## A. Project Title: Cerebral neuronal dysfunction associated with human prion diseases.

## B. Principal Investigator (PI) name, direct collaborators and full affiliation (10 lines max)

**PI**: Simote T. Foliaki; **Direct collaborators:** Cathryn Haigh, Bradley Groveman

The PI and collaborators are from the National Institute of Allergy and Infectious Diseases, NIH.

## C. Specific Aims

Using a combination of human cerebral organoid cultures generated from induced pluripotent stem cells and electrophysiology, we aim to;
(1) investigate the influence of genetic mutations that pre-dispose individuals to prion disease on neuronal electrical signaling and health;
(2) identify the changes in neuronal electrical signaling that are induced by exposure to human prions;
(3) determine whether proposed anti-prion compounds can reverse or prevent prion-induced changes in neuronal signaling and identify new pathway targets for therapeutics.

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## D. Results to date

***Aim 1: To investigate the influence of genetic mutations that pre-dispose individuals to prion disease on neuronal electrical signaling and health.***

Our lab has successfully developed an organoid model of a genetic form of Creutzfeldt Jakob disease (CJD) caused by a E200K mutation within the prion protein gene. Dermal fibroblasts from two donors with the E200K mutation (line #1 and line #2) were used to generate induced pluripotent stem cells (iPSCs) that were subsequently differentiated into cerebral organoids. The controls were cerebral organoids differentiated from iPSCs made from donors' fibroblasts without the E200K mutation or any other known mutations causing genetic neurodegeneration. We first tested if the E200K organoids displayed any of the pathology hallmarks of prion diseases such as the presence of insoluble protease-resistant prion protein species as well as prion species capable of seeding propagation of the misfolded forms *in vitro* (usually detected by RT QuIC assay). We found that up to 12 months old, these pathological features were still absent, suggesting that just the presence of the mutation is insufficient to cause prion disease in these organoids even over a long period of time in culture.

We next sought to determine if the presence of the mutation has other influences on organoid function or health. For this, we measured the ability of the E200K organoids to spontaneously generate neuronal electrical signaling (i.e the baseline neuronal activity without any stimulant/treatment) as compared with control organoids. We found that at the baseline level, the E200K organoids produced weaker

electrical signaling compared to the control organoids. This finding was demonstrated by the E200K organoids exhibiting significantly slower rate of neuronal spike (electrical signaling or action potential) relative to the control organoids (Figure 1 A). Consistently, the E200K organoids produced weaker neuronal communication compared with the controls. This was demonstrated by the E200K organoids exhibiting significantly lower rate of neuronal burst (Figure 1 B), a measure of neuronal firing. These results were evident at 3-4 and 6-7 months old, respectively (Figure 1).

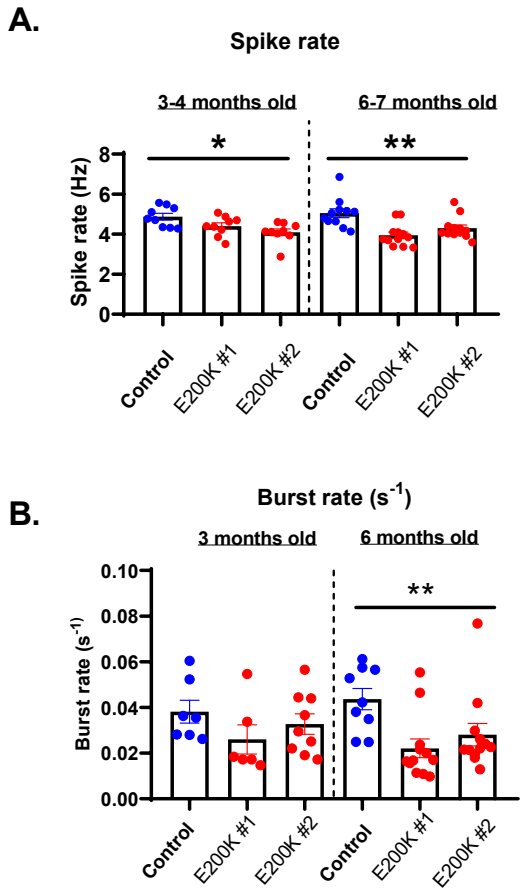


***Figure 1:*** *Baseline neuronal function (i.e without applying any stimulant) was significantly lower in the E200K organoids compared with the control organoids. (A & B) The spontaneous spike rate (A) and burst rate (B) of E200K organoids (two lines from two different donors) at 3-4 months old and 6-7 months old compared with the age matched control organoids.*

We next measured the ability of the E200K organoids to respond to increasing doses of glycine, a neuronal transmitter known to stimulate excitatory neurons (i.e excitable neurons that are responsible for the brain activity). We conducted this study in two age groups, 3-4 and 6-7 months old. The degree of the response to the treatment was measured as how much the spike rate and burst rate changed after the glycine treatment relative to the baseline. In the controls, low levels of glycine stimulated an increased spike rate as expected, regardless of the age of the organoids, and became significantly lowered by the higher doses of glycine (Figure 2 A & B). The E200K organoids did not consistently show

the same glycine dose response (Figure 2 C -F). We obtained analogous results with the rate of neuronal burst in 6-month-old organoids (Figure 2 G). Taken together, despite the poor neuronal functions of the E200K organoids at the baseline level, they appeared to have higher tolerance to neuronal inhibition upon exposure to higher doses of glycine. Hence, we hypothesized that E200K organoids might lack protective mechanisms that prevent neurons from becoming overexcited, a potential dysfunction that could directly lead to excitotoxicity and neuronal death.

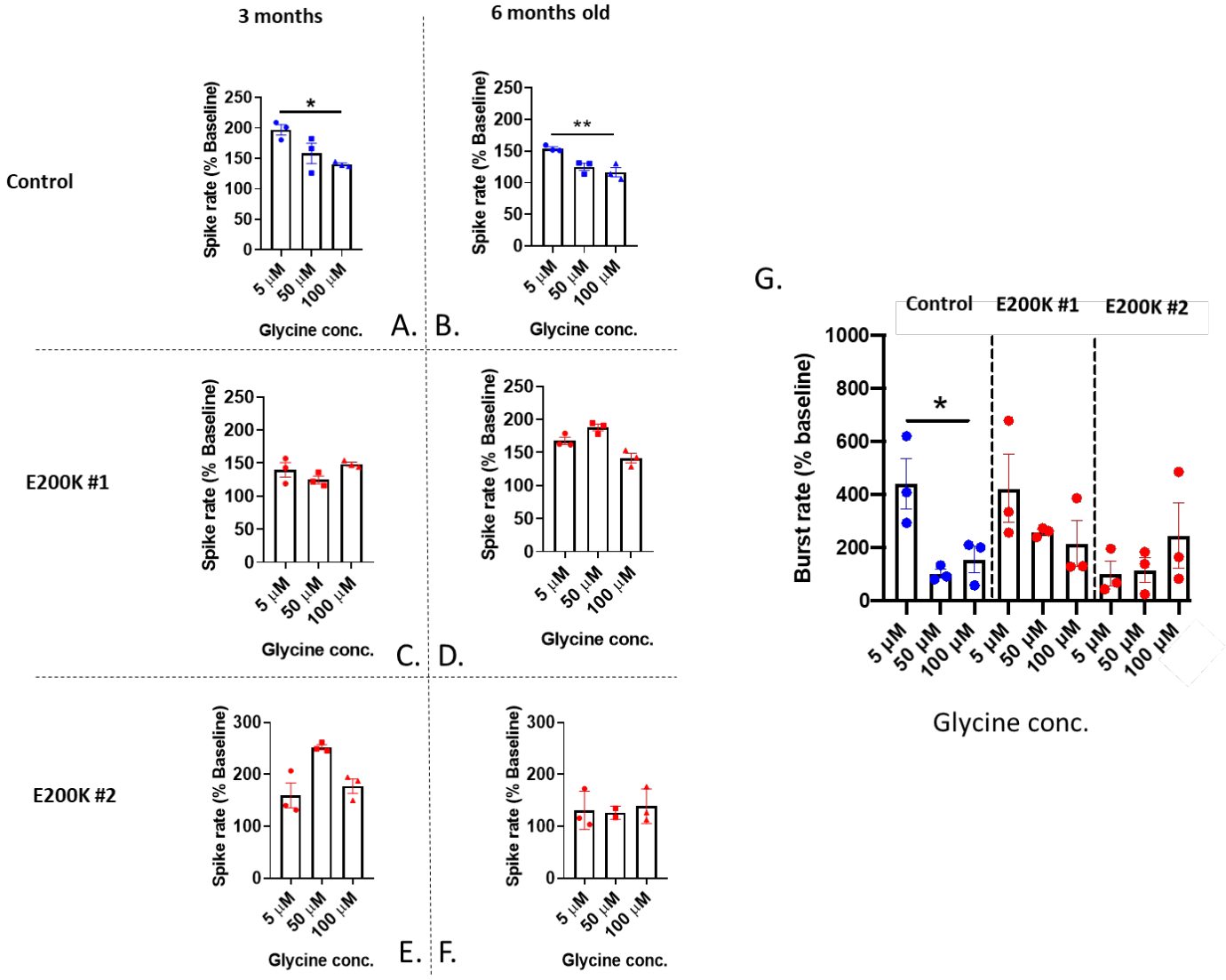


***Figure 2:*** *The ability of the cerebral organoids to cope with high excitability. The response (spike rate) of control organoids (A-B) and E200K organoids (line 1: C-D; line 2: E-F) at 3-4 months old (A, C, D) and 6-7 months old (B, D, F) to an increasing dose of glycine. G. The burst rate response of control and E200K organoids to increasing doses of glycine.*

Before further investigating if the E200K organoids lacked protective mechanisms against hyperexcitability, we first determined if the glycine treatment caused neuronal death. To do this, we

measured the level of active caspases in these organoids after treatment with the increasing doses of glycine. While a baseline level of caspase activation is required for some cellular functions unrelated to cell death, heightened activation of caspases is a physiological correlate of programmed cell death. We found that the high dose of glycine significantly reduced the levels of active caspases in control organoids (Figure 3). This dose-dependent effect was not significantly evident in the E200K organoids. The results did not indicate a consistent (or statistically significant) elevation in active caspases in the E200K organoid to suggest extensive neuronal death. Interestingly, the reduced neuronal function in the control organoids after exposure to high doses of glycine strongly correlated with the glycine dose-dependent decrease in active caspases, suggesting that the control organoids may have protective mechanisms that prevent overexcitability and neuronal death. These protective mechanisms are likely absent or deficient in the E200K organoids.

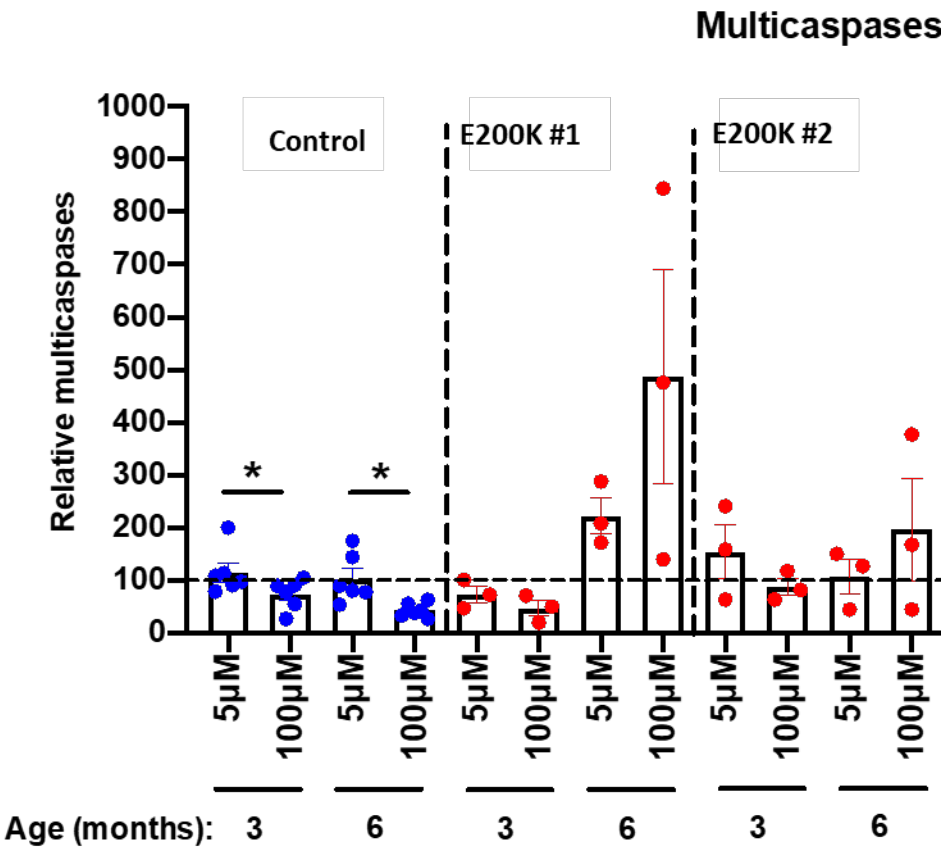


***Figure 3: Active caspases in control and E200K organoids (at 3-4 ad 6-7 months old) after treatment with increasing doses of glycine.***

A neuronal population can prevent excitability by expressing a specialized type of neurons called the inhibitory neurons. These neurons inhibit the excitatory neurons when they are hyperactive. We determined the levels of these inhibitory neurons by measuring the levels of RNA encoding a receptor called the GABA receptor, which is specifically expressed on these neurons. We found that the inhibitory neurons were significantly lower in the E200K organoids compared with the control organoids (by at least 2 folds).

Excitatory neurons themselves can reduce their own activity to prevent being hyperactive by removing a key receptor (ion channel) called the AMPA receptor from their surface. When AMPA receptors are attached to the surface of excitatory neurons in high levels, the neurons become highly active. Hence, removing AMPA receptors from the neuronal surface reduces the neuronal activity. This process is highly dynamic, thus making it a suitable mechanism to actively regulate neuronal excitability. We measured how much AMPA receptor was on the surface of the organoids following treatments with increasing doses of glycine. We found that unlike the control organoids (at 3-4 and 6-7 months old) where the surface AMPA receptor became depleted in response to the high dose of glycine, the E200K organoids exhibited variable levels of surface AMPA receptor (Figure 4). E200K organoids line #1 had very low levels of surface AMPA receptor at both 3-4 and 6-7 months old (Figure 4). The E200K organoid line #2 at 3-4 and 6-7 months old appeared to have reduced surface AMPA receptors after treatment with the high dose of glycine, similar to the control organoids, albeit not reaching statistical significance (Figure 4). Interestingly, despite harboring the same disease-causing mutation, the two lines (# 1 and #2) of E200K organoid exhibited different levels of surface AMPA receptor, thus supporting why the disease manifests differently between individuals.

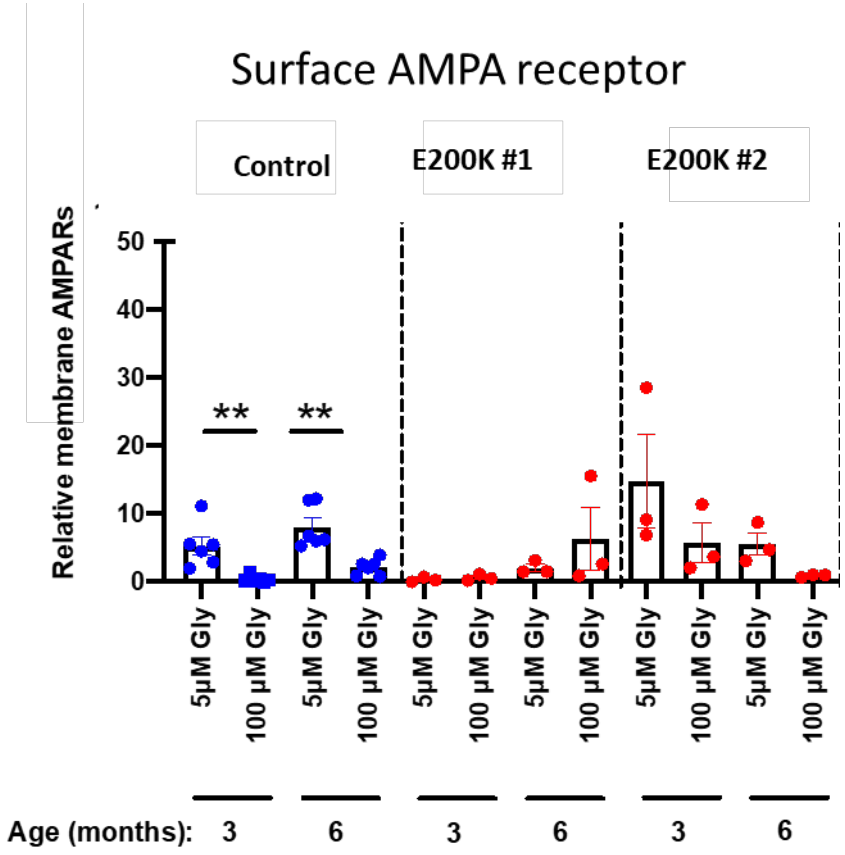


***Figure 4: Levels of surface AMPA receptor in control and E200K organoids (3-4 and 6-7 months old) after treatment with increasing doses of glycine.***

***Summary of Aim 1:***

E200K organoids exhibited weaker neuronal functions than control organoids at the baseline level. Under stimulated, enhanced excitability, control organoids appeared to protect themselves from neuronal death by reducing their neuronal function, whereas the E200K organoids lacked those protective mechanisms. Further investigations are being undertaken to determine the specific protective mechanisms lacking in E200K organoids.

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***Aim 2: To identify the changes in neuronal electrical signaling that are induced by exposure to human prions***

We previously reported that brain extracts from patients who died from sporadic CJD successfully infected healthy organoids (Groveman et al., 2019 Acta Neuropathologica communications). These organoids produced the pathogenic forms of prion protein. After a couple of months post inoculation, the organoids became positive for seeding activity as measured by RT-QuIC, and after ~170 days of the inoculation, substantial levels of insoluble protease-resistant prions were detectable

Here we infected organoids (made from cells with no background of genetic neurodegeneration) with prions from sporadic CJD brains, and from ~8 weeks post inoculation, we assessed their neuronal functions (spike rate and burst rate) every week until the week 22. We found that the neuronal activity of the control organoids progressively increased after the ~16$^{th}$ week, suggesting that they formed advanced neuronal communication as they got older (Figure 5). Relative to the uninfected organoids, the neuronal functions (spike rate and burst duration) of the infected organoids became significantly impaired after ~16 weeks post inoculation (Figure 5).

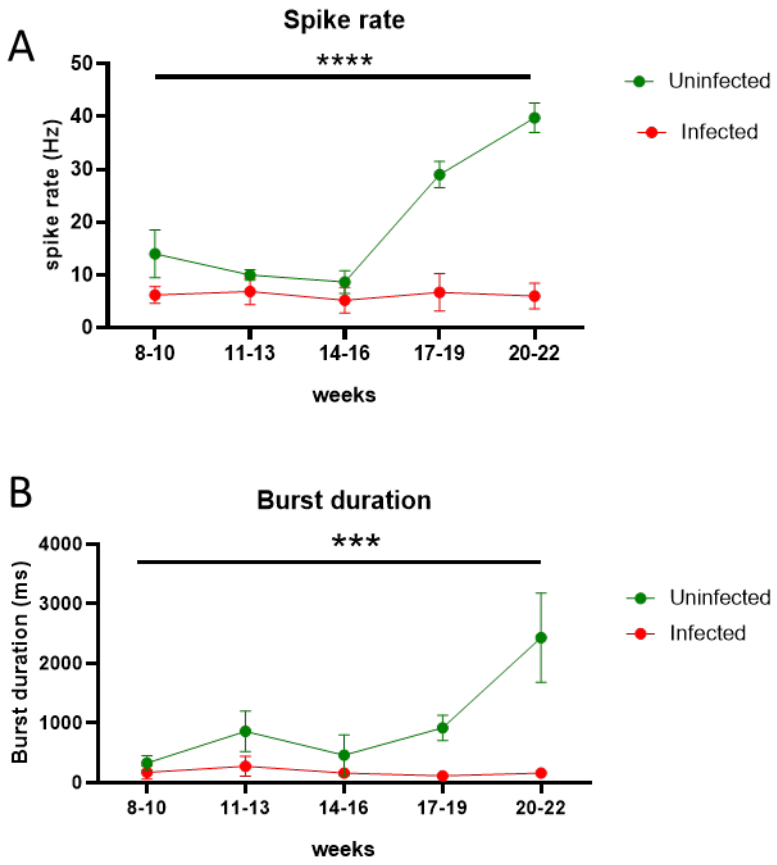


***Figure 5: Neuronal functions (measured as spike rate [A] and burst duration [B]) of cerebral organoids infected with prions from sporadic CJD brains.***

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***Aim 3: To determine whether proposed anti-prion compounds can reverse or prevent prion-induced changes in neuronal signaling and identify new pathway targets for therapeutics.***

One of the therapeutic drugs of prion diseases that has been shown to prolong disease duration in mouse is Pentosan polysulphate (PPS).

We infected organoids with prions from sporadic CJD as described in Aim 2, and after ~25 weeks of the infection, at which time point the neuronal functions became persistently impaired, we continuously treated the organoids for 6 weeks with a high dose of PPS that was previously determined non-toxic. Neuronal functions were monitored weekly. We found that the PPS treatment enhanced the spike rate of the uninfected organoids but not the infected organoids (Figure 6 A). Similarly, the PPS treatment did not rescue the impairment of the burst duration in the infected organoids (Figure 6 B).

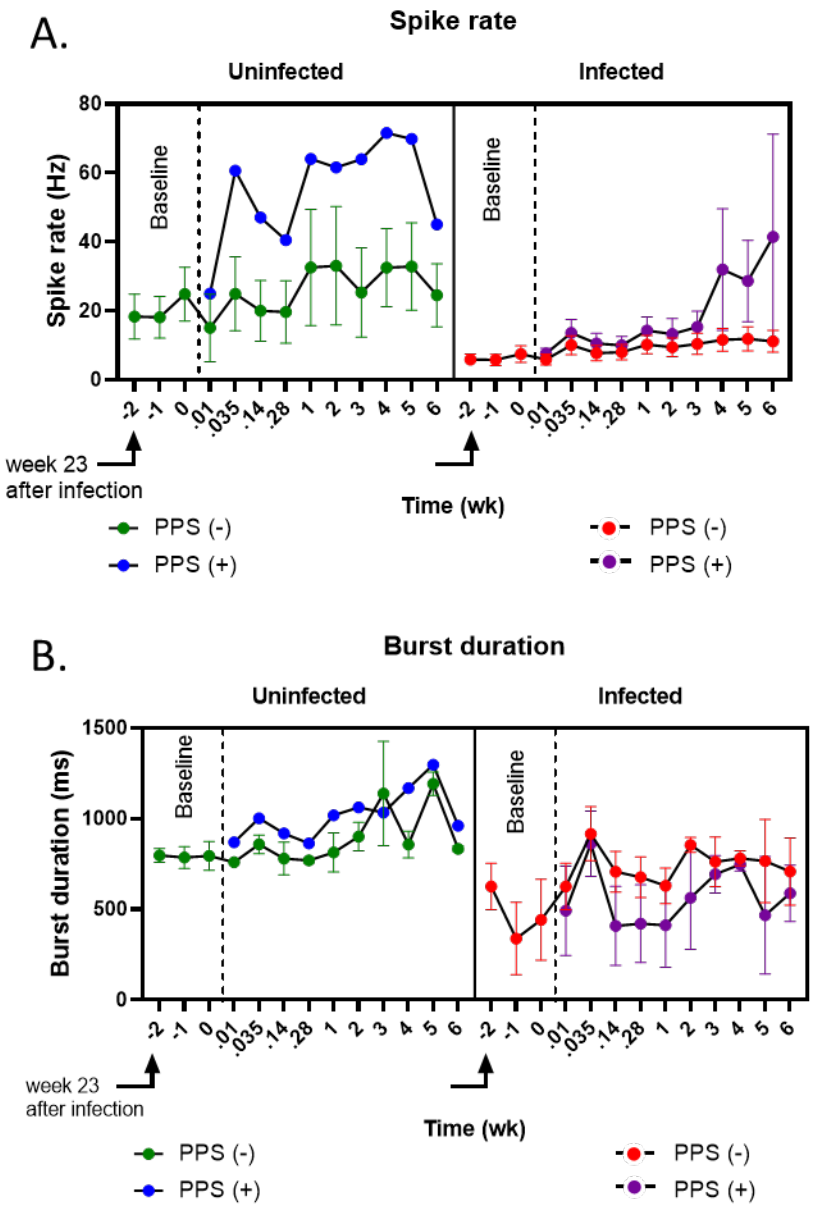


***Figure 6: PPS failed to rescue neuronal functions (measured by spike rate [A] and burst duration [B]) in organoids infected with prions from sporadic CJD.*** *The recording started at 23 weeks post infection with a 3-week baseline recording prior to the PPS treatment.*

**Conclusion:**

We have successfully completed all experiments outlined in the grant proposal, and the funding gratefully received has produced interesting results and new avenues of investigation. We found that our cerebral organoid model is a great tool to study how neuronal functions, associated with cognition and behavior, become impaired in human prion diseases. Our model supports that some therapeutic drugs like PPS are ineffective in human, while effective in mouse. This finding supports that our model

best represents human prion disease. Importantly, this model opens up the possibility of therapeutic restoration of neuronal function, which may significantly reduce the severity of cognitive and behavioral deficits in human prion diseases.

Lastly, I am very thankful for the financial support and the all the words of motivation that I received while in DC for the Conference. Because of those, I have tried my best to address all the aims in the proposal although there are still more works to be completed.