Article

Differences between cerebral organoids derived from a non-PD and a Parkinson's patient: a potential vulnerability for neuroinvasive viruses.

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Simple Summary: Research has shown that that there are significant differences in neurotransmission and immunity between normal humans and those with Parkinson's disease. The number of people being diagnosed with Parkinson's disease is rapidly increasing. At the same time, climate change is causing an increase in mosquito-borne infections that can damage the brain. We wondered if the brains of Parkinson's patients could have differences in neurotransmission and immunity that relate to the antiviral response when compared with a normal brain. Human stem cells were used to generate miniature brains. We measured differences in neurotransmission and immunity. The data show that there are differences in gene expression that could contribute to a dysfunctional antiviral response in the brains of Parkinson's patients. The need for more investigation is highlighted.

Abstract: The development of 3D cerebral brain organoids which accurately resemble aspects of the human brain permits a more accurate characterization of physiological processes and neurological diseases. Cerebral organoids can be grown from stem cell lines with various genetic backgrounds allowing multiple neurodegenerative diseases to be modeled. While dysfunction in neurotransmission of patients with neurodegenerative diseases is expected, the impact of chronic neurodegeneration on the response to viral infection of the CNS is poorly understood. For instance, several mosquito-borne viruses like Dengue virus and West Nile Virus cause post-viral parkinsonism. How CNS infection might impact a host with inherent CNS dysfunction such as Parkinson's Disease in poorly understood. This preliminary, observational study aimed to understand dysfunction in intrinsic and innate expression of a patient with a neurodegenerative disease and a non-affected individual in relation to potential viral infection in the CNS. Cerebral organoids were generated from human induced pluripotent stem cells with a normal genetic background or with idiopathic Parkinson's Disease. After differentiation and maturation, organoid size, gene expression and immunofluorescence were evaluated to assess neurotransmission and innate immunity. While there was no significant difference in size of the organoids with a normal or Parkinson's genetic background, gene expression studies identified multiple differences in innate immunity and neurotransmission. Immunofluorescence also identified differences in protein expression related to neurotransmission and innate immunity. Of note, organoids derived from a Parkinson's patient exhibited endogenous up-regulation of dopamine and muscarinic acetylcholine receptors, GABA, glycine, and glutamate targets, and the majority of cytokines. This expression pattern suggests a chronic state of neuroexcitation and neuroinflammation in this population of organoids.

Keywords: organoid, induced pluripotent stem cell, Parkinson's disease, neurotransmission, neuroimmunity, neuroinflammation, viral infection

1. Introduction

The use of human induced pluripotent stem cells (hiPSC) in disease research is increasing, not only because relevant data can be generated, but because these cells can be differentiated systems that directly translate to a human model. The use of hiPSCs reduce the need for animal models, thereby reducing the costs to perform studies. Hundreds of hiPSC cell lines are available, originating from normal "disease free" patients or patients with various forms of disease. Research on different hiPSCs and organoids has shown that products derived from patients with a "disease" state exhibit different morphology and gene expression compared with products derived from a "normal" patient [1; 2; 3; 4]. This research has deepened the understanding of disease states and highlighted potential issues for diseased persons [4; 5; 6; 7].

Neuroinvasive infections and Parkinson's Disease (PD) diagnoses are both increasing and the impact of viral infection in a compromised CNS is relatively unexplored. Thus, in light of recent genetic characterization of PD neuronal cell types (reviewed by Tran et al. 2020), it was hypothesized that endogenous differences in markers related to viral pathogenesis would exist between normal and diseased genotypes [8]. Studies have shown that neuronal hyperexcitability is found in organoids derived from patients with Alzheimer's disease [9]. Unfortunately, most studies utilizing PD cerebral brain organoids evaluate morphology but not systemic differences in innate immunity and neurotransmission [10; 11]. Most organoid modeling of PD is based on midbrain organoids that recapitulate PD pathologies of the dopaminergic networks, neurite disfunction and abnormal localization of α-synuclein [11].

For this preliminary, observational study, cerebral organoids were utilized because they have cortical neurons that contain functional synapses that produce Ca+ surges with glutamate release which can be affected when virus is present [12]. Cerebral organoids were also chosen because they develop immunocompetent astrocytes that are key players in neuroinvasive disease response [13]. While mid-brain organoids are standard for PD research, they omit the cerebral cortex that is integral to viral pathogenesis in the human CNS [14]. Thus, the goal of this study was to define differences in cerebral organoids derived from an individual with PD versus an individual without PD so that these model systems may be used to inform future studies of viral CNS infections. Significant differences were observed in neurotransmission and immunity even though the organoids were physically indistinguishable. This preliminary, observational study, shows the differences in gene and protein expression of a normal and PD IPSC derived cerebral organoids and discusses how dysregulation could impact viral infection in the brain.

2. Methods

2.1. Cell Culture

Human Induced Pluripotent Stem Cells (hiPSC) (ATCC ACS-1019) and hiPSC with a genetic background of Parkinson's Diseases (ATCC ACS-1013) were cultured in mTeSR1 media (StemCell Technologies) on plates coated with vitronectin XF (Stemcell Technologies) prior to organoid formation. The genotype for ACS-1013 is unknown and for the purposes of this manuscript, it is assumed that the patient had sporadic PD.

2.2. Generation of Human Cerebral Organoids

Cerebral organoids were formed from hiPSC ACS-Normal and hiPSC ACS-Parkinson's using the StemDiff Cerebral Organoid Kit (StemCell Technologies Cat #08570) and StemDiff Cerebral Organoid Maturation Kit (Cat. #08571 StemCell Technologies) following the manufacturer's directions. This methodology has been used for exploring pathologies for Alzheimer's disease [9] brain development [15] and a host of other applications [16]. Briefly, hiPSC were harvested with Gentle Cell Dissociation Reagent (StemCell Technologies Cat #07174) and the seeded into ultra-low attachment 96 well plates (Corning #7007) at a density of 9000 cells/well. Cells were seeded in seeding media containing Y-27632. On days 2 and 4, 100ul of EB formation media was added to the wells. On day 5, EBs were observed to be rounded and tightly packed spheres about 200nm in size. EBs were embedded in Matrigel and incubated at 37°C for 1 hour. Embedded EBs were then placed in a 6 well, ultra-low attachment plate (StemCell Technologies Cat #3471) containing organoid expansion media. After three days, media was changed to maturation media. Media changes then occurred

twice per week and cerebral organoids were matured for 53 days before data collection to allow for full maturation and to best resemble an adult brain [17; 18].

2.3. Cerebral Organoid Size Measurements

Organoids were imaged using ImageQuant LAS 4000 with the bright field filter under high-resolution with automatic exposure. Organoid size was determined by using ImageJ (National Institutes of Health). The scale of the program was set to 13.9327 pixels/mm, and the area of each organoid was recorded. Results are expressed as an average between at least 12 organoids per treatment.

2.4. Cerebral Organoid mRNA Extraction and Gene Expression

mRNA was extracted using Zymo Quick-RNA Kit (Zymo Research #R1052), and cDNA was generated using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814). Gene expression studies were then conducted using TaqMan Array Human Neurotransmitters (Applied Biosystems #4414094), TaqMan Array Human Immune Response (Applied Biosystems #4414204), and TaqMan Array Human Alzheimer's Disease (Applied Biosystems #4414070) with Applied Biosystems TaqMan Gene Expression Master Mix (Applied Biosystems #4369016). The ΔCT was obtained for all targets using GUSB as the housekeeping gene.   Here CT values for GUSB was subtracted from the CT value of each target. This provided a baseline expression for each organoid type. The $\Delta\Delta CT$ method was used to calculate relative fold-difference in gene expression between PD and non-PD organoids. This allowed us to compare how much PD and non-PD organoids differed from each other for each target.
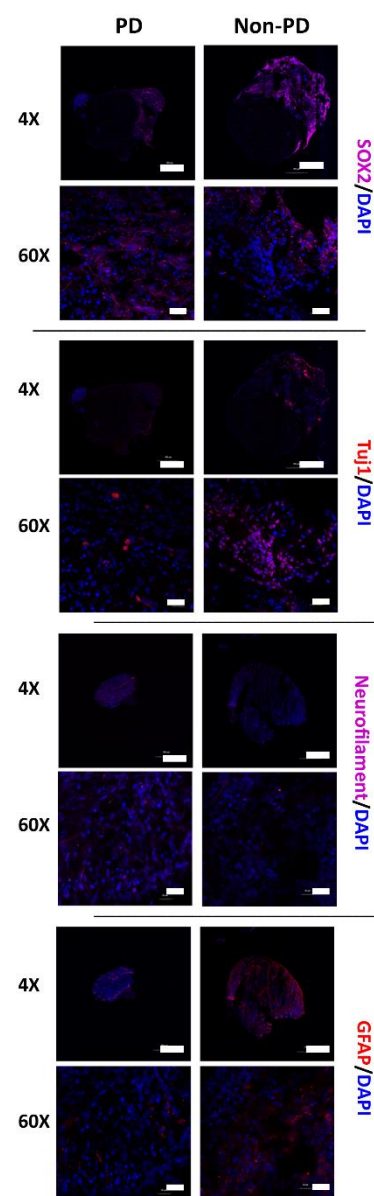
2.5. Immunofluorescence

Immunofluorescence was used to document morphology and validate gene expression. Organoids were fixed in 4% paraformaldehyde in PBS (ThermoScientific Cat# J19943-K2) overnight at 4°C then cryoprotected in 30% sucrose prior to sectioning. After freezing samples at -80°C, organoid sections of 18 micrometers thick were produced using a cryomicrotome (CryoStar NX50, Thermo Fisher Scientific, Waltham, MA, USA). Afterwards organoid sections were blocked in 5% fetal sheep serum and primary staining was conducted overnight at 4°C (Table 1). Secondary staining was then conducted using fluorescent antibodies (Table 1) for 1 hour at 25°C. Slides were mounted with ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology #8961S). Organoids were imaged using an Olympus Fluoview 3000 Confocal Laser Scanning Microscope (Olympus America Inc., Center Valley, PA, USA). All images were obtained using the same parameters including slices, gain, and offset. Individual tiles for all images can me found in the supplementary material.

3. Results

3.1. Size and morphology are similar between organoids from normal and diseased backgrounds

Morphological differences between the organoids were not visually apparent. The size of organoids derived from PD and Non-PD was calculated using ImageJ.   The average size of a PD organoid was 4.33 mm2 (±1.3 mm2) and the average size of a non-PD organoid was 5.1 mm2 (±2.0 mm2) Pairwise comparisons of the measurements using Student's t-test produced a p-value >0.05.

Immunofluorescence for morphology markers showed that both PD and normal organoids expressed SOX2, Tuj1, neurofilament, and GFAP. SOX2 is expressed in proliferating neural progenitors and Tuj1 is a neuron-specific β-Tubulin, as expected. Both of these markers had increased fluorescence on the outer margins of the organoids (Figure 1, S1, S2). Neurofilament is a component of mature neuronal cytoskeleton often found in high concentrations in axons. In growing or developing neurons, neurofilament may not be readily apparent since younger axons are much smaller than mature neurons [19].   GFAP or Glial fibrillary acidic protein is expressed by astrocytes and was found throughout both PD and normal organoids (Figure 1, S1, S2).

3.2. Parkinson's patients exhibit unique endogenous gene expression

ΔCt analysis showed that of a total 276 genes, 171 were down-regulated in PD organoids comparison with non-PD organoids (Figure 2), while 44 genes were up-regulated in PD organoids compared to non-PD organoids. The remaining 63 genes had no significant differences between PD and non-PD organoids (Figure 2).
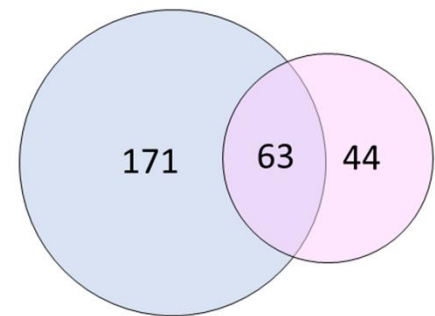
3.3. Neurotransmission is significantly different between the organoids
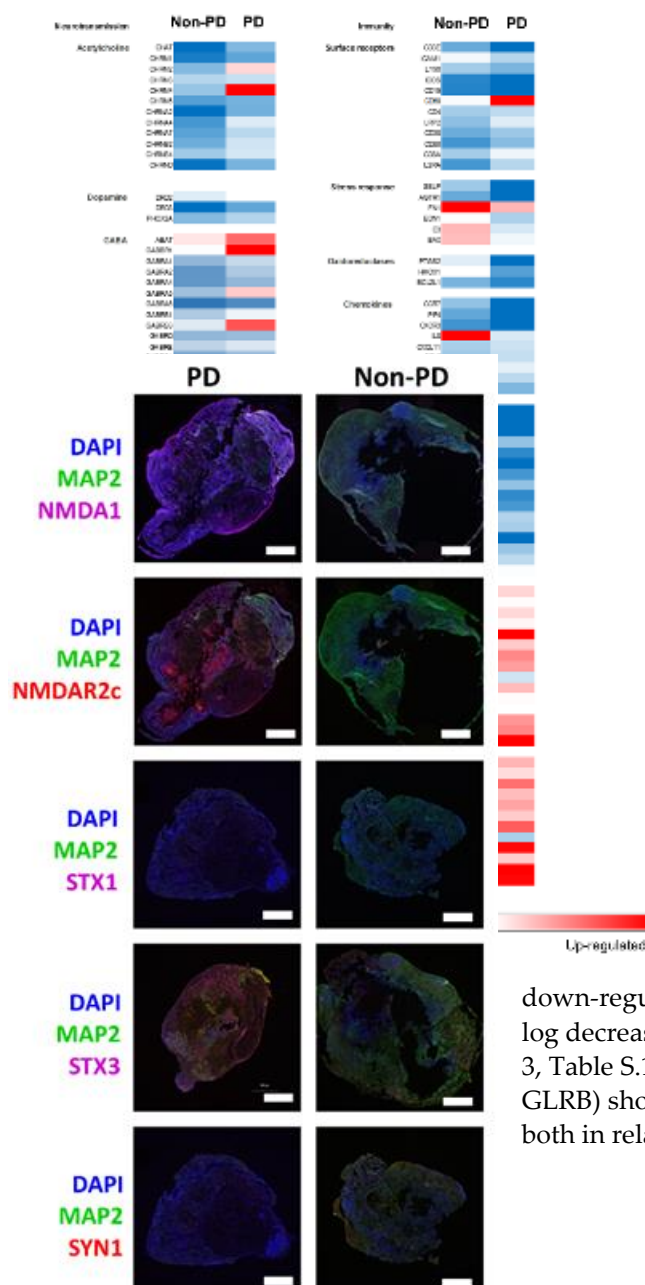
Expression of nicotinic and muscarinic receptors was down-regulated in both PD and non-PD derived organoids in relation to the housekeeping gene (Figure 3). Parkinson's organoids also exhibited decreased expression of nicotinic receptors, although the relative expression was significantly higher when compared with non-PD (Figure 3, Table S.1). ΔΔCt comparison, where ΔCt of PD organoids was subtracted from non-PD organoids, showed that relative expression of muscarinic receptors in PD organoids was up-regulated from 1.5-fold (CHRM3) to 22-fold (CHAT) (Table S.1). Expression of nicotinic receptors ranged from 0.69-fold (CHRNE) to 37.37-fold (CHRNA4) (Table S.1).

Expression of dopamine receptors (DRD2, DRD3, and PHOX2A) was down-regulated in both organoids in relation to their housekeeping genes (Figure 3). Here too, PD organoids exhibited increased expression of dopamine receptors in relation to non-PD organoids as determined by ΔΔCt comparison as described above (Figure 3, Table S.1).

Seventeen GABA receptors were evaluated for differential expression. GABRD and GABRB2 displayed the same magnitude of expression for PD and non-PD organoids (Figure 3, Table S.1). All other genes except GABRP were up-regulated ranging from 3.99-fold (GABRE) to 150.08-fold (GABRA5) (Figure 3, Table S.1). GABRP was down-regulated in PD organoids 0.002-fold translating to an 8.43 log decrease in expression compared to non-PD organoids (Figure 3, Table S.1). Four glycine receptors (GLRA1, GLRA2, GLRA3, and GLRB) showed significantly less expression in non-PD organoids, both in relation to housekeeping gene and in relation to PD or-

ganoids (Figure 3). Eight Glutamate receptors were evaluated and, as the trend suggests, PD organoids exhibited increased expression of all targets over non-PD when comparing their ΔCt values (Figure 3, Table S.1).

Six genes associated with serotonin neurotransmission were examined. Both PD and non-PD organoids displayed decreased expression of HTR1B, HTR2A, HTR3A, HTR3B, and HTR7 when compared with their housekeeping genes.   However, Tryptophan hydroxylase 1 (THP1) was up-regulated 10-fold in non-PD organoids in relation to PD organoids (Figure 3, Table S.1).

Thirty targets representing a spectrum of transporters involved in neurotransmission were examined. ΔCt values showed that non-PD organoids exhibited reduced expression of all transporter genes except COMT, which was up-regulated (Figure 3, Table S.1).   Synaptophilin (SNPH) and Syntaxins (STX) 1A, 1B, and 3 were significantly down-regulated in PD organoids compared with non-PD organoids by 4.87 to 11.38 logs as determined by ΔΔCt analysis (Figure 3, Table S.1).   Synapsins SYN1 and SYN3 were up-regulated 5.21 and 10.74-fold, respectively in PD organoids (Figure 3, Table S.1).   Synaptophysin (SYP) and Synaptotagmin (SYT1) were also up-regulated in PD organoids 16.32 and 4.05-fold, respectively (Figure 3, Table S.1).

Immunofluorescence for neurotransmission markers of glutamate receptors NMDA1 and NMDAR2c were used to validate gene expression patterns and reflected gene expression data where PD organoids had greater fluorescence of both markers compared to non-PD organoids (Figure 4, S2, S3). STX1 fluorescence mirrored gene expression data of decreased expression in PD organoids however STX3 appeared to have increased fluorescence on PD organoids at 4X magnification. Increasing magnification to 60X showed that that fluorescence covered much less area than in non-PD organoids (Figure S4). PD organoids also had increased fluorescence of SYN1 that was visible under 60X magnification (Figure S4).


3.4. Alterations in Immune Regulation

Eleven of 12 surface receptors important for antigen recognition, expression, or presentation exhibited decreased expression in PD organoids by ΔCt analysis. CD68, a scavenger receptor, was up-regulated in PD organoids 2.75-fold over non-PD organoids. Six genes involved in cellular stress response were significantly different between PD and non-PD organoids with SELP, AGTR1, FN1, EDN1, C3, and BAX were all expressed at higher levels in non-PD organoids. Three genes encoding oxidoreductases were significantly down-regulated in PD organoids as determined by ΔΔCt analysis (Figure 3, S2, S3, S4, Table S.1).

Seventeen cytokines exhibited differential expression between PD and non-PD organoids. Six cytokines were down-regulated in PD organoids including: CSF2, CSF3, CD80, SELE, FAS , and LIF (Figure 3, S3, S4, Table S.1).   Five chemokines exhibited differential expression between PD and non-PD organoids including: CCR7, PF4, CXCR3, CCL19 and CCL3 (Figure 3 S2, S3, S4, Table S.1). Six interleukins were down-regulated in PD organoids for both ΔCt and ΔΔCt analysis. However, IL-8 was up-regulated 5.48-fold in non-PD organoids (Figure 3, S3, S4, Table S.1).

Immunofluorescence for immunological markers showed that ICAM was not detected by fluorescence in PD organoids even at 60X magnification (Figure 5, S3, S4,). CCR7, CYP46A1 and SELE had increased expression in non-PD organoids reflecting gene expression data (Figure 5, S3, S4,).

3.5. Markers for Neurodegeneration were as expected
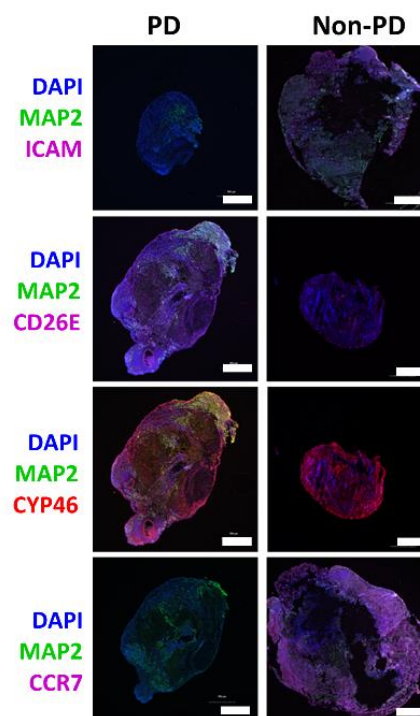
In contrast to the previous pattern of down-regulation of neurotransmitters and immune targets, PD organoids exhibited an overall pattern of up-regulation of genes associated with APP, Tau, and other neurodisease markers, as reported in other research [2; 20; 21] (Figure 3).

Twelve genes related to APP displayed differential expression both via ΔCt and ΔΔCt analysis. PSEN1 NAE1, PSENEN, NCSTN, BACE1 and BACE2 were up-regulated (Figure 3, Table S.1). In addition, APP, APH1A, APH1B, APLP1, APBA1, APBB1 and APBA2 were up-regulated in PD organoids (Figure 3, Table S.1). Up-regulation of Tau proteins were found for MAPK1, MAPK3, and MAPT with fold changes of 10.61, 8.88, and 349.57, respectively (Figure 3, Table S.1).

Thirteen genes associated with neurodegenerative disorders were differentially expressed in both PD and non-PD cells via ΔCt analysis. ΔΔCt analysis showed that APH1A, CDK5R1, HSD17B10, IDE, SERPINA3, SNCA, VSNL1, CSNK1D, GAP43, CYP46A1, and UBQLN1 were upregulated in PD organoids while CTSD and CAPNS2 were down-regulated in PD organoids compared to non-PD organoids (Figure 3, Table S.1).

4. Discussion

Here, the endogenous expression pattern of cerebral organoids derived from a PD and a non-PD patient is described. The goal of this study was to characterize these model systems so that they can be used for future studies on viral infections. Vector-borne viruses of humans can be acquired all over the globe and many cause neurological symptoms and sequelae. For instance, West Nile virus causes encephalitis as well as long term neurological deficits [22; 23; 24; 25]. Equine Encephalitis viruses cause neuro-cognitive sequalae over 5 years after infection [26; 27; 28]. Chikungunya virus causes persistent (and likely permanent) mood disorders including depression and anxiety [29; 30; 31]. Several arboviruses like Dengue virus and West Nile virus cause post-viral parkinsonism that are effectively treated with Parkinson's Disease (PD) therapeutics [28; 32; 33; 34; 35; 36].

While there are several animal models that recapitulate specific types of PD, there are none that replicate spontaneous or idiopathic PD [8]. Compounding that, there are no suitable models for studying viral encephalitis or neurological sequalae in rodents [37; 38; 39; 40; 41]. Most rodents must be genetically modified to exhibit symptoms of infection, and usually die as a result of infection in a few days. Large animal (sheep[37], horse[42] and pig[43]) and primate models are costly and labor prohibitive. This has hindered the development of vaccines and therapeutics for viral infections in the CNS.

The use of organoids for viral research is growing as a model for understanding viral pathogenesis. For any virus, a spectrum of disease ranging drastically from asymptomatic to death could present in infected individuals. Research has shown that this spectrum of response can reflect viral exposure history [44], age [45; 46] and environment [47; 48]. Recently, the role of chronic diseases on intrinsic and innate defense is emerging as a significant player in a patient's ability to deal with viral infections [49; 50; 51; 52]. For neuroinvasive viruses, cerebral organoids have been used to study viral pathogenesis [53; 54] and microcephaly caused by Zika virus infection [55]. Cerebral organoids recapitulate the epigenetic signatures of the human brain [56] and have cortical neurons that produce Ca+ surges with glutamate release via functional synapses [12]. When mature, cerebral organoids develop immunocompetent astrocytes that are key players in neuroinvasive disease response [13]. While mid-brain organoids are available, their use for delineating viral pathogenesis is limited.

The data support that cerebral organoids generated from PD patients exhibit significant dysregulation of neurotransmission and immune markers, which is expected for these patients in general, but this also highlights pathways that may negatively affect PD patients upon infection with neuroinvasive viruses. Multiple studies have provided evidence that PD patients have impaired neural function [57], oxidative stress response [58], and neuroinflammation [59] at basal levels of expression.

Data also reflects what has been reported in other research using human and animal models. For instance, PTSG2 has increased production in Alzheimer's patients as it contributes to microglial dysfunction, amyloid-β plaque deposition, and cognitive impairment [60]. The data reflects this with the overall increase in amyloid-β expression in organoids derived from a PD patient.

Overall, PD organoids exhibited increased expression of neurotransmitters in comparison to non-PD organoids. Studies have reported neurite alterations [58; 61] and impaired neurotransmitter regulation in sporadic and LRRK2 PD phenotypes at a basal level [58; 62; 63]. The increased expression of dopamine and muscarinic acetylcholine receptors reflects research on PD and suggests an intrinsic dysfunction of acetylcholine regulation [64; 65; 66]. Muscarinic acetylcholine receptors act specifically on dopaminergic neurotransmission such that increased expression of muscarinic acetylcholine receptors can cause either increases or decreases in dopamine transmission depending on which muscarinic acetylcholine receptor was activated. The data show that CHRM2 and CHRM4 were up-regulated over the non-PD organoids which has been shown to inhibit dopamine transmission in other research [67]. Additionally, CHRM1 expression is associated with long-term potentiation between synapses and cognition [65; 68]. Dysfunction of CRHM2 and CHRM3 are associated with movement disorders [65; 68]. CHRM4 has been shown to contribute to PD [69; 70; 71]. When it comes to viral infections, infection with Japanese encephalitis virus results in decreased expression of CHRM2 resulting in neurological sequalae of deficits in spatial memory and learning [72].

The increased expression of GABA, glycine, and glutamate targets suggests that PD organoids exist in a state of increased neuroexcitation compared to non-PD organoids. Over time, neuroexcitation leads to loss of motor control, neuronal burn-out and cell death which contributes to the pathology of neurodegeneration [73]. Dysfunction on these targets has been shown to contribute to loss of neuronal plasticity [74; 75; 76] and to have detrimental effects on learning, memory, and cognitions [77]. Increased dopaminergic activity is also associated with neurological-viral infections that produce neurocognitive and degenerative syndromes [78]. Current data suggests infection induces a state of neuroexcitation through up-regulation of glutamate neurotransmission [79; 80]. An individual with an intrinsic state of neuroexcitation could be vulnerable to exacerbated pathogenesis of over excitation of glutamate neurotransmission if infected with a mosquito-borne virus [79; 81]. It will be critical to understand how viral infection of the CNS affects PD patients since mosquito-borne viruses are endemic throughout the world.

BAX, BCL2 and BCL2L1 are regulators of intrinsic apoptosis.   Normally, BCL2 and BCL2L1 function to inhibit the action of BAX which is to release calcium and activate caspase and other apoptotic proteins. Reports have shown that viral infection in the CNS causes up-regulation of BAX leading to brain injury [82; 83; 84]. The decreased expression of these proteins in PD organoids could render the intrinsic apoptotic pathway more vulnerable to apoptotic triggers from viral insult and unnecessary cell death. This agrees with other studies that report significant impairment of cellular waste recycling in PD patients at a basal level [8].

The data show that PD organoids have reduced expression of NFKB2, a transcription control protein that functions in the innate antiviral response [85].   In PD patients, NFKB is activated along with IL-17 when co-cultured with T-lymphocytes[86]. Under normal circumstances, activation of NFKB2 results in the production of interferons which play a significant role in the innate antiviral response [85]. A down-regulation of proinflammatory cytokines in PD organoids when compared with non-PD organoids was observed.   IL-1, IL-12, TNF, CSF2, and CSF3 respond to infection by inducing inflammation and recruiting lymphocytes to the site of infection. Several viruses evade the innate immune response by blocking autophagy, thereby blocking monocyte differentiation and apoptosis [87; 88].

Cytokines function as messengers that direct the innate immune response and play an important role in regulating the adaptive response. The expression of specific cytokines can serve as biomarkers for certain viral infections [89]. Our The data generated in this study supports that PD organoids do not differ from non-PD organoids in their expression of IL-4, IL-5, IL-6, and IL-10, indicating that there is no activation of a Th2 response.   However, IL-12 was significantly up-regulated in PD organoids, which would favor a cell-mediated inflammatory response to stress or infection, as well as the activation of cytotoxic T lymphocytes. Significant down regulation of IL-18 and IL-1B in PD organoids was also observed which catalyze the production of several proinflammatory cytokines and recruitment of immune cells to the site of microbial infections. Research supports that IL-12 promotes protective immunity to a variety of viruses and that IL-12 and IL-18 work together during the antiviral response [90]. With expression of IL-18 and IL-1B down-regulated by 4-5 log-fold, PD organoids could be more vulnerable to infection than non-PD organoids. For instance, patients with endogenous down regulation of IL-1 and IL-10 can have exacerbated mental illness or psychotic episodes following infection with Chikungunya virus [29; 91; 92].

Chemokines are a subset of cytokines which specifically function in the innate immune response to tissue damage as well as foreign proteins and antigens. Chemokines also recruit and assist white blood cells in crossing the endothelial blood-vessel barrier into target tissues.   Overproduction of chemokines is associated with a variety of autoimmune diseases. The majority of chemokines examined in this study were expressed at greater levels in PD organoids than non-PD.   This suggests a chronic state of inflammation in this population of organoids, potentially causing complications for responding to viral infections. CCL19 is a chemokine that binds to the CCR7 receptor and acts to recruit dendritic cells.

CCL19 was up-regulated 11.72-fold in PD organoids, but CCR7 was down-regulated 400.47-fold. This expression profile suggests potential compensation by CCL19 for CCR7 and reflects expression profiles documented from cerebrospinal fluid from patients infected with Varicella-Zoster virus [93]. Also, studies in CCR7 deficient mice reported increased death from West Nile virus infection via over-recruitment of leukocytes and inflammation [94]. The reduced activity of CCR7 observed could make PD cells vulnerable to neuropathogens due to enhanced expression towards an inflammatory response.

CCL3 interacts with CCR4 and CCR5 during the acute inflammatory response and functions to recruit mast cells, and other monocytes which can have an impact on the neuroimmune response. Increased expression of CCL3 and CCL5 occurs during infection with respiratory pathogens and is associated with severe manifestations of disease [95]. Animal studies support that expression of CCR5 is up-regulated in CNS infections with Japanese Encephalitis Virus and positively correlated with increased pathogenesis [96]. The increased expression of these targets suggests that the PD organoids may exist in an inflammatory state, contributing to neuro-dysfunction over time.

The complement system is a part of the innate immune response that can lyse cells, activate inflammation, target virus to phagocytic cells, and clear non-cytopathic viruses from the circulatory system. Here, the expression of C3 was evaluated as it functions in both classical and alternative complement activation pathways. Deficiency of C3 can make humans more susceptible to viral and bacterial infections [97; 98]. In this study, PD organoids had a 22.04-fold reduction in C3 expression compared with non-PD organoids suggesting an innate impairment of the complement system. The down-regulation of C3 has been reported in patients with Hepatitis C infection [97]. Functional expression of C3 is necessary to neutralize West Nile and other flaviviruses which are causes of acute neurological infections and death [98; 99]. This poses an important question to be addressed in future research: could an endogenous reduction in C3 leave patients with PD disease primed for infection by a neuroinvasive virus?

Oxidoreductases communicate ER stress to the immune system via multiple readouts: they determine MHC class I surface exposure, they influence the activation of inflammation, but they also signal the intracellular stress status to the immune system when found on the plasma membrane [100]. In particular, HMOX1 has antiviral activity with increased levels associated with clearance of infection [101; 102; 103].   PTGS2 (COX2) is an inflammatory marker and in non-PD patients, increased COX2 production is associated with the antiviral response [104; 105]. Studies have shown that oxidative stress response is impaired during both basal and stressed conditions in PD patients [106]. Data generated show that PD organoids had decreased expression of HMOX1 and PTGS2 (COX2). When present, HMOX1 interacts with IL-10 (also down-regulated) as an anti-inflammatory mechanism of the innate immune response [60]. Research has shown that Zika and Dengue viruses decrease host expression of HMOX1 as part of their antiviral response [101; 107]. The endogenous deficit of COX2 and HMOX1 could render PD patients more vulnerable to viral infection.

For this study, normal and diseased stem cells were utilized from ATCC which are readily available and require no IRB approval. The PD type of the diseased cells is not described and may not reflect the response of other PD types based on genetic mutations. The use of only 2 cell lines (1 non-PD, 1 PD) is a limitation of this study. The genetic variation of PD is staggering and there are nearly 400 hiPSC cell lines derived from PD patients available for research [8]. A recently published report showed that neuronal cell studies focused on disease use 5 cell lines per study (3 diseased, 2 control) however, the use in brain organoids was not discussed [8]. PD research utilizing organoids typically differentiate from 1 health and 1 diseased hiPSC line [11]. In depth analysis of preliminary concepts requires substantial resources and time that is not justifiable for pilot studies, especially when generating organoids [8]. Thus, preliminary data is often limited to 2 cell

lines (control and diseased) [108; 109; 110]. Regardless, the findings here need substantiation in organoids derived from additional cell lines.

5. Conclusions

What is known regarding neuropathogenesis of viral infections in the human CNS is limited. The impact of neuroinvasive infection in a host with a neurodegenerative background has not been explored. Alternative models for the study of neurological diseases and infection are critical to fully understand mechanisms of neuropathology that can't be accurately recapitulated in animal models. The data from this study supports the use of organoid models for these studies, with evidence that the differential gene expression of cerebral organoids derived from a PD patient could contribute to a more severe disease in response to neurological assault from pathogens. There are significant discrepancies in the gene expression of neurotransmitters, immunity genes, and markers associated with neurodegenerative disorders. Many of these genes have been implicated in host antiviral response and viral countermeasures for evading host defense mechanisms. More work is needed to delineate the mechanisms of viral pathology in the human CNS and the impact of variability of endogenous gene expression in the neurodegenerative host.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

KLB and EMS conceived and designed the experiments. KLB, EMS, DDD, BZ, SX, and TJJ performed experiments and analysed data. KLB and EMS drafted the manuscript and all other authors edited and approved the text.

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Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1. Gene expression data of PD and non-PD organoids. Figure S1. 4X split channel magnification of morphological markers. Figure S2. 60X split channel magnification of morphological markers. Figure S3. 4X split channel magnification of neurological and immunological markers. Figure S4. 60X split channel magnification of neurological and immunological markers.

Data Availability Statement: All supporting data are provided in the manuscript.

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1.1 Figures

Figure 1. Immunofluorescence of selected targets to observe organoid morphology. Images of organoids were obtained at 4X and 60X magnification using an Olympus Fluoview3000 confocal microscope. Scale bar represents 500nm/20nm. Individual tiles can be found in the supplementary data.

Figure 2. Venn diagram showing the overlap of differentially expressed genes between non-PD and Parkinson's. Number of genes up- or down-regulated by at least 2-fold in relation to the housekeeping gene (GUSB) are presented in the Venn diagram. Data represents 12 pooled organoids from each cell line.

Figure 3. Differential expression of PD and non-PD derived organoids. Heatmap and density color code of the genes showing differential expression for PD and non-PD cerebral organoids. Genes are grouped according to function in the human brain. ΔCt for each cell line was calculated using GUSB housekeeping gene. Data represents ΔCt from 12 pooled organoids from each cell line.

Figure 4. Immunofluorescence of neurotransmission targets in PD and non-PD organoids at 4X magnification. Images were obtained to validate gene expression data. Images of organoids were obtained at 4X magnification using an Olympus Fluoview3000 confocal microscope. Scale bar represents 500nm. Individual tiles along with images obtained at 60X magnification of these targets can be found in the supplementary data.

Figure 5. Immunofluorescence of immunological targets in PD and non-PD organoids at 4X magnification. Images were obtained to validate gene expression data. Images of organoids were obtained at 4X magnification using an Olympus Fluoview3000 confocal microscope. Scale bar represents 500nm. Individual tiles along with images at 60X magnification of these targets can be found in the supplementary data.

1.2 Tables

Table 1. Antibodies used to visualize production of proteins associated with neurotransmission and innate immunity.

Antibody	Host	Type	Source	Dilution
Primary Antibodies				
Syntaxin 1A	Mouse	Monoclonal	Novus Biologicals	1:1000
Syntaxin 3	Rabbit	Polyclonal	Novus Biologicals	1:500
CCR7	Mouse	Monoclonal	Novus Biologicals	1:1000
NMDAR2C	Rabbit	Polyclonal	Novus Biologicals	1:1000
MAP2	Chicken	Polyclonal	Novus Biologicals	1:5000
NMDAR1	Mouse	Monoclonal	Novus Biologicals	1:1000
Synapsin 1	Rabbit	Polyclonal	Invitrogen	1:1000
SOX2	Mouse	Polyclonal	EMD Millipore	1:1000
Tuj1	Rabbit	Monoclonal	EMD Millipore	1:1000
Neurofilament	Mouse	Monoclonal	EMD Millipore	1:1000
GFAP	Rabbit	Polyclonal	Novus Biologicals	1:1000
CD62/SELE	Mouse	Monoclonal	R&D Systems	1:1000
CYP46A1	Rabbit	Polyclonal	Invitrogen	1:500
ICAM1	Mouse	Monoclonal	R&D Systems	1:1000
Secondary Antibodies				
Anti-Chicken	Goat	Alexaflour 488	Novus Biologicals	1:3000
Anti-Rabbit	Goat	Alexaflour 594	Novus Biologicals	1:1000
Anti-Mouse	Goat	Alexaflour 647	Novus Biologicals	1:2000

2. **Supplementary Material**

Table S1. Gene expression data of PD and non-PD organoids.

Figure S1. 4X split channel magnification of morphological markers. Scale bar represents 500nm.

Figure S2. 60X split channel magnification of morphological markers. Scale bar represents 20nm.

Figure S3. 4X split channel magnification of neurological and immunological markers. Scale bar represents 500nm.

Figure S4. 60X split channel magnification of neurological and immunological markers. Scale bar represents 20nm