Evaluating the Influence of HCMV Infection on Alzheimer's Disease Pathology

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BACKGROUND

Alzheimer's Disease (AD) is a common, debilitating form of dementia typically characterized by a progressive decline in neuronal function that ultimately results in memory loss, unpredictable behavior, and death. Decades of research have uncovered associations between AD and a host of factors (ex. synaptic deterioration, amyloid plaque/neurofibrillary tangle accumulation, functional defects, etc.), though little definitive information exists regarding the disease's underlying mechanisms and potentiating factors. However, a developing body of literature describes the potential effects that viral infection has on AD progression.

Several members of the viral family Herpesviridae demonstrate the potential to induce altered cellular phenotypes relevant to AD pathology. Among these herpesviruses is Human Cytomegalovirus (HHV-5; HCMV), a common pathogen found in 40-70% of the US adult population. Previous studies demonstrate overlap between AD and infection via HCMV-mediated increases in amyloid beta (Aß, in fibroblasts) and associations with increased rates of neurofibrillary tangles (NFTs, phospho-Tau, pTau) in post-mortem brain tissue.[1] Aß and pTau accumulation is known to drive the synaptic dysfunction prevalent AD. Further, preliminary differential RNAseq data comparing HCMV-infected and mock-treated AD organoids expands upon HCMV's potential to dysregulate synapses, with infection-dependent downregulation of key synaptic transcripts. Together, these data highlight HCMV's ability to structurally dysregulate the synaptic compartment and worsen AD pathology. Interestingly, HCMV-infected cerebral organoids demonstrate decreased calcium signaling (baseline calcium; response to KCl) and neurotransmission when compared to mock conditions.[2,3] This contradicts the effects commonly ascribed to AD neurons: increased calcium signaling and hyperexcitability. Considering the existing data, the relationship between AD and HCMV is likely complex, with different aspects of AD pathology being altered independently.

Here, we capitalize on the use of patient-derived human induced pluripotent stem cells (iPSCs) to generate 2D forebrain neuron cultures that model the sporadic and familial forms of AD. Then, using the TB40/E-eGFP clinical variant of HCMV, we will assess HCMV's effects on two aspects of AD pathology: Aß/pTau protein accumulation and synaptic function.

METHODS

Forebrain Neuron Differentiation: Control and AD iPSC lines were used in conjunction with the STEMdiff™ Forebrain Neuron Differentiation/Maturation kits (STEMCELL Technologies; #08600, #08605) to generate neuronal cultures representative of the human forebrain region.

HCMV Infection: Forebrain neurons were infected with one of two sub-variants of the HCMV clinical strain TB40/E (TB40/E-eGFP or TB40/E-eGFP+mCherry) at a multiplicity of infection (MOI) of 3. Infection was propagated for different lengths, dependent on assay.

Live Cell Imaging: At D77 of differentiation (D53 post-neuronal platedown), cells were infected with HCMV (TB40/E-eGFP+mCherry) or mock-treated. Subsequently, infection progress was observed over the next 7 days using an Incucyte imaging system (Sartorius) to observe infection progression.

Viral Titering: Conditioned media from HCMV-infected neuronal cultures (D98 of differentiation, 14 DPI) was collected and applied to ARPE-19 epithelial cells. Media was allowed to remain on the epithelial cells for 7 days. After this period, ARPE-19 cells were fixed with methanol, probed for HCMV Immediate Early gene 1, and counted to determine titers.

Viral Infectivity: Using conditioned media from viral titering experiments, copies of the viral immediate early gene UL123 were determined via qPCR (vDNA isolated via phenol-chloroform method with linear acrylamide) relative to a known UL123 standard. This value derived from this process was then compared to the number of infectious units obtained from viral titering experiments. The resulting ratio denotes viral infectivity.

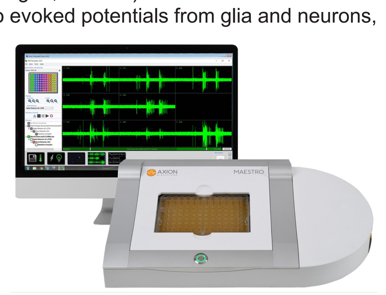
Fixation and Immunofluorescence: Neuronal coverslips were fixed in 4% PFA for 15 minutes. Subsequently, coverslips were washed 2x with Dulbecco's PBS prior to storage at 4°C (in dPBS). Antibodies used: GABA (Enzo, GA1159), VGLUT2 (Synaptic Systems, 135403), Vimentin (Abcam, ab24525), and Ki67 (Vector, VP-K451). Imaging was conducted using a Zeiss LSM980 confocal microscope.

Aß Ratio Analysis: Neuronal cultures were either infected (TB40/E-eGFP) or mock-treated at D54, D84, and D114 of differentiation. Infection was allowed to persist for 14 days in all cases, and conditioned media (CM) was collected at D68, D98, and D128, respectively. CM was then used to assess concentrations of secreted amyloid products. $Aß_{1-40}$ and $Aß_{1-42}$ values were determined via species-specific ELISA assays (Invitrogen; #KHB3481, #KHB3544). Data is presented as standard amyloid ratio (42/40).

Soluble/Insoluble Western Blots: Forebrain neurons were infected or mock-treated at D54 of differentiation. After allowing the infection to persist for 14 days, cells were collected and pelleted. Pellets were lysed and underwent ultracentrifugation steps to separate soluble and insoluble proteins, as described by Santarriaga et. al.[4] Subsequently, soluble/insoluble proteins were resolved on a 12% polyacrylamide gel before being transferred to PVDF membrane. Membranes were probed using an antibody against $pTau_{Ser262}$ (Invitrogen, 44-750G) and data analysis was conducted in ImageJ.

Calcium Imaging: After HCMV (TB40/E-eGFP) exposure or mock-treatment at D77, infection in neuronal coverslips was allowed to persist for 7 days (D84 of differentiation). At 7 DPI, coverslips were washed with extracellular normal HEPES (ENH) and bathed in FURA 2-AM ratiometric calcium dye (Invitrogen, #F1221) for 45 minutes. Before imaging, cells were again washed with ENH to remove excess FURA 2-AM. Cells were stimulated using 10µM ATP and 50µM KCl to evoked potentials from glia and neurons, respectively. Appropriate washout times were implemented between stimulant administrations. Presented data is limited to KCl-sensitive cells (neurons).

Microelectrode Array (MEA) Assay: After initial progenitor differentiation, neuron cultures were plated onto 48-well Cytoview MEA plates (Axion BioSystems, M768-tMEA-48W) using poly-L-lysine and laminin as substrates. Care was taken to ensure cell plating was above electrode cluster. Neurons were maintained for 50 days of differentiation before infection with HCMV (TB40/E-eGFP) or mock-treatment. Throughout culturing, recordings were taken 3x weekly using a Maestro MEA system (Axion BioSystems). MEA recording paradigm consisted of 6 minutes of spontaneous recordings, followed by 2 minutes of evoked (0.5V stimulation, 10s intervals). Neurons were maintained for 50+ DPI.



Data Analysis: Statistical comparisons drawn from one- and two-way ANOVAs, as appropriate. All statistics utilize a significance value of p<0.05.

REFERENCES/ACKNOWLEDGEMENTS:

References:

[1] Lurain et al., J Infect Dis. 2013
[2] Sison et al., J Virol. 2019
[3] Sun et al., Cell Rep. 2020
[4] Santarriaga et. al., 2022

● This work is funded by NIH/NIAID R01 AI132414 (SST, ADE)
● Thank you to Dr. Eain Murphy for the fluorescent viruses.
● MCW Neuroscience Doctoral Program

RESULTS

Forebrain Neuron Cultures Permit HCMV (TB40/E) Infection

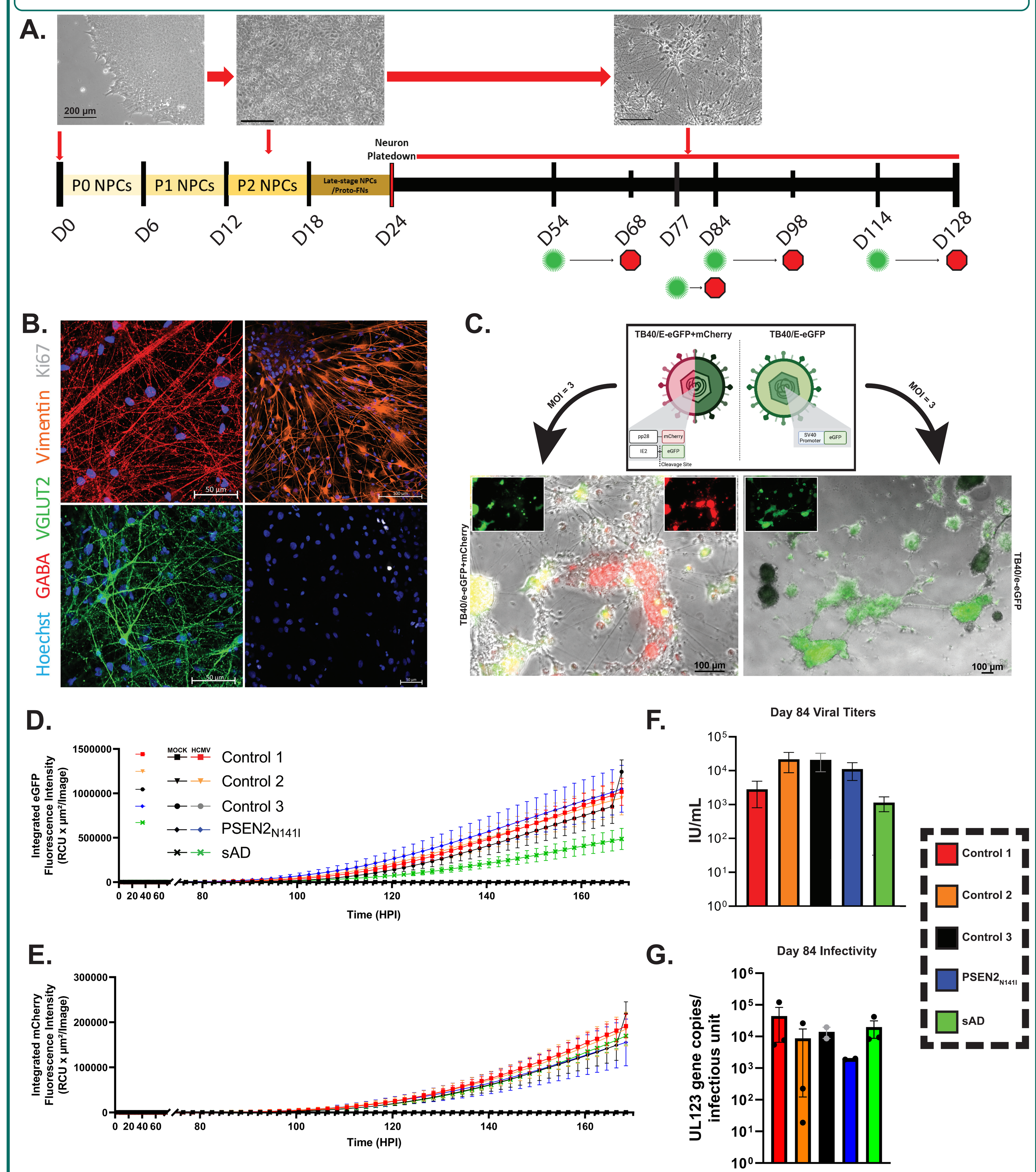


Figure 1: Model System Characterization and HCMV Permissivity. (A) Timeline for generating electrophysiologically-mature forebrain neurons from patient-derived iPSCs with included infection schedule and experimental endpoints. **(B)** Immunofluorescence demonstrates culture system is composed primarily of excitatory (VGLUT2+) and inhibitory (GABA+) neurons, with small populations of astrocytes (Vimentin+) and neural progenitor cells (Ki67+). **(C)** Imaging details HCMV infection of neuronal cultures at 15 DPI, using two clinical subtypes: TB40/E-eGFP and TB40/E-eGFP+mCherry. **(D,E)** Timecourse fluorescence of forebrain cultures infected with TB40/E-eGFP+mCherry characterizing immediate-early **(D)** and late **(E)** stages of infection. Viral titering data **(F)** and infectivity data **(G)** highlight infected neurons' ability to produce competent virus and relative functional to non-functional virus production, respectively.

HCMV Alters $Aß_{1-42}$ and pTau Amounts in Forebrain Neurons

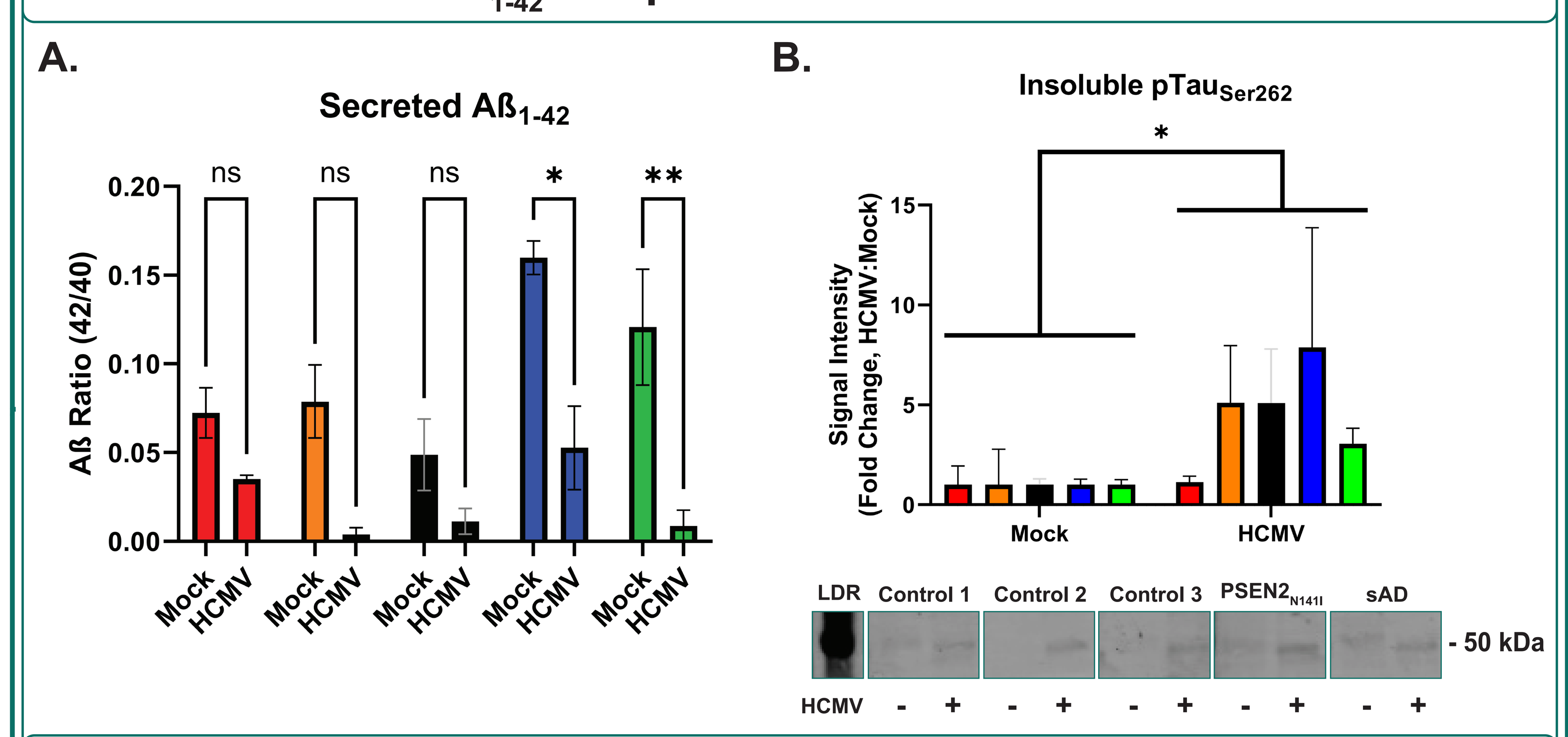


Figure 2: HCMV infection alters quantities of AD-associated peptides Aß and pTau. (A) Secreted amyloid beta species were quantified using neuron conditioned media and ELISAs (D98 of differentiation, 14 DPI). While not significant, HCMV appears to drive down Aß ratios in control cell lines. In neurons derived from AD patient iPSCs, secreted Aß ratios are significantly decreased. (n=3-4) **(B)** Collectively, HCMV demonstrates the ability to increase the insoluble phospho-Tau burden (observed via $pTau_{Ser262}$) within forebrain neuron cultures. (n=3) *p<0.05; **p<0.01.

Functional Implications of HCMV Infection in Forebrain Neurons

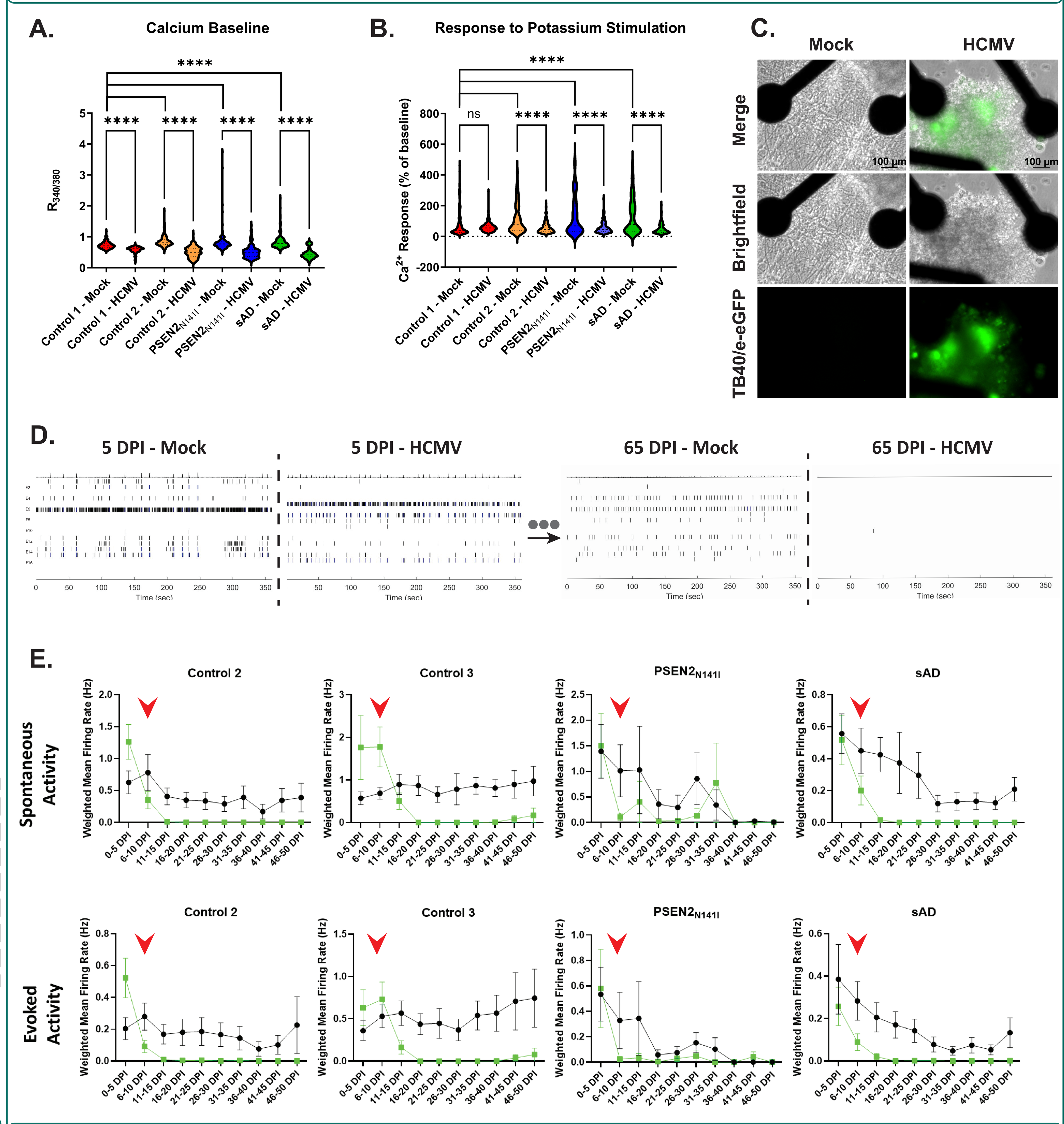


Figure 3: HCMV infection affects neuronal capacity for calcium signaling and action potential generation. (A) In calcium imaging, HCMV infected, KCl-sensitive neurons demonstrated lower basal calcium levels. **(B)** Infection also dampened calcium influx in response to KCl response. **(C)** Representatve images showing HCMV-infected and mock-treated forebrain neurons plated over the electrodes of an MEA plate. **(D)** Example raster plots demonstrating the amount of spontaneous action potential generation in cultures +/- HCMV at 5 DPI and 65 DPI. **(E)** Timecourse data highlighting the detrimental effects of HCMV infection on neuronal firing, regardless of AD background, from 0 to 50 DPI. ****p<0.0001

SUMMARY and CONCLUSIONS

- **iPSC-derived forebrain neurons are permissive to infection by HCMV clinical strain TB40/E.**
- **HCMV-infected neurons demonstrate *decreased* secreted $Aß_{1-42}$ relative to mock-treated controls and *increased* levels of $pTau_{Ser262}$ overall.**
- **Infection alters neuronal function via (1) decreasing calcium baseline and KCl-stimulated influx and (2) diminishing action potential generation by 15 DPI.**

Collectively, these data highlight mechanisms whereby active HCMV infection can potentially alter aspects of Alzheimer's Disease pathogenesis.