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CHARACTERIZATION OF APOPTOSIS AND INHIBITORY NEURONS IN CORTICAL ORGANOIDS GENERATED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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ABSTRACT

Modeling human brain diseases in a laboratory setting is critical to understanding the underlying pathology and developing new treatments for patients. Organoids are 3D tissue cultures that provide a promising way to model human brain development *in vitro* and have been used previously to gain novel insights into diseases unique to humans. We are examining a method for generating cortical organoids with a single internal lumen from induced pluripotent stem cells. It is still unclear how well these organoids represent the many cell types present in the brain. To establish cortical organoids as a model for the developing human cortex, my study focused on the characterization of inhibitory neuron subtypes in cortical organoids and the origins of the ventricle-like lumen. The primary aims were to determine the role cell death and apoptosis played in creating the organoid structure, and to determine the nature of inhibitory neurons present within that structure. I analyzed confocal microscopy images of immunostainings to determine cell identities and distributions. First, in examination of apoptotic cells, we showed cell death was occurring in a low proportion of cells (1.10±0.54%) and that apoptotic cells were distributed away from the center of the organoid. Second, by examining stains unique to specific subtypes of inhibitory neurons, we showed the presence of parvalbumin (PV) (4.41±2.65% of inhibitory neurons), somatostatin (SST) (6.15±2.81%), calretinin (CR) (3.85±0.19%), and calbindin (CB) (4.65±2.91%) expressing subtypes. Together, these results indicate that the organoids' internal lumen is not an area of excessive cell death and that the organoids contain of a diverse population of inhibitory neurons. This broadens current understanding of the connections between *in vivo* and *in vitro* growth and the capabilities organoids can provide for understanding neurodevelopmental disorders.

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INTRODUCTION

One of the challenges in understanding and developing treatments for human diseases is reproducing the disease in a laboratory setting accurately enough that tests can produce relevant information. *In vivo* animal studies and *in vitro* cell culture both provide advantages and limitations. The advantage of animal modeling is that neural cells develop in a natural environment, complete with support tissue such as blood vessels. However, animal models of the brain can be hindered by differences in cell types [1]–[5], and development [6], [7] between the mouse, non-human primate, and human brain. One way to gain the human-specific information missing from animal studies is by using cortical organoids. Cortical organoids are three-dimensional (3D), self-organized, tissues grown from human pluripotent stem cells [8]–[11]. These organoids recapitulate features of cerebral cortex structure and function with multiple cell and tissue types [10].

Although organoids provide the advantage of using human cells, studying the brain *in vitro* raises a different set of issues that must be addressed to validate them as a model for brain development. One concern is that organoids contain only brain tissue, and therefore lack blood vessels and other structures that support the brain *in vivo* [8], [12]. Although culture media attempts to provide the nutrients required for cell growth, the current diversity of culture media used in the literature and the lack of a completely standardized culture protocol means investigating cell death in the organoids is necessary to confirm the health of the tissue [10], [12]–[14].

Another factor to consider is that organoids are most often used to model only one region of the brain at a time [9], [10], [12]–[15]. For example, several studies have been conducted using organoids that model the dorsal telencephalon [11], [13]–[15]. One effect of focusing on an organoid representing the dorsal telencephalon may be the exclusion of cells that are not generated in the dorsal region itself but migrate from ventral areas [9], [14], [16]. This includes at least a portion of inhibitory neurons, which often migrate from the ganglionic eminences (GEs) to the dorsal telencephalon during development [17]. However, whether all inhibitory neurons come from outside the dorsal telencephalon [18] or whether a majority are generated in the dorsal telencephalon itself [6] remains undetermined. Studying what types of inhibitory neurons are present in dorsal organoids could help answer this question [9], [14].

In this study, we aimed to improve the understanding of dorsal cortical organoids as a model for the brain by examining two features: cell death and the subtypes of inhibitory neurons present in the organoid. This was done by analyzing microscope images of immunostained organoid sections. Our results show that the organoids seemed to be healthy, with only small amounts of cell death. We also showed that a variety of inhibitory neurons types were present, supporting the idea that the development of inhibitory neurons can occur from progenitors within the dorsal telencephalon. This adds an additional data point to the body of research connecting how organoid systems develop in comparison to *in vivo* growth and encourages further investigation into the use of organoids to understand disorders that may be related to inhibitory neurons.

BACKGROUND

There are a variety of disorders affecting human brain development that have yet to be fully understood because of their intricacy and the complexity of the brain itself. These disorders can have a damaging effect on mental health [19], as well as general quality of life [20] [21] across patients' lifespans. Caring for children with neurodevelopmental disorders also takes a physical and psychological toll on caregivers [22]. Given that these disorders have been shown to affect about 15% of children in the US, improving our knowledge of how the brain develops is an important goal [23].

In order to develop treatments for neurological disorders, we need to first advance our understanding of their physiological characteristics. Only limited information can be gained from clinical studies using techniques such as medical imaging [24]–[29]. To glean a deeper understanding, it is essential to model both healthy and diseased states in a laboratory setting.

A large amount of what we know about brain development has come from experiments on animal models. In particular, mice and rats are commonly used because of many practical advantages [7] despite the fact that their brains are not as similar to human brains as brains from primates [30]. Mouse and rat brains can be used because many pivotal events in development are conserved across mammals, albeit on varying time and spatial scales [7]. Modeling the brain *in vitro* can provide some advantages over animal models, along with a different set of drawbacks.

A. Cell Differentiation and in vitro *modeling*

An important tool for the production of relevant human tissue in the lab is the use of stem cells to produce a variety of other cell types. Embryonic stem cells have the ability to become any type of cell in the body under the right conditions [31], [32]. As the body develops, embryonic stems cells become more specific stem cells that can only produce, for example, brain cells. Once a cell has become fully differentiated it only fulfills a certain purpose, such as in the role of a neuron. This process of more and more precise specialization occurs as cells respond to signals that they receive from nearby or sometimes distant cells [11], [33], [34]. A single cell may respond to a complex combination of signal molecules, usually proteins, secreted by many cells around it. One response a cell may have to the signals it receives is the production of certain proteins that perform a role in that cell's structure, movement, chemical processes, or subsequent signaling [35]. If expression of a particular protein is somewhat unique, it can be used to define cell types and characteristics that are relevant to research [36].

Fully differentiated cells do not under normal circumstances go back to producing other cell types as a stem cell could [35], [37]. However, Takahashi et al. showed that previously differentiated cells, such as fibroblasts, could in fact be made into stem cells with the application of four transcription factors [35]. The resulting cells, called induced Pluripotent Stem Cells (iPS cells) are functionally equivalent to embryonic stem cells [35]. Furthermore, by additionally controlling the signals these cells receive, researchers are able to control their differentiation towards desired cell types, such as neural progenitors able to produce cells of the cerebral cortex [16]. This opens up exciting possibilities for taking a small sample of patient cells, creating iPS cells, and then developing tissue in the lab that replicates that same patient's disorder, allowing researchers to better understand it and develop treatments [10], [24], [38], [39].

This is the goal that organoids aim to fulfill. Organoids are 3D tissues that model either a full or partial organ [8], [9], [40]. Organoids have been used to model a variety of tissues in the body, including muscle [41], the kidney [42], and the stomach [43]. A variety of approaches have been developed to model the cerebral cortex with cortical organoids. One of the first was by Lancaster et al. [10], who were able to show different regions of the brain with cell types organized in layers as *in vivo*. They also were able to derive iPS cells and then organoids from

the skin cells of a patient with genetically-based microcephaly, a disease that has been difficult to reproduce in animals [44]. In doing so, they were able to replicate characteristics of the patient's disorder such as reduced volume of neuroepithelium and poor orientation of radial glial cells [10]. Microcephaly caused by ZIKA virus infection was additionally recapitulated in cortical organoids by Qian et al. [14], showing the ability to model diseases from a variety of causes. Qian et al. also developed organoids specific to different regions of the brain [14].

B. Overview of brain development

In order to make these organoids useful models of the brain, it is necessary to replicate some of the steps of normal brain development in the lab. The brain forms from neuroectodermal cells that develop during gastrulation [45]. These cells create a hollow structure called the neural tube early in development. The cells on the outside of this tube give rise to the entire brain and spinal cord, while the hollow cavity becomes the equivalently hollow ventricles in the mature brain [45].

After the neural tube is formed, three initial brain regions develop by the eighth week of human development: precursors to the forebrain, midbrain, and hindbrain. The forebrain region splits into the telencephalon and diencephalon. We will concentrate on the telencephalon here, because it is the precursor to the focus of this work, the cerebral cortex. Dorsal and ventral regions of the telencephalon develop respectively into dorsal and ventral regions of the mature cortex [45].

In forming all of these regions, the original neuroectodermal cells that outlined the neural tube divide to produce more specific progenitors which in turn produce the many different cell types of the brain. In general, the dividing progenitor cells remain nearest the hollow of the ventricle, while mature neurons move outward, forming the bulk of the cerebral cortex in layers [45].

C. Inhibitory neurons in humans and animal models

Within the cerebral cortex, there are several broad cell types, the distribution of which is mostly shared across mammalian species [7]. These include glial cells, which perform a variety of support functions for the brain, and two main types of neurons: excitatory and inhibitory. The balance of excitatory and inhibitory neurons is important to proper functioning of neural circuits [46]. Inhibitory neurons, also called GABAergic because of their production of the neurotransmitter γ-aminobutric acid (GABA), only comprise about a fifth of neurons in the cortex [18]. However, they have important roles in regulating excitatory neurons [46] and have been implicated in multiple disorders including epilepsy [47] and autism [48]. Inhibitory neurons also present an area where we see marked differences between humans and other mammals, with the human brain having more GABAergic neurons than even other primates [4], [49].

Across many species, inhibitory neurons can be classified into three major groups, designated by the expression of somatostatin (SST), parvalbumin (PV) or 5-hydroxytryptamine 3A (5-HT3A) [50]. In mice, the origins of each group of inhibitory neurons have been thoroughly characterized [50]–[54] and can be used as the basis of what we understand to occur in humans [7], [18]. The earlier developing groups, PV and SST, migrate from the medial ganglionic eminence (MGE) to the dorsal cortex [51]. The later developing group, expressing 5-HT3A, migrates from the caudal ganglionic eminence (CGE) [50]. These three groups originating in the ganglionic eminences produce all inhibitory neurons in mice [51]. Within the three groups, there are additional divisions, just a few of which will be considered here. Vasointestinal peptide (VIP) is expressed in a subset of cells that are part of the 5-HT3A group

[50] while calbindin (CB) expression occurs in a subset of the SST group [17]. Calretinin expression seems to overlap with both SST and 5-HT3A groups [17].

Unlike mice, humans [55] and other primates [30] generate some inhibitory neurons in the dorsal telencephalon itself. Multiple studies show that inhibitory neurons from the late developing group (marked by 5-HT3A and originating in the CGE in mice), including those expressing CR, can come directly from the dorsal telencephalon in humans [17], [30], [55]. Letenic found that as many as 65% of inhibitory neurons came from the VZ and SVZ of the dorsal telencephalon, leaving only 35% to originate in the ganglionic eminences [6]. Others have argued that all inhibitory neurons come from outside the dorsal cortex, with some migrating from the GEs early in development and others coming later [18]. Because of the differences between rodents and humans, this is one area where animal models can only go so far in improving our understanding of the human brain, and techniques developed to study human tissue *in vitro* may provide better insight. One goal of this study is to contribute to the understanding of inhibitory neuron development in the dorsal cortex using organoids that model this brain region.

D. Cortical Organoids

The dorsal prefrontal cortex region of the brain has been shown to have a role in a broad array of functions such as working memory [56], pain perception [57], and sense of self [58]. Changes in this region have been found in psychiatric disorders such as autism [59], bipolar [60], schizophrenia [61], and depression [62]. Because of these important roles several studies have attempted to model the dorsal prefrontal cortex or, more specifically, its embryonic precursor, the dorsal telencephalon, using cortical organoids [9]–[13], [15]. These studies have been successful in showing layered organization of neuronal cell types [10], along with some glial cells [13], and have modeled cellular changes associated with disorders such as autism [15]. However, there are many neuronal and glial cell types that have yet to be characterized thoroughly in cortical organoids and which need to be understood in order to study complex diseases.

E. Inhibitory neurons in cultured cortical tissue

Inhibitory neurons were lacking in some initial studies of dorsal cortical organoids [16]. This seemed to support the hypothesis that most inhibitory neurons originate in the subcortex and migrate to the dorsal cortex later in development [18]. Migration of interneurons was additionally shown *in vitro* by Birey et al., using separate ventral and dorsal organoids that were grown, respectively, from ventral and dorsal neural progenitors. When they later fused these separate organoids, Birey et al. observed migration of GABAergic neurons from the ventral subcortex to the dorsal cortex, as *in vivo* [9]. However, in work by Lee et al. both inhibitory and excitatory neurons were found in dorsal cortical organoids [12]. Inhibitory neurons subtypes are one feature that was considered in this study. Analyzing the subtypes of inhibitory neurons present could help determine how dorsal cortical organoids are capable of producing the variety of interneurons present in the developing brain or, alternatively, whether some neurons must migrate from the subcortex as proposed by others [9].

F. Apoptosis and cell death in cultured cortical tissue

Another feature we examined was cell death and the arrangement of cells around an inner lumen. Zhang et al. showed differentiation of pluripotent stem cells into neural precursor cells results in the formation of neural-tube-like structures called neural rosettes. Cells in each rosette are oriented around a central lumen [63]. Many previous studies have used cortical organoids that develop from clusters of these rosettes [10], [14]. In the neural tube and, correspondingly,

the neural rosette, molecular signaling between cells is an important way that unique brain regions and cell types develop [45]. This molecular signaling is a concern in the development of organoids from rosette clusters, since interactions between rosettes could create signaling patterns dissimilar from normal brain development. In fact, Gessi et al. have described the occurrence of multiple rosettes *in vivo* as a hallmark of a particularly malignant form of CNS Primitive Neuroectodermal Tumor. These extremely proliferative tumors most often result in death within a year of diagnosis [64]. To avoid the complications arising from multiple rosettes, organoids that grow around a single rosette lumen have recently been generated and may better recapitulate healthy development [12].

The inner lumen of cortical organoids was hypothesized to be a feature arising from development, similar to the rosette lumen and brain ventricle. This could be fully confirmed by showing the arrangement of cells around the lumen matched that of the brain, including ependymal cells lining the cavity and neuronal cells forming concentric layers around it based on maturity [45]. Alternatively, the organoid inner lumen could be a cavity left by many cells dying in the organoid center as an artifact of *in vitro* growth conditions. Therefore, a major goal of this research was to investigate ventricular characteristics of the inner lumen by examining cell death in the organoids. Cell death was examined using the marker anti-active caspase 3. Caspase 3 is essential in cellular processing leading to apoptosis [65]. Because of this role in the mechanism of apoptosis, it has been detected in a variety of cell death conditions, such as those following chemical injury [66], endoplasmic reticulum stress [67], and ischemia [68], [69]. In this study, caspase 3 was used to examine whether excessive cell death was occurring in the cortical organoids.

METHODS

A. Study Design

The goal of this study was to understand cell characteristics in cortical organoids. To accomplish this, we analyzed confocal microscopy images of immunostained organoid sections generated from stem cells *in vitro*. First, to determine whether the inner lumen present in the center of the organoid was an artifact of cell death, the quantity and distribution of apoptotic cells were examined in four sections from organoids from two different cell lines. Using an ANOVA test, the positions of apoptotic cells were compared to the positions of all non-apoptotic cells, and the results were also compared between the cell lines.

To analyze the types of inhibitory neurons present, five inhibitory neuron subtypes were considered in relation to total inhibitory neurons, as well as organoid cells in general. The subtypes were examined in two organoid sections each from organoids derived from four different cell lines. An ANOVA test was used to compared the results from the four cell lines.

B. Cortical Organoid Model

The cortical organoids used were generated in the Shcheglovitov Laboratory (University of Utah), grown as follows with methods similar to published procedures [10], [16]. Inhibitory neuron analysis was completed using organoids from four different human stem cell lines which originated as follows. An embryonic stem cell (ES cell) healthy control line was purchased (H9 from WiCell WA09). An induced pluripotent stem cell (iPS cell) healthy control line was generated by electroporating plasmids with SOX2, KLF4, OCT3/4, L-MYC, LIN28, and shRNA factors into fibroblasts from Stanford University as described by Okita et al [70]. In addition to these two healthy control lines, we used CRISPR/Cas9 modified versions of both lines with SHANK3 gene deletions generated as described previously [71], [72]. SHANK3 is important for synaptic transmission between excitatory neurons, but is not important for the early development of cortical structure or for the formation of inhibitory neurons, so for the sake of this study all four lines were expected to develop similarly [72]. In the caspase 3 analysis, organoids from the unedited iPS cell line and the edited ES cell line were used. Pluripotency was maintained in all cell lines using Essential 8 media (ThermoFisher A1517001) and was confirmed by the University of Utah Cellular Translational Research Core when beginning differentiation.

All cell lines underwent the same differentiation process to obtain organoids. Neural induction was performed with 1:1 N2/B27 media and dual SMAD inhibition using 2 μM dorsomorphin dihydrochloride (Tocris 3093) and 10 μM SB 431542 (Tocris 1614) as described previously [16], [73], [74]. N2 media was made from 0.1% N2 supplement (ThermoFisher A1370701), 0.1% non-essential amino acids (ThermoFisher 11140050), 2 μg/mL heparin (Stem Cell 07980), and 50 mg/ml penicillin/streptomycin (ThermoFisher 150700) in DMEM/F12 medium (ThermoFisher 11320082). B27 media was made from 0.1% B27 supplement (ThermoFisher 17504044), 0.5% GlutaMAX™ (ThermoFisher 35050061) and 50 mg/ml penicillin/streptomycin (ThermoFisher 15070063) in Neurobasal™-A medium (ThermoFisher 10888022). After neuroepithelial cells were present [16] the media was switched to 1:1 N2/B27 (as before) with 10 ng/ml EGF (Stem Cell 78006) and 10 ng/ml FGF (ThermoFisher 68-8785-82) to promote formation of rosettes [63]. Once 2D neural rosettes were visible [63], individual rosettes were mechanically cut away from surrounding cells, scooped off the bottom of the plate with a bent glass pipette, and placed in individual wells in suspended culture to allow growth in 3D [12]. After 14 days, rosettes were embedded in undiluted Matrigel® (Thomas Scientific B003T15) and grown up to four months [10]. All differentiation and culture was performed by members of the Shcheglovitov Laboratory (University of Utah).

For apoptosis analysis, a total of 4 sections (sectioning described below) from 2 organoids were analyzed. These included 2 sections each from organoids derived from the edited ES cell line and from the unedited iPS cell line. For inhibitory neuron analysis 8 sections total were analyzed for each of 5 staining conditions. These included 2 sections each from organoids derived from the 4 different cell lines described above.

C. Sectioning, Immunohistochemistry and Imaging

Four-month-old organoids were fixed and sectioned for use in immunohistochemistry. Organoids were first fixed in 4% paraformaldehyde (Thomas Scientific C993M24) in PBS (Caisson Labs 1951, diluted from 10x in water) and stored overnight at 4^{o}C. After rinsing 3x with PBS, the organoids were suspended overnight in 10%, 20%, and then 30% sucrose (by weight also in PBS from Caisson Labs 1951). Following the sucrose gradient, organoids were embedded in OCT compound (Fisher Scientific 23-730-571) before being frozen to -80^{o}C. Sections 16 μm thick were cut on a Leica cryostat machine, adhered to positively charged microscope slides (Fisher Scientific 22-037-246), and stored at -20^{o}C. Sections through near the center of the organoid were selected for immunostaining and imaging to obtain a large cross-section.

Immunohistochemistry was performed according to established protocols as follows [10]. Samples were permeabilized and blocked using 0.3% Triton X-100 and 5% bovine serum albumin (BSA) (Sigma-Aldrich A4919) before applying primary antibodies in 1% BSA solution (all in PBS). For apoptotic analysis, the primary antibody was anti-active caspase 3 (rabbit, BD-Pharmingen 559565, 1:500). For inhibitory neuron analysis, anti-calbindin (CB) (mouse, Swant 300, 1:500), anti-parvalbumin (PV) (mouse, Swant 235, 1:5000), or anti-somatostatin (SST) (rat, Chemicon Ab5494, 1:500) subtype stains were used in combination with anti-GABA (rabbit, Sigma Aldrich Z031129-2, 1:500) to show all inhibitory neurons, and anti-Vasoactive Intestinal Peptide (VIP)(rabbit, Immunostar 20077, 1:500), or anti-calretinin (CR) (rabbit, Swant 7699/4, 1:2000) subtype stains were used in combination with anti-GAD67 (mouse, EMD Millipore MAB5406, 1:1000) to show major subtypes of inhibitory neurons. Primary antibodies were incubated overnight at 4^{o}C and then rinsed off with Dulbecco's Phosphate Buffered Saline (DPBS) (HyClone SH30378.03). Secondary antibodies, Alexa fluor anti-mouse 488 (goat, Invitrogen A11029, 1:500), Alexa fluor anti-mouse 594 (goat, Invitrogen A11005, 1:500), Alexa fluor anti-rabbit 488 (goat, Invitrogen A11034, 1:500), Alexa fluor anti-rabbit 568 (goat, ThermoFisher Scientific A11036, 1:500), and Alexa fluor anti-rat 488 (goat, Invitrogen A21212, 1:500) were then applied for 1 hour and rinsed off with DPBS. Finally, Hoechst 33342 dye (ThermoFisher Scientific H1399, 1:1000) was applied for 5 minutes, sections were rinsed, and a 0.15 mm coverslip was applied (Thomas Scientific 1217N68). 3 channel images of fully stained sections were collected. using a Nikon A1 confocal microscope with LASER illumination, a 20X objective (in air), and NIS elements software (Nikon). Staining and imaging was also performed on adult mouse tissue for isotype controls and to understand specific versus nonspecific staining. Immunohistochemistry and confocal microscopy were performed by Chad Russell (Shcheglovitov laboratory, University of Utah).

D. Caspase 3 analysis

In order to determine whether the inner lumen resulted from cell death in the center of the organoid, we examined both the proportion and position of apoptotic cells in 4 sections. Each section had a caspase 3 stain to show apoptotic cells and a Hoechst stain to show all nuclei. Nuclei were selected from the Hoechst stain using the spot detection feature of the Mosaic plugin for ImageJ [75]. Caspase 3-positive cells were selected manually. The proportion of apoptotic

cells was calculated and the means were compared between the two cell lines using a Student's T-test with p=0.05.

The inner and outer edges of the organoid were designated manually and saved as a set of coordinates. A custom program written in Python (Python Software Foundation) was used to determine the radial distance from all of the Hoechst-stained cells and from the caspase 3-positive cells to the inner and outer edges of the cultured organoid in each image. Fractional position was defined as follows to quantify the relative positions of the cells in the organoids:

$$Fractional\ position = \frac{radial\ distance\ to\ inner\ edge}{distance\ to\ inner\ edge + distance\ to\ outer\ edge} \quad (1)$$

This number describes the proportional distance between edges, with a fractional position near 0 describing cells close to the inner lumen and a fractional position near 1 describing cells close to the outer edge. The objective was to determine whether apoptotic cells were evenly distributed among the other cells, or whether they were disproportionately clustered near the central lumen.

A two-factor ANOVA test with p=0.05 was conducted to determine whether the average fractional position of apoptotic and non-apoptotic cells was different, and whether the positions of both groups of cells differed between the unedited iPS cell line and the edited ES cell line.

E. Inhibitory neuron analysis

Inhibitory neuron analysis compared the specific subtype stains: CR, CB, SST, PV, and VIP, to stains for all inhibitory neurons: GABA or GAD67, in order to find the proportion of inhibitory neurons accounted for by each subtype. Each image had 3 different color channels corresponding to stains for all cells (Hoechst), inhibitory neurons, and specific subtypes. Positive cells for each stain were selected using Volocity software (PerkinElmer), which was manually calibrated to find positively stained cells on each channel using size and intensity ranges. First, a region of interest including all cells was designated using the Hoechst channel. Next, all inhibitory neurons were found by selecting positively stained cells on the GABA or GAD67 channel. Finally, cells positive for a specific subtype were selected within the inhibitory neurons using the subtype channel. The percentage of inhibitory neurons belonging to each subtype was determined from examining the output counts. An ANOVA test with p=0.05 was completed to compare ES cell vs iPS cell-derived organoids and control vs CRISPR/Cas9 edited lines with respect to the 5 subtype stain.

RESULTS

A. Caspase 3 analysis

To evaluate the proportion of apoptotic cells in 4 month old organoids, we performed immunostaining with anti-caspase 3 antibodies (Figure 1A) and counted caspase 3-expressing cells across the entire section. The organoids did not exhibit a large amount of apoptosis, as shown by the occurrence of the caspase 3 marker in only 1.10 ± 0.54 % (SEM) of cells (Figure 1C). No significant difference between mean rates of apoptosis was found in comparing the unedited iPS cell line-derived organoids and the edited ES cell line-derived organoids with a Students T-test ($p=0.22$), although it must be noted that statistical power was low given only 2 organoid sections were used for each case.

Despite the small number of apoptotic cells, it was important to know whether they were clustered around the inner lumen. To determine this, we measured the distance from each cell to the inner lumen edge of the organoid. The results showed apoptotic cells were more populous near the outer edge than near the center of the organoid, as shown by the histogram in Figure 2. In order to confirm that this did not simply result from there being more total cells near the outer edge of the organoid, the positions of apoptotic and non-apoptotic cells were compared in all sections (Figure 3). A two-factor ANOVA was used to compare the average positions of caspase 3-positive cells and caspase 3-negative cells, as well as to compare the positions of cells between the edited ES cell line and unedited iPS cell line. The ANOVA indicated a significant difference in the positions of apoptotic and non-apoptotic cells ($p=0.0003$) and was not able to show a difference in the cell distributions between the two cell lines ($p=0.876$). This indicates that there is not significant apoptosis occurring in the center of the organoid. Together, the proportion and distribution of apoptosis in the organoids suggest the internal lumen is not a product of cell death and, albeit within our small sample, this holds regardless of organoid cell line origin.

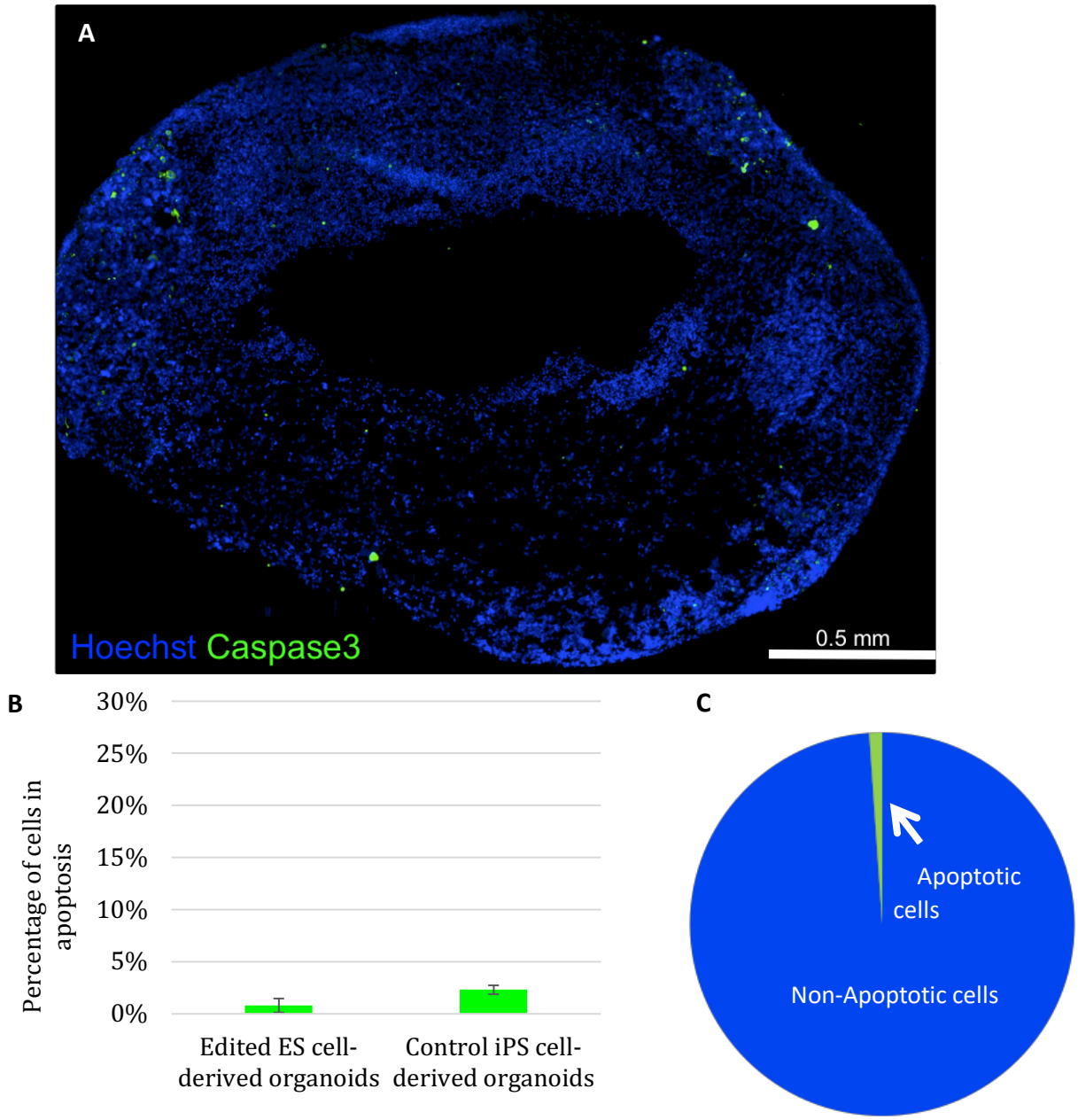

Figure 1. Proportion of cells in apoptosis. A**:** Example image of organoid cross-section (Chad Russell, Shcheglovitov Lab, University of Utah). B: With 2 organoid sections used per cell line, a significant difference between the mean proportion of apoptotic cells was not found using a Students T-test ($p=0.22$). C: A small percentage of cells, 1.10±0.54% were apoptotic on average.

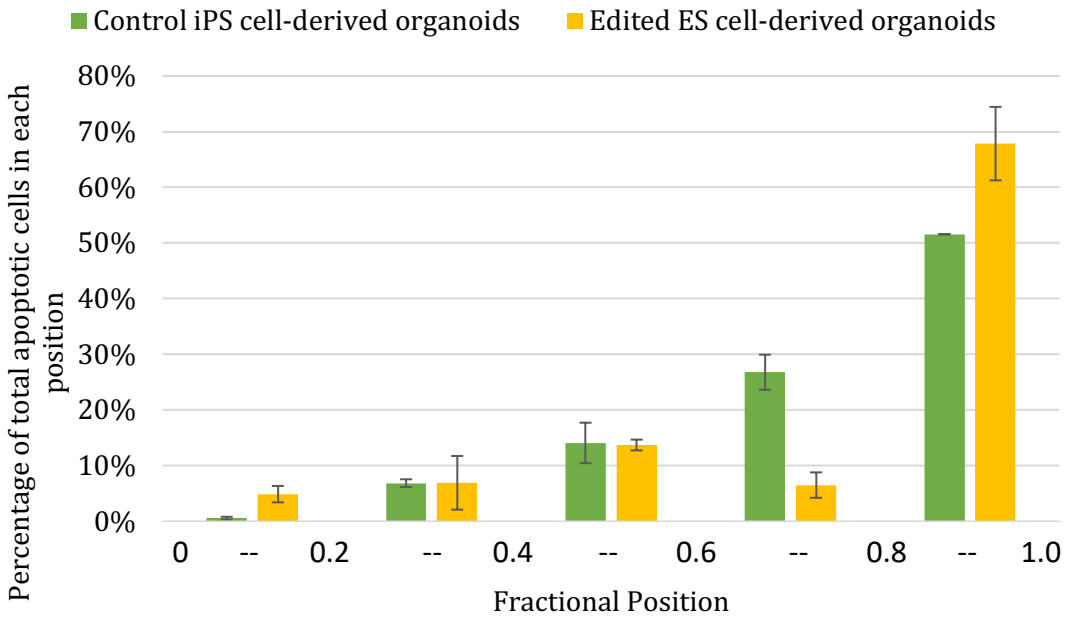

Figure 2. Histogram of apoptotic cells positions. Fractional position is defined as (Distance from inner lumen edge)/(Distance between inner and outer edges). Apoptotic cells were more common near the outer edge of the organoid than near the organoid inner lumen. Distribution of apoptotic cells was similar between the two cell lines examined.

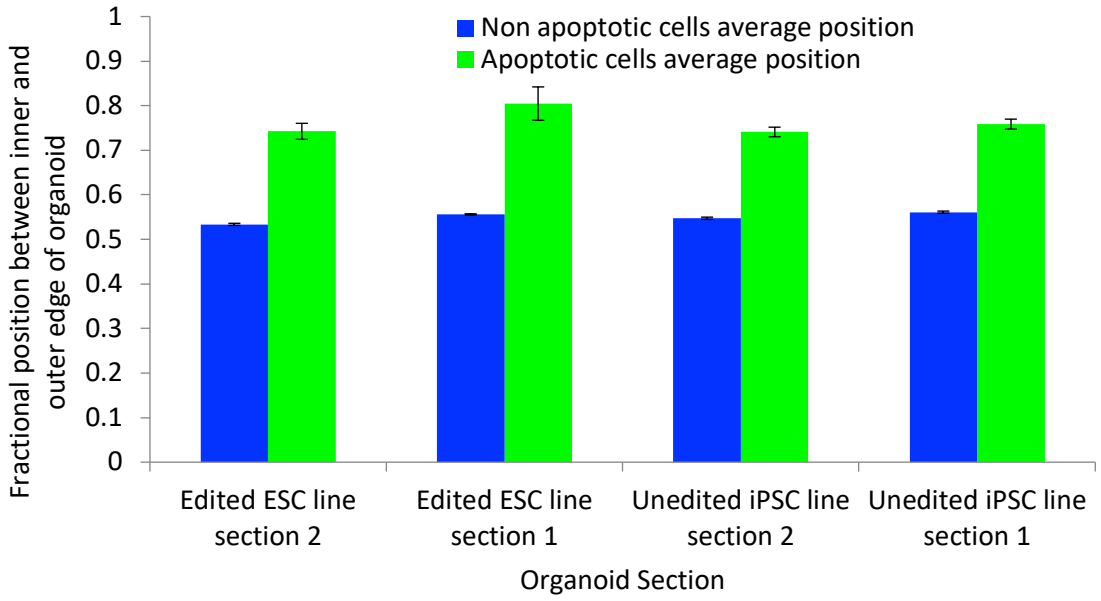

Figure 3. Apoptotic cells were positioned further away from the organoid inner lumen on average than non-apoptotic cells. Fractional position is defined as (Distance from inner lumen edge)/(Distance between inner and outer edges). The average position of apoptotic, caspase 3-positive, cells and non-apoptotic cells is shown for all four organoid sections. A two-factor ANOVA was used to compare both the average positions of apoptotic versus non-apoptotic cells and the edited ES cell line-derived organoids versus unedited iPS cell line-derived organoids. The ANOVA showed there was a significant difference between the positions of apoptotic and non-apoptotic cells ($p=0.0003$), but not between cell lines ($p=0.876$).

B. Inhibitory neuron analysis

To evaluate the presence of inhibitory neurons in cortical organoids, we performed immunostaining (Figures 4 and 5) on 4-month-old organoids with anti-calbindin (CB), anti-parvalbumin (PV), anti-somatostatin (SST), anti-calretinin (CR) and anti-vasointestinal peptide (VIP) antibodies and counted cells expressing each protein. The proportion of inhibitory neurons expressing CB, PV, or SST each was found by comparing their presence to a GABA stain for all inhibitory neurons. Similarly, CR and VIP stains were compared to a Gad67 stain for all inhibitory neurons. At least 4 of the 5 inhibitory neuron subtype markers tested were found (Figures 4 and 5), showing a broad range of cell types present in the organoids. CB, PV, SST, and CR each composed 4–6% on average of the inhibitory neurons in the organoid sections. VIP was only found in one of the 12 sections where it had been stained. However, the proportion of inhibitory neurons accounted for by each cell type was highly variable between sections, as shown in Figure 6. Given this high variability and the fact that only two sections from each cell line were used for each subtype, an ANOVA could not show any significant differences for ES cell-derived organoids versus iPS cell-derived organoids or edited versus unedited lines, using the 5 subtypes' outcomes. These results suggest the human dorsal cortex may be capable of producing more diverse inhibitory neurons than previously shown, but the consistency of this outcome cannot be confirmed based on the data collected.

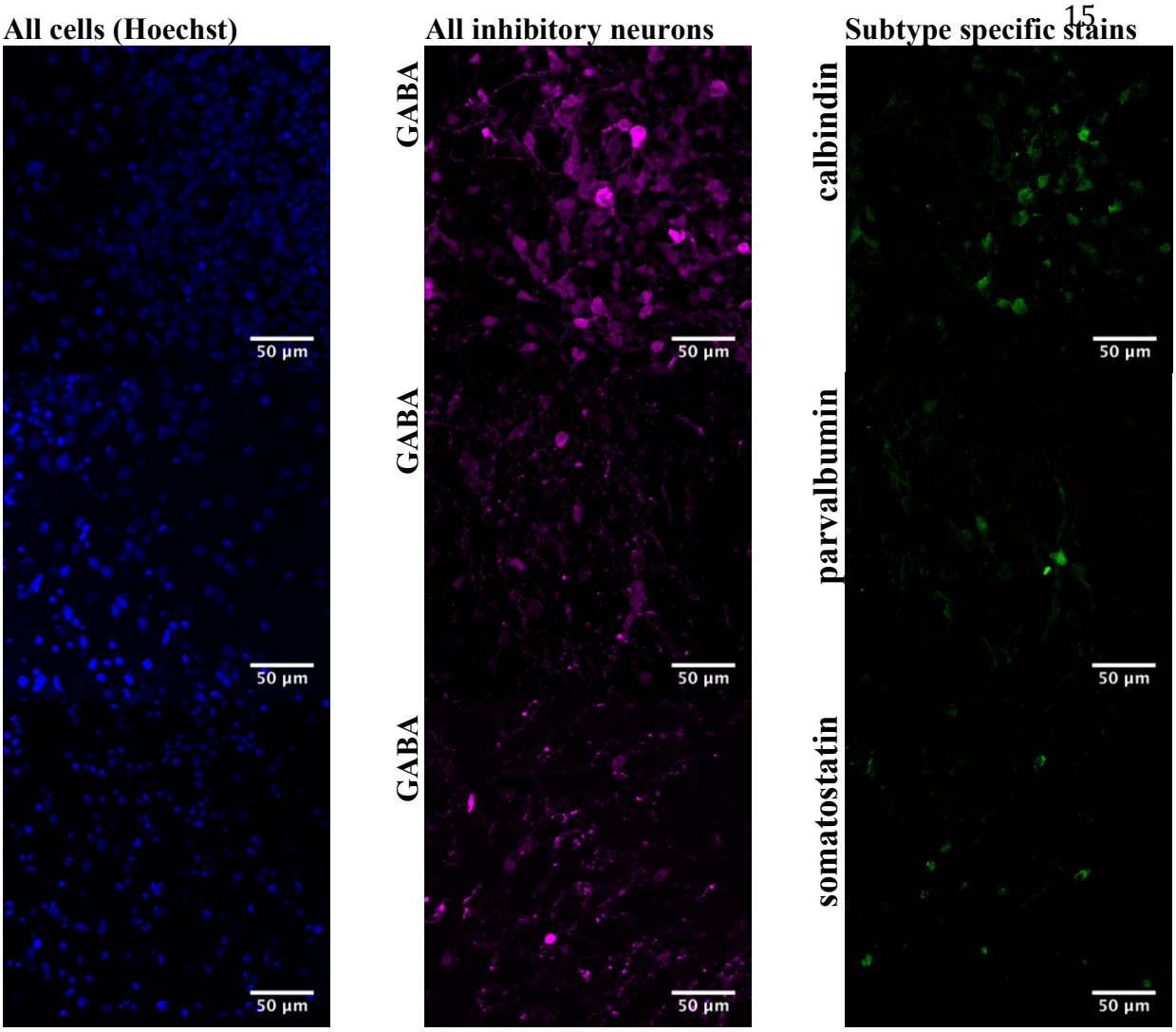

Figure 4. Example stains for inhibitory neurons. Each section had a Hoechst stain for nuclei, GABA for inhibitory neurons, and a subtype stain with anti-calbindin (CB), anti-parvalbumin (PV), or anti-somatostatin (SST) antibodies. (Chad Russell, Shcheglovitov Lab, University of Utah, unpublished data). First row: CB was expressed in 4.7±2.9% of inhibitory neurons. Second Row: Parvalbumin PV was expressed in 4.4±2.7% of inhibitory neurons. Third Row: Somatostatin SST was expressed in 6.2±2.8% of inhibitory neurons.

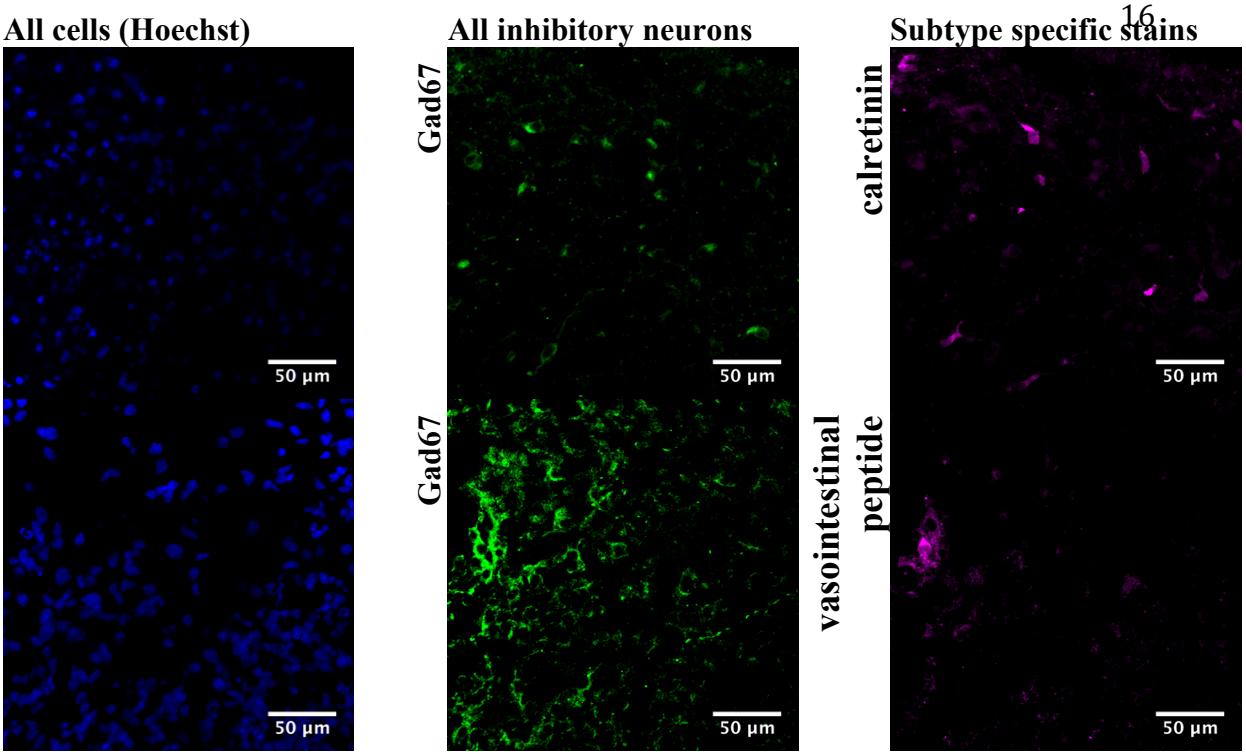

Figure 5. Example stains for inhibitory neurons. Each section had a Hoechst stain for nuclei, Gad67 for inhibitory neurons, and a subtype stain with anti-calretinin (CR) or anti vasointestinal peptide (VIP) (Chad Russell, Shcheglovitov Lab, University of Utah). First row: CR was expressed in 3.8±0.2% of inhibitory neurons. Second Row: VIP was seen in only one section, 0.3±0.3% of inhibitory neurons.

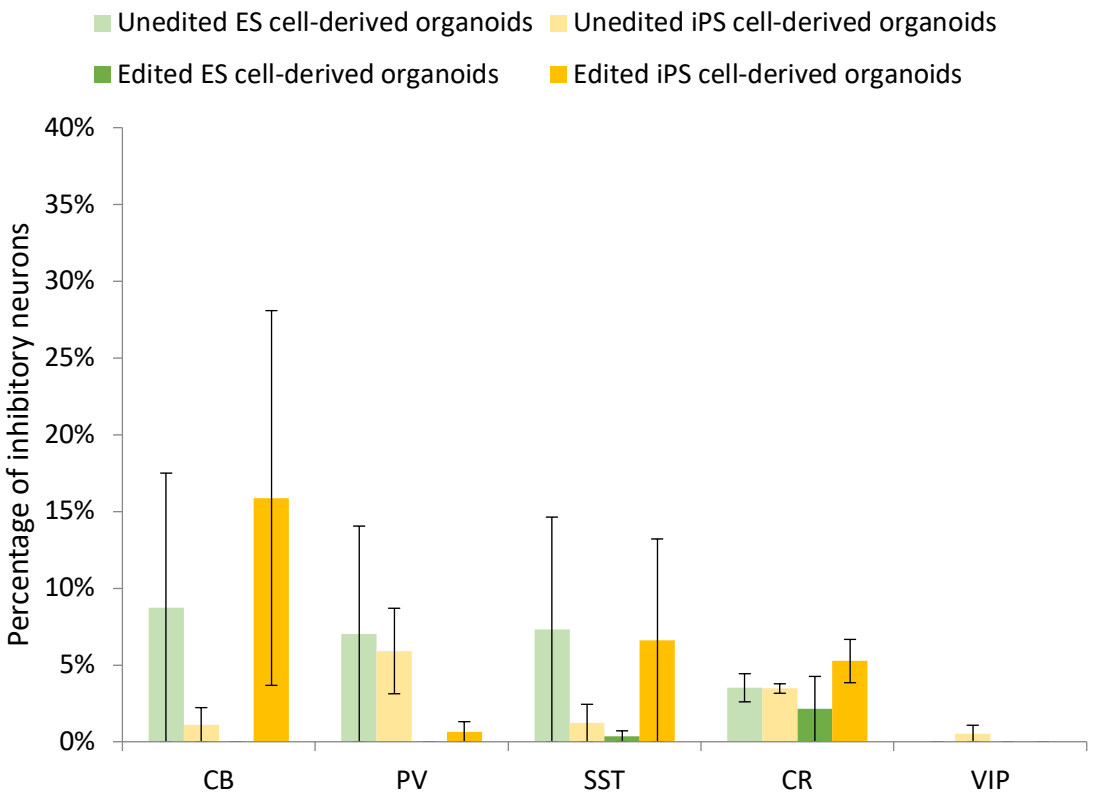

Figure 6. Inhibitory neurons subtypes were present in highly variable amounts among the sections analyzed. For each subtype, 8 sections from 4 different cell lines were analyzed. Bars show the mean of data between the 2 sections analyzed in each condition, with error bars indicated standard error between those two sections. An ANOVA test did not show significant differences between ES and iPS cell-derived organoids or edited and unedited lines.

DISCUSSION

The aim of this study was to investigate two features of cortical organoids in relation to brain development: subtypes of inhibitory neurons and cell death in relation to an inner lumen. These two characteristics were examined by analyzing confocal microscopy images of immunostained organoid sections. The results showed that the ventricle-like cavity in the center did not result from cell death, as evidenced by the small number of apoptotic cells (Figure 1) and their positioning away from the cavity (Figures 2 and 3). We also found that a variety of inhibitory neurons were present, including 4 of the 5 subtypes for which we stained, but the proportion of each inhibitory neuron subtype was highly variable (Figure 6).

A. Cell death analysis

Since cortical organoids lack the vascular supply of the brain, understanding whether the cavity in the center might result from ischemic-like conditions was a particular concern. In fact, studies of non-vascularized tumor spheroids have shown that, both *in vivo* and *in vitro*, oxygen can be reduced to zero in the center of spheroids greater than 0.5 mm in diameter [76], smaller than our organoids. Moreover, Bell et al. showed that the central area of spheroid tumor models exhibited necrotic death [77], which would not involve, for instance, the caspase 3 activation we looked for in this study. However, they also saw apoptosis, which occurred almost exclusively in the area immediately surrounding the necrotic center, and likely in response to it [77]. In contrast, more than half of the apoptotic cells in our study were in the outer 20% of the organoid (Figure 2), making it unlikely that they represent the peri-necrotic apoptosis described by Bell et al. [77]. From this information we concluded that the organoids in this study must have better nutrient distribution than, for instance, the tumor spheroids described above, and so do not undergo ischemic-like cell death in the center. This supports the idea that the inner cavity could be a structural feature functionally resembling the ventricle.

The pattern of apoptosis we observed in 4-month-old organoids is closer to the distribution of apoptotic cells seen *in vivo* by Rakic et al. near the 4 month time point of normal brain development. They reported 0.6% apoptotic cells in the ventricular and subventricular zones and 1.15% in the subplate and intermediate zones [78]. Although we do not have the ability to compare our data exactly in terms of individual zones, our 1.10% total rate of apoptosis (Figure 2) with a greater distribution away from the center of the organoid (Figure 3) seems reasonably similar to Rakic et al.'s findings *in vivo* [78]. Although our conclusions are limited by the use of only 4 organoid sections, the distribution of apoptosis was similar across those 4 sections, indicating the organoids are consistently healthy.

A limitation of our cell death analysis is that we only considered a single time point, 4 months. Since active caspase 3 is only present in currently apoptotic cells, our staining would not be able to detect if there had been significant apoptosis that had previously completed. However, if there was a problem with, for instance, nutrient distribution in the organoids, we would expect ongoing apoptosis near the center that could be detected. It is possible that the cells in our organoids are undergoing necrosis without any of them expressing caspase 3, unlike in Bell et al. [77], [79]. It is also possible that the cells in the center remain alive despite deficiencies in oxygen or glucose through metabolism regulation and quiescence as seen in some tumor cells [80]. Otherwise, these findings suggest that the inner cavity of the cortical organoids does not result from cell death and could be a structure similar to the ventricle of the brain. This could make it useful as a landmark to study the 3D cortical structure and layering of developing tissue. Future work examining this cavity at different time points and looking for the presence of ventricular characteristics such as ependymal cells could further inform our conclusions.

B. Inhibitory neuron analysis

In the second part of this study, we showed four of the five inhibitory neuron subtype markers, parvalbumin (PV), somatostatin (SST), calretinin (CR), and calbindin (CB), were frequently present in the organoids, while vasoactive intestinal peptide (VIP) was only seen in one section. While some studies have argued that all human inhibitory neurons, like those of mice, migrate from the ganglionic eminences in the ventral cortex (GEs) [18], the presence of four inhibitory neuron subtypes in our dorsal organoids is supported by others who have indicated inhibitory neurons in humans can originate in the dorsal telencephalon [6], [17], [55], [81].

The inhibitory neurons that have been shown most frequently to develop within the dorsal cortex in humans [55] and other primates [30] are the late-developing group expressing 5-HT3A and CR [17]. This made it unsurprising that CR was the subtype present in most consistent quantities across our samples (Figure 6).

The origins of the early developing groups of inhibitory neurons in vivo are less clear. Fertuzinhos et al. showed that SST neurons are absent from the cortex in cases of Severe Striatal Hypoplasia, while CR neurons are always present, implying that the SST group cannot come from the dorsal cortex itself [81]. The CB subgroup of SST neurons have also been seen to migrate from the MGE to the cortex *in vivo* [17]. Birey et al. showed migration of SST, CB, CR, and PV cells between separate dorsal and ventral spheroids *in vitro* [9]. These findings make the presence of SST and CB neurons in our dorsal cortical organoids surprising (Figure 6). However, while some studies indicate that only the late developing inhibitory neurons come from the cortex and early groups come from the MGE, Letenic found that 65% of inhibitory neurons came from the VZ and SVZ of the dorsal telencephalon, leaving only 35% to originate in the ganglionic eminences [6]. This is supported by the presence of PV and SST inhibitory neurons in the forebrain specific organoids of Qian et al. They additionally showed that these inhibitory neurons appeared without the typical Nkx2.1-expressing ventral progenitors, indicating that a dorsal progenitor cell may be able to produce inhibitory neurons [14]. Since our dorsal cortical organoids also exhibited PV, SST, and CB expressing inhibitory neurons, they would support the possibility that dorsal progenitors have the ability to produce inhibitory neurons.

It is important to note that, assuming CB expressing neurons are a subset of the SST expressing cells, we have only accounted for 10-15% of total inhibitory neurons detected (Figure 6). The most obvious explanation for this would be that a large proportion of the inhibitory neurons in these organoids are from the subset of the 5-HT3A group that do not express any of the markers we examined. Further immunostaining would be needed to examine this possibility.

These findings are limited by the large variability between our samples and the fact that we only examined inhibitory neurons at a single time point. In the future, considering both inhibitory neurons and their precursors over a longer time period would allow a better understanding of their development. In particular, our analysis thus far relies on knowing that the pluripotent (embryonic or induced) cells we began with were fully converted into dorsal neural progenitors as expected before differentiating further into excitatory and inhibitory neurons. This assumption was based on the fact that neural induction using dual SMAD inhibition along with N2/B27 media has been shown repeatedly to promote efficient loss of pluripotency and the rise of dorsal neural identity shown by the expression of markers such as Pax6 and FoxG1 [12], [73], [74], [82]–[84]. However, if the organoids did not start out from pure dorsal progenitor cells but had even a few contaminating ventral progenitor cells, the conclusion that the 4 inhibitory neuron types came from cells of the dorsal cortex would be invalid.

In addition, research connecting *in vivo* and *in vitro* results needs to continue in order to determine whether the cells formed in this experiment followed a natural development trajectory

or were artificially pushed towards abnormal differentiation by the *in vitro* environment. In particular, showing dorsal neural progenitors can produce inhibitory neurons *in vitro* does not mean that they follow this path *in vivo* and may indicate a response to artificial signaling conditions rather than the existence of a development path that has not been observed before. Examining the morphology of individual cells and their complete protein expression over time would enhance our understanding of the subtypes present. Determining the sources of variation in these organoids in order to improve their consistency may also shed light on the exact proportions of cells being produced and their origins, as well as if there are differences between cell lines that are currently being obscured by the high variation among individual sections.

In summary, our study of inhibitory neurons shows multiple subtypes may be produced by dorsal cortical organoids. This is corroborated by previous work indicating that inhibitory neurons in the human brain, unlike in mice, have origins in both dorsal and ventral telencephalic regions [55]. Continued investigation into the development of inhibitory neurons will improve our insight into human diseases that may be affected by inhibitory neuron dysfunction, such as epilepsy [47], autism [15], and schizophrenia [46].

CONCLUSION

Overall, this work adds a new piece in understanding the development of cortical organoids as *in vitro* models of the embryonic dorsal telencephalon. Having a model that successfully replicates some of the cellular diversity and 3D structure in the brain opens up possibilities for better understanding the complex networks that develop in the cerebral cortex. Continuing to advance these models can improve our knowledge of healthy brain development, effectiveness at modeling disease states, and ability to test treatments for neurological disorders.

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