute (since 2021) which has received funding from Innova and CSL Seqirus and Aviva and DAM Health (unpaid). All other authors declare no competing interests.

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Contributions

C.D.H. and B.D.M. designed the research, and C.D.H., F.N.E, L.B-M., L.H., and J.G-J. conducted the experiments. C.D.H., F.N.E, L.H., N.M.M. and L.L. conducted data analysis, and C.D.H wrote the paper, with significant critical input from F.N.E, A-C.M., C.D., S.A.B., T.S., Y.H., M.E., A.D.L., and B.D.M. N.M.M. contributed voluntarily and outside the scope of their technical role at LIV-SRF. Funding was acquired by C.D.H, F.N.E and B.D.M. B.D.M. supervised the research.

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Abstract

Seizures are a frequent and severe complication of herpes simplex virus type 1 (HSV-1) encephalitis which are associated with increased disease severity and poor outcome. However, the underlying mechanisms driving neuronal hyperexcitability are unclear. Using human iPSC-derived cortical neurons, we show that HSV-1 infection induces spontaneous hyperexcitability and enhances evoked network responses before significant cell death. Interleukin-6 (IL-6) was elevated in HSV encephalitis patients who had seizures and IL-6 secretion correlated with neuronal network activity in vitro. Exogenous IL-6 increased spontaneous and evoked activity in human cortical networks, and this was prevented by blockade of IL-6 signalling with anti-human IL-6R. Additionally, treatment with an IL-6R antagonist prevented HSV-1 associated hyperexcitability. HSV-1 infection caused neuronal downregulation of KCNQ2, which is prevented by IL-6R blockade. These findings identify IL-6-dependent neuroimmune signalling as a key contributor to HSV-1-associated neuronal hyperexcitability and suggest a modifiable pathway underlying seizure phenotype in viral encephalitis.

Introduction

Herpes Simplex Virus type 1 (HSV-1) is the leading cause of sporadic viral encephalitis (HSE) globally^{1,2}. HSE is an acute haemorrhagic inflammation of the brain parenchyma, with predominant involvement of the temporal and frontal lobes, regions that are highly epileptogenic^{1,3}. While aciclovir has significantly reduced mortality from approximately 70% to 10–20%, HSE remains a major clinical challenge as approximately 48% of survivors are left with severe, permanent neurological deficits including memory loss, cognitive impairment and epilepsy^{1,4,5}.

Acute seizures occur in 40–75% of patients and are associated with greater extent of brain injury and worse functional recovery^{3,6,7}. Up to 29% of patients develop status epilepticus, which is often refractory to traditional antiseizure medications^{6,8–10}. Clinical and translational evidence suggests that an excessively pro-inflammatory host immune response, rather than viral burden alone, drives this pathology^{11–13}. Hence,

modulation of the immune response might be an emerging option as an adjunctive therapy to aciclovir to prevent seizure and resultant excitotoxic brain injury that precipitates post-encephalitic epilepsy.

Both pathogen-associated molecular patterns and pro-inflammatory cytokine secretion within the central nervous system (CNS) have previously been associated with the development of seizures^{13–17}. During viral encephalitis or brain injury, interleukin-6 (IL-6) is secreted predominantly by activated microglia, astrocytes, and infiltrating innate immune cells; but also by neurons themselves^{18–21}, which in HSE likely occurs soon after active viral replication begins. In patients with HSE, higher concentrations of IL-6 in the CSF are associated with a worse prognosis and the occurrence of status epilepticus^{4,22}. Elevated IL-6 concentrations have also been detected in both serum and cerebrospinal fluid (CSF) in individuals with epilepsy^{13,23–25}. The clinical significance of IL-6 and its relationship to seizure is supported by work *in vivo* and *in vitro*. IL-6-deficient mice exhibit a significant decrease in seizure incidence following viral infection, whereas exogenous administration or transgenic overexpression of IL-6 in the brain is pro-convulsive and can induce spontaneous seizures as well as worsen seizures initiated by epileptogenic agents^{12,25–29}. Together, these findings suggest that IL-6 may be a driver of ictogenesis during the acute phase of neurotropic viral infection. While *in vivo* models of HSE develop seizures, these models do not allow for the precise examination of the underlying cellular and network drivers³⁰. Consequently, it is difficult to resolve how specific cell subtypes or individual soluble mediators coordinate the transition to hyperexcitability within human cortical tissue using traditional models.

Thus, humanised *in vitro* models have become useful for investigating the inflammatory cascades and network-level physiology that drive viral- and cytokine-induced hyperexcitability. Human induced pluripotent stem cell (hiPSC)s provides a scalable and cost-effective method for generating potentially limitless numbers of live human cortical neurons. hiPSC-derived neurons are electrically active, generating repetitive action potentials and forming functional synaptic contacts within complex networks. By employing differentiation protocols that produce highly enriched cortical neuron populations, it is possible to investigate whether inflammatory signalling drives neuronal hyperexcitability independently of

interactions involving glial or peripheral immune cells. Multi-electrode arrays (MEAs) are a non-invasive platform that record extracellular field potentials from neuronal populations^{11,31}. Unlike traditional electrophysiological methods, such as single-cell patch-clamping, MEAs facilitate non-invasive monitoring of the functional activity of entire neuronal populations and networks^{32–34}. This approach allows for characterisation of population-level spiking, bursting and network synchrony during viral infection.

We aimed to determine how HSV-1 infection and associated inflammatory signalling affect the electrophysiological activity of human iPSC-derived cortical neurons using multielectrode array recordings, informed by data from clinical samples and alongside an established model. Secondly, we sought to identify candidate cytokines that are a tractable therapeutic target for preventing downstream neuronal hyperexcitability and seizure in primary HSV-1 infection.

Results

Differentiation of cortical neurons from hiPSCs and characterisation of markers and electrophysiology

Differentiation of human induced pluripotent stem cells (hiPSCs) into cortical neurons was performed as previously described by Karch et al. (2019)³⁵. A schematic of the overall experimental protocol is shown in Fig. 1a. During and after differentiation, the hiPSC-CNs were characterised through microscopy at days 0–35 to confirm the identity and maturation level of the cortical neurons (Fig. 1b–d), as well as the absence of glial cells. On days 15 (Fig. 1c) and 35 (Fig. 1d), robust neuronal process extension was observed, and neural and neuronal morphology was present. Phase image in Fig. 1e demonstrates an example of the distribution of cortical neurons cultured on microelectrode array. Neurons were observed to be present on the surfaces of electrodes, and axon bundles forming between cell clusters, including between cells clustered on electrodes. Immunocytochemistry confirmed that neural progenitors were positive for PAX6 expression and negative for mature neuronal marker NeuN and glial marker GFAP prior to commencing cortical neuron differentiation (Fig. 1f). NeuN was examined at D0 and D15 to assess whether expression was detectable during the earlier stages, whereas Tuj1 was used at D35 to visualise neuronal morphology

and neurite development. After 15 days of differentiation, cultures showed expanded numbers of PAX6+ neural progenitors with no positive staining for GFAP or NeuN at this stage (Fig. 1g). Conversely, by day 35 of differentiation, PAX6 expression was reduced in intensity whilst long axon-like projections were positive for the mature neuronal marker Tuj1 (Fig. 1h). This confirmed neuronal identity. Further immunofluorescence characterisation demonstrated expression of receptors involved in inhibitory and excitatory neurotransmission; GABA-B receptors (Fig. 1i) and NMDA receptors (Fig. 1j), alongside the presynaptic marker synaptophysin (Fig. 1k) in hiPSC-CNs after 35 days of differentiation. To validate that hiPSC-CNs were functional, hiPSC-CNs were plated onto multi-electrode array (Axion Biosystems) (Fig 1e). Neurons were found to be spontaneously electrically active, with a representative heat map of a single well shown in Fig 1l as well as a continuous spike trace from a single electrode and representative spike waveforms from day 35 of differentiation shown in Fig 1m-n. From day 7 of differentiation, numbers of spikes and average resistance (impedance) was measured for 100 seconds every 6 hours up to 33 days of differentiation, as a proxy for cell viability and proliferation. This confirmed that spontaneous spiking had occurred in 100% of wells the end of the differentiation protocol (Fig. 1o), whilst overall impedance remained stable (Fig 1p), confirming maintenance of cell viability and no significant proliferation.

HSV-1 infection induces spontaneous hyperexcitability and hypersynchrony in hiPSC-CNs

To evaluate the effect of HSV-1 infection on spontaneous activity of the hiPSC-CNs on the MEAs, hiPSC-CNs were infected with 2.5×10^3 PFU of HSV-1 KOS per well and the activity was analysed at 20 hours post-infection. To provide a positive control for seizure-like activity *in vitro*, uninfected HiPSC-CNs were treated with kainic acid (KA) at the same time as viral inoculation, and exposed to KA for the whole 20 hour period, as described previously³⁶. Fig. 2a depicts representative spike raster plots of activity recorded at baseline and then at 20 hours post-infection in one well. Fig. 2b shows a representative heat map of activity in one infected well, whilst Fig. 2c-d depicts a representative single electrode trace and waveforms at 20 hours post-infection with HSV-1. Videos of baseline activity and spontaneous activity at 24 hours post-infection are included in the supplement (Supplementary Videos S1-S2). At 20 hours post-infection

there was not yet any overt cytopathic effect of the viral infection as there was no significant difference in average resistance recorded across the electrodes (Fig. 2e). HSV-1 infected neurons showed a significant increase of recorded spontaneous activity, comparable to treatment with the kainate receptor agonist kainic acid, while there was no significant change in uninfected control. There was a significant increase in spontaneous spike frequency (Fig. 2f), as well as an increased frequency of bursting activity (Fig 2g) and network bursts (Fig 2h). Synchrony, measured as the area under the normalised cross-correlation curve, was also significantly increased (Fig. 2i). HSV-1 infection in the hiPSC-CN MEA cultures was confirmed by performing RT-qPCR for HSV-1 polymerase mRNA on infected cell lysate, to detect only actively replicating virus. HSV-1 polymerase mRNA was detected in 6/6 inoculated samples at 24 hours post-inoculation and was not detected in 6/6 mock-infected control sample (Fig. 2j). Together, these data indicate that HSV-1 infection is associated with an early increase in spontaneous and coordinated neuronal network activity prior to overt cytopathic change.

HSV-1 infection enhances evoked response to electrical stimulus

As spontaneous activity at 20 hours-post infection was increased, we hypothesised that infected neuronal networks would also exhibit an exaggerated response to excitatory input. We therefore measured electrically evoked activity to determine whether HSV-1 infection enhanced network responsiveness to stimulation. hiPSC-CNs were infected with 2.5×10^3 PFU per well of HSV-1 KOS. Impedance and response to a stimulation sequence across ten seconds was recorded at baseline and 24, 48 and 72 hours post-infection. Fig. 3a depicts a raster plot of evoked spike and burst activity recorded from one infected well at 72 hours post-infection (with stimulations indicated at times designated by black arrows, each black arrow represents one stimulation block of 3 events, 9 total stimulation events). Fig. 3b depicts a representative single electrode continuous spike trace and waveform spike plots at 72 hours post-infection with HSV-1. Relative impedance decreased significantly in infected cultures compared with mock-infected controls from 48 hours post-infection onwards and continued to decline through to 72 hours (Fig. 3c). To account for infection-associated reductions in culture impedance, evoked activity measures were expressed

relative to their pre-infection baseline and normalised to the corresponding fold change in impedance at each timepoint. Evoked spike frequency increased significantly from 24 hours post-infection and remained elevated at all time points up to 72 hours post-infection (Fig 3d). Average spikes per trial of stimulation were significantly increased from 48 hours post-infection onwards (Fig. 3e), while synchrony of evoked responses was also significantly elevated compared to baseline at 72h post-infection (Fig. 3f).

Exposure to inactivated supernatant from infected neurons enhances evoked response to stimulus

We next evaluated the ability of neuron-derived soluble mediators to reproduce the hyperexcitable phenotype observed in infected cells. hiPSC-CNs on MEA were exposed to UV-inactivated and filtered supernatants taken from hiPSC-CN cultures 24 hours post-infection with 2.5×10^5 PFU HSV-1, and evoked responses assessed. Representative raster plots for baseline (Fig. 4a) and after 24 hours of exposure (Fig. 4b) are shown (with stimulation at times designated by black arrows), with representative electrode trace (Fig. 4b). At 24 hours post-treatment, there was no significant decrease in impedance, indicating no significant cell death (Fig. 5c). Supernatant-treated hiPSC-CNs showed a significant increase in mean firing rate (Fig. 5d), burst frequency (Fig. 4e), spikes per trial (Fig. 4f) and synchrony (Fig. 4g) when stimulated, compared to mock. We also considered the effect of astrocyte cytokine secretion on network activity but found that it had a comparatively modest effect compared to both direct infection and conditioned neuronal supernatant (Figure S1). These data indicate that virus-free neuronal supernatant from infected cells was sufficient to increase multiple parameters of evoked excitability, indicating a role for neuron-derived soluble mediators in regulating neuronal network response.

IL-6 is associated with seizure in patients with HSV encephalitis, and IL-6 production by neurons *in vitro* is correlated with hyperexcitability

As infection-conditioned medium was able to increase excitability *in vitro*, supporting the involvement of a soluble mediator, we then examined whether patients with HSV encephalitis who experienced acute seizure or status epilepticus at admission showed differences in their intrathecal cytokine profiles compared

with those who remained seizure-free. CSF samples were obtained from 55 participants upon admission, and their seizure history was also recorded at this time. CSF samples were assessed for a broad panel of mediators using the Bio-Rad multiplex Luminex array panel. Pairwise analysis revealed positive associations between CSF concentrations of cytokines IL-6, IL-8, CCL2, and CCL27 and seizures, whilst there was a negative association between CCL5 and seizure (Fig. 5a and Fig. 5b). We then screened for neuronal production of these cytokines using Luminex *in vitro* and using ELISA and found that several of these cytokines were either not secreted *in vitro* or were not correlated with electrophysiological activity (Figure S2). However, at 24 hours post-infection, supernatant from HSV-1 infected and hyperexcitable neurons showed a significant increase in IL-6 production compared to both mock-infected and KA-treated neurons (Fig. 5c). IL-6 was also significantly elevated in supernatant from infected neurons at 72 hours post-infection compared to mock-infected and KA-treated neurons (Fig. 5d). Other cytokines identified from our patient cohort analysis were either not secreted by infected neurons such as CCL2 (Fig. 5e) and CCL5 (Fig. 5f). We then examined how burst frequency, as a measure of the seizure-like bursting phenotype observed, was related to IL-6 secretion in the same wells. IL-6 was significantly positively correlated with burst frequency in its paired wells (Fig. 5g).

HSV infection induces neuronal expression IL-6 receptor alpha binding protein in a murine model

Given the association between levels of IL-6 cytokines with seizure in our HSV encephalitis patient cohort, and the correlation between neuronal IL-6 production and hyperexcitability in the *in vitro* experiments, we hypothesised that IL-6 and/or its binding subunits would be upregulated *in vivo* by neurons following HSV infection. To test our hypothesis, we performed single-cell RNA sequencing (scRNA-seq) to quantify heterogeneity of mRNA expression in brain cell types, including neurons, subject to HSV infection, using an established HSV encephalitis murine model³⁰. Single-cell RNA-seq data was mapped to Reference UMAP embeddings using the TransferData and MapQuery functions from Seurat package to identify cell types (Fig. 6a). Then, we determined which brain cell types expressed gene transcripts for HSV-1 proteins UL24, UL30, UL29, UL42 and UL5 to denote active viral replication. We noted the presence of HSV gene

transcripts in various mouse brain cells, including neurons, microglia, and astrocytes, but predominantly neurons (Fig. 6b). Having confirmed the expression of HSV gene transcripts in neurons, we compared the expression levels of the mRNA for the IL-6R receptor alpha subunit (Il6ra) and IL-6 mRNA transcripts in neurons from the HSV-1-infected and sham-control mice. We noted that Il6ra was highly expressed in infected neurons (log2fold change = 1.35, *P* value = 0.014) (Fig. 6c). IL-6 was also expressed by neurons, microglia and astrocytes, as well as ependymal cells and oligodendrocytes in infected animals. IL-6 upregulation in neurons during infection was not significant at five days post-infection (Fig. 6d). This indicates that neurons express IL-6, and that HSV infection is associated with increased neuronal expression of the IL-6R alpha subunit, suggesting that neurons may have been exposed to increased IL-6 signaling during an earlier stage of infection.

Exogenous IL-6 increases spontaneous and evoked activity which can be rescued by treatment with anti-IL-6R

To determine whether IL-6 was driving hyperexcitability in our model, we treated hiPSC-CNs with 100 ng/mL exogenous recombinant IL-6 a high concentration selected to elicit a robust functional response. Neurons were also concurrently treated with 20 µg/mL anti-IL-6R to see if any resultant functional phenotype could be prevented. Representative raster plots, electrode traces and waveforms for spontaneous activity of isotype control, IL-6 and IL-6 + anti-IL-6R at 48 hours post-treatment are shown in Fig. 7a-c. At 48 hours post-treatment onwards, cumulative spike counts across the experiment (normalised to baseline activity) in IL-6 exposed hiPSC-CNs showed a significant increase in overall spiking, as compared to isotype control and anti-human IL-6R treated cells (Fig 7d). IL-6 treated hiPSC-CNs showed a significant fold increase in spontaneous spike frequency, whereas isotype control and concurrently anti-human IL-6R treated cells showed no significant increase (Fig. 7e). We then assessed evoked activity and found that, similarly, IL-6 treated hiPSC-CNs showed a significant fold increase in spike frequency at 48hrs (Fig. 6f) indicating a higher frequency of firing in response to stimulus.

Anti-IL-6R treatment prevents hyperexcitability and hypersynchrony caused by HSV-1 infection

We next aimed to determine whether blocking IL-6 signalling is a promising strategy for preventing neuronal hyperexcitability and hypersynchrony observed in our *in vitro* HSV-1 infection experiments. As such, we treated hiPSC-CNs with 20 µg/mL anti-IL-6R or left hiPSC-CNs untreated before inoculating with 2.5×10^3 PFU HSV-1 and recording evoked responses at baseline and then every 24 hours, to assess the fold change from baseline in network activity parameters. Increased network responsiveness was observed from 48 hours, with significant elevation in fold change in number of spikes recorded (Fig. 8a) and spike frequency (Fig. 8b) for infected neurons, compared to both IL-6R treated infected neurons and mock-infected controls. Fold change in numbers of bursts in infected neurons was significantly elevated compared to mock-infected from 48 hours but was not significantly different to anti-IL-6-R treated infected neurons (Fig. 8c). Similarly, infected neurons showed significantly increased spikes per burst (Fig. 8d), synchrony (Fig. 8e) and spikes per trial (Fig. 8f), compared to mock infected cells. Infected neurons, whether treated with anti-IL-6R or not, showed a significant decrease in impedance over 72 hours, plausibly reflecting lytic cell death that was unable to be prevented by anti-IL-6R treatment (Fig 8g). These data indicate that IL-6R blockade prevents HSV-1-induced increase in neuronal network responsiveness but does not prevent neuronal death.

HSV-1 infection is associated with KCNQ2 downregulation that can be prevented with IL-6R blockade.

To determine possible candidate mechanisms by which IL-6 signalling induces neuronal hyperexcitability, we compared mRNA for various neuronal excitability markers in HSV-1 infected neurons to mock-infected and infected neurons treated with anti-IL-6-R. Firstly, infection status in infected neurons was confirmed by calculating the relative viral load of HSV-1 polymerase mRNA, compared to mock infected and infected anti-IL-6R treated neurons 24 hours post-infection. Viral mRNA was not detected in mock-infected but was detected in both infected conditions, with no significant difference between the relative viral load between infected conditions (Fig. 9a). There was no significant difference between conditions for GRIN1

(GluN1), GABBR1 (GABAB1) or SLC32A1 (VGAT) expression (Fig. 9b-d). KCNQ2 (Kv7.2) was downregulated in infected neurons but was not significantly different to control when infected neurons were treated with anti-IL-6R (Fig. 9e). SLC17A7 (VGlut1) was upregulated in infected neurons compared to mock-infected, but fold change was not significantly different to infected neurons when treated with anti-IL-6R (Fig. 9f). These data implicate IL-6 signalling in altered transcriptional regulation of the Kv7.2 potassium channel and VGlut1 glutamate transporter.

Discussion

We have identified that HSV-1 infection of hiPSC-CNs *in vitro* drives spontaneous hyperexcitability, hypersynchrony and increased response to electrical stimulus. This effect was partially recapitulated by exposure to infection-conditioned neuronal supernatant, indicating that soluble mediators contribute to the observed network dysfunction. Treatment with infected (and inactivated & filtrated) neuron-derived supernatants also increased network responsiveness, suggesting a role for neuron-derived signals that participate in regulating neuronal excitability during HSV-1 infection. In our HSV encephalitis patient cohort, several cytokines associated with seizure were identified, including IL-6, and hiPSC-CNs produced IL-6 *in vitro* in response to HSV-1 infection. In our murine model of HSV encephalitis, at five days post-infection, the IL-6R alpha subunit (also known as gp130) was significantly upregulated in infected neurons, which is the binding site of the anti-IL-6R antibody tocilizumab³⁷. When hiPSC-CNs were treated with exogenous IL-6, this increased spontaneous and evoked activity which was rescued with anti-human IL-6R treatment. Further, IL-6R blockade prevented the phenotype of increased network activity associated with HSV-1 infection from appearing and prevented alteration of HSV-1 associated KCNQ2 transcription, suggesting IL-6 signalling is a tractable target for preventing neuronal hyperexcitability. Our data supports a model in which HSV-1-associated neuronal hyperexcitability is driven not only as a direct consequence of infection, but also through secondary neuroimmune signalling within the CNS microenvironment.

The observation in the present study that HSV-1 drives neuronal hyperexcitability in cortical neurons is aligned with previous studies showing hyperexcitability of neurons *in vivo* and in organotypic cultures when infected with HSV-1^{38,39}. Cuddy et al. demonstrated that IL-1 β can induce neuronal hyperexcitability and promote HSV-1 reactivation, providing evidence that cytokines modulate neuronal activity and that such neuronal activity can also directly influence the HSV-1 life cycle⁴⁰. Though we did not test the ability of exogenous IL-1 β to stimulate hyperexcitability, we did not find IL-1 β to be significantly produced by infected neurons at 24 hours post-infection, which may be due to its short half-life and autocrine function (Fig. S2).

We have identified IL-6 as a mediator linking neuroinflammation to seizure-associated neuronal dysfunction in HSV-1 infection. In the patient cohort, IL-6 was among the cytokines in patient CSF associated with seizure. This was reinforced by the observations that exogenous human IL-6 was able to increase network excitability and responsiveness *in vitro* in human iPSC-derived cortical neurons, and that this effect could be attenuated by anti-human IL-6R treatment. Further, treating iPSC-CNs *in vitro* with anti-human IL-6R prevented the hyperexcitable phenotype induced by HSV-1 infection. This provides evidence for a contributory mechanistic role of IL-6 in modulating neuronal excitability during infection. Most importantly, IL-6 is a tractable therapeutic target, particularly in the context of neuroinflammatory seizure. The humanised anti-IL-6R monoclonal antibody tocilizumab has been reported to effectively manage new-onset refractory status epilepticus (NORSE) and febrile infection-related epilepsy syndrome (FIRES)^{41,42}. Lipopolysaccharide and IL-6 are known to induce inflammation that increases absence seizure activity in rat epilepsy models, that is effectively prevented by treatment with tocilizumab⁴³. Moreover, intranasal human IL-6 administration and human IL-6 overexpression in the brain profoundly increase susceptibility to drug-induced seizures^{28,44}. We found that IL-6 is secreted by infected human neurons *in vitro*, in the absence of glia. Although intrathecal IL-6 production following HSV-1 infection has traditionally been attributed to non-neuronal cells, particularly microglia, IL-6 expression has also been reported in mouse and rat neurons during states of injury or inflammation, including cortical neurons,

306 demonstrating cell-autonomous neuronal IL-6 signalling^{18,21,45,46}. We found that neurons in our murine
307 model of HSV encephalitis express IL-6. We also found that at 5 days post-infection, neurons have
308 upregulated the IL-6R alpha subunit, suggesting increased responsiveness to IL-6. Our data suggest a need
309 for further *in vivo* and clinical studies to determine the efficacy of IL-6 blockade in treating infection-related
310 seizure.

311 We have also identified a potential pathway that may be differentially regulated under conditions of
312 infection-related intrathecal IL-6 production, which may lead to seizure genesis. IL-6, amongst other
313 cytokines produced in response to viral infection, has been found to depolarise neurons and increased
314 baseline excitability in mouse neocortical neurons⁴⁷. IL-6 has also been found to inhibit voltage-gated
315 sodium channel activity through IL-6st/gp130⁴⁸. There is limited evidence on the connection between IL-6
316 signalling and potassium channel regulation. In our cultures, HSV-1 infected neurons downregulated
317 KCNQ2 after 24 hours of infection, and this downregulation was prevented by treatment with human anti-
318 IL-6-R. KCNQ2 encodes the Kv7.2 protein which composes the Kv7.2/7.3 neuronal ‘M channel’ with
319 Kv7.3. Loss-of-function mutation in this gene is associated with a range of neonatal epileptic disorders⁴⁹.
320 KCNQ2 is positively regulated by the transcription factor Specificity protein 1 (SP1), and negatively
321 regulated by the transcription factor RE1-Silencing Transcription Factor (REST)⁵⁰. REST is increased in
322 response to sustained neuronal hyperactivity⁵¹ but has also been found to be increased in dorsal root ganglia
323 neurons in response to inflammatory mediators produced by neurons in the presence of damage-associated
324 molecular patterns, which leads to KCNQ2 downregulation and reduced M-current density in these
325 neurons⁵⁰. Hyperexcitability in such peripheral sensory neurons contributes to chronic pain, and
326 hyperexcitability in the brain leads to seizures and epilepsy. REST is also known to regulate HSV-1 gene
327 expression during the latent phase of the infection⁵². Though we did not directly assess REST in our model,
328 its role in KCNQ2 downregulation in response to neuronal hyperexcitability and inflammatory signalling
329 raises the possibility that REST-mediated transcriptional regulation may be a result of IL-6 signalling,

leading to loss of KCNQ2 during HSV-1 infection. Further studies are required to determine whether this is the case.

An advantage of our model is that it is entirely human iPSC-derived, providing a uniform genetic background at scale which may not be possible using primary human cortical neurons which are also difficult to source and unable to be propagated. In comparison, iPSC-derived cell types are both accessible, reproducible, and can be scaled to higher-throughput studies. Further, the homogenous neuronal population derived from this differentiation protocol provides the ability to study the effect of HSV-1 and cytokines on cortical neuron function without input from other cell types. Whilst the present study utilised iPSCs from a healthy donor, our model also establishes the possibility of using patient-derived iPSCs to model genetic susceptibility to infection-induced seizure and potential therapeutics. Electrophysiology studies of HSV-1-infected neurons has been reported previously, particularly in rodent dorsal root ganglion cultures using intracellular recording and voltage clamp^{28,53,54}, and more recently in human iPSC-derived cortical neurons using MEA. However, relatively little work has examined HSV-1-induced hyperexcitability in human cortical neuronal networks in an HSV encephalitis-relevant context. Bergström et al. reported reduced synaptic activity of iPSC-neurons on MEA after HSV-1 infection rather than hyperexcitability⁵⁵. However, HSV-1 in this study did not reduce iPSC-neuron viability at 48 hours post-infection, which was consistently observed in the present study. Differences in the viral strain used, multiplicity of infection, neuronal maturity, and the presence of glia across HSV-1 neuronal models may influence the observed phenotype and could contribute to differences between studies. This study has several limitations. First, the 2D hiPSC-CN model lacks the cellular complexity of the *in vivo* CNS microenvironment, including astrocytes, microglia, infiltrating immune cells, and blood-brain barrier components, all of which undoubtedly contribute to inflammatory signalling during HSV encephalitis. We did consider possible effects of astrocyte cytokine secretion during infection; however we found that astrocyte conditioned medium only had a modest effect on network activity compared to direct infection. Secondly, this study used only a single hiPSC line and differentiation protocol, and the findings should therefore be interpreted with

recognition that genetic background may influence neuronal susceptibility to infection and hyperexcitability. Nevertheless, the use of one well-characterised line allowed tighter experimental control and reduced variability arising from differences in differentiation efficiency, maturation state, and baseline electrophysiological behaviour between lines. Third, the incomplete rescue observed with IL-6R blockade following exogenous IL-6 exposure suggests that exogenous cytokine stimulation may not fully recapitulate the broader endogenous signalling cascades associated with HSV infection. Lastly, our *in vivo* analysis used data from a separate study not designed to investigate seizures. As seizure status was not recorded, we could not assess associations between cytokine or receptor subunit expression and seizure frequency or severity in this post-hoc analysis.

In conclusion, we report that acute HSV-1 infection induces neuronal network hyperexcitability in human iPSC-derived cortical neurons, accompanied by IL-6 secretion and altered expression of excitatory genes, including KCNQ2, which may be reversed by blocking IL-6 signalling. Future work is required to define the molecular mechanisms linking IL-6 signalling to transcriptional regulation of physiological signalling pathways and gene expression involved in regulating neuronal excitability.

Methods

Study participants and sample collection

Samples were collected from adults with PCR-confirmed Herpes Simplex Virus (HSV) encephalitis from 53 National Health Service hospitals in the UK. The participants were recruited in a clinical trial and randomised to receive either adjunctive dexamethasone plus intravenous acyclovir (treatment group) or acyclovir alone (control group). The clinical trial is described in detail elsewhere⁴. Informed consent was provided for all participants before they were recruited into the study. All ethical approvals were in place before the start of the clinical trial. Ethics approval was obtained from the UK National Research Ethics Committee (reference number: 15/NW/0545). From each participant, up to 2.5 mL of CSF was collected in a universal container, and 5 mL of venous blood in a BD Vacutainer Serum Separator Tube. Serum was

separated by centrifugation, and both samples were frozen at the sites per local hospital policy. All frozen specimens were couriered in batches to the Brain Infection and Inflammation Group, Clinical Science Centre, University of Liverpool, for analysis. The specimens were stored at -80°C until analysed, and freeze-thaw cycles were minimised. CSF and blood samples were obtained at recruitment, 14 and 30 days after randomisation in the study or when the patient was discharged from the hospital.

HSV-1 Strain for *in vitro* and *in vivo* models

Human herpesvirus 1 strain KOS (ATCC #VR-1493) was propagated, purified and titrated using Vero cells (Sigma #84113001) grown in Dulbecco's modified Eagle's medium (DMEM, Gibco # 10741574) supplemented with 10% foetal bovine serum (Gibco # 15555309), 1% HEPES (Gibco # 15630080), 1% GlutaMAX (Gibco #35050061), and penicillin-streptomycin (Merck #15140122) as previously described.⁵⁶

iPSC culture

The iPSC line HPSI0214i-kucg_2 (ECACC #77650065) was obtained from the HipSci collection (donor was a White British male 65-69 years old and clinically unaffected). iPSCs were plated on Vitronectin XF (Stemcell Technologies #07180) using Essential 8 Flex (Essential 8 Flex Medium Kit #A2858501). For the first 24 hours post-thaw, cells were supplemented with Y-27632 (Strattech Scientific #B1293-APE) before this was withdrawn. Cells were passaged with ReLesr dissociation reagent according to manufacturer protocol (StemCell Technologies #100-0483) and maintained in E8 Flex medium. Cells were maintained at 37°C with 5% CO_2 .

Cortical neuron differentiation

iPSCs were differentiated into neural progenitors using a protocols modified from those previously described by Karch et al. and TCW et al.^{35,57}. Briefly, iPSCs were dissociated using Accutase (Gibco #A1110501) and resuspended in either STEMDIFF Neural Induction Medium with Smadi supplement (StemCellTechnologies #08581) or alternatively in neural induction medium (DMEM:F12 (Gibco #10565018), 1X N2 supplement (Gibco #17502048), 1X B27-VitA (Gibco #15934204), 0.1 μM LDN193189 (Apollo Scientific #BUP04416), 10 μM SB431542 (Apollo Scientific #BIS4592), 1X MEM

NEAA (Gibco #11140050) and 25 µg/mL Gentamicin (Capricorn Scientific #CSR353). Cell suspensions were then dispensed into a 96-well round-bottom plate and centrifuged at 130 x g for 3 minutes to form aggregates of 50,000 cells. After 5 days, neural aggregates were then plated in 6-well plates coated with poly-l-ornithine (Merck #P4957) and laminin (Merck #L2020). After 7 days neural rosettes were selected using Neural Rosette Selection Reagent (StemCellTechnologies #5832) and replated in Neural Induction Medium with added 20 ng/mL FGF-2 (Gibco #17821163) on poly-L-ornithine and laminin-coated 6 well plates to expand neural progenitors. For differentiation of neural progenitors to cortical neurons, 24 hours after plating, the medium was replaced with cortical neuron differentiation medium (Neurobasal Plus medium with 1x B27 Plus (Gibco #A3653401), 20ng/mL BDNF (ProteinTech #HZ-1335), 20ng/mL GDNF (ProteinTech #HZ-1311), 50 µM cAMP (Merck #D0260), 1X Glutamax (Gibco #35050061), 100 µM Abscorbic acid (Merck #1043003) and 25 µg/mL gentamicin) and cells were maintained in this medium for 6 weeks before and during experiments. For the first 7 days of differentiation, 2.8 µM DAPT (Merck #D5942) was used as a pulse treatment before being withdrawn.

For multi-electrode array experiments, neural progenitors were then plated in Neural Induction Medium onto 24-well Cytoview MEA plates (Axion Biosystems #1000548) at a density of 8×10^4 cells per array/well and after 24 hours switched to, and maintained in, cortical neuron differentiation medium for 6 weeks.

For immunostaining experiments, neural progenitors were plated in standard 24 well tissue culture plates at a density of 8×10^4 cells per well coated with poly-l-ornithine and laminin and maintained in cortical neuron differentiation medium for 6 weeks.

For RT-qPCR experiments, neural progenitors were plated in standard 6 well tissue culture plates at a density of 4×10^5 cells per well coated with poly-l-ornithine and laminin and maintained in cortical neuron differentiation medium for 6 weeks prior to infection and lysis for RNA extraction.

Immunofluorescent staining and confocal microscopy

Cultures were fixed with 4% paraformaldehyde (Thermo Scientific #28908) for 15 minutes and then washed three times with 1X PBS with calcium and magnesium (+/+) (Gibco #15450594). Cells were then incubated for 1 hour with Dako-block (Agilent #X090930-2). Cells were then washed once with PBS +/+ and incubated with primary antibody (See Supplementary Methods Table 1) diluted in antibody diluent (1X PBS +/+, 1% BSA (Sigma #A2153), 10% donkey serum (Sigma #D9663), 0.1% Triton-X (Sigma #X100)) overnight at 4°C. Cells were then washed 3x with 1x PBS +/+ and incubated with secondary antibody (See Supplementary Methods Table 1) and DAPI (Thermofisher #P36962) in antibody diluent and incubated in the dark for 2 hours at room temperature. Cells were then washed 3x with PBS +/+. Cells grown on coverslips were inverted onto 20 µL droplets of Vectashield (Vector Laboratories #H-1200-10) on microscope slides and sealed with nail polish. Cells were imaged at 63x magnification on a Leica DMI8 on an Andor Dragonfly spinning disk confocal microscope. Images were then processed in Fiji/ImageJ (v 1.54)⁵⁸ for addition of scale bars and forming composite/split channel images.

In vitro infection of hiPSC-cortical neurons

For hiPSC-cortical neurons plated on 24-well tissue culture plates: cells were infected with HSV-1 KOS diluted in DMEM:F12 (Gibco #11039021) at 8×10^3 PFU per well for 2 hours. Following incubation with the virus, the cells were washed twice with DMEM:F12 and then switched to the appropriate growth medium. For mock infection, sonicated uninfected Vero supernatant was diluted into DMEM:F12 at the same dilution and applied to cells for 2 hours.

For iPSC-cortical neurons on multi-electrode array: neurons were infected as described above. Electrophysiological recordings were taken at baseline before infection or treatment, and before supernatants were harvested at later timepoints. Supernatants were collected from MEA cultures after recording at appropriate timepoints and transferred to a 6-well plate (for UV inactivation) before filtering (see below). After UV-inactivation and filtration, supernatants were transferred to a 1 mL cryovial and stored immediately at -80°C for later analysis of cytokine and chemokine secretion using ELISA. For collection of conditioned neuronal supernatants for inactivation and use in further experiments, neural

progenitors were seeded at 4×10^5 cells per well into 6 well plates and differentiated for 30 days before being infected as described above with 4×10^6 PFU of HSV-1 KOS. Infection-conditioned supernatants were collected 24 hours post-infection. For mock infection, cells were incubated with DMEM:F12 for 2 hours before medium was replaced, and supernatants were taken 24 hours after from uninfected cells.

Inactivation and filtration of supernatant from infected cells

Supernatants were transferred into wells of an open 6 well plate were placed directly underneath a Fusion 11W G23 2 Pin UV Lamp mounted on a XX15 Exposure UV Lamp Stand (Analytik Jena #UVP-18-0062-01) that generates 254 nm light from ~5 cm above the samples. Samples were irradiated with a fluence of 18 J/cm² at 5 cm over 30 minutes. Samples were then subsequently passed twice through a 20 nm filter using a syringe.

Treatment of hiPSC-cortical neurons with IL-6 and anti-human IL-6R

Recombinant human IL-6 (Proteintech #HZ-1019) was diluted in cortical neuron differentiation medium to a concentration of 100 ng/mL and applied to hiPSC-CNs for the whole experiment. Cells were fed every 2 days as follows: 100% of IL-6 containing medium was removed and replaced with the same volume of cortical neuron differentiation medium containing 100 ng/mL IL-6. For anti-human IL-6R treatment, hiPSC-CNs were pretreated with 20 µg/mL anti-human IL-6R (BioXcell #SIM0014) for 2 hours prior to infection with IL-6 treatment and were concurrently treated with anti-human IL-6R and IL-6 for the whole period of the experiment. Isotype control was human IgG1 (RecombiMAb human IgG1 isotype control #CP174) and was added at the start of the IL-6 treatment to untreated cells. For concurrent HSV-1 infection and anti-IL-6R treatment, anti-IL-6R treatment was commenced as above for 2 hours immediately preceding the 2-hour incubation period with HSV-1, and after infection cells were washed once with PBS -/- before medium containing 20 µg/mL anti-human IL-6R was replaced.

Multi-electrode array electrophysiological functional assays

For spontaneous recordings, spontaneous activity and viability were recorded for 100 seconds. For each evoked recording, cultures were stimulated for a total of 9 stimulation events over a period of ten seconds, using the Brain stimulation module in the Neural module of Axis Navigator (v3.9, Axion Biosystems). Spontaneous and evoked recordings of MEA cultures were taken at baseline immediately before infection or treatment. MEA cultures were then infected as described previously or exposed to virus-filtrated supernatants for the same period. Mock-infected controls were treated with diluted Vero supernatant or uninfected conditioned medium for the same period. Positive controls for spontaneous hyperexcitability were treated with 200 μ M kainic acid (Sigma-Aldrich #K0250) throughout the experiment. Recordings of spontaneous and evoked activity were then made at 24, 48 and 72 hours, respectively. Impedance, spontaneous and evoked activity data were extracted from Axion spike (.spk) files using Neural Metrics Tool (v.4.0.5, Axion Biosystems). Where specified, resistance and firing data were normalised to baseline, and firing data normalized to fold change in viability, using the following formula:

$$fold\ change = \frac{X_{Ti}}{X_0}$$

Measurement of Cytokines in CSF

A Bio-Plex Pro Human Cytokine 48-plex Assay (Bio-Rad Laboratories #12007283) and a Bio-plex 200 Luminex analyzer (Bio-Rad Laboratories #171000201) was used for mediators profiling. 50 μ L of undiluted sample or standard was pipetted into each well. The samples were analysed in triplicate. Plates were processed according to the manufacturer's instructions.

ELISA of cell culture supernatants

Supernatants were collected as described above and stored at -80 °C prior to cytokine levels being measured using ELISA. Kits were purchased for human IL-6 (Invitrogen #88-7066-88), IFN γ (Invitrogen #88-7316-88) and CCL2 (Invitrogen #88-7399-88), and Bio-technique for MIF (Bio-technique #DMF00B) and CCL5 (Bio-technique #DRN00B) and performed according to manufacturer's protocol. Absorbance values of blank wells were subtracted from raw absorbance values, and a standard curve was generated using absorbance values

for known concentrations. Absorbance values for unknown concentrations were then interpolated from the standard curve using simple linear regression.

RT-qPCR of cell cultures

Total RNA from cells was extracted by using TRIzol reagent and the manufacturers RNA extraction protocol, before being treated with DNase I. RNA was quantified using the Qubit HS RNA assay (Invitrogen #Q32852) and diluted in nuclease-free water to an appropriate concentration for downstream RT-qPCR. RT-qPCR was carried out using the GoTaq 1-Step RT-qPCR kit (Promega #A6020) according to manufacturers protocol using an Agilent AriaMx Real-Time PCR System and gene expression quantitated using the ddCq method relative to 18S or RPLP0 from the same sample and RT-qPCR run. 20 ng of RNA was used in each experiment in duplicate. Master mix only and -RT controls were run for each experiment. For HSV-1 relative viral load, dCq method was used. Where Cq was undetected, the sample was assigned a Cq of the total number of cycles +1. Primer/Probes used are listed in Supplementary Methods Table 2.

Herpes simplex virus encephalitis mouse model

C57BL/6 female mice aged 8 -12 weeks (Charles River Laboratories #556) were intracranially infected with 20 µL of 10⁴ PFU HSV-1-KOS or sham-infected with sterile PBS. Mice were euthanised three days post-infection, and death was confirmed by transection of the great arteries. About 0.5 cm of the cerebral cortex was sampled into RNAlater (Invitrogen #10564445) and immediately processed for single-nuclei isolation. All animal work was carried out at Harvard University under IACUC number: 2016N000521.

Single Nuclei Isolation

Samples were homogenised in 2ml of ice-cold Nuclei EZ lysis buffer (Sigma, #EZ PREP NUC-101), transferred into a different tube containing an additional 2ml of ice-cold Nuclei EZ lysis buffer and incubated on ice for 5min. Samples were then filtered through a 30µm cell strainer (MACS Smart Strainer #130-098-458) and centrifuged at 500g for 5min at 4°C. The supernatant was discarded, and the pellet was

resuspended in fresh 4mL of Nuclei EZ lysis buffer. This process was repeated three times. After the last centrifugation, the pellets were resuspended in 300 μ L of NSB (1xPBS, 5% BSA, 0.25% Glycerol and 40U/ μ L RNase inhibitor from Clontech Takara #2313A/B) and filtered through a 40 μ m Cell Strainer (Flowmi #H13680-0040), followed by filtration through a 10 μ m cell strainer. The single nuclei suspension was stained with 1 μ g/ml DAPI (Fisher #D1306) and DAPI+ events were sorted. The concentration of single nuclei was adjusted to 1000 single nuclei per μ L and immediately loaded on the 10X Chromium controller instrument (10X Genomics) for sequencing.

Single-cell RNA sequencing

Cell suspensions were loaded along with reverse transcriptase reagents, 3' gel beads, and emulsification oil onto separate channels of a 10X Single Cell B Chip, which was loaded into the 10X Chromium instrument to generate emulsions. Emulsions were transferred to polymerase chain reaction (PCR) strip tubes for immediate processing and reverse transcription. Library preparation was performed according to the manufacturer's recommendations. Expression libraries were generated using Chromium Single Cell Next GEM 3'V3.1 chemistry (10X Genomics #PN-1000268). DNA and library quality were evaluated using an Agilent 2100 Bioanalyzer, and concentration was quantified using the Qubit dsDNA high-sensitivity reagents (Thermo Fisher Scientific #Q33231). Gene expression libraries were sequenced on the Illumina NextSeq 500/550 High Output Kit v2.5 (75 Cycles) (Illumina #20024906).

Statistical information

For *in vitro* data: Data were tested for normality using Shapiro-Wilk test prior to statistical analysis. Statistical tests for each analysis can be found in the figure legend. Normal data are presented as mean $\pm$ SEM, whereas non-normal data are presented as median $\pm$ IQR. Statistical tests and data visualizations were performed in GraphPad Prism (v11.0.0). Significance was defined as $p < 0.05$. The difference between two normally distributed groups was tested using two-tailed unpaired t-test or Welch's two-tailed t-test (where SD ratio was greater than 2). The difference between two non-normally distributed groups was tested using Mann-Whitney U test. The difference between >2 normally distributed groups was tested using one-way ANOVA with Tukey's post-hoc test. The difference between >2 non-normally distributed groups was tested using Kruskal-Wallis test. Where experiments had two dependent variables (resistance or activity and time), 2-way ANOVA with Dunnett's or Bonferroni post-hoc correction was used.

For the human data, numerical variables were compared between seizure groups using the independent samples Wilcoxon rank-sum (Mann-Whitney) test. Graphs were generated using the `ggplot2` package⁵⁹ and statistical annotations were added using the `ggpubr` package⁶⁰ in R software version 4.5.0⁶¹.

For single-cell RNA-seq data: data were mapped to Reference UMAP embeddings using the TransferData and MapQuery functions from Seurat package to identify cell types. Differential gene expression analysis of murine data was done in R statistical software version 4.5.0⁶¹ using the pseudobulk approach with DESeq2() function⁶². Gene expression counts were first aggregated across cells within each biological sample and pre-determined cell cluster using the AggregateExpression() function. Differential gene expression between infected and control groups was then assessed using DESeq2. P values were adjusted for multiple comparisons using the Benjamini–Hochberg correction.

Data Availability

All *in vitro*, *in vivo*, human cytokine data reported in this paper will be shared by the lead contact upon request. Original code used for analysis will be shared by the lead contact upon request. Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

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Acknowledgements

We thank the tissue donors, patients and their carers who volunteered to contribute to these studies, and the research staff in every hospital who recruited patients. B.D.M., is supported by NIHR Health Protection Research Unit in Emerging and Zoonotic Infections at University of Liverpool. B.D.M. is supported for additional neurological inflammation research due to viral infection by grants from: the NIHR (award CO-CIN-01), the MRC (MC_PC_19059), the UKRI/MRC (MR/V007181/1), MRC (MR/T028750/1) and the Wellcome Trust (ISSF201902/3). F.N.E. is supported for neurological inflammation research by grants from the Wellcome Trust (grant number 315711/Z/24/Z). The DexEnceph trial was funded by the National Institute for Health and Care Research (NIHR) Efficacy and Mechanism Evaluation Programme for the Department of Health (reference 12/205/28). T.S. is supported by the NIHR Health Protection Research Unit in Emerging and Zoonotic Infections (grant number IS-HPU-1112-10117), NIHR Global Health Research Group on Brain Infections (grant number 17/63/110), and The Pandemic Institute, Liverpool, UK. A-C.M. is supported by a grant from Encephalitis International (E-26/202.313/2026). The publication contents are solely the responsibility of the authors and do not necessarily represent the official views of the UKRI, MRC, NIHR or supporting organizations. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. The authors acknowledge the Liverpool Centre for Cell Imaging, University of Liverpool, for access to the Andor Dragonfly spinning-disk confocal

733 microscope funded by Biotechnology and Biological Sciences Research Council (BBSRC) grant
734 BB/R01390X/1. The authors acknowledge the technical assistance and computational resources provided
735 by the Computational Biology Facility (CBF) at the University of Liverpool, part of the Liverpool Shared
736 Research Facilities (LIV-SRF). The authors acknowledge use of the Liverpool University Biobank and
737 GCP Laboratory Facility provided by the Faculty of Health and Life Sciences, University of Liverpool.
738 We would like to acknowledge the Liverpool Experimental Cancer Medicine Centre for providing
739 infrastructure support for this research (Grant Reference: ECMCQQR-2022/100004).

740 **Competing Interests**

741 T.S. has been a member of Encephalitis International since 1998 and President of Encephalitis International
742 since 2019 (unpaid). T.S. is the Director of The Pandemic Institute (since 2021) which has received funding
743 from Innova and CSL Seqirus and Aviva and DAM Health (unpaid). All other authors declare no competing
744 interests.

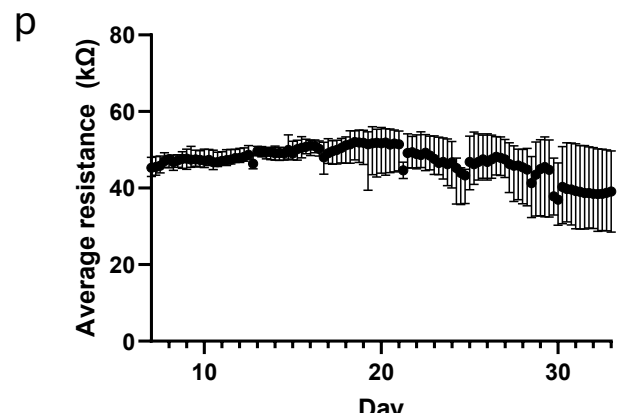
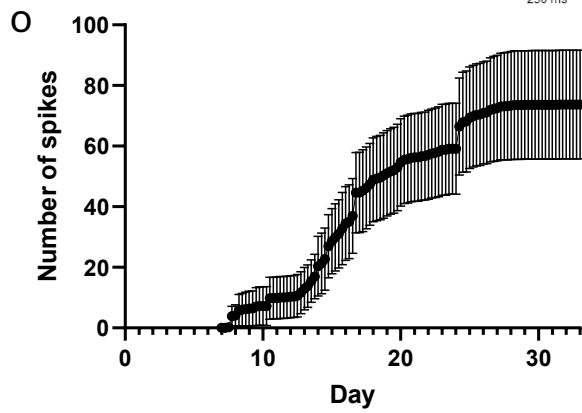
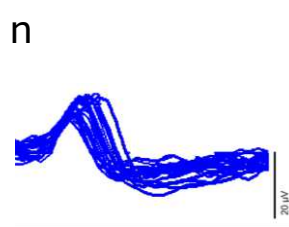
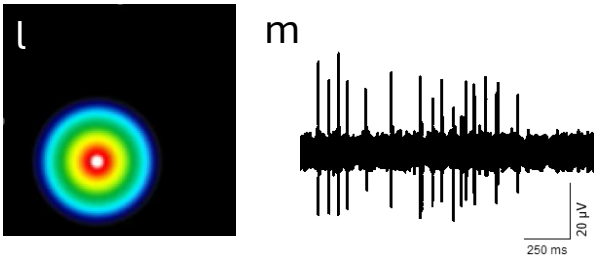
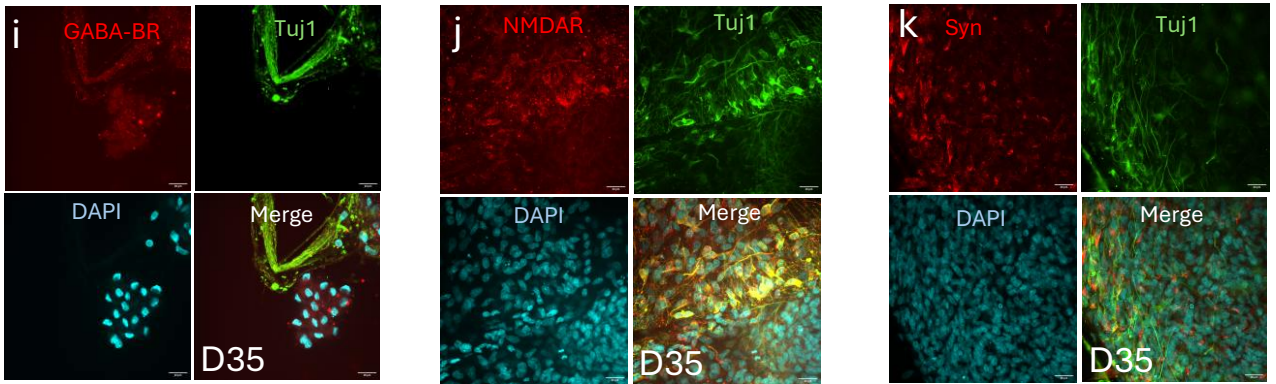
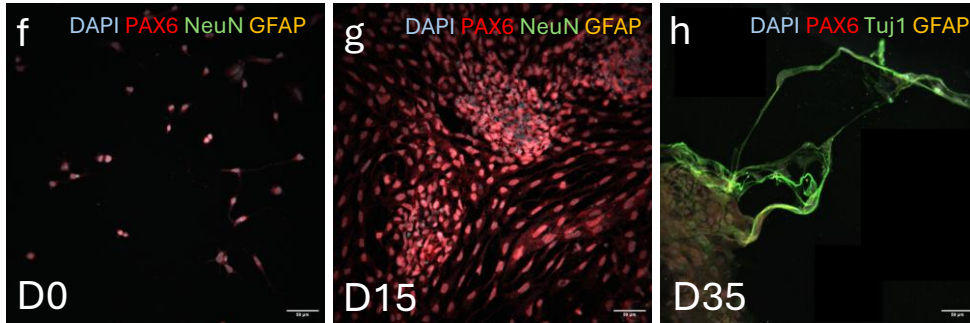
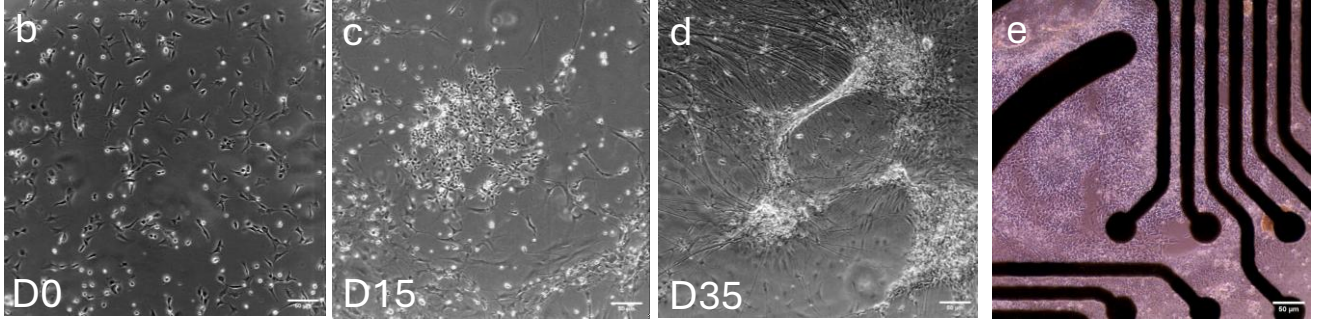
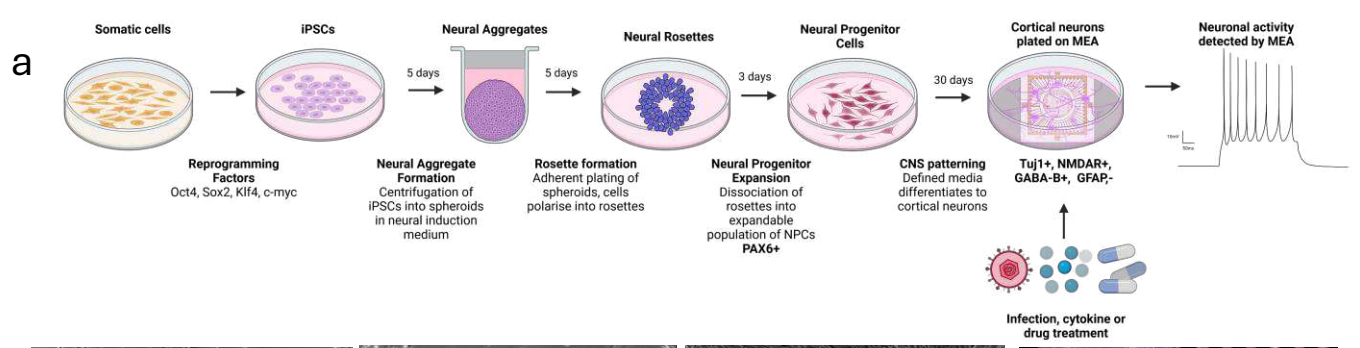
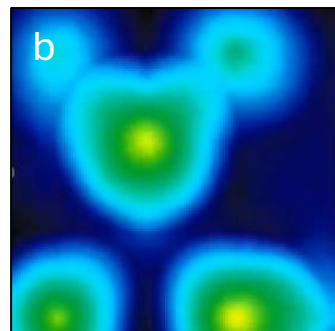
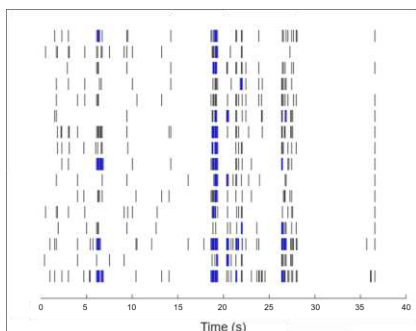
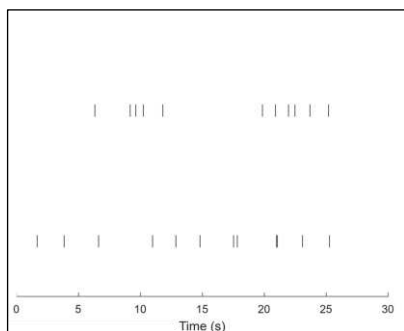


Figure 1. Differentiation and characterisation of cortical neurons. a) Schematic for differentiation of cortical neurons from human iPSC and downstream experiments. Made with Biorender.com. b-d) Phase contrast images of NPCs at day 0 (b), Day 15 (c) and Day 35 (d) of terminal cortical neuron differentiation. e) Phase contrast image of hiPSC-CNs on multi-electrode array plate. Scale Bars = 50 μm . f) At day 0, NPCs were stained for NPC marker PAX6, mature neuron marker NeuN and glial marker GFAP. There was no NeuN or GFAP positive staining whereas cells were predominantly PAX6 positive, confirming NPC identity. g) At Day 15 of terminal differentiation, PAX6+ NPCs had expanded in population, whilst there was still no positive staining of NeuN and GFAP, indicating NPC identity at this stage. h) At day 35, PAX6 staining was reduced, and long projections with axon-like morphology were positive for mature neuron marker B-tubulin III. No GFAP positive cells were detected at any timepoint. Scale Bars = 50 μm . i-k) Clustered neurons at day 35 of differentiation show immunopositive staining for inhibitory neuron marker GABA-BR in the cell bodies (i), and diffusely for excitatory marker NMDAR (j) and synapse marker synaptophysin (k). Scale Bars = 25 μm . l) Heat map representation of spontaneous spike activity recorded from cultured cortical neurons after 35 days of differentiation. m-n) Representative continuous spike plot and spike waveform from spontaneous spike activity on the same single electrode. o) Number of spikes recorded cumulatively per well over the period of differentiation from day 7 to day 35. By day 35 all wells had shown baseline spontaneous activity. p) Average resistance of the wells over the same period. N = 24 wells.

Baseline

20 hpi

a



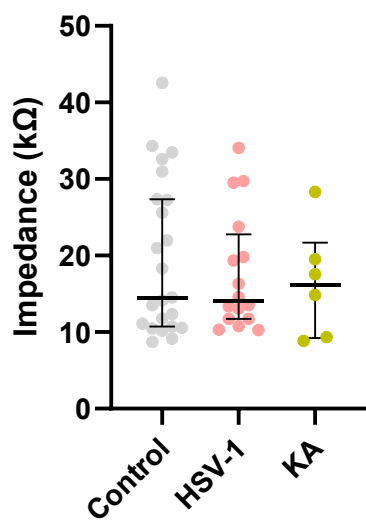
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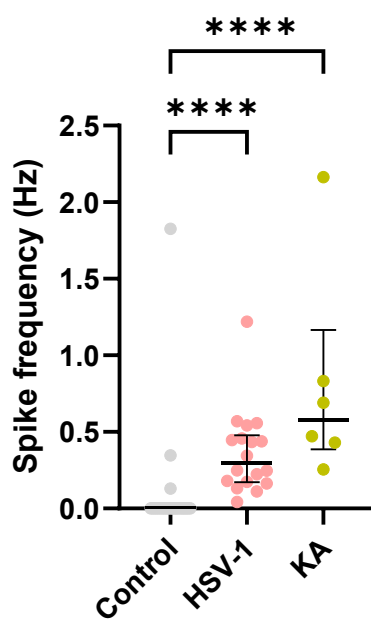
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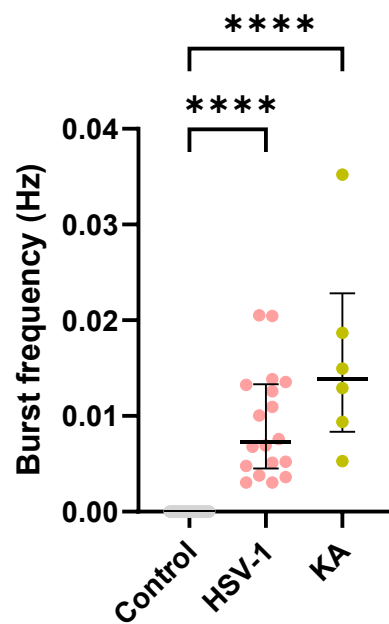
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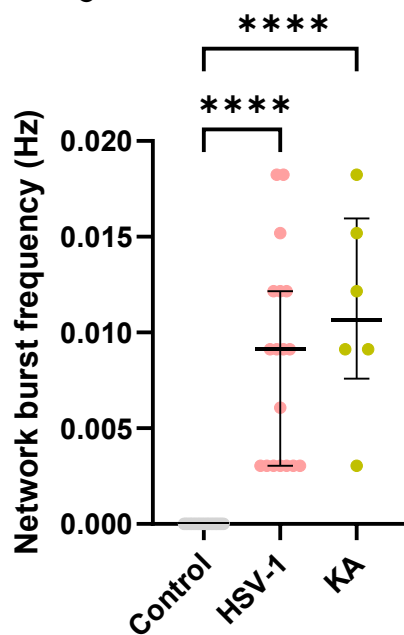
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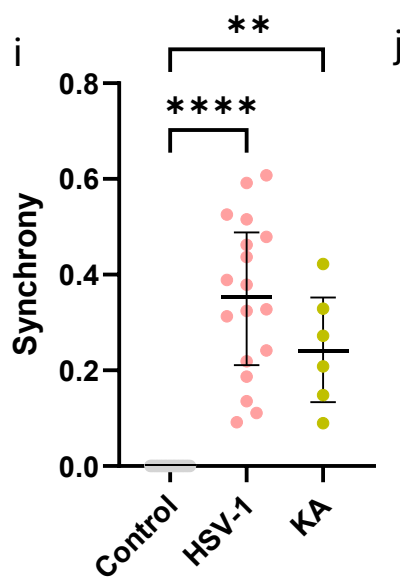
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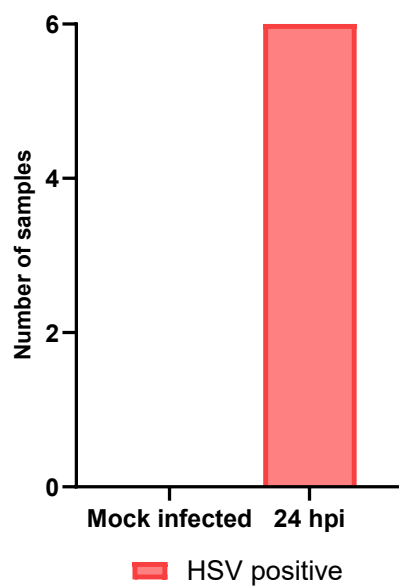
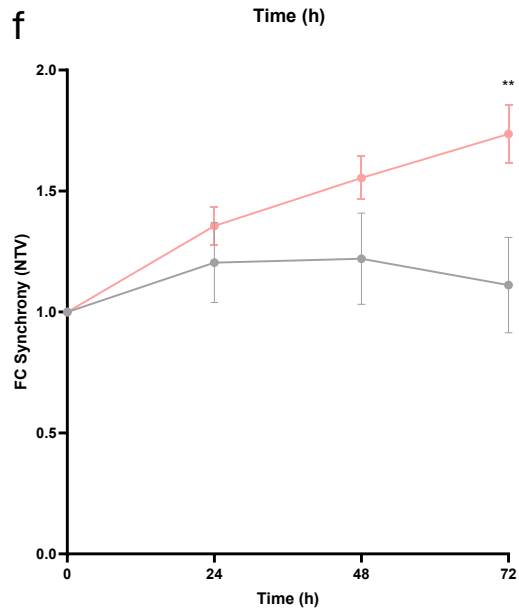
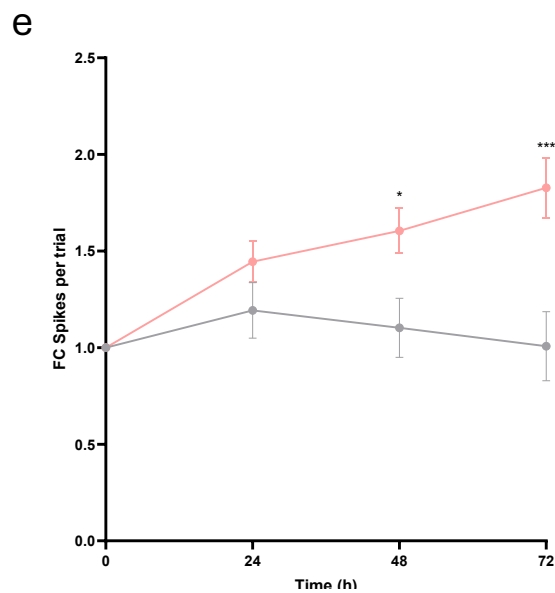
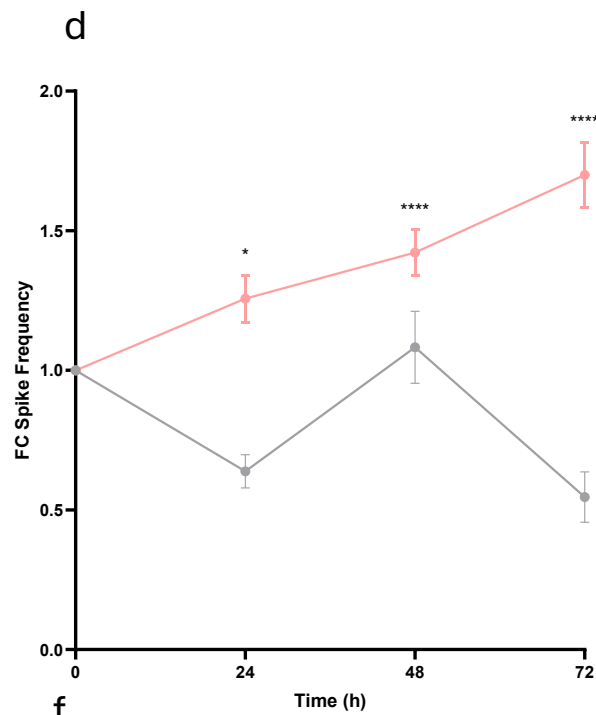
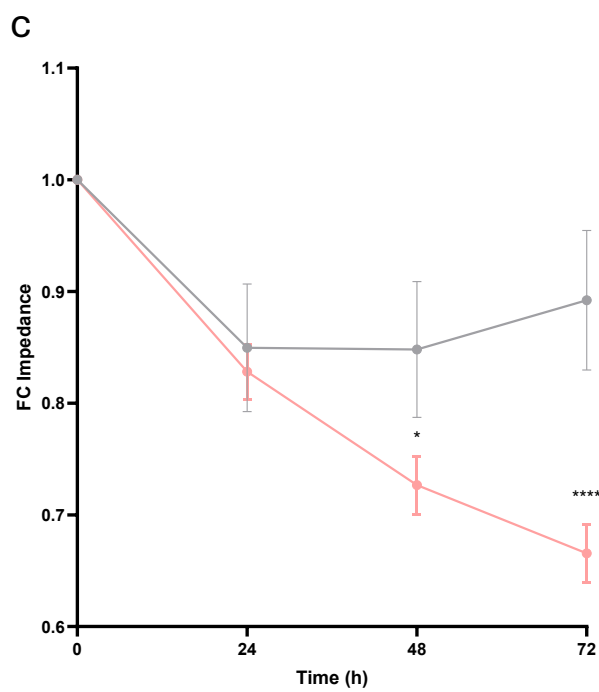
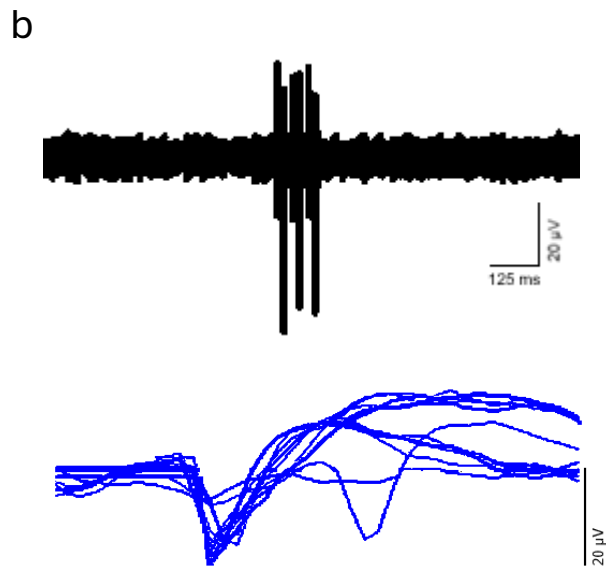
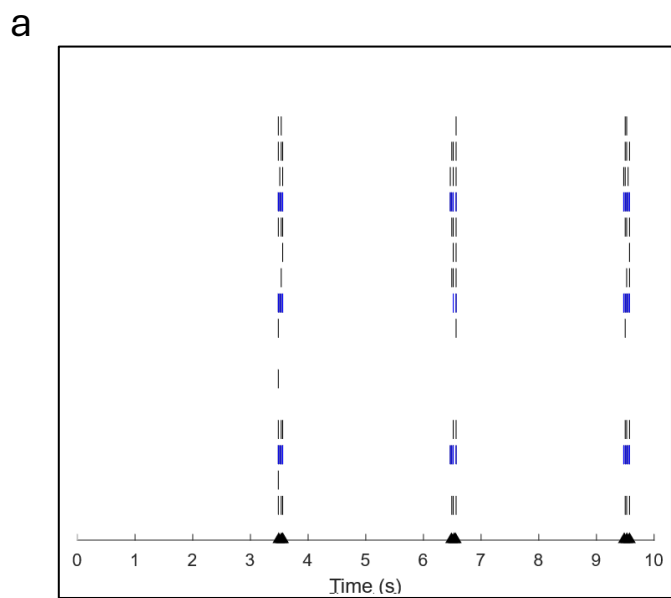


Figure 2. Increased spontaneous firing of HSV-1 infected hiPSC-CNs prior to cell death. a) Representative spike raster plots are shown for a single well at baseline and the same well 20 hours post-infection with HSV-1 at 2.5×10^3 PFU. b) Representative heat map of spontaneous spike activity recorded from hiPSC-CNs at 20 hours post-infection. c-d) Representative continuous spike plot and spike waveform from spontaneous spike activity on the same single electrode after infection. e) At 20 hours post-infection/treatment, there was no significant decrease in cell impedance (viability). f) Spontaneous spike frequency as well as burst and network burst frequency (g-h) was significantly increased in infected cells compared to control and was not significantly different to neurons exposed to the seizuregenic compound kainic acid. i) Network synchrony was also significantly increased. N=24 wells for control, n=18 and 6 for HSV-1 and KA respectively. Kruskal-Wallis test with Dunn's post-hoc correction was used. ****, ***, ** and * represent $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively. j) RT-qPCR for HSV-1 polymerase was used to confirm infection status in mock- and HSV-1 infected lysate of hiPSC-CNs at 20 hours post-infection. 6/6 mock- infected samples were negative for HSV-1 (No Cq) and 6/6 infected samples were positive for HSV-1 (Cq detected). Data are shown as median $\pm$ IQR.



● Control
● HSV-1 2.5 $\times 10^3$ PFU

Figure 3. HSV-1 infection increases evoked response to electrical stimulation. hiPSC-CNs were infected with 2.5×10^3 PFU HSV-1 per well or with equivalent dilution of Vero supernatant (mock-infection), and evoked response to stimulation assessed at baseline and then every 24 hours after infection. a) Representative evoked spike raster plot for a single well at baseline and the same well 72 hours post-infection with HSV-1 at 2.5×10^3 PFU. b) Representative continuous spike plot and spike waveform from evoked spike activity on the same single electrode 72 hours after infection. c) At 24 hours post-infection, there was no significant decrease in cell impedance (viability) from baseline, but there was a significant decrease from 48 hours post-infection up to 72 hours. d) Fold change in spike frequency across the evoked recording was significantly elevated from baseline in response to the same stimulus sequence, compared to mock-infected control. e) Spikes per trial (average number of spikes provoked by each individual stimulation) was significantly elevated at 48h-72h post-infection. f) Synchrony of evoked activity was significantly elevated from baseline at 72h post-infection. N= 6 wells for mock-infected and 24 for HSV-1. 2-Way ANOVA test with Dunnett's post-hoc correction was used. ****, ***, **, and * represent $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively. Data are shown as mean $\pm$ SEM.

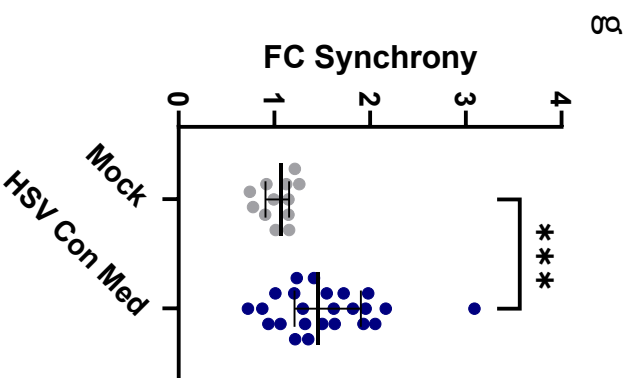
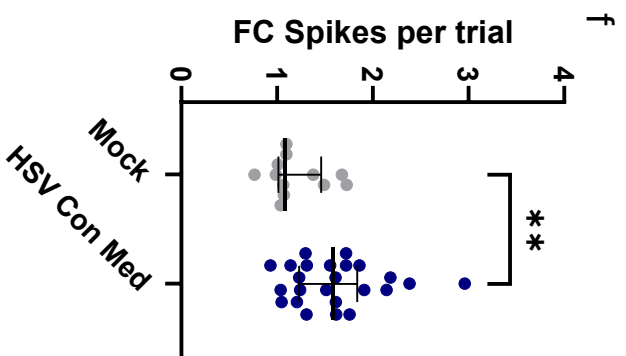
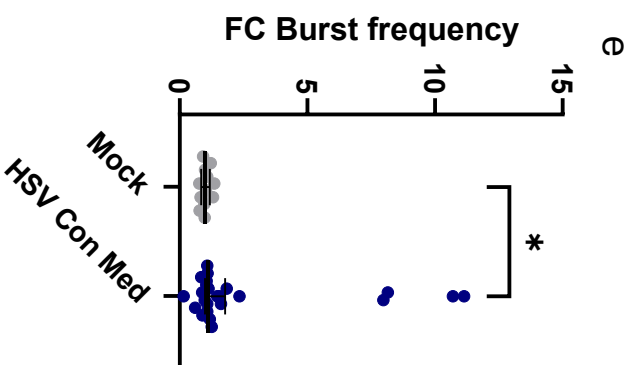
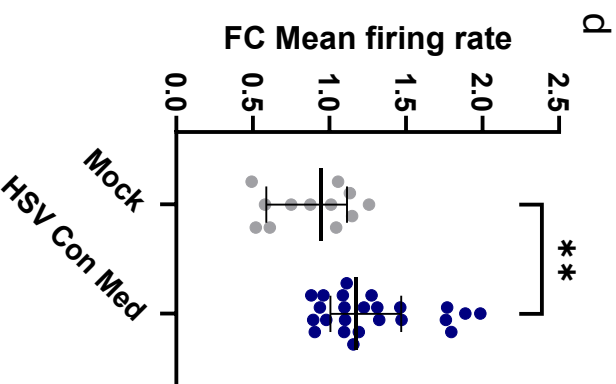
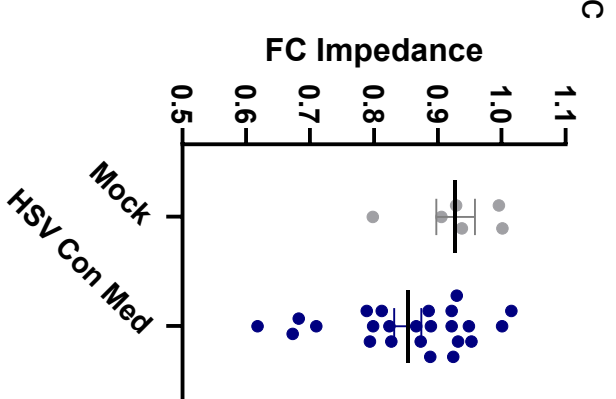
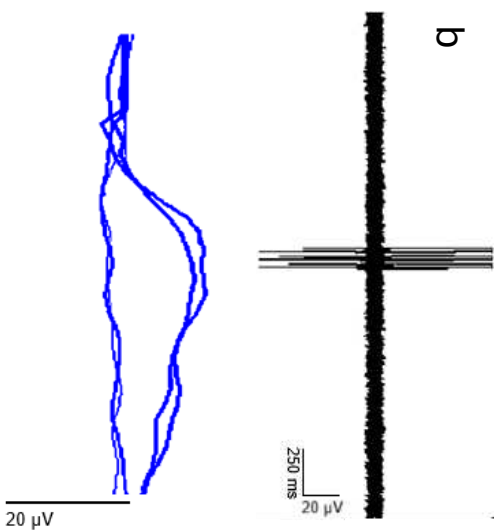
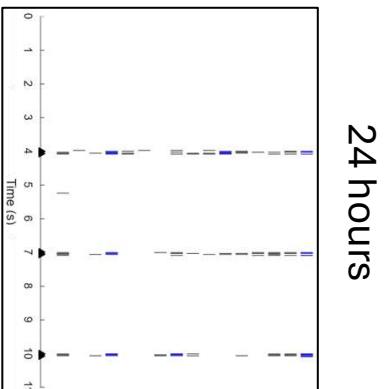
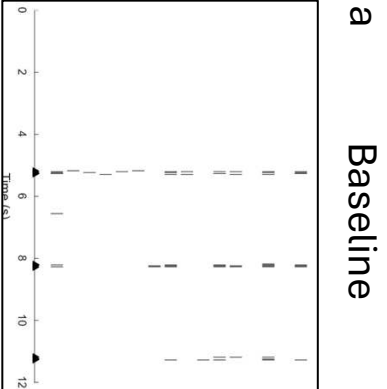
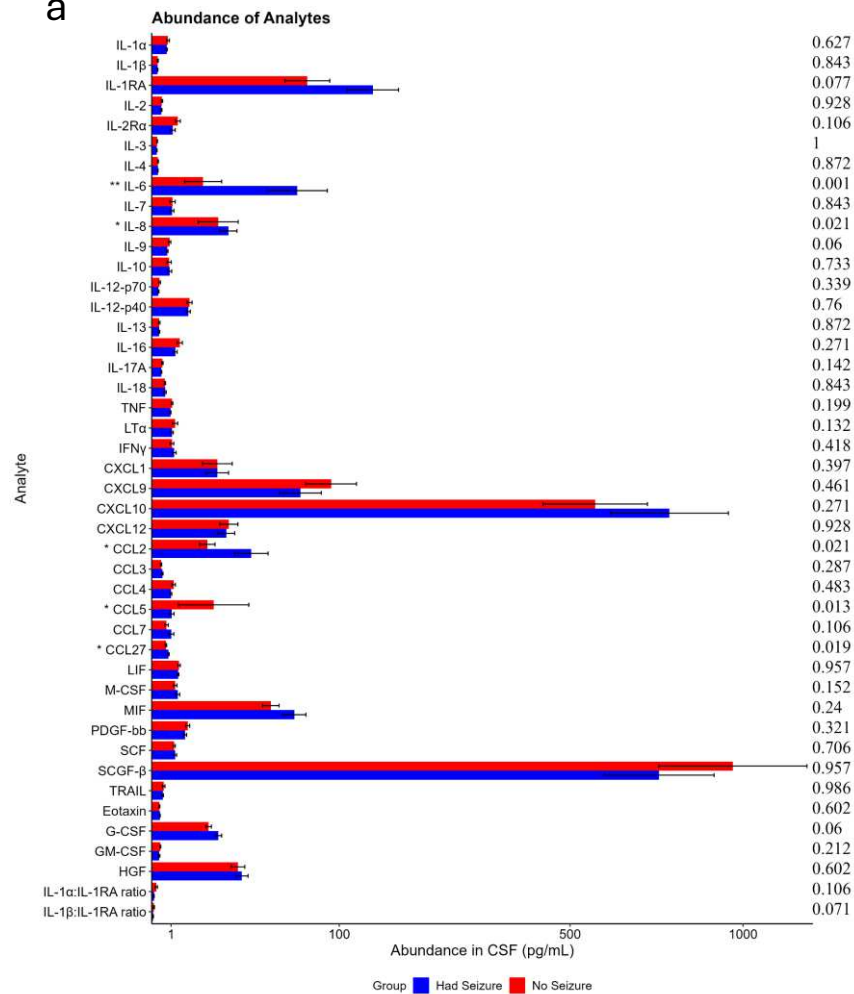
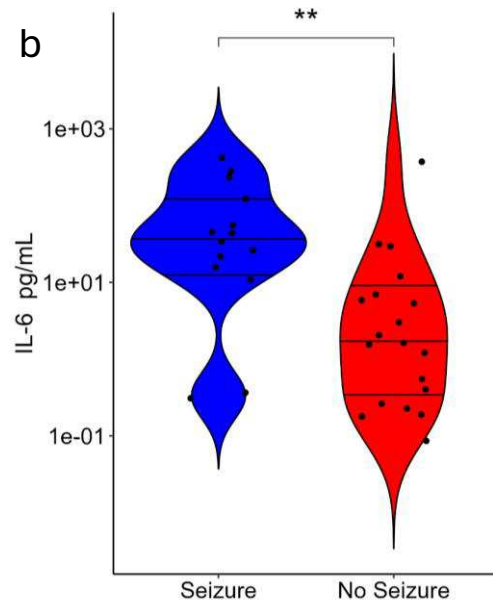


Figure 4. Exposure to inactivated conditioned medium from infected neurons increases evoked responses to electrical stimulus. hiPSC-CNs plated on MEA were incubated with (inactivated and filtrated) conditioned medium collected from HSV-1 infected hiPSC-CNs at 24 hours post-infection. Evoked recordings were then made 24 hours post-treatment. a) Representative evoked spike raster plot for a single well at baseline and the same well 24 hours post-treatment with conditioned medium from infected neurons at 24 hours post-infection. b) Representative continuous spike plot and spike waveform from evoked spike activity on the same single electrode 24 hours after treatment. c) There was no significant decrease in viability at 24 hours post-treatment with conditioned medium. d-g) Neurons exposed to conditioned medium from infected neurons showed significant fold increases from baseline in spike frequency (d), burst frequency (e), spikes per trial and synchrony (g). N = 6 wells for mock and 24 for HSV conditioned medium. Where data was normal (c, e-g) Welch's two-tailed t-test was used, and where data was non-normal (d) Mann-Whitney U test was used. ****, ***, ** and * represent $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively. Data are shown as median $\pm$ IQR (c-f) and mean $\pm$ SEM (g).

a



b



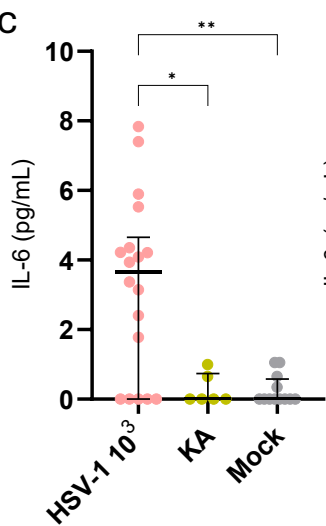
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72 hpi

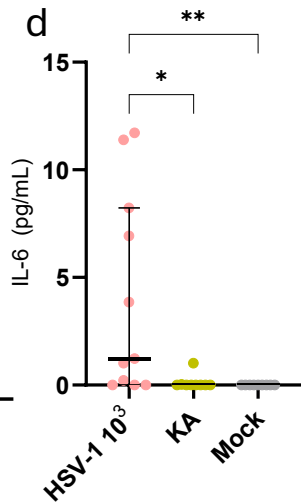
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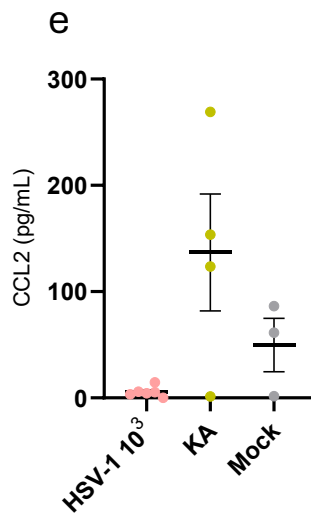
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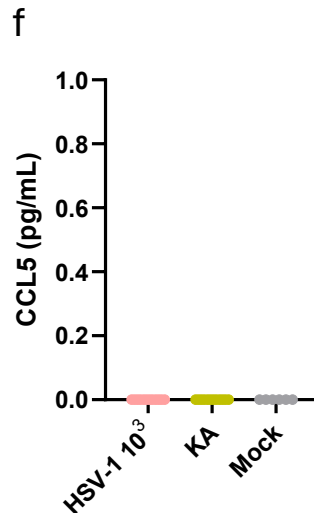
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e



f



g

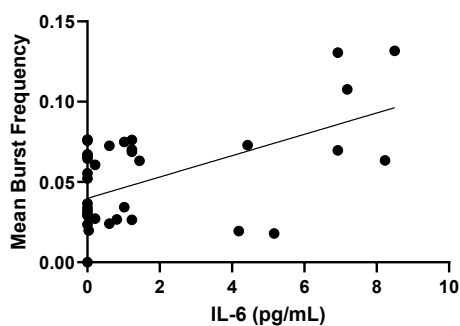
R² = 0.2864, p = 0.0004

Figure 5. CSF IL-6 is associated with seizure in HSV encephalitis patients, and neuronal secretion is correlated with excitability *in vitro*. a) Analyte abundance plot showing concentrations of mediators in CSF from encephalitis patients upon admission to hospital according to seizure status upon admission to hospital. IL-6, IL-8, CCL2 and CCL27 were found to be elevated in patients with seizure, whilst CCL5 was found to be lower in patients with seizure. Each analyte represents median-centred and normalised values. *P* values are displayed on the right. b) Concentration of IL-6 in CSF stratified by seizure status. Mean was compared using the Mann-Whitney test. The violin plot shows the median and interquartile values; each data point represents one patient's clinical sample. Seizure status on admission was recorded as 'seizure' or 'no seizure' by treating clinicians.

c) Supernatant from neurons plated on MEA after 24 and 72 hours post-infection with 2.5×10^3 PFU of HSV-1, or after 24 and 72 hours post-mock infection or KA treatment, was harvested and IL-6 concentration was measured by ELISA. At 24 hours post-infection, IL-6 was significantly elevated in infected neurons compared to those treated with KA or mock-infected. d) At 72 hours, IL-6 remained elevated compared to mock infected and KA-treated neurons. *N* = 12 for HSV-1 and 9 for mock and KA. e) CCL2 was not secreted by infected neurons and was not significantly different to controls. *N* = 6 for CCL2, 3 for mock and 4 for KA. f) CCL5 secretion was not detected in any condition. *N* = 12 for HSV-1 and 6 for mock and KA. Data are shown as median $\pm$ IQR. Kruskal-Wallis with Dunn's post-hoc test was used to compare groups. g) IL-6 secretion at 72 hours post-infection was positively correlated with burst frequency in infected neurons ($R^2 = 0.2864$, $p = 0.0004$). ****, ***, ** and * represent $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively.

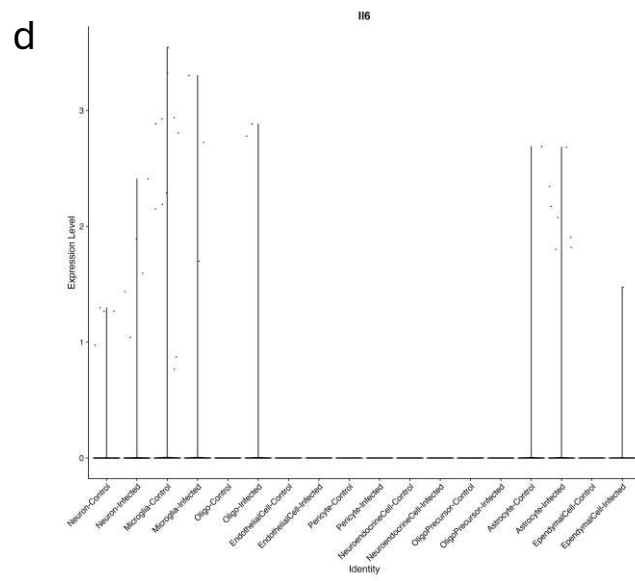
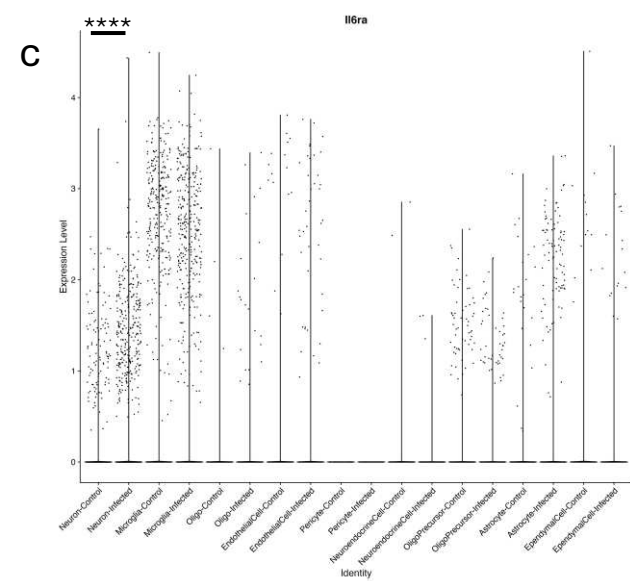
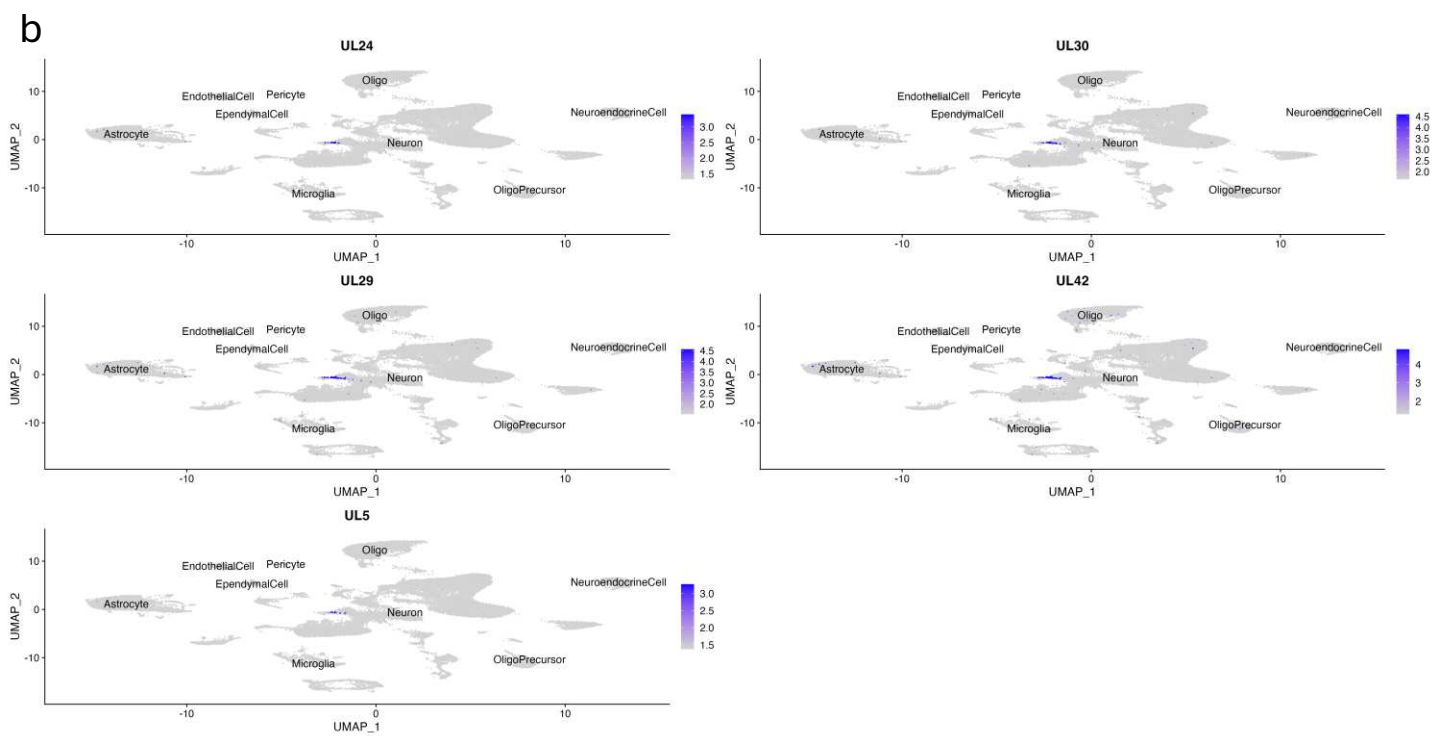
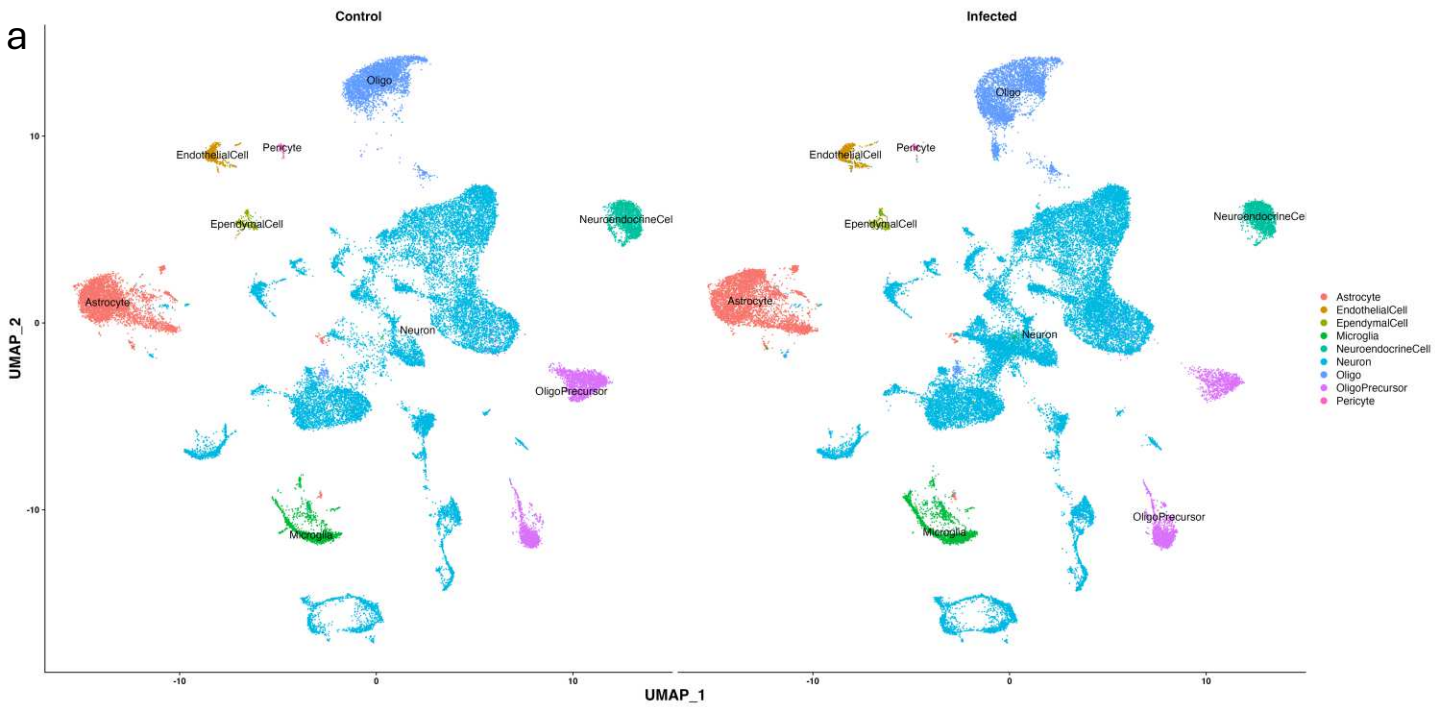


Figure 6: HSV infection induced neuronal expression of IL-6ra in a murine model. a) UMAP plot of scRNAseq data from mouse cortex in control and uninfected animals, labelled by manual annotation. Colours are mapped to the clusters (annotated on the right); broader cell-type definitions are overlayed on the plot. b) Active viral infection (as defined by expression of HSV-1 genes UL24, UL30, UL29, UL42 and UL5) were observed in neuronal and glial cells, but predominantly neurons. c) HSV infection induced neuronal and astrocyte expression of IL-6ra, and in infected animals, neuronal expression of IL-6ra was significantly upregulated. d) IL-6 expression was detected in both control and infected neurons, microglia and astrocytes, and also in infected oligodendrocytes and ependymal cells. IL-6 expression was not significantly upregulated in neurons of infected mice at day 5 post-infection in the pseudobulk analysis ($p = 0.973$). P values were determined by Bonferroni corrected non-parametric Wilcoxon rank sum test. Bar plots show mean expression levels. Each dot represents expression in one cell.

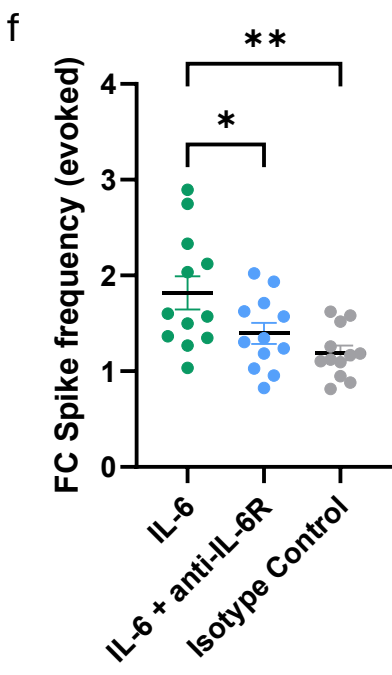
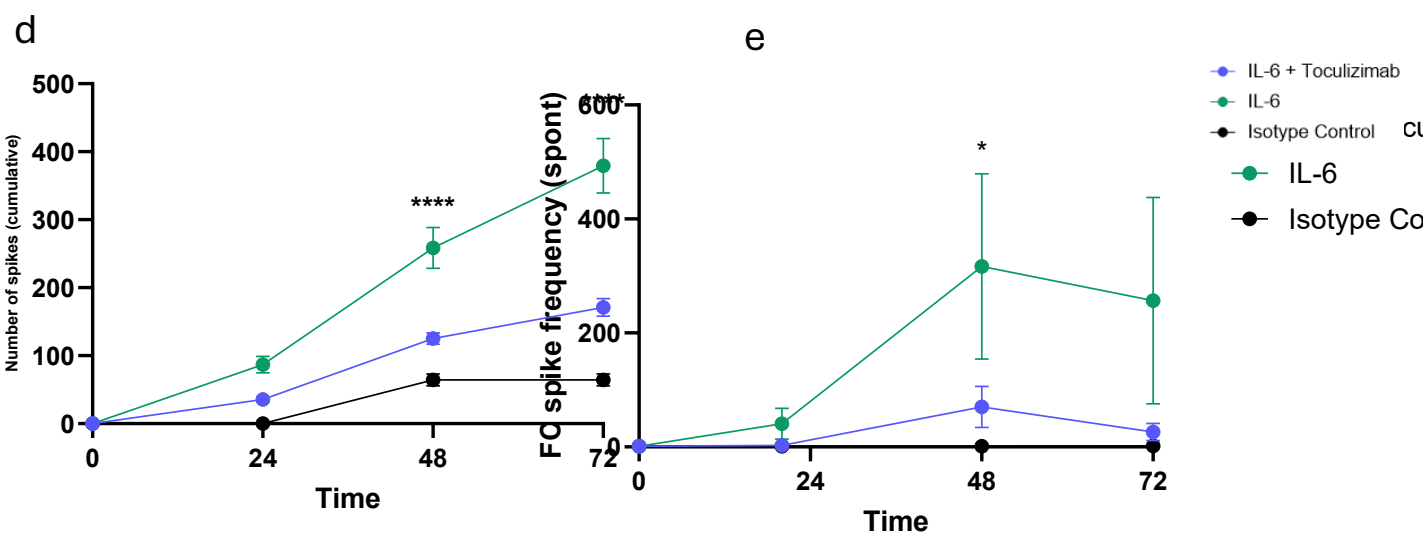
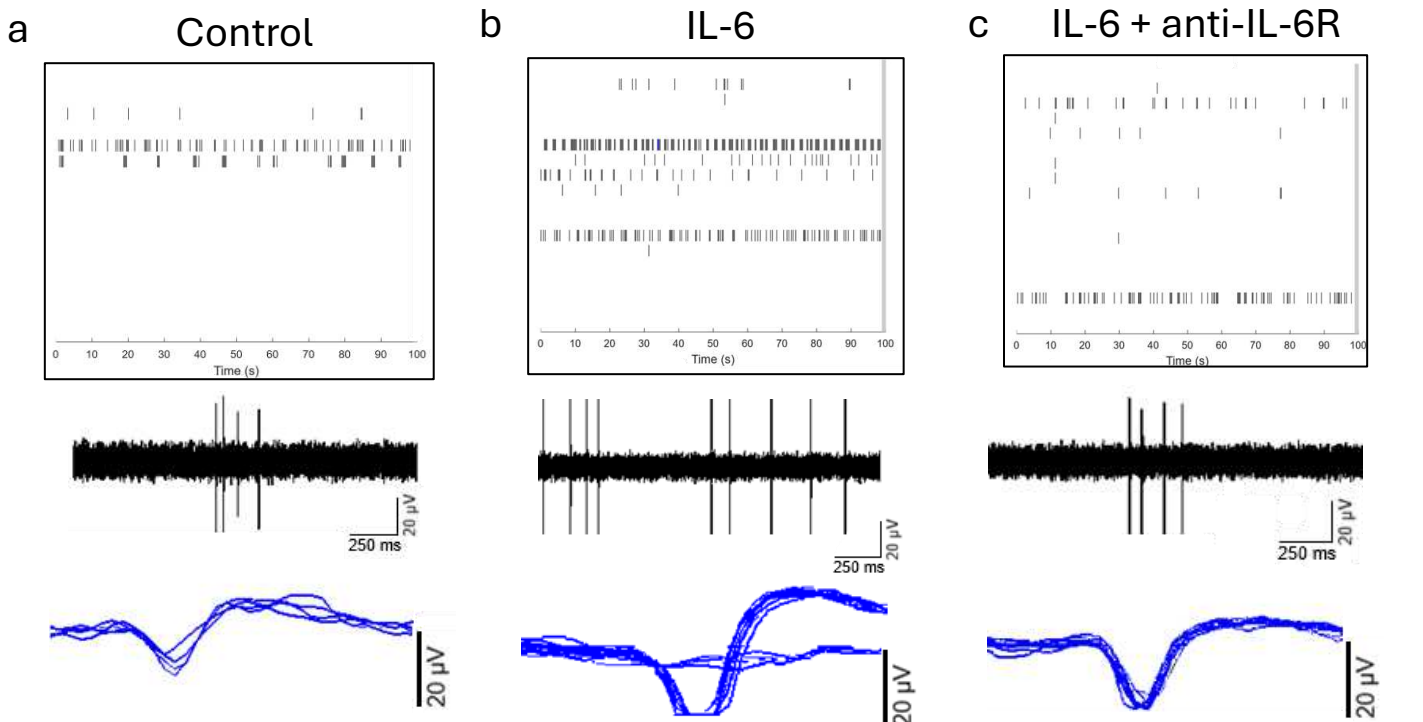


Figure 7. Increased spontaneous and evoked firing of hiPSC-CNs treated with human IL-6 that is partially rescued by treatment with anti-IL-6R. hiPSC-CNs were either left untreated or pre-treated with 20 $\mu\text{g/mL}$ anti-IL-6R and then incubated with 100 ng/mL IL-6, and spontaneous and evoked responses were assessed every 24 hours. a-c) Representative spike raster plots, continuous spike plots and spike waveform plots for spontaneous activity are shown for a single control well, a single well treated with human IL-6 (b) and a single well treated with IL-6 and anti-IL-6R (c) at 24 hours post-treatment. d) Number of spikes counted cumulatively over the 72-hour period was significantly elevated at 48 and 72 hours post-treatment with IL-6, compared to control and anti-IL-6R treated neurons. e) 48 hours after treatment with human IL-6, fold change in spontaneous spike frequency was significantly elevated compared to control and cells also treated with anti-IL-6R. 2-way ANOVA with Bonferroni post-hoc tests was used. f) Fold change in evoked spike frequency was also increased at 48 hours compared to control and anti-IL-6R treated neurons and was also elevated in anti-IL-6R treated neurons compared to control. N = 12 wells for IL-6 and IL-6 + IL-6R treated wells as well as control. 1-way ANOVA was used. ****, ** and * represent $p < 0.0001$, $p < 0.01$ and $p < 0.05$ respectively. Data are shown as mean $\pm$ SEM.

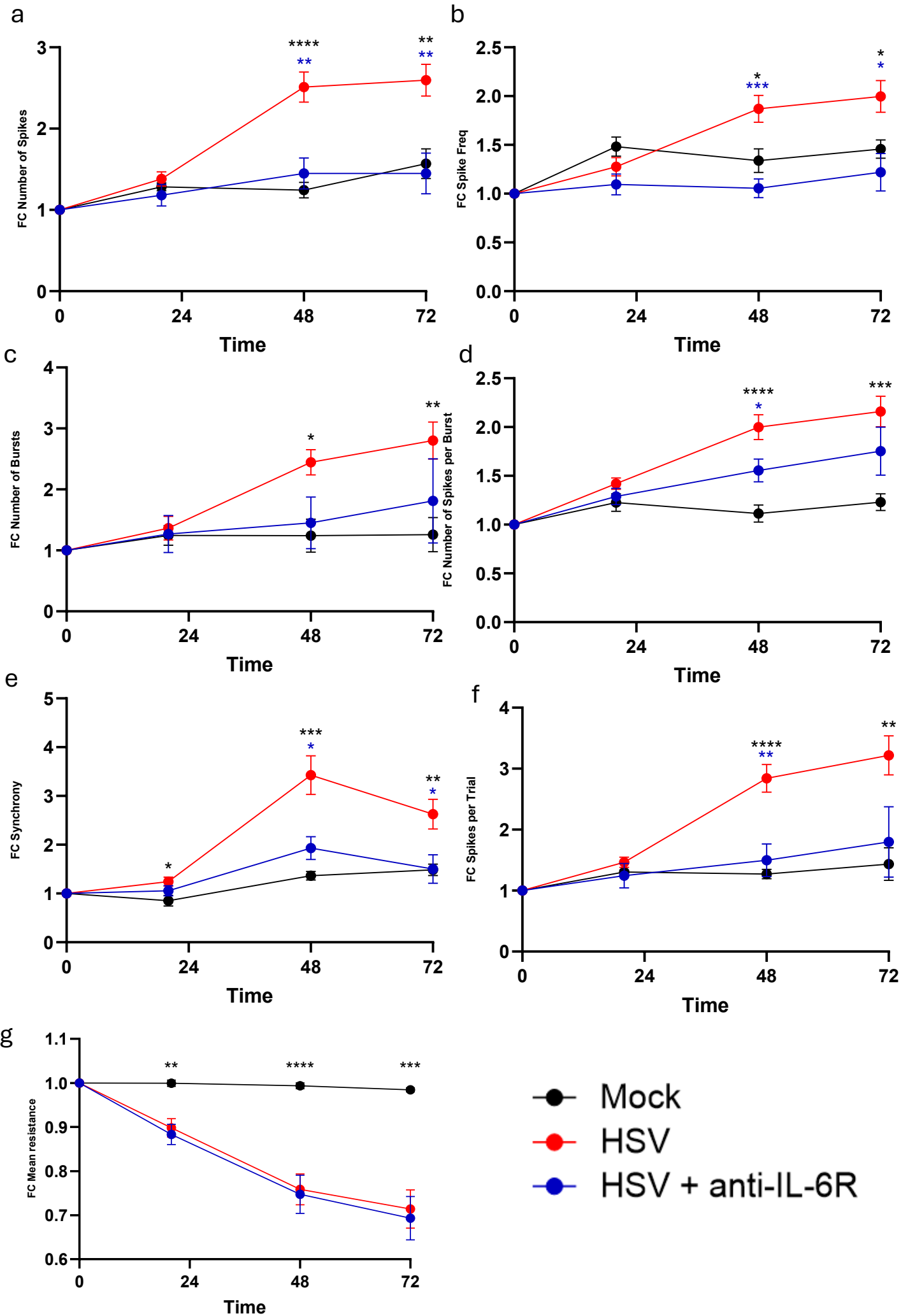


Figure 8. Anti-IL-6R pre-treatment prevents hyperexcitability and hypersynchrony caused by HSV-1 infection. hiPSC-CNs were either left untreated or pre-treated with 20 $\mu\text{g/mL}$ anti-IL-6R and then infected with 2.5×10^3 PFU HSV-1 per well or with equivalent dilution of Vero supernatant (mock-infection), and evoked response to stimulation assessed at baseline and then every 24 hours after infection. a) Fold change in the number of evoked spikes as well as spike frequency (b) was elevated at 48 and 72 hours post-infection in HSV-1 infected neurons compared to mock-infected and infected neurons pre-treated with anti-IL-6R. c) Fold change in the number of bursts was also increased at 48 and 72 hours for infected neurons compared to mock-infected, but this was not significantly different to anti-IL-6R treated neurons. d) FC in spikes per burst was elevated in HSV infected compared to HSV + anti-IL-6R and mock but only compared to mock at 72 hours. e-f) FC in spikes per trial (e) and synchrony (f) was elevated in infected neurons at 48 and 72 hours compared to mock and IL-6R treated neurons. g) Fold change in viability was significantly decreased in both HSV-1 infected and HSV-1 + anti-IL-6-R neurons compared to mock infected from 24 hours post-infection. There was no significant difference in fold change of viability between HSV-1 and HSV-1 + anti-IL-6R. $N = 12$ wells for HSV and HSV + IL-6R wells and $N = 6$ for mock-infected. 2-Way ANOVA with Tukey's post-hoc correction was used. ****, ***, ** and * represent $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively. Data are shown as mean $\pm$ SEM.

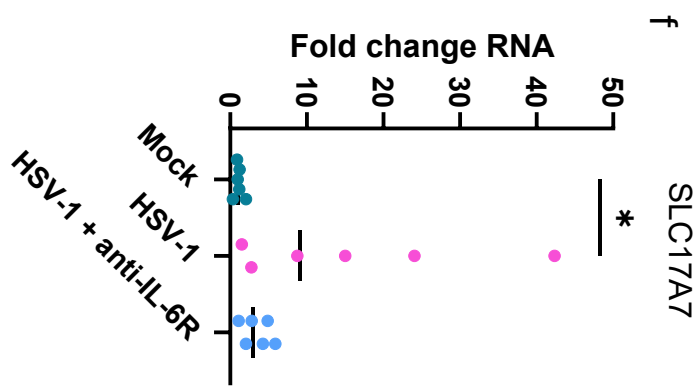
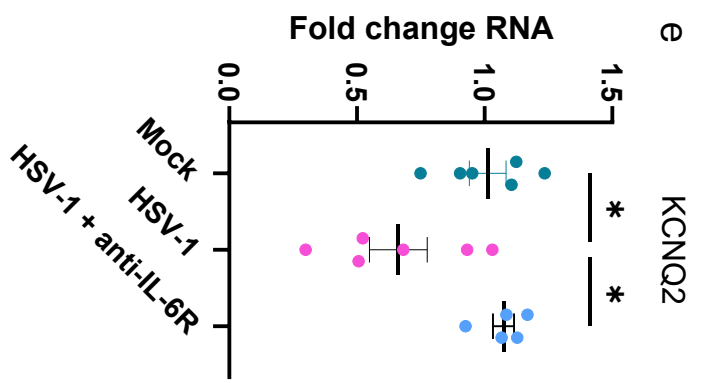
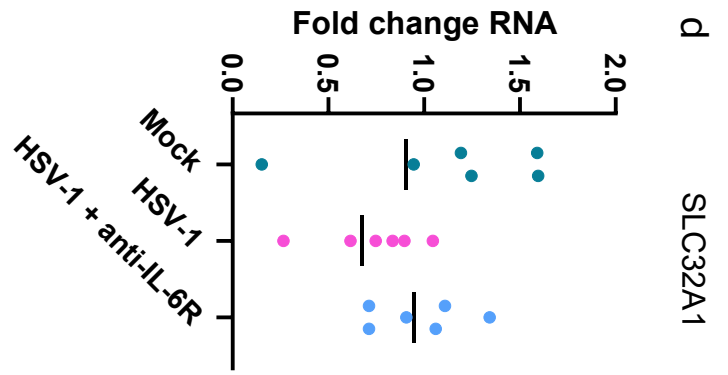
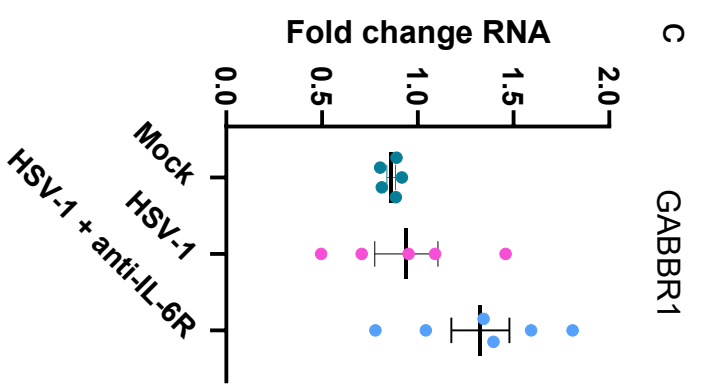
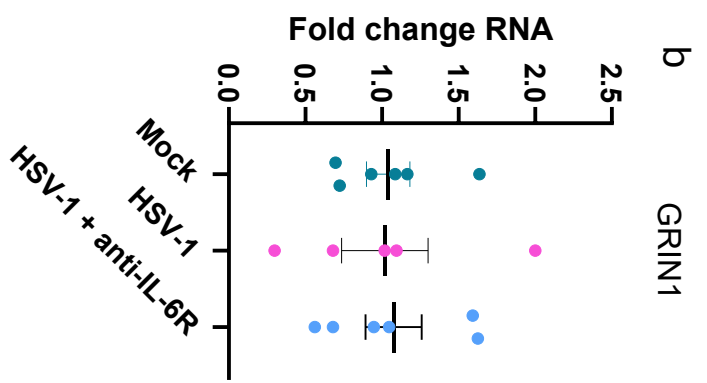
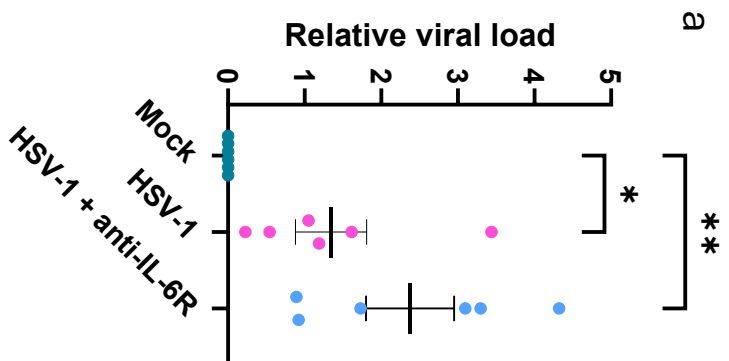


Figure 9. HSV-1 infection in hiPSC-CNs is associated with downregulation of KCNQ2 which is prevented by anti-IL-6R treatment. hiPSC-CNs were either left untreated or pre-treated with 20 $\mu\text{g/mL}$ anti-IL-6R and then infected with 2.5×10^3 PFU HSV-1 per well or with equivalent dilution of Vero supernatant (mock-infection), and lysate for RNA extraction was taken at 24 hours post-infection and RT-qPCR performed for viral mRNA and relevant neuronal markers. $\Delta\Delta\text{C}_q$ method was used to calculate fold change. a) HSV-1 polymerase mRNA was not detected in mock-infected neurons and was detected in HSV-1 infected neurons. Mock-infected = 0 for the purposes of visualisation. Anti-IL-6R treated neurons did not have a significantly different relative viral load to untreated infected neurons. b-d) Fold change in expression for Grin1 (NMDA subunit) (b), GABBR1 (GABA-BR subunit) (c) and SLC32A1 (VGAT) was not significantly different between conditions. d) KCNQ2 (Kv7.2 subunit) expression was downregulated in HSV-1 infected neurons, and this downregulation was not observed in HSV-1 infected neurons that were treated with anti-IL-6R. e) SLC17A7 was upregulated in HSV-1 infected neurons compared to mock-infected, but this was not significantly different to infected neurons treated with anti-IL-6R ($p = 0.0781$). $N = 6$ wells for all conditions. Data are shown as the mean $\pm$ SEM. One-way ANOVA with Tukey's post-hoc tests was used. ** and * represent $p < 0.01$ and $p < 0.05$ respectively.

Supplementary Files

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- [HSVSeizureOctober2026SupplementaryInfo.pdf](#)
- [SupplementaryVideoS2.mp4](#)
- [SupplementaryVideoS1.mp4](#)